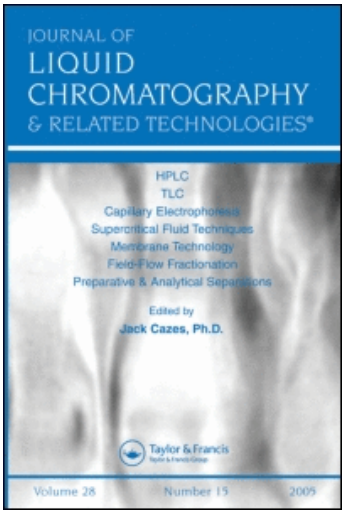

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Reversed Phase Liquid Chromatographic Method for Separation and Determination of Positional Isomeric Mono- and Di-substituted Anilines and Phenols on an *R,S*-Hydroxypropyl Ether β-Cyclodextrin Column

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ABSTRACT

A reversed-phase high performance liquid chromatographic (HPLC) method was developed for the simultaneous analysis of a group of fourteen positional isomeric di- and tri-substituted benzenes; including *o*-, *m*-, and *p*-aminophenol, *o*-, *m*-, and *p*-nitrophenol, *o*-, *m*-, and *p*-nitroaniline, 2-chloro-4-nitroaniline, 4-chloro-2-nitroaniline, 2,4-dinitroaniline, 2,6-dinitro-

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aniline, and 3,5-dinitroaniline. Baseline separation was achieved within 40 min using a Cyclobond I RSP (*R*,*S*-hydroxypropyl ether-*β*-cyclodextrin) stationary phase in conjunction with gradient elution with methanol–water as the mobile phase. All compounds were detected at 254 nm. The quantitation limits ranged from 0.80 to 5.6 ng/mL.

Key Words: Isomeric anilines and phenols; Reversed phase liquid chromatographic separation; Cyclodextrin bonded phases.

INTRODUCTION

In the context of research aimed at evaluation of the efficacy of utilizing the surfactant-mediated extraction (a.k.a. cloud point extraction, see [1–5] for recent reviews) technique for the extraction of some priority pollutants, including substituted aniline and phenolic compounds, from aqueous sample matrices, it was necessary to first develop a HPLC method for their separation and determination. Previously, several HPLC methods for separation of the positional isomers of the nitrophenols (reversed phase mode using C-18, fluorocarbon-, or phenylaminopropyl- bonded silica, or poly(styrene-divinylbenzene) resins as the stationary phase),[6–11] nitroanilines (normal phase mode using silica, amino, or diamine bonded phases),[12,13] and aminophenols (reversed phase mode using a polystyrene Chromalite 5HGN or LiChrospher RP Select B stationary phase)[14,15] had been reported. In addition, an open-tubular capillary electrochromatography method has been employed to separate the positional isomers of nitrophenols and aminophenols using a capillary column coated through sol-gel chemistry with a macrocyclic dioxopolyamine.[16]

In addition, various native and derivatized cyclodextrin stationary phases have been employed.[17–36] While the majority of published reports employing cyclodextrins in chromatography have focused on the resolution of optical isomers (enantiomeric separations),[17–19,34,36] they have excellent utility for separation of closely related analytes, such as positional or geometric isomers as well.[19–33] For instance, the isocratic reversed phase HPLC separation of the positional isomers of nitrophenols,[21–23,29,30,33] nitroanilines[21,22,25–27,32,33] and aminophenols[26,29,30] among others,[19,28] on underivatized native or derivatized *β*-CD bonded (or *β*-CD polymer coated) stationary phases has been reported. However, almost all of such reports concerned the separation of the *o*, *m*, and *p* isomers of a specific disubstituted benzene, not the simultaneous separation of all of the positional isomers of many such components present in a mixture.

This paper describes a method for the separation and determination of fourteen positional isomeric components (nitrophenols, aminophenols,

nitroanilines, dinitroanilines and chloronitroanilines) on a hydroxypropyl ether β-cyclodextrin (HP-β-CD) stationary phase in 40 min using a gradient elution and UV detection.

