Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.63222

Quantitative determination of biphenyls and their metabolites in cell cultures of *Comamonas thiooxydans* N1 using high-performance liquid chromatography

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Abstract

Biphenyl-degrading bacteria were isolated from contaminated soil and identified as *Comamonas* species. Among these strains, *C. thiooxydans* N1 was the most suitable for biphenyl degradation. When 1200 μg/mL of biphenyl was used, it was completely degraded within 24 h. A simple, rapid, and precise, high-performance liquid chromatography (HPLC) method for quantifying biphenyls and their metabolites in the culture medium was optimised and validated. The proposed method was performed using reverse-phase HPLC equipped with a Luna C18 column. The mobile phase was water and acetonitrile (30/70, v/v) under isocratic conditions, and biphenyls and their metabolites were quantified on a SHIMADZU HPLC system with a diode-array detection detector at 254 nm. Peak identity was confirmed by ultraviolet spectroscopy and gas chromatography-mass spectrometry. Validation was performed according to European Union requirements (2002/657/EC) for linearity, accuracy, repeatability, reproducibility, limit of detection, limit of quantification, and detection capability. Linearity was observed in the concentration range of 0.04-20 μg/mL with a high coefficient of determination ($R^2 \geq 0.999$). The validated method was applied to determine the content of biphenyl and its metabolites in the culture medium.

Keywords: biphenyl-degrading bacteria; 2,3-dihydroxy-biphenyl; high-performance liquid chromatography; method validation; *Comamonas thiooxydans*.

Practical Application: A validated HPLC method can be used to rapidly quantify biphenyl and their metabolites.

1 Introduction

Hydrophobic organic pollutants, biphenyls, and their metabolites are serious environmental issues. Biphenyls and their metabolites, most of which are toxic aromatic hydrocarbons, tend to persist in the environment for a long time (Pieper, 2005; Sharma et al., 2014). Furthermore, they have been reported to cause several adverse health effects (Aoki, 2001; Faroon et al., 2000). Biphenyl chemicals have been used in various commercial and industrial applications (Abraham et al., 2002; Pieper & Seeger, 2008). Polychlorinated biphenyls (PCBs) have been shown to cause tumours in laboratory animals following high-dose lifetime exposure (Cogliano, 1998; Robertson & Ludewig, 2011; Liu et al., 2010). Many studies consider PCBs as recalcitrant toxic compounds because of their chemical stability, poor water solubility, and toxicity (Quinete et al., 2014). These deleterious effects on humans have motivated the search for methods to remove these organic pollutants from contaminated soil. For instance, PCBs are a common contaminant of agricultural crop soil fertilised over a long term with organic waste (Antolín-Rodríguez et al., 2016). Further, four strains of biphenyl-degrading bacteria were isolated from sewage and identified to belong to genera *Rhodococcus* and *Aquamicrobium* (Chang et al., 2013). Thus, it is necessary to screen strains that have a superior ability to degrade biphenyl, PCBs, and their intermediates. Several biphenyl-utilising bacteria have been isolated and characterised in terms of the ability to degrade PCBs, including both gram-negative and gram-positive bacteria (Bedard & Haberl, 1990; Kim et al., 1996). Biphenyl can be mineralised under aerobic conditions by either a single microorganism or a microbial consortium. The gram-negative bacterium *C. testosteroni* TK102, which was isolated from soil contaminated with PCBs, has the ability to degrade high concentrations of a commercial PCB mixture (Fukuda et al., 2014). Biological degradation of PCBs is an attractive clean-up strategy because it is environmentally friendly and cost-effective (Ohtsubo et al., 2004). The complete biphenyl catabolic pathway includes the biphenyl upper pathway (transformation of biphenyl/PCBs into benzoate/chlorobenzoates and aliphatic acids) and biphenyl lower pathway (Aken et al., 2010).

To date, the analysis and characterisation of biphenyl and its metabolites from culture medium have relied mainly on gas chromatography (GC)-based methods. However, the use of GC techniques for PCB separation has many difficulties, including the instability of derivatised compounds (Sarkhosh et al., 2012). A gas chromatography-mass spectrometry (GC-MS) method that does not require derivatisation was thus developed.

