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Optimization and intralaboratorial validation of an analytical method by HPLC/UV for identification and quantification of p-phenylenediamine in henna dye hair and eyebrows

Otimização e validação intralaboratorial de método analítico por CLAE/UV para identificação e quantificação de p-fenilenodiamina em tinturas de hena para cabelos e sobrancelhas

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ABSTRACT

Introduction: p-Phenylenediamine (PPD), aromatic sensitizing amine, has been added to henna powder to modify its natural color to black, increasing its fixation time, a practice that is prohibited in eyelash and eyebrows dyes. Objective: The objective of this study was to optimize and validate, at an intra-laboratory level, an analytical method by HPLC/UV for identification and quantification of PPD in henna dyes for hair and eyebrows. Method: In the method, C8 reverse phase column, mobile phase 1% triethanolamine (pH 8.4) and acetonitrile (99: 1, v/v), detection 280nm, injection volume: 10uL, flow 1.0 mL/min, column temperature 32°C, run time 10 min, linearity 5-45 µg/mL (n = 5) with correlation coefficient 0.9982, were used. For Cochran test (homoscedasticity): 0.3350 and critical (0.3934) with 99% confidence. Limits of detection 1.17 µg/mL and quantification 3.54 µg/mL. The coefficient of variation of repeatability was 0.12% and the intermediate precision by F-test yielded p value of 0.283 with 95% of confidence. Accuracy results comprised acceptance criteria of 90%-107%. Results: Of the 19 analyzed samples, 14 presented PPD content between 1.74 and 3.65% w/w, in disagreement with Legislation. Conclusions: The proposed method can contribute to monitoring of quality and safety of use of these products.

KEYWORDS: p-fenilenodiamina; Henna; Method Validation

RESUMO

Introdução: O p-fenilenodiamina (PPD), amina aromática sensibilizante, vem sendo adicionado ao pó de hena para modificar sua cor natural para preta, aumentando seu tempo de fixação, prática proibida em tinturas para cílios e sobrancelhas. Objetivo: O objetivo deste estudo foi otimizar e validar, em níveis intralaboratoriais, um método analítico por CLAE/UV para identificação e quantificação de PPD em tinturas de hena para cabelos e sobrancelhas. Método: Foi utilizada coluna em fase reversa C8, fase móvel trietanolamina 1% (pH 8,4) e acetonitrila (99:1, v/v), detecção a 280 nm, volume de injeção de 10 µL, fluxo 1,0 mL/min, temperatura da coluna 32°C, tempo de corrida 10 min, linearidade 5-45 µg/mL (n = 5) com coeficiente de correlação de 0,9982. Para o teste de Cochran (homocedasticidade), 0,3350 e o $C_{crítico}$ (0,3934), com 99% de confiança. Limites de detecção, 1,17 µg/mL e quantificação, 3,54 µg/mL. O coeficiente de variação da repetibilidade 0,12% e na precisão intermediária pelo teste F obteve-se p-valor de 0,283 com 95% de confiança. Resultados: Os resultados da exatidão compreenderam os critérios de aceitação (90%-107%). Das 19 amostras analisadas, 14 apresentaram teor de PPD entre 1,74% a 3,65% p/p, em desacordo com a Legislação. Conclusões: O método proposto poderá contribuir com o monitoramento da qualidade e segurança de uso destes produtos.

PALAVRAS-CHAVE: PPD; Hena; Validação de Método

INTRODUCTION

P-Phenylenediamine (PPD) is an aromatic amine, considered by the European Union Scientific Committee on Consumer Safety (CCSC) to be one of the five chemical compounds identified as potent sensitizers[1]. It was synthesized in Germany by Hofmann in 1863 in order to create a substance with antioxidant and dyeing properties[2]. It is a prohapten and its intraepidermal oxidation produces benzoquinone, which is the substance responsible for contact allergy[3].

The use of these pigments in hair dyes began in 1883, and from that date on, there has been an increase in hair dyeing by both women and men[3]. In Brazil, according to data from the National Institute of Metrology, Quality and Technology (Inmetro), 26% of the adult population uses hair dyes: 85% of the users are women and 15% are men.