EXPERIMENTAL

Materials

The analytes resolved in this study included *o*-aminophenol, *m*-aminophenol, *p*-aminophenol, 2-cyanophenol, 3-cyanophenol, 4-cyanophenol, 2,6-dinitroaniline, 3,5-dinitroaniline, *o*-nitroaniline, *m*-nitroaniline, 4-chloro-2-nitroaniline, 2-chloro-4-nitroaniline (all received from Aldrich Chemical Co., Milwaukee, WI), *m*-nitrophenol and *p*-nitrophenol (both obtained from Fisher Scientific Co., Raleigh, NC), *p*-nitroaniline (Sigma Chemical Co., St. Louis, MO), and 2,4-dinitroaniline and *o*-nitrophenol (both obtained from Eastman Kodak, Rochester). Calibration standard 2-nitrophenol and *p*-nitroaniline solutions, 50.0 ng/μL in methylene chloride, (NSI Solutions, Inc., Research Triangle Park, NC) were employed for the recovery experiments. The solvents employed for preparation of the mobile phase included HPLC grade methanol or acetonitrile and deionized, ultra filtered water (Fisher Scientific Co.).

Safety Considerations

The phenols and anilines are all toxic and caution should be exercised with these compounds. All handling of the pure compounds, concentrated stock solutions, and standards should be performed in a well-ventilated hood and appropriate gloves should be worn in order to avoid inhalation or skin contact.

Apparatus and Procedures

A Cyclobond I 2000 RSP HPLC (250 × 4.6 mm, serial #12928) column (Advanced Separation Technologies, Inc., Whippany, NJ) was employed in this work. The liquid chromatograph system (Waters Associates, Milford, MA) consisted of a Model 680 automated gradient controller, Model 501 and 510 pumps, U6K injector, and Model 484 tunable absorbance detector (14 μL, 1 cm path length). Detector response was monitored using a Hewlett Packard Model 3396 Series II Integrator Recorder. The analytes were dissolved in methanol; with 25 μL of a known analyte solution or mixture being directly

injected to initiate each run. Mobile phases were prepared by mixing appropriate amounts of methanol and water. The mobile phase mixtures were degassed by ultrasonication and filtered (0.45 μ filter) prior to use. Preliminary isocratic runs were conducted with aqueous methanolic mobile phases containing 25.0, 30.0, 35.0, and/or 40.0% methanol. The final optimized HPLC gradient runs were conducted by using a methanol–water mobile phase with a linear gradient from 25.0% methanol to 40.0% methanol over a 15 min period, followed by continued elution with the 40% methanol for an additional 25 min. The total analysis time was, thus, 40.0 min. At the conclusion of each run, a linear gradient from 40% methanol back to 25% methanol was run over a ten min period prior to the next injection. All separations were carried out at a mobile phase flow rate of 1.00 mL/min at room temperature (ca. 25.0°C) with ultraviolet detection at 254 nm. The elution order was established from consecutive injections of the individual pure isomers. Calibration curves for the fourteen analytes were obtained by plotting the peak area versus known injected amounts of standard solutions of the compounds, using 8 to 22 data points in the 2.0 to 20.0 ppb concentration range. Recovery data were obtained by analyzing diluted (1/10) calibration standard (50.0 ng/mL) solutions of *p*-nitroaniline and *o*-nitrophenol. Statistical evaluation of the data was carried out using the Excel 2000 spreadsheet software (Microsoft).

RESULTS AND DISCUSSION

Each of the 17 positional isomers selected for evaluation was initially chromatographed on the HP-*β*-CD column, individually, under isocratic conditions with aqueous mobile phases containing different amounts of organic modifier, methanol, in water in order to determine their retention times and the elution order. Representative data for some of the isomers eluted under isocratic conditions is shown in Table 1. As can be observed in this reversed phase mode, retention of all components decreased as the percent organic modifier (methanol) in the mobile phase was increased (Table 1), as expected, based on prior literature reports.[19,22,25–27,29,30,32,33] The final gradient elution protocol was selected after several trials, in which the mobile phase composition and change with respect to time, was optimized in order to achieve baseline separation of the maximum number of isomeric components in the shortest time frame.

The final optimized gradient elution conditions lead to the separation of fourteen selected components in 40 min with good resolution (Fig. 1). The obtained separation permits good peak area integration for all components. The repeatability of the retention times was investigated by repeated injection

Table 1. Retention times of several isomeric substituted anilines and phenols on the cyclobond I 2000 RSP column under isocratic elution[a].