PCB quantitation in environmental samples has been reported, including sampling techniques, extraction, and clean-up procedures. Selected ion monitoring MS or ion-trap MS detectors have sensitivities lower than those of electron capture detection but greater selectivity for PCBs, and can distinguish

Received 27 Apr., 2022
Accepted 27 June, 2022
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and individually measure homologs that may be co-eluted on high-resolution GC columns (United States Environmental Protection Agency, 1999).

Previously, cell cultures of *Comamonas* spp. were reported to contain both biphenyls and their metabolites (Fukuda et al., 2014). Therefore, this study describes a rapid high-performance liquid chromatography (HPLC)-based method coupled with diode-array detection (DAD) and MS for the simultaneous detection and identification of biphenyl and its metabolites from cell cultures of *C. thiooxydans* N1. Identifying each PCB was accomplished by comparing the retention time (RT) and ultraviolet (UV) spectral properties with known standards. The molecular structure of biphenyl, 2-phenylphenol, and 2,3-dihydroxy-biphenyl was confirmed by GC-MS analysis.

Overall, this study aimed to describe the analytical methods and validate the method using HPLC coupled with UV detection, which was suitable for determining biphenyls and their metabolites in cell cultures of *C. thiooxydans* N1.

2 Materials and methods

2.1 Chemicals and reagents

Analytical standards of biphenyl (≥ 99% purity) and 2-phenylphenol (≥ 99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 2,3-dihydroxybiphenyl (≥ 98% purity) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Water for the preparation of all samples was purified using a Milli-Q system (Millipore, Burlington, MA, USA). HPLC-grade acetonitrile was purchased from J. T. Baker (Phillipsburg, NJ, USA). All other chemicals and solvents were of HPLC-grade.

2.2 HPLC analysis

Chromatographic analyses were performed using a SHIMADZU HPLC-DAD system (LC System; Shimadzu Corporation, Kyoto, Japan) equipped with a quaternary pump (LC-20AD), thermostat-controlled column oven (CTO-20A) with a 10 µL loop injector, and a SHIMADZU DAD (SPD-M20A). Data were processed using Chemstation software (Shimadzu). Separation was achieved using a Luna C18 reversed-phase column (250 × 4.6 mm; Phenomenex, Torrance, CA, USA). The mobile phase was degassed and vacuum filtered through 0.45 µm nylon membranes. The detection wavelength was set at 254 nm with a mobile phase composed of an isocratic solvent mixture comprising water-acetonitrile (30:70, v/v) at a 1.0 mL/min flow rate.

2.3 GC-MS conditions

GC-MS analyses were performed on a GCMS-TQ8050 system (Shimadzu). GC separation was performed using a DB-5 capillary column with a length of 30 m × 0.25 mm internal diameter and a film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA). GC was run for 1 min with pulsed splitless injection at 29 psi, and the oven temperature gradient was programmed from 60 °C (1 min) to 125 °C at 25 °C/min, and then to 250 °C (18.07 min) at 15 °C/min. The inlet temperature was 250 °C. Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min.

2.4 Growth conditions for biphenyl degradation test and cell counting

Basal salt medium (BSM) was used to test the degradation ability of *C. thiooxydans* N1 (GenBank accession number CP079703). BSM was composed of K_2HPO_4 (4.3 g/L), KH_2PO4 (3.4 g/L), $(NH_4)_2SO_4$ (2 g/L), $MgCl_2$ (0.16 g/L), $MnCl_2{\cdot}4H_2O$ (1 µg/L), $FeSO_4{\cdot}7H_2O$ (6 µg/L), $CaCl_2{\cdot}2H_2O$ (26 µg/L), and $NaMoO_4{\cdot}2H_2O$ (2 µg/L) (Lee et al., 1997). Controls to which *C. thiooxydans* N1 was not added were incubated for the same time to confirm the relationship between the presence of *C. thiooxydans* N1 and the degradation of biphenyls. The first treatment sample (N1-BP) was composed of BSM (4.9475 mL), 50 µL of 100 mg/mL biphenyl (final concentration = 1000 ppm), 2.5 µL of a 10% yeast extract (Becton, Dickinson and Company, East Rutherford, NJ, USA), and 100 µL of *C. thiooxydans* N1 cell culture. Each control and N1-BP sample was prepared using cells cultured for seven different time points, namely 0, 2, 4, 6, 8, 10, and 12 h. The mixture was incubated at 30 °C with shaking (175 rpm) in a 50 mL conical tube (SPL Life Sciences, Pocheon-si, Korea). The incubated mixtures were collected at 2 h intervals up to 12 h. Subsequently, 100 µL of the incubated mixture was collected and immediately used for cell counting, and the rest was stored in a refrigerator (< 5 °C).

