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Review

Mercury speciation in seafood using isotope dilution analysis: A review

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ABSTRACT

Mercury is a toxic compound that can contaminate humans through food and especially via fish consumption. Mercury's toxicity depends on the species, with methylmercury being the most hazardous form for humans. Hg speciation analysis has been and remains a widely studied subject because of the potential difficulty of preserving the initial distribution of mercury species in the analysed sample. Accordingly, many analytical methods have been developed and most of them incur significant loss and/or cross-species transformations during sample preparation. Therefore, to monitor and correct artefact formations, quantification by isotope dilution is increasingly used and provides significant added value for analytical quality assurance and quality control.

This review presents and discusses the two different modes of application of isotope dilution analysis for elemental speciation (i.e. species-unspecific isotope dilution analysis and species-specific isotope dilution analysis) and the different quantification techniques (i.e. classical and multiple spike isotope dilution analyses). Isotope tracers are thus used at different stages of sample preparation to determine the extent of inter-species transformations and correct such analytical artefacts. Finally, a synthesis of the principal methods used for mercury speciation in seafood using isotope dilution analysis is presented.

Contents

Abbreviations: Hg°, elemental mercury; iHg, inorganic mercury; MeHg, methylmercury; THg, total mercury; EC, external calibration; M, methylation; D, demethylation; ID–MS, isotope dilution – mass spectrometry; IDA, isotope dilution analysis; SU-IDA, species-unspecific isotope dilution analysis; SS-IDA, species-specific isotope dilution analysis; S-IDA, simple isotope dilution analysis; M-IDA, multiple isotope dilution analysis; D-IDA, double isotope dilution analysis; D-SS-IDA, double species-specific isotope dilution analysis; IPD, isotope pattern deconvolution; CV, cold vapour; EI-MS, electron impact mass spectrometry; ETV–ICP-MS, electrothermal vaporisation – inductively coupled plasma mass spectrometry; GC, gas chromatography; HPLC, high performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; MAE, microwave assisted extraction; MC-ICP-MS, multicollector inductively coupled plasma mass spectrometry; MIP-AES, microwave induced plasma-atomic emission spectrometry; SPME, solid phase micro-extraction; US, ultrasound.

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1. Introduction

Seafood is the primary source of human contamination by MeHg [1–3]. Over 90% of MeHg is absorbed through the gastrointestinal tract, then transferred into the bloodstream due to its complexation with thiol and to active transport, and distributed throughout the body. With oral exposure, the central nervous system is organic mercury's main target organ, particularly during foetal development. The toxic effects are alteration of sensory functions (sight, hearing), motor coordination, memory, attention and learning [3–5]. The toxicity of inorganic mercury causes renal lesions, neurotoxicity and cardiovascular disorders. In the most extreme cases, it can cause death as was the case in Minamata, Niigata and Iraq [6,7].

As a safeguard for human health, maximum permissible levels of Hg in fish (0.50 or 1 mg kg^{-1} essentially for predatory fish) and shellfish (0.50 mg kg^{-1}) have been set by Regulation (EC) No 629/2008 to limit dietary exposure of consumers [8]. Furthermore, in 2003 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) of 1.6 μg MeHg/kg bw and 5 μg THg/kg bw [9]. In 2010, the PTWI for THg was withdrawn by the Committee and replaced by a PTWI for inorganic mercury (iHg) of 4 μg kg^{-1} bw [10]. This new PTWI is applicable to dietary exposure to total mercury from foods other than fish and shellfish. For MeHg in these foods, the previously established PTWI should be applied.

Currently available contamination values in seafood are based on THg concentrations and evaluation of MeHg exposure is based on a simplifying assumption which considers that the average percentage of Hg present as methylated mercury in the flesh of fish is 84% of THg (MeHg = 0.84 THg) with a 15% variation around this value between species and within them, according to the fishing area [11]. This hypothesis was challenged by studies which have shown that MeHg may represent a smaller share of THg in some fish [12,13]. This discovery confirms the need to estimate levels of MeHg, in addition to THg concentrations, to obtain more specific toxicological reference values and recommendations.

After a short summary of the most widely developed Hg speciation analysis methods, isotope dilution analysis (IDA) will be introduced. The advantages and principal applications of this innovative quantification technique will then be presented and discussed.

2. Mercury speciation analysis

The methods developed to perform Hg speciation analysis involve the coupling of a powerful separation technique (liquid or gas chromatography, capillary electrophoresis) with a selective and sensitive (elemental) detection method (fluorescence spectrometry, plasma source coupled with emission spectrometry or mass spectrometry) [14–16]. Gas chromatography coupled with inductively coupled plasma mass spectrometry (GC/ICP-MS) has rapidly conquered the field of trace and ultra-trace elemental analysis due to the higher resolving power of the capillary GC columns compared to the packed columns, which offers excellent separation of Hg species and the sensitive multi-elemental and multi-isotopic detection capabilities of ICP-MS. Furthermore, this analytical set-up offers the best opportunity to perform speciated isotope dilution mass spectrometry [15,17].

