# Mercury

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# CHAPTER 8

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**Abstract:** Mercury (Hg) pollution is an acknowledged major environmental problem. Considering its extreme toxicity, Hg has recently been included in the top ten list of chemicals of major public health concern according to the World Health Organization. Once released into the environment, it is transformed in aquatic ecosystems by microorganisms into the neurotoxic methylmercury. The hazardous effect is then biomagnified through the trophic/food chain. Diet is considered the main exposure pathway of Hg in humans. Therefore, safety values have been established by food safety authorities in order to protect consumers. Seafood, followed by rice, is the primary source of Hg in the human diet. A variety of analytical methodologies is available for the analysis of Hg and its species in food. This chapter presents recent advances in the determination of Hg in foodstuffs. Special attention is given to innovative Hg (species) extraction and preconcentration systems assisted by nanoparticles. Non-chromatographic approaches, as an alternative to classical chromatographic approaches used for speciation are detailed. The potential and limitations of Hg isotopic analysis in food is also discussed.

**Keywords:** certified reference materials, diet, fish, food, GC, HPLC, ICP-MS, isotopic dilution analysis, isotopic fractionation, MC-ICP-MS, methylmercury, mercury, mercury species, non-chromatographic methods, rice, speciation.

## INTRODUCTION

Mercury (Hg) pollution is considered a major environmental and public health concern. Because of its toxicity, Hg has been recently included in the top ten hazardous chemicals by the World Health Organization (WHO). Pregnant women and children in early life are considered the most vulnerable population to Hg harmfulness. Toxic effects can be lethal and include affections of the nervous, digestive and immune systems, and on lungs and kidneys.

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Taking into account the capability of Hg to bind thiols [1], its interaction with essential proteins and enzymes leading to their dysfunction seems to be the origin of such toxicity.

Despite occupational exposure (*i.e.*, miners, dentists) - dental amalgams being undisputable sources of Hg - diet appears as the main exposure pathway of Hg in humans. In general, seafood consumption is recognized as the most common pathway of Hg human exposure. It is especially troubling considering the recent and important increase of Hg in oceanic waters [2]. Anthropogenic activities, such as mining and coal burning are responsible for the increased Hg levels in the atmosphere and in oceanic surfaces [3]. Microorganisms in aquatic ecosystems play a crucial role since they biotransform inorganic Hg (iHg) into methylmercury (MeHg which is present in its free form as $CH_3Hg^+$). The latter exhibits high levels of toxicity and it is easily bioaccumulated through the food chain resulting in serious social and health effects.

Considering Hg toxicity, food safety authorities set the maximal acceptable levels for Hg in food. The established Hg values in foodstuffs depend on their nature. For food supplements, the maximum level is as high as 0.10 mg $kg^{-1}$ in the final product. In the case of fishery products comprising crustaceans and muscle meat of fish (except predatory ones), it is fixed at 0.5 mg $kg^{-1}$ wet weight and for predatory fish species as bonito, eel, marlin, sharks and tuna, among others, it is 1 mg $kg^{-1}$ wet weight (COMMISSION REGULATION (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs). These regulations are effective for fresh and processed fish.

A tolerable weekly intake (TWI) has been set as a safe consumption threshold in order to avoid Hg exposure risks and is regularly reevaluated. The European Food Safety Authority set in 2012 a new MeHg TWI at 1.3 µg $kg^{-1}$ bodyweight, lower than that established by the Joint Food and Agriculture Organization (FAO)/WHO Expert Committee on Food Additives of 1.6 µg $kg^{-1}$ bodyweight. However, due to seafood consumption, a significant part of the global population, mainly from developing countries, is exposed to higher Hg levels than the thresholds established by food safety authorities [3].

Taking into account that diet is the principal source of exposure to Hg, its toxicity strongly related to its speciation, and Hg species are toxic at low concentrations, the analytical chemistry community is continuously seeking for advances in foodstuffs analysis methods. Currently, the main goals of the new and trendy analytical approaches are the development of sensitive, cost-effective and green

methods for the determination of Hg and its species. Isotopic fractionation analysis also appears as a fresh strategy for the identification and discrimination of Hg sources in food products, adding another dimension to total Hg quantification and speciation. In this chapter, current and promising approaches for Hg analysis in food are described.

## FOOD MATRICES WHERE Hg IS OFTEN DETERMINED

According to a recent report of FAO (2016), fisheries and aquaculture are very important sources of food, nutrition, income and livelihood for hundreds of millions of people around the world. Driven by rising domestic income, consumers in emerging economies (where consumption was previously based on locally available products) are experiencing a diversification of the types of available fish through an increase in fishery imports. The significant growth in fish consumption has enhanced people's diets around the world through diversified and nutritious food. Fish consumption represents in many countries the dominant source of proteins. Therefore, the high seafood consumption could lead to significant risks due to MeHg ingestion.

Since seafood is considered a major contributor of Hg through diet, the quantification of Hg and its species in such products grabbed the attention of the analytical chemistry community. As a consequence, most of the Hg speciation studies in foodstuffs correspond to the analysis of fish and other seafood. The new methodologies developed for Hg speciation in fish and seafood are presented all along the text.

Rice is a dominant global crop, recognized to be one of the most important sources of Hg in human diet. Microbial Hg methylation is considered the main source of this organomercurial species in paddy soils. The traditional rice culture practice involves several flooding processes, which lead to anaerobic conditions facilitating iHg methylation by sulfate reducing bacteria [4]. In addition, the use of iodomethane as fumigant, enhances Hg methylation in soil under sunlight, increasing MeHg exposure from rice [5]. After soil uptake by the plant, Hg is transported to the edible part [6-7]. It constitutes a potential risk in Hg polluted areas like Hg-contaminated mining regions, where Hg values reach up to 500 ng $g^{-1}$ [7]. In such regions, the most important MeHg exposure source is not fish, but rice consumption [8]. MeHg intake through rice ingestion has been reflected on the levels of MeHg in hair of inhabitants of such areas [7, 9].

Rice seeds consist of a hull and a nutritious bran coat layer surrounding the endosperm and inner embryo. Brown rice is the result of removing the hull (inedible) and can be consumed in this state. Further processing yields to “white” or “polished” rice. The distribution of Hg species varies according to the fraction of the rain grains. Mostly, iHg is located in hull and bran, while MeHg is found in edible white rice. Rice processing leads to a release of up to 78% of iHg, which is contrasting with MeHg that remains almost untouched [10]. The analysis of commercial rice and baby-food rice from European markets (2 samples of each type) reveals a higher proportion of MeHg in babyfood. It is attributed to the processing practice that includes rice washing and cooking. It is suggested that iHg could be lost during these steps, leading to higher MeHg percentage [11].

Hg speciation in rice is a challenge principally taking into consideration the difficult extraction from the starch matrix of Hg species without provoking their degradation/interconversion and the lack of rice CRMs (Certified Reference Materials) for MeHg. In absence of certified MeHg values in rice matrices, other biological CRMs are used for method validation despite the remarkable matrix mismatching [12-13]. Species specific isotope dilution is definitely an elegant solution for the validation of analytical methods in rice. The addition of the isotopically enriched species allows tracking potential species degradations/transformations and losses during the analytical procedure [11, 14].

Alkaline and acid digestion including several subsequent extractions and back-extractions steps are commonly used [13]. Two fast and sensitive methodologies based on both, acid and basic microwave (MW) extraction of Hg species followed by their derivatization (ethylation) and analysis by gas chromatography (GC) coupled to Atomic Fluorescence Spectrometry (AFS) via pyrolysis, were developed. Basic digestion uses 25% tetramethylammonium hydroxide while 6N $HNO_3$ is chosen for acid treatment. Compared to traditional methods for Hg speciation in this matrix, it implies a noticeable time reduction (it is less time consuming) (from sample preparation to analysis). The sum of Hg(II) (being present in its free form as $Hg^{2+}$) and MeHg in the CRMs was similar for acid and basic digestions. However, it should be taken into account that adequate blanks are only obtained for acid MW digestion, being more suitable for samples with low Hg contents. Limits of detection (LODs) were in the range of ng $g^{-1}$ (0.1-0.7 ng $g^{-1}$) [13].

Considering the economic rises on developing countries, there is an urgent need for developing inexpensive and simple but accurate and sensitive Hg speciation

analysis methods [15]. Inspired by this urgency, a fresh ultrasensitive Hg speciation method by headspace solid-phase microextraction (HS-SPME) using porous carbons (PCs) and GC-Dielectric Barrier Discharge (DBD) Optical Emission Spectrometry (OES) was developed.

The applicability of the method was demonstrated by the analysis of 12 rice samples from both, unpolluted and abandoned Hg mining districts in China. The protocol for Hg species extraction from rice comprised the addition of 10.0 mL of 25% (w/v) potassium hydroxide-methanol to 1 g of grounded samples and the mixture was maintained in a water bath at 65 °C during 4 h. The resulting digest was diluted to 25 mL with methanol. Hg(II), ethylmercury (EtHg, $CH_3CH_2Hg^+$) and MeHg were derivatized with $NaBPH_4$ to their volatile species and preconcentrated by HS-SPME assisted by PCs. In-house prepared PCs SPME presented superior qualities than commercial SPME considering the simultaneous and outstanding extraction efficiency for the three Hg species that was maintained even after 200 extraction cycles. The comparison of Hg species quantification by the new GC-DBD-OES method and conventional high-performance liquid chromatography (HPLC)-inductively coupled plasma mass spectrometry (ICP-MS) showed evidence of a complete matching of the results. The sum of the Hg(II) and MeHg coincided with the total Hg concentration determined by ICP-MS after MW assisted acid digestion. LODs were 0.5, 0.75, and 1.0 μg $kg^{-1}$ for Hg(II), MeHg, and EtHg, respectively, with a recovery between 90 and 105% [15].

DBD-OES benefits from low power consumption, simple set up, high electron temperature and weak background radiation. Considering the small size and compactness of DBD-OES, it can be directly connected to the GC capillary column by a T tube conveniently introducing Ar discharge gas. Direct coupling without using transport tubes prevents loss of Hg and memory effect. This represents a pioneer application of GC-DBD-OES on elemental speciation, used before for volatile carbon containing compounds and halohydrocarbons [15]. Despite the PCs HS-SPME-GC-DBD-OES method was developed and applied on rice samples, its accuracy validation using fish protein (DORM-4) and lobster hepatopancreas (TORT-3) CRMs reveals the potential of this approach on the analyses of complex matrices as seafood and other foodstuffs. The potential coupling of DBD-OES with miniaturized GC should be promising in the field of Hg speciation due to its compactness, and low power and gas consumption [15].

Rice is one of the scarce food matrices where the analysis of Hg is not restricted to the quantification of its most common species, Hg(II) and MeHg. In view of the

public health concern associated to the consumption of this staple product, several studies focus on the understanding of Hg accumulation pathways by different approaches as molecular speciation [10, 14], imaging [10], and isotopic analysis [9, 14].

Hg imaging analyses in food are not common and one of the rare examples corresponds to Hg characterization in rice. Synchrotron radiation microscopic X-ray fluorescence (SR-μXRF) provides the Hg map in rice grains, as well as for other elements as Ca, Cd, Se, Cu, Fe, Mn, Zn and K. The principal Hg location is the surface of brown rice grains, corresponding to the pericarp and aleurone layer. The elements K, Ca, Fe, Mn and Cd are co-localized at the surface of the rice grain. It is attributed to the absorption of atmospheric iHg [10]. Speciation by X-ray absorption near-edge spectroscopy (XANES) is limited to bran samples due to their higher Hg content. Results indicate that Hg(II) and MeHg are both binding cysteine (Cys). Although the structure of the Cys-rich bioligands binding the species are not identified, it has been suggested that Hg(II) could be associated to phytochelatins and MeHg to proteins. These differences on speciation could justify the higher mobility of MeHg-Cys, actively transported to the endosperm during seed ripening [10].

