POLITECHNIKA GDAŃSKA

Imię i nazwisko autorki rozprawy: Katarzyna Owczarek

Dyscyplina naukowa: Chemia

ROZPRAWA DOKTORSKA

Tytuł rozprawy w języku polskim: Analityka związków chemicznych z grupy bisfenoli w próbkach o skomplikowanym składzie matrycy, w tym pochodzenia biologicznego

Tytuł rozprawy w języku angielskim: *Analytics of bisphenol A analogues in the samples characterized by complex matrix, including biological ones*

Promotor *podpis*	Drugi promotor *podpis*
Dr hab. inż. Błażej Kudłak	Prof. dr hab. inż. Zofia Mazerska

Gdańsk, rok 2022

Składam serdeczne podziękowania

Moim promotorom,

Pani prof. dr hab. inż. Zofii Mazerskiej
oraz Panu dr. hab. inż. Błażejowi Kudłakowi
za cierpliwość, za przekazaną wiedzę,
udzielone wsparcie
i nieocenioną pomoc w przebiegu realizacji części badawczej
i redagowaniu niniejszej pracy.

Dziękuję za owocną współpracę również
Panu prof. dr. hab. inż. Andrzejowi Wasikowi
oraz dr. inż. Pawłowi Kubicy,
którzy zawsze wykazywali się życzliwością
i udzielili wielu cennych wskazówek
w trakcie prowadzenia części eksperymentalnej mojego projektu doktorskiego.

Szczególny wpływ na całokształt mojego dorobku miał mój mentor,
inspirujący naukowiec
oraz niepodważalny autorytet w dziedzinie chemii analitycznej,
ś.p. Pan prof. dr hab. inż. Jacek Namieśnik.

Wyjątkowe podziękowania należą się moim Bliskim,
za wsparcie, pomoc i motywację.

Spis treści

Wykaz skrótów i akronimów

Akronim/Skrót	Termin anglojęzyczny	Termin polskojęzyczny
ACN	*Acetonitrile*	Acetonitryl
ANOVA	*Analysis of variance*	Analiza wariancji
BADC	*Bisphenol A Cyanate esters*	Estry dicyjanianowe bisfenolu A
BECy	*Bisphenol E Cyanate esters*	Estry cyjanianowe bisfenolu E
BMDC	*Bisphenol M Dicyanate Esters*	Estry dicyjanianowe bisfenolu M
BMI	*Mody mass indeks*	Wskaźnik masy ciała
BPDC	*Bisphenol P Cyanate esters*	Estry dicyjanianowe bisfenolu P
BPA	*Bisphenol A*	Bisfenol A
BSTFA	*N,O-Bis(trimethylsilyl)trifluoroacetamide*	N,O-Bis(trimetylsilyl)trifluoroacetamid
CA	*Concentration Addition*	Model addycji stężeń
DLLME	*Dispersive liquid-liquid microextraction*	Dyspersyjna mikroekstrakcja w układzie ciecz-ciecz
EC	*Effective Concentration*	Stężenie efektywne
EDC	*Endocrine Disrupting Compounds*	Związki endokrynnie czynne
EI	*Electron Ionization*	Jonizacja elektronami
EFSA	*European Food Safety Authority*	Europejski Urząd ds. Bezpieczeństwa Żywności
ERα	*Estrogen receptor α*	Receptor estrogenowy α
ERβ	*Estrogen receptor β*	Receptor estrogenowy β
ESI	*Electrospray ionization*	Jonizacja w trybie elektrorozpylania
FAI	*Free Androgen Index*	Wskaźnik wolnych androgenów
FSH	*Follicle-stimulating hormone*	Folitropina
GC	*Gas chromatography*	Chromatografia gazowa
HPLC	*High Performance Liquid chromatography*	Wysokosprawna Chromatografia Cieczowa
IA	*Independent Action*	Model działań niezależnych
ISTD	*Internal Standard*	Wzorzec wewnętrzny
IUPAC	*International Union of Pure and Applied Chemistry*	Międzynarodowa Unia Chemii Czystej i Stosowanej
LC	*Liquid Chromatography*	Chromatografia cieczowa
LLE	*Liquid-liquid Exctration*	Ekstrakcja w układzie ciecz-ciecz
LOD	*Limit of Detection*	Granica wykrywalności
LOQ	*Limit of Quantitation*	Granica oznaczalności
MeOH	*Methanol*	Metanol
MDR	*Model Deviation Ratio*	Współczynnik Odchylenia Modelu
MIP	*Moleculary Imprinted Polymers*	Polimer z odciskiem molekularnym
MRM	*Multiple Reaction Monitoring*	Tryb monitorowania wielu przejść jonowych
MS	*Mass spectrometry*	Spektrometria mas
MS/MS	*Tandem Mass Spectrometry*	Tandemowa spektrometria mas

MSTFA	*N-Methyl-N-(trimethylsilyl)-trifluoroacetamide*	N-metylo-N-(trimetylosilyl)trifluoroacetamid
NHANES	*National Health and Nutrition Examination Survey*	Narodowego Badania Zdrowia i Żywienia w Stanach Zjednoczonych
NSCLC	*Non Small Cell Lung Cancer*	Niedrobnokomórkowy rak płuc
PCOS	*Policystic Ovary Syndrome*	Zespół policystycznych jajników
SIM	*Single Ion Monitoring*	Tryb monitorowania pojedynczego jonu
SBSE	*Stir Bar Sorptive Extraction*	Ekstrakcja za pomocą ruchomego elementu sorpcyjnego
SPE	*Solid phase extraction*	Ekstrakcja do fazy stałej
SPME	*Solid phase microextraction*	Mikroekstrakcja do fazy stacjonarnej
TDI	*Tolerable Daily Intake*	Dopuszczalna dzienna dawka
TG	*Triglicerides*	Triglicerydy
UPLC	*Ultrahigh Performance Liquid Chromatography*	Ultrasprawna Chromatografia Cieczowa
UV – Vis spectroscopy	*Ultraviolet – visible spectroscopy*	Spektroskopia UV-Vis

Wprowadzenie

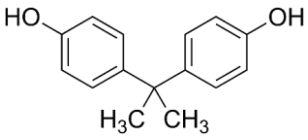
1. Wprowadzenie

Bisfenole stanowią szeroką klasę związków chemicznych, powszechnie zaangażowanych w procesach produkcji poliwęglanowych tworzyw sztucznych i żywic epoksydowych. Najbardziej rozpowszechnioną i stosowaną na największą skalę cząsteczką, przynależącą do tej grupy, jest bisfenol A (BPA). Szacuje się, że światowa produkcja BPA w roku 2020 wynosiła około 6,2 mln ton [1]. Ogromna skala produkcji i powszechne zastosowania w przemyśle sprawiły, że związek ten stał się wszechobecny w elementach składowych środowiska. Jego negatywny wpływ na organizmy żywe oraz całe ekosystemy został potwierdzony licznymi badaniami naukowymi i stosunkowo dobrze poznany oraz opisany w literaturze specjalistycznej na przestrzeni ostatnich dwóch dekad. Sztandarowym przykładem negatywnego oddziaływania BPA na zdrowie jest jego zdolność do wiązania się z receptorami estrogenowymi i androgenowymi dzięki strukturalnemu podobieństwu do naturalnych hormonów, przez co zalicza się go do grupy związków endokrynnie czynnych [2]. Co więcej, wiele wyników badań sugeruje, że istnieje korelacja pomiędzy stężeniem BPA w tkankach a rozwojem nowotworów hormonozależnych, takich jak rak piersi lub prostaty [3,4], a także iż związek ten niekorzystnie wpływa między innymi na neurorozwój dzieci, funkcje seksualne i reprodukcyjne czy zaburzenia metaboliczne [5-8].

Z tego powodu Agencja Ochrony Środowiska Stanów Zjednoczonych (US EPA) - opierając się na dostępnych badaniach naukowych - wyznaczyła referencyjną dopuszczalną dzienną dawkę (TDI) dla bisfenolu A wynoszącą 50 µg/kg masy ciała/ dzień [9]. Natomiast ze względu na niezwykle szerokie zastosowanie i coraz większą ilość doniesień naukowych dotyczących powiązania BPA z wyżej wymienionymi zaburzeniami zdrowotnymi, w 2015 roku Europejski Urząd ds. Bezpieczeństwa Żywności (EFSA) obniżył dopuszczalną dzienną dawkę do 4 µg/kg masy ciała/dzień [10], a wiele krajów wprowadziło do swojej legislacji przepisy ograniczające jego zastosowanie. Pierwszym państwem, które sklasyfikowało BPA jako toksyczny związek chemiczny i zakazało jego stosowania w produkcji butelek dla dzieci w 2010 roku była Kanada [11]. Za jej przykładem poszły Stany Zjednoczone, niektóre państwa Unii Europejskiej i kraje azjatyckie, w tym Chiny i Japonia.

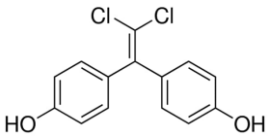
Efektem zmian i regulacji prawnych było wycofanie się niektórych producentów tworzyw sztucznych z użycia BPA lub znaczące jego ograniczenie, co w konsekwencji doprowadziło do poszukiwania zamienników tej cząsteczki. Naturalnie, najbardziej odpowiednie okazały się analogi BPA, w których strukturze również występują dwa pierścienie aromatyczne, połączone poprzez mostek węglowy lub inny element struktury chemicznej oraz grupy hydroksylowe -OH ustawione względem tego mostka w pozycji *-para*. Ze względu na podobną budowę, a także fakt, iż to grupy hydroksylowe odgrywają kluczową rolę w wiązaniu się tych molekuł z receptorami estrogenowymi, można podejrzewać, iż większość bisfenoli będzie wykazywało mniejsze lub większe powinowactwo do wspomnianych receptorów, a co za tym idzie analogi BPA również będą związkami endokrynnie

czynnymi. Na rysunku 1. przedstawiono struktury chemiczne, nazwy IUPAC oraz akronimy (BPx) związków z grupy bisfenoli, które były przedmiotem badań opisanych w niniejszej rozprawie.



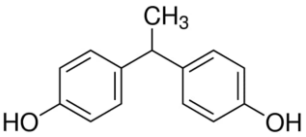
2,2-bis(4-hydroksyfenylo)propan

BPA (nr CAS 80-05-7)



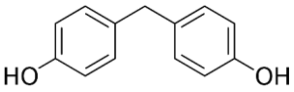
Bis(4-hydroxyphenyl)-2,2-dichloroethylene

BPC (nr CAS 14868-03-2)



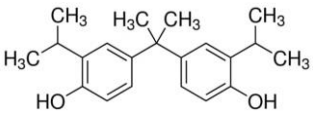
1,1-Bis(4-hydroxyphenyl)ethane

BPE (nr CAS 2081-08-5)



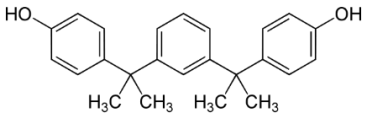
4,4′-Methylenediphenol

BPF (nr CAS 620-92-8)



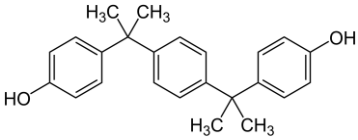
2,2-Bis(4-hydroxy-3-isopropylphenyl)propane

BPG (nr CAS 127-54-8)



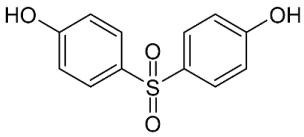
4,4′-(1,3-Phenylenediisopropylidene) bisphenol

BPM (nr CAS 13595-25-0)



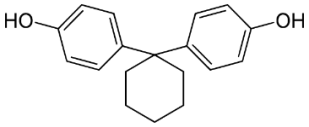
4,4′-(1,4-Phenylenediisopropylidene) bisphenol

BPP (nr CAS 2167-51-3)



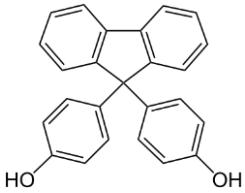
4,4′-Sulfonyldiphenol

BPS (nr CAS 80-09-1)



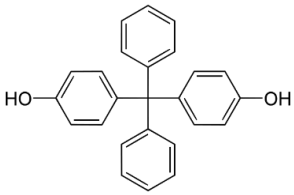
4,4′-Cyclohexylidenebisphenol

BPZ (nr CAS 843-55-0)



4,4′-(9-Fluorenylidene)diphenol

BPFL (nr CAS 3236-71-3)



1,1-Bis(4-hydroxyphenyl)-1,1- diphenylmethane

BPBP (nr CAS 1844-01-5)

Rysunek 1. Uproszczone struktury chemiczne bisfenolu A oraz innych wybranych związków z grupy bisfenoli (wraz z numerami CAS), które były przedmiotem badań podjętych w projekcie doktorskim.

O ile BPA, jego toksyczność ogólna, ekotoksyczność, potencjał endokrynny i wiele innych szkodliwych właściwości są, tak jak już wspomniano, dobrze scharakteryzowane, o tyle badania dotyczące analogów strukturalnych są tematem stosunkowo nowym w literaturze. Wraz z ich rosnącym rozpowszechnieniem w środowisku i stale powiększającą się świadomością społeczeństw o szkodliwości BPA i jego analogów, można dostrzec wyraźny wzrost zainteresowania środowisk naukowych tą grupą związków. Ilustruje to liczba dostępnych publikacji i doniesień w uznanych czasopismach z dziedzin medycyny, toksykologii, chemii, ekotoksykologii i oczywiście analityki chemicznej. Ponieważ ostatnia z wymienionych dziedzin jest niezbędnym i nieodłącznym narzędziem badawczym w pozostałych obszarach nauk technicznych i o życiu, uzasadnione jest opracowywanie nowoczesnych procedur analitycznych, umożliwiających jakościowe i ilościowe oznaczanie zawartości związków z grupy bisfenoli w matrycach różnorodnego pochodzenia.

Dodatkowo z analitycznego punktu widzenia tematyka ta stanowi niekiedy niemałe wyzwanie, choćby ze względu na to, że w większości opiera się o analizę śladów. Tu nie tylko wybór techniki końcowych oznaczeń jest kluczowy, ale przede wszystkim odpowiednio opracowane i zoptymalizowane procedury przygotowywania próbek do analizy są w stanie zagwarantować odpowiednią czułość i selektywność metody, a co za tym idzie miarodajność otrzymanych wyników. Nie bez znaczenia jest też obecność bisfenolu A i jego analogów w wielu przedmiotach codziennego użytku, a także w przyrządach laboratoryjnych i ich elementach zużywalnych, co rodzi konieczność pozbywania się analitów ze wszystkich składowych procedury analitycznej poza próbką badaną. Bez wątpienia zatem bisfenole w szeroko pojętym środowisku stanowią ważny temat, w ramach którego można pogłębiać wiedzę z zakresu chemii analitycznej, a to z kolei stanowi istotny element niniejszej pracy w ramach podejścia opartego na analizie instrumentalnej.

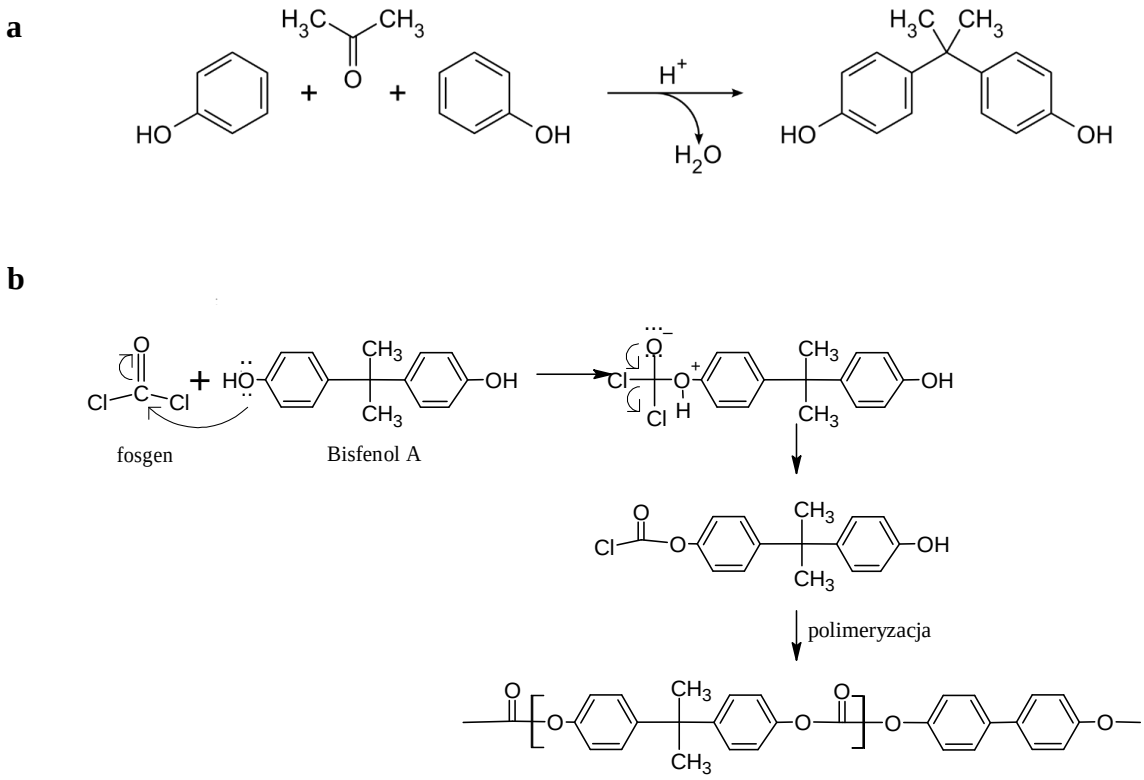
Drugim obszarem poruszonym podczas rozwijania projektu doktorskiego była część bioanalityczna stosująca biotesty do ewaluacji wzajemnych oddziaływań pomiędzy bisfenolami. Połączenie tych dwóch obszarów stanowi w przekonaniu autorki ciekawe i wyczerpujące studium problematyki związków z grupy bisfenoli, ich właściwości i rozpowszechnienia w różnych elementach środowiska, z jakimi na co dzień ma kontakt człowiek.

Część literaturowa

2. Część literaturowa

2.1. Zastosowanie bisfenolu A w przemyśle

Bisfenol A został po raz pierwszy zsyntetyzowany w 1891 roku przez rosyjskiego chemika, Aleksandra Dianina na drodze reakcji fenolu z acetonem, w obecności kwaśnego katalizatora. W latach 50-tych XX wieku odkryto, że reakcja tego związku z dichlorkiem karbonylu (fosgenem) w środowisku silnie zasadowym prowadzi do polimeryzacji i powstania twardej, przezroczystej żywicy – poliwęglanu [12]. Reakcje syntezy BPA oraz jego polimeryzacji zostały przedstawione odpowiednio na Rysunku 2a i 2b.



Rys. 2. Schematyczne przedstawienie (a) reakcji kondensacji prowadzącej do powstania cząsteczki bisfenolu A oraz (b) reakcji polimeryzacji prowadzącej do powstania łańcucha poliwęglanowego.

Od tamtej pory światowa produkcja BPA systematycznie rosła, by osiągnąć obecnie około 6 milinów ton rocznie w skali świata [1]. Związek ten stosowany jest przede wszystkim do produkcji wspomnianych wcześniej polimerów poliwęglanowych, co stanowi około 65% jego ogólnego zastosowania produkcyjnego, w około 30% używany jest w wytwórstwie żywic epoksydowych, a pozostałe 5% stanowią jego zastosowania jako stabilizator i przeciwutleniacz w produkcji polichlorku winylu czy poliuretanu [12,13].

Poliwęglanowe tworzywa sztuczne na bazie BPA charakteryzują się właściwościami korzystnymi z punktu widzenia wielu gałęzi przemysłu, takimi jak wysoka udarność, twardość,

sztywność, przezroczystość i odporność na stosunkowo szeroki zakres temperatur w przedziale od około -40°C do 145°C oraz odporność chemiczną na wiele kwasów i olei [14]. Z tego powodu znajdują zastosowanie jako materiał do produkcji między innymi bidonów, opakowań do żywności, materiałów konstrukcyjnych w budownictwie, płyt CD, sprzętu elektronicznego oraz szerokiej gamy przedmiotów codziennego użytku spotykanych w gospodarstwach domowych i miejscach pracy, takich jak sprzęt AGD [15]. Żywice epoksydowe z kolei stosowane są przy otrzymywaniu różnego rodzaju lakierów stanowiących powłoki mebli, powłoki znajdujące się wewnątrz opakowań żywności czy klejów i spoiw. Ponadto BPA często stosowany jest także w produkcji papieru termalnego jako wywoływacz koloru, tak jak ma to miejsce np. w przypadku paragonów fiskalnych [13-16]. W ostatnich latach pojawiły się również doniesienia na temat wykrywalności BPA w ubraniach – zarówno nowych jak i używanych, co może wskazywać na jego obecność w barwnikach do koloryzacji tkanin, samych tkaninach czy w detergentach do prania [17,18].

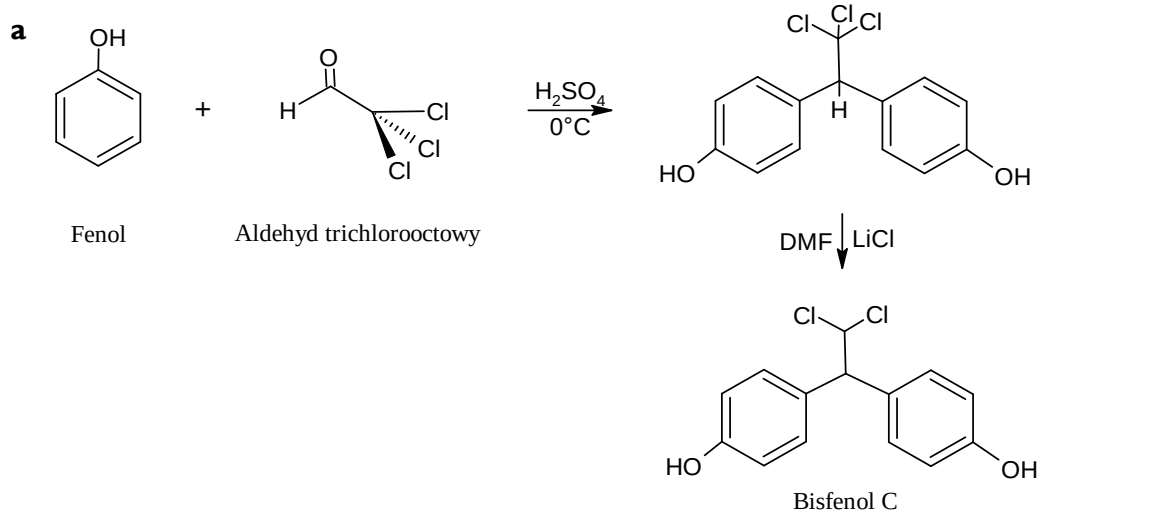
2.2. Zastosowanie przemysłowe innych związków z grupy bisfenoli

W związku z narastającym zaniepokojeniem dotyczącym negatywnego wpływu BPA na zdrowie człowieka i środowisko oraz ograniczeniami prawnymi nałożonymi na zastosowanie jego w produkcji m.in. butelek dla dzieci, wytwórcy tworzyw sztucznych rozpoczęli poszukiwanie substytutów, których wprowadzenie wiązałoby się z mniejszą presją społeczną na wycofanie potencjalnie szkodliwych produktów z rynku. Zastosowanie bisfenolu AF, bisfenolu F czy bisfenolu S stało się najpopularniejszą alternatywą i bardzo często produkty wytworzone z polimerów na bazie tych bisfenoli, oznaczane są przez producentów jako „*BPA free*". W przypadku niektórych bisfenoli dostęp do informacji na temat ich przemysłowego zastosowania jest bardzo ograniczony, natomiast istnieje wiele doniesień literaturowych wskazujących na ich występowanie w różnych elementach środowiska.

BPC jest analogiem znajdującym zastosowanie przede wszystkim w syntezie polimerów służących do produkcji materiałów ogniotrwałych [19,20]. Po raz pierwszy uzyskano BPC na drodze reakcji kondensacji fenolu z aldehydem trichlorooctowym – chloralem i następującej później dehydrohalogenacji produktu (Rysunek 3a) [20]. Początki syntezy polimerów opartych na tym związku sięgają wczesnych lat 60-tych XX wieku, natomiast reakcja polimeryzacji jest analogiczna do przedstawionej wcześniej reakcji BPA z fosgenem (Rysunek 3b).

Poza poliwęglanami, bisfenol C jest składnikiem budulcowym innych ognioodpornych i samogaszących polimerów, takich jak poliaryletery, poliuretany, epoksydy, poliamidy, policyjanurany lub żywice fenolowe [19, 21-24]. Za unikalne właściwości w zakresie ognioodporności BPC i materiałów polimerowych na jego bazie odpowiedzialne są prawdopodobnie oddziaływania pomiędzy atomami chloru a pierścieniami aromatycznymi [25].

BPE również jest związkiem chemicznym istotnym z punktu widzenia wytwórstwa polimerów. Szczególne zastosowanie mają estry cyjanianowe bisfenolu E (BECy), czyli żywice termoutwardzalne, które tworzą polimery o wysokiej termostabilności i doskonałych właściwościach mechanicznych. Dzięki absorbowaniu wilgoci w niewielkim stopniu i dużej sile klejenia polimery estrów cyjanianowych zastępują w niektórych zastosowaniach epoksydy. Dodatkowo żywice BECy są bardzo dobrym kandydatem nadającym się do napraw iniekcyjnych, np. kompozytów z włókna węglowego, przez to, że charakteryzują się wysoką temperaturą zeszklenia i niską lepkością w temperaturze pokojowej [26-29].



Rys.3 Reakcje chemiczne prowadzące do (a) powstania BPC oraz (b) jego polimeryzacji.

BPF jest związkiem o najmniejszej masie cząsteczkowej spośród wszystkich członków rodziny bisfenoli. Podobnie jak w przypadku wcześniej opisanych bisfenoli, jego produkcja oparta jest na reakcji kondensacji fenolu. Jako drugi substrat reakcji stosuje się formaldehyd, natomiast katalizatorem jest zwykle silny kwas – np. szczawiowy, chlorowodorowy lub siarkowy [30]. BPF stał się głównym substytutem BPA w produkcji żywic epoksydowych. Żywice takie charakteryzują się podobnymi lub nawet lepszymi właściwościami mechanicznymi, większą odpornością na działanie rozpuszczalników oraz niższą lepkością w porównaniu do epoksydów opartych na BPA [30,31]. Ze względu na wypieranie BPA z rynku, BPF znajduje zatem coraz szersze zastosowanie w produkcji opakowań do żywności, wypełnień dentystycznych, powłok polimerowych, lakierów czy spoiw [31,32].

W przypadku bisfenolu G znalezienie informacji (w tym publikacji naukowych) o konkretnych zastosowaniach tego związku w przemyśle jest wyjątkowo trudne. Z całą pewnością jest on również użyteczny w przemyśle produkcji tworzyw sztucznych, ponieważ jego nazwa chemiczna pojawia się w wielu patentach. BPG jest wykrywany w różnego rodzaju matrycach biologicznych i środowiskowych, coraz częściej badana też jest jego aktywność biologiczna, co zostanie opisane w kolejnych sekcjach niniejszej pracy.

BPM stosowany jest w największej mierze do produkcji polimerów termoutwardzalnych opartych na estrach dicyjanianowych bisfenolu M (BMDC) [33]. Możliwe jest również tworzenie cyklicznych poliwęglanów bisfenolu M, na drodze reakcji polikondensacji z fosgenem, katalizowanej przez trietyloaminę oraz mieszanek estrów dicyjanianowych bisfenolu A (BADC) oraz bisfenolu M [34]. Podobnie jest z diastereoizomerem BPM, czyli bisfenolem P – w tym przypadku również wytwarza się żywice estrów dicyjanianowych bisfenolu P (BPDC), które można łączyć z BADC, tworząc materiały kompozytowe o ulepszonych właściwościach i/lub trwałości [35]. Polimery takie znajdują zastosowanie w zaawansowanych technologicznie gałęziach przemysłu takich jak elektronika lub inżynieria lotnicza i kosmiczna, gdzie ważną cechą stosowanego materiału jest jego odporność termiczna [36,37].

Podobnie jak BPF, bisfenol S jest często stosowanym substytutem BPA. W produktach codziennego użytku w zastępstwie BPA zaczął pojawiać się na samym początku XXI w. [38]. Znalazł zastosowanie również jako inhibitor procesu korozji i składnik szybkoschnących klejów epoksydowych [39], a także jako wywoływacz koloru w papierze termicznym [38].

BPZ syntetyzowany jest, podobnie jak reszta związków z tej grupy, na drodze reakcji kondensacji fenolu z odpowiednim ketonem, w tym przypadku cykloheksanonem w środowisku kwaśnym. Wprowadzany jest też między innymi jako substrat do wytwarzania poliestrów aromatycznych, które charakteryzując się doskonałymi parametrami, takimi jak odporność chemiczna, stabilność termiczna i dobre właściwości fizykochemiczne, znajdują zastosowanie jako polimery w zaawansowanych rozwiązaniach inżynieryjnych w lotnictwie, elektronice czy branży samochodowej [40].

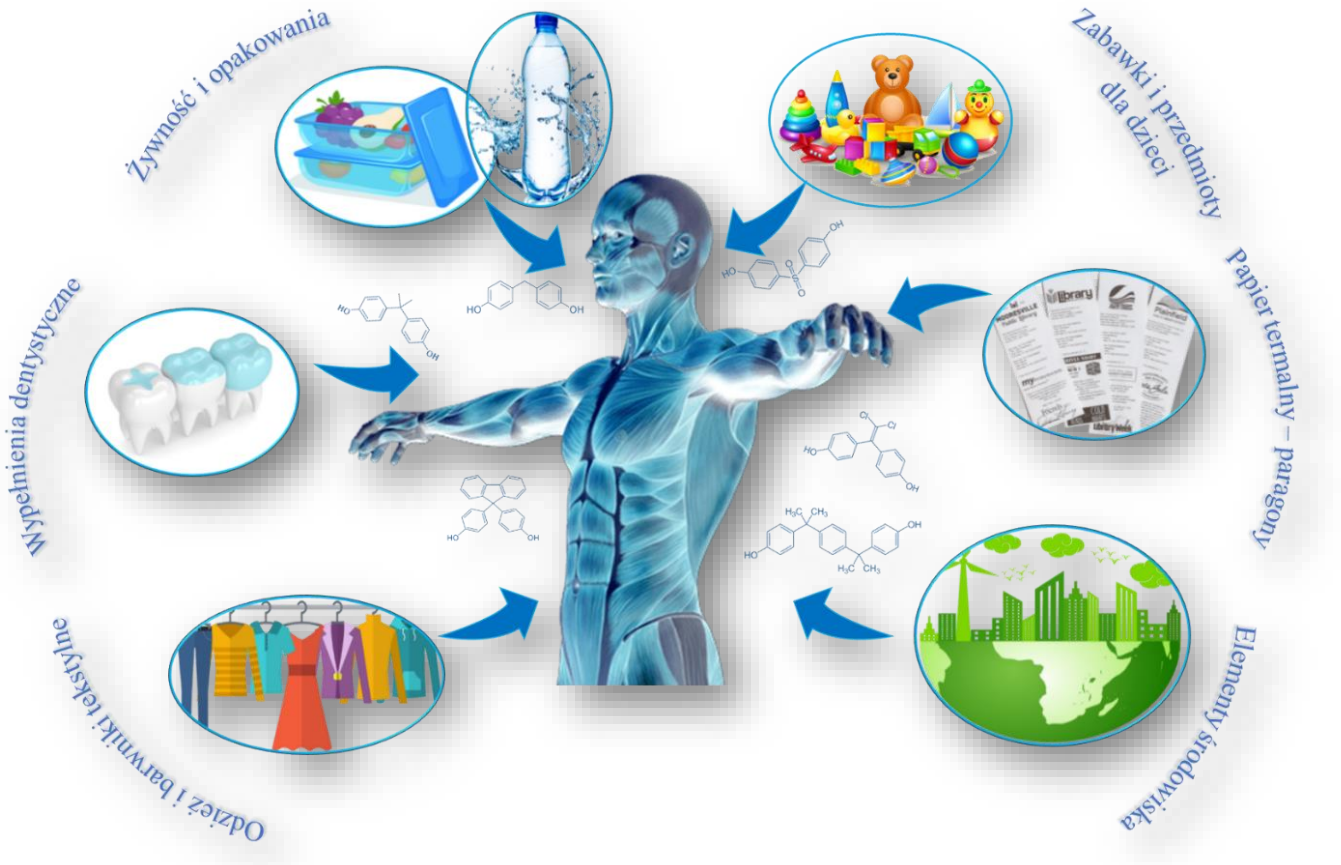
BPBP oraz BPFL są bisfenolami, których zastosowanie nie zostało dotąd szeroko opisane w literaturze; tak jak cała gama związków z tej grupy są substratami do produkcji polimerów, np. poliarylanów [41].

2.3. Drogi narażenia człowieka na BPA i jego analogi

Biorąc pod uwagę liczbę zastosowań bisfenoli, nie powinien dziwić fakt, iż istnieje wiele doniesień naukowych udowadniających ich obecność w elementach środowiska (takich jak powietrze wewnętrzne, wody powierzchniowe, osady denne, gleba czy też żywność, opakowania do żywności i cała gama przedmiotów codziennego użytku) [13, 42]. Z tego względu organizm człowieka może być narażony na kontakt z tymi ksenobiotykami poprzez układ pokarmowy, układ oddechowy oraz transdermalnie, z czego główną drogą narażenia jest droga pokarmowa. Ze względu na produkcję opakowań do żywności głównie z tworzyw sztucznych oraz lakierowanie między innymi metalowych puszek do konserw specjalnymi powłokami zawierającymi bisfenole, zanieczyszczenia żywności pochodzą przede wszystkim z wycieków tych związków z materiałów opakowaniowych. Przykłady kontaktu człowieka z elementami zawierającymi bisfenole poglądowo przedstawiono na Rysunku 4.

Jak już wcześniej wspomniano, liczne doniesienia wskazują na obecność bisfenoli w próbkach powietrza wewnętrznego i kurzu pobranych w wielu krajach Europy, Azji, obu Ameryk oraz Oceanii. W przeważającej większości próbek oznaczany był BPA, co wskazuje, że wysiłki badawcze dotyczące oznaczania BPA są prowadzone na szeroką skalę i na brak dostatecznej uwagi poświęconej innym związkom z tej grupy. Obecność w powietrzu takich bisfenoli jak BPB, BPS, BPF, BPAP, BPAF, BPZ czy BPP może być wynikiem wprowadzanych regulacji prawnych i stopniowego zastępowania BPA alternatywnymi substancjami [43-45]. Średnia zawartość BPA mieści się w przedziale od poniżej 1 ng/m^3 w domach i biurach do kilkunastu ng/m^3 w miejscach takich jak fabryki części elektronicznych czy wytwórnie tworzyw sztucznych [43-48].

Wody i osady ściekowe są doskonałym indykatorem i źródłem informacji o obecności różnego rodzaju ksenobiotyków, ponieważ oczyszczalnie ścieków są niejako punktem zbiorczym dla zanieczyszczeń uwalnianych z gospodarstw domowych, placówek biznesowych i usługowych oraz z przemysłu. Niestety, liczne doniesienia literaturowe sugerują, że BPA oraz szereg jego analogów nie są w oczyszczalniach ścieków eliminowane całkowicie, co prowadzi do dalszego uwalniania tych związków do środowiska [45, 49]. W przypadku ścieków i osadów ściekowych najwyższe stężenia obserwowano w przypadku BPA, BPF i BPS, natomiast analogi takie jak BPZ, BPE, BPB i BPAF wykrywane były w znacznie niższych stężeniach [45, 50-52]. Stężenie BPA mieściło się w zakresie około kilkuset ng/L, natomiast inne związki z tej grupy wykrywane były w przedziale od kilku do kilkudziesięciu ng/L. Zbiorniki wodne oraz osady denne są z punktu widzenia monitorowania zanieczyszczeń i ich wpływu na ekosystem, niezwykle ważnym źródłem informacji. W wodach powierzchniowych najbardziej rozpowszechnionym bisfenolem jest ponownie BPA. Na przestrzeni ostatnich kilku lat powstały prace naukowe (oryginalne i przeglądowe) podsumowujące występowanie tej klasy związków m.in. w wodach na terytorium Chin, Indii, Kanady i Azji południowo-wschodniej [45, 53-56].



Rys. 4. Przykłady narażenia człowieka na kontakt ze związkami z grupy bisfenoli.

Bisfenole są wykrywane i oznaczane w bardzo szerokim spektrum produktów spożywczych oraz przedmiotów codziennego użytku, takich jak zabawki, butelki dla niemowląt, opakowania żywności, paragony (i inne wydruki, gdzie używany jest papier termalny), a nawet bielizna i rajstopy. W przypadku żywności najwyższe stężenia BPA, BPF i BPS znajdują się w produktach puszkowanych [57-59].

Przytoczone powyżej informacje zostały przedstawione w sposób bardziej szczegółowy i usystematyzowany w Tabeli 1 poniżej.

Tabela 1. Zestawienie danych na temat występowania i poziomów BPA i innych bisfenoli w środowisku oraz produktach spożywczych

	Matryca	Region geograficzny	Okres prowadzenia badań	Zakres stężenia analitów	Źródło
Elementy środowiska	Powietrze wewnętrzne – kurz	USA Chiny Japonia Korea	2012	BPA: 0,2 – 39,1 µg/g BPAF: nd – 0,091 µg/g BPAP: nd – 0,0071 µg/g BPB: nd – 0,030 µg/g BPF: nd – 107 µg/g BPS: 0,00083−26,6 µg/g BPP: nd−0,63 µg/g BPZ: nd	[47]
	Powietrze wewnętrzne	Argentyna	2016-2017	BPA: 84 – 2454 pg/m^3	[48]
	Ścieki	USA	2013-2015	Pomiar przed oczyszczalnią BPA: nd – 8550 ng/L BPF: nd – 1170 ng/L BPS: nd – 301 ng/L Pomiar za oczyszczalnią BPA: nd – 3380 ng/L BPF: nd – 325 ng/L BPS: nd – 199 ng/L	[51]
	Osady ściekowe			BPA: 50,6 – 1910 ng/g suchej masy BPF: nd – 249 ng/g suchej masy BPS: nd – 70 ng/g suchej masy	[51]
	Ścieki	Chiny	2016	Pomiar przed oczyszczalnią BPA: 189 – 20400 ng/L BPAF: nd – 12,6 ng/L BPE: nd –97,6 ng/L BPF: nd – 166 ng/L BPS: nd – 746 ng/L Pomiar za oczyszczalnią BPA: 16,2 – 1100 ng/L BPAF: nd – 16,6 ng/L BPE: nd – 75,2 ng/L BPF: nd – 35,4 ng/L BPS: nd – 3,7 ng/L	[52]

Tabela 1. c.d.

Elementy środowiska	Wody powierzchniowe	Indie	2017-2018	BPA: nd – 14800 ng/L BPF: nd – 333 ng/L BPS: nd – 438 ng/L BPAF: nd BPB: nd BPZ: nd BPP: nd	[53]
	Wody powierzchniowe	Chiny	2015	BPA: 19,0 – 2180 ng/L BPAF: nd – 6,6 ng/L BPBP: nd – 0,43 ng/L BPE: nd – 2,69 ng/L BPF: 0,24 – 255 ng/L BPG: nd – 2,47 ng/L BPP: nd – 1,93 ng/L BPPH: nd – 1,03 ng/L BPS: 0,07 – 133 ng/L BPTMC: 0,10 – 101 ng/L BPZ: nd – 1,09 ng/L	[54]
	Osady denne	Chiny	2015	BPA: 26,6 – 1970 ng/g BPAF: nd – 4,40 ng/g BPBP: nd – 0,35 ng/g BPC: nd – 2,67 ng/g BPE: nd – 2,55 ng/g BPF: 16,0 – 1390 ng/g BPG: nd – 210 ng/g BPP: nd – 2,78 ng/g BPPH: nd BPS: nd – 5,60 ng/g BPTMC: nd – 150 ng/g BPZ: nd – 2,32 ng/g	[54]

Tabela 1. c.d.

Produkty spożywcze	Napoje	USA	2008 – 2012	Średnia zawartość [ng/g świeżej masy]: BPA: 0,235 BPAF: 0,006 BPAP: 0,005 BPB: 0,013 BPF: 0,025 BPP: 0,025 BPS: 0,007 BPZ: 0,025	[57]
	Nabiał	USA	2008 – 2012	BPA: 2,55 BPAF: 0,028 BPAP: 0,185 BPB: 0,014 BPF: 0,134 BPP: nd BPS: 0,040 BPZ: nd	[57]
	Warzywa, w tym puszkowane	USA	2008 – 2012	BPA: 8,99 BPAF: 0,009 BPAP: 0,124 BPB: 0,013 BPF: 1,00 BPP: 0,473 BPS: 0,018 BPZ: 0,076	[57]
	Pasztet	Hiszpania	2019	Średnia zawartość [µg/kg]: BPA: 13,39 (puszka), 5,10 (słoik) BPB: nd BPE: nd	[59]
	Szparagi	Hiszpania	2019	BPA: 80,66 (puszka), nd (słoik) BPB: nd BPE: nd	[59]
	Kurczak	Hiszpania	2019	BPA: 20,91 (puszka), 1,41 (świeży) BPB: 3,86 (puszka), 4,19 (świeży) BPE: nd	[59]

nd – *dot detected*

2.4. Wykrywalność związków z grupy bisfenoli w ludzkim organizmie

Bezpośrednią miarą narażenia człowieka oraz innych organizmów na związki z grupy bisfenoli jest częstość ich wykrywania oraz stężenie w poszczególnych tkankach i płynach ustrojowych. Bez wątpienia najlepiej zbadany pod tym względem jest bisfenol A, natomiast na przestrzeni ostatnich lat pojawia się coraz więcej doniesień związanych z oznaczaniem innych bisfenoli w zróżnicowanych matrycach biologicznych.

Wskaźnik wykrywalności (definiowany jako iloraz liczby próbek, w których związek był obecny w stężeniu powyżej granicy wykrywalności, do całkowitej liczby przebadanych próbek) BPA w moczu, zarówno wśród kobiet jak i mężczyzn, zawiera się w przedziale 70 - 100% [60]. Często wykrywane są także BPF i BPS, jako popularne zamienniki BPA stosowane w produktach konsumenckich. W mniejszym stopniu w moczu pojawiają się takie analogi jak BPAF, BPAP, BPB, BPP czy BPZ [61,62]. Ponadto niektóre bisfenole wykrywane są w surowicy krwi, w tym w próbkach pobranych od kobiet ciężarnych [63,64], w mleku karmiących matek [65,66,67], płynie owodniowym oraz surowicy pępowinowej [68]. Dane te skłaniają do wyciągnięcia wniosku, iż ekspozycja człowieka na BPA (oraz jego analogi) ma charakter stały i uporczywy. Co więcej, ich wykrywanie w mleku oraz płynach owodniowych wskazuje na prenatalne narażenie i niesie ze sobą bardzo niebezpieczne ryzyko związanych z tym implikacji zdrowotnych i zaburzeń rozwojowych.

2.5. Aktywność biologiczna

Związki endokrynnie czynne (EDC) są grupą substancji pochodzenia egzogennego, które mają zdolność naśladowania lub hamowania działania naturalnych funkcji układu dokrewnego – w tym hormonów, takich jak estradiol, testosteron lub hormony tarczycy [69]. Pierwsze doniesienia na temat możliwości występowania zaburzeń endokrynnych w wyniku ekspozycji w środowisku na związki endokrynnie czynne sięgają lat 70-tych XX wieku i związane były z niepełną eliminacją hormonów steroidowych z wód ściekowych w procesie ich oczyszczania. W tamtym czasie uwaga środowiska naukowego nie była skupiona na temacie EDC, ponieważ związki takie jak ludzkie hormony lub farmaceutyki o właściwościach endokrynnych wykrywane były w śladowych ilościach, natomiast wiedza na temat ich znaczenia w toksykologii oraz ekotoksykologii nie była zbyt obszerna [69]. Opinia publiczna skupiła się na problemie związków zaburzających równowagę hormonalną w momencie, kiedy nagłośniony został problem korelacji stosowania środków antykoncepcyjnych z obserwowaną feminizacją w populacjach dziko żyjących fok i ryb [69-71].

Bisfenol A już dawno temu został zakwalifikowany jako związek wchodzący w interakcje z hormonalnym. Wskazano, że cząsteczka, w której występują dwa pierścienie układem aromatyczne

z przyłączonymi grupami hydroksylowymi, wpasowuje się w miejsce aktywne receptorów estrogenowych zarówno ERα jak i ERβ, przy czym wykazuje około 10-krotnie większe powinowactwo względem receptora β [72]. Poza oddziaływaniem z receptorami jądrowymi, BPA zaburza także procesy steroidogenezy, czyli naturalnej syntezy hormonów steroidowych [73]. Ze względu na podobieństwo strukturalne, inne związki z grupy bisfenoli mogą również wywoływać niepożądane reakcje systemu endokrynnego.

2.5.1. Aktywność endokrynna

Ze względu na to, że BPA zyskał swą niechlubną sławę właśnie za sprawą oddziaływania na układ dokrewny, potencjał endokrynny pozostałych analogów jest kwestią intensywnie badaną, przede wszystkim w testach *in vitro*. Obiektem zainteresowania w tym obszarze jest zwłaszcza aktywność estrogenowa i antyandrogenowa [42]. Poza BPF i BPS, analogami wykazującymi podobny lub silniejszy potencjał estrogenny niż BPA są BPAF i BPB [42,72,74,75], natomiast aktywność antyandrogenną przejawiają BPF, BPE, BPAF i BPB [42]. W przypadku BPB, BPE, BPF i BPS potencjał aktywności endokrynnej w stosunku do receptorów estrogenowych i androgenowych jest tego samego rzędu wielkości jak w przypadku BPA [74,75]. Ponadto wykryto, iż BPAF, BPAP, BPB, BPC i BPZ, podobnie jak BPA, cechują się znacząco większym powinowactwem do receptorów ERβ niż ERα i wykazują wobec tych receptorów działanie antagonistyczne, które jednak jest słabsze niż w przypadku BPA [72].

W badaniu przeprowadzonym na linii komórkowej ludzkiego raka nadnerczy H295R, która charakteryzuje się zachowaniem kompletnej ścieżki steroidogenezy, uzyskano wyniki wskazujące na znaczące podwyższenie stężenia estradiolu (E2) po ekspozycji komórek na działanie BPA, BPF, BPM i BPS. Natomiast stężenie testosteronu (T) istotnie spadło w wyniku ekspozycji na działanie BPA, BPB, BPF, BPS, BPZ i BPAP. Poza bisfenolami P i AF, wszystkie wymienione uprzednio związki miały wpływ na podwyższenie stosunku produkcji estradiolu do testosteronu (E2/T), w następującej kolejności: BPZ>BPA>BPF>BPM>BPB>BPAP>BPS [76].

2.5.2. Wpływ na płodność

Biorąc pod uwagę potencjał endokrynny związków z grupy bisfenoli i ich wpływ m.in. na syntezę hormonów płciowych nie powinien dziwić fakt, iż narażenie na ich działanie ma wpływ na zdrowie reprodukcyjne. Stosunkowo niedawno zostały przeprowadzone eksperymenty, w których wzięło udział odpowiednio 351 oraz 450 kobiet poddających się procedurze zapłodnienia *in vitro*. Jednorazowo została od nich pobrana próbka moczu w dniu ekstrakcji oocytów. W obu badaniach u kobiet, w których moczu wykryto najwyższe stężenia BPA, uzyskano najmniejszą ilość komórek jajowych, natomiast nie zanotowano korelacji pomiędzy jakością embrionów i wskaźnikiem udanych zapłodnień a stężeniem tego ksenobiotyku [77,78].

Inny eksperyment stanowiło szeroko zakrojone badanie dotyczące poszukiwania korelacji stężenia BPA, BPAP, BPAF, BPB, BPP, BPS w moczu pacjentek u których wystąpiły nawracające poronienia. W przypadku wszystkich analogów odnotowano wysoki współczynnik wykrywalności, mieszczący się w przedziale 77,8 - 99,3%, a wykryte poziomy stężeń tych związków były statystycznie znacząco wyższe niż wśród grupy kontrolnej, gdzie poronienia nie występowały. Wyniki te wskazują na pozytywną korelację pomiędzy narażeniem na związki z grupy bisfenoli a podwyższonym ryzykiem poronienia [79].

Ponadto bisfenol A od długiego czasu łączony był z wpływem na rozwijanie się zespołu policystycznych jajników (PCOS) u kobiet i liczne doniesienia naukowe wskazują na istotnie podwyższony poziom BPA w organizmach pacjentek cierpiących na to schorzenie, w porównaniu do grupy kontrolnej, w skład której wchodzą kobiety zdrowe [80-82]. Istnieją również przesłanki pozwalające stwierdzić, że poza BPA również BPS może mieć znaczący wpływ na wystąpienie i rozwój PCOS [83].

Systematycznie rośnie również liczba badań na temat wpływu bisfenoli na męskie funkcje rozrodcze. Niepłodność wywołana zachwianiem równowagi hormonalnej oraz stresem oksydacyjnym należy do głównych implikacji narażenia męskiej części populacji na działanie tych związków. Większość danych literaturowych wskazuje przede wszystkim na spadek wytwarzania testosteronu i wzrost wytwarzania estrogenu i progesteronu w modelach zwierzęcych i liniach komórkowych, spadek produkcji nasienia i pogorszenie jego jakości oraz cytotoksyczność w stosunku do komórek Leydiga [84-88].

2.5.3. Wpływ na rozwój nowotworów

Wobec właściwości endokrynnych, a w szczególności poprzez działanie estrogenopodobne BPA jest istotnym czynnikiem ryzyka mającym wpływ na rozwój nowotworów hormonozależnych, takich jak rak jajników czy rak piersi [89]. Wiele badań *in vitro* przeprowadzonych na liniach komórkowych raka jajnika wskazało na występowanie pozytywnej korelacji między występowaniem wybranych zanieczyszczeń z grupy EDC a wzrostem intensywności proliferacji komórek nowotworowych [45,90] oraz stymulacją komórek do migracji i tworzenia ognisk przerzutów [91,92].

W 2019 roku ukazała się praca, w której autorzy wykryli działanie BPS stymulujące rozwój nowotworu piersi, poprzez jego wpływ na poziom metylacji DNA w regionie genów promotorowych, związanych z nowotworami piersi, t.j. CDH1, SFN oraz TNFRSF10C. Odbiegający od normy poziom metylacji tych regionów może prowadzić do wyciszenia genów, a to z kolei wpływa na rozwój guza. Badanie zostało przeprowadzone *in vitro* przy użyciu linii komórkowej MCF-7 [93]. Nowotwory hormonozależne nie są jedynym typem raka, na którego rozwój i inwazyjność mogą mieć wpływ związki z grupy bisfenoli. Nanomolowe stężenia BPS w badaniach *in vitro* przeprowadzonych wobec

komórek niedrobnokomórkowego raka płuc (NSCLC) powodowały zwiększenie mobilności komórek nowotworowych oraz podwyższenie ich zdolności do proliferacji [94]. W przypadku raka gruczołu krokowego wykryto, iż ekspozycja linii komórkowej LnCaP oraz C4-2 na działanie BPS powoduje wzmocnienie centrosomów [95]. BPA z uwagi na swoją aktywność estrogenową także może mieć wpływ na rozwój nowotworu prostaty, głównie poprzez modyfikację tempa proliferacji komórek [96].

2.5.4. Wpływ na rozwój zaburzeń metabolicznych

Zaburzenia metabolizmu glukozy, objawiające się jako cukrzyce różnego typu, to choroby metaboliczne na które według danych udostępnionych przez WHO cierpi około 420 milionów osób na świecie i jest to liczba, która systematycznie wzrasta [97]. Bisfenol A oraz jego analogi od dłuższego czasu były podejrzewane o wpływ na rozwój tego schorzenia. BPA został powiązany z wywoływaniem cukrzycy typu drugiego i ze zwiększonym ryzykiem wystąpienia choroby [98-100]. Ponadto, stężenie BPA w moczu u dzieci zostało powiązane dodatkowo z występowaniem otyłości (w badaniu przeprowadzonym na dużej - 2664 dzieci - reprezentatywnej grupie badawczej w Stanach Zjednoczonych) [101]. Wyniki te są spójne z wynikami uzyskanymi w grupie dorosłych mężczyzn i kobiet, gdzie autorzy znaleźli statystycznie znaczącą, liniową korelację pomiędzy stężeniem BPA w moczu a wskaźnikiem masy ciała BMI [102,103].

Także inne cząsteczki z rodziny bisfenoli, takie jak BPAF czy BPS, zostały powiązane z występowaniem cukrzycy typu drugiego [104]. BPAF, jak wykazały badania dużej grupy kobiet ciężarnych w Chinach (1841 pacjentek), ma wpływ na pojawianie się cukrzycy ciążowej. Kobiety, których indeks masy ciała mieścił się w normie przed ciążą i u których wykryto podwyższone stężenie BPAF w moczu w pierwszym trymestrze ciąży były narażone na podwyższone ryzyko wystąpienia cukrzycy ciążowej [105]. W badaniach *in vitro* prowadzonych na linii komórek wątroby HepG2 zbadany został potencjał wpływania na metabolizm glukozy dla BPA, BPF, BPS i BPAF. Związki te modulują szlaki metaboliczne w różnym stopniu, przy czym BPF oddziałuje najsilniej, wpływając na największą ilość tych szlaków. Podczas pomiaru poziomu ekspresji genów w następstwie ekspozycji na bisfenole ustalono, że BPAF zaburza ekspresję genu kodującego kinazę pirogronianową, natomiast pozostałe z badanych analogów obniżają ekspresję genu kodującego glukokinazę [106].

