ACTA ABP BIOCHIMICA POLONICA

Vol. 67, No 3/2020
409–415
https://doi.org/10.18388/abp.2020_5368

Regular paper

A novel method for rapid and quantitative detection of bisphenol A in urine*

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Bisphenol A (BPA) is classified as an endocrine disruptor (ED) and can interact with a variety of hormone receptors leading to hormonal disruption and increased risk of numerous adverse health effects. Reducing human exposure to BPA is one of the main challenges of public health, as it is constantly present in the daily life. A low-cost and commonly applied method to enable determination of BPA in the patient's body has yet to be developed. Currently available techniques are expensive, time-consuming, and require access to highly equipped analytical chemistry laboratories. Here, we describe a fast and inexpensive engineered lateral flow assay of our design, to detect BPA in urine samples. This technology provides an opportunity to perform rapid biomonitoring. Addition of β-glucuronidase improves sensitivity of detection, as it releases free BPA from glucuronide complexes in the urine. This invention may become a commonly used analytical tools for lowering human exposure to BPA and probably to other EDs as well, and consequently it may be useful in decreasing the risk for several lifestyle diseases.

Key words: Bisphenol A, BPA, lateral flow assay, biomonitoring, endocrine disruptors

Received: 15 May, 2020; revised: 28 June, 2020; accepted: 28 June, 2020; available on-line: 30 July, 2020

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*A patent for this invention has been granted by the Polish Patent Office on the 23rd of April 2020.
Acknowledgements of Financial Support: This study was funded by resources of the Department of Analytical Chemistry, Gdańsk University of Technology, Poland
Abbreviations: BPA, bisphenol A; BSA, bovine serum albumin; CALUX, Chemically Activated Luciferase gene eXpression; ED, endocrine disruptor; EDC, endocrine disrupting chemical; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; GC, gas chromatography; LC-MS/MS, liquid chromatography combined with tandem mass spectrometry; LFA, lateral flow assay; LOD, limit of detection; PBS, Phosphate-Buffered Saline; PPARγ2-CALUX, proliferator-activated receptor γ2-Chemically Activated LUciferase gene eXpression; YES, Yeast Estrogen Screen

INTRODUCTION

Bisphenol A (BPA) is one of numerous endocrine disrupting chemicals (EDCs) or the so-called endocrine disruptors (EDs). The United States Environmental Protection Agency defines ED as an "exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock *et al.*, 1996). Similarly to some steroid hormones, the structure of BPA contains aromatic rings; hence, it can interact with a variety of receptors for hormones and in consequence lead to hormonal disruption and increase the risk of various adverse health effects (Di Donato *et al.,* 2017; Sifakis *et al.,* 2017).

Widespread use of BPA-containing products, together with its ability to migrate from these materials, may explain the detectable levels of BPA in laboratory specimens that include serum, urine, amniotic fluid, milk, placenta, and neonatal blood (Vandenberg *et al.,* 2010; Mendonca *et al.,* 2014; Lee *et al.,* 2018). Since humans are permanently exposed, there is an indisputable need for reducing the exposure to BPA (Gore *et al.,* 2015). In addition, special attention should be given to pregnant women, as elevated concentrations of EDs in the amniotic fluid may disrupt the endocrine balance crucial for proper development of the gonads and can increase the risk of congenital anomalies of the urogenital tract (Braun, 2017; Pergialiotis *et al.*, 2018). Permanent exposure may also alter epigenetic mechanisms, resulting in a higher risk of alterations in the offsprings even in the third generation (Mileva *et al.*, 2014, Rutkowska *et al.*, 2016; Ideta-Otsuka *et al.*, 2017).

Despite the high relevance of biomonitoring and efforts aimed at reducing the exposure, no low-cost and easily applied method for rapid determination of BPA levels in a patient's body has been yet developed. Currently available methods for quantitative detection of EDs can be divided into three groups. The first consists of highly sensitive chromatographic techniques, such as gas chromatography (GC) or high-performance liquid chromatography (HPLC); often coupled with mass spectrometry, tandem mass spectrometry, or solid-phase microextraction (Sun *et al.*, 2016; Jurek *et al.*, 2017; Jurek *et al.*, 2018; Owczarek *et al*, 2018; Dreolin *et al.*, 2019).