Breast milk is a food matrix particularly interesting that allows the Hg exposure estimation of lactating women and newborns, who constitutes an extremely sensitive population. MeHg is able to cross the mammary gland, being present in the colostrum and breast milk [16]. Hg content in breast milk is strongly linked to maternal exposure. Mothers' diet and their dental amalgams are the main sources of Hg in breast milk [17-19]. The percentage of MeHg varies in a wide range according to the diet, where highest values correspond to fish-consuming populations [19-20].

Most of the analytical methods for Hg quantification in breast milk involved acid digestions and relatively long sample treatments [21-22]. The characterization of Hg content and speciation in large sets of samples (*i.e.*, breast milk banks, epidemiological studies) claims for sensitive, cost-effective and validated analytical approaches. Hg quantification by advanced mercury analyzer demonstrated to be a good alternative to expensive ICP-MS analysis, with a simple sample treatment based on freeze-drying [23].

GC coupled to different detection systems like cold vapor (CV)-AFS [19, 20, 22] and electron capture detection [21] has been proposed for the separation of Hg species in breast milk after their derivatization into volatile forms. ICP-MS has

recently been hyphenated to chromatographic techniques for Hg speciation in breast milk in order to exploit the advantages of isotopic dilution (ID) analysis, which enhance accuracy by the correction of potential losses and/or species transformation during the analytical procedure [23].

MeHg speciation methods in breastmilk based on both, GC-isotopic dilution (ID)-ICP-MS and HPLC-ID-ICP-MS, exhibit similar quantification limits (~1 μg $kg^{-1}$) and accuracy (94-97%). Although GC is more efficient considering the time required for species separation, this method involves a tedious derivatization step. In contrast, sample treatment for HPLC is simpler, limited to a preconcentration by freeze-drying and dissolution of the dried breastmilk on a minimum volume of water [23]. Taking into account the similarity on the analytical characteristics, both approaches can be used for the analysis of this complex matrix. HPLC-ID-ICP-MS was chosen in a recent French epidemiological study of 180 breast milk samples. Hg and MeHg values reach up to 16.9 μg Hg $kg^{-1}$ and 0.43 μg Hg $kg^{-1}$ wet weight, respectively [23].

In addition to quantification of Hg and its species, a metalloproteomic study has also been carried on breast milk. Quantification of Hg in the breast milk proteome, obtained using two-dimensional electrophoresis after protein precipitation with acetone, indicates that Hg is principally associated to proteins with molecular masses in the range from 14 to 26 kDa [24].

Nuts in general are an important source of essential minerals, proteins and unsaturated fat. They are consumed worldwide and often recommended to be included in diet in order to reduce risks of coronary diseases. They are comprised in the list of foodstuffs analyzed in national dietary studies since they became habitually consumed [25]. Hg content in such products can be higher when grown in contaminated regions. The content of Hg is attributed to the soil-plant interaction since nuts are isolated by the shell, therefore excluding the potential Hg atmospheric contribution. The concentration in nut peel is up to 10 times higher than in the enclosed nuts, endorsed to the shield role of peel, preventing the transfer of toxic elements as Hg and Cd to nuts [26]. Acid digestion by using $HNO_3$ or $HNO_3$/HF mixture of nuts samples in a MW system is largely used for the quantification of Hg, as part of a multi-elemental analysis, by ICP-MS [25-26]. The fat fraction in edible nuts varies between 25 to 60% and does not contain Hg [27]. Defatting strategies are often included in the sample treatment protocol. For ICP-MS analysis, a defatting step consisting of vigorously shaking 20 g of grounded

sample with 100 mL of a (2:1, v/v) chloroform-methanol solution, repeated three times, is included before acid digestion on a MW system [28].

Direct mercury analyzer (DMA) appears as an alternative for Hg quantification by a cost-effective method. Despite one of the main advantages of this technique is the non-requirement of sample pretreatment, in the case of nuts, the presence of fat causes interferences during the pyrolysis processes due to the production of high volumes of fumes, conducing to erroneous Hg measurements. Defatting is guaranteed by vigorous shaking during 10 min of 3 g of samples with 15 mL of (2:1, v/v) chloroform-methanol solution. The LOD for the analysis of 100 mg sample by DMA is 0.08 μg $kg^{-1}$ (LOQ: 0.3 μg $kg^{-1}$ and RSD 2-14%) [27].

Hg concentration in diverse types of nuts from different geographical origin is commonly lower than 5 μg $kg^{-1}$ [25-27]. In Brazil nuts, Hg reaches up to 8000 μg $kg^{-1}$. It is principally associated (72%) to the NaOH extractable fraction, mainly binding biomolecules between 0.36 and 14.4 kDa [28].

Honey's benefits as antioxidant and anti-inflammatory raise the interest in its consumption. Honey is worldwide largely consumed due to its exceptional nutritional value. The increase on popularity of such natural product is also accompanied by authenticity and geographical origin concerns. The safety quality of honey depends, among others, on the level of some toxic elements as Hg. Honey, considered a well-known environmental indicator, is able to accumulate metals and metalloids. Hg is often included in a wide list comprising other mineral and trace elements analyzed in honey in order to determine the quality and safety of honey, but also for the assessment of biological and geographical origin by chemometric methods. It is therefore usually analyzed by multi-elemental analytical techniques like ICP-MS after nitric acid MW digestion [25, 29-31]. The DMA alternative avoids any sample treatment and a complete analysis of Hg in five min [32]. According to the Hg values reported in honey, an ordinary consumption of this product does not seem to represent a risk for human health.

## ADVANCES ON THE DETERMINATION OF TOTAL Hg IN FOOD

Hg usually belongs to a long list of toxic elements to be analyzed in food products in order to evaluate the dietary exposure of a population to food contaminants. ICP-MS is therefore the most popular technique chosen by food control laboratories/institutions due to its multi-elemental character and its sensitivity. Hg is commonly quantified, after MW assisted digestion, in a wide type of matrices including cereals, eggs (and eggs products), baby food, drinks, snacks, ice creams,

spices, meat, sweeteners, honey, and confectionery [25, 29, 33]. In spite of the undeniable capacity of this technique for Hg determination in food, the analytical community actively works on the development of innovative approaches dealing with specific situations like the treatment of great numbers of samples, and environmentally friendly and miniaturized solutions, among others. Representative advances on the analysis of Hg in food are presented hereafter.

Advanced or direct mercury analyzers (AMA or DMA) are techniques for Hg quantification in food that can be used for solid and liquid samples. One of the main advantages of direct mercury analyzer compared with the primary techniques used for Hg analysis in food -atomic absorption spectrometry (AAS), AFS, inductively coupled plasma atomic emission spectrometry (ICP-AES) and mass spectrometry (ICP-MS)- is the possibility of analyzing Hg at ultra-trace levels without any sample pre-treatment. Short analysis time, easy use, and cost effectiveness associated are some of the main advantages of the method. DMA is based on the thermal decomposition of the sample followed by Hg amalgamation by gold system subsequent atomic absorption detection. It has successfully been applied to diverse food matrices like honey, nuts, infant food, seafood and animal edible tissues as equine muscle, bovine kidney and swine kidney, and poultry muscle [34].

DMA is the technique chosen by European countries authorities to carry out national diet studies on infant and toddlers, resulting on the analysis of more than 370 infant food samples [35-36]. Accurate dietary exposure assessment claims for the detection and quantification limits as low as possible. The most recent DMA method, developed and validated for (infant) food analysis, exhibits detection and quantification limits of 0.3 μg $kg^{-1}$ fresh weight and 0.6 μg $kg^{-1}$, respectively [35].

Although DMA is principally associated to solid samples, it has been validated for the quantification of Hg in breast milk. The comparison of ICP-MS and DMA in this food matrix shows that quantification limits for both analytical approaches are in the range between 2 and 5 μg Hg $kg^{-1}$, tending to be lower by DMA. Considering the similar sensitivity, Hg quantification by direct analysis represents a better option due to the superior sample throughput. ICP-MS analysis implies a previous digestion of the sample, in this case based on mineralization with aqua regia on a heating block during 3 h. In contrast, DMA does not require any sample treatment. Breast milk samples are exclusively freeze-dried before analysis and Hg is quantified by the standard addition method [23].

Unquestionably, DMA has an enormous potential for Hg analysis in food. Nevertheless, the main limitation of this technique is its exclusive success on total

Hg concentration, not extended to speciation. A few recent examples suggest the possibility of using thermal release for speciation in solid samples [37], not yet widely extended. The recent combination of a thiourea derivative polymer with DMA leads to the screening of Hg(II) and MeHg in food, specifically in fish [38].

Electro thermal vaporization (ETV) appears as a direct sampling technique with a versatile formation and high sample introduction efficiency. It has been coupled to diverse detection systems as AAS, AFS, ICP-OES/MS. The gas phase enrichment method guarantees an optimum interference separation by trapping the analyte. A gold amalgamator is frequently used for selective Hg trapping, separating the element from other pyrolysis gases and concentrating it before thermal induced release into the detector. In a similar way, a tungsten coil is used for the separation of Cd from the matrix interferences [39].

Gold and tungsten coil traps were integrated for the first time into one ETV-AAS system for the quantification of two toxic elements usually simultaneously tracked in foodstuffs. Considering the noticeable differences on vaporization temperature between Hg and Cd, a sequential vaporization of these elements in food matrices was obtained by using an improved on-line ashing furnace. The whole analytical procedure is carried out within 10 min, including sample pre-treatment, with a LOD of 0.7 pg for Hg (and 0.5 pg for Cd). Mushrooms, green tea, corn, milk and spinach are some of the food samples already analyzed using this method. The coupling to miniaturized detection AAS or AFS systems will promote the application of this easy, green and digestion free method for *in situ* monitoring of Hg (and Cd) in food [39].

Electrochemistry represents a fast, simple, and sensitive alternative, suitable for Hg routine analysis. In addition to the low cost, compared with other analytical techniques (ICP-MS, CVAAS, CVAFS), it is portable and appropriate for *in situ* determination. In general terms, Hg is preconcentrated on the electrode followed by stripping, mainly by anodic stripping voltammetry (ASV). Electrochemical techniques for Hg detection are a hot topic. They are characterized by a high pre-concentration capacity, achieving low LODs. Most of the recent advances on this subject correspond to the development of electrode surfaces allowing the preconcentration of lowest amounts of Hg. DNA-based assays and nanostructured electrodes are the most used methodologies in the last years [40].

DNA-based electrodes exploit the strong bound between Hg(II) and thymine (T) DNA bases, allowing the preconcentration of Hg. DNA-based electrodes exhibit extremely low LODs, but they are less stable and more time-consuming than

nanostructured electrodes. In general, the longer analysis time and the stability difficulties of DNA-based assays hamper their application in complex samples [40].

Electrodes modified with lead nanoparticles with thiol-functionalized polysiloxilane [41], nickel nanoparticles with carbon composites [42], among a long list, have been used for Hg determination. The high affinity of gold towards Hg justifies the larger application of gold nanoparticles (AuNPs) on electrode surface for Hg determination [40]. In general, nanostructured electrode surfaces with high electroactive areas and excellent surface properties guarantee a sensitive detection in a short time. Nanoporous materials appear as a prominent approach, characterized by shorter analysis times and relatively simpler procedures. Screen-printed electrodes are expected to be used for the development of future commercial Hg sensors, principally due to their low-cost, disposable and miniaturized properties [40].

