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Direct spectrofluorimetric methods as alternatives to compendial ones used for the quality control of biopharmaceuticals: development, validation and application

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A simple, rapid, precise, accurate and sustainable spectrofluorimetric method (SFM) was developed, validated and applied for the determination of 4-aminobenzoic acid and aromatic amino acids (phenylalanine, tryptophan and tyrosine). These compounds are used in biopharmaceutical formulations and therefore must be analyzed by quality control laboratories to meet the criteria established in pharmacopoeias. In general, potentiometric titration (PT) is described in the compendia as the official analytical technique. However, this method showed low sensitivity and selectivity, and moreover was performed with a non-aqueous solvent (acetic acid), which led to higher consumption of reagents and consequently to the formation of residues. Therefore, the SFM was developed in aqueous medium at pH 7.2 using phosphate buffer. It was successfully validated according to the ICH guidelines and showed good linearity range (r > 0.999), specificity, accuracy and precision (within and between days) and robustness. The test results were compared between the SFM and PT using raw material samples, while according to the F- and t- tests at 95% confidence level, no statistical difference was found between the methods.

Keywords: 4-aminobenzoic acid. Analytical validation. Aromatic amino acids. Fluorescence spectroscopy. Raw material.

INTRODUCTION

Biopharmaceuticals (BPs) are biologically derived drugs obtained from organisms or cells by the use of biotechnology. They can be classified into three main groups: therapeutic proteins, monoclonal antibodies, and vaccines, all of which have great relevance to public health, with applications ranging from prophylactic tools to the treatment, remediation and cure of diseases (Pimenta, Monteiro, 2019).

Due to complexity and fragility of the BP structures, a wide variety of compounds are used in their formulation, such as: amino acids (AAs), sugars, alcohols, and vitamins (Sun *et al.*, 2022; Wang, Ohtake, 2019).

The main applications of AAs are as adjuvants, to improve the immunological response to a given antigen (Azuar *et al.*, 2021; Skwarczynski *et al.*, 2020; Lim *et al.*, 2019); stabilizers, to avoid losses and degradation of BPs during the lyophilization process (Castro *et al.*, 2021; Idrees *et al.*, 2020; Mohammed, Coombes, Perrie, 2007); nanoparticles (Vrieling *et al.*, 2019); and buffers (Wlodarczyk *et al.*, 2018).

Among the vitamins, 4-aminobenzoic acid (PABA) (Figure 1a) is classified as vitamin B10, although it is structurally a non-protein amino acid. It is known as an intermediate substance in the folate synthesis route (Mirzaei,

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Khayat, Saeidi, 2012). In addition, it has been used as an important antioxidant, especially against UV radiation (Schnellbaecher *et al.*, 2019), and for auxotrophic mutant production during the development of live attenuated vaccines (Cohen *et al.*, 2012; Chimalapati *et al.*, 2011).

To apply these compounds as raw materials for BP production, it is mandatory to analyze them by a quality control laboratory to guarantee the quality, safety and efficacy according to best good manufacturing practices (Ramos-Martínez, Alonso-Herreros, Rosales-Cabrera, 2020).

Potentiometric titration is the official test method according to the British Pharmacopoeia (2020), European Pharmacopoeia (2020), Japanese Pharmacopoeia (2021) and United States Pharmacopoeia (2022). However, some aspects of this method can be improved, such as: (i) analytical aspects, since the method does not have high selectivity and specificity, involves difficult solubilization for sample preparation of AA assays, and is performed using a non-aqueous solvent (glacial acetic acid), which is strongly influenced by atmospheric CO_2, H_2O or O_2 (Kratochvil, 1976); (ii) economic aspects, since in comparison with methods in aqueous media, it requires high consumption of reagents, especially solvents; and (iii) environmental aspects, due to the high generation of waste.

Fluorescence spectroscopy, also known as spectrofluorimetry, is an analytical technique based on the luminescence of aromatic molecules that can be carried out directly (without the need for a derivatization step). Derivatizing reagents are useful for molecules that are non-fluorescent or have a weak fluorophore group. In this case, the quantum fluorescence yield is improved by the reaction between analyte and derivatizing reagent (Andrade-Eiroa *et al.*, 2010).

This technique has been described as a good alternative to overcome the limitations of other analytical techniques with poor sensitivity and selectivity, like potentiometric titration. The principal advantages of spectrofluorimetry are speed and the ability to use inexpensive aqueous media that are easily available and have high sensitivity and selectivity (Lakowicz, 2006). Spectrofluorimetric methods (SFMs) have hence been applied to quantify a wide range of compounds in pharmaceutical science (El Sharkasy *et al.*, 2022; Abo Shabana, Elmansi, Ibrahim, 2022; Elama *et al.*, 2022).