The second treatment sample (N1-PP) comprised BSM (4.97 mL), 25 µL of 100 mg/mL 2-phenylphenol (final concentration = 500 ppm), 5 µL of 5% casamino acids (Becton, Dickinson and Company), and 100 µL of the N1 cell culture. The control and each N1-PP sample were prepared using cells cultured for nine different time points (0, 12, 24, 36, 48, 60, 72, 84, and 96 h). The mixture was incubated at 30 °C with shaking (175 rpm) in a 50-mL conical tube (SPL Life Sciences). The incubated mixtures were collected at 12 h intervals up to 96 h. Subsequently, 100 µL of the incubated mixture was collected and immediately used for cell counting; the rest was stored in a refrigerator and then filtered through 0.45 µm nylon membranes for HPLC analysis.

2.5 HPLC method validation

The HPLC method for the quantitative determination of PCBs was validated in terms of linearity, specificity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision according to Commission Decision 168 2002/657/EC (European Commission, 2002).

2.6 Statistical analysis

Graphs were generated using GraphPad Prism 8.4.3 for Windows (GraphPad, Inc., San Diego, CA, USA). Data are expressed as the mean ± standard deviation of three replicates. Statistical comparisons were performed using one-way analysis of variance followed by Tukey's honestly significant difference test ($p < 0.05$) using JMP 15 (SAS Institute Inc., Cary, NC, USA).

3 Results and discussion

3.1 Optimisation of chromatographic conditions and separation of target analytes

To choose the best extraction solvent, various isocratic mixtures of acetonitrile and water were used to determine the optimum chromatographic conditions for biphenyls and their metabolites in cell cultures of *C. thiooxydans* N1. From the obtained results, 70% acetonitrile was optimal compared with the other tested solvent concentrations (0, 30, 50, and 70%). The chromatograms of standard biphenyl, 2-phenylphenol, and 2,3-dihydroxybiphenyl were obtained using the optimised flow rate of 1.0 mL/min (Figure 1). The separation of the three target analytes was achieved within 15 min. The RTs for 2,3-dihydroxybiphenyl, 2-phenylphenol, and biphenyl were 4.2, 5.4, and 11.0 min, respectively.

3.2 Method validation

Specificity, linearity, and range

The HPLC method was validated for specificity, linearity, LOD, LOQ, accuracy, and precision. In addition, specificity was evaluated by comparing the chromatograms of the mobile phase blank, standard solution, and sample solution (Figure 1). The linearity study was performed in triplicate with standard solutions corresponding to each point on the calibration curves. Good linear regression was observed between the peak area and concentration at six concentrations ranging from 0.04 to 12.8 μg/mL for biphenyl and from 0.0625 to 20 μg/mL for 2-phenylphenol and 2,3-dihydroxy biphenyl. From the regression analysis, all calibration curves showed good linearity ($R^2 > 0.999$) within their respective linearity ranges (Table 1).

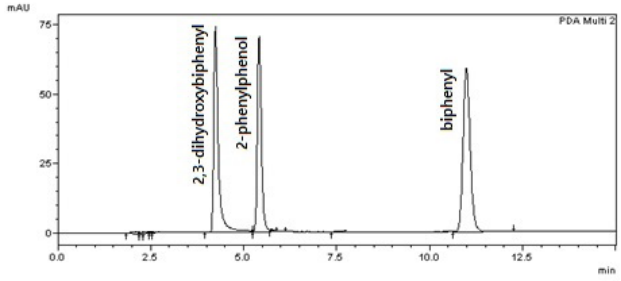

Figure 1. Chromatograms of biphenyl and metabolite standards from culture medium samples using high-performance liquid chromatography.

Table 1. Regression parameters of the calibration curve (n = 3).