In the United Arab Emirates (UAE), the use of henna is part of their tradition and culture. Women of all ages use henna on their skin as adornment. This is considered an essential part of wedding ceremonies and other celebrations[4]. A 2010 study in this country has shown that henna samples obtained from salons contained high concentrations of PPD. People involved in the study developed contact dermatitis because of its use, leading researchers to further recommend that the addition of PPD to natural henna be prohibited in the UAE[4].

The natural henna powder is reddish-brown in color and sensitive to light[5]. Its use is relatively safe due to its low allergenic potential. There are still few reports in the literature about the emergence of allergic reactions, although there are in vitro and in vivo studies on its potential genotoxicity/mutagenicity[6,7,8].

The addition of PPD to henna powder is intended to change the reddish-brown coloration of its pigments to black or ebony, increasing tattoo time by up to 6 weeks, although this practice is prohibited in eyelash and eyebrow dyes, as ruled by RDC n. 03 of January 20, 2012[9]. This Resolution only sets the maximum limit of up to 6% PPD in hair dye formulations.

PPD used in dyes has molecular characteristics that provide satisfactory aesthetic results. However, because it is easily absorbed by the skin, it can cause sensitization and lead to allergic contact dermatitis[10]. In studies conducted with people who underwent contact tests with PPD, the percentage of allergic dermatitis obtained was about 4%. The literature reports adverse reactions related to the exposure to products containing PPD in hair dyes and henna tattoos[3].

High concentrations of PPD and other components of tattoo dyes, when in contact with one's skin during the tattooing process, may also induce an intense immune response that favors concomitant sensitization[7]. PPD can cause systemic lesions and induce immediate hypersensitivity, causing skin rash, angioedema, and respiratory distress. It may also have delayed hypersensitivity mechanisms, with reactions that sometimes appear years after the tattoo[11].

Eyebrow dye products are becoming increasingly popular, hence allergic contact dermatitis in this area has become an emerging problem over the last decade. PPD and its derivatives are the most common allergens in eyelashes and eyebrow dyes[12]. The literature also reports adverse reactions related to the exposure to products containing PPD in hair dyes and henna tattoos[13,4].

Because of the increasing incidence of cases of allergies to the product, several analytical procedures have been designed to separate, identify and quantify intermediate amines and PPD in hair and skin dyes. The literature reports techniques of High Performance Liquid Chromatography (HPLC) combined with several detectors: HPLC/ultraviolet (UV), HPLC/mass spectrometry (MS), HPLC/photodiode array (DAD)[14]. In addition to methods like HPLC/DAD combined with ion chromatography[15], gas chromatography/mass spectrometry (GC/MS)[16]. Some of these techniques involve complex processes for the extraction and chemical derivatization of hair dye pigments. These are also costly and time-consuming techniques.

In this scenario, the objective of the present study was to optimize and validate, at intra-laboratory level, an HPLC/UV analytical method for identification and quantification of PPD in henna dyes for hair and eyebrows.

The method was applied to the dosing of products acquired in the market, by the Health Surveillance and by the Forensic Institute of São Paulo.

METHOD

Sigma-Aldrich p-phenylenediamine standard, batch #WXBC1642V, 99.4% pure. Acetonitrile and methanol chromatographic grade (Merck). Triethanolamine, PA phosphoric acid (Merck) and analytical grade sodium sulfite (Vetec), Certified Reference Material 4.0, 7.0 and 10.0 buffer solutions (Digimed) and ultrapure water. PVDF HV membranes with 0.45 µm pores and 0.45 µm pore filter units (both Durapore, Millipore®). The chromatographic columns we used were reverse phase and capped, Purospher Star® RP8 (Merck) column with 5 µm, 125 x 4 mm long and internal diameter particles, Agilent Poroshell HPH-C18 and X-Terra C18 column of 5 µm, with 250 x 4 mm, by Waters. We used calibrated glassware consisting of amber volumetric flasks, volumetric pipettes, amber vials and Falcon tubes. For the henna matrix, without the analyte, a colorless natural henna sample without the addition of PPD was purchased in the market.