Most of the applications of electrochemical detection correspond to Hg analysis in water so far [40, 43]. An ultrasensitive and reusable electrochemical biosensor for the determination of mercury ions (Hg(II)) in water in the range of 10 ng $L^{-1}$ to 1 µg $L^{-1}$ has been developed based on thymine modified gold nanoparticles/reduced graphene oxide nanocomposites [44]. The development of new assays and the analytical improvement of electrochemical methods should open new perspectives for the analysis of more matrices, like biological and food samples. Some examples are already present in the literature, describing successful Hg characterization in urine and blood [40, 43]. Indium-tin oxide surfaces have been used to develop nanoporous gold electrodes for the detection of Hg. They have been primarily used in water but also in milk analysis achieving a detection limit of 0.03 µg $L^{-1}$ [45].

The application of electrochemical Hg detection in food is incipient. A variety of food samples comprising cockles [46-47], seaweed [47], milk [45], breast milk [48] and vegetable origin samples like red chili and tomato [46] has been successfully analyzed. However, the existing studies on foodstuffs focus mainly in fish samples [46, 49-51]. Under optimal conditions, anodic stripping voltammetry gold nanoparticle-modified glassy carbon electrode achieves a detection limit of 0.001 µg $L^{-1}$ with a short deposition time of 2 min in fish. The analytical performance of the electrochemical approach is comparable to conventional DMA analysis [50] Compared to DMA, the main drawback of electrochemical techniques is the need of longer analysis time and a wet sample digestion which implies the generation of waste solution with possible loss of analyte by vaporization [50].

## Sample Treatment for Total Hg Determination Assisted by Solid-Phase Extraction

Effective isolation of the analyte and preconcentration are common sample treatment requirements for trace element analysis. In general terms, solid-phase extraction (SPE) is the technique that conquered the preconcentration of Hg from real samples. Low reagent consumption, simplicity and minimum cost are their main advantages. Assisted by the tremendous progress in nanomaterials, there are noticeable advances on SPE for Hg determination in food.

Dispersed solid-phase extraction represents an alternative to classical SPE. It consists in the direct addition of the solid phase to the agitated sample solution during an optimized time. Therefore, no special equipment is required for sample treatment. The enriched solid phase is mechanically recovered (*i.e.*, centrifugation, filtration) and the analyte is therefore eluted with an adequate solvent for further analysis. This SPE alternative is robust, fast, low cost and simply operated. Matrix solid-phase dispersion (MSPD) is particularly interesting in sample preparation of solid, semisolid and highly viscous samples. The sample is blended with a solid support in a mortar with a pestle. The solid support plays an abrasive role leading to the disruption of sample architecture. Sample components are consequently dispersed on the surface of the support particles. The solid homogeneous mixture is commonly packaged into a cartridge for analyte elution with a suitable solvent [52].

The association of matrix solid phase dispersion assisted by multiwall carbon nanotubes (MWCNTs-MSPD) with single drop solute on electrode glow discharge induced (SD-SEGD) chemical vapor generation constitutes an important advancement on Hg quantification in micro amount samples. The MWCNTs-MSPD consists in blending 1 mg of the solid sample with 0.5 mg of MWCNTs for 5 min using an agate pestle. The homogeneous solid mixture is placed in the MSPD column and Hg species retained are extracted by 100 μL of eluent containing 0.5% L-Cys and 4% HCOOH. Hg species are efficiently converted to $Hg^0$ by SD-SEGD-CVG and further transported to AFS for detection. The accuracy of the method was validated by the analysis of several biological CRMs and it was applied to fish samples [53]. LOD improved 100 fold with this robust SD-SEGD-CVG-AFS method, in comparison to conventional CVG-AFS where the micro amount of sample is significantly diluted by the carrier solution. In addition, memory effect, typically associated to CVG or pneumatic nebulization ICP-MS is not observed. The absence of memory effect is attributed to several factors as: the low sample volume (20μL), the inhibition of adsorption in connecting tubes by the presence of

L-Cys and the minimized transport tube length [53]. The combination of MWCNTs-MSPD and SD-SEGD-CVG-AFS is an absolutely promising approach for Hg quantification in foodstuffs. It could be particularly interesting for Hg analysis in rare and/or luxurious food products such as caviar, saffron and truffles, where minimal amounts of samples are available for analysis.

Magnetic SPE (MSPE) is a new kind of solid-phase extraction based on the combination of a non-magnetic adsorbent with a magnetic material [54]. Magnetic nanoparticles (MNPs) have a high surface area and sorption capacity. Iron, nickel, cobalt and their oxides constitute the core of most of the MNPs. The surface of the MNPs is frequently covered by molecular imprinted polymers, silica or graphene, in order to avoid nanoparticles aggregation [55]. Once the sorption of the analyte is complete on the magnetic sorbent, they are separated by an external magnetic field. Therefore, any additional step of physical separation (*i.e.*, centrifugation, filtration) is required. The analyte is eluted by magnetic separation of the regenerated solvent, resulting in a simple and fast methodology [55]. Considering the multiple advantages of MSPE, there is an unquestionable trend on its application to Hg (and other heavy metal ions). So far, it has been particularly used for water analysis, but several examples of Hg analysis in food show the analytical potential of the method (Table **1**) [56].

$Fe_3O_4$ nanoparticles are excellent supports for SPE due to their magnetic properties, making easy the separation from the solution by using a magnet. They exhibit high thermal and chemical stability and can be modified to develop selective sorbents [57]. However, they tend to form aggregates and are susceptible to oxidation. Surface decoration with specific coating avoids these drawbacks and potentially increases their selectivity even in complex matrices. Several examples show its potential for the analysis of Hg in food [57-60].

Magnetized polymers, including both ion imprinted and non-imprinted polymers have been used to determine Hg(II) in food [57, 61]. Uptake time, sample pH, and nanosorbent amount influence the sorption procedure. Type and concentration of the eluent, volume and time of elution are the main parameters to be considered for elution optimization.

Silica is one of the most ideal shells, since it enhances mechanical and chemical stability in extreme acid/basic media. Polymeric materials have been used to modify the surface of such MNPs used on the extraction and preconcentration of Hg(II). They have been applied to the determination of Hg in complex food matrices [61]. The surface of $Fe_3O_4@SiO_2$ NPs was modified by polythiophene using a self-

assembly of thiophene onto $Fe_3O_4$ NPs. The sorbent, resulting on the modification of $Fe_3O_4$@$SiO_2$ presents a sorption capacity of 59 mg $g^{-1}$. Quantification by CV-AAS of the eluted Hg(II) provides a detection limit of 0.02 ng $mL^{-1}$. The method was validated by the good correlation between the Hg certified and experimentally obtained values in fish CRMs, DOLT 4 and DORM 2. The magnetic nanosorbent was therefore used for the quantification of Hg in shrimp, fish and canned tuna [61].

Ion imprinted polymers (IIPs) appear as an advanced generation of polymeric materials. The presence of specific binding sites from the metal ion/ligand guarantees an exceptional selectivity. IIPs particles are grafted on various substrates in order to increase active surface area and favor the extraction efficiency. IIPs are obtained by several polymerization methods as precipitation, suspension and bulk polymerization [56]. Surface imprinting of MNPs is a modern and promising alternative for Hg analysis that combines a magnetic core with a highly selective surface (IIP) [57].

A novel IIP based on N-(pyridin-2-ylmethyl)ethenamine (V-Pic) was grafted on $Fe_3O_4$ NPs. It was used with success for the quantification of Hg in fish. Hg was extracted from the sample solution (pH adjusted at 8) by 2 min contact with 0.001 g of the sorbent. After isolation of the MNPs containing Hg, by the action of a magnet, Hg ions were eluted with 5 mL solution of 0.1 mol $L^{-1}$ of HCl and 0.1 mol $L^{-1}$ of EDTA. Finally, the eluent was analyzed by ICP-OES. The procedure exhibited excellent analytical performance, with a relative standard deviation (RSD) of 1.5% and a 0.03 ng $mL^{-1}$ detection limit [57].

Metal organic frameworks (MOF) have also been used to modify $Fe_3O_4$ NPs, for the extraction of several toxic metal ions [56]. MOFs have highly porous-crystalline structures based on various coordinative connections among metal ions and organic linkers, resulting on materials of outstanding features such as diverse geometrical configurations, tunable pore size, large surface area, and mechanical/thermal stability. The shapes and sizes of the porous determine their selectivity, which justify the application on SPE of such nanocomposites, relatively quickly prepared [56].

The combination of magnetic SPE and MOF lead to superior high-yield extraction in complex matrices. Magnetic MOFs are easily dispersed and magnetically isolated from the extraction medium. $Fe_3O_4$ NPs have been modified with 4-(5)-imidazoledithiocarboxylic acid and then reacted with trimesic acid and Cu(II) acetate to form the metal-organic framework capable of extracting Hg(II). This material has been employed for the separation and preconcentration of Hg(II) in

fish and canned tuna samples. It has a great sorption capacity (254 mg $g^{-1}$) and is reusable during up to 12 sorption/elution cycles, without decreasing extraction efficiency. Hg(II) is eluted with a 1.1 mol $L^{-1}$ thiourea solution and subsequently analyzed by CV AAS. The detection limit and the relative standard deviation of the method are 10 ng $L^{-1}$ and <8.3%, respectively [59].

Magnetic carbon nanomaterials exhibit exclusive physicochemical properties, exceptional thermal/chemical stability and large sorption capacity [56]. The versatile structure of graphene and graphene-based materials explains the exceptional adsorption of metal ions (*i.e.*, Hg(II)), among others [54, 58]. The most recent example of magnetized carbonaceous materials corresponds to the incorporation of $ZnFe_2O_4$ nanoparticles on graphene sheets. The integration of this mixed oxide, $ZnFe_2O_4$, prevents agglomeration of graphene sheets and improves their stability and adsorption capacity. This material, with a preconcentration factor of 30 and a high reusability (50 cycles) was used for the fast extraction of Hg(II) from water and fish samples. Hg was quantified by CV-AAS exhibiting a detection limit of 10 ng $L^{-1}$ and a precision (RSD) of 2.7% [54].

The application of MSPE *on column* minimizes reagent volume consumption and sample handling in comparison to MSPE in batch. However, there is a limited number of applications so far, using MSPE *on column*. Hg isolation from food of animal and vegetable origin (*i.e.*, fish, cabbage, potatoes, green pepper, etc.) was achieved by an *on column* method with a new biosorbent based on the use of fungi *Coprinus micaceus* and $\gamma$-$Fe_2O_3$. Biomagnetic-sorbents, that match with low cost and green procedures, have been used in MSPE. Bacteria, yeast, fungi, and algae are some of the biomaterials employed. In general terms, structure stability is one of the main limitations of this approach. These materials are not widely used for Hg(II) extraction up to now. The fungal biomass immobilized with $\gamma$-$Fe_2O_3$ NPs with a sorption capacity of 26.2 mg $g^{-1}$was incorporated to a polyethylene column (1x10 cm size). The sample solution, resulting on the digestion of the mentioned foodstuffs, was passed through the MSPE column by using a peristaltic pump. Hg(II) ions eluted with 5 mL of 1 mol $L^{-1}$ HCl and were analyzed by ICP-OES. The method, validated with water and fish muscle CRMs presents excellent LODs [62].