Therefore, in this work we aimed: (i) to develop and validate a SFM for PABA and aromatic AAs (L-phenylalanine (PHE), L-tryptophan (TRP) and L-tyrosine (TYR)) (Figure 1) used as raw materials for the BP formulation; (ii) to apply them in the assays typically carried out by quality control laboratories; and (iii) to compare the results with those obtained by compendial methods.
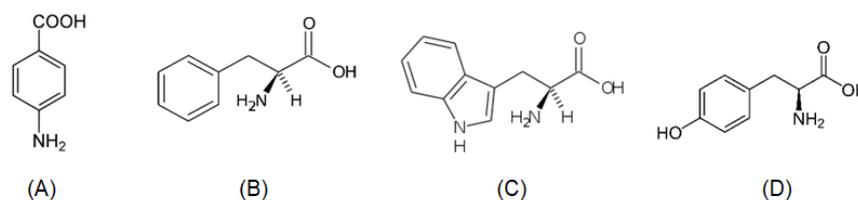
FIGURE 1 - Chemical structures of 4-aminobenzoic acid (PABA) (A); L-phenylalanine (PHE) (B); L-tryptophan (TRP) (C); and L-tyrosine (TYR) (D).

MATERIAL AND METHODS

Instrumentation

Fluorescence spectra and measurements were recorded using a Shimadzu RF-6000 spectrofluorophotometer (Japan) equipped with a 150 W xenon arc lamp and a 1 cm quartz cell, controlled by a computer running the Lab Solutions RF software for Windows®. The slit widths for the excitation and emission monochromators were set to 3.0 nm. A pH meter (Metrohm, Switzerland) was used for pH adjustment. Titrations in non-aqueous media were performed in an 836 Titrando system (Metrohm, Switzerland) using a Solvotrode® electrode (LiCl saturated in ethanol). Karl Fischer titrations were performed with an

870 KF Titrino Plus (Metrohm, Switzerland). For drying loss (LDRY) analyses, a forced-air oven (Velticell®, Medcenter Einrichtungen GmbH, Germany) was used.

All analyses were performed at the Physicochemical Control Laboratory, Quality Control Department, Institute of Immunobiological Technology (Bio-Manguinhos), Oswaldo Cruz Foundation (Fiocruz).

Chemicals and solutions

The aqueous solutions were prepared with deionized water using a Milli-Q® purification system. The following chemical reference standards (CRS) were used: (i) PABA (USP catalog number 1019803; lot R095Q0; with content of 0.999 mg PABA per mg of material on an "as is" basis); (ii) PHE (USP catalog number 1530503; lot R030D0; with content of 1.00 mg PHE per mg of as-is material); (iii) TYR (USP catalog number 1705006; lot R08390; with content of 1.00 mg TYR per mg of as-is material); and (iv) TRP (EDQM catalog number T2610000; lot 3, with content of 100.0% TRP).

Glacial acetic acid, anhydrous formic acid, methanol, CombiTitrant 5 Keto (reagent for Karl Fischer volumetric titration), potassium dihydrogen phosphate (KH_2PO_4), and solutions (perchloric acid ($HClO_4$) 0.1 mol L^{-1}, sodium hydroxide (NaOH) 0.1 mol L^{-1}, and hydrochloric acid (HCl) 0.1 mol L^{-1}) were purchased from Merck (Darmstadt, Germany).

Potentiometric method (PM)

The potentiometric method (PM) was performed in triplicate at room temperature (18 ± 1 °C) using raw material samples of PABA, PHE, TRP, and TYR according to the methods described in the European Pharmacopoeia (2020) and presented in Table I.

TABLE I - Experimental conditions used in the potentiometric determination of 4-aminobenzoic acid (PABA), L-phenylalanine (PHE), L-tryptophan (TRP) and L-tyrosine (TYR) (European Pharmacopoeia, 2020)

Analyte	Weight (mg)	Titrand solution	Titrant solution
PABA	100	50 mL of water + heating*	NaOH 0.1 mol L^{-1}
PHE	100	3.0 mL of formic acid + 30 mL of acetic acid	$HClO_4$ 0.1 mol L^{-1}
TRP	150	3.0 mL of formic acid + 50 mL of acetic acid	$HClO_4$ 0.1 mol L^{-1}
TYR	150	5.0 mL of formic acid + 30 mL of acetic acid	$HClO_4$ 0.1 mol L^{-1}

*heating (40 °C) was used in the solubilization step.

Spectrofluorimetric method (SFM)

Phosphate buffer (2.50×10^{-4} mol L^{-1}) was prepared by dissolving KH_2PO_4 in water and pH was adjusted to 7.2 using NaOH 0.1 mol L^{-1}. Stock standards and sample solutions (100 mg L^{-1}) were individually prepared by weighing 20.0 mg of solid and then adding 1.0 mL of NaOH 0.1 mol L^{-1} to solubilize it. The solution was transferred to a 200 mL volumetric flask, followed by adding 10 mL of phosphate buffer, and the volume was completed with deionized water. All the solutions were transferred to amber flasks immediately after preparation.

The pH of all diluted solutions was measured before and after fluorescence measurements to ensure that it was 7.2. The analyses were performed at room temperature (18 ± 1 °C).

The effect of pH on the fluorescence intensity was evaluated to determine the maximum emitted fluorescence using stock solutions of PABA, PHE, TRP and TYR at pH 7.2.

Water determination and loss on drying (LDRY) analyses

All results of the PM and SFM analyses of the raw material samples were corrected to PHE, TRP and TYR by subtracting the LDRY results and the water values determined by Karl Fischer volumetric titration of PABA to express them in dry weight. Both analyses were performed in triplicate according to the corresponding monographs of the European Pharmacopoeia (2020).

