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DOCTORAL THESIS

2020 Ing. Marek Reichstädter

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ANALYTICAL, ENVIRONMENTAL AND GEO- CHEMISTRY

APPLICATION OF DIFFUSIVE GRADIENTS IN THIN FILMS TECHNIQUE IN FOOD- AND ENVIRONMENTAL ANALYSIS

VYUŽITÍ TECHNIKY DIFÚZNÍHO GRADIENTU V TENKÉM FILMU V ANALÝZE POTRAVIN A V ENVIRONMENTALNÍ ANALÝZE

DOCTORAL THESIS
DISERTAČNÍ PRÁCE

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Application of diffusive gradients in thin films technique in food and environmental analysis

Doctoral Thesis assignment:

1) Laboratory preparation of sorbent containing immobilized cysteine
2) Validation of DGT technique with binding gel containing laboratory prepared sorbent for determination of mercury event. other elements in the aqueous medium in conjunction with spectroscopic / spectrometric methods
3) Validation of DGT technique with binding gel containing Purolite S924 for determination of mercury event. other elements in the aqueous medium in conjunction with spectroscopic / spectrometric methods
4) Validation of new types of DGT for the determination of selected metals in liquid flavorings in conjunction with spectroscopic / spectrometric methods
5) Analysis of real samples using new types of DGT and spectroscopic / spectrometric methods
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DECLARATION

I declare that the doctoral thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the doctoral thesis is the property of the Faculty of Chemistry of Brno University of Technology and Faculty of Sciences and Bioengineering Sciences of Vrije Universiteit Brussel and all commercial use is allowed only if approved by both the supervisors and the deans of both institutions.

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ABBREVIATIONS

3-MFS	3-mercaptopropyl functionalised silica
AAS	atomic absorption spectroscopy
AES	atomic emission spectroscopy
AGE	agarose diffusive gel
ANOVA	analysis of variance
APA	agarose-derivative crosslinked polyacrylamide diffusive gel
APS	ammonium persulfate
ASV	anodic stripping voltammetry
b.w.	body weight
BCZ	Belgian coastal zone
CAPS	cysteine-immobilised aminopropyl functionalised silica
CAS	cysteine-immobilised amino functionalised silica
CV-AFS	cold-vapour atomic fluorescence spectroscopy
DBL	diffusive boundary layer
DGT	diffusive gradients in thin films technique
DNA	deoxyribonucleic acid
DRIFT	diffuse reflectance infrared Fourier transform spectroscopy
EC	European Commission
EEC	European Economic Community
EFSA	European Food Safety Authority
ET-AAS	electrothermal atomic absorption spectroscopy
EU	European Union
F-AAS	flame atomic absorption spectroscopy
FAO	Food and Agriculture Organisation of the United Nations
FDA	United States Food and Drug Administration
GC	gas chromatography
GSH	glutathione
HG-AFS	hydride-generation atomic fluorescence spectroscopy
HPLC	high-performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectroscopy
IR	infrared radiation
ISE	ion-selective electrode

IUPAC	International Union of Pure and Applied Chemistry
LA-ICP-MS	laser ablation inductively coupled plasma mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MeHg	methylmercury
MQ	deionised water (MilliQ)
MS	mass spectrometry
MWD	microwave digestion method
RES	bisacrylamide-crosslinked polyacrylamide (restrictive) diffusive gel
ROS	reactive oxygen species
TCD	thermal conductivity detector
TD-AAS	thermal decomposition atomic absorption spectroscopy
WHO	World Health Organisation

SUMMARY

This work studies development of Diffusive Gradients in Thin films (DGT) technique for determination of mercury (Hg) and other trace metals and further application possibilities of this technique. In this work, the DGT technique is developed for the determination of Hg and other trace metals in various liquid media. Two different Hg-specific ion-exchange resins were evaluated for application in the DGT technique – Purolite S924 and Cysteine-Modified Amino-Propyl silica (CAPS). The Purolite S924 is commercially available chelating resin, the CAPS resin was prepared under laboratory conditions by glutaraldehyde-mediated immobilisation of cysteine onto 3-aminopropyl functionalised silica. Both resins showed promising application potential in the DGT technique thanks to their reliable performance in solutions of a broad range of pH and ionic strength. The performance of the DGTs with the new resins was compared with the performance of the DGTs with the commonly used Chelex-100 and 3-mercaptopropyl silica resins. The major advantage of the S924 and CAPS resin is the ability of simultaneous assessment of Hg and other trace metals (Cu, Ni, Pb, Cd, Co). Due to different requirements on the resins used in the DGT technique for Hg and other trace metals, the DGT technique or simultaneous quantitative determination of Hg and other trace metals was not reported yet. Until now, the assessment of Hg and other trace metals have been performed by two separated types of the DGT samplers – one for Hg and one for other trace metals. That increased the number of samples produced, and chemical reagents and material used. The DGT technique with the CAPS resin was used for determination of metals in Oostende and Zeebrugge marine harbours in the Belgian coastal zone. Although the DGT technique was originally introduced as an environmental analysis tool, the application of the DGT technique in food analysis was also studied in this work. The performance of the DGT technique was validated in fish sauces and the effective diffusion coefficients of Hg and trace metals in the fish sauce were determined. Subsequently, the DGT technique was successfully applied to determine the concentration of mercury and other trace metals in fish sauce samples. To compare the new analytical procedure using DGT technique, fish sauces were also analysed directly by thermal decomposition gold amalgamation atomic absorption spectrometry (TD-AAS) and also after microwave decomposition by sector field inductively coupled plasma mass spectrometry (SF-ICP-MS). Due to the preconcentration ability of the DGT technique, lower detection limits were achieved in comparison with the TD-AAS or the SF-ICP-MS. Moreover, the wear and corrosion of metal parts of the analytical instruments were eliminated by the ability of the DGT technique to separate the trace metals from the complex matrix of fish sauce.

KEYWORDS: cadmium, lead, mercury, trace metals, food analysis, diffusive gradients in thin films

ABSTRAKT

Tato disertační práce se zabývá vývojem techniky difúzního gradientu v tenkém filmu (DGT) pro stanovení rtuti (Hg) a dalších prvků (zejména Cd a Pb) ve vodném prostředí, a dále rozšířením aplikačních možností této techniky. V rámci vývoje techniky DGT byly testovány dva typy nových sorpčních gelů – sorpční gel s komerčně dostupným sorbentem Purolite S924 a sorpční gel s laboratorně připraveným sorpčním gelem obsahujícím imobilizovaný cystein na 3-aminopropyl funkcionalizovaném silikagelu (CAPS). Oba testované sorpční gely vykazovaly slibný potenciál pro použití v technice DGT díky funkčnosti v roztocích o širokém rozsahu pH i iontové síly. Výkonnost techniky DGT s novými sorpčními gely byla porovnána s výkonností běžně používané techniky DGT využívající sorpční gely se sorbenty Chelex-100 a 3-merkaptopropyl funkcionalizovaným silikagelem. Hlavní výhodou sorpčních gelů s Purolite S924 a CAPS se ukázala být schopnost současného stanovení Hg a dalších prvků (Cd, Pb, Co, Ni, Cu). Vzhledem k odlišným požadavkům na sorbenty používané v technice DGT nebylo dosud současné kvantitativní stanovení Hg a dalších prvků možné. Doposud bylo pro stanovení rtuti a dalších prvků nutné využít vždy dva typy techniky DGT – DGT pro stanovení Hg a DGT pro stanovení dalších prvků, což zvyšovalo množství vzorků pro analýzu a množství použitého spotřebního materiálu. Technika DGT s CAPS sorpčním gelem byla použita pro stanovení koncentrací kovů v mořské vodě v námořních přístavech v Oostende a Zeebrugge ležících na belgickém pobřeží Severního moře. Přestože byla technika DGT původně vyvíjena pro použití v analytické chemii životního prostředí, v této práci bylo zkoumáno i použití této techniky v analýze potravin. V prostředí rybí omáčky byla ověřena funkčnost techniky DGT se sorpčním gelem Purolite S924 a byly stanoveny difúzní koeficienty Hg a dalších prvků. Technika DGT byla následně použita pro stanovení koncentrace rtuti a dalších prvků v různých komerčně dostupných vzorcích rybí omáčky. Výsledky nově navrhnutého analytického postupu využívajícího techniku DGT byly porovnány s výsledky přímé analýzy rybích omáček atomovou absorpční spektroskopií s termální dekompozicí a s amalgamací (TD-AAS) a po mikrovlnném rozkladu vzorků rybí omáčky také s výsledky získaných analýzou pomocí sektorové hmotnostní spektrometrie s indukčně vázanou plazmou (SF-ICP-MS). Díky schopnosti techniky DGT prekoncentrovat analyt v sorpčním gelu bylo díky nově navrženému postupu analýzy rybí omáčky dosaženo nižších detekčních limitů ve srovnání s přímou analýzou rybích omáček pomocí TD-AAS nebo SF-ICP-MS. Technika DGT díky schopnosti extrahovat analyt z komplexní matrice rybí omáčky rovněž snížila riziko koroze kovových částí použitých přístrojů a výrazně snížila servisní intervaly u používaných instrumentálních technik.

KLÍČOVÁ SLOVA: kadmium, olovo, rtuť, stopové kovy, analýza potravin, technika difúzního gradientu v tenkém filmu

SAMENVATTING

In deze thesis wordt de ontwikkeling van Diffusieve Gradienten in Dunne films (DGT) gebruikt in de bepaling van kwik (Hg) en andere sporen van metalen zoals Cu, Ni, Pb, Cd en Co, hoofdzakelijk in verschillende vloeibare media, bestudeert en besproken. Twee verschillende Hg-specifieke ionen-uitwisselingsharsen, Purolite S924 en cysteïne-gemodificeerd amino-propyl silica (CAPS), werden geëvalueerd voor gebruik aan de hand van de DGT-techniek. Purolite S924 is een commercieel verkrijgbaar chelerend hars terwijl CAPS een onder laboratoriumomstandigheden vervaardigd hars is datverkregen werd door de glutaraldehyde-gemedieerde immobilisatie van cysteïne op 3-aminopropyl gefunctionaliseerd silica. Beide harsen lieten een veelbelovend resultaat zien voor het gebruik met DGT, dankzij hun betrouwbare prestaties in oplossingen met zeer verschillende pH-waarden en ionconcentraties. De prestaties van DGTs met Purolite S924 en CAPS werden vergeleken met de prestaties van DGTs met de typisch gebruikte Chelex-100 en 3-mercaptopropyl silica harsen. Het voornaamste voordeel van de Purolite S924 en CAPS-harsen is de mogelijkheid om simultaan de hoeveelheden van Hg en andere sporenmetalen vast te stellen. Tot voorkort werd de vaststelling van Hg en andere sporenmetalen uitsluitend met twee verschillende DGT-samplers – één voor Hg en één voor de andere sporenmetalen – uitgevoerd, deze methode vergt een tweevoud aan geproduceerde stalen en chemische reagentia ten opzichte van simultane determinatie. Echter, doordat de Purolite S924 en CAPS-harsen aan verschillende voorwaarden moeten voldoen om de simultane kwantitatieve determinatie van Hg en de andere sporenmetalen te meten dan wanneer de elementen apart op de harsen worden gemeten, worden deze resultaten hier (nog) niet behandeld. De DGT-techniek met CAPS-hars werd gebruikt voor het bepalen van metalen in de mariene havens van Oostende en Zeebrugge in de Belgische kuststreek. DGTs zijn in eerste instantie geïntroduceerd als technieken om milieuvraagstukken te beantwoorden, echter in dit werk is ook de toepassing in de voedingsindustrie onderzocht. De prestaties van DGTs zijn gevalideerd door onderzoek naar vissaus, waarin de diffusiecoëfficiënt van Hg en sporenmetalen in vissaus werd bepaald en de concentraties van Hg en sporenmetalen werden vastgesteld. Deze resultaten zijn gevalideerd door ze te vergelijken met thermische decompositie goud amalgamatie atomisch absorptie spectrometrie (TD-AAS) en microgolf decompositie door sector veld inductief gekoppelde plasma massa spectrometrie (SF-ICP-MS). Wegens het concentratievermogen van de DGT techniek, werden lagere detectielimieten bereikt dan met TD-AAS of SF-ICP-MS. Daarnaast zorgt de scheiding van Hg en sporenmetalen via DGT voor een verlaging van de slijtage en corrosie van de massaspectrometers aangezien de complexe matrix van de vissaus vooraf verwijderd wordt.

SLEUTELWOORDEN: cadmium, lood, kwik, spoormetalen, voedselanalyse, diffuse gradiënten in dunne films

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

The trace elements undergo biogeochemical cycles in the ecosystem, during which they are changing their forms. Some trace elements (e.g. Cu, Co, Fe or Zn) are important nutrients in low dose, however, they might be harmful in higher concentrations. Others, e.g. mercury of arsenic have no metabolic function and are toxic to organisms (Azeh Engwa et al. 2019). In dependence on the physical and chemical forms, the trace elements also have different reactivity, toxicity and bioavailability (Ali et al. 2019). Plants and aquatic organisms are also part of the biogeochemical cycles, and through them, the trace elements are entering the food chain (Ali and Khan 2018). During the trophic transfers in the food chain, the bioaccumulation and biomagnification of trace elements occur (Jaishankar et al. 2014). The position of humans is on the top levels of this food chain, and consumption of foodstuffs contaminated with trace elements can notably contribute to the total exposure of these toxic metals with a possible negative impact on human health (Jaishankar et al. 2014).

Typical methods of trace elements analysis are spectroscopic and spectrometric techniques, which provide accurate results about the total concentration of an element but not about its different forms. After taking the sample and before its analysis, many changes to the form of the element can occur (Divis et al. 2005a). That can be avoided by using *in situ* techniques, which allow processing of the sample at the sampling spot, however, the number of such available techniques is still limited to electrochemical methods (Gao et al. 2019). Further advantages offered thin films gel techniques – diffusive equilibrium in thin-films technique (DET) and diffusive gradients in thin-films (DGT) technique. The DET technique was introduced to measure the concentrations of trace elements on the phase sediment-water column and works on a similar principle as dialysis. In the DET technique, the analytes are forming an equilibrium between the concentration in the environment and the polyacrylamide gel in the DET sampler. After the equilibrium is formed, the gel is cut into pieces and analysed to obtain a vertical concentration profile of the analyte in the aquatic sediment. The DET technique has several disadvantages, also for use at low concentrations, therefore the DGT technique was introduced (Divis et al. 2005a). The DGT technique is an *in situ* passive sampling technique used to assess the concentration of labile trace elements in different environmental matrices (Davison and Zhang 1994; Zhang and Davison 2015). The labile forms of trace metals are often understood as bioavailable (Degryse and Smolders 2016; Linnik et al. 2018). The DGT technique consists of a diffusive domain backed up by a resin that binds all free metals and metalloids and their complexes that dissociate in the diffusive domain. The DGT technique has several unique features, as it allows *in situ* application, assessment of bioavailable fraction of trace elements, speciation analysis or preconcentration and separation of analytes from the matrix (Divis et al. 2005a; Zhang and Davison 2015). The last two features are also especially useful for food chemistry (Chen et al. 2014). Despite the recent advances, the routinely used methods of the food analysis are still facing difficulties with the low concentration of trace elements together with strong matrix effects (Reilly 2006). Food samples also require difficult pre-treatment steps and the application of the DGT technique in food analysis could overcome these issues (Biziuk and Kuczynska 2006; Chen et al. 2014). The DGT technique has different requirements for the assessment of mercury (Hg) and the other trace elements (Docekalova and Divis 2005). That requires the deployment of two different DGT assemblies simultaneously, which increases the number of samples and consumables used. Also, most sorbents for the Hg is no longer commercially available because of discontinued production (Diviš 2013). Therefore, there is an urgent demand for novel resins allowing simultaneous quantitative binding with Hg and other trace elements. The potential solutions are using novel commercially available resins or laboratory preparation of custom resin.

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2 THEORETICAL PART

2.1 TRACE ELEMENTS

By the definition of IUPAC, a trace element is a chemical element with an average concentration of less than about 100 ppm (parts per million) atoms or less than 100 $\mu g \cdot g^{-1}$ (Nič et al. 2009). That includes many elements of which most frequently monitored are antimony (Sb), arsenic (As), bismuth (Bi), cadmium (Cd), cerium (Ce), chromium (Cr), cobalt (Co), copper (Cu), gallium (Ga), gold (Au), iron (Fe), lead (Pb), mercury (Hg), manganese (Mn), nickel (Ni), platinum (Pt), silver (Ag), tellurium (Te), thallium (Tl), tin (Sn), uranium (U), vanadium (V) and zinc (Zn) (Azeh Engwa et al. 2019). The older unspecific term “heavy metals” is also used to describe this group of elements, based on their high specific density, atomic weight and/or other chemical and biological properties, however, some of these elements are not metals but metalloids (As, Sb and Se) or are not matching the density criteria for heavy metals (e.g. As). Therefore, the term “trace elements” recommended by IUPAC is more suitable (Duffus 2002; Hubner et al. 2010). Term “trace metals” can be also used, if arsenic and other metalloids are excluded.

Some trace elements, such as Cu, Co, Fe, Ni, Se, Mn and Zn are micro-nutrients at low levels with functional roles in living organisms, which are essential in various diverse physiological and biochemical processes and deficiencies of these elements result in diseases. However, high doses of the same trace metals are harmful and cause acute or chronic toxicities (Azeh Engwa et al. 2019). Other elements, such as Cd, Hg and Pb do not possess any biological function and are toxic even in low concentrations (Jaishankar et al. 2014). Although the essentiality of As at trace concentrations for some animals and humans are the subject of scientific discussions (Uthus 2003; Zeng et al. 2005), As is still considered as toxic in its inorganic form by WHO (Ng et al. 2001).

2.1.1 Sources of trace elements in the environment

The trace elements are naturally present in the environment and their sources could be both natural and anthropogenic origin (Bradl 2005). The natural origin includes geological sources such as volcanic activities, magmatic, sedimentary and metamorphic rocks and their subsequent weathering, soil formation and release of the trace elements (Siegel 2002; Bradl 2005). However, with increasing industrialisation since the 18th century, the contribution of anthropogenic sources has risen drastically. These sources are linked with human activities, including agricultural production and using trace elements-based fertilisers and pesticides (Bradl 2005). Similarly, the industrial activities notably contribute to the trace element emissions. Trace elements are extracted from metal ores as by-products in mining and metallurgy processes (Cullen and Maldonado 2013). Another source is emissions from coal and petroleum combustion for heat and energy production. Formerly used Pb-based fuel additives are the additional source of pollution (Jaishankar et al. 2014). In urban areas, the pipes made of lead and lead-based alloys, household products and paints can increase the contamination of water. The roadside soils and vegetation are facing increased contamination by trace elements from exhausts and catalytic converters of cars (Bradl 2005; Jaishankar et al. 2014). Using colour- and antifouling paints on the hulls of ships has a negative influence on the water quality in harbours (Warnken et al. 2004). Also, the use of waste incinerators is a minor source of atmospheric pollution in cities. Landfill sites of waste from agronomic activities (e.g. slag, animal manure) and domestic and industrial waste (e.g. batteries, tires, automobiles) lead to soil and groundwater contamination, because of unsuitable treatment, often combined with unsuitable geological and hydrogeological conditions (Bradl 2005).

The European environmental policies have implemented strategies against the chemical pollution of surface-, drinking- and wastewaters in the EU Water Framework Directive (Directive 2000/60/EC 2000). In the EU Environmental Quality Standards Directive (Directive 2008/105/EC 2008), the lists of dangerous and priority substances were introduced, including (but not limited only to) Cd, Pb, Hg, Ni and their compounds; as well as Cu, Co and Zn.

2.1.2 Biogeochemical cycle of trace elements

The trace elements in the environment are distributed in the atmosphere, hydrosphere and pedosphere and undergo biogeochemical cycles between them as they change their forms. These cycles are different for every element, but Figure 1 shows a simplified schematic of a typical biogeochemical cycle. The flux exchanges between the earth and the atmosphere are mostly in the form of airborne particles – along with rains, which includes dust, dirt, and the emissions from burning the fossil fuels and industry. Also, if an element that has a vapour phase, it may be released to the atmosphere from the surface of the earth or the oceans as a gas phase. River streams take the substances with high solubility in water to the oceans, which are (along with the sediments) the final reservoirs. From the oceans, the trace elements undergo deposition in sediments. From the soil and the oceans, the trace elements could enter the food chain (Tessier and Turner 1995; Selin 2009; Wuana and Okieimen 2011; Cullen and Maldonado 2013).

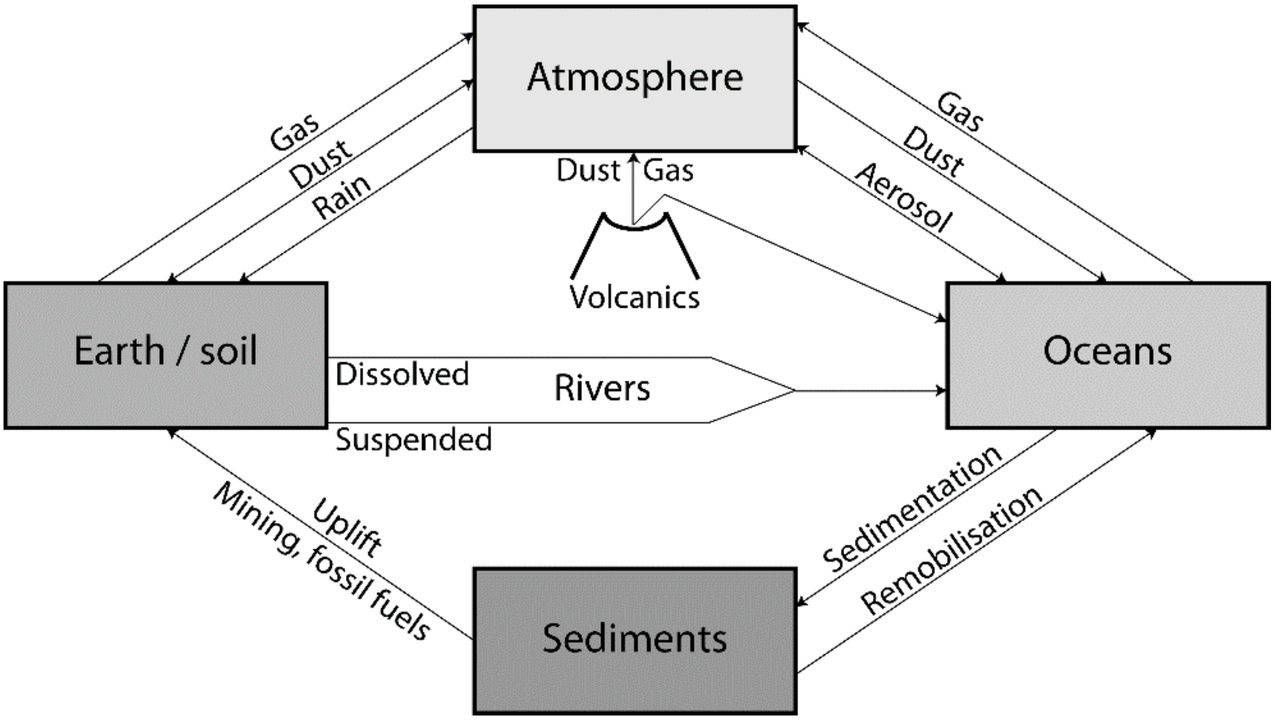

Figure 1 General geochemical cycle of trace elements. Adapted from Mackenzie et al. (1979) and Förstner and Wittmann (1981).

The cycles of some elements, such as As, Hg or Se, are more complex, as these also transform inorganic species to organic species, i.e. by methylation (Selin 2009; Hall and Gamble 2012; Mason 2012). The methylation occurs by both biotic and abiotic processes and the biogeochemical cycle of Hg is an excellent example (Craig 1980; Barkay et al. 2003; Mason 2012). Besides the cycling between the soil, water and atmosphere, the methylation of Hg also transforms its chemical species into more toxic compounds, which can enter the living organisms more easily. Microbial uptake of Hg is a key step both in its methylation and its bioaccumulation (Barkay et al. 2003). The major transformations are methylation/demethylation and oxidation/reduction of Hg species. The methylation of ionic Hg^{II} to methylmercury (MeHg) occurs mostly by sulphate-reducing bacteria, by methyltransferase-mediated transfer of methyl group from methylcobalamin via the acetyl-coenzyme A pathway (Morel et al. 1998; Barkay et al. 2003). Fulvic, humic and carboxylic acids and alkylated tin compounds are the principal factors for abiotic methylation of Hg. Biotic reductive demethylation by anaerobic bacteria yields methane and elemental Hg^0. Oxidative biotic demethylation by uncharacterised microbial processes was also reported, yielding carbon dioxide and unidentified Hg species. Demethylation is also occurring abiotically in wetlands and lakes by photodegradation at 200-400 nm. The biotic reduction of ionic Hg^{II} to elemental Hg^0 takes place in algae and it significantly contributes to the flux of Hg from natural waters into the atmosphere. Abiotic photochemical reactions with organic and inorganic free radicals reduce

Hg^{II} to Hg^0, because of the high volatility of Hg^{II} and its low solubility in water. The least explored step is the biotic oxidation of elemental Hg, caused by hydroperoxidases in organisms. Abiotic oxidation occurs in the atmosphere, natural waters and soils, mainly by photooxidation by various oxidants and free radicals and by dark oxidation in oceans (Barkay et al. 2003). This biogeochemical cycling is the reason for trace elements entering the food chain at different stages of these cycles. The soil-to-plant transfer of trace elements is the major entry route for most of the toxic trace elements to the terrestrial food chain (Ali and Khan 2018). Mercury is more linked with the aquatic environment and food chain, because of the microbial uptake of Hg and subsequent trophic transfers (Morel et al. 1998; EFSA 2012). Planktonic bacteria and microalgae bioaccumulate many trace elements, but the levels of trace element contamination are not increasing at the higher levels of the food chain. Mercury, however, does not only bioaccumulate but during the trophic transfers in the food chain it also biomagnifies its concentration, for both Hg and MeHg (Morel et al. 1998).

2.1.3 Human exposure to trace elements

Human exposure to trace elements in the environment by three major routes – ingestion, inhalation, and dermal absorption. The rate of exposure is different for every person based on the local habitat, occupational exposure at work and the local and the individual diet and lifestyle habits. The awareness, knowledge and precautions about trace elements exposure is poor in the developing countries (Anyanwu et al. 2018). The occupational and habitational exposures account for the third and fourth highest contribution to total trace elements intake, right after the food and drinking water sources (EFSA 2009a; EFSA 2010; EFSA 2012; Jaishankar et al. 2014; EFSA 2015; Anyanwu et al. 2018). People living and working close to the sources of trace elements (e.g. mining and metallurgy industry) are facing higher exposure to all trace elements. Smoking also contributes significantly to the exposure to Cd and it can prevail the intake by food (EFSA 2009a; Ali et al. 2019). Household products, such as colours and paint, can contribute to the exposure to Pb slightly by hand-to-mouth activities. Several regulations about using of tetraethyllead fuel additive adopted since the 1970s significantly decreased the environmental pollution by Pb, however households near busy highways or old orchard lands might still contain elevated levels of Pb (EFSA 2010; Ali et al. 2019). Household accidents with fluorescent light bulbs or thermometers are a further minor contribution to Hg exposure (EFSA 2012). The contributions of outgassing of dental amalgam (Barkay et al. 2003; Clarkson et al. 2007; Richardson et al. 2011) and use of organomercury preservatives in some vaccines are questionable compared to the total exposure to mercury compounds (Clarkson et al. 2007; Barregard et al. 2011; EFSA 2012).

2.1.4 Trace elements in the foodstuffs

As described in section 2.1.3, foodstuffs are the most common exposure route to trace elements for the majority of the population. Out of all types of the food, the fish and fishery products have the highest occurrence of trace elements, due to the connection with the aquatic environment (Olmedo et al. 2013; Jia et al. 2017; Rajeshkumar and Li 2018; FDA 2020), especially for the Hg and As (Reilly 2006; EFSA 2012). Predatory fish in the aquatic food chain (e.g. shark, swordfish tuna and pike species), contain more trace elements due to more extensive bioaccumulation and biomagnification (Falco et al. 2006; Bilandžić et al. 2011; EFSA 2012; Olmedo et al. 2013; Jia et al. 2017; Rajeshkumar and Li 2018; FDA 2020). Another group with a high concentration of trace elements are edible offal and offal products, due to being the target toxicity organs (chapter 2.1.3), where the accumulation occurs, e.g. liver and kidney (Reilly 2006; EFSA 2009a; EFSA 2010; Jaishankar et al. 2014). Cereals, cereal products, vegetables and fruit are also a source of trace elements due to the uptake of these metals and metalloids by plants and crops directly from the soil (Intawongse and Dean 2006; Reilly 2006; EFSA 2009a; EFSA 2010; EFSA 2012) or as a part of agrochemical pollution (Reilly 2006; Manzoor et al. 2018). Milk and dairy products are also sources of trace elements due to the trophic transfer of trace elements from soil to crops and agricultural feed (Ziarati et al. 2018). Wine and similar products can also contain increased levels of trace elements due to fungicides used in vineyards (Naughton and Petroczi 2008; Voica et al. 2009).

The absolute concentration of trace elements in foodstuffs is not the only factor affecting the total exposure by alimentation route. Seaside and inland countries have different consumption of fish products, which affects the total exposure to trace elements (EFSA 2009a; EFSA 2010; EFSA 2012). The cereals and cereal products have relatively low levels of trace elements but are consumed frequently, therefore they usually contribute the most to total exposure (Reilly 2006; EFSA 2009a; EFSA 2010; EFSA 2012). The individual eating preferences can also affect the exposure to trace elements. The literature suggests that vegetarians and vegans could face increased levels of Cd due to consumption of more Cd rich food and methionine deficiency, compared to the regular population (Krajčovičová-Kudláčková et al. 2005; Krajčovičová-Kudláčková et al. 2006; Rossi et al. 2016).

Most concerned trace metals in foodstuffs are Hg, Cd and Pb (WHO 2011; Olmedo et al. 2013; WHO 2013). For Cd and Pb, the maximum allowed levels in food products have been established by Commission Regulation 1881/2006/EC (2006) in certain foods, including red meat, fish, dairy products, fruits and vegetables. The same regulation also sets maximal limits of Hg and MeHg, however in fish meat and fishery products only, although the increased concentration of Hg species were reported also in other foodstuffs (da Silva et al. 2010; Chudzyński et al. 2011). The missing maximal permitted levels of other trace elements in the fishery products might harm the consumers' health.

The requirements on the quality of drinking water are set by the EU Drinking Water Directive (Council Directive 98/83/EC 1998). That directive sets the parameter values at 10 $\mu g \cdot L^{-1}$ for As, 5 $\mu g \cdot L^{-1}$ for Cd, 50 $\mu g \cdot L^{-1}$ for Cr, 2 $mg \cdot L^{-1}$ for Cu, 10 $\mu g \cdot L^{-1}$ for Pb and 20 $\mu g \cdot L^{-1}$ for Ni, as most of these elements are naturally occurring groundwater contaminants, industrial source water pollutants and are used in water-piping directly or in metal alloys (EFSA 2010; WHO 2017).

Joint FAO/WHO Expert Committee on Food Additives sets the tolerable intake values of 25 $\mu g \cdot kg^{-1}$ body weight (b.w.)/month for Cd (WHO 2013), 4 $\mu g \cdot kg^{-1}$ b.w./week for Hg (WHO 2011) and the European Food Safety Authority sets the tolerable intake value of 0.63 $\mu g \cdot kg^{-1}$ b.w./day for Pb (EFSA 2010), 2.8 $\mu g \cdot kg^{-1}$ b.w./day for Ni (EFSA 2015) and 2 $\mu g \cdot kg^{-1}$ b.w./day for As (EFSA 2009b). The tolerable daily intakes (TDI) are then 0.83 $\mu g \cdot kg^{-1}$ b.w./day, 0.53 $\mu g \cdot kg^{-1}$ b.w./day, 0.63 $\mu g \cdot kg^{-1}$ b.w./day for Cd, Hg and Pb respectively, or 50 µg Cd/day, 32 µg Hg/day, 38 µg Pb/day, 168 µg Ni/day and 120 µg As/day, if assuming a body weight of 60 kg (WHO 2013).

2.1.5 Toxicity of trace elements

The toxicity of trace elements involves various toxicological mechanisms of action, which are different for each element. All of the trace elements do, however, have certain similarities in their toxicity mechanisms, which can be split into two groups – toxicity induced via free radicals formed by the presence of trace elements and toxicity induced directly by trace elements (Tchounwou et al. 2012; Azeh Engwa et al. 2019).

The toxicity induced vide free radicals is based on generating of reactive oxygen species (ROS), that may lead to oxidative stress and oxidation of biological molecules (Jaishankar et al. 2014). The mechanism of free radical generation is specific to the type of element, including Haber-Weiss and Fenton reactions and other reactions, which generate superoxide-, peroxyl- and hydroxyl-radicals (Valko et al. 2005). The ROS are causing single-strand DNA breaks and oxidations, distortions of nucleic acids and proteins and lipid peroxidation, causing cell membrane damage as shown in Figure 2 (Tchounwou et al. 2012; Azeh Engwa et al. 2019). This type of toxicity is induced by most of the trace elements.

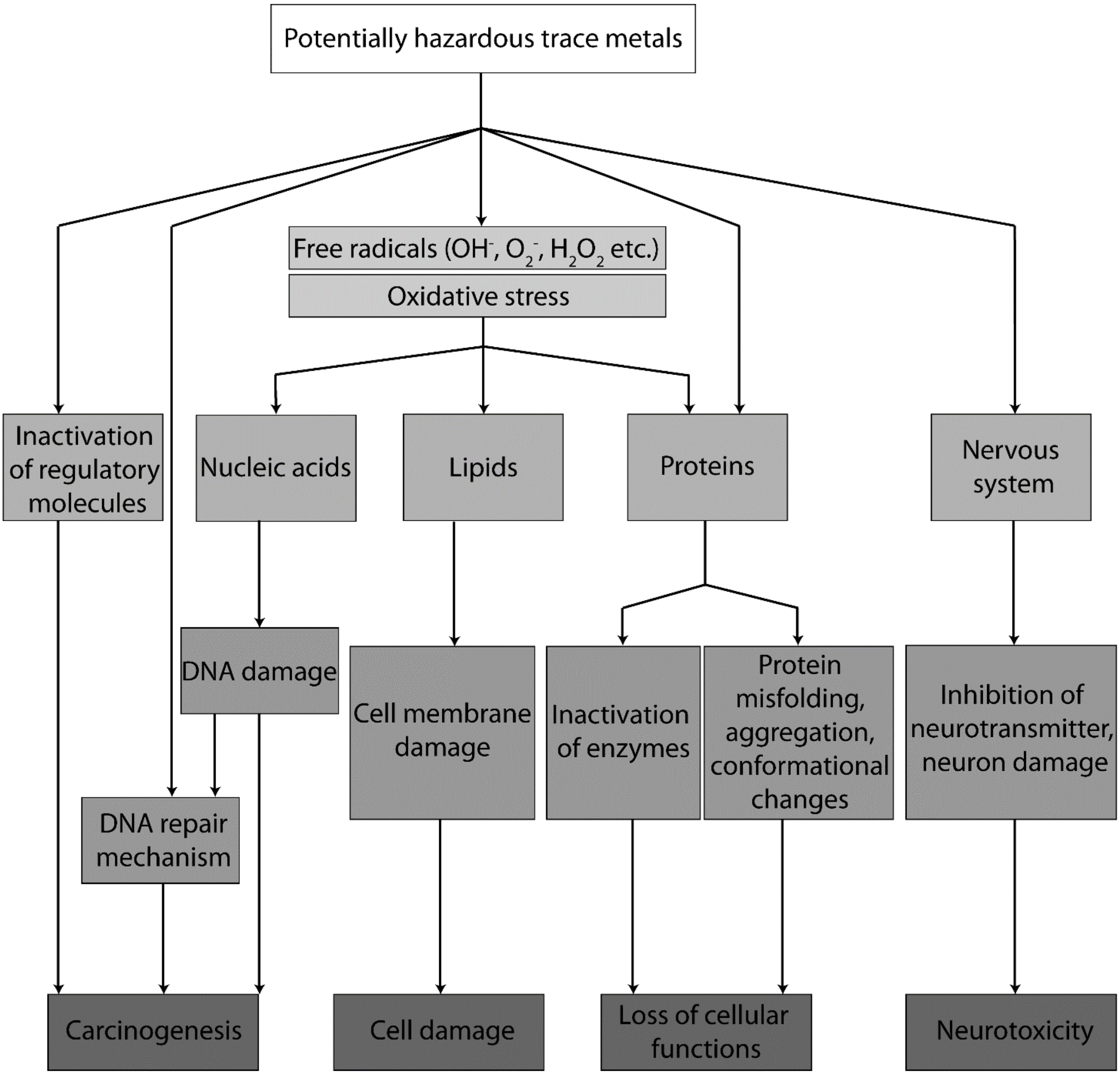

Figure 2 Toxic effects mechanism of trace elements. Adapted from Azeh Engwa et al. (2019)

The toxicity induced directly by trace elements is caused by their high affinity towards functional groups of vital biomolecules in the living organisms, especially to ones containing sulphur, nitrogen and oxygen (Valko et al. 2005; Tchounwou et al. 2012; Jaishankar et al. 2014; Jan et al. 2015; Azeh Engwa et al. 2019), Several signalling or regulatory proteins participate in apoptosis, cell cycle regulation, DNA repair, cell growth and transcription factors (Valko et al. 2005). By targeting the functional groups of these proteins by trace elements, the metabolic pathways and gene expression could be altered, and carcinogenic effects induced (Valko et al. 2005). Trace elements also cause proteins to aggregate and inhibit protein folding, resulting in loss of cellular functions. Similarly, the trace elements are bound to the functional groups of enzymes, resulting in inhibition of enzymatic activity. Trace elements can also bind to substrates, subsequently forming a nonstandard enzyme-substrate complex and blocking the enzyme, with the trace element embedded in the tissue, leading to dysfunctions and abnormalities and damages (Azeh Engwa et al. 2019). The toxic effect may get also induced by the replacement of metalloenzymes by another metal ion of similar size, e.g. Cd displaces Zn and Cd from Zn-finger proteins and metalloproteins (Valko et al. 2005; Azeh Engwa et al. 2019). Some trace metals as Pb, Mn and Hg can inhibit the neurotransmitter release, cause neuron and brain-specific oxidative damage (Ralston and Raymond 2018; Azeh Engwa et al. 2019).

