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Determination of Para-Phenylenediamine (PPD) in Henna Samples Collected from Libyan Local Markets Using HPLC

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Abstract: Henna is frequently used as body adornment in Libya, the Arabic world, and some African and Asian countries. It is part of the traditions and cultures. The addition of para-phenylenediamine (PPD) to the natural henna increases the risk of allergic contact dermatitis as well as toxic effects, which sometimes lead to severe toxicity and death. Cases have already been reported in Al-Bayda city - Libya. This study aimed to identify the presence and determination of PPD in henna by High-Performance Liquid Chromatography (HPLC). In the current study, eleven henna samples were collected from local markets in Al-Bayda - Libya. A rapid, simple, and reliable method is developed and validated for the determination of PPD in henna samples using 50% methanol solution as solvent. The method is validated over a wide linear range of 5 – 25 µg/ml with correlation coefficients being consistently greater than 0.997. The minimum PPD level was observed in a random sample of mixed henna (paste2) (0.0104 % w/w), while the Royal Black Henna sample showed the highest PPD content (11.9107 % w/w). The HPLC measurements indicated that the results of PPD concentrations in the Royal Red henna sample and Shikha henna (natural henna) were PPD free (ND) (Not Detected). The concentrations in Black henna samples were higher than Red henna samples. The PPD content in red henna samples analyzed in this study is below the allowable limits set by the Scientific Committee on Consumer Products (SCCP) and the US Food and Drugs Administration. While in Black henna samples it was higher than that recommended limit.

Keywords: Black Henna; Red Henna; PPD; Methanol; HPLC

INTRODUCTION

Henna or Hina *Lawsonia inermis* .L, family Lythraceae is a flowering plant or shrub native to tropical and subtropical regions of Africa and Southern Asia. Henna is commercially cultivated in Morocco, Sudan, India, Pakistan, Yemen, and other countries. Henna body art is done by putting henna paste on the skin. The henna paste is made by drying the henna leaves and crushing them to powder, and then this powder is combined with oil or water to form the paste. The henna paste is applied to the skin, the dye called lawsone present in henna leaves extract

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would migrate from the paste to the skin outermost layer, and when the paste is kept on the skin for longer, more lawsone migrates, resulting in a red-brown stain (Robert, 2002). Since the bronze era in the eastern Mediterranean, henna has been used to beautify women's bodies during wedding celebrations and other social gatherings. Henna is used for skin adornment and hair coloring during social events in the Arab world and the Indian subcontinent, particularly during wedding celebrations, people adorn the bride and sometimes groom with henna (Doumas and Doumas, 1992). Paraphenylenediamine (PPD) is a chemical substance that is widely used as a permanent hair dye. It is an organic compound with a chemical formula of $C_6H_4(NH_2)_2$. This derivative of aniline is a white solid, but samples can darken due to air oxidation (Puri and Puri, 2013). It is mainly used as a component of engineering polymers and composites, and also an ingredient in hair dyes as well as used occasionally as a substitute for henna (Puri and Puri, 2013; HSDB, 1993). Paraphenylenediamine (PPD) is a monocyclic arylamine compound. It is a white to light purple powder that oxidizes, turning first to red, then brown, and finally to black on exposure to air (HSDB, 1993). In addition to hair dyes and henna, PPD may also be found in textile or fur dyes, photographic developing agents, dark coloured cosmetics, temporary tattoos, photocopying and printing inks, black rubber, oils, greases, gasoline, and as an antioxidant in rubber compounds (Puri and Puri, 2013; HSDB, 1993). Its use is being supplanted by other aniline analogues and derivatives such as 2,5-diamino hydroxyethylbenzene and 2,5-diaminotoluene. Other popular derivatives include tetraaminopyrimidine, indoanilines, and indophenols. Derivatives of diaminopyrazole give red and violet colours (Thomas et al., 2007). Individuals may be occupationally exposed to PPD during its manufacture or use, and the exposure may occur through inhalation, skin and/or eye contact, and ingestion (SCCP, 2006). In the case of short-term exposure to high amounts of PPD (acute toxicity), the symptoms include severe dermatitis, eye irritation with tearing, asthma, gastritis, renal failure, vertigo, tremors, convulsions, coma, and death. On the other hand, long-term exposure (chronic effect) might lead to eczematous contact dermatitis (Kind et al., 2012; Jacob et al., 2008; Lepoittevin and LeCoz, 2007).