	Retention time[a] (min)		
Analyte	30.0	35.0	40.0
o-Nitrophenol	20.8	15.4	12.4
m-Nitrophenol	29.8	21.8	16.1
p-Nitrophenol	40.4	29.5	22.1
m-Nitroaniline	22.0	17.2	14.0[b]
o-Nitroaniline	25.1	18.1	14.0[b]
p-Nitroaniline	49.9	34.5	26.6
4-Chloro-2-nitroaniline	34.7	23.9	17.6
2-Chloro-4-nitroaniline	57.2	37.5	28.0
o-Cyanophenol	15.8[b] [7.5][c]	11.3[b]	8.9[b]
m-Cyanophenol	15.8[b] [9.6][c]	11.3[b]	8.9[b]
p-Cyanophenol	18.2 [11.4][c]	13.3	10.4

[a]As a function of % methanol in water of the mobile phase, isocratic elution with indicated methanol:water (V/V) mobile phase, flow rate 1.00 mL/min, 25.0°C, detection wavelength 254 nm.
[b]Indicated isomers co-elute.
[c]The isomeric cyanophenols can be separated on a 250 × 4.6 mm Cyclobond II (native β-cyclodextrin) column using a 20 : 80 acetonitrile : water mobile phase as shown by the bracketed data.

($n = 62$) of a solution mixture containing all of the positional isomeric components. The results are summarized in Table 2 and are indicative of excellent reproducibility; the relative standard deviations were all better than 3.9% for the fourteen di- or tri-substituted benzene test components.

The elution order observed for the positional isomeric nitroanilines, i.e., meta < ortho < para (Table 2), on the HP-β-CD column is the same as had been previously reported for the separation of these isomers on native β-CD[21,22,25,27,32,33,36] or tyrosine derivatized β-CD[26] stationary phases. For comparison, the elution order of nitroanilines on an ODS stationary phase with acetonitrile:water mobile phase was para < meta < ortho.[27,33] For normal phase separations on silica, amino or diamine bonded phases, the opposite elution order is reported: ortho < meta < para.[12,13]

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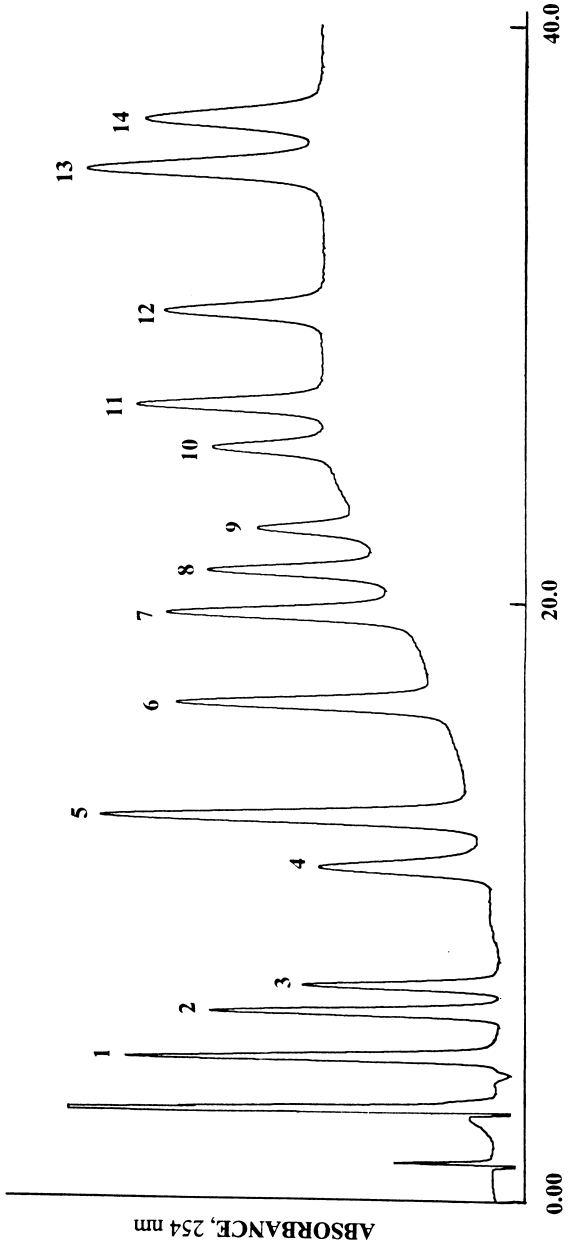

