Heliyon 5 (2019) e02978

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Improvised method for urinary *p*-cresol detection and measurement using high performance liquid chromatography/mass spectrometry

E. Nandini [a], B. Arunraj [b], N. Rajesh [b], Vidya Rajesh [a,*]

[a] Department of Biological Sciences, Birla Institute of Technology and Science, Pilani-Hyderabad Campus, Hyderabad, 500 078, India
[b] Department of Chemistry, Birla Institute of Technology and Science, Pilani-Hyderabad Campus, Hyderabad, 500 078, India

ARTICLE INFO

Keywords:
Analytical chemistry
Biochemistry
Metabolite
HPLC
LC-MS
Urinary *p*-cresol

ABSTRACT

Gut microbiota has been implicated in many disorders including Autism Spectrum Disorder (ASD). ASD is a neurodevelopmental brain disorder affecting individuals leading to restricted and repetitive pattern of behaviour and disruption of communication and social interactions. Altered microbiome and the presence or absence of key species capable of affecting specific responses in levels of their fermentation products are reflected in the urinary metabolite profile of patients. The aim of our study is to develop an improvised method for the detection and quantification of urinary *p*-cresol levels which could serve as an indicator for GI microbial dysbiosis. The *p*-cresol analysis was achieved using HPLC by a reverse phase C18 column with mobile phase composition of Acetonitrile/water/formic acid (10:90:0.05, v/v/v) in an isocratic mode of elution with a flow rate of 1.0 mL/min. The mass analysis of *p*-cresol was performed using LC-MS [Triple Quadrupole Liquid Chromatography Mass Spectrometer] in negative ESI mode with electron multiplier detector. *p*-cresol was eluted at a retention time of approximately 3.4 min. The standard calibration curves had a superior regression coefficient of greater than 0.99 ($R^2 > 0.99$) and were linear over a range from 0.0005 mg/mL to 0.015 mg/mL. The method was validated by analysis of six replicates with 0.08% relative standard deviation and method detection and quantification limits were 20 ng/mL and 50 ng/mL respectively. Further validation of method on real urine samples from two groups of children (Control population:< 10 years of age; 5M: 3F and ASD individuals: <10 years of age; All males) showed that detection was effective over a wide range of metabolite at levels as high as 149.73 μg/mL to as low as 0.897 μg/mL. This study reports a rapid, validated and sensitive method for the detection of *p*-cresol in urine samples.

1. Introduction

Interest toward the human microbiome – the diverse and niche-specialized microbial communities that develop in the human host, particularly, the gut microbiome has increased in the recent past and their role in disease aetiologies have gained attention. The interactions between gut microbiota and the host have helped in understanding their roles and their unique metabolites and their implications in metabolism, development and homeostasis of immunity and the nervous system. Perturbation to the composition and function of the gut microbiota (gut microbial dysbiosis) may lead to dysfunction of host systems, thereby contributing to pathogenesis and/or progression toward a broad spectrum of conditions like inflammatory bowel disease, celiac disease, colorectal cancer, obesity, diabetes and Autism Spectrum Disorder (ASD) [1, 2, 3, 4, 5]. ASD is a collection of various neurodevelopmental disorders which comprises Autism Disorder (AD), Asperger syndrome (AS) and Pervasive developmental disorder (PDD) that begins early in the childhood [6]. Disturbances in multiple human systems including gastrointestinal disturbances are reported in the multifactorial manifestations of ASD symptoms [7].

The human gut is inhabited by a complex and metabolically active microbial ecosystem which contributes to the intestinal metabolites of hosts, thus influencing the metabolome. Intestinal metabolites are shown to affect various physiological processes, including energy metabolism, cell-to-cell communication, and host immunity [8]. Altered GI microbiota and the alteration in levels of their fermentation products based on the presence and absence of key microbes needs attention. Gut dysbiosis and difference in the metabolite profiles are likely to be reflected in the bio fluids like urine. Detection and measurement of gut metabolites in urine and other bio fluids becomes critical.

One such metabolite is *p*-cresol (C_7H_8O: 4 - methylphenol), an organic aromatic compound with a molecular weight of 108 kDa, which is produced by the gut microbes and has been implicated in neurological conditions like Autism Spectrum Disorder (ASD). It is

* Corresponding author.
E-mail address: vidya_nrajesh@yahoo.com (V. Rajesh).

https://doi.org/10.1016/j.heliyon.2019.e02978
Received 21 October 2019; Received in revised form 13 November 2019; Accepted 29 November 2019