The second group consists of bioassays used to determine the endocrine disruption potential of chemicals, their damaging effect on DNA (genotoxicity), and generation of oxidative stress. It includes various methods and the most commonly used are CALUX (Chemically Activated LUciferase gene eXpression), E-SCREEN, and YES (Yeast Estrogen Screen) (Park *et al.*, 2008; Valitalo *et al.*, 2016; Aneck-Hahn *et al*, 2018; Owczarek *et al.*, 2018). The third group includes enzyme immunoassays used to visualize and quantify antigens, such as ELISA (enzyme-linked immunosorbent assay) (Lee *et al.*, 2017).

All of the above-mentioned techniques require access to a fully equipped specialized laboratory and involve experienced chemists; hence, they are also expensive and time-consuming.

Another method that can be used for EDs determination is immunochromatography. Using it in a lateral flow rapid test makes it a low-cost and simple-to-use method that can easily be performed outside a laboratory, even by the patients themselves in the outpatients' clinics or at home.

Although analytical methods are being developed with greater simplicity and efficiency, higher selectivity and sensitivity, lower sample and solvent consumption, and greater automation routine, analysis of BPA levels in patients still poses great challenges (Sun *et al*., 2016; Dhanjai *et al*., 2018), especially since the serum/whole blood concentrations may often not predict the total body burden.

Here, we describe an engineered fast and inexpensive lateral flow assay (LFA) of our design for BPA detection in urine samples. This is a first powerful analytical tool that can be ready-to-use for biomonitoring without the need for an access to a laboratory and scientific equipment. Its unique advantage is the possibility to determine if the individual exposure to BPA is low, medium, or high, which may be of key importance from the public health perspective and human biological monitoring.

MATERIALS AND METHODS

Materials. **Chemicals**. Phosphate-Buffered Saline (PBS), Bovine Serum Albumin (BSA), β-glucuronidase enzyme, as well as the BPA standard and all LC chromatography reagents were ordered from Sigma Aldrich (USA). Innova Bioscience Latex Conjugation Kit was from Expedeon (formerly Innova BioScience, United Kingdom).

Antibodies. Rabbit polyclonal antibodies to BPA, and goat polyclonal antibodies to the rabbit antibodies were from Antibodies-online GmbH (Germany).

LFA constructions. All membranes: nitrocellulose, glass and cotton used in LFA were purchased from Ahlstrom-Munksjö (Finland). Color latex beads were from Expedeon (formerly Innova BioScience, United Kingdom).

Urine samples. Forty healthy volunteers (age range 2–40 years old) including males, females (also pregnant), and children had mid-stream urine samples collected into glass vials (previously autoclaved and sterilized) after an overnight fast. All samples were frozen at –70°C until analysis.

Preparation of the lateral flow strips and test run. The lateral flow strips were created as a combination of different types of membranes, polyclonal antibodies to BPA – both adsorbed on membranes and on color carrier beads, and antibodies to the anti-BPA antibodies. A urine sample was being applied onto the sample pad (1) and then it flowed through an overlapping conjugate pad (2) onto which color carrier beads, coated with polyclonal antibodies to BPA, had been placed earlier. Further, the sample flowed through a membrane within which the urine sample and the color carrier beads, including those bound to the BPA present in the sample, can freely migrate (3). The membrane contains three indication areas covered with immobilized polyclonal antibodies to BPA (5) and one dedicated control area covered with immobilized polyclonal antibodies to antibodies to BPA, i.e. the control line (6). The first BPA indication area (the closest to the sample pad) detects a concentration that corresponds to low BPA exposure. Appearance of color in the first two areas indicates intermediate BPA exposure, whereas its appearance in the three consecutive BPA indication areas corresponds to high BPA exposure. A control area (the control line) contains antibodies to anti-BPA antibodies and the appearance of color in these lines upon application of the biological material indicates a proper test functioning. Migration of the sample and the unbound latex beads coated with antibodies stops at the termination area of the test (4) – the absorbent pad, where the adsorption of the sample occurs. The backing card, free from BPA and its derivatives (7), is an additional element that supports the membranes and facilitates the performance of the test outside a laboratory; however, it was skipped during laboratory validation.