For the LDRY analyses used in this work, 1,000 g of each sample was weighed with an analytical balance. Subsequently, the samples were kept at over 105 ± 2 °C until reaching constant mass, i.e., when the difference between the mass after and before drying did not exceed 0.5 mg after one hour under the temperature conditions specified in the respective monographs (Brazilian Pharmacopoeia, 2019).

After this interval, the samples were cooled to room temperature in a desiccator containing silica and then reweighed. The results were expressed according to equation 1 below:

$$LDRY(\%) = \frac{(b_i - b_f)}{m_i} \times 100 \qquad (1)$$

Where LDRY (%) is the drying loss result in percentage; b_i and b_f are the initial and final mass of the bottle containing the sample before and after drying, respectively, in grams; and mi is the sample mass in grams.

Water measurement, expressed as percentage, was performed using 1.00 g of the sample directly in the instrument with automatic end-point detection by reacting water in the sample with sulfur dioxide and iodine in a suitable anhydrous medium, in the presence of a base (imidazole) with sufficient buffer capacity (European Pharmacopoeia, 2020).

Method validation

Validation of direct SFM for PABA, PHE, TRP and TYR tests was based on the procedure described by the International Conference on Harmonization (ICH) (2022a). Some criteria for acceptability were based on Brazilian legislation (ANVISA, 2017; Brasil, 2017) and the Guidelines for Standard Methods Performance Requirements (AOAC, 2016). The validation parameters evaluated were specificity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness.

Specificity

Specificity was determined by the excitation (λ_{EX}) and emission (λ_{EM}) wavelengths at which the maximum fluorescence intensity was observed according to screening spectra obtained from standard stock solutions of each analyte.

Linearity and range

The linearity was determined by successive dilutions from CRS stock solutions of the analytes. Each concentration level was prepared and measured in triplicate.

The results of the relationship between fluorescence intensity versus concentration were used to determine the parameters of the linear regression by the least squares method. The equation which describes this relationship was $y = a + bx$, where *a* and *b* are the linear and angular coefficients, respectively. A correlation coefficient (r) greater than 0.990 was considered appropriate for these methods (ICH, 2022b).

All statistical analyses were performed at a 95% confidence level, and regression significance, normality and homoscedasticity were assessed by ANOVA (F-test), Shapiro-Wilk and Cochran tests, respectively.

After the linearity parameters were established, the target concentration (TC) was chosen to be near the central point of the linearity range. Calibration curves were then plotted with five concentration levels in an interval between 80 and 120% of the TC.

Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ were determined using equations (2) and (3), respectively:

$$LOD = 3.3 \times \frac{s_{10}}{b} \tag{2}$$

$$LOQ = 10 \times \frac{s_{10}}{b} \tag{3}$$

Where s_{10} is the standard deviation obtained from 10 measurements of the lowest concentration level found in the linearity analysis, and b is the angular coefficient of the analytical curve (Miller, Miller, 2010).

Precision

Precision was assessed by the dispersion of the results of 3 measurements for the low, mean, and maximum values of the calibration curve, corresponding to TC values of 80, 100 and 120%, respectively. These analyses were carried out in triplicate and the results were expressed as percentages by the relative standard deviation (RSD).

Repeatability (intra-day precision) was evaluated under the same conditions within a day (operation, analyst and instrumentation), while the intermediate precision (inter-day precision) was evaluated under different conditions (day and analyst) with the same instrumentation.

Accuracy

Accuracy was determined in triplicate with the same solutions used for precision analyses. The results were expressed as percent recovery, calculated by the ratio between the concentration determined in the linear regression and the theoretical concentration.

Robustness

The robustness of SFM was examined in triplicate and demonstrated by the scattering of the fluorescence intensity results, expressed as RSD, with the same solutions used for precision analysis. The reliability of the analyses was evaluated with respect to small variations in experimental (pH buffer 7.2 ± 0.2) and instrumental ($\lambda_{EM} \pm 1$ nm) parameters.

RESULTS AND DISCUSSION

Effect of pH on the fluorescence properties

In the development of a SFM, pH is an important parameter to evaluate because the luminescence process is directly affected by the charge distribution in the molecules. Figure 2 shows the effects of pH conditions from 2 to 11 for each analyte from its respective stock solution.

PABA showed two important groups as possible protonation sites: the amino group, which is a proton acceptor, and benzoic acid, which is a donor. Thus, this compound can present intramolecular charge transfer after photoexcitation associated with dependence on the solvents' characteristics, particularly in aqueous solutions, being strongly affected by pH levels (Chan *et al.*, 2020).

The results of fluorescence intensity of PABA in the analyzed pH range showed a sigmoidal plot profile, which agreed with the measurements performed by Tanojo, Junginger, Boddé (1997) (Figure 2). The lowest fluorescence intensity was observed at pH around the pI (3.69) (Table II), since the protonic and neutral species are not able to emit significant amounts of fluorescence. Therefore, this condition was disregarded in the PABA determination.

The maximum fluorescence was observed between pH 6 and 8. This result can be attributed to the high charge distribution in the molecules due to the inductive effect of the amino group combined with the resonance effect of the aromatic ring. A slight decrease was observed from pH 8 upward (Figure 2).