Currently, PPD is added to henna and more than 1000 hair dye formulations marketed all over the world (Stanley et al., 2005). Epidemiologic studies demonstrated that workers in the textile dye and rubber industries, hair dye users, and barbers incurred a high risk of bladder cancer, non-Hodgkin's lymphoma, multiple myeloma, and hematopoietic cancers (Thun et al., 2005). Carcinogens usually cause genomic damage to expose cells which may either undergo apoptosis or proliferation with genomic damage and potentially lead to transformation in cancerous cells (Steller, 1995). Some analytical methods are developed for the determination of PPD in henna, hair dyes, and other products by HPLC (Elmanfe et al., 2019; Al-Suwaidi and Ahmed, 2010; Ursula et al., 2002; Yoshiaki and Masa-aki, 2000), GC/MS (Stambouli et al., 2014; Di Gioiaa et al., 2005), voltammetric method (Inzhang et al., 2011), emission spectroscopy (Kessarin et al., 2012) and some spectrophotometric methods are reported.

The spectrophotometric methods have their relative merits. But the methods are carried out with time-consuming diazotization followed by coupling with N-(1-naphthyl) ethylenediamine (Nitin et al., 2010), which involves oxidation of the compound converted into salt measured colorimetrically (Hilton, 1960), and coupling of triclosan with reagent 2-aminonaphthalene-4,8-disulfonic acid with low-level detection (Olexander and Natalia, 2010). Another method is based on the reaction of sodium nitrite with p-

sulfanilic acid in an acidic medium to form a diazonium ion. Triclosan then further formed an azo compound in an alkaline medium (Huihui et al., 2009). Determination of triclosan in antiperspirant gels by first-order derivative spectrophotometry was also developed (Du et al., 2011).

The present study was aimed to develop a simple, sensitive, rapid, reproducible, precise, and accurate spectrophotometric method and HPLC techniques for the analysis of PPD. This method was based on other methods recommended by other researchers with some modifications, in order to isolate and determine PPD in hair dyes and henna (Elmanfe et al., 2019; Latha et al., 2014; Al-Suwaidi and Ahmed, 2010). The method used is more rapid and simple when compared with other methods (Latha et al., 2014; Al-Suwaidi and Ahmed, 2010). The objectives of this study were to detect the presence and concentration of PPD in henna available in the Libyan market because of the increased risk of toxicity caused by this ingredient, and several death cases have been reported in the past few years in Libya, especially in El-Bieda city.

MATERIALS AND METHODS

Chemicals and reagents: All chemicals, analytical standards, reagents, and solvents used throughout this study were analytical grade and highly pure. PPD (Para-Phenylenediamine) was purchased from (India-ResearchLab) with a purity of 97 % (for research and development). Also, other chemicals and solvents were used, including Methanol (Riedel-Dehaen AG Seelze Hannover) with 99.9 % purity (for HPLC) as a solvent, Ammonia (BDH-Laboratory), and Acetic acid (Riedel-Dehaen AG Seelze Hannover) with 99.8 % purity.

Chemicals : Standard PPD solution : 0.01 g in 100 ml (0.10 mg ml^{-1}) solution was prepared. Working standards were prepared by appropriate dilution of the stock. (5, 10, 15, 20, and 25 µg ml^{-1}). Aqueous methanol solution. 50 %. Acetic acid solution 0.05 M: 2.88 ml of acetic acid in 1000 ml distilled water and adjusted to a pH of 5.9 with ammonia. This solution was used for HPLC as the aqueous mobile phase.

Instrumentation: The HPLC system (Thermo Series 2000 Pump) Autosampler, Series 200 UV/Vis Detector (from 190 to 1000 nm, The Series 200 Autosampler, Series 2000 Analytical Pump, Series 200 Column Oven, and 20 µl loop injector. The stationary phase represents the analytical column was a Brownlee Bio C18 column of 250x4.6 mm and 5 µm particle size.
HPLC operating conditions:
Instrument*:* The HPLC system (Thermo Series P2000 Pump)
Column*:* Brownlee Bio C18 column of 250x4.6 mm and 5 µm particle size.
Mobile Phase: A: 85% acetic acid buffer; pH ≈ 5.9; B: 15% methanol
Flow rate: 1.5 ml/min. Injection: 20 µl. *Tr*: 2.1 min for PPD.