A nitrocellulose membrane of 240–280 μm in thickness was selected for the membrane of sample migration (3). Rabbit polyclonal antibodies to BPA, previously placed in PBS with 2% BSA, were applied onto the membrane. The amount of the antibodies corresponded to the detection of BPA concentrations of the maximum values of 2 ng/mL for the first, 3 ng/mL for the second, and 10 ng/mL for the third indication area. Goat polyclonal antibodies to rabbit antibodies to BPA were applied at the end of the membrane to form the control line of the test. A glass fiber membrane was selected for the sample pad (1) and the conjugate pad (2), whereas cotton fiber membrane was selected for the absorbent pad (4). The membrane was impregnated with PBS with 2% BSA and dried for 24 hours with subsequent application of 40 μL of 1% solution of latex beads coated with rabbit antibodies to BPA (Expedeon, formerly Innova BioScience, United Kingdom).

Conjugation of color carries beads to antibodies. The process of conjugation was conducted by using the reagents and protocol according to the Innova Bioscience Latex Conjugation Kit manual. Briefly, the stock of polyclonal antibodies to BPA was diluted to 0.1 mg/mL and then added to 400 nm blue latex nanoparticles. After 15 minutes of incubation at room temperature, the conjugation reaction was quenched. Then, the samples were centrifuged at 10 000 rpm for 6 minutes. The conjugation buffer was removed, and the pellet was resuspended in a buffer with the addition of 0.1% BSA.

Determination of BPA concentration in urine samples and estimation of individual exposure. A small volume of urine was poured into a container made from materials free from BPA and its derivatives. Then, 2 mL were transferred into a glass receptacle included in the test kit with a micropipette, up to the level marked with a horizontal line visible on the side wall of the receptacle. ß–glucuronidase enzyme was sprayed onto the bottom and walls of the receptacle in order to catalyze hydrolysis of BPA glucuronide from urine; hence, to obtain the free form of BPA. The enzyme activity was at least 20 000 units per gram, and it was added at a concentration of 20 μL per 1 mL of urine. Afterwards, 100 μL of the sample collected from the receptacle was applied on the sample pad and flowed to the area with immobilized color carrier beads where free BPA was bound by antibodies on the color latex beads. The urine sample, the unbound BPA, the beads with bound BPA, and the beads that did not bind BPA migrated onto the nitrocellulose membrane. According to the parameters of the membrane, the average migration rate was 150 s/4 cm.

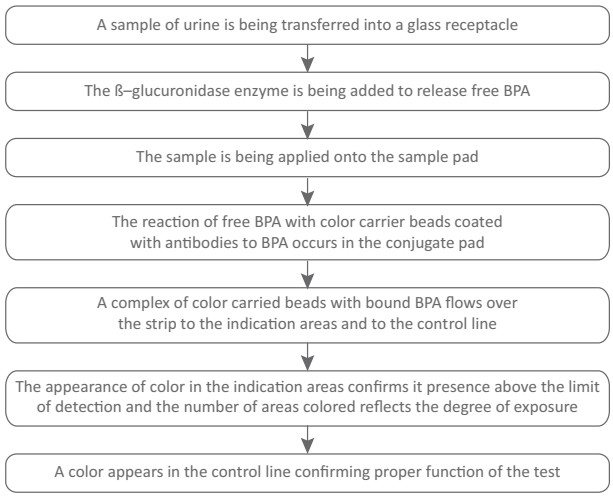

Figure 1. A scheme of using lateral flow assay for detection of BPA in urine samples.

A scheme of the procedure of urinary BPA determination using LFA is shown in Fig. 1.

The BPA quantification results using lateral flow strips were verified and validated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Shimadzu LCMS-8050, Japan). Each determination of BPA concentration in the urine sample was conducted in triplicate. The final results of BPA quantification in urine samples were expressed as an arithmetic mean of three measurements and the uncertainty of measurement; the procedure has been already published elsewhere (Wilczewska *et al.*, 2016).