The experience related with the use of on-line SPE for Hg detection is scarce, but based on the examples corresponding to other elements some challenges have been identified. The main limitations are the high backpressure on the SPE column and the feasibility of reconditioning/exchange after degradation [56]. In general, backpressure difficulties are due to decreased column permeability with NPs. Off-

line Hg detection, performed by a large choice of analytical techniques (Table **1**), is commonly used after MSPE. However, the handling could lead to sample contamination and there is also a risk of non-quantitative recovery of the analyte due to losing aliquots of the sorbent. The on-line hyphenation of SPE, is expected to allow automatization and consequently accuracy and precision. The on-line coupling minimizes the use of solvents and the efficient factor is favored.

The immobilization of the magnetic SPE material onto the inner walls of a knotted reactor placed in the injection valve of the flow injection manifold allows the development of an *on-line* detection system [63]. The flow injection magnetic solid-phase microextraction coupled to CV-ETAAS was used for Hg determination in seafood (fish, mussels). The proposed configuration does not present backpressure problems and represents a promising solution in routine analysis. MNPs were coated with silica, which is recognized by its unique features of ordered pore network, high surface area and versatility regarding further functionalization. The resulting silica coated MNPs were modified with 1,5-bis(di-2-pyridyl)methylene thiocarbohydrazide. The Hg(II) adsorption capacity of the material was 5.22 mg $g^{-1}$. This green analytical approach is rapid, selective, easy to use and the analytical performances (LOD: 7.8 ng $L^{-1}$; RSD: 1.7%) are comparable to similar methods for Hg determination [63].

Despite the irrefutable popularity of MSPE for the analysis of Hg(II) in food and environmental samples, its use in Hg speciation is limited to a few applications in food analysis [11, 60, 64, 65].

## RECENT ADVANCES IN SPECIATION ANALYSIS OF MERCURY IN FOOD

Hg speciation in food is principally restricted to the quantification of two species: Hg(II) and MeHg, since food safety authorities exclusively regulate the content of such species. In general terms, the most common analytical methods for Hg speciation consist in the hyphenation of a highly effective separation technique and a sensitive elemental detection (*i.e.*, ICP-MS, ICP-OES, AAS, AFS). The most popular separation techniques are unequivocally GC and HPLC. ICP-MS detection is favored by the possibility of multi-elemental analysis, high sensitivity, wide linear range, and isotopic information.

**Table 1:** Application of magnetized nanomaterials for MSPE of Hg in food.

| Magnetic solid-phase | Analyte | Isolation | Detection | Elution solvent | Contact time (min) | Elution time (min) | pH | Nano-sorbent amount (mg) | Sorption capacity (mg $g^{-1}$) | LOD (ng $L^{-1}$) | RSD (%) | Cycles | Matrix | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **$Fe_3O_4$@$SiO_2$@polythiophene** | Hg(II) | batch | CV-AFS *(off-line)* | 3.0 mL, 0.3 mol $L^{-1}$ thiourea in 0.34 mol $L^{-1}$ HCl solution | 6.8 | 8.4 | 5.2 | 26 | 59 | 20 | < 9.2 | - | Shrimp, fish, canned tuna | [61] |
| **$Fe_3O_4$@IIP based on N-(pyridin-2-ylmethyl)ethenamine (V-Pic)** | Hg(II) | batch | ICP-OES *(off-line)* | 5 mL solution 0.1 mol $L^{-1}$ HCl and 0.1 mol $L^{-1}$ EDTA | 2 | 10 | 8 | 10 | 147 | 30 | 1.47 | | Fish | [57] |
| **GO/$Fe_3O_4$@ polythiophene** | Hg(II) | batch | FI-CV-AAS *(off-line)* | 2.5 mL HCl 1.7 mol $L^{-1}$ | 21 | 2 | 6.5 | 20 | 1 | 25 | <10 | - | Seafood | [58] |
| **MNPs functionalized with 1,5-bis(di-2-pyridyl)methylene thiocarbohydrazide** | Hg(II) | on column | CV-ETAAS (*on-line*) | 0.6 mL $min^{-1}$, thiourea 6%, $NaBH_4$ 0.002%, NaOH 0.5% | 2 | 1.7 | 5 | 50 | 5.22 | 7.8 | 1.7 | At least 550 | Mussel, fish | [63] |
| **graphene/$ZnFe_2O_4$** | Hg(II) | batch | CV-AAS *(off-line)* | 5 mL 2.0 mol $L^{-1}$ HCl | 3 | 3 | 6 | 100 | 4.1 | 10 | 2.7 | 50 | Fish | [54] |
| **$Fe_3O_4$@DTIM-HKUST-1(Cu)** | Hg(II) | batch | CV-AAS (*off-line*) | 3.5 mL 1.1 mol $L^{-1}$ thiourea | 8 | 11 | 6 | 24 | 254 | 10 | <8.3 | 12 | Fish, canned tuna | [59] |
| **bio-MSPE (*C. micaceus* immobilized with γ-$Fe_2O_3$)** | Hg(II) | on column | ICP-OES *(off-line)* | 5 mL 1.0 mol $L^{-1}$ HCl | - | 1.7 | 5 | 100 | 26.2 | 40 | - | 35 | Potato, cabbage, ketchup, green pepper, meat, fish, chicken, milk | [62] |
| **$Fe_3O_4$@$SiO_2$@γ-mercaptopropyltrimethoxysilane** | Hg(II), MeHg, PhHg | batch | HPLC-ICP-MS *(off-line)* | 0.5 mL 0.1 mol $L^{-1}$ $HNO_3$ and 4% (m/v) thiourea | 15 | 2 | 4 | 5 | 29.8 (for Hg(II)), 98.1 (for MeHg) | 0.74 (for Hg(II)), 0.67 (for MeHg), 0.49 (for PhHg) | <10 | | Fish | [60] |
| **poly-*L*-methionine-MWCNTs** | Hg(II), MeHg | on column | HPLC-ICP-MS *(on-line)* | 10% HCl + 20% methanol | - | 10 | 9 | 3 | - | 15 (for Hg(II)), 17 (for MeHg) | <5 | 575 | Fish oil based dietary supplements | [64] |

There is a clear upward trend in analytical chemistry towards the development of green and environmentally friendly methods. Hg analysis in food is also concerned by this purpose. The progress on nanomaterials undeniably assists with the development of miniaturized, sensitive and green approaches for the speciation of Hg in foodstuffs. Several non-chromatographic methods have recently been developed for Hg speciation in food (Fig. **1**).

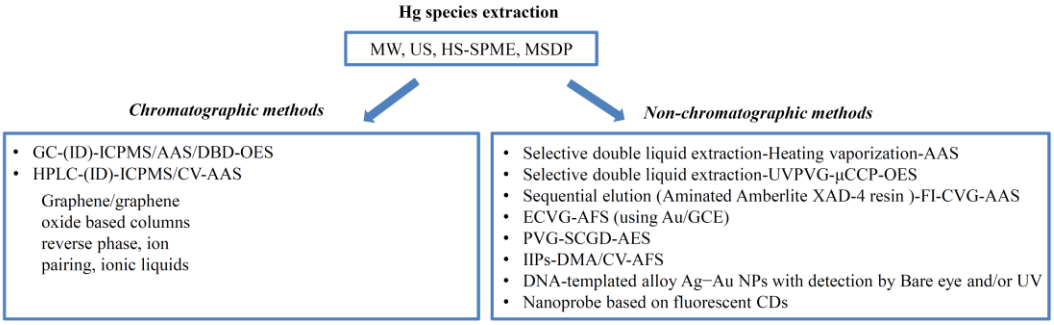


**Figure 1:** Chromatographic and non-chromatographic approaches for Hg speciation in food.

GC is one of the traditional separative techniques used for Hg speciation. The hyphenation of GC-ICP-MS is recognized as a powerful tool for Hg speciation. The detection by ICP-MS allows isotopic dilution analysis enhancing accuracy and precision on Hg species determination [23, 66]. A recent scheme for ultrasensitive Hg speciation (Hg(II), MeHg, and EtHg) analysis in rice proposes the hyphenation of GC to DBD-OES. Compactness and cost-effectiveness are some of the main advantages of this approach thanks to the small size, low power, and low gas consumption of DBD-OES [15].

In general terms, GC is characterized for shorter separation times and higher resolution in comparison to HPLC. Nevertheless, contrary to HPLC separation, GC requires the derivatization of the Hg species present in food to volatilize them. This crucial step of sample preparation processes is long, tedious and strongly influenced by the sample matrix. For these reasons, HPLC is often chosen for Hg speciation in food [23].

## Hg Speciation in Food by Using High Pressure Liquid Chromatography

HPLC is widely used for Hg speciation in food. The main advances in this regard are largely assisted by nanomaterials, from species extraction [53, 64, 67] to interfaces on the detection system [68-70].

### *Chromatographic Separation Assisted by Solid-Phase Extraction*

The key role of SPE on recent methods for Hg analysis in food is not limited to total Hg quantification approaches, but extended to Hg speciation studies. Dispersed solid-phase extraction has a recognized potential on the extraction of elemental species despite being principally applied in the isolation of organic compounds. In comparison to conventional species extraction methods like sonication, heating treatments or MW radiation, MSPD is carried out at room temperature and atmospheric pressure reducing the risks of species instability/degradation. Additionally, this quantitative and fast extraction approach can be considered environmentally friendly due to the low waste generated and the use of reagents with low toxicity. A few but promising analytical methods exploit the advantages of this sample treatment approach for Hg speciation [52, 67].

The first Hg speciation study using MSPD, achieved in 2013, consisted in blending for 3 min 0.2 g of fish sample with 0.5 g of $SiO_2$, acting as solid support. Hg species were quantitatively extracted with 5 mL of 0.5 mol $L^{-1}$ NaCl and 4.2 mol $L^{-1}$ HCl solution with 1 min of stirring time. After derivatization with sodium tetraphenylborate, separation and determination of Hg(II) and MeHg were performed by GC coupled to mass spectrometry (MS). Considering the matrix effect, quantification was carried out by matrix-matched calibration. After method validation with CRMs, Hg speciation was carried out in tuna, shark and guitar fish. The relative standard deviation was lower than 9.5% and LODs for MeHg and Hg(II) were 0.06 and 0.12 μg $g^{-1}$, respectively [52].

The hyphenation of MSPD with HPLC represents an evolution with respect to GC speciation considering the non-requirement of derivatization steps. The coupling of these techniques constitutes the basis for a simple on-line solid sampling platform for Hg speciation in fish. MSPD step was improved by the use of multiwall carbon nanotubes (MWCNTs) considered an excellent solid support for Hg species extraction [53, 64, 67]. This carbonaceous nanomaterial is characterized by a large surface area and outstanding mechanical properties. 4 mg of MWCNTs and 1 mg of fish were blended for MSPD, using a solution containing 2% (v/v) HCl and 1.5% (m/v) L-Cys as eluent. Extracted Hg species were directed to the HPLC-ICP-MS system and separated by reversed-phase liquid chromatography. The LODs were 9.9 ng $g^{-1}$ for Hg(II) and 8.4 ng $g^{-1}$ for MeHg, whereas the LOQs were 21.5 ng $g^{-1}$ for Hg(II) and 18.3 ng $g^{-1}$ for MeHg (RSD <10% for Hg(II) and <12.5% for MeHg) [67]. The *on-line* speciation method assisted by MWCNTs is superior to previous MSPD approaches from several points of view. It involves high sample throughput,

a low sample (1 mg) and solid support (4 mg) consumption without any derivatization of the extracted species minimizing the contamination risk [67].