Analytes	Linear range (μg/mL)	Determination coefficient
Biphenyl	0.04-12.8	1.000
2-Phenylphenol	0.0625-20	1.000
2,3-dihydroxy biphenyl	0.0625-20	0.999

LOD and LOQ

The LOD and LOQ of the proposed analytical method were calculated based on 3.3 r/S and 10 r/S, respectively, where r is the standard deviation of the intercept of regression analysis, and S is the average slope of the calibration curve. The LOD and LOQ for biphenyl and their metabolites ranged from 0.02-0.04 μg/mL and 0.07-0.12 μg/mL, respectively (Table 2).

Precision and accuracy

The accuracy of analytical data for biphenyl, 2,3-dihydroxybiphenyl, and 2-phenylphenol (Table 3) showed percentage recovery at all three levels in the range of 92.3-112.7%, and the relative standard deviation values were in the range of 0.41-8.49% (Table 3). The results of recovery and precision were within the acceptable limits of 70.0% to 120.0%, respectively, with RSD < 15.0%, indicating the applicability of the method for the analysis of food samples contaminated with soil.

3.3 Degradation of biphenyl by Comamonas thiooxydans N1

To verify the simultaneous HPLC analysis method determined previously, changes in biphenyl and 2-phenylphenol and production of 2,3-dihydroxybiphenyl were quantitatively analysed. Furthermore, *C. thiooxydans* N1, isolated from strawberry farm soil, was grown for 24 h using biphenyl and 2-phenylphenol as the sole carbon source. Biphenyl concentration decreased sharply after 6 h and reached about 100 μg/mL after 12 h (Figure 2). Compared with the growth *of C. thiooxydans* N1, the cell count grew slowly up to 12 h; more than 90% of biphenyl content was decomposed during this period. Subsequently, logarithmic growth was observed for 24 h, after which a plateau was reached. These results confirm that *C. thiooxydans* N1 uses biphenyl as a carbon source for growth. In addition, 2,3-dihydroxybiphenyl, an intermediate metabolite of biphenyl degradation, was detected after 8 h of incubation, reached the maximum level after 10 h, and remained detectable at a concentration of about 0.8 ppm after 24 h of incubation (Figure 2). It was not rapidly degraded in the slow early stage but was presumed to be used as a carbon source together with biphenyl in the logarithmic growth stage of *C. thiooxydans* N1. The same experiment was performed using 2-phenylphenol for 24 h, but the growth of *C. thiooxydans* N1, which had grown by degrading biphenyl, was not observed, and degradation of 2-phenylphenol could not be confirmed (data not shown).

Table 2. Performance characteristics.

Compound	Slope	Intercept	LOD_{approx} (ug/mL)	LOQ_{approx} (ug/mL)
Biphenyl	68667.86	476.91	0.02	0.07
2-Phenylphenol	34452.09	165.82	0.02	0.05
2,3-dihydroxy biphenyl	30764.00	366.61	0.04	0.12

Slope: the slope of the calibration curve; Intercept: standard deviation of the *y*-intercept on the regression line; LOD: limit of detection; LOQ: limit of quantification.

Table 3. Recovery, precision (intra and inter-day), and accuracy (%) data for the simultaneous determination of biphenyl, 2-phenylphenol, and 2,3-dihydroxy biphenyl using high-performance liquid chromatography. Concentrations are expressed as the mean ± standard deviation of three replication.

Analyte	Concentration (μg/mL)	Detection concentration (μg/mL) (% RSD)†	Recovery (%)	Intra-day precision	Inter-day precision (% RSD)†
Biphenyl	0.05	0.050.00	109.64	5.11	13.07
	0.5	0.490.00	98.80	0.85	8.49
	1	1.040.02	104.05	1.51	10.42
	2	2.130.02	106.39	0.78	2.44
	4	4.310.01	107.68	0.31	6.75
	8	8.470.03	105.82	0.41	3.26
2-Phenyl phenol	0.1	0.130.00	99.82	3.54	14.26
	1	1.060.01	105.76	0.96	8.26
	2	2.190.05	109.45	2.24	10.59
	4	4.510.03	112.66	0.75	3.54
	8	8.890.04	111.16	0.46	6.92
	16	17.340.08	108.37	0.46	3.32
2,3-dihydroxy biphenyl	0.1	0.050.00	81.66	3.40	9.65
	1	0.990.02	99.31	3.10	5.83
	2	1.890.10	94.72	6.42	14.88
	4	3.690.06	92.29	1.71	5.77
	8	8.150.16	101.92	2.02	7.90
	16	15.730.13	98.34	0.84	3.87

†RSD: relative standard deviation.