RESULTS

The colored indication area in LFA pointed to BPA detection in the urine sample. The concentrations of detected BPA sum up; thus, the appearance of color in one, two, or three BPA indication area(s), starting from the sample pad, corresponds to a level of up to 2 ng/mL (low BPA concentration), up to 5 ng/mL (moderate BPA concentration), and up to 15 ng/mL (high BPA concentration), respectively. Proper functioning of the test (migration of carrier beads correctly coated with antibodies to BPA) was confirmed by the appearance of blue color in the control area. Figure 2 illustrates the principle of the strip test and exemplary results of the test for various BPA concentrations in the samples. The examples of different results for BPA concentration analysis in urine samples are illustrated in Fig. 3. The first strip (LFA 1) illustrates an example of the negative result of the test which means BPA concentration in the patient's urine was below LOD (<1 ng/mL). The sec-

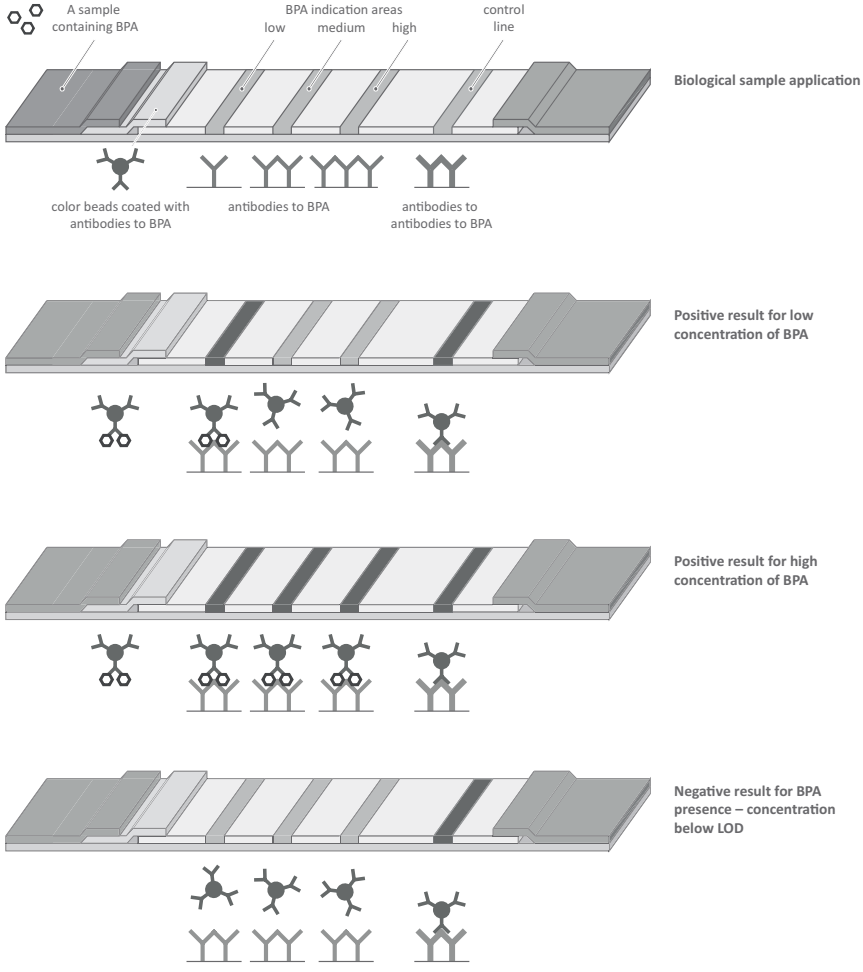

Figure 2. Determination of BPA concentration in urine samples by lateral flow strips and estimation of different levels of individual exposure

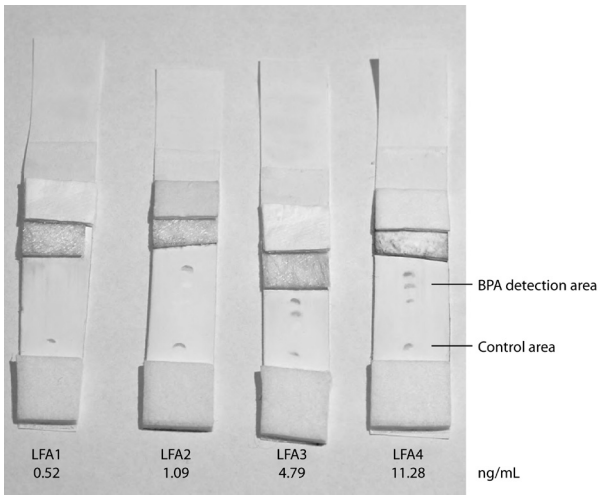

Figure 3. Examples of results of the lateral flow assay for different BPA concentrations in the urine samples, with results validated by LC-MS/MS.