Among the aromatic AAs, PHE had the lowest molar absorptivity (173 cm^2 mol^{-1}), while TRP and TYR exhibited values of 5,600 and 1,400 cm^2 mol^{-1}, respectively (Xiong *et al.*, 2021). Also, the quantum yield, which is defined by the ratio between emitted and absorbed photons by the molecule, followed the same order of TRP > TYR > PHE (Lakowicz, 2006).

These profiles are consistent with the data depicted in Figure 2, where the fluorescence intensity of PHE exhibited the lowest magnitude among the compounds. In the studied pH range, no significant variation in the

fluorescence emission intensity was observed. This result indicated that in this case, pH was not a critical parameter to consider in the development of the analytical method to determinate PHE content.

TRP has one of the strongest fluorescence properties among AAs, and due to the presence of the indole group, it has the greatest absorptivity and quantum yield. Unlike PHE and TYR, the TRP emission profile differs significantly according to the solvent's polarity, so that in polar solvents it has an emission wavelength around 350 nm, whereas the wavelength is different in nonpolar solutions (Hellmann, Schneider, 2019).

The pH effect involved in the decrease of TRP fluorescence can be explained by the dissociation of the carboxylate ions at pH < pKa_1 (2.83) (Table II). On the other hand, a slight increase of fluorescence was observed from pH 8 upward, with a maximum at pH 10, attributed to the deprotonation of amino groups (Cowgill, 2022).

The fluorescence property attributed to TYR is associated with phenol in the fluorophore group of its structure (Figure 1D). For this reason, this AA can absorb UV radiation through $\pi \rightarrow \pi^*$ transition to the excited state (Xiong et al., 2021).

In contrast to PHE, TYR had the lowest solubility in water compared to the other aromatic AAs. Also, it had the highest fluorescence intensity, which was greatly affected by pH, so that the maximum values were observed in the pH range of 4 to 8 (Figure 2) (Martín-Tornero et al., 2019). The decrease of fluorescence can be explained by the protonation of the carboxylate ions close to pKa_1 (2.20), and by phenol ionization around pKa_3 (10.07) (Table II) (Xiong et al., 2021).
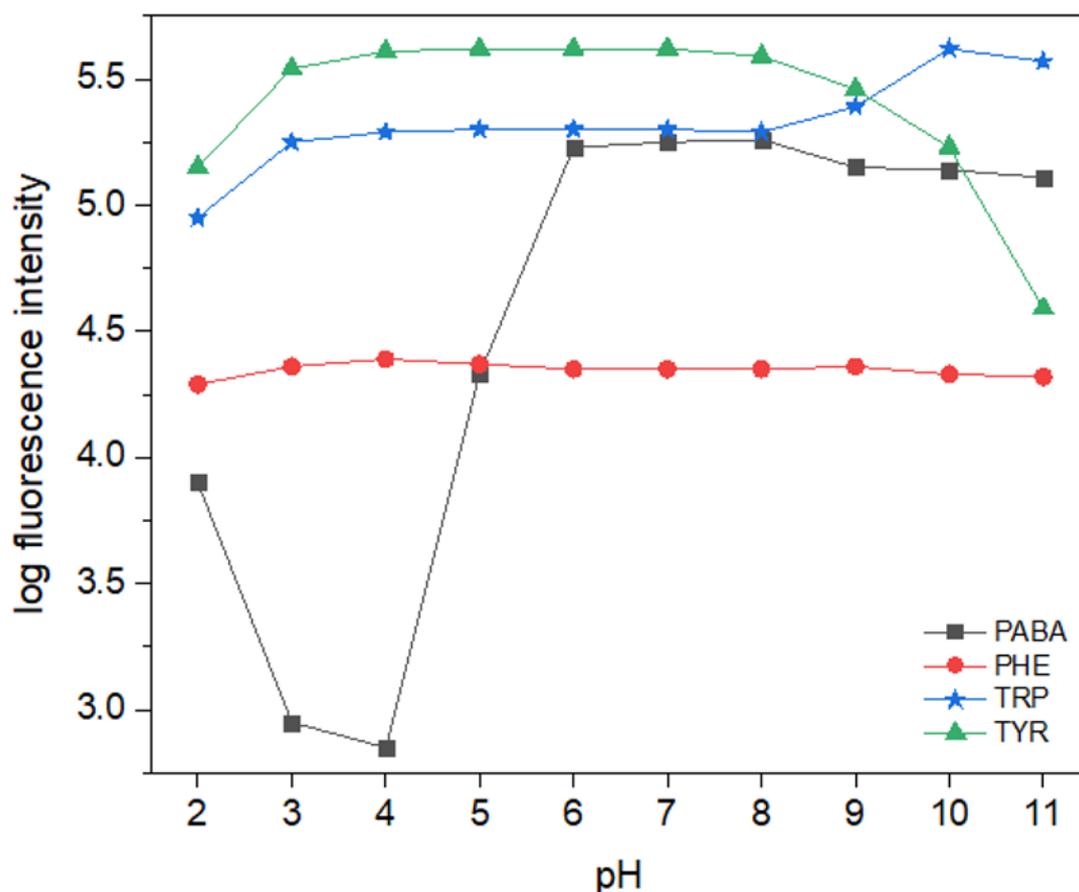
FIGURE 2 - Effect of pH on the emission fluorescence intensity (expressed by the respective log-transformed value) to 4-aminobenzoic acid (PABA); L-phenylalanine (PHE); L-tryptophan (TRP); and L-tyrosine (TYR).

