Poly (styrene-divinyl benzene-glycidylmethacrylate) stationary phase grafted with poly amidoamine (PAMAM) dendrimers for rapid determination of phenylene diamine isomers in HPLC

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Abstract

A novel high performance liquid stationary phase was prepared by grafting poly amidoamine (PAMAM) dendrimers to poly (styrene-divinyl benzene-glycidylmethacrylate) matrix in this work. Different generations of PAMAM dendrimers grafted to polymer matrices containing different glycidylmethacrylate amounts by reacting with epoxy groups in glycidylmethacrylate molecules. The synthesized PAMAM dendrimers and stationary phase were characterized by fourier transform infrared spectroscopy, scanning electron microscope, N₂ adsorption/desorption, elemental analysis and thermo gravimetric analyzer. The proposed stationary phase was suitable for rapid separation of phenylene diamine isomers and aminobenzene. The separation mechanism of benzene rings attraction and amino repulsion was verified by retention behaviors of methylamine, phenylamine, phenylene diamine, naphthylamine and toluene. The reliability of the stationary phase was demonstrated by separation and determination of phenylene diamine isomers including *m*-phenylene diamine, *o*-phenylene diamine and *p*-phenylene diamine in hair dye precursors within 10 min. Satisfactory resolution and short analysis time make the columns promising for determination of aniline compounds in various fields.

Keywords: HPLC column; grafted stationary phase; poly amidoamine dendrimers; phenylene diamine isomers; hair dye precursors

1. Introduction:

Nowadays, phenylene diamine is widely used as an important chemical constituent along with other chemical compounds in various kinds of dyes, such as hair dyes, henna and azo dyes[1-3]. These toxic compositions are always responsible for many serious diseases like allergic dermatitis and cancers [4-6]. The abuse of these dyes has increased the health risk. Thus, a large amount of analytical methods have been developed for the separation and detection of this toxic substance, mainly including gas chromatography(GC), mass spectrometer(MS) [7, 8], gas chromatography-mass spectrometer (GC/MS) [3, 9, 10], capillary electrophoresis (CE) [11, 12] and high performance liquid chromatography (HPLC) [2]. All of the proposed methods have separated phenylene diamine with other compounds successfully. However, there are three phenylene diamine isomers with different structures and toxicities. Dyes containing *m*-phenylene diamine (MPD) may have greater toxicity than these of *o*-phenylene diamine (OPD) and *p*-phenylene diamine (PPD). Rapid separation of phenylene diamine isomers is of great significance.

HPLC is one of the most useful methods for analysis of organic samples in various fields, such as environment, food, medicine and chemical engineering [13-16]. As the core component of HPLC system, columns packed with different particles have become a research focus [17-19]. Since the earliest day of HPLC column technology, silica is recognized as the most popular chromatographic support [20-22]. Stationary phase manufactured from silica matrix always has excellent mechanical properties and is easy for further derivatization. However, silica columns always have short service life in base mobile phase and high temperature, which limits the serviceable range of the columns [23, 24]. To improve chemical and thermal stabilities, organic polymer supports have been developed as HPLC stationary phase [25, 26]. Organic polymers used for HPLC matrices are mainly synthesized from styrene, divinyl benzene, methacrylate, acrylates and acrylamide monomers [27-29].