The occurrence of toxic effects depends on the presence of antioxidants (e.g. glutathione, L-cysteine, α-tocopherol, ascorbic acid etc.), which are responsible for the scavenging of free radicals, interrupting radical chain reactions, the formation of stable metal-complexes, resulting to the decrease of oxidative stress (Valko et al. 2005; Clarkson et al. 2007; Jan et al. 2015). Especially the glutathione, glutamic acid-glycine-cysteine tripeptide, plays a major role in the cellular defence system, as it also directly binds the trace elements in cells (Valko et al. 2005; Jozefczak et al. 2012; Jan et al. 2015)

Trace elements have several different target organs in the body, depending on the element, its form and the type of exposure – e.g. while Cr^{III} or metallic Hg^0 possess none or low risk during dermal exposure, the Cr^{VI} or organic compounds of Hg are extremely toxic (Clarkson et al. 2007; Azeh Engwa et al. 2019). The toxic effects can be either acute or chronic, and due to long biological half-life of trace elements, even low long-term exposure can progressively lead to muscular, physical and neurological degenerative effects. The most common target organs for many trace elements are liver, kidneys, heart and lungs. Pb causes infamously known heavy negative effects on the brain and the central nervous system, similarly as Hg and As (Azeh Engwa et al. 2019). Both As and Pb are causing dermatological problems and due to disturbances in calcium metabolism, bone damage is often caused by Cd (Jaishankar et al. 2014). A special category is Hg and its organic species, which are lipophilic and easily surpass the cell membranes. That results in high bioaccumulation and extremely long biological half-life in organs like the brain (Clarkson et al. 2007; Ralston and Raymond 2018).

The toxic effects are well described for individual elements, but in reality, the exposure to a mixture of contaminants is more common (Anyanwu et al. 2018). This leads to "cocktail effect", where the joint toxic effects are significantly different and in some cases could increase the health risks (Whittaker et al. 2011; Zhou et al. 2017).

Also, the toxicity and negative effects of these trace elements are not only dependent on their overall concentration, but also on the chemical form under which they are present and their bioavailability. For example, in the water column, out of free ions, labile and non-labile dissolved fraction (together as a total dissolved fraction), colloid or particulate form – only the labile fraction and free ions are considered as the bioavailable and therefore with the most negative impact on the environment (Tessier and Turner 1995; Baeyens et al. 2018).

2.2 METHODS FOR ANALYSIS OF TRACE ELEMENTS

There are many available analytical techniques allowing analysis of trace elements and the choice of the right technique varies in dependence on the required selectivity, sensitivity and the budget available. Most of the techniques for determination of trace elements are spectroscopic and spectrometric methods, however, some electrochemical techniques are also used. Due to many difficulties in sample introduction or spectral and non-spectral interferences in measurement by spectroscopic and spectrometric methods, before the instrumental analysis, extensive pre-treatment of the sample in complex matrices is often required e.g. extraction, digestion, mineralisation or dilution of the sample (Biziuk and Kuczynska 2006; Bulska and Ruszczyńska 2017).

2.2.1 Spectroscopic and spectrometric methods

Spectroscopic methods are used to study physical systems by the electromagnetic radiation with which they interact or that they produce. Spectrometric methods are based on the measurement of electromagnetic radiation as a means of obtaining information about physical systems and their components. Among the most used spectroscopic methods are ultraviolet and visible light spectroscopy and atomic spectroscopy. The use of ultraviolet and visible light spectroscopy is limited by lower sensitivity and selectivity. In the case of atomic spectroscopy, there are different variations, including absorption, emission and fluorescence spectroscopy. Spectrometric methods for the analysis of trace elements include mass spectrometry. The in-depth principles of all mentioned spectroscopic and spectrometric methods were already described many times (Harvey 2000; Bulska and Ruszczyńska 2017; Yeung et al. 2017).

One of the most common spectroscopic method used for the analysis of trace elements is atomic absorption spectroscopy (AAS) (Gilmutdinov 2017). AAS is based on the interaction of the probing radiation beam from a primary source with gas-phase analyte atoms (Gilmutdinov 2017). Two different types of primary source are used in AAS: line sources emitting narrow spectral lines of the element to be analysed and a continuum source (normally a high-pressure xenon arc lamp) that produces radiation from below 180 nm to over 800 nm (Hill and Fisher 2017a). In principle, the continuum source is best suited for AAS. It has a unique capability for multielement determinations and provides extended analytical range and inherent background correction (Hill and Fisher 2017a; Yeung et al. 2017). However, the lack of intensity found below 280 nm still limits the use of the source in research laboratories (Gilmutdinov 2017). According to the type of atomisation, AAS can be divided into flame AAS or graphite furnace AAS. Special techniques of AAS are generating cold vapours or hydrides (Gilmutdinov 2017). AAS normally allows the analysis of liquid samples or suspensions. The analysis of solid samples is achieved by thermal decomposition AAS described in chapter 4.3.1.

Atomic emission for spectrochemical analysis is the oldest of modern atomic spectroscopic techniques (Holcombe and Farnsworth 2017). Although excitation by combustion flames and electrical arcs were methods of choice in the early days of spectrochemical analysis and have been replaced generally by modern plasmas, flames are still routinely used for determinations of elements of group I and II of the periodic table, and high-voltage sparks still find niche applications in, for example, metallurgical industries (Holcombe and Farnsworth 2017). Modern plasmas, especially inductively coupled plasmas, are the dominant excitation source in today's laboratories and provide detection capabilities in the range of parts per billion to subparts per billion for many trace elements (Bonchin et al. 2017; Holcombe and Farnsworth 2017; Yeung et al. 2017). With modern detectors (e.g. charge coupled devices), simultaneous multielement detection is also easily accomplished, and the dynamic range can easily cover 4–5 orders of magnitude in concentration for any given element (Bonchin et al. 2017; Bulska and Ruszczyńska 2017; Holcombe and Farnsworth 2017; Yeung et al. 2017).

The use of atomic fluorescence spectroscopy for routine analysis has been limited because competing techniques, most notably inductively coupled plasma mass spectrometry (ICP-MS), which provides comparable sensitivity and much greater versatility with simpler instrumentation. One exception is the use of cold-vapour atomic fluorescence spectroscopy (CV-AFS) for the determination of mercury. Because atomic vapour of Hg is easily generated at room temperature, relatively simple dedicated Hg detectors based on CV-AFS are capable of achieving subparts per trillion detection limits (Hill and Fisher 2017b; Holcombe and Farnsworth 2017).

Inductively coupled plasma mass spectrometry (ICP-MS) combines plasma with a mass spectrometer. It features a multielement capability, good precision, a long linear dynamic range, simple spectra, low detection limits and the ability to do rapid isotopic analysis (Beauchemin 2017; Bulska and Ruszczyńska 2017). With such advantages, ICP-MS has found wide application to the analysis of a variety of samples, including water and food, as well as geochemical, environmental and biological samples (Biziuk and Kuczynska 2006; Bulska and Ruszczyńska 2017; Wilkins 2017). Similar to spectroscopic methods, ICP-MS analyses liquid samples, however, laser ablation enables highly sensitive elemental and isotopic analysis to be performed directly on solid samples (Brenner 2017; Hill and Fisher 2017a; Koch and Günther 2017).

2.2.2 Electrochemical methods

The two principal electrochemical methods used for the analysis of trace elements are potentiometry and voltammetry. Potentiometric methods usually do not have limits of detection much below the micromolar level, so in the area of trace analysis, voltammetry generally holds the edge (Von Wandruszka 1994). Voltammetric methods include polarography, cyclic voltammetry, pulse voltammetry and stripping voltammetry (Mendoza et al. 2015). The main virtue of voltammetric techniques is their good accuracy, excellent precision, sensitivity, and wide dynamic range. One of the most widely used voltammetric methods for trace metal determination is anodic stripping voltammetry (ASV). In ASV metal ions in the sample solution are concentrated into a mercury electrode during a given time by application of sufficiently negative potential (Biziuk and Kuczynska 2006; Bulska and Ruszczyńska 2017). These amalgamated metals are then stripped (oxidised) out of the mercury by scanning the applied potential in the positive direction. The resulting peak currents are proportional to the concentration of each metal in the sample solution, with the position of the peak potential specific to each metal (Mendoza et al. 2015; Bulska and Ruszczyńska 2017).

The most common potentiometric method usable for determination of trace metals is potentiometry with an ion-selective electrode (ISE). Potentiometry with ISE is based on the measurement of the electrical potential difference between two electrodes, which, in contact with one or more electrolyte solutions, form an electrochemical cell (Biziuk and Kuczynska 2006). A high-impedance voltmeter measures the electrical potential difference between the two electrodes. The major disadvantages of ISE are the relatively high LODs and the susceptibility to the other components in the sample (e. g. complexions or interfering ions), the pH limitation, the slow response in diluted solutions or the dependence of the response on the temperature of the solution (Bulska and Ruszczyńska 2017).

2.2.3 Sample pre-treatment steps and specifics of trace elements analysis

When measuring trace elements in solid samples by spectroscopic, spectrometric or electrochemical methods, a prior sample pre-treatment stage is mandatory. The main treatment consists in destroying the organic matter of the sample and in converting the analyte into the solution. This process is called mineralisation or ashing (Andrade Korn et al. 2008). The two most common ashing procedures are wet ashing and dry ashing procedures. Wet ashing may be done using a typical hot plate or heating blocks or it may be done using microwaves under increased pressure (Biziuk and Kuczynska 2006). Many modern microwave systems are now under temperature and pressure control and various methods are available in the literature (Srogi 2006;

Andrade Korn et al. 2008; da Silva et al. 2010; Gholami et al. 2016; Godshaw et al. 2017). Dry ashing digestion procedures use a high-temperature muffle furnace capable of maintaining temperatures of between 200 and 600 °C to remove the organic matrix in samples, then the residues are dissolved with an appropriate solvent (Biziuk and Kuczynska 2006). Dry ashing digestion procedures require low volumes of reagents and provide low blank values, but during that process, some elements might volatilise or react with the digestion vessel, resulting into a low recovery rate (Freire and Santelli 2012; Dehouck et al. 2016).

Sample pre-treatment is a key step before the analysis of trace elements also in the case of liquid samples. The simplest method of liquid sample treatment is dilution or filtration of the sample (Godshaw et al. 2017). The dilution could, however, lower the concentrations of analytes below the working range of the instrumental technique used (Srogi 2006).

By analysis of non-homogeneous solutions, the instruments are facing an increased risk of mechanical complications, e.g. blocking the pneumatic nebuliser or the central quartz torch tube of ICP-MS (Subramanian 2006). Corrosive samples (e.g. with high salt content) are also exposing the metallic parts of the instruments to increased wear and corrosion (Dehouck et al. 2016).

Both environmental and food analysis procedures should follow the standards published by the National Standards Committee, International Standard Organisation or European Committee of Standardisation, which offer a different approach based on the available instruments and equipment (Biziuk and Kuczynska 2006).

2.2.3.1 Environmental analysis specifics

The bioavailability, mobility and toxicity of trace elements is not influenced only by the total concentration in the system, but primarily by the specific forms (species) of the elements present (Ure and Davidson 2002; Pawlaczyk et al. 2018). In past the term “speciation” has been used in numerous publications in different ways, describing the transformation or distribution of the species or the process of their determination. To avoid misinterpretation, this work will follow the IUPAC recommendations (Templeton et al. 2000):

- Speciation analysis should be the assessment of chemical species (defined by their isotopic composition, electronic or oxidation state and complex or molecular structure).
- Speciation should be the distribution of chemical species in a system.
- Fractionation should be a process of analyte classification based on its physical (e.g. size or solubility) or chemical (e.g. bonding or reactivity) properties.

2.2.3.1.1 Trace element fractionation

The aquatic environment involves two parts, the water column and the sediment. These parts can be further split into more sub-groups, the solid particulate phase (consisting of the suspended particulate matter and the sediment) and the dissolved phase (consisting of the water column and the sediment pore waters). In the water column, it is standard practice to filter the sample to split the sample into insoluble and soluble (the filtrate) fractions. Although the average size of different chemical species varies widely, the fraction discrimination is usually done by 0.45µm filtration (Pickering 2002; Menegario et al. 2017). The filtrate is usually analysed directly by spectroscopic or spectrometric technique, although the salt matrix increases the risk of corrosion or clogging the instrument (Freire and Santelli 2012; Dehouck et al. 2016). The total dissolved fraction of a trace element is not representative for the bioavailable fraction, which is directly accessible for organisms and therefore is more dangerous. Bioavailability depends on the lability (the ability of complex to dissociate) and the mobility of trace element (Tessier and Turner 1995; Linnik et al. 2018). The labile fraction than includes free (hydrated) metal and metalloid ions, their hydroxy complexes, complexes with inorganic ligands, and part of unstable complexes with organic ligands (Linnik et al. 2018). The possibilities that assess the labile fraction of trace elements are limited to the application of micro-electrodes, anodic stripping voltammetry and dialysis (Divis et al. 2005a; Gao et al. 2019). Further possibilities and advances of *in situ* determination of bioavailable fraction are the diffusive equilibrium in thin films technique and the diffusive gradients in thin films technique (Divis et al. 2005a; Gao et al. 2019). The suspended particulate matter (fraction caught on the 0.45µm filter) and the sediments are usually digested by microwave extraction with a mixture of acids, with or without HF (Rao et al. 2007). If more detailed fractionation of the solids is required, sequential chemical extractions are used instead, to divide the particulate trace elements into exchangeable, bound to carbonates, Fe-Mn oxides or organic matter and the residual particulates (Tessier et al. 2002). A similar approach is applied to the fractionation of soils (Rao et al. 2007).

2.2.3.1.2 Trace element speciation analysis

The different oxidation state in which is a trace element present is crucial for the toxicity, for example, Cr^{VI} is highly toxic, compared to essential Cr^{III} (Pawlaczyk et al. 2018). Differentiation between organic and inorganic species of trace elements is also important for the assessment of toxicity, as inorganic As species are more toxic compared with organic As species (Pawlaczyk et al. 2018). In opposite, the organomercury compounds are much more toxic than inorganic mercury species (Bernhoft 2012; Ali et al. 2019). Therefore, the speciation of trace elements is crucial when assessing their toxicity and risks. The spectroscopic and spectrometric analytical techniques themselves do not provide chemical or structural information about trace elements, since all forms are atomised or ionised during the analysis. The most common approach for speciation is then a combination of a separation technique, e.g. HPLC or GC, with a spectroscopic or a spectrometric technique, which then acts as a detector, e.g. ICP-MS, HG-AFS or CV-AFS – depending on the analyte (de Brauwere et al. 2009; Pawlaczyk et al. 2018; Ardini et al. 2020).

Despite the late instrumentation advances, the speciation analysis is still facing difficulties, especially during the analysis of complex matrices. Many trace element species are not stable enough and might change their form during the sample collection, handling, storage, pre-treatment or analysis (Pickering 2002; Pawlaczyk et al. 2018).

2.2.3.2 Food analysis specifics

The analysis of foodstuffs is challenging, as not many food samples are in the form of water solution. For direct analysis by spectroscopic or spectrometric technique, low surface tension and low viscosity of the solution is required for successful nebulisation in the instrument. From foodstuffs, only mineral water and clear non-carbonated soft drinks have such properties (Biziuk and Kuczynska 2006; Andrade Korn et al. 2008). Dissolved analytes (e.g. salt, sugar or extracts) can be separated from the food matrix by simple filtration. The filtrate could still require further steps, such as dilution, due to the matrix effects which could cause issues during analysis (e.g. ethanol content) (Andrade Korn et al. 2008; da Silva et al. 2010; Gholami et al. 2016). The insoluble analytes of solutions and solid samples require pre-treatment steps, usually more than one. Most often, the sample needs to be washed, processed as usual for the respective food type (e.g. peeled or eviscerated), homogenised and dried (by lyophilisation or oven drying) (Godshaw et al. 2017). These pre-steps are followed by either more gentle extraction technique step or more aggressive mineralisation step and the choice depends on analyte and food matrix (Voegborlo and Akagi 2007; da Silva et al. 2010). The extraction techniques, as liquid extraction, accelerated solvent extraction, supercritical fluid extraction and solid-phase extraction are used for speciation analysis and determination of organometallic compounds. The routinely used mineralisation step is dry ashing, which is cheap and fast, but it cannot be used for the determination of volatile elements, e.g. Hg, Se or As, Alkali fusions could be used for the geological samples, but are not recommended for the decomposition of biological samples. Therefore, the most common technique for mineralisation of foodstuffs is microwave acid digestion, performed in closed vessels of PTFE to avoid any losses or contamination (Falco et al. 2006; Julshamn et al. 2007; Voica et al. 2009). There are available instrumental methods, capable of analysis of trace elements in solids samples, namely TD-AAS for Hg and ET-AAS and LA-ICP-MS for all trace elements, however, they possess many limitations. TD-AAS and most of ET-AAS are analysing only one element at a time, which results in slow sample throughput. LA-ICP-MS is capable of analysing solids but due to high acquisition and operating costs is not frequently used for the analysis of trace elements in foodstuffs (Kitawaki and Abduriyim 2006).

In addition to the previous, the speciation analysis is also often performed in food analysis, as the organic species (e.g. Sn and Hg) have different toxicity and different legislative limit than the inorganic counterparts (Commission Regulation 1881/2006/EC 2006; Polatajko and Szpunar 2006).

2.2.4 Diffusive gradients in thin films technique

Diffusive gradients in thin films (DGT) technique is an environmental analysis tool, used as *in-situ* passive sampling technique. The DGT technique was first introduced in 1994 for environmental analysis, as it greatly simplified the determination of zinc in seawater since it effectively separated the analytes from the matrix, which otherwise caused specific problems during the analysis (Davison and Zhang 1994).

2.2.4.1 Principle

The DGT technique uses a diffusion-based mass transport of analytes through a porous diffusive hydrogel layer and accumulates the analytes on binding hydrogel layer which contains specific resins with functional groups for analytes (Davison and Zhang 1994; Zhang and Davison 1995).

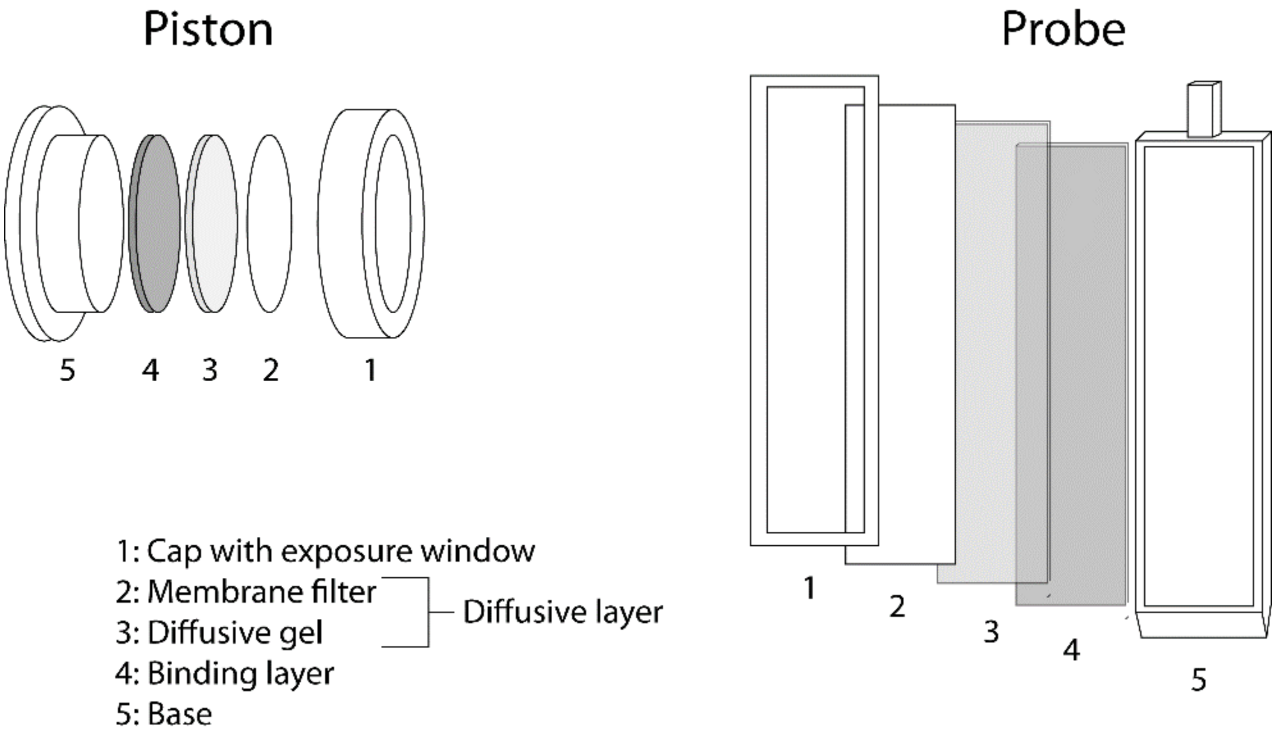

Figure 3 Schematic depictions of the layers in DGT mouldings. Adapted from Davison et al. (2007) and Gao et al. (2019).

The layers are enclosed in acrylonitrile butadiene styrene moulding, referred as a piston (for water, soil and sediments) or as a probe (for soil and sediment profiles), as shown in Figure 3.

After immersion of the DGT in the measured solution, the ions diffuse through a well-defined layer of diffusion gel of known thickness Δg to the binding hydrogel. The most widely used binding hydrogel for the determination of trace metals is the gel containing chelating resin Chelex-100 with bound iminodiacetic acid groups. The ions passed through the diffusion gel are bound to the functional groups on the surface of the resin until its capacity is saturated. Thus, a linear concentration gradient is established in the diffusion gel in a very short time (Figure 4). If the gradient remains constant during the deployment time t, by following the Fick's first law of diffusion (Davison and Zhang 2016b) it is possible to calculate the flow of analytes F from the bulk solution through the diffusive layer Δg [cm] by Equation 1:

$$F = D \cdot \frac{\mathrm{d}c}{\mathrm{d}x} = \frac{D \cdot (c - c_0)}{\Delta g}$$

Eq. 1,

where D is the diffusion coefficient [$\mathrm{cm}^2 \cdot \mathrm{s}^{-1}$] of analyte in the diffusive layer, c [$\mathrm{ng} \cdot \mathrm{cm}^{-3}$] is the concentration is the bulk solution and c_0 [$\mathrm{ng} \cdot \mathrm{cm}^{-3}$] is the concentration of the analyte on the surface of the binding layer (Figure 4). The thickness of Δg consists of the diffusive gel thickness and the membrane filter thickness (Figure 4). Assuming that the resin in the binding gel is not saturated, all the analyte is bound in the binding gel and therefore $c_0 = 0$. Close to any solid surface in a flowing aqueous environment, there is a solely diffusion-controlled thin layer, known as the diffusive boundary layer (DBL, δ). This layer appears even at vigorously stirred solutions between the membrane filter and bulk solution and it can affect the results by increasing the thickness of the diffusive barrier, where analytes must surpass to reach the accumulation layer (Figure 4). The parameter Δg is measurable, the DBL is determinable experimentally and usually negligible in well-stirred solutions (in laboratory conditions and flowing natural waters). In still natural waters, the DBL is in the range of 0.1-1 mm (Diviš 2013; Davison and Zhang 2016b).

The analyte flow through Δg is be also defined by Equation 2 (Diviš 2013; Davison and Zhang 2016b):

$$F = \frac{M}{A \cdot t}$$

Eq. 2

where M [ng] is the mass of analyte in the binding gel, A is the exposure area [cm^2] and t the exposure time [s] (Diviš 2013; Davison and Zhang 2016b).

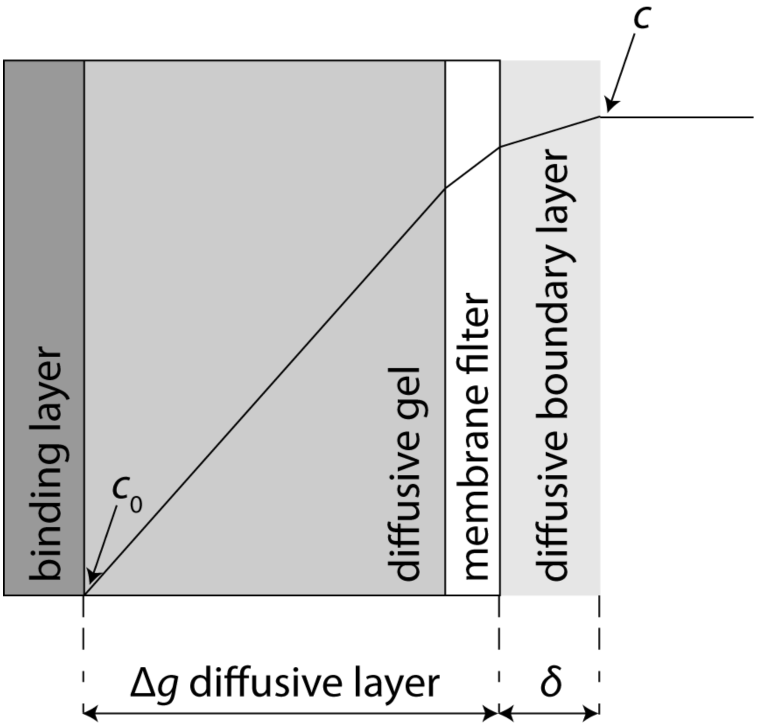

Figure 4 Schematic representation of the steady-state concentration gradient of the analyte through the binding and diffusion layers withing a DGT device. Adapted from Zhang and Davison (1995).

Equation 1 combined with Equation 2 forms Equation 3 (Diviš 2013; Davison and Zhang 2016b):

$$\frac{D \cdot c}{\Delta g} = \frac{M}{A \cdot t}$$

Eq. 3,

which after adjustment forms Equation 4 used to calculate the concentration of the analyte in the bulk solution (Diviš 2013; Davison and Zhang 2016b):

$$c = \frac{M \cdot \Delta g}{D \cdot t \cdot A}$$

Eq. 4.

The mass of analyte in the binding gel can be either measured directly by proton-induced X-ray emission or by LA-ICP-MS for trace elements (Davison et al. 1997; Gao and Lehto 2012) or by TD-AAS for Hg (Divis et al. 2005b). *M* can also be analysed indirectly, after the exposure. In that case, the resin gel is eluted by suitable eluent and the eluate is analysed by one of the spectroscopic or spectrometric methods, e.g. by AAS (Diviš et al. 2012) or by ICP-MS (Gao et al. 2019). For the determination of organic compounds, HPLC, GC or tandem techniques with mass spectrometry are required (Gao et al. 2019).

2.2.4.2 Advantages

The main advantages of the DGT technique are *in-situ* sampling nature, passive sampling, preconcentration of analyte and matrix effect removal, which are useful benefits for environmental application. Also, the numerous modification possibilities offer wide specificity and application range, by choosing a suitable combination of the DGT components (Garmo et al. 2003; Bennett et al. 2016).

The major advantage of in situ measurement compared with laboratory analysis is the elimination of artefacts due to sample handling. These artefacts include adsorption of compounds to any surface used during sampling, gaseous re-equilibration of the sample with the atmosphere, coagulation of colloidal matter or changing of trace compounds because of microbial activity. Other advantages of in situ measurement are the possibility of real-time analysis or measuring concentration gradients and fluxes at environmental interfaces (Buffle et al. 1997).

The passive sampling nature of the DGT technique offers a great advantage for environmental monitoring, as it presents the time-weighted concentration average. That provides more representative results than the instantaneous concentration peaks of contaminants during grab-sampling, e.g. caused by rainfall or tidal changes (Gong et al. 2018).

The pre-concentration feature of the DGT technique eliminates the requirement for the difficult pre-concentration steps of large volumes of grab-sampled water, which is usually required to perform the trace-level analysis (Gong et al. 2018). This lower LOD goes along with the matrix effect removal, which further simplifies the analysis procedures (Uher et al. 2012), especially for analysis in the environments with the complicated matrix.

2.2.4.3 Application

At present, the DGT technique is routinely used for assessment of more than 55 elements (Garmo et al. 2003). By proper choice of the components, the DGT technique also allows the speciation between As^{III} and As^{V} (Bennett et al. 2011; Sun et al. 2014; Guan et al. 2015) or between inorganic and organic Hg species (Clarisse and Hintelmann 2006; Hong et al. 2011; Gao et al. 2014; Ren et al. 2018a).

From the assessment of trace metals and metalloids, the application possibilities have expanded widely and nowadays, the DGT technique is also applied with suitable instrumental method to determine pesticides (Guibal et al. 2017), antibiotics (Ren et al. 2018b), endocrine-disturbing compounds (Guo et al. 2017; Guo et al. 2019) and drugs (Zhang et al. 2018) in many different environments, e.g. seawaters (Gao et al. 2019), freshwaters (Divis et al. 2007), wastewaters (Challis et al. 2020), river and marine sediments (Divis et al. 2005b) or soil (Liu et al. 2012a).

The DGT technique assesses the free ions and most of the naturally occurring metal and metalloid complexes in the water. These metal and metalloid complexes are labile species, which will dissociate in the diffusive layer of the DGT sampler (Zhang and Davison 2015). The results of the DGT technique correlate well with the concentration of bioavailable species because the DGT technique mimics the diffusion limiting uptake conditions of trace elements from soils by plants (Degryse and Smolders 2016). Even in the aquatic environment, the uptake by aquatic organisms is strongly correlated with the concentrations of free ions and labile complexes of trace elements obtained by the DGT technique (Lehto et al. 2006; Davison et al. 2007). Despite the bioavailability of trace elements is not only affected by the presence of labile complexes but also by competing ions, the DGT technique is used to assess the bioavailable species and bioavailability predictions (Pelcova et al. 2018).

2.2.4.4 Diffusive and binding layers used in DGT

2.2.4.4.1 Diffusive layer

The key feature of DGT is that the solutes are transported through the diffusive layer in a controlled and predictable manner, during which they should not interact with it (Davison 2016). In the DGT technique, the diffusive layer consists of a membrane filter and diffusive gel (Figure 3). The membrane filter protects the DGT assembly from mechanical damage and serves also as a filtration step. Most used filter membranes are made of polyethersulphone or hydrophilic polyvinylidene fluoride with 0.45μm pore size and 125μm thickness (Wang et al. 2016).

The diffusive gels are hydrogels, where the transport of solutes occurs solely by free diffusion. Most common diffusive gels are made of polyacrylamide cross-linked with a commercial agarose derivative (APA). Another alternative is polyacrylamide crosslinked with N,N'-methylene-bis-acrylamide, also known as a restrictive gel (RES). The polymerisation of both diffusive gel types is initiated by adding ammonium sulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) and temperature of 45 °C is maintained during that process (Davison and Zhang 2016a). The effective pore size of these gels depends on the proportion of reagents, however, the pore sizes of RES are generally smaller (Zhang and Davison (1999) reported <2 nm) than in APA (Davison and Zhang (1994); Zhang and Davison (1999) reported 5-10 nm). Polyacrylamide gels contain in their structure free primary amino groups, which disallows free diffusion of some analytes, especially Hg, which is getting accumulated in these diffusive gels (Docekalova and Divis 2005). For these reasons, agarose diffusive gel (AGE) is used instead during the determination of Hg. AGE is made of agarose dissolved in hot water to form a 1.5% solution, after cooling down this solution forms a gel with an average pore size of 35-47 nm (Fatin-Rouge et al. 2003). The use of AGE bears some disadvantages, as the naturally occurring agarose is degrading during long term field deployment (Challis et al. 2018; Stroski et al. 2018) and as it can contain some impurities, which could also affect the free diffusion process, e.g. sulphonates, ester sulphate, ketal pyruvate and carboxyl groups (Fatin-Rouge et al. 2003).

2.2.4.4.2 Binding layer

The binding layer consists of hydrogel, which contains analyte-specific cation- or anion exchange resin, metal (hydr)oxides or functionalised silica beads, which accumulates the analytes. Since the resins are in the DGT technique used more often, the binding layer is commonly referred as a resin gel. The main requirement on the resin is to bind the analytes rapidly and irreversibly to achieve the ideal conditions for the functioning of the DGT technique (Bennett et al. 2016).

For every resin used in the resin gel, laboratory validation must be performed, following a series of experiments (Divis et al. 2010; Bennett et al. 2016). First, the suitability for the uptake of the analytes and ability to quantitatively recover the analytes back by elution is evaluated, followed by testing the ability of linear accumulation of analytes over time. The DGT technique is based on the stable flux of analytes through the diffusive layer, therefore, the linear accumulation of analytes over time and the capacity of resin gel are tested to avoid the saturation and loss of constant flow of analytes. The linear accumulation is verified by performing time-series experiments (e.g. 6, 12, 18, 24 h) and evaluating the mass of accumulated analytes in the resin gel. Capacity is assessed by deployment in solution with a high concentration of target analyte and an evaluation of equilibria states. Since the conditions in natural systems vary significantly, the resin gel requires a wide working range of ionic strength and pH to maintain its performance. That is evaluated by deployment the resin gels into model solutions of different ionic strength and pH and comparing the performance.

The first resin gel used in the DGT technique held Chelex-100 resin and it is still routinely used for determination of many divalent or trivalent metals in DGT technique. Chelex-100 is a styrene-divinylbenzene copolymer-based ion exchanger with iminodiacetate functional groups and binds a wide range of elements (Davison 2016; SenGupta 2017). Lowered performance and non-linear uptake of Hg was reported due to iminodiacetic functional groups of Chelex-100 and their low affinity towards Hg and Hg-chloride complexes (Divis et al. 2009; Gao et al. 2011; Hong et al. 2011).

The mechanism of Hg-selective resins is based on the high affinity of Hg towards sulphur. Many commercially available resins were introduced for use in the DGT technique, e.g. dithiocarbamate-based Sumichelate Q10R (Gao et al. 2011) or thiol-based Duolite GT73 and Spheron-Thiol (Divis et al. 2010). However, the availability of such resins is limited, as the production of most of them has been discontinued. Currently, only 3-mercaptopropyl silica (3-MFS) still being routinely used

(Divis et al. 2009; Divis et al. 2010; Gao et al. 2011). However, the laboratory practice shows that the preparation of 3-MFS resin gels could require a lot of manual experience. Another possibility is a modification of commercially available resin, e.g. Iontosorb AV modification with 6-mercaptopurine or imidazole (Divis et al. 2009; Divis et al. 2010). Production of novel thiol-functionalised silicas was also reported for use with the DGT technique (Gao et al. 2011). More Hg-selective resins are currently commercially available, with thiourea, isothiouronium or thiol functional groups on different matrices, however, their particle sizes are higher than the thickness of resin gel, therefore their performance in the DGT technique was not yet tested (Zaganiaris 2016; SenGupta 2017), except for resin with isothiourea groups (Ren et al. 2018a). The DGTs with 3-MFS resin is also used for As^{III} speciation (Bennett et al. 2011), while for As^{V} Amberlite IRA 910 is used (Rolisola et al. 2014) and metal oxides are used for total As (Panther et al. 2008; Price et al. 2013; Sun et al. 2014). Use of liquid binding phases (Liu et al. 2012b), chromatography papers (Larner and Seen 2005; Almeida et al. 2012), and other binding agents (Bennett et al. 2016) as the alternative binding layers are also reported in the literature, however many of these do not offer any significant advantage to the layers routinely for trace metals assessment.

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3 AIMS AND OBJECTIVES

The goal of this work is to study the novel possibilities for the determination of Hg along with other trace metals (metalloids excluded) by the DGT technique and the application of the DGT technique for food analysis.

Some of the resins formerly used in the DGT technique for determination of Hg are no longer available, while other synthesised or modified resins and new commercially available resins have appeared as alternatives. These novel resins are also offering a promising potential for simultaneous determination of Hg along with other trace metals by DGT technique, which has been not reported yet. Therefore, synthesis and characterisation of novel resin and its application in the DGT technique simultaneous determination of Hg and other trace metals is the first objective of this work. Additionally, the application of novel commercially available resins in the DGT technique for simultaneous determination of Hg and other trace metals is also studied.

The possibilities of the application of the DGT technique for food analysis are also studied, using fish sauce as a typical example of a complex food matrix with contamination by trace metals. The benefits of the DGT technique could offer an advantage compared to routinely used analysis methods.