Standard Solutions (Calibration curve): Figure (1) shows the RP-HPLC chromatograms of different concentrations of PPD (5 - 25 µg/ml). To determine the standard curve of PPD concentration. Standard solution of PPD (0.1 mg/ml) was prepared by weighing pure PPD substance (0.011 gm) and dissolving it in 50 % aqueous methanol solution (100 ml). Five standard solutions of PPD were prepared by dilution of stock PPD solution using a concentration in the range of 5 -25 µg/ml, as shown in figure (2).

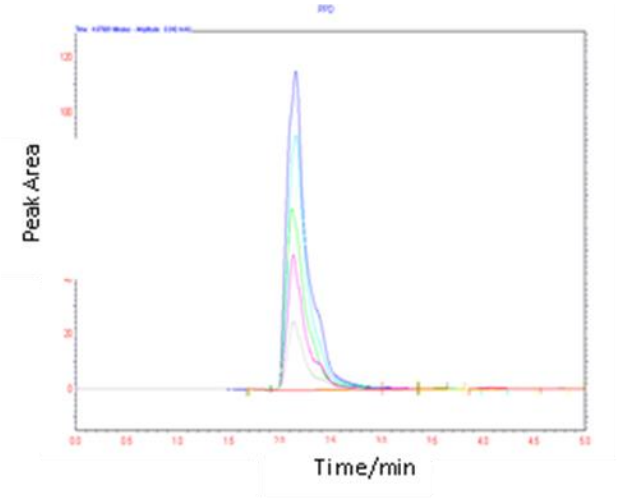

Figure: (1). Chromatograms of different concentrations of PPD by HPLC.

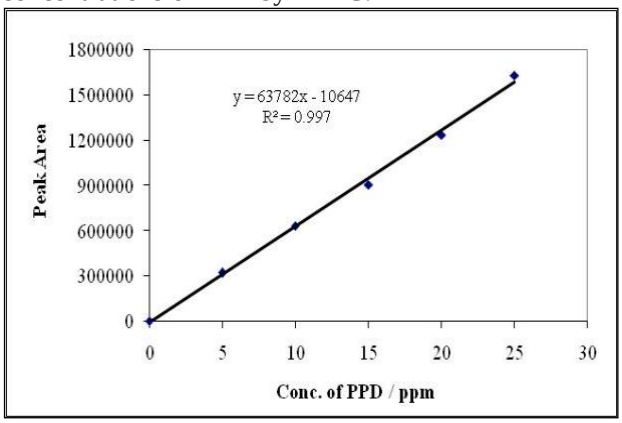

Figure: (2). Calibration curve for standard solutions of PPD, expressed on a linear scale.

The standard linear calibration curves obtained from the analysis of the standard solutions presented in figure (2) showed a good linear relationship between the peak area and concentrations of the standard solutions of PPD.

Sample collection: Eleven samples were collected from local markets in El-Bieda-Libya. Four of these samples were Libyan products, and two were random samples of mixed henna. The rest of the samples were imported from different countries, as shown in table (1).

Sample preparation (PPD extraction procedure): The extraction procedures were carried out with some modification and development, based on the other studies in order to isolate and determine PPD in henna and hair dyes (Elmanfe et al., 2019; Mounika and Kinnera, 2015; Latha et al., 2014; Al-Suwaidi and Ahmed, 2010). One gram of each of the collected samples was weighed and transferred into a 50 ml volumetric flask and diluted with 50 ml of 50% aqueous methanol solution, then filtered after 15 minutes. Finally, one ml of filtrate was diluted to 5 ml with 50% aqueous methanol solution and analyzed for PPD by RP-HPLC. To confirm the identity of PPD in samples, one ml of the standard was diluted to 5 ml with 50% aqueous methanol solution and analyzed before analyzing any sample to determine its retention time.

Table: (1). Henna samples characteristics

N^0	Sample Name	Source
S1	Royal Black Henna (For Hair)	Top Line Exam Inc,(India)
S2	Royal Chestnut Colour Henna (For Hair)	Top Line Exam Inc,(India)
S3	Royal Red Henna (For Hair)	Top Line Exam Inc,(India)
S4	Aroos Alhelal Henna	Indu Alhilal Perfumes Factory. Makkah Almukarama-Saudi Arabia
S5	Shikha Henna (Nutural Henna)	Al-Madina Company - Libya
S6	Sabaia Henna (Nutural Henna)	Al-Madina Company - Libya
S7	Tag Henna	Omdurman_ Sudan
S8	Tayeba Henna (Kone Poste)	Pakistan
S9	- Rani Kone Henna Paste	Saudi Arabia
S10	Random Samples of Mixed Henna (Paste)1 .	Made in Libya (Home Made)
S11	Random Samples of Mixed Henna (Paste)2	Made in Libya (Home Made)

Statistical analysis: The data were analyzed using Minitab version 18 software and Microsoft Office Excel. The statistical differences in some henna samples were tested using one-way ANOVA, subsequently followed by LSD test (the least significant difference) to determine significant

differences between the concentrations of PPD in different samples p values ≤ 0.05 is considered significant.