2.5.5. Wpływ na rozwój innych schorzeń

Bisfenole na przestrzeni ostatnich lat coraz częściej kojarzone są z chorobami sercowo-naczyniowymi, natomiast do tej pory ukazało się stosunkowo niewiele pozycji literaturowych z tego zakresu. Po raz pierwszy skorelowano podwyższony poziom BPA w moczu z zawałem mięśnia sercowego oraz anginą serca, analizując dane uzyskane podczas przeprowadzonego w latach 2003 - 2004 Narodowego Badania Zdrowia i Żywienia w Stanach Zjednoczonych (NHANES) [107]. Podobne wnioski wysnuto również badając populacje europejskie w Niemczech i we Francji [108]. Korelację

pomiędzy stężeniem BPA oznaczonym w moczu i surowicy krwi wyznaczono również w odniesieniu do ryzyka związanego z wystąpieniem choroby niedokrwiennej serca, miażdżycą tętnic szyjnych u dorosłych i zwiększeniem grubości ścian tętnic szyjnych u młodzieży i młodych dorosłych [109-111]. W przypadku innych bisfenoli, ilość dostępnych danych jest ograniczona i są to najczęściej badania prowadzone na modelach zwierzęcych. Podobnie jak BPA, BPS podawany myszom lub szczurom wywołał skutki arytmogenne [112] i w znaczącym stopniu spowolnił odzyskanie sprawności po zawale serca, w szczególności u samców [113, 114].

W niektórych źródłach można znaleźć także informacje dotyczące związku bisfenoli, przede wszystkim BPA, BPF i BPS, z zaburzeniami układu immunologicznego. Jest to istotny kierunek opisu relacji bisfenole – organizmy żywe. Odkryto na przykład, iż podwyższone poziomy tych substancji w moczu pacjentów związane są z przypadkami występowania alergii typu astmy i kataru siennego [115]. Co ciekawe, również układ nerwowy podatny jest na negatywne oddziaływanie BPA, którego obecność została powiązana z chorobami neurodegeratywnymi, takimi jak choroba Parkinsona lub Alzheimera. BPA może bowiem modyfikować szlaki dopaminergiczne w mózgu, co w efekcie jest jednym z czynników pojawiania się chorób tego typu. Jednak warto podkreślić, że jest dotychczas niewiele badań przeprowadzonych na reprezentatywnie licznych grupach pacjentów [116]. Poza tym, w przypadku ekspozycji prenatalnej oraz w wieku wczesnodziecięcym, długotrwałe narażenie na BPA może być przyczyną zaburzeń neurobehawioralnych, takich jak hiperaktywność, agresja czy niepokój, a efekty zależne są między innymi od płci dziecka [117-119].

2.6. Analityka związków z grupy bisfenoli

Biorąc pod uwagę powyższą wiedzę, uzasadnionym staje się pytanie, czy substytuty bisfenolu A na pewno są rozwiązaniem przynoszącym holistycznie więcej „korzyści" niż „szkód". Z całą pewnością ważne jest dalsze poszerzanie wiedzy na temat mechanizmów działania, poziomu narażenia i wzajemnych oddziaływań związków z grupy bisfenoli, a w szczególności tych analogów, którym do tej pory poświęcano znacznie mniej uwagi niż BPA. Narzędziem niezbędnym do tego celu jest między innymi chemia analityczna oraz jej nowoczesne procedury i metodyki, pozwalające jakościowo i ilościowo oznaczać te związki w śladowych stężeniach w różnego rodzaju matrycach.

2.6.1. Właściwości fizykochemiczne bisfenoli

Z punktu widzenia opracowywania i rozwoju efektywnych metod analitycznych służących do identyfikacji i ilościowego oznaczania dowolnego analitu, jego właściwości fizykochemiczne są kwestią kluczową. Znajomość parametrów takich jak polarność, kwasowość (rozumiana jako ujemny logarytm ze stałej kwasowości – pK_a) czy współczynnik podziału oktanol-woda (K_{ow}) pozwala analitykowi przewidzieć zachowanie danej cząsteczki w układzie chromatograficznym czy ekstrakcyjnym, a co za

tym idzie oszczędzić czas i zasoby bez konieczności stosowania podejścia opartego na metodzie prób i błędów.

Bisfenole na których skupiono się w niniejszej pracy, pomimo oczywistego faktu przynależności do konkretnej rodziny związków, znacząco różnią się od siebie właściwościami fizykochemicznymi typu Kow czy rozpuszczalność, co nie powinno dziwić po bliższym przyjrzeniu się ich strukturom chemicznym (por. Rysunek 1.). Jednak większość BPx wykazuje charakter słabego kwasu z wartościami pKa około 10. Wybrane właściwości analitów zebrano w Tabeli 2.

Tabela 2. Właściwości fizykochemiczne wybranych związków z grupy bisfenoli

Nazwa	**Wzór cząsteczkowy**	**Masa molowa [Da]**	**pK_a [a]**	**log K_{ow} [b]**	**Rozpuszczalność w wodzie [mg/L]**
BPA	$C_{15}H_{16}O_2$	228,29	10,29 -10,93	3,64	172,7
BPC	$C_{14}H_{10}Cl_2O_2$	281,13	9,71 – 10,01	3,75	37,9
BPE	$C_{14}H_{14}O_2$	214,26	10,00 - 10,10	3,19	265,0
BPF	$C_{13}H_{12}O_2$	200,23	9,81 – 10,01	3,06	542,8
BPG	$C_{21}H_{28}O_2$	312,45	10,14 – 10,32	6,55	0,10
BPM	$C_{24}H_{26}O_2$	346,46	10,21 – 10,41	6,25	0,11
BPP	$C_{24}H_{26}O_2$	346,46	10,21 – 10,41	6,25	0,11
BPS	$C_{12}H_{10}O_4S$	250,27	7,46 - 8,23	1,65	3518,0
BPZ	$C_{18}H_{20}O_2$	268,35	9,91-10,51	5,00	3,8
BPBP	$C_{25}H_{20}O_2$	352,43	10,01-10,91	6,08	0,15
BPFL	$C_{25}H_{18}O_2$	350,41	9,58-10,19	6,08	0,012

[a] Obliczone przy użyciu oprogramowania EPISuite™ US EPA, [b] Dane pochodzące z ogólnodostępnej bazy *on-line* - ChemSpider

2.6.2. Metodyki i narzędzia analityczne stosowane do oznaczania bisfenoli

Fakt, iż poszczególne bisfenole posiadają różne właściwości fizykochemiczne oraz najczęściej występują w śladowych ilościach w matrycach o zróżnicowanym składzie, sprawia, że analiza mieszanin tych związków może stanowić pewnego rodzaju wyzwanie analityczne. Stwarza to konieczność opracowywania wysoce czułych i selektywnych, specyficznych metod umożliwiających ich ilościowe oznaczanie. Z tego względu analizy takie wykonywane są niemal wyłącznie z zastosowaniem spektrometrii mas sprzężonej z wysokosprawną chromatografią cieczową lub - w mniejszym zakresie - gazową (po uprzedniej derywatyzacji analitów). W literaturze można natknąć się również na prace, gdzie zastosowana została detekcja fluorescencyjna, jednak z techniką tą spotykamy się zdecydowanie rzadziej. Z uwagi na wcześniej wspomniane niskie stężenia bisfenoli (zwykle rzędu pikogramów i nanogramów na mililitr) najszersze zastosowanie znajdują techniki oparte na zastosowaniu tandemowej spektrometrii mas w trybie MRM (*Multiple Reaction Monitoring*).

W przypadku metod angażujących chromatografię cieczową, zdecydowanie przeważa zastosowanie jonizacji typu elektrorozpylania w trybie ujemnym (ESI -). Wynika to przede wszystkim z obecności w cząsteczce grup hydroksylowych, które, w przypadku tej grupy związków, są miejscem najłatwiej ulegającym jonizacji zachodzącej poprzez odszczepienie protonu. Fazy ruchome w przeważającym stopniu bazują na mieszaninie metanolu i wody, często buforowanych octanem lub mrówczanem amonu z dodatkiem amoniaku. Kolumny chromatograficzne najczęściej pojawiające się w doniesieniach literaturowych dotyczących podziału i analiz bisfenoli w układach LC-MS to kolumny wypełnione krzemionką modyfikowaną grupami oktadecylowymi, czyli popularne i niezwykle szeroko stosowane złoża typu C18.

Metody oparte o chromatografię gazową, jak już wspomniano, wymagają przeważnie wcześniejszej derywatyzacji, zwykle z zastosowaniem odczynników sililujących MSTFA lub BSTFA, bądź bezwodnika octowego służącego do acylacji analitów. Najczęściej stosowaną techniką jonizacji jest jonizacja elektronami – EI.

Stosowane procedury przygotowania próbek najczęściej obejmują takie etapy jak ekstrakcja analitów z matrycy, oczyszczenie ekstraktu w celu usunięcia substancji interferujących oraz jego zatężenie by uzyskać odpowiednią czułość analizy. Próbki żywności przygotowywane są w różny sposób, w zależności od postaci w jakiej się znajdują. Próbki stałe najczęściej są w pierwszym etapie homogenizowane lub liofilizowane [122]. Dominującym sposobem ekstrakcji jest ekstrakcja za pomocą rozpuszczalnika, gdzie w przypadku bisfenoli acetonitryl jest rozpuszczalnikiem preferowanym [122]. Wspomaganie ekstrakcji, poprzez użycie zewnętrznego źródła energii takiej jak mikrofale lub ultradźwięki, może w znaczący sposób poprawić jej efektywność oraz zminimalizować objętość zużywanego w tym procesie rozpuszczalnika [122 – 124]. W przypadku próbek pochodzenia zwierzęcego i takich, które zawierają znaczne ilości tłuszczów, niezbędne jest dodatkowe oczyszczenie próbki. Lipidy bowiem negatywnie wpływają na sprawność chromatograficzną, osadzając się na wypełnieniu kolumny i redukując jej zdolność rozdzielczą. Dodatkowo w przypadku technik stosujących spektrometrię mas, tłuszcze zanieczyszczają źródło jonów prowadząc do znaczącej supresji sygnału. Aby je usunąć z ekstraktów, najczęściej stosowane są do przemywania wysoce niepolarne rozpuszczalniki takie jak *n*-heksan lub *n*-heptan [125]. Natomiast żywność w formie płynnej lub próbki wód powierzchniowych są często poddawane oczyszczeniu wstępnemu za pomocą techniki SPE (*Solid Phase Extraction*), opartej na nieselektywnych złożach sorpcyjnych, takich jak C18. Ekstrakcja może bazować na dwufazowym układzie ekstrakcyjnym ciecz-ciecz lub być prowadzona z zastosowaniem sorbentu w postaci SPME (*Solid Phase Microextraction*), gdzie włókna wykonane są z np. poliakrylanów [126] lub SBSE (*Stir Bar Sorptive Extraction*) z sorbentem polidietylosiloksanowym [127]. Następnie anality uwalniane są drogą termicznej desorpcji lub za pomocą odpowiednio dobranego rozpuszczalnika [122].

Inną zminiaturyzowaną metodą ekstrakcji związków z grupy bisfenoli, która regularnie pojawia się w literaturze jest dyspersyjna mikroekstrakcja w układzie ciecz-ciecz (DLLME), pozwalająca na zredukowaniu zastosowanej ilości toksycznych rozpuszczalników organicznych. W przypadku tej techniki specjalnie dobrany układ rozpuszczalnika dyspergującego i ekstrahującego jest wstrzykiwany do ciekłej próbki. Tworzy się mętny roztwór złożony z mikrokropel rozpuszczalnika rozproszonych w fazie wodnej próbki, przez co anality niemal natychmiast przechodzą do fazy organicznej. Mikrokrople charakteryzują się bardzo dużą powierzchnią właściwą, co sprzyja wzrostowi efektywności podziału analitów pomiędzy obie fazy [128]. DLLME jest techniką najczęściej stosowaną w przypadku matryc wodnych, takich jak różnego rodzaju napoje oraz w analizie moczu lub osocza [122]. W przypadku próbek biologicznych, bisfenole jak każde ksenobiotyki występują w formie wolnej i zmetabolizowanej, jako koniugaty. Dlatego przed analizą zwykle konieczne jest przeprowadzenie hydrolizy enzymatycznej, by oznaczyć stężenie całkowite analizowanej substancji. Najbardziej popularnymi koniugatami takich metabolitów są glukuroniany i siarczany [122].

Przegląd metod analitycznych wraz z etapem przygotowania próbek oraz wyróżnieniem matrycy, w której bisfenole były oznaczane, został przedstawiony i usystematyzowany w Tabeli 3.

Tabela 3. Przegląd metod służących do oznaczania szeregu bisfenoli w próbkach różnego pochodzenia

Rodzaj próbki	Anality	Przygotowanie próbki	Technika oznaczeń końcowych	Odzysk [%]	LOD/LOQ [ng/mL]	Źródło
Mocz	BPA, BPAF, BPAP, BPF, BPS, BPP, BPZ	Hydroliza enzymatyczna z użyciem β-glukuronidazy, rozcieńczenie próbki 10% roztworem NaCl, następnie DLLME: aceton 750 µL – rozpuszczalnik dyspergujacy, 1,2-dichloroetan 500 µL – rozpuszczalnik ekstrahujący; wytrząsanie 10 s; wirowanie 20 min; odparowanie ekstraktu, rozpuszczenie w mieszaninie MeOH:H_2O	LC-MS/MS ESI (-) (MRM); kolumna: Supelco Ascentis Express C18 75 mm × 2,1 mm × 2,7 µm; Układ faz ruchomych: MeOH:H_2O + 0,1% amoniaku (v/v), ISTD: BPA-*d*16	90 – 105	LOD: 0,005 – 0,2 LOQ: 0,02 – 0,5	[129]
Mocz	BPA, BPAF, BPAP, BPB, BPE, BPF, BPS, BPP, BPZ	Hydroliza enzymatyczna z użyciem β-glukuronidazy; Ekstrakcja DLLME: ACN 1325 - µL, Tetrachloroetylen – 85 µL i bezwodnik octowy - 125 µL (upochodnienie). Wirowanie 20 min;	GC-MS (SIM) Kolumna: DB-5_{MS} 30 m × 0,25 mm × 0,25 µm ISTD: BPA- *d*16	62 – 99	LOD: 30 – 4550	[130]
Mocz	BPA, BPB, BPE, BPF, BPS, BPZ, BPAF	Rozcieńczenie próbki buforem fosforanowym pH=7, SPE na złożu PDVB-QPEI (polidivinylbenzene – quarternary polyethyleneinine): przygotowanie MeOH – 5 mL, H_2O – 5 mL, przemycie wodą amoniakalną pH=12, wymycie próbki za pomocą MeOH;	HPLC - UV Kolumna: Agilent ZORBAX SB-C18 250 mm × 4.6 mm × 5 µm; Układ faz ruchomych: ACN: 10 mM bufor foforanowy pH=2,7	82,4 – 99,4	LOD: 3 – 6	[131]
Osocze	BPA, BPAF, BPF, BPS	Rozcieńczenie próbki solą fizjologiczną, ekstrakcja LLE: Eter metylowo-tertbutylowy – 2 mL, zebranie fazy organicznej, odparowanie do sucha, rozpuszczenie w 10 mM mrówczanie amonu	LC – MS/MS ESI (-), MRM Kolumna: Kinetex C18 150 mm × 3,0 mm × 1,7 µm Układ faz ruchomych: MeOH: H_2O ISTD: BPA- *d*16, BPS- *d*4	93,0 – 113,1	LOQ: 0,042 – 0,151	[132]

Tabela 3. c.d.

Mleko ludzkie	BPA, BPB, BPAP, BPAF, BPBP, BPC, BPE, BPPH, BPS, BPF, DHDPE, BPFL, BPZ, BPM, BPP,	Hydroliza enzymatyczna z użyciem β-glukuronidazy/ sulfatazy; Odbiałczenie: Aceton, 5 + 3 mL Odparowanie warstwy organicznej Ekstrakcja: SPE, CHROMABOND HR-X; Elucja: ACN, 14 mL Oczyszczenie: SPE, MIP(MACHEREY BPA); Elucja: MeOH, 10 mL Odparowanie rozpuszczalnika Rozpuszczenie w ACN Derywatyzacja za pomocą MSTFA	GC-EI-MS (SRM) Kolumna: Optima®-17-MS 30 m × 0,25 mm × 0,25 mm	90 - 109	LOD: 0,01- 0,09 LOQ: 0,4	[67]
Warzywa Napoje Nabiał, Owoce morza	BPA, BPF, BPS	Homogenizacja próbek stałych, Ekstrakcja: Octan etylu 5 mL, Oczyszczenie: SPE Oasis HLB, kondycjonowanie MeOH 3 mL, H_2O 3 mL, Elucja: MeOH 5 mL, Odparowanie, rozpuszczenie w mieszaninie ACN:H_2O (1:1 v/v) Napoje: ogdazowanie na łaźni ultradźwiękowej, Ekstrakcja: octan etylu 6 mL, oczyszczenei SPE – jak wyżej	LC – MS/MS ESI (-) (MRM) Kolumna: Aqcuity BEH C18 2,1 mm × 50 mm × 1,7 µm Układ faz ruchomych: ACN: H_2O ISTD: BPA- *d*16	80,3 – 103,8	LOD: 0,003–0,015 ng/ mg LOQ: 0,01 – 0,05 ng/ mg	[133]
Napoje alkoholowe i bezalkoholowe	BPA, BPAF, BPAP, BPB, BPE, BPF, BPP, BPS, BPZ	Ekstrakcja SPE: Złoże Oasis MCX, kondycjonowanie: octan etylu 5 mL, metanol 5 mL, 0,1% FA 5 mL, elucja mieszaniną octanu etylu/metanolu/0,1% FA. Derywatyzacja: chlorek pyridino-3-sulfonylu	LC – MS/MS ESI (+) MRM Kolumna: Ascentis Express F5 100 mm × 2,1 mm × 2,7 µm Układ faz ruchomych: H_2O + 0,2% FA, ACN/H_2O + 0,2% FA d (98:2, v/v), ISDT: BPS-^{13}C12	>94	LOD: 0,0016 – 0,028 LOQ: 0,0052 – 0,093	[134]
Mleko modyfikowane dla niemowląt	BPA, BPAF, BPC, BPE, BPFL, BPS, BPZ	Rozpuszczenie mleka w proszku w wodzie MilliQ – 5 mL, dodatek ACN – 5 mL, dodatek bezwodnego $MgSO_4$ – 2 g, dodatek NaCl – 1 g. Wirowanie – 10 min, zamrożenie -20°C, ponowne wirowanie, przeniesienie ekstraktu na płytkę filtracyjną, wirowanie 10 min;	LC-MS/MS ESI (-) MRM Kolumna: Phenonemex Kinetex C18 100 mm × 2,1 mm × 1,7 µm Układ faz ruchomych: MeOH/H_2O ISDT: BPA-*d*10, BPAF-*d*4, BPE-*d*12 BPS-*d*8	91,7 – 106,5	LOQ: 0,09 – 0,29 [ng/g]	[135]

Tabela 3. c.d.

Papier	BPA, BPB, BPF, BPS	Ekstrakcja: dodatek metanolu i wspomaganie ultradźwiękami – 20 min. Zatężenie próbki w łagondym strumieniu azotu, rozpuszczenie w H_2O.	LC – MS/MS ESI (-) MRM Kolumna: Phenomenex Kinetex C18 2,1 mm × 100 mm × 1,7 µm Układ faz ruchomych: MeOH/H_2O ISTD: BPA-^{13}C12	71 - 133	LOD: 0,29 - 0,40 [ng/g] LOQ: 1,09 – 1,32 [ng/g]	[136]
Środki higieny osobistej: pasty do zębów, szampony, żele do mycia twarzy, żele pod prysznic, kosmetyki z filtrem przeciw-słonecznym, kremy do rąk	BPA, BPF, BPP, BPS, BPZ, BPAP, BPAF	Ekstrakcja za pomocą MTBE – 5 mL, 30 min, wirowanie 10 min, zebranie supernatantu, ponowna ekstrakcja, połączenie ekstraktów, odparowanie do sucha pod łagodnym strumieniem azotu; Rozpuszczenie w mieszaninie DCM/heksan (9:1); Oczyszczenie ekstraktu przy pomocy SPE: złoże krzemionkowe, kondycjonowanie: DCM/octan etylu (1:1) – 5 mL, heksan – 5 mL; Elucja: DCM/octan etylu (1:1) – 10 mL, odparowanie ekstraktu do 1mL.	LC – MS/MS ESI (-) MRM Kolumna: Atlantis C18 2,1 mm × 150 mm × 5 µm Układ faz ruchomych: MeOH/ 10 mM octan amonu ISTD: BPA-^{13}C12	70 - 93	LOQ: 0,25 – 1 ng/g	[137, 138]

2.6.3. Źródła zewnętrznych zanieczyszczeń bisfenolami i metody ich usuwania

Z uwagi na duże rozpowszechnienie bisfenoli, zanieczyszczenie tymi związkami może nastąpić na etapie pobierania próbki, zabezpieczania jej, przygotowania do analizy jak i samego oznaczenia. W środowisku laboratoryjnym, gdzie całe spektrum materiałów zużywalnych oraz aparatury (np. rękawiczki jednorazowe, końcówki pipet, próbówki wirówkowe, kolumny do SPE, woda o wysokiej czystości, gdzie bisfenole mogą przenikać z plastikowych elementów systemów oczyszczających czy wszelkiego rodzaju łączeń w aparaturze chemicznej [139-141]) wykonane jest z tworzyw poliwęglanowych lub żywic epoksydowych, ryzyko przedostania się bisfenoli do próbki jest znaczące. Biorąc pod uwagę przypadkowy z reguły charakter tego typu zanieczyszczenia próbki, wskazanie konkretnego jej źródła jest najczęściej niezwykle trudne. Aby uniknąć takiej kontaminacji należy przede wszystkim unikać kontaktu próbki z materiałami wykonanymi z wcześniej wymienionych rodzajów tworzyw sztucznych. Ponadto wysoce wskazane jest przygotowywanie i analizowanie ślepej próby, poddawanej dokładniej tym samym procesom przygotowania, co próbki badane, aby na bieżąco monitorować obecność i poziom zawartości bisfenoli w tle.

Procedura przygotowania materiałów laboratoryjnych, najczęściej opisywana w literaturze, wskazuje na konieczność kilkukrotnego przemywania szkła laboratoryjnego rozpuszczalnikiem organicznym, takim jak np. metanol oraz późniejsze wypiekanie szkła w wysokiej temperaturze rzędu 400 – 500^{o}C przez 2 do 4 godzin. W przypadku stosowania ekstrakcji SPE, bisfenole są efektywnie wymywane z kolumienki podczas etapu jej kondycjonowania rozpuszczalnikiem organicznym [142, 143].

Podsumowanie problemów analitycznych najczęściej napotykanych podczas opracowywania metod służących do ilościowego oznaczania śladowych ilości analitów w próbkach o skomplikowanym składzie matrycy opisano w pracy przeglądowej opublikowanej w czasopiśmie *Trends in Analytical Chemistry* [144]. Poruszono w niej zagadnienia dotyczące między innymi zewnętrznego zanieczyszczania próbek najbardziej rozpowszechnionym związkiem z grupy bisfenoli – BPA oraz przytoczono przegląd doniesień literaturowych opisujących sposoby eliminacji źródeł tego typu zanieczyszczeń. W przypadku związków z rodziny bisfenoli mowa jest zazwyczaj o śladowych lub ultra-śladowych poziomach stężeń, w próbkach pochodzenia biologicznego. Dlatego każde zanieczyszczenie próbki jest wysoce niepożądane, ponieważ prowadzi do przeszacowania zawartości lub wyników fałszywie pozytywnych, a w konsekwencji do wysnucia błędnych wniosków.

Trends in Analytical Chemistry 105 (2018) 173–184

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Main complications connected with detection, identification and determination of trace organic constituents in complex matrix samples

N. Szczepańska, M. Rutkowska, K. Owczarek, J. Płotka-Wasylka*, J. Namieśnik

Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, 11/12 G. Narutowicza Street, 80-233 Gdańsk, Poland

ARTICLE INFO

Article history:
Available online 30 May 2018

Keywords:
Analysis of trace constituents
Background problems in analysis
Error sources in sample preparation
Types of background problems
Blank problems

ABSTRACT

It is well known that some problems with the determination of organic analytes at trace level can occur. This issue is connected with contamination during each stage of the analytical procedure from sampling to sample preparation up to chromatographic analysis, which often leads to false-positive or overestimated results. Another problem associated with determination of analytes occurs at trace- and ultra-trace level is a background problem which is mainly dictated by techniques, glassware and solvents. This review provides information on main complications connected with determination of trace organic constituents in complex matrix samples. Error sources in the field of determination of trace analytes are described in detail. In addition, the type of the background in each of the stapes of analytical procedure is summarized.

1. Introduction

In the end of XIX century, Wilhelm Ostwald, defined analytical chemistry as the art of separating, recognizing different substances and determining the constituents of a sample [1]. Since this time, analytical chemistry evolved from art to a branch of chemical science of greatest theoretical and practical utility for medicine, industry and in general for all applied sciences [2]. Over the years, applications of analytical chemistry in many different areas such as food science, pharmaceutical science, wastewater technology, etc. led to the development of analytical approaches aimed to the global characterization of a thousand type of samples to define their physico-chemical and sensory characteristics as well as to ensure their quality and safety for consumers, supporting and implementing the laws and regulations of the many fields.

Nowadays it can be stated that analytical chemists are philosophizing and moreover, they are using many new logical terms and ideas to describe population distributions at near zero concentrations, where the quantized nature of matter dominates [3]. These new definitions and concepts are required to estimate the limits of detection and quantification and the capability of an analytical method, equipment, and connect them with its selectivity, sensitivity and optimization [3,4]. The evolution and development of separation techniques like extraction, cloud point extraction, solid and liquid phase microextraction techniques were also of high importance [5].

In recent years, design and discover completely new analytical methodologies and reference materials that will constitute the tools for obtaining reliable analytical information become of major importance. This is due to the fact that analytical chemistry frequently requires the determination of sub-ultra-trace xenobiotics in samples, which are very often characterised by a complex matrix composition (Fig. 1).

Without a doubt it need to be stated that in the last 7 decades, analytical chemists succeeded to introduce many improvements in their work as well as cost and time saving procedures. And so, several classical methods such as gravimetric and titrimetric analyses are now in most cases replaced by instrumental techniques which can produces two- and even multi-dimensional information [6]. Moreover, several new approaches with advance analytical features have been introduced. Old applications which let the determination of a single or at most two analytes have been improved or replaced by others which allow to simultaneous determination of dozens of analytes at quantities of a few nanograms, picograms or even femtograms [6].

This improvement is of high importance as the application area of trace- and ultra-trace analysis is extensive (Fig. 2). In addition,

* Corresponding author.
E-mail addresses: plotkajustyna@gmail.com, juswasyl@pg.edu.pl (J. Płotka-Wasylka).

https://doi.org/10.1016/j.trac.2018.05.005
0165-9936/

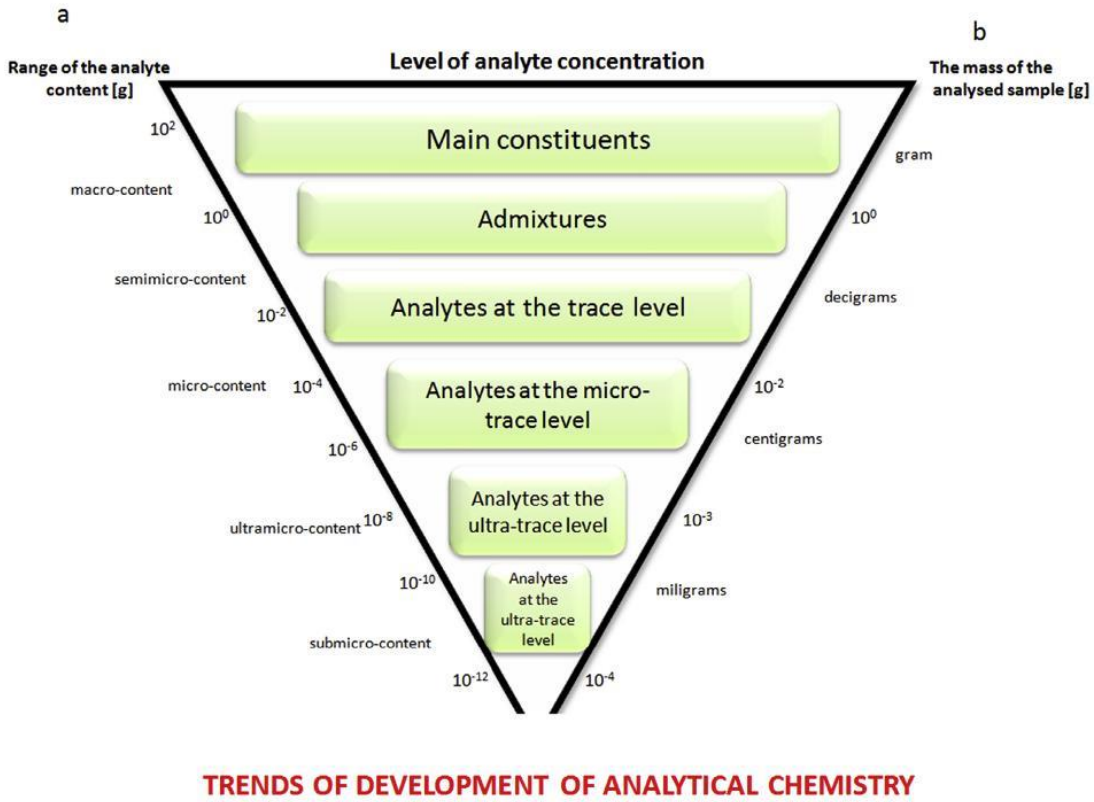


Fig. 1. Schematic classification of different analytical approaches from the point of view of: a) range of analyte content in different types of sample of representation of directions in the development of analytical chemistry; b) size (mass or volume) of sample for analysis.

the consideration of noises and interferences of the analytical signal, the strategies of their removal as well as the concepts of sensitivity, limit of detection (LOD), limit of decision, response time, etc. are treated in some cases mainly by applying logical ideas, for example different tools of chemometric analysis [7].

It is well known that some problems with determination of analytes that occur in a sample at trace-level of concentration exist. The various operations carried out during sample preparation procedure which is very often required prior to final determination, can be a source of many errors crucially affecting the final result of analysis [7,8].

Another problem associated with determination of analytes occurs at trace- and ultra-trace level is a background problem. Two types of background problem are known: an instrumental or constants background, and a sample- or volume-dependent background [9]. The later is the most important and problematic.

This review provides information on an important topic which is the problem associated with determination of organic compounds occur in the sample at trace- and ultra-trace level of concentration. Error sources in the field of determination of trace analytes are described in detail. In addition, the type of the background in each of the stages of analytical procedure is summarized.

To the best of our knowledge, no review article related to problem of background and its value estimation in determination of trace organic analytes published in last few years exist. It will be useful for the readers not only because it presents the problem of background but mainly, it shows how to solve this problem.

2. Type of the background in each of the stage of analytical procedure

It is well known that different types of blanks exist and can be distinguished on every stage of analytical method existed [5]. These are presented on Fig. 3. A proper preparation and analysis of these blanks may provide valuable information on characteristics of overall background noise and interferents. Field blank and trip blank contribute to evaluating contamination connected to sampling technique, preservatives applied and transportation. Field blank should be prepared on matrix matched to the sample type, without addition of analytes standard solution. Trip blanks may be prepared in the field or in laboratory and then transported along with samples.

Method blanks provide information on contamination that may occur during sample preparation techniques under laboratory conditions. These kind of blanks are prepared in the same way as actual samples, without analyte addition. Reagent blanks are useful for determination of analytes concentration in specific reagents used at sample preparation stage. Finally, instrument blank is used to identify contamination that origins from system components. General characteristics of samples used in the stage quality control of analytical results are presented in Fig. 4. More detailed description of each blank and background problems are discussed in the next paragraphs of this paper.

It also needs to be mentioned that the important issue that impact on the background error is the location of the analytical instrument in relation to the tested object/sample. The following

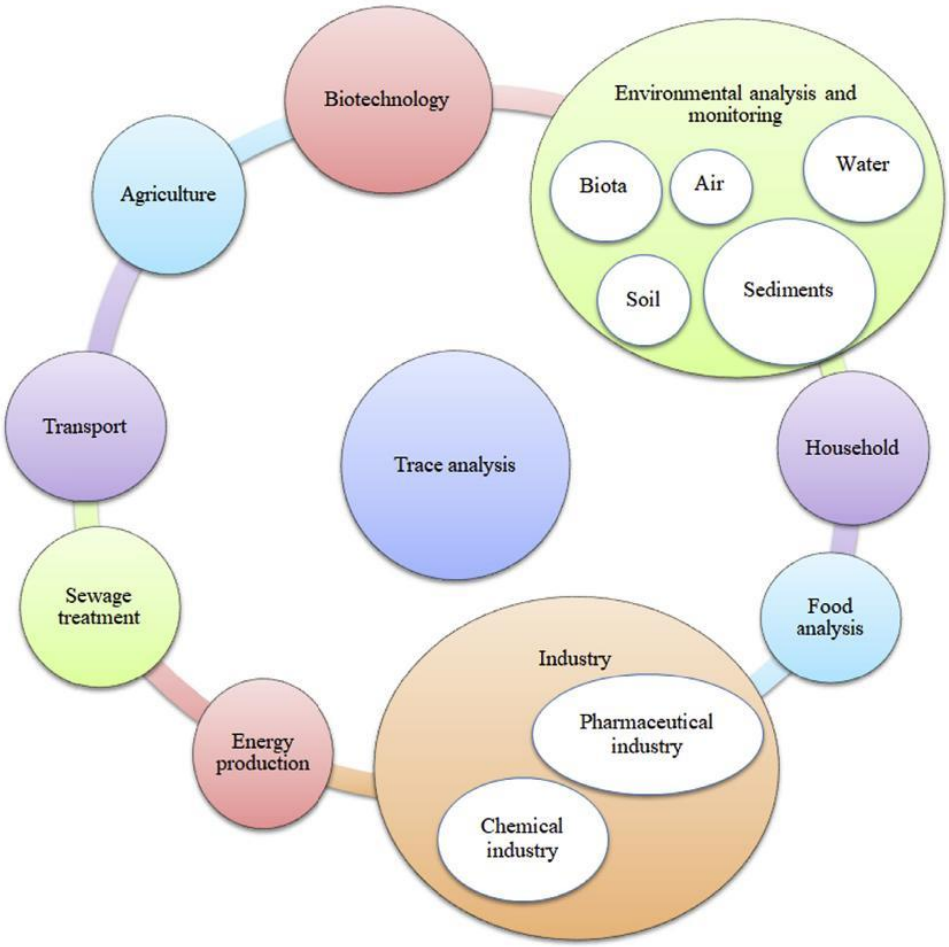


Fig. 2. Different subject areas which require trace or ultra-trace detection of the analytes.

locations should be listed here: in-line, on-line, at-line and off-line [10]. This is very important because it affect the transport time and thus required storage conditions, preservation, what in sum can have negative impact on loss of analytes.

3. Error sources in the field of determination of trace analytes concentration

Organic compounds are widely used and they are present in air, water, organic solvents or plastics. Ubiquitous presence generated the interest in the determination of organic compounds at relatively low levels. The determination of those components in samples of different matrix composition is becoming ever more common therefore more attention should be paid to problems involved in this type of activity [5]. It is widely recognised that one of the major problems in trace analysis of organic analytes is contamination during each stage of the analytical procedure from sampling to sample preparation up to chromatographic analysis, which often leads to false-positive or overestimated results. Factors which may affect the level of the traces in the liquid sample are presented in a schematic way in Fig. 5. It is certainly not surprising that strict control of the "blanks" is a primary requirement.

3.1. Blank problems – methods of elimination or reduction

It is common that analytical results are too high due to contamination during sample preparation and determination of analytes steps, or no satisfactory limits of detection (LODs) can be achieved due to too high background signals. Therefore, each procedure should help to ensure the transparency of the blank problem, provide methods for identifying background sources and ways to reduce or eliminate system contamination in order to design methods keeping blanks below a critical threshold. Solving blind sampling problems can be difficult to start if several sources contribute to the contamination of the system. Elements that may have an impact on the background value in the analysis of trace organic analytes are presented in a schematic way in Fig. 3.

3.2. Sampling

Sampling is the first key step in the analytical procedure. In most cases, the samples are collected in the field and then transported to a laboratory where they are further processed in the determination procedure. The background of the field operation can generally be described as field blank and trip blank. Field blank is the specification of the contamination related to the sampling technique, the

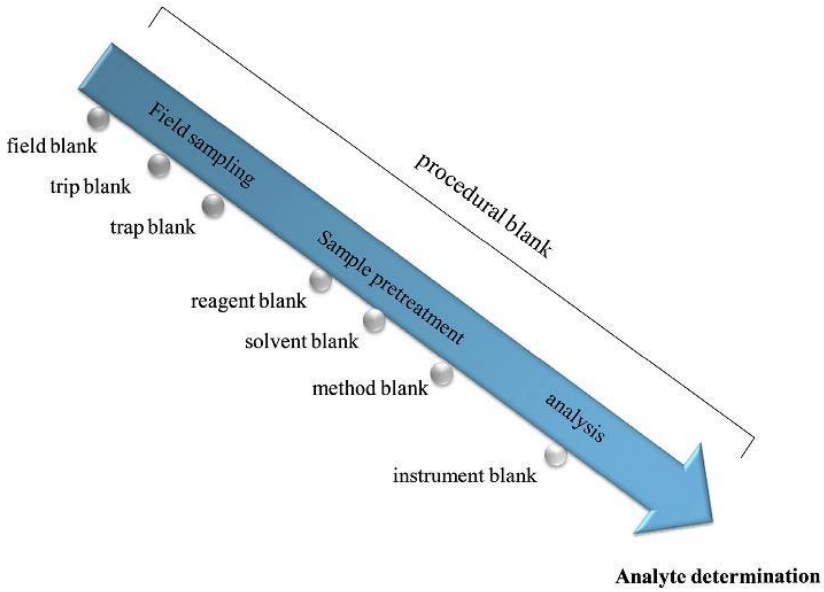


Fig. 3. Blank types concerning different stages of analytical method from sampling to final analysis.

analytical procedure, field operations, sample containers and storage of samples. Trip blank specifies the contamination of the sample due to the samplers, preservatives, transport and also storage of the samples.

To avoid the input of contaminants into the sample in which trace amounts of organic compounds will be determined, sampling of liquids and solids samples should be carried out in glass containers. In addition, the glass containers are rinsed with the solvent and dried or heated out. It is also very important to clean the stoppers and caps and make sure that they are leak proof.

Contact of the sample with the skin, plastic gloves or other plastic parts such as spatulas (it is suggested to use metal parts) should be eliminated. The temperature at which the samples are stored is very important in terms of background value of the reprocessed analysis. Solid samples (biota, soil, sediments) should be stored in a freezer (−20°C) and liquid samples in a refrigerator (4°C) [11].

3.3. Sample preparation

The most important principle is that the risk of contamination is reduced if sample preparation is kept to a minimum (extraction steps, extract concentration, glassware) [11]. The background value is mainly dictated by techniques, glassware and solvents. It can generally be described as method blank and reagent blank.

The method blank determines the contamination related to the techniques for sample preparation and determination of analytes. Samples shall be prepared on the basis of a standard matrix without addition of analyte. They are used for the detection of contamination that may have entered into the sample in the laboratory. Reagent blank specifies the level of contaminants associated with specific reagents used in sample preparation and determination of analytes. To obtain reagent blank value, the analytical determination of analytes in samples of reagents is carried out.

Testing and verification of solvent and other reagents purity is an extremely important step in the analytical procedure [12]. Purity ratings are applied to indicate the possible impurities in a measured amount of a pure chemical reagent (gas, solvents, etc.), or it mixture. Impurities can be introduced in a number of ways, but primarily they are inherent in the raw material in its pure form [5,12,13]. There are several terms applied to indicate the purity of a given reagent, however, these terms are not absolute, thus variance in the same term can be found across different industries. One of purity ratings way is based on the number of nines in the purity percentage. Some examples are presented in Fig. 6.

To exclude interference by the injection process, solvent can be aspirated directly into the inlet of the pre-column of an appropriate chromatograph. Pure solvent can also been obtained by dispersive solid phase extraction using aluminum oxide which can be added to the vessel. It absorbs from apolar solvents (such as pentane and hexane) all materials with a specific polarity (e.g. phthalates), but can contaminate the solvent with components for which it is a poor adsorbent, e.g. hydrocarbons [14,15].

Depending on the type of analytes, the glassware can be flushed with a given solvent or heated out at 400°C [14]. After cooling, it is recommended that the glass should be stored in a closed container. Sometimes memory effects were observed. Rinsing is proposed to determine the residual analyses on the walls of glass vessels or septa for autosampler vials. In this solution, relevant items are rinsed out with the same solvent and the determined amount of analyte is divided by the number of elements [14].

For the better understanding of the blank problems in trace analysis, analytes concentrations (especially volatile analytes) in the laboratory air can be also measured. Due to the fact that laboratories use different devices, equipment, reagents (liquids, solids and gases), the analytes may be at very high concentration levels.

Depending on the type of compounds being determined, factors such as plastic materials, laboratory cleaning agents or personal hygiene products may have an equal influence on background value in the analysis, which should be eliminated if possible [11].

3.4. Determination of analytes

The most significant factor that may influence background value at the determination stage of the analytes is measurement system

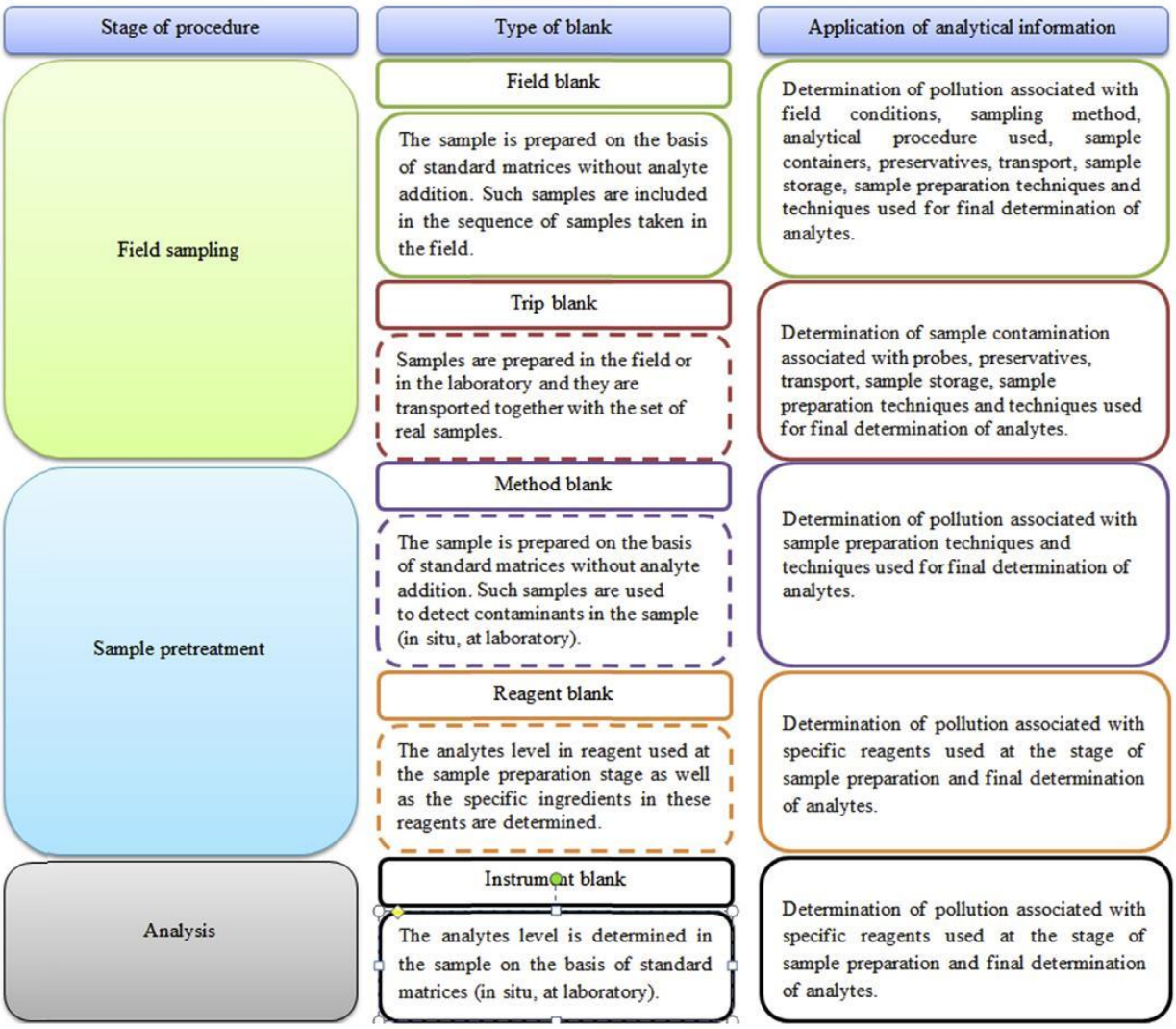


Fig. 4. Schematic representation of general characteristics of samples used in the stage quality control of analytical results.

and it may be described as instrument blank. The instrument blank describes the contaminants associated with the measurement system which is used. Thus, the analytes level is determined in the sample on the basis of standard matrices (*in situ*, laboratory).

The most commonly used techniques in the case of analysis of trace organic analytes are chromatographic techniques. In these solutions the most important contamination is located in the inlet and gas supply system.

In some cases "heating out blanks", as testing tool in GC system, can be used. This can be achieved by cooling the oven to ambient temperature for a longer period of time during which the trace organic analytes (entered by the carrier gas) were accumulated in the column by cold trapping [11]. It was examined that during the "heating out blanks" stage of phthalates determination using the GC-MS technique, substantial peaks were observed and in this case a carbon filter was installed after the gas control system [14].

Problem of the background and its value estimation in analysis of trace organic analytes is extremely common and meets daily laboratory practice. Therefore, methods for identifying sources and ways of reducing or eliminating system contamination should be understood and applied. Examples of methods of elimination or reduction of intensity of influence of various factors on the concentration of traces in the liquid sample are presented in Fig. 5.

3.5. Determination of contaminants – evaluation of purity of the reference samples

Sampling, including extraction and purification, is still a crucial step. Since the aim is to obtain extracts suitable for quantitative analysis, the extraction of analytes and the elimination of potential contaminants are the main objectives of sample pretreatment [16]. The purity of a sample under chemical analysis is the most important factor for accurate and precise quantitative (direct) measurements [17]. However, it is very difficult to determine the purity of the samples in the isolation process, particularly when the retention times are quite similar for the two substances desired in HPLC. On the other hand, the purity of the isolated samples has a significant impact on the subsequent structural characteristics [18].

No.	Factor affecting the concentration of the trace element in the liquid sample	Methods of elimination or reduction of factor intensity
1.	Contact with laboratory air	- Hermetization of all steps and operations; - Use of a clean box and clean room to carry out sample preparation operations for analysis;
2.	Residues of components of dishwashing mixtures	- Use of the correct cleaning products and adequate (tried) cleaning, washing and drying procedures;
3.	Water used in sample preparation operations	- Correct water preparation techniques (deionisation, distillation, etc.);
4.	Reagents and solvents used	- Use of high quality reagents (High Purity Reagent – HPR); - Use reagents from the same batch; - Addition of reagents only in reasonable excess; - Use of so-called "solvent-free" sample preparation techniques;
5.	Contact with an analyst	- Use of protective clothing (headgear, gloves, etc.);
6.	Evaporation of the volatile components	- Hermetization of sample preparation operations; - Storage of solutions and samples in containers filled up' under the cork'; - The use of adequate volume vessels;
7.	Adsorption-desorption processes (wall memory effect)	- Use of vessels made of relevant materials; - Special surface preparation of vessels (deactivation) by the following: ✓ electropolishing, ✓ electropassivation, ✓ silanisation;
8.		- Reducing the storage temperature of samples and solutions; - Washing the vessels with part of the sample or the solution;
9.	Adsorption of analytes on slurry	- Preliminary removal of the slurry by the following: ✓ decantations, ✓ filtration, ✓ centrifuging;
10.	Precipitation of sludge	- Acidification of the sample;
11.	Leaching of ingredients from the material of the vessel	- Use of vessels made of proper materials;
12.	Permeation (penetration of ingredients) air into the solution	- Use of vessels of materials with a low value of constant permeability compared to gases;
13.	Permeation of the solution components to the outside air	- Use of vessels of materials with a low value of constant permeability compared to the components of the solution; - Use of thick-walled synthetic vessels;
14.	Reaction of the analyte with the vessel material	- Special preparation of the surface of vessels - see point 9.
15.	Chemical reaction between the solution components	- Decrease the solution temperature; - Derivatization of analytes.

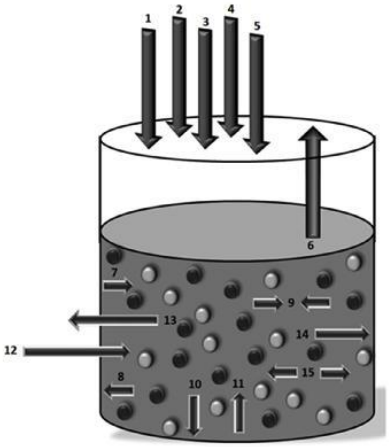


Fig. 5. A schematic presentation of the factors which may affect the level of the traces in the liquid sample and methods of its elimination or reduction.

The practical purpose of determining purity is usually to decide whether a given sample fulfils certain specifications which are defined by the intended subsequent use of the sample [19]. For example, in most cases 'pure' reference samples are used for calibration. The common methods for identifying the purity of prepared samples are thin layer chromatography (TLC), HPLC-UV–Vis, MS, or LC-MS, and LC coupled with nuclear magnetic resonance spectroscopy (LC-NMR) [18–21].

TLC has been used for many years and is a useful and simple technique for basic separation and analysis. However, the sensitivity of the method is quite limited; therefore it is often very difficult to assess whether or not the sample is pure using only the TLC [18].

Most HPLC separations use only one UV–VIS wavelength, which sometimes does not provide sufficient information to evaluate the purity of the samples. In this example, what seemed to be a single ideal peak in HPLC is generally indicated as a mixture in subsequent MS or NMR measurements. MS/LC-MS or LC-NMR, on the other hand, provides a universal method of evaluation of purity of the samples under investigations. There are also examples of research when the HPLC coupled with chemiluminescence (CL) and evaporative light scattering (ELC) detectors [22,23], have been applied in routine assessment of the purity of natural products. Relatively new, efficient, convenient, fast, and inexpensive approach for on-line determination of the purity of samples isolated from natural products is system coupling HPLC and a CCD (Charge Coupled Device) spectrometer. This solution has been successfully used to differentiating Tanshinone I, Tanshinone IIA, and their mixture [18].

Quantitative nuclear magnetic resonance spectroscopy (qNMR) has been proposed to determine the purity of analytical standard samples [24]. Due to the unique combination of both a powerful structural analysis method (for example, to determine the structure of organic molecules [24]) and a competitive quantitative analytical tool qNMR has been in used for many years [17]. Despite the disadvantage of qNMR technique, which is high costs of devices, it has following advantages:

- ability to determine structures at molecular level;
- no need to calibrate the light intensity if ratios are determined;
- relatively short measurement times;
- non-destructive character;
- easy sample preparation (no previous isolation of the analyte from mixture)
- ability to perform treatment operations at the same time; and the determination of more than one analyte in a mixture.

These advantages allow qNMR common use [25]. Quantitative qNMR in liquid samples can be used in pharmacy [26], agriculture [27], material science [28] and for military purposes [29], etc.

Completely different examples (whether in terms of type of samples, tests or analytes) for the evaluation of the purity could be the use of popular analytical techniques, including scanning and transmission electron microscopy (SEM and TEM), thermogravimetric analysis (TGA), and Raman and near-infrared (NIR) spectroscopy for the evaluation of the purity of bulk quantities of single-walled carbon nanotubes (SWNTs) [30] or differential scanning

Purity degree determination		The sum of pollutions [ppm]
The number of nines "N"	% [m/m]	
Standard purity		
3N	99,9	1000
3N7	99,97	300
4N	99,99	100
2N5	99,5	5000
2N85	99,85	1500
High Purity Materials, Ultra High Purity Materials		
5N	99,999	10
6N	99,999	1
11N	99,999999999	10^{-5}

Fig. 6. Information on purity degree determination with the number of nines.

calorimetry (DSC) for measurement of the depression of the freezing point of a sample to determine its purity [19].

4. Problem of the background – examples

In this section several examples of the background problems connected with such compounds determination as phthalates, alkylphenols and bisphenol A and its solutions are described.