Functionalization of MWCNTs is a simple process that increases their selectivity. MWCNTs are decorated with poly-L-methionine, a sulfur-based substrate, on a novel *on-line* SPE-HPLC-ICP-MS system for Hg(II) and MeHg preconcentration from fish muscle and fish oil-based dietary supplements [64]. The preconcentration microcolumn contained a mixture of the functionalized MWCNTs with inert particles of a low density polyethylene wax (Epolene) (1:10) to avoid aggregation and back pressure. The stable sorbent has a life time of at least 575 cycles with an enrichment factor up to 190. Hg(II) and MeHg eluted from the preconcentration microcolumn were separated by HPLC on a C18 column in approximately 10 min using a ternary mixture of 0.5% formic acid, 0.2% 2-mercaptoethanol and 20% methanol as mobile phase [64]. This approach is very attractive considering the LODs (15-17 ng $L^{-1}$) and the high sample throughput (5 samples $h^{-1}$), using minimum amount of substrate without derivatization of the mercurial species [64].

MNPs are suitable adsorbents for micro MSPE of different Hg species depending on the reagent used for functionalization [65]. Magnetic core-modified silver nanoparticles ($Fe_3O_4$@Ag) functionalized with the sodium salt of 2-mercaptoethane-sulphonate adsorb Hg(II) selectively. However, a functionalization of the NPs with L-Cys ($Fe_3O_4$@Ag@Cys) leads to the retention of the inorganic species, but also monomethyl mercury, dimethyl mercury, ethyl mercury, phenyl mercury and diphenyl mercury. Once eluted with KI, total Hg was measured by ETAAS using silver nitrate and potassium permanganate solutions for chemical modification. Organomercurial species are desorbed in two fractions (MeHg + dimethylmercury ($Me_2Hg$, $(CH_3)_2Hg$) + EtHg and phenylmercury (PhHg, $C_6H_5Hg^+$) + diphenylmercury ($Ph_2Hg$, $(C_6H_5)_2Hg$)). Therefore, this method cannot be considered a complete Hg speciation tool, but an easy screening of the organic and inorganic fractions. It was applied to the analysis of edible oils and the low amount of sorbent required (3 mg) facilitated the miniaturization of the procedure. Inorganic and organic Hg levels in vegetal edible oils are lower than the LOD (0.01 µg $L^{-1}$), contrasting with fish oils where the concentration ranged between 0.08 and 0.45 µg $kg^{-1}$ [65].

An advanced method, by using the same magnetic-core NPs, enabled precise quantification of organomercurial species. $Fe_3O_4$@$SiO_2$@γ-mercaptopropyltrimethoxysilane MNPs were used for Hg speciation in water and fish samples. After ultrasound-based extraction of Hg species from fish muscle, the

MNPs efficiently retained Hg(II), MeHg and PhHg. A solution of 0.1 mol $L^{-1}$ $HNO_3$ and 4% (m/v) thiourea was used for analytes desorption. After magnet isolation of the sorbent, Hg species were quantified by HPLC-ICP-MS. The proposed method is characterized by outstanding preconcentration factors (around 175 enhancement factor), fast separation (8 min) and high sensitivity (LOD: 0.49-0.74 ng $L^{-1}$) [60].

A robust, cost-effective, and sensitive method was developed for MeHg speciation in rice and rice products without derivatization. It consists of a simple sample preparation followed by on-line SPE preconcentration coupled to HPLC-CV-AFS [11]. Rice and baby-food rice samples (0.3 g) were digested with 3 mL THAMH (25% (m/v)) for 20 min at 55 °C and 20 min at 60 °C and 1600 W in open vessels. After addition of 2 mL HCl, the mixture was heated again in the MW for 20 min at 55 °C and 20 min at 60 °C. Approximately 60% of the filtered supernatant was diluted up to 50 mL, of which 35 mL were loaded on the SPE column (2.1 x 3 mm) at a speed of 5 mL $min^{-1}$. A commercial thiol/thiourea silica material was used as sorbent for SPE. Hg species were eluted with 1.5 mmol $L^{-1}$ ammonium pyrrolidine dithiocarbamate in 75% (v/v) methanol and separated *on-line* by a C8 HPLC column. Post column addition of bromine as oxidant and UV irradiation assured oxidation of MeHg to Hg(II). Conventional reduction with acidic tin (II) chloride was used to convert Hg(II) to $Hg^0$. The sample throughput of the SPE preconcentration coupled to HPLC-CV-AFS method is four per hour [11].

Contrary to fish Hg (speciation) analysis that benefits from a wide range of CRMs for method validation, there are no CRMs for MeHg in rice. Therefore, an elegant solution was cross-validation against standard addition and species specific isotope dilution (SSID)-GC-ICP-MS, which showed no significant differences versus the external calibration with SPE-HPLC-CV-AFS [11, 12]. Isotopically enriched $^{199}$Hg(II) spike was added to the sample in order to track abiotic MeHg formation in this organic sample. The isotopic standard was enriched in isotope 199 at 98% and was spiked in excess (20-folds) in order to clearly see any alteration on the natural Hg isotopic ratio, attributed to abiotic MeHg formation. The natural isotopic ratio of MeHg remained unalterable, which guaranteed the absence of artificial MeHg formation during the whole analytical procedure- from digestion until determination. Therefore, the MeHg found in rice corresponded to the occurrence of this organomercurial species in food products [11].

The LOD for MeHg by this simple and reliable approach is 0.4 ng $L^{-1}$. In the analyzed rice grains and baby-food rice samples from local European shops, the

concentration varied between 1.56 and 2.69 µg $kg^{-1}$ with a RSD ranging between 5.2 and 16.7%. It is a cost effective method for Hg speciation based on the detection by CV-AFS in comparison with GC/HPLC-ICP-MS approaches. Therefore, it is attractive for routine food control laboratories [11].

### *Trends in the Choice of Stationary and Mobile Phases in HPLC for Hg Analysis*

HPLC is largely used for Hg speciation analysis, mainly using reversed-phase (RP) chromatographic mode (Table **2**). Mobile phases in RP may contain high salt levels and considerable amount of organic solvents. It has negative consequences on the operations of some detectors, such as nebulizer obstruction and interface deposition of carbon and salt in ICP-MS. Species separation by HPLC implies long separation times reducing analytical efficiency and throughput [71]. The large volumes of mobile phase waste, rich on toxic organic compounds, place this technique at a great distance of green chemistry. Hg speciation by ion-pairing-RP-ICP-MS using aqueous mobile phases containing minimal quantities of salts, promises to solve the previously mentioned problems.

Two advanced ion-pairing HPLC methods were developed based on positively and negatively charged ion-pairing reagents (tetrabutylammonium hydroxide –TBAH and sodium dodecylbenzene sulfonate –SDBS), where sodium 3-mercapto-1-propylsulfonate (MPS) and L-Cys were included respectively in the mobile phases to transform mercury species into negative and positive Hg-complexes for good resolution. The addition of phenylalanine (Phe) enhanced the analytical efficiency and reduced waste production through reducing the baseline separation time in combination with short C18 guard columns (5 µm, 12.5 mm × 2.1 mm i.d.) [71]. Sample preparation consisted of ultrasonication (15 min at 40 °C) of 0.5 g of fish with two successive additions of 10 mL of a solution containing 5 mol $L^{-1}$ HCl and 2.0 mmol $L^{-1}$ Cys. After centrifugation and 0.45 µm filtration, 5 µL of the extract were injected. The analysis of five freshwater fish samples evidenced that this green and efficient method could be exploited for (food) routine analysis laboratories [71]. Hg(II), MeHg, EtHg and PhHg were baseline separated by two consecutive C18 guard columns with optimized mobile phases (2.0 mmol $L^{-1}$ SDBS + 2.0 mmol $L^{-1}$ Cys + 1.0 mmol $L^{-1}$ Phe (pH 3.0) and 4.0 mmol $L^{-1}$ TBAH + 2.0 mmol $L^{-1}$ MPS + 2.0 mmol $L^{-1}$ Phe (pH 6.0)). The LODs were 0.015, 0.014, 0.028 and 0.042 µg $L^{-1}$ for Hg(II), MeHg, EtHg and PhHg, respectively. The analysis of fish tissues CRMs demonstrated a good recovery of Hg species (91-106%) and the accuracy of the method [71].

Rice consumption is one of the most important sources of exposure to arsenic (As). Therefore, speciation methods enabling the simultaneous speciation of As and Hg in this food product are particularly interesting. Ion-pairing reversed-phase liquid chromatography coupled to ICP-MS was used for the simultaneous speciation of four As species as well as Hg(II), EtHg and MeHg. Taking into account the compromise of best extraction for Hg (60% of total Hg) and As species, 1% $HNO_3$ MW assisted extraction was chosen. The species were separated by ion pairing-RP liquid chromatography within 20 min (including 10 min of re-equilibration) by using tetrabutylammonium hydroxide (TBAH) as ion-pairing reagent [72]. Hg species were quantified by external calibration. The LODs of the simultaneous As/Hg speciation method were 0.04, 0.01 and 0.03 µg $L^{-1}$ for Hg(II), EtHg, and MeHg, respectively [72]. The main advantage of this approach is the simultaneous speciation of three Hg and four As species in a single extraction and chromatographic run without any requirement of organic solvents in the mobile phase.

Ionic liquids (ILs) have demonstrated to be a fresh alternative to conventional ion-pairing agents, such as tetraalkylammonium salts, for Hg speciation by RP-HPLC-CV-AFS. MeHg, EtHg, and Hg(II) were separated using a C18 column and a gradient developed by mixing methanol and a solution composed of 0.4% (v/v) 1-octyl-3-methylimidazolium chloride [C8mim]Cl, 100 mmol $L^{-1}$ NaCl and 20 mmol $L^{-1}$ buffer citric acid/citrate (pH 2.0) within 12 min. LODs were 0.05, 0.06 and 0.11 µg $L^{-1}$ for MeHg, EtHg and Hg(II), respectively, which are comparable to other methods [73]. The method was applied to a variety of samples, including fresh seafood, canned fish, yeast and garlic. Hg species were extracted from food samples (300 mg) by sonication during 10 min with 5 mL of a 5 mol $L^{-1}$ solution. 5-fold dilution for seafood samples and 10-fold for yeast and garlic was proposed to avoid possible matrix effect. The extraction yield varied according to the matrix, from 51% for yeast up to 87% for seafood. Hg species were separated by HPLC and organic species were photo-oxidized *on-line* in a UV digestion unit by using a 0.75% (w/v) $K_2S_2O_8$ solution [73]. In the analyzed foodstuffs, Hg levels were in a range between 0.3 and 0.7 µg $g^{-1}$ and Hg species distribution varied according to the food sample. MeHg was the major species in seafood while Hg(II) dominated in yeast and garlic [73].

For HPLC analysis, the development of new stationary phases based on nanomaterials opens new perspectives on Hg speciation. A recent example is the successful use of graphene as stationary phase in HPLC. Graphene is a new class of carbon-based nanomaterial, commonly obtained by graphite oxidation and

subsequent reduction processes; it does not contain metal impurities. This nanomaterial has outstanding sorptive properties and therefore a great trace metal preconcentration capacity [74]. Both surfaces of the planar sheet are accessible for sorption, resulting in a huge specific surface area. Graphene oxide is oxidized graphene, keeping the singular structures and properties of graphene.

In general terms, the application of graphene to trace metal speciation is rare so far. The unique adsorption properties of graphene and graphene oxide have been exploited for the first time on the development of HPLC stationary phase for Hg speciation. Hg species (inorganic and organic ones) complexes with 2-thiosalicylic acid were attracted by graphene oxide due to π electron stacking. The coupling of the graphene oxide based column to ICP-MS provided LODs of 0.016, 0.027, 0.032 and 0.068 μg $L^{-1}$, for Hg(II), MeHg, EtHg and PhHg, respectively [75]. Graphene and graphene oxide based columns (4.6 mm i.d. and 5 cm long) did not show different results regarding retention time and peak resolution. The separation of inorganic and organic mercury species was achieved in 12 min, by using 2 mmol $L^{-1}$ TSA and 5 mmol $L^{-1}$ phenylalanine solution at pH 8 with a flow rate of 1.0 mL $min^{-1}$ [75].