- Application of new resin in the DGT technique
    - Synthesis and characterisation of novel resin for use in the DGT technique (chapter 5.1.1)
    - Application of novel commercially available resins in the DGT technique (chapters 5.2.1 and 5.2.2)
- Application of the DGT for food analysis
    - DGT method development and validation for determination of Hg in fish sauces (chapter 5.2.1)
    - DGT method development and validation for multi-trace metal determination in fish sauces (chapter 5.2.2)

4 MATERIALS AND METHODS

4.1 CHEMICALS AND MATERIALS[1]

Acrylamide solution (40%, p.a., Merck, Darmstadt, Germany), agarose powder (Biorad, Hercules, USA), 3-aminopropyl-functionalised silica gel (40-63 µm particle size, Sigma-Aldrich, Darmstadt, Germany), amino-functionalised silica gel (40-75 µm particle size, Sigma-Aldrich, Darmstadt, Germany), ammonium iron (III) phosphate dodecahydrate (ACS, Sigma-Aldrich, Darmstadt, Germany), ammonium persulfate (>98%, Merck, Darmstadt, Germany), cadmium stock solution 1000 $mg \cdot L^{-1}$ (VWR, Haasrode, Belgium), certified reference material DORM-4 (fish protein, National Research Council Canada, Ottawa, Canada), certified reference material ERM-B422 (fish muscle, Joint Research Centre, Geel, Belgium), certified reference material ERM-CC580 (estuarine sediment, Joint Research Centre, Geel, Belgium), Chelex-100 (200–400 mesh, Biorad, Hercules, USA), copper stock solution 1000 $mg \cdot L^{-1}$ (VWR, Poole, UK), L-cysteine (BioUltra, Sigma-Aldrich, Darmstadt, Germany), DGT cross-linker (DGT Research Ltd, Lancaster, UK), DGT pistons (DGT Research Ltd, Lancaster, UK), Durapore membrane filters (0.45 µm pore size, 125µm thickness, Merck, Darmstadt, Germany), ethanol (99 %, Sigma-Aldrich, Darmstadt, Germany), fish sauce samples (various retails and countries of origin), formaldehyde (37%, VWR, Leuven, Belgium), glutaraldehyde solution (25 %, Sigma-Aldrich, Darmstadt, Germany), hydrochloric acid (optima grade, Fisher Scientific, Leicestershire, UK), lead stock solution 1000 $mg \cdot L^{-1}$ (VWR, Poole, UK), 3-mercaptopropyl-functionalised silica gel (Sigma-Aldrich, Darmstadt, Germany), mercury stock solution 1000 $mg \cdot L^{-1}$ (Alfa Aesar, Kandel, Germany), MilliQ water (MQ, Millipore, >18 $M\Omega \cdot cm$, Merck, Darmstadt, Germany), N,N,N,N-tetraethylenediamine (99%; p.a., Merck, Darmstadt, Germany), nickel stock solution 1000 $mg \cdot L^{-1}$ (Merck, Darmstadt, Germany), nitric acid (optima grade, Fisher Scientific, Leicestershire, UK), nitrogen gas (Linde, Brno, Czech Republic), oxalic acid dihydrate (p.a., Merck, Darmstadt, Germany), oxygen gas (Linde, Brno, Czech Republic), potassium bromide (FT-IR grade, Sigma-Aldrich, Darmstadt, Germany), potassium chromate (p.a., Penta, Prague, Czech Republic), potassium thiocyanate (p.a., Penta, Prague, Czech Republic), Purolite® S924 resin (Purolite Ltd), silver nitrate (p.a., Sigma-Aldrich, Sigma-Aldrich, Darmstadt, Germany), sodium bicarbonate (p.a., Merck, Darmstadt, Germany), sodium chloride (extra pure, Sigma-Aldrich, Seelze, Germany), sodium hydroxide (p.a., Merck, Darmstadt, Germany), sodium phosphate dibasic (p.a., Sigma-Aldrich, Darmstadt, Germany), sodium phosphate monobasic (p.a., Sigma-Aldrich, Darmstadt, Germany), sulphanilamide (analytical

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[1] The supplier could vary based on the location where the experiments were conducted (Czech Republic or Belgium).

standard, Supelco, Darmstadt, Germany), Teflon foil (0.25/0.50/0.75mm thickness, Bohlender GmbH, Grünsfeld, Germany).

All glassware and plasticware were immerged in 1 mol·L^{-1} HNO_3 overnight and rinsed three times by MQ water before use. The solution of 0.6% glutaraldehyde was prepared in 0.1 mol·L^{-1} mono-/dibasic sodium phosphate buffer (pH 7), solutions with different cysteine concentration were prepared by dissolving in 0.1 mol·L^{-1} mono-/dibasic sodium phosphate buffer (pH 6) (chapter 4.2.1.1). Solutions of $AgNO_3$, NaCl, KSCN and HCl in MQ water and $NaHCO_3$ in ethanol were prepared for the titrimetric analysis and standardisation and saturated solution of $NH_4(FeSO_4)_2$ in 1.2 mol·L^{-1} HNO_3 was prepared as the indicator for the thiol groups quantification (chapter 4.2.1.2). Purolite S924 resin was ground and sieved through 50μm nylon sieve (chapters 5.2.1 and 0). Polyacrylamide gel solution was prepared by mixing 15 % acrylamide and 0.4 % agarose-derived crosslinker, ammonium persulfate was mixed with MQ water to prepare 10% solution and agarose was mixed with MQ water to prepare 1.5% solution (chapter 4.2.2.1). Solutions of 0.01 mol·L^{-1} NaCl and 0.01 mol·L^{-1} $NaNO_3$ were prepared for storage of the diffusive and resin gels (chapter 4.2.2.1). Uptake kinetics solutions were prepared as 1 mg·L^{-1} of trace metals in 0.01 mol·L^{-1} NaCl solution and *aqua regia* solution (optima HCl and optima HNO_3 in 3:1 molar ratio) and 1 mol·L^{-1} HNO_3 (optima grade) solution were prepared for elution experiments (chapter 4.2.2.2.1). Solutions for testing the linear accumulation in time (chapter 4.2.2.2.2) and the influence of pH and ionic strength (chapter 4.2.2.2.4) were prepared as 20-50 μg·L^{-1} of trace metals in 0.001-1.0 mol·L^{-1} NaCl solution and the pH was adjusted in the range of 4.5-8.5 by HNO_3/NaOH. Fish sauce solutions for the DGT deployment were prepared by diluting fish sauce samples were diluted 5 times with MQ water. For the test of uptake kinetics in fish sauce, the fish sauce solution was spiked to 1 mg·L^{-1} of trace metals (chapter 4.2.2.2.1). For the test of linear accumulation in fish sauces, the fish sauce solutions were spiked to 20-50 μg·L^{-1} of trace metals (chapter 4.2.2.2.2).

4.2 METHODS

4.2.1 Cysteine modified silica preparation and characterisation

4.2.1.1 Modification of silica

Synthesis of novel resin allowing simultaneous binding of Hg and other trace metals can resolve different and complicated requirements for resins in the DGT technique. One of the possible designs comes from cellular defence mechanisms against hazardous metals. The major scavenging and disposal of hazardous trace metals at the cellular level occur by antioxidant glutathione (GSH) (Ali et al. 2019). GSH is a tripeptide, biosynthesised of cysteine bound to glycine and a side chain of glutamate (Jozefczak et al. 2012). However, the cysteine was found more effective at extracting Pb than GSH during experiments with trace metals extraction from soils (Vadas and Ahner 2009), wherefore similar could be expected for other trace metals. Using the detoxifying biomolecules-based resin could offer interesting insight on bioavailability as it contains molecules with functional groups present in the environment and can closely mimic the hazardous metals binding and adsorption processes occurring in the organisms (Bridges and Zalups 2005; Hoffman and Hubbell 2013; Jan et al. 2015; SenGupta 2017).

Preparation of different cysteine-functionalised particles was previously reported by immobilisation techniques (Dakova et al. 2011; Upadhyay and Verma 2014; Verma et al. 2017). Immobilisation is a physical or chemical attachment of molecules in/on certain space. Out of three major immobilisation methods, the immobilisation based on the physical interactions and the immobilisation by entrapment is not suitable for further application in the DGT technique, with only immobilisation by covalent attachment remaining. In that case, a covalent bond is formed between the support and immobilised molecule, often using a mediator. The functionality and activity of immobilised biomolecule can be affected, based on the functional groups used for forming the bond (Hoffman and Hubbell 2013). The distance between the carrier and the immobilised molecule is known as a spacer. The length of the spacer is also affecting the activity of the immobilised molecule because too short spacer can lead to steric hindrance (Zhang et al. 2014). The use of glutaraldehyde and supports containing primary amino groups is one of the most frequently used enzyme and biomolecule immobilisation techniques (Betancor et al. 2006). Due to its sensitivity for storage, oxygen, pH changes and easy ongoing autopolymerisation, controlled reaction conditions are required and there is still no agreement about the main reactive species in glutaraldehyde solutions during the binding process despite its frequent usage (Margel and Rembaum 1980; Migneault et al. 2004; Betancor et al. 2006; Cheung and Nimni 2009). In brief, working with diluted solutions in the temperature up to 25 °C in neutral/slightly acidic pH should result into the high occurrence of the monomeric form (Rasmussen and Albrechtsen 1974; Margel and Rembaum 1980; Migneault et al. 2004). Working in an oxygen-free atmosphere is also recommended (Dakova et al. 2011), although the conclusions about the influence of oxygen are not consistent (Gillett and Gull 1972).

Resin modification was performed by glutaraldehyde-mediated immobilisation of cysteine on the surface of various silicas with amino functional groups, according to the procedure from Dakova et al. (2011). Amino-functionalised silica and 3-aminopropyl-functionalised silica were used as carriers and two modified resins were called Cysteine-modified Amino-functionalised Silica (CAS) and Cysteine-modified 3-AminoPropyl-functionalised Silica (CAPS). In the first step, the amino groups of silica reacted with 40 mL 0.6% glutaraldehyde solution in 0.1 mol·L^{-1} mono-/dibasic sodium phosphate buffer (pH 7) for 1 hour under nitrogen atmosphere to avoid auto-polymerisation of glutaraldehyde (Margel and Rembaum 1980) and finally formed an activated glutaraldehyde-silica intermediate (Migneault et al. 2004; Dakova et al. 2011). After glass microfibre filtration and MQ water washing, cysteine dissolved in 0.1 mol·L^{-1} mono-/dibasic sodium phosphate buffer (pH 6) was added. The mixture was stirred and slowly bubbled through by nitrogen. The activated glutaraldehyde-silica intermediate formed a bond with the functional groups of cysteine (Dakova et al. 2011; Verma et al. 2017). After 48 hours of stirring, the solid product was collected by glass microfibre filtration and washed by MQ water. Products were dried under vacuum for 72 hours and sieved by a nylon sieve to obtain the fraction of particles below 75 µm. By changing the weight ratio of cysteine and silica, in total 22 resin variants were prepared including 8 variants of CAS and 14 variants of CAPS, which were further characterised.

4.2.1.2 Characterisation of the modified silica

To optimise the modification process, different reaction conditions were set by changing the ratio of cysteine and silica. The resins prepared at different conditions were analysed by CHNS elemental analysis (chapter 4.3.3) and the variants with the highest total sulphur and nitrogen content were chosen. The functional groups on the final CAS and CAPS were first qualitatively characterised by DRIFT spectroscopy (chapter 4.3.4). Subsequently, the detected functional groups of the CAS and the CAPS were further quantified by titrimetry. The load of reachable thiol groups on the modified resins and in cysteine for verification was determined by argentometry – Volhard's titration (Vogel 1939; Gao et al. 2011). The thiol groups react with a known excess amount of $AgNO_3$ (standardised against NaCl) and the remaining Ag is titrated by standardised KSCN solution. The saturated solution of $NH_4Fe(SO_4)_2$ in 1.2 mol·L^{-1} HNO_3 was used as the indicator (United States Department of Agriculture 2009; Gao et al. 2011). The amount of reachable carboxylic groups on the modified silica was determined by Sørensen formol titration (Kolthoff and Stenger 1942; Akluwalia and Aggarwal 2007). In this method, the amino groups react with excess formaldehyde (neutralised to pH 7), forming a methylene-amino group. The remaining carboxylic acid groups are then titrated by NaOH (standardised against oxalic acid). The amount of reachable primary amino groups on the native and modified silica was estimated by the method described by Foreman and Harris (Foreman 1920; Harris 1924; Szabadváry 2013). After neutralising the carboxyl groups by soda titration in the ethanolic environment, the primary amino groups were then back-titrated by hydrochloric acid. The titration methods were in this work used for characterisation and quantification of functional groups in modified silicas to optimise the resin synthesis procedure and achieve as the highest load of functional groups as possible.

4.2.2 Diffusive gradients in thin films (DGT) technique

4.2.2.1 DGT preparation and assembly

4.2.2.1.1 Diffusive gels

The polyacrylamide with agarose derivative crosslinker (APA) diffusive gels were prepared of gel solution mixture, consisting of 15 % acrylamide and 0.4 % agarose-derived crosslinker. The polymerisation was initiated by 7 µL of fresh ammonium persulfate solution and 2.5 µL of N,N,N',N'-tetramethylethylendiamine (TEMED) per millilitre of gel solution. After shaking, the mixture was cast between two glass plates with a spacer of different thickness 0.25/0.50/0.75 mm. After the polymerisation was finished, the APA gel sheet was hydrated in three batches of MQ water for 24 hours. During the hydration, the APA gel expanded by ~60 % to 0.4/0.8/1.2mm thickness. After the hydration, the gel discs were cut with plastic cutter (2cm diameter) and stored in containers with 0.01 $mol \cdot L^{-1}$ NaCl solution in a fridge (Docekalova and Divis 2005).

The agarose (AGE) diffusive gels were prepared by casting 1.5% hot agarose solution between two glass plates with a spacer of different thickness (0.25/0.50/0.75 mm). For the preparation of the resin gels with the 3-MFS, the S924, the CAS and the CAPS resins, the resin was mixed with 1.5% hot agarose solution and the gel solution was cast in two glass plates with a spacer of 0.5 mm. After cooling down, the gel sheets were washed three times by MQ water and gel discs were cut with plastic cutter and stored separately in containers with 0. 01 $mol \cdot L^{-1}$ NaCl solution in a fridge (Docekalova and Divis 2005).

4.2.2.1.2 Resin gels

Chelex resin gels were prepared of gel solution mixture, consisting of 15 % acrylamide and 0.4 % agarose-derived crosslinker and 0.35 g of the Chelex resin was added to the gel solution. The polymerisation was initiated by 7 µL of fresh ammonium persulfate solution and 2.5 µL of TEMED per millilitre of gel solution. After shaking, the mixture was cast between two glass plates with a spacer of 0.25mm thickness. After the polymerisation was finished, Chelex resin gel sheets were separately hydrated in three batches MQ water for 24 hours and gel discs were cut with plastic cutter (2cm diameter) and stored separately in containers with 0.01 $mol \cdot L^{-1}$ NaCl or solution in a fridge (Docekalova and Divis 2005).

For the preparation of the resin gels with the 3-MFS, the S924, the CAS and the CAPS resins, the resin was mixed with 1.5% hot agarose solution and the gel solution was cast in two glass plates with a spacer of 0.5 mm. After cooling down, the gel sheets were washed three times by MQ water, gel discs were cut with plastic cutter and stored separately in containers with 0.01 mol·L^{-1} NaCl solution in a fridge (Docekalova and Divis 2005).

4.2.2.1.3 DGT assembly

For the assembly of the DGT piston, the resin gel was placed on the top of the piston. The resin gel was covered by diffusive gel and by a 0.45μm pore size membrane filter. The front cap was pressed tightly (Docekalova and Divis 2005). The AGE diffusive gel was used in the combination with 3-MFS, S924, CAS and CAPS resin gels, hereafter referred as “3-MFS-DGT”, “S924-DGT”, “CAS-DGT” and “CAPS-DGT”. The APA diffusive gel was used in the combination with Chelex resin gels because none of them is applicable for the Hg assessment and it is the common DGT setup (Docekalova and Divis 2005).

4.2.2.2 Basic DGT tests

Whenever a new resin is used in the DGT technique, a set of experiments is required for its validation. When an already validated DGT technique is used in a different deployment condition (e.g. different pH, ionic strength, salinity) the validation is also recommended, because the composition of the deployment solution can also affect the diffusion and binding processes.

The DGT experiments were performed at 20 °C in a glass beaker (volume of 4L, stirred at 400 rpm by Teflon stirrer bar, chapter 5.2.1) or in polypropylene containers with a lid and a polycarbonate DGT piston rack inside (volume of 1.75 L stirred at 1200 rpm by Teflon stirrer bar, all other chapters).

4.2.2.2.1 Uptake kinetics and elution

The uptake kinetics of the resin gels were evaluated in 1 mg·L^{-1} mixture solution of different analytes and different matrixes, based on the topic of the work. The solution was equilibrated for 12 h in the lab, and afterwards, the resin gels were separately immersed in the solutions and were then mildly shaken for 24 h. Subsamples from the solutions were collected every 5 min for the first hour, and then every hour to monitor the concentration change. Then the subsamples were pre-treated (if required) and analysed by TD-AAS or ICP-MS. By plotting the concentration changes of the solutions versus time, the uptake kinetics (the uptake as a function of the deployment time) was determined by mass balance calculation (Mason et al. 2005).

Whenever a new resin is used in the DGT technique and the direct analysis of resin gel is not possible (e.g. for ICP-MS analysis), a suitable elution reagent is needed and the elution factor f_e must be calculated using Equation 5:

$$f_e = \frac{M_e}{(M_0 - M_T)} \qquad \text{Eq. 5,}$$

where M_e is the mass of analyte eluted from the resin gel, M_0 is the mass of the analyte in the stock solution at the beginning of the experiment, and M_T is the mass of analyte in the same solution at the end of the experiment (Bennett et al. 2016). For the determination of elution factors, the resin gels were retrieved at the end of the exposure experiment and rinsed by MQ water. For the S924-DGT, the CAS-DGT and the CAPS-DGT, two different eluents were tested, 1 mL of 1 mol·L^{-1} HNO_3 for 24 h (Panther et al. 2014) and 1 mL of *aqua regia* heated at 70 °C for 24 h (Abdulbur-Alfakhoury et al. 2019; Bratkic et al. 2019). The elution method with higher f_e was further used. The elution of the 3-MFS-DGT and the Chelex-DGT followed the routine procedures and 70°C *aqua regia* (Fernandez-Gomez et al. 2011; Bratkic et al. 2019) and 1 mol·L^{-1} HNO_3 (Panther et al. 2014) was used, respectively. All the eluates were diluted ten times before the ICP-MS analysis.

4.2.2.2.2 Linear accumulation in time

To ensure accurate measurements of solutes by the DGT technique, linear accumulation of the target analyte must be met to ensure the fundamental assumption of the DGT technique.

Nine DGT pistons were deployed in 20-50 µg·L^{-1} mixture solution of different analytes and different matrixes, based on the topic of the work. The batches of pistons were retrieved at three different time intervals. The pistons were disassembled, and the resin gels washed by MQ water and analysed by TD-AAS or eluted and analysed by ICP-MS. The effective diffusion coefficients D_e were then calculated using the linear accumulation curve. The slope (k) of the linear regressions of the accumulated mass normalised over the solution concentration (M/c) as a function of time was used to determine the D_e [cm^2·s^{-1}] using Equation 6 (Docekalova and Divis 2005), where Δg is the thickness of the diffusive layer, A is the exposure are of the DGT piston and t is the DGT deployment time:

$$D_e = \frac{k \cdot \Delta g}{A \cdot t} \qquad \text{Eq. 6.}$$

4.2.2.2.3 Diffusive boundary layer

A diffusion-controlled thin layer, known as the diffusive boundary layer (DBL, δ), exists at any solid surface in an aqueous environment, and its thickness depends on the turbulence or flow pattern close to that surface. This layer even exists in vigorously stirred solutions and it can affect the DGT results. The higher the turbulence in the solution, the thinner the DBL and the more analyte will accumulate on the DGT resin in the same period. The DBL was assessed by using different thickness of diffusive gels in the DGT pistons (Davison and Zhang 2016b). The experiment was performed by the deployment of nine DGT pistons, with three different diffusive gel thickness in a solution for 4 h. After retrieving, the resin gels were analysed by TD-AAS or eluted and analysed by ICP-MS. The reciprocal mass of analytes accumulated on the binding gel (M^{-1}) is plotted versus the thickness of the diffusion layer (Δg). Using the slope k and intercept q of the linear curve, the thickness of the DBL was calculated using Equation 7 (Warnken et al. 2006), where D_e is the measured effective diffusion coefficient of the analytes in this study and D_w is the diffusion coefficient of the analytes in water obtained from literature:

$$\delta = \frac{k}{q} \cdot \frac{D_w}{D_e} \qquad \text{Eq. 7.}$$

4.2.2.2.4 Influence of pH and ionic strength

To ensure accurate performance of the DGT technique in a range of environmental conditions, the influence of pH and ionic strength was tested, since that can impact the interactions between the resin gel and the analytes (Bennett et al. 2016).

The performance of the DGT technique was evaluated by deploying the DGT pistons into solutions spiked with the analytes at different pH and ionic strength. The pH was adjusted by HNO_3/NaOH in the range of 4.5-8.5, which is the typical pH value of natural waters and fish sauce (Park et al. 2001; Bennett et al. 2016; Nakano et al. 2017). The ionic strength was adjusted in the range of 0.01-1.0 $mol \cdot L^{-1}$ represented by NaCl, which is the typical ionic strength in natural waters and 5-fold diluted fish sauce. After the deployment, the pistons were disassembled, the resin gels washed by MQ water and analysed by TD-AAS or eluted and analysed by ICP-MS. Subsamples of the deployment solutions were taken at the beginning and the end of the deployment and were analysed by TD-AAS or ICP-MS. The DGT measured concentrations (cDGT) were calculated by using Equation 4. The cDGT was compared with the cSOL (results of direct TD-AAS or ICP-MS analysis of the solution) and the ratios of cDGT/cSOL were calculated to check the performance variation (Divis et al. 2010). A ratio in the range of 0.9-1.1 indicates an accurate DGT performance (Bennett et al. 2016).

4.2.3 Sample preparation of foodstuffs

As described in section 2.2.3.2, most of the food samples require mineralisation as a pre-treatment step. Most often, closed Teflon vessel digestion using acid or a mixture of acids with microwave heating is used (Biziuk and Kuczynska 2006). In this work, the microwave-assisted digestion of food samples was performed by Anton Paar Multiwave Go (Anton Paar, Graz, Austria), following EPA3052 method for microwave-assisted acid digestion of siliceous and organically based matrices (U.S. EPA 1996; Agazzi and Pirola 2000; Biziuk and Kuczynska 2006) before the ICP-MS analysis. The efficiency of microwave digestion was validated by the digestion of the CRM DORM-4 (fish protein).

4.3 INSTRUMENTAL METHODS

4.3.1 Thermal Decomposition Atomic Absorption Spectroscopy

Thermal decomposition atomic absorption spectrometer (TD-AAS) is a special, single-purpose instrument for trace level determination of total Hg, routinely used for the analysis of samples with complex matrix and solids in both environmental and food analysis and its detailed principle has been described many times (Száková et al. 2004; Diez et al. 2007; Pelcova et al. 2014; Kwaansa-Ansah et al. 2016; Pelcova et al. 2018). In brief – the sample is introduced as a solid or liquid and present Hg species are transformed by thermal decomposition of the sample in oxygen atmosphere into Hg^0 vapours. The vapours are brought by the carrier gas (O_2 or N_2) through the heated catalyst tube towards the golden amalgamator, where the Hg is bound. All other compounds are eliminated and washed off the system. After the accumulation of the Hg, amalgamator is heated and Hg is released into the optical system of the instrument, where the Hg vapours absorb radiation with wavelength 253.7 nm, allowing the quantification by Lambert-Beer law. Multiple accumulation phases on the amalgamator are possible, increasing the sensitivity of the measurements and allowing the analysis of low-concentration samples (Diez et al. 2007; Altec Ltd. 2012).

The TD-AAS used in this work was AMA-254 (Altec, Prague, Czechia). AMA-254 contains two heated optical cells of different lengths (Figure 5), allowing it to work in two calibration ranges (Altec Ltd. 2012). Oxygen was used as the carrier gas. TD-AAS was validated by direct analysis of CRM ERM-CC580 (estuarine sediment) and CRM ERM-BB422 (fish muscle).

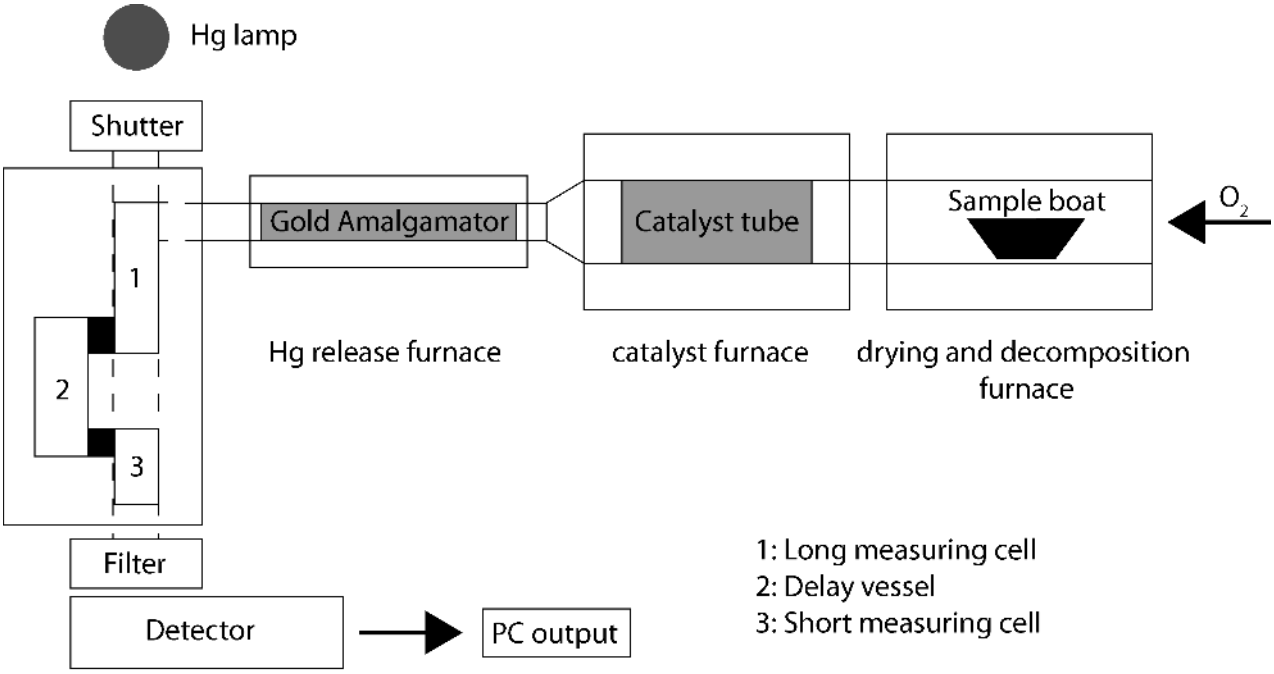

Figure 5 Scheme of TD-AAS (AMA-254). Adapted from Diez (2007).

4.3.2 Inductively Coupled Plasma Mass Spectrometry

4.3.2.1 Principle

Inductively coupled plasma mass spectrometry (ICP-MS) is one of the most commonly used techniques for elemental analysis on the trace and ultra-trace levels due to its low detection limits, high sensitivity, low consumption of the sample and wide concentration range scaling from $mg \cdot L^{-1}$ to $ng \cdot L^{-1}$ levels. It is routinely used for trace element determination in both environmental and food analysis (Falco et al. 2006; Julshamn et al. 2007; Hong et al. 2011; Kwaansa-Ansah et al. 2016; Choi et al. 2018).

First, the liquid sample is transferred in tubing by a peristaltic pump to a nebuliser, where is it transformed into an aerosol by the flow of carrier gas argon. Since the plasma is not efficient at dissociating large droplets, they are separated in a gas chamber based on their different mass. The separated fine aerosol is then carried by the carrier gas towards the plasma torch. The plasma torch consists of three concentric tubes. One is for the carrier gas, which is responsible for the transport of the aerosol, the second is for the plasma gas and the third is for auxiliary gas, which allows changes the position of plasma in the torch. At the end of the torch, the coil is placed, connected to a radio-frequency (RF) generator. That creates an RF field in the plasma gas flow. The plasma is initiated by a high voltage spark into the torch, which strips some of the electrons from the Ar atoms. Because of the RF field, the electrons are accelerated and start to collide with other atoms, causing a chain reaction and ionisation, until the stable inductively coupled plasma is formed. Different zones in plasma possess different temperatures in the range of 6000-10000 K. As long as the plasma gas input and RF field remain, the plasma remains too. When the fine aerosol droplets enter the plasma, the solvent is evaporated, and the analytes are atomised and ionised. The generated ions are then carried to the interface. Ions then enter the mass spectrometer through the interface zone with lower pressure (150-250 Pa). It lies between the atmospheric pressure part of the torch and the spectrometer under high vacuum (0.1 mPa). It consists of sampler and skimmer cones with 0.6-1.2 mm slices, transporting the ions into the ion optics. The ion optics serve to focus and direct the ion stream into the mass separator. Furthermore, ion optics prevents the entry of photons, solids and neutral particles into the mass spectrometer, thereby minimising the background noise. The focused ion beam is brought to a mass separator section, where a turbomolecular pump is generating high vacuum.

There are several types of mass separators, while the most commonly used are quadrupole, followed by magnetic sector field and time of flight analyser. They separate the input ions based on the mass-charge ratio and to filter the interfering and matrix ions. To achieve high resolution of the measurements, sector field combining the magnetic and the electric field is routinely used (Bateman 2017). One of the most common detectors is a discrete dynode electron multiplier. Incoming ion lands a conversion dynode and produces electrons, which are accelerated to the next dynode, where they generate more electrons. By repeating that process, a pulse of electrons is generated and detected at the collector (Wilschefski and Baxter 2019).

4.3.2.2 Specifications of the instrument

In this work, high-resolution double-focusing sector field ICP-MS (SF-ICP-MS, Element II, Thermo Fisher, Bremen, Germany) was used with ESI fast autosampler. The combination of the magnetic and electrostatic field of this instrument results in double focusing and high-resolution properties, compared to the routinely used quadrupole.

4.3.3 Elemental Analysis CHNS

The CHNS elemental analysers are routinely used for the fast quantitative determination of total carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) in many solid or liquid matrices in different application fields (Enev et al. 2018; Kovalcik et al. 2018; Pernicova et al. 2019). The instruments are constructed in different configurations, depending on the required analytes and their expected concentrations. The basic principles of this technique were described in detail several times (Kirsten 1983a; Kirsten 1983b; Bird et al. 2017). In brief, CHNS analysis is based on Pregl-Dumas total nitrogen determination combustion method and requires high-temperature combustion (~1000 °C) in an oxygen atmosphere. By that, all present carbon is converted to CO_2, hydrogen to H_2O, nitrogen compounds to N_2 or oxides of nitrogen (NO_x) and sulphur to SO_2. (Kirsten 1983b). This mixture of gasses is transported by an inert carrier gas (He) through different absorbents and columns to remove additional combustion products and through a heated copper trap to remove any surplus oxygen and convert NO_x to N_2 (Figure 6). The detection of CO_2, H_2O, N_2 and SO_2 mixture is commonly performed by automated gas chromatography (GC) separation followed by thermal conductivity detection (TCD). Other possibilities include frontal GC partial separation followed by TCD (not applicable for S) or sequenced TCD and infrared radiation (IR) cells for individual compounds determination (Thompson 2008).

In this work, CHNS elemental analysis was used for quantitative characterisation of modified silica, proposed as a novel resin for DGT technique. EuroVector EA3000 EA 3000 CHNS/O analyser (Euro Vector, Pavia, Italy) with TCD detection was used. Samples were packed in tin capsules and combusted at 980 °C, using pure oxygen as the combustion gas and pure helium as the carrier gas. Calibration curves for N and S were obtained using sulphanilamide as a reference standard sample.

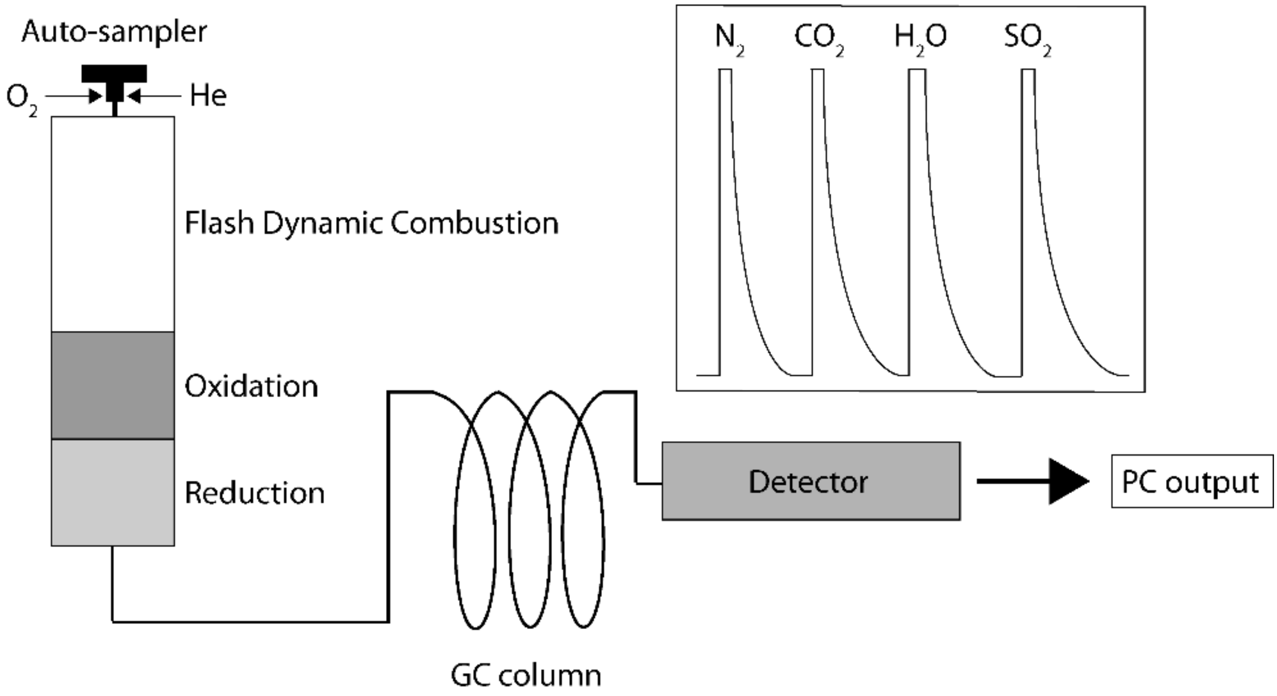

Figure 6 Scheme of CHNS elemental analyser. Adapted from Thompson (2008).

4.3.4 Diffusive Reflectance Infrared Fourier Transform spectroscopy

Diffusive Reflectance Infrared Fourier Transform (DRIFT) spectroscopy is a rapid and sensitive spectral technique used to obtain otherwise difficult infrared radiation (IR) spectra of samples with minimum sample preparation (Murthy and Leyden 2002). DRIFT spectroscopy is used for selective and non-destructive surface analysis of powders, particles, fibres and films. Surface characterisation of modified silica particles is also routinely performed by this technique since the other infrared spectroscopy techniques are unsuitable for this type of matrix (Fuchsgruber et al. 1988; Vansant et al. 1995; Murthy and Leyden 2002). The sample preparation for transmission spectroscopy changes the surface functional groups of silica and attenuated total reflectance spectroscopy requires good contact of the sample with an infrared crystal, which is difficult for silica microspheres (Mitchell 1993). The basic principles of IR spectroscopy in general and DRIFT spectroscopy were both described in detail in many works (Fuller and Griffiths 1978; Mitchell 1993; Murthy and Leyden 2002; Accardo et al. 2014; Renner et al. 2017). In brief, the sample is mixed with a non- or low-IR-absorbing powder (e.g. KCl or KBr) and placed into a sample cup. The IR passes through the mixture and when it reaches a sample surface, different scenarios can occur. The DRIFT spectroscopy is based on the principle, that the IR can either get reflected from the surface (specular reflection) or absorbed and reflected internally (diffuse reflectance) multiple times on the particle surfaces (Figure 7). During these processes, the emerging IR is lowered, subsequently collected by converging lens and guided to the IR detector, resulting into a Fourier-transformed infrared spectrum (Fuller and Griffiths 1978; Mitchell 1993; O'Neill and Fielden 1993).

In this work, DRIFT spectroscopy was used for qualitative characterisation of modified silica, proposed as a novel resin for DGT technique. The spectra were obtained using Nicolet *i*S50 spectrometer (Thermo Fisher Scientific, Waltham, USA). All spectra were recorded over the 4000-400 cm^{-1} range at a resolution of 8 cm^{-1} and with an average of 512 scans.