4.1. Phthalates

As mentioned before, the subject matter of background problems is inextricably linked to issues regarding the presence of phthalates in analytical laboratories. Phthalates (PAEs) are the esters of phthalic acid and they were introduced in the 1920s. Since that time, a constant growth in the use of those compounds in various branches of industry has been observed. Phthalates are commonly used as plasticizers to increase flexibility, durability, workability, transparency and longevity of materials [31,32]. There is no doubt that their use brings numerous benefits. Unfortunately, due to the fact that PAEs do not bind with plastics, they can easily migrate to the medium with which they are in contact [14]. The most frequently identified contaminants include compounds such as diisobutyl phthalate (DIBP), dibutyl phthalate (DEP), di(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DINP) [33–35]. Literature indicates that they have been found in laboratory air, dust, solvents, sorbents, plastic materials and glassware [31]. Although the blank problems are well-known, unfortunately the solutions to these problems are not yet well established.

The first and the most obvious potential source of phthalates in an analytical laboratory are all plastic materials. Many instruments parts used at each stage of analysis are made of polyethylene or polypropylene, from solid-phase extraction cartridges, pipette tips, filters, syringes, collection tubes, cups, to septa for auto-sampler vials. Testing of storage containers and sample collections, as well as preparation devices before their use is therefore required. Numerous information can be found in the literature that blank polypropylene SPE cartridges extracted with ethyl acetate contained 3–14 ng of DIBP, DBP and DEHP [33]. Moreover, research aimed at determining the content of phthalates in pipette tips showed that they contained trace amounts of DEP, DIBP (diisobutyl phthalate), DBP and DCHP (dicyclohexyl phthalate) [33,36]. In other studies, it was shown that filters made of glass fibre released trace amounts of DBP, while the ones made of nylon released DEHP [35]. Detailed information on the content of phthalates in blank samples is provided in Table 1.

Table 1
Information on concentration of different background types in various medium.

Sample		Analyte	Concentration	Ref
Laboratory environment	Air	DBP	1.2–3.0 [ug/m^3]	[14]
		DEHP	0.9–2.4 [ug/m^3]	
		DIBP, DBP, DEHP	0.3–0.7 [ug/m^3]	[33]
		DIBP	2.2 [ug/m^3]	[34]
		DBP	0.65 [ug/m^3]	
		DEHP	0.96 [ug/m^3]	
		DIBP DBP	0.5–1 [ug/m^3]	[35]
		DEP,	0.2 [ug/m^3]	
		DEHP	0.1 [ug/m^3]	
	Dust	BBP	15.2 [mg/kg]	[37]
		DBP	87.4 [mg/kg]	
		DEHP	604 [mg/kg]	
		DIDP	33.6 [mg/kg]	
		DINP	129 [mg/kg]	
		DEP	8.81–300 [ng/g]	[36]
		DBP	297–2120 [ng/g]	
		DEHP	15.000–46.500 [ng/g]	
		DOP	421–4960 [ng/g]	
		MeP	22.9–128 [ng/g]	[39]
		BuP	14.2–116 [ng/g]	
		BADGE	5.43–73.1 [ng/g]	[39]
		BADGE·2H2O	73.9–452 [ng/g]	
Solvents	Hexane	DEHP, DBP	100 [ug/L]	[38]
		DEHP, DNBP	100 [ug/L]	[31]
		DEP	0.029 ± 0.024 [ng/ml]	[35]
		DIBP	0.044 ± 0.048 [ng/ml]	
		DBP	0.45 ± 0.53 [ng/ml]	
		BBP	0.057 ± 0.064 [ng/ml]	
		DEHP	0.59 ± 0.69 [ng/ml]	
		DNOP	0.002 ± 0.005 [ng/ml]	
	Cyclohexane	DEP, DEHP	0.5 [ng/ml]	[35]
		DIBP, DBP	1.5 [ng/ml]	
	Methyl tert-butyl ether	DEP, DEHP	0.5 [ng/ml]	[35]
		DIBP	0.4 [ng/ml]	
		DBP	0.1 [ng/ml]	
	Acetone	DEP	0.010 ± 0.005 [ng/ml]	[35]
		DIBP	0.002 ± 0.0007 [ng/ml]	
		DBP	0.010 ± 0.005 [ng/ml]	
		BBP	0.023 ± 0.001 [ng/ml]	
		DEHP	0.58 ± 0.004 [ng/ml]	
		DNOP	0.022 ± 0.003 [ng/ml]	
		DEHP	9.8 [ug/kg]	[39]
		DNOP	3.0 [ug/kg]	
	dichloromethane	DEP	0.019 ± 0.017 [ng/ml]	[35]
		DIBP	0.026 ± 0.034 [ng/ml]	
		DBP	0.27 ± 0.35 [ng/ml]	
		BBP	0.074 ± 0.10 [ng/ml]	
		DEHP	6.39 ± 0.64 [ng/ml]	
		DnOP	0.006 ± 0.009 [ng/ml]	
	acetonitrile	DEP	0.017 ± 0.006 [ng/ml]	[35]
		DIBP	0.21 ± 0.009 [ng/ml]	
		DBP	0.080 ± 0.068 [ng/ml]	
		BBP	0.027 ± 0.010 [ng/ml]	
		DEHP	0.28 ± 0.089 [ng/ml]	
	Mili-Q-water	DBP	0.59 ± 0.04 [ng/ml]	[32]
		DEHP	1.14 ± 0.78 [ng/ml]	
		DnOP	0.47 ± 0.43 [ng/ml]	
	UHQ water	BPA	20–200 [ng/L]	[38]
	Ultra resi-analysed water	DBP	0.32 ± 0.28 [ng/ml]	[32]
		DEHP	0.81 ± 0.51 [ng/ml]	
		DnOP	0.83 ± 0.68 [ng/ml]	
Sorbents	Florisil	DEHP	5–45 [ng/g]	[35]
	Florisil heated at 450°C	DEHP	4.81 ± 1.33 [ng/g]	[35]
		DNOP	0.05 ± 0.03 [ng/g]	
	Alumina	DEP	0.01 ± 0.01 [ng/g]	[35]
		DIBP	0.14 ± 0.01 [ng/g]	
		DEHP	6.55 ± 3.41 [ng/g]	
		DNOP	0.24 ± 0.26 [ng/g]	
	Anhydrous sodium sulfate	DEP	0.007 ± 0.003 [ng/g]	[35]
		DIBP	0.05 ± 0.09 [ng/g]	
		DEHP	2.70 ± 2.32 [ng/g]	
		DNOP	0.03 ± 0.01 [ng/g]	
Laboratory utensils	Plastic tips	DEP	0.007 ± 0.005 [ng/tip]	[35]
		DIBP	0.03 ± 0.04 [ng/tip]	

Table 1 (*continued*)

Sample		Analyte	Concentration	Ref
		DBP	0.01 ± 0.02 [ng/tip]	
		BBP	0.001 ± 0.001 [ng/tip]	
Personal care products	Hand cream	BPA	30.2 [ng/g]	[40]
		BPAF	100 [ng/g]	
	Body cream	DMP	1295 [ng/g]	[41]
		DEP	80.15 [ng/g]	
		DBP	1805 [ng/g]	
		DOP	4484 [ng/g]	
	Deodorant	DMP	6255 [ng/g]	
		DEP	50.38 [ng/g]	
		DBP	1618 [ng/g]	
		DOP	439 [ng/g]	
	Liquid soap	MeP	969 [μg/g]	[42]
		nPP	917 [μg/g]	

BADGE – bisphenol A diglycidyl ether; BADGE·2H2O – Bisphenol A bis(2.3-dihydroxypropyl) ether; BPA – bisphenol A; BuP-monobutyl phthalate; BzBP-benzylbutyl phthalate; DBP – dibutyl phthalate; DEHP – Bis(2-ethylhexyl) phthalate; DEP – diethyl phthalate; DIBP – diisobutyl phthalate; DIDP – diisodecyl phthalate; DMP – dimethyl phthalate; DINP – diisononyl phthalate; DNBP-di-n-butyl phthalate; DnOP-di-n-octyl phthalate. DOP-dioctyl phthalate; MeP-monoethyl phthalate; nPP – n-propyl paraben; UHQ-Ultra High Quality.

Another source of the blank problem is the presence of phthalates in organic solvents. Phthalate esters have been found in most reagent-grade, high-purity organic solvents. Probable sources of these contaminants were technological processes related to the production and storage of solvents (they were mainly stored in PVC tanks). The information on the content of phthalates in the solvents most frequently used for research was gathered in Table 1. As it is clearly visible in the data provided in the table, the content of phthalates in various solvents varies from 0.001 ng/ml to even 6 ng/ml. Unfortunately, due to the fact that at various stages of the analysis large quantities of solvents are used (generally hundreds of millilitres), even a trace presence of phthalates in the solvent may result in the introduction of 10 to even 100 ng of phthalates into the sample [33,37,38]. To increase their purity, organic solvents may be distilled, or they may be passed through sorbents. In studies carried out by researchers, the addition of 3% deactivated alumina to hexane resulted in a reduction in the content of phthalates by as much as 99.8% [33,39]. Attention should also be paid to the use of water, as even ultrapure water from the Milli-Q systems can contain some amounts of phthalates and bisphenol A because of the plastic equipment [31,32]. Another factor which is of major importance for preserving the highest purity of solvents used is the necessity to avoid exposition to laboratory air and dust. As shown in numerous research results, phthalate concentration in indoor air is often elevated. Concentrations of DBP and DEHP are reported to range from 1.2 to 3 $\mu g/m^3$, while another study indicated that they may range between 0.3 and 0.7 $\mu g/m^3$ for DIBP, DBP and DEHP [14,33]. Of course, the content of impurities in the air depends on the presence of phthalate-containing sources, such as: building materials, furniture, cables, as well as the temperature and ventilation. For example, research done by Marega and co-workers showed that concentrations of DIBP, DBP and DEHP in the air with closed windows were approximately doubled when a window was open [34]. Phthalates present in the air may contaminate glassware and solvents by absorption. Literature indicates that exposure of hexane to laboratory air for only 1 h resulted in an almost threefold increase in DBP and DEHP in the solvent [33]. Table 1 contains information on the phthalates content in commercially available solvents.

Finally, it should be noted that personal hygiene materials (soap, hand cream, cosmetics, perfumes) often contain phthalates (mainly DMP and DOP), bisphenol A and their analogues and parabens. Therefore they must be avoided during sample handling. More detailed information on concentration of analytes are summarized in Table 1.

4.2. Alkylphenols and bisphenol A

Similar to phthalates analysis, BPA and alkylphenols determination is one of the most problematic issue, due to a ubiquitous occurrence of these chemicals. Bisphenol A (BPA) and alkylphenols (APs) are widespread chemicals known for their endocrine disrupting nature [43,44]. BPA is commonly used in production of e.g. epoxy and phenol resins, polyesters, plasticizers or flame retardants [45]. Alkylphenols are used in agriculture and industry, and may be also products of non-ionic surfactants degradation. Taking under consideration adverse health and environmental effects exerted by these compounds and their abundant occurrence, they are strictly regulated. To quantitate low concentration levels of BPA and APs, highly sensitive and selective analytical methods are in demand. In case of such methodologies background contamination observed as large peaks of analytes in procedure blanks is an important problem. It may result in obtaining high limits of detection and quantitation, and when the concentrations of analytes in blank are not constant, its subtraction usually lead to quantitation errors [46]. In 2012 a comprehensive study of possible sources of these chemicals and methods to avoid the blank contamination in water samples analysis was made [46].

Authors described important aspects of sample handling and analysis that may influence BPA and APs presence in blanks, namely:

- Preparing of laboratory glassware
- Contamination originating from mobile phases and LC system
- Filtration prior to LC system injection.

4.2.1. Laboratory glassware preparation

In case of determination of bisphenol A and alkylphenols the use of plasticware should be avoided and the glassware should be properly cleaned. Three different cleaning procedures were compared (cleaning with alkaline soap and rising with solvents, cleaning with acetone and methanol and cleaning with acetone and baking at 350°C overnight). Blank contamination was minimal only for 2 of 5 selected analytes (4-octylphenol and 4-*n*-nonylphenol). A significant amount of BPA, nonylphenol (NP) and 4-*t*-octylphenol (4tOP) were found in blanks for all studied cleaning procedures. In case of BPA, the highest contamination was observed for cleaning with alkaline soap. Procedure based on

cleaning with acetone and methanol was chosen to be most optimal one [46].

4.2.2. Instrument and solvents blanks

Substantial peaks of NP and 4tOP were found after injecting pure MeOH to LC system. After comparing the results with an instrument blank no significant difference was observed indicating that contaminations source is LC-MS system. Replacing water used as mobile phase (Milli-Q Gradient A10) with water obtained from system with minimum contact with plastic reservoirs (Milli-Q Direct 5) resulted in reduction of above mentioned analytes concentration in blanks. Also application of LC-MS grade organic phase influenced 4tOP occurrence. Septa vials and LC mobile phase tubing were tested as well and concluded to have minimal influence on background contamination with BPA and Aps [46,47].

4.2.3. Filtration

Three type of syringe filters were tested, namely nylon 0,2 μm, PTFE 0,2 μm and PVDF 0,45 μm filters. The lowest blanks were observed after application of PTFE filters.

In 2016 a study investigating problems with determination of trace levels of BPA in human serum was introduced. Authors observed that BPA peak area in blanks is proportional to the column conditioning time while gradient elution was applied. It was concluded that bisphenol A, present in mobile phase, is accumulating in the column and then is eluted while gradient increased. To solve this problem, isocratic elution with high content of organic phase was used [47].

4.3. Atmospheric pressure ionization LC-MS

Another interesting and frequent occurring example of background interference problem concerns liquid chromatography coupled to electrospray ionization (ESI) and atmospheric pressure ionization (API), that are of the most versatile analytical techniques. In both cases, background noise ions are very commonly observed interference, regardless of application of high purity HPLC solvents and improved interface for declustering or desolvation [48]. Even though chemical background is unavoidable, it may lead to a number of undesirable effects including low quality of obtained MS spectra and chromatograms, affecting detection and quantitation limits and the contribution to the drifting and high TIC (the total ion current) baseline. It may generate additional obstacles with application of API LC-MS to analysis of trace analytes concentrations, especially in the case of biological samples characterized by complex matrices [48]. Chemical background noise may interfere by overlapping the analyte signal in chromatograms and mass spectra. Although many efforts including hardware and software improvement have been made to reduce background noise it is still a problem due to a variety of chemical interferences origin. Some of the trace contaminants are present in HPLC mobile phase solvents, laboratory air, plastic materials such as rubber stoppers, O-rings etc.

In 2006, a systematic characterization of typical chemical interferences present in API LC-MS has been made [48]. Authors focused on chemical structures and possible origins of positive charged background ions commonly found in mass spectrum. Major ions groups that contribute in background noise are summarized in Fig. 7. It is important to note that interferences may vary significantly depending on the eluents and additives, columns, chromatographic system and laboratory atmosphere.

The experimental study was conducted using two types of mobile phase solvents: ACN/H_2O/formic acid (50:50:0,1%) and MeOH/H_2O/acetic acid (50:50:0,1%) and under constant ionization condition. The results indicated that main background ions were [48]:

- protonated phthalic acid anhydride (precursor ion at *m/z* 149 and product ions characterized by consecuting loss of neutral molecules of 28) originating from phthalate esters.
- protonated dibutyl, dioctyl or ethylhexyl phthalate esters (precursor ion at *m/z* 149)
- sebacates such as dibutyl sebacate (product ion at *m/z* 315 and some fragment ions indication this group of compounds).
- Protonated phosphoric acid and ions derived from phosphate esters
- adipates
- silicone-related ions

Most of the above mentioned ions that contribute to chemical background in positive mode of API-LC/MS are unavoidable. They are commonly used as plasticizers abundant in laboratory disposable materials, hardware parts and tubing. They may also be found in mobile phases and additives.

The other group of interferents contributing in chemical background are stable cluster ions originating from clustering and solvation reactions of solvents and additives used in LC analysis. The examples of such interferents are sodiated molecules and protonated phosphoric acid, forming clusters with acetic acid used as mobile phase additive. The possible sources of H_3PO_4 may be inorganic contaminants or degradation of phosphate plasticizers [48].

A method focused on reducing chemical background associated with above mentioned cluster ions occurring in API-LC-MS analysis was developed in 2007. The method is based on cluster-type ions reactions with neutral reagent dimethyl disulphide (DMDS) in gas phase. The reagent was introduced into the collision cell. It resulted in shift of the mass-to-charge- ratios of interfering background ions, which reacted efficiently with DMDS. On the other hand very

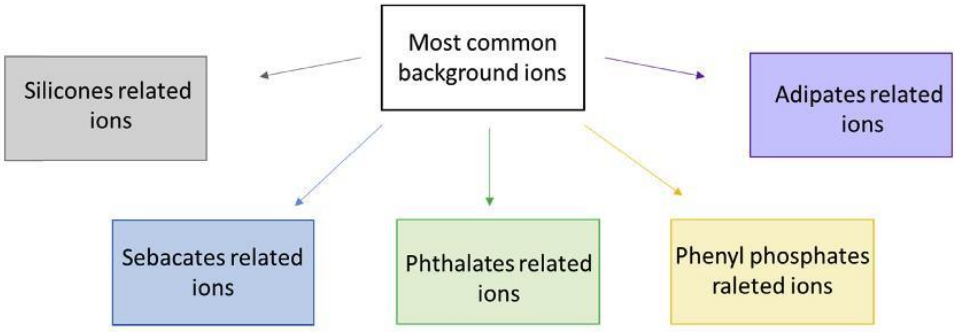


Fig. 7. Scheme of the most common chemical background interfering ions present in positive ionization mode of API-LC/MS technique.

low reactivity of neutral reagent towards protonated analytes was observed [49]. The method may be successfully used for efficient reduction of background noise and improvement of S/N ratios in trace analysis conducted with use of API-LC/MS.

5. Final conclusions

In recent years, analytical chemists perform research to discover completely new principles of measurements and are at the forefront of the utilization of major discoveries. This is due to many reasons, one of them is the course of analytical chemistry development in direction of the analysis of sub-ultra-trace analytes in sample characterized by complex matrix composition. However, this is well known that some problems with determination of analytes can occur at trace-level of concentration, among which background problem is one of the bigger issue. This issue is connected with contamination during each stage of the analytical procedure from sampling to sample preparation up to chromatographic mass spectrometric analysis, which often leads to false-positive or overestimated results.

The problem of background value of sample preparation step is mainly dictated by techniques, glassware and solvents. It can generally be described as method blank and reagent blank. Another significant factor that may influence background value at the determination stage of the analytes is measurement system and it may be described as instrument blank. The instrument blank describes the contaminants associated with the measurement system which is used. To determine the instrument blank, the level of analytes in the sample on a standard matrix basis (without analytes) is determined.

The authors hope that this article will be useful in pointing out issues in determining analytes at trace- and ultra-trace level in sample characterized by complex matrice composition. Moreover, authors believe that some examples given in this review help readers in solving issues connected with the background problems.

Acknowledgments

The work has been co-financed by the National Science Center of Poland, grant no. 2015/17/N/ST4/03835.

References

[1] B.S. Bari, L.L. Williams, Y.S. Jaiswal, Analytical methods – an overview, in: B.S. Bari, L.L. Williams, Y.S. Jaiswal (Editors), Theory and Practice of Chromatographic Techniques, BS Publications, 2018.
[2] M. Gallo, P. Ferranti, The evolution of analytical chemistry methods in foodomics, J. Chromatogr. A 1428 (2016) 3–15.
[3] M.I. Karayannis, C.E. Efstathiou, Significant steps in the evolution of analytical chemistry—is the today's analytical chemistry only chemistry? Talanta 102 (2012) 7–15.
[4] C.E. Efstathiou, On the sampling variance of ultra-dilute solutions, Talanta 52 (2000) 711–715.
[5] J. Namieśnik, Modern trends in monitoring and analysis of environmental pollutants, Pol. J. Environ. Stud. 10 (2001) 127–140.
[6] J. Namieśnik, Trace analysis - challenges and problems, Crit. Rev. Anal. Chem. 32 (2002) 271–300.
[7] J. Namieśnik, T. Górecki, Preparation of environmental samples or the determination of trace constituents, Pol. J. Environ. Stud. 10 (2001) 77–84.
[8] M. Hoenig, Preparation steps in environmental trace element analysis – facts and traps, Talanta 54 (2001) 1021–1038.
[9] G.L. McClure, Computerized Quantitative Infrared Analysis: a Symposium, ASTM Special Technical Publication, 1987.
[10] L. Dagge, K. Harr, Marcus Paul, G. Schnedl, Classification of process analysis: offline, atline, online, inline, Cement Int. 7 (2009) 72–81.
[11] F. David, P. Sandra, B. Tienpont, F. Vanwalleghem, M. Ikonomou, in: C.A. Staples (Editor), Phthalate Esters, Part Q (The Handbook of Environmental Chemistry), vol. 3, Springer-Verlag, Berlin, Heidelberg, 2003, p. 9.
[12] S. Noack, Of what purity are pure substances, GIT Fachz. Lab. 40 (1996) 771–775.
[13] K. Clark, M. Retzik, D. Darbouret, Measuring TOC to maintain high-purity water, Ultrapure Water (February 1997) 21–24.
[14] A. Fankhauser-Noti, K. Grob, Blank problems in trace analysis of diethylhexyl and dibutylphthalate: investigation of the sources, tips and tricks, Anal. Chim. Acta 582 (2007) 353–360.
[15] A. Fankhauser-Noti, K. Grob, Injector-internal thermal desorption from edible oils performed by programmed temperature vaporizing (PTV) injection, J. Sep. Sci. 29 (2006) 2365–2374.
[16] M. Núñez, F. Borrull, E. Pocurull, N. Fontanals, Sample treatment for the determination of emerging organic contaminants in aquatic organisms, Trends Anal. Chem. 97 (2017) 136–145.
[17] F. Malz, H. Jancke, Purity assessment problem in quantitative NMR—impurity resonance overlaps with monitor signal multiplets from stereoisomers, Anal. Bioanal. Chem. 385 (2006) 760–765.
[18] B. Chen, X. Shen, J. Kong, Determining the purity of samples from natural products by coupling HPLC and CCD spectrometry, J. Sep. Sci. 28 (2005) 286–290.
[19] M.E. Brown, Determination of purity by differential scanning calorimetry (DSC) 56 (1979) 310–313.
[20] S.J. Cho, S.K. Lee, B.J. Cha, Y.H. Kim, K.S. Shin, Detection and characterization of the Gloeosporium gloeosporioides growth inhibitory compound iturin A from Bacillus subtilis strain KS03, FEMS Microbiol. Lett. 223 (2003) 47–51.
[21] A. Randazzo, G. Bifulco, C. Giannini, M. Bucci, C. Debitus, G. Cirino, L. Gomez-Paloma, Halipeptins a and B: two novel potent anti-inflammatory cyclic depsipeptides from the Vanuatu marine sponge haliclona species, J. Am. Chem. Soc. 123 (2001) 10870–10876.
[22] M. Mäkinen, V. Piironen, A. Hopia, Postcolumn chemiluminescence, ultraviolet and evaporative light-scattering detectors in high-performance liquid chromatographic determination of triacylglycerol oxidation products, J. Chromatogr. A 734 (1996) 221–229.
[23] A. Dapkevicius, T.A. van Beek, H.A.G. Niederlander, On-line detection of antioxidative activity in high-performance liquid chromatography eluates by chemiluminescence, Anal. Chem. 71 (1999) 736–740.
[24] T. Schoenberger, Determination of standard sample purity using the high-precision ^{1}H-NMR process, Anal. Bioanal. Chem. 403 (2012) 247–254.
[25] F. Malz, H. Jancke, Validation of quantitative NMR, J. Pharmaceut. Biomed. Anal. 38 (2005) 813–823.
[26] J. Forshed, F.O. Andersson, S.P. Jacobsson, NMR and Bayesian regularized neural network regression for impurity determination of 4-aminophenol, J. Pharmaceut. Biomed. 29 (2002) 495–505.
[27] R.J. Wells, J.M. Hook, T.S. Al-Deen, D.B. Hibbert, Quantitative nuclear magnetic resonance (QNMR) spectroscopy for assessing the purity of technical grade agrochemicals: 2,4-dichlorophenoxyacetic acid (2,4-D) and sodium 2,2-dichloropropionate (dalapon sodium), J. Agric. Food Chem. 50 (2002) 3366–3374.
[28] N. Papke, J. Karger-Kocsis, ^{13}C- und ^{1}H-NMR analyse eines Nitrilkautschuks mit und ohne Glycidylmethacrylat Pfropfung, Eur. Polym. J. 37 (2001) 547–557.
[29] T.J. Henderson, Quantitative NMR spectroscopy using coaxial inserts containing a reference standard: purity determinations for military nerve agents, Anal. Chem. 74 (2002) 191–198.
[30] M.E. Itkis, D.E. Perea, R. Jung, S. Niyogi, R.C. Haddon, Comparison of analytical techniques for purity evaluation of single-walled carbon nanotubes, J. Am. Chem. Soc. 127 (2005) 3439–3448.
[31] S. Net, A. Delmont, R. Sempéré, A. Paluselli, B. Ouddane, Reliable quantification of phthalates in environmental matrices (air, water, sludge, sediment and soil): a review, Sci. Total Environ. 515–516 (2015) 162–180.
[32] M. Fernández-Amado, M.C. Prieto-Blanco, P. López-Mahía, S. Muniategui-Lorenzo, D. Prada-Rodríguez, A comparative study of extractant and chromatographic phases for the rapid and sensitive determination of six phthalates in rainwater samples, Chemosphere 175 (2017) 52–65.
[33] B. Tienpont, F. David, E. Dewulf, P. Sandra, Pitfalls and solutions for the trace determination of phthalates in water samples, Chromatographia 61 (2005) 365–370.
[34] M. Marega, K. Grob, S. Moret, L. Conte, Phthalate analysis by gas chromatography-mass spectrometry: blank problems related to the syringe needle, J. Chromatogr. A 1273 (2013) 105–110.
[35] Y. Guo, K. Kannan, Challenges encountered in the analysis of phthalate esters in foodstuffs and other biological matrices, Anal. Bioanal. Chem. 404 (2012) 2539–2554.
[36] T.M. Tran, T.B. Minh, T.A. Kumosani, K. Kannan, Occurrence of phthalate diesters (phthalates), p-hydroxybenzoic acid esters (parabens), bisphenol A diglycidyl ether (BADGE) and their derivatives in indoor dust from Vietnam: implications for exposure, Chemosphere 144 (2016) 1553–1559.
[37] M. Abb, T. Heinrich, E. Sorkau, W. Lorenz, Phthalates in house dust, Environ. Int. 35 (2009) 965–970.
[38] H. Gallart-Ayala, O. Núñez, P. Lucci, Recent advances in LC-MS analysis of food- packaging contaminants, TrAC - Trends Anal. Chem. 42 (2013) 186–204.
[39] R. Gibson, M.J. Wang, E. Padgett, A.J. Beck, Analysis of 4-nonylphenols, phthalates, and polychlorinated biphenyls in soils and biosolids, Chemosphere 61 (2005) 1336–1344.
[40] S. Lu, Y. Yu, L. Ren, X. Zhang, G. Liu, Y. Yu, Estimation of intake and uptake of bisphenols and triclosan from personal care products by dermal contact, Sci. Total Environ. 621 (2018) 1389–1396.

[41] P. Viñas, N. Campillo, M. Pastor-Belda, A. Oller, M. Hernández-Córdoba, Determination of phthalate esters in cleaning and personal care products by dispersive liquid-liquid microextraction and liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1376 (2015) 18–25.
[42] C. Esteve, L. Herrero, B. Gómara, J.E. Quintanilla-López, Fast and simultaneous determination of endocrine disrupting compounds by ultra-high performance liquid chromatography-tandem mass spectrometry, Talanta 146 (2016) 326–334.
[43] B.S. Rubin, Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects, J. Steroid Biohem. Mol. Biol. 127 (2011) 27–34.
[44] L.B. Barber, J. Loyo-Rosales, C.P. Rice, T.A. Minarik, A.K. Oskoiue, Endocrine disrupting alkylphenolic chemicals and other contaminants in wastewater treatment plant effluents, urban streams, and fish in the Great Lakes and Upper Mississippi River Regions, Sci. Total Environ. 517 (2015) 195–206.
[45] K. Mikołajewska, J. Stagierowicz, J. Gromadzińska, Bisphenol A – application, sources of exposure and potential risks in infants, children and pregnant women, Int. J. Occup. Med. Environ. Health 28 (2015) 209–241.
[46] N. Salgueiro-Gonzalez, E. Concha-Grana, I. Turnes-Carou, S. Muniategui-Lorenzo, P. Lopez-Mahia, D. Prada-Rodriguez, Blank and sample handling troubleshooting in ultratrace analysis of alkylphenols and bisphenol A by liquid chromatography tandem mass spectrometry, Talanta 101 (2012) 413–419.
[47] K. Wilczewska, J. Namieśnik, A. Wasik, Troubleshooting of the determination of bisphenol A at ultra-trace levels by liquid chromatography and tandem mass spectrometry, Anal. Bioanal. Chem. 408 (2016) 1009–1013.
[48] X. Guo, A.P. Bruins, T.R. Covey, Characterization of typical chemical background interferences in atmospheric pressure ionization liquid chromatography-mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 3145–3150.
[49] X. Guo, A.P. Bruins, T.R. Covey, Method to reduce chemical background interference in atmospheric pressure ionization liquid chromatography – mass spectrometry using exclusive reactions with the chemical reagent dimethyl disulphide, Anal. Chem. 79 (2007) 4013–4021.

OŚWIADCZENIE WSPÓŁAUTORÓW PRACY ZESPOŁOWEJ WSKAZUJĄCE, CO STANOWI ICH WŁASNY WKŁAD

N. Szczepańska, M. Rutkowska, K. Owczarek, J. Płotka – Wasylka, J. Namieśnik, *Main complication connected with detection, identification and determination of trace organic constituents in complex matrix samples,* TrAC, 105, 173, 2018

Nazwisko współautora	**Wkład**	**Podpis**
N. Jatkowska (Szczepańska)	Studium literaturowe, współudział w tworzeniu tekstu manuskryptu, współudział w przygotowaniu odpowiedzi na uwagi recenzentów	
M. Rutkowska	Studium literaturowe, współudział w tworzeniu tekstu manuskryptu, współudział w przygotowaniu odpowiedzi na uwagi recenzentów	
K. Owczarek	Studium literaturowe, współudział w tworzeniu tekstu manuskryptu, współudział w przygotowaniu odpowiedzi na uwagi recenzentów	
J. Płotka – Wasylka	Opracowanie konspektu pracy, studium literaturowe, współudział w tworzeniu tekstu manuskryptu, współudział w przygotowaniu odpowiedzi na uwagi recenzentów	
J. Namieśnik	Koncepcja pracy, sprawdzenie manuskryptu pod względem merytorycznym	* Profesor Jacek Namieśnik zmarł 14 kwietnia 2019 r.

Cel oraz zakres badań

3. Cel oraz zakres badań

Pomimo wielu przesłanek skłaniających do stwierdzenia, że analogi, BPA, nie stanowią dla niego bezpiecznej alternatywy, nadal jedynie niektóre z tych związków podlegają regulacjom prawnym. Od niedawna tematyka obecności i toksyczności analogów analogów BPA stanowi istotne zagadnienie dla środowiska naukowego, podczas gdy jeszcze kilka lat temu informacje dotyczące potencjału endokrynnego, kancerogenności czy monitorowania ich zawartości w próbkach pochodzenia biologicznego były ograniczone. Obecnie, jak już wcześniej wspomniano, nadal niełatwe jest odszukanie chociażby wzmianek o konkretnych zastosowaniach mniej popularnych bisfenoli, takich jak BPE, BPG, BPBP czy BPFL. Dodatkowo w niewielu pracach badawczych poświęcono uwagę szerokiej gamie tych substancji, zwykle skupiając się jedynie na popularnych lub najczęściej pojedynczo oznaczanych analogach, takich jak BPAF, BPF lub BPS. Tym niemniej kluczowe jest opracowywanie nowych metodyk analitycznych umożliwiających oznaczanie tychże analitów obok siebie w przypadku rozwoju przemysłu, zastosowania na masową skalę ww związków a zatem również zagrożenia środowiska ze strony możliwości skażenia różnego typu analogami bisfenolu A.

Wobec powyższego pogłębienie ogólnej wiedzy o tej grupie cząsteczek, w tym o ich potencjale biologicznym oraz poziomach zawartości w próbkach różnego pochodzenia, stanowiło według autorki dysertacji atrakcyjne zadanie badawcze spełniające kryteria nowości naukowej. Realizując tak postawiony cel pracy doktorskiej postanowiono zagadnienie to opracować stosując dwa podejścia – podejście oparte o narzędzia bioanalityczne oraz drugie, oparte o analitykę z zastosowaniem zaawansowanych rozwiązań instrumentalnych.

Biorąc pod uwagę fakt, że bisfenole – tak jak i inne zanieczyszczenia - rzadko występują niezależnie/oddzielnie od siebie należy założyć, że ich toksyczność jest pewnego rodzaju wypadkową wzajemnych interakcji, które mogą prowadzić do zjawisk znanych w ekotoksykologii jako synergizm, działanie addytywne oraz antagonizm. Dlatego w początkowym etapie pracy eksperymentalnej skupiono się na wyznaczeniu wzajemnych binarnych interakcji pomiędzy bisfenolami stanowiącymi przedmiot badań, stosując proste narzędzia analityczne, oparte na testach biologicznych. W czasie kiedy niniejsza praca powstawała, w literaturze naukowej nie były dostępne tego typu opracowania. Wobec powyższego zaproponowane badania wchodziły w obszar bioanalitycznego podejścia będącego wstępem do dalszego opracowania zagadnień dotyczących analizy związków z grupy bisfenoli.

Kolejnym, aczkolwiek równolegle realizowanym, etapem pracy było opracowanie nowych metodyk analitycznych, bazujących na wyjątkowo czułych i selektywnych narzędziach instrumentalnych, m. in.tandemowej spektrometrii mas sprzężonej z zaawansowanymi technikami separacyjnymi, takimi jak chromatografia cieczowa i gazowa. Szczególną uwagę w podejściu instrumentalnym poświęcono analityce próbek surowicy pobranych od pacjentów cierpiących na

różnego rodzaju zaburzenia natury hormonalnej. Celem postawionym sobie realizując badania instrumentalne było opracowanie nowych metod pozwalających na oznaczenie wybranych związków z grupy bisfenoli w śladowych stężeniach w próbkach charakteryzujących się złożonym składem matrycy. W metodach tych koniecznym jest zachowanie zasad zielonej chemii analitycznej, gdzie na etapie przygotowania próbek stosuje się niewielkie ilości szkodliwych rozpuszczalników organicznych. Podejściu temu każdorazowo towarzyszyła próba postawienia dodatkowych wniosków i korelacji uzyskanych wyników z dostępnymi danymi medycznymi pacjentów poddanych badaniu.

Przedmiotem zainteresowania w niniejszej pracy było również oznaczanie wybranych analitów w próbkach charakteryzowanych innymi niż biologicznymi matrycami oraz opracowanie nowej metody, alternatywnej do powszechnie używanej (ze względu na bardzo wysoką czułość, brak konieczności upochodnienia analitów i znaczną elastyczność) w oznaczeniach bisfenoli techniki LC-MS/MS. Z tego względu skupiono się na zbadaniu zawartości pięciu analogów bisfenolu A w odżywkach i preparatach dla sportowców przy pomocy techniki GC-MS. Temat ten stanowił ciekawe wyzwanie analityczne, ponieważ opierając się na przeglądzie literatury nie natrafiono na pracę opisującą oznaczanie szeregu bisfenoli w tego typu matrycach.

Wszystkie opracowane procedury zostały zwalidowane, a ich kluczowe parametry każdorazowo spełniały wymagania stawiane metodom analitycznym przez wytyczne międzynarodowe. Z tego względu po przetransferowaniu mogłyby być one stosowane rutynowo w innych laboratoriach.

Część doświadczalna

4. Część doświadczalna

4.1. Toksyczność i wzajemne oddziaływania bisfenoli w mieszaninach binarnych

Przyglądając się danym dostępnym w literaturze, można zauważyć, że badane próbki rzeczywiste zawierają zwykle więcej niż jeden związek z grupy bisfenoli [47 – 59]. Co za tym idzie warte sprawdzenia jest czy substancje te wykazują wzajemne interakcje, mogące wpływać na ich toksyczność bądź potencjał endokrynny. Przedstawiona publikacja stanowi podsumowanie badań związanych z analizą toksyczności związków z grupy bisfenoli, która była prowadzona z zastosowaniem metod biologicznych. W badaniach tych skupiono się na wyznaczeniu stężenia EC_{50} (*Effective Concentration causing 50% inhibition*) dla każdego z badanych związków *in vitro* przy pomocy testu Microtox®, w którym organizmem modelowym są bakterie *Aliivibrio fischeri* oraz wyznaczeniu toksyczności ostrej dla dwuskładnikowych mieszanin związków z grupy bisfenoli. Na tej podstawie podjęto próbę określenia charakteru i typu interakcji zachodzących pomiędzy związkami w ich mieszaninach, stosując do tego narzędzia modelowania takie jak: analiza regresji, metoda addycji stężeń - CA (*Concentration Addition*), metoda działań niezależnych - IA (*Independant Action*) zaś zgodność modelu z wartością rzeczywistej odpowiedzi określono stosując wartość numeryczną parametru MDR (*Model Deviation Ratio*).

Ponadto, celem oszacowania potencjału endokrynnego każdego z analitów przeprowadzono badania z zastosowaniem testu XenoScreen YES/YAS. Jest to test bazujący na transaktywacji receptorów w specjalnie zmodyfikowanych komórkach drożdży *Saccharomyces cerevisiae*, do których chromosomów wprowadzono sekwencje DNA kodujące ludzkie receptory estrogenowe i androgenowe. Komórki te zawierają także gen reporterowy lac*Z*, kodujący enzym β-galaktozydazę. W sytuacji, gdy dany związek chemiczny wiąże się z receptorami hormonalnymi, modulacji ulega transkrypcja genu lacZ, co doprowadza do produkcji β-galaktozydazy. Enzym następnie przedostaje się do medium wzrostowego i przekształca obecny tam substrat w produkt o czerwonej barwie, którego stężenie może następnie być zmierzone kolorymetrycznie [144].

Biorąc pod uwagę strukturalne podobieństwo wszystkich badanych związków oraz fakt, iż wywoływały one różne efekty toksyczne i endokrynne, zastosowano także modelowanie zależności struktura – toksyczność by ocenić które elementy struktury chemicznej mają na te efekty największy wpływ.

Związkiem cechującym się największą toksycznością ostrą w stosunku do organizmów testowych okazał się bisfenol S (EC_{50} = 61,6±5,6 μM), natomiast wartości EC_{50} dla BPZ, BPM i BPP były dużo wyższe niż możliwy do zbadania przedział stężeń. Ograniczenie to wynikało z konieczności przygotowywania roztworów modelowych w wodzie, w której BPZ, BPM i BPP są słabo rozpuszczalne. Stosując podejście oparte na parametrze MDR, uzyskano wyniki wskazujące na to, iż większość z badanych związków cechuje się działaniem wpisującym się w model *IA*. BPA, BPC i BPFL były substancjami o największym potencjale endokrynnym.

Pomimo iż badania zmian toksyczności oraz interakcji zachodzących pomiędzy związkami chemicznymi w mieszaninach wciąż są stosunkowo mało popularne, w ostatnich latach w literaturze naukowej można zaobserwować zwiększone zainteresowanie tą tematyką. Informacje uzyskane w tych badaniach są z całą pewnością niezwykle cenne z punktu widzenia współczesnej ekotoksykologii, ze względu na to, iż dają możliwość dużo lepszego odzwierciedlenia rzeczywistych efektów wywoływanych przez ksenobiotyki.

Praca została opublikowana w czasopiśmie *Molecules* [145].

molecules

MDPI

Article

Binary Mixtures of Selected Bisphenols in the Environment: Their Toxicity in Relationship to Individual Constituents

Katarzyna Owczarek [1], Błażej Kudłak [1,*], Vasil Simeonov [2], Zofia Mazerska [3] and Jacek Namieśnik [1]

[1] Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, 11/12 Narutowicza Str., 80-233 Gdańsk, Poland; katarzyna.owczarek.90@gmail.com (K.O.); jacek.namiesnik@pg.edu.pl (J.N.)

[2] Department of Analytical Chemistry, Faculty of Chemistry and Pharmacy, University of Sofia, St. Kliment Ohridski, 1164 Sofia, 1 James Bourchier Blvd., Bulgaria; vsimeonov@chem.uni-sofia.bg

[3] Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, 11/12 Narutowicza Str., 80-233 Gdańsk, Poland; zofia.mazerska@pg.edu.pl

* Correspondence: blakudla@pg.edu.pl; Tel.: +48-58-347-2128

Received: 18 November 2018; Accepted: 3 December 2018; Published: 6 December 2018

Abstract: Bisphenol A (BPA) is one of the most popular and commonly used plasticizer in the industry. Over the past decade, new chemicals that belong to the bisphenol group have increasingly been used in industrial applications as alternatives to BPA. Nevertheless, information on the combined effects of bisphenol (BP) analogues is insufficient. Therefore, our current study aimed to find the biological response modulations induced by the binary mixtures of BP compounds. We determined the toxicity levels in Microtox and XenoScreen YES/YAS assays for several BP analogs alone, and for their binary mixtures. The results obtained constituted the database for chemometric intelligent data analysis to evaluate the possible interactions occurring in the mixtures. Several chemometric/biophysical models have been used (concentration addition—CA, independent action—IA and polynomial regression calculations) to realize this aim. The best fitting was found for the IA model and even in this description strong evidence for synergistic behaviors (modes of action) of some bisphenol analogue mixtures was demonstrated. Bisphenols A, S, F and FL were proven to be of significant endocrine threat (with respect to XenoScreen YES/YAS assay); thus, their presence in mixtures (including presence in tissues of living organisms) should be most strictly monitored and reported.

Keywords: bisphenol A analogues; Microtox®; XenoScreen YES/YAS; model deviation ratio

1. Introduction

Over the past decade, new chemicals that belong to the bisphenol group have increasingly been used in industrial applications as an alternative to bisphenol A, but current knowledge of their environmental and biological impact is still limited. Initially, the occurrence of bisphenol analogues was poorly researched, but in recent years, this chemical group has been attracting more scientific attention. The number of studies proving that bisphenols are present in different elements of the environment is growing, but to the best of our knowledge, there is no study focused on the assessment of the possible interactions occurring between these chemicals.

The group of bisphenols consists of chemicals that contain two p-hydroxyphenyl functionalities in their molecular structure. The most widely known analogue—BPA—Is a synthetic chemical used for a broad spectrum of commercial applications worldwide. BPA was synthesized for the first time

in 1891, and it has been used in industries for the commercial production of epoxy resins since the early 1950s. In the polymerization process, BPA is used to create polycarbonate plastic—A very durable and hard material [1]. BPA has reached an annual global production of six million metric tons and this is predicted to increase. Epoxy resins and polycarbonates are used in the manufacturing of a large number of everyday items; the human exposure routes to BPA include dermal, oral and inhalation intake.

BPA has been comprehensively studied for its impact on human health and is well known for its estrogenic activity. Furthermore, a vast number of other adverse effects have been proven, including neural and developmental disorders [2,3], alternation of thyroid function [4], metabolic disorders [5], and suspicion of increasing the risk of Parkinson disease [6]. Moreover, bisphenol A is also a hazard to the environment, especially to aquatic ecosystems, due to its ubiquitous presence. Because of ecological and health concerns regarding BPA, new BPA-related chemicals were considered to be safer alternatives to partially replace BPA in industrial applications. A total of 16 bisphenols have been documented to be industrially applied [7–9].

Bisphenol S and bisphenol F are currently the most commonly used BPA substitutes, predominantly in the manufacturing of epoxy resins, polyesters and polycarbonate plastics. Other bisphenol analogues are also used in the plastic industry to produce a broad spectrum of products, such as dental sealants, pesticides, thermal papers, food containers' inner coatings, toys lacquers, and powder paints. Supplementary Table S1 presents basic information about tested bisphenols, their chemical structures, IUPAC names, and most common applications.

The first regulatory standard for BPA was established by the Environmental Protection Agency (EPA) in 1988, and the oral reference admissible dose was assessed to be 50 mg/kg/day [10]. Because of growing concerns about health and developmental issues, and continually increasing occurrence in the environment, in 2010, baby bottles containing BPA were prohibited by the Canadian government; the European Union followed suit in 2011 [11]. Since BPA is permitted to be used in food contact materials (under the regulation framework 10/2011/EU), in 2015, the tolerable daily intake (TDI) of BPA was reduced from 50 mg per kg to 4 mg per kg of body mass.

These regulations on BPA use and production created the necessity to develop and produce safer alternatives [12,13] that can serve as plasticizers, especially in the case of BPA-free products. BPS and BPF are the most broadly used analogues, but recently there is a growing amount of scientific data, indicating that humans may be exposed to other analogues such as BPE, BPBP, BPM, BPP, BPZ, BPAF [14–16]. Environmental issues are emerging as well, since a vast number of bisphenol analogues has been found in different elements worldwide. BPA, BPS, BPF, BPAF, TCBPA, BPAP, BPFL, and BPZ were detected in the environmental samples of water bodies and sediments [17,18]; BPA, BPE, BPF, BPS, BPP, BPZ TBBPA, TCBPA, and BPAF were detected in sludge and indoor dust [7,19–21]; and BPA, BPB, BPE, BPF, BPP, BPS, BPZ BPAF, and BPAP were detected in foodstuff [21,22]. Even though other bisphenol analogues show many similarities to BPA, these chemicals do not fall under any legislative regulations. It is important to highlight that in the past decade, scientific knowledge about the modes of action of many of these chemicals has expanded significantly. Despite this fact, relatively old safety standards, based on a threshold-dose model, are still valid. There is also strong evidence indicating that other chemicals that belong to the bisphenol group exert similar or even stronger endocrine and toxic effects than BPA, but their use in manufacturing everyday use products is still not regulated.

In most cases, modern analytical chemistry plays a key role in identifying and quantifying bisphenols in different matrices. High performance liquid chromatography or gas chromatography coupled with tandem mass spectrometry are commonly used for this purpose [15,16,21]. On the other hand, when the main goal is to assess the biological risk, bioassays are the best tools to distinguish the types of interactions and the modes of action of chemicals towards living organisms. Available scientific data indicate various toxic effects of bisphenol analogues. These data include endocrine effects, genotoxic action, neurotoxicity, and reproductive disruption [7]. Most of the BPs exhibit estrogenic potential similar to or even stronger than that of bisphenol A (except from BPF, BPS and

BPC) [23]. An antiandrogenic mode of action has also been observed for BPA, BPF, BPE and BPB [7]. Most bisphenols may also have an impact on gene expression processes, especially those associated with enzymatic proteins, which influence fetal development. BPA was also proven to affect genes related to the immune system [24].

As confirmed by numerous studies, bioassays are the most efficient effect-based tools to learn about the combined biological effects of complex mixtures. They have the potential to elucidate the relationship between chemical contamination and ecological status of a given object of interest [25]. They can also be easily used as a regulatory reference for deriving environmental quality standards under the Water Framework Directive [26].

The model deviation ratio approach is a reliable tool with which to determine the possible synergistic or antagonistic effects of environmental stressors studied. To assess the toxicity of bisphenol A analogues (when present in binary mixture with BPs), the MDR approach was applied to the experimental results of ecotoxicity studies with Microtox®; subsequently, a correlation with XenoScreen YES/YAS was performed [27–29].

Considering the referenced information given before, the problem of BPs co-occurrence in the environment is clearly visible. Unfortunately, the numerous implications of their presence in complex mixtures with other environmental stressors are not understood yet. Bearing in mind all above, the goal of this study was to evaluate the toxicity of the series of BPs (alone and in their binary mixtures) with the *Vibrio fischeri* bacteria (as a non-target environmental prokaryote model) and gather data regarding plausible endocrine potentials. Simple analysis of the structure-activity led to preliminary conclusions on the role of bisphenol structure elements in their toxicity. Furthermore, the reliable modeling techniques that were used in this study allowed us to determine the possible interactions (antagonistic or synergistic) between the analytes that may occur in the real environmental samples and that have an influence on their final biological potency. To the best of our knowledge, the approach to evaluate the environmental toxicity of the single analytes and binary mixtures of the broad spectrum of the chemicals belonging to the bisphenols group was undertaken for the first time in the presented study. As structure-activity studies (performed by us) searching for plausible impact of BPs on observed toxicity failed, it even more strongly justifies the necessity to perform bioassays in order to study the real impact of such toxins and their mixtures on living organisms.

2. Results

The results of studies on toxicity and endocrine potential (and to subsequently select C_1, C_2 and C_3 at, respectively, 33, 66 and 100% of EC_{50} of the given analytes) and determined impact of their co-presence on toxicity levels are listed in Table 1. Data on LOEC (lowest observable effect concentration) and NOEC (no-observed effect concentration) of a few given chemicals with respect to XenoScreen YES/YAS are also provided.

2.1. Structure-Toxicity Relationship

The results of bisphenol toxicity presented in Table 1 indicated that only 7 of 10 compounds expressed the detectable toxicity in the Microtox® test, whereas BPM, BPP and BPZ were inactive. Therefore, this question was asked: What elements of the structure are responsible for the toxicity of these environmental pollutants? The results of molecular modeling performed with molecular dynamics are presented in Supplementary Table S2.

Table 1. Concentration levels of bisphenol analogues studied during the research and EC_{50} values calculated for respective compounds.

Analyte	Microtox® *					XenoScreen YES/YAS				
	Concentration Ranges Tested	$EC_{50} \pm SD$ (n = 3)	C_1	C_2	C_3	Concentration Ranges Tested	Effect (n = 3)			
	[μM]					[μM]				
							YES+	YES−	YAS+	YAS−
BPA	8.96–71.68	37.7 ± 6.7	12.44	24.88	37.69	1.71–1752.15	173.9 ^{L}	>1752 ^{N}	>1752 ^{N}	>1752 ^{N}
BPC	6.33–63.84	30.8 ± 2.1	10.17	20.34	30.82	1.53–1560.43	15.4 ^{L}	>1560 ^{N}	>1560 ^{N}	>1560 ^{N}
BPE	38.19–305.49	57.9 ± 4.4	19.12	38.24	57.94	1.83–1866.89	>1886 ^{N}	1.83 ^{L}	>1886 ^{N}	1.83 ^{L}
BPF	10.22–81.72	28.3 ± 1.5	9.34	18.68	28.31	1.95–1997.70	>1997 ^{N}	>1997 ^{N}	>1997 ^{N}	>1997 ^{N}
BPG	2.60–26.19	14.85 ± 0.98	4.90	9.80	14.85	1.25–1280.21	>1280 ^{N}	>1280 ^{N}	>1280 ^{N}	12.6 ^{L}
BPM	70.84–566.77	>566.77 #	-	-	-	1.13–1154.53	>1154 ^{N}	>1154 ^{N}	>1154 ^{N}	11.5 ^{L}
BPP	70.84–566.77	>566.77 #	-	-	-	1.13–1154.53	>1154 ^{N}	>1154 ^{N}	>1154 ^{N}	>1154 ^{N}
BPS	32.69–261.54	61.6 ± 5.6	20.34	40.68	61.64	1.56–1598.27	>1598 ^{N}	>1598 ^{N}	>1598 ^{N}	>1598 ^{N}
BPZ	75.62–762.23	>762.23 #	-	-	-	1.46–1490.60	>1490 ^{N}	>1490 ^{N}	>1490 ^{N}	>1490 ^{N}
BPFL	0.58–58.40	3.31 ± 0.52	1.09	2.18	3.31	1.12–1141.52	1141.5 ^{L}	113.3 ^{L}	1141.5 ^{L}	>1141 ^{N}

* C_1, C_2 and C_3 stand for 33, 66 and 100% of EC_{50} of respective analyte. # solubility limit reached under conditions of the experiment, ^{L} LOEC, ^{N} NOEC.

2.2. CA Studies

Results of CA studies as a function of MDR parameter values are summarized in Table 2a below.

2.3. IA Studies

Results of IA studies as a function of MDR parameter values are summarized in Table 2b below.

2.4. Regression Studies

Supplementary Table S3 presents the results of the comparison of the best-fit polynomial models ($y = ax^2 + bx + c$) for toxicity studies of each couple of bisphenol analogues in the mode "effect of A on B" and "effect of B on A".

2.5. MDR Uncertainties

The MDR values presented enable us to provide uncertainties for the frequency distribution of the results provided (please refer to Table 3 for details) and to determine safety factors in cases of pollutants present in complex mixtures. In the case of substances with similar activity, the CA model had been shown to accurately predict the toxicity of the mixtures. Therefore, it can be considered as an advantage in environmental studies, as the most toxic/dangerous components of mixture can be easily targeted in this way.

Table 2a. MDR values for bisphenol A analogues binary mixtures toxicity studies performed with Microtox® assay (for both CA modelling) (red—Synergism, blue—Antagonism, green—Overestimation, yellow—Underestimation, C_1, C_2 and C_3 stand for 33, 66 and 100% of EC_{50} of respective analogue as presented in Table 1. #—data inconclusive).