Table 1. Results of chromatographic analysis of BPA concentration in urine samples without and with incubation with β-glucuronidase.

Sample	BPA concentration without β-glucuronidase (ng/mL)	BPA concentration with β-glucuronidase (ng/mL)
1.	1.15±0.13	10.22±1.5
2.	0.19±0.02	1.89±0.09
3.	0.81±0.07	1.35±0.08
4.	<LOD	1.01±0.08
5.	2.46±0.29	4.79±0.36

LOD – limit of detection

ond strip (LFA 2), with one colored indication area illustrates low BPA concentration in the patient's urine, i.e. up to 2 ng/mL. The result with two blue lines in the indication area of the third strip (LFA 3) demonstrates moderate BPA concentration in the sample, i.e. up to 5 ng/mL. The last strip (LFA 4) presents the possibility to evaluate high exposure to BPA and detection values up to 15 ng/mL. The values of BPA concentrations presented in Fig. 3 were obtained for the same samples of urine using LC-MS/MS during assay validation to confirm the results of the assay.

Addition of β-glucuronidase to urine samples improved the LFA sensitivity. An example of chromatographic analysis results for the content of free BPA in the urine samples from healthy women (aged 21 to 24) before and after addition of β-glucuronidase (incubation with 20 μL per 1 mL of urine) are summarized in Table 1. Table 2 presents concentrations of BPA in all urine samples measured *via* chromatography after addition of the enzyme, divided into levels with clear and unclear LFA results. LFA could give unclear/false results for the lowest concentrations of BPA detected in the samples. Although addition of β-glucuronidase has increased the level of free BPA in the sample and the sensitivity of the assay, there was still a risk for troubles in detection of the lowest BPA concentrations. Nevertheless, we believe that the uncertainty of some of the LFA results may be explained by manual application of antibodies on the membrane.

Table 2. BPA concentrations in the collected urine samples assessed with chromatography for validation.

Clear results for BPA detection [ng/mL]	Uncertain /false results for BPA detection in the samples [ng/mL]
10.22	1.05
1.89	1.01
1.35	1.6
4.79	1.2
3.3	2.6
8.25	
2.14	
5.12	
5.2	
8.9	
4.2	
10.15	
3.79	
5.2	
3.19	
9.14	
4.15	
3.99	
12.4	
10.5	
2.19	
2.85	
1.43	
1.92	
3.8	
1.85	
1.16	
3.6	
4.09	
4.85	
1.41	
3.88	
3.5	
4.25	
3.82	
3.2	
11.2	
4.21	
14.2	

DISCUSSION

Constant human exposure to BPA may increase the risk of developing endocrine and metabolic disorders, obesity, diabetes mellitus, cardiovascular disease, infertility, and endocrine-dependent tumors in both, women and men (Street *et al.*, 2018). In 2015 EFSA suggested

Table 3. A comparison of advantages and drawbacks of the currently known analytical methods and the test that we developed.