The comparison with a commercial C8 column (5μm, 4.6 mm i.d. and 5 cm long) demonstrated the superior analytical performance of the graphene based developed column [75]. The GO@$SiO_2$ based column, successfully used for Hg speciation in fish, showed its feasibility for routine Hg speciation analysis by HPLC-ICP-MS. The success of the graphene oxide based column on the retention of Hg species evidenced the potential of this carbon-based nanomaterial as a powerful adsorbent in preconcentration procedures [75].

### *Vapor Generation of Hg Species Assisted by Nanomaterials*

A considerable number of applications of nanomaterials on Hg speciation corresponds to their use in efficient interfaces between liquid chromatography and the detection by CV-AFS (Table **3**). Photochemical vapor generation is an advanced approach that can also be successfully hyphenated to chromatographic and flow injection on one side and atomic detection methods, as AFS, on the other side. It consists of the generation of reducing radicals from low molecular weight organic compounds under UV irradiation. Laser and microwave irradiation are also effective on the generation of free radicals with similar analytical purposes [76-77].

AFS is a convenient method for Hg determination characterized by its sensitivity, selectivity, and relatively low cost instrumentation. The sensitivity, and

consequently the LODs, are strongly influenced by the sample introduction efficiency. Vapor/hydride generation is largely used, guaranteeing matrix separation and an efficient analyte transport. Vapor/hydride generation by using tetrahydroborate is the most frequent approach for Hg determination [68]. The chemical vapor generation method based on the use of tetrahydroborate suffers of interferences that degrade the sensitivity and reproducibility of the method, and the large amount of hydrogen generated can dramatically affect the plasma/flame source.

**Table 2:** Some recent examples of Hg speciation in food by methods based on HPLC separation.

| Chromatographic method | Column | Mobile phase | Hg species | Detection system | Time (min) | LOD (ng $L^{-1}$) | Samples | Ref. |
|---|---|---|---|---|---|---|---|---|
| **RP** | C8 (4.6 x 150 mm, 5 µm) | 1.5 mmol $L^{-1}$ ammonium pyrrolidine dithiocarbamate in 75% (v/v) methanol | MeHg | CV-AFS | 14 | 0.4 (MeHg) | Rice, baby-food rice | [11] |
| **Ion-pairing-RP** | C18 (4.6 x 150 mm, 5 µm) | Gradient: (A) 5 mmol $L^{-1}$ TBAH, 10 mmol $L^{-1}$ $NH_4H_2PO_4$; (B) 5% (v/v) methanol, 0.1% (m/v) L-Cys, 0.06 mmol $L^{-1}$ $CH_3COONH_4$ (pH 7.1) | Hg(II), EtHg, MeHg | ICP-MS | 20 | 10 (EtHg), 40 (Hg(II)), 30 (MeHg) | Rice, rice flour standard | [72] |
| **Ion-pairing-RP** | C18 guard columns (5 µm, 12.5 mm x 2.1 mm i.d.) | 2.0 mmol $L^{-1}$ SDBS + 2.0 mmol $L^{-1}$ Cys + 1.0 mmol $L^{-1}$ Phe (pH 3.0); 4.0 mmol $L^{-1}$ TBAH + 2.0 mmol $L^{-1}$ MPS + 2.0 mmol $L^{-1}$ Phe (pH 6.0) | Hg(II), MeHg, EtHg, PhHg | ICP-MS | 3 | 15 (Hg(II)), 14 (MeHg), 28 (EtHg), 42 (PhHg) | Fish | [71] |
| **Ion-pairing-RP** | C18 (150 mm x 4.6 mm) | Gradient: (A) 100% (v/v) methanol (B) 0.4% (v/v) [C8mim]Cl; pH 2.0; 0.02 mol $L^{-1}$ citric acid/citrate buffer; 0.1 mol $L^{-1}$ NaCl | Hg(II), MeHg, EtHg | CV-AFS | 12 | 11 (Hg(II)), 50 (MeHg), 60 (EtHg) | Fresh seafood, canned fish, yeast, garlic | [73] |
| **Graphene oxide column** | GO@$SiO_2$ column (500 mm x 4.6 mm) | 2 mmol $L^{-1}$ TSA and 5 mmol $L^{-1}$ phenylalanine (pH 8) | Hg(II), MeHg, EtHg, PhHg | ICP-MS | 12 | 16 (Hg(II)), 27 (MeHg), 32 (EtHg), 68 (PhHg) | Fish | [75] |

The photocatalytic properties of $TiO_2$ are exploited on the development of an efficient interface for Hg speciation by HPLC-CV-AFS [70]. The method allows direct vapor generation of Hg species on nano $TiO_2$ under UV irradiation in the presence of a mixture of formic acid and sodium formate, acting as a hole scavenger. Compared to the most frequently used system $KBH_4$/NaOH-HCl, the direct photocatalysis vapor generation on nano $TiO_2$ clearly exhibited better analytical performance [70]. The method was applied on the investigation of Hg species (Hg(II), MeHg, EtHg and PhHg) in fish, oysters and others seafood, with a LOD between 10 and 70 ng $L^{-1}$, according to the mercurial species. The principle of *on-line* photocatalyst-assisted vapor generation using nano $TiO_2$, has also successfully been used in the hyphenation of HPLC with ICP-MS [78].

Nanosized $TiO_2$ has also been included in the integrated MW/UV interface for Hg species determination by HPLC-AFS. Hg(II), EtHg, MeHg were converted to $Hg^0$ by the combined action of UV and MW in presence of formic acid and Cys in the mobile phase. This method allows the replacement of the commonly used tetrahydroborate system by an environmentally friendly alternative for Hg speciation with LODs between 0.15 and 0.35 μg $L^{-1}$ in fish [68].

The use of $ZrO_2$-NPs improves the efficiency of the photocatalytic system, compared to nanosized $TiO_2$ [55]. A UV/nano-$ZrO_2$/HCOOH photocatalytic reduction unit was developed as an on-line interface between HPLC and AFS for Hg speciation. Hg species were transformed into atomic Hg on nanosized $ZrO_2$ upon UV irradiation. The sensitive novel system reached LODs of 13, 16 and 24 ng $L^{-1}$ for MeHg, EtHg and Hg(II), respectively, and was successfully applied to the analysis of fish and other seafood samples [69].

Advanced oxidation using $Fe_3O_4$ MNPs constitutes another example of how nanoparticles assist Hg speciation. Hydride generation/cold vapor inactive species were transformed in active ones by efficient *on-line* post-column advanced oxidation using $Fe_3O_4$ MNPs without MW/UV irradiation. The LODs of this simple, efficient and green approach were 0.7, 0.8, 0.9 and 1.1 μg $L^{-1}$, for Hg(II), EtHg, PhHg and MeHg, respectively. Peak broadening was reduced due to the very short length of $Fe_3O_4$ MNPs column (2.5 cm). This approach, which was applied to Hg speciation in fish, does not require the complex experimental set up of conventional oxidation techniques and eliminates the use of toxic oxidation reagents [79].

**Table 3:** Some examples of nanomaterials used as efficient interfaces between liquid chromatography and CV-AFS detection for Hg speciation in foodstuffs.

| Hg species | Matrix | NPs (action) | Analytical technique | Mobile phase | LOD (μg $L^{-1}$) | Ref. |
|---|---|---|---|---|---|---|
| **Hg(II), EtHg, MeHg** | Fish | $TiO_2$ (photocatalysis) | HPLC-AFS | 1% methanol, 1% formic acid, 5 mmol $L^{-1}$ Cys, 0.15 mol $L^{-1}$ $NaClO_4$. | 0.15-0.35 | [68] |
| **Hg(II), EtHg, MeHg, PhHg** | Fish, oysters and others seafood | $TiO_2$ (UV/$TiO_2$ photocatalysis reaction device) | HPLC-AFS | acetonitrile and water (65:35) containing 1.5 mmol $L^{-1}$ ammonium pyrrolidine dithiocarbamate (pH 5.5) | 0.01-0.07 | [70] |
| **Hg(II), EtHg, MeHg** | Fish, oysters and others seafood | $ZrO_2$ (UV/$ZrO_2$/HCOOH photocatalytic reduction unit) | HPLC-AFS | 10% HCOOH, pH 3 | 0.013-0.024 | [69] |
| **Hg(II), MeHg, EtHg, PhHg** | Fish | $Fe_3O_4$ (*on-line* advanced oxidation) | HPLC-AFS | acetonitrile and water (10:90) at pH 6.8 containing 0.12% (m/v) L-Cys | 0.7-1.1 | [79] |

## Hg Speciation in Food by Non-Chromatographic Methods

Chromatographic methods are absolutely well established on Hg speciation in food. However, the high cost of the instrumentation and analysis as well as the complex sample pre-treatment used in these conventional methods motivated the development of non-chromatographic approaches. Non-chromatographic methods are based on the individual chemical and physical properties of the different species. Low solvent consumption, promptness, and preconcentration are some of the advantages of such methods. The functionalization of nanomaterials opens new perspectives on the development and application of non-chromatographic approaches. Some of these recent and promising methods are presented hereafter.

Electrochemical vapor generation (ECVG) is an important sample introduction technique based on the reaction between H· derived from the cathode of the electrolytic cell and the analyte. In comparison to photochemical vapor generation (PVG), ECVG is more efficient and environmentally friendly considering that it only requires a simple electrolytic cell and a power supply [80]. ECVG has been coupled to spectroscopic techniques as AAS and AFS for Hg quantification in a wide type of samples. However, its application to speciation is relatively new [81].

The first example corresponds to the development of a L-Cys modified graphite cathode, which efficiently converts Hg(II) and MeHg to Hg vapor [80]. Taking into account that at low current mode (0.2 A) exclusively Hg(II) is efficiently converted to Hg vapor and at high current mode (2.2 A), both MeHg and Hg(II) can be reduced efficiently, a sensitive speciation method was established relying on the current control. The novel non-chromatographic speciation method was validated by the analysis of several CRMs and applied on Hg speciation in seafood. The superiority of ECVG, compared to conventional CVG was demonstrated since it avoids sodium tetraethylborate and oxidizing agents consumption, minimizing costs and potential environmental pollution risks [80].

An innovator compact method based on flow injection photochemical vapor generation (PVG) coupled with miniaturized solution cathode glow discharge atomic emission spectroscopy (SCGD-AES) was developed for the determination of Hg and its species [82]. This novel method combines the small size, independence of compressed gases and low power consumption of SCGD-AES with the simplicity, miniaturization feasibility and green analytical characteristic of PVG. The SCGD was generated between a miniature hollow titanium tube and a solution emerging from a glass capillary. An automated stopped-flow operation allowed increasing the UV irradiation time on-line. Hg vapor, generated by PVG, was transported to the SCGD for excitation, and finally the emission signals were recorded by a miniaturized spectrograph. Inorganic and organic Hg species (MeHg and EtHg) exhibited dissimilar responses at different wavelengths and powers from the UV lamp. Therefore, Hg speciation was possible by the variation of wavelengths and powers from the UV lamp and irradiation times. Inorganic and organic species were converted to $Hg^0$ for the determination of total Hg with 8 W/254 nm UV lamp and 60 s irradiation time; while only Hg(II) can be reduced to $Hg^0$ and determined selectively with 4 W/365 nm UV lamp and 20 s irradiation time. Consequently, organic mercury concentration was calculated by the difference on total Hg and inorganic Hg concentrations. Hg(II), less toxic than organic species, was successfully used as a primary standard for the quantification of Hg considering the similar sensitivity at 8 W/254 nm. This pioneer PVG-SCGD-AES approach results on a 365-fold improvement in Hg(II) LOD (0.2 μg $L^{-1}$) compared to SCGD-AES. The method was validated for water and biological samples including fish and could be extrapolated to other elements [82]. Nickel demonstrated to be an optimum enhancement reagent for the PVG efficiency of Hg(II) and MeHg. Under optimized conditions, the addition of nickel (10 mg $L^{-1}$) increased the signal intensity of Hg(II) and MeHg around 1.5 times compared to that without nickel ion. Separative techniques like GC and HPLC could be

potentially combined to PVG-SCGD-AES, opening new possibilities for Hg speciation analysis [82].