Concentration Addition

		BPA																				
		C_1	C_2	C_3																		
C_1	BPC	0.76	0.71	0.74																		
C_2	BPC	0.79	0.79	0.75	BPC																	
C_3	BPC	0.90	0.81	0.59	C_1	C_2	C_3															
C_1	BPE	0.28	0.30	0.30	0.60	0.64	0.63															
C_2	BPE	0.74	0.65	0.77	0.84	0.71	0.58	BPE														
C_3	BPE	0.78	0.71	0.46	0.78	0.81	0.56	C_1	C_2	C_3												
C_1	BPF	0.80	0.76	0.79	0.74	0.77	0.81	0.68	0.73	0.86												
C_2	BPF	0.82	0.81	0.82	0.76	0.90	0.79	0.70	0.72	0.83	BPF											
C_3	BPF	0.87	0.82	0.64	0.75	0.80	0.63	0.69	0.73	0.63	C_1	C_2	C_3									
C_1	BPG	0.64	0.66	0.70	0.49	0.61	0.50	0.42	0.47	0.53	#	1.73	1.24									
C_2	BPG	0.67	0.68	0.69	0.52	0.72	0.57	0.53	0.63	0.69	0.67	1.32	0.55	BPG								
C_3	BPG	0.74	0.71	0.69	0.56	0.68	0.53	0.52	0.61	0.61	1.34	0.75	0.37	C_1	C_2	C_3						
C_1	BPS	0.54	0.67	0.71	0.46	0.45	0.42	0.48	0.46	0.51	0.11	0.22	0.41	0.70	0.60	0.55						
C_2	BPS	0.36	0.44	0.40	0.75	0.67	0.64	0.52	0.63	0.67	0.09	0.12	0.39	0.62	0.53	0.58	BPS					
C_3	BPS	0.55	0.58	0.35	0.73	0.71	0.47	0.75	0.74	0.56	0.08	0.12	0.17	0.99	0.76	0.58	C_1	C_2	C_3			
C_1	BPFL	1.03	1.33	0.9	0.73	0.64	0.71	1.00	0.87	0.88	0.21	0.42	0.84	0.93	0.65	0.70	0.35	0.85	0.68			
C_2	BPFL	0.77	0.79	0.79	0.54	0.6	0.68	0.72	0.77	0.83	0.04	0.17	0.31	0.48	0.53	0.59	0.29	0.48	0.74			
C_3	BPFL	0.66	0.64	0.61	0.37	0.52	0.65	0.52	0.6	0.59	0.02	0.09	0.16	0.44	0.51	0.57	0.21	0.35	0.44			

Table 2b. MDR values for bisphenol A analogues binary mixtures toxicity studies performed with Microtox® assay (for both CA modelling) (red—Synergism, blue—Antagonism, green—Overestimation, yellow—Underestimation, C_1, C_2 and C_3 stand for 33, 66 and 100% of EC_{50} of respective analogue as presented in Table 1. #—data inconclusive).

Independent Action

		BPA			BPC			BPE			BPF			BPG			BPS			BPFL		
		C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3	C_1	C_2	C_3
C_1	BPC	1.31	1.23	1.29																		
C_2	BPC	1.11	1.19	1.17																		
C_3	BPC	1.09	1.09	0.83																		
C_1	BPE	1.58	1.44	1.32	1.07	1.27	1.10															
C_2	BPE	1.41	1.22	1.20	1.18	1.10	1.16															
C_3	BPE	1.26	1.40	0.76	1.14	0.89	0.80															
C_1	BPF	1.22	1.18	1.19	1.24	1.20	1.10	1.14	1.16	1.10												
C_2	BPF	1.10	1.16	1.14	1.15	1.31	1.13	1.09	1.11	1.10												
C_3	BPF	1.09	1.14	0.88	1.14	1.11	0.87	1.15	1.13	0.86												
C_1	BPG	1.13	1.01	1.04	0.95	0.91	0.95	0.87	0.95	0.96	#	1.61	3.40									
C_2	BPG	1.00	0.96	0.97	1.28	1.06	0.98	0.90	0.95	0.95	2.50	2.80	1.72									
C_3	BPG	0.98	0.92	0.91	0.87	0.83	0.77	0.94	0.94	0.86	1.64	1.17	0.87									
C_1	BPS	0.95	0.72	0.89	0.90	1.11	1.08	1.05	0.87	1.11	0.63	0.49	0.48	1.17	1.01	1.31						
C_2	BPS	0.72	1.14	1.07	0.98	1.10	1.09	0.94	1.05	1.10	0.67	0.37	0.40	1.09	0.98	1.09						
C_3	BPS	1.24	1.08	0.68	0.96	1.08	0.73	1.04	1.10	0.81	0.94	0.85	0.32	1.05	1.16	0.88						
C_1	BPFL	1.38	1.10	0.96	1.08	0.99	0.85	1.36	1.20	1.02	0.98	0.96	1.13	1.48	0.80	0.76	0.78	0.91	0.83			
C_2	BPFL	1.67	1.04	0.90	0.85	0.86	0.85	1.06	1.07	0.95	0.78	1.06	1.09	0.90	0.77	0.76	1.28	0.90	0.78			
C_3	BPFL	1.08	0.98	0.80	0.89	0.86	0.88	1.03	1.07	0.85	1.19	0.99	0.87	0.91	0.80	0.79	0.85	1.05	0.70			

Table 3. Percentile values for model deviation ratios (MDR) and numbers of cases for each group of CA and IA experiments of BP toxicity studies.

Model	No. of Cases							
	Synergism	Under-Estimation	Over-Estimation	Antagonism	Percentile			
					80	90	95	99
CA	45	78	1	0	0.464	0.338	0.170	0.075
IA	5	4	8	3	0.860	0.780	0.720	0.396

3. Discussion

3.1. Discussion of Structure-Toxicity Relationship Results

We observed that the compounds active in the Microtox® test expressed similar 3D structures (please refer to Supplementary Table S2 for details) for which:

(i) the distance between central carbon atom and oxygen atoms was close to 5.7 Å,
(ii) the distance between phenol oxygen atoms was equal to 9.3 Å,
(iii) the angle between phenol rings was near 109°.

On the other hand, bisphenols BPM and BPP with the additional aromatic ring between phenol fragments kept different shape of the molecules. They were nontoxic in the Microtox® test; only slightly toxic BPZ out-stood the presented rule. Accordingly, 7 of 10 compounds were qualified to the following studies on the co-presence of analyte pairs in the Microtox test.

Results on the endocrine action obtained in XenoScreen YES/YAS endpoints/tests (Table 1) were difficult for the description with structure-activity relationships. Four compounds of different structures did not express activity in any of the performed tests. There were BPF, BPP, BPS and BPZ. Thus, only six compounds were active in one of the applied endocrine tests. BPA and BPC were the antagonist of estrogen receptor (YES+), whereas BPFL turned out to be the agonist of this receptor (YES−). On the other hand, two compounds, BPG and BPM expressed the agonistic action towards androgen receptor (YAS−). Compound BPE was extremely active in endocrine tests (the lowest value of LOEC), being a highly active agonist of estrogen as well as of androgen receptor.

3.2. Discussion on CA Studies

Studies with CA prove underestimation and synergism in most of the studied cases (only seven cases of overestimation were found, refer to Table 2a,2b for details). BPA combined with BPC, BPF, BFG and BPFL shows underestimation; however, interestingly, synergism with BPS is strongly present. Similar situations could be observed in the case of BPC's impact when co-present with BPE, BPF and, in most cases, other analogues. Interestingly, again, single synergism is detected with BPG and BPFL, but most importantly in combination with BPS. BPE impact is synergistic with BPG and, again, with BPS at the lowest concentrations studied.

Binary mixtures of BPF and BPG show that there is a tendency to overestimate the behavior of these chemicals (with strong trend of increasing mutual impact with increase of concentrations of given compounds). BPF in the co-presence of BPS and BPFL shows very strong synergistic potential, even at the lowest concentration; also noticeable is a clear trend of concentrations impact on toxicity levels. Additionally, BPG in combination with BPS and BPFL in most cases shows concentration-dependent trend of signal underestimation (and synergism in the lowest concentration of BPG). A similar situation is observed for BPS-BPFL mixtures, where the impact of concentration is even more pronounced.

3.3. Discussion on IA Studies

As already stated, the results of MDR for IA modeling calculations reach generally higher values than CA models for similar mixtures [30,31]; this finding is confirmed in this study (refer to Table 2a,2b for details). When analyzing the toxicity of analogues, IA models seem to deny the methodological background of the approach; even so, the results are presented to confirm the hypothesis stated. Model studies on BPA impact on other analogues' toxicity quite accurately reflect the observed toxicity results. Despite studies with BPS, no clear impact of modulation of BPA concentrations on MDR is noticeable. The same holds true for the impact of BPC and BPE on other analogues studied.

Discrepancies are again noticeable in the studies on BPF impact on BPG and BPS solutions where antagonism and synergism, respectively, were noted with strong dependence on varying concentrations of analytes in binary mixtures. As already stated in CA studies, a mixture of BPF and BPG has a strong tendency for antagonistic behavior (certainly in studies with *Vibrio fischeri*), and a decrease of MDR is observable only for the highest concentration level of binary mixture ingredients in both CA and IA studies. The IA model seems to correctly predict the impact of BPF-BPFL mixture, as its MDR values oscillate around a value of "1.0". Similar conclusions can be drawn in studies of BPG impact on BPS and BPFL and in the latter ones (BPS and BPFL) when present in a binary mixture.

3.4. Discussion of Regression Studies

Based on the results presented (ref. to Supplementary Table S3) it could be concluded that 18 out of 21 interactions displayed effects of A on B and B on A in similar manner; an independent mode of action is thus determined. In the remaining 3 out of 21 cases, the mode of action is different from

the independent one. In general, these results coincide with the very significant number of cases with independent action found with application of the MDR approach.

The values and differences in the regression coefficients a, b and c and R^2 for each one of the bisphenol analogue binary mixtures are presented in Supplementary Table S3. It is readily seen that the similarity between regression coefficients and R^2 values within a binary mixture could be accepted as the "screening indication" for independent action mode. The differences in coefficient sign (change of slope) and differences higher than 0.1 in R^2 are indications of behavior different from independent action.

Although the approach is semi-quantitative, it could be concluded that it might be of use for rapid estimation of the interactions, as follows:

Independent action: no differences in coefficients signs and model validity (R^2 as measure) for the compared couples of bisphenols

Different from the independent action: differences in coefficients signs and model validity (differences in R^2 higher than 0.1) for the compared couples of bisphenols

3.5. Environmental Impact

If one compares the toxicity ranges of the levels of different BPs studied in the present study (Table 1) with the concentration ranges found in environmental monitoring (surface an wastewater water in Asia [17,20,24], indoor dust in USA, China, Japan and Korea [32], it can be readily seen that the environmental levels are generally lower than those used in the present study. Certainly, the data presented refer to acute toxicity exposure, while environmental exposures are assumed to be of chronic character. The results of endocrine potential presented in this study for selected analytes reflect those reported by [24]. BPF and BPS are the most commonly used substitutes of BPA and, as confirmed in the present study, constitute similar threats to ecosystems, especially when their presence in complex mixtures with other pollutants is considered. Although the determining mode of action of the analytes of interest was not the aim of this research, one may conclude (in relation to other toxicity studies) that a competitive receptor-mediated mode of action for bisphenol analogues is very plausible. Although XenoScreen YES/YAS is a very potent tool for endocrine potential determination, one must consider its limitations resulting from the complexity of the procedure, which is of particular importance when ultra-low concentration levels of toxicants are studied. In such cases—To reflect the environmental threat of chronic exposure to low levels of stressors—it seems to be reasonable to introduce the study of toxicity of mixtures of pollutants (with a properly selected battery of bioassays) to routinely conducted environmental monitoring. In this study, we confirmed the impact of bisphenol A and its analogues on endocrine receptors—Even at 1.83 μM concentration levels. As expected, mostly estrogenic agonistic and androgenic antagonistic behaviors were observed. The levels studied reflect the environmentally stated levels of most BPs and constitute important prerequisite to run complex studies on the endocrine impact of these compounds, when present in binary (or higher) mixtures.

4. Materials and Methods

The experimental design and approach was previously described in greater detail in [29], while the basics of the research performed are described below to assure easy access to a wider audience.

4.1. Chemicals and Reagents

Model substances selected for the study, BPA (CAS no. 80-05-7), BPC (CAS no. 79-97-0), BPE (CAS no. 2081-08-5), BPF (CAS no. 620-92-8), BPG (CAS no. 127-54-8), BPM (CAS no. 13595-25-0), BPP (CAS no. 2167-51-3), BPS (CAS no. 80-09-1), BPZ (CAS no. 843-55-0), and BPFL (CAS no. 3236-71-3) of analytical (>99%) purity were purchased from Sigma Aldrich (Darmstadt, Germany), as were HPLC grade methanol (CAS no. 67-56-1) and dimethyl sulfoxide (DMSO, CAS no. 67-68-5). Ultra-pure water was obtained using a grade A10 Milli-Q system (Millipore, Darmstadt, Germany) equipped with

EDS-PAK® Polisher cartridge (Merck, Darmstadt, Germany) to remove trace levels of bisphenol A and other endocrine-disrupting chemicals from water.

4.2. Standards and Mixtures Preparation

A standard stock solution of each compound was prepared separately by dissolving the given standard (to reach the concentration of 4 mg/mL) in HPLC grade methanol and stored in −20 °C. Various working solutions were obtained by serial dilution of the stock solutions with HPLC-grade methanol or ultrapure Milli-Q water (maximum methanol content in standard solutions for biological assays was 5%). The concentration ranges [μM] for bisphenol A analogues studied to determine their respective EC_{50} data and subsequently to select C_1, C_2 and C_3 (being 33, 66 and 100% of EC_{50} of respective analyte) and to determine the impact of their co-presence on toxicity levels are listed in Table 1 (together with LOEC (lowest observable effect concentration) and NOEC (no-observed effect concentration) of given chemicals with respect to XenoScreen YES/YAS).

4.3. Microtox® Reagents and Methodology

The Microtox® test acute reagent (lyophilized *Vibrio fischeri*), osmotic adjustment solution (OAS, 22% solution of sodium chloride), reconstitution solution (RS), and diluent (2% solution of sodium chloride) were purchased from Modern Water (Cambridge, UK). The study was conducted using Microtox® analyzer model 500 (M500, Modern Water, Cambridge, UK). The apparatus was equipped with 30 incubation wells as well as reagent (bacterial suspensions) and read wells. Temperatures were assigned to the corresponding type of performed test (in this case acute toxicity test) and internally maintained at 5.5 ± 1.0 °C for reagent well and 15.0 ± 0.5 °C for both the incubator part and the read well. pH was adjusted to fall within the 6.5–7.5 range with concentrated NaOH (CAS no. 1310-73-2) and HCl (CAS no. 7647-01-0) (purchased from Avantor Performance Materials S.A. (Poznań, Poland)) using Metrohm pH-meter model 827 (Metrohm, Opacz-Kolonia, Poland).

The EC_{50} parameter for each analyte of interest separately was determined by standard protocol using the Microtox® Analyzer Model 500 and serial dilutions. Lyophilized reagent with *Vibrio fischeri* bacteria was hydrated with 1 mL of RS and maintained at 5.5 ± 1.0 °C, subsequently 100 μL of bacterial solution and a pre-made samples of standard dissolved in distilled water (made from stock solutions of given analyte dissolved in ethanol) were added into the vials. To produce a suitable osmotic pressure (above 2%), OAS was added to the vial with the highest concentration and proper dilutions and ions additions were prepared. The incubation time was 30 min. Range-screening test for insoluble substance was also performed to narrow the range of concentrations tested; afterwards, proper tests were performed in triplicates to determine the range of linearity and calculate particular analytes EC_{50} values.

In order to determine whether the addition of one BP to solution of another one would change the bioluminescence of bacterial suspension, concentrated solutions of the compounds were prepared. Test mixtures were prepared in such a way that the compounds were present in an appropriate ratio: 100% of the first model substance and the second substance with a reduced effect to 33% and 66% of EC_{50}. Incubation time of samples with bacteria for all of the tests was 30 min.

4.4. XenoScreen YES/YAS Reagents and Methodology

A set of XenoScreen YES/YAS reagents was purchased from Xenometrix AG (Allschwil, Switzerland), namely vial with hERα yeasts (to determine estrogenic activity) and hAR (to determine androgenic activity) settled on the filtration paper, basal medium, vitamin solution, L-aspartic acid solution, L-treonine solution, $CuSO_4$, 17β-estradiol (E2, YES+ control), 5α-dihydrotestosterone (DHT, YAS+ control), 4-hydroksytamoxyphene (HT, YES− control), flutamide (FL, YAS− control), DMSO. CPRG (chlorophenol red-β-D-galactopyranoside) was purchased from Sigma Aldrich (Hamburg, Germany). Measurement of cell density (wavelength 690 nm) and the intensity of the

CPRG transformation product (wavelength 570 nm) was performed with a TECAN Infinite M200 spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland).

To investigate endocrine potential of bisphenol analogues, a slightly modified protocol of XenoScreen YES/YAS was utilized, which uses genetically modified yeast cells of *Saccharomyces cerevisiae*. For this purpose, the DNA sequence of human estrogen hERα or androgen hAR receptors was stably integrated into the main chromosome of the yeast cells. Yeasts exposed to compounds that act endocranially produce β-galactosidase, which oxidizes the dye CPRG in growth medium. The interpretation occurs by measuring the density of the cell suspension and the color saturation of the oxidized dye. Furthermore, the cells also contain an expression plasmid carrying the lacZ reporter gene encoding the enzyme β-galactosidase and means responsive to estrogens (YES) or androgen (YAS). The yeast cells were cultured from the filter papers in growth medium (basic medium with a vitamin solution, solution of L-threonine, L-aspartic acid and copper (II) sulfate (VI)). 5 mL of growth medium was transferred to a labeled culture bottles with caps with a gas permeable filter; afterwards, the yeast disks were sterilely transferred and placed on an orbital shaker set at 32 °C and 100 rpm for 48 h. 100 µL of DMSO was added to each control vial containing standards: E2 (17β-estradiol control of YES agonist), DHT (5α-dihydrotestosterone control of YAS agonist), HT (4-hydroxytamoxifen control of YES antagonist), and FL (flutamide control of YAS antagonist). Test plates were prepared in such a way that the controls were in duplicate in eight serial dilutions, respectively:

- YES Agonist plate E2 (min. concentration 1×10^{-11} M, max. concentration 1×10^{-8} M).
- YES Antagonist plate HT (min. concentration 1×10^{-8} M, max. concentration 1×10^{-5} M, additionally in the entire plate E2 was present at constant concentration of 1×10^{-9} M).
- YAS Agonist plate DHT (min. concentration 1×10^{-9} M, max. concentration 1×10^{-6} M).
- YAS Antagonist plate FL (min. concentration 1×10^{-7} M, max. concentration 1×10^{-4} M, additionally in the entire plate DHT was present at constant concentration of 3×10^{-8} M).

The addition of E2 or DHT present at the same concentration to the entire YES or YAS antagonist plate, respectively, is intended to examine (confirm/deny) andro- and estrogenic antagonistic activity of samples. A substance with the antagonist properties competes with E2 or DHT present on the plate and binds to the receptor without inducing the expression of β-galactosidase. Without the enzyme, substrate staining does not occur; however, if the test sample does not contain antagonistic substances, then E2 and DHT present in the wells bind with the receptor expressing β-galactosidase and staining of the substrate occurs.

60 µL of 6 mM CRPG dye was added to each assay well. BPs' serial dilutions were studied to detect a broad range of possible interactions. All of the studies on mixtures were performed in triplicates; furthermore, controls were made for pure substances in duplicates. 100 µL of YES and YAS suspension of yeast culture (yeast cells density > 0.3 OD_{690}) was added into agonist and antagonist YES and YAS plates, respectively. Assay plates were sealed with semi-permeable membranes and placed in a zipper bag moistened with watered gauze on an orbital shaker for 48 h at 32 °C 100 rpm. After 48 h of incubation, a cell density (by OD) was read at a wavelength of 690 nm, and color intensity at a wavelength of 570 nm was determined. Afterwards, the activity of β-galactosidase was calculated as ratio of $[(OD_{570} - OD_{690})/OD_{690}]$.

4.5. Calculations of Model Deviation Ratios (MDRs)

The two most exploited models for environmental hazard and risk assessments of mixtures are Concentration Addition (CA) and Independent Action (IA) [29]. These two approaches could assess the combined toxicological effect of chemicals assuming similar mode of action (CA) or dissimilar mode of action (IA). In the environmental risk assessment, CA models are more frequently applied, since they are slightly more conservative than IA models and could be used as a precautious first tier for environmental hazard and risk assessment of mixtures, irrespective of the modes of action of their components.

In this study, the combined toxicological effect of mixture was assessed by a CA model using Equation (1) [27]:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \quad (1)$$

where ECx_{mix} is the total concentration of the mixture that causes x effect; p_i indicates the proportion of component i in the mixture; n indicates the number of components in the mixture; ECx_i indicates the concentration of component i that would cause x effect.

The independent action (IA) model is used to test toxicants in a mixture for a dissimilar mode of action. The concept is that they act independently. In fact, the IA model is a statistical approach to predict the chance that one of multiple events will occur. The total mixture effect is calculated using Equation (2):

$$(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \quad (2)$$

where $E(c_{mix})$ is the total concentration of the mixture; $E(c_i)$ is the concentration expected from component i.

The CA model does not count for possible interaction between different chemicals in the mixture and deviations of tested mixture toxicity from the predicted one could be evidence for synergistic or antagonistic interaction between chemicals. To outline significant deviations (interactions between chemicals), the model deviation ratio (MDR) approach proposed by [31] is applied. MDR (unitless) is defined as Equation (3):

$$\text{MDR} = \frac{Expected\ toxicity}{observed\ toxicity} \quad (3)$$

where *Expected toxicity* is the effective concentration toxicity for the mixture predicted by CA/IA model; *Observed toxicity* is the effective concentration toxicity for the mixture obtained from toxicity testing.

The MDR values are easily applicable to reflect the impact of toxicants mixture, when compared to predictive models. MDRs can be also presented in a plot form on a logarithmic scale to visualize the predicted toxicity in comparison to an observed one. The mixtures with MDR values falling outside the range from 0.5 to 2.0 have a high probability for biologically significant, respectively, synergistic or antagonistic interactions between chemicals. The underestimated or overestimated toxicity mixtures close to these levels also most likely include possible synergistic or antagonistic interactions [31]. In current research, it was arbitrarily assumed that MDR falling within 0.50–0.71 and 1.40–2.00 justify the concluding on, respectively, possible under- and overestimation of presented models.

Since CA and IA models are one of many options for assessing possible interactions between the chemicals involved in the ecotoxicity study, we have tried another approach for estimation of the possible independent action and action different from independent. This simple mode of assessment requires calculation of the regression function A = f(B), where A and B are notations for two different bisphenol analogues as well as regression B = f(A). If in the concentration intervals of A and B experimentally studied, the slope and the offset of the polynomial equation do not differ significantly, one can accept that there is independent action mode at hand. Different mode of action (dependent mode) is ascribed if the regression parameters are significantly different. A similar approach (best-fit modeling) proved to be effective in the assessment of the bisphenol analogues' interactions [29].

4.6. Molecular Modeling Calculations

To determine the most probable conformations of the studied bisphenols and its analogues, two molecular modeling techniques were used. Each compound first underwent molecular dynamics calculation for at least 200 ps after thermal equilibration at 300 K and the most popular conformer was selected. The geometry of this conformer was next optimized by molecular mechanics to minimize its potential energy. The Polak-Ribiere algorithm with termination at 0.05 kcal/(Å·mol) RMS (root mean squared) gradient was used in molecular mechanics' optimization. The modeling calculations were

done with HyperChem 8.0 software (Hypercube, Inc., Gainesville, FL, USA) using Bio+(CHARMM) empirical force field.

4.7. Quality Assurance/Quality Control

For quality assurance of running the proper test, the following parameters were used, according to the manufacturers' guidelines: for Microtox®, I_0 of bacterial suspension >70 U (chromium sulfate was used as a positive control in the bacterial stock suspension test run), and for XenoScreen YES/YAS, the OD_{690} of yeast cultures should be >0.3. In all cases presented, these criteria were fulfilled.

5. Conclusions

The correct evaluation of the interactions between potentially toxic (ecotoxic) materials is a significant challenge to all professionals dealing with hazardous materials. In the present work, two simple options for rapid assessment of the ecotoxicity of BPs in their binary mixtures were studied. According to the MDR approach, most of the binary mixtures revealed predominantly independent modes of action; however, several cases showed a typical synergistic or antagonistic effect. We confirmed that it is possible to introduce a relative scale for the calculated MDR values to better distinguish (even qualitatively rather than quantitatively) the independent, synergistic or antagonistic effects.

The calculation of the best-fit polynomial regression models for the impact of A member of the mixture on the B member (and vice versa) makes it possible to compare/distinguish independent interactions from dependent ones. In principle, the calculation of the linear regression models approach confirmed the dominant number of independent action in binary mixtures of bisphenol analogues (18 out of 21 mixtures).

Since studies on mixture toxicity of newly synthesized chemicals remain scarce, the data presented constitute an important record for environmental toxicologists. It should be highlighted that these analytes are of the highest probability of synergistic or antagonistic interactions. Moreover, there is a risk that BPs can be present in some environmental compartments at higher concentrations that have not been examined so far. The results presented here offer clear guidance on how to predict the combined effect of BPs (to endocrine systems and bacteria) in their binary mixtures. Further investigations are required to better understand and mathematically describe the behaviors of pollutants present in environmentally relevant mixtures, which lead to various acute/chronic endpoints for communities of geographically variable characteristics for given regions.

Supplementary Materials: The following are available online, Supplementary Table S1: Basic information about analytes studied, Supplementary Table S2: Structural parameters of bisphenol analogs calculated with molecular dynamics, Supplementary Table S3: Comparison of the polynomial models for BPs studied with Microtox®.

Author Contributions: Conceptualization, K.O., B.K. and Z.M.; methodology, K.O., B.K., V.S. and Z.M.; software, K.O., B.K. and Z.M.; validation, K.O., B.K., V.S., Z.M. and J.N.; formal analysis, K.O., B.K. and Z.M.; investigation, K.O., B.K. and V.S.; resources, K.O., B.K. and J.N.; data curation, K.O., B.K. and V.S.; writing—original draft preparation, K.O., B.K., V.S., Z.M. and J.N.; writing—review and editing, K.O., B.K., V.S., Z.M. and J.N.; visualization, K.O. and B.K.; supervision, K.O., B.K., Z.M. and J.N.; project administration, K.O., B.K. and J.N.; funding acquisition, K.O., B.K., V.S. and J.N.

Funding: The support of the H2020 programme of the European Union (project Materials Networking Horizon 2020 project ID: 692146-H2020-eu.4.b) is gratefully acknowledged by one of the authors (V. Simeonov).

Acknowledgments: Acknowledgments are given to Jan Mazerski from the Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry of Gdańsk University of Technology for help in structure-activity studies.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Vogel, S. The Politics of Plastics: The Making and Unmaking of Bisphenol A "Safety". *Am. J. Publ. Health* **2009**, *99*, 559–566. [CrossRef] [PubMed]

2. Kundakovic, M.; Gudsnuk, K.; Franks, B.; Madrid, J.; Miller, R.L.; Perera, F.; Champagne, F. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9956–9961. [CrossRef] [PubMed]
3. Arbuckle, T.; Davis, K.; Boylan, K.; Fisher, M.; Fu, J. Bisphenol A, phthalates and lead and learning and behavioral problems in Canadian children 6–11 years of age: CHMS 2007–2009. *Neurotoxicology* **2016**, *54*, 89–98. [CrossRef] [PubMed]
4. Ahmed, R.G. Maternal bisphenol A alters fetal endocrine system: Thyroid adipokine dysfunction. *Food Chem. Toxicol.* **2016**, *95*, 168–174. [CrossRef] [PubMed]
5. Stojanoska, M.M.; Milosevic, N.; Milic, N.; Abenavoli, L. The influence of phthalates and bisphenol A on the obesity development and glucose metabolism disorders. *Endocrine* **2016**, *55*, 666–681. [CrossRef] [PubMed]
6. Huang, B.; Jiang, C.; Luo, J.; Cui, Y.; Qin, L.; Liu, J. Maternal exposure to bisphenol A may increase the risks of Parkinson's disease through down-regulation of fetal IGF-1 expression. *Med. Hypotheses* **2014**, *82*, 245–249. [CrossRef] [PubMed]
7. Chen, D.; Kannan, K.; Tan, H.; Zheng, Z.; Feng, Y.L.; Wu, Y.; Widelka, M. Bisphenol analogues other than BPA: Environmental occurrence, human exposure and toxicity—A review. *Environ. Sci. Technol.* **2016**, *50*, 5438–5453. [CrossRef] [PubMed]
8. Niederst, J.; Evans, R.H.; O'Brien, R.M.; Romagnoli, K.; Killilea, H.T.; Von Maier, M.S.; Deng, L. Developer for Thermally Responsive Record Materials. U.S. Patent 14,418,014, 15 March 2013.
9. Hsieh, B.Y.; Hsu, J.P. Photosensitive Resin Composition, Color Filter and Method for Manufacturing the Same, and Liquid Crystal Display Apparatus. U.S. Patent 14,554,067, 26 November 2014.
10. Bisphenol A; CASRN 80-05-7. Available online: https://cfpub.epa.gov/ncea/iris/iris_documents/documents/subst/0356_summary.pdf (accessed on 5 July 2017).
11. Rochester, J. Bisphenol A and human health: A review on the literature. *Reprod. Toxicol.* **2013**, *42*, 132–155. [CrossRef]
12. Rosenmai, A.K.; Dybdahl, M.; Pedersen, M.; van Vught-Lussenburg, B.; Wedebye, E.; Taxvig, C.; Vinggaard, A. Are Structural Analogues to Bisphenol A Safe Alternatives? *Toxicol. Sci.* **2014**, *139*, 35–47. [CrossRef]
13. Eladak, S.; Grisin, T.; Moison, D.; Guerquin, M.J.; N'Tumba-Byn, T.; Pozzi-Gaudin, S.; Benachi, A.; Livera, G.; Rouiller-Fabre, V.; Habert, R. A new chapter in the bisphenol A story: Bisphenol S and bisphenol F are not safe alternatives to this compound. *Fertil. Steril.* **2015**, *103*, 11–21. [CrossRef]
14. Xue, J.; Kannan, P.; Kumosani, T.; Al-Malki, A.; Kannan, K. Resin-based dental sealants as a source of human exposure to bisphenol analogues, bisphenol A diglycidyl ether, and its derivatives. *Environ. Res.* **2018**, *162*, 35–40. [CrossRef] [PubMed]
15. Niu, Y.; Wang, B.; Zhao, Y.; Zhang, J.; Shao, B. Highly Sensitive and High-Throughput Method for the Analysis of Bisphenol Analogues and Their Halogenated Derivatives in Breast Milk. *J. Agric. Food Chem.* **2017**, *65*, 10452–10463. [CrossRef] [PubMed]
16. Owczarek, K.; Kubica, P.; Kudłak, B.; Rutkowska, A.; Rachoń, D.; Namieśnik, J.; Wasik, A. Determination of trace levels of eleven bisphenol A analogues in human blood serum by high performance liquid chromatography–tandem mass spectrometry. *Sci. Total Environ.* **2018**, *628–629*, 1362–1368. [CrossRef] [PubMed]
17. Yamazaki, E.; Yamashita, N.; Taniyasu, S.; Lam, J.; Lam, P.; Moon, H.B.; Jeong, Y.; Kannan, P.; Achyuthan, H.; Manuswamy, N.; et al. Bisphenol A and other bisphenol analogues including BPS and BPF in surface water samples from Japan, China, Korea and India. *Ecotoxicol. Environ. Saf.* **2015**, *122*, 565–572. [CrossRef] [PubMed]
18. Yang, Y.; Lu, L.; Zhang, J.; Yang, Y.; Wu, Y.; Shao, B. Simultaneous determination of seven bisphenols in environmental water and solid samples by liquid chromatography–electrospray tandem mass spectrometry. *J. Chromatogr. A* **2014**, *1328*, 26–34. [CrossRef] [PubMed]
19. Lee, S.; Liao, C.; Song, G.J.; Ra, K.; Kannan, K.; Moon, H.B. Emission of bisphenol analogues including bisphenol A and bisphenol F from wastewater treatment plants in Korea. *Chemosphere* **2015**, *119*, 1000–1006. [CrossRef] [PubMed]
20. Song, S.; Song, M.; Zeng, L.; Wang, T.; Liu, R.; Ruan, T.; Jiang, G. Occurrence and profiles of bisphenol analogues in municipal sewage sludge in China. *Environ. Pollution* **2014**, *186*, 14–19. [CrossRef]

21. Caballero-Casero, N.; Lunar, L.; Rubio, S. Analytical methods for the determination of mixtures of bisphenols and derivatives in human and environmental exposure sources and biological fluids: A review. *Anal. Chim. Acta* **2016**, *908*, 22–53. [CrossRef]
22. Liao, C.; Kannan, K. Concentrations and Profiles of Bisphenol A and Other Bisphenol Analogues in Foodstuffs from the United States and Their Implications for Human Exposure. *J. Agric. Food Chem.* **2013**, *61*, 4655–4662. [CrossRef]
23. Rivas, A.; Lacroix, M.; Olea-Serrano, F.; Laios, I.; Leclercq, G.; Olea, N. Estrogenic effect of a series of bisphenol analogues on gene and protein expression in MCF-7 breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **2002**, *82*, 45–53. [CrossRef]
24. Fic, A.; Mlakar, S.; Juvan, P.; Mlakar, V.; Marc, J.; Dolnec, M.; Broberg, K.; Masic, L. Genome wide gene expression profiling of low-dose, long-term exposure of human osteosarcoma cells to Bisphenol A and its analogs. *Toxicol. In Vitro* **2015**, *29*, 1060–1069. [CrossRef] [PubMed]
25. Wieczerzak, M.; Namieśnik, J.; Kudłak, B. Bioassays as one of the Green Chemistry tools for assessing environmental quality: A review. *Environ. Int.* **2016**, *94*, 341–361. [CrossRef] [PubMed]
26. Water Framework Directive. Available online: http://www.waterframeworkdirective.wdd.moa.gov.cy/docs/pdf/wfd.pdf (accessed on 5 October 2017).
27. Faust, M.; Altenburger, R.; Backhaus, T.; Bodeker, W.; Scholze, M.; Grimme, L.H. Predictive assessment of the aquatic toxicity of multiple chemical mixtures. *J. Environ. Qual.* **2000**, *29*, 1063–1068. [CrossRef]
28. Szczepańska, N.; Kudłak, B.; Namieśnik, J. Assessing ecotoxicity and the endocrine potential of selected phthalates, BADGE and BFDGE derivatives in relation to environmentally detectable levels. *Sci. Tot. Env.* **2018**, *610–611*, 854–866. [CrossRef] [PubMed]
29. Wieczerzak, M.; Kudłak, B.; Yotova, G.; Nedyalkova, M.; Tsakovski, S.; Simeonov, V.; Namieśnik, J. Modeling of pharmaceuticals mixtures toxicity with deviation ratio and best-fit functions models. *Sci. Total Environ.* **2016**, *571*, 259–268. [CrossRef] [PubMed]
30. Altenburger, R.; Nendza, M.; Schuurmann, G. Mixture toxicity and its modeling by quantitative structure–activity relationships. *Environ. Toxicol. Chem.* **2003**, *22*, 1900–1915. [CrossRef] [PubMed]
31. Belden, J.B.; Gilliom, R.J.; Lydy, M.J. How well can we predict the toxicity of pesticide mixtures to aquatic life? *Integr. Environ. Assess. Manag.* **2007**, *3*, 364–372. [CrossRef] [PubMed]
32. Liao, C.; Liu, F.; Guo, Y.; Moon, H.-B.; Nakata, H.; Wu, Q.; Kannan, K. Occurrence of Eight Bisphenol Analogues in Indoor Dust from the United States and Several Asian Countries: Implications for Human Exposure. *Environ. Sci. Technol. Lett.* **2012**, *46*, 9138–9145. [CrossRef]

Sample Availability: Samples of the compounds are not available from the authors.

Electronic supplementary material for Article

Binary mixtures of selected bisphenols in the environment: their toxicity in relationship to individual constituents

Katarzyna Owczarek[1], Błażej Kudłak[1,*], Vasil Simeonov[2], Zofia Mazerska[3], Jacek Namieśnik[1]

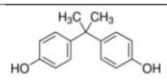
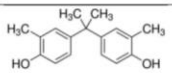
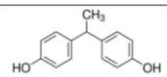
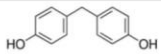
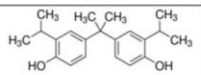
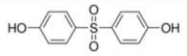
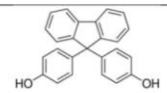
[1] Department of Analytical Chemistry, Faculty of Chemistry, Gdansk University of Technology, 11/12 Narutowicza Str., Gdańsk 80-233, Poland; blakudla@pg.edu.pl

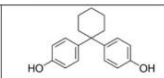
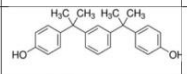
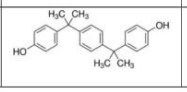
[2] Department of Analytical Chemistry, Faculty of Chemistry and Pharmacy, University of Sofia „St. Kliment Ohridski", 1164 Sofia, 1 James Bourchier Blvd., Bulgaria; vsimeonov@chem.uni-sofia.bg

[3] Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, Gdansk University of Technology, 11/12 Narutowicza Str., Gdańsk 80-233, Poland; zofia.mazerska@pg.edu.pl

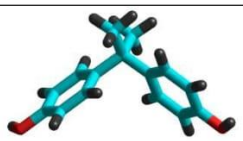
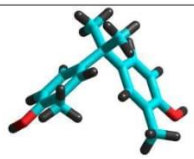
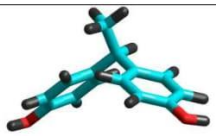
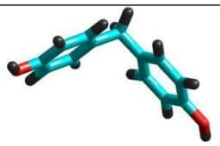
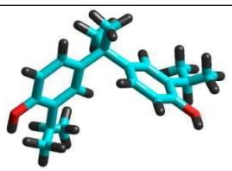
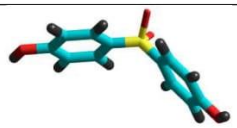
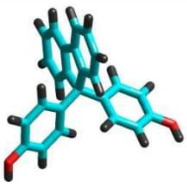
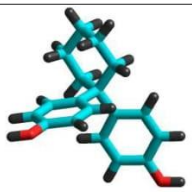
* Correspondence: blakudla@pg.edu.pl; phone no.: +48-58-347-2128, ORCID no. 0000-0002-2237-2927

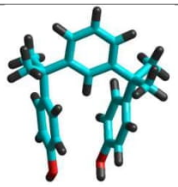
Supplementary Table S1. Basic information about analytes studied

Analyte /CAS no./ molecular weight [g/mol]	Structure	IUPAC name	Application (if known)
BPA / 80-05-7/ 228.29		2,2-bis(4-hydroxyphenyl) propane	Food packaging coatings, plastic bottles, plastic items (toys, every-day use products) [I]
BPC /79-97-0/ 256.34		2,2-bis(4-hydroxy-3-methylphenyl) propane	Used as intermediate in pesticide and pharmaceuticals production [II]
BPE /2081-08-5/ 214.26		1,1-bis(4-hydroxyphenyl) ethane	Polymer production, flame retardants resin composite, heat sensitive recording material [III, IV]
BPF /620-92-8/ 200.23		4,4′-methylenediphenol	BPA substitute, plastic items (toys, every-day use products)
BPG /127-54-8/ 312.45		2,2-bis(4-hydroxy-3-isopropylphenyl) propane	Thermally responsive recording materials [V]
BPS /80-09-1/ 250.27		4,4′-sulfonyldiphenol	Dental sealants, thermal papers production, electronics [VI]
BPFL /3236-71-3/ 350.41		4,4′-(9-fluorenylidene)diphenol	Photosensitive resins [VII]

BPZ /843-55-0/ 268.35		4,4′-cyclohexylidene-bisphenol	Polycarbonates plastic production, thermosensitive materials, optical composites [VIII, IX]
BPM /13595-25-0/ 346.46		4,4′-(1,3-phenylenediisopro-pylidene)bisphenol	Flame retardants, polycarbonate plastics production, aromatic co-polyesters manufacturing, thermosensitive materials [X, XI]
BPP /2167-51-3/ 346.46		4,4′-(1,4-phenylenediisopropylidene) bisphenol	Found in food packaging, polycarbonate plastics production, flame retardant production, thermoplastic resins production [XII, XIII, XIV]

Supplementary Table S2. Structural parameters of bisphenol analogs calculated with molecular dynamics

Analyte	Structure	Distance [Å]		Angle [°]
		O-C[1]	O-O[2]	O-C-O[3]
BPA		5.74	9.25	107.26
BPC		5.74	9.19	106.38
BPE		5.72	9.29	108.47
BPF		5.71	9.29	109.00
BPG		5.74	9.19	106.55
BPS		5.67	10.26	129.41
BPFL		5.74	9.70	115.40
BPZ		5.76	9.11	104.36

BPM		5.92	3.50	34.41
BPP		6.38	9.73	99.2

[1] Distance between central carbon atom and left branch oxygen atom forming phenol
[2] Distance between both branches oxygen atoms forming phenols.
[3] The angle between phenolic rings

Supplementary Table S3. Comparison of the polynomial models for BPs studied with Microtox®

BPs binary combination y/x	Regression coefficients: a, b, c	Correlation R^2	Mode of action
BPA/BPC	0.06; -0.16; 0.71	0.97	IA
BPC/BPA	0.07; -0.21; 0.72	0.98	
BPA/BPE	0.12; -0.44; 0.84	0.94	IA
BPE/BPA	0.08; -0.34; 0.52	0.97	
BPA/BPF	0.07; -0.27; 0.93	0.98	IA
BPF/BPA	0.06; -0.21; 0.85	0.98	
BPA/BPG	0.005; 0.08; 0.58	0.97	IA
BPG/BPA	0.007; 0.12; 0.61	1.00	
BPA/BPS	0.11; -0.42; 0.87	0.97	IA
BPS/BPA	0.11; -0.12; 0.36	0.96	
BPA/BPFL	0.06; -0.17; 075	0.98	IA
BPFL/BPA	0.04; -0.27; 0.16	0.98	
BPC/BPE	0.03; 0.002; 0.48	0.96	IA
BPE/BPC	0.07; 0.05; 0.60	0.94	
BPC/BPF	0.04; -0.06; 0.66	0.96	IA
BPF/BPC	0.05; -0.12; 0.73	0.97	
BPC/BPG	-0.003; 0.17;0.36	0.99	Different from IA
BPG/BPC	0.05; -0.16; 0.39	0.97	

BPC/BPS	0.06; -0.19; 0.66	0.94	IA
BPS/BPC	0.08; -0.24; 0.65	0.97	
BPC/BPFL	-0.007; 0.16; 0.49	0.99	IA
BPFL/BPC	-0.005; 0.26; 0.36	0.99	
BPE/BPF	0.05; -0.15; 0.75	0.97	IA
BPF/BPE	0.05; -0.14; 0.72	0.97	
BPE/BPG	0.02; 0.02; 0.55	0.99	IA
BPG/BPE	0.03; 0.11; 0.49	0.99	
BPE/BPS	0.07; -0.24; 0.79	0.96	IA
BPS/BPG	0.07; -0.18; 0.65	0.96	
BPE/BPFL	0.07; -0.19;0.73	0.98	IA
BPFL/BPE	0.06; -0.11; 0.62	0.99	
BPF/BPG	0.04; -0.10; 0.72	0.99	IA
BPG/BPF	0.04; -0.15; 0.92	0.99	
BPF/BPS	0.15; -0.48;0.58	0.95	IA
BPS/BPF	0.11; -0.25; 0,41	0.96	
BPF/BPFL	0.12; -0.32; 0.34	0.99	IA
BPFL/BPF	0.08; -0.33; 0.92	0.99	
BPG/BPS	0.06; -0.22; 0.92	0.77	Different from IA
BPS/BPG	0.07; -0.20; 0.68	0.98	
BPG/BPFL	-0.02; 0.22; 0.38	0.99	IA
BPFL/BPG	-0.01; 0.59; -0.005	0.99	
BPS/BPFL	0.05; -0.11; 0.53	0.86	Different from IA
BPFL/BPS	0.01; 0.10; 0.34	0.99	

References for electronic supplement:

[I] Vogel, S. The Politics of Plastics: The Making and Unmaking of Bisphenol A "Safety". *Am. J. Publ. Health* **2009**, *99*, 559–566.

[II] Rudd, J.F. Blends of Phenolphthalein Polycarbonates with Rubber-Modified Monovinylidene Aromatic Copolymers, Eur. Pat. Appl. 0000146, **1979**.

[III] Hada, T.; Shimizu, M.; Aoki, Y.; Endo, N.; Ohhashi, H.; Ishiguro, T., Heat-Sensitive Recording Material and Method for Producing Same. U.S. Patent, 13,254,045, 16 February **2010**.

[IV] Zouta, K.; Tokita, A. Polycarbonate Resin Composition, and Fluorescence Detection/analysis Substrate Produced using Polycarbonate Resin Composition. U.S. Patent, 14,901,210, 19 June **2014**.

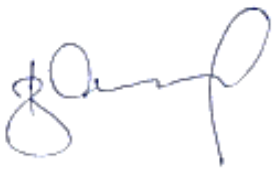
[V] Niederst, J.; Evans, R.H.; O'Brien, R.M.; Romagnoli, K.; Killilea, H.T.; Von Maier, M.S.; Deng, L. Developer for Thermally Responsive Record Materials. U.S. Patent 14,418,014, 15 March **2013**.

[VI] Chen, D.; Kannan, K.; Tan, H.; Zheng, Z.; Feng, Y.L.; Wu, Y.; Widelka, M. Bisphenol analogues other than BPA: Environmental occurrence, human exposure and toxicity—A review. *Environ. Sci. Technol.* **2016**, *50*, 5438–5453.
[VII] Hsieh, B.Y.; Hsu, J.P. Photosensitive Resin Composition, Color Filter and Method for Manufacturing the Same, and Liquid Crystal Display Apparatus. U.S. Patent 14,554,067, 26 November **2014**.
[VIII] Ochiai, T.; Masuda, T. Thermal Recording Material and Method for Producing the Same. U.S. Patent 13,132,984, 21 November **2009**.
[IX] Coleman, Z. **2011**. Method of manufacturing an optical composite, U.S. Patent 13/163,938.
[X] Zouta, K.; Nakao, K.; Takagi, Y.; Ishii, H. Flame-Retardant Sheet or Film, Products Comprising the Same and Process for Production Thereof, U.S. Patent 14,900,386, 20 June **2011.**
[XI] Weiss, J. Process for Improving the Performance of a Marine Antifouling Paint, and Top Coat Composition Employed in this Process. Eur. Pat. Appl. 0005771, **1979**.
[XII] Wagner, S.; Kramer, R.H.; Konig, A.; Roth, M. Flame-Retardant Polyesters. U.S. Patent 14,917,039, 3 December **2015**.
[XIII] Tamura, M.; Ohashi, T.; Enomoto, T. Thermosetting Resin Composition Containing Polymer Having Specific Terminal Structure. U.S. Patent 14,900,489, 4 June **2014**.
[XIV] Teichert, D.; Conrad, L.; Grotzinger, J. Epoxy Resin-Upgraded Cement-Bound Composition as Coating or Seal. U.S. Patent 14,904,884, 14 July **2014**.

DECLARATION OF THE PERSONAL INPUT TO THE ORIGINAL ARTICLE

OŚWIADCZENIE WSPÓŁAUTORÓW PRACY ZESPOŁOWEJ WSKAZUJĄCE, CO STANOWI ICH WŁASNY WKŁAD

K. Owczarek, B. Kudłak, V. Simeonov, Z. Mazerska, J. Namieśnik, *Binary Mixtures of selected bisphenols in the environment: their toxicity in relationship to individual constituents*, Molecules, 23, 3226, 2018

Name *Nazwisko współautora*	**Input** *Wkład*	**Signature** *Podpis*
K. Owczarek	Conceptualization, methodology, validation, investigation, data curation, original draft preparation, manuscript rewiev and editing *Koncepcja pracy, opracowanie metodologii, walidacja wyników, przeprowadzanie eksperymentów, analiza danych, współudział w tworzeniu tekstu manuskrytu i odpowiedzi na uwagi recenzentów*	
B. Kudłak	Conceptualization, methodology, validation, investigation, supervision, data curation, original draft preparation, manuscript rewiev and editing, project administration *Koncepcja pracy, opracowanie metodologii, walidacja wyników, nadzór nad prowadzeniem eksperymentów, analiza danych, współudział w tworzeniu tekstu manuskryptu i odpowiedzi na uwagi recenzentów, nadzór nad projektem*	
V. Simeonov	Methodology, validation, investigation, data curation, original draft preparation, manuscript rewiev and editing *Opracowanie metodologii, nadzór nad zapewnieniem jakości , analiza danych, współudział w tworzeniu tekstu manuskryptu i odpowiedzi na uwagi recenzentów*	
Z. Mazerska	Conceptualization, methodology, validation, supervision, original draft preparation, manuscript rewiev and editing, project administration *Koncepcja pracy, opracowanie metodologii, nadzór nad zapewnieniem jakości, współudział w tworzeniu tekstu manuskryptu i odpowiedzi na uwagi recenzentów, nadzór nad projektem*	
J. Namieśnik	Validation – QA/QC, manuscript rewiev and editing, project administration, supervision *Nadzór nad zapewnieniem jakości, sprawdzenie manuskryptu pod względem merytorycznym, nadzór nad projektem;*	* Professor Jacek Namieśnik died 14.04.2019 * *Profesor Jacek Namieśnik zmarł 14.04.2019*

4.2. Oznaczanie związków z grupy bisfenoli w próbkach surowicy ludzkiej

Analiza ksenobiotyków w próbkach pochodzenia biologicznego jest ważnym elementem biomonitoringu i szacowania ekspozycji. Bisfenol A oraz jego najpowszechniej stosowane substytuty, tj. BPF i BPS, są związkami często oznaczanymi w tego typu matrycach. W przypadku mieszaniny pozostałych substancji z grupy bisfenoli obecnych w ludzkich płynach ustrojowych ilość danych wciąż jest relatywnie niewielka, co uniemożliwia kompleksową ocenę narażenia człowieka na ich działanie. Ze względu na coraz częstsze doniesienia o występowaniu analogów BPA w elementach środowiska i produktach żywnościowych (por. Tabela 1.) na przestrzeni ostatnich lat można zaobserwować rosnące zainteresowanie tym tematem, przekładające się również na zwiększoną liczbę doniesień naukowych [63, 65 – 68, 129, 130].

Wartości liczbowe parametru log K_{ow} dla bisfenoli będących przedmiotem zainteresowania mieszczą się w przedziale 1,65 – 6,55 (Tabela 2), co pozwala zakwalifikować je do grupy substancji średnio i silnie lipofilowych. Wskazuje to na znaczący potencjał do bioakumulacji w tkankach organizmów żywych [146]. Chociażby z tego względu wielkość ekspozycji oraz mechanizmy transportu i przemian, jakim związki te podlegają w organizmie, powinna być przedmiotem szczególnej uwagi.

Celem opisanych badań opisanych w przedmiotowej publikacji było opracowanie toku postępowania analitycznego, który umożliwi identyfikację oraz ilościowe oznaczenie śladowych ilości jedenastu związków z rodziny bisfenoli w próbkach surowicy krwi. Krew i jej pochodne stanowią cenne źródło informacji o krótkotrwałym, bieżącym narażeniu na działanie ksenobiotyków ze względu na to, że pozostaje w ciągłej styczności ze wszystkimi tkankami. Większość dostępnych metod z zakresu analityki mieszanin bisfenoli skupiona jest na analizie próbek moczu, dlatego też nowe procedury mające na celu oznaczanie tych analitów we krwi stanowią cenne uzupełnienie obecnego stanu wiedzy w tym zakresie.

Etap przygotowania próbek do analizy oparty był na ekstrakcji w układzie ciecz – ciecz, zoptymalizowanej tak, aby zużycie rozpuszczalników organicznych było minimalne a analizowana objętość próbki jak najmniejsza. Dodatkowo cała procedura była prosta i mało czasochłonna. Jako technikę oznaczeń końcowych wybrano ultrasprawną chromatografię cieczową sprzężoną z tandemową spektrometrią mas (UPLC-MS/MS) w trybie monitorowania wielu przejść jonowych (MRM), co zapewniło możliwość uzyskania niskiej granicy wykrywalności (LOD) i oznaczalności (LOQ) mieszczących się w przedziałach, odpowiednio, 0,0079 ÷ 0,039 ng/mL i 0,024 ÷ 0,12 ng/mL. Aby zminimalizować efekty matrycowe, kalibrację przeprowadzono stosując krzywe wzorcowe z odwzorowaniem matrycy oraz metodę wzorca wewnętrznego. Na drodze optymalizacji procedury udało się uzyskać na tyle dobrą sprawność układu chromatograficznego, że możliwe było niezależne oznaczenie BPM i BPP, które są izomerami konfiguracyjnymi. Dużą role odegrało w tym przypadku zastosowanie kolumny chromatograficznej z ziarnem typu porowato-rdzeniowego, które

w przypadku układów pracujących w warunkach UPLC zapewniło obniżenie wartości ciśnienia wstecznego oraz znaczącą poprawę kształtu pików. Możliwe było również skrócenie czasu retencji, które nie nastąpiło kosztem pogorszenia rozdzielczości.