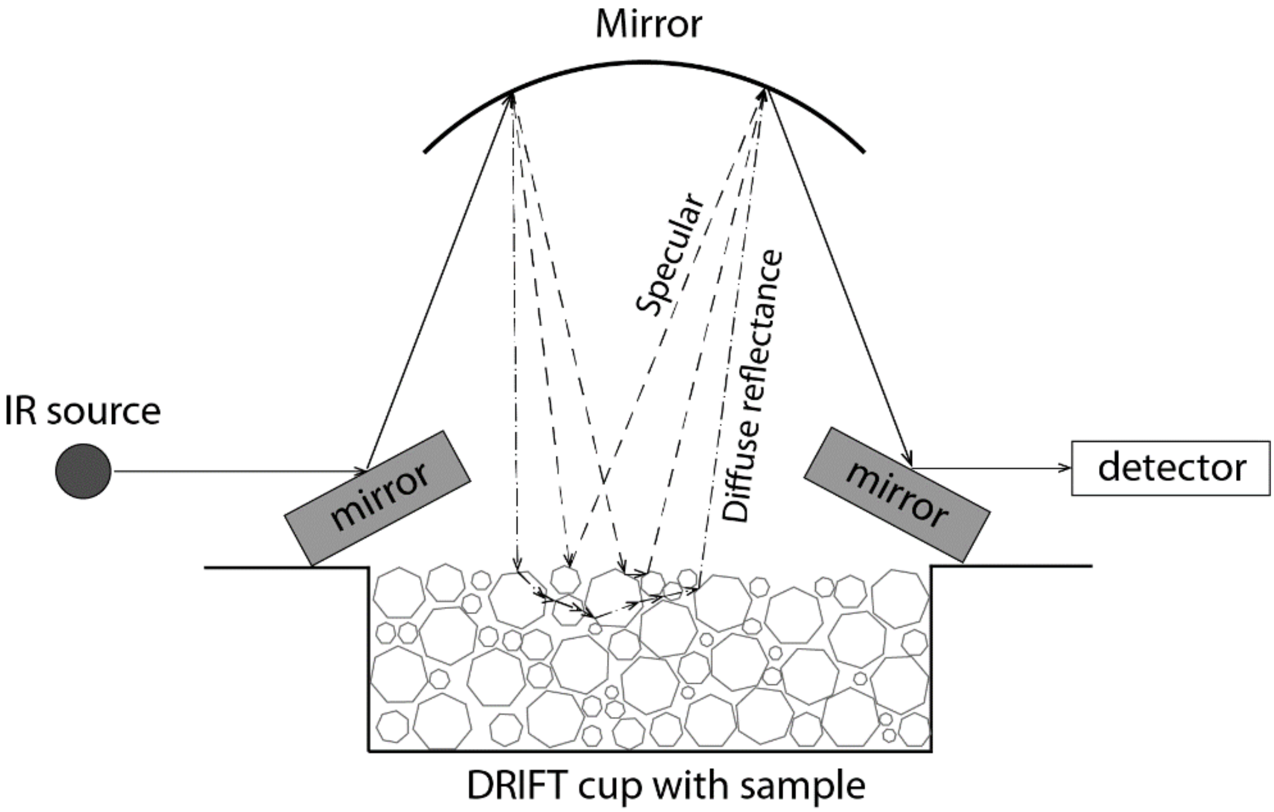

Figure 7 The principle of DRIFT spectroscopy with specular and diffuse reflectance visualisation. Adapted from Mitchell (1993) and Renner et al. (2017)

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5 RESULTS AND DISCUSSION

5.1 NEW SORBENTS FOR DGT

In past, many different Hg-specific resins were routinely used in the DGT technique for Hg assessment (e.g. Spheron-Thiol, Duolite GT73, Amberlite GT73 or Ambersep GT74), mostly with thiol functional groups (Docekalova and Divis 2005; Diviš 2013). Majority of these resins is no longer commercially available due to discontinued production (Diviš 2013). Nowadays, only relatively expensive 3-mercaptopropyl functionalised silica (3-MFS) is routinely used for the assessment of Hg and the preparation of 3-MFS resin gel requires certain experience (Bratkic et al. 2019). For most of the other trace metals, the DGT technique with Chelex-100 resin is used, however, it is not applicable for Hg (Docekalova and Divis 2005). Due to the different requirements on the resin used in the DGT technique for Hg and the other trace metals, simultaneous quantitative determination of Hg and other trace metals by the DGT technique was not reported yet. That increases the number of produced samples and the amount of material used, which increases the cost of such determination as two parallel DGT methods are necessary. Therefore, there is an urgent demand for novel resins allowing simultaneous quantitative binding with Hg and/or other trace metals.

There are two possibilities how to resolve that issue – laboratory preparation of a resin or testing new commercially available resins. Both options have several advantages and disadvantages. The main advantage of laboratory-prepared resins is the ability to influence their structure, which allows preparation of a resin most suitable for the given application. Synthesised mercaptopropyl nanoporous resins accumulated Hg slightly better than the commercially available 3-MFS (Gao et al. 2011). Chemical modifications of commercially available resins and their application in the DGT technique for Hg also brought promising results (Divis et al. 2009; Divis et al. 2010). However, 3-MFS was never replaced in the routine application by any of the synthesised or modified resins, probably due to the long and expensive synthesis or modification process and not enough significant advantages of synthesised or modified resins over 3-MFS. The new commercially available resins have particle size bigger than the usual thickness of the resin gel, therefore their direct application in the DGT technique is not possible, e.g. Purolite S924 with thiol functional groups and average particle size up to 0.8 mm (Lenntech 2019). Mechanical pre-treatment (grinding and sieving) of such resins does not usually decrease their functionality, which allows their application in the DGT technique (Diviš 2013; Abdulbur-Alfakhoury et al. 2019).

In this work, two different resins were tested – one prepared in the laboratory (chapter 5.1.1) and one commercially available (chapter 5.2). The laboratory prepared resin was biomolecule-based resin prepared by glutaraldehyde immobilisation of cysteine onto amino-functionalised silica, inspired in the literature (Dakova et al. 2011). The design of the prepared resin comes from the cellular defence mechanisms against hazardous trace elements – the major scavenging and disposal of hazardous metals at the cellular level occurs by antioxidant glutathione (GSH) (Ali et al. 2019). Glutathione is a tripeptide, biosynthesised of cysteine bound to glycine and a side chain of glutamate (Jozefczak et al. 2012). However, cysteine was found more efficient than GSH at the extraction of trace metals from soils (Vadas and Ahner 2009), therefore cysteine was used instead of GSH. The commercially available resin tested in this work was Purolite S924, polystyrene-based resin with thiol groups, used in the waste treatment and hydrometallurgical processes for selective removal of Hg (Lenntech 2019). Purolite S924 was ground and sieved for fraction below 50-100 μm before use in the DGT technique.

5.1.1 Cysteine-modified silica resin in DGT samplers for mercury and trace metals assessment

In this work, two variants of biomolecule-based resin were prepared by glutaraldehyde immobilisation of Cysteine onto Amino-functionalised Silica (hereafter referred as "CAS") and 3-AminoPropyl-functionalised Silica (hereafter referred as "CAPS"). The total content of nitrogen and sulphur in the resins prepared at different reaction conditions was monitored by CHNS elemental analysis to optimise the modification conditions. The final CAS and CAPS were qualitatively characterised by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy and observed functional groups quantitatively titrimetrically characterised. Immobilisation of cysteine introduced thioether bond, primary and secondary amino groups, and carboxylic group to the structure of the CAS and the CAPS. That combined the features of another Hg-specific resin with isothiouronium groups and Chelex-100 resin used for other trace metals. The functional groups on modified resins were qualitatively and quantitatively characterised.

During uptake kinetics study, the DGT with the CAPS showed faster uptake of Hg, Cd, Pb, Co, Ni and Cu than the DGTs with the CAS (hereafter referred as "CAPS-DGT" and "CAS-DGT", respectively). The analytes in the resin gels of the CAS-DGT and the CAPS-DGT were eluted by *aqua regia*, and the eluates analysed by SF-ICP-MS. All analytes accumulated linearly on both CAS-DGT and CAPS-DGT. Due to Cl-complexation of analytes (Gao et al. 2012), the effective diffusion coefficients D_e obtained by CAPS-DGT in 0.01 $mol \cdot L^{-1}$ NaCl solution were slightly lower than the literature values for all metals (obtained in $NaNO_3$ solutions). The D_e obtained by CAS-DGT for all metals were much lower than those obtained by CAPS-DGT, due to lower load of functional groups and slower uptake kinetics of the CAS compared to the CAPS. The capacity of CAS resin gel disc was 25 μg Hg and 40 μg for other metals. The capacity of CAPS resin gel disc was > 50 μg of every metal. The actual *D*$_e$ for all metals is listed, compared with the literature and further discussed in the manuscript attached below. The pH in the range of 4.5-8.5 and ionic strength in the range of 0.001-1.0 mol·L^{-1} NaCl did not affect the performance of the CAS-DGT and the CAPS-DGT by more than 10 %. That provides a significant advantage for fieldwork application since the pH and ionic strength matched the typical conditions range in both freshwater and seawater. During the fieldwork application, the standard DGTs with 3-mercaptopropyl functionalised silica for Hg and with Chelex-100 for the other trace metals (hereafter referred as "3-MFS-DGT" and "Chelex-DGT", respectively) were deployed simultaneously and their elution followed the standard DGT procedures (Gao et al. 2012; Bratkic et al. 2019). Analysis of variance with post-hoc Tukey Honest Significant Difference comparison method was used for the statistical analysis. For the results of the fieldwork application, there was no statistically significant difference between the results of the CAPS-DGT and the 3-MFS-DGT for Hg ($p = 0.746$, $\alpha = 0.05$). The statistical differences between the CAPS-DGT and the Chelex-DGT for the other trace metals were also not significant ($p = 0.893$, $\alpha = 0.05$). The differences between CAS-DGT and all other DGT techniques were significant for all metals. The concentrations of all metals during the fieldwork application were influenced by the traffic in the sampling sites of the Belgian Coastal Zone and are comparable with the data of previous years. The concentrations are summarised, compared and further discussed in the manuscript attached below.

5.1.1.1 Conclusions

The new design of resin successfully combined the functional groups of isothiouronim Hg-specific resin and the Chelex-100 (routinely used for the other trace metals). The performance of novel the CAPS-DGT corresponded well with the 3-MFS-DGT for Hg and the Chelex-DGT for other trace metals – in both laboratory and fieldwork conditions. Compared to the CAPS, the CAS had a lower load of functional groups, which caused the lowered performance of the CAS-DGT compared to the CAPS-DGT during uptake kinetics studies, linear accumulation and fieldwork applications. The higher performance of the CAPS could be also credited to the longer length of the "spacer" between the cysteine and silica (formed by the propyl group), which could suppress some steric hindrances.

This work introduces the first simultaneous determination of Hg together with Cd, Pb, Co, Ni and Cu by the DGT technique. The structure of modified resins from this work could further contribute to the design of universal resin used in the DGT technique for multi-trace metal assessment by the DGT technique.

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Cysteine-modified silica resin in DGT samplers for mercury and trace metals assessment

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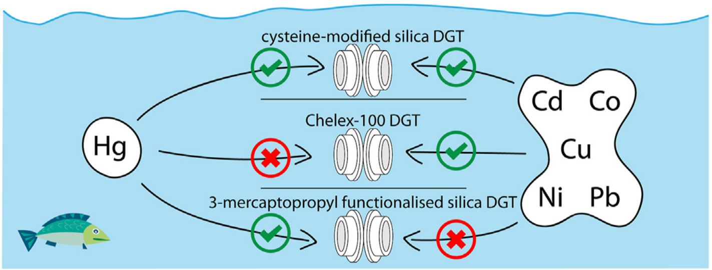
HIGHLIGHTS

- Silica resins were modified by cysteine for Hg and trace metal assessment using DGT.
- Functional groups of modified resins were characterised using CHNS/O and DRIFT.
- Modified silica resin (CAS and CAPS) owns the features of Chelex-100 and 3-MFS.
- The DGT using CAS and CAPS resin can simultaneously bind Hg and Cd, Co, Cu, Ni, Pb.
- The DGT using CAS and CAPS resin is successfully applied in marine aquatic systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Diffusive gradients in thin-films (DGT) is an *in situ* passive sampling technique to assess labile trace metal concentrations in different environmental matrix. The technique is consisting of a diffusive domain backed up by a resin gel that binds free metals and metal complexes that dissociate in the diffusive domain. This technique requires specific resin for special metals, for example mercury (Hg), since the classic resin (Chelex-100) gel is not applicable for Hg measurement. A simultaneous determination of Hg with other metals by the DGT was not yet reported. Two biomolecule-based resins were prepared by glutaraldehyde immobilisation of cysteine onto 3-amino-functionalised silica and 3-aminopropyl-functionalised silica, respectively. The load of functional groups on modified resins was qualitatively and quantitatively characterised. The modified resins were applied in the DGT technique and the uptake efficiency, elution efficiency, and linear accumulation of analytes of the DGT were tested. This novel DGT technique, using two cysteine-modified resins, can accumulate Hg and other metals in a broad range of pH and ionic strength in solutions. In the Belgian coastal zone (BCZ), the concentrations of Hg and other trace metals sampled by cysteine-modified resin-DGTs were similar as those by the other two DGT assemblies for Hg and other trace metals, respectively. The cysteine-modified silica resin combined the features of Chelex-100 resin and 3-mercaptopropyl silica resin and allowed simultaneous determination

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of labile Hg and other trace metals. The resin with a higher load of functional groups also showed higher performance in the further application in the DGT technique.

1. Introduction

Diffusive gradients in thin-films technique (DGT) is an *in-situ* passive sampling technique for assessment of solutes in various environmental matrix (Davison and Zhang, 1994). It uses a diffusion-based mass transport of analytes through a porous diffusive hydrogel layer, accumulates the analytes on resin gels which contains specific functioning groups (Davison and Zhang, 1994; Zhang and Davison, 1995; Divis et al., 2005). The diffusive and resin gels covered by membrane filter are enclosed in a plastic moulding. When the DGT samplers are deployed in aquatic systems, a concentration gradient is established between the bulk solution and the resin gel through the diffusion process in the diffusive layers. The analytes accumulate continuously on the resin gel if the saturation of the resin gel is not achieved. After the deployment, the resin gel is peeled off from the DGT unit and eluted with elution solution. Afterwards, the eluate is analysed by spectroscopic methods, e.g. atomic absorption spectrometry (AAS) (Diviš et al., 2012) or inductively coupled plasma mass spectrometry (ICP-MS) (Gao et al., 2019). In some cases, the resin gel can be analysed directly, e.g. by thermal decomposition atomic absorption spectrometry for Hg (Divis et al., 2005) or laser ablation-ICP-MS for trace metals (Gao and Lehto, 2012). By choosing a suitable combination of diffusive and resin gels, the DGT can monitor different analytes including trace metals (Gao et al., 2019; Gaulier et al., 2019), pesticides (Guibal et al., 2017), antibiotics (Guibal et al., 2019) and endocrine disturbing compounds (Guo et al., 2017, 2019). This technique can also be applied in different environments, e.g. freshwater (Divis et al., 2007; Menegario et al., 2017), estuarine and seawater (Bratkic et al., 2019; Gaulier et al., 2019), aquatic sediments (Divis et al., 2005; Gao et al., 2009, 2015) or food matrices (Chen et al., 2014; Reichstädter et al., 2020). Most diffusive gels are made of polyacrylamide linked with an agarose-derived crosslinker (APA) or agarose (AGE). Both APA and AGE diffusive gels have been used for determination of many elements in the DGT technique (Scally et al., 2006). However, the APA contains amide groups, which interact and form bonds with Hg, therefore the APA is not recommended for monitoring of Hg (Docekalova and Divis, 2005; Gao et al., 2011). In the DGT, the combination of the APA diffusive gel and the Chelex-100 resin gel is commonly used for assessment of the most trace metals (Garmo et al., 2003; Scally et al., 2006; Gao et al., 2019). However, it was reported previously (Divis et al., 2009; Gao et al., 2011) when the Chelex-DGT was used for Hg measurement, lower performance and non-linear uptake were observed because iminodiacetic functional groups of Chelex-100 have low affinity towards Hg-complexes. The majority of the reported Hg-specific resins are those with thiol functioning groups (Divis et al., 2009, 2010; Gao et al., 2011). However, many of them are not commercially available due to discontinued production, with only 3-mercaptopropylsilica (3-MFS) being used with high price (Gao et al., 2011; Bratkic et al., 2019).

Due to different requirement on the diffusive and the resin gels, the DGT technique for a simultaneous quantitative determination of Hg and other trace metals was not reported yet. However, it is possible to deploy two types of DGT samplers separately for Hg and for other trace metals, which increases the number of samples and consumables. It was also reported that the DGT technique equipped with Whatman P81 cellulose ion exchange membrane as a resin layer was used either for Hg determination using cold vapour atomic fluorescence spectrometry (Colaco et al., 2014) or for other trace metals (Mn, Co, Ni, Cu, Zn and Pb) assessment using energy dispersive X-ray fluorescence spectrometry (Almeida et al., 2012), but never for simultaneous determination Hg and the other trace metals.

Therefore, there is an urgent demand for novel resins allowing simultaneous binding with Hg and other trace metals like Co, Ni, Cu, Cd and Pb. In this work, the design of such resin comes from the concept of cellular defence mechanisms against hazardous trace elements. The major scavenging and disposal of hazardous metals at the cellular level occurs by antioxidant glutathione (GSH) (Ali et al., 2019), a tripeptide formed by cysteine, glycine and glutamate (Jozefczak et al., 2012). However, cysteine itself was more efficient than GSH for trace metal extraction from soils (Vadas and Ahner, 2009). The DGT technique using the detoxifying biomolecules-based resin can mimic the processes, how hazardous metals bind with organisms and gives insights in bioavailable fractions of these toxicants (Bridges and Zalups, 2005; Hoffman and Hubbell, 2013; Jan et al., 2015; SenGupta, 2017).

This work aims at modification of silica-based resins by immobilisation of cysteine for simultaneous binding of Hg and other metals, characterisation of the modified resins, and their application in the DGT technique under the laboratory and fieldwork conditions. Optimisation of the modification process was performed to achieve the highest load of the functional groups. When the load of the immobilised molecules is too dense, the performance of the resin could be lowered due to steric effect or steric hindrance, which resulted in inaccessibility of the functional groups to the target analytes (Zhang et al., 2014). Steric effect can be avoided by using a spacer between the carrier and the immobilised molecule, which increases the accessibility of the target analyte to the functional groups (Hage and Cazes, 2005). Therefore, two different silica-based resins with different load and spacer length were tested, one with primary amino groups bound directly to the silica and the other one with primary amino groups bound to the silica via a propyl spacer. The modified silica-based resins with the highest load of the functional groups were then chosen for the further DGT experiments. Whenever a new resin is used in the DGT technique, its performance must be tested including uptake efficiency, capacity, elution factor, linear accumulation of analytes over time and influence of different pH and ionic strength. After laboratory validation, the DGT pistons containing the modified silica-based resin gels were applied in the harbours of Oostende and Zeebrugge at the Belgian coastal zone (BCZ). To validate the performance of these novel DGTs, the 3-MFS-DGT for Hg and the Chelex-DGT for the other metals (Co, Ni, Cu, Cd and Pb) were also deployed simultaneously.

2. Materials and methods

2.1. Reagents and materials

Acrylamide solution (40%, p. a., Merck, Darmstadt, Germany), agarose powder (Biorad, Hercules, USA), amino-functionalised silica gel (40–75 μm particle size, Sigma-Aldrich, Darmstadt, Germany), 3-aminopropyl-functionalised silica gel (40–63 μm particle size, Sigma-Aldrich, Darmstadt, Germany), ammonium iron (III) phosphate dodecahydrate (ACS, Sigma-Aldrich, Darmstadt, Germany), ammonium persulfate (10%, 0.1 g in 1 g of water; >98%, Merck, Darmstadt, Germany), cadmium stock solution 1000 mg L^{-1} (VWR, Haasrode, Belgium), Chelex-100 (200–400 mesh, Biorad, Hercules, USA), cobalt stock solution 1000 mg L^{-1} (Supelco, Darmstadt, Germany), copper stock solution 1000 mg L^{-1} (VWR, Poole, UK), L-cysteine (BioUltra, Sigma-Aldrich, Darmstadt, Germany), DGT cross-linker (DGT Research Ltd, Lancaster, UK), ethanol (99%, Sigma-Aldrich, Darmstadt, Germany), DGT pistons (DGT Research Ltd, Lancaster, UK), Durapore membrane filters (0.45 μm, Merck, Darmstadt, Germany) formaldehyde (37%, VWR, Leuven, Belgium), glutaraldehyde solution (25%, Sigma-Aldrich, Darmstadt, Germany), hydrochloric acid (optima grade, Fisher Scientific, Leicestershire, UK), lead stock solution 1000 mg L^{-1} (VWR, Poole, UK), mercury stock solution 1000 mg L^{-1} (Alfa Aesar, Kandel, Germany), MilliQ water (MQ, Millipore, >18 MΩ cm, Merck, Darmstadt, Germany), N,N,N,N-tetraethylenediamine (99%; p. a., Merck, Darmstadt, Germany), nickel stock solution 1000 mg L^{-1} (Merck, Darmstadt, Germany), nitric acid (optima grade, Fisher Scientific, Leicestershire, UK), nitrogen gas (Linde, Brno, Czech Republic), oxalic acid dihydrate (p.a., Merck, Darmstadt, Germany), oxygen gas (Linde, Brno, Czech Republic), potassium bromide (FT-IR grade, Sigma-Aldrich, Darmstadt, Germany), potassium chromate (p.a., Penta, Prague, Czech Republic), potassium thiocyanate (p.a., Penta, Prague, Czech Republic), silver nitrate (p.a., Sigma-Aldrich, Sigma-Aldrich, Darmstadt, Germany), sodium bicarbonate (p.a., Merck, Darmstadt, Germany), sodium chloride (extra pure, Sigma-Aldrich, Seelze, Germany), sodium hydroxide (p.a., Merck, Darmstadt, Germany), sodium nitrate (Suprapur, Merck, Darmstadt, Germany), sodium phosphate dibasic (p.a., Sigma-Aldrich, Darmstadt, Germany), sodium phosphate monobasic (p.a., Sigma-Aldrich, Darmstadt, Germany), sulphanilamide (analytical standard, Supelco, Darmstadt, Germany).

All glassware and plasticware were immerged in 1 mol L^{-1} HNO_3 overnight and rinsed three times by MQ water before use. The modification of resins took place in a glove box under a nitrogen atmosphere using a magnetic stirrer plate.

2.2. Apparatus and instruments

The elemental composition of the modified resins was determined by EA 3000 CHNS/O analyser (Euro Vector, Pavia, Italy). The FTIR spectra were obtained by Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy using a Nicolet *i*S50 spectrometer (Thermo Fisher Scientific, Waltham, USA). The content of cobalt (^{59}Co), nickel (^{60}Ni), copper (^{63}Cu), cadmium (^{114}Cd), mercury (^{202}Hg) and lead (^{208}Pb) was analysed by sector-field inductively coupled plasma mass-spectrometry (SF-ICP-MS, Element II, Thermo Fisher Scientific Bremen GmbH, Germany), indium (^{115}In) was used as an internal standard.

2.3. Resin modification

Resin modification was performed by glutaraldehyde-mediated immobilisation of cysteine on the surface of various silicas with amino functional groups, according to the procedure from Dakova et al. (2011). Amino-functionalised silica and 3-aminopropyl-functionalised silica were used as carriers and two modified resins were called Cysteine-modified Amino-functionalised Silica (CAS) and Cysteine-modified 3-AminoPropyl-functionalised Silica (CAPS). In the first step, the amino groups of silica (0.5 g) reacted with 40 mL 0.6% glutaraldehyde solution in 0.1 mol L^{-1} mono-/dibasic sodium phosphate buffer (pH 7) for 1 h under nitrogen atmosphere to avoid auto-polymerisation of glutaraldehyde (Margel and Rembaum, 1980) and finally formed an activated glutaraldehyde-silica intermediate (Migneault et al., 2004; Dakova et al., 2011). After glass microfibre filtration and MQ water washing, cysteine dissolved in 0.1 mol L^{-1} mono-/dibasic sodium phosphate buffer (15 mL, pH 6) was added. The mixture was stirred and slowly bubbled through by nitrogen. The activated glutaraldehyde-silica intermediate formed a bond with the functional groups of cysteine (Dakova et al., 2011; Verma et al., 2017). After 48 h of stirring, the solid product was collected by glass microfibre filtration and washed by MQ water. Products were dried under vacuum for 72 h and sieved by a nylon sieve to obtain the fraction of particles below 75 μm. By changing the weight ratio of cysteine and silica, in total 20 resin variants were prepared in triplicates, including 7 variants of CAS and 13 variants of CAPS, which were further characterised.

2.4. Resin characterisation

2.4.1. Elemental analysis

The amounts of N and S in the modified silicas represent the total content of all amino and thiol/thioether groups, respectively. Therefore, the total content of nitrogen (N) and sulphur (S) was quantified by CHNS/O elemental analysis for every experiment. The resin samples (12–15 mg) were packed in tin capsules, which were placed in an oven for combustion at 980 °C, using pure oxygen as the combustion gas and pure helium as the carrier gas. Calibration curves for nitrogen (N) and sulphur (S) were obtained using sulphanilamide as a reference sample (Enev et al., 2018). The CAS and the CAPS with the highest load of N and S were chosen for further characterisation and the DGT experiments.

2.4.2. Characterisation and quantification of the resin functional groups

The selected CAS and the CAPS were further qualitatively characterised by DRIFT spectroscopy. Approximately 25 mg of samples were homogenised with 100 mg of KBr in an agate mortar and then transferred to the sample holder cup of the diffuse reflectance accessory. All spectra were recorded in a range of 4000–400 cm^{-1} at a resolution of 8 cm^{-1} with an average of 512 scans. The spectra of KBr, cysteine, amino-functionalised silica and 3-aminopropyl-functionalised silica were also obtained. The KBr spectrum was used as the background for the measurements of cysteine, amino-functionalised silica, and 3-aminopropyl-functionalised silica. The spectrum of amino-functionalised silica and 3-aminopropyl-functionalised silica was used as the background for measurement of the CAS and the CAPS, respectively.

The amount of reachable thiol groups in the modified resins was determined by Volhard's silver titration (Vogel, 1939; Gao et al., 2011). The thiol groups reacted with a known amount of silver nitrate solution standardised against sodium chloride and the excess was titrated by standardised potassium thiocyanate solution. Saturated solution of ammonium iron (III) sulphate in 1.2 mol L^{-1} nitric acid was used as the indicator (United States Department of Agriculture, 2009; Gao et al., 2011). The amount of reachable carboxylic groups in the modified resins was determined by Sørensen formol titration (Kolthoff and Stenger, 1942; Akluwalia and Aggarwal, 2007). The amino groups reacted with neutralised formaldehyde in surplus, forming a methylene-amino group. The remaining carboxylic acid groups were then titrated by sodium hydroxide, standardised against oxalic acid. The amount of reachable primary amino groups in native silicas and the modified resins was determined by the method described by Harris (Harris, 1924; Szabadváry, 2016). After neutralising the carboxyl groups in ethanolic solution by soda titration, the primary amino groups were then back titrated by hydrochloric acid.

2.5. Preparation of the DGT pistons

For the preparation of the CAS, the CAPS and the 3-MFS resin gels, briefly 1 g of resin mixed with 10 mL of 1.5% agarose solution and the gel solution was cast in two glass plates with a spacer of 0.5 mm. The preparation of Chelex-100 resin gels followed the routine procedure as described by Gaulier et al. (2019). The preparation of agarose (AGE) diffusive gels and polyacrylamide (APA) diffusive gel can be found elsewhere (Docekalova and Divis, 2005; Gao et al., 2011). The assembling of all DGT pistons followed the routine procedures as described previously (Divis et al., 2009; Gao et al., 2011). The four types of DGT pistons are further referred as “CAS-DGT”, “CAPS-DGT”, “Chelex-DGT” and “3-MFS-DGT”.

2.6. DGT performance tests

2.6.1. Resin gel uptake, capacity, and elution

The DGT experiments were performed in polypropylene containers (volume of 1.75 L stirred at 1200 rpm by Teflon stirrer bar) at 20 °C with a lid and a polycarbonate DGT piston rack inside.

To test the uptake kinetics, 1 mg L^{-1} of Co, Ni, Cu, Cd, Hg, and Pb mixture was made in 0.01 mol L^{-1} NaCl solution at pH 6 and was stabilised for 24 h at 20 °C. The CAS and the CAPS resin gels were immersed separately in 20 mL of this mixture for 24 h and were mildly shaken. Subsamples of the mixture solution were taken every 5 min for the first hour, then every 30 min for the following 5 h and then after 12, 18 and 24 h. The uptake kinetics were determined by plotting the concentration change over time (Mason et al., 2005; Reichstädter et al., 2020). The uptake factor and the capacity of the binding gel discs were calculated by the concentration difference of the solution at the beginning and the end of the exposure.

When a new resin is used in the DGT technique, a suitable elution reagent has to be found and the elution factor can be calculated using Equation (1) (Zhang and Davison, 1995; Davison and Zhang, 2012):

$$f_e = M_e/(M_0 - M_T) \tag{1}$$

where M_e [ng] is the mass of analyte obtained by elution, M_0 [ng] is the beginning mass of the analyte in the deployment solution, M_T [ng] is the end mass of the analyte in the deployment solution. To determine the elution factor, the CAS and the CAPS resin gels were retrieved at the end of the experiment, rinsed by MQ water and eluted in 1 mL of *aqua regia* (made of HNO_3 and HCl in 1:3 M ratio) for 24 h at 70 °C (Abdulbur-Alfakhoury et al., 2019; Reichstädter et al., 2020).

2.6.2. Linear accumulation and determination of diffusion coefficient

The linear accumulation of all metals (Hg, Co, Ni, Cu, Cd and Pb) was evaluated for the CAS-DGT and the CAPS-DGT. For comparison, the 3-MFS-DGT was used for Hg accumulation and the Chelex-DGT for Co, Ni, Cu and Cd. The test was performed in the mixture of 30 μg L^{-1} of Co, Ni, Cu, Cd, Hg, and Pb in 0.01 mol L^{-1} NaCl solution (referred as the mixture solution hereafter) at pH 6 at 20 °C, and the solution was left to equilibrate for 12 h before experiments. In total, 48 DGT pistons were deployed in the solution. At each time interval (4, 8, 24 and 48 h), three pistons of each type of DGTs were retrieved, disassembled and the resin gels were eluted. The elution of the 3-MFS resin gels was also performed by 1 mL of *aqua regia* (Bratkic et al., 2019). The Chelex-100 resin gels were eluted by 1 mL of 1 mol L^{-1} HNO_3 for 24 h (Panther et al., 2014; Gao et al., 2019). Prior to the ICP-MS analysis, all eluates were diluted 10 times by MQ water.

The mass of the accumulated metals (M) normalised for their concentration in solution (c) was plotted against the exposure time (t). The slope k of the linear regression was used to calculate the effective diffusion coefficients (D_e) of Co, Ni, Cu, Cd, Hg, and Pb in the AGE diffusive gel using Equation (2) (Davison and Zhang, 1994), where Δg was the thickness of the diffusive layers (diffusive gel + membrane filter) and A [cm^3] was the exposure area of the piston:

$$D_e = k \cdot \Delta g/(A \cdot t) \tag{2}$$

2.6.3. Assessment of diffusive boundary layer

A thin diffusion-controlled layer, known as diffusive boundary layer (DBL), exists at any solid surface in an aqueous environment, even at vigorously stirred solutions. The DBL can affect the DGT results (Garmo et al., 2006; Warnken et al., 2006) and its thickness can be assessed by using different thickness of diffusive layers in the DGT pistons. The CAPS-DGT pistons equipped with three different thickness (0.25/0.50/0.75 mm) of AGE diffusive gels were prepared in four replicates for each thickness and deployed for 4 h in the mixture solution mentioned above stirred at 1200 rpm. After the retrieval, the pistons were disassembled, and the resin gels were processed as described above. The reciprocal mass of analytes (M^{-1}) is plotted versus the thickness of the diffusive layers (Δg). Using the slope k, the intercept q and the diffusion coefficients of analytes in water D_w (Davison and Zhang, 2016) and the effective diffusion coefficient D_e measured in this work, the DBL thickness was calculated using Equation (3) (Warnken et al., 2006):

$$\delta = k \cdot D_w/q \cdot D_e \tag{3}$$

2.6.4. Effect of pH and ionic strength on the DGT performance

The performance of the CAS-DGT and the CAPS-DGT was tested at different pH and ionic strength. The pH of natural waters is usually in a range 4.5–8.5 and the ionic strength is in a range 0.0001–0.7 mol L^{-1} (Ren et al., 2018; Guibal et al., 2019). These two types of DGT pistons were deployed in the mixture solution and the pH and ionic strength of the solution were adjusted by using HNO_3/NaOH and NaCl, respectively. The pH of the solution was in the range of 4.5–8.5 with the interval value of 0.5, at the fixed ionic strength of 0.01 mol L^{-1} (NaCl). The ionic strength was adjusted in the range of 0.001, 0.01, 0.03, 0.05, 0.1, 0.5 and 1 mol L^{-1} NaCl, at the fixed pH of 6. Five mL subsamples were taken from the beginning deployment solution and the end solution and analysed by ICP-MS. The DGT measured concentrations (cDGT) were calculated by using Equation (4) (Zhang and Davison, 1995):

$$cDGT = M \cdot \Delta g/(D_e \cdot t \cdot A) \tag{4}$$

The cDGT was compared with the cSOL (results of direct ICP-MS analysis of the solution) and the ratios of cDGT/cSOL were calculated to check the performance variation (Divis et al., 2010; Reichstädter et al., 2020).

2.7. DGT field application

In April 2019, four sampling stations in Belgian coastal zone (BCZ) including HO-1, HO-2 in Oostende harbour and HZ-1, HZ-2 in Zeebrugge harbour were selected for the DGT deployment. These sampling stations host diverse industrial harbour activities for several decades, which could offer promising high metal loads. Five replicates of every type of DGTs (CAS-DGT, CAPS-DGT, 3-MFS-DGT and Chelex-DGT) were deployed in each station for 14 days. After the retrieval, the pistons were washed properly by MQ water and stored at 4 °C during transportation. In the laboratory, the resin gels from the CAS-DGT, the CAPS-DGT and the 3-MFS-DGT were peeled off and eluted by 1 mL *aqua regia*; while the resin gels from Chelex-DGT were peeled off and eluted by 1 mL 1 mol L^{-1} HNO_3.

3. Results and discussion

3.1. Characterisation of modified resins

3.1.1. Elemental analysis

The goal of the elemental analysis was to select the cysteine-modified silica-based resins with the highest N and S content, which indicates the highest loads of functional groups with N and S.

By increasing the ratio of cysteine/silica, the total content of N and S also increased (Fig. 1), which confirmed the successful immobilisation of cysteine. The maximum content was 0.3% (*w*/*w*) for N and 1.1% (*w*/*w*) for S in the CAS and 2% (*w*/*w*) for N and 4% (*w*/*w*) for S in the CAPS, respectively. Further increasing of cysteine/silica ratio did not result in higher contents of N and S, suggesting the saturation of the glutaraldehyde-activated primary amino groups. At higher cysteine/silica ratio (e.g. 1.2), the N and S contents decreased which was also observed by Dakova et al. (2011). Such decrease is caused by the change of the stoichiometric ratio of glutaraldehyde-activated primary amino groups and cysteine in the mixture because cysteine was in surplus (Okuda et al., 1991) and will precipitate. Despite working in a nitrogen atmosphere, cysteine could be oxidised to cystine quickly (Routh, 1939; Walter, 2019). This phenomenon can be observed at the start of the reaction by the appearance of a small amount of less soluble cystine. In aqueous solutions, glutaraldehyde occurs in different forms with various reactivity (Migneault et al., 2004), however, consistent results were obtained for different batches in this study (Fig. 1). Therefore, the final CAS resin was prepared at a ratio of 0.3 (*w*/*w*, cysteine/silica) and the final CAPS resin was prepared at a ratio of 1.1 (*w*/*w*) and they were chosen for the further characterisation and the DGT experiments.

3.1.2. DRIFT spectroscopic characterisation of functional groups

The spectra were interpreted based on the spectral tables of Simons (Sadtler Research Laboratories, 1978) and Nyquist (2001). The KBr spectrum was used as the background for the measurement of cysteine, amino-functionalised silica, and 3-aminopropyl-functionalised silica. The spectrum of amino-functionalised silica and 3-aminopropyl-functionalised silica was used as the background for measurement of the CAS and the CAPS, respectively.

Due to the higher content of N and S, the CAPS was chosen as an example for comparison and description of the spectrum. Fig. 2 shows the DRIFT spectra of cysteine (blue line), 3-aminopropyl functionalised silica (black line) and the CAPS (red line).

The spectrum of cysteine shows the typical features of aliphatic amino acid containing thiol. The sharp peaks at 2595 cm^{-1} and 2552 cm^{-1} are specific for thiols, caused by S—H stretching vibrations (ν). The other two strong merged peaks at 1610 cm^{-1} and 1589 cm^{-1} represent in-plane scissoring vibration (δ) of N—H in primary amines and asymmetric stretching vibration in amino acids. The peaks at 1430 cm^{-1} and 1270 cm^{-1} stand for C—O stretching vibration coupled with O—H in-plane deformation vibration, characterising carboxyl groups of organic acids. The peak at 1065 cm^{-1} was recognised as C—N stretching vibrations of in primary amines.

The presence of immobilised cysteine was confirmed by the intense peak at 3340 cm^{-1} marking N—H stretching vibration in secondary amines. The three intense merged peaks at 1666 cm^{-1}, 1612 cm^{-1} and 1581 cm^{-1} were identified as in-plane scissoring vibration of secondary amines. These peaks, together with the less intense peak at 1200 cm^{-1} (C—N stretching vibration in secondary amines and amino acids) also confirm the successful immobilisation of cysteine. The weaker peaks at 685 cm^{-1} and 655 cm^{-1} are typical for C—S stretching vibrations in thiols or S—S stretching vibrations in thioethers, confirming the sulphur content in the modified silica. It suggests that the thioether bond was formed to immobilise the cysteine, hence the typical peak for thiol groups is completely missing in the spectrum of the CAPS.

3.1.3. Quantification of functional groups and structure of modified resins

The functional groups observed in DRIFT spectra were titrimetrically quantified. The load of thiol groups was

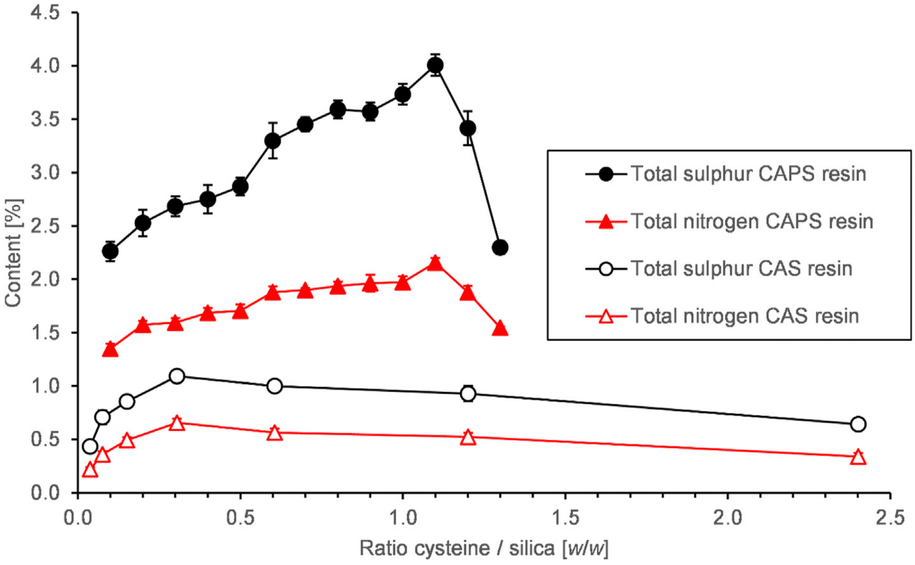

Fig. 1. Total sulphur and nitrogen content in different variants of the CAS and the CAPS by changing the cysteine/silica ratio, the error bars display the standard deviation (n = 3).

