Journal of Chromatography B, 879 (2011) 1795–1801

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid determination of *para*-phenylenediamine by gas chromatography–mass spectrometry with selected ion monitoring in henna-containing cosmetic products

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ARTICLE INFO

Article history:
Received 23 February 2011
Accepted 21 April 2011
Available online 6 May 2011

Keywords:
Para-phenylenediamine
Henna tattoo products
GC/MS-SIM
1,4-Phenylenediamine-2,3,5,6-d_4

ABSTRACT

A rapid method for the determination of *para*-phenylenediamine (PPD) in cosmetic products, such as henna tattoos has been developed and evaluated. This analytical procedure involved extracting a 10 mg test portion of cosmetic product in 10 mL of ethyl acetate, followed by determination by gas chromatography–mass spectrometry in the selected ion monitoring mode (GC/MS-SIM). 1,4-Phenylenediamine-2,3,5,6-d_4 was selected as an internal standard that was added at the beginning of the extraction procedure and used to correct for recovery and matrix effects. The linearity ranged from 1.0 to 1275 μg/mL with a coefficient of determination (r^2) greater than 0.999. LOQ and LOD were 1.0 and 0.10 μg/mL, respectively. The recovery in a tattoo product containing PPD was 94% and that for a tattoo product containing no PPD reached 105%. Extraction efficiency of 98% was obtained. This method has been successfully applied to henna temporary tattoo and other henna-related cosmetic products for the determination and quantitation of PPD.

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1. Introduction

Decorative tattoos have existed for thousands of years. Tattooing was primitively practiced for embellishment. However, it can represent a sign of distinction or social rank in some cultures and customs. Today, temporary henna tattoos applied to the skin are very fashionable and have become globally popular [1,2], but this has been accompanied by more allergic reactions [3]. Henna is a natural product and itself has a relatively low allergic potential. It was reported that the allergic reactions are mostly caused by some ingredients added by the so-called "artists" [1]. The ingredients are generally coloring agents such as diaminotoluenes and diaminobenzenes. *Para*-phenylenediamine (PPD) is a typical diaminobenzene (Fig. 1) and is one of the most popular ingredients [4].

The allergic reactions caused by PPD include acute inflammatory reactions, eczematous hypersensitivity reactions, photoaggravated reactions, granulomatous reactions, lichenoid reactions and pseudolymphomatous reactions [1,2,4–7]. It was reported that the long duration of skin contact, the high concentrations of sensitizing materials, and the lack of a neutralizing agent dramatically increases the risk of skin sensitization [1,2,4,5,7]. Therefore, the Food and Drug Administration (FDA) approves henna only for use as a hair dye, not for direct application to the skin, as in the body-decorating process known as mehndi [8]. The unapproved use of a color additive makes these products adulterated and therefore illegal. PPD is a chemical substance, which is widely used as a permanent hair dye. It may also been found in textile or fur dyes, dark colored cosmetics, temporary tattoos, photographic developer and lithography plates, photocopying and printing inks, intermediates in the manufacture of antioxidants and accelerators for rubbers, oils, greases and gasoline. This chemical has traditionally been used for the coloration of hair and temporary tattoo [1]. The use of PPD as a hair dye is popular because it is a permanent dye that gives a natural look. Due to its high toxicity (lethal dose in rats is 80 mg/kg orally), PPD has been controlled for sale to the public [3,9].