Extraction and derivatisation steps are common for most sample preparation methods involved in Hg speciation analysis by GC–ICP-MS. The main extraction method used is microwave assisted extraction (MAE) due to its speed, efficiency and low occurrence of methylation (M) and demethylation (D) reactions [18–20]. For the derivatisation of Hg species, alkylating reagents such as sodium tetrapropylborate ($NaBPr_4$) and sodium tetraethylborate ($NaBEt_4$) are mainly used because derivation takes place in an aqueous medium, the natural environment of most biological samples [19,21]. Such derivatisation procedures avoid additional solvent extraction step needed for example when Grignard reagents are used.

The recent development of IDA has drastically improved the accuracy and quality of Hg speciation analysis results [15,22,23]. Indeed, the use of isotopically enriched species (i.e. spikes) as tracers overcame the traditional problems related to non-quantitative recoveries and the formation of Hg artefacts that particularly occur during the extraction and derivatisation steps [19,24–28].

3. Isotope dilution analysis

Despite the many improvements made in sample preparation procedures and analytical techniques for the speciation analysis of Hg, quantification is still a difficult step due to potential losses, non-quantitative yields or species transformation reactions that may throughout the entire analytical method. When conventional quantification techniques such as external calibration or standard additions are used, losses or transformations of Hg species cannot be measured leading to erroneous results. In IDA, the initial isotopic composition of the sample is altered by the addition of known amounts of one or more isotopic labelled species. The quantification is then based only on the measurement of isotopic ratios, making quantitative recoveries unnecessary. Provided that spikes are in forms equivalent to those of natural Hg species, and that they reach complete isotopic equilibrium, they will act as "ideal" internal standards and rearrangement reactions will be easily detected.

Only mass spectrometry allows the use of IDA because of its specificity (detection based on the ratio m/z). ID–MS is considered to be a definitive method, offering accurate determination of Hg species with only small uncertainties and has been the subject of numerous review articles [16,17,22,29,30]. Speciation analysis of Hg in fish products by IDA is carried out by EI-MS or ICP-MS. The main advantage of EI-MS detection is to partially atomize the sample, which preserves the structural integrity of compounds. Moreover, this technique is less expensive to purchase, operate and maintain and is more frequently encountered in laboratories than ICP-MS. However, sample preparation is generally simpler when using ICP-MS as detector, avoiding the additional sample clean-up step often needed when using EI-MS detection, which can be disturbed by matrix components. Furthermore, ICP-MS has a capacity of multi-element analysis and offers better sensitivity with absolute detection limits up to the femtogram, as against the picogram for EI-MS.

The use of spikes for trace element speciation analysis in biological and environmental matrices has increased considerably since the first experiments in the 1950s [31–33]. There are two different modes of IDA application, i.e. species-unspecific (SU) and species-specific (SS) spiking mode.

3.1. Species-unspecific spiking mode isotope dilution analysis

The SU spiking mode was generally used because of a lack of commercially available isotopically enriched mercury species identical to the species of interest, and is exclusively limited to the correction of errors derived from the detection step. Indeed, after a complete separation of endogenous species, isotopically enriched spike was added to the separated natural species, before the ionisation and detection process, in order to accurately measure the corresponding isotope ratio [17]. This technique can only correct for matrix effects during detection and save analysis time compared to standard addition quantification, which first requires the

preparation and analysis of an internal standard calibration. Nowadays, many Hg enriched isotope tracers are available or are synthesised in laboratories and the SU spiking mode is usually replaced by the SS spiking mode.

3.2. Species-specific spiking mode isotope dilution analysis

In SS spiking mode, species of interest are marked, during the sample preparation, by analogue species that have been isotopically modified. In this way, IDA is applied specifically to one or more species, depending on whether the single or multiple IDA technique is used. This powerful approach was first employed in 1985 by Unger and Heumann [34] to determine inorganic compounds (nitrite and nitrate traces) in food samples. The SS spiking mode has since mainly been used to analyse organometallic compounds such as MeHg [20,25–28,35,36].

The prerequisites for using the SS spiking mode have previously been discussed in detail [23]. Briefly, the chemical identities of the sample and spike(s) must be fully known and a complete isotopic equilibrium between endogenous species and spike(s) must be quickly reached. Isotope tracers are most often added at the beginning of sample preparation to ensure optimum control of the whole analytical procedure.

Classical or simple isotope dilution analysis (S-IDA) consists of the addition of one isotopically enriched species to the sample to alter the natural isotopic abundance of the endogenous species. From the mixed isotope ratio obtained and the S-IDA equation, quantification becomes possible [17,25,38], provide that the spike behaves chemically the same way as the endogenous Hg species, S-IDA enables the final result to be corrected for any loss or non-quantitative extraction and therefore achieves excellent accuracy and precision, improving relative standard deviation of the method by a factor of four, on average, compared to external calibration [39–41]. However, inter-conversion reactions between species are not taken into account as only one spike is added. To evaluate and correct the final result for methylation and demethylation reactions, multiple-isotope dilution analysis (M-IDA) must be used [27,28,39,42]. In double isotope dilution analyses (D-IDA), the sample is spiked with known amounts of two isotope tracers (e.g. ^{199}iHg and Me^{201}Hg) that will react identically to the studied species (^{202}iHg and Me^{202}Hg). Quantification is then based on the measurement of the mixed isotope ratios. D-IDA is established as a baseline approach that allows the main analytical bias to be corrected and permits an accurate quantification of Hg species [25,26,28,42–45].