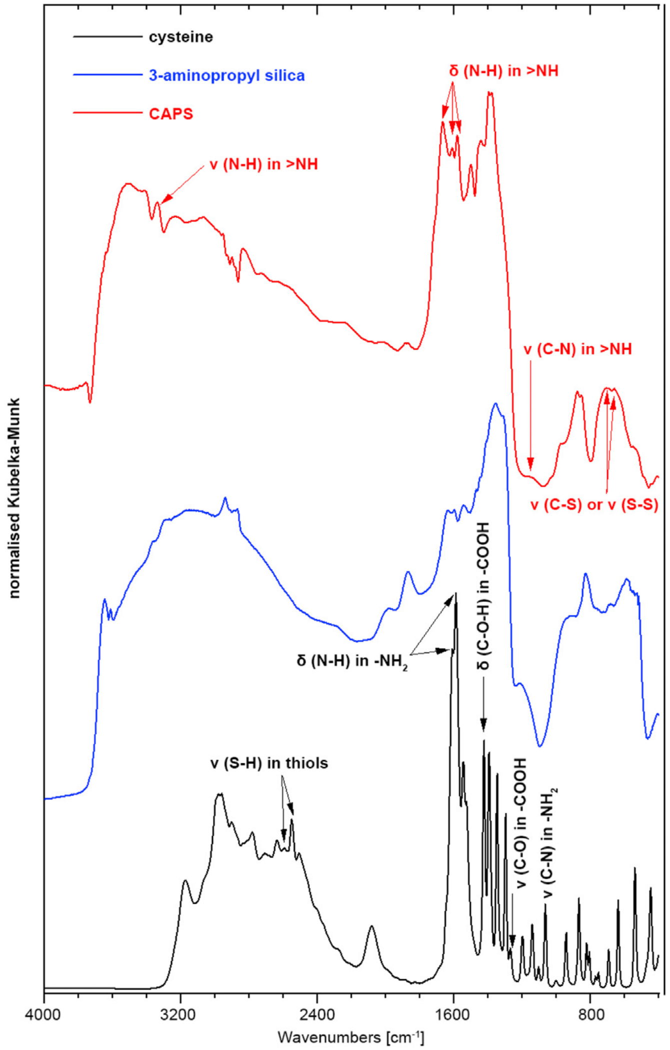

Fig. 2. The DRIFT spectra of cysteine (black), 3-aminopropyl silica (blue) and CAPS (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

0.04 ± 0.003 mmol g^{-1} for the CAPS and 0.05 ± 0.01 mmol g^{-1} for the CAS. The amount of thiol functional groups was negligible on these two new resins. The load of carboxylic groups was 0.85 ± 0.03 mmol g^{-1} and 0.56 ± 0.02 mmol g^{-1} for the CAPS and the CAS, respectively. The original load of primary (1°) amino groups on 3-aminopropyl-functionalised silica was 0.93 ± 0.08 mmol g^{-1}, which agrees with the value of ~1 mmol g^{-1} from the supplier. The load of 1° amino groups on the amino-functionalised silica was 0.65 ± 0.08 mmol g^{-1} and no comparative value was provided by the supplier. After modification, the load of 1° amino groups was 0.81 ± 0.03 mmol g^{-1} for the CAPS and 0.58 ± 0.03 mmol g^{-1} for the CAS. The glutaraldehyde-mediated immobilisation is not fully quantitative, due to complex structure and reactivity of glutaraldehyde in solutions (Migneault et al., 2004), which explains the slight decrease of 1° amino group load on the two modified resins. However, the immobilisation introduced additional 1° amino and carboxyl groups from the molecule of cysteine to the resins. The molar ratio of carboxylic and 1° amino, carboxylic and thiol groups was close to 1:1:0 in both the CAPS and the CAS. For comparison, this molar ratio in native cysteine was 1:1:1 (1° $-NH_2$: $-COOH$: $-SH$). Together with the results of DRIFT spectrometry, it suggests that the activated glutaraldehyde-silica intermediate formed a bond with the thiol group of cysteine, introducing thioether (R-S-R') group to the structures of both the CAPS and the CAS. This thioether group was present in the DRIFT spectra of both the CAPS and the CAS and agreed with the literature (Upadhyay and Verma, 2014; Verma et al., 2017). The modification process and the expected structure of cysteine-modified silica resin are shown in Fig. 3.

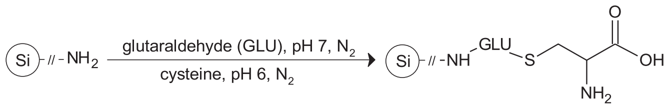

Fig. 3. Modification process and general structure of cysteine-modified silica-based resin.

The modification of silica with cysteine was inspired by the literature (Dakova et al., 2011), however, the results in our study are in contrary to that work, as the presence of thiol functional groups in the final product was refuted by both DRIFT spectroscopy and titrimetric analysis. The results of our study are also supported by the literature (Upadhyay and Verma, 2014; Verma et al., 2017).

The cysteine-modified silica resins (Fig. 3) carry three types of functional groups: 1° amino, thioether and carboxylic groups. The amino and the thioether groups of both CAPS and CAS are also present in the structure of the isothiouronium resin used for Hg — in the wastewater treatment (Zaganiaris, 2016; SenGupta, 2017) and in the DGT technique (Ren et al., 2018). The 1° amino group of both the CAPS and the CAS appears naturally in amino acids or chitosan, which were used for the uptake of hazardous trace metals (Ishii et al., 1995). The carboxylic group of both the CAPS and the CAS is also present in natural humic acids, amino acids and alginate, used for trace metals in wastewater treatment (Vandenbossche et al., 2014) and is also in the structure of Chelex-100, routinely used in the DGT technique for trace metals (Zhang and Davison, 1995). Since the cysteine-modified silica resins showed the potential for metal uptake, these resins were further tested in the DGT technique.

3.2. DGT performance test results

3.2.1. Uptake kinetics and elution factors of resin gels

Within 24 h of deployment, the CAS resin gels showed high uptake (around 80%) for all metals beside Hg, reaching a plateau within 4 h (Fig. 4A). The uptake of Hg was much lower than other metals and reached around 50% after 24 h. When the CAPS resin gel was used, the uptake of all metals reached around 100% within 3–4 h (Fig. 4B). The better performance of the CAPS resin gel could be credited to the higher load of functional groups than the CAS. In addition, these two resins possessed different length of the spacer between the silica carrier and the glutaraldehyde-immobilised cysteine, which also influences the reactivity and accessibility of the functional groups (Hage and Cazes, 2005).

The elution factor of each metal was calculated using Equation (1) and very high elution factors around 100% were obtained for all metals, Hg having the lowest of 95%, which is in agreement with the previously published results (Ren et al., 2018).

Based on the results of uptake test in 50 mL of 1 mg L^{-1} mixture solution (Fig. 4), the maximal capacity of the CAS resin gel was calculated and 40 μg/disc for Cd, Co, Ni and Cu, 35 μg/disc for Pb and 25 μg/disc for Hg was obtained. The capacity of the CAPS resin gel was at least 50 μg/disc for each metal. Since the highest concentrations of labile trace metals in the marine systems were 0.007 μg L^{-1} for Hg (Bratkic et al., 2019), 0.05 μg L^{-1} for Cd, 0.02 μg L^{-1} for Pb, 0.11 μg L^{-1} for Co, 0.59 μg L^{-1} for Ni and 0.60 μg L^{-1} for Cu (Gaulier et al., 2019), both types of DGTs using

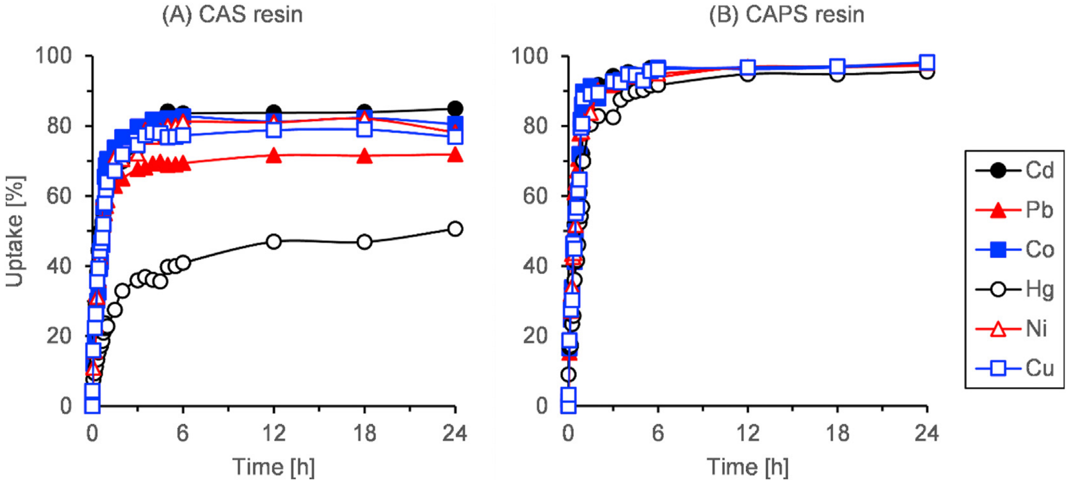

Fig. 4. Uptake efficiency of the CAS resin gel (A) and the CAPS resin gel (B).

two resins have sufficient capacity for fieldwork application.

3.2.2. *Effective diffusion coefficients and diffusive boundary layer*

The mass of all analytes accumulated on the CAS-DGT (Fig. 5A), the CAPS-DGT (Fig. 5B), the Chelex-DGT (Fig. 5C) and the 3-MFS-DGT (Fig. 5D) increased linearly with the deployment time up to 48 h. The effective diffusion coefficients D_e of the analytes were then calculated using Equation (2) and are summarised in Table 1.

In the case of Hg, the D_e obtained by the CAPS-DGT was much higher than the D_e obtained by the CAS-DGT (Table 1), as the uptake ratio of the CAS was not high enough (Fig. 4) to crease a zero concentration at the interface of the diffusive and resin gel, as required for the correct performance of the DGT technique (Zhang and Davison, 1995). The D_e obtained by the CAPS-DGT also agreed well with the D_e obtained by the 3-MFS-DGT (Table 1). The D_e of Hg reported previously by Gao et al. (2011) was slightly higher than both CAPS-DGT and 3-MFS-DGT (Table 1), but it was obtained in $NaNO_3$ solution. In our study, NaCl was used to prepare the deployment solution and Hg forms relatively stable Hg-chloride complexes (Pelcova et al., 2018; Ren et al., 2018) with lower affinity to the resins, which caused the slightly decreased D_e of Hg for both CAPS-DGT and 3-MFS-DGT (Docekalova and Divis, 2005; Ren et al., 2018). The DGTs equipped with Whatman P81 membrane obtained slightly lower D_e for Hg than our results and have problems when ionic strength is above 0.1 mol L^{-1} (Colaco et al., 2014).

For all other trace metals, the D_e obtained by the CAPS-DGT was always higher than the D_e obtained by the CAS-DGT (Table 1). The diffusive coefficients in the AGE diffusive gel are well-studied for Hg, but not for the other trace metals. The D_e of Co, Ni and Cu obtained by the CAPS-DGT (Table 1) agreed well with the previously published results of Wang et al. (2016), but the D_e obtained by the CAS-DGT were far below those D_e (Table 1). The D_e of Cd obtained by the CAPS-DGT (Table 1) was close to the values reported previously by Fatin-Rouge et al. (2003), Wang et al. (2016) and Reichstädter et al. (2020). Similar for Pb, the D_e obtained by the CAPS-DGT (Table 1) was close to the values reported previously by Fatin-Rouge et al. (2003) and Reichstädter et al. (2020), however much higher D_e of Pb (Table 1) was reported by Wang et al. (2016). In our study, NaCl was used to prepare the deployment solutions. In aqueous solutions with NaCl, Pb is forming lead-chloride complexes with low solubility (Holdich and Lawson, 1987; Byrne et al., 2010), which can explain the decreased D_e of Pb.

The D_e of trace metals obtained by the Chelex-DGT was different from the D_e obtained by the other DGT because the standard Chelex-DGT is using the APA diffusive gel instead of the AGE diffusive gel. The D_e for all the trace metals obtained by the Chelex-DGT (Table 1) corresponded well with the literature (Scally et al., 2006).

The D_e in different matrixes should theoretically descend: water > AGE diffusive gel > APA diffusive gel > bisacrylamide-crosslinked polyacrylamide (restrictive) diffusive gel, due to the decrease of gel pore size (Davison and Zhang, 2016; Österlund et al., 2016). The pore size of the APA is in a range of 5–20 nm (Zhang and Davison, 1995; Scally et al., 2006) and the one of the AGE is in a range of 35–47 nm (Zhang and Davison, 1999; Fatin-Rouge et al., 2004). In our study, the D_e values of Co, Ni and Cu in the AGE followed this trend, however not for Cd and Pb. That might be because the diffusion of these metals is not affected only by the pore size but also by the dissociation rates of metal complexes in the diffusive layer. At different pH and NaCl concentration, trace metals form hydroxide- or chloride-complexes with different stability and solubility (Byrne et al., 2010; Pelcova et al., 2018), which can affect the DGT results. Additionally, the agarose gel has slightly negative charge, which could also influence the diffusion process negatively (Fatin-Rouge et al., 2003, 2004). The impurities present in the agarose structure (e.g. sulphonate, ester sulphate, ketal pyruvate and carboxyl groups) can influence the diffusion process negatively too (Fatin-Rouge et al., 2003).

The thicknesses of DBL (δ) was measured in the solution stirred at 1200 rpm in the lab condition. For the calculation, the Δg was expanded by plus the value of δ in Equation (4). The calculated average DBL was 0.05 ± 0.01 mm in the lab condition.

3.2.3. *Effect of pH and ionic strength on the DGT performance*

The performance of the CAS-DGT and the CAPS-DGT was not strongly influenced by the pH and ionic strength changes. For all metals (Fig. 6), the *c*DGT/*c*SOL ratio was in a range of 0.9–1.0 during the pH changes from 4.5 to 8.5 and the ionic strength changes from 0.001 to 1.0 mol L^{-1} NaCl. The lowest *c*DGT/*c*SOL ratio was obtained at pH above 8 for Pb (*c*DGT/*c*SOL = 0.92, Fig. 6C), caused by the formation of insoluble $Pb(OH)_2$ in more basic conditions (Perera et al., 2001). For ionic strength changes, the most affected element was Hg in 1.0 mol L^{-1} NaCl solution (*c*DGT/*c*SOL = 0.92, Fig. 6A). Despite these two slight influences, the performance of the CAS-DGT and the CAPS-DGT did not vary more than 10% (Fig. 6) which is the basic requirement of the DGT technique (Davison and Zhang, 1994; Zhang and Davison, 1995). These novel DGTs provide a significant advantage for fieldwork application since the typical

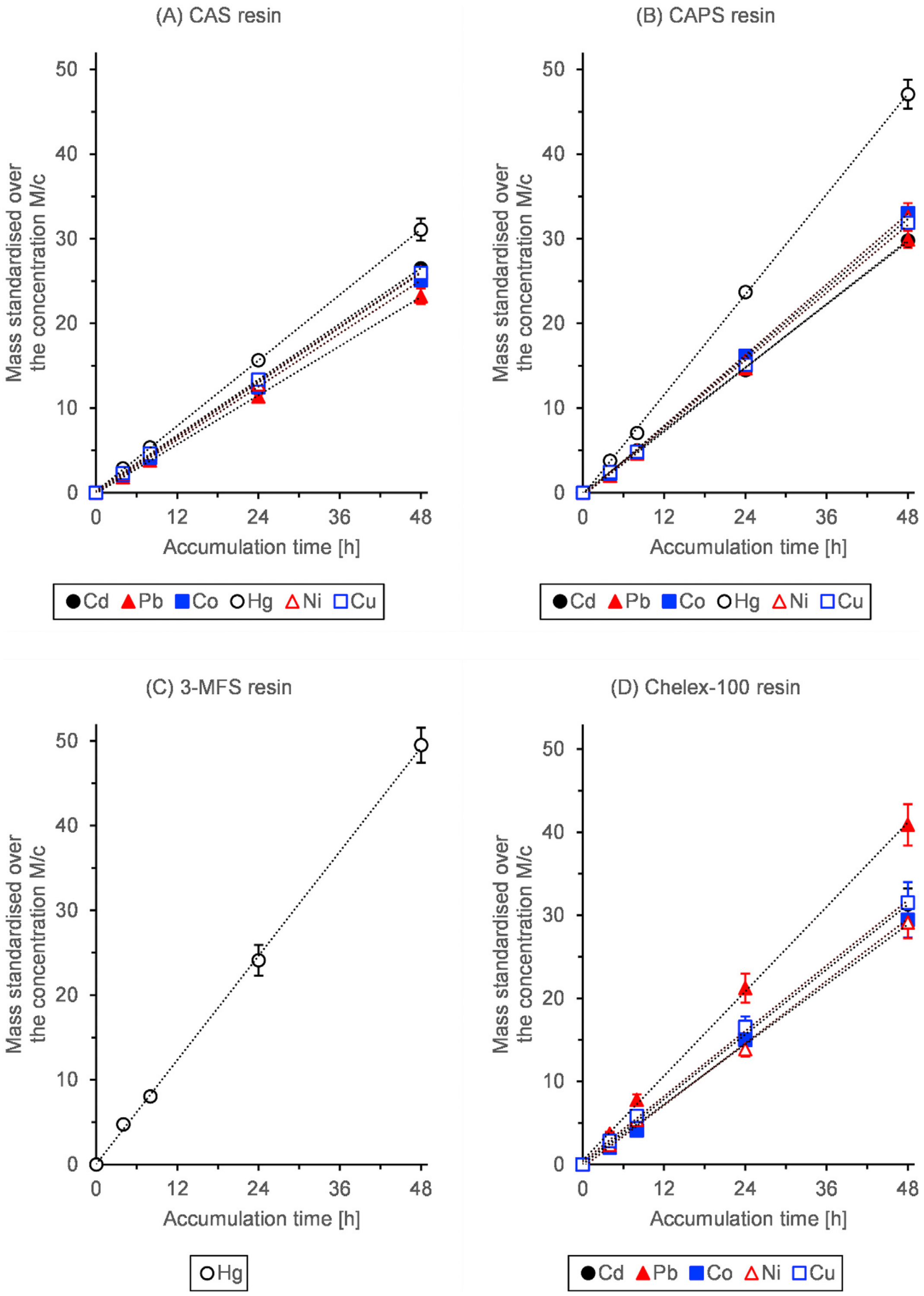

Fig. 5. Accumulation of analytes by (A) CAS resin, (B) CAPS resin, (C) 3-MFS resin and (D) Chelex-100 resin. The error bars display the standard deviation (n = 3).

ionic strength in both freshwater and seawater varies in a range of 0.001–0.7 mol L^{-1} (Noh et al., 2016).

3.3. Fieldwork results

The coordination of sampling stations, the average deployment temperature, pH, and salinity are summarised in Table 2. To calculate the metal concentrations in the field, the D_e for all metals (Table 1) was recalculated using the average deployment temperature (Zhang and Davison, 1995). Then, the DGT measured concentration of analytes at each sampling station was calculated by Equation (4).

For Hg assessment in all stations, the CAPS-DGT and the 3-MFS-DGT produce comparable results, which were higher than those from the CAS-DGT. The statistical analysis using analysis of variance (ANOVA) with post-hoc Tukey's Honest Significant Difference

Table 1
Summary and comparison of diffusion coefficients at 20 °C using different experimental setup.

Detailed setup	Hg	Cd	Pb	Co	Ni	Cu	References
AGE, CAS resin	4.99 ± 0.35	4.24 ± 0.28	3.74 ± 0.41	4.04 ± 0.43	4.18 ± 0.33	4.23 ± 0.36	This work
AGE, CAPS resin	7.64 ± 0.48	4.78 ± 0.21	4.86 ± 0.26	5.38 ± 0.35	5.26 ± 0.29	5.51 ± 0.21	This work
AGE, 3-MFS resin	7.93 ± 0.30	–	–	–	–	–	This work
AGE, 3-MFS resin	8.44	–	–	–	–	–	Gao et al. (2011)
AGE, Purolite S924 resin	6.19 ± 0.36	4.44 ± 0.26	3.67 ± 0.27	–	–	–	Reichstädter et al. (2020)
AGE, Whatman P81	7.18[a]	–	–	–	–	–	Colaco et al. (2014)
AGE, diffusion cell	–	4.62	4.03	–	–	–	Fatin-Rouge et al. (2003)
AGE, Chelex-100 resin	–	5.57[a]	7.35[a]	5.71[a]	5.71[a]	5.76[a]	Wang et al. (2016)
APA, Chelex-100 resin	–	5.33 ± 0.36	6.94 ± 0.23	5.10 ± 0.33	4.92 ± 0.35	5.40 ± 0.37	This work
APA, Chelex-100 resin	–	5.30	6.99	5.17	5.05	5.42	Scally et al. (2006)

[a] Was obtained at other temperature and was corrected to 20 °C (Zhang and Davison, 1995). All values are in [$(\cdot 10^{-6})$ cm$^2\cdot$s^{-1}].

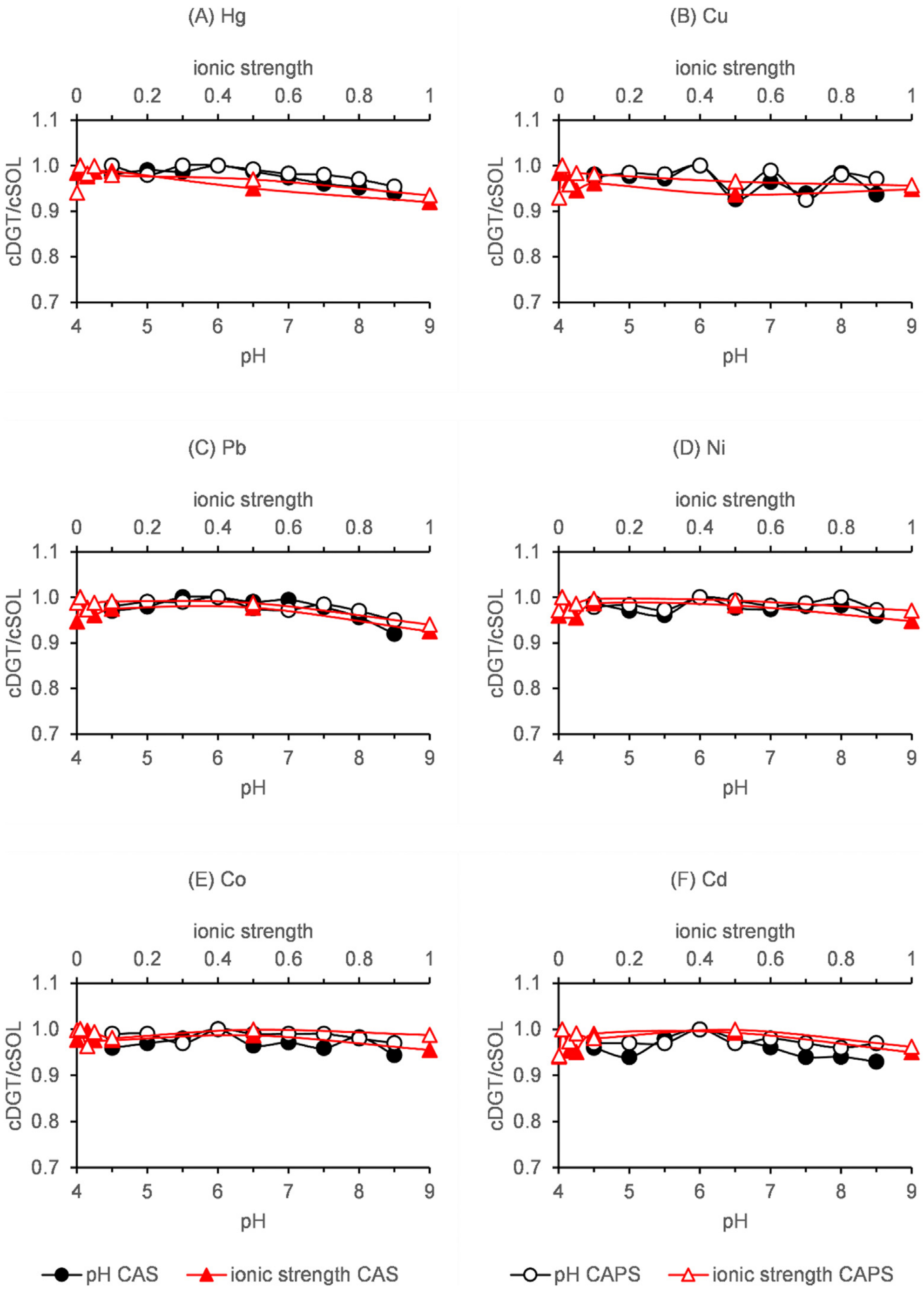

Fig. 6. Effect of pH and ionic strength on the performance of the CAS-DGT and the CAPS-DGT.

Table 2
The locations of the stations and the sampling conditions.

Station	Latitude	Longitude	T_{AVG} [°C]	pH_{AVG}	Sal_{AVG} [PSU]
HO-1	51.2343 N	2.9268 E	11.7	7.2	11
HO-2	51.2264 N	2.9361 E	11.4	7.2	20
HZ-1	51.3404 N	3.2030 E	9.7	7.0	29
HZ-2	51.3111 N	3.1995 E	9.8	7.2	29

(Tukey's HSD) comparisons (R Core Team, 2013) showed the difference between the 3-MFS-DGT and the CAPS-DGT was not significant ($p = 0.746$, $\alpha = 0.05$), but the differences between the 3-MFS-DGT and the CAS-DGT and between the CAS-DGT and the CAPS-DGT were all significant ($p < 0.0001$, $\alpha = 0.05$). Hg concentrations in station HO-2, HZ-1 and HZ-2 were around 1.5 ng L^{-1} (Fig. 7A), which is higher than in station HO-1, which can be credited to the sludge disposal near these stations (Leermakers et al., 2001).

For the other trace metal determination, the CAPS-DGT and the Chelex-DGT produced comparable results in all stations. The statistical analysis using ANOVA with post-hoc Tukey's HSD showed the difference between the Chelex-DGT and the CAPS-DGT was not significant ($p = 0.893$, $\alpha = 0.05$). However, the differences between the Chelex-DGT and the CAS-DGT and between the CAS-DGT and the CAPS-DGT were all significant ($p < 0.0001$, $\alpha = 0.05$). Trace metal concentrations measured with the CAPS-DGT and the Chelex-DGT in this study were in good agreement with the results from Gaulier et al. (2019). Cu concentration around 0.6 μg L^{-1} in Station HZ-2 was the highest among all stations, and then the concentration decrease followed the sequence of HO-1 > HZ-1 > HO-2 (Fig. 7B). The highest concentration of Cu in HZ-2 is due to the antifouling paints of the vessels anchored in this harbour,

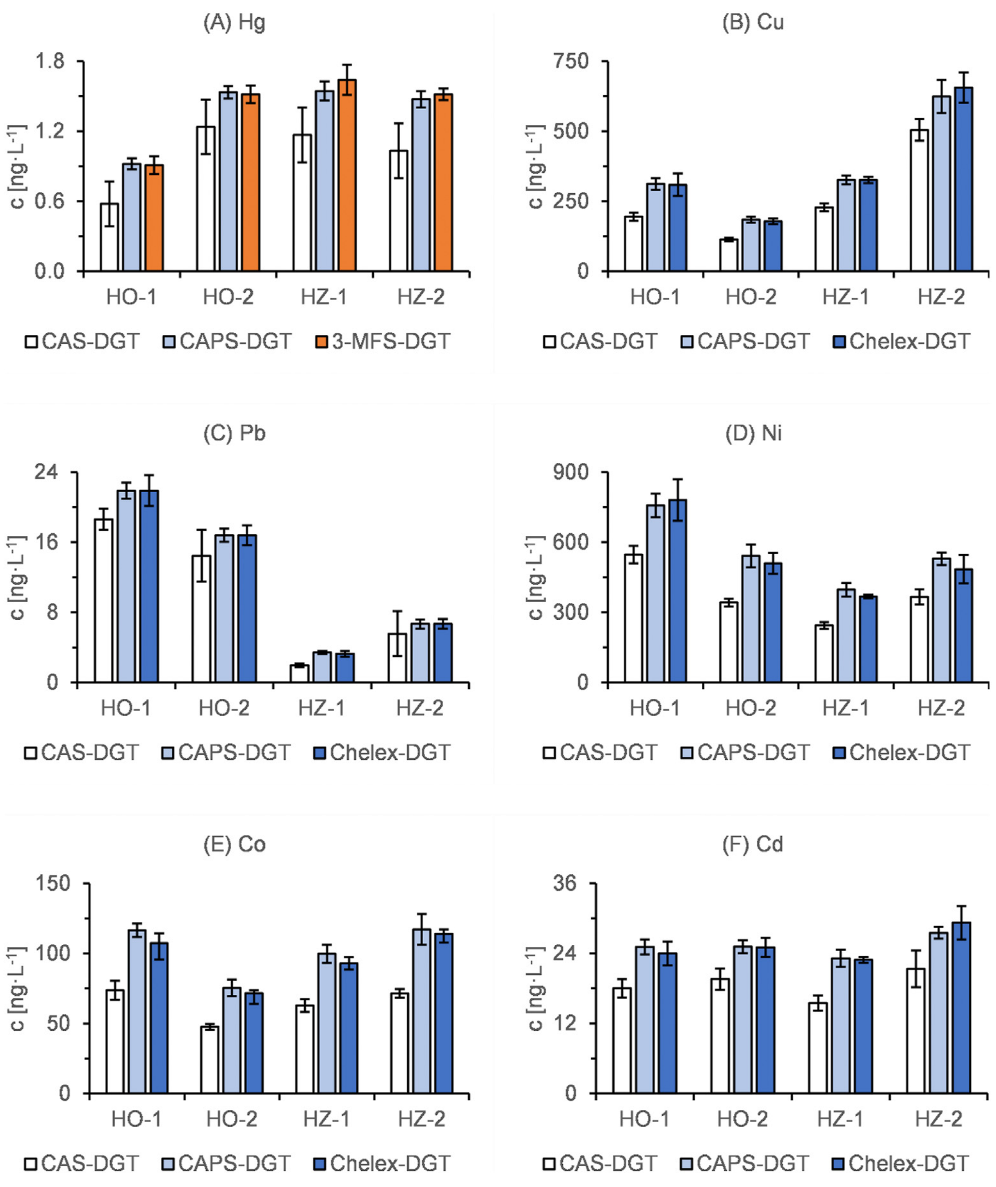

Fig. 7. Comparison of concentrations obtained by different DGT methods during fieldwork application in the sampling stations HO-1, HO-2, HZ-1, and HZ-2. The error bars display the standard deviation ($n = 5$). The statistical analysis using ANOVA with post-hoc Tukey's HSD, showed for Hg that the difference between the 3-MFS-DGT and the CAPS-DGT was not significant ($p = 0.746$, $\alpha = 0.05$), but the difference between the 3-MFS-DGT and the CAS-DGT and between the CAS-DGT and the CAPS-DGT was significant ($p < 0.0001$, $\alpha = 0.05$). For the other trace metals, the difference between the Chelex-DGT and the CAPS-DGT was not significant ($p = 0.893$, $\alpha = 0.05$). The differences between the Chelex-DGT and the CAS-DGT and between the CAS-DGT and the CAPS-DGT were significant ($p < 0.0001$, $\alpha = 0.05$).

which was reported previously (Warnken et al., 2004; Dunn et al., 2007; Costa et al., 2013). Pb and Ni concentrations showed the similar trend with the highest value found in station HO-1 and the lowest ones in station HZ-1 (Fig. 7C and D). Ni concentrations in HO-2 and HZ-2 are around 0.5 μg L^{-1}, however, Pb concentration in station HO-2 was much higher than in station HZ-2. The higher concentration of Pb in HO-1 and HO-2 in the Oostende harbour can be credited to the heavier traffic than in HZ-1 in marine military base and HZ-2 in the anchorage of the recreational vessels. The resuspension of sediments caused by boat traffic can contribute to the total Pb concentration in the water column (Superville et al., 2014). Co and Cd concentrations in all stations (Fig. 7E and F) were quite content with the lowest concentration of Co found in station HO-2.

The deployment conditions of the DGTs are summarised in Table 2. The concentration of Hg was the lowest among all metals and was in a range of 0.9–1.6 ng L^{-1} for 4 stations (Fig. 7A). The use of the CAS-DGT acquired lower concentration with higher average relative standard deviation (RSD) of 24%, than the CAPS-DGT (RSD 5%) and the 3-MFS-DGT (RSD 6%). The concentration of Cu (Fig. 7B) was in a range of 0.31–0.66 μg L^{-1}, with the low RSD 7% for all three types of DGTs. The concentration of Pb was observed in a wide range of 0.003–0.022 μg L^{-1} (Fig. 7C).

4. Conclusions

In this study, the cysteine-modified silica resins were prepared successfully with one using amino-functionalised silica and the other using 3-aminopropyl-functionalised silica as carriers. The modified resins were qualitatively and quantitatively characterised by CHNS elemental analysis, DRIFT spectrometry and titrimetric analysis. The modified resin based on 3-aminopropyl-functionalised silica possessed a higher load of carboxyl and primary amino groups. The modified resins were further used in the DGT technique (CAS-DGT and CAPS-DGT) for simultaneous assessment of Hg and other trace metals. The performance of the novel DGTs corresponded well with the 3-MFS-DGT for Hg determination and the Chelex-DGT for other trace metal assessment. The DGT techniques using both modified resins were capable to assess all analytes in a broad range of pH and ionic strength, however the CAPS-DGT presented higher performance than the CAS-DGT. During the fieldwork application in the harbours at the Belgian Coastal Zones (BCZ), there were no significant differences between the CAPS-DGT and the 3-MFS-DGT for Hg, and between the CAPS-DGT and the Chelex-DGT for the other trace metals. The CAPS-DGT successfully combined the advantages of the 3-MFS-DGT and the Chelex-DGT and allowed simultaneous determination of all analytes by a unique DGT technique. Future perspectives can expand to the application of these modified resins for determination of mercury speciation.

Credit author statement

Marek Reichstädter, Methodology, Software, Validation, Formal analysis, Investigation, Writing – Original Draft, Visualization. Pavel Diviš, Conceptualization, Investigation, Supervision, Writing – Review & Editing. Camille Gaulier, Formal analysis, Investigation, Visualization. Tianhui Ma, Investigation, Visualization. Martine Leermakers, Formal analysis, Investigation. Yue Gao, 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.

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5.2 APPLICATION OF DGT WITH NEW SORBENTS FOR ANALYSIS OF TRACE METALS IN FISH SAUCE

Despite the recent advances in food analysis, the determination of trace metals in foodstuffs is still a challenging process (chapter 2.2.3.2). Even the modern routinely used methods are still facing difficulties with the low concentration of trace metals, also negatively influenced by the matrix effects of the food samples. Due to this complex matrix, several pre-treatment steps are often required before the sample analysis (e.g. extraction procedures or mineralisation). Special instrumentation for direct analysis of trace metals is available, however, the complex matrix is still causing issues (e.g. corrosion or interferences of smoke). The DGT technique has some unique features (chapter 2.2.4.2), of which the preconcentration and separation of analytes from the matrix are especially useful for application in food analysis and it could overcome the issues of the routine methods. This work builds on the previous work (Chen et al. 2014), where poly(aspartic acid) was used as the binding agent in the DGT for the determination of Pb in soybean sauce.

In this work, special attention was given to fish sauces, which are the typical example of processed fishery product (Mizutani et al. 1992). Fish sauce is a liquid seasoning, consisting of fishes mixed with salt and other additives, e.g. sugar, rice or tamarind. Most commonly used fishes are different genera of mackerels and anchovies (Lopetcharat et al. 2001). Therefore, an increased occurrence of trace metals is expected in fish sauce, especially Hg, as it is more connected with the aquatic food chain and its concentrations can biomagnify (chapter 2.1.2). Although fish sauce has origins in ancient Rome (Lopetcharat et al. 2001), it is nowadays not very typical for European cuisine, but it is an essential part of eastern and southeastern Asian cuisine (Park et al. 2001; Ardiansyah et al. 2015). The consumption of fish sauce in these regions reaches up to 4 kg per capita per year as a national average, considerably contributing to the total consumption of fish-related food (Needham and Funge-Smith 2015). With the increasing global popularity of these cuisines, the global consumption of fish sauce is increasing as well, requiring the same awareness of trace metals content as in fish (YouGov 2018). Fish sauce is food with complex chemical composition, which is mainly influenced by the type of fish used for the production and by the fermentation conditions (Lopetcharat et al. 2001). Main components of fish sauce are amino acids, organic acids, major elements and water-soluble vitamins (Park et al. 2001; Gildberg et al. 2007). The typical characteristic of fish sauces is a high salt content of up to 300 $g \cdot L^{-1}$ (Park et al. 2001; Nakano et al. 2017). That properties make it a perfect example of a food with a complex matrix, in which the trace metals analysis could be challenging (chapter 2.2.3.2). The typical content of Cd, Pb and Hg in fish sauces is hard to find in literature, characteristically below the very high LOD of the used method (Mabesa et al. reported the concentrations below the LOD - 20 μg·L^{-1}, 400 μg·L^{-1} and 20 μg·L^{-1} for Cd, Pb and Hg respectively, using wet ashing and AAS). The few available works reported concentration of Hg in the range of 0.001-0.09 mg·L^{-1}, Cd 0.3-14 mg·L^{-1} and Pb 0.1-0.7 mg·L^{-1} (Mabesa et al. 1985; Nakazato et al. 2000; Funatsu Y. et al. 2001; Yang et al. 2015). As the fish sauce is a fishery product but not fish meat, so it also stands aside from the regulations for the content of trace metals in foodstuffs. The only maximal level is set for Hg in fishery products by Commission Regulation 1881/2006/EC (2006). The other regulations (Joint FAO/WHO 2019; FDA 2020) do not set any maximal level of trace metals for fish sauce type of food.