As far as analytical methods for PPD are concerned, gas chromatography (GC) has some restrictions for the determination of the hydrophilic substance in cosmetic products due to its high polarity and low volatility unless PPD is subject to derivatization, which is a tedious process [5,10]. In recent years, high performance liquid chromatography (HPLC) was the most convenient method for the determination of these dye intermediates. However, the mobile phase contains 1,8-diaminooctane or triethylamine as an amine modifier and sodium heptane sulfonate as counterion to solve asymmetric and tailing problems [10]. For the liquid chromatographic (LC) separation, if an ultra visible/visible (uv/vis) detector is used, interference will affect the detection. If a liquid chromatography/mass spectrometry (LC/MS) method is used, the buffer agents

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1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2011.04.030

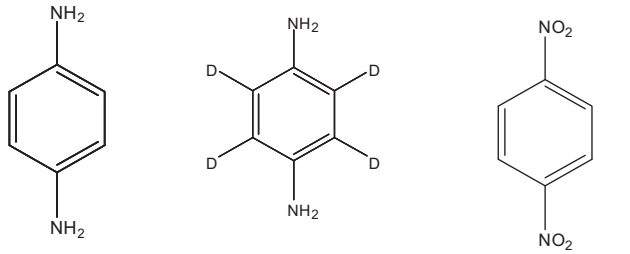

Fig. 1. Chemical structures of PPD, PPD-d_4 and 1,4-dinitrobenzene.

are not compatible. Therefore, HPLC methods have some limitations as well.

The aim of this study was to develop a novel gas chromatography–mass spectrometry with selected ion monitoring (GC/MS-SIM) method to identify and quantitate the PPD in henna tattoo products. Since stable isotopically labeled PPD-d_4 was used, any loss in the sample preparation and signal instability during a GC/MS run were compensated. A significant benefit of this method is that no time-consuming clean-up process is involved. This is the first method that uses a stable isotopically labeled standard for PPD in cosmetic products. This method can be also applied to other cosmetic products that contain PPD.

2. Experimental

2.1. Chemicals

Reagent grade ethyl acetate was purchased from Fisher Scientific (Pittsburgh, PA, USA). *Para*-phenylenediamine, and *para*-phenylenediamine-2,3,5,6-d_4, were purchased from Sigma–Aldrich (St. Louis, MO, USA). All of the chemicals were used without further purification.

2.2. Stock solution preparation for PPD and PPD-d_4

A solid standard was accurately weighed into a volumetric flask to which ethyl acetate was added. Sonication was needed to accelerate the dissolution. A working PPD solution was used to make calibration standards. The stock solution and working solution were prepared as follows:

$[PPD]_{stock}$ = 2125 μg/mL;
$[PPD]_{working}$ = 106.25 μg/Ml;
$[PPD\text{-}d_4]_{stock}$ = 428 μg/mL.

2.3. Calibration standard preparation

For each standard, 100 μL of PPD-d_4 stock solution was added into a 2-mL autosampler vial. For standard 1 through 3, 10 μL, 20 μL and 200 μL of PPD working solutions were respectively added into the corresponding vials; for standard 4 through 9, 20 μL, 50 μL, 150 μL, 300 μL, 600 μL and 900 μL of PPD stock solution were respectively added into the corresponding vials. They were summarized in Table 1.

Table 1 shows an example of preparation of a set of calibration standards.

2.4. Extraction solution preparation

To quantify the content of PPD in a henna product and also to determine the recovery of PPD in the henna product, two kinds of extraction solutions were generally prepared as follows:

Un-spiked extraction solution: A fixed amount of internal standard (PPD-d_4) stock solution at 428 μg/mL (e.g., 25 mL) was transferred into a 250 mL volumetric flask to which ethyl acetate was added to mark. The final PPD-d_4 concentration was 42.8 μg/mL.
Spiked extraction solution: The same amount of internal standard (PPD-d_4) stock solution and certain amount of PPD stock solution were transferred into a volumetric flask to which ethyl acetate was added to mark. PPD-d_4 had the same concentration in both extraction solutions.

2.5. Sample preparation

Ten (10) mg of test portion of a henna product was accurately weighed into a 10-mL centrifuge tube in 12 replicates. 5 mL of unspiked extraction solution was added into 6 replicates and 5 mL of spiked extraction solution was added into another 6 replicates. The tubes were first vortexed for approximately 1 min to "rinse" the wall and then sonicated for approximately 10 min. Extracts were centrifuged for 10 min at 4500 revolutions per minute (rpm). The supernatant was filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter and 1.0 μL of the filtered extract was injected into the GC/MS.