RESULTS AND DISCUSSION

The results indicate that the PPD levels in henna samples were in the range of 0.0104 % w/w - 11.9107 % w/w. These results are shown in table (2) and figure (3). The modified extraction method used in this study provides many advantages, including cheap cost and simplicity. Furthermore, it is easier than SFE techniques. The minimum PPD level was observed in the random sample of mixed henna (paste2) (0.0104 % w/w), while the highest PPD level was observed in the Royal Black Henna sample from India (11.9107 % w/w) as shown in table (2) and figure (3). While some of these samples were free from PPD (not detected), for example, the Royal red henna sample and Shikha henna (natural henna). The results indicate that PPD concentrations were in Black henna samples higher than Red henna samples. In general, there were different concentrations of PPD in henna samples, and these results indicate that there were significant differences between sample (1) and other samples at ($p < 0.05$). While for samples (3) and (5), there are no significant differences at ($p < 0.05$). The PPD content in red henna samples analyzed in this study is well below the allowable limits set by the US Food and Drugs Administration and the Scientific Committee on Consumer Products, which is (4 - 6 %) (SCCP, 2006), while in Black henna samples were higher than that recommended (Al-Suwaidi and Ahmed, 2010; SCCP, 2006).

Validation of the used Method: There are different factors that are used in the validation of the analytical methods including: Linearity, Accuracy, Precision, RSD%, Recovery, LOD, LOQ, etc.

Table: (2). Concentrations (% w/w) of PPD in different samples by HPLC

n°	Sample Name	PPD(% w/w)
S1	Royal Black Henna	11.9107
S2	Royal Chestnut Colour Henna	3.1768
S3	Royal Red Henna	ND
S4	Aroos Alhelal Henna	0.4810
S5	SHIKHA HENNA (Nutural Henna)	ND
S6	SABAIA HENNA (Nutural Henna)	1.2115
S7	Tag Henna	0.3329
S8	Tayeba Henna(Kone Poste)	0.2778
S9	- Rani Kone Henna Paste	0.04367
S10	Random Samples of Mixed Henna (Paste)1 .	0.0165
S11	Random Samples of Mixed Henna (Paste)2	0.0104

ND = (Not Detected)

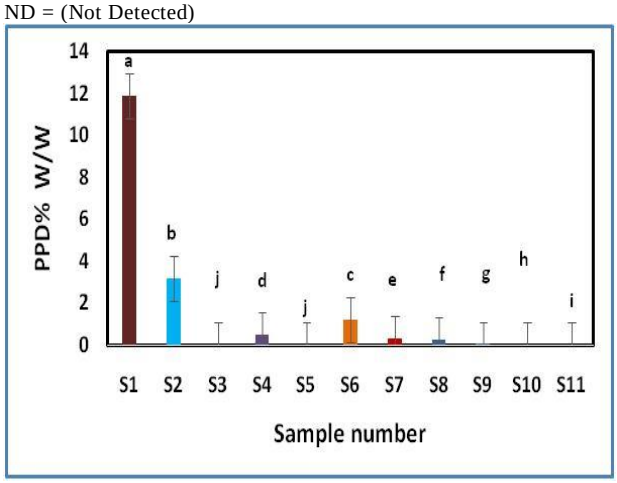

Figure: (3). Analysis of the concentration (% w/w) of PPD in different samples by LSD

Linearity: To determine the linearity of HPLC response, standard solutions of PPD were prepared. Good linear correlations were obtained between peak areas and concentrations in the selected range of 5– 25 µg/ml. Characteristic parameters for regression equations and correlation coefficients are given in table 3. The linearity of the calibration curve was validated by the high value of correlation coefficients of the regression graph. A peak tailing (As ~1.94) is observed in this case. Calibration standards (calibration curves) were prepared and demonstrated a linear response (R^2 =0.997)

over a 5 -25 µg/ml.