Figure 1. Representative chromatograph showing the HPLC separation of 14 positional isomeric mono-, and di-substituted phenols and anilines on the Cyclobond I 2000 RSP column with a methanol : water mobile phase under gradient program control (i.e., the mobile phase composition was changed in a linear fashion from 25 : 75 (v/v) methanol : water to 40 : 60 (v/v) methanol : water over a 15 min period followed by continued elution with the 40 : 60 methanol : water for an additional 25 min, the flow rate was 1.00 mL/min, absorbance detection at 254 nm). Peak identification (and concentration): 1: *p*-aminophenol (12 ppb); 2: *m*-aminophenol (20 ppb); 3: *o*-aminophenol (16 ppb); 4: 2,6-dinitroaniline (4 ppb); 5: 3,5-dinitroaniline (4 ppb); 6: 2,4-dinitroaniline (4 ppb); 7: *o*-nitrophenol (14 ppb); 8: *m*-nitroaniline (4 ppb); 9: *o*-nitroaniline (4 ppb); 10: *m*-nitrophenol (6 ppb); 11: 4-chloro-2-nitroaniline (4 ppb); 12: *p*-nitrophenol (20 ppb); 13: *p*-nitroaniline (21 ppb); and 14: 2-chloro-4-nitroaniline (21 ppb).

Table 2. Repeatability of retention times under gradient elution.

Analyte	Mean value[a] (min)	Standard deviation (min)	RSD[b] (%)
p-Aminophenol	4.73	0.184	3.88
m-Aminophenol	6.48	0.103	1.59
o-Aminophenol	7.27	0.164	2.26
2,6-Dinitroaniline	11.3	0.276	2.45
3,5-Dinitroaniline	13.0	0.293	2.26
2,4-Dinitroaniline	17.0	0.376	2.22
o-Nitrophenol	20.0	0.450	2.25
m-Nitroaniline	21.5	0.456	2.12
o-Nitroaniline	23.0	0.467	2.03
m-Nitrophenol	25.9	0.501	1.94
4-Chloro-2-nitroaniline	27.3	0.487	1.79
p-Nitrophenol	30.5	0.656	2.15
p-Nitroaniline	35.4	0.887	2.50
2-Chloro-4-nitroaniline	37.0	0.867	2.34

[a]Mean value of 62 separate runs of the component mixture. Chromatographic conditions are as noted in the experimental section under optimized gradient runs. The problem of estimation of void (dead) volumes for cyclodextrin phases has been previously noted (34–36). The average retention time for elution of reference (void) compounds such as methanol or ethanol was 2.68 ± 0.10 min, while that of water was 2.25 min. Use of such reference void markers allows for conversion from the reported retention times to retention (capacity) factors if desired.
[b]Percent relative standard deviation.

In the case of the positional isomers of aminophenol, the elution order on the HP-β-CD phase was para < meta < ortho (Table 2). This is the same elution order as had been reported for their separation on a tyrosine derivatized β-CD stationary phase.[26] However, Crini, et al. observed an elution order of meta < ortho < para for the separation on polymeric β-CD coated silica stationary phases.[29–31] An elution order of para < meta < ortho had also been reported for aminophenols separated on a hypercross-linked polystyrene Chromalite 5HGN (Purolite) column in conjunction with aqueous acetonitrile mobile phases,[14] or a LiChrospher RP Select B column with a buffered aqueous methanol mobile phase.[15] Wang et al. observed an elution order of ortho < meta < para on an OTCEC column modified with a macrocyclic dioxopolyamine, using an aqueous sodium phosphate (pH 5.3) mobile phase.[16]