2.6. Instrumentation

The study was carried on an Agilent's 6890N Network GC System coupled to 5973 Network Mass Selective Detector (Santa Clara, CA, USA). The data were acquired and processed using MSD ChemStation E.0200.493. The capillary column was Restek Rtx®-5 Amine by 30 m × 0.25 mm ID × 0.5 μm df (Bellefonte, PA, USA). The following capillary column was also tested: Rxi®-MS, 30 m × 0.25 mm ID × 0.5 μm df (Bellefonte, PA, USA). The oven temperature was held at 100 °C for 2 min, then ramped at 25 °C/min to 250 °C, and held at this temperature for 3 min. The total run time is 11 min. Injector temperature was fixed at 250 °C, and the split mode was used with helium carrier gas. The temperature for MS Source and MS Quad was set at 230 °C and 150 °C, respectively. The split ratio was 50:1 with a column flow of 0.5 mL/min. Selected ion monitoring (SIM) mode was used to acquire data. The following *m*/*z* ions

Table 1
Calibration standard preparation (final volume: 1000 μL).

Standard order	I.S. (μL) Needed	PPD (μL) Stock/working	Et. Acetate (μL)	PPD-d_4 (μg/mL)	PPD (μg/mL)
1	100	10 working	890	42.8	1.06
2	100	20 working	880	42.8	2.13
3	100	200 working	700	42.8	21.25
4	100	20 stock	880	42.8	42.50
5	100	50 stock	850	42.8	106.05
6	100	150 stock	750	42.8	318.75
7	100	300 stock	600	42.8	637.50