Therefore, the DGT technique using novel resin Purolite S924 with thiol functional groups (hereafter referred as "S924-DGT") was tested for determination of Hg in fish sauces (chapter 5.2.1). The reference method was the routine direct analysis of foodstuffs by TD-AAS (Senila et al. 2016).

This concept was further expanded for simultaneous determination of Hg, Cd and Pb in fish sauces (chapter 5.2.2). The routine DGT technique with Chelex-100 resin was also studied for the determination of Cd and Pb in fish sauces. Microwave-assisted digestion combined with the ICP-MS was used (hereafter referred as "MWD") as the reference method (U.S. EPA 1996; Agazzi and Pirola 2000).

5.2.1 Determination of mercury in fish sauces by TD-AAS after preconcentration by DGT technique

Thermal decomposition gold amalgamation atomic absorption spectrometry (TD-AAS; AMA-254, Altec, Prague, Czech Republic) was validated by direct analysis of CRM ERM-CC580 (estuarine sediment) and CRM ERM-BB422 (fish muscle) with no statistically significant differences between the obtained and the certified values ($\alpha = 0.05$; $p = 0.088$ and $p = 0.381$, respectively). The methodology limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the background concentration for TD-AAS and the blank resin gels for the S924-DGT (using Equation 4 and assuming 24 hours deployment). Both LOD and LOQ of S924-DGT was about 10 times lower compared to the direct TD-AAS analysis.

The S924-DGT accumulated Hg linearly in time in 0.01 mol·L^{-1} NaCl solution and the effective diffusion coefficient D_e was slightly lower than the one reported in the literature in 0.01 mol·L^{-1} $NaNO_3$ solution (Docekalova and Divis 2005). Such decrease of D_e was already described previously (Hong et al. 2011), due to the formation of $[Hg(Cl)_x]^{2-x}$ complexes (Pelcova et al. 2018). The pH in the range of 3-6 and NaCl content in the range of 0.01–1 mol·L^{-1} did not affect the performance of the S924-DGT by more than 10 %. The D_e obtained in spiked fish sauce 5-fold diluted with MQ water was lower than in the 0.01 mol·L^{-1} NaCl solution due to formation of Hg-amino acid complexes with relatively higher stability constants (Dočekal et al. 2005;

Fernandez-Gomez et al. 2011; Pelcova et al. 2018). The actual D_e are listed and further discussed in the manuscript attached below. During the kinetic studies, Hg was bound fast and quantitatively in both 0.01 mol·L^{-1} NaCl solution and the fish sauce solution. The capacity of S924 resin gel disc was >50 µg Hg.

In total, 10 fish sauce samples of various origin and anchovy content were analysed by S924-DGT in the combination with TD-AAS and directly by TD-AAS. Both methods obtained results with no significant difference between them ($p > 0.05$). The Hg content of two samples was below the LOQ of TD-AAS, but it was possible to analyse them using S924-DGT. The actual Hg content in all samples obtained by both methods is in the manuscript attached below. All samples matched the criteria about the maximal permitted level of contaminants in food (Commission Regulation 1881/2006/EC 2006).

5.2.1.1 Conclusions

In this study, novel resin Purolite S924 was introduced for use in the DGT technique for determination of Hg in fish sauces. The S924-DGT was based on analysis of the resin gels with preconcentrated Hg by TD-AAS and was compared with routine direct analysis by TD-AAS. Both methods obtained statistically same results, but S924-DGT had 10 times lower LOQ and LOD than TD-AAS. That allowed assessment of Hg by S924-DGT even in two fish sauce samples with concentration below the LOQ of TD-AAS. The S924-DGT has the ability of matrix removal and preconcentration of analytes, which is useful for analysis of trace metals in foodstuffs. Additionally, this work has also proven the shortening of the service intervals of TD-AAS during the direct analysis of fish sauce due to excessive corrosion damage (Figure 8), compared to the analysis of the resin gels of the DGT technique. Therefore, the DGT technique offers remarkable advantages for the use in food analysis despite the longer analysis time.

Mercury is a highly studied metal and the concentration of Hg in fish products is monitored and regulated. This newly designed analytical method can be an interesting tool for monitoring of food quality and safety. Future research could investigate the application possibility of S924-DGT for methylmercury or other trace metals in fish sauces and other food matrices.

Figure 8 The corrosion and wear effects to TD-AAS during the direct analysis of fish sauce samples. Sample boat (A) and sample boat holder (B) before (left) and after (right) several runs of direct analysis.

5.2.1.2 References

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DETERMINATION OF MERCURY IN FISH SAUCES BY THERMAL DECOMPOSITION GOLD AMALGAMATION ATOMIC ABSORPTION SPECTROMETRY AFTER PRECONCENTRATION BY DIFFUSIVE GRADIENTS IN THIN FILMS TECHNIQUE

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Abstract

The analysis of mercury in food presents a challenge for analytical chemists. Due to the low concentration of mercury in many types of samples, as well as complicated sample matrices causing suppression or enhancement of the analyte signal, sample pre-treatment, preconcentration, and separation of mercury prior to measurement are required, even when highly sensitive analytical methods are used. In this work, the Diffusive Gradients in Thin Films technique (DGT) combined with thermal decomposition gold amalgamation atomic absorption spectrometry (AAS) was investigated for determination of the total dissolved mercury in fish sauces. Moreover, a new type of binding gel with Purolite S924 resin was used in DGT. Linearity assays for DGT provided determination coefficients around 0.995. Repeatability tests ($n = 3$) showed a relative standard deviation less than 10%. pH values in the range of 3–6, as well as NaCl concentrations up to 50 $g \cdot L^{-1}$, did not affect the performance of DGT. The effective diffusion coefficient of mercury in 5-fold diluted fish sauce was determined to be $3.42 \times 10^{-6} \pm 0.23$ $cm^2 \cdot s^{-1}$. Based on 24 h deployment time of DGT, the limit of detection (LOD) for the investigated method was 0.071 $\mu g \cdot L^{-1}$. The proposed method, which combines DGT and AAS, allows the analysis of fish sauces with mercury concentrations below the LOD of AAS, and significantly reduces the wear and corrosion of the AAS components.

Keywords: mercury; fish sauce; flavouring; Diffusive Gradients in Thin Films; atomic absorption spectroscopy; preconcentration

Introduction

Fish sauce is an amber-coloured liquid that is produced by fermenting fish with sea salt and is used as a flavouring, mainly in South East Asian cuisine (Lopetcharat et al. 2001). Fish sauce is a food with a complex chemical composition, mainly influenced by the type of fish used for production and by the fermentation conditions. The main components of fish sauce are amino acids, organic acids, major elements, and water-soluble vitamins (Park et al. 2001; Gildberg et al. 2007). A typical characteristic of fish sauces are high salt content, ranging up to 300 $g \cdot L^{-1}$ (Park et al. 2002; Nakano et al. 2017).

Fish used in the production of fish sauce are harvested from waters contaminated by varying amounts of metals, and these metals may accumulate in the fish at such a high level as to cause human health problems (FDA 2011); one such problematic metal is mercury (Hg). The total Hg content of fish used in the production of fish sauce is reported to be within the range of 0.04 to 0.1 $mg.kg^{-1}$ (Falco et al. 2006; Bilandzic et al. 2011; Miklavcic et al. 2011). These concentrations are relatively low compared to the maximum permitted Hg content determined by Commission Regulation (EC) No. 1881/2006 (Commission Regulation, 2006), however, it is important to note that large quantities of these fish are used to produce these sauces. Chronic Hg poisoning causes hair loss, digestive disorders, neurological and psychological problems, and other serious health problems such as anaemia, rheumatic diseases, and kidney damage. Chronic exposure to Hg can also cause tooth decay, rashes, muscle tremors, memory loss, behavioural changes, and central nervous system damage (Bernthoft, 2012). Therefore, to protect consumer health, it is important to control the amounts of Hg in foods, a task which requires sensitive methods for analysing Hg levels in food products.

The most common methods used to determine total Hg in various types of samples are spectroscopic methods, including cold vapour atomic absorption spectrometry, cold vapour atomic fluorescence spectroscopy, and inductively coupled plasma mass spectrometry (Gao et al. 2012). A cost-effective alternative to these conventional techniques is thermal decomposition gold amalgamation atomic absorption spectrometry, which additionally allows direct determination of Hg in solid matrices (Costley et al. 2000). Due to the low concentration of Hg in many types of samples and complicated matrices causing suppression or enhancement of the analyte signal, sample pre-treatment (Gao et al. 2012), preconcentration, and separation of Hg prior to measurement are required, even when highly sensitive analytical methods are used (Leopold et al. 2009). The most widely used techniques for separation and preconcentration of Hg at low concentrations are classic extraction techniques (Ali et al. 2016; Krawczyk and Stanisz 2016; Kallithrakas-Kontos and Foteinis 2016; Altunay 2018).

In 1994, the Diffusive Gradients in Thin Films technique (DGT) was introduced by Davison and Zhang. This technique has greatly simplified the determination of zinc in seawater, as it effectively separates the analyte from the matrix, which otherwise causes specific problems during analysis (Davison and Zhang 1994). The principle of DGT has been described extensively in the literature (Zhang and Davison 1995; Divis et al. 2005). Although DGT was originally designed for environmental analysis, several other applications of DGT are currently being developed (Pelferne et al. 2011; Chen et al. 2014). At present, in combination with instrumental methods, more than 50 elements can be determined by DGT (Garmo et al. 2003); studies have also been performed exploring its use in Hg determination (Docekalova and Divis 2005; Divis et al. 2010; Fernandez-Gomez et al. 2011; Gao et al. 2011; Hong et al. 2011; Szkandera et al. 2013; Gao et al. 2014; Hlodak et al. 2015). The major problem encountered when using DGT to determine Hg levels is that most of the previously tested Hg-selective sorbents are no longer commercially available; only 3-mercaptopropyl silica gel particles are currently available to assess Hg by DGT.

This work builds on the results obtained by Chen et al. (2014) investigating the use of a poly(aspartic acid) binding agent in DGT for the determination of lead in soybean sauce. In their results, the authors recommended focusing on new opportunities for DGT application in the analysis of high salinity condiments. In the current work, a new type of binding gel containing Purolite S924 resin was tested for application in DGT. Purolite S924 is a polystyrene-based chelating resin designed for selective removal of Hg. After validating the use of DGT with Purolite S924 binding gel, this technique was used in combination with thermal decomposition gold amalgamation atomic absorption spectrometry for the determination of total dissolved Hg in fish sauce samples.

Material and methods

Chemicals, solutions, and other material

Mercury standard solution (Astasol, 1000 mg·L^{-1}, Analytika, Prague, Czech Republic) was used to prepare all model solutions containing different concentrations of Hg. Nitric acid (Analpure, Analytika, Prague, Czech Republic) and sodium hydroxide (Penta, Prague, Czech Republic) were used to adjust the final pH of the model solutions. Sodium chloride (Penta, Prague, Czech Republic) was used to prepare solutions with different salt content. Purolite S924 chelating resin with thiol functional groups (Purolite, Paris, France) was used to prepare the binding gel. Since the mean size of these resin particles was 0.5–0.8 mm, an unsatisfactory size for preparing thin, compact binding gels, the resin was milled to an average particle size of 0.1 mm and sieved through a 0.1 mm sieve prior to use. The binding gel was then prepared by mixing 0.35 g of Purolite S924 resin with 4 mL of hot 1.5% agarose (Merck, Darmstadt, Germany) solution and pipetting the mixture between two glass plates that were separated from each other using 0.05 cm thick Teflon foil. The diffusive gel was prepared in the same way by only using 1.5% hot agarose solution. To prepare diffusive gels with thicknesses other than 0.05 cm, Teflon foils with thicknesses of 0.025, 0.075 and 0.1 cm were used. The empty

DGT pistons were purchased from DGT Research Ltd. (DGT Research, Lancaster, United Kingdom) and assembled as described by Docekalova and Divis (2005). The layer of binding gel and diffusive gel was covered with a membrane filter (Pall, USA, diameter 2.5 cm, thickness 0.014 cm, pores 0.45 μm). The basic model solutions and spiked 5-fold diluted fish sauce solutions were all prepared as 4 L (basic model solution) or 2 L (5-fold diluted fish sauce) solutions containing 25 $\mu g \cdot L^{-1}$ Hg^{2+}. In addition, the basic model solutions contained 0.01 $mol \cdot L^{-1}$ NaCl (Penta, Prague, Czech Republic). In order to test the influence of differing pH and salt content, these properties of the basic solution were modified. Final DGT performance testing was conducted on a 5-fold diluted fish sauce spiked with 5 $\mu g \cdot L^{-1}$ Hg^{2+}. The concentration of Hg in all solutions was monitored for the duration of all performed tests using an Advanced Mercury Analyser AMA 254. All water used in this study was ultrapure water (Elga Purelab, High Wycombe, United Kingdom, resistivity 18 $M\Omega \cdot cm$). All solutions were stirred at 400 rpm using a magnetic stirrer (IKA, Germany). Temperature and pH measurements during the experiments were performed using a pH probe (WTW GmbH, Germany).

Instrumentation

An Advanced Mercury Analyser AMA 254 (Altec, Prague, Czech Republic) was used for Hg analysis in the model solutions, binding gels, and commercially available fish sauce samples. This instrument is principally based on combustion of the sample in a combustion chamber flooded with oxygen. The released gases are then removed from the combustion chamber and passed over a gold amalgamation trap to separate the atomic Hg. The amalgamation cell is subsequently heated to release the Hg, which is finally measured by atomic absorption spectroscopy (Szakova et al. 2004). The working program of the Advanced Mercury Analyser consisted of 60 sec of drying, 150 sec of decomposition and 45 sec of waiting. The Advanced

Mercury Analyser is further referred hereto as thermal decomposition gold amalgamation atomic absorption spectrometry, abbreviated as AAS.

Method validation

The validation of AAS was performed via evaluations of the limit of detection (LOD), limit of quantification (LOQ), trueness, and precision. The LOD of AAS was calculated from the average background signal obtained from 10 parallel analyses of 100μL ultrapure water and raised by three times of standard deviation (SD). The LOQ of AAS was calculated from the average background signal obtained from 10 parallel analyses of 100μL ultrapure water and raised by ten times of SD. Trueness and precision were evaluated by analyses of ERM-BB422 (fish muscle meat), a certified reference material (0.601 ± 0.030 mg·kg^{-1} Hg). The long-term stability of AAS performance was evaluated through analysing the certified reference material, ERM-CC580 (132 ± 3 mg·kg^{-1} Hg), every day for the duration of the experimental period leading up to sample analysis, and a control chart was generated from these measurements. Additionally, AAS performance was further validated through participation in an interlaboratory trial (PT/CHA/2/2017, CSlab, Czech Republic), in which samples of wastewater were analysed.

The validation of DGT was performed both using a model solution and a diluted fish sauce solution. The linearity of Hg accumulation in the binding gel over time, value of the effective diffusive coefficient (D_e), thickness of the diffusive boundary layer (DBL, δ), performance of DGT in solutions with different pH and NaCl concentrations were all evaluated, along with the LOD, LOQ, trueness, and precision. LOD was calculated from the average mass of Hg in binding gel blanks raised by three times of SD and by taking into account the determined effective diffusion coefficient, the standard parameters of the DGT unit, and the exposure time of 24 hours, according to Equation 1, where: M = mass of analyte; Δg = diffusive layer thickness; D_e = effective diffusion coefficient of analyte in the diffusive gel; t = deployment time, and A = exposure area.

$$c_{\mathrm{DGT}} = (M \cdot \Delta g) / (D_e \cdot t \cdot A) \qquad \text{Eq. (1)}$$

The LOQ was calculated in a similar way using the average mass of Hg in the binding gel blanks raised by ten times of SD. The trueness of DGT was evaluated using the recovery for a 5-fold diluted fish sauce sample spiked with a known amount of Hg. The precision of DGT was assessed by calculating the relative standard deviation (RSD) of DGT concentrations calculated from Equation 1 in the analysis of 6 parallel samples of binding gel peeled off from DGT pistons at the end of the deployment time in 5-fold diluted fish sauce.

The effective diffusion coefficient, D_e, was calculated after performing tests on the linear accumulation of Hg during the exposure time. Ten DGT pistons were immersed in the model solution and two units were removed from the solution after 2, 4, 6, 8, and 24 hours. The DGT pistons were then dismantled and the binding gel was analysed using AAS. By plotting the dependence of $M.c^{-1}$ on time, the slope k of linear regression was obtained and D_e was calculated using Equation 2 (Docekalova and Divis 2005), where t_s is the conversion factor of hours to seconds.

$$D_e = k \cdot \Delta g \cdot A^{-1} \cdot t_s^{-1} \qquad \text{Eq. (2)}$$

This procedure was also used to calculate the D_e of Hg in diluted fish sauce. The D_e can be affected by the uptake efficiency of the analyte on the binding gel, therefore, it is essential to verify uptake efficiency, especially in complex matrices. In this work, we adopted our procedure for determining the uptake efficiency of Hg on the binding gel from the work of Abdulbur-Alfakhoury et al. (2019).

The thickness of the DBL was calculated from the simultaneous deployment of eight DGT pistons with different thicknesses of diffusive layer (diffusive gel and membrane filters 0.039, 0.064, 0.089, and 0.114 cm, tested in duplicate) in the model solution. After plotting M^{-1} against Δg, the DBL was calculated from the slope k and intercept q obtained from the regression equation of dependence of M^{-1} to Δg (Warnken et al. 2006). The diffusion coefficient of Hg in water, D_w, was taken from the work of Docekalova and Divis (2005).

$$\delta = q \cdot k^{-1} \cdot (D_w / D_e) \qquad \text{Eq. (3)}$$

All experiments were performed at the laboratory temperature of 20 °C and were repeated three times.

The effect of pH of the model solution on DGT performance was evaluated by immersing four pistons into model solution with a pH of 3 for 4 hours. This test was then repeated in model solutions adjusted to pH 4, 5, and 6. The concentration of Hg in the model solution was monitored for the duration of the test by analysis on AAS and recorded as c_{SOL}. This concentration was then compared with the concentration determined by DGT (c_{DGT}) and recovery R was calculated as $R = (c_{\mathrm{DGT}}/c_{\mathrm{SOL}}) \times 100$. To evaluate the effects of NaCl concentration of the solution on DGT performance, DGT was used on model solutions with NaCl concentration ranging from 2–50 $\mathrm{g \cdot L^{-1}}$, corresponding to the salt concentration in five-fold diluted fish sauce. The test arrangement was similar to that used in the testing of pH influence on DGT performance, and the R value was again calculated.

Analysis of fish sauce samples

Samples of fish sauce were purchased at Asian markets in Brno, Czech Republic, and in Brussels, Belgium. Fish sauces were diluted with ultrapure water to a volume ratio of 1:4 and stirred for a total of 1 hour in a 2 L glass beaker. Subsequently, four DGT pistons were inserted into the solution for a total time of 24 hours. After the exposition, DGT units were dismantled, and the binding gels were directly analysed using AAS. The DGT concentration was calculated using Equation 1. The concentration of Hg in all undiluted samples was measured directly at the same time in triplicate by AAS. Concentrations were obtained in μg·L^{-1} (from both methods) and recalculated to the average concentrations (μg·L^{-1}) and then recalculated to mass concentrations in mg·kg^{-1} using the average fish sauce density of 1.2 g·mL^{-1} (Lopetcharat et al. 2001). All experimental data were statistically processed using XLstat software (Addinsoft, New York, USA).

Results and discussion

Validation of AAS

The values for the LOD and LOQ of AAS were determined to be 0.068 ng Hg (0.68 μg.L^{-1}) and 0.13 ng (1.3 μg.L^{-1}), respectively. The LOD of new AAS (AMA 254), as stated by the manufacturer, is 0.01 ng Hg. Most studies using this type of instrument for determination of Hg report this LOD (0.01 ng) without further verification (Rozanski et al. 2016, Celechovska et al. 2007, Durrieu et al. 2005). Gao et al. (2010) reported a LOD of 0.03 ng Hg for AAS, however, in our study, such an LOD could not be achieved through repeating the instrument's cleaning program or replacing all of the consumables (seals, boat, boat holders). Thus, the LOD determined in this study is more realistic for a normally loaded device. The LOD of AAS should be sufficient for the analysis of Hg in most fish sauces, however, samples of fish sauces containing Hg concentrations below the LOD of AAS were also analysed in this study (Table 1). Thanks to the possibility of direct analysis of fish sauce samples, the AAS technique used in this study provides a better LOD than inductively coupled plasma mass spectrometry, for which the decomposition of a complex matrix of fish sauce is required (Reichstadter et al. 2020).

Trueness and precision of AAS were verified through analyses of ERM-BB422 reference material. The difference between the determined and declared concentrations in the certified reference material was not significant (determined Hg concentration = 0.589 ± 0.017

mg·kg^{-1}, n = 6, p = 0.3808, α = 0.05). The coefficient of variation calculated from the obtained results was 3%. Based on the measured results, it can be concluded that AAS used in this study meets the performance requirements for analytical methods set by Commission Decision 2002/657/EC (Commission Decision, 2002).

The capability of AAS to provide accurate and reliable results within permissible levels of uncertainty was also tested by participating in an interlaboratory comparison (during which a sample of wastewater with a reference value of 5 μg·L^{-1} was analysed) and by evaluation of the long-term behaviour of AAS. The achieved Z-score in an interlaboratory comparison, Z = 0.15, did not exceed the limit for satisfactory results ($|Z| \leq 2$). To evaluate the long-term behaviour of AAS for Hg determination, control charts of Hg contents were obtained through analysis of ERM-CC580 certified reference material. During all the analyses performed on the ERM-CC580 certified reference material, no out of control signals were recorded, proving good stability of the analytical system. The difference between the determined average concentration (130.8 ± 2.6 mg·kg^{-1}) and the declared concentration (132 ± 3 mg·kg^{-1}) of Hg in the certified reference material was not significant (n = 53, p = 0.0880, α = 0.05).

Validation of DGT

Standard DGT testing procedures include time series accumulation experiments, during which the linear accumulation of the analyte on the DGT binding gel is monitored as a function of time (Zhang and Davison, 1995). In the model solution, the mass of accumulated Hg in the binding gel increased linearly with increasing exposure time (r^2=0.9991, **Figure 1**), while the RSD for DGT deployment was <10 %, confirming proper DGT function. From the time series experiment, the effective Hg diffusion coefficient, D_e, was calculated using Equation 2. The calculated D_e of Hg in the model solution was $6.23 \times 10^{-6} \pm 0.19$ cm^2·s^{-1}. This value is lower than the diffusion coefficient of Hg in water (Docekalova and Divis 2005), however, values of

D_e lower than D_w have already been described in NaCl solutions, owing to formation of $[Hg(Cl)_x]^{2-x}$ complexes (Fernandez-Gomez et al. 2011, Hong et al. 2011).

When DGT is immersed in solution, a region is created close to the exposition window of the DGT piston where the transport of metal ions and complexes undergoes a rapid transition from advective to diffusive control. This region is known as the diffusive boundary layer (DBL). The thickness of the DBL may bias the determined concentrations if it is not considered in the calculations and, therefore, should be verified with each application of DGT (Warnken et al. 2006). The higher the DBL in the solution due to insufficient mixing of the solution, the less analyte that will accumulate in the binding gel in the same period. To estimate DBL thickness in DGT, several different procedures can be used (Garmo et al. 2006). In this study, DBL was estimated using the inverse plot method. The inverse of the Hg mass was linearly correlated with Δg ($r^2=0.9956$, **Figure 2**), which is consistent with the theory of DGT. Under the experimental conditions in this study, the DBL thickness was calculated to be 50 μm. In other studies, DBL measurements in experiments have ranged from 30 to 200 μm (Reichstadter et al. 2020, Warnken et al. 2006, Garmo et al. 2006, Zhang and Davison 1995). In this study the pistons were mounted on a nylon cord (no plastic holder was used) and deployed in a larger volume of solution, which, together with intensive mixing, seems to have led a lower DBL thickness than those published by other authors. Although the determined DBL thickness is small compared to the standard diffusion gel thickness (0.05 cm), this thickness was included in the DGT calculations in the form of extended Δg.

The parameter c_{DGT} can be influenced by changes in the pH of the solution. Changes in pH can affect the speciation of the analyte in solution and, thus, can affect the ability of the analyte to bind to the binding gel (Panther et al. 2012, Luo et al. 2010). The pH of the solution may also change the structure of the functional groups of the resin used in the binding gel (Atzei et al. 2010), thus influencing the performance of DGT (Gimpel et al. 2001, Davison and Zhang, 1995). Speciation of the analyte in solution is also affected by the presence of inorganic or organic ligands in the solution. In the case of Hg, the effect of humic substances and chlorides on the performance of DGT is frequently monitored. Low DGT performance using a binding gel containing Chelex 100 resin to determine Hg content in the presence of chlorides or humic substances has already been described in the literature (Szkandera et al. 2013, Divis et al. 2010). The performance of the DGT in solutions of different compositions is commonly performed by investigation of *R* value. In this study, good agreement between c_{DGT} and c_{SOL} was obtained over the pH range investigated, resulting in *R* values between 93% and 98% (Fig. 3). Because the typical pH value of fish sauce is approximately 5 (Nakano et al. 2017), it can be concluded that DGT is suitable for application in this matrix. Due to the fact that fish sauces contain high levels of salt, DGT performance in solutions containing NaCl concentrations up to 50 $\mathrm{g \cdot L^{-1}}$ was also investigated. As seen in **Figure 3**, high salt content solutions (at levels corresponding 5-fold diluted fish sauce) did not have a major impact on the performance of DGT.

Tests in the model solution were performed in order to compare DGT performance with other studies. However, the tests had to be repeated in a diluted fish sauce solution as this solution has a completely different composition. Plots of the inverse Hg mass versus time gave good correlation coefficients in the diluted fish sauce solution (r^2=0.9992, **Figure 4**). From the measured slope of the linear regression line, the D_e of Hg in diluted fish sauce was calculated to be $3.42\times10^{-6} \pm 0.23$ $\mathrm{cm^2 \cdot s^{-1}}$ (Equation 2). This calculated D_e is lower than D_e values determined during the tests on model solutions (6.23×10^{-6} $\mathrm{cm^2 \cdot s^{-1}}$), a finding which can be explained by the presence of substances other than NaCl diluted in the fish sauce solution, especially proteins and amino acids (Park et al. 2001) that can bind Hg to their structure. To be sure that the D_e of Hg is affected only by a change of Hg species in diluted fish sauce and not by decreases in adsorption efficiency, the uptake efficiency was verified in the model solution. As shown in **Figure 5**, the uptake of Hg from diluted fish sauce by the binding gel is a continuous and fast process, such that the influence of D_e by adsorption processes can be avoided.

The LOD and LOQ of DGT were 0.071 $\mu g \cdot L^{-1}$ and 0.116 $\mu g \cdot L^{-1}$, respectively. These values are comparable to the LODs and LOQs achieved using DGT with other binding gels containing Hg-selective resins and AAS as an instrumental method (Divis et al. 2010), as well as to methods requiring elution of Hg from the binding gel, such as inductively coupled plasma mass spectrometry and liquid chromatography coupled to cold vapour atomic fluorescence spectrometry methods (Reichstadter et al. 2020, Pelcova et al. 2015). Compared to direct analysis of fish sauce on AAS, LOD ten-times lower were achieved when preconcentrating the Hg by DGT.

Since no certified reference material corresponding to the composition of dilute fish sauce was available at the time of this study, trueness was evaluated using a recovery test. The background concentration of Hg in the fish sauce used for the test was 2.5 ± 0.3 $\mu g \cdot L^{-1}$. After dilution and spiking, the final concentration of Hg in the fish sauce was 5.6 ± 0.3 $\mu g \cdot L^{-1}$ (measured directly by AAS). The determined DGT concentration of Hg in the spiked 5-fold diluted fish sauce was 5.3 ± 0.4 $\mu g \cdot L^{-1}$ ($n = 6$), which corresponds to 95% recovery. The precision of DGT expressed as RSD was 7.5 %. These results pass the standards recommended by DGT Research Ltd., indicating good accuracy by DGT utilising binding gel with Purolite S924 resin.

Analysis of fish sauce samples

After all validation testing, DGT was used to extract Hg from 10 different fish sauces (originating in Vietnam and Thailand) and, through AAS analysis of binding gels from DGT, the Hg concentrations of the fish sauce samples were calculated. Mercury concentration was also determined in parallel by direct analysis of fish sauces by AAS. Analytical results are summarized in **Table 1**. Through direct analysis of fish sauce samples using AAS, it was possible to determine the concentration of Hg in eight of the 10 samples of fish sauce. The concentration of Hg in two of the samples was below the LOD of AAS. The content of Hg in the fish sauces ranged from 0.8 to 42.8 $\mu g \cdot kg^{-1}$ and none of the samples examined exceeded the maximum authorized concentration of 500 $\mu g.kg^{-1}$ that is set for fish products (Commission Regulation, 2006). Mercury content in fish sauces is difficult to find in the literature. Mabesa et al. (1985) and Funatsu et al. (2001) reported concentrations of Hg in different types of fish sauces ranging from 1–90 $\mu g \cdot kg^{-1}$, which is consistent with the concentrations found in this work. Differences between Hg concentrations determined by direct analysis of the sample using AAS and by analysis of the sample using AAS after a DGT extraction step were not significant for all samples ($p > 0.05$).

Although it is possible to use AAS for direct analysis of fish sauce samples, the complicated salt matrix of the analysed samples produces a heavy burden on the components of the AAS and may eventually damage the catalyst of the instrument. Corrosion of the metal parts of the device are observable after only a few samples have been analysed. Some of the biggest advantages of the validated DGT are the preconcentration of Hg from a sample of fish sauce, which allows concentrations under the LOD of AAS to be determined, and the ability to separate Hg from the complex matrix of fish sauce, which results in longer service intervals of AAS. Thanks to the preconcentration ability of DGT, the Hg concentration in all 10 fish sauce samples was able to be determined.

Conclusion

In this work, DGT with Purolite S924 binding gel and agarose diffusive gel were successfully used in combination with AAS in the determination of total dissolved Hg in fish sauce. All validation parameters of the investigated method were within acceptable limits, indicating that the method has good accuracy and sensitivity. The results of this study provide a gentle and sensitive method for the determination of total dissolved Hg in complex high salt matrices such as fish sauce.

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Compliance with Ethical Standards

Conflict of Interest Pavel Divis declares that he has no conflict of interest. Marek Reichstadter declares that he has no conflict of interest. Yue Gao declares that she has no conflict of interest. Aneta Habartova declares that she has no conflict of interest. Martine Leermakers declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent not applicable.

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Tables

Table 1 Results from analysis of fish sauce samples

	Sample information	c_{DGT} µg.L^{-1}	c_{AAS} µg.L^{-1}	$c_{m\ av.}$ µg.kg^{-1}
FS1	Vietnam, 95 % anchovy content	5.2±0.4	4.5±0.3	4.1
FS2	Vietnam, 97 % anchovy content	3.0±0.3	2.5±0.3	1.3
FS3	Vietnam, 70 % anchovy content	2.3±0.2	1.9±0.3	1.8
FS4	Thailand, 70 % anchovy content	1.3±0.1	< LOD	1.1
FS5	Thailand, 77 % anchovy content	2.0±0.2	1.6±0.3	1.5
FS6	Thailand, 55 % anchovy content	0.9±0.1	< LOD	0.8
FS7	Thailand, 63 % anchovy content	29.9±2.1	32.9±1.5	26.2
FS8	Thailand, n.a. % anchovy content	5.4±0.5	6.4±0.6	4.9
FS9	Thailand, 70 % anchovy content	33.9±3.1	35.7±2.0	29.0
FS10	Thailand, 70 % anchovy content	50.6±4.6	52.2±3.6	42.8

Figure captions

Figure 1 Measured mass of mercury in the binding gels immersed in Hg model solution over time.

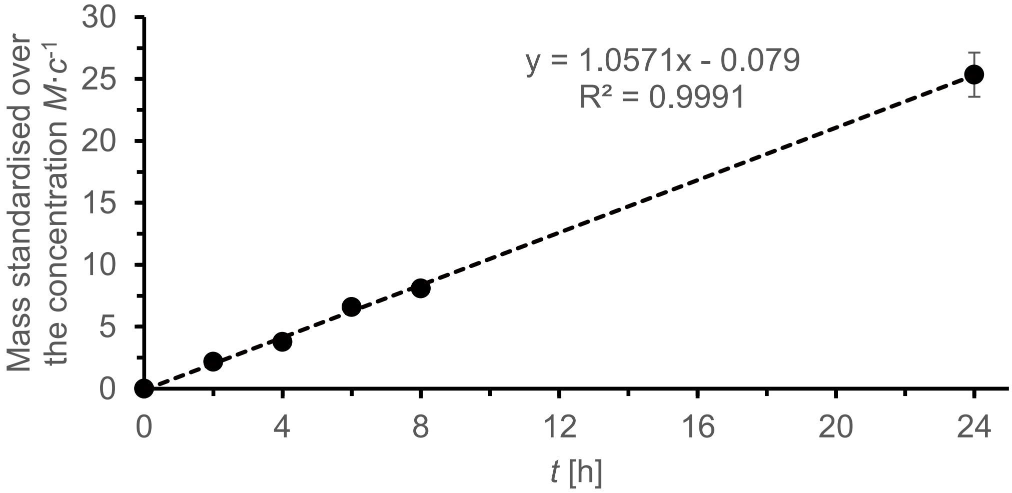


Figure 2 Measured mass of mercury in the binding gels immersed in Hg model solution for agarose gel layers of various thickness.

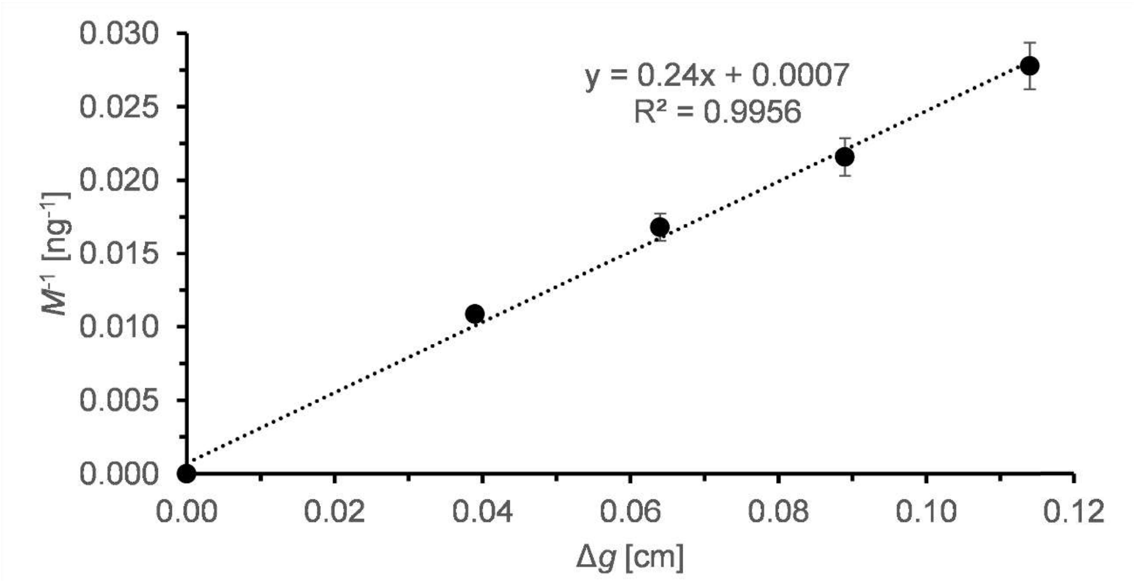


Figure 3 Influence of pH and NaCl concentration on recovery. RSD<10 %.

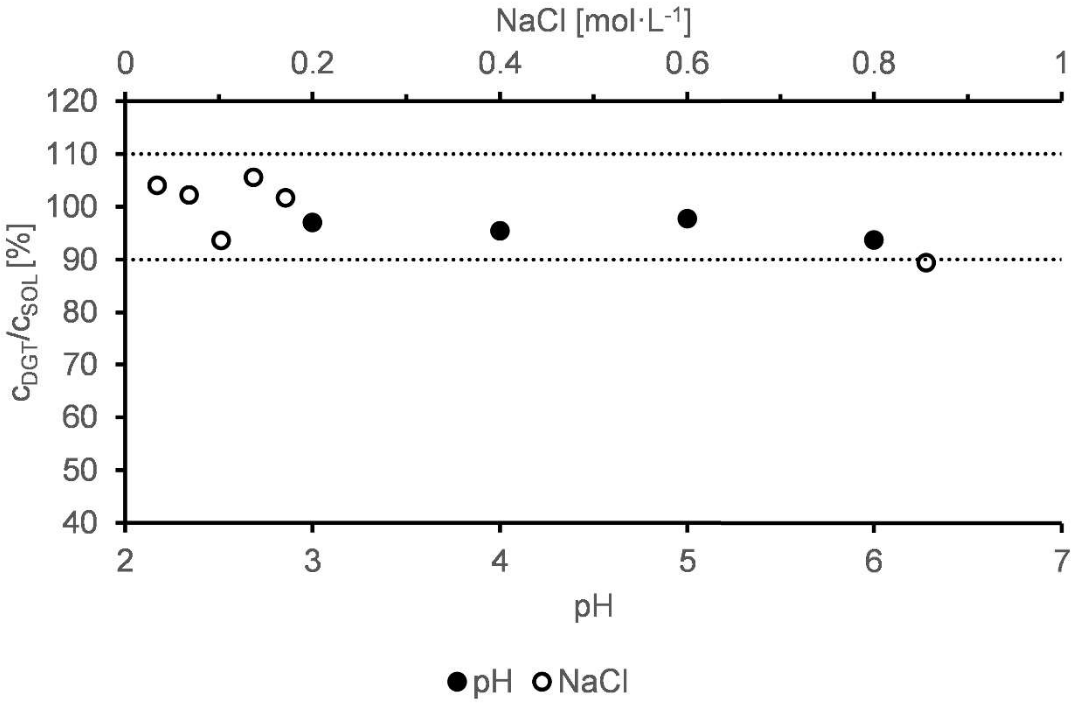


Fig. 4 Measured mass of mercury in the binding gels immersed in diluted fish sauce for various periods.