The equipment we used was: Mettler Toledo MT5 analytical balance, Metrohm pH meter, Quimis® refrigerated centrifuge, Unique Ultrasonic Cleaner ultrasound, Purilab Classic (Elga) system, Waters Alliance model 2695 (Mildford, MA, USA) liquid chromatograph with degasser, column oven, quaternary pump, diode array detector controlled by the Empower software.

Of the 19 samples of henna powder analyzed in this work, seven were randomly acquired in regular stores in the city of São Paulo, Brazil, two were submitted by the Brazilian National Health Surveillance Agency (Anvisa) and ten by the Forensic Institute of São Paulo. The samples were henna dyes for eyebrows and hair from several brands, coming from four manufacturers, of different shades and batches.After several analytical tests, the final condition of the method was obtained: C8 reverse phase chromatographic column, 1% triethanolamine mobile phase in water (pH adjusted to 8.4 with phosphoric acid) and acetonitrile (99: 1, v/v), detection at 280 nm, injection volume of 10 µL, 1 mL/min flow, column temperature at 32°C, run time of 10 min. The PPD standard was prepared with 0.1% (w/v) aqueous Na_2SO_3 solution at a concentration of 30 µg/mL.

Once the method optimization was completed, it was subjected to the system suitability test as set forth in RDC n. 166 of July 24, 2017[17]. The chromatographic parameters of tail factor, resolution, system efficiency (number of theoretical plates) and standard deviation were evaluated.

We ran some tests to check the stability of the PPD standard solution with the optimized method, at three different concentrations, stored in amber glass vials and in a refrigerator. Next, we checked peak purity in the standard PPD solution through the diode array detector in the wavelength range of 210 to 295 nm, and in the matrix (henna without PPD analyte)[18].

Henna samples were processed so that 200 mg were solubilized with 10 mL of 0.1% w/v sodium sulfite solution in falcon tubes, manually shaken for 1 min, centrifuged at 4,000 rpm for 30 min and filtered on filter units for amber vials.

The validation of the method was done according to RDC n. 166/2017[17] and as established by the DOQ-CGCRE-008 document of Inmetro[19]. The parameters we evaluated were: selectivity, linearity, precision (intermediate and repeatability), accuracy (recovery), detection limit, quantification limit.

Selectivity: this was determined based on the henna solution (matrix without PPD analyte). Two solution groups were prepared: group 1 and group 2. Both with 6 independent replicates, and the addition of 1 concentration level of 40 µg/mL of PPD.

- Group 1: assay blank + addition of analyte standard.
- Group 2: matrix without analyte (blank sample) + addition of analyte standard.

Calibration analytical curve: prepared with five PPD concentrations: 5, 15, 25, 30 and 45 µg/mL in independent triplicates, divided into two groups.

- Group 1: blank (0.1% sodium sulfite solution) plus the addition of the PPD standard.
- Group 2: matrix without analyte (henna sample without PPD) plus the addition of the PPD standard.

We used the paired sample T test for the statistical treatment of the curve. The limits of detection and quantification were obtained by the analytical curve.

Precision: assessed for repeatability and intermediate accuracy. Repeatability was performed with the same measurement procedure, the same analyst, the same instrument and in the same environment conditions. The replicates were independent with nine 35 µg/mL determinations of a sample (A) containing the PPD analyte and the coefficient of variation (CV%) was calculated.

Intermediate precision was performed on the same sample, with the same analytical conditions but varied analysts and days. The parameter was evaluated using the F test and analysis of variance.

Accuracy: performed in terms of recovery by the standard analyte-free matrix addition method, with three concentration levels: 15, 30 and 45 µg/mL (low, medium and high) of the PPD standard in triplicate.

After the method was validated, it was applied to identify and quantify PPD in 19 samples of henna for various purposes and by different brands.