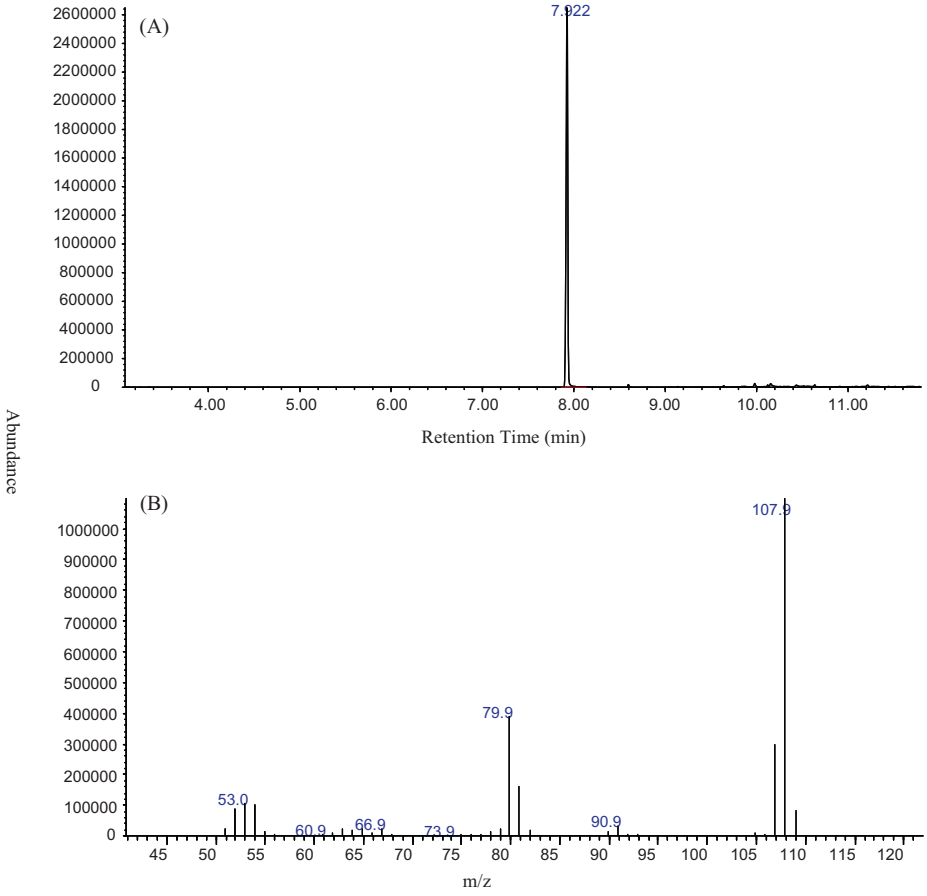

Fig. 2. Total ion chromatogram and ion spectra of PPD. (A) Total ion chromatogram, (B) Ion spectra.

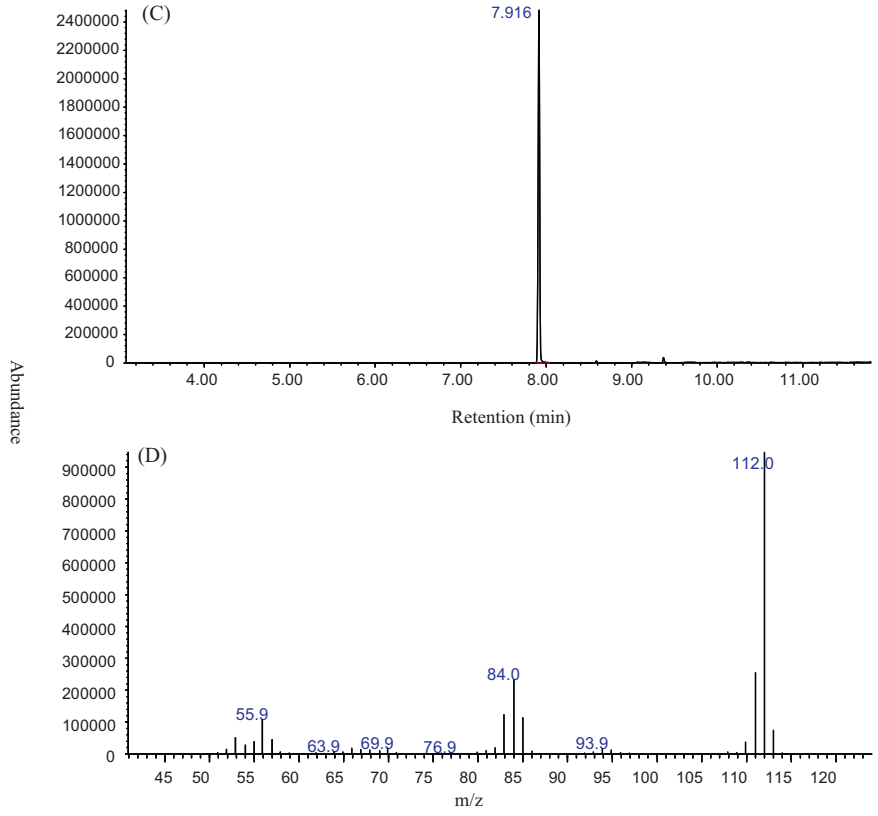

Fig. 3. Total ion chromatogram and ion spectra of PPD-d_4. (C) Total ion chromatogram; (D) Ion spectra.

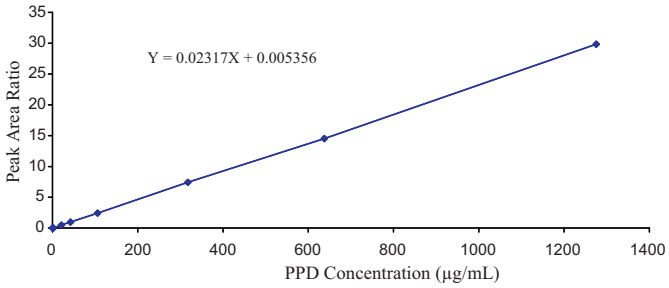

Fig. 4. Calibration curve of PPD using PPD-d_4 as an internal standard. $r = 0.999$ with a weighting factor $1/x^2$ (Internal standard was "assigned" as 1).

were monitored: 112, 84, and 56 for PPD-d_4 and 108, 80, and 53 for PPD.