Opracowana metoda posłużyła do detekcji i ilościowego oznaczenia szeregu bisfenoli w 245 próbkach rzeczywistych surowicy ludzkiej. Związkiem pojawiającym się w próbkach najczęściej był oczywiście BPA (zidentyfikowano go w ponad 90% próbek), a związkiem najmniej rozpowszechnionym okazał się BPFL (zawierało go nieco powyżej 20% próbek). Należy podkreślić, że wszystkie z oznaczanych związków wykrywano w badanym materiale biologicznym, co wskazuje na nieustanny kontakt człowieka z tą grupą substancji. Ze względu na dużą czułość i selektywność tej metody zapewniła ona monitorowanie niskich zawartości bisfenoli w surowicy. Krótki czas potrzebny na przygotowanie próbek do analizy jest istotną zaletą dla zastosowania tej procedury w rutynowych i przesiewowych badaniach, gdzie liczba próbek jest bardzo duża.

Praca została opublikowana na łamach czasopisma *Science of the Total Environment* i była jednym z pierwszych opracowań, opisujących oznaczanie szerokiego spektrum bisfenoli w elementach próbek krwi [147].

Science of the Total Environment 628–629 (2018) 1362–1368

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Determination of trace levels of eleven bisphenol A analogues in human blood serum by high performance liquid chromatography–tandem mass spectrometry

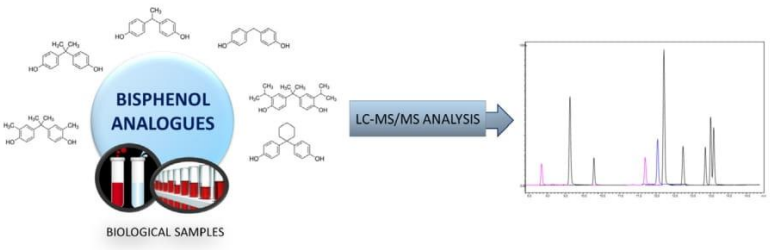
Katarzyna Owczarek [a], Paweł Kubica [a], Błażej Kudłak [a], Aleksandra Rutkowska [b], Aleksandra Konieczna [b], Dominik Rachoń [b], Jacek Namieśnik [a], Andrzej Wasik [a,*]

[a] Department of Analytical Chemistry, Chemical Faculty, Gdańsk University of Technology, Narutowicza 11/12 str, 80-223 Gdańsk, Poland
[b] Department of Clinical and Experimental Endocrinology, Medical University of Gdańsk, Dębinki 7, 80-210 Gdańsk, Poland

HIGHLIGHTS

- Chromatographic separation of bisphenol A and 10 bisphenol A analogues
- Liquid-liquid extraction of bisphenol A analogues from 245 human serum samples
- Matrix-matched calibration to compensate for matrix effects

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 September 2017
Received in revised form 12 February 2018
Accepted 12 February 2018
Available online xxxx

Editor: Yolanda Picó

Keywords:
Bisphenol A
BPA analogues
Blood serum analysis
Liquid chromatography
Tandem mass spectrometry
Endocrine disrupting chemicals

ABSTRACT

Chemicals showing structural or functional similarity to bisphenol A (BPA), commonly called BPA analogues, have recently drawn scientific attention due to their common industrial and commercial application as a substitute for BPA. In the European Union, the use of BPA has been severely restricted by law due to its endocrine disrupting properties. Unfortunately, it seems that all BPA analogues show comparable biological activity, including hormonal disruption, toxicity and genotoxicity. Until now, the knowledge about human exposure to BPA analogues is scarce, mainly due to the lack of the data concerning their occurrence in human derived biological samples. This study presents the development of an analytical method for determination of trace levels of eleven BPA analogues in human blood serum samples. The method involves fast and simple liquid-liquid extraction, using low sample and solvent volumes. Chromatographic separation of analytes was optimized using one-factor-at-a-time approach (mobile phase composition, gradient shape, chromatographic column selection, separation temperature, etc.).
The method allows for effective separation of the analytes, even in the case of configurational isomers (bisphenol M and bisphenol P). The calibration curves for all analytes were linear in the range tested. The limits of detection and quantitation were in the range of 0.0079 ÷ 0.039 ng/mL and 0.024 ÷ 0.12 ng/mL respectively. Compound-dependent recovery values were in the rage of 88 ÷ 138%. Matrix effects were mitigated with the help of matrix-matched calibration curves prepared for every batch of samples. Results obtained after the analysis of 245 real human blood serum samples indicate that human beings are exposed to different BPA analogues, that are present in the environment and in common, daily use products.

* Corresponding author.
E-mail address: wasia@pg.edu.pl (A. Wasik).

https://doi.org/10.1016/j.scitotenv.2018.02.148
0048-9697/

1. Introduction

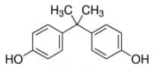
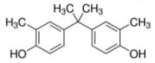
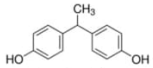
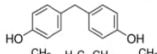
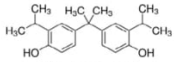
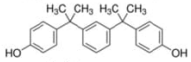
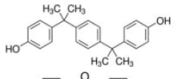
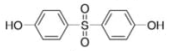
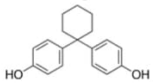
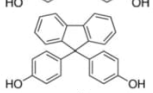
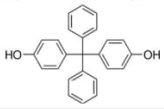
In the last years endocrine disrupting compounds (EDCs) have become the chemical group of special concern due to their ability to interfere with hormonal system and ubiquitous presence (Rissman and Adli, 2014). Bisphenols (BPs) are the chemicals that have recently been the subject of growing interest due to their endocrine disrupting properties (Konieczna et al., 2015). BPs contain two *p*-hydroxyphenyl functionalities in their molecular structure and include several analogues, of which bisphenol A (BPA) is the most commonly used and known. Since early 1950s, BPA has been used in plastic industry for the production of epoxy and polycarbonate resins (Vogel, 2009) being commonly used as raw materials in the manufacturing of a broad spectrum of everyday use products (i.e. tin linings and other food contact materials, water pipes, powder paints, toys etc.). Nowadays, the annual production of BPA reaches over 7.7 million tons and the demand for this compound is predicted to increase over the next years (Industry Experts, 2016).

The effects exerted by BPA on human health have been extensively studied and its estrogenic activity is one of the best known upshots. Due to its phenolic structure BPA is able to interact with estrogen receptors and therefore may exert estrogenic actions (Konieczna et al., 2015) leading to ovarian dysfunction (Rutkowska and Rachoń, 2014) or even estrogen dependent cancers (Rachoń, 2015). Besides that, the vast number of other adverse effects have been proven, including neural and developmental disorders (Arbuckle et al., 2016; Kundakovic et al., 2013), alternation of thyroid function (Ahmed, 2016), metabolic disorders (Stojanoska et al., 2017) and suspicion of increasing the risk of Parkinson disease (Huang et al., 2014). Hazardous implications of BPA presence are not only limited to humans. Especially, the homeostasis of aquatic ecosystems can be disrupted in various ways such as feminization of many wildlife species or developmental and behavioral alternations (Bhandari et al., 2015). Therefore, several endocrine societies have published their position statements on its adverse health risks and stressed the need of governmental authorities in establishing the laws of minimizing the exposure to these endocrine disrupting chemicals (Grob et al., 2015; Hunt et al., 2016; Rutkowska et al., 2015).

Detailed information including chemical structures and IUPAC names of BPA analogues under the study are given in Table 1.

Due to the growing doubts concerning ecological and long-term health implications, new BPA-related chemicals were considered to be safer alternatives for industrial applications. The total number of 16 bisphenols has been documented to be commercially applied (Chen et al., 2016). Bisphenol S (BPS) and bisphenol F (BPF) are nowadays the most commonly used BPA substitutes, predominantly in the manufacturing of epoxy resins, polyesters and polycarbonate plastics. Other analogues are also used in plastic industry to produce dental sealants, pesticides, thermal papers, food container's inner coatings, toys, lacquers, powder paints, flame retardants, personal care products, thermosensitive materials and others (Hada et al., 2013; Hsieh and Hsu, 2015; Ochiai and Masuda, 2013; Teichert et al., 2014; Wagner et al., 2015; Zouta et al., 2014). Currently, only the BPA applications are regulated by legislative standards in European Union, United States and Canada (Yang et al., 2014). BPA, BPE, BPF, BPS, BPP, BPZ, TBBPA, TCBPA, and BPAF were detected in sludge, surface water and indoor dust (Bhandari et al., 2015; Lee et al., 2015; Song et al., 2014; Yamazaki et al., 2015). BPA, BPB, BPE, BPF, BPP, BPS, BPZ BPAF, and BPAP were detected in foodstuffs (Liao and Kannan, 2012; Yang et al., 2014). Unfortunately, the understanding of the environmental, biological and the health impact of BPA analogues is still very scarce. The environmental abundance of BPs undoubtedly indicates that humans are

Table 1
Basic information on bisphenol A analogues.

Compound/molecular weight [g/mol]	CAS number	Structure	IUPAC name
BPA 228.29	80-05-7		2,2-Bis(4-hydroxyphenyl)propane
BPC 256.34	79-97-0		2,2-Bis(4-hydroxy-3-methylphenyl)propane
BPE 214.26	2081-08-5		1,1-Bis(4-hydroxyphenyl)ethane
BPF 200.23	620-92-8		4,4′-Methylenediphenol
BPG 312.45	127-54-8		2,2-Bis(4-hydroxy-3-isopropylphenyl)propane
BPM 346.46	13,595-25-0		4,4′-(1,3-Phenylenediisopropylidene)bisphenol
BPP 346.46	2167-51-3		4,4′-(1,4-Phenylenediisopropylidene)bisphenol
BPS 250.27	80-09-1		4,4′-Sulfonyldiphenol
BPZ 268.35	843-55-0		4,4′-Cyclohexylidenebisphenol
BPFL 350.41	3236-71-3		4,4′-(9-Fluorenylidene)diphenol
BPBP 352.43	1844-01-5		1,1-Bis(4-hydroxyphenyl)-1,1-diphenylmethane

constantly exposed to the wide spectrum of these chemicals, but the data concerning the presence of BPA analogues in human-derived samples is still very limited.

Human exposure to mentioned chemicals include dietary (as a most probable) and non-dietary (inhalation, dermal) routes. Human exposure to bisphenol analogues other than BPA is not well characterized. Available data concerning bisphenols levels in body fluids and tissues is very limited so far. Majority of studies has been conducted on the urine where BPA, BPAF, BPB, BPF, BPS, BPP and BPZ have been measured.

Among sampling material of human origin, blood (and its derivatives - plasma and serum) is a matrix that carries most valuable information about short-term exposure due to its contact with all body cells and tissues. Due to the of lack of scientific data concerning analytical methods for the determination of a wide spectrum of BPA analogues, the aim of this research was to develop an easy, fast, highly sensitive and robust method for human biomonitoring of these chemicals. To encompass the range of bisphenols that are commonly present in the environment constituents and may pose health risk, the total number of 11 bisphenols was determined in human serum samples using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). Analytes have been selected on the basis of the probability of their occurrence in both the environment and common goods and previous research concerning their genotoxicity, estrogenicity or toxicity (Chen et al., 2002; Rivas et al., 2002). Taking into consideration that most of bisphenols other than BPA may also exhibit similar biological activity (Chen et al., 2002; Rivas et al., 2002; Rosenmai et al., 2014; Sui et al., 2012) and number of their industrial and commercial applications is increasing, introducing new methods for BPs biomonitoring seems to be justified due to the constant human exposure to them. In addition, the data concerning the occurrence and concentration levels of BPA analogues in human derived samples is very limited or absent. To the best of authors' knowledge, this is the first report on the determination of eleven BPA analogues in human blood serum samples.

2. Experimental

2.1. Materials and standards

Analytical standards of BPA, BPC, BPE, BPF, BPG, BPM, BPP, BPS, BPZ, BPFL and BPBP were purchased from Sigma-Aldrich (St. Louis, USA, 99% purity). Acetic acid, formic acid and ammonia were purchased from Sigma-Aldrich (St. Louis, USA). $MgSO_4$ was obtained from Eurochem BGD (Tarnów, Poland). Internal standard ^{13}C-labeled BPA (ring-13C12) was supplied by Cambridge Isotope Laboratories Inc. (UK). Normal human serum (cat. no. S1-M EMD Millipore) was also obtained from Sigma-Aldrich (St. Louis, USA). Acetonitrile (ACN) and methanol (MeOH), used during the sample preparation procedure and as a mobile phase components, were LC-MS grade, obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water was produced by the Milli-Q Gradient A10 system equipped with an EDS-Pak cartridge for removing endocrine disrupting compounds (Merck-Millipore).

2.2. Samples

The study has been conducted according to the Declaration of Helsinki for Medical Research involving human subjects and was approved by the Ethics Committee of the Medical University of Gdańsk (permission number NKBBN 198/2012). In total 245 women aged 18–40 years took part in the study. They were all informed about the purpose of it and gave a written consent to participate. Venous blood samples were drawn after an overnight fast (>8 h) between 7 am and 9 am at the medical diagnostic laboratory based in the city centre of Gdańsk (Bruss, ALAB group, Poland), which possesses current ISO certificates and has an accreditation in the field of medical laboratory diagnostics. All the procedures were conducted with precautions intended to minimize the risk of sample contamination with BPA analogues. Blood was drawn from the antecubital vein using a vacuum blood collection system directly into a 7 mL glass tubes (no additives) and within 30–90 min was centrifuged at the speed of 2500 rpm for 15 min. After the centrifugation process, sera were transferred into 2 mL glass vials using a plastic disposable (Pasteur) transfer pipette made of BPA-free low-density polyethylene (LDPE) and were closed with a polypropylene (PP) screw cup with a polytetrafluoroethylene (PTFE) septum. The samples were then stored at −70 °C for further analyses.

2.3. Preparation of standards and calibration

Individual stock solutions (0.5 mg/mL) of all analytes were prepared by dissolving accurately weighted amounts of analytical standards in ACN. Working solution was obtained by mixing the stock solutions and diluting the mixture with ACN. All solutions were stored in a freezer (−20 °C). All glassware was pre-washed with methanol. Seven-point (0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 ng/mL) matrix-matched calibration curves were prepared using normal human blood serum. Before use, the serum used for preparation of the calibration curves, was analyzed for the presence of analytes under the study. It was found free from all studied bisphenols except for small amounts of BPBP (see Supplementary Fig. 1), therefore the calibration data for this compound were corrected to account for this fact, i.e. average blank peak area of BPBP was subtracted from average peak areas of other calibration points. Internal standard (IS) concentration was kept at 25 ng/mL in all calibration samples. Fresh calibration solutions were prepared for every batch of samples.

2.4. Sample preparation

Sample preparation step was conducted with precautions intended to minimize sample contamination. All glassware was washed with MeOH and all plastics were made of high quality polypropylene to avoid contamination of the samples by bisphenols. In order to extract the analytes from serum samples, the following liquid-liquid extraction method was used. 500 μL of serum was placed in a clean glass tube and mixed with 1.5 mL of ACN and 10 μL of IS solution (2.5 μg/mL). Samples were shaken for 30 s and left for 10 min in room temperature to complete protein precipitation. After that, 250 mg of anhydrous $MgSO_4$ was added and each sample was vortexed to remove water. After centrifugation for 2 min (6000 rpm, 3864*g*) supernatants were transferred to clean glass tubes and evaporated under the gentle stream of nitrogen in a water bath (42 °C), to the final volume of about 150 μL. The residue was mixed with 250 μL of mobile phase (MeOH:H_2O, 50:50, 0.01% v/v NH_3), vortexed again and transferred to chromatographic vials for analysis. Procedural blanks spiked with IS, along with system blanks were prepared for every batch in triplicate in the same way as other samples. Example of a procedural blank chromatogram is given in Supplementary Fig. 1.

2.5. MS/MS and separation conditions

All analyses were performed with Shimadzu triple quadrupole LC-MS/MS system (LCMS-8060, Shimadzu, Japan) equipped with an electrospray ionisation source (ESI) working in the negative multiple reaction mode (MRM). LabSolutions v.5.85 software suite was used for data acquisition and processing. Detailed information on ion transitions, MS/MS operational parameters and ion source parameters are given in Supplementary material (Supp. Table 1). Optimal conditions for MRM transitions were established using an automated procedure built into the LabSolutions software suite. In short, it consisted of injecting standard solutions of analytes (one at a time) into the spectrometer, finding a precursor ion ($[M-H]^-$), fragmentation of precursor, selecting most intense product ions and finally automatic optimisation of detector voltages. Ion source parameters (gas flows, temperatures) were adjusted

manually using one-factor-at-a-time approach to obtain the best signal intensity for all analytes.

2.6. Separation conditions

Chromatographic separation was carried out using the UPLC Nexera ×2 system (Shimadzu, Japan) consisting of degasser DGU-20A5R, controller CBM-20A, binary pump LC-30AD, autosampler SIL-30AC and column oven CTO-20AC. Two chromatographic methods were applied to determine the analytes. BPC, BPE, BPF, BPG, BPM, BPP, BPZ, BPFL, BPBP were separated using gradient of H_2O (mobile phase A) and MeOH (mobile phase B), both modified with 0.01% v/v of ammonia. Initial conditions of 5% B were kept for 1.5 min, then the content of B component was increased to 75% over 10.5 min and further increased to 100% over 4 min. Following this, mobile phase composition was set-back to starting conditions and maintained for 5 min for column re-equilibration. For graphical illustration of mobile phase gradient see inset of Fig. 1.

During the determination of BPA and BPS the mobile phase consisted of H_2O (component A) and MeOH (component B) without additives. Isocratic elution using 1:1 mixture of components A and B was used.

Ascentis® Express (C18 15 cm × 2.1 mm, 2.7 μm) with guard column (0.5 cm × 2.1 mm, 2.7 μm), mobile phase flow of 0.55 mL/min, 50 °C of thermostated column compartment and injection volume of 5 μL were applied for separation of analytes in case of both methods.

Examples of chromatograms obtained after analysis of calibration solutions using both methods are given in the Fig. 1. More detailed information on separation conditions will be discussed in the next section.

3. Results and discussion

3.1. Extraction conditions

General problem in bisphenol analysis is ion suppression or enhancement, resulting from the presence of matrix components. In case of such complex biological matrix as blood serum, procedure was focused on removal of its elements. ACN and MeOH were tested as the extraction solvents that are also needed for precipitation of endogenous proteins. Eventually, ACN was chosen due to significantly higher recovery of analytes. Drying of the extract with anhydrous $MgSO_4$ enhanced peaks intensities due to precipitation of water soluble interferences which could caused signal suppression. Salting-out effect could also help to improve the transport of analytes from aqueous phase into the organic solvent.

3.2. Separation and detection of the analytes

The goal of the conducted research was to develop analytical method for separation and determination of 11 bisphenols in blood serum samples. Ascentis® Express C18 (15 cm × 2.1 mm, 2.7 μm) column with guard column (0.5 cm × 2.1 mm, 2.7 μm), packed with core-shell technology particles, was chosen due to its high separation efficiency and relatively short analysis time.

MeOH and ACN were tested as the main organic components of the mobile phase, however, in the case of ACN, peak broadening, peak shape deterioration along with the much smaller response were noted. This phenomenon has been observed for several other compounds determined in ESI negative ion formation mode (Gioumouxouzis et al., 2015). 5, 10, 20 and 25 mM ammonium acetate, 0.01, 0.05 and 0.1% v/v formic acid, acetic acid and ammonia were tested separately as the mobile phase additives. The most promising results (in terms of response and peak shapes) were obtained when applying the latter. Buffer and acid solutions have caused signal suppression.

In case of BPS the addition of ammonia to the mobile phase resulted in decrease of sensitivity and shifting the BPS signal towards system void time. Moreover, low initial content of methanol as well as long analysis and conditioning time caused the enrichment of BPA, derived from system elements, on the front of separation column (Wilczewska et al., 2016).

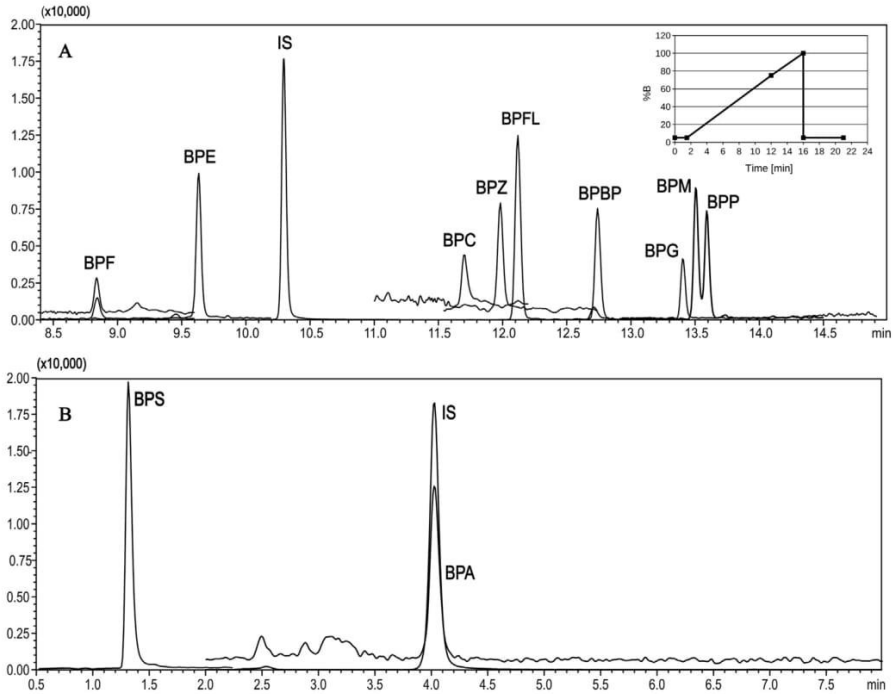


Fig. 1. Chromatograms obtained after analysis of 1 ng/mL calibration solution; (A) separation of 9 bisphenols (mobile phase gradient in the inset), (B) separation of BPA and BPS. For clarity IS trace has been scaled down by the factor of 0.05.

Therefore BPA and BPS were determined with separate method using a mobile phase consisting of MeOH:H_2O without additives. Isocratic flow of relatively high elution strength (50% v/v MeOH) mobile phase provided accurate results and good linearity of the calibration curves.

3.3. Method validation

The performance of both analytical methods was evaluated in terms of linearity, limits of detection (LODs) and quantitation (LOQs) and recoveries. The obtained results are presented in Table 2 and in Suppl. Table 2 of Supplementary material. For both methods the linear calibration equations were obtained from 7-point calibration curves, that were made by plotting the ratios of analyte peak area to IS peak area versus corresponding concentrations. Calibration curves were linear in the tested concentration range from 0.05 to 5 ng/mL. To increase the accuracy at the lowest concentration range, the weighting factor 1/x was applied to every calibration curve.

The LOD values were evaluated on the basis of matrix-matched calibration curves analyzed in triplicates, using the LOD = $(3.3S_b)/a$ equation, where: S_b – standard deviation of intercept of the calibration curve, a – slope of the calibration curve (ICH Harmonized Guidelines, 2005).

In further calculations of LOQ values it was assumed that LOQ = 3 × LOD. LOD values were in the range from 0.0079 ng/mL for BPG to 0.039 ng/mL for BPBP, which indicate that proposed analytical method is highly sensitive towards BPA analogues. More detailed information on validation parameters are given in Suppl. Table 2 of Supplementary material.

To evaluate the recoveries, spiked samples were prepared according to the described procedure using normal human blood serum. Six independent chromatographic runs were carried out for each of three concentration levels. Obtained recoveries varied from 88% for BPC up to 138% for BPZ. All relative standard deviations were below 10% and are within the range of 1.2% to 7.8%. Recovery discrepancies observed for some bisphenol analogues confirm, that preparing matrix-matched calibration curves during analysis of real samples is highly justified in order to minimize its impact on the end results.

Total matrix effects (i.e. comprising of recovery and ion suppression/enhancement) for each analyte were also evaluated and are given in Suppl. Table 2 (Supplementary material). In case of some compounds noticeable enhancement of the signal was observed (27% for BPC and 29% for BPP). The reason for this effect could be a coelution with some unknown compound enhancing ionisation efficiency for these analytes.

Despite the fact that recoveries and magnitude of matrix effects differ among analytes the use of matrix-matched calibration curves allows us to compensate for these phenomena and obtain reliable results.

4. Analysis of real world samples

Proposed methods were successfully used to analyse 245 real human blood serum samples in order to determine the analytes' content and to assess the human exposure to 11 BPA analogues for the first time. The results are summarized in Table 3 and examples of real world sample chromatograms are given in the Fig. 2. Analytes were found in over 50% of serum samples except for BPC, BPZ, BPFL and BPBP. BPA, BPG and BPS were the most often occurring analogues. Beyond the problem of constant human exposure, the presence of bisphenols in blood is important in the terms of possible adverse health issues (Konieczna et al., 2015; Rachoń, 2015). There are scientific proofs that these compounds have the ability to induce eryptosis (suicidal death of erythrocytes) (Maćczak et al., 2016) or biochemical and morphological alternations in mononuclear cells of peripheral blood (Michałowicz et al., 2015).

5. Conclusions

Presence of BPA and its analogues in different environmental matrices has been well studied by now, but there is still an insufficient scientific data on the occurrence of bisphenol analogues in the human and animal tissues/fluids. Bisphenols other than BPA doesn't seem to be its safer alternatives, therefore the interest in this research area has increased in the last decade. In the field of biomonitoring of BPs in human fluids and tissues the scientific data is still scarce although already noticeable. In 2016 Asimakopoulos et al. determined 8 bisphenols in human urine samples along with 49 other xenobiotics. Mean analytes concentrations varied from 0.05 ng/mL (for BPB and BPAF) up to 13.3 ng/mL (for BPS) (Asimakopoulos et al., 2016). Also in 2016 BPA and six other analogues (BPF, BPS, BPAP, BPAF, BPP, BPZ) were determined in human urine by applying novel DLLME technique coupled to LC-MS/MS. Mean BPA concentration was 2.8 ng/mL, while other analogues were found at much lower rates (2–10% of samples) (Rocha et al., 2016). In case of serum, plasma and blood, scientific data is even more limited. In 2009 BPA and BPB were determined in human serum at 0.79–7.12 ng/mL (BPA) and 0.88–11.94 ng/mL (BPB) concentration ranges (Cobellis et al., 2009). A broader range of analogues (BPA, BPB, BPC, BPE, BPF, BPPH, BPS, BPFL, BPP, BPM and BPZ) was determined in human breast milk in 2015. However, only BPA and BPS were found in concentrations 0.002–1.16 ng/g and 0.23 ng/g respectively (Deceuninck et al., 2015). More information on the comparison of the results obtained in this study to other studies are given in Supplementary Table 3. Because of very limited data on analogues selected for this research, this comparison has been extended to bisphenol derivatives and other biological matrices. Most of the analytes have been found in concentrations similar to the results presented.

In this paper the development of a novel analytical LC-MS/MS method for determination of a broad spectrum of BPA analogues was

Table 2
Recovery values obtained for three independent concentrations of spiked quality control (QC) samples.

Analyte	Recovery (RSD) [%] of analyte (n = 6)			Detection and quantitation limits (n = 3)	
	0.05 ng/mL	0.5 ng/mL	1 ng/mL	LOD [ng/mL]	LOQ [ng/mL]
BPC	89.8 (3.4)	87.6 (1.9)	88.7 (2.3)	0.021	0.061
BPE	96.0 (3.7)	95.5 (3.0)	106.1 (2.9)	0.011	0.032
BPF	123.4 (4.2)	120.7 (3.6)	118.7 (2.8)	0.012	0.037
BPG	103.7 (1.4)	104.4 (1.7)	103.9 (1.2)	0.0080	0.024
BPM	90.3 (2.3)	90.7 (3.2)	93.4 (4.8)	0.018	0.054
BPP	105.1 (6.5)	103.5 (7.8)	105.8 (2.6)	0.019	0.056
BPZ	132.4 (1.5)	138.2 (1.9)	134.6 (1.9)	0.017	0.051
BPFL	98.3 (3.3)	99.1 (2.7)	99.6 (2.8)	0.014	0.041
BPBP	99.2 (2.7)	98.9 (1.9)	99.0 (1.7)	0.039	0.12
BPA	103 (12)	106.0 (2.9)	101.0 (2.6)	0.0090	0.028
BPS	96 (15)	96.5 (3.7)	101.6 (3.0)	0.022	0.067

Table 3
Information on the results obtained for the real world samples analysis.

Analyte	Quantification rate [% of all samples]	Detection rate > LOD [% of all samples]	Median [ng/mL]	Concentration range [ng/mL]
BPC	27.2	40.7	0.177	0.071 ÷ 3.800
BPE	55.1	59.7	0.154	0.053 ÷ 0.828
BPF	49.8	65.0	0.115	0.052 ÷ 0.845
BPG	60.5	70.4	0.188	0.050 ÷ 1.190
BPM	58.8	65.8	0.212	0.057 ÷ 1.104
BPP	52.7	66.3	0.142	0.057 ÷ 0.917
BPZ	37.5	45.3	0.235	0.053 ÷ 1.415
BPFL	7.8	23.5	0.070	0.050 ÷ 1.597
BPBP	47.8	53.9	0.37	0.12 ÷ 2.98
BPA	86.4	91.4	0.124	0.050 ÷ 4.017
BPS	68.7	72.0	1.135	0.073 ÷ 4.844

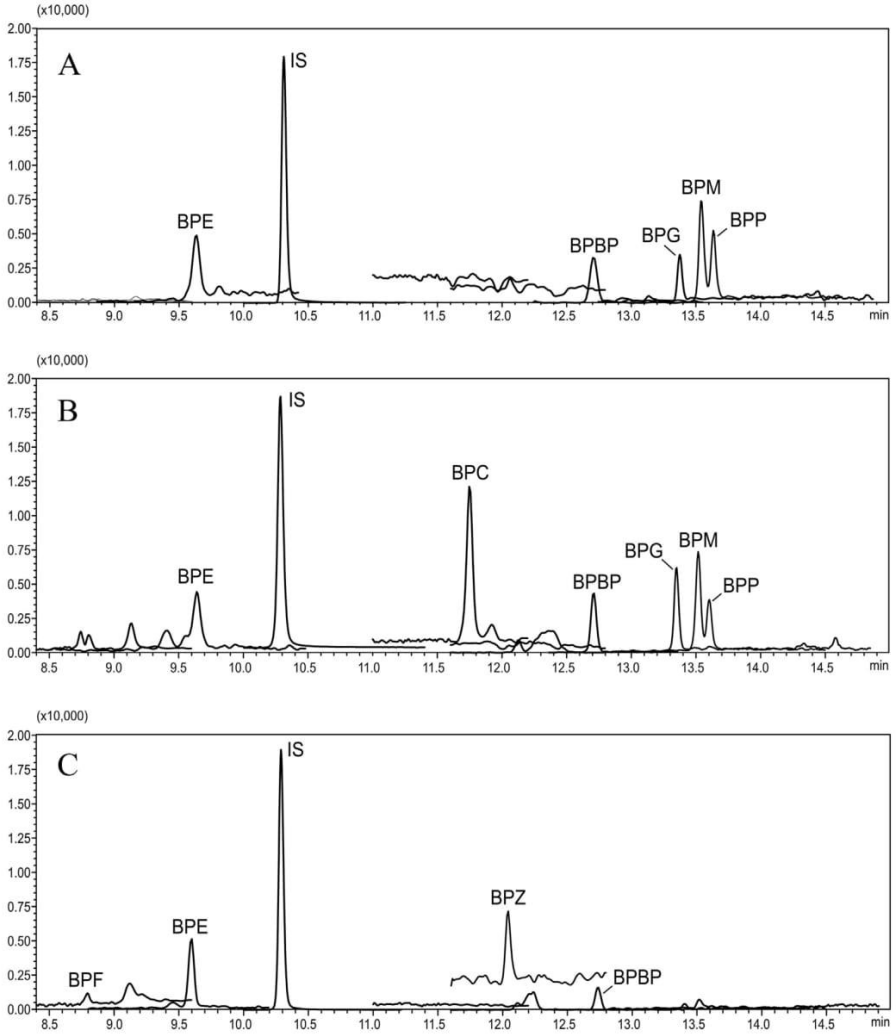


Fig. 2. Examples of chromatograms obtained after the analysis of a real world blood serum samples. For clarity IS trace has been scaled down by the factor of 0.05.

described. Sample preparation procedure consisting of liquid-liquid extraction is a routine and inexpensive approach that consumes low volumes of the sample (500 μL) and relatively low volumes of organic solvent. An addition of anhydrous $MgSO_4$ provided better peak shapes and response, due to removing water soluble interfering matrix compounds. The method is suitable to identify and effectively separate compounds of interest, even constitutional isomers (BPM and BPP), as well as to obtain very low detection and quantification limits. The developed method was successfully applied for the analysis of real human blood serum samples. To the best of authors' knowledge, this study is the first attempt to determine selected 11 bisphenol A analogues in human derived serum samples. Results indicate that the problem of the bisphenols occurrence in body fluids is still underestimated, and may lead to some adverse health issues. For this reason, the development and application of novel analytical procedures focused on bisphenols' human biomonitoring are of high scientific importance.

Acknowledgements

This study was financed from the funds of the Polish National Centre for Science (grant number 2014/15/B/NZ7/00999).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.02.148.

References

Ahmed, R., 2016. Maternal bisphenol A alters fetal endocrine system: thyroid adipokine dysfunction. Food Chem. Toxicol. 95, 168–174.

Arbuckle, T.E., Davis, K., Boylan, K., Fisher, M., Fu, J., 2016. Bisphenol A, phthalates and lead and learning and behavioral problems in Canadian children 6–11 years of age: CHMS 2007–2009. Neurotoxicology 54, 89–98.

Asimakopoulos, A.G., Xue, J., De Carvalho, B.P., Iyer, A., Abualnaja, K.O., Yaghmoor, S.S., Kumosani, T.A., Kannan, K., 2016. Urinary biomarkers of exposure to 57 xenobiotics and its association with oxidative stress in a population in Jeddah, Saudi Arabia. Environ. Res. 150, 573–581.

Bhandari, R.K., Deem, S.L., Holliday, D.K., Jandegian, C.M., Kassotis, C.D., Nagel, S.C., Tillitt, D.E., vom Saal, F.S., Rosenfeld, C.S., 2015. Effects of the environmental estrogenic contaminants bisphenol A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. Gen. Comp. Endocrinol. 214, 195–219.

Chen, M.-Y., Ike, M., Fujita, M., 2002. Acute toxicity, mutagenicity, and estrogenicity of bisphenol-a and other bisphenols. Environ. Toxicol. 17, 80–86.

Chen, D., Kannan, K., Tan, H., Zheng, Z., Feng, Y.-L., Wu, Y., Widelka, M., 2016. Bisphenol analogues other than BPA: environmental occurrence, human exposure, and toxicity: a review. Environ. Sci. Technol. 50, 5438–5453.

Cobellis, L., Colacurci, N., Trabucco, E., Carpentiero, C., Grumetto, L., 2009. Measurement of bisphenol A and bisphenol B levels in human blood sera from healthy and endometriotic women. Biomed. Chromatogr. 23, 1186–1190.
Deceuninck, Y., Bichon, E., Marchand, P., Boquien, C.-Y., Legrand, A., Boscher, C., Antignac, J.P., Le Bizec, B., 2015. Determination of bisphenol A and related substitutes/analogues in human breast milk using gas chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 407, 2485–2497.
Gioumouxouzis, C.I., Kouskoura, M.G., Markopoulou, C.K., 2015. Negative electrospray ionization mode in mass spectrometry: a new perspective via modeling. J. Chromatogr. B 998, 97–105.
Grob, K., Gürtler, R., Husøy, T., Mennes, W., Milana, M.R., Penninks, A., Roland, F., Silano, V., Smith, A., Poças, M. de F.T., et al., 2015. Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: executive summary. EFSA J. 13, 3978–4599.
Hada, T., Shimizu, M., Aoki, Y., Endo, N., Ohhashi, H., Ishiguro, T., 2013. Heat-sensitive Recording Material and Method for Producing Same (Google Patents).
Hsieh, B.-Y., Hsu, J.-P., 2015. Photosensitive Resin Composition, Color Filter and Method for Manufacturing the Same, and Liquid Crystal Display Apparatus (Google Patents).
Huang, B., Jiang, C., Luo, J., Cui, Y., Qin, L., Liu, J., 2014. Maternal exposure to bisphenol A may increase the risks of Parkinson's disease through down-regulation of fetal IGF-1 expression. Med. Hypotheses 82, 245–249.
Hunt, P.A., Sathyanarayana, S., Fowler, P.A., Trasande, L., 2016. Female reproductive disorders, diseases, and costs of exposure to endocrine disrupting chemicals in the European Union. J. Clin. Endocrinol. Metab. 101, 1562–1570.
ICH Harmonized Guidelines, 2005. Validation of Analytical Procedures: Text and Methodology. Q2 R1 p. 1.
Industry Experts, 2016. Bisphenol-A — A Global Market Overview.
Konieczna, A., Rutkowska, A., Rachon, D., 2015. Health risk of exposure to bisphenol A (BPA). Rocz. Państw. Zakl. Hig. 66.
Kundakovic, M., Gudsnuk, K., Franks, B., Madrid, J., Miller, R.L., Perera, F.P., Champagne, F.A., 2013. Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Proc. Natl. Acad. Sci. 110, 9956–9961.
Lee, S., Liao, C., Song, G.-J., Ra, K., Kannan, K., Moon, H.-B., 2015. Emission of bisphenol analogues including bisphenol A and bisphenol F from wastewater treatment plants in Korea. Chemosphere 119, 1000–1006.
Liao, C., Kannan, K., 2012. Determination of free and conjugated forms of bisphenol A in human urine and serum by liquid chromatography–tandem mass spectrometry. Environ. Sci. Technol. 46, 5003–5009.
Maćczak, A., Cyrkler, M., Bukowska, B., Michalowicz, J., 2016. Eryptosis-inducing activity of bisphenol A and its analogs in human red blood cells (in vitro study). J. Hazard. Mater. 307, 328–335.
Michałowicz, J., Mokra, K., Bąk, A., 2015. Bisphenol A and its analogs induce morphological and biochemical alterations in human peripheral blood mononuclear cells (in vitro study). Toxicol. in Vitro 29, 1464–1472.
Ochiai, T., Masuda, T., 2013. Thermal Recording Material and Method for Producing the Same (Google Patents).
Rachoń, D., 2015. Endocrine disrupting chemicals (EDCs) and female cancer: informing the patients. Rev. Endocr. Metab. Disord. 16, 359–364.
Rissman, E.F., Adli, M., 2014. Minireview: transgenerational epigenetic inheritance: focus on endocrine disrupting compounds. Endocrinology 155, 2770–2780.
Rivas, A., Lacroix, M., Olea-Serrano, F., Laios, I., Leclercq, G., Olea, N., 2002. Estrogenic effect of a series of bisphenol analogues on gene and protein expression in MCF-7 breast cancer cells. J. Steroid Biochem. Mol. Biol. 82, 45–53.
Rocha, B.A., da Costa, B.R.B., de Albuquerque, N.C.P., de Oliveira, A.R.M., Souza, J.M.O., Al-Tameemi, M., Campiglia, A.D., Barbosa Jr, F., 2016. A fast method for bisphenol A and six analogues (S, F, Z, P, AF, AP) determination in urine samples based on dispersive liquid-liquid microextraction and liquid chromatography-tandem mass spectrometry. Talanta 154, 511–519.
Rosenmai, A.K., Dybdahl, M., Pedersen, M., Alice van Vugt-Lussenburg, B.M., Wedebye, E.B., Taxvig, C., Vinggaard, A.M., 2014. Are structural analogues to bisphenol A safe alternatives? Toxicol. Sci. 139, 35–47.
Rutkowska, A., Rachoń, D., 2014. Bisphenol A (BPA) and its potential role in the pathogenesis of the polycystic ovary syndrome (PCOS). Gynecol. Endocrinol. 30, 260–265.
Rutkowska, A., Rachoń, D., Milewicz, A., Ruchała, M., Bolanowski, M., Jędrzejuk, D., Bednarczuk, T., Górska, M., Hubalewska-Dydejczyk, A., Kos-Kudła, B., et al., 2015. Polish Society of Endocrinology Position statement on endocrine disrupting chemicals (EDCs). Endokrynol. Pol. 66, 276–285.
Song, S., Song, M., Zeng, L., Wang, T., Liu, R., Ruan, T., Jiang, G., 2014. Occurrence and profiles of bisphenol analogues in municipal sewage sludge in China. Environ. Pollut. 186, 14–19.
Stojanoska, M.M., Milosevic, N., Milic, N., Abenavoli, L., 2017. The influence of phthalates and bisphenol A on the obesity development and glucose metabolism disorders. Endocrine 1–16.
Sui, Y., Ai, N., Park, S.-H., Rios-Pilier, J., Perkins, J.T., Welsh, W.J., Zhou, C., 2012. Bisphenol A and its analogues activate human pregnane X receptor. Environ. Health Perspect. 120, 399.
Teichert, D., Conrad, L., Grötzinger, J., 2014. Epoxy Resin-Upgraded Cement-Bound Composition as Coating or Seal (Google Patents).
Vogel, S.A., 2009. The politics of plastics: the making and unmaking of bisphenol A "safety". Am. J. Public Health 99, S559–S566.
Wagner, S., Kramer, R.H., Konig, A., Roth, M., 2015. Flame-retardant Polyesters (Google Patents).
Wilczewska, K., Namieśnik, J., Wasik, A., 2016. Troubleshooting of the determination of bisphenol A at ultra-trace levels by liquid chromatography and tandem mass spectrometry. Anal. Bioanal. Chem. 408, 1009–1013.
Yamazaki, E., Yamashita, N., Taniyasu, S., Lam, J., Lam, P.K., Moon, H.-B., Jeong, Y., Kannan, P., Achyuthan, H., Munuswamy, N., et al., 2015. Bisphenol A and other bisphenol analogues including BPS and BPF in surface water samples from Japan, China, Korea and India. Ecotoxicol. Environ. Saf. 122, 565–572.
Yang, Y., Guan, J., Yin, J., Shao, B., Li, H., 2014. Urinary levels of bisphenol analogues in residents living near a manufacturing plant in south China. Chemosphere 112, 481–486.
Zouta, K., Nakao, K., Takagi, Y., Ishii, H., 2014. Flame-Retardant Sheet or Film, Products Comprising the Same and Process for Production Thereof (Google Patents).

ELECTRONIC SUPPLEMENTARY MATERIALS

Determination of trace levels of eleven bisphenol A analogues in human blood serum by high performance liquid chromatography – tandem mass spectrometry.

Katarzyna Owczarek[a], *Paweł Kubica*[a], *Błażej Kudłak*[a], *Aleksandra Rutkowska*[b], *Aleksandra Konieczna*[b], *Dominik Rachoń*[b], *Jacek Namieśnik*[a], **Andrzej Wasik*[a]

[a] Department of Analytical Chemistry, Chemical Faculty, Gdańsk University of Technology, Narutowicza 11/12 str., 80-223, Gdańsk, Poland

[b] Department of Clinical and Experimental Endocrinology, Medical University of Gdańsk, Dębinki 7, 80-210 Gdańsk, Poland

* - corresponding Author (wasia@pg.edu.pl)

Suppl. Tab. 1 Optimized MS/MS conditions for negative mode MRM analysis for target analytes

Compound	Precursor ion M-H [m/z]	Quantitation ion [m/z]	Confirmation [m/z]	Q1 PreRod bias* [V]	Collision energy [V]*	Q3 PreRod bias* [V]
BPC	255.3	240.15	147.1	13	20	10
BPE	213.0	198.15	119.1	10	18	18
BPF	199.3	93.0	105.1	13	23	13
BPG	311.1	295.3	175.2	22	35	29
BPM	345.0	330.2	133.1	17	29	14
BPP	345.0	330.2	133.1	17	29	14
BPZ	267.1	173.1	145.1	12	27	10
BPFL	349.1	256.1	215.1	12	27	11
BPBP	351.1	274.2	258.2	12	25	25
BPA	227.3	212.1	133.1	11	20	13
BPS	249.0	108.1	92.0	12	27	10
13C-BPA	239.1	224.0	138.0	11	20	13

Nebulizing gas flow [L/min]	Heating gas flow [L/min]	Interface temperature [ºC]	DL temperature [ºC]	Heat block temperature [ºC]	Drying gas flow [L/min]
3	10	300	250	400	10

* – presented values refer to quantitation ion only

Suppl. Tab. 2 Regression equations, LOD and LOQ for each analyte

Analyte	Calibration curve equation (7 points, n=3)	R^2	LOD [ng/mL]	LOQ [ng/mL]	Matrix effect [%]
BPC	y=0.0052x+0.00035	0.9975	0.021	0.061	27
BPE	y= 0.015x+0.00077	0.9992	0.011	0.032	7
BPF	y=0.0035x+0.00051	0.9996	0.012	0.037	17
BPG	y= 0.0046x+0.00034	0.9991	0.0080	0.024	15
BPM	y=0.010x+0.00023	0.9986	0.018	0.054	15
BPP	y=0.0088x+0.000070	0.9997	0.019	0.056	29
BPZ	y=0.0082x+0.00097	0.9993	0.017	0.051	6
BPFL	y=0.024x+0.00079	0.9988	0.014	0.041	13
BPBP	y=0.0068x+0.0035	0.9968	0.039	0.12	19
BPA	y=0.092x+0.0015	0.9997	0.0093	0.028	6
BPS	y=0.065x+0.017	0.9999	0.022	0.067	12

For a given analyte total matrix effects were calculated according to the following equation:

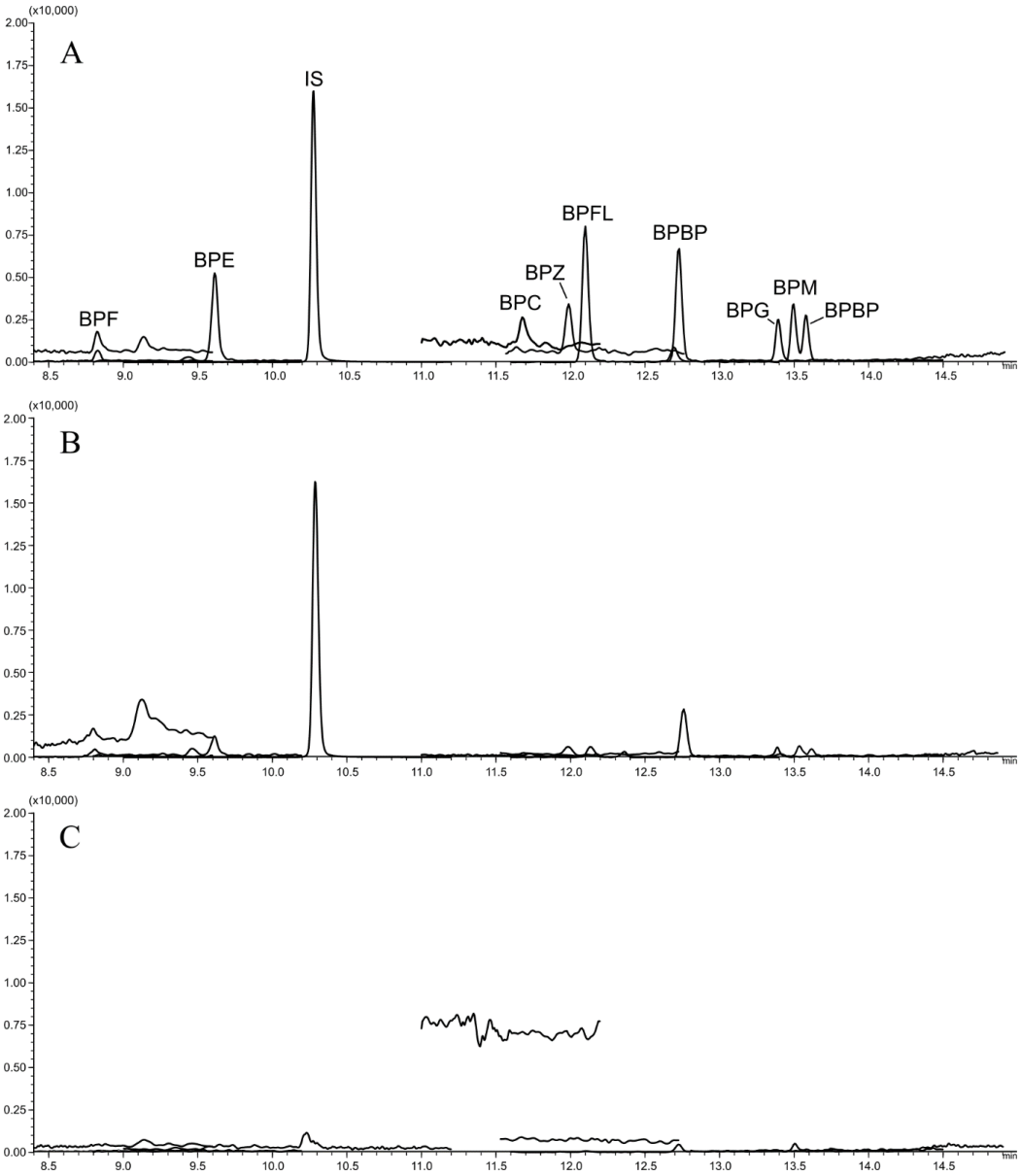
$$M_e = \left(\frac{a_m}{a_s} - 1 \right) \times 100\%$$

where:
a_m – slope of the matrix matched calibration curve prepared using blood serum,
a_s – slope of the calibration curve, obtained after injecting pure standard solutions.

Suppl. Tab. 3 Comparison of LOD and LOQ values for different studies concerning determination of bisphenol analogues and derivatives in biological samples of human origin.

Analytes	Matrix	Sample volume [µL]	Determination method	LOD and LOQ [ng/mL]	Measured concentrations [ng/mL]	Reference
BPA, BPC, BPE, BPF, BPG, BPM, BPP, BPS, BPZ, BPFL, BPBP	Serum	500	HPLC-ESI-MS/MS	LOD = 0.0079 - 0.039 LOQ = 0.024 - 0.12	0.05- 4.8	**This study**
BPA, BPADS, BPAG, BPAMC, DCBPA, TCBPA,	Serum	500	LC-ESI-MS/MS	LOD = 0.003 – 0.02 LOQ = 0.01 – 0.05	BPA <LOQ- 0.588 BPAG<LOQ – 11.9 BPADS <LOQ- 1.77	(Liao and Kannan, 2012)
BPA, BPB	Serum	300	LC-FD	LOD = 0.15 – 0.18 LOQ = 0.5 – 0.6	BPA 0.79-7.12 BPB 0.88-11.94	(Cobellis et al., 2009)
BPA, BPB, BPAP, BPAF, BPBP, BPC, BPE, BPPH, BPS, BPF, BPFL, BPZ, BPM, BPP	Breast milk	3 g	GC-ESI-MS	MDL = 0.001 – 0.03 MQL = 0.003 – 0.1	BPA 0.02-1.16 BPS 0.23	(Deceuninck et al., 2015)
BPA	Breast milk	1000	LC-MS/MS	LOD = 0.22	0.22-10.8	(Zimmers et al., 2014)
BPA, BPB, BPF, BPS, BPAF,TCBPA	Urine	-	LC-MS/MS	LOQ = 0.024-0.310	BPA <LOQ – 8.07 BPAF<LOQ – 0.217	(Yang et al., 2014)
BPA, BPF, BPP, BPS, BPZ, BPAF, BPAP	Urine	5000	LC-MS/MS	LOD = 0.005 – 0.2 LOQ = 0.02 – 0.5	BPA (mean) 2.8 Other analytes were found in very limited range of samples	(Rocha et al., 2016)
BPA	Water, Mouse serum	100	ELISA	LOD = 0,1 (water) LOD = 2 (serum)		(Zhao et al., 2002)

BPAMC – BPA monochloride, BPADC – BPA dichloride, BPATC – BPA trichloride, BPADG – BPA glucuronide, BPADS – BPA disulfate, TCBPA – tetrachloro-BPA



Supplementary Figure 1. Example chromatograms for; blank human serum spiked with a mixture of analytes at 0.5 ng/mL level (**A**), procedural blank i.e. blank human serum extract (**B**), system blank (**C**).
For clarity **IS** trace has been scaled down by the factor of 0.05.

References

Yang, Y., Lu, L., Zhang, J., Yang, Y., Wu, Y., Shao, B., 2014. Simultaneous determination of seven bisphenols in environmental water and solid samples by liquid chromatography–electrospray tandem mass spectrometry. J. Chromatogr. A, seria 1328, 26–34.

Zhao, M.-P., Li, Y.-Z., Guo, Z.-Q., Zhang, X.-X., Chang, W.-B., 2002. A new competitive enzyme-linked immunosorbent assay (ELISA) for determination of estrogenic bisphenols, Talanta 57, 1205–1210.

Zimmers, S.M., Browne, E.P., O'Keefe, P.W., Anderton, D.L., Kramer, L., Reckhow, D.A., Arcaro, K.F., 2014. Determination of free bisphenol A (BPA) concentrations in breast milk of US women using a sensitive LC/MS/MS method. Chemosphere 104, 237–243.