Analytical method	Chromatographic analysis (e.g. HPLC, GC)	Bioassay (e.g. CALUX, E-SCREEN, YES)	Immunoassay (e.g. ELISA)	Lateral flow assay and sensors	Developed device with addition of β-glucuronidase
Advantages	Ultra-high sensitivity of detection. Possibility to measureconcentration of a specific compound in a mixture.	High sensitivity of detection. Ability to estimate the endocrine potential of a mixture.	High sensitivity of detection. Simple sample preparation. Rapidity. Ease of operation of portable devices.	Simplicity. Rapidity. Cost-effectiveness	Simplicity. Ready to use at home. Rapidity. Cost-effectiveness. Higher sensitivity (free BPA release from glucuronide complexes). Possibility to estimate BPA level exposure.
Drawbacks	Higher acquisition and operational costs. Highly specialized personnel and laboratory setting. Long procedure of sample preparation. High cost of analysis.	Cell-based assay (analytical results may depend on the cells' condition). Highly specialized research equipment. Time-consuming procedure. High cost of analysis.	Semi-quantitative. Risk of false positives. Specialized research equipment.	Lower sensitivity. Higher cross-reactivity. Risk of false positives.	Semi-quantitative.
Protocols	Ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS), LOD 0.13 ng/mL, recoveries of 73.02-108.76% (Yao *et al*., 2018). Dispersive liquid-liquid microextraction (DLLME) and heart-cutting multidimensional gas chromatography coupled to mass spectrometry (MDGC/MS), LOD 0.03 ng/mL (Cunha *et al*., 2010). Liquid chromatography--tandem mass spectrometry (LC-MS/MS), LOD 0.4 ng/mL (Chen *et al*., 2012). Micro-QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method coupled to gas chromatography-mass spectrometry (GCMS), LOD 0.13ng/mL, recoveries >74% (Correia-Sa *et al*., 2018). High performance liquid chromatography tandem mass spectrometry (HPLC--MS/MS), LOD 0.048 μg/L, recoveries of 82.2-88.9% (Wang *et al*., 2017). Solid phase extraction and liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS), LOD 0.10 ng/mL, recoveries of 80.1%-108% (Jing *et al*., 2011). Porous organogel materials in combination with liquid chromatography–tandem mass spectrometry (LC–MS–MS), LOD 5 ng/mL, recovery >80 % (ter Halle *et al*., 2015).	PPARγ2-CALUX cells (Dusserre *et al*., 2018). E-SCREEN (Soto *et al*., 1995; Wu *et al*., 2012). Xenoscreen YES/YAS (Dvorakova *et al*., 2016; Owczarek *et al*., 2018). Microtox (Owczarek & Kudlak et al., 2018).	Direct- and indirect competitive enzyme--linked immunosorbent assay (ELISA), LOD 7.0 ng/mL and 0.08 ng/mL, respectively (Yajing Lei *et al*., 2013) Radioimmunoassay, LOD 0.2 ng/mL (Harthe *et al*., 2012).	Aptamer-functionalized magnetic nanoparticles (AMNPs) combined with high performance liquid chromatography (HPLC), LOD 1.0 ng/mL, recoveries of 90.8-93.8 % (Su *et al*., 2018).	

tenfold lowering of the Tolerable Daily Intake (TDI) for BPA – from 50 μg/kg of body weight/day to 4 μg/kg of body weight/day (establishing it as a temporary Tolerable Daily Intake – t-TDI) (Cwiek-Ludwicka, 2015; EFSA Panel on Food Contact Materials, 2015). A new EFSA assessment is planned to be ready by 2020 (EFSA's Panel on Food Contact Materials EaPAC, 2018). Although BPA has been removed from most consumer products and human exposure should be dropping among the population in the future, it is still present in the daily lifestyle; thus, biomonitoring is of human and environmental benefit. Table 3 presents a comparison of advantages and drawbacks of the currently known and

used analytical methods and of the test that we developed.

Lateral flow immunochromatographic assay may be an easy, and yet still valuable tool for determining the exposure to EDs, including BPA. To the best of our knowledge, there is no similar assay designed, nor was one patented (Mei *et al.*, 2013; Maiolini *et al.*, 2014; Sun *et al.,* 2016).

The LFA technology presented here makes it possible to conduct a rapid diagnostic test without access to a central laboratory and it can be performed either by medical staff or by the patient at home. The addition of β-glucuronidase improves the sensitivity of detection, as it leads to a release of free BPA from glucuronide complexes in the urine. It is estimated that in the European Union only, exposure to EDs may substantially contribute to diseases and dysfunction across individual life courses, with costs of hundreds of billions of EUR per year. Without a doubt, its prevention can benefit the economy, but most importantly- the human life (Trasande *et al.*, 2015). Thus, our invention may be a powerful and helpful analytical device for biomonitoring, as well as a concept to be used for preparing other LFAs for EDs detection; and therefore, it may help lowering the risk of some lifestyle diseases that have been linked with exposure to BPA (Gerona *et al.*, 2020).

Acknowledgements

The authors are grateful to Prof. Błażej Kudłak, PhD Eng. from the Department of Analytical Chemistry, Gdańsk University of Technology, Poland, for his great contribution in the LFA development.

Conflict of Interest

None

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