A volume of 1.0 μL of calibration mixtures containing the standard PPD solutions at concentrations ranging from 1.0 to 1275 μg/mL with a fixed concentration of internal standard, PPD-d_4 (at 42.8 μg/mL), was injected into the GC–MS system.

2.7. Recovery determination

PPD content in henna products was determined in six replicates by using un-spiked extraction solution (refer to Section 2.4 for details). Extraction recovery was determined by comparing a PPD amount in spiked samples with that in un-spiked samples. Spiked samples were prepared using spiked extraction solution and un-spiked samples were prepared using un-spiked extraction solution. The recovery calculation for a product containing PPD is expressed as follows:

$$\text{Recovery}\ (\%) = \frac{\text{PPD}_{\text{total}}\,[\text{mg/mL}] - (\text{Wt.} \times \text{PPD in sample})/\text{Extraction Sol. Vol.}\,[\text{mg/mL}]}{\text{Spiked PPD concentration}\,[\text{mg/mL}]} \times 100$$

where $\text{PPD}_{\text{total}}$ was the PPD concentration determined from the sample prepared using spiked extraction solution. Spiked PPD concentration was the PPD concentration in a spiked extraction solution; Extraction Sol. Vol. is the volume of spiked extraction solution for sample preparation, Wt. was the weight of henna product. PPD in sample was PPD content determined using un-spiked extraction solution.

3. Results and discussion

3.1. Ionization of PPD and PPD-d_4

Total ion chromatogram (TIC) and electron impact (EI) spectra for PPD and PPD-d_4 are presented in Figs. 2 and 3, respectively.

Table 2
A market survey for henna products.

Products	Description	PPD (g/kg)
1	Hair dye	97.34 ± 0.05
2	Black powder	42.78 ± 0.13
3	Shelly Powder	102.49 ± 0.08
4	Grey Powder	272.44 ± 0.02
5	Henna Powder	N/D
6	Henna	N/D
7	Temporary Tattoo	N/D
8	Nature Powder	N/D
9	Henna Paste	N/D
10	Henna Kit	N/D
11	Henna Tattoo	N/D
12	Henna Design	N/D
13	Body Ink	N/D
14	Black Henna Paste	N/D

N/D: none detected at LOD = 0.10 μg/mL.

Table 3
Extraction recovery for a hair dye product containing PPD at 97.34 ± 0.05 g/kg.

Replicates	Added (μg/mL)	Determined (μg/mL)	Recovery (%)
1	201.60	186.92	92.72%
2	201.60	189.31	93.91%
3	201.60	189.26	93.88%
4	201.60	188.74	93.62%
5	201.60	192.35	95.41%
6	201.60	195.10	96.78%
		Mean	94.39%
		RSD	1.55%

For PPD quantitation, selected ion monitoring mode (SIM) was applied. The selected ions (m/z) for PPD were 108, 80 and 53; and the ions m/z 112, 84 and 56 were selected for PPD-d_4. The peak area of the selected ions was used in calculation of concentration.

3.2. Calibration and determination of PPD in henna products

A representative calibration curve (Fig. 4) was established by plotting the ratio of the peak areas of PPD and PPD-d_4 versus the concentrations of PPD. The internal standard concentration was "assigned" as 1.0 μg/mL. The linearity and accuracy of the calibration curve were evaluated. The coefficient of determination (r^2) was greater than 0.999 and the accuracy was within 100 ± 5% for the whole calibration range.

A market survey of 14 henna products was conducted. PPD was detected in 4 of them. PPD was quantified for these 4 products. The results were summarized in Table 2.

A recovery test for one product containing PPD was performed and the result was summarized in Table 3.