Limit of detection (LOD) and Limit of quantitation (LOQ): The limits of detection and quantification were determined to be 1.21 and 3.67 µg/ml for PPD respectively, which was more sensitive than the previously reported method (Mounika and Kinnera, 2015).

Accuracy and precision: The precision of the proposed method were also determined by running calibration series solutions at 5-25 µg/ml and then was evaluated in term of repeatability and expressed as the relative standard deviation (RSD,%). The result of precision ranged between 0.09 and 1.15 %, indicating good repeatability. The validation parameters for PPD are summarized in table (3).

Table: (3). Summary of validation data for the quantification of PPD using Brownlee BIO C18 column (250 mm x 4.6 mm i.d., particle size 5 µm); mobile phase: Acetic acid buffer and methanol (85:15); detector wavelength: 240 nm.

Parameter	PPD
t_R (min) (t_0 = 1.95 min[a])	2.13
Capacity factor (k')	0.12
Symmetry factor (A_s)	1.94
LOD (µg /ml)	1.21
LOQ (µg/ ml)	3.67
N (plates) 148.14	(592.56) *N expressed in plates per metre*
Coefficient of regression (R^2)	0.997 (y = 63782 x - 10647)
Precision (%RSD) N=3	
25 µg/ ml	0.23
20 µg /ml	0.29
15 µg /ml	0.09
10 µg / ml	0.33
5 µg / ml	1.15

CONCLUSION

In this study, a new method was developed to determine the concentration of PPD, which is reliable and may be used for further investigations. The validated method was successfully applied for the determination of PPD in different brands of commercial hhenna. In summary, these results show that analysis of PPD in henna samples using HPLC shows differences in concentrations of PPD. The PPD content in most of the henna samples in the study was below the allowable limits set by the US Food and Drugs Administration and the Scientific Committee on Consumer Products, while in Black henna samples, they were higher than that recommended. More caution must be taken when using henna and other dyes, especially those of black color and unknown origin.

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تقدير مادة بارافينيلين داي امين (PPD) في عينات حناء جمعت من الأسواق المحلية الليبية باستخدام كروماتوجرافيا السائل عالي الكفاءة (HPLC)

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المستخلص: الحناء تحظى بشعبية كبيرة كزينة للجسم في ليبيا، والدول العربية، وبعض الدول الأفريقية، والآسيوية، فهي جزء من التقاليد والثقافات. تؤدي إضافة بارا–فينيلين دي امين (PPD) إلى الحناء الطبيعية إلى زيادة خطر الإصابة بالتهاب الجلد التماسي، بالإضافة الى التأثيرات السامة، وقد تؤدي أحيانًا إلى حالات الوفاة، والتي تم الإبلاغ عنها بالفعل في السنوات القليلة الماضية في مدينة البيضاء – ليبيا. تهدف هذه الدراسة إلى الكشف عن وجود مادة PPD في الحناء، وتقديرها بواسطة الكروماتوغراجيا السائلة عالية الأداء (HPLC). في هذه الدراسة تم جمع أحد عشر عينة حناء من الأسواق المحلية في البيضاء – ليبيا. تم تطوير طريقة سريعة، وبسيطة، وموثوق بها لتقدير PPD في عينات الحناء باستخدام محلول ميثانول بنسبة 50٪ بوصفه مذيبا. تم التحقق من صحة الطريقة على مدى واسع من الخطية يتراوح من 5 إلى 25 ميكروغرام / مل بمعامل ارتباط أكبر من 0.997. لوحظ أن الحد الأدنى لمستوى PPD كان في عينة من خليط الحناء تصنيع محلي (معجون 2) (0.0104 ٪)، بينما أظهرت عينة حناء رويال السوداء (Royal Black Henna) أعلى نسبة من مادة PPD (11.9107%). القياسات الكروماتوجرافية (RP-HPLC) أشارت إلى أن نتائج تراكيز PPD في عينة الحناء الحمراء الملكية، والشيخة (الحناء الطبيعي) كانت خالية من PPD (أقل من حدود الكشف). كانت التركيزات في عينات الحناء السوداء أعلى من عينات الحناء الحمراء. محتوى PPD في عينات الحناء الحمراء التي تم تحليلها في هذه الدراسة أقل بكثير من الحدود المسموح بها التي وضعتها إدارة الغذاء، والدواء الأمريكية، بينما كانت عينات الحناء السوداء أعلى من تلك الموصي بها.

الكلمات المفتاحية: الحناء السوداء؛ الحناء الحمراء، PPD، الميثانول، HPLC.

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