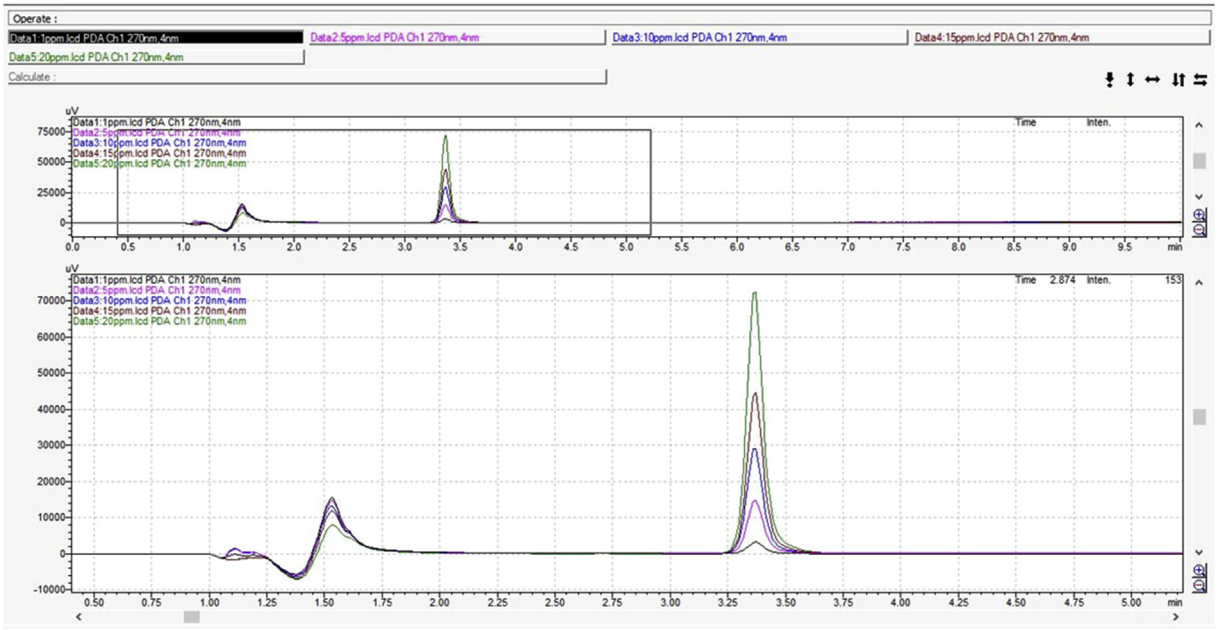

Figure 1. Comparison of *p*-cresol standards at different concentrations.

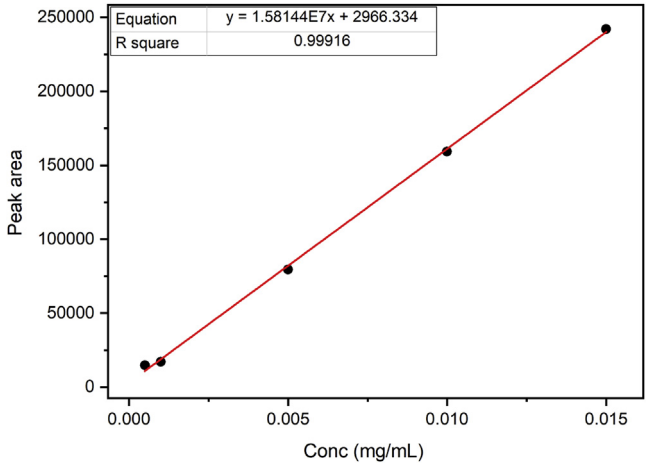

Figure 2. Linearity standard calibration curve of *p*-cresol.

hypothesized that *p*-cresol may contribute to worsen autism severity and gut dysfunction, often present in autistic children [2]. *p*-cresol is one of the metabolites of the amino acid tyrosine, and to a certain extent also of phenylalanine, which is converted to 4-hydroxyphenylacetic acid by intestinal bacteria, before being decarboxylated to *p*-cresol (putrefaction) [9]. The synthesis of *p*-cresol can happen in two distinct pathways in the gut microbiome. *Clostridium difficile* which is an anaerobic spore-forming bacteria expresses an enzyme namely *p*-hydroxy phenyl acetate decarboxylase which causes conversion of tyrosine to *p*-cresol [2]. Also, *Pseudomonas stutzeri, Pseudomonas mendocina* etc. produces *p*-cresol from toluene by toluene monooxygenase [3, 4]. The former pathway is significantly more important compared to the latter due to the increased availability of L-tyrosine when compared to toluene. *p*-cresol is metabolized through conjugation [10, 11] removal of the unconjugated *p*-cresol is, at least in part, via the urine [12]. Zeng et. al., (2017), have reported the detection of *p*-cresol levels and other metabolites in rat serum and urine [13].

However according to recent studies, the frequency of GI tract symptoms and affected mechanisms in children with ASD has attracted attention [14, 15, 16, 17, 18, 19] and ranges from 9% - 84%. A number of studies in a subgroup of ASD patients have documented the overgrowth of an unusual microflora population namely the *Clostridium* species. A study by Altieri et. al., (2011), observed an elevated level of *p*-cresol metabolite in urine samples of young children with ASD compared with control [20]. They also reported a positive correlation between *p*-cresol and ASD severity. *p*-cresol has stimulated interest as a biomarker with a potential to contribute to a multi-biomarker diagnostic panel for ASD [2]. Hence, urinary *p*-cresol as a metabolite, is important for conditions like ASD and its detection and evaluation merits attention.

In this communication, we propose an effective method for urinary *p*-cresol detection and measurement with validation for urinary levels of *p*-cresol using control (<10 years of age; 5 Males: 3 Females) and ASD samples (<10 years of age; All males) from representative Indian population.

2. Materials and methods

2.1. Chemicals and reagents

Standard GI metabolite *p*-cresol was procured from Sigma-Aldrich. Milli-Q water (MQW) was used for the preparation of all the solutions. All HPLC grade of Acetonitrile, Methanol, Trifluoroacetic acid, Diethylether were purchased from Sisco Research Laboratories Pvt. Ltd. India. Analytical grade Formic acid was procured from Sisco Research Laboratories Pvt. Ltd. India. Sodium hydroxide was obtained from Merck Specialities Private Limited, India.