TABLE II - Physical and chemical properties of the compounds determined in this study (Lundblad, MacDonald, 2010)

Compounds	Molar weight (g mol^{-1})	Solubility* (g kg^{-1})	pKa$_1$	pKa$_2$	pKa$_3$	pI
4-aminobenzoic acid	137.14	5.40	2.50	4.79	-	3.69
L-phenylalanine	165.19	27.9	1.83	9.13	-	5.48
L-tryptophan	204.23	13.2	2.83	9.39	-	5.89
L-tyrosine	181.19	0.46	2.20	9.11	10.07	5.66

*in water at 25 °C.

Method validation

Considering the application of these SFMs for routine analyses in physicochemical laboratories, we chose pH 7.2 as the condition for analysis of PABA, PHE, TRP and TYR. Since these raw materials are not analyzed at trace levels, the maximum fluorescence intensity was not the criterion for TRP (Figure 2). However, the most important aspect was the simplification of the procedure for sample preparation, by standardizing the method using phosphate buffer (pH 7.2) for all analytes.

The specificity of the methods was verified by the pairs of maximum $\lambda_{EX}/\lambda_{EM}$ obtained from stock solutions at pH 7.2 of PABA, PHE, TRP and TYR, which were 304/336, 261/283, 288/352 and 275/312 nm, respectively. The spectra of these results are shown in Figure 3. Blank spectra (water and buffer) showed no interference at the maximum λ_{EX} and λ_{EM} values of the analytes.

Since linearity was present within a range of application of the analytical technique, the regression significance was certified by ANOVA (F-test) at 95% confidence. The concentration ranges observed were 0.050-2.5, 10-135, 0.025-5.0 and 0.25-10 mg L^{-1} for PABA, PHE, TRP and TYR, respectively. All the calculated r values were satisfactory (values above 0.990) according to ICH (2022b), for a wide range of concentrations. These data exhibited homoscedasticity and normality, as verified by the Cochran and Shapiro-Wilk tests, respectively, at 95% confidence level (Table III).

Although LOD and LOQ are not required by the ICH guidelines for the test method, the values were calculated to complement the study and to provide more information about SFM. As expected, among the aromatic AAs, the LOD values were inversely proportional to the molar absorptivity coefficient, so that PHE (1.8 mg L^{-1}) > TYR (0.061 mg L^{-1}) > TRP (0.008 mg L^{-1}) (Table III).

Calibration curves were constructed with 5 concentration levels, considering TC as the central point, and the other points as TC ± 20%. Thus, the following values were used: 0.8, 0.9, 1.0, 1.1 and 1.2 mg L^{-1} for PABA and TYR; 1.6, 1.8, 2.0, 2.2 and 2.4 mg L^{-1} for TRP; and 40, 45, 50, 55 and 60 mg L^{-1} for PHE. Of all the analytes, PHE exhibited the widest linearity range (from 10 to 135 mg L^{-1}), so its TC was highest among them (50 mg L^{-1}).

The data exhibited homoscedasticity and linearity as indicated by the r-values, which were acceptable according to ICH (2022b). Using these concentrations, the data dispersion of the figures of merit (precision, accuracy and robustness) were calculated using low, medium and high values, corresponding to 80, 100 and 120% of TC, respectively (Table III).

Considering all developed SFMs, the maximum results, expressed in RSD, of intra- and inter-day precision were 2.1 and 3.9%, respectively (Table IV). These results are consistent with the guidelines described by AOAC (2016), which establishes RSD of less than 11% for a range of analyte concentrations around 1.0 mg L^{-1}.

The accuracy showed good recovery between the true value, obtained from the CRS solutions, and the value found experimentally. The maximum RSD was 2.6%, while the recovery percentages were between

97 and 104% (Table IV), well within the interval recommended by AOAC (2016), which is from 80 to 110%.