1 Recently, glycidylmethacrylate (GMA) has attracted the attention of researchers as a
2 new kind of monomers for polymer matrices. These polymers can be easily modified
3 by various reagents and reactions, because of the large amount of epoxy groups on
4 their surface [30, 31].
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8 Dendrimers [32-34] are high branched tree like monodispersed macromolecules
9 with a regular three-dimension structure. In general, dendrimers are composed of an
10 initial core, a number of repeated units and a high density of surface groups. They
11 have been extensively applied in various fields due to these special structures. For
12 instance, as a result of their unique initial core structure, dendrimers are widely used
13 in encapsulation and carriers in new catalysts [35]. They are perfect research subject
14 for host-guest chemistry because of their repetitive hyperbranched structure and lots
15 of space between branches [36]. The rich surface groups enable them good functional
16 materials in synthesis. In addition, other merits such as excellent optical and electrical
17 performance, good dispersion, low toxicity and biological compatibility make them
18 more and more popular in electrochemistry, materials chemistry, nanotechnology,
19 detection, and biological medicine areas [37, 38]. Nowadays, most research in
20 dendrimers has focused on a special dendritic macromolecule named poly
21 (amidoamine) (PAMAM) [39, 40]. PAMAM is one of the most common dendrimers
22 because of its simple structure and easy synthesis. Synthesis procedure of PAMAM
23 involves two steps: Michael addition and amidation reaction. Each step will produce a
24 half generation (G). Terminal group of half generation and whole generation of
25 PAMAM are ester base and amino, respectively. These groups may play an important
26 role in liquid chromatography separations. Thus, PAMAM may have extensive
27 application prospects in modification of liquid chromatographic stationary phase.
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50 In this study, a new kind of HPLC column modified by PAMAM was proposed
51 for rapid separation of phenylene diamine isomers. The column was based on
52 polystyrene-divinylbenzene-glycidylmethacrylate (PS-DVB-GMA) stationary phase.
53 PAMAM was grafted to the stationary phase by direct reaction with epoxy group on
54 surface of the matrix. Reliability of the column was demonstrated by separating OPD,
55 PPD and MPD in hair dyes. The application of PAMAM dendrimers in HPLC
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polymer stationary phase was explored for the first time.

2. Material and methods

2.1 Apparatus and instruments

All chromatographic investigations were carried out on the Thermo Fisher Scientific Ultimate 3000 HPLC system, which was equipped with a LPG-3400SD pump, a TCC-100 thermostatted column compartment, a six-port valve fitted with a 20 μ L sample loop and a VWD-3400 variable-wavelength UV detector. The eluent flow rate was 1.2 mL min⁻¹. The system temperature of all chromatographic tests was set at 30 °C. Data were collected by Chromeleon 6.80 chromatography data management software (Thermo Fisher Scientific).

The scanning electron microscope (SEM) images were obtained using Hitachi SU-70 (Hitachi Japan). Fourier transform infrared (FTIR) spectroscopy was performed on a Bruker Vector 22 spectrometer (Bruker, Germany). Thermogravimetric analysis (TGA) was carried out on DSCQ1000 differential scanning calorimetry (TA, New Castle, Delaware, USA). The content of nitrogen in stationary phase was analyzed by utilizing an elemental analyzer (Flash EA1112, Thermofinnigan). The surface characteristics were investigated on a surface area and porosity analyzer ASAP 2020 HD88 (Micromeritics, Atlanta, Georgia, USA). The homemade columns were packed by the QP 6000 packing pump (Chuang Xin Tong Heng Science and Technology Co., Ltd., Beijing, China). All of the organic compounds were emulsified by an SB-5200DT ultrasonic equipment (Scientz, China). Water for all eluents and standard solutions was purified by a water purification system (Thermo Fisher Scientific) to a specific resistivity of 18.2 M Ω cm.

2.2 Reagents

Styrene (St), Azobisisobutyronitrile (AIBN), Dibutyl phthalate (DBP), sodium dodecylsulfonate (SDS), polyvinyl alcohol (PVA) and benzyl peroxide (BPO) were purchased from Huipu Chemical Reagent Co. Ltd. (Hangzhou, China). Ethylenediamine, methyl acrylate and 1, 4-butanediol diglycidyl ether (BDDE, 60% in H₂O, v/v) were obtained from Aladdin Chemical Co. Ltd. (Shanghai, China).

1 Styrene, DVB (55%, Zhengguang Chemical Plant, Hangzhou, China) and GMA (97%,
2 Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) were used after washing with 10% (w/v)
3 sodium hydroxide aqueous solution. AIBN and BPO were recrystallized before use.
4 All of the standard solutions were prepared by dissolving appropriate amount of
5 reagents in methanol (chromatographically pure). Hair dye samples were purchased
6 from online shopping market. All reagents employed for the synthesis were of
7 analytical grade.

14 2.3 Preparation of chromatographic stationary phase

16 2.3.1 The production of PAMAM

18 Different generations of PAMAM were synthesized according to the method
19 described by Tomalia et al[32]. Ethylenediamine was used as a nitrogen core in
20 synthetic procedure. As shown in Fig. 1, it involved 2 repeated steps: Michael
21 addition and amidation reaction. Each step reacted for 24 h at 30 °C. Production from
22 each step was purified by rotary evaporation device.