RESULTS AND DISCUSSION

The chromatographic conditions described in the method of Fu and Lei[14] and Almeida et al.[6] have shown retention time of PPD shorter than 2 min and tail factor greater than 2. This retention time reveals little interaction between the analyte and the active sites of the column because of deviations from the normal dispersion of the analyte at the stationary phase. As a result, the low effectiveness of the column impacted the tail factor. The optimization of the method involved several steps: use of C18 columns from several manufacturers and C8 column; pH change from 7.7 to 8.4 to minimize analyte ionization; mobile phase tests of different polarities, until we obtained satisfactory results that met the suitability of the system.

The diluent described in the method of Fu and Lei[14] and Almeida et al.[6] was replaced with the 0.1% sodium sulfite solution, which achieved excellent results in PPD solubility in both standard and PPD-containing henna samples evaluated in the recovery test of samples with and without PPD. The matrix - colorless natural henna - was evaluated at the same concentration of the sample solutions and did not peak at the same retention time as the standard solution.

After optimization of the method, the results of the stability studies of the PPD standard solution demonstrated that the stability was not longer than 8 hours under the established conditions.

The results of the suitability test of the optimized method chromatographic system were: 1.26 tail factor, 4375.4 number of theoretical plates and standard deviation of 1.28%. This way, the

optimized method fulfilled the parameters established in the suitability of the system.

The method selectivity was verified by the analysis of the standard solution and the samples by molecular absorption in the UV region with the aid of DAD. For peak purity verification, the scanning spectra in the UV region (240–400 nm) of the asset signal were extracted at the respective retention times in the chromatograms of the standard and of the analyzed samples, as shown in Figure 1. We used the Empower software for both standard solution and sample and obtained values of purity angle lower than the purity threshold, thus confirming the purity of the peaks. We determined the similarity between the spectra of 0.99 and the selectivity was considered adequate.

Figure 2 shows chromatograms A, B and C done in the optimized and validated method. Chromatogram A corresponds to the development of a PPD-free henna (matrix) sample and, in the time of 2.65 min, the presence of a possible matrix interferer in the PPD determination in the chromatographic system is not observed. Chromatogram B corresponds to the development of thePPD standard solution, in which the retention time of 2.65 min shows good interaction between the active sites of the column and the PPD analyte. Chromatogram C corresponds to the henna sample added with PPD solution. Thereby it is shown that the method is suitable for the intended analytical purposes.

The literature reports various procedures for clean-up of vegetables, like the use of Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) mixture of salts. Purification tests of henna samples were run with PPD. With these salts, the results presented were unsatisfactory due to the adsorption of PPD pigments by the active carbon, one of the components of QuEChERS[20].

The validation of the method started with the selectivity test to ensure that the henna components, as well as other pigments present in the formulation, did not interfere with PPD identification and quantification. The results obtained in the comparison of groups 1 and 2, through the Grubbs statistical test with 99% confidence, have not shown dispersed values. F and T tests with 95% confidence have shown heterogeneous variances and equivalent means, respectively, thus demonstrating that there is no interferenceof the henna matrix.

The method presented linearity in the range of 5 to 45 µg/mL (n = 5) with correlation coefficient (r) equal to 0.9982 and line equation y = 4350.2x + 2077.5. The statistical treatment of the calibration curve done through the Cochran test has shown results for homoscedasticity of 0.3350 and the $C_{critical}$ (0.3934) with 99% confidence. We concluded that the variances are homogeneous.

Thelimit of detection (LoD) of 1.17 µg/mL and the limit of quantification (LoQ) of 3.54 µg/mL were obtained by statistical treatment of the analytical curve. It was based on the residual standard deviation of the analytical curve (Sx/y) and the slope of the analytical curve. The LoQ value equivalent to the lowest concentration level of the curve was determined with acceptable precision and accuracy.

The method precision results were obtained through the coefficient of variation (CV%), which was less than 1% (0.12%). This was calculated by the standard deviation ratio with the mean of the obtained values, showing that the method is accurate.

The intermediate precision results evaluated by the F test for six determinations were equivalent, p-value = 0.283 with 95% confidence.