The SFM can be considered robust, as the maximum RSD was less than 4.0% with regard to the instrumental and experimental parameters (Table IV).
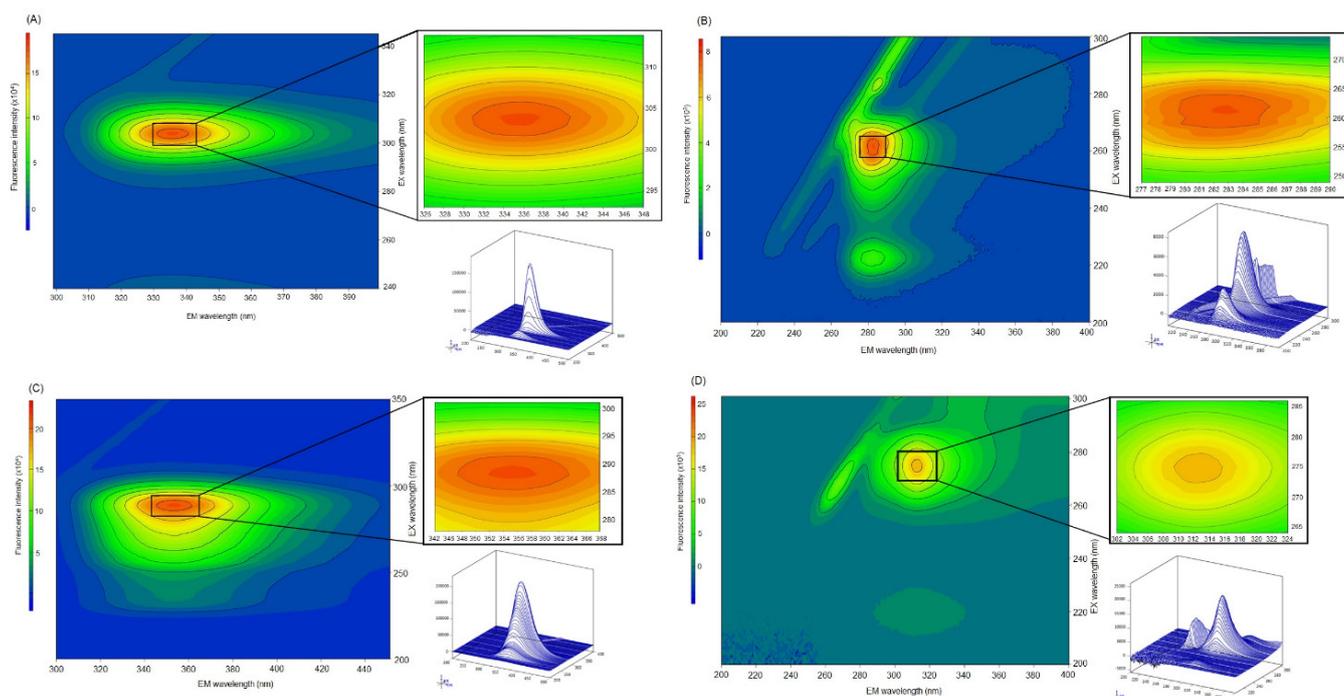
FIGURE 3 - Fluorescence spectra expressed by the fluorescence intensity depending on the emission (x-axis) and excitation (y-axis) wavelengths, in nm, obtained for 4-aminobenzoic acid (PABA) (A); L-phenylalanine (PHE) (B); L-tryptophan (TRP) (C); and L-tyrosine (TYR) (D) solutions of 100 mg L^{-1} (pH 7.2).

TABLE III - Analytical performance data for the determination of 4-aminobenzoic acid (PABA), L-phenylalanine (PHE), L-tryptophan (TRP) and L-tyrosine (TYR) by spectrofluorimetric method

Parameters	PABA	PHE	TRP	TYR
λ_{EX} / λ_{EM} (nm)	304/336	261/283	288/352	275/312
Linearity range (mg L^{-1})	0.050 to 2.5	10 to 135	0.025 to 5.0	0.25 to 10
Correlation coefficient (r)	1.000	0.999	0.999	0.999
Shapiro-Wilk test[a]	0.793 (0.788)[b]	0.937 (0.842)[d]	1.184 (0.818)[c]	0.855 (0.788)[b]
Cochran test[a]	0.242 (0.616)[b]	0.282 (0.445)[d]	0.338 (0.516)[c]	0.277 (0.616)[b]
Regression significance[a] (ANOVA F-test)	5,859 (4.45)[e]	3,853 (4.18)[f]	2,451 (4.28)[g]	2,168 (4.45)[e]
LOD (mg L^{-1})	0.013	1.8	0.008	0.061
LOQ (mg L^{-1})	0.045	5.4	0.025	0.18
Target concentration (mg L^{-1})	1.0	50	2.0	1.0
Calibration curve (mg L^{-1})	0.80 to 1.2	40 to 60	1.6 to 2.4	0.8 to 1.2
Intercept (a)	4.89	-29.9	2.53	-13.1

TABLE III - Analytical performance data for the determination of 4-aminobenzoic acid (PABA), L-phenylalanine (PHE), L-tryptophan (TRP) and L-tyrosine (TYR) by spectrofluorimetric method

Parameters	PABA	PHE	TRP	TYR
Slope (b)	135	8.40	362	209
Correlation coefficient (r)	0.997	0.999	1.000	1.000

[a]Critical values in parentheses (P=0.05); [b]n=6; [c]n=8; [d]n=10; [e]$F_{1,17}$; [f]$F_{1,23}$; [g]$F_{1,29}$.

LOD: limit of detection; LOQ: limit of quantification; λ_{EX}: excitation wavelength; λ_{EM}: emission wavelength.

TABLE IV - Validation parameters (precision, accuracy and robustness) obtained by the spectrofluorimetric methods for 4-aminobenzoic acid (PABA), L-phenylalanine (PHE), L-tryptophan (TRP) and L-tyrosine (TYR)