29 2.3.2 Preparation of PS-GMA beads

31 Polymer seeds were firstly prepared by dispersion polymerization method. The
32 typical procedure was as follows[41]: The reaction was conducted in a 250 mL
33 four-necked round-bottomed flask. 3 g PVP and 100 mL mixture of ethanol and water
34 (95:5, V/V) were added to the flask with 70 °C temperature-controlled water bath.
35 After then, mixture of 18 g styrene and 0.8 g AIBN were added into the flask
36 dropwise in 30 min. The seeds were washed with excess water and stored in 1% SDS
37 after reaction for 24 h. Secondly, PS-GMA beads were synthesized by swelling and
38 polymerization procedure. Polymer seeds were dispersed in 15 mL SDS aqueous
39 (0.2%, w/v) and placed in a 500 mL three neck flask along with 30 mL SDS aqueous
40 solution (0.2%, w/v) containing 4 g DBO. After stirring overnight, another mixture
41 which was consisted of 30 g organic compounds dispersed in 250 mL PVA aqueous
42 solution (1.0%, w/v) was added and swelled. The above organic compounds consisted
43 of ST, DVB, GMA, toluene, BPO and SDS. The amount of toluene was equal to the
44 sum of ST and DVB. The content of BPO and SDS were 0.6% and 2.5% (w/w) of
45 organic compounds. Different amount of GMA (3%, 6% and 12%, w/w) were added
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in different stationary phase. After 24 h, the mixture were heated by water bath at 70 °C under nitrogen atmosphere and lasted for 24 h. Subsequently, the resulting beads were washed successively with hot water and alcohol. Then, the beads were purified by soxhlet extraction with toluene for 48 h. After washing by water and alcohol, the PS-GMA beads were dried under vacuum at 60 °C for 24 h.

2.3.3 Preparation of PS-GMA-PAMAM stationary phase

PS-GMA-PAMAM stationary phase was obtained by grafting PAMAM to the surface of PS-GMA beads. Specific as follows: 3.0 g PS-GMA beads were dispersed in 30 mL alcohol by ultrasonication for 10 min. Then, the excessive ethylenediamine and PAMAM with different generations (1.0G and 2.0G) were added to the mixture separately and stirred at 70 °C for 1 h. After being washed by water and methanol, the PS-GMA-PAMAM HPLC stationary phases were obtained (Fig. 2).

2.4 Column packing procedure

The columns were packed by a slurry method. 3.0 g PS-GMA-PAMAM stationary phases described above were dispersed in 50 mL deionized water to form slurry by ultra-sonication. The slurry consisting of modified matrix was packed into a column (stainless steel, 150 mm × 4.6 mm i.d.) at the pressure of 200 bar. The volume of packing solvent passing through the column should be more than 350 mL. The columns were flushed by methanol overnight before being connected to HPLC instrument.

2.5 Sample preparation

An accurate amount of hair dye precursors were dissolved in 5 mL methanol and sonicated for 5 min. The solution was injected into HPLC system directly after being filtered through a 0.45 µm membrane filter.

3. Result and Discussion

3.1 FTIR spectra of PAMAM dendrimers

The structure of the synthesized 0.5G PAMAM and 1.0G PAMAM dendrimers was confirmed by FTIR. As shown in Fig. 3, Absorption peaks at 2954 cm⁻¹ and 2829 cm⁻¹ were corresponding to -CH₃ and -CH₂- groups in 0.5G PAMAM, respectively.

The absorption bands at 1747 cm^{-1} and $1336\text{-}1042\text{ cm}^{-1}$ could be attributed to the stretching vibrations of C=O and -C-O-C groups in 0.5G PAMAM. The absorption peak at 1462 cm^{-1} in 0.5G PAMAM was related to the -CH₂- bending vibration. Double absorption peaks in 3309 cm^{-1} and 3085 cm^{-1} were attributed to stretching vibration of -NH₂ groups in 1.0G PAMAM. The significant peaks at 1642 cm^{-1} and 1573 cm^{-1} in 1.0G PAMAM were corresponding to -CONH- groups. The C-N stretching vibration in 1.0G PAMAM dendrimers can be observed at 1322 cm^{-1} and 1469 cm^{-1} . No double absorption peaks of -NH₂ was found in 0.5G PAMAM. And a wide band of -C-O-C groups between 1336 cm^{-1} and 1042 cm^{-1} was not observed in 1.0G PAMAM dendrimers, which is exactly consistent to PAMAM structures in Fig. 1[42].