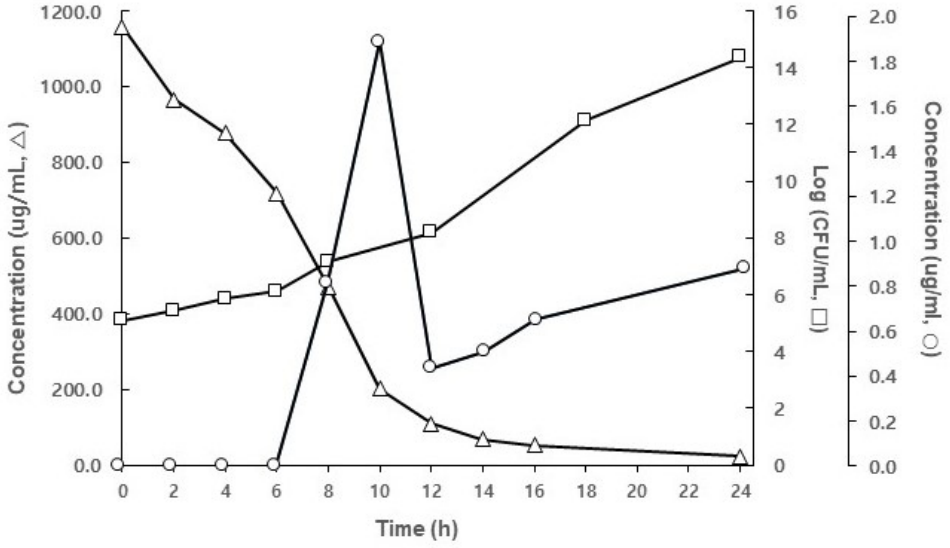

Figure 2. Growth curve and biphenyl degradation curve of *Comamonas thiooxydans* N1. Error bars represent the standard deviation of the mean (n = 3). Symbols: □ growth curve; △ biphenyl; ○ 2,3 dihydroxy biphenyl.

Some species belonging to the genus *Comamonas*, such as *Comamonas testosteroni* TK102, have been reported to possess the ability to degrade biphenyl and their metabolites (Hiraoka et al., 2002). In this study, *Comamonas thiooxydans* N1, isolated from contaminated strawberry farm soil, could deplete biphenyl and its metabolites within 24 h. Previous studies have shown that *Dyella ginsengisoli* LA-4 degraded 95% of 100 mg/L biphenyl within 36 h (Li at al., 2009), *Achromobacter* sp. BP3 completely degraded 50 mg/L biphenyl within 28 h (Hong et al., 2009), *Mycobacterium* sp. PYR-1 could degrade over 98% of 80 mg/L biphenyl within 72 h (Moody et al., 2002), and HC3 grew on 1000 mg/L biphenyl (Hu et al., 2015).