Accuracy test results were obtained by the recovery assay of samples fortified with PPD standard at three concentration

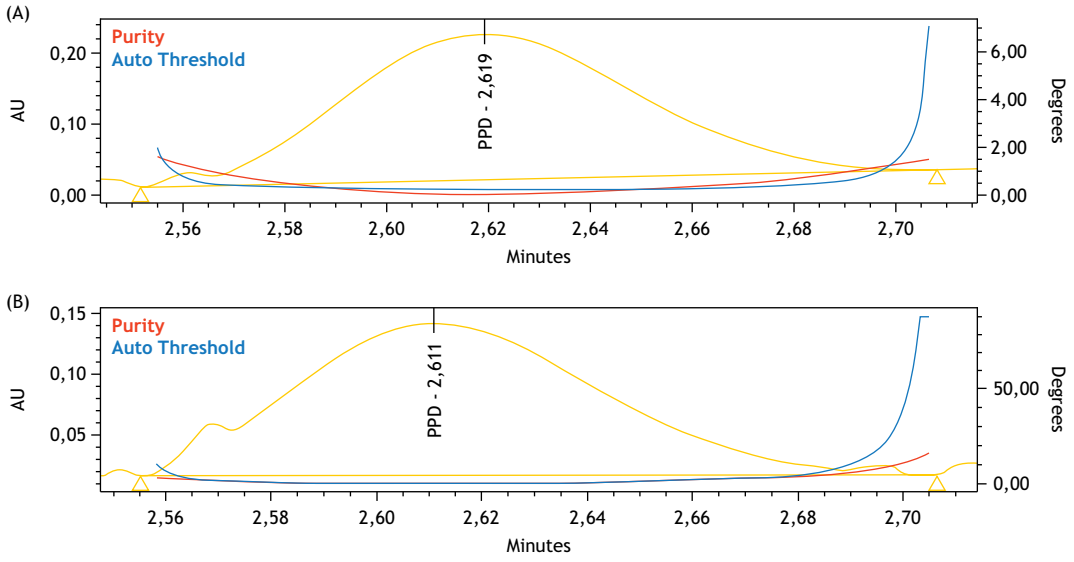

Figure 1. Peak purity chromatograms A and B done in the optimized and validated method represent PPD standard and sample solutions, respectively.