2.2. Collection of urine samples from ASD and control population

Early morning urine samples were collected from neuro-typical, high functioning, normal, faculty children at BITS, Pilani-Hyderabad campus after getting a signed informed consent from the parent of the children. This acted as the normal control population for the study. Similarly, patient samples were collected from the "Autism Ashram" Center at Keshavaram,

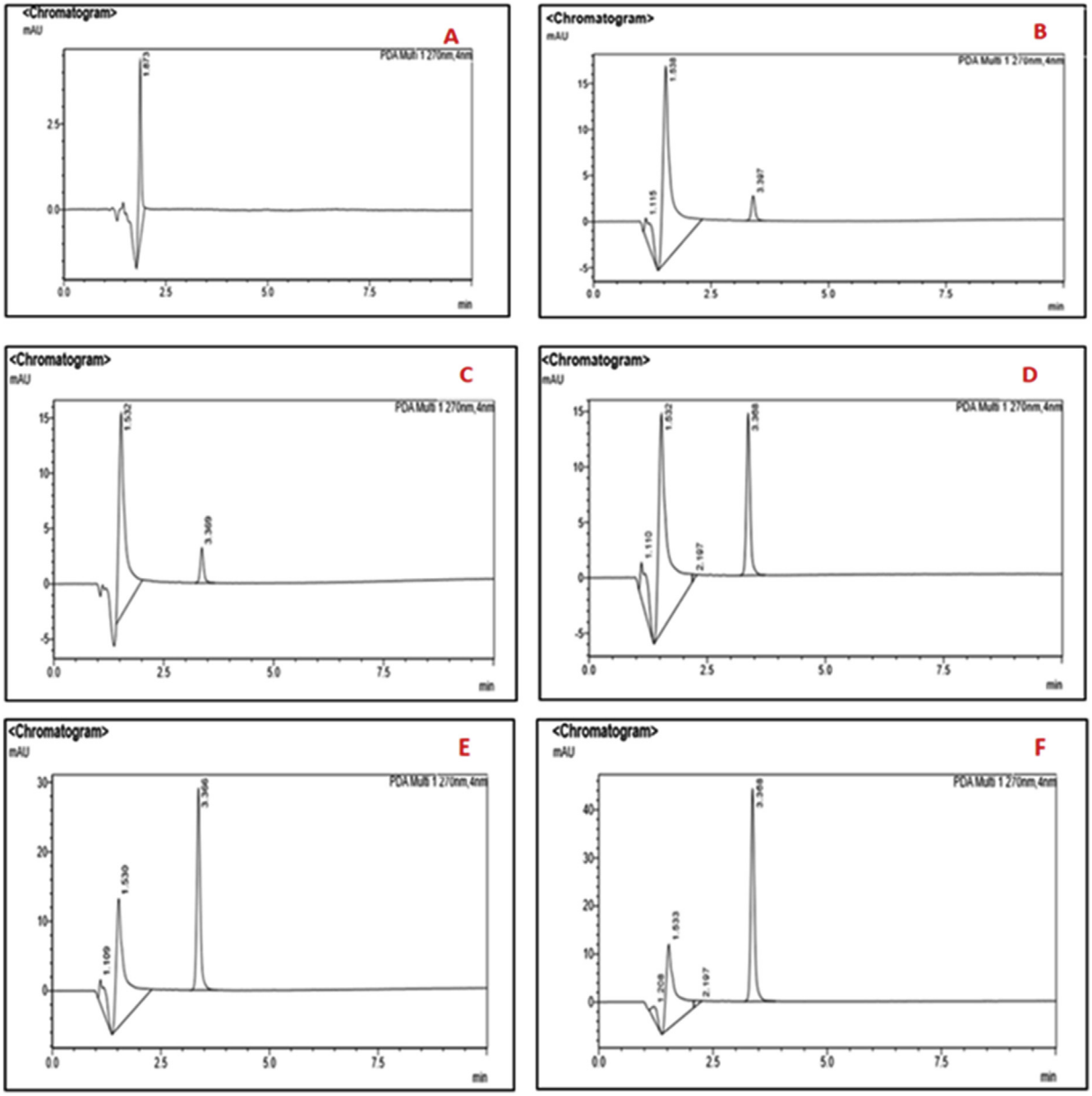

Figure 3. A- Blank; B-F- *p*-cresol standards at 0.0005 mg/mL, 0.001 mg/mL, 0.005 mg/mL, 0.01 mg/mL, 0.015 mg/mL concentration respectively with PDA detection at 270 nm (PDA Multi 270 nm,4 nm) with a retention time of approximately 3.4 min.

Table 1. Evaluation of concentration versus peak area using HPLC for the *p*-cresol standards in improvised method.

S.NO	Concentration (mg/mL)	Peak area	Retention time (mins)
1	0.0005	14845	3.397
2	0.001	17087	3.369
3	0.005	79531	3.368
4	0.010	159350	3.366
5	0.015	242172	3.368

Hyderabad, India. In both controls and ASD children, early morning urine sample was taken for analysis. The ASD children were on vegetarian Jain food diet (no onion, garlic and less spice simple nutritious food) on a regular basis. The control children had normal Indian diet. Sample size of four children diagnosed with ASD (<10 years of age; All males) and 8 controls (<10 years of age; 5 Males: 3 Females) each were considered and their urine samples were used for validation of the developed method. Labelled vials were provided for the urine sample collection. A sample amount of 10–15 mL was collected and reproducibility of the results was also confirmed. Proper care was taken to follow all ethical and standard guidelines for the collection and storage of urine samples. The study was approved by the Institutional Human Ethics Committee (BITS-HYD/IHEC/2019/01). The collected urine samples were stored at -20 °C in order to minimize the variations arising during the collection.

2.3. Sample preparation for the determination of urinary p-cresol levels in normal and ASD individuals

The samples were taken from -20 °C and thawed for further sample processing. To 1.0 mL of the urine sample in the glass vials, 400 µL of