The elution order of ortho < meta < para for nitrophenols on the HP-β-CD column was the same as that reported on heptakis-2,3-O-dimethyl-β-CD or s-triazine modified β-CD phases.[22,23] However, the elution order on these derivatized β-CD phases is different from that reported on native β-CD stationary phases,[21,22,29,30,33] which was meta < ortho < para. This same meta < ortho < para elution order has been observed for the OTCEC separation using a column modified with a macrocyclic dioxopolyamine, with an aqueous sodium phosphate (pH 8.0) mobile phase.[16] In contrast, the order of elution observed for the reversed phase HPLC separation of nitrophenols on C-18 or polyfluorocarbon bonded silica phases was para < meta < ortho.[6,7,11,29,32,33] Most recently, the nitrophenols have been resolved on CALTREX-BI columns (based on bonded calixarenes, formed by condensation of phenol with formaldehyde to form four-, six-, or eight-member ring molecules) with water:acetonitrile mobile phases.[37] The elution order observed was ortho < meta < para, which is the same as observed with the HP-β-CD. However, with the positional isomeric nitroanilines, the elution order was meta < para < ortho on the CALTREX column.[37]

Although, positional isomeric dimethylanilines have been separated on cyclodextrin columns,[36] we are not aware of any prior reports concerning the baseline HPLC separation of 2,6-, 3,5-, and 2,4-dinitroanilines or 4-chloro-2-nitroaniline and 2-chloro-4-nitroaniline on any cyclodextrin bonded stationary phase.

The formation of guest (analyte)-host (bonded cyclodextrin) inclusion complexes is known to be the most important factor that dictates retention when using CD stationary phases with aqueous based mobile phases.[21,22,28,34,36] The magnitude of the binding interaction is determined by such factors as the analyte molecules hydrophobic association with the interior of the CD cavity, hydrogen bonding possibilities between appropriate functional groups of the guest with the hydroxyl groups present on the rim of the CD (or hydroxyl group of the hydroxypropyl moiety), and steric considerations, i.e., degree of fit between the dimensions (size) of the guest in relation to the cavity size of the cyclodextrin molecule among others.[20–22,26] The elution order should parallel the strength of the binding interaction between the analytes and the CD. For instance, binding constants reported for the interaction of nitroanilines and native (underivatized) β-CD[38] follow the order: meta ($K_b = 25\,M^{-1}$) < ortho ($K_b = 47\,M^{-1}$) < para ($K_b = 117\,M^{-1}$), which is the same as the order of elution observed in this and other studies.[21,22,25,27,32,33,36] Binding constants between β-CD and 4-nitrophenol and 3-nitrophenol were reported as $350\,M^{-1}$ and $274\,M^{-1}$, respectively,[39] which parallels the fact that the meta isomer elutes prior to that of the para for these two components.

For the separation of the cyanophenols on the native β-CD phase, the elution order was ortho < meta < para (Table 1). Binding constants for the interaction of the meta- and para-cyanophenol isomers with β-CD are reported to be 36–90 M^{-1} and 330–400 M^{-1}, respectively.[40] Thus, the observed elution order of the meta and para isomers are in agreement with this data.

However, it is important to point out that differences in elution order can be observed for analytes separated on derivatized β-CD phases compared to that observed on native β-CD phases under otherwise identical conditions.[22,36] This just reflects the fact that the replacement of the H of the hydroxyl group of the native cyclodextrin by the R functional group of the derivatized CD can alter the strength of the binding interaction.[22] Thus, the elution order observed on native β-CD stationary phases for the *o*-, *m*-, and *p*-nitrophenols is different compared to that observed on derivatized phases such as the β-hydroxypropyl ether β-CD phase in this work, or dimethylated or s-triazine modified β-CD phases by others.[22,23]

Calibration plots were constructed by plotting the peak area vs. the analyte concentration (typically in the 2.0–20.0 ppb range) using a detection wavelength of 254 nm. The specific calibration equations and parameters of merit for the substituted phenols and anilines are summarized in Table 3. Linear relationships with $R^2 > 0.99$ were obtained, in all cases, with detection limits ranging between 0.30 and 1.7 parts per billion (ppb) [the quantitation limits ranged from 0.8 to 5.7 ppb].

For comparison, for HPLC methods with UV detection, detection limits of 0.16, 0.11, and 0.05 ppm (detection at 280 nm) had been previously reported for the isomeric *o*-, *m*-, and *p*-aminophenols,[14] respectively, or 1.02 and 1.14 ppm (detection at 235 nm) for *m*- and *p*-aminophenol,[15] while for *p*-nitrophenol, a value of 0.37 ppb (monitored at 270 nm) was reported.[42] Detection limits of 36.1, 45.9, and 4.9 ppb were recently reported for *o*-, *m*-, and *p*-nitrophenol, using a spectroscopic multicomponent sequential injection method with on-line liquid-liquid extraction and preconcentration.[43] Via use of a SPME–HPLC–UV–ED method, detection limits of 3.6 (0.05) and 1.6 (0.03) ppb were reported for 4-nitrophenol and 2-nitrophenol, respectively, using UV detection at 280 nm (or electrochemical detection).[44] The detection limits of the phenolic or aniline derivatives achieved by the proposed method are as good, or better, than those reported in this prior literature, with the exception of that for *o*- and *p*-nitrophenol, as determined by the electrochemical detector.