Double liquid-liquid extractions and/or selective derivatization are commonly used for Hg speciation by non-chromatographic methods [83]. A recent example is the introduction of methyl isobutyl ketone on the sample treatment of seafood as degassing agent leaving Hg into the water phase. MeHg was extracted from an aliquot of the aqueous phase with HBr, $CuCl_2$ and toluene, and back-extracted into a L-Cys–sodium acetate solution. This method was proposed for quantification of both total Hg and MeHg in two consecutive steps using a single Hg commercial standard solution by heating vaporization AAS. Despite most analytical studies focused on fish muscle, liver and gonads are also consumed. The analysis revealed remarkable differences between the MeHg fraction in sea urchins and squids glands [84]. This protocol, validated by the analysis of several CRMs, is much simpler and cost-effective in comparison with chromatographic techniques [84].

A non-chromatographic Hg speciation method based on double liquid-liquid extraction and measurement by flow injection UV photochemical vapor generation capacitively coupled plasma microtorch optical emission spectrometry (UVPVG-μCCP-OES) was developed for Hg determination in fish and others seafood [83]. In brief, Hg species extracted from 0.2 g of lyophilized fish and seafood samples were confined in 10 mL hydrobromic acid (47%), followed by MeHg extraction in toluene and back-extraction in 1% L-Cys solution. MeHg was quantified by UV-PVG-μCCP-OES and external calibration against Hg(II) standards in formic acid. LOD and LOQ (2 μg $kg^{-1}$ and 6 μg $kg^{-1}$, respectively) were improved in comparison to thermal decomposition atomic absorption spectrometry (TDAAS) reference method and other methods used for Hg speciation in seafood [83]. Hg(II) was determined by the difference between total Hg and MeHg concentrations [85]. This new miniaturized approach involves a low power/Ar consumption plasma microtorch (100 mL $min^{-1}$) and a low resolution microspectrometer. The developed method is simple and safe concerning sample preparation through elimination of conventional, harmful reductants and attractive for using unsophisticated instrumentation [85].

A modification of this method, based on ultrasound assisted extraction of Hg(II) species with formic acid go further on its green concept. In that case the use of toluene is no longer required, formic acid being the unique reagent. Concentration of MeHg was determined by subtraction of Hg(II) amount from total Hg. The eco-

scale non-chromatographic method, with LODs and LOQs of 4.8 and 14.4 μg $kg^{-1}$ respectively, was applied on Hg speciation in fish [86].

***Non-Chromatographic Speciation Assisted by SPE***

Amberlite XAD-series resins, easy derivatized and highly stable in a wide pH range, are on the top of sorbents used for speciation. Aminated Amberlite XAD-4 resin was proposed as a solid-phase extractant for Hg speciation in seafood (fish and mussel). Hg(II) and MeHg were sequentially eluted from the aminated XAD-4 resin column coupled to flow injection cold vapor generation AAS. 400 mg of AAXAD-4 were placed into a SPE cartridge column (3 mL volume, 8 mm i.d., 65 mm length) and sample solution passed through the column at a flow rate of 1.5 mL $min^{-1}$. Hg(II) and MeHg eluted consecutively with 10 mL of 0.1% (w/v) thiourea in 3% (v/v) HCl and 10 mL of 6.0 mol $L^{-1}$ HCl solution at 1.5 mL $min^{-1}$ flow rate, respectively. Hg(II) was directly quantified by FI-CVG-AAS, while MeHg was previously oxidized before analysis with 20 μL of 1% (m/v) $KMNO_4$. The enhancement factor for Hg(II) and MeHg was 11.7 and 17.2, respectively, and the column did not show significant decay in the Hg species extraction efficiency up to 60 cycles. This method (LOD: 0.148 and 0.157 μg $L^{-1}$ for Hg(II) and MeHg, respectively) allowed semiautomatic quantification of Hg species in seafood [87].

IIPs also assist Hg speciation in food [38, 88, 89]. For example, one of the few Hg speciation studies in wine consists of the high selectivity and preconcentration towards Hg(II) of a non-chromatographic method by using a core-shell ion-imprinted sorbent. Airborne particulate matter deposition and the grapevine intake from the soil/groundwater system explained the presence of Hg, and other toxic elements in wine [88]. Considering the low Hg content, usually at sub μg $L^{-1}$, the speciation required preconcentration steps.

The Hg(II) ion-imprinted sorbents were synthesized via copolymerization using MAA as a functional monomer, as a crosslinking agent in the presence of Hg-complexes with 1-pyrrolidinedithiocarboxylic acid, as chelating agent. Silica gel acted as supporting material, chemically modified with 3-(trimethoxysilyl)propyl methacrylate. The sorbent exhibited a high specificity for the retention of Hg(II) without interferences of the organic matrix and benefits from a fast kinetics of sorption and desorption processes. Hg speciation in white and red wines (20-50 mL) was carried out in a column solid-phase extraction scheme using 100 mg of sorbent. Hg(II) was quantified in column eluates by CV-AAS and MeHg concentration was determined by the difference between total Hg and Hg(II) concentrations [88]. The LOD for Hg(II) (0.02 μg $L^{-1}$), was comparable to those

obtained by GC-ICP-MS in wine without requirement of derivatization steps. This sorbent exhibited a great chemical and mechanical stability, unalterable after more than 50 cycles of sorption/desorption [88].

The combination of a thiourea derivative polymer with DMA was proposed for screening and pre-concentration of Hg species. The method was based on the use of a new selective polymer, synthesized by using 2-(methacryloylamino) ethyl 2-methyl acrylate as cross-linker, 1-phenyl-3-(-3-vinyl phenyl) thiourea as monomer and azobisisobutyronitrile as initiator. Hg species were extracted from fish by ultrasonication and loaded on the highly selective polymer. The speciation strategy consisted of the specific release of MeHg at 120 °C, followed by the standard DMA temperature ramp up to 600 °C for the quantification of Hg(II). On the first heating step (at 60 °C), MeHg was recovered up to 50%, being the rest of the organomercurial species, measured in conjunction with Hg(II) after application of higher temperatures [38]. The method was therefore considered for rapid screening, and not for precise quantification of Hg species. This innovative approach provides a fast MeHg screening in food with simple handling and sample pretreatment.

A more advanced approach was based on the use of a new polymeric material that allowed, for the first time, the retention of both species, Hg(II) and MeHg. After sequential elution, Hg(II) and MeHg were quantified by CV-AFS. The polymer was synthesized by using vinyl derivative of 8-hydroxiquinoline as monomer, and 2-(methacryloylamino) ethyl 2-methyl acrylate as co-monomer in presence of azobisisobutyronitrile, as initiator. At least 20 cycles of Hg species sorption/desorption can be carried out, with a reproducibility of 5% [89]. The analytical procedure, with a quantification limit of 0.015 mg $kg^{-1}$, was validated with a tuna CRM (BCR 464) and applied to commercial fish. Hg species extraction from lyophilized fish samples was achieved with 5 mol $L^{-1}$ HCl under ultrasonication. Highly acidic fish extracts were diluted before loading on the polymer cartridge in order not to disturb the optimum sorption loading, at neutral pH. 1 mL of the fish extract was loaded on a conventional glass solid-phase extraction cartridge. Hg(II) was eluted by the addition of 3 mL of 2 mol $L^{-1}$ HCl in methanol for Hg (II), followed by the MeHg elution with 3 mL of NaClO 20% in 1 mol $L^{-1}$ HCl. The proposed method involves minimum handling and could be exploited for Hg speciation in routine laboratories [89]. This rapid, low cost, and simple Hg speciation approach also reduces considerably the reagents waste compared with conventional speciation approaches.

### *Non-Chromatographic Methods Assisted by Nanomaterials*

An innovative recent technology proposes the use of gold particles deposited glassy carbon electrode (Au/GCE) as cathode for ECVG combined with AFS for Hg speciation. The Au/GCE exhibited outstanding catalytic property for electrochemical conversion of MeHg to gaseous mercury. Highly consistent or distinct difference between the AFS signals of MeHg and Hg(II) could be achieved by control of ECVG electrolytic parameters. The method, based on the divergent electrochemical reactivity of Hg(II) and MeHg showed high sensitivity (LOD 0.44 μg $kg^{-1}$) and good repeatability (<6%, RSD) in fish analysis [81]. This sensitive, green and simple technique has an enormous potential in food routine laboratories.

A visual detection method of MeHg and EtHg in fish samples has been developed based on DNA-templated alloy Ag−Au NPs. The speciation approach relies on the ability of DNA sequences containing T to combine with organomercurial species. The different and tunable binding force of DNA sequence to Hg species by modifying the number and locations of T bases explains the application of T-rich DNA on Hg speciation [90].

Two T-rich aptamers were designed: $H_{T5}$ for exclusive recognition of MeHg and $H_{T7}$ that recognizes MeHg and EtHg. Therefore, quantification of EtHg was carried out by subtraction of MeHg obtained with $H_{T5}$ from the total MeHg and EtHg concentration obtained with $H_{T7}$. In presence of $Ag^{+}$ and $Au^{+}$, the aptamers preferentially bind the organomercurial species and consequently the formation of alloy Ag-Au NPs after reduction producing the color change of the solution (from colorless to purple). The presence of Hg(II) and other metallic ions, even at higher concentration than MeHg and EtHg did not interfere the detection of these organic species. It demonstrates the excellent recognition specificity of the designed aptamers [90].

This method allows bare eye detection of the organomercurial species at 1.0 mg $kg^{-1}$. LODs are around 10 times lower by using UV-Vis spectrometer as detector. The method was applied to the detection and quantification of organomercurial species in fish, extracted by MW acid digestion with a recovery of 101-109% (RSD <8%). The instrument-free visual discrimination and detection of MeHg and EtHg constitutes a simple, rapid, and cost effective alternative for Hg speciation in food [90].

Carbon dots (CDs), a new alternative to quantum dots, are fluorescent nanoparticles with excellent optical probes (highly photo stables, strong fluorescence and tunable

color emission). They are attractive also considering that in contrast to quantum dots, their simple synthesis, using eco-friendly carbon sources (*i.e.*, glucose, sucrose) does not involve precursors containing toxic elements, becoming a green alternative [74, 91].

The applications of CDs in elemental speciation are still scarce but promising. A fast and simple fluorescent assay has been recently developed for MeHg detection using D-fructose as carbon source. Ultrasonication was used for simultaneous CDs fabrication and selective recognition of the target analyte. The assay was accomplished within 1 min, exhibiting a LOD of 5.9 nmol $L^{-1}$ of MeHg (RSD 2.2%). Hg species were extracted from marine animal tissues CRMs (0.4 g) by ultrasonication during 4 min at 12% of amplitude in 5 mol $L^{-1}$ HCl solution. MeHg was quantified by the fluorescent assay after filtration (0.45 μm) and clean up (C18 cartridges) of the fish extracts. MeHg recognition was attributed to the hydrophobicity of this species and its capability to facilitate a non-radiative electron/hole recombination [91]. The validation of this environmentally friendly method with water and marine animal tissues CRMs guarantees its successful application in (sea)food and beverages.