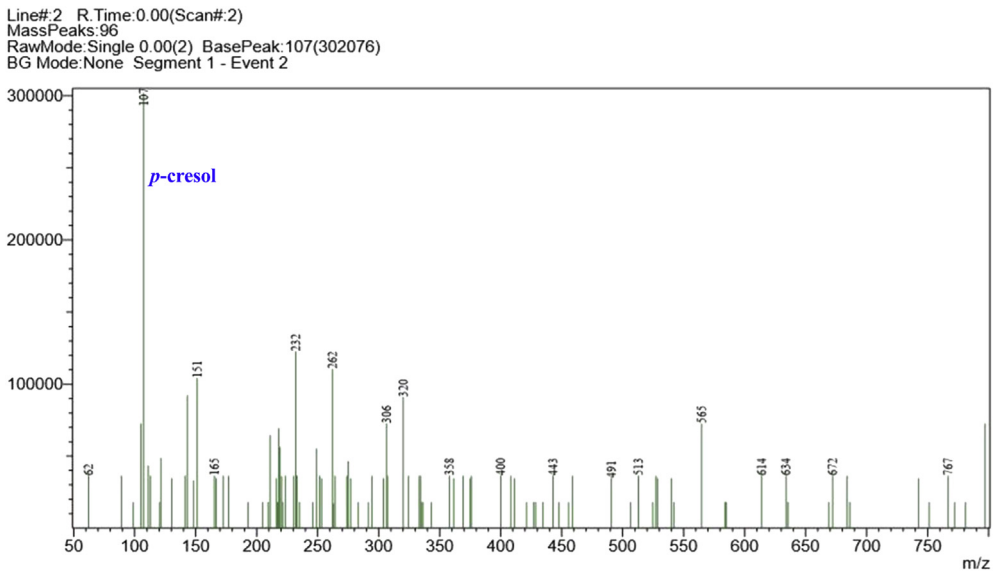

Figure 4. LC-MS fragmentation pattern of *p*-cresol with major m/z of 107.

concentrated hydrochloric acid was added. The above mixture was boiled at 90 °C for 60 min [21]. The mixture was cooled down and the phenols were extracted with 800μL of diethylether. It was mixed by repeated inversions in glass vials for 1 min. This was then centrifuged at 500 g for 10 min (Eppendorf centrifuge 5430R). After the clear distinction of the aqueous and organic phase, the organic phase was transferred into 2 mL amber glass HPLC vials. Later the organic phase was aspirated into 600 μL of 0.05M NaOH in methanol. The resultant solution was evaporated to dryness under vacuum condition at 45 °C. The residue was dissolved in 1 mL MilliQ water, filtered by syringe filter (PVDF syringe filter 13mm, 0.45 μm, Axiva) and analysed to HPLC system for *p*-cresol determination.

2.4. Instrumentation

The *p*-cresol was measured by High performance liquid Chromatography (HPLC) connected with Photo Diode Array (PDA) detection. C18, 5μm particle size (Sigma, Ascentis, ODS, 150 × 4.6mm) column was used in HPLC Shimadzu Corporation system (MODEL NO. CTO-10ASVP). The column temperature was maintained at 28 °C with a UV detection wavelength of 270nm. The mobile phase acetonitrile/water/formic acid (10:90:0.05, v/v/v) was prepared. The mobile phase was filtered through 0.22μm membrane filter and sonicated to remove air bubbles. The HPLC was performed under isocratic mode of elution with a flow rate of 1.0 mL/min. The mass analysis of the standard was performed using LC-MS [LCMS-8040, Triple Quadrupole Liquid Chromatography Mass Spectrometer, SHIMADZU Corporation] in ESI mode. The liquid chromatograph had an inbuilt on-line degasser, UFLCXR autosampler, a binary gradient elution pumping system and an electron multiplier detector. All parameters were controlled by LabSolutions LCMS software, version 5.4 (SHIMADZU Corporation).

2.5. Preparation of standard solutions

The stock standard solution of *p*-cresol was prepared at a concentration of 1.0 mg/mL using acetonitrile/water (5:95, v/v) and stored at 4 °C. Further working standard solution of 0.0005 mg/mL, 0.001 mg/mL, 0.005 mg/mL, 0.01 mg/mL, 0.015 mg/mL were prepared from stock standard solution using acetonitrile/water (5:95, v/v). The standard solution was filtered using 0.45μm syringe filter and filled in amber vials for HPLC analysis. 30 μL of each linearity standard solution was injected into HPLC. These working standard solutions were used to check the detection of the compound at various concentration by HPLC and also to determine the linearity range of compound from the regression coefficient value in standard calibration plot. The standard calibration curve was developed by plotting the peak areas of *p*-cresol against the set of concentration used for standard preparation.

3. Results and discussion

3.1. Method development and standardization for the detection of p-cresol levels using HPLC

Different concentration of *p*-cresol analysis was performed using a C18 column, 5μm particle size (Sigma, Ascentis, ODS, 150 × 4.6mm) in HPLC Shimadzu Corporation system. PDA detection for *p*-cresol was done at wavelength 270nm. The retention time was approximately 3.4 min (Figure 1). The standard calibration curve was developed by plotting the peak areas of *p*-cresol against the set of concentration used for standard preparation, to obtain a standard curve equation $y = mx + c$ with a regression value of $R^2 = 0.999$ (Figures 2 and 3). Here, 'y' corresponds to the peak area and 'x' corresponds to the set of concentrations used for standard development. Table 1 shows the concentration of *p*-cresol versus the peak area using HPLC with PDA detection at 270 nm. The method was successfully used for the detection of *p*-cresol at various concentrations using HPLC. The LOD (Limit of detection) and LOQ (limit of Quantification) of *p*-cresol was found to be 20 ng/mL and 50 ng/mL respectively.

Limit of detection (LOD-3σ) was calculated as three times the standard deviation of the lowest concentration by the slope (m) of the calibration curve. Limit of quantification (LOQ-10σ) was determined as 10 times the standard deviation of the lowest concentration by the slope (m) of the calibration curve.