OŚWIADCZENIE WSPÓŁAUTORÓW PRACY ZESPOŁOWEJ WSKAZUJĄCE, CO STANOWI ICH WŁASNY WKŁAD

K. Owczarek, P. Kubica, B. Kudłak, A. Rutkowska, A. Konieczna, D. Rachoń, J. Namieśnik, A. Wasik, *Determination of trace levels of eleven bisphenol A analogues in human blood serum by high performance liquid chromatography – tandem mass spectrometry*, Sci. Total Environ. 628, 1362, 2018

Nazwisko współautora	Wkład	Podpis
K. Owczarek	Opracowanie metody analitycznej, opracowanie etapu przygotowania próbek, analiza uzyskanych wyników, przygotowanie tekstu manuskryptu	
P. Kubica	Opracowanie metody chromatograficznej, wybór warunków pracy tandemowego spektrometru mas, analiza próbek rzeczywistych, obróbka danych, współudział w tworzeniu tekstu manuskryptu	
B. Kudłak	Współudział w pozyskaniu środków finansowych w postaci grantu na prowadzenie badań, współudział w wykonaniu części badań, współudział w opracowaniu odpowiedzi na uwagi recenzentów	
A. Rutkowska	Współudział w prowadzeniu badań, współudział w przygotowaniu odpowiedzi na uwagi recenzentów	
A. Olsson (poprzednio Konieczna)	Zabezpieczenie i przygotowanie materiału badawczego	
D. Rachoń	Zrekrutowanie pacjentek od których pochodziły próbki surowicy, korekta ostatecznej wersji manuskryptu	
J. Namieśnik	Nadzór nad zapewnieniem jakości, sprawdzenie manuskryptu pod względem merytorycznym, nadzór nad projektem;	* Profesor Jacek Namieśnik zmarł 14 kwietnia 2019 r.
A. Wasik	Współudział w opracowaniu metodologii, udział w tworzeniu tekstu manuskryptu oraz przygotowaniu odpowiedzi dla recenzentów	

4.3. Określenie korelacji pomiędzy stężeniami BPA a poziomem testosteronu w surowicy krwi kobiet cierpiących na PCOS

Schorzenie znane pod nazwą zespołu policystycznych jajników (PCOS) jest dolegliwością o podłożu hormonalnym, dotykającym kobiet w wieku rozrodczym. Charakteryzuje się ono wzmożonym wydzielaniem i aktywnością androgenów, zahamowaniem owulacji oraz insulinoopornością [148,149]. PCOS ma negatywny wpływ na wiele parametrów zdrowotnych i jest powiązany z występowaniem hirsutyzmu, trądziku, niepłodności czy otyłości, może też prowadzić do rozwinięcia się cukrzycy typu II i wzrostu związanego z tym ryzyka pojawienia się chorób układu sercowo-naczyniowego [149]. Złożona natura tego schorzenia sprawia, że jego zdiagnozowanie jest najczęściej trudne, a ponadto środowisko medyczne nie jest jednomyślne jeżeli chodzi o jego patogenezę. Podejrzewa się, że dużą rolę odgrywają tu przede wszystkim czynniki genetyczne i metaboliczne, ale także środowiskowe, takie jak obecność związków endokrynnie czynnych, do których zalicza się między innymi BPA i jego analogi [150, 151]. Z tego względu elementem celu pracy było oznaczenie zawartości BPA w próbkach surowicy pacjentek u których zdiagnozowano PCOS oraz próba określenia jego potencjalnego wpływu na to zaburzenie.

Próbki krwi pobrane od kobiet cierpiących na PCOS oraz od kobiet zakwalifikowanych do grupy kontrolnej (zdrowych w aspekcie występowania PCOS) zostały przeanalizowane pod kątem oznaczenia szeregu parametrów biochemicznych, takich jak między innymi poziomy: triglicerydów (TG), cholesterolu o wysokiej gęstości i całkowtego (HDL i TCh), folitropiny (FSH), testosteronu całkowitego (TST) czy 17β-estradiolu (E2). Zawartość BPA została zbadana z zastosowaniem metody LC-MS/MS, której opracowanie opisano w przytoczonej w punkcie 4.2. pracy [147]. Wykazano jej bardzo wysoką czułość i precyzję. Analizie poddano w sumie 186 próbek rzeczywistych, z czego 106 pobranych zostało od kobiet z zespołem policystycznych jajników, a 80 od kobiet zdrowych. Wszystkie wyniki poddane zostały analizie statystycznej z użyciem takich narzędzi jak test D'Agostino-Pearson'a (badanie dystrybucji uzyskanych wyników) czy analiza wariancji ANOVA (porównanie stężenia BPA w obrębie trzech różnych fenotypów PCOS). Do analizy korelacji pomiędzy stężeniem BPA a wynikami otrzymanymi dla innych zmiennych zastosowano obliczony współczynnik korelacji Pearsona.

Otrzymane wyniki pozwoliły na wysnucie wniosków, iż w przypadku kobiet u których stwierdzono występowanie PCOS zawartość BPA w ich surowicy była znacząco wyższa niż w przypadku kobiet z grupy kontrolnej, co znajduje potwierdzenie w innych doniesieniach naukowych, w których również skupiono się na tematyce wpływu BPA na PCOS. [152]. Co więcej, stężenie BPA było również pozytywnie skorelowane z podwyższoną zawartością testosteronu oraz wskaźnikiem wolnych androgenów (*Free Androgen Index*, FAI). Biorąc pod uwagę powyższe fakty, BPA może być jednym z ważniejszych czynników w procesie rozwoju PCOS.

Praca ta została opublikowana w czasopiśmie *Reproductive Toxicology* [149]. W chwili publikacji, zgodnie z najlepszą wiedzą autorów, była to jedna z pierwszych prac, gdzie do oznaczenia stężenia BPA w surowicy pacjentek z PCOS zastosowano metodę analityczną opartą o technikę chromatografii cieczowej sprzężonej z tandemową spektrometrią mas. Metoda ta może z powodzeniem zostać wdrożona do laboratoriów analitycznych w celu prowadzenia rutynowych analiz zawartości bisfenolu A lub – jeśli zajdzie taka potrzeba - jego analogów.

Reproductive Toxicology 82 (2018) 32–37

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome

Aleksandra Konieczna[a], Dominik Rachoń[a,*], Katarzyna Owczarek[b], Paweł Kubica[b], Agnieszka Kowalewska[c], Błażej Kudłak[b], Andrzej Wasik[b], Jacek Namieśnik[b]

[a] Department of Clinical and Experimental Endocrinology Medical University of Gdańsk, Dębinki 7, 80-210 Gdańsk, Poland
[b] Department of Analytical Chemistry, Chemical Faculty, Gdańsk University of Technology, Narutowicza 11/12, 80-223 Gdańsk, Poland
[c] Outpatient Gynecological Practice, Parkowa 6, 81-549 Gdynia, Poland

ARTICLE INFO

Keywords:
Bisphenol A
Polycystic ovary syndrome (PCOS)
Endocrine disrupting chemicals (EDC)
Reproductive endocrinology
Environmental exposure
Toxicology

ABSTRACT

The aim of this study was to determine serum bisphenol A (BPA) concentrations using high performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) in women with polycystic ovary syndrome (PCOS) (n = 106, age range 18–40 yrs) and to evaluate its potential impact on their hormonal and metabolic profile. The control group consisted of age- and BMI-matched 80 eumenorrheic women with no clinical or biochemical hyperandrogenism. Our results showed that women with PCOS had significantly higher serum BPA concentrations than healthy controls (geometric mean and [95% CI]: 0.202 ng/mL [0.150; 0.255] vs. 0.154 ng/mL [0.106; 0.201], $P = 0.035$), which correlated positively with serum total testosterone (TST) ($R = 0.285$, $P = 0.004$) and the free androgen index (FAI) ($R = 0.196$, $P = 0.049$). There were no significant correlations between serum BPA and BMI, waist circumference, serum glucose, insulin and lipids. These results point to the potential role of BPA in the pathogenesis of the ovarian hyperandrogenism in women with PCOS.

1. Introduction

Polycystic ovary syndrome (PCOS) in recent years has been attracting enormous attention, not only among reproductive medicine specialists, but also endocrinologists, diabetologists as well as dieticians and mental health providers (psychological therapists), which point to the complex nature of this syndrome. Although its prevalence is estimated to be up to 16% among women before menopause [1,2], the exact incidence is difficult to estimate due to its heterogeneity and lack of consensus on its diagnostic criteria [3,4]. PCOS is characterized by anovulation, androgen excess and insulin resistance [5], which lead to the development of obesity, hypertension, and type 2 diabetes mellitus (T2DM) [6]; therefore, increasing the risk of cardiovascular disease [7–9]. Due to ovulatory dysfunction, PCOS is also the major cause of fertility problems [10,11]. Not only is the diagnosis of PCOS still debatable, but also its pathogenesis; hence, together with the genetic and metabolic predispositions [12,13], environmental factors, such as endocrine disrupting chemicals (EDCs), may contribute to its development [14,15]. EDCs have recently gained a lot of attention and have been pointed out as the potential cause of the ovarian dysfunction [16,17] observed in women with PCOS [18,19]. Bisphenol A (BPA) is one of the most common industrial compound with endocrine disrupting potential, which is used to bind or harden plastic [20] in packaging, food cans, bottles, plastic water pipes, thermal paper (receipts), cosmetics and healthcare equipment, as well as toys and articles for children [21]. Human exposure to BPA includes various routes such as oral – mainly by consumption of food products, inhalation of the dust, and transdermal contact e.g. with plastic products or thermal paper [19]. Due to its phenolic structure, BPA has been shown to interact with estrogenic receptors. Therefore, exposure to this EDC may impact fertility [22], lead to the development of estrogen dependent neoplasms (i.e. breast and uterine cancer) [23,24] and also several metabolic disorders such as insulin resistance, obesity, and T2DM [22,25]. Therefore, the aim of our study was to determine serum concentrations of BPA using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) in women with PCOS and to study its potential impact on their hormonal and metabolic profile.

2. Subjects and methods

2.1. Subjects

This cross-sectional study was performed between [the] 16th of January 2016 the and 21st of December 2017 and was approved by the

* Corresponding author.
E-mail address: dominik.rachon@gumed.edu.pl (D. Rachoń).

https://doi.org/10.1016/j.reprotox.2018.09.006
Received 5 July 2018; Received in revised form 7 September 2018; Accepted 19 September 2018
Available online 25 September 2018

Ethics Committee of the Medical University of Gdańsk (permission number NKBBN/198/2012). In total, 304 female participants (age range: 18–40 yrs) were enrolled into the study. Women were recruited at the endocrinology outpatient practice by a specialist in internal medicine and endocrinology (D.R.), where they were referred by other clinical practitioners mostly due to clinical signs of hyperandrogenism (hirsutism, acne, androgenic alopecia) and/or menstrual disturbances and/or fertility problems. They were all informed about the purpose of the study and signed a written consent to participate. Women, who were taking oral contraceptives, anti-androgens (e.g. spironolactone) or glucocorticosteroids, were excluded from the study. The control group consisted of healthy women with regular menses and no biochemical or clinical signs of hyperandrogenism, which were referred to the same practice in order to exclude some of the common endocrine disorders (e.g. hypothyroidism, hyperprolactinemia).

2.2. Gynecological examination and ultrasonography

All the participants were also examined by a specialist in gynecology and endocrinology (A.K.). Gynecological examination, together with a transvaginal or transabdominal ultrasonography, was performed using the Accuvix V20 Ultrasound Machine (Medison, Seoul, South Korea), 4–9 MHz and 4–8 MHz, respectively. The ovarian volume was calculated and the total number of antral follicles was counted. The presence of an ovulatory follicle (> 10 mm) was also noted.

2.3. Study protocol

An interview-based medical form was used to acquire the information regarding gynecological and obstetric history (*menarche*, menstrual regularity, time of last period, number of pregnancies and oral contraceptive use). Then a physical examination was performed where the height, body weight, waist circumference and blood pressure were measured. Also the presence of hirsutism, acne or androgenic alopecia was recorded.

2.4. Sample collection and preparation

Venous blood was obtained during the follicular phase (days 6–10) of a spontaneous or a progestin induced menstrual cycle (oral dydrogesterone: 10 mg twice daily for 10 days). All the hormonal and biochemical analyses were performed at the clinical diagnostic laboratory (ALAB-Bruss Laboratories, Gdynia, Poland), which possesses current ISO certificates and has an accreditation in the field of medical laboratory diagnostics. The blood was collected into three polypropylene tubes: one on the clot, one containing sodium citrate for the analysis of glucose concentrations and another containing EDTA for the evaluation of total blood count. In order to analyze serum BPA, blood was additionally collected into a glass tube with no additives and was centrifuged within 30–90 min at the speed of 2500 rpm for 15 min. After the centrifugation, serum samples for the BPA analyses were collected using a disposable transfer (Pasteur) pipette made of BPA-free low-density polyethylene (LDPE) with precautions intended to minimize the risk of sample contamination with BPA and were then stored at −70 °C for further analysis.

2.5. Biochemical and hormonal analyses

Total blood count was evaluated automatically using XE-2100 D analyzer (Sysmex, Kobe, Japan). Serum C-reactive protein (CRP), glucose, triglycerides (TG), HDL-Cholesterol (HDL-C) and total cholesterol (TCh), follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyrotropin (TSH), prolactin (PRL), 17β-estradiol (E2), total testosterone (TST), dehydroepiandrosterone sulphate (DHEA-S), progesterone, 17OH-progesterone (17OH-P), and insulin concentrations were measured in an electrochemiluminescence immunoassay (Roche Diagnostics – Cobas E601, Mannheim, Germany).

The intra-assay coefficients of variation (CVs) for CRP, glucose and TG were: 3.5%, 1.5% and 2%, whereas the limits of detection (LODs) were 0.3 mg/L, 0.11 mmol/L and 9 mg/dL, respectively. The intra-assay CVs for HDL-C and TCh were 2% and LODs were 4 mg/dL and 3 mg/dL, respectively. The intra-assay CVs for gonadotropins (FSH and LH), TSH and PRL were 2% and LODs for these parameters were 0.1 mIU/mL, 0.005 μIU/mL, 1.0 μIU/mL, respectively. The intra-assay CVs for E2, TST and DHEA-S were 3.5%, 3.0% and 3.5% and LODs were 18.5 pmol/L, 0.087 nmol/L and 0.1 μg/dL, respectively. The intra-assay CVs for progesterone, 17OH-P and insulin were 2.1%, 4.6% and 3.5%, whereas LODs were 0.159 nmol/L, 0.11 ng/mL and 0.2 mIU/L, respectively. Serum sex hormone binding globulin (SHBG) concentrations were measured in an immunoassay (Access 2 Immunoassay System, Beckman Coulter, Brea, United States) with the intra-assay CV of 4.15% and LOD of 0.35 nmol/L. Serum androstenedione concentrations were evaluated using the chemiluminescent immunoassay (CLIA) method on a Liaison analyzer (Diasorin, Saluggia, Italy), with the intra-assay CV of 3.6% and LOD 0.3 mg/mL. The inter-assay CVs for: CRP, glucose, TG, HDL-C, TCh, FSH, LH, TSH, PRL, E2, TST, DHEA-S, progesterone, 17OH-P, insulin, SHBG and androstenedione were 2.0%, 2.5%, 1.4%, 1.8%, 1.7%, 1.9%, 1.9%, 1.0%, 1.8%, 1.0%, 2.5%, 3.2%, 2.1%, 4.6%, 1.8%, 1.2%, 3.6%, respectively. Serum LDL-Cholesterol (LDL-C) concentrations were calculated using the Friedewald equation taking into the account its limitations (e.g. serum TG > 4.5 mmol/L). Free androgen index (FAI) was calculated according to the formula: FAI = TSTx100/SHGB. Insulin resistance was assessed using the Homeostasis Model of Assessment-Insulin Resistance (HOMA-IR) according to the formula: fasting insulin (μU/mL) x fasting glucose (mmol/L)/22.5.

2.6. Diagnosis of PCOS

The diagnosis of PCOS was made according to the criteria of the Androgen Excess and PCOS (AE&PCOS) Society where apart from the ovulatory dysfunction (OvD) or the polycystic ovarian morphology (PCOM) the presence of clinical or biochemical hyperandrogenism (HA) is mandatory [26].

Biochemical HA was defined as having TST, DHEA-S and/or androstenedione and/or FAI above the 95th percentile of the values recorded among the women from the control group (n = 80). The threshold levels for TST, DHEA-S, androstenedione, and FAI were 1.54 nmol/L, 8.75 μmol/L, 13.65 nmol/L, and 3.32, respectively.

OvD was defined as having menstrual cycles lasting for less than 25 or more than 35 days or anovulation. Therefore, in women who had apparently regular menses, serum progesterone concentrations were measured 7 days before the end of their menstrual cycle. The threshold value for the presence of ovulation was > 5 ng/mL. PCOM was defined as an antral follicle count (AFC) of ≥ 12 in 2–9 mm diameter and/or ovarian volume of ≥ 10 ml at least in one ovary [27]. When an ovulatory follicle was present, the volume of that ovary was not taken into consideration. In order to exclude other causes of ovulatory dysfunction or in the case of severe signs of hyperandrogenism, serum concentrations of TSH and PRL as well as 17OH-P, were measured respectively. Women with overt thyroid dysfunction (n = 36), hyperprolactinemia (n = 14) or a suspicion of non-classical congenital adrenal hyperplasia (serum 17OHP > 2 ng/dL – n = 3) were excluded from further analysis. Women who were morbidly obese (body mass index (BMI) > 40 – n = 5), underweight (BMI < 18.5 – n = 2), had laboratory signs of an infection (serum CRP > 15 mg/L – n = 2) or were diagnosed with T2DM (n = 2) were also excluded from the study. Some women were also excluded due to the lack of some laboratory results (n = 36), or the lack of TV USG (n = 18). After these exclusions, the results from 186 subjects were taken into the consideration. Among them 106 women were diagnosed with PCOS and 80 were considered healthy (the control group) [26]. The AE&PCOS Society diagnostic criteria yield three separate PCOS phenotypes (A–C). Phenotype A includes all the three

features (HA, OvD and PCOM) whereas phenotype B and C only two (HA and OvD or HA and PCOM, respectively). The prevalence of the A, B and the C phenotype among women with PCOS in our cohort was 38%, 33% and 29%, respectively.

2.7. Serum BPA analyses

The evaluation of serum BPA concentrations were performed using high performance liquid chromatography method with tandem mass spectrometry (HPLC–MS/MS) on the Shimadzu triple quadrupole LC–MS/MS system (LCMS-8060; Shimadzu, Japan) equipped with an electrospray ionization source (ESI) working in the negative multiple reaction mode (MRM). Detailed description of the whole method has been already published by our group elsewhere [28]. This method was chosen as it provides extra precision and higher sensitivity in comparison to MS [29]. Additionally, HPLC-MS/MS is believed to be a selective and sensitive method to determine phenol concentrations in serum samples [30]. The limit of quantification (LOQ) and LOD of BPA in sera was 0.028 ng/mL and 0.0093 ng/mL, respectively.

2.8. Statistical analyses

All the statistical analyses were performed using the GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, La Jolla, California USA, www.graphpad.com). The D'Agostino–Pearson test was used to determine the distribution of the measured variables. Non-normally distributed variables were log-transformed before the analyses and are presented as geometric means and their 95% confidence interval (CI). Normally distributed variables are presented as arithmetic means ± SD. Due to the possibility of serum sample contamination with BPA during the procedures of sample preparation and analysis, BPA results were also analysed for outliers using the ROUT method with the Q value set at 1%. Differences between the groups were compared using an unpaired Student's t-test. Serum BPA concentrations among the three PCOS phenotypes (A, B and C) were compared using the one-way analysis of variance (ANOVA). Correlation analyses between the selected variables and serum BPA concentrations in women with PCOS were performed using Pearson's correlation calculations where in the case of large samples (n > 100) the assumption that both X and Y variables are sampled from populations that follow a Gaussian distribution is not too important. P value of less than 0.05 was considered statistically significant.

3. Results

Clinical, biochemical, and metabolic characteristics of women with PCOS and healthy controls are presented in Table 1. There were no significant differences in age, BMI, waist circumference, serum glucose and insulin concentrations or HOMA-IR among the studied groups. Women with PCOS had also significantly higher serum LH concentrations ($P = 0.007$) and greater LH/FSH ratio ($P = 0.044$). Serum concentrations of E2 were lower, and TST, androstenedione, and DHEA-S along with FAI were significantly higher in women with PCOS ($P < 0.001$). Compared with the control subjects, women with PCOS had significantly higher serum LDL-C concentrations ($P = 0.008$). There were no differences in serum TCh, HDL-C nor TG concentrations between the studied groups.

BPA was detectable in 99% and 92% of serum samples from the women with PCOS and controls, respectively. Outliers were excluded from further analyses (n = 4 in PCOS group and n = 5 in the control group). Women with PCOS had significantly higher serum BPA concentrations than the control subjects (geometric mean and [95% CI] −0.202 ng/mL [0.150; 0.255] vs. 0.154 ng/mL [0.106; 0.201], $P = 0.035$) (Fig. 1). Moreover, in women with PCOS serum BPA concentrations correlated positively with FAI (R = 0.196, $P = 0.049$) and serum total TST concentrations (R = 0.285, $P = 0.004$) (Fig. 2). There were no significant differences in serum BPA concentrations between the A, B and C phenotypes within the PCOS group (geometric means and [95% CI]: 0.136 ng/ml [0.101; 0.182] vs. 0.224 ng/ml [0.140; 0.358] vs. 0.185 ng/ml [0.116; 0.295] respectively, P = 0.157).

Table 1
Clinical, hormonal, and metabolic characteristics of women with PCOS (n = 106) and healthy controls (n = 80).

Variable	PCOS (n = 106)	Control group (n = 80)	*P*-value
Age (yrs)	26.9 ± 5.2	28.2 ± 5.7	0.123
BMI[a]	24.8 (23.9; 25.7)	24.1 (23.1; 25.1)	0.273
Waist circumference (cm)	88.2 ± 11.9	85.6 ± 12.2	0.270
Fasting plasma glucose (mmol/L)[a]	4.83 (4.76; 4.9)	4.79 (4.71; 4.87)	0.483
Serum Insulin (mIU/L)[a]	8.6 (6.8; 10.4)	7.8 (7.0; 8.6)	0.097
HOMA-IR[a]	1.84 (1.43; 2.25)	1.67 (1.47; 1.86)	0.094
Serum LH (IU/L)[a]	8.1 (7.3; 9.0)	6.6 (5.7; 7.4)	0.007
Serum FSH (IU/L)[a]	6.7 (6.2; 7.2)	6.4 (5.9; 6.8)	0.284
LH/FSH[a]	1.22 (1.08; 1.35)	1.0 (0.90; 1.18)	0.044
Serum E2 (pmol/L)[a]	200.6 (174.4; 226.7)	250.7 (184.4; 317.1)	0.005
Serum PRL (mIU/L)	18.5 ± 6.8	18.0 ± 7.2	0.623
Serum DHEA-S (μmol/L)[a]	297.1 (275.1; 319.1)	194.3 (178.7; 209.8)	< 0.001
Serum androstenedione (nmol/L)[a]	11.2 (10.4; 12.1)	7.1 (6.5; 7.7)	< 0.001
Serum total TST (nmol/L)	1.79 ± 0.5	1.08 ± 0.3	< 0.001
Serum SHBG (nmol/L)[a]	54.0 (48.11; 59.85)	68.9 (62.87; 74.90)	0.002
FAI	3.97 ± 2.9	1.68 ± 0.8	< 0.001
Serum TCh (mmol/L)	5.04 ± 0.97	4.82 ± 0.8	0.089
Serum HDL-C (mmol/L)[a]	1.67 (1.59; 1.75)	1.76 (1.66; 1.85)	0.170
Serum LDL-C (mmol/L)[a]	2.80 (2.63; 2.96)	2.47 (2.30; 2.63)	0.008
Serum TG (mmol/L)[a]	0.87 (0.78; 0.95)	0.83 (0.72; 0.95)	0.642

Normally distributed variables are presented as arithmetic means ± SD.
BMI – body mass index, LH – luteinizing hormone, FSH – follicle-stimulating hormone, E2 – 17β-estradiol, PRL – prolactin, DHEA-s – dehydroepiandrosterone sulphate, TST – testosterone, SHBG – sex hormone binding globulin, FAI – free androgen index, TCh – total cholesterol, HDL-C – HDL-cholesterol, LDL-C – LDL-cholesterol, TG – triglycerides, HOMA-IR – Homeostasis Model of Assessment-Insulin Resistance.
[a] Non-normally distributed variables are presented as geometric means (95% CI).

4. Discussion

The aim of the present study was to evaluate serum BPA concentrations among women with PCOS and to determine, if they may have an impact on the hormonal and metabolic parameters in these patients.

Women with PCOS had significantly higher indices of hyperandrogenemia (TST, DHEA-S, androstendione and FAI), which well characterizes the hormonal profile of these patients [31]. Although women with PCOS are usually characterized by atherogenic dyslipidemia (low HDL-C and high TG) [32,33] there were no significant differences in serum TCh, HDL-C or TG concentrations between our studied groups. Nevertheless, women with PCOS had significantly higher serum LDL-C levels than healthy controls. This is consistent with the results of a study conducted by Couto et al. [34] where after adjusting for age and BMI, also serum LDL-C concentrations remained increased in women with PCOS. It's well known that many factors, including body composition, physical activity, intake of dietary macronutrients or the presence of the metabolic syndrome affect the cholesterol efflux and lipid metabolism [35,36]. It is also believed that higher LDL-C concentrations in women with PCOS may be linked to [the] androgen excess [32]. Nevertheless, in our study serum LDL-C concentrations did not correlate with serum androgen levels (data not shown).

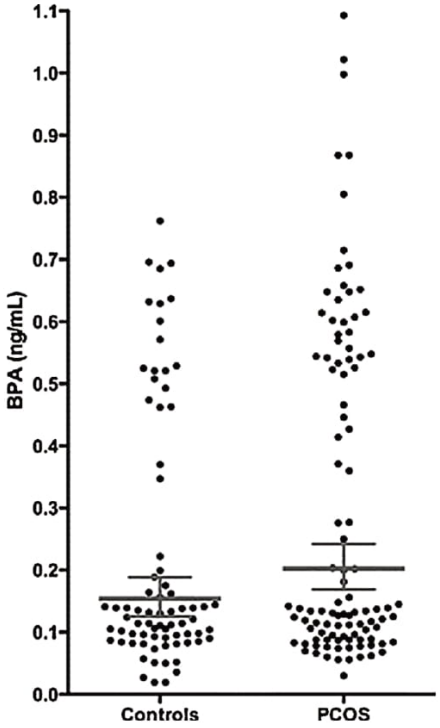


Fig. 1. Serum BPA concentrations in studied groups. The horizontal lines represent geometric mean with whiskers as 95% confidence interval.

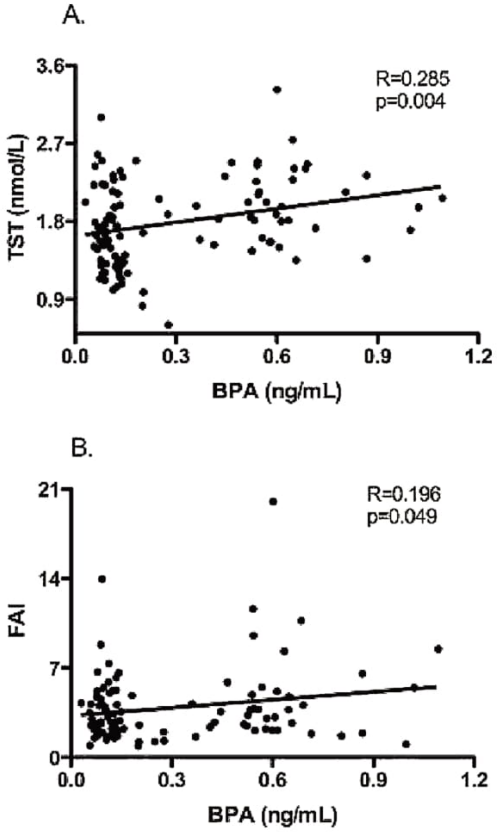


Fig. 2. Correlations of BPA concentrations with total TST and FAI in women with PCOS.

Serum BPA concentrations in women with PCOS were significantly higher than in age- and BMI- matched healthy controls. Analogous results were found in studies conducted by other researchers [37–39], even though serum BPA concentrations were measured using the immunoenzymatic assay and not the HPLC-MS/MS method. In the study of Vahedi et al. [40], serum BPA concentrations were also higher compared to the control subjects although they were determined using the HPLC method only, without the concomitant use of MS. To our best knowledge, our study is the first one where this very reliable and sensitive method has been used for BPA quantification in sera of women with PCOS. Similarly to other studies, BPA was detectable in almost all of the of the analyzed serum samples (99% in the PCOS and 92% in the control group), which point to the unavoidable exposure to this EDC in our daily life [41].

Moreover, likewise in other studies [37,42] among women with PCOS, serum BPA concentrations correlated positively with both testosterone concentrations as well as FAI. The possible mechanism involves both the direct stimulation of the ovarian theca cells to the exaggerated androgen synthesis [43,44] as well as the potential of displacing sex hormones from SHBG binding sites. On the other hand, BPA has been shown to possess estrogenic effects [45] and as such could stimulate the production of SHBG in the liver. However, studies from women who use estrogen replacement therapy show that only oral administration increases the hepatic production of SHBG, whereas transdermal regimen do not have such an effect [46]. Therefore, it might be possible that the exposure to BPA does not occur mainly through food consumption but through transdermal contact with materials containing that compound such as plastic furniture and decorations, receipts, and packages [21]. Nevertheless, it has been already shown *in silico* [47] and in a study using a competitive binding assay that BPA has a binding affinity to SHBG [48]. Hence, we might speculate that BPA by binding with SHBG may displace TST, and thus increase FAI. Nevertheless, in our study we did not find any correlation between serum BPA concentrations and the SHBG levels.

Serum BPA concentrations in women with PCOS did not correlate with BMI ($R = -0.072$, $P = 0.473$), waist circumference ($R = 0.039$, $P = 0.716$), serum insulin ($R = 0.033$, $P = 0.742$), glucose ($R = 0.047$, $P = 0.642$) or HOMA-IR ($R = 0.038$, $P = 0.710$). Furthermore, no correlations were found between serum BPA and serum lipids in this group (TCh: $R = -0.061$, $P = 0.545$; HDL-C: $R = -0.019$, $P = 0.853$; LDL-C: $R = -0.071$, $P = 0.480$; TG: $R = 0.065$, $P = 0.515$).

Our study design has some limitations. First of all, serum BPA concentrations may not necessarily reflect the actual exposure to this EDC. It has been shown that when present in blood, BPA is rapidly metabolized in the liver and has a half-life of several hours, and some researchers suggest that urine might be a better material to determine the actual exposure risk [49]. Nevertheless, it is alarming that BPA was detectable in almost all of the serum samples from our studied subjects.

Another limitation of our study is that in our cohort serum testosterone concentrations were measured using an automated electrochemiluminescence immunoassay. Although, these measurements were performed in the clinical diagnostic laboratory, evaluating serum testosterone concentrations in women is inherently problematic [50]. The novel LC–MS/MS methods, which have been shown to be superior over the immuno-metric platforms, would probably be more appropriate [51]. Regrettably, we were not able to validate our serum testosterone measurements against this method. Nevertheless, the diagnosis of PCOS in our cohort was made according to the AE&PCOS Society criteria [26] where not only laboratory indices of hyperandrogenism were present but also clinical (i.e. hirsutism). Therefore, it is very unlikely that we have misdiagnosed these subjects.

In summary, the results of our study show that not only serum BPA concentrations in women with PCOS are significantly higher than in

age- and BMI-matched controls but they also correlate positively with serum total TST and FAI which points to the potential role of this EDC in the pathogenesis of the ovarian hyperandrogenism.

Conflict of interests

The authors declare no conflict of interests related to the content of this manuscript

Funding

This study was financed from the funds of the Polish National Centre for Science (grant number 2014/15/B/NZ7/00999).

References

[1] M.P. Lauritsen, J.G. Bentzen, A. Pinborg, A. Loft, J.L. Forman, L.L. Thuesen, A. Cohen, D.M. Hougaard, A. Nyboe Andersen, The prevalence of polycystic ovary syndrome in a normal population according to the Rotterdam criteria versus revised criteria including anti-Mullerian hormone, Hum. Reprod. 29 (4) (2014) 791–801.
[2] G. Bozdag, S. Mumusoglu, D. Zengin, E. Karabulut, B.O. Yildiz, The prevalence and phenotypic features of polycystic ovary syndrome: a systematic review and meta-analysis, Hum. Reprod. 31 (12) (2016) 2841–2855.
[3] L.C. Varanasi, A. Subasinghe, Y.L. Jayasinghe, E.T. Callegari, S.M. Garland, A. Gorelik, J.D. Wark, Polycystic ovarian syndrome: prevalence and impact on the wellbeing of Australian women aged 16-29 years, Aust. N. Z. J. Obstet. Gynaecol. 58 (2) (2017) 222–233.
[4] A. Dokras, S. Saini, M. Gibson-Helm, J. Schulkin, L. Cooney, H. Teede, Gaps in knowledge among physicians regarding diagnostic criteria and management of polycystic ovary syndrome, Fertil. Steril. 107 (6) (2017) 1380–1386 e1.
[5] H. Mani, M.J. Levy, M.J. Davies, D.H. Morris, L.J. Gray, J. Bankart, H. Blackledge, K. Khunti, T.A. Howlett, Diabetes and cardiovascular events in women with polycystic ovary syndrome: a 20-year retrospective cohort study, Clin. Endocrinol. (Oxf.) 78 (6) (2013) 926–934.
[6] P. Anagnostis, B.C. Tarlatzis, R.P. Kauffman, Polycystic Ovarian Syndrome (PCOS): long-term metabolic consequences, Metab. Clin. Exp. 86 (2017) 33–43.
[7] A.S. de Melo, S.V. Dias, C. Cavalli Rde, V.C. Cardoso, H. Bettiol, M.A. Barbieri, R.A. Ferriani, C.S. Vieira, Pathogenesis of polycystic ovary syndrome: multifactorial assessment from the foetal stage to menopause, Reproduction (Cambridge, England) 150 (1) (2015) R11–24.
[8] M. Ebrahimi-Mamaghani, M. Saghafi-Asl, S. Pirouzpanah, A. Aliasgharzadeh, S. Aliashrafi, N. Rezayi, M. Mehrzad-Sadaghiani, Association of insulin resistance with lipid profile, metabolic syndrome, and hormonal aberrations in overweight or obese women with polycystic ovary syndrome, J. Health Popul. Nutr. 33 (1) (2015) 157–167.
[9] K.H. Rubin, D. Glintborg, M. Nybo, B. Abrahamsen, M. Andersen, Development and risk factors of type 2 diabetes in a nationwide population of women with polycystic ovary syndrome, J. Clin. Endocrinol. Metab. 102 (10) (2017) 3848–3857.
[10] I. Brosens, G. Benagiano, Menstrual preconditioning for the prevention of major obstetrical syndromes in polycystic ovary syndrome, Am. J. Obstet. Gynecol. 213 (4) (2015) 488–493.
[11] Consensus on infertility treatment related to polycystic ovary syndrome, Hum. Reprod. 23 (3) (2008) 462–477.
[12] L.J. Moran, R.J. Norman, H.J. Teede, Metabolic risk in PCOS: phenotype and adiposity impact, Trends Endocrinol. Metab. 26 (3) (2015) 136–143.
[13] J. Bellver, L. Rodriguez-Tabernero, A. Robles, E. Munoz, F. Martinez, J. Landeras, J. Garcia-Velasco, J. Fontes, M. Alvarez, C. Alvarez, B. Acevedo, Polycystic ovary syndrome throughout a woman's life, J. Assist. Reprod. Genet. 35 (1) (2018) 25–39.
[14] E. Palioura, E. Diamanti-Kandarakis, Polycystic ovary syndrome (PCOS) and endocrine disrupting chemicals (EDCs), Rev. Endocr. Metab. Disord. 16 (4) (2015) 365–371.
[15] A.Z. Rutkowska, E. Diamanti-Kandarakis, Polycystic ovary syndrome and environmental toxins, Fertil. Steril. 106 (4) (2016) 948–958.
[16] A. Ziv-Gal, J.A. Flaws, Evidence for bisphenol A-induced female infertility: a review (2007–2016), Fertil. Steril. 106 (4) (2016) 827–856.
[17] M.S. Bloom, E. Mok-Lin, V.Y. Fujimoto, Bisphenol A and ovarian steroidogenesis, Fertil. Steril. 106 (4) (2016) 857–863.
[18] A. Rutkowska, D. Rachon, Bisphenol A (BPA) and its potential role in the pathogenesis of the polycystic ovary syndrome (PCOS), Gynecol. Endocrinol. 30 (4) (2014) 260–265.
[19] L. Correia-Sa, M. Kasper-Sonnenberg, A. Schutze, C. Palmke, S. Norberto, C. Calhau, V.F. Domingues, H.M. Koch, Exposure assessment to bisphenol A (BPA) in Portuguese children by human biomonitoring, Environ. Sci. Pollut. Res. Int. 24 (35) (2017) 27502–27514.
[20] E. Jackson, R. Shoemaker, N. Larian, L. Cassis, Adipose tissue as a site of toxin accumulation, Compr. Physiol. 7 (4) (2017) 1085–1135.
[21] A. Konieczna, A. Rutkowska, D. Rachon, Health risk of exposure to Bisphenol A (BPA), Roczniki Panstwowego Zakladu Higieny 66 (1) (2015) 5–11.
[22] F. Acconcia, V. Pallottini, M. Marino, Molecular mechanisms of action of BPA, Dose 13 (4) (2015) 1559325815610582.
[23] A.Z. Rutkowska, A. Szybiak, K. Serkies, D. Rachon, Endocrine disrupting chemicals as potential risk factor for estrogen-dependent cancers, Pol. Arch. Med. Wewn. 126 (7–8) (2016) 562–570.
[24] D. Rachon, Endocrine disrupting chemicals (EDCs) and female cancer: informing the patients, Rev. Endocr. Metab. Disord. 16 (4) (2015) 359–364.
[25] D. Petrakis, L. Vassilopoulou, C. Mamoulakis, C. Psycharakis, A. Anifantaki, S. Sifakis, A.O. Docea, J. Tsiaoussis, A. Makrigiannakis, A.M. Tsatsakis, Endocrine disruptors leading to obesity and related diseases, Int. J. Environ. Res. Public Health 14 (10) (2017).
[26] R. Azziz, E. Carmina, D. Dewailly, E. Diamanti-Kandarakis, H.F. Escobar-Morreale, W. Futterweit, O.E. Janssen, R.S. Legro, R.J. Norman, A.E. Taylor, S.F. Witchel, The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report, Fertil. Steril. 91 (2) (2009) 456–488.
[27] D.K. Song, J.Y. Oh, H. Lee, Y.A. Sung, Differentiation between polycystic ovary syndrome and polycystic ovarian morphology by means of an anti-Mullerian hormone cutoff value, Korean J. Intern. Med. 32 (4) (2017) 690–698.
[28] K. Owczarek, P. Kubica, B. Kudłak, A. Rutkowska, A. Konieczna, D. Rachoń, J. Namieśnik, A. Wasik, Determination of trace levels of eleven bisphenol A analogues in human blood serum by high performance liquid chromatography–tandem mass spectrometry, Sci. Total Environ. 628–629 (2018) 1362–1368.
[29] W.M.A. Niessen, MS–MS and MSn, in: J.C. Lindon, G.E. Tranter, D.W. Koppenaal (Eds.), Encyclopedia of Spectroscopy and Spectrometry, third edition), Academic Press, Oxford, 2017, pp. 936–941.
[30] J. Li, T. Chen, Y. Wang, Z. Shi, X. Zhou, Z. Sun, D. Wang, Y. Wu, Simple and fast analysis of tetrabromobisphenol A, hexabromocyclododecane isomers, and polybrominated diphenyl ethers in serum using solid-phase extraction or QuEChERS extraction followed by tandem mass spectrometry coupled to HPLC and GC, J. Sep. Sci. 40 (3) (2017) 709–716.
[31] M.W. O'Reilly, A.E. Taylor, N.J. Crabtree, B.A. Hughes, F. Capper, R.K. Crowley, P.M. Stewart, J.W. Tomlinson, W. Arlt, Hyperandrogenemia predicts metabolic phenotype in polycystic ovary syndrome: the utility of serum androstenedione, J. Clin. Endocrinol. Metab. 99 (3) (2014) 1027–1036.
[32] R.A. Wild, M. Rizzo, S. Clifton, E. Carmina, Lipid levels in polycystic ovary syndrome: systematic review and meta-analysis, Fertil. Steril. 95 (3) (2011) 1073–1079 e1-11.
[33] P. Pinola, K. Puukka, T.T. Piltonen, J. Puurunen, E. Vanky, I. Sundstrom-Poromaa, E. Stener-Victorin, A. Linden Hirschberg, P. Ravn, M. Skovsager Andersen, D. Glintborg, J.R. Mellembakken, A. Ruokonen, J.S. Tapanainen, L.C. Morin-Papunen, Normo- and hyperandrogenic women with polycystic ovary syndrome exhibit an adverse metabolic profile through life, Fertil. Steril. 107 (3) (2017) 788–795 e2.
[34] A. Couto Alves, B. Valcarcel, V.P. Makinen, L. Morin-Papunen, S. Sebert, A.J. Kangas, P. Soininen, S. Das, M. De Iorio, L. Coin, M. Ala-Korpela, M.R. Jarvelin, S. Franks, Metabolic profiling of polycystic ovary syndrome reveals interactions with abdominal obesity, Int. J. Obes. (Lond.) 41 (9) (2017) 1331–1340.
[35] A. Roe, J. Hillman, S. Butts, M. Smith, D. Rader, M. Playford, N.N. Mehta, A. Dokras, Decreased cholesterol efflux capacity and atherogenic lipid profile in young women with PCOS, J. Clin. Endocrinol. Metab. 99 (5) (2014) E841–7.
[36] Y. Ma, Y. Li, D.E. Chiriboga, B.C. Olendzki, J.R. Hebert, W. Li, K. Leung, A.R. Hafner, I.S. Ockene, Association between carbohydrate intake and serum lipids, J. Am. Coll. Nutr. 25 (2) (2006) 155–163.
[37] E. Kandaraki, A. Chatzigeorgiou, S. Livadas, E. Palioura, F. Economou, M. Koutsilieris, S. Palimeri, D. Panidis, E. Diamanti-Kandarakis, Endocrine disruptors and polycystic ovary syndrome (PCOS): elevated serum levels of bisphenol A in women with PCOS, J. Clin. Endocrinol. Metab. 96 (3) (2011) E480–4.
[38] G. Tarantino, R. Valentino, C. Di Somma, V. D'Esposito, F. Passaretti, G. Pizza, V. Brancato, F. Orio, P. Formisano, A. Colao, S. Savastano, Bisphenol A in polycystic ovary syndrome and its association with liver-spleen axis, Clin. Endocrinol. (Oxf.) 78 (3) (2013) 447–453.
[39] Y. Wang, Q. Zhu, X. Dang, Y. He, X. Li, Y. Sun, Local effect of bisphenol A on the estradiol synthesis of ovarian granulosa cells from PCOS, Gynecol. Endocrinol. 33 (1) (2017) 21–25.
[40] M. Vahedi, A. Saeedi, S.L. Poorbaghi, M. Sepehrimanesh, M. Fattahi, Metabolic and endocrine effects of bisphenol A exposure in market seller women with polycystic ovary syndrome, Environ. Sci. Pollut. Res. Int. 23 (23) (2016) 23546–23550.
[41] L.N. Vandenberg, I. Chahoud, J.J. Heindel, V. Padmanabhan, F.J. Paumgartten, G. Schoenfelder, Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A, Cien. Saude Colet. 17 (2) (2012) 407–434.
[42] T. Takeuchi, O. Tsutsumi, Y. Ikezuki, Y. Takai, Y. Taketani, Positive relationship between androgen and the endocrine disruptor, bisphenol A, in normal women and women with ovarian dysfunction, Endocr. J. 51 (2) (2004) 165–169.
[43] W. Zhou, J. Liu, L. Liao, S. Han, J. Liu, Effect of bisphenol A on steroid hormone production in rat ovarian theca-interstitial and granulosa cells, Mol. Cell. Endocrinol. 283 (1–2) (2008) 12–18.
[44] J. Peretz, R.K. Gupta, J. Singh, I. Hernandez-Ochoa, J.A. Flaws, Bisphenol A impairs follicle growth, inhibits steroidogenesis, and downregulates rate-limiting enzymes in the estradiol biosynthesis pathway, Toxicol. Sci. 119 (1) (2011) 209–217.
[45] A. Leonardi, M. Cofini, D. Rigante, L. Lucchetti, C. Cipolla, L. Penta, S. Esposito, The effect of bisphenol A on puberty: a critical review of the medical literature, Int. J. Environ. Res. Public Health 14 (9) (2017).
[46] A. Ropponen, K. Aittomaki, V. Vihma, M.J. Tikkanen, O. Ylikorkala, Effects of oral and transdermal estradiol administration on levels of sex hormone-binding globulin in postmenopausal women with and without a history of intrahepatic cholestasis of pregnancy, J. Clin. Endocrinol. Metab. 90 (6) (2005) 3431–3434.
[47] I.A. Sheikh, I.A. Tayubi, E. Ahmad, M.A. Ganaie, O.S. Bajouh, S.F. AlBasri, I.M.J. Abdulkarim, M.A. Beg, Computational insights into the molecular interactions of environmental xenoestrogens 4-tert-octylphenol, 4-nonylphenol, bisphenol

A (BPA), and BPA metabolite, 4-methyl-2, 4-bis (4-hydroxyphenyl) pent-1-ene (MBP) with human sex hormone-binding globulin, Ecotoxicol. Environ. Saf. 135 (2017) 284–291.
[48] H. Hong, W.S. Branham, H.W. Ng, C.L. Moland, S.L. Dial, H. Fang, R. Perkins, D. Sheehan, W. Tong, Human sex hormone-binding globulin binding affinities of 125 structurally diverse chemicals and comparison with their binding to androgen receptor, estrogen receptor, and alpha-fetoprotein, Toxicol. Sci. 143 (2) (2015) 333–348.
[49] A.M. Calafat, H.M. Koch, S.H. Swan, R. Hauser, L.R. Goldman, B.P. Lanphear, M.P. Longnecker, R.A. Rudel, S.L. Teitelbaum, R.M. Whyatt, M.S. Wolff, Misuse of blood serum to assess exposure to bisphenol A and phthalates, Breast Cancer Res. 15 (5) (2013) 403.
[50] W. Rosner, R.J. Auchus, R. Azziz, P.M. Sluss, H. Raff, Position statement: Utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement, J. Clin. Endocrinol. Metab. 92 (2) (2007) 405–413.
[51] F. Fanelli, A. Gambineri, M. Mezzullo, V. Vicennati, C. Pelusi, R. Pasquali, Revisiting hyper- and hypo-androgenism by tandem mass spectrometry, Rev. Endocr. Metab. Disord. 14 (2) (2013) 185–205.

Lund, dnia 22.06.2022 r.

Dr n. o zdr. Aleksandra Olsson (poprzednio Konieczna)
Wydział Zdrowia
Wyższa Szkoła Bankowa w Gdańsku
al. Grunwaldzka 238A, 80-266 Gdańsk
E-mail: aolsson@wsb.gda.pl

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na współopracowaniu koncepcji badania, zabezpieczeniu i przygotowaniu materiału badawczego, analizie danych, współudziale w tworzeniu tekstu manuskryptu oraz odpowiedzi na uwagi recenzentów.

Konieczna A., Rachoń D., Owczarek K., Kubica P., Kowalewska A., Kudłak B., Wasik A., Namieśnik J. 2018. *„Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome"* Reprod Toxicol (accepted for publication).

(podpis współautora)

Gdańsk, dnia 24 września 2018

Dr hab. n. med. Dominik Rachoń
Zakład Endokrynologii Klinicznej
i Doświadczalnej GUMed
ul. Dębinki 7, 80-210 Gdańsk
E-mail: dominik.rachon@gumed.edu.pl

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na **zrekrutowaniu badanych kobiet oraz merytorycznej korekcie ostatecznej wersji manuskryptu.**

Konieczna A., **Rachoń D.**, Owczarek K., Kubica P., Kowalewska A., Kudłak B., Wasik A., Namieśnik J. 2018. *„Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome"* Reprod Toxicol (accepted for publication).

.....

(podpis współautora)

Gdańsk, dnia 24 września 2018

Mgr inż. Katarzyna Owczarek
Katedra Chemii Analitycznej
Wydział Chemiczny
Politechnika Gdańska
ul. Narutowicza 11/12, 80-223 Gdańsk
E-mail: katowcza1@student.pg.edu.pl

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na **przeprowadzeniu części analitycznej badania.**

Konieczna A., Rachoń D., **Owczarek K.**, Kubica P., Kowalewska A., Kudłak B., Wasik A., Namieśnik J. 2018. *„Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome"* Reprod Toxicol (accepted for publication).

K. Owczarek

(podpis współautora)

Gdańsk, dnia 24 września 2018

Dr inż. Paweł Kubica
Katedra Chemii Analitycznej
Wydział Chemiczny
Politechnika Gdańska
ul. Narutowicza 11/12, 80-223 Gdańsk
E-mail: pawel.kubica@pg.edu.pl

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na **przeprowadzeniu części analitycznej badań.**

Konieczna A., Rachoń D., Owczarek K., **Kubica P.**, Kowalewska A., Kudłak B., Wasik A., Namieśnik J. 2018. *„Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome"* Reprod. Toxicol. (accepted for publication).

.....

(podpis współautora)

Gdańsk, dnia 24 września 2018

Dr n. med. Agnieszka Kowalewska
Indywidualna Specjalistyczna Praktyka Lekarska
ul. Parkowa 6, 81-549 Gdynia
E-mail: akw@poczta.onet.eu

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na **udziale w rekrutowaniu i wykonywaniu badania USG TV u pacjentek**.

Konieczna A., Rachoń D., Owczarek K., Kubica P., **Kowalewska A.**, Kudłak B., Wasik A., Namieśnik J. 2018. *„Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome"*. Reprod Toxicol (accepted for publication).

Agnieszka Kowalewska

.....

(podpis współautora)

lek. med. Agnieszka Kowalewska
specjalista położnictwa i chorób kobiecych
specjalista endokrynolog
tel. 602 72 63 62

Gdańsk, dnia 23 września 2018

Dr hab. inż. Błażej Kudłak
Katedra Chemii Analitycznej
Wydział Chemiczny
Politechnika Gdańska
ul. G. Narutowicza 11/12, 80-223 Gdańsk
E-mail: blakudla@pg.edu.pl

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na **przeprowadzeniu części analitycznej badania.**

Konieczna A., Rachoń D., Owczarek K., Kubica P., Kowalewska A., **Kudłak B.**, Wasik A., Namieśnik J. 2018. *„Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome"* Reprod Toxicol (accepted for publication).

(podpis współautora)

Gdańsk, dnia 24 września 2018

Dr hab. inż. Andrzej Wasik
Katedra Chemii Analitycznej
Wydział Chemiczny
Politechnika Gdańska
ul. Narutowicza 11/12, 80-223 Gdańsk
E-mail: wasia@pg.edu.pl

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na nadzorze nad przeprowadzeniem części analitycznej badania oraz korekcie tekstu.

Konieczna A., Rachoń D., Owczarek K., Kubica P., Kowalewska A., Kudłak B., **Wasik A.**, Namieśnik J. 2018. *„Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome"* Reprod Toxicol (accepted for publication).

.....
(podpis współautora)

Gdańsk, dnia 24 września 2018

Prof. dr hab. inż. Jacek Namieśnik
Katedra Chemii Analitycznej
Wydział Chemiczny
Politechnika Gdańska
ul. Narutowicza 11/12, 80-223 Gdańsk
E-mail: jacek.namiesnik@pg.edu.pl

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie poniższej publikacji polegał na **ocenie systemu kontroli i zapewnieniu jakości wyników badań (QA/QC) oraz merytorycznej korekcie maszynopisu.**

Konieczna A., Rachoń D., Owczarek K., Kubica P., Kowalewska A., Kudłak B., Wasik A., **Namieśnik J**. 2018. *„Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome"* Reprod Toxicol (accepted for publication).

.....

(podpis współautora)

4.4. Oznaczenie poziomu zawartości wybranych związków z rodziny bisfenoli w próbkach odżywek i preparatów sportowych

Rynek produktów dietetycznych oraz suplementów diety przeznaczonych dla profesjonalnych sportowców oraz osób uprawiających sport amatorsko od lat stale się poszerza. Odżywki sportowe zażywane są różnych celach, w tym dla poprawy regeneracji organizmu po wysiłku, zwiększenia jego wydolności i efektywności działania, lepszego budowania mięśni, redukcji zmęczenia czy poprawy odporności. Suplementy tego rodzaju zazwyczaj przyjmowane są przez dłuższy okres czasu, co sprawia że są potencjalnym źródłem długofalowego narażenia odbiorcy na obecne w nich ksenobiotyki.