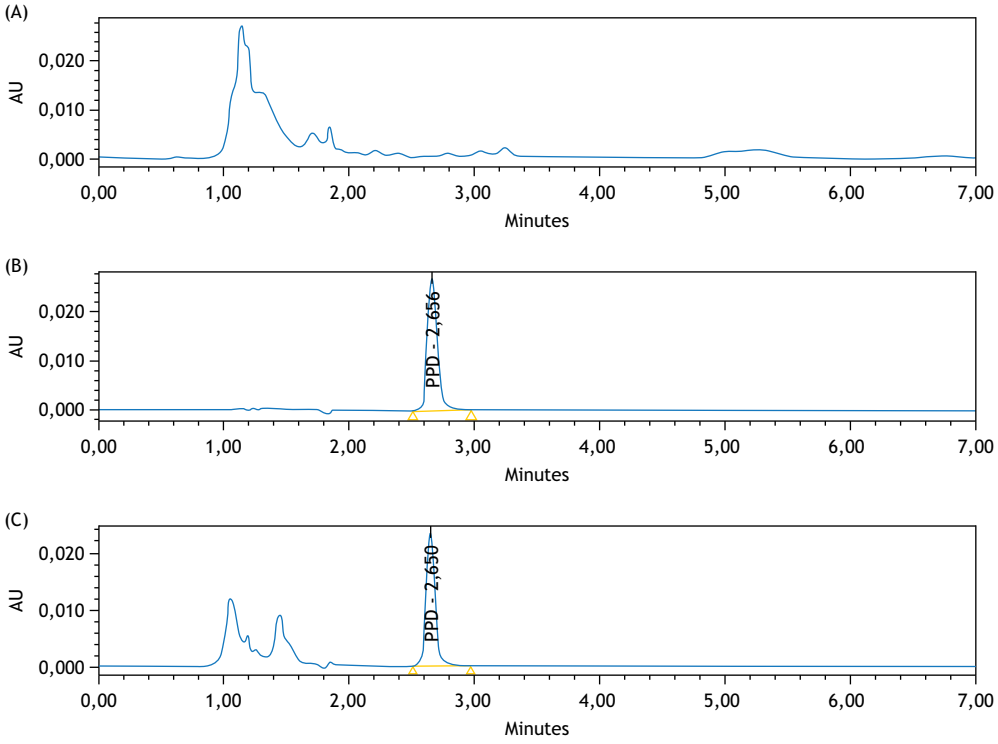

Figure 2. Chromatograms A, B and C, done in the optimized and validated method, represent henna solutions (matrix) without addition of PPD; PPD standard and added sample of the PPD standard.

levels as shown in Table 1. They met the acceptance criteria of 90% to 107%, established by Inmetro DOQ-CGCRE-008, as it is a topical cosmetic product, considering that the legislation allows the use of PPD in hair dyes but has banned its use for eyelash and eyebrow dyes[19].

The results of the validation parameters proved appropriate for use in the qualitative and quantitative determination of PPD in hair and eyebrow henna products, as shown in Table 2.

According to the results shown in Table 2, 14 analyzed samples composed of eyebrow henna have shown values of PPD content between 1.74% and 3.65% w/w. Therefore they are in disagreement with RDC n. 03/2012[6], which prohibits the use of PPD in henna dyes for eyebrows and does not regulate its use in henna dyes for hair. Of the analyzed samples, one declared the presence of PPD in its formulation on the label, but it did not indicate its content, which shows the manufacturer's non-compliance with the legislation.

Table 1. Accuracy of the chromatographic method used in the PPD analysis.

Accuracy			
n (triplicate)	3	3	3
Theoretical concentration µg/mL	15.0	30.0	45.0
Concentration obtained µg/mL	14.9	27.5	40.5
% Recovery	99.8	91.8	90.1
Coefficient of variation (%)	2.4	2.4	1.2

In the henna samples we analyzed (Table 2), we found no higher concentration of PPD in any different shades of henna dyes. That is contrary to the literature reported by researchers who

Table 2. Determination of PPD in henna samples.

Henna samples	Indication of use	Color	PPD content (% w/w)
A	Eyebrows (hair and skin)	Light brown	2.95
B	Eyebrows (hair and skin)	Light brown	3.28
C	Eyebrows (hair and skin)	Light brown	3.23
D	Eyebrows (hair and skin)	Brown	1.80
E	Eyebrows (hair and skin)	Blond	2.52
F	Eyebrows (hair and skin)	Dark brown	2.96
G	Eyebrows (hair and skin)	Medium brown	2.96
H	Eyebrows (hair and skin)	Dark blond	2.93
I	Eyebrows (hair and skin)	Brown	1.80
J	Eyebrows (hair and skin)	Medium brown	3.39
K	Eyebrows (hair and skin)	Light brown	2.96
L	Eyebrows (hair and skin)	Brown	1.80
M	Eyebrows (hair and skin)	Brown	1.74
N	Eyebrows (hair and skin)	Black	3.65
O	Eyebrows (hair and skin)	Burgundy	0
P	Hair	Black	0
Q	Hair	Grayish	0
R	Hair	Natural	0

PPD: p-phenylenediamine.

found high concentrations of PPD in black henna[21]. As for the samples of hair dye henna we analyzed, no PPD was found in their formulation.

CONCLUSIONS

The optimized and validated method is simple, fast and sensitive to identify and quantify PPD by HPLC/UV. Because this technique is versatile, it enabled us to change the chromatographic conditions to obtain a method that met the requirements established in the suitability of the system, showing the efficacy of its application to determine PPD in hair and eyebrow henna samples. The results of the samples we analyzed suggest the need to establish monitoring programs for these products with the Health Surveillance, since they may contain PPD, known as a potent allergen and related to the onset of allergic dermatitis.

Considering that the role of Health Surveillance is to reduce the exposure of the population to non-compliant products that may damage or harm health, future results obtained from monitoring programs will subsidize the marketing of these products and even the improvement of health-related legislation.

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.