3.2. Mass spectrometry of standard p-cresol

Standard *p*-cresol was analysed using Mass spectrometry in ESI (Electron Spray Ionization) mode with Electron multiplier detector. The

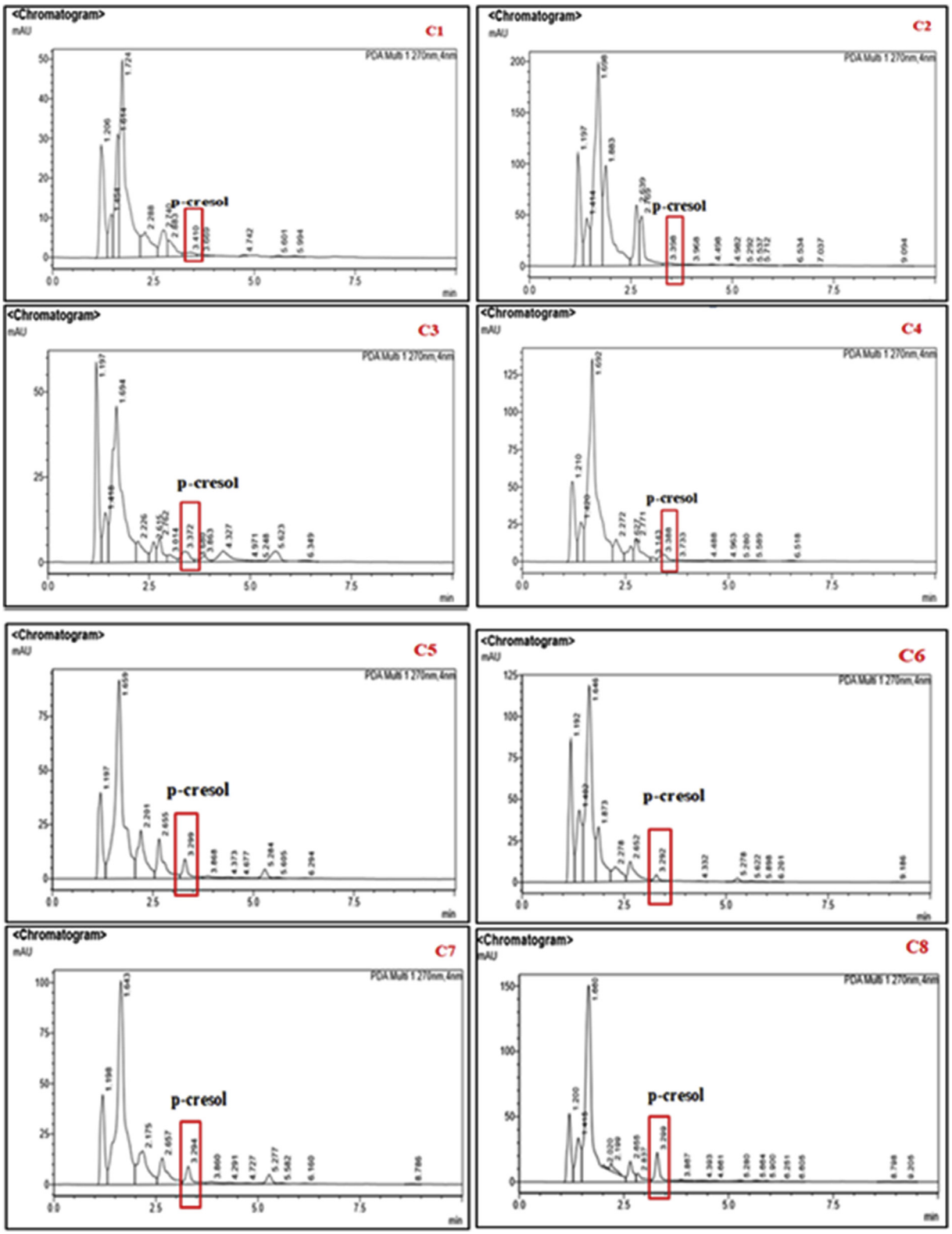

Figure 5. *p*-cresol detection (PDA Multi 1 270 nm,4 nm) and quantification in normal control urine sample with a retention time of approximately 3.4 min. (C1 to C8 are control urine samples from children of the age group between 3-9).

analysis in mass spectrometry is usually done in both positive as well as the negative mode of ESI ionization where the ionic form of the compound that is either protonated $[M\text{-}H]^+$ or deprotonated form $[M\text{-}H]^-$ is obtained [13]. The organic compound *p*-cresol (m/z = 108) [22] is deprotonated and is obtained in the negative mode of ESI with an m/z ratio of 107 (C_7H_7O) (Figure 4). This acted as the reference for the detection of *p*-cresol in urine samples.