3.2 Characterization of stationary phase

3.2.1 Surface feature and diameter of the particles

Surface and morphology characterizations of the stationary phase were characterized with SEM (Fig.4) and BET. The PS-GMA beads are uniform and monodisperse porous microspheres with average diameter of 5 μm . Surface of the beads is smooth with no sags or crests. The pore on the surface of the beads can be seen clearly under the electron microscope. Pore size of the PS-GMA beads investigated by BET was 5.98 nm. After grafting with PAMAM, the morphology and size of the beads are basically the same, while the pore size on the surface increased to 6.28 nm. The chemical modification of PAMAM dendrimers has no significant impact on the particle surface morphology.

3.2.2 Element analysis

Five different columns were synthesized in this work, as shown in Table 1. Ethylenediamine and different generations of PAMAM (1.0G, 2.0G) grafted to the surface of PS-GMA beads with different content of GMA (3%, 6%, 12%, w/w). Element analysis was performed to evaluate the content of nitrogen in grafting beads (shown in Table 2). Because PS-DVB beads have no nitrogen, nitrogen content in stationary phase is proportional to the ratio of grafted PAMAM dendrimers. Comparing 6G1P stationary phase with 3G1P and 12G1P, the percentages of nitrogen

increased with more GMA content in PS-GMA beads. Meanwhile, increased nitrogen contents in 6G0P, 6G1P and 6G2P modified beads suggested that the percentages of nitrogen increased with the increase of PAMAM generations. Therefore, more functional groups will be obtained by increasing GMA content and PAMAM generations.

3.2.3 Thermal stability of the beads

The thermodynamic stability of PS-GMA beads and PS-GMA-PAMAM stationary phase were studied by Thermo Gravimetric Analyzer (TGA). As shown in Fig. 5, there is almost no weight loss for both PS-GMA beads and PS-GMA-PAMAM beads under 300 °C, which verified that the beads can keep good thermal stability after being grafted with PAMAM.

3.3 Retention behavior of phenylene diamine isomers in synthetic HPLC columns

There are three different phenylene diamine isomers with amino groups located in ortho-position, para-position and meta-position. A mixture containing *o*-phenylene diamine, *p*-phenylene diamine, *m*-phenylene diamine and phenylamine was used to evaluate the separation effect of the column after modified by PAMAM dendrimers. 20 mg L⁻¹ standard solution was separated by PS-GMA beads and PS-GMA-PAMAM beads, respectively. A mixture of methanol/water (50/50, v/v) was utilized as mobile phase in isocratic mode with a flow rate at 1.2 mL min⁻¹. The wavelength for detection was 280 nm. As shown in Fig. 6, retentions of the analytes in PS-GMA column were between 6-18 min with two wide peaks. However, phenylene diamine isomers in 6G1P column were separated with satisfactory resolution (Table 3). Grafting with PAMAM has improved the performance of the PS-GMA beads.

PAMAM was synthesized with ethylenediamine as a core. Each repeat unit in PAMAM was called a generation (G). Columns modified by different generations of PAMAM were investigated under the same chromatography condition by phenylene diamines isomers. Representative chromatograms were shown in Fig. 7. All of the columns can separate the isomers successfully. Phenylene diamines have less retention time in 6G1P than 6G0P and 6G2P column. Besides, retention behaviors on stationary phase with different content of GMA were also evaluated under the same

condition by isomers. The result was shown in Fig. 8, retention time in columns decreased with the increasing of GMA contents.

3.4 Separation mechanism

According to retention behaviors in different columns, separation mechanism of the stationary phase was explored. Generally, there are 3 kinds of groups in the PS-GMA-PAMAM columns, including benzene rings, carbonyl groups and amino groups. Theoretically, retention ability is influenced by attraction forces between benzene rings in stationary phase and phenylene diamines, and repulsive forces between amino groups in PAMAM and amino groups in analytes. Thus, more GMA content in beads should graft more PAMAM. Much PAMAM on the surface of beads would decrease attraction force between benzene rings. Besides, large amount of PAMAM terminal amino groups in stationary phase will increase amino repulsion. Consequently, retention time of phenylene diamines decreased with increasing GMA contents, which is consisted with the result in Fig. 8. Similarly, different generations of PAMAM grafted to PS-GMA beads will cover the benzene ring at different degrees. Attraction forces between benzene rings will be lower in beads grafted with high generation of PAMAM. In addition, high generation of PAMAM will have more amino groups and increase amino repulsion. As 6G0P and 6G1P curves shown in Fig. 7, with increasing generation of PAMAM grafted to the beads, retention time in stationary phase will be decreased. However, retention time of phenylene diamines in 6G2P is longer than 6G1P in Fig. 7, which is account for π - π conjugate effect between benzene rings in phenylene diamines and carbonyl groups in PAMAM. With increase of PAMAM generations, carbonyl groups increase rapidly and π - π conjugate will have a greater impact than amino repulsion.