Parameters	PABA	PHE	TRP	TYR
Precision (%RSD; n=3)				
Repeatability (intra-day)				
80% TC	2.4	3.4	1.7	1.6
100% TC	1.4	3.2	2.1	0.79
120% TC	1.8	1.5	2.5	1.0
Intermediate precision (inter-day)				
80% TC	0.92	1.1	0.07	0.78
100% TC	2.6	0.38	3.6	1.0
120% TC	2.0	1.5	2.4	1.2
Accuracy (%recovery mean±SD; n=3)				
80% TC	96.8 ± 1.6	100.7 ± 3.3	103.2 ± 0.9	99.3 ± 2.4
100% TC	99.8 ± 2.5	100.0 ± 3.0	103.5 ± 1.6	100.4 ± 1.6
120% TC	100.0 ± 2.8	99.5 ± 1.4	102.1 ± 1.0	99.9 ± 2.3
Robustness (%recovery mean±SD; n=9)				
Instrumental (λ_{EM} ± 1 nm)				
80% TC	96.8 ± 1.7	99.8 ± 4.4	102.9 ± 1.1	99.0 ± 4.9
100% TC	99.8 ± 2.1	99.5 ± 4.3	103.1 ± 1.9	99.1 ± 4.9
120% TC	100.0 ± 1.9	99.7 ± 3.6	102.1 ± 1.2	98.6 ± 5.4
Experimental (pH 7.2 ± 0.2)				
80% TC	97.7 ± 3.4	99.2 ± 2.1	102.2 ± 1.2	102.0 ± 2.4
100% TC	101.0 ± 0.4	99.5 ± 0.9	101.6 ± 1.4	101.8 ± 2.2
120% TC	101.1 ± 1.7	97.3 ± 4.8	100.8 ± 2.2	102.6 ± 2.8

Target concentration (TC): 1.0 mg L^{-1} (PABA); 50 mg L^{-1} (PHE); 2.0 mg L^{-1} (TRP); 1.0 mg L^{-1} (TYR). SD: standard deviation; RSD: relative standard deviation

Comparison between potentiometric (PM) and spectrofluorimetric (SFM) methods

The PM methods, described in the European Pharmacopoeia (2020), were selected for comparison with our proposed SFM because they are applied in physicochemical laboratories and are part of the technology transfer between Bio-Manguinhos and its partners. Likewise, Karl Fischer titration and LDRY analyses were employed based on the same reference.

PM has been described by several pharmacopoeias as an official method to be applied in quality control laboratories for the analysis of raw materials. The aromatic AS method has the main disadvantages of high consumption of reagents and thus high cost of analysis, and the large amount of waste generated, since it is performed in a non-aqueous medium (acetic acid).

Although the PABA assay is performed in an aqueous medium, neither method has sufficient selectivity and specificity for its respective analytes. Moreover, the USP describes the PABA assay by liquid chromatography with UV detection, and the SFM may be faster and less complex because it does not require using organic solvents (United States Pharmacopoeia, 2022).

Thus, the SFM is an efficient alternative to those methods by not only overcoming these limitations but also increasing the speed and sustainability of the laboratory quality control routine.

Table V shows the analytical results, expressed as percentages of dried substances, whose values were corrected using the results of Karl Fischer titration (for PABA) and LDRY (for aromatic AA).

In general, LDRY analysis is used to determine volatile compounds or any other compound eliminated under the conditions described in the monograph. Nevertheless, for the assay to define the CRS, the correction of the content was performed using the corresponding data indicated in the manufacturing certificate, which were 99.9% for PABA and 100% for PHE, TRP and TYR. Samples from different manufacturers of PABA, PHE, TRP and TYR were analyzed considering their respective TC. These results were obtained using the SFM developed in this work and the PM according to the European Pharmacopoeia (2020).

Initially, the results were compared using both methods based on the acceptance criteria of the British Pharmacopoeia (2020), European Pharmacopoeia (2020), Japanese Pharmacopoeia (2021) and United States Pharmacopoeia (2022). All test results, expressed on a dry basis, were consistent with the recommended intervals described in the respective monographs, as were the moisture levels and LDRY results (Table V).

Moreover, the comparison of the statistical data between the SFM and the PM tests showed no statistically significant difference, considering a 95% confidence level. The t-test used to compare the means obtained by both methods, we observed that the null hypothesis was accepted for all the analyzed samples ($t_{calculated} < t_{critical}$). Likewise, the F-test indicated that both methods have similar precision ($F_{calculated} < F_{critical}$). The tests to compare the means between the SFM and CRS also showed statistical equivalence (Table VI).

In terms of reagent consumption, the SFM also was better than the PM. For example, in the routine analysis of a batch of TRP, performed in triplicate (blank and sample) and using the PM, 300 mL of acetic acid and 18 mL of formic acid were consumed, not considering the volume of titrant solution (Table I). Consequently, the occurrence of residues cannot be excluded, which reinforces the need to develop a method that uses a more sustainable analytical tool.

However, considering the previous example of SFM, whose analyses are performed in aqueous media using diluted solutions and compounds with low toxicity (phosphate buffer, HCl and NaOH), we can conclude that these methods are more feasible than the PM, because their results are statistically similar while they are faster, more selective and have lower analytical cost and reagent consumption.