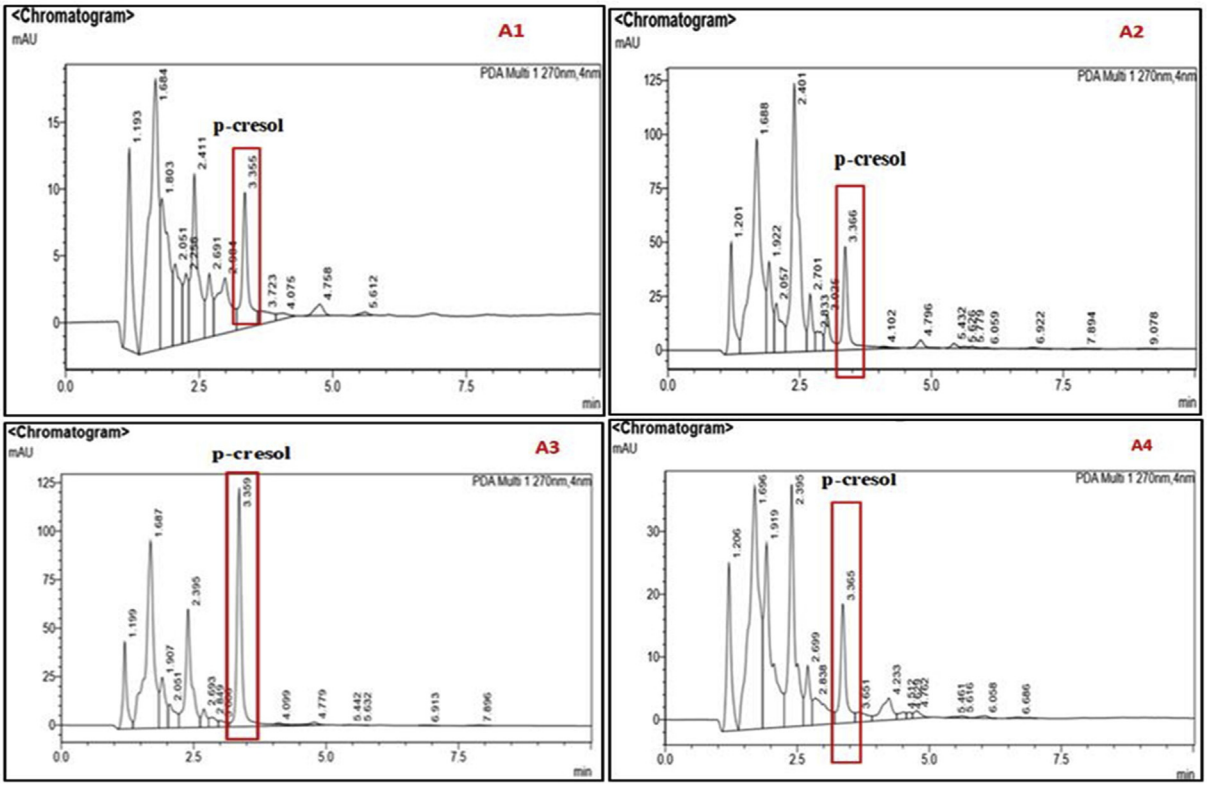

Figure 6. *p*-cresol detection (PDA Multi 1 270 nm,4 nm) and quantification in Autistic urine samples with a retention time of approximately 3.4 min. (A1-A4 are urine samples of children with ASD from the age group between 8-9).

3.3. Validation of the method for urinary p-cresol detection in normal and ASD individuals

After the successful optimization of the proposed method, the same set of HPLC parameters as that of the standard was followed for real urine samples. Biological fluid such as urine was used to check the validation of *p*-cresol detection due to its complexity caused by the interference of other metabolites. The processed urine samples of both normal and autistic population were analysed by HPLC system for detection of *p*-cresol. It was confirmed that *p*-cresol is present in the urine sample based on the retention time of the standard. It was further validated by LC-MS analysis which confirmed the presence of *p*-cresol based on reference standards. Hence after the completion of analysis, the peak area, for both normal and ASD samples, was integrated (Figure 5 and 6). Table 2 depicts the concentration of urinary *p*-cresol obtained in normal and autistic individuals from the Indian population.

Table 2. Urinary *p*-cresol peak area and concentration in normal and autistic individuals.

Nature of the sample	Sample identifier	Age/Sex	Peak area	*p*-cresol concentration (μg/mL)
Control	C1	7 years, Female	17586	0.897
	C2	3 years, Female	56256	2.812
	C3	9 years, Female	26007	1.300
	C4	5 years, Male	32390	1.620
	C5	9 years, Male	85520	4.270
	C6	7 years, Male	29762	1.488
	C7	8 years, Male	47967	2.390
	C8	9 years, Male	123040	6.152
Children with ASD	A1	8 years, Male	60232	3.011
	A2	9 years, Male	71663	35.830
	A3	9 years, Male	299469	149.730
	A4	9 years, Male	89305	4.460

3.4. Mass spectrometry of urinary p-cresol

Urine samples were analysed by Mass spectrometry in ESI (Electron Spray Ionization) mode with electron multiplier detector to detect the presence of urinary *p*-cresol. *p*-cresol is obtained in the negative mode of ESI with an m/z ratio of 107 as that of the standard (Figure 7). This confirms the presence of *p*-cresol in both normal control population as well as the autistic population.

Based on the results obtained, the proposed method was successfully able to detect and quantify urinary *p*-cresol levels in human urine samples. The efficiency of the method was validated by evaluating the levels of urinary *p*-cresol between ASD and control population in India. As the range of detection was broad, it was able to distinguish urinary *p*-cresol levels as high as 149.73 μg/mL to as low as 0.897 μg/mL. Elevated levels of urinary *p*-cresol as seen in patient 2 and 3 were in accordance with previously published report [16]. The method was further validated by using validation parameters such as Linearity, LOD, LOQ etc.

3.5. Validation parameters for the proposed method

The ICH (International Council for Harmonisation) guidelines recommend evaluating a minimum of five concentrations in order to determine the linearity range. Calibration plots were constructed for five different concentrations of *p*-cresol and it was linear over a range from 0.5 mg/L to 15 mg/L with a regression coefficient of always R^2>0.99. The repeatability of the proposed method was ascertained by injecting six replicates of known sample concentration and determining the variations in peak area, retention time and peak height in percentage Relative Standard deviation (%RSD). To ensure the precision of the