## ADVANCES AND TRENDS IN THE USE OF Hg STABLE ISOTOPES

The use of stable isotope tracers is a great support for developing analytical methods for Hg speciation. The addition of isotopically enriched species, usually $^{199}$Hg(II) and $^{201}$MeHg, allows monitoring and correction of losses and/or species transformation from the sample preparation steps. Isotopic dilution analysis is a powerful tool in analytical quality assurance and quality control. Its application in Hg analysis in food is extended to a wide variety of samples as seafood [66, 92], rice [11, 14] and breast milk [23].

Species specific isotopic dilution is a smart solution to the quantification of Hg species in absence of CRMs. In complex rice and breast milk samples, it proves the validation of speciation methods based on both GC and HPLC hyphenated to ICP-MS [11, 14, 23].

The potential of Hg stable isotopes in food is not restricted to isotopic dilution analysis. Hg isotopic fractionation-the natural isotopic abundance variation-can be also exploited in dietary studies.

Hg is recognized as a “model element” for isotopic fractionation studies. It has several stable isotopes that exhibit mass-dependent and mass-independent

fractionation [93], which, combined, provide precious information on sources discrimination, reactivity and fate of Hg in a biogeochemical context. The improvement of analytical methods and the development of multiple collector inductively coupled mass spectrometry (MC-ICP-MS) has drastically expanded the research on non-traditional stable isotope deviated from traditional geological applications.

The great relevance of Hg isotopic signature on a large variety of subjects such as the elucidation of metabolic pathways [14, 94-97], tracking of pollution sources [98-99], discrimination of human exposure pathways [100-103], and biogeochemical processes [93, 104-107] has been demonstrated. The potential of the Hg isotopic pattern can be extended to food products.

Hg isotopic composition has been determined in rice and fish. Hg isotopic pattern in fish is an excellent tool for tracking Hg (pollution) sources in aquatic ecosystems. Moreover, the isotopic pattern of Hg in this foodstuff is extremely useful for the determination of Hg human exposure. In general, an enrichment of heavier isotopes of approximately 2‰ ($\delta^{202}$ Hg) is observed in hair as compared to the levels of these isotopes in consumed fish. Additionally, the preservation of mass independent fractionation signatures in human's hair and urine allows the discernment between occupational exposure (gold mining, dental professionals) and fish ingestion [100, 102]. The clear differences between Oceanic and coastal fish Hg isotopic patterns provide the hints to successfully identify Oceanic and mixed seafood consumers by the analysis of their hair [103]. More recently, the characterization of Hg stable isotopes enabled the discernment of different populations of European seabass, *Dicentrarchus labrax*, what can be extrapolated to high resolution dietary discrimination in a local scale [108].

In the case of rice, it has provided, among others, crucial information about sources of Hg contamination. The fraction of atmospheric Hg in the plants follows the trend: leaf > stem > seeds > roots [97]. The combination of Hg speciation and isotopic composition revealed the dissimilar uptake and translocation pathways of Hg species from paddy soil to the edible part. The fact that seeds, containing higher MeHg proportion, exhibit significantly heavier Hg isotopes is understood as a facilitated MeHg uptake and transport to the seed in comparison to inorganic Hg species [14].

In general, most of Hg isotopic studies are so far restricted to elemental isotopic pattern determination. The major drawbacks of Hg isotopic analysis are: 1) the high cost of the multicollector instrument, being exclusive of few laboratories; 2) the

reduced number of CRM for the validation of Hg elemental isotopic characterization and the absolute lack of such materials regarding the isotopic composition of Hg species and 3) the elevated concentrations usually required for a precise analysis.

The analysis is based on a complete digestion of the sample, usually by acid digestion, followed by isotopic determination through continuous CV-MC-ICP-MS. In order to diminish the problems associated with Hg isotopic characterization at ultra-trace levels, an on-line pre-concentration method was developed based on the hyphenation of a commercially available cold vapor generation dual gold amalgamation system to a multi-collector ICP-MS. The automated method supports a rapid throughput and consumes less than 100 mL of sample, representing a reduction of the sample volume of more than two orders of magnitude in comparison to existing off-line pre-concentration strategies. This method proved to be accurate and precise for the determination of total Hg isotopic composition of fish [109].

The relatively recent coupling of GC to MC-ICP-MS [110] constitutes a remarkable evolution on isotopic analysis since it allows the characterization of both Hg species, named compound-specific stable isotope analysis (CSIA) method. An automated on-line preconcentration system, which allows injections volumes up to 100 folds higher than in a conventional GC-MC-ICP-MS scheme has been introduced in order to be able to analyze samples with low Hg content. Preconcentration and transfer of Hg species (Hg(II) and MeHg) to the column was achieved with a programmed temperature vaporization injector. Hg species were efficiently preconcentrated and fish reference materials with concentrations down to 150 ng $g^{-1}$ were successfully analyzed.

Another alternative to the isotopic characterization of MeHg is via a selective extraction method for MeHg, followed by continuous CV-MC-ICP-MS [111]. It has been applied to the analysis of rice samples. Briefly, 0.25-0.5 g were extracted with 5 mL of 30% w/w NaBr in 4 mol $L^{-1}$ $H_2SO_4$, 10 mL of 2.5% w/w $CuSO_4$ and 10 mL of toluene. The mixture was shaked for 1 h at 420 rpm, in order to transfer MeHgBr from the matrix to the toluene phase, followed by a back-extraction with aqueous sodium thiosulfate solution (0.005 mol $L^{-1}$) to convert the MeHgBr into a stable MeHg−thiosulfate complex. Hg isotopic characterization of MeHg was then carried out by CV-MC-ICP-MS after dilution of the MeHg-thiosulfate solution with a 3:1 v/v mixture of $HNO_3$ and HCl. Concerning the stable isotopic composition of Hg(II), it was determined by isotopic mass balance difference between total Hg and

MeHg isotopic patterns. The method was validated with a fish CRM and applied to the analysis of rice and human hair samples in order to understand human exposure pathways [9].

A promising chromatographic methodology has recently been validated for Hg isotope ratio measurement of MeHg in fish tissues at environmentally relevant levels. It is based on the isocratic separation of Hg(II) and MeHg in a reversed-phase HPLC column with a mobile phase containing L-Cys as complexing agent. One of the main advantages of this approach is the non-requirement of derivatization steps. The MeHg fraction isolated from the chromatographic columns was completely oxidized, prior to CV-MC-ICP-MS, due to the challenges driven by the non-reactivity of MeHg with $SnCl_2$ and, interferences caused by the presence of reduced sulfur in the mobile phase [112]. Considering that for the moment there are not commercially available CRMs for isotopic composition of Hg species, it is extremely valuable to have the possibility of contrasting the measurements obtained by different methods, such as GC and HPLC coupled to MC-ICP-MS.

## CONCLUDING REMARKS

The urgency to develop sensitive and environmentally friendly methodologies promotes the advances in the analysis of Hg in food. Such development is greatly supported by the progress in nanomaterials that assist in the whole procedure comprising Hg (species) extraction, preconcentration, separation and detection. Hg isotopic fractionation emerges as a powerful tool on dietary studies, allowing the discernment among several Hg sources of exposure. In addition, the identification of pollution sources in food is also possible through the Hg isotopic signature. It would be extremely valuable on the development of strategies that alleviate the impact of Hg contamination sources in foodstuffs.

Seafood monopolizes the attention of the analytical community that validated a wide variety of analytical approaches for such matrix. However, the global consumption of rice, considered an important source of Hg, claims for adequate methodologies to this complex samples.

Hg analysis in food is motivated by the human risk associated to dietary exposure. Consumer's guidelines are based on the assumption that MeHg from food is absolutely absorbed after human ingestion. However, it seems to overestimate the real assimilation of MeHg, since the nature of the matrix, fish species, cooking processes and the co-ingestion of certain food components seems to play a relevant

role on the potential human assimilation of ingested Hg [113-118]. For example, cooking practices like, steaming, boiling, grill and frying have been evaluated [113, 114, 118, 119], resulting in a reduction of the potential absorption at the intestinal epithelium level up to 90% [114], The co-ingestion of polyphenol-rich beverages like tea and coffee or pure polyphenols are leading to a decrease on Hg bioaccessibility [116, 118, 119].

The existing methods for Hg analysis in food focus on the quantification of Hg and its species based on the consumer guidelines, which set safe consumption thresholds. Nevertheless, some new risk assessment criteria are under development. A new seafood safety index named Selenium health benefit value (HBVSe) is a good case in point, which is based on the observation that Se is an antagonist of Hg toxicity. It considers both, MeHg and Se concentrations in fish, and shows more reliable accuracy than predictions based exclusively on MeHg [120]. In summary, the future and perspectives of Hg analysis in food will be absolutely influenced by the trends and evolution of consumption guidelines.

## CONFLICT OF INTEREST

None Declared


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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AAS | atomic absorption spectrometry |
| AFS | atomic fluorescence spectrometry |
| Au/GCE | gold particle deposited glassy carbon electrode |
| ASV | anodic stripping voltammetry |
| CDs | carbon dots |
| CRMs | certified reference materials |
| CSIA | compound-specific stable isotope analysis |
| CV | cold vapor |
| Cys | cysteine |
| DMA | direct mercury analyzer |

| | |
|---|---|
| DBD | dielectric barrier discharge |
| ECVG | electrochemical vapor generation |
| ETAAS | electro thermal atomic absorption spectrometer |
| ETV | electro thermal vaporization |
| GC | gas chromatography |
| GFAAS | graphite furnace AAS |
| HPLC | high performance liquid chromatography |
| HS-SPME | headspace solid-phase microextraction |
| ICP-AES | inductively coupled plasma atomic emission spectrometry |
| ICP-MS | inductively coupled plasma mass spectrometry |
| ID | isotopic dilution |
| IIP | ion imprinted polymers |
| ILs | ionic liquids |
| LOD | limit of detection |
| LOQ | limit of quantification |
| MS | mass spectrometry |
| MPS | 3-mercapto-1-propylsulfonate |
| MSPE | magnetic solid-phase extraction |
| MSPD | matrix solid-phase dispersion |
| MC-ICP-MS | multiple collector inductively coupled mass spectrometry |
| MW | microwave |
| MWCNTs | multiwall carbon nanotubes |
| NPs | nanoparticles |
| OES | optical emission spectrometry |
| PCs | porous carbons |
| RSD | relative standard deviation |
| TWI | tolerable weekly intakes |
| PVG | photochemical vapor generation |
| RP | reversed phase |
| SD-SEGD | single drop solution electrode glow discharge induced |
| SDBS | sodium dodecylbenzene sulfonate |
| HBVSe | Selenium health benefit value |
| SPE | solid-phase extraction |
| SSID | species specific isotope dilution |
| SR-μXRF | synchrotron radiation microscopic X-ray fluorescence |
| T | thymine |
| TBAH | tetrabutylammonium hydroxide |
| T-Hg | total mercury |
| UV | ultraviolet |

| | |
|---|---|
| UVPVG-μCCP-OES | UV photochemical vapor generation capacitively coupled plasma microtorch optical emission spectrometry |
| WHO | World Health Organization |
| XANES | X-ray absorption near-edge spectroscopy |


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