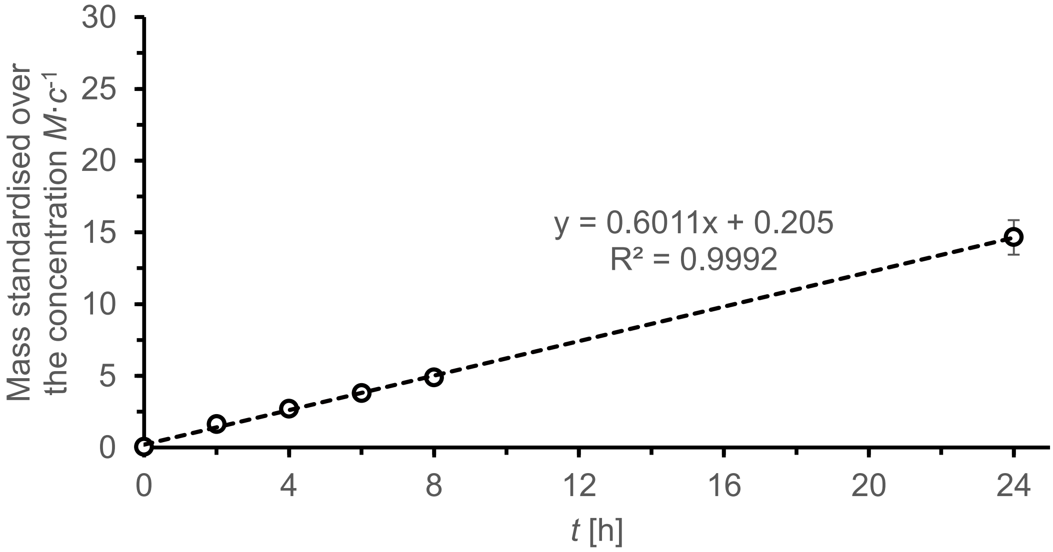
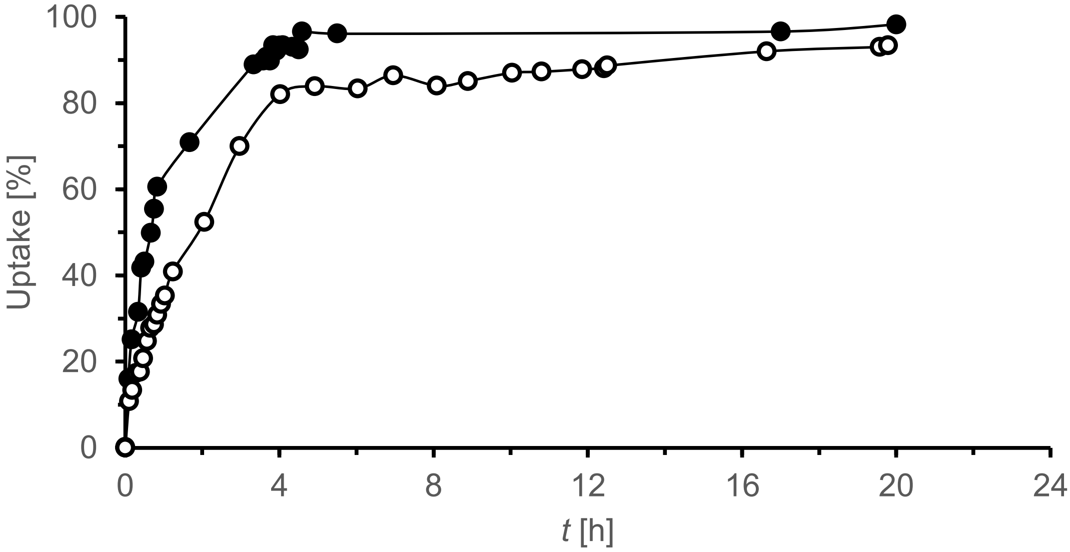


Figure 5 Uptake efficiency of Hg (1 mg·L^{-1}) on binding gel. ● 0.3 M NaCl, ○ diluted fish sauce.



5.2.2 Simultaneous determination of mercury, cadmium and lead in fish sauce using Diffusive Gradients in Thin-films technique

The MWD (microwave oven - Anton Paar Multiwave Go, Graz, Austria - combined with SF-ICP-MS - Element2, Thermo Fisher, Bremen, Germany) was validated by digestion of DORM-4 CRM (fish protein) with no statistically significant differences between the obtained and reference values ($\alpha = 0.05$; Hg: $p = 0.439$, Cd: $p = 0.591$ and Pb: $p = 0.667$). The limits of detection (LOD) and limits of quantification (LOQ) were calculated based on blank runs for the MWD and on resin gel blanks for the S924-DGT and the Chelex-DGT, as the average plus three times and ten times the standard deviation, respectively (using Equation 4 and assuming 24 hours deployment). The S924-DGT provided comparable but slightly lower LOD and LOQ for all metals than the Chelex-DGT. The DGT technique provided ~100 times lower LOD and LOQ than the MWD.

In both 0.03 $\text{mol}\cdot\text{L}^{-1}$ NaCl solution and spiked fish sauce solution, all three metals linearly accumulated on the S924-DGT, which was also the case for Cd and Pb on the Chelex-DGT. Due to Cl-complexation of metals (Morel et al. 1998; Selin 2009; Cullen and Maldonado 2013), the effective diffusion coefficients D_e obtained in 0.03 $\text{mol}\cdot\text{L}^{-1}$ NaCl solution were slightly lower than the literature values for all metals (obtained in $NaNO_3$ solutions) in both agarose diffusive gel used by S924-DGT (Tchounwou et al. 2012; Ali et al. 2019) and polyacrylamide diffusive gel used by Chelex-DGT (Garmo et al. 2003). For both S924-DGT and Chelex-DGT, the D_e obtained in fish sauce solution was lower than the D_e obtained in 0.03 $\text{mol}\cdot\text{L}^{-1}$ NaCl solution, due to complexation of trace metals by the wide spectrum of amino acids present in the fish sauce (Park et al. 2001). The actual D_e are listed and further discussed in the manuscript attached below. The pH in the range of 3.5-8.5 and NaCl content in the range of 0.01-1.0 $\text{mol}\cdot\text{L}^{-1}$ did not affect the performance of the S924-DGT and the Chelex-DGT by more than 10 %, matching the requirements for the correct performance of DGT technique (Zhang and Davison 1995) in the matrix of 5-fold diluted fish sauce (Nakano et al. 2017). The binding of metals to the resin gels was examined by kinetics study, where all analytes were bound fast and quantitatively, except for Pb in the fish sauce solution by Chelex-DGT, where the uptake was only around 50 % within 24 hours due to complexation with organic matter (Farkas and Buglyó 2017). Therefore, Purolite S924 resin is more suitable for the use with the DGT technique for simultaneous measurement of Hg, Cd and Pb in food with a highly complexed matrix and in the marine environment. The capacity of S924 resin gel disc was > 50 µg of Hg, Cd and Pb each. The elution of S924-DGT was tested for *aqua regia* (elution efficiency f_e ~100 %) and 1 $\text{mol}\cdot\text{L}^{-1}$ HNO_3 (f_e ~ 70 %). The elution of Chelex-DGT was standardly performed by 1 $\text{mol}\cdot\text{L}^{-1}$ HNO_3 (Zhang and Davison 1995).

Four fish sauce samples with different anchovy content were analysed by the S924-DGT (for Hg, Cd, Pb) and by the Chelex-DGT (for Cd and Pb) with the ICP-MS detection. The MWD was the reference method. The differences between results obtained by the S924-DGT, the Chelex-DGT and the MWD were not statistically significant for all metals (multi-way ANOVA, $\alpha = 0.05$, $p = 0.193$). The actual content of Hg, Cd and Pb in all samples obtained by all methods is in the manuscript attached below. All samples matched the criteria about the maximal permitted level of contaminants in food (EC Commission Regulation No 1881/2006). By performing a risk assessment calculations from the available data (Whittaker et al. 2011), the consumption of fish sauce matching the EC 1881/2006 criteria could contribute to the tolerable daily intake of Hg, Cd and Pb by 1-2 %, which is negligible to the total occupational and dietary exposure.

5.2.2.1 Conclusions

In this study, the DGT technique using S924 resin gel was developed for simultaneous measurements of Hg, Pb and Cd in fish sauce. The performance of S924-DGT corresponded well with the standard Chelex-DGT for Cd and Pb. The S924-DGT obtained statistically same results as the MWD, routinely used in the analysis of trace metals in foodstuffs. However, the MWD method requires sample pre-treatment and much higher dilution factors. These added steps prolonged the total analysis time and increased the contamination risk of the sample. The DGT technique also provides much lower LOD and LOQ, no sample pre-treatment, preconcentration ability and the ease of sample matrix removal compared to the MWD method. Combining the abilities of preconcentration, matrix effect removal and simultaneous determination of the most hazardous metals in fish and fishery products, S924-DGT is useful to determine hazardous trace metals in fish sauce or other complexed food matrices.

The S924-DGT also allowed simultaneous determination of Hg, Cd and Pb by the DGT technique in general, which offers further wide application in different environments which are more typical for the DGT technique.

5.2.2.2 References

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Simultaneous determination of mercury, cadmium and lead in fish sauce using Diffusive Gradients in Thin-films technique

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ABSTRACT

Fish sauce is a popular seasoning liquid originating from southeastern Asian cuisine, consisting of fermented fish, salt and additional ingredients. Fish can contain high amounts of metals, some of which are hazardous for human health. Therefore, authorities responsible for food safety and quality should monitor the levels of these contaminants in fish and fish deviated products. In this work, the passive sampling technique of Diffusive Gradients in Thin-films (DGT) containing Chelex-100 and Purolite S924 resin gels, is used for the determination of dissolved mercury (Hg), cadmium (Cd) and lead (Pb) in fish sauce. The DGT performance test showed linear accumulation of Hg, Cd and Pb on the binding gels versus deployment time. A wide range of pH and salt concentration did not affect the performance of the DGT. The effective diffusion coefficients of Hg, Cd and Pb in diffusive gels were determined by applying a series of deployments in fish sauce solution. Besides the direct sampling with the DGT technique, fish sauce samples were also digested using a microwave oven. Analyses of DGT and microwave oven digested samples were performed with Sector Field Inductively Coupled Plasma Mass Spectrometry (SF-ICP-MS). Both methods were then used for the analysis of fish sauces from local retail stores. Due to the preconcentration ability of DGT, lower detection limits of Hg, Cd and Pb could be achieved compared to the microwave digestion method. The DGT technique offers a more sensitive method for trace element analysis in complex food matrices.

Credit author statement

Marek Reichstädter: Methodology, Software, writing original draft preparation. Pavel Divis: Visualization, Supervision, Investigation. Ehab Abdulbur-Alfakhoury: Methodology. Yue Gao: Conceptualization, Supervision, Writing- Reviewing and Editing.

1. Introduction

Fishes and fish-related products are generally considered as a part of a healthy diet, containing many beneficial nutrients like high-quality proteins, iodine, vitamins, omega-3 fatty acids and minerals. However, fishes live in an aquatic environment, where exposure to many trace metals is a reality [1–3]. Some of these trace metals are harmful and toxic, even at low concentrations, e.g. Hg, Pb and Cd [4,5]. These toxic trace metals enter the aquatic food chain, where their accumulation and biomagnification occur [6]. As the humans are positioned on the top of this food chain [2,7], frequent consumption of contaminated fish or fishery products can considerably contribute to the total exposure of these toxic trace metals, with a possible negative impact on human health [8,9].

Mercury (Hg), cadmium (Cd) and lead (Pb) are three of the most common metal contaminants in fishery products [10], that can potentially cause harmful effects on humans. These metals can accumulate in human brain, liver and bones due to their long biological half-life [11–13]. Consequently, they can inhibit major metabolic pathways [14] and cause damage to DNA and its repair mechanisms [11,12] since they can bind strongly with sulfhydryl groups in many proteins and enzymes [15,16]. Mutual interaction can magnify the individual toxic effect of Hg, Cd and Pb in mixtures [17]. Therefore, it becomes essential to monitor the level of the toxic trace elements in fish considering food safety and quality. However, fish meat is not the only fish-related product that is consumed, another example of a fishery product is fish sauce [18]. Fish sauce is a liquid seasoning, consisting of fishes mixed with salt and other additives, e.g. sugar, rice or tamarind. Commonly used fishes are different genera of mackerels and anchovies [19]. Although fish sauce has origins in ancient Rome [19], it is not very typical for the European cuisine, but it is an essential part of the eastern and

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southeastern Asian cuisine [20,21]. The consumption in these regions reaches up to 4 kg per capita per year as a national average, considerably contributing to the total consumption of fish-related food products [22]. With the increasing global popularity of these cuisines [23], the global consumption of fish sauce is increasing as well, requiring the same awareness of toxic trace metals as in fish.

The analysis of fish meat is usually a relatively straightforward process consisting of the following steps - sample homogenisation, microwave-assisted digestion, filtration and assessment by analytical instruments, e.g. inductively coupled plasma mass spectrometry (ICP-MS) [10,24]. However, the determination of toxic trace metals in fishery products is more challenging than in fish meat. The classic analytical instruments cannot analyse fish sauce directly due to the low concentrations and high salt content in the samples. Mechanical complications are also common for direct analysis of such complexed organic matrix with high salt and protein contents, e.g. blocking the pneumatic nebuliser or the central quartz torch tube of ICP-MS [25]. Because of the high salt concentration, metallic parts of the instruments are also facing increased damage and corrosion problems [26,27]. The microwave-assisted acid digestion can solve both mechanical and corrosion issues. However, the dilution of sample caused by the microwave digestion can decrease the concentration of analytes close to or below the limits of detection (LOD).

Diffusive Gradients in Thin-films (DGT) is a passive sampling technique with the original application for trace metal sampling in the aquatic system, where it greatly simplified Zn determination in marine waters, thanks to preconcentration and matrix effect removal [28]. However, it is nowadays widely applied to determine pesticides [29], antibiotics [30], endocrine-disturbing compounds [31,32] and drugs [33] in various environments [34–37]. The principles of the DGT technique were already described in detail in many studies [28,38–40]. Briefly, the DGT technique makes use of two hydrogel layers: a polyacrylamide or agarose gel covered with a membrane filter is used as the diffusive layer, which is backed up by a binding layer to accumulate solutes. The binding layer consists of specific resins selective for the analytes of interests and is also referred as the resin gel. In the DGT assembly, a filter membrane, a diffusive gel and a resin gel are inserted into a plastic moulding. During the deployment in aqueous environments, the concentration gradient is established between the bulk solution and the resin gel through the diffusion process in the diffusive layer. The analytes of interest will accumulate continuously on the resin gel as long as the saturation of the resin gel is not achieved. The DGT technique can only assess the metals species that are able to pass through the diffusive gel and get bound by resin gel, i.e. labile species including free ions, and some metal complexes which can dissociate during the diffusive transport [39]. In environmental studies, these species are considered as bioavailable and with the most possible negative impact on health and environment [41,42]. The DGT technique has been successfully applied in many marine systems with a range of salinities [36,39,43], because this technique can potentially avoid the matrix effect of the solution [44].

Since the transport of the analytes occurs through diffusion, the concentration of analytes in the bulk solution can be calculated using Fick's first law:

$$c\mathrm{DGT} = (M \cdot \Delta g) / (D_{\mathrm{e}} \cdot t \cdot A) \quad (1)$$

where Δg is the thickness of the diffusive layer (the diffusive gel and the filter) [cm], D_{e} is diffusion coefficient of the analyte in diffusive gel [$\mathrm{cm}^2 \cdot \mathrm{s}^{-1}$], t is the deployment time [s] and A is the exposure area of the DGT sampler [cm^2] [45]. M [ng] can be either measured directly by proton-induced X-ray emission (PIXE) but this is only possible for major elements [45], thermal decomposition atomic adsorption spectrometry (TD-AAS), e.g. AMA-254 for mercury [34] or laser ablation ICP-MS for trace elements [46]. M can also be measured indirectly after the elution of resin gels by ICP-MS [39].

This work focuses on the simultaneous determination of dissolved Hg, Cd and Pb in fish sauces using the DGT technique containing Purolite S924 resin gels. Purolite S924 is a polystyrene-based resin with thiol functional groups, initially designed for mercury removal in wastewater treatment and hydrometallurgical process water [47]. However, the thiol functional group makes the resin also selective for a few other elements such as Cd and Pb [48]. Following the previous work [49], agarose was used to prepare diffusive gel in the DGT because of its lower adsorption of Hg. Another binding layer containing Chelex-100 resin is routinely used for determination of divalent or trivalent metals in the DGT technique, including Cd and Pb. Chelex-100 contains iminodiacetate functional groups and binds a wide range of metal elements [45,47], however, its lower interaction with Hg has been reported [50,51], especially in solutions with higher salt content [52]. Therefore, it is not possible to determine Pb, Cd and Hg simultaneously with the Chelex-DGT, however the use of Purolite S924 resin could solve that problem. In the classic Chelex-DGT [45], a polyacrylamide diffusive gel and a Chelex-100 resin gel were assembled in the DGT piston, which was used for the determination of Cd and Pb in this study. These two types of DGT samplers were validated in aquatic solutions and fish sauce solutions, and they were subsequently used for the determination of Hg, Cd, and Pb in fish sauce. The measurements of all the fish sauce samples were also performed by combining the microwave digestion method (MWD) with the ICP-MS and the results were compared with the DGT results.

2. Materials and methods

2.1. Chemicals and materials

Acrylamide solution (40%; p. a., Merck), agarose powder (Biorad), ammoniumpersulfate (10%, 0.1 g in 1 g of water; >98%, Merck), cadmium stock solution 1000 mg L^{-1} (AVR Titrinorm, VWR), Chelex-100 (200–400 mesh, Biorad), DGT cross-linker (DGT Research Ltd), DORM-4 (National Research Council Canada, fish protein certified reference material for trace metals), ethanol (99%, VWR), hydrochloric acid (optima grade, Fisher Scientific), lead stock solution 1000 mg L^{-1} (SpectrosoL, BDH), mercury stock solution 1000 mg L^{-1} (Alfa Aesar), MilliQ water (MQ, Millipore, >18 MΩ cm), N,N,N,N-tetraethylenediamine (99%; p. a., Merck), nitric acid (optima grade, Fisher Scientific), nitric acid distilled (certified AR, Fisher Chemical), Purolite® S924 resin (Purolite Ltd), sodium chloride (extra pure, Riedel-de Haën), sodium hydroxide (p.a., Merck), Teflon foil spacers 0.25/0.50/0.75 mm thickness (Bohlender GmbH), Durapore membrane filters (0.45 µm pore size, 125 µm thickness, Merck), DGT plastic pistons (DGT Research Ltd).

2.2. Experimental design and analysis

Laboratory experiments were performed in 0.03 mol L^{-1} NaCl solution consisting of 50 µg L^{-1} of Hg, Cd and Pb, and this solution is referred as “standard solution” hereafter. The pH of the standard solution was adjusted to 6 by nitric acid and sodium hydroxide. For the validation of the methods in fish sauce samples, the five-fold diluted fish sauce sample 1 (one of the four fish sauce samples purchased for the further analysis) was spiked by a mixture of 50 µg L^{-1} of Hg, Cd and Pb, which was further referred as “spiked fish sauce” (42 g L^{-1} of NaCl, pH 6). Before the DGT deployment, these solutions were stirred for 12 h for equilibration.

Preparation, conditioning, storage and assembling of all polyacrylamide (APA) and agarose (AGE) diffusive gels and Chelex-100 resin gels followed the routine procedures as described elsewhere [38,53]. Purolite S924 resin was ground and sieved through 50 µm nylon sieve. The preparation of S924 resin gels in 1.5% agarose followed the procedure described previously [54]. The S924 resin gel, the AGE diffusive gel and the membrane filter were enclosed in the DGT piston which was further referred as "S924-DGT". The Chelex-100 resin gel, the APA diffusive gel and the membrane filter were enclosed in the DGT piston, which was further referred as "Chelex-DGT". The diffusive gels with different thickness 0.25/0.50/0.75 mm were prepared using different thickness of Teflon foil spacers.

1 mol L^{-1} nitric acid was prepared from optima nitric acid for the elution of Chelex-DGT and S924-DGT resin gels. Another 1 mol L^{-1} nitric acid was prepared by diluting distilled nitric acid for cleaning all glassware and plasticware. The *aqua regia* solution was prepared by mixing 1 mL optima nitric acid with 4 mL optima hydrochloric acid. For the microwave-assisted digestion (MWD) method, the distilled nitric acid was used for the digestion of the fish sauce samples.

The Anton Paar Multiwave Go (Germany) was used to digest fish sauce samples applying the EPA3052 method [55,56]. 5 mL of concentrated distilled HNO_3 was added to the sample and the digestion was performed. After the digestion, the MQ water was added to the total volume of 50 mL. Prior to ICP-MS analysis, this solution was diluted 10 times, with the final elution factor of 500. The MWD efficiency was validated by the digestion of the certified reference material DORM-4. The DGT experiments were performed in 1.75 L polypropylene containers (stirred at 1200 rpm by a Teflon stirrer bar) with a lid and a polycarbonate DGT piston rack inside.

Hg, Pb and Cd in all samples were analysed by SF-ICP-MS (Element II, Thermo Fisher Scientific Bremen GmbH, Germany). As an internal standard, ^{115}In was used.

2.3. Quality control

The limits of detection (LOD) of the methodologies were calculated based on the average concentration of ten blanks plus three times the standard deviation. The limits of quantification (LOQ) of the methodologies were calculated based on the average concentration plus ten times the standard deviation. For the MWD method, the limits were calculated based on the concentrations observed during blank digestion runs. For the DGT methods, the limits were calculated based on the Chelex and S924 resin gel blanks.

2.4. Binding gel uptake, capacity and elution

In order to test the uptake kinetics of the resin gels in different matrixes, 1 mg L^{-1} mixture solution of Hg, Cd and Pb was made in 0.03 mol L^{-1} NaCl solution at pH 6, further referred as the "uptake solution". Fish sauce solution (prepared of fish sauce sample 1 with 63% anchovy content) was also spiked with the same concentration of these three elements, further referred as the "uptake fish sauce solution" (NaCl content 42 g L^{-1}, pH 6). All solutions were equilibrated for 12 h in the lab, and afterwards, the Chelex-100 and S924 resin gels were immersed in the solutions and were then mildly shaken for 24 h. Subsamples from the solutions were collected every 5 min for the first hour, and then every hour to monitor the concentration change. Then the subsamples of fish sauce solutions were digested by the MWD and analysed by ICP-MS. The subsamples of Hg, Pb and Cd solutions were further diluted and analysed by ICP-MS. By plotting the concentration changes of the solutions versus time, the uptake kinetics (the uptake as a function of the deployment time) was determined by mass balance calculation, as described by Mason [57].

Whenever a new resin is used in the DGT technique, first a suitable elution reagent is needed and secondly, the elution factor f_e must be calculated using Equation (2):

$$f_e = M_e / (M_0 - M_T) \tag{2}$$

where M_e [ng] is the mass of analyte eluted from the resin gel, M_0 [ng] is the mass of the analyte in the uptake solution or the uptake fish sauce solution at the beginning of experiment [45], and M_T [ng] is the mass of analyte in the same solution at the end of the experiment. For the determination of elution factors, the Chelex-DGT and S924-DGT binding gels were retrieved at the end of the exposure experiment, rinsed by MQ water and first eluted by 1 mL of 1 mol L^{-1} HNO_3 for 24 h [39,45,58]. Another elution method [54] was also applied to elute Hg, Cd and Pb from S924 resin gels using 1 mL *aqua regia* heated at 70 °C for 24 h. All the eluates were diluted ten times before the ICP-MS analysis.

2.5. Measurements of diffusion coefficients in standard and fish sauce solution

The S924-DGT was validated by testing the linear accumulation of Hg, Cd, and Pb versus time in a standard solution. Nine S924-DGT pistons (using AGE) were deployed in the standard solution. At each time interval (2 h, 4 h and 6 h), three pistons were retrieved, and the resin gels were peeled off from the pistons, eluted by 1 mL *aqua regia* heated at 70 °C for 24 h and analysed by ICP-MS. At the same time, 5 mL of the deployment solution were also taken and diluted and analysed by ICP-MS. The same process was followed to assess the linear accumulation of Cd and Pb on Chelex-DGT. Nine Chelex-DGT pistons (using APA) were deployed in the standard solution and were retrieved at three different time intervals. Chelex-100 resin gels were peeled off and eluted by 1 mL 1 mol L^{-1} HNO_3 for 24 h and analysed by ICP-MS.

Similarly, the linear accumulation of Hg, Cd, and Pb on S924-DGT and Cd and Pb on Chelex-DGT was evaluated in the spiked fish sauce, following the previously mentioned steps. Subsamples of spiked fish sauce were collected when the pistons were retrieved. Next, they were analysed by ICP-MS after MWD digestion.

The effective diffusion coefficients D_e of Hg, Cd and Pb were then calculated using the linear accumulation curve. The slope (k) of the linear regressions of the accumulated mass normalised over the solution concentration (M/c) as a function of time was used to determine the effective diffusion coefficients of Cd and Pb in APA and Hg, Cd and Pb in AGE using Equation (3) [45], where D_e is the effective diffusion coefficient ($cm^2 \cdot s^{-1}$), Δg is the thickness of the diffusive layer [cm], A is the exposure are of the DGT piston [cm^2] and t is the DGT deployment time [second]

$$D_e = k \cdot \Delta g / (A \cdot t) \tag{3}$$

2.6. Assessment of diffusive boundary layer

A diffusion-controlled thin layer, known as the diffusive boundary layer (DBL, δ), exists at any solid surface in an aqueous environment, and its thickness depends on the turbulence or flow pattern close to that surface. This layer even exists in vigorously stirred solutions and it can affect the DGT results. The higher the turbulence in the solution, the thinner the DBL and the more analyte will accumulate on the DGT resin in the same period.

DBL can be assessed by using different thickness of diffusive gels in the DGT pistons. The experiment was performed by the deployment of nine DGT pistons, with three different thicknesses of hydrogels in the standard solution for 4 h. After retrieving, the S924 resin gels were processed as described above, and the eluates were analysed by ICP-MS. The reciprocal mass of analytes accumulated on binding gel (M^{-1}) is plotted versus the thickness of diffusion layer (Δg). Using the values of the slope k and intercept q of the linear curve, the thickness of the DBL was calculated using Equation (4) [59]:

$$\delta = k \cdot D_w / (q \cdot D_e) \tag{4}$$

where D_e is the measured effective diffusion coefficient of the analytes in this study [$cm^2 \cdot s^{-1}$] and D_w is the diffusion coefficient of the analytes in water [$cm^2 \cdot s^{-1}$] obtained from literature ($9.13 \cdot 10^{-6}$ cm^2 s^{-1}, $7.19 \cdot 10^{-6}$ cm^2 s^{-1} and $9.45 \cdot 10^{-6}$ cm^2 s^{-1} for Hg, Cd and Pb respectively [35,60]).

2.7. Effect of pH and ionic strength on the DGT performance

The DGT performance was evaluated at different pH and ionic strength (represented as the salt content in case of fish sauce) in this study. The pH value of fish sauces usually ranges between 4.5 and 6.5 and the ionic strength between 2.5 and 4.0 mol L^{-1} of NaCl [19,21]. The Chelex-DGT works in the range of pH 5–9 [61,62], and the ionic strength between 0.001 and 0.7 mol L^{-1} [58], which is matching the conditions of 5 times diluted fish sauce. According to the literature, the resin S924 works in the pH range of 1–11 [47,48]. However, the influence of ionic strength or salt content on the performance of this resin is unknown.

Therefore, the performance of S924-DGT was tested in 50 μg L^{-1} of Hg, Cd and Pb mixture solutions at the pH range of 4.5–8.5 and ionic strength range of 0.03–1.0 mol L^{-1} of NaCl. Subsamples of 5 mL were taken from the deployment solution at the beginning and the end of each experiment and analysed by ICP-MS.

2.8. Analysis of commercial fish sauces

Four fish sauce samples were purchased from local retail stores. All samples originated from Thailand and had various anchovy content (up to 70%), salt content (150–250 g L^{-1}), and fish used (farmed or wild fish), however not all information was provided on the packaging of the samples. The concentrations of Cd, Pb and Hg in these fish sauce samples were determined using two methods, first with the MWD method using 5 mL of distilled HNO_3. After digestion, the sample was diluted to 50 mL with MQ water. Before ICP-MS analysis, this solution was diluted 10 times, with the final elution factor of 500. Secondly, with the DGT techniques, 5 pistons of each Chelex-DGT and S924-DGT were immersed in these four fish sauce solutions (5-fold diluted) for 4 h at 20 °C. After the deployment, Chelex and S924 resin gels were eluted for 24 h by 1 mol L^{-1} HNO_3 and *aqua regia* respectively. Prior to the ICP-MS measurement, the eluates were diluted 10 times and then the sample concentrations were calculated using Equation (1).

3. Results and discussion

3.1. Quality control of the methodology

The certified concentrations of Hg, Cd and Pb in DORM-4 are 0.412 ± 0.036 mg kg^{-1}, 0.299 ± 0.018 mg kg^{-1} and 0.404 ± 0.062 mg kg^{-1} respectively. The concentrations obtained by the MWD method were 0.40 ± 0.03 mg kg^{-1}, 0.29 ± 0.02 mg kg^{-1} and 0.39 ± 0.02 mg kg^{-1} for Hg, Cd and Pb respectively. Based on the t-test results [63], the differences between the determined and the certified values are not statistically significant at $\alpha = 0.05$: $p = 0.439$ (Hg), $p = 0.591$ (Cd) and $p = 0.667$ (Pb). The results of the MWD method show good trueness and accuracy.

The LOD and LOQ of MWD method were calculated from the concentrations obtained from blank runs. The LOD and LOQ of the DGT methods were calculated on of 6 blank resin gels (each DGT type was tested, see results in Table 1). In analytical studies, the instrumental LOD and LOQ can be much lower than methodological ones due to the impurities of reagents, complex analytical procedures etc. [64,65]. The LOD and LOQ obtained from the DGT technique were much lower than the MWD method, which is an advantage of the passive sampling technique compared to routine methodologies. However, the Chelex-DGT method showed higher LOD and LOQ for Cd and Pb than S924-DGT despite the thiol functional groups of S924 resin were more susceptible to contamination during the preparation of the binding gel than Chelex-100.

Table 1
Summary and comparison of calculated methodology LOD and LOQ.

Element	Method	LOD [$\mu g \cdot L^{-1}$]	LOQ [$\mu g \cdot L^{-1}$]
Cd	S924-DGT	0.01	0.01
	Chelex-DGT	0.04	0.06
	MWD	2.11	3.25
Pb	S924-DGT	0.07	0.11
	Chelex-DGT	0.12	0.19
	MW	13.98	21.50
Hg	S924-DGT	0.04	0.06
	MW	5.85	9.00

3.2. Uptake kinetics and elution factors of binding gels

Both types of binding gels showed an uptake efficiency around 100%. The actual concentration of Hg, Cd and Pb in environmental and food matrices is far below our experimental concentrations, but at these high concentrations both resins still show sufficient capacity for the uptake of the elements. Overall, the uptake of Hg, Cd and Pb by S924 resin gels are more efficient in the “uptake solution” than in the “uptake fish sauce solution” since the uptake of these three elements reached around 100% within 4 h in the “uptake solution” (Fig. 1A, B and C), but only 90% within the same time period in the “uptake fish sauce solution”. However, the uptake further increased after 4 h to reach a plateau of 100% within 24 h. When Chelex-100 resin gel was used in these two solutions, the uptake of Cd and Pb was more efficient and faster in the “uptake solution” than in the “uptake fish sauce solution (Fig. 1D), however the uptake of Pb showed a completely different trend than Cd in “uptake fish sauce solution” wherein it only reached around 50% within 24 h (Fig. 1E). The slower uptake by both resins from “uptake fish sauce solution” compared to “uptake solution” was probably caused by metal complexation with ligands present in the matrix of fish sauce. Especially the binding affinity of Chelex-100 resin towards Pb was strongly influenced by high salt contents. Moreover, organic matter (OM) can be found in fish sauce (around 10% proteins and amino acids and around 7% carbohydrates [18,20,21]), which could influence the uptake kinetics of the DGT. The difference between “uptake solution” and “uptake fish sauce solution” was not obvious for Hg, Cd and Pb when S924 resin gel was used. In this test, S924 resin gel showed stronger resistance to high salt contents than Chelex-100 resin gel. This is further discussed in section 3.3. Therefore, Purolite S924 resin is a better choice in the DGT technique for simultaneous measurement of Hg, Cd and Pb in food with a high salt matrix and in the marine environment.

The elution factors (f_e) of the three elements were calculated by using Equation (2). In this study, the f_e was 96 ± 4% for Cd and 93 ± 3% for Pb when 1 mol L^{-1} HNO_3 was used to elute Chelex-100 resin gel. These values correspond well with f_e values published by Panther et al., 2014 [58]. The f_e was 98 ± 2% for Hg, 96 ± 2% for Cd and 97 ± 2% for Pb when the *aqua regia* solution was used to elute the S924 resin gel at 70 °C for 24 h. The f_e values obtained by using 1 mol L^{-1} HNO_3 for the elution of S924 resin gel were much lower (around 70%) than those obtained by using the *aqua regia* elution method, which was finally chosen.

3.3. Effective diffusion coefficients and diffusive boundary layer (DBL)

The mass of Hg, Cd and Pb accumulated on the S924-DGT and Cd and Pb on the Chelex-DGT increased linearly with increasing deployment time both in standard solution (Fig. 2) and in spiked fish sauce (Fig. 3). The effective diffusion coefficients of Hg, Cd and Pb were calculated using Equation (3), the results were summarised in Table 2. The effective diffusion coefficients of Cd and Pb obtained in standard solution were $(4.44 \pm 0.26) \cdot 10^{-6}$ cm^2 s^{-1} for Cd and $(3.67 \pm 0.27) \cdot 10^{-6}$ cm^2 s^{-1} for Pb and agreed well with the previously published ones of $4.74 \cdot 10^{-6}$ cm^2 s^{-1} for Cd and $4.03 \cdot 10^{-6}$ cm^2 s^{-1} for Pb at 20 °C in 0.1 mol L^{-1} $NaNO_3$ solution [45,66]. The effective diffusion coefficient of Hg was $(6.19 \pm 0.36) \cdot 10^{-6}$ cm^2 s^{-1} using the

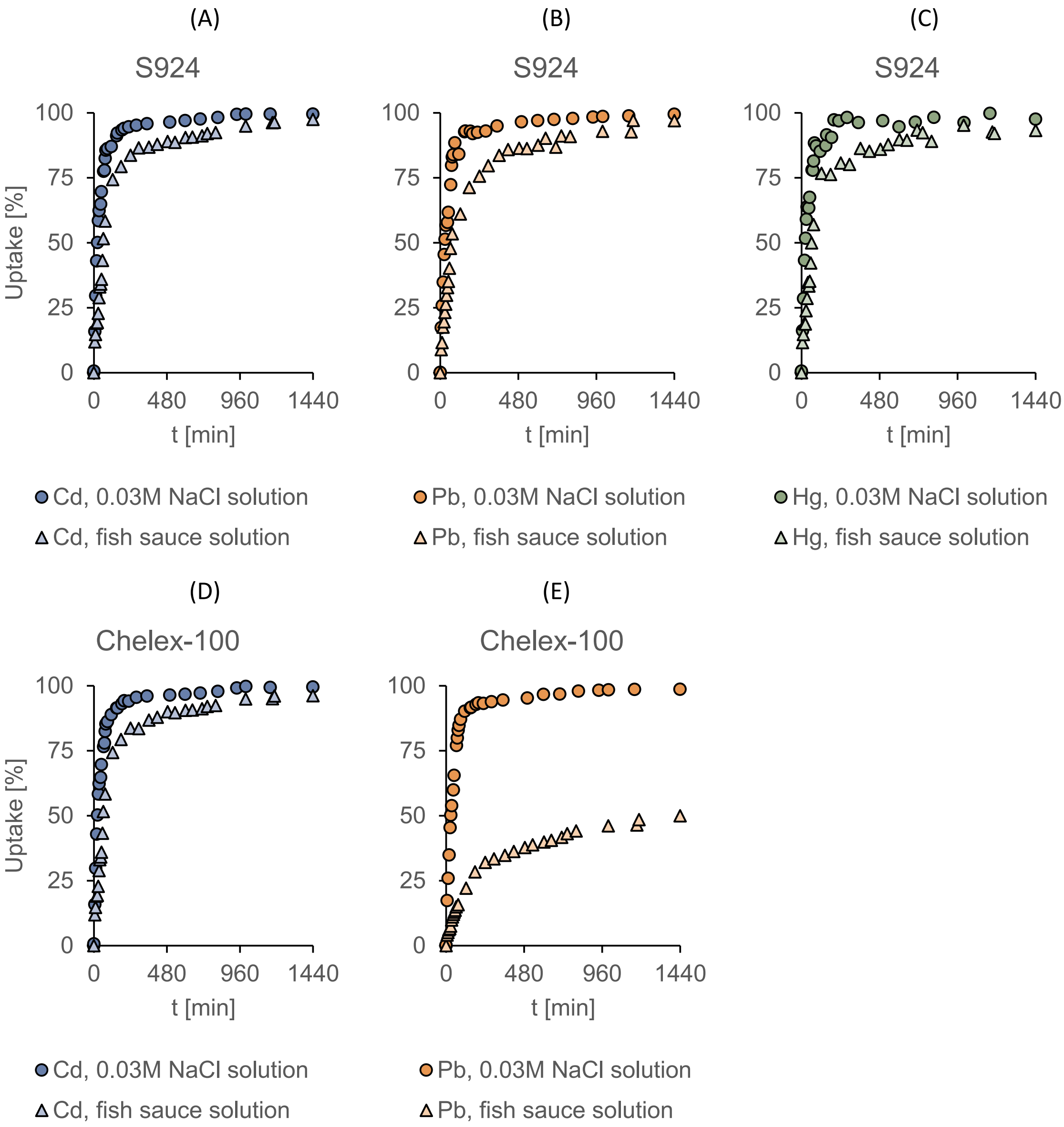

Fig. 1. Uptake efficiency of Cd (A), Pb (B) and Hg (C) by S924 resin gel; and Cd (D) and Pb (E) by Chelex resin gel of in 20 mL of aquatic solution and fish sauce solution, both spiked 1 mg L^{-1} Cd, Pb and Hg mixture, 0.03 M NaCl, pH 6, 20 °C for 24 h deployment.