W literaturze naukowej można odnaleźć doniesienia na temat kontrowersji związanych z rozbieżnością pomiędzy deklarowanym i rzeczywistym składem tych produktów [153-155], co więcej, informacje na temat zawartości ksenobiotyków, w tym związków o działaniu endokrynnym w tych matrycach, są wciąż trudnodostępne. Przyczyna pojawiania się w odżywkach sportowych substancji z grupy bisfenoli nie jest jasno określona, chociaż przypuszcza się, że są one zanieczyszczeniem powstałym na etapie produkcji lub, przede wszystkim, przenikają do produktu z materiałów opakowaniowych, podobnie jak ma to miejsce w przypadku żywności.

Biorąc pod uwagę powyższe fakty, w niniejszej pracy podjęto próbę opracowania prostej metodyki analitycznej opartej na zastosowaniu techniki chromatografii gazowej sprzężonej ze spektrometrią mas w celu oznaczenia szeregu związków z grupy bisfenoli w próbkach odżywek i suplementów przeznaczonych dla sportowców. Anality wybrano na podstawie poprzednich badań prowadzonych w ramach pracy doktorskiej (między innymi opisanych w sekcji 4.2. i 4.3.) oraz przeglądu literatury, opierając się na zaobserwowanej częstości ich występowania. Technika GC-MS w przypadku analizy bisfenoli jest zdecydowanie mniej popularna niż chromatografia cieczowa, ze względu na konieczność derywatyzacji analitów. Jest to natomiast technika zdecydowanie mniej kosztowna i ogromna liczba laboratoriów analitycznych jest wyposażona w aparaty GC-MS. W związku z rosnącym zainteresowaniem tematyką występowania bisfenoli w próbkach różnego pochodzenia, stosownym wydawało się zaproponowanie prostej i stosunkowo taniej alternatywy dla metod opartych na analizach LC-MS/MS, która mogłaby być zastosowana rutynowo w danych warunkach.

Opracowana metoda pozwoliła na detekcję i ilościowe oznaczenie szeregu sześciu bisfenoli – BPA, BPE, BPF, BPP, BPS i BPZ w próbkach odżywek sportowych o różnorodnym składzie. Dzięki odpowiedniej optymalizacji charakteryzowała się ona krótkim czasem przygotowania próbek, niskim zużyciem rozpuszczalników organicznych oraz stosunkowo niską granicą oznaczalności (50 ng/mL), co w przeliczeniu na masę próbki wynosi 1ng/mg produktu). Spektrometr mas pracował w trybie monitorowania pojedynczego jonu (SIM), natomiast potwierdzenie jakościowe zostało zapewnione poprzez monitorowanie dodatkowych charakterystycznych jonów dla każdego związku. Zastosowanie metody dodatku wzorca wewnętrznego pozwoliło na kompensację strat podczas etapu

ekstrakcji oraz różnic w odpowiedzi detektora. Metoda została zwalidowana zgodnie z międzynarodowymi wytycznymi, a wszystkie kluczowe parametry spełniły założone kryteria akceptacji tj.: współczynnik regresji dla liniowości $r \geq 0,99$, odzysk mieszczący się w przedziale 70% ÷ 130%, precyzja wyrażona jako względne odchylenie standardowe ≤ 10%, brak interferencji w próbach ślepych.

Analizie poddano piętnaście próbek odżywek i preparatów sportowych dostępnych handlowo, z których każdy charakteryzował się odmiennym składem. Związkami wykrytymi w największej liczbie próbek był BPA (7 próbek), następnie BPS (5 próbek) oraz BPE (4 próbki). BPZ nie został wykryty w żadnej z badanych próbek. Źródłem tych substancji, jak już wcześniej wspomniano, były najprawdopodobniej materiały opakowaniowe wykonane z tworzyw sztucznych lub zostały wyemitowane na etapie produkcji i konfekcjonowania produktów na różnych etapach ich produkcji.

W chwili powstawania niniejszej pracy, badania przedstawione w załączonej publikacji były pierwszą próbą oznaczenia szeregu analogów bisfenolu A w próbkach odżywek sportowych. Tematyka ta może w szczególności zainteresować osoby uprawiające sport zawodowo, ze względu na dużą wagę, jaką przykłada się w tej grupie do dbałości o zdrowie. Biorąc pod uwagę udowodniony negatywny wpływ bisfenoli na funkcjonowanie organizmu oraz potencjalny udział w wywoływaniu chorób układu sercowo-naczyniowego, analityka tych związków w produktach dla sportowców może przyczynić się do wzrostu świadomości społecznej, przy wyborze suplementów diety specjalnego przeznaczenia. Publikacja ukazała się w czasopiśmie *Microchemical Journal* [156].

Microchemical Journal 180 (2022) 107643

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Validated GC–MS method for determination of bisphenol a and its five analogues in dietary and nutritional supplements

Katarzyna Owczarek [a,b,*], Emilia Waraksa [c], Ewa Kłodzińska [c,d], Yaroslav Zrobok [d], Mariusz Ozimek [e], Dominik Rachoń [f], Błażej Kudłak [a], Andrzej Wasik [a], Zofia Mazerska [g]

[a] Gdańsk University of Technology, Faculty of Chemistry, Department of Analytical Chemistry, G. Narutowicza 11/12 Street, 80-233 Gdańsk, Poland
[b] Medical University of Gdańsk, Faculty of Medicine, Depertment of Biochemistry, Bioanalytical Laboratory, Dębinki 1 Street, 80-211 Gdańsk, Poland
[c] Institute of Sport - National Research Institute, Department of Analytical Chemistry and Instrumental Analysis, Trylogii 2/16 Street, 01-982 Warsaw, Poland
[d] Alpinus Chemia Ltd., Garbary 5 Street, 86-050 Solec Kujawski, Poland
[e] Jagiellonian University in Krakow, Jagiellonian University Sports Centre, Piastowska 26 street, 30-067, Krakow, Poland
[f] Medical University of Gdansk, Institute of Maritime and Tropical Medicine, Division of Clinical & Experimental Endocrinology, Dębinki 7 Street, 80-211, Gdańsk, Poland
[g] Gdańsk University of Technology, Faculty of Chemistry, Department Pharmaceutical Technology and Biochemistry, G. Narutowicza 11/12 Street, 80-233 Gdańsk, Poland

ARTICLE INFO

Keywords:
Bisphenol A analogues
Dietary exposure
Emerging environmental contaminants
Endocrine disrupting compounds
Sport supplements

ABSTRACT

Bisphenol A (BPA) and its analogues showing structural and functional similarity to BPA are commonly applied in various industrial applications and thus are becoming ubiquitous in the environment. At the same time there is increasing scientific evidence that exposure to these chemicals may lead to adverse health effects in human and wildlife. In recent years dietary and nutritional supplements dedicated for athletes have become more popular and are widely used even by people who are not professionals. This study presents the development, optimization and validation of an analytical procedure for determination of six bisphenol analogues in dietary supplements using gas chromatography – mass spectrometry technique. All validation parameters met the established acceptance criteria in accordance with international guidelines. The method was linear within the tested range of 50–1000 ng/mL, the limit of quantitation was set as the lowest calibration point 50 ng/mL, detection limit for each bisphenol was calculated as standard estimation error on the basis of the linearity testing and was in the range of 8.73–15.44 ng/mL. Method trueness, accuracy (within and between runs) and precision were also verified and were in the range of 78.8 – 100.4%, 94.3 – 103.1%,

0.5 – 9.6% respectively.

The developed procedure was successfully applied for real samples analysis, namely for fifteen sport supplements of different composition and designated for various purposes, i.e. for increasing effectiveness, promoting muscle recovery and endurance, reducing tiredness and fatigue or increasing immune ability. For the majority of samples, bisphenols were either not detected or detected at the LOD level except bisphenol A which was quantitated in few samples at LOQ level or higher (in the range 0.852–2.892 ng/mg)

The field of bisphenol analogues analytics has increased in recent years due to law regulations becoming more strict, thus the development of new analytical tools for quality control of dietary products is needed and fully justified.

1. Introduction

The topic of bisphenol A (BPA) has been present in the scientific literature since a long time, mainly because of increasing evidence that exposure to this chemical may lead to adverse health effects in human and wildlife. As bisphenol A has one of the highest production volume of all chemicals produced globally by plastic industry [1], it is ubiquitous in the environment. During the production, usage and finally the

* Corresponding author at: Gdańsk University of Technology, Faculty of Chemistry, Department of Analytical Chemistry, G. Narutowicza 11/12 Street, 80-233 Gdańsk, Poland.
E-mail address: katowcza1@student.pg.edu.pl (K. Owczarek).

https://doi.org/10.1016/j.microc.2022.107643
Received 9 May 2022; Received in revised form 25 May 2022; Accepted 26 May 2022
Available online 30 May 2022

disposal of consumer products containing BPA, it is inevitably released into the environment and may be detected in a broad range of matrices i.e. water samples [2,3], sludge and sediment [4,5], and aquatic wildlife [6]. The widespread presence of BPA leads to a high risk of chronic human exposure to this chemical, which raises concerns due to its endocrine-disrupting properties [7–9], a role in causing metabolic (obesity, diabetics) and neurological disorders (autism spectrum and attention deficit hyperactivity disorders) [10–13].

Even though the topic of BPA has been investigated and well recognized in recent years, other bisphenol analogues also may exert adverse health effects, similar to these related to BPA. Whereas bisphenol A is still the most abundant xenobiotic, a total of 16 other bisphenols have been recognized as being used for various industrial applications [14,15]. The presence of BPA analogues is subject to governmental regulations in European Union, Canada, United States and some Asian countries like China, Malaysia or Philippines. Concerning production and usage of BPA, is also regulated by other authorities like i.e. European Food Safety Authority (EFSA) [16–18]. These regulations result in a gradual decrease of the number of applications or Total Daily Intake values established for BPA. That is the reason why there is a push to find a replacement for this substance for certain industrial branches [19,20].

With regard to the replacement of bisphenol A by other analogues, the second most abundant chemicals applied in the industry are bisphenol S (BPS), bisphenol F (BPF), bisphenol B (BPB) and bisphenol AF (BPAF). BPF and BPB are the alternative raw materials for the production of epoxy resins and polycarbonates, applied in the manufacturing of food contact materials. BPAF is used for the production of phenolic resins and in the synthesis of fluoroelastomers (as a crosslinker). BPF has also a broad range of applications and can be found in lacquers, varnishes, liners, adhesives plastics and water pipes. It is also used for some applications related to the healthcare, such as in dental sealants, oral prosthetic devices, tissue substitutes.

BPS is used in the production of in epoxy glues, as monomer in the synthesis of polythersulfosane and increasingly as an additive in thermal papers, substituting BPA as colour developer [14,21–24].

Other bisphenol analogues like bisphenol E (BPE), bisphenol M (BPM), bisphenol P (BPP) or bisphenol Z (BPZ) have also been reported in the literature as trace level environmental contaminants, found i.e. in water bodies, indoor dust, sediments or sludge, personal care products, food and beverages or biological samples [25–31]. Their origin is still not recognized, as the application of abovementioned chemicals is not specified in the literature. To the best of authors' knowledge, the only source that documents industrial application of other bisphenol analogues are some patents [32–36].

In recent years, performance enhancing dietary and nutritional supplements, dedicated for athletes and sports animals have attracted attention focused on verifying the accordance of the actual composition of the product with the composition declared by the manufacturer or testing these products for the substances illicit in sport competitions that may be considered as doping [37–39]. On the other hand the knowledge of the occurrence of xenobiotics or endocrine disruptors in such products is still scarce. Dietary supplements when used regularly, may be an additional potential long term exposure source of compounds altering the proper hormonal system function for sportsmen and sports animals. Bisphenols if present in such products, besides known effects on human organism mentioned before, may exert additional health problems taking under consideration that sportsmen body is constantly subjected to the greater than normal physical effort.

In the case of bisphenols analytics LC-MS and LC-MS/MS techniques are most frequently selected by specialists due to high sensitivity, selectivity and relatively easy sample preparation step [36,40–42]. Even though in recent years there has been a number of publications describing usage of gas chromatography for bisphenols determination, liquid chromatography is still the first choice for this topic. Although GC–MS technique requires derivatization step during sample preparation to enhance thermal stability and volatility of analytes, it is a very popular laboratory equipment in many laboratories because of its economic attractiveness. This in turn leads to procedures based on GC–MS technique as being highly desirable, especially for routine analyses.

Sample preparation approaches applied prior to bisphenols GC–MS analysis are dependant mainly on the matrix type. For biological samples (like breast milk) usually cleanup and concentration step is applied using solid phase extraction (SPE) [43]. Effective and clean sample preparation option is also using stationary phase microextration (SPME) coupled to direct thermal desorption of analytes from the fiber [44]. For urine analysis, as the matrix is mainly water, the application of dispersive liquid – liquid microextration (DLLME) is very effective and eco-friendly miniaturized extraction technique allowing to achieve satisfactory enrichment factors [45]. For foodstuffs such as canned meat a combination of QeChERS (quick, easy, cheap, effective, rugged, and safe) extraction and DLLME with simultaneous derivatization with acetic anhydride in DLLME has been recently reported [46].

Taking all into consideration, the aim of this study is focused on developing and validating an easy, fast and sensitive chromatographic method for identification and quantitative determination of six most frequently occurring bisphenols – BPA, BPE, BPF, BPS, BPP and BPZ in the samples of dietary supplements dedicated to sportsmen and sports animals.

2. Material and methods

2.1. Standards and reagents

Analytical standards of BPA (CAS no. 80–05-7, purity 99.0%), BPE (CAS no. 2081–08-5, purity 99%), BPF (CAS no. 620–92-8, purity 98.9%), BPP (CAS no. 2167–51-3, purity 99.9%), BPS (CAS no. 80–09-1, purity 99.0%), BPZ (CAS no. 843–55-0, purity 99.4%), were purchased from Sigma-Aldrich (St. Louis, USA). Internal standard (ISTD) 13C-labeled BPF (ring-^{13}C12) (CAS no. 1410794–08-9, purity 99.0%) was supplied by Cambridge Isotope Laboratories Inc. (UK).

Diethyl ether, methanol (MeOH), ethyl acetate, acetonitrile (ACN), *n*-pentane, used during the sample preparation procedure, were LC-MS grade, obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water was produced by the Milli-Q Gradient A10 system equipped with an EDS-Pak cartridge for removing endocrine disrupting compounds (Merck-Millipore). N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Wherever possible, the glassware was used to avoid contamination of samples with bisphenols during the sample preparation procedure. Any plastic consumables had been washed with mixture of MeOH: ACN (1:1 *v/v*) twice before use.

2.2. Stock and working solutions, calibrators and quality control samples

Stock solutions of each respective bisphenol were prepared by dissolving accurately weighted solid certified standard and dissolving in MeOH to obtain concentration of 1 mg/mL. Working solutions were prepared as a mixture of analytes, by diluting stock solutions with MeOH to concentrations of 100 μg/mL and 10 μg/mL. A solution of 13C-labeled BPF at concentration of 100 μg/mL in pure MeOH was used as ISTD. All stock and working solutions were stored at −20 °C prior to the analysis.

Calibration solutions (n = 3) were prepared by dissolving in water an accurately weighted amount of blank supplement sample (50 mg) and then spiked with an appropriate volumes of analyte working solutions to obtain concentrations of 50, 100, 200, 500, 1000 ng/mL for all of the analytes with the addition of 10 μL ISTD solution. More detailed information on the calibrants and sample preparation procedures is given in the paragraph 2.4. All of the calibration curves were prepared on a daily basis, prior to analysis and prepared according to the sample

preparation procedure, then analyzed by GC–MS system.

For evaluation of precision and accuracy quality control (QC) samples were prepared at three concentration levels (n = 3) in the range of calibration curves: 100, 500 and 1000 ng/mL.

2.3. Real samples

Fifteen different dietary supplements were obtained at local gym stores and analyzed. The nominal compositions of the tested samples are given in the Supplementary Table 1 in the electronic supplementary materials.

2.4. Sample preparation

2.4.1. Calibrants and QC samples

50 mg of blank matrix was accurately weighted to the 15 mL test tube, dissolved with 1 mL of water and vortexed (1 min). Appropriate volume of the stock solution containing bisphenols mixture and 10 μL of ISTD solution was added to each tube, the sample was vortexed and left for 15 min to allow the internal standard and analytes stabilize.

Then, 2 mL of diethyl ether was added, each sample was vortexed (2 min) and shaken automatically with laboratory shaker (6 rpm, 15 min) to enhance the extraction process. Samples were centrifuged (5 min, 1500 × g) to accelerate phase separation. 1 mL of the upper organic phase (diethyl ether) was collected and evaporated to dry under gentle nitrogen stream. 50 μL of MSTFA was added to the dry residue, samples were thermostated in 60 °C and derivatization process was conducted for 45 min. All calibrants were prepared in triplicates, while for QC samples each concentration, namely, 50 ng/mL (low QC), 500 ng/mL (medium QC) and 1000 ng/mL (high QC) was prepared in six independent repetitions.

2.4.2. Double blank solution

Taking into consideration that bisphenols, especially BPA, may be present in the laboratory environment, e.g. in the plasticware used for the samples preparation, a double blank sample was prepared to determine possible background.

1 mL of MilliQ pure water was transferred to the 15 mL test tube and vortexed (1 min). Then, 2 mL of diethyl ether was added, sample was vortexed (2 min) and shaken automatically with laboratory shaker (6 rpm, 15 min) to enhance the extraction process. Samples were centrifuged (5 min, 1500 × g) to accelerate phase separation. 1 mL of the upper organic phase (diethyl ether) was collected and evaporated to dry under gentle nitrogen stream. 50 μL of MSTFA was added to the dry residue, samples were thermostated in 60 °C and derivatization process was conducted for 45 min. Blank samples were prepared in triplicates.

2.4.3. Blank solution

Blank solution was prepared to verify is there are no interferences originating from matrix.

50 mg of blank matrix was accurately weighted to the 15 mL test tube, dissolved with 1 mL of water and vortexed (1 min). 10 μL of ISTD solution was added, the sample was vortexed and left for 15 min to allow the internal standard stabilize.

Then, 2 mL of diethyl ether was added, each sample was vortexed (2 min) and shaken automatically with laboratory shaker (6 rpm, 15 min) to enhance the extraction process. Samples were centrifuged (5 min, 1500 × g) to accelerate phase separation. 1 mL of the upper organic phase (diethyl ether) was collected and evaporated to dry under gentle nitrogen stream. 50 μL of MSTFA was added to the dry residue, samples were thermostated in 60 °C and derivatization process was conducted for 45 min. All calibrants were prepared in triplicates, for QC samples each concentration, namely, 50 ng/mL (low QC), 500 ng/mL (medium QC) and 1000 ng/mL (high QC) was prepared in six independent repetitions.

Table 1
SST results.

Analyte	RSD of A/A_{ISTD} (n = 6) [%]
BPA	2.77
BPE	2.44
BPF	2.42
BPP	2.27
BPS	1.04
BPZ	3.17

2.4.4. Reference solution a (500 ng/mL)

1 mL of pure water was transferred to the 15 mL test tube and the appropriate volume of the stock solution containing bisphenols mixture and 10 μL of ISTD solution was added, the sample was vortexed (1 min) and left for 15 min to allow the internal standard and analytes stabilize.

Then, 2 mL of diethyl ether was added, sample was vortexed (2 min) and shaken automatically with laboratory shaker (6 rpm, 15 min) to enhance the extraction process. Samples were centrifuged (5 min, 1500 × g) to accelerate phase separation. 1 mL of the upper organic phase (diethyl ether) was collected and evaporated to dry under gentle nitrogen stream. 50 μL of MSTFA was added to the dry residue, samples were thermostated in 60 °C and derivatization process was conducted for 45 min. Blank samples were prepared in triplicates.

2.4.5. Reference solution B (500 ng/mL)

For the method optimization purpose.

50 μL of the stock solution 10 μg/mL was diluted with 950 μL of the MeOH to obtain concentration of 500 ng/mL, then evaporated to dry and 50 μL of MSTFA was added to the dry residue. The solution was thermostated in 60 °C and derivatization process was conducted for 60 min.

2.4.6. Test samples

50 mg of the supplement powder was accurately weighted to the 15 mL test tube, dissolved with 1 mL of water and vortexed (1 min). 10 μL of ISTD solution was added, the sample was vortexed and left for 15 min to allow the internal standard stabilize.

Then, 2 mL of diethyl ether was added, each sample was vortexed (2 min) and shaken automatically with laboratory shaker (6 rpm, 15 min) to enhance the extraction process. Samples were centrifuged (5 min, 1500 × g) to accelerate phase separation. 1 mL of the upper organic phase (diethyl ether) was collected and evaporated to dry under gentle nitrogen stream. 50 μL of MSTFA was added to the dry residue, samples were thermostated in 60 °C and derivatization process was conducted for 45 min.

2.5. Optimized GC–MS conditions

The GC–MS analyses were performed on a GC System 6890 coupled with a 5973 MS (Agilent Technologies, Santa Clara, CA, USA). A ZB-5MSi column (30 m × 0.25 mm × 0.25 μm; Zebron, Phenomenex, Barcelona, Spain) was used for chromatographic separation.

The following oven temperature program was applied: 120 °C to 250 °C at rate 20 °C/min, then to 310 °C at rate 5 °C/min (held 2 min), with a total run time of 20.5 min. Helium (99.9995%) was used as a carrier gas (flow rate 2.0 mL/min). The injection port temperature was set at 250 °C, and the injection volume was 2 μL. MSD transfer line temperature was set at 250 °C. The splitless mode was applied. The MS was operated in selected ion monitoring (SIM) mode. The EI ion source temperature was set at 250 °C, electron energy was set at 70 eV. The analytes were identified on the basis of characteristic peaks for ions at *m/z* 357.2 at the retention time of 7.310 min (BPA); at *m/z* 343.2 and 358.2 at the retention time of 7.119 min (BPE), at *m/z* 329.2 and 344.2 at the retention time of 6.981 min (BPF), at *m/z* 475.2 and 490.2 at the retention time of 13.953 min (BPP); at *m/z* 379.0 and 394.1 at the

retention time of 10.478 min (BPS); at *m/z* 369.1 and 412.2 at the retention time of 9.864 min (BPZ); and the ISTD peak was monitored for ions at *m/z* 341.2 and 356.2 at the retention time of 6.981 min.

2.6. Method validation

Validation of an analytical method is performed to determine if the performance characteristic of given procedure meets the requirements of the intended analytical applications.

Here, the validation tests were carried out according to the developed method described in detail in the paragraphs 2.2–2.5. In order to validate GC–MS method for determination of bisphenols A, E, F, P, S and Z content in dietary supplements, the following method parameters were checked:

2.6.1. Carry-over, specificity and system suitability (SST)

To verify specificity double blank and blank solutions, reference solution of bisphenols at 500 ng/mL (injected six times) were analyzed. Acceptance criterion for SST was relative standard deviation (RSD) calculated from six injections of reference solution 500 ng/mL should be less than 10%. For carry over effect verification blank solution was injected after the highest concentration standard. In accordance to the FDA guidelines, acceptance criterion adapted for carry over effect was that the analyte peak area in the chromatogram of the blank sample should not exceed 20% of the peak area in the lowest calibrator chromatogram.

2.6.2. Linearity and range, limit of detection (LOD), limit of quantitation (LOQ)

Linearity test was performed using matrix-matched 6-point calibration curves. A sample pre-checked for the presence of any of the analytes was used as the matrix and spiked with bisphenols. All calibration samples were prepared according to the procedure given in the paragraph 2.4. Calibration curves were constructed plotting the ratio of the analyte response and response of the internal standard versus analyte concentration. Method linearity was verified within the range of 50–1000 ng/mL for each bisphenol.

2.6.3. Recovery, accuracy and precision

Theoretical recovery (trueness) of the method was evaluated by calculating the ratio of pre-extraction A/A_{ISTD} value to the post-extraction A/A_{ISTD} value. Accuracy was verified at concentration levels of 100 ng/mL, 500 ng/mL and 1000 ng/mL. Pre-extraction samples of blank matrix were spiked with the analytes before extraction process. Post-extraction samples of blank matrix were spiked with the analytes after the extraction. In both cases internal standard was added to the samples after the extraction process, to compensate the MS detector discrepancies. Then, the samples were derivatized as described in paragraph 2.5.

To evaluate accuracy and precision, QC samples at the concentration levels of 100 ng/mL, 500 ng/mL and 1000 ng/mL were prepared in 6 independent repetitions each. Then, the concentration was calculated against the calibration curve and precision was expressed as the RSD [%] value. Accuracy was calculated against expected concentration.

Precision was demonstrated within a single run and between runs analyzed on the three different days.

3. Results and discussion

3.1. Method optimization

To optimize GC method, instrumental parameters such as oven temperature gradient program, injection mode, injection volume and carrier gas flow were evaluated. The described conditions in the section *2.5 GC–MS conditions* provided the best sensitivity and selectivity at shorter analysis time.

3.1.1. Optimization of the extracting solvent

Four organic solvents immiscible with water were tested to select the optimal one: ethyl acetate, diethyl ether, hexane and dichloromethane. Samples were prepared as given in the paragraph 2.4 for Reference solution A at the concentration of 500 ng/mL. All the results were presented as [%] of recovery in relation to reference solution B 500 ng/mL (2.4.). Graphical representation of the extracting solvent optimization is given in the Fig. 1.

Recoveries obtained for dichloromethane and diethyl ether were in most cases comparable. Diethyl ether was eventually chosen as the optimal one, because as an organic solvent which is lighter than water, it was easier to collect than DCM.

3.1.2. Optimization of derivatization process

During the pre-elimination studies silylation and acylation of the target compounds were checked. Due to the presence of an active hydrogen in –OH groups in the target molecules both way may be suitable. Acylation enhances GC performance as the analyte volatility is increased, moreover chromatography and peak shapes may be improved because of reduced surface adsorption. Some acylated derivatives are more stable than corresponding silylated compounds. On the other hand sylilation is versatile derivatization tool for the GC–MS purposes leading to, as well as acylation, increase of volatility and thermal stability of the analytes.

MSTFA was selected for the sylilation and acetic anhydride was chosen for acylation. The quality of the chromatography was similar. Using acetic anhydride was more labour and time -consuming procedure. Using MSTFA time necessary to obtain full derivatization was 45 min, for acetic anhydride it was 2.5 h. Additionally, responses obtained with MSTFA were significantly higher. The data concerning optimization of derivatization agent is depicted in the Fig. 2a and 2b.

3.1.3. GC – MS optimization

To achieve the best instrument performance expressed in the sensitivity GC–MS parameters were optimized. For the evaluation of the characteristic ions, each of the certified standard solutions after derivatization was introduced to the detector in full scan mode in the range of 100 – 700 *m/z* using standard equipment parameters, and most abundant ion was chosen for each bisphenol. Various chromatographic conditions combinations were tested, including injector temperature, temperature gradient optimization and gas flow rate to achieve satisfactory analytes separation. Optimized conditions are given in the paragraph 2.5.

The optimization of mass detector parameters is also crucial for achieving good ion intensity. In this study ion source temperature was optimized in the way of testing temperature range of 220 – 270 °C using 10 °C intervals. Temperature of 250 °C provided the best sensitivity for all six analytes and was a compromise between the best peak shapes and bisphenols fragmentation degree in the MS source.

3.2. Validation results

3.2.1. Selectivity, system suitability and carry over

Acceptance criteria for specificity is absence of interfering peaks in the analytes retention times in chromatograms of blank and double blank samples.

For double blank sample (no matrix and no ISTD) neither analyte peaks nor interference of ISTD were observed, which indicate that bisphenols that may be present in the laboratory glassware and plasticware do not transfer to the sample during extraction process. For blank sample (blank matrix and ISTD) no analyte peaks were observed in the chromatograms (Fig. 3). Therefore it may be stated that matrix constituents or reagents do not interfere with target compounds. RSD for all analytes calculated from six following standard injections were less than 10%. This SST check is at the same time an injection precision test. No carry over effect was observed for the blank sample analyzed after

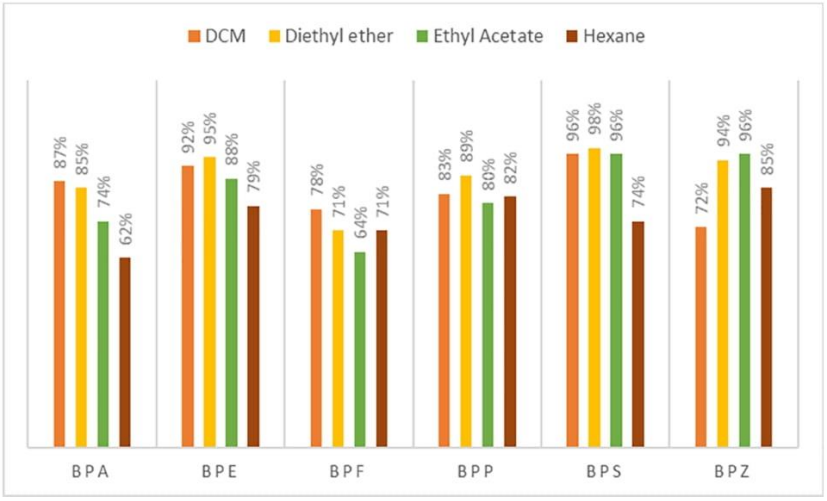


Fig. 1. Representation of extracting solvent optimization.

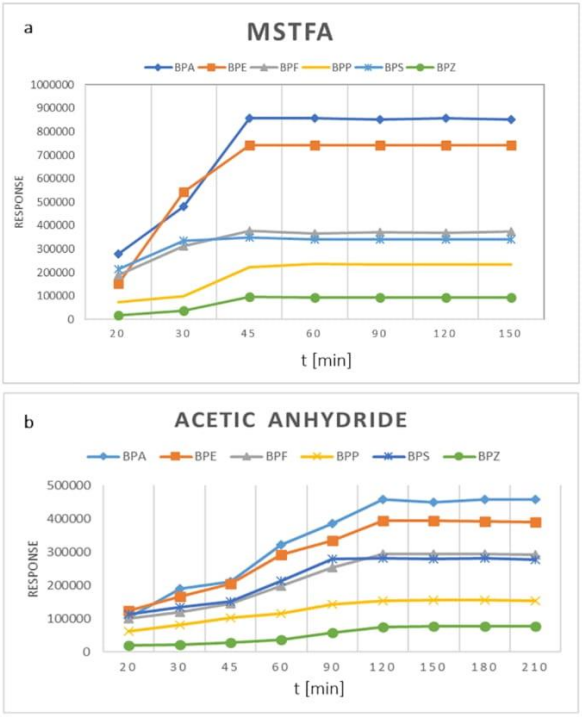


Fig. 2. Charts plotting the time needed for derivatization with analytes' responses for the process carried out with MSTFA agent (a) and acetic anhydride (b).

the 1000 ng/mL standard injection. Specificity and SST criteria were met. Injection precision (SST) results are summarized for each analyte in the Table 1.

3.2.2. Linearity, limit of detection, limit of quantitation.

The developed method is shown to be linear within the range of 50–1000 ng/mL for each analyte. The exemplary chromatogram of calibration solution is given in the Fig. 4.

The LOD values were calculated on the basis of the linearity testing results, as standard error of estimate y for × (S_{yx}). For LOD calculation following equation (Eq.1) was used:

$$LOD = (3.3 \times S_{xy})/a \quad (1)$$

where:

S_{xy} – standard deviation of calibration curve intercept,

a – calibration curve slope.

The LOQ was assumed as the lowest point of the calibration curves and unified as 50 ng/mL for all analytes, which gives 1 ng/mg of a sample.

The six-point calibration curves constructed by plotting relative ratio of analyte and internal standard areas to analyte concentration were prepared. The last square regression weighting (1/x) was applied for all curves to achieve better fit, improving an accuracy, especially at the low ends of the concentration range. All of the obtained calibration curves were characterized by good correlation coefficient R^2, which was above 0.990 for each analyte. Relative standard deviation for regression slope was calculated on the basis of 3 independent repetitions of calibration curves and in all cases it did not exceed the value of 10% which was adopted as acceptance criterion. Achieved calibration parameters meet the acceptance criteria according to international guidelines provided by ICH, FDA or EMA.

Achieved LOD and LOQ values are satisfactory, taking under consideration simple sample preparation procedure that does not include any analyte enrichment e.g. solid phase extraction (SPE). Single quadrupole mass detection does not have as much sensitivity as tandem mass spectrometry, thus ultra-trace level analysis is not possible. For that reason SPE is a common but laborious sample preparation step in the case of GC–MS analysis. In this study relatively simple sample preparation, that might be used in routine dietary supplements analysis was a goal. More detailed information on the method linearity characteristics is given in the Table 2.

3.2.3. Accuracy, precision and recovery

The accuracy and precision parameters were tested as within run and between-run assays (intra and interday). Between run experiments were performed on three different days. Obtained values were within the range 0.5–9.6% for precision and 93.7–103.1% for accuracy and meet the acceptance criteria set as ± 15% for both. The investigated method is sufficiently accurate and precise. At the same time, the trueness of the developed procedure, expressed as recovery, was in the range of 78.8–100.4%. The lowest recoveries were observed for bisphenol Z and

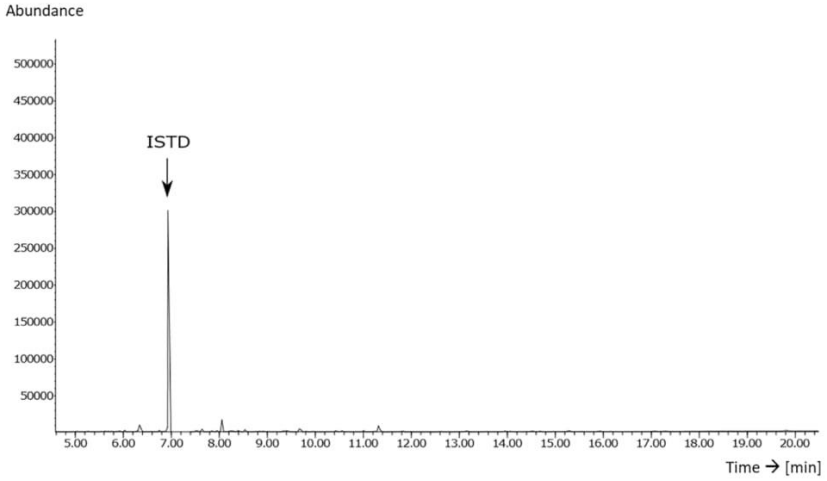


Fig. 3. The chromatogram obtained for blank solution.

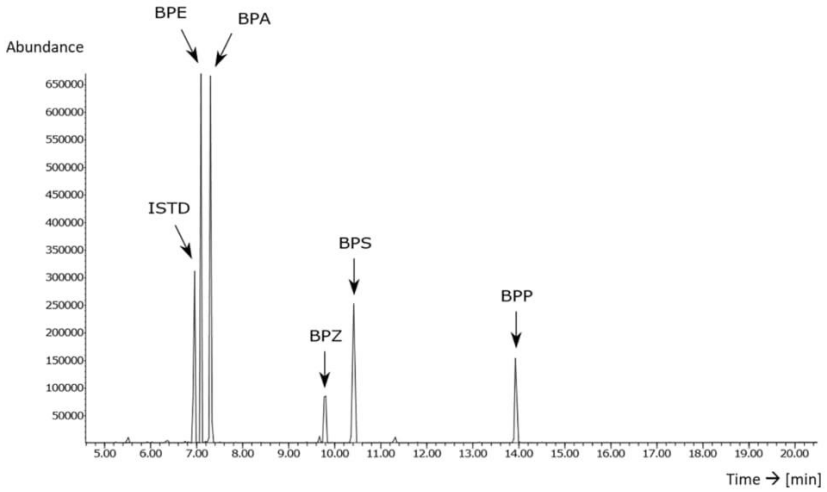


Fig. 4. The chromatogram obtained for the calibration solution containing standards at the 500 ng/mL level.

Table 2
A summary of the linearity test results.

Analyte	R^2	Slope RSD [%]	LOD [ng/mL]	LOD [ng/mg]
BPA	0.9992	0.50	8.73	0.17
BPE	0.9911	5.95	4.60	0.09
BPF	0.9989	0.56	10.01	0.20
BPP	0.9989	2.96	15.44	0.31
BPS	0.9983	0.90	11.43	0.23
BPZ	0.9992	3.86	13.43	0.27

bisphenol P (78.8–80.1% and 86.2 – 90.1%, respectively). That might be caused by the chemical structures of these compounds which differs from other tested bisphenols by the presence of other constituents in the BPP and BPZ molecules. BPP contains more aromatic rings and is similar to doubled bisphenol A molecule. For BPZ cyclohexane moiety is present in the molecule. These differences may affect molecules' polarity resulting in worsened diffusion to the extracting solvent. Maximum RSD for recovery was 3.8% and it was observed also in the case of BPP and BPZ. Despite this, RSD values were all below 10% thus results indicate that the method is reproducible. All accuracy, precision and trueness results were described in detail in the Table 3.

3.3. Real samples analysis

Validated analytical procedure was used for real samples of sport dietary supplements analysis for bisphenols content quantitation. Obtained results are summarized in the Table 4, and calculated in relation to tested substance. All the values given in the Table 4 below 1 ng/mg are smaller than limit of quantitation but are higher than limit of detection. They can be detected in the sample, but given numerical values are only estimated by extrapolation of calibration curve. The highest content may be noted for the most ubiquitous bisphenol A. BPA was quantitated in five out of 15 samples and detected in two other samples.

BPS was detected in five out of 15 samples, BPP in three samples, BPE in two samples, BPF in one sample and BPZ was not detected in any samples. More detailed data representing the obtained results with standard deviation are given in the Table 4. The exemplary chromatogram of the real sample containing bisphenols is given in the Fig. 5.

The origin of the bisphenols present in the dietary supplements may be discussed. Taking under consideration that these compounds are

Table 3
Results obtained for accuracy, precision and trueness verification.

Concentration spiked [ng/mL]	BPA				BPE				BPF			
	Mean concentration calculated [ng/mL]	**Precision RSD [%]**	**Accuracy [%]**	**Recovery [%]**	**Mean concentration calculated [ng/mL]**	**Precision RSD [%]**	**Accuracy [%]**	**Recovery [%]**	**Mean concentration calculated [ng/mL]**	**Precision RSD [%]**	**Accuracy [%]**	**Recovery [%]**
Within run												
100	95.0	4.0	95.0	91.3 ± 1.2	93.7	2.0	93.7	94.2 ± 1.2	101.0	2.5	101.0	100.2 ± 3.1
500	494.5	1.2	98.9	96.4 ± 1.2	506.9	2.4	101.4	100.4 ± 1.1	495.3	1.9	99.1	98.1 ± 2.4
1000	1002.5	0.9	100.2	98.7 ± 1.7	1012.2	3.1	101.2	99.5 ± 2.0	1016.5	2.7	101.7	98.5 ± 1.5
Between run												
100	94.3	5.4	94.3		96.8	7.6	96.8		99.6	4.6	99.6	
500	498.9	2.8	99.8		502.2	2.7	100.4		499.5	2.4	99.9	
1000	1007.8	3.0	100.8		992.4	3.3	99.2		1010.9	2.0	101.1	
Concentration spiked [ng/mL]	**BPP**				**BPS**				**BPZ**			
	Mean concentration calculated [ng/mL]	**Precision RSD [%]**	**Accuracy [%]**	**Recovery [%]**	**Mean concentration calculated [ng/mL]**	**Precision RSD [%]**	**Accuracy [%]**	**Recovery [%]**	**Mean concentration calculated [ng/mL]**	**Precision RSD [%]**	**Accuracy [%]**	**Recovery [%]**
Within run												
100	97.3	4.7	97.3	86.2 ± 3.8	101.4	3.1	101.4	98.8 ± 1.3	96.2	4.8	96.2	79.1 ± 1.3
500	498.9	1.7	99.8	88.6 ± 3.2	495.0	1.7	99.0	97.6 ± 2.6	502.0	1.3	100.4	78.8 ± 1.6
1000	999.4	1.7	100.0	90.1 ± 0.8	1023.7	1.6	102.4	99.1 ± 2.6	1013.6	0.5	101.4	80.0 ± 3.6
Between run												
100	100.6	4.2	100.6		97.7	5.4	97.7		103.1	9.6	103.1	
500	499.7	1.7	99.9		498.6	2.0	99.7		499.2	2.4	99.8	
1000	997.3	3.2	99.7		1006.7	2.1	100.7		1012.2	2.4	101.2	

Table 4
Results of bisphenols' determination in the real samples.

Sample	Analyte concentration in relation to sample weight [ng/mg] ± standard deviation					
	BPA	BPE	BPF	BPP	BPS	BPZ
1	<LOD	0.362 ± 0.022	<LOD	<LOD	<LOD	<LOD
2	<LOD	0.320 ± 0.011	<LOD	<LOD	<LOD	<LOD
3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
5	1.1983 ± 0.0011	<LOD	<LOD	<LOD	<LOD	<LOD
6	1.049 ± 0.054	<LOD	<LOD	<LOD	<LOD	<LOD
7	2.892 ± 0.089	<LOD	<LOD	<LOD	<LOD	<LOD
8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
9	0.444 ± 0.017	<LOD	<LOD	<LOD	<LOD	<LOD
10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
11	1.863 ± 0.096	0.3308 ± 0.0041	<LOD	0.3287 ± 0.0099	0.369 ± 0.019	<LOD
12	1.10 ± 0.10	0.3174 ± 0.0084	0.2184 ± 0.0056	0.336 ± 0.028	0.334 ± 0.016	<LOD
13	0.852 ± 0.056	<LOD	<LOD	0.354 ± 0.029	0.326 ± 0.017	<LOD
14	<LOD	<LOD	<LOD	<LOD	0.396 ± 0.020	<LOD
15	<LOD	<LOD	<LOD	<LOD	0.464 ± 0.020	<LOD

broadly used for plastic production the most probable source is the packaging of the tested supplements but other sources like production process or raw materials contamination cannot be excluded.

Following other scientific publications concerning bisphenol A and its analogues determination in various matrices it can be observed that the BPA is the one most frequently present in the samples. At the same time it is not surprising taking under consideration that BPA is broadly used raw material in many industry branches. Its concentration varies significantly, depending on the sample type. Results presented in this study may be compared to the results obtained for canned foods or beverages. BPF and BPS that were introduced to the industry as bisphenol A replacement are the second most common analogues, present in the beverages. Other bisphenol analogues are detected in small amounts and are detected rarely [14]. To the best of authors knowledge there is no publication on the bisphenols content in sport dietary supplements so the direct comparison of the obtained results to other scientists' work is not possible. It is important to note that except for BPA, other bisphenols were detected below lowest calibration point so given concentrations are estimated to specify that they were present in the tested sample and indicative only.

4. Conclusions

The developed procedure allows to determine six bisphenol analogues in the dietary and nutritional sport supplements using small amount of the test sample (50 mg) and relatively small amount of the organic solvent for extraction (2 mL). The method was successfully validated, all test were performed in accordance with international guidelines. With all acceptance criteria concerning accuracy, precision, linearity and repeatability fulfilled it can be stated that the method is suitable for detection and quantitation of the bisphenol A, E, F, P, S and Z in the dietary supplements. It was successfully applied for determination of BPA analogues in real samples of 15 dietary supplements.

The proposed analytical procedure may be an alternative for a majority of the methods in the field of bisphenol determination based on an expensive liquid chromatography – tandem mass spectrometry, thus may be implemented by laboratories that are not equipped with LC-MS.

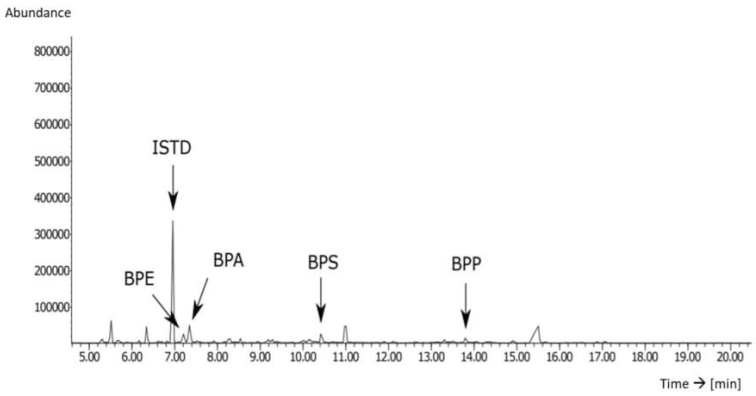


Fig. 5. The chromatogram obtained for the real sample solution.

Moreover, gas chromatography offers better separation efficiency and allows to avoid using significant amounts of organic solvents as mobile phases, so may be considered as more environmental friendly.

Presented methodology is easy, labour-saving and utilizes materials and reagents commonly used in almost every analytical laboratory. It offers quite low quantitation limit without including time-consuming SPE clean up. On the other hand derivatization step that is crucial for BPs determination with GC–MS technique is conducted using harmful and carcinogenic reagent, MSTFA, which requires careful handling. Above mentioned LOQ is satisfactory taking under consideration single quad MS, but for future perspective and samples containing trance levels of bisphenol analogues, it would be worth transferring to tandem mass spectrometry equipment to gain more selectivity and sensitivity.

Because the field of bisphenol analogues analytics has increased in last years due to law regulations becoming more strict, thus the development of new analytical tools for dietary products quality control seems to be needed and fully justified. Another future perspective in this topic might be developing qualitative screening methods for various matrices, detecting broader spectrum of bisphenols and its derivatives. Taking under consideration the fact, that a lot of studies indicate that BPA substitutes are not safer alternatives, this kind of analytical tool certainly would be valuable.

CRediT authorship contribution statement

Katarzyna Owczarek: Conceptualization, Methodology, Writing – original draft, Data curation, Visualization, Investigation. **Emilia Waraksa:** Validation, Data curation, Writing – original draft, Resources, Investigation. **Ewa Kłodzińska:** Resources, Writing – review & editing. **Yaroslav Zrobok:** Investigation. **Mariusz Ozimek:** Writing – review & editing. **Dominik Rachoń:** Writing – review & editing, Funding acquisition, Resources. **Błażej Kudłak:** Supervision, Writing – review & editing. **Andrzej Wasik:** Writing – review & editing. **Zofia Mazerska:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The work has been co-financed by the National Science Center, Poland, grant no 2014/15/B/NZ7/00999 (OPUS 8).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2022.107643.

References

[1] L.N. Vandenberg, R. Hauser, M. Marcus, N. Olea, W.V. Welshons, Human exposure to bisphenol A (BPA), Reprod Toxicol. 24 (2) (2007) 139–177.
[2] H. Jina, L. Zhu, Occurrence and partitioning of bisphenol analogues in water and sediment from liaohe river basin and taihu lake, China, Water Res. 103 (2016) 343–351.
[3] L. Renz, C. Volz, D. Michanowicz, K. Ferrar, C. Christian, D. Lenzner, T. El-Hefnawy, A study of parabens and bisphenol A in surface water and fish brain tissue from the Greater Pittsburgh Area, Ecotoxicol. 22 (4) (2013) 632–641.
[4] Y. Kitada, H. Kawahata, A. Suzuki, T. Oomori, Distribution of pesticides and bisphenol A in sediments collected from rivers adjacent to coral reefs, Chemosphere 71 (11) (2008) 2082–2090.
[5] Y.Q. Huang, C.K.C. Wong, J.S. Zheng, H. Bouwman, R. Barra, B. Wahlström, L. Neretin, M.H. Wong, Bisphenol A (BPA) in China: A review of sources, environmental levels, and potential human health impacts, Environ Int. 42 (2008) 91–99.
[6] N. Ismail, S. Wee, A. Aris, Bisphenol A and alkylphenols concentrations in selected mariculture fish species from Pulau Kukup, Johor, Malaysia, Mar. Pollut. Bull. 127 (2018) 536–540.
[7] M. Di Donato, G. Cernera, P. Giovannelli, G. Galasso, A. Bilancio, A. Migliaccio, G. Castoria, Recent advances on bisphenol-A and endocrine disruptor effects on human prostate cancer, Mol. Cell. Endocrinol. 457 (2017) 35–42.
[8] J.A. Rogers, L. Metz, V.W. Yong, Review: endocrine disrupting chemicals and immune responses: A focus on bisphenol-A and its potential mechanisms, Mol. Immunol. 53 (4) (2013) 421–430.
[9] M. Perrot-Applanat, M. Kolf-Clauw, C. Michelc, C. Beausoleil, Alteration of mammary gland development by bisphenol a and evidence of a mode of action mediated through endocrine disruption, Mol. Cell. Endocrinol. 475 (2018) 29–53.
[10] M.M. Stojanoska, N. Milosevic, N. Milic, L. Abenavoli, The influence of phthalates and bisphenol A on the obesity development and glucose metabolism disorders, Endocrine 55 (3) (2017) 666–681.
[11] C. Fang, B.o. Ning, A.B. Waqar, M. Niimi, S. Li, K. Satoh, M. Shiomi, T. Ye, S. Dong, J. Fan, Bisphenol A exposure induces metabolic disorders and enhances atherosclerosis in hyperlipidemic rabbits, J. Appl. Toxicol 35 (9) (2015) 1058–1070.
[12] C.S. Rosenfeld, Neuroendocrine disruption in animal models due to exposure to bisphenol A analogues, Front. Neuroendocrinol. 47 (2017) 123–133.
[13] T.P. Stein, M.D. Schluter, R.A. Steer, L. Guo, X. Ming, Guo L, Ming X, bisphenol a exposure in children with autism spectrum disorders, Autism Res. 8 (3) (2015) 272–283.
[14] D.a. Chen, K. Kannan, H. Tan, Z. Zheng, Y.-L. Feng, Y. Wu, M. Widelka, Bisphenol analogues other than bpa: environmental occurrence, human exposure, and toxicity - a review, Environ. Sci. Technol. 50 (11) (2016) 5438–5453.
[15] B. Baradie, M.S. Shoichet, Novel fluoro-terpolymers for coatings applications, Macromolecules 38 (13) (2005) 5560–5568.
[16] Efsa, Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA J. 13 (2015) 3978–4000.
[17] EFSA. 2012. Report of ESCO WG on non-plastic Food Contact Materials [Internet]. Available from: http://www.efsa.europa.eu/en/supporting/doc/139e.pdf.
[18] EFSA [Internet]. 2015b. Parma: European Food Safety Authority; Available from: http://www.efsa.europa.eu/de/corporate/pub/factsheetbpa150121.htm European Commission. 2011. Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food. Off. J. of the European Union 2011 vol.54 L12/1.