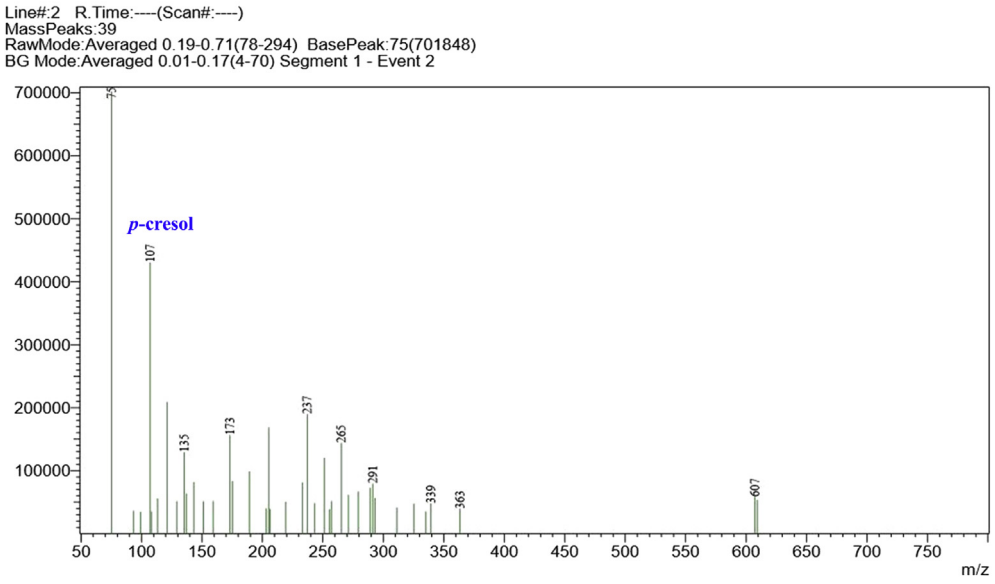

Figure 7. LC-MS fragmentation pattern of *p*-cresol in autistic urine sample.

Table 3. System Suitability Test parameters for the improved method.

SST parameter	Acceptable range	Results
Repeatability (% RSD)	<1	Retention time: 0.077 Peak Area: 0.081 Peak height: 0.403
Plate count (N)	>2000	7500–7600
Tailing factor (T_f)	<2.0	1.1–1.2

method, %RSD should be < 2. The precision of the proposed method was always less than %RSD <0.5. Limit of detection is defined as the lowest concentration of the analyte which can be detected but not necessarily quantified. It was determined to be 20 ng/mL. Limit of Quantification is defined as the lowest amount of the analyte which can be quantified with accuracy and precision. It was determined to be 50 ng/mL. Systems suitability parameters are the most integral part of any given analytical procedure. The ideology is that the analytical operations, equipment and sample play a crucial role in the system. The primary SST parameters are % RSD, Plate count (N), Tailing factor (T_F). The acceptable range for each parameter is shown in Table 3. All the SST parameters for the proposed method were found within range of guidelines from FDA's Center for Drug Evaluation and Research (CDER) [13].

3.6. Comparison with previously reported methods

The proposed method was compared with previous reports and the comparative parameters are listed in Table 4. The method developed uses a unique combination of solvent i.e. Acetonitrile, water and formic acid, not reported earlier in any study. In the isocratic mode, earlier methods developed by, Birkett et. al. (1995) [21], Schlatter et al. (1995) [23], Yoshikawa et. al. (1986) [24] had run times of 40, 20 and 16 min respectively while the proposed method had a greater efficiency with a run time of 10 min only. An earlier report from Lee et. al. (2009) [25] in the binary gradient mode had a low run time of 5 min, however, the LOD/LOQ outcomes of the developed method in this study were better. Comparative analysis of LOD/LOQ across previously reported methods in Isocratic/Binary gradient modes, clearly indicate the proposed method to be superior.

4. Conclusions

This study reports a rapid, validated and sensitive method for the detection of *p*-cresol. The efficiency of the method was validated by detection and quantification of urinary *p*-cresol levels between normal and autistic individuals. With six replicates, using acetonitrile (ACN), water, formic acid as eluents under isocratic mode, the LOD and LOQ were found to be 20 ng/mL and 50 ng/mL respectively. Urine being a complex biological fluid, the addition of internal standard and performing the validation on fortified urine would have helped judge the precision of the method better. Definitive conclusions cannot be arrived for levels of *p*-cresol between control and ASD samples, due to the smaller number of samples analysed in the Indian population and the intent was only for the validation of the developed method. Direct outcomes on *p*-cresol levels between controls and ASD patients need more follow up studies and remains inconclusive.

Table 4. Comparison of the improvised method with other reported method.

S.No	Compound	Sample Type	Mobile phase composition	Run time (min)	LOD, LOQ	Mode of elution	Ref
1	*p*-cresol	Human urine	ACN/H_2O/Formic acid	10	20 ng/mL, 50 ng/mL	Isocratic	Present work
2	*p*-cresol	Human urine	ACN/H_2O/TFA	25	20 ng/mL, 70 ng/mL	Binary gradient	[20]
5	*p*-cresol	Urine/faeces	Methanol/Phosphate buffer	40	0.8 μg/mL, N.A*	Isocratic	[21]
3	*p*-cresol	Human Urine	ACN/H_2O/Phosphoric acid	20	0.2 μg/mL, N.A*	Isocratic	[23]
4	*p*-cresol	Human urine	ACN/H_2O/β-cyclodextrin	16	15 ng/mL, N.A*	Isocratic	[24]
6	*p*-cresol	Human urine	Ethanol/acetic acid/β-cyclodextrin	5	0.8 μg/ml, N.A*	Binary gradient	[25]

Declarations

Author contribution statement

Vidya Rajesh: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

E Nandini: Performed the experiments; Wrote the paper.

B Arunraj: Analyzed and interpreted the data; Wrote the paper.

N Rajesh: Conceived and designed the experiments; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors acknowledge the Central Analytical Laboratory, BITS Pilani, Hyderabad Campus, India for providing the analytical instrumentation facilities throughout this work.

References

[1] Z.Y. Kho, S.K. Lal, The human gut microbiome - a potential controller of wellness and disease, Front. Microbiol. 9 (2018) 1–23.

[2] A.M. Persico, V. Napolioni, Urinary *p*-cresol in autism spectrum disorder, Neurotoxicol. Teratol. 36 (2013) 82–90.

[3] V. Cafaro, E. Notomista, P. Capasso, A. Di Donato, Mutation of glutamic acid 103 of toluene o-xylene monooxygenase as a means to control the catabolic efficiency of a recombinant upper pathway for degradation of methylated aromatic compounds, Appl. Environ. Microbiol. 71 (2005) 4744–4750.