Another recovery test was performed using a product that contains no PPD at different spiked concentration levels. They are summarized in Table 4. The recovery for the product containing no PPD was calculated as follows:

$$\text{Recovery}\,(\%) = \left[\frac{\text{Detected Amount}}{\text{Spiked Amount}}\right] \times 100$$

3.3. Extraction efficiency

An extraction efficiency test was designed as follows: after centrifugation (refer to Section 2.5 for details), the supernatant was

Table 4
Recovery test for a product containing no PPD.

Weight (mg)	Spiked PPD (μg/mg)	Detected PPD (μg/mg)	Recovery (%)
21.3	0.50	0.51	103.06
35.2	0.30	0.32	104.94
30.1	0.35	0.36	103.06
18.9	0.56	0.57	101.18
21.6	0.49	0.50	102.12
61.2	0.17	0.19	111.53
31.3	13.67	14.28	104.48
79.9	5.32	5.64	106.06
50.1	8.48	8.98	105.82
26.3	16.16	16.86	104.34
25.3	16.80	17.52	104.29
36.2	11.74	12.33	105.01
		Average	104.66
		RSD	2.49%

Table 5
Extraction efficiency.

Sample preparation	Weight (mg)	First extraction (μg/mg)	Second extraction (μg/mg)	Third extraction (μg/mg)
1	10.8	269.91	3.56	2.03
2	9.7	283.76	3.64	1.20
3	10.6	270.92	3.11	2.78
4	10.5	275.52	3.12	2.13
5	11.9	267.96	3.19	2.52
6	10.8	275.13	3.95	2.93
	Average	273.87	3.43	2.26
	RSD	2.08%	10.02%	27.73%

Table 6
Weighting factor for calibration.

Theoretical (μg/mL)	Equal weighting r^2 = 0.999968 a = 0.02263 b = 0.001432		$1/x$ r^2 = 0.999947 a = 0.02265 b = 0.007819		$1/x^2$ r^2 = 0.999834 a = 0.02286 b = 0.006677	
	Calculated (μg/mL)	Error%	Calculated (μg/mL)	Error%	Calculated (μg/mL)	Error%
1.06	1.31	23.03	1.02	−3.61	1.06	0.20
2.13	2.37	11.32	2.08	−2.04	2.11	−0.59
21.25	22.15	4.23	21.85	2.81	21.70	2.10
42.50	43.54	2.44	43.22	1.69	42.87	0.87
106.05	107.88	1.73	107.51	1.37	106.57	0.49
318.75	321.49	0.86	320.92	0.68	318.02	−0.23
637.50	632.22	−0.83	631.38	−0.96	625.63	−1.86
1275.50	1277.87	0.22	1276.37	0.11	1264.70	−0.81

Error% = (([Calculated concentration] − [Theoretical concentration])/[Theoretical concentration]) × 100%.

Table 7
Standard stability at room temperature.

Theoretical (μg/mL)	Day 0		Day 1		Day 2		Day 3		Day 4		Day 5	
	Quantified	Accuracy	Quantified	Accuracy	Quantified	Accuracy	Quantified	Accuracy	Quantified	Accuracy	Quantified	Accuracy
1.06	1.08	102%	1.10	104%	1.09	103%	1.11	104%	1.11	104%	1.11	104%
2.13	2.10	99%	2.18	103%	2.11	99%	2.13	100%	2.14	101%	2.13	100%
21.25	21.41	101%	21.36	101%	21.34	100%	21.43	101%	21.4	101%	21.43	101%
42.50	42.57	100%	42.43	100%	42.43	100%	43.29	102%	42.32	100%	42.39	100%
106.05	105.62	100%	105.59	100%	105.12	99%	105.27	99%	105.17	99%	105.04	99%
318.75	319.35	100%	319.24	100%	314.59	99%	319.14	100%	317.81	100%	317.26	100%
637.50	625.59	98%	625.49	98%	623.11	98%	627.77	98%	629.64	99%	626.11	98%
1275.00	1286.97	101%	1286.76	101%	1260.63	99%	1289.78	101%	1245.27	98%	1264.89	99%