[19] C. Liao, F. Liu, Y. Guo, H.-B. Moon, H. Nakata, Q. Wu, K. Kannan, Occurrence of eight bisphenol analogues in indoor dust from the United States and several Asian countries: implications for human exposure, Environ Sci Technol. 46 (16) (2012) 9138–9145.
[20] A. Jurek, E. Leitner, Analytical determination of bisphenol A (BPA) and bisphenol analogues in paper products by GC-MS/MS, Food Addit. Contam. Part A 34 (7) (2017) 1225–1238.
[21] N. Cabaton, C. Dumont, I. Severin, E. Perdu, D. Zalko, M. Cherkaoui-Malki, M. C. Chagnon, Genotoxic and endocrine activities of bis(hydroxyphenyl)methane (bisphenol F) and its derivatives in the HepG2 cell line, Toxicology 255 (2009) 15–24.
[22] H. Gallart-Ayala, E. Moyano, M.T. Galceran, Analysis of bisphenols in soft drinks by on-line solid phase extraction fast liquid chromatography-tandem mass spectrometry, Anal. Chim. Acta. 683 (2011) 227–233.
[23] T. Geens, L. Goeyens, K. Kannan, H. Neels, A. Covaci, Levels of bisphemol-A inthermal paper receipts from Belgium and estimation of human exposure, Sci. tot. Environ. 435–436 (2012) 30–33.
[24] Y. Konno, H. Suzuki, H. Kudo, A. Kameyama, T. Nishikubo, Synthesis and properties of fluorine-containing poly(ether)s with pendant hydroxyl groups by the polyaddition of bis(oxetane)s and bisphenol AF, Polym. J. 36 (2004) 114–122.
[25] C. Liao, F. Liu, Y. Guo, H. Moon, B, Nakata H, Wu Q, Kannan K, Occurrence of eight bisphenol analogues in indoor dust from the United States and several Asian countries: implications for human exposure, Environ. Sci. Technol. 46 (2012) 9138–9145.
[26] S. Song, M. Song, L. Zeng, T. Wang, R. Liu, T. Ruan, G. Jiang, Occurrence and profiles of bisphenol analogues in municipal sewage sludge in China, Environ. Pollut. 86 (2014) 14–19.
[27] S. Lee, C. Liao, G.J. Song, K. Ra, K. Kannan, H. Moon, B, Emission of bisphenol analogues including bisphenol A and bisphenol F from wastewater treatment plants in Korea, Chemosphere 119 (2015) 1000–1006.
[28] C. Liao, K. Kannan, A survey of alkylphenols, bisphenols, and triclosan in personal care products from China and the United States, Arch. Environ. Contam. Toxicol. 67 (2014) 50–59.
[29] C. Liao, K. Kannan, Concentrations and profiles of bisphenol A and other bisphenol analogues in foodstuffs from the United States and their implications for human exposure, J. Agric. Food Chem. 61 (2013) 4655–4662.
[30] A.G. Asimakopoulos, J. Xue, B.P. De Carvallo, A. Iyer, K.O. Abualnaja, S. S. Yaghmoor, T. Kumosani, A, Kannan K, Urinary biomarkers of exposure to 57 xenobiotics and its association with oxidative stress in a population in Jeddah, Saudi Arabia. Environ. Res. 150 (2015) 573–581.
[31] K. Owczarek, P. Kubica, B. Kudłak, A. Rutkowska, A. Konieczna, D. Rachoń, J. Namieśnik, A. Wasik, Determination of trace levels of eleven bisphenol A analogues in human blood serum by high performance liquid chromatography-tandem mass spectrometry Sci, Total Environ. 628–629 (2018) 1362–1368.
[32] Hada T, Shimizu M, Aoki Y, Endo N, Ohhashi H, Ishiguro T, Heat-sensitive Recording Material and Method for Producing Same (Google Patents), 2013.
[33] B.-Y. Hsieh, J.-P. Hsu, Photosensitive Resin Composition, Color Filter and Method for Manufacturing the Same, and Liquid Crystal Display Apparatus (Google Patents) (2015).
[34] Ochiai T, Masuda T, Thermal Recording Material and Method for Producing the Same (Google Patents), 2013.
[35] Teichert D, Conrad L, Grötzinger J, Epoxy Resin-Upgraded Cement-Bound Composition as Coating or Seal (Google Patents), 2014.
[36] Zouta K, Nakao K, Takagi Y, Ishii H, Flame-Retardant Sheet or Film, Products Comprising the Same and Process for Production Thereof (Google Patents), 2014.
[37] E. Waraksa, K. Kowalski, R. Rola, E. Kłodzińska, T. Bieńkowski, J. Namieśnik, Determination of free tyrosine in equestrian supplements by LC–MS/MS and comparison of its quantity with total free amino acids content in view of doping control, Microchem. J. 146 (2019) 56, https://doi.org/10.1016/j.microc.2018.12.033.
[38] E. Waraksa, K. Kowalski, E. Kłodzińska, R. Rola, J. Ciekot, W. Filipiak, T. Bieńkowski, J. Namieśnik, A rapid and eco-friendly method for determination of the main components of gamma-oryzanol in equestrian dietary and nutritional supplements by liquid chromatography—Tandem mass spectrometry, J. Pharm. Biomed. Anal. 172 (2019) 339, https://doi.org/10.1016/j.jpba.2019.04.029.
[39] E. Waraksa, A. Pokrywka, M. Konop, J. Namieśnik, E. Kłodzińska, Comparison of GC–MS and MEKC methods for caffeine determination in preworkout supplements, Electrophoresis 40 (2019) 1540, https://doi.org/10.1002/elps.201800512.
[40] R. Silveira, B. Rocha, J.L. Rodrigues, F. Barbosa Jr, Rapid, sensitive and simultaneous determination of 16 endocrine-disrupting chemicals (parabens, benzophenones, bisphenols, and triclocarban) in human urine based on microextraction by packed sorbent combined with liquid chromatography tandem mass spectrometry (MEPS-LC-MS/MS), Chemosphere 240 (2020), 124951.
[41] X.-L. Cao, I. Kosarac, S. Popovic, S. Zhou, D. Smith, R. Dabeka, LC-MS/MS analysis of bisphenol S and five other bisphenols in total diet food samples, Food Addit. Contam. 36 (2019) 1740–1747.
[42] N. Venisse, G. Cambien, J. Robin, S. Rouillon, C. Nadeau, T. Charles, S. Rabouan, V. Migeot, A. Dupuis, Development and validation of an LC–MS/MS method for the simultaneous determination of bisphenol A and its chlorinated derivatives in adipose tissue, Talanta 204 (2019) 145–152.
[43] Y. Deceuninck, E. Bichon, P. Marchand, C. Boquien, A. Legrand, C. Boscher, J. P. Antignac, B.L. Bizec, Determination of bisphenol A and related substitutes/analogues in human breast milk using gas chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 407 (2015) 2485–2497.
[44] M. del Olmo, A. Zafra, B. Suarez, A. Gonzalez-Casado, J. Taoufiki, J. Vilchez, Use of solid-phase microextraction followed by on-column silylation for determining chlorinated bisphenol A in human plasma by gas chromatography-mass spectrometry, J. Chromatogr. B 817 (2005) 167–172.
[45] N. González, S.C. Cunha, C. Monteiro, O. Fernandes, M. Marques, J. Domingo, L, Nadal M, Quantification of eight bisphenol analogues in blood and urine samples of workers in a hazardous waste incinerator, Environ. Res. 176 (2019), 108576.
[46] S.C. Cunha, T. Inácio, M. Almada, R. Ferreira, J.O. Fernandes, Gas chromatography–mass spectrometry analysis of nine bisphenols in canned meat products and human risk estimation, Food Res. Int. 135 (2020), 109293.

SUPPLEMENTARY MATERIALS

Validated GC-MS method for determination of bisphenol A and its five analogues in dietary and nutritional supplements

Katarzyna Owczarek [a,b*], Emilia Waraksa [c], Ewa Kłodzińska [c,d], Yaroslav Zrobok [d], Mariusz Ozimek [e] Dominik Rachoń [f], Błażej Kudłak [a], Andrzej Wasik [a], Zofia Mazerska

Supplementary Table 1. Characteristics of investigated supplements.

Supplement name	Intended purpose	Composition declared on the label
Nox pro	Promoting increased endurance, the growth of muscles and focus on training.	Nutritional information: Total carbohydrates: 5 g; Sugars: 0 g; Vitamin B6: 2 mg; Vitamin B12: 2.8 mg; Sodium: 70 mg; Potassium: 145 mg; Calcium: 140 mg; Magnesium: 150 mg; Phosphorus: 400 mg Pump matrix: Citruline DL-Malate: 5000 mg; Beta Alanine: 2000 mg Power boost matrix: Taurine: 2000 mg; Caffeine Anhydrous: 300 mg; L-Tyrosine: 150 mg; Cayenne Pepper (95%): 50 mg; Black Pepper (95%): 50 mg; Bitter orange (98%): 30 mg Performance & endurance matrix: MEX HDP-SR™ (Hudroxypropyl Distarch Phosphate): 5000 mg; Core-Creat™ (Creatine HCL): 1000 mg; Tri-Creatine Malate: 1000 mg Electrolyte matrix: Sodium Bicarbonate; Di-Calcium Phosphate; Di-Sodium Phosphate; Di-Potassium Phosphate; Trisodium Citrate; Magnesium Citrate

Nox pump	Promoting increased energy levels.	Nutritional information: Protein: 0.0 g; Carbohydrate: 6.8 g (Of which sugars: 6.3 g); Fat: 0.0 g; Fibre: 0.1 g; Sodium: 0.2 g; Vitamin C (as Calcium Ascorbate): 250 mg; Vitamin B1 (as Thiamin Hydrochloride): 25 mg; Vitamin B3 (as Niacin and Niacinamide): 25 mg; Vitamin B5 (as Calcium D-Pantothenate): 100 mg; Vitamin B6 (with Co-enzyme B6): 10 mg; Vitamin B12 (as Methylcobalamin): 50 µg; Biotin (150 µg); Ultimate cell-volumizing matrix L-Arginine Alpha-Ketoglutarate (2:1); L-Citruline DL Malate (2:1); Choline Citrate; Taurine, L-Phenylalanine; Buffered Creatine Monohydrate; Phosphates, N-Acetyl L-Tyrosine; GlycoCarn™; Trimethylglycine; Ultimate pro-energy matrix Sugars (Fructose; Trehalose; Ribose); Guarana Seed Extract (Caffeine); Glucuronolactone; Maltodextrin; Caffeine; Grape Seed Extract; Ginger Root Extract; Vinca Minor L Extract (Vincamine); Crioceras Longiflorus Extract (Vinpocetone); Hunteria Ebumea P. Extract (Vinbumine)
Work it Lady	Reducing tiredness and fatigue. The product is intended for active women to use before physical activity.	Nutrition information: Niacin: 16 mg NE (100%); Vitamin B6: 0.7 mg (50%); Calcium: 120 mg (15%); Magnesium: 75 mg (20%); Chromium: 39 µg (97.5%); L-carnitine: 1g; Branched chain amino acids: L-leucine: 0.5 g; L-valine: 0.25 g; L-isoleucine: 0.25 g; L-tyrosine: 0.28 g; Beta alanine: 0.25 g; L-glutamine: 0.5 g; Glucuronolactone: 150 mg; Green tea extract: 100 mg (of which: EGCG: 55 mg; Caffeine (from all sources): 61.5 mg; Raspberry fruit extract: 70 mg)
Iron pump	Promoting increased efficiency and maximal muscular endurance.	The complex components of the action supporting the efficiency of CNS: L-taurine: 2000 mg; N-acetyl L-carnitine: 1000 mg; L-tyrosine: 1000 mg; phosphatidylcholine: 200 mg; magnesium: 140 mg; caffeine: 100 mg; Schzandrol A: 50 mg; Vitamin B1: 2.5 mg Matrix stimulating maximal muscular endurance: Beta-alanine: 5000 mg; Alpha ketoglutarate arginine: 5000 mg; citruline malate: 2500 mg; tricreatine malate: 1000 mg; L-norvaline: 200 mg; Vitamin B3: 30 mg; Vitamin B6: 2.5 mg; Vitamin B12: 10 µg
Cell pump	Promoting increased energy levels.	Nutrition information: Tricreatine malate: 2450 mg and Creatine monohydrate: 750 mg (of which: creatine: 2500 mg); L-arginine alpha-ketogluturate: 750 mg and L-arginine HCl: 620 mg (of which: L-arginine: 996 mg); Taurine: 500 mg; Caffeine: 145 mg; Niacin (mg NE): 16 mg (100% NRV)
BCAA	Enable faster recovery after exercises	rosemary, glutamine, leucine, isoleucine, carnitine, valine, citric acid, flavor, crude fat, raw protein, crude fiber, crude ash, moisture, sugars

Sedemag	exhibit sedative properties vitamin-rich preparation	sugar, dextrose, magnesium fumarate, magnesium oxide, linseed oil, crude protein, crude fat, crude ash, calcium, magnesium, tryptophan, vitamin B1, B2, B6, B12, niacin, folic acid, calcium D-pantothenate, manganese
Yarrowia Equinox	supports the immune system	crude protein, crude fat, crude fiber, moisture, ash, calcium, magnesium, phosphorus, sodium, iron,
Superjoint	enable faster recovery after injury or exercises	oil, protein, fibre, ash, moisture, glycine, glutamine, soya isolate, dextrose, calcium carbonate, mucopolysaccharides, sulphur, vitamin C, hyaluronic acid, boron, glucuronic acid, glutamic acid, vitamin B6, copper sulphate, alganine, arginine, cysteine, histidine, isoleucine, tryptophan, lysine, methionine,
Myostem mass	enhance performance enable faster recovery after excercise	potato protein, fenugreek, calcium carbonate, extract walls of specific yeasts molasses, L-histidine, leucine, L-phenylalanine, L-arginine, L-lysine
Dermasept	support the natural processes of skin regeneration	aqua, cooper gluconate, colloidal silver, arylates/ palmeth-25 acrylate copolymer, glycerin, propylene glycol, Peg-20 glyceryl laurate, allantoin, isopropyl alcohol, tea tree (melaleuca alternifolia), leaf oil, propolis cera, methylchloroisothiazolinone, methylisothiazolinone
Pro-Am Prep	source of concentrated hydrolyzed proteins enhance performance	hydrolysed vegetable protein, sodium chloride, arginine, leucine, isoleucine, tryptophan, threonine, histidine, methionine, phenylalanine, valine, lysine, glutamic acid, aspartic acid, glycine, proline, tyrosine, serine, alanine, cysteine
Redplex	stimulate the production of red blood cells improve the efficiency of the effort increase the immune ability vitamin-rich preparation	monopropylene glycol, potassium chloride, magnesium chloride, angelica, vitamin A, D3, E, B1, B2, B6, B12, choline chloride, pantothenic acid, folic acid, biotin, iron, zinc, copper, manganese, cobalt, selenium, iodine, propyl gallate
Muscle Toner	enhance performance enable faster recovery after exercises	extract from rice bran oil, gamma-oryzanol, creatine, citric acid
Excel muscle	enhance performance	stabilized rice bran, calcium, vitamin E, phosphorus

OŚWIADCZENIE WSPÓŁAUTORÓW PRACY ZESPOŁOWEJ WSKAZUJĄCE, CO STANOWI ICH WŁASNY WKŁAD

K. Owczarek, E. Waraksa, E. Kłodzińska, Y. Zrobok, M. Ozimek, D.Rachoń, B. Kudłak, A. Wasik, Z. Mazerska, *Validated GC–MS method for determination of bisphenol a and its five analogues in dietary and nutritional supplements,* Microchem. J. 180, 107643, 2022

Nazwisko współautora	Wkład	Podpis
K. Owczarek	Koncepcja pracy, metodologia, współudział w tworzeniu tekstu manuskryptu, przeprowadzenie eksperymentów, analiza wyników;	
E. Waraksa	Zaplanowanie walidacji wyników, analiza wyników, współudział w tworzeniu tekstu manuskryptu, przeprowadzanie eksperymentów;	
E. Kłodzińska	Zapewnienie zasobów, współudział w przygotowaniu odpowiedzi dla recenzentów	
Y. Zrobok	Udział w planowaniu części eksperymentalnej	
M. Ozimek	Współudział w przygotowaniu odpowiedzi dla recenzentów	
D. Rachoń	Współudział w przygotowaniu tekstu manuskryptu oraz odpowiedzi dla recenzentów, współudział w finansowaniu zasobów	
B. Kudłak	Nadzór nad projektem, udział w tworzeniu tekstu manuskryptu oraz przygotowaniu odpowiedzi dla recenzentów	
A.Wasik	Udział w tworzeniu tekstu manuskryptu oraz przygotowaniu odpowiedzi dla recenzentów	
Z. Mazerska	Nadzór nad projektem, udział w tworzeniu tekstu manuskryptu oraz przygotowaniu odpowiedzi dla recenzentów	

Podsumowanie

5. Podsumowanie

Cele postawione na etapie planowania niniejszej pracy doktorskiej obejmowały realizację podejścia bioanalitycznego, w którym stosuje się jako narzędzia analityczne biotesty, oraz podejścia instrumentalnego, gdzie w przypadku nowoczesnych technik analitycznych bazuje się na nowoczesnych metodach chromatograficznych sprzężonych z tandemową spektrometrią mas.

Podejście bioanalityczne stanowiło istotny wstęp do dalszych badań i miało na celu oszacowanie toksyczności i potencjału endokrynnego wybranych związków z grupy bisfenoli oddzielnie i jako mieszaniny substancji. W przypadku podejścia instrumentalnego nadrzędnym celem było opracowanie nowych procedur analitycznych, które musiały charakteryzować się wysoką czułością i selektywnością, tak aby umożliwić oznaczanie BPA i związków będących jego analogami w matrycach o skomplikowanym składzie - przede wszystkim w próbkach biologicznych.

W toku rozwoju projektu doktorskiego oba założone cele udało się zrealizować. W przypadku części bioanalitycznej, wykorzystując test Microtox® wyznaczono toksyczność ostrą wobec organizmów testowych indywidualnie dla każdego z wybranych analitów oraz dla mieszanin dwuskładnikowych bisfenoli. Ponadto przy użyciu testu XenoScreen YES/YAS oszacowano aktywność estrogenową, androgenową, antyestrogenową oraz antyandrogenową dla wszystkich związków, które stanowiły przedmiot badań. W oparciu o otrzymane wyniki wyznaczono wartości stężenia efektywnego EC_{50} hamującego bioluminescencję organizmów testowych dla każdego bisfenolu oraz dla ich mieszanin binarnych oraz wartości NOEC i LOEC dla każdego z badanych związków w kontekście ich aktywności hormonalnej (a także NOAEL i LOAEL). Określono również typ i charakter interakcji zachodzących w mieszaninach binarnych stosując różne narzędzia modelowania (analiza regresji, modele addycji stężeń i działań niezależnych, które zweryfikowano z użyciem parametru MDR). Podjęto także próbę wyznaczenia zależności struktura – toksyczność. Analizując otrzymane wyniki można było zauważyć, iż bisfenole inne niż BPA, np. BPE i BPC, wykazały większą aktywność endokrynną i charakteryzowały się dużo wyższą toksycznością. Wzajemne interakcje związków z grupy bisfenoli najlepiej wpisywały się w model numeryczny działań niezależnych.

W przypadku realizacji części drugiej, bazującej na narzędziach instrumentalnych opracowano i zwalidowano czułą, selektywną i relatywnie prostą w aplikacji procedurę analityczną opartą na technice LC-MS/MS. Została ona z powodzeniem zastosowana do oznaczenia śladowych stężeń BPA i jego dziesięciu analogów w rzeczywistych próbkach surowicy ludzkiej. Następnie metoda ta stała się narzędziem przy detekcji i ilościowym oznaczeniu BPA w badaniu skupionym na oszacowaniu korelacji pomiędzy zawartością BPA i testosteronu we krwi pacjentek cierpiących na zespół policystycznych jajników. By zaproponować mniej kosztowną i wymagającą niewielkiego nakładu pracy alternatywę do oznaczania bisfenoli techniką LC-MS/MS opracowano także procedurę analityczną, gdzie narzędziem

dla oznaczeń końcowych była chromatografia gazowa sprzężona ze spektrometrią mas. Metodę tę zastosowano do analizy prób odżywek i preparatów przeznaczonych dla sportowców. Pomimo zastosowania spektrometrii mas z pojedynczym kwadrupolem pracującym w trybie SIM, udało się uzyskać satysfakcjonujący poziom czułości metody. Naturalnie poziom ten nie może być porównywany z możliwościami jakie oferują urządzenia wyposażone w tandemowe spektrometry mas, natomiast procedura ta może być z powodzeniem wykorzystana na etapie analiz przesiewowych.

Przeprowadzone badania wykazały, że bisfenole obecne były w większości badanych próbek, ale na różnych poziomach stężeń. Najbardziej rozpowszechniony był BPA oraz jego najszerzej stosowane substytuty, czyli BPF i BPS. Niepokojący jednakże jest fakt, iż również inne związki z tej grupy obecne były przede wszystkim w próbkach surowicy ludzkiej. Wskazuje to na bieżącą i nieustanną ekspozycję człowieka na tego typu substancje, przenikające z elementów otaczającego nas środowiska. Coraz silniejsze przesłanki wskazują też na powiązanie podwyższonych poziomów stężeń bisfenoli z różnego typu schorzeniami, takimi jak zespół policystycznych janików czy rozwój nowotworów i cukrzycy.

Z punktu widzenia chemii analitycznej, substancje będące przedmiotem zainteresowania niniejszej pracy stanowią pewnego rodzaju wyzwanie. Z jednej strony ich zawartość w próbkach różnego pochodzenia, w tym biologicznych, jest śladowa - co samo w sobie wymaga odpowiedniego wyboru i optymalizacji zarówno samej procedury przygotowania próbek jak i warunków pracy układu chromatograficznego. Z drugiej zaś strony są to związki, a w szczególności dotyczy to bisfenolu A, które ze względu na niezwykle szerokie zastosowanie obecne są powszechnie również w sprzęcie laboratoryjnym i materiałach zużywalnych. Dlatego należy zachować szczególną ostrożność aby nie doszło do zanieczyszczenia próbki na jakimkolwiek etapie pracy, zaczynając od jej pobierania, aż po wykonanie oznaczeń końcowych.

Bisfenole stanowią charakterystyczną klasę substancji, która obecna jest w bardzo wielu elementach otoczenia człowieka i która od długiego czasu wzbudza kontrowersje, przenikając do świadomości społecznej. W świetle coraz bardziej restrykcyjnych uregulowań prawnych, dotyczących możliwości aplikacji BPA, przemysł poszukuje substytutów w postaci mniej toksycznych analogów tego związku. Elementem tych poszukiwań są wyniki niniejszej pracy. Chemia analityczna oferuje bowiem bogatą gamę narzędzi, które można zastosować celem identyfikacji potencjalnych źródeł narażenia, aktywności biologicznej oraz poziomów zawartości BPA i ich analogów w różnorodnych matrycach. Dlatego też nowoczesne procedury analityczne i bioanalityczne pomogą znaleźć odpowiedź na pytanie: czy analogi BPA są dla człowieka i środowiska „lepszą" alternatywą? Mając na uwadze powyższe, pozwalam sobie stwierdzić, że przedstawiona dysertacja zawiera liczne wytyczne i wskazówki umożliwiające uzyskanie odpowiedzi na to pytanie oraz pokazuje drogę jaką

inne ośrodki mogą podjąć, aby te informacje uzyskać w przypadku poszerzenia zakresu badanych związków w wielorakich próbkach chrakteryzujących się złożonym składem matryc.

Bibliografia

6. Bibliografia

[1] Global Industry Analysts Inc., Bisphenol A Global Market Trajectory & Analytics, Raport, 2021, dostęp 30.05.2022, dostępny pod adresem https://www.researchandmarkets.com/reports/1227819/bisphenol_a_global_market_trajectory_ and

[2] BS. Rubin, *J. Steroid. Biochem. Mol. Biol*., 127, 27, 2011

[3] L.A. Tse, P.M.Y. Lee, W.M. Ho, A.T. Lam, M.K. Lee, S.S.M. Ng, Y. He, K.S. Leung, J.C. Hartle, H. Hu, H. Kan, F. Wang, C.F. Ng, *Environ. Int.*, 107, 1, 2017

[4] Y.K. Leung, V. Govindarajah, A. Cheong, J. Veevers, D. Song, R. Gear, X. Zhu, J. Ying, A. Kendler M. Medvedovic, S. Belcher, S.M. Ho, *Endocr. Relat. Cancer*, 24, 365, 2017

[5] T.K. Jensen, V. Mustieles, D. Bleses, H. Frederiksen, F. Trecca, G. Schoeters, H.R. Andersen, P. Grandjean, H.B. Kyhl, A. Juul, N. Bilenberg, A.M. Andersson, *Environ. Res.*, 170, 398, 2019

[6] E. Den Hond, H. Tournaye, P. De Sutter, W. Ombelet, W. Baeyens, A. Covaci, B. Cox, T.S. Nawrot, N. Van Larebeke, T. D'Hooghe, *Environ. Int*., 84, 154, 2015

[7] J.L. Carwile, K.B. Michels, *Environ. Res*., 111, 825, 2011

[8] Y. Duan, Y. Yao, B. Wang, L. Han, L. Wang, H. Sun, L. Chen, *Environ. Pollut.*, 243, 1719, 2018

[9] US Environmental Protection Agency (USEPA) Bisphenol A. (CASRN 80-05-7) USEPA Integrated Risk Information System (IRIS), Washington, DC, 1993 Raport dostępny pod adresem: http://www.epa.gov/iris/subst/0356.htm , dostęp 30.05.2022

[10] EFSA Panel on Food Contact Materials & Aids Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs 13, 3978, 2015

[11] Raport Summary of Bisphenol A (BPA) Regulation (2nd Edition) (CPIE-018-13), dostęp 30.05.2022, dostępny pod adresem: https://www.mts-global.com/en/news_details.html?lang=en&id=122

[12] S.A. Vogel *Am. J. Public Health*, 99, S55, 2009

[13] T. Vasiljevic, T. Harner, *Sci. Total. Environ*., 789, 148013, 2021

[14] J-H. Kang F. Kondo, Y. Katayama, *Toxicology*, 226, 79, 2006

[15] T. Geens, L. Roosens, H. Neels, A. Covaci, *Chemosphere*, 76, 755, 2009

[16] E. Ribeiro, C. Ladeira, S. Viegas, *Toxics*, 5, 1, 2017

[17] L. Wang, Y. Zhang, Y. Liu, X. Gong, T. Zhang, H. Sun, *Environ. Sci. Technol*., 53, 7095, 2019

[18] J.C. Xue, W.B. Liu, K. Kannan, *Environ. Sci. Technol*., 51, 5279, 2017

[19] K. Ellzey, A Fire-resistant polymers containing bisphenol C and deoxybenzoin derivatives. University of Massachusetts Amherst, Rozprawa doktorska, 2004.

[20] R. E. Lyon, Fire Research Program Fire Safety Section AAR-422 FEDERAL AVIATION ADMINISTRATION W.J. Hughes Technical Center, Raport, 1998

[21] J.L. Jurs, J. M. Tour, *Polymer*, 44, 3709, 2003

[22] Z.K. Brzozowski, J. Kusmierek, S. Porejko, US Pat. 3,786,019, 1972.

[23] Z.K. Brzozowksi, Z. P. Grabowska-Rostek, *J. Makromol. Sci*., 16, 1207, 1981

[24] M. L. Ramirez, R. Walters, R. E. Lyon, E. P. Savitski, *Polym. Degrad. Stab*., 78, 73, 2002

[25] S. I. Stoliarov, P. R. Westmoreland, *Polymer*, 44, 5469, 2003

[26] A. Bauer, M. Thunga, K. Obusek, M. Akinc, M. R. Kessler, *Polymer*, 54, 3994, 2013

[27] M. Thunga, W. Y. Lio, M. Akinc, M. R. Kessler, *Compos .Sci. Technol*., 71, 239, 2011

[28] M. Thunga, A. Bauer, K. Obusek, R. Meilunas, M. Akinc, M. R. Kessler, *Compos .Sci. Technol*., 100, 174, 2014

[29] M. Thunga, M. Akinc, M. R. Kessler, *Express Polym. Lett*., 8, 336, 2014

[30] S.K. Jana, T. Kugita, S. Namba, *Catal. Lett*., 90, 3, 2003

[31] A. Usman, S. Ikhlas, M. Ahmad, *Toxicol. Lett*., 312, 222, 2019

[32] J.R. Rochester, A.L. Bolden, *Environ. Health Perspect.*, 123, 643, 2015
[33] L. Simon, S. Simon, *Macromolecules*, 41, 1310, 2008
[34] H. R. Kricheldorf, S. Böhme, G. Schwarz, C-L. Schultz, *J. Polym. Sci. A Polym. Chem.*, 43, 1248, 2005
[35] E. Baştürk, F. Şen, M. V. Kahraman, S. Madakbaş, *Polymer Bulletin*, 72, 1611, 2015
[36] R.R Mukhametov, A.A. Shimkin, E.V. Dolgova, Y.I. Merkulova, *Russ. J. Appl. Chem.*, 87, 1908, 2014
[37] R.R. Mukhametov, K.R. Akhmadieva, M.A. Kim, A.N. Babin, *Aviats. Mater. Tekhnol.*, 2012
[38] L.H. Wu, X.M. Zhang, F. Wang, C.J. Gao, D. Chen, J.R. Palumbo, Y. Guo, E.Y. Zeng, *Sci. Total. Environ*., 615, 87, 2018
[39] Strona internetowa OEHHA, dostępna pod adresem: https://oehha.ca.gov/chemicals/bisphenol-s-bps
[40] A.N. Al Balawi, N.A. Yusof, S. Kamaruzaman, F. Mohammad, H. Wasoh , K.F. Al Abbosh, H.A. Al-Lohedan, *Biomed. Res. Int*., 2019
[41] Y-T. Zhou, S-R. Sheng, C-C. Tang, C. Song, Z-Z. Huang, X-L. Liu, *High Perform. Polym.*, 30, 1203, 2018
[42] D. Chen, K. Kannan, H. Tan, Z. Zheng, Y.L. Feng, Y. Wu, M. Widelka, *Environ. Sci. Technol.*, 50, 5438, 2016
[43] G. Russo, F. Barbato, D.G. Mita, L. Grumetto, *Food Chem. Toxicol*., 131, 110575, 2019
[44] W. Wang, K.O. Abualnaja, A.G. Asimakopoulos, A. Covaci, B. Gevao, B. Johnson-Restrepo, T.A. Kumosani, G. Malarvannan, T.B. Minh, H.B. Moon, H. Nakata, R.K. Sinha, K. Kannan, *Environ. Int.*, 83, 183, 2015
[45] C.J. Catenza, A. Farooq, N.S. Shubear, K.K. Donkor, *Chemosphere*, 268, 129273, 2021
[46] A. Cecinato, P. Romagnoli, M. Perilli, C. Balducci, *Environ. Toxicol. Stud., J.* 1, 1, 2017
[47] C. Liao, F. Liu, Y. Guo, H.B. Moon, H. Nakata, Q. Wu, K. Kannan. *Environ, Sci. Technol.*, 46, 9138, 2012
[48] N.S. Graziani, H. Carreras, E. Wannaz, *Heliyon*, 5, 9138, 2019
[49] Y. Luo, W. Guo, H. H. Ngo, L.D. Nghiem, F. I. Hai, J. Zhang, S. Liang, X. C. Wang, *Sci. Total Environ.* 473–474, 619, 2014
[50] M. Česen, K. Lenarčič, V. Mislej, M. Levstek, A. Kovačič, B. Cimrmančič, N. Uranjek, T. Kosjek, D. Heath, M. S. Dolenc, E. Heath, *Sci. Total Environ.*, 616–617, 744, 2018
[51] J. Xue, K. Kannan, *Sci. Total Environ.*, 648, 442, 2019
[52] Q. Sun, Y. Wang, Y. Li, M. Ashfaq, L. Dai, X. Xie, C-P. Yu, *Environ. Pollut*., 225, 542, 2017
[53] D. Lalwani, Y. Ruan, S. Taniyasu, E. Yamazaki, N. J.I. Kumar, P. K.S. Lam, X. Wang, N. Yamashita, *Ecotoxicol. Environ. Saf.*, 200, 110718, 2020
[54] Z. Huang, J.-L. Zhao, Y-Y. Yang, Y-W. Jia, Q-Q. Zhang, C-E. Chen, Y-S. Liu, B. Yang, L. Xie, G-G. Ying, *Environ. Pollut*., 263, 114361, 2020
[55] K. Goeury, S. Vo Duy, G. Munoz, M. Prévost, S. Sauvé, *J. Chromatogr. A*, 1591, 87, 2019
[56] P. Šauer, H. Švecová, K. Grabicová, F. Aydın, T. Mackuľak, V. Kodeš, L.D. Blytt, L. Henninge, R. Grabic, H. Kroupová, *Sci. Total Environ.*, 751, 141801, 2021
[57] C. Liao, K. Kannan, *J. Agric. Food Chem.*, 61, 4655, 2013
[58] J.A. Adeyemi, M. Gallimberti, C.C. Olise, B.A. Rocha, C.O. Adedire, F. Barbosa, *Environ. Sci Pollut. Res*., 27, 37645, 2020
[59] N. González, S. C. Cunha, R. Ferreira, J. O. Fernandes, M. Marquès, M. Nadal, J. L. Domingo, *Food Chem. Toxicol*, 136, 110992, 2020
[60] I. Rebai, J.O. Fernandes, M. Azzouz, K. Benmohammed, G. Bader, K. Benmbarek, S.C. Cunha, *Environ. Res*., 202, 111666, 2020

[61] A.G. Asimakopoulos, J. Xue, B.P. De Carvalho, A. Iyer, K.O. Abualnaja, S.S. Yaghmoor, T.A. Kumosani, K. Kannan, *Environ. Res*., 150, 573, 2016
[62] Y. Chen, J. Fang, L. Ren, R. Fan, J. Zhang, G. Liu, L. Zhou, D. Chen, Y. Yu, S. Lu, *Environ. Pollut*., 238, 299, 2018
[63] A. Li, T. Zhuang, W. Shi, Y. Liang, C. Liao, M. Song, G. Jiang, *Sci. Total Environ*., 707, 136100, 2020
[64] D. Tan, J. Jin, L. Wang, X. He, C. Guo, X. Lu, J. Chen, *J. Sep. Sci*., 42, 991, 2019
[65] Y. Niu, B. Wang, Y. Zhao, J. Zhang, B. Shao, *J. Agri. Food Chem*., 65, 10452, 2017
[66] H. Jin, J. Xie, L. Mao, M. Zhao, X. Bai, J. Wen, T. Shen, P. Wu, *Environ. Pollut*., 259, 113779, 2020
[67] Y. Deceuninck, E. Bichon, P. Marchand, C.Y. Boquien, A. Legrand, C. Boscher, J.P. Antignac, B. Le Bizec, *Anal. Bioanal. Chem.*, 407, 2485, 2015
[68] B. Zhang, Y. He, H. Zhu, X. Huang, X. Bai, K. Kannan, T. Zhang, *Environ. Int.*, 136, 105407, 2020
[69] H.S. Chang, K.H. Choo, B. Lee, S.J. Choi, *J. Hazard. Mat*., 172, 1, 2009
[70] C.E. Purdom, P.A. Hardiman, V.J. Bye, N.C. Eno, C.R. Tyler, J.P. Sumpter, *Chem. Ecol*., 8, 275, 1994
[71] V.J. Kramer, S. Miles-Richardson, S.L. Pierens, J.P. Giesy, *Aquat. Toxicol*., 40, 335, 1998
[72] J.C. Gould, L.S. Leonard, S.C. Maness, B.L. Wagner, K. Conner, T. Zacharewski, S. Safe, D.P. McDonnell, K.W. Gaido, *Mol. Cell Endocrinol.*, 142, 203, 1998
[73] X. Zhang, H. Chang, S. Wiseman, Y. He, E. Higley, P. Jones, C.K. Wong, A. Al-Khedhairy, J.P. Giesy, M. Hecker, *Toxicol. Sci.*, 121, 320, 2011
[74] F. Stossi, M. J. Bolt, F.J. Ashcroft, J.E. Lamerdin, J. Melnick, R.T. Powell, R. D. Dandekar, M.G. Mancini, C.L. Walker, J.K. Westwick, M.A. Mancini, *Chem. Biol*., 21, 743, 2014
[75] A.K. Rosenmai, M. Dybdahl, M. Pedersen, A. van VugtLussenburg, E.B. Wedebye, C. Taxvig, A.M. Vinggaard, *Toxicol. Sci*., 139, 35, 2014
[76] J. Lin, L. Deng, M. Sun, Y. Wang, S. Lee, K. Choi, X. Liu, *Steroids*, 169, 108826, 2021
[77] J. Shen, Q. Kang, Y. Mao, M. Yuan, F. Le, X. Yang, X. Xu, F. Jin, *Ecotoxicol. Environ. Safe.*, 187, 24, 2020
[78] P. Radwan, B. Wielgomas, M. Radwan, R. Krasiński, A. Klimowska, D. Kaleta, J. Jurewicz, *Reprod. Toxicol.*, 96, 216, 2020
[79] J. Ao, X. Huo, J. Zhang, Y. Mao, G. Li, J. Ye, Y. Shi, F. Jin, S. Bao, J. Zhang, *Environ. Res.*, 204, 112293, 2022
[80] S. Akgül, U. Sur, Y. Düzçeker, A. Balcı, M.P. Kızılkan, N. Kanbur, G. Bozdağ, P. Erkekoğlu, E. Gümüş, B. Kocer-Gumusel, *Gynecol. Endocrinol*., 35, 1084, 2019
[81] M. Vahedi, A. Saeedi, S.L. Poorbaghi, M. Sepehrimanesh, M. Fattahi, *Environ. Sci. Pollut. Res.*, 23, 23546, 2016
[82] R. Mukhopadhyay, N.B. Prabhu, S.P. Kabekkodu, P.S. Rai, P. S. *Environ. Sci. Pollut. Res*., 29, 326311, 2022
[83] J. Jurewicz, J. Majewska, A. Berg, K. Owczarek, R. Zajdel, D. Kaleta, A. Wasik, D. Rachoń, *Environ. Pollut*., 272, 115962, 2021
[84] A.A. Shamhari, A. Z. Abd Hamid, S.B. Budin, N.J. Shamsudin, I.S. Taib, *Biomedicines*, 9, 1744, 2021
[85] P. Wisniewski, R.M. Romano, M.M.L Kizys, K.C. Oliveira, T. Kasamatsu, G. Giannocco, M.I. Chiamolera, M. R. Dias-da-Silva, M.R. M.A. Romano, *Toxicology*, 329, 1, 2015
[86] Y. Feng, Z. Jiao, J. Shi, M. Li, Q. Guo, B. Shao, *Chemosphere*, 147, 9, 2016
[87] H.F. Wang, M. Liu, N. Li, T. Luo, L.P. Zheng, X.H. Zeng, *Toxicol. Sci*., 152, 145, 2016
[88] X. Li, Z. Wen, Y. Wang, J. Mo, Y. Zhong, R.S. Ge, *Front. Endocrinol*., 11, 447, 2020

[89] D.D. Seachrist, K. Bonk, S.M. Ho, G.S. Prins, A.M. Soto, R.A. Keri, *Reprod. Toxicol*., 59, 167, 2016
[90] X-Y. Shi, Z. Wang, L. Liu, L-M. Feng, N. Li, S. Liu, H. Gao, *Chemosphere*, 185, 361, 2017
[91] Y.S. Kim, K.A. Hwang, S.H. Hyun, K.H. Nam, C.K. Lee, K.C. Choi, *Chem. Res. Toxicol.*, 28, 662, 2015
[92] A. Ptak, M. Hoffmann, I. Gruca, J. Barc, *Toxicol. Lett.*, 229, 357, 2014
[93] W. Huang, C. Zhao, H. Zhong, S. Zhang, Y. Xia, Z. Cai, *Environ. Pollut.*, 246, 697, 2019,
[94] P. Song, K. Fan, X. Tian, J. Wen, *Toxicol. in Vitro*, 54, 224, 2018
[95] S.M. Ho, R. Rao, S. To, E. Schoch, P. Tarapore, *Endocr. Relat. Cancer*, 24, 83, 2017
[96] M. Di Donato, G. Cernera, P. Giovannelli, G. Galasso, A. Bilancio, A. Migliaccio, G. Castoria, *Mol. Cell. Endocrinol*., 457, 35, 2017
[97] Strona internetowa WHO dostępna pod adresem https://www.who.int/health-topics/diabetes#tab=tab_1
[98] R. Rezg, S. El-Fazaa, N. Gharbi, B. Mornagui, *Environ. Int.*, 64, 83, 2014
[99] A. Shankar, S. Teppala, *J. Clin. Endocrinol. Metab*., 96, 3822, 2011
[100] M.K. Silver, M.S. O'Neill, M.R. Sowers, S.K. Park, *PLoS ONE,* 6, 26868, 2011
[101] R. Bhandari, J. Xiao, A. Shankar, *Am. J. Epidem.*, 177, 1263, 2013
[102] I.A. Lang, T.S. Galloway, A. Scarlett, W.E. Henley, M. Depledge, R.B. Wallace, D. Melzer, *JAMA*., 300, 1303, 2008
[103] J.L. Carwile, K.B. Michels, *Environ. Res.*, 111, 825, 2011
[104] Y. Duan, Y. Yao, B. Wang, L. Han, L. Wang, H. Sun, L. Chen, *Environ. Pollut*., 243, 1719, 2018
[105] W. Zhang, W. Xia, W. Liu, X. Li, J. Hu, B. Zhang, S. Xu, Y. Zhou, J. Li, Z. Cai, Y. Li, *Front. Endocrinol*., 10, 262, 2019
[106] S. Yue, J. Yu, Y. Kong, H. Chen, M. Mao, C. Ji, S. Shao, J. Zhu, J. Gu, M. Zhao *Environ. Int.*, 129, 59, 2019
[107] D. Melzer, N.E. Rice, C. Lewis, W.E. Henley, T.S. Galloway, *PloS ONE*, 5, 8673, 2010
[108] C.Y. Hu, B. Schottker, N. Venisse, F. Limousi, P.J. Saulnier, M. Albouy-Llaty, A. Dupuis, H. Brenner, V. Migeot, S. Hadjadj, *Environ. Sci. Technol*., 53, 9876, 2019
[109] D. Melzer, N.J. Osborne, W.E. Henley, R. Cipelli, A. Young, C. Money, C., P. McCormack, R. Luben, K-T. Khaw, N.J. Wareham, T.S. Galloway, *Circulation*, 125, 1482, 2012
[110] P.M. Lind, L. Lind, *Atherosclerosis*, 218, 207, 2011
[111] C.Y. Lin, F.Y. Shen, G.W. Lian, K.L. Chien, F.C. Sung, P.C. Chen, T.C. Su, *Atherosclerosis*, 241, 657, 2015
[112] X. Gao, J. Ma, Y. Chen, H.S. Wang, *Environ. Health Perspect.*, 123, 571, 2015
[113] A. Kasneci, J.S. Lee, T.J. Yun, J.J. Shang, S. Lampen, T. Gomolin, C.C. Cheong, L.E. Chalifour, *Toxicol. Sci.*, 159, 189, 2017
[114] Y-F. Zhang, C. Shan, Y. Wang, L.L. Qian, D.D. Jia, Y-F. Zhang, X-D. Hao, H-M. Xu, *Sci. Total Environ*., 723, 137952, 2020
[115] A. Mendy, P.M. Salo, J. Wilkerson, L. Feinstein, K.K. Ferguson, M.B. Fessler, P.S. Thorne, D.C. Zeldin, *Environ. Res*. 183, 108944, 2020
[116] D. Rebolledo-Solleiro L.Y. Castillo Flores, H. Solleiro-Villavicencio, *Front. Biosci.,* 26, 363, 2021
[117] K. Yolton , Y. Xu, D. Strauss D, M. Altaye, A.M. Calafat, J. Khoury, *Neurotoxicol. Teratol.,* 33, 558, 2011
[118] H. Inadera, *Int. J. Med. Sci*., 12, 926, 2015
[119] S.F. Evans, R.W. Kobrosly, E.S. Barrett, S.W. Thurston, A.M. Calafat, B. Weiss, R. Stahlhut, K. Yolton, S.H. Swan, *Neurotoxicol.,* 45, 91, 2014

[120] L. Cobellis, N. Colacurci, E. Trabucco, C. Carpenteiro, L. Grumetto, *Biomed. Chromatogr.*, 23, 1186, 2009
[121] A. Moral, M.D. Sicilia, S. Rubio, D. Perez-Bendito, *J. Chromatogr. A*, 1100, 8, (2005)
[122] N. Caballero-Casero, L. Lunar, S. Rubio, *Anal. Chim. Acta*, 908, 22, 2016
[123] A. Goodson, W. Summerfield, I. Cooper, *Food Addit. Contam.*, 19, 796, 2002
[124] N. Casajuana, S. Lacorte, *J. Agric. Food Chem.*, 52, 3702, 2004
[125] L. Grumetto, D. Montesano, S. Seccia, S. Albrizio, F. Barbato, *J. Agric. Food Chem*., 56, 10633, 2008
[126] P. Vinas, N. Campillo, N. Martínez-Castillo, M. Hernandez-Cordoba, *Anal. Bioanal. Chem*., 397, 115, 2010
[127] J.I. Cacho, N. Campillo, P. Vinas, M. Hernandez-Cordoba, *J. Chromatogr. A*, 1247, 146, 2012

[128] A. Quigley, W. Cummins, D. Connolly, J. Chem., 2016
[129] B.A. Rocha, B.R.B. da Costa, N.C.P. de Albuquerque, A.R.M. de Oliveira, J.M.O. Souza, F. Barbosa, *Talanta*, 196, 511, 2016
[130] N. González, S.C. Cunha, C. Monteiro, J.O. Fernandes, M. Marques, J.L. Domingo, M. Nadal, *Environ. Res*., 176, 108576, 2019
[131] C. Huang, J. Yang, J. Ma, W. Tan, L. Wu, B. Shan, S. Wang, J. Chen, Y. Li, *Microchem. J*., 180, 107536, 2022
[132] L.K. Sosvorova, T. Chlupacova, J. Vitku, M. Vlk, J. Heracek, J., L. Starka, D. Saman, M. Simkova, R. Hampl, *Talanta*, 174, 21, 2017
[133] H. Shaaban, A. Mostafa, A.M. Alqarni, Y. Almohamed, D. Abualrahi, D. Hussein, M. Alghamdi, *J. Food Compos. Anal*., 110, 104549, 2022
[134] J. Regueiro, T. Wenzl, *J. Chromatogr. A*, 1422, 230, 2015
[135] K. Karsauliya, M. Bhateria, A. Sonker, S.P. Singh, *J. Agric. Food Chem.*, 69, 3932, 2021
[136] A. Jurek, E. Leitner, *Food Addit. Contam.*, 35, 2256, 2018
[137] S. Lu, Y. Yu, L. Ren, X. Zhang, G. Liu, Y. Yu, *Sci. Total Environ.*, 621, 1389, 2018
[138] C. Liao, K. Kannan, *Arch. Environ. Contam. Toxicol*., 67, 50, 2014
[139] H. Gallart-Ayala, E. Moyano, M.T. Galceran, *J. Chromatogr. A*, 1218, 1603, 2011
[140] N.C. Twaddle, M.I. Churchwell, M. Vanlandingham, D.R. Doerge, *Rapid Commun. Mass Spectrom.* 24, 3011, 2010
[141] K. Aalto-Korte, K. Alanko, M. Henriks-Eckerman, T. Estlander, R. Jolanki, *Contact. Derm.*, 49, 202, 2003
[142] S.C. Cunha, J.O. Fernandes, *Food Control*, 33, 549, 2013
[143] Y. Watabe, T. Kondo, H. Imai, M. Morita, N. Tanaka, K. Hosoya, *Anal. Chem.*, 76, 105, 2004
[144] Strona internetowa Xenometrics, dostępna pod adresem: https://www.biotoxik.it/wp-content/uploads/2017/01/XenoScreen-XL-YES_YAS-short-protocol-3_01.pdf
[145] K. Owczarek, B. Kudłak, V. Simeonov, Z. Mazerska, J. Namieśnik, *Molecules*, 23, 3226, 2018
[146] Q. Wang, M. Chen, G. Shan, P. Chen, S. Cui, S. Yi, L. Zhu, *Sci. Total Environ.*, 598, 814, 2017
[147] K. Owczarek, P. Kubica, B. Kudłak, A. Rutkowska, A. Konieczna, D. Rachoń, J. Namieśnik, A. Wasik, *Sci. Total Environ.*, 628, 1362, 2018
[148] R.J. Norman, D. Dewailly, R. S. Legro, T. E. Hickey, *The Lancet*, 370, 685, 2007
[149] A. Konieczna, D. Rachoń, K. Owczarek, P. Kubica, A. Kowalewska, B. Kudłak, A. Wasik, J. Namieśnik, *Reprod. Toxicol.*, 82, 32, 2018
[150] J. Bellver, L. Rodriguez-Tabernero, A. Robles, E. Munoz, F. Martinez, J. Landeras, J. Garcia-Velasco, J. Fontes, M. Alvarez, C. Alvarez, B. Acevedo, *J. Assist. Reprod. Genet.*, 35, 25, 2017
[151] E. Palioura, E. Diamanti-Kandarakis, *Rev. Endocr. Metab. Disord*., 16, 365, 2015

[152] E. Kandaraki, A. Chatzigeorgiou, S. Livadas, E. Palioura, F. Economou, M. Koutsilieris, S.Palimeri, D. Panidis, E. Diamanti-Kandarakis, J. *Clin. Endocrinol. Metab*., 96, 480, 2011
[153] E. Waraksa, K. Kowalski, R. Rola, E. Kłodzińska, T. Bienkowski, J. Namieśnik, *Microchem. J.*, 146, 56, 2019
[154] E. Waraksa, K. Kowalski, E. Kłodzińska, R. Rola, J. Ciekot, W. Filipiak, T. Bienkowski, J. Namieśnik, *J. Pharm. Biomed. Anal.*, 172, 339, 2019
[155] E. Waraksa, A. Pokrywka, M. Konop, J. Namieśnik, E. Kłodzińska, Electrophoresis 40, 1540, 2019
[156] K. Owczarek, E. Waraksa, E. Kłodzińska, Y. Zrobok, M. Ozimek, D.Rachoń, B. Kudłak, A. Wasik, Z. Mazerska, *Microchem. J.*, 180, 107643, 2022

Wykaz dorobku naukowego

7.1. Artykuły w czasopismach indeksowanych w *Journal Citation Report*

[1] **K. Owczarek,** E. Waraksa, E. Kłodzińska, Y. Zrobok, M. Ozimek, D. Rachoń, B. Kudłak, A. Wasik, Z. Mazerska, *Microchem. J.*, 180, 107643, 2022

[2] J. Jurewicz, J. Majewska, A. Berg, **K. Owczarek**, J. Zajdel, D. Kaleta, A. Wasik, D. Rachoń, *Environ. Pollut.*, 272, 115962, 2021

[3] E. Waraksa, **K. Owczarek**, P. Kubica, E. Kłodzińska, M. Ozimek, R. Wrzesień, B. Bobrowska-Korczak, J. Namieśnik, *J. Chrom. B*, 1151, 122197, 2020

[4] **K. Owczarek**, P. Kubica, B. Kudłak, A, Rutkowska, A. Konieczna, D. Rachoń, J. Namieśnik, A. Wasik, *Sci. Tot. Environ.*, 628, 1362, 2018

[5] **K. Owczarek**, B. Kudłak, V. Simeonov, Z. Mazerska, J. Namieśnik, *Molecules*, 23, 3226, 2018

[6] A. Konieczna, D. Rachoń, **K. Owczarek**, P. Kubica, A. Kowalewska, B. Kudłak, A. Wasik, J . Namieśnik, *Reprod. Toxicol.*, 82, 32, 2018

[7] N. Szczepańska, M. Rutkowska, **K. Owczarek**, J. Płotka-Wasylka, J. Namieśnik, *Trends Anal. Chem.*, 105, 173, 2018

[8] M. Rutkowska, **K. Owczarek.** M. de la Guardia, J. Płotka-Wasylka, J. Namieśnik, *Trends Anal. Chem.*, 97, 104, 2017

[9] J. Płotka-Wasylka, M. Rutkowska, **K. Owczarek**, M. Tobiszewski, J. Namieśnik, *Trends Anal. Chem.*, 91, 12, 2017

[10] J. Płotka-Wasylka, **K. Owczarek**, J. Namieśnik, *Trends Anal. Chem.* 85, 46, 2016

[11] N. Szczepańska, **K. Owczarek**, B. Kudłak, A. Pokrywka, Z. Mazerska, A. Gałuszka, J. Namieśnik, *Pol. J. Environ. Stud*., 25, 45, 2016

[12] A. Bejrowska, B. Kudłak, **K. Owczarek**, N. Szczepańska, Z. Mazerska, J. Namieśnik *Trends Anal. Chem.*, 74, 109, 2015

[13] B. Kudłak, **K. Owczarek**, J.Namieśnik, *Environ. Sci. Pollut. Res.*, 22, 11975, 2015

[14] B. Kudłak, **K. Owczarek**, N. Szczepańska, Z. Mazerska J. Namieśnik, *Crit. Rev. Anal. Chem.*, 45, 191, 2015

Pozostałe publikacje

[1] **K. Owczarek**, N. Jatkowska, J. Płotka-Wasylka, M. Rutkowska, P. Konieczka, J. Namieśnik, Analityka: Nauka I Praktyka, 3, 12, 2017

[2] **K. Owczarek**, B. Kudłak, J. Namieśnik, Analityka: Nauka i Praktyka, 4, 42, 2016

[3] **K. Owczarek**, B. Kudłak, J. Namieśnik, Analityka: Nauka i Praktyka, 1, 46, 2016

[4] **K. Owczarek**, N. Szczepańska, J. Płotka-Wasylka, M. Rutkowska, O. Shyshchak, M. Bratychak, J. Namieśnik, Chem. *Chem. Technol.*, 10, 601, 2016

[5] K. Yavir, **K. Owczarek**, Ł. Marcinkowski, A. Kloskowski, J. Namieśnik, Analityka: Nauka i Praktyka, 4, 12, 2016

[6] K. Yavir, **K. Owczarek**, Ł. Marcinkowski, A. Kloskowski, J. Namieśnik, Analityka: Nauka i Praktyka, 3, 4, 2016

[7] **K. Owczarek**, Z. Mazerska, B. Kudłak, J. Namieśnik, *J. Anal. Bioanal. Tech.*, 6, 47, 2015

[8] N. Jatkowska, **K. Owczarek**, B. Kudłak, Z. Mazerska, J. Namieśnik, Analityka: Nauka i Praktyka, 4, 32, 2014

[9] N. Jatkowska, **K. Owczarek**, B. Kudłak, Z. Mazerska, J. Namieśnik, Analityka: Nauka i Praktyka, 3, 37, 2014

7.2. Rozdziały w opracowaniach książkowych

[1] B. Kudłak, N. Szczepańska, **K. Owczarek,** Z. Mazerska, J. Namieśnik, „*ENDOCRINE DISRUPTING COMPOUNDS – PROBLEMS AND CHALLENGES". Emerging Pollutants in the Environment - Current and Further Implications*, Intech, Rjeka, ISBN 978-953-51-4232-4

[2] J. Płotka-Wasylka, N. Szczepańska, **K. Owczarek**, J. Namieśnik, *Miniaturized solid phase extraction. Green Extraction Techniques: Principles, Advances and Applications*, 76, 279-318, 2017, ISBN: 978-0-12-811082-9

[3] **K. Owczarek,** N. Szczepańska, J. Płotka-Wasylka, J. Namieśnik, *New Achievements in the Field of Extraction of Trace Analytes from Samples Characterized by Complex Composition of the Matrix. Green Analytical Chemistry. Green Chemistry and Sustainable Technology*. Springer, Singapore, 2019, ISBN**:** 978-981-13-9104-0

7.3. Wystąpienia konferencyjne

[1] **K. Owczarek,** *Assessment of cytotoxic and endocrine potential of selected xenobiotics commonly present in food products, 6th International Conference and Exhibition on Analytical & Bioanalytical Techniques*, 1-3.09.2015, Walencja, Hiszpania

[2] **K. Owczarek,** *Analytical aspects of model compounds toxicity assessment using MTT and Microtox bioanalytical assays, 11th International Students Conference "Modern Analytical Chemistry*, 22-23.09.2015, Praga, Czechy