Based on this assumption, methylamine, phenylamine, naphthylamine, phenylene diamines and toluene were separated by the 6G1P stationary phase to verify the mechanism above. The chromatograms are shown in Fig. 9. The mixture of methanol/water (70/30, v/v) was used as eluent under isocratic mode with flow rate at 1.2 mL min⁻¹. Methylamine, phenylamine and naphthylamine were separated in Fig. 9a. The result was detected by a UV detector with the wavelength at 215 nm.

Mathylamine without benzene rings has almost no retention in column. Retention time of naphthylamine is longer than phenylamine because of more benzene rings. As shown in Fig. 9b, phenylene diamine, phenylamine and toluene were separated by the column and detected by a UV detector with the wavelength at 280 nm. Phenylene diamine with two amino groups was eluted out firstly. Phenylamine with one amino group was eluted out subsequently. Toluene cannot be eluted out with 70% methanol eluent until 20 min because of no amino group in structure. Therefore, more benzene rings in structure will result in longer retention time, while more amino groups in molecular will result in less retention time. The results are consistent with the mechanism of benzene rings attraction and amino repulsion.

3.5 Determination of phenylene diamine isomers in hair dye precursors

Reliabilities of the synthetic HPLC columns were investigated by determination of phenylene diamine isomers in hair dye precursors. Series standard solutions with concentrations varying from 1 mg L^{-1} to 20 mg L^{-1} were detected under isocratic mode. The mobile phase was consisted of methanol/water (50/50, v/v) mixture and the flow rate was 1.2 mL min^{-1} . A UV detector was used to detect the results with the wavelength at 280 nm. Linearity, limits of detection and limits of quantification of phenylene diamine isomers are shown in Table 4. The result showed a good linearity with correlation coefficient more than 0.9989. The limit of detection (LOD) was determined based on the signal to noise (S/N) ratio of 3 and the limit of quantification (LOQ) was determined based on the signal to noise (S/N) ratio of 10. The relative standard deviations (RSDs) for retention time, peak area and peak height were in the range of 0.4% - 2.3%. The chromatograms of hair dye precursors and standard solution were shown in Fig. 10. Phenylene diamine isomers in practical samples are separated successfully by the synthetic column. 5 mg L^{-1} standard solutions were added into the samples, the average recovery of MPD, PPD and OPD were 87% - 104%. Only 0.0152 mg/g MPD was detected in sample 1. The detected concentrations of MPD, PPD and OPD in sample 2 were 0.343 mg/g, 0.0100 mg/g and 0.0205 mg/g, respectively.

4. Conclusion

A novel PAMAM grafting HPLC column based on PS-GMA stationary phase was artificially synthesized for the separation of phenylene diamine isomers. Different generations of PAMAM and matrix containing various GMA amounts were utilized in synthesis procedure. The structure, chemical composition and thermodynamic stability of PAMAM dendrimers and the columns were verified by FTIR, SEM, BET, element analysis and TGA. All of the homemade columns have rapidly separated the phenylene diamine isomers with good symmetry and satisfactory resolution. Retention ability of the columns was closely related to GMA contents and PAMAM generations. Mechanism for phenylene diamine isomers separations in columns were explored and explained. According to the proposed mechanism, the homemade column is expected to have a broad prospect in separation of aniline category matters.