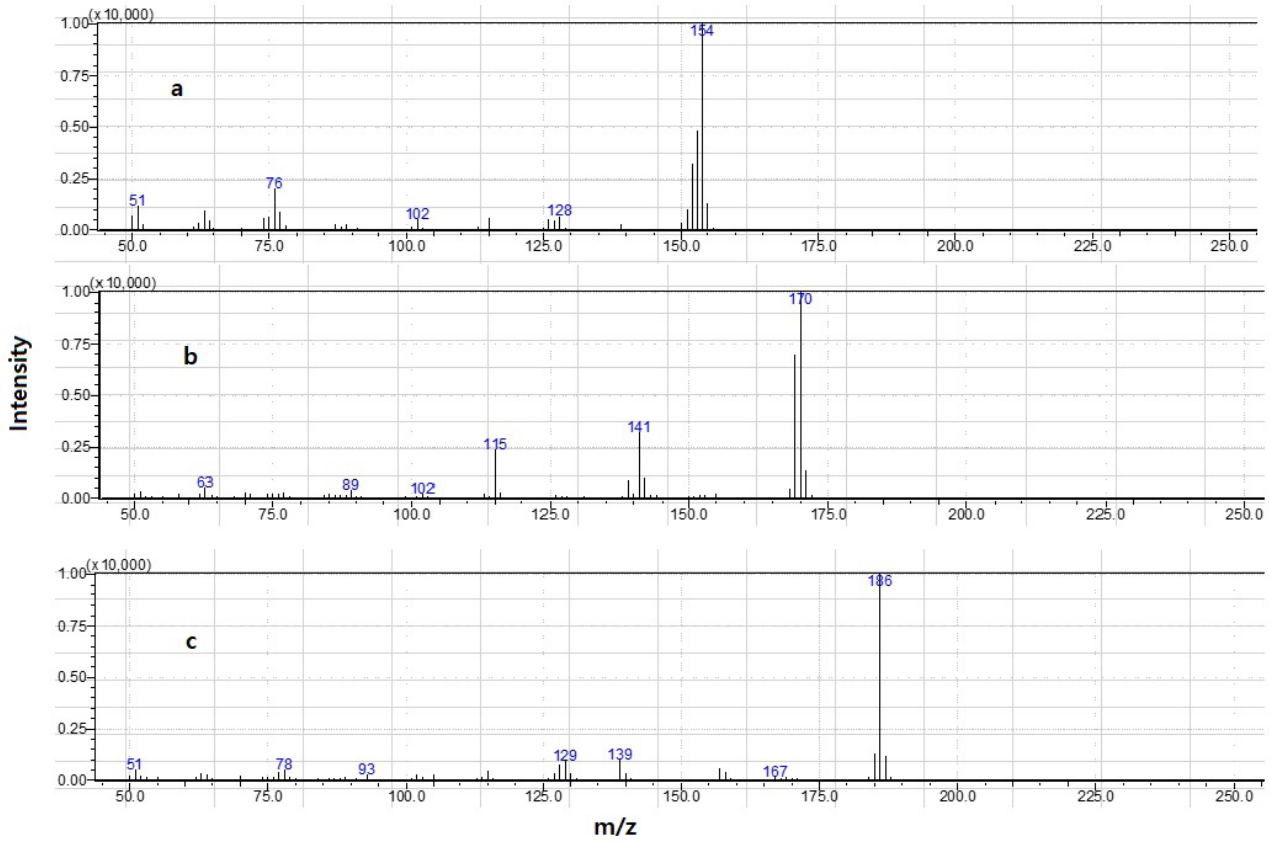

Figure 3. The mass spectra of the main metabolites in cell cultures. (a) biphenyls; (b) 2-phenylphenol; (c) 2,3-dihydroxy biphenyl.

In conclusion, the strain *C. thiooxydans* N1, isolated from contaminated strawberry farm soil, showed efficient degradation ability toward biphenyls. In this study, a validated, 15-min HPLC method was established to rapidly quantify biphenyl, 2-phenylphenol, and 2,3-dihydroxybiphenyl from cell cultures using a simple and accurate method. The RT for biphenyl, 2-phenylphenol, and 2,3-dihydroxybiphenyl were 3.5, 4.2, and 7.4 min, respectively. The peak identities were identified using GC-MS analyses (Figure 3). This method offers the following advantages: simultaneous analysis of three substances, isocratic run conditions, and easy sample preparation. The proposed method can be conveniently used for analysing similar classes of biphenyls from culture medium samples.

4 Conclusions

Comamonas thiooxydans N1 was isolated from contaminated strawberry farm soil and proved effective for biphenyl degradation in BSM, ultimately reducing the biphenyl content within 24 h. A reversed-phase HPLC method with DAD and MS proved efficient for the separation and quantification of biphenyl, 2-phenylphenol, and 2,3-dihydroxybiphenyl in the culture medium. Using optimised analytical conditions, HPLC-DAD analysis revealed good linearity with a correlation coefficient of 0.999. The estimated LOD and LOQ values were in the range of 0.02-0.04 and 0.05-0.12 µg/mL, respectively. The proposed analytical method was thus applied to analyse the biphenyl content in culture medium samples.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

Acknowledgements

This research was supported by the Main Research Program of the Korea Food Research Institute (KFRI), funded by the Ministry of Science and ICT.

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