Commercial certified standard solutions of two of the analytes were analyzed by the proposed HPLC method in order to assess its effectiveness and accuracy. Recovery from these samples was calculated by interpolating the peak areas on the calibration curves. The results (Table 4) show average

Table 3. Analytical calibration parameters for the substituted benzene analytes.

Analyte	Calibration equation coefficients[a]		Regression coefficient (R^2)	Detection limit[b] (ng/mL)	Quantitation limit[c] (ng/mL)
	$a \times 10^3$ [a]	$b \times 10^4$ [a]			
p-Aminophenol	31.7	3.82	0.9933 ($n=9$)[d]	0.78	2.6
m-Aminophenol	3.50	1.97	0.9962 ($n=21$)	1.7	5.6
o-Aminophenol	3.65	1.76	0.9937 ($n=18$)	1.7	5.7
2,6-Dinitroaniline	−102	21.5	0.9923 ($n=22$)	0.30	1.0
3,5-Dinitroaniline	349	6.06	0.9939 ($n=11$)	0.63	2.1
2,4-Dinitroaniline	−70.9	19.0	0.9975 ($n=10$)	0.23	0.76
o-Nitrophenol	−517	10.5	0.9949 ($n=18$)	0.66	2.2
m-Nitroaniline	−76.6	16.9	0.9937 ($n=15$)	0.36	1.2
o-Nitroaniline	91.5	5.41	0.9884 ($n=8$)	0.36	1.2
m-Nitrophenol	−62.6	11.4	0.9934 ($n=9$)	0.52	1.7
4-Chloro-2-nitro-aniline	149	15.8	0.9923 ($n=11$)	0.39	1.3
p-Nitrophenol	57.5	2.73	0.9940 ($n=8$)	1.1	3.7
p-Nitroaniline	−32.2	3.94	0.9922 ($n=22$)	1.2	4.1
2-Chloro-4-nitro-aniline	18.4	2.87	0.9930 ($n=22$)	1.2	3.9

[a]a and b are coefficients of the calibration regression equation of the dependence of the peak area (S) on concentration (ng/mL) of the substance (C): $S = a + b \cdot C$. The chromatographic conditions are as noted in the experimental section for gradient runs; detection wavelength 254 nm.

[b]Limit of detection (LOD); calculated as $3(1/b)\,(S_{R,c})\,[(n-2)/(n-1)]^{1/2}$, where b is the slope of the regression equation, $S_{R,c}$ the error of the estimate, and n is the number of points in the calibration equation (41).

[c]Limit of quantification; calculated as the LOD except that the 3 in the numerator is replaced by 10 (41).

[d]Number in parenthesis indicates the number of data points upon which the calibration equation was based.

Table 4. Recovery data from analysis of commercial calibration standard solutions.

Analyte	Known concentration (ng/mL)	Amount found (ng/mL)	Recovery (%)	Mean (%)
2-Nitrophenol	5.00	4.82	96.4	
($k' = 6.5$)	5.00	4.78	95.6	96.3
	5.00	4.85	97.0	
2-Nitroaniline	5.00	4.62	92.4	
($k' = 12.2$)	5.00	4.44	88.8	92.3
	5.00	4.78	95.6	

recoveries of 96% (RSD = 0.8%) for *o*-nitrophenol and 92% (RSD = 3.7%) for 4-nitroaniline; while the relative errors were 3.6% and 7.8%, respectively.

CONCLUSIONS

The use of the *R*,*S*-hydroxypropyl ether β-cyclodextrin allows for the direct, and simultaneous, gradient elution reversed phase liquid chromatographic separation and determination of the fourteen positional isomeric anilines and phenols examined with good selectivity and sensitivity. The method should be applicable to the analysis of these components in a variety of samples.

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