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Tables

Table 1

5 columns synthesized with different generations of PAMAM and contents of GMA

PAMAM generations GMA contents	0G PAMAM (ethylenediamine)	1.0G PAMAM	2.0G PAMAM
3% GMA	*	3G1P	*
6% GMA	6G0P	6G1P	6G2P
12% GMA	*	12G1P	*

Table 2

Element analysis of stationary phases

Stationary phase	Carbon (%)	Nitrogen (%)	Hydrogen (%)
PS-6% GMA	87.29	0	8.15
PS-3% GMA-1.0G PAMAM	84.52	0.13	7.637
PS-6% GMA-0G PAMAM	81.47	0.34	7.409
PS-6% GMA-1.0G PAMAM	86.00	0.39	7.969
PS-6% GMA-2.0G PAMAM	85.12	0.44	7.806
PS-12% GMA-1.0G PAMAM	81.94	0.76	7.981

Table 3

The separation of phenylene diamine isomers with 6G1P columns

	Resolutions	Asymmetry factor	RSD (n=6) %		
			Peak area	Peak height	Retention time
<i>p</i> -phenylene diamine	2.17	1.06	2.53	2.62	0.00
<i>m</i> -phenylene diamine	3.08	1.25	2.13	2.84	0.25
<i>o</i> -phenylene diamine	n.a	1.31	0.52	2.18	1.69

Table 4

Calibration parameters (five data points) for the three analytes in standard solutions.

	linear equation	Correlation coefficient (R ²)	LOD(μg L ⁻¹) (S/N=3)	LOQ(mg L ⁻¹) (S/N=10)
<i>p</i> -phenylene diamine	y = 0.1967x - 0.1086	0.9989	57.9	0.193
<i>m</i> -phenylene diamine	y = 0.1391x + 0.0206	0.9999	53.1	0.177
<i>o</i> -phenylene diamine	y = 0.1858x + 0.1320	0.9995	59.7	0.199

Captions

Fig. 1 The synthesis procedure of different generations of PAMAM dendrimers

Fig. 2 Preparation of PS-GMA-PAMAM stationary phase by grafting with different PAMAM generations

Fig. 3 FTIR of 0.5G PAMAM and 1.0G PAMAM dendrimers

Fig. 4 SEM images of PS-GMA beads (a) $\times 2000$ times, (b) $\times 10000$ times, (c) $\times 40000$ times and PS-GMA-PAMAM stationary phase (d) $\times 2000$ times, (e) $\times 10000$ times, (f) $\times 40000$ times

Fig. 5 Thermogravimetric analysis of PS-GMA beads (A) and PS-GMA-PAMAM (B) particles, conducted in nitrogen atmosphere: heating rate: $10^{\circ}\text{C min}^{-1}$

Fig. 6 Separation of phenylene diamine isomers and phenylamine with 6G1P column and PS-GMA beads. Column dimensions: 4.0 mm I.D. by 150 mm; eluent: methanol/water (50/50, v/v); flow rate: 1.2 mL min^{-1} ; detection wavelength: 280 nm; (1) PPD, (2) MPD, (3) OPD, (4) phenylamine.

Fig. 7 Separation of phenylene diamine isomers with columns modified by different generations of PAMAM. Column dimensions: 4.0 mm I.D. by 150 mm; eluent: methanol/water (50/50, v/v); flow rate: 1.2 mL min^{-1} ; detection wavelength: 280 nm; (1) PPD, (2) MPD, (3) OPD.

Fig. 8 Separation of phenylene diamine isomers with modified columns containing different GMA amounts. Column dimensions: 4.0 mm I.D. by 150 mm; eluent: methanol/water (50/50, v/v); flow rate: 1.2 mL min^{-1} ; detection wavelength: 280 nm; (1) PPD, (2) MPD, (3) OPD.

1 Fig. 9 Separation of (a) methylamine, phenylamine, naphthylamine and (b)
2 phenylamine, phenylene diamines, toluene in 6G1P column. Column dimensions: 4.0
3 mm I.D. by 150 mm; eluent: methanol/water (70/30, v/v); flow rate: 1.2 mL min⁻¹;
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5 detection wavelength: (a) 215 nm, (b) 280 nm.
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10 Fig. 10 Detection of PPD, MPD and OPD in hair dye precursors with 3G1P column.
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12 Column dimensions: 4.0 mm I.D. by 150 mm; eluent: methanol/water (50/50, v/v);
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14 inject volume: 20 µL; flow rate: 1.2 mL min⁻¹; detection wavelength: 280 nm.
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Fig 1

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Fig 6

Fig 7

Fig 8

Fig 9a

Fig 9b

Fig10