Accuracy = ([quantified concentration]/[theoretical concentration]) × 100%.

decanted into a clean vial. This was the first extract. 5 mL unspiked extraction solution with internal standard was added to the tube. Vortexing was needed to break the cake at the bottom of the tube. After sonicating for 10 min, the tubes were centrifuged for 10 min at 4500 rpm. The supernatant was decanted into another clean vial. This was the second extract. The above procedures were repeated to obtain the third extract. The extracts were injected into the GC/MS to quantify PPD. The results were summarized in Table 5.

It can be seen that one-time extraction should be sufficient in that 98% of the analyte was recovered in the first extraction.

3.4. The importance of application of stable isotopically labeled internal standard

When the GC/MS method was initially developed, a structurally similar analog, 1,4-dinitrobenzene (Fig. 1), was selected as an internal standard.

Even though 1,4-dinitrobenzene had acceptable chromatographic behavior and high precision, it could not adequately correct for matrix effects due to the different chemical properties between PPD and 1,4-dinitrobenzene and also they eluted at different retention times. However, once the internal standard was changed to 1,4-phenylenediamine-2,3,5,6-d_4, the matrix affects were corrected and linearity was significantly improved (Fig. 5).

3.5. The role of weighting factor for calibration

Since the calibration curve covers three orders of magnitude, the weighting factor plays an important role for the calibration (Table 6).

A set of standards was processed using different weighting factors: equal weighting, inverse of concentration ($1/x$), and inverse square of concentration ($1/x^2$). Three (3) equations were created in the following form:

$$y = ax + b$$

where x and y are the peak-area ratio and concentration ratio between PPD and PPD-d_4. "a" is slope and "b" is the intercept. Once

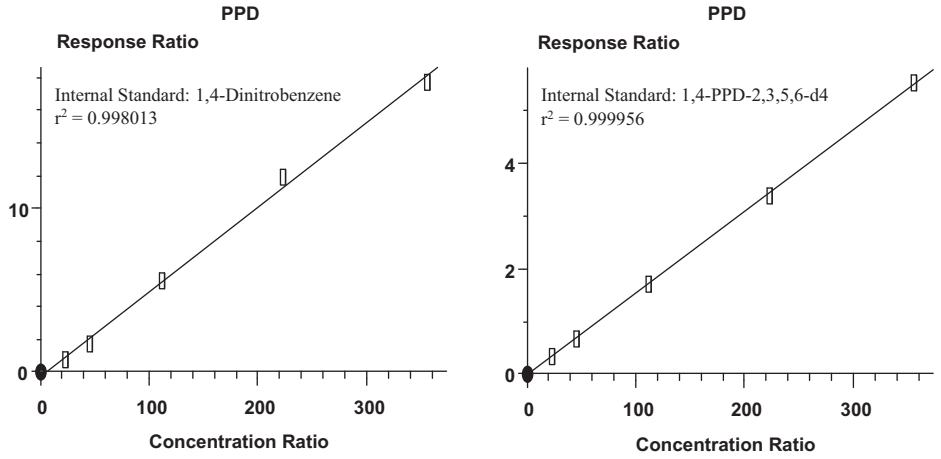

Fig. 5. Linearity comparison of 1,4-dinitrobenzene with PPD-d_4 as an internal standard.

the equation was established, the concentrations at different levels were calculated. The error% was calculated as follows:

$$\text{Error}\,\% = \left[\frac{\text{Calculated concentration} - \text{Theoretical concentration}}{\text{Theoretical concentration}}\right] \times 100$$

Even though coefficients of determination (r^2) were all greater than 0.999 for three weighting factors, their errors were significant. Therefore, it is misleading just to report coefficients of determination (r^2) or correlation coefficient (r) without considering accuracy because a reported concentration at the lower end may be erroneous. Inverse square of concentration ($1/x^2$) was applied for this study because it gave least errors.