TABLE V - Percentage results (mean ± standard deviation; n=3), on dry basis, obtained from the raw material samples and chemical reference standards (CRS) of 4-aminobenzoic acid (PABA), L-phenylalanine (PHE), L-tryptophan (TRP) and L-tyrosine (TYR) by the potentiometric (compendial) and spectrofluorimetric (this study) methods

Samples and standards	Water and volatile compounds[a] (%)		Assay (% on dry basis)		Acceptance criteria[a,b] (%)
	(mean ± SD)	Acceptance criteria[a]	Spectrofluorimetric method (mean ± SD)	Potentiometric method[a] (mean ± SD)	
PABA-Sample 1	0.19 ± 0.006[c]		99.9 ± 0.2	98.7 ± 0.7	BP and Ph. Eur.: 99.0–101.0
PABA-Sample 2	0.19 ± 0.006[c]	≤0.2%	100.1 ± 1.9	99.7 ± 0.8	USP: 98.0–102.0
PABA-Sample 3	0.18 ± 0.008[c]		100.4 ± 0.1	99.0 ± 0.1	
PABA-CRS	-		100.6 ± 0.6	-	
PHE-Sample 1	0.12 ± 0.006[d]		100.0 ± 1.2	100.3 ± 0.6	BP and Ph. Eur.: 98.5–101.0
PHE-Sample 2	0.03 ± 0.000[d]		100.1 ± 0.8	99.3 ± 1.1	JP: ≥98.5
PHE-Sample 3	0.39 ± 0.006[d]	≤0.5%	100.1 ± 0.9	99.7 ± 0.6	USP: 98.5–101.5
PHE-Sample 4	0.01 ± 0.000[d]		100.0 ± 0.6	99.9 ± 0.8	
PHE-CRS	-		99.4 ± 0.4	-	
TRP-Sample 1	0.37 ± 0.001[d]		100.0 ± 0.8	99.9 ± 0.3	BP: 98.5–101.0
TRP-Sample 2	0.38 ± 0.006[d]	≤0.5%	99.9 ± 1.2	100.1 ± 0.9	JP and Ph. Eur.: 99.0–101.0
TRP-Sample 3	0.00 ± 0.000[d]		100.3 ± 0.6	100.1 ± 0.8	
TRP-CRS	-		100.0 ± 0.8	-	
TYR-Sample 1	0.27 ± 0.006[d]		99.9 ± 1.2	99.3 ± 0.2	BP: 99.0 – 101.0
TYR-Sample 2	0.00 ± 0.010[d]		99.8 ± 0.4	99.6 ± 0.6	Ph. Eur.: 98.5–101.0
TYR-Sample 3	0.20 ± 0.008[d]	≤0.5%	99.5 ± 0.4	99.3 ± 0.2	JP: ≥98.5
TYR-CRS	-		100.4 ± 0.7	-	USP: 98.5–101.5

SD: standard deviation; [a] European Pharmacopoeia, 2020 (Ph. Eur.); [b] British Pharmacopoeia, 2020 (BP), Japanese Pharmacopoeia, 2021 (JP) and United States Pharmacopoeia, 2022 (USP); [c] water determined by Karl Fischer titration; [d] loss on drying (at 105 ± 2 °C).

TABLE VI - Statistical evaluation of the results of PABA, PHE, TRP and TYR assays obtained by the comparison between the same sample by the spectrofluorimetric (SFM) and potentiometric (PM) methods, and between standards (CRS) and samples by the SFM

Samples	Comparison between samples by the SFM and PM		Comparison between sample and CRS by SFM	
	t-test[a]	F-test[b]	t-test[a]	F-test[b]
PABA-Sample 1	2.4	1.3	2.0	12
PABA-Sample 2	1.2	1.5	0.29	1.4

Thallis Martins Souza, Robert da Silva Angelino, Larissa Manhães Ornella, Eduardo dos Santos Ribeiro, Simone Ferreira Rodrigues Fernandes

TABLE VI - Statistical evaluation of the results of PABA, PHE, TRP and TYR assays obtained by the comparison between the same sample by the spectrofluorimetric (SFM) and potentiometric (PM) methods, and between standards (CRS) and samples by the SFM

Samples	Comparison between samples by the SFM and PM		Comparison between sample and CRS by SFM	
	t-test[a]	F-test[b]	t-test[a]	F-test[b]
PABA-Sample 3	1.5	5.7	1.5	17
PHE-Sample 1	0.33	4.5	1.0	11
PHE-Sample 2	1.0	2.1	2.3	5.1
PHE-Sample 3	0.87	2.3	1.7	6.4
PHE-Sample 4	0.29	1.7	2.6	3.1
TRP-Sample 1	0.02	13	0.46	2.0
TRP-Sample 2	0.29	1.8	0.45	2.1
TRP-Sample 3	0.57	2.2	0.29	2.0
TYR-Sample 1	0.80	1.8	2.0	1.1
TYR-Sample 2	0.97	3.3	2.2	1.8
TYR-Sample 3	2.2	3.3	2.3	3.8

Critical values (P=0.05): [a]2.78 (n=6; degrees of freedom = 4); [b]19.0 ($F_{2,2}$).

CONCLUSION

Rapid, selective, sensitive, robust, and sustainable analytical methods based on spectrofluorimetric were satisfactory developed to determinate PABA, PHE, TRP and TYR, used as raw materials in the BP formulation. The figures of merit, determined in the validation step, can be efficient alternatives to the compendial methods, which are based in potentiometric titration in acetic acid medium (non-aqueous). Moreover, the assay results showed no statistical differences in comparison with the results obtained by the methods described in different pharmacopoeias.

The methods described here are more efficient and faster, and also less expensive due to the lower cost of and consumption of reagents. Consequently, there will be less waste in the routine analyses of quality control laboratories.

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