[4] G.M. Whited, D.T. Gibson, Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to *p*-cresol in Pseudomonas mendocina KR1, J. Bacteriol. 173 (1991) 3010–3016.

[5] J. Durack, S.V. Lynch, The gut microbiome: relationships with disease and opportunities for therapy, J. Exp. Med. 216 (2019) 20–40.

[6] L. Wing, The Autism Spectrum: A Guide for Parents and Professionals, Constable Publications, London, 1996.

[7] C.J. McDougle, C.A. Erickson, K.A. Stigler, D.J. Posey, Neurochemistry in the pathophysiology of autism, J. Clin. Psychiatry 66 (2005) 9–18.

[8] Z. Li, G. Quan, X. Jiang, Y. Yang, X. Ding, D. Zhang, X. Wang, P.R. Hardwidge, W. Ren, G. Zhu, Effects of metabolites derived from gut microbiota and hosts on pathogens, Front. Cell. Infect. Microbiol. 8 (2018) 314.

[9] H.C. Curtius, M. Mettler, L. Ettlinger, Study of the intestinal tyrosine metabolism using stable isotopes and gas chromatography-mass spectrometry, J. Chromatogr. A 126 (1976) 569–580.

[10] T. Niwa, K. Maeda, T. Ohki, A. Saito, K. Kobayashi, A gas chromatographic-mass spectrometric analysis for phenols in uremic serum, *Clinica chimica acta*, Int. J. Clin. Chem. 110 (1981) 51–57.

[11] N. Ogata, N. Matsushima, T. Shibata, Pharmacokinetics of wood creosote: glucuronic acid and sulfate conjugation of phenolic compounds, Pharmacology 15 (1995) 195–204.

[12] B.A. Geypens, D. Claus, P. Evenepoel, M. Hiele, B. Maes, M. Peeters, Y. Ghoos, Influence of dietary protein supplements on the formation of bacterial metabolites in the colon, Gut 41 (1997) 70–76.

[13] Y. Zeng, L. Luo, W. Hou, B. Lu, J. Gong, J. Chen, X. Zhang, B. Han, Z. Xie, Q. Liao, Targeted metabolomics analysis of aromatic amino acids and their gut microbiota–hosts cometabolites in rat serum and urine by liquid chromatography coupled with tandem mass spectrometry, J. Sep. Sci. 40 (2017) 3221–3230.

[14] T. Buie, D.B. Campbell, G.J. Fuchs, G.T. Furuta, J. Levy, J. Vandewater, A.H. Whitaker, D. Atkins, M.L. Bauman, A.L. Beaudet, E.G. Carr, M.D. Gershon, S.L. Hyman, P. Jirapinyo, H. Jyonouchi, K. Kooros, R. Kushak, P. Levitt, S.E. Levy, J.D. Lewis, K.F. Murray, M.R. Natowicz, A. Sabra, B.K. Wershil, S.C. Weston, L. Zeltzer, H. Winter, Evaluation, diagnosis, and treatment of gastrointestinal disorders in individuals with ASDs: a consensus report, Pediatrics 125 (Supplement 1) (2010) S1–S18.

[15] C. Black, J.A. Kaye, H. Jick, Relation of childhood gastrointestinal disorders to autism: nested case-control study using data from the UK General Practice Research Database, BMJ 325 (2002) 419–421.

[16] K. Horvath, J.A. Perman, Autistic disorder and gastrointestinal disease, Curr. Opin. Paediatr. 14 (2002) 583–587.

[17] S.E. Levy, M.C. Souders, R.F. Ittenbach, E. Giarelli, A.E. Mulberg, J.A. Pinto-Martin, Relationship of dietary intake to gastrointestinal symptoms in children with autistic spectrum disorders, Biol. Psychiatry 61 (2007) 492–497.

[18] L. Qinrui, H. Ying, B.C.D. Angel, J.H. Randi, The gut microbiota and autism spectrum disorders, Front. Cell. Neurosci. 11 (2017) 120.

[19] W. Jolanta, K. Mark, Gastrointestinal symptoms and autism spectrum disorder: links and risks – a possible new overlap syndrome, Pediatr. Health Med. Therapeut. 6 (2015) 153–166.

[20] L. Altieri, C. Neri, R. Sacco, P. Curatolo, A. Benvenuto, F. Muratori, R. Rigardetto, Urinary *p*-cresol is elevated in small children with severe autism spectrum disorder, Biomarkers 16 (2011) 252–260.

[21] A.M. Birkett, G.P. Jones, J.G. Muir, Simple high-performance liquid chromatographic analysis of phenol and *p*-cresol in urine and feces, J. Chromatogr. B Biomed. Sci. Appl. 674 (1995) 187–191.

[22] R. Vanholder, R. De Smet, G. Lesaffer, *p*-cresol: a toxin revealing many neglected but relevant aspects of uraemic toxicity, Nephrol. Dial. Transplant. 14 (1999) 2813–2815.

[23] J. Schlatter, A. Astier, Rapid determination of o- and *p*-cresol isomers in urine from workers exposed to toluene bv *u* high-performance liquid chromatography using a graphitized carbon column, Biomed. Chromatogr. 9 (1995) 302–304.

[24] M. Yoshikawa, Y. Taguchi, K. Arashidani, Y. Kodama, Determination of cresols in urine by high-performance liquid chromatography, J. Chromatogr. A 362 (1986) 425–429.

[25] C.W. Lee, J. Lee, J. Lee, H.Y. Eom, M.K. Kim, J.H. Suh, H. Yeom, U. Kim, J.R. Youm, S.B. Han, Rapid HPLC method for the simultaneous determination of eight urinary metabolites of toluene, xylene and styrene, Bull. Korean Chem. Soc. 30 (9) (2009) 2021–2026.