3.6. Stability of standards at room temperature

The stability study of standards at room temperature was performed by injecting the same standards on six consecutive days. The results are summarized in Table 7. All the quantified concentrations were calculated using the standard curve created on day zero. The accuracy was the ratio of the quantified concentration and the theoretical concentration. The results showed that a set of standards was stable at room temperature for at least five days.

3.7. Column selection

Due to the polarity of PPD and PPD-d_4, column chemistry plays an important part for this method. Two kinds of column were

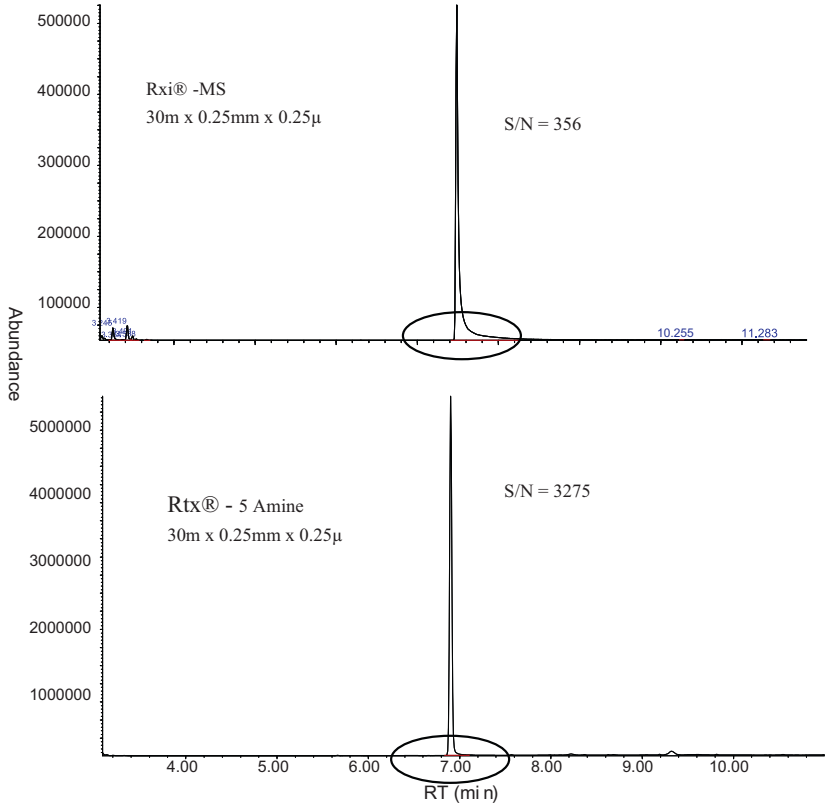

Fig. 6. Column comparison.

evaluated with a PPD concentration of 200 μg/mL and their chromatograms were presented in Fig. 6:

It can be seen that use of the Rtx®-5 Amine column not only improved the peak shape but also significantly increased the sensitivity.

4. Conclusion

It is necessary that the stable isotopically labeled internal standard for PPD be used to provide accurate quantitation by compensating for matrix effects and recovery loss without developing laborious cleanup procedures or performing standard additions for each sample extract. To obtain high accuracy and avoid misleading values for the lower concentrations, a weighting factor $1/x^2$ (inverse square of concentration) should be applied to generate a calibration curve. The stability of standards at room temperature may be extended if additional data are obtained. One-time extraction efficiency reached 98%. Extraction recovery reached 94% and 105% for a product containing PPD and containing no PPD, respectively. This method has been successfully applied to henna temporary tattoo and other henna-related cosmetic products for the identification and quantitation of PPD. This is the first method that uses a stable isotopically labeled standard for PPD in cosmetic products. It can be used to determine and quantify PPD for other products.

Acknowledgement

Authors wish to thank Wayne Wamer, Jon Wong, Robert Bronaugh, and Don Havery (all from U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition) for helpful discussions.

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