S924 resin gel and AGE as the diffusive gel, which was lower than the previously published value of $8.44 \cdot 10^{-6}$ cm^2 s^{-1} [35], however, a decrease of the effective diffusion coefficient in NaCl solution was already reported and was due to the strong complexation of mercury with chloride [51].

All three elements were linearly accumulated on S924-DGT, which was also the case for Cd and Pb on Chelex-DGT in fish sauce solution (Fig. 3), however Cd showed stronger accumulation than Hg and Pb on both types of resins. Especially the weaker affinity of Pb for both types of binding gels in fish sauce solution, as illustrated by the lowest slope value of Pb accumulation curve (Fig. 3), is again due to high salt and OM levels [51,67]. The effective diffusion coefficients of Cd are similar in the fish sauce and the standard solution when S924-DGTs are used, but that of Pb is lower in the fish sauce than in the standard solution (Table 2). The effective diffusion coefficient of Hg in the fish sauce was much lower than that obtained in the standard solution, which could be attributed to the effect of dissolved OM according to Hong et al. [51]. The effective diffusion coefficient of Cd in fish sauce agreed well with that obtained in the standard solution, indicating that the Cd flux is much less influenced by the dissolved OM compared to Pb and Hg since Cd is preferably forming labile and mobile complexes with chlorides, which are able to dissociate in the diffusive gel [45].

In fact, both Hg and Pb have a high affinity for many elements such as oxygen, nitrogen and sulphur, which are the functional groups of proteins, peptides and amino acids [15]. The fish sauce contains a wide range of various amino acids at different concentrations [19] that form strong bonds with Hg [16] and Pb [68]. For example, one of many organic compounds in fish sauce is reduced glutathione containing a thiol functional group: the stability constant of Pb thiol complex (single bound) is 9.67 at 25 °C [68]. The stability constant of the Pb iminodiacetic acid (Chelex-100 functional group) complex (single bound) is lower than 7.3 at 25 °C [69], implying Pb to stay bound with the glutathione. Lead can also form multiple bonds with the different functional groups of proteins, peptides and amino acids [68]. All these stable metallo-organic complexes of Pb and Hg will hinder their dissociation during the diffusive transport through the hydrogel and their binding on the resin gel [51,70]. A similar principle applies for the Hg-chlorides complexes [51]. On the other hand, resins with thiol

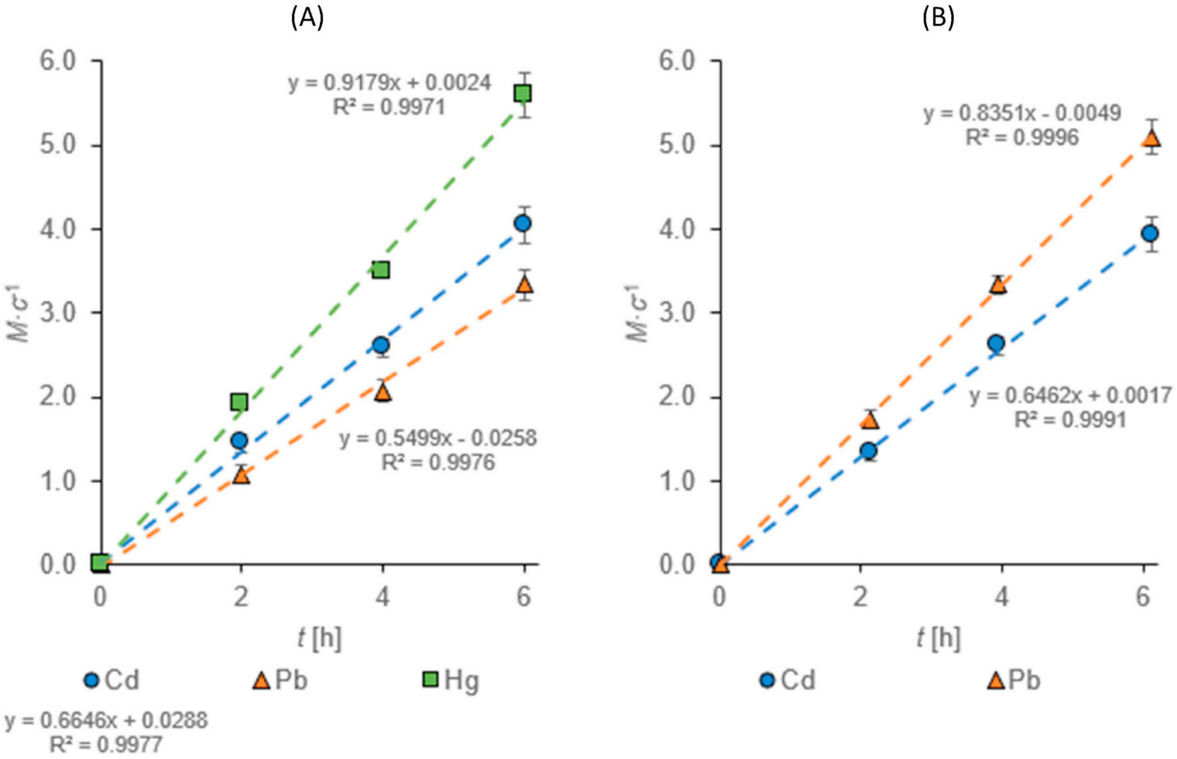

Fig. 2. Linear accumulation of Cd, Pb and Hg on S924-DGT (A) and Chelex-DGT (B) in standard solution.

functional groups (similar to the S924 resin) were reported to form very strong binds with Hg, even though there is strong competitions with other natural ligands, inert organic species and some colloids [37,71], due to a very high affinity of Hg for thiol groups, which is a certain manner also applicable for Cd and Pb [8,72].

In the standard solution, the diffusion coefficients of Cd and Pb measured with the Chelex-DGT were all higher than those using the S924-DGT, which is also the case for Cd in fish sauce solution. The values of diffusion coefficients in different matrixes should theoretically decrease in the order of: water > AGE diffusive gel > APA diffusive

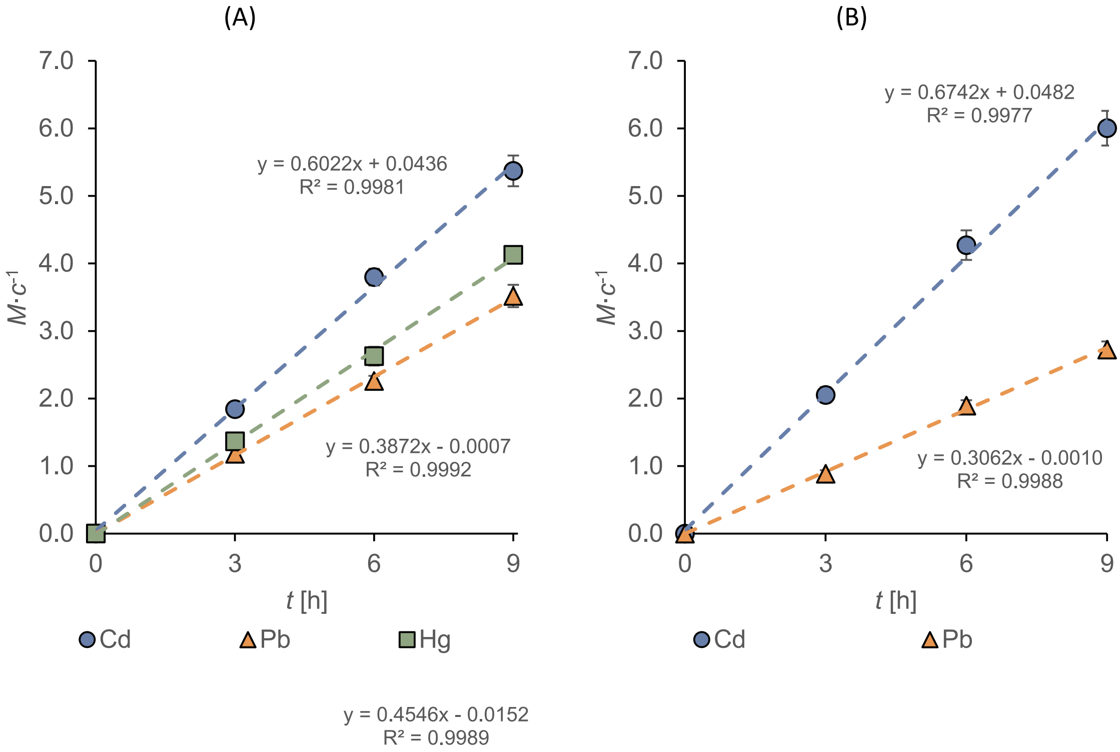

Fig. 3. Linear accumulation of Cd, Pb and Hg on S924-DGT (A) and Chelex-DGT (B) in fish sauce solution (5-fold diluted fish sauce sample 1, spiked to 50 μg L^{-1} of Hg, Cd and Pb with final NaCl concentration of 42 g L^{-1} at pH 6).

Table 2
Summary and comparison of calculated diffusion coefficients (n = 3).

Method name	Diffusive gel material	Element	Diffusion coefficient (× 10^{-6}) [$cm^2 \cdot s^{-1}$]	
			Standard solution	Fish sauce
S924-DGT	AGE	Cd	4.44 ± 0.26	4.05 ± 0.43
		Pb	3.67 ± 0.27	2.61 ± 0.49
		Hg	6.19 ± 0.36	3.52 ± 0.19
Chelex-DGT	APA	Cd	5.33 ± 0.33	4.85 ± 0.24
		Pb	6.94 ± 0.54	2.20 ± 0.11

gel > bisacrylamide-crosslinked polyacrylamide (restrictive) diffusive gel, due to the decreasing pore size [73]. The reported pore size of AGE is around 35–47 nm [73,74] while that of APA is, usually around 2–10 nm [40,53,73,75]. The diffusion coefficients in AGE are well investigated for Hg but not for the other elements. In literature we found following ranges of diffusion coefficients for Cd (~$5 \cdot 10^{-6}$ cm^2 s^{-1} [60] and ~$6 \cdot 10^{-6}$ cm^2 s^{-1} [76]) and Pb (~$4 \cdot 10^{-6}$ cm^2 s^{-1} [60] and ~$8 \cdot 10^{-6}$ cm^2 s^{-1} [76]). In this work, the obtained diffusion coefficients for Cd and Pb were close to the results reported by Fatin-Rouge et al. (2003) [76]. The diffusion of these analytes is not only affected by the pore size, but also limited by the dissociation rates of their complexes in the diffusive domain. Additionally, agarose gel has more small negative charges than APA gels [60,74], which could influence the diffusion process too.

The thickness of the DBL (δ) was measured in a standard solution, which was stirred at 1200 rpm. The obtained DBL was 30 µm under these laboratory conditions.

3.4. Effect of pH and ionic strength on the DGT performance

To quantify the pH and ionic strength effects, the concentration obtained by the DGT technique cDGT was compared to the concentration obtained by direct ICP-MS analysis of the solution cSOL. The ratio of both concentrations (*R*) is given using Equation (5) and is close to 1 if pH and ionic effects are small:

$$R = cDGT/cSOL \quad (5)$$

The performance of S924-DGT was almost not influenced by pH and ionic strength changes (Fig. 4A–C). The *R*-value was in the range of 0.95–1.0 for Cd (Fig. 4A), Pb (Fig. 4B) and Hg (Fig. 4C) for pH changes from 4.5 to 8.5 and ionic strength changes from 0.03 to 1.0 mol L^{-1} NaCl, and this is also the case for Chelex-DGT for both Cd (Fig. 4D) and Pb (Fig. 4E), which is in a good agreement with previously published results [45]. The slight decrease of *R* for Cd at pH < 5 (Fig. 4D) was already known and is due to the lower selectivity of Chelex for Cd at a pH below 5 [61,62]. The decrease of *R* for Pb at pH > 8 (Fig. 4E) was caused by the increasing formation of insoluble $Pb(OH)_2$ at pH 8–10 [77]. The observed variation of *R* did not affect the performance by more than 10%, as it is required for the correct use of the DGT technique [45].

3.5. Fish sauce analysis

After the validation of the S924-DGT and the Chelex-DGT, they were applied for the measurement of Hg, Cd and Pb in 4 commercial fish sauces. DGT results were compared with those obtained by the MWD method (Table 3). Generally, the two DGT techniques produced similar results as the MWD method, but the MWD results showed higher standard deviation, especially for samples with lower concentrations. The MWD method also produced much higher LOD and LOQ than the DGT techniques (Table 1).

Among all fish sauce samples, the second fish sauce sample showed the lowest element concentration, however the origin of the fish is unknown. The third and fourth fish sauce samples with a higher percentage of anchovy contents showed the highest metal concentrations. The highest concentrations of Cd (14 µg L^{-1}) and Hg (52 µg L^{-1}) were found in sample 4 and the highest Pb concentration (47 µg L^{-1}) was observed in sample 3.

When performing multi-way variance analysis on all results using the R software [63], significant differences between the samples ($\alpha = 0.05$, $p < 0.0001$) were observed. However, the differences between concentrations of the metal assessed by different methods (S924-DGT, Chelex-DGT, MWD) were not significant ($p = 0.193$). This confirms that the S924-DGT, the Chelex-DGT and the MWD methods provide similar results, however, the MWD method produced more variance and much higher LODs and LOQs. The DGT technique is generally used to determine the concentration of labile metal species, while the MWD method assesses the content of all metal species. Although the complexation of species (and therefore the lability) is depending on the pH, NaCl and OM content, the concentrations obtained by S924-DGT and by MWD were similar. Thus, with the S924-DGT even stable complexes can be measured, as it was described earlier for Hg and thiol-based resins [37,71].

The European Commission (EC) Regulation No. 1881/2006 sets the maximum permitted level of 0.5 mg kg^{-1} (w.w.) for fish sauce [13]. Fish sauce has an average density of 1.2 kg L^{-1} [19], so the maximum permitted level of Hg for the fish sauce is about 600 µg L^{-1}. The concentration of Hg in all samples (Table 3) was by far lower than the allowed level. Mercury is thus not a concern in the fish sauces we studied. There are no maximum permitted levels of Cd and Pb in fishery products, so it is not possible to compare their concentrations with the current legislation. However, the EC permitted levels of Cd and Pb in fish meat are 0.05–0.25 mg kg^{-1} w. w [11] and 0.3 mg kg^{-1} w. w. respectively [12]. The concentration (c_F) of metals in the fish meat (used to produce fish sauce) can be estimated from the individual metal concentration in fish sauce (c_{FS}), knowing the average density of fish sauce $\rho_{avg} = 1.2$ kg L^{-1} [19] and the average production yield of fish sauce $f_y = 0.31$ (1 kg of fish is required for 310 g of fish sauce) [78]. If the transfer of Cd and Pb from fish to the fish sauce is assumed as 100%, i.e. $f_T = 1.0$, then c_F can be calculated using the equation below:

$$c_F = c_{FS} \cdot f_T \,/\, (\rho_{avg} \cdot f_Y) \quad (6)$$

The estimated concentration of Cd and Pb in fish meat (used to produce the fish sauce) is 0.04 mg kg^{-1} for Cd in sample 4 and 0.1 mg kg^{-1} for Pb in sample 3. These estimated concentrations are still below the maximum allowed concentration for Cd at 0.05 mg kg^{-1} and Pb at 0.3 mg kg^{-1} in anchovy muscle meat in the EC regulation [11–13].

The fish sauces we sampled are matching the EC regulation for Hg content in fishery products. They were probably also matching the EC regulation for Cd and Pb content in fish meat. In this study, the fish sauce samples with higher concentrations of Hg, Cd, and Pb were made of wild fish, as declared by the manufacturers on the package. However, it is not possible to control the conditions and the contamination of the aquatic environment where wild fish grows. For the least contaminated sample, the origin of fish is not available (Table 3), but it is expected that this sample is produced from farmed fish with a more controlled environment. The safety concern for fish sauces is especially related to the use of fish growing in heavily contaminated aquatic systems. Therefore, studies should be enlarged (1) to monitor metal contamination in aquatic system and (2) to trace the transfer route of metals from solution to fish and eventually to fish sauces. Furthermore, the sample size in our study was rather limited.

3.6. Daily intake and risk assessment

Joint FAO/WHO Expert Committee on Food Additives (JECFA) sets the tolerable intake values at 25 µg kg^{-1} body weight (b.w.)/month for Cd [79], and at 4 µg kg^{-1} b. w./week for Hg [80], while the European

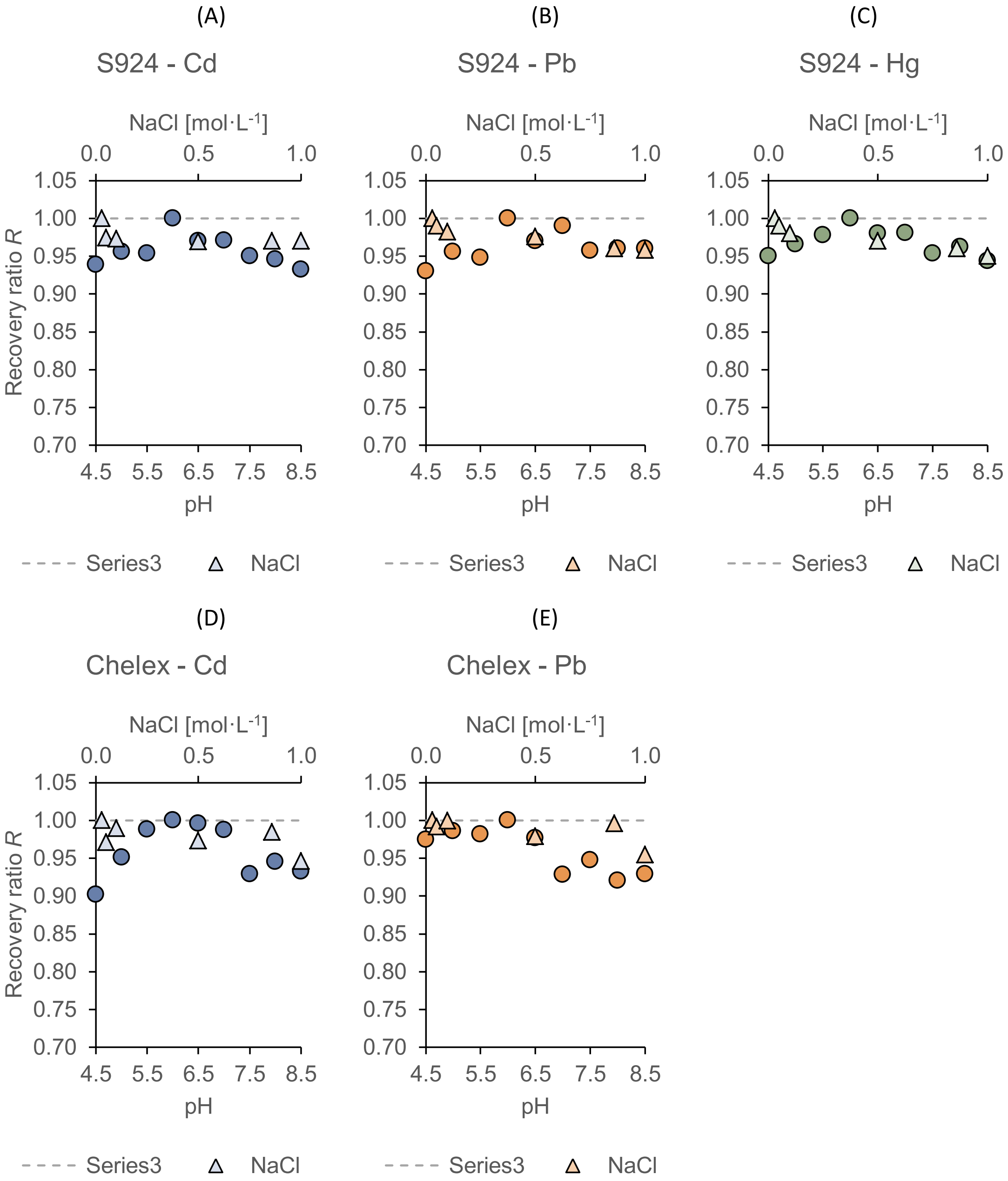

Fig. 4. Effect of pH and NaCl on the performance of S924-DGT for (A) Cd, (B) Pb and (C) Hg; and Chelex-DGT for Cd (D) and Pb (E).

Table 3
Summary and comparison of concentrations [$\mu g \cdot L^{-1}$] obtained by different methods in fish sauce samples (n = 5).

Element	Method	Sample 1 63% anchovy content, wild	Sample 2 N/A anchovy content	Sample 3 70% anchovy content, wild	Sample 4 70% anchovy content, wild
Cd	Chelex-DGT	10.6 ± 0.5	5.6 ± 0.3	11.6 ± 0.6	12.9 ± 0.7
	S924-DGT	11.4 ± 0.9	5.8 ± 0.4	11.4 ± 0.6	14.0 ± 0.7
	MW	11 ± 1	6.0 ± 0.9	11 ± 2	13 ± 1
Pb	Chelex-DGT	26 ± 2	25 ± 1	45 ± 3	39 ± 2
	S924-DGT	27 ± 2	26 ± 1	46 ± 2	40 ± 2
	MW	28 ± 2	25 ± 2	48 ± 5	40 ± 3
Hg	S924-DGT	30 ± 1	5.4 ± 0.4	34 ± 2	51 ± 3
	MW	33 ± 2	7 ± 1	34 ± 3	49 ± 4

Food Safety Authority sets this tolerable intake value at 0.63 μg kg^{-1} b. w./day for Pb [12]. The tolerable daily intakes (TDI) are then 0.83 μg kg^{-1} b. w./day, 0.53 μg kg^{-1} b. w./day, 0.63 μg kg^{-1} b. w./day for Cd, Hg and Pb respectively, or 50 μg Cd/day, 32 μg Hg/day and 38 μg/day, when assuming a body weight of 60 kg [79].

It is difficult to perform an accurate risk assessment, as the global consumption data of fish sauce are not available. The available data for the Asia-Pacific region published by FAO correspond to the consumption of up to 4 kg/year per capita as a national average [22]. In our study, the highest concentrations of 14 μg L^{-1} for Cd, 50 μg L^{-1} for Hg and 46 μg L^{-1} for Pb were found. Consumption of these samples at a rate of 4 kg/year results in average daily intake of 0.13 μg Cd/day, 0.46 μg Hg/day and 0.42 μg Pb/day or 0.3% Cd, 1.4% Hg and 1.1% Pb of the total TDI. The daily exposure is thus by far lower than the TDI, especially when compared to other alimentary sources, e.g. meat, dairy products, cereals and vegetables [11–13]. Moreover, for the majority of the population, the dietary exposure of Cd and Pb is lower than their exposure to air, water, soil and household products [11,12], while for Hg the situation is in the opposite [13]. Finally, our study confirmed that the exposure of Cd, Pb and Hg by normal ingestion of the selected fish sauces is negligible.

4. Conclusions

In this study, the DGT technique using Purolite S924 resin gel was developed for simultaneous measurements of Hg, Pb and Cd in fish sauce. The performance of the novel DGT corresponded well with the classic Chelex- DGT method for the determination of Cd and Pb, but not for the information of Hg. At the same time, Hg, Cd and Pb in 4 commercial fish sauce samples were determined by the S924-DGT, the Chelex-DGT and the MWD method combined with ICP-MS analysis. The results of the S924-DGT and the Chelex-DGT closely correspond with those obtained by the MWD method. However, the MWD method requires sample pre-treatment and much higher dilution factors. These added steps prolonged the total analysis time and increased the contamination risk of the sample. The DGT technique also provides much lower LOD and LOQ, no sample pre-treatment, preconcentration ability and the ease of sample matrix removal compared to the MWD method.

The S924-DGT method is more suitable for simultaneous determination of Hg, Cd and Pb in fish sauces compared to Chelex-DGT, due to its high affinity to Hg and its fast uptake of three analytes from the solution. Combining the abilities of preconcentration, matrix effect removal and simultaneous determination of the most hazardous metals in fish and fishery products, S924-DGT is useful to determine hazardous trace metals in fish sauce or other complexed food matrices.

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.

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5.3 COMPARISON OF CAPS-DGT AND S924-DGT

The blank values of the CAPS-DGT from Chapter 5.1.1 and the S924-DGT from Chapter 5.2.2 for Hg, Cd and Pb are compared in Table 1. The limits of detection (LOD) were calculated as described in Chapter 5.2.2 and are compared in Table 1.

Table 1 Comparison of blanks and LODs for new DGTs.

Trace metal	DGT type	Blank [ng]	LOD [$\mu g \cdot L^{-1}$]
Hg	S924-DGT	0.06	0.004
	CAPS-DGT	0.02	0.002
Cd	S924-DGT	0.06	0.011
	CAPS-DGT	0.03	0.005
Pb	S924-DGT	0.34	0.091
	CAPS-DGT	0.25	0.032

For all metals, the CAPS-DGT had lower blanks and LOD than the S924-DGT. The slightly higher blanks of S924-DGT are credited to contamination during the mechanical pre-treatment of Purolite S924.

6 GENERAL CONCLUSIONS

In this work, two novel resins were tested and successfully applied for the DGT assessment of Hg – the laboratory prepared CAPS and the commercially available Purolite S924. Moreover, both resins performed well also for other trace metals, which allowed the first simultaneous determination of Hg and other trace metals by the DGT technique. The use of CAPS resin in the DGTs allowed simultaneous determination of Hg together with Cd, Pb, Co, Ni and Cu thanks to the combination of several functional groups – thioether bridge, primary and secondary amines and carboxylic groups. The use of commercially available thiol-based Purolite S924 resin in the DGTs was validated and successfully applied for simultaneous determination of Hg, Cd and Pb. The use of novel resins tested in this work provides significant advantages over the routinely used DGT methods, where two separated DGTs are required for assessment of Hg (with 3-mercaptopropyl silica) and the other trace metals (with Chelex-100 resin). The use of unique DGT sampler for both Hg and other trace metals lowers the number of samples produced, consumables used and therefore lowers the overall price of such analysis. The DGTs with CAPS resin allowed determination of a wider range of trace metals, however, the production of CAPS resin is a long, subtle and relatively expensive process. Higher accessibility of the DGT technique using CAPS resin could be achieved by upscaling of the resin production process. On the other hand, the Purolite S924 resin requires mechanical pre-treatment before use in the DGT technique, however, it is available in the identical form worldwide, which makes it a more accessible option. The slightly higher blanks and LOD of the S924-DGT can be improved by more careful mechanical pre-treatment to avoid contamination. Additionally, the Purolite S924 shows promising application potential for also other trace metals and metalloids than Hg, Cd and Pb, which can also further increase its chances for adaptation in routine use of the DGT technique.

The DGT technique was in this work also applied for food analysis – determination of Hg and other trace metals in fish sauce. The DGT technique was validated for use in fish sauce, first for Hg only and later expanded for more trace metals. In both cases, the DGT technique acquired statistically the same results as the routinely used methods of food analysis. However, thanks to the preconcentration of analytes and matrix effect removal of the DGT technique, lower detection limits (LOD) were achieved and the wear and corrosion of the used instruments also lowered. Thanks to the lower LOD of the DGT technique, analysis of fish sauce samples with the concentration below the LOD of the direct determination was possible. During the development part, the preparation of DGT assemblies is as time-consuming as the pre-treatment steps required by routinely used analytical procedures for food analysis. However, that can be sped up by the adoption of the gel preparation procedures. Then, the DGT technique could offer a faster approach to analysing the liquid food matrices, while providing advantages of the matrix effect removal, higher specificity and much LOD than the routinely used methods. The DGT technique could be applied in batch during transport of the liquid foodstuffs in the tanks or potentially in flow systems, similarly to rivers in the environmental application, however, these long-term outlooks would require further deep research and examinations.

7 FUTURE PERSPECTIVES

The DGT technique is routinely used in many different environments, while in this work, only the application in the water column and foodstuffs was evaluated. The CAPS resin can be further validated for the DGT application in aquatic sediments and also soils, as the studied trace metals are also heavily linked with the terrestrial food chain. That would allow easier and more efficient studies of trace metal bioavailability to plants and crops by one unique type of DGT. The commercially available Purolite S924 resin offers ever more future perspectives. The functionality of Purolite S924 in the DGT technique for Hg, Cd and Pb was already proven by this work. However, it has the same functional groups (thiol) as the resins routinely used for the DGT assessment of methylmercury and As^{III}. Some new preliminary results are also showing the promising potential of Purolite S924 for more trace metals than in this work. If Purolite S924 resin would quantitatively accumulate all mentioned analytes without competition effects, it could find numerous application possibilities in all fields of the DGT technique and maybe become a comparable alternative to the most used Chelex-100 resin. That, however, will require an enormous amount of laboratory experiments, validations and a careful choice of a suitable elution and analysis steps.

The successful application of the DGT technique in food analysis opens the potential to further expansion of the application range to other food matrices. One of the possible examples is milk, with complicated water-protein-fat matrix, where the DGT technique could be used for determination of trace element nutrients and contaminants, avoiding the tremendous pre-treatment steps. Milk also offers other possibilities for the DGT application, such as determination of iodine species by specific resins or determination of organic pollutants in milk, e.g. antibiotics.

8 CURRICULUM VITAE

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Education

2019 – present: Joint PhD: Brno University of Technology (BUT), Faculty of Chemistry (FCH), Czech Republic – Vrije Universiteit Brussel (VUB), Analytical, Environmental and Geo- Chemistry (AMGC), Brussels, Belgium

2015 – present: PhD in Food Science, BUT, FCH, Brno, Czech Republic

2010 – 2015: Master’s degree in Food Science and Biotechnology, BUT, FCH, Brno, Czech Republic

2010 – 2013: Bachelor’s degree in Food Science, BUT, FCH, Brno, Czech Republic

Relevant work & traineeship experience:

3/2018 – 5/2019: PhD traineeship, VUB, AMGC, Brussels, Belgium

10/2017 – 12/2017: Laboratory technician, Materials Research Centre, BUT, FCH, Brno, Czech Republic

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9 AUTHOR’S PUBLISHING ACTIVITIES

- **Research Outputs**
  - **Peer-reviewed publication (published)**
    - Reichstädter, M.; Diviš, P.; Abdulbur-Alfakhory, E.; Gao, Y. Simultaneous determination of mercury, cadmium and lead in fish sauce using Diffusive Gradients in Thin-films technique. *Talanta*, 2020, 217. https://doi.org/10.1016/j.talanta.2020.121059
    - Reichstädter, M.; Gao, Y.; Diviš, P.; Ma, T.; Gaulier, C.; Leermakers, M. Cysteine-modified silica resin in DGT samplers for mercury and trace metals assessment. *Chemosphere*, 2021, 263. https://doi.org/10.1016/j.chemosphere.2020.128320
  - **Peer-reviewed publications (under review)**
    - Diviš, P.; Reichstädter, M.; Gao, Y.; Habartová, A.; Leermakers, M. Determination of mercury in fish sauces by thermal decomposition gold amalgamation atomic absorption spectrometry after preconcentration by diffusive gradients in thin films technique. *Food Analytical Methods*.
    - Gaulier C.; Zhou C.; Gao Y.; Guo W.; Reichstädter M.; Ma T.; Baeyens W.; Billon G. Investigation on trace metal speciation and distribution in the Scheldt estuary. *Science of the Total Environment*.
  - **Abstracts**
    - Reichstädter, M.; Diviš, P.; Gao, Y. Simultaneous Determination of Trace Mercury, Cadmium and Lead using Diffusive Gradients in Thin Films Technique in Fish Sauce Food Matrix. ChemCYS 2020 - Book of abstracts. 1. Blankenberge, Belgium: Royal Flemish Chemical Society, Belgium, 2020. p. 41-41.
    - Reichstädter, M.; Diviš, P.; Gao, Y. Simultaneous Determination of Trace Mercury, Cadmium and Lead in Fish Sauce Food Matrix by Diffusive Gradients in Thin Films Technique. Chemistry is Life 2019 – Book of abstracts. Brno: Brno University of Technology, Faculty of Chemistry, 2019. p. 61-61. ISBN: 978-80-214-5807-9.
    - Reichstädter, M.; Diviš, P.; Leermakers, M.; Gao, Y. Determination of potentially hazardous metals in fish sauces using DGT technique. 6th Conference on Diffusive Gradients in Thin Films 2019. 1. Vienna, Austria: University of Natural Resources and Life Sciences, Vienna (BOKU), 2019. p. 33-33.

        - Diviš, P.; Reichstädter, M.; Leermakers, M.; Gao, Y.; Habartová, A. Determination of mercury in fish sauces using diffusive gradients in thin films technique. 2nd Food Chemistry Conference, Seville 17-19.9. : Book of abstracts. 2019. p. 2.2.10 (2.2.10 p.)
        - Diviš, P.; Reichstädter, M.; Habartová, A.; Křikala, J. Validation of diffusive gradient in thin films technique for determination of mercury in fish sauce. 7th Meeting on Chemistry and Life 2018. Book of abstracts. 2018. p. 62-62. ISBN: 978-80-214-5488-0.
        - Diviš, P.; Reichstädter, M.; Habartová, A.; Křikala, J. Determination of Mercury in Fish Sauces using DGT Technique and TD-AAS. Proceedings of the Fourth International Conference on Food Chemistry & Technology (FCT-2018). *Journal of Food Chemistry and Nanotechnology*. 2018. p. S20 ISSN: 2471-4291. https://doi.org/10.17756/jfcn.2018-suppl1
        - Reichstädter, M.; Diviš, P.; Pořízka, J. Development of DGT technique for determination of hazardous metals in food matrixes: Step I - preparation of sorption gel. Chemistry is Life 2017, Book of abstracts. 1. Brno University of Technology, Faculty of Chemistry, Purkyňova 464/118, 612 00 Brno, 2017. s. 71-71. ISBN: 978-80-214-5488- 0
        - Diviš, P.; Reichstädter, M. Development of DGT technique for determination of mercury in various matrix. 10th International Conference on Instrumental Methods of Analysis: Modern Trends and Applications. Heraklion, Greece, 2017. s. 261
        - Reichstädter, M.; Diviš, P.; Pořízka, J. Preparation of new sorption gel discs for determination of mercury in food matrixes by diffusive gradient in thin film technique. 39. International Symposium on Environmental Analytical Chemistry, Book of abstracts. Hamburg, Germany, 2016.

- **Participation to conferences**
    - **Presentation at conferences (oral)**
        - Determination of potentially hazardous metals in fish sauces using DGT technique.
          DGT Conference 2019, BOKU, Vienna, Austria.
        - Simultaneous Determination of Trace Mercury, Cadmium and Lead in Fish Sauce Food Matrix by Diffusive Gradients in Thin Films Technique.
          Chemistry is Life 2019, Brno, Czech Republic.
        - Simultaneous Determination of Trace Mercury, Cadmium and Lead using Diffusive Gradients in Thin Films Technique in Fish Sauce Food Matrix.
          ChemCYS 2020, Blankenberge, Belgium.

  - **Presentation at conferences (poster)**
    - Development of DGT technique for determination of hazardous metals in food matrixes: Step I - preparation of sorption gel.
      Chemistry is Life 2017, Brno, Czech Republic.
    - Preparation of new sorption gel discs for determination of mercury in food matrixes by diffusive gradient in thin film technique.
      International Symposium on Environmental Analytical Chemistry 2016, Hamburg, Germany.

- ## Doctoral training and activities
  - **Domain-specific skills**
    - Belgian continental shelf and Scheldt estuary campaign 2020 on board of the RV Belgica
    - North Sea Campaign 2019 on board of the RV Belgica
    - Confucius Institute activities 2018 - Strategy of Sustainable Development of China: Environmental Aspects workshop
  - **Transferable skills**
    - Slide Clinic: trim the inefficient, emphasize your message (2020)
    - Research presentations and posters with impact: Basics (2019)

- ## Awards and grants
  - Award for the best oral submission
    Chemistry is Life 2019, Brno, Czech Republic
    Section: Organic chemistry, environmental chemistry and biochemistry
    "*Simultaneous Determination of Trace Mercury, Cadmium and Lead in Fish Sauce Food Matrix by Diffusive Gradients in Thin Films Technique*"
  - VUB Doctoral School Travel Grant 2019
    Travel Grant (NSE-TG-2019-62) for ChemCYS2020
    Awarded by the Doctoral School NSE of the VUB