Data obtained by D-IDA can be processed specifically for two species (i.e. double species-specific isotope dilution analysis or D-SS-IDA) or for the whole system (i.e. isotope pattern deconvolution or IPD). For example, the D-SS-IDA model can look at 3 isotopes (e.g. 199, 201 and 202) and 6 species (^{199}iHg, ^{201}iHg, ^{202}iHg, ^{199}MeHg, ^{201}MeHg and ^{202}MeHg). In this case, 6 mass conservation balances are established according to the equations below:

For iHg:

$$N_m^{iHg} = (N_s^{iHg} + N_{sp}^{iHg})(1 - M) + (N_s^{MeHg} + N_{sp}^{MeHg})D(1 - M)$$

For MeHg:

$$N_m^{MeHg} = (N_s^{MeHg} + N_{sp}^{MeHg})(1 - D) + (N_s^{iHg} + N_{sp}^{iHg})M(1 - D)$$

For example, for ^{199}iHg in the mixture (m), total mass (N_m^{iHg}) consists of the mass of iHg initially present in the sample (N_s^{iHg}) and the iHg from the spike (N_{sp}^{iHg}), taking into consideration M (loss of iHg by methylation) and D (gain in iHg by demethylation of ^{199}MeHg) reactions.

From the 6 mass balances, 4 mixed isotope ratios can be calculated with $R_{m,\,iHg}^{202/201}$ and $R_{m,\,Hg(II)}^{202/199}$ depending on the demethylation of MeHg and $R_{m,\,MeHg}^{202/201}$ and $R_{m,\,MeHg}^{202/199}$ and depending on the methylation of iHg. By a mathematical rearrangement as described in the publication of Monperrus et al. [42], M and D rates can be determined independently of the amount of endogenous species and final Hg species concentrations will be corrected for them.

In IPD, all the different isotopic patterns of both spikes and endogenous species are considered [23,28,46]. The combination of isotope ratios of natural species, spikes and mixtures enable two systems of equations to be established for each species that can be expressed in matrix form. In contrast to D-SS-IDA, this method makes it possible to use more isotopes (and therefore more equations) than unknowns. The measured isotopic distribution of the Hg species in the mixture is deconvoluted by multiple linear regression, applying least squares fitting to obtain the molar fractions for each Hg species from the three isotope patterns considered (i.e. the natural pattern, the iHg pattern and the MeHg pattern) and the interconversion factors M and D [47]. Quantification is thus more accurate as potential cross-species transformations on unmarked species are corrected. However, the mathematical resolution becomes particularly complex and results are less precise than with D-SS-IDA, because errors become more numerous when the number of parameters is increased [48]. D-SS-IDA and IPD differ in their mathematical complexity and their ability to expand to deal with a large number of species, but the processing of all the element's isotopes allows the calculation of variance-covariance to determine the instrumental mass bias as effectively as by analysis of thallium, which is a significant advantage over other techniques of quantification by isotope dilution [47].

To conclude, despite the apparent mathematical complexity of the M-IDA techniques (SS-IDA and IPD), these approaches retain all the advantages of S-IDA while providing powerful capabilities for quantification and correction of inter-conversion reactions.

3.3. Advantages and limitations of IDA

IDA can be only applied to elements with at least two stable isotopes that can be analysed by mass spectrometry without spectral interference [17]. IDA is accurate only if a complete isotopic equilibrium between endogenous species and spikes is achieved. In practice, this can be difficult as spikes tend to be extracted more efficiency than endogenous species because they are unrelated to the matrix [35,45].

For Hg speciation analysis, many enriched standards have been developed and are available commercially, permitting the application of SS spiking mode. The exact isotopic composition of all added spikes is determined by monitoring the stability of concentrations by reverse isotope dilution analysis and the stability of isotopic abundances by isotope abundance analysis [17].

Uncertainty measurements associated with IDA depend essentially on the uncertainty measurement of the mixed isotope ratio "R_m" [35,49]. This parameter is calculated by dividing the peak area obtained for the most abundant natural isotope by the peak areas measured for the spike (e.g. $R_{Hg}^{202/201}$ for MeHg, $R_{Hg}^{202/199}$ for iHg, etc.). It is based on a multitude of other parameters such as detector mass bias, detector dead time, sensitivity of the detector, data acquisition parameters, peak shape. This ratio is constant throughout the sample, which explains why quantitative extraction is not needed [17]. In return, "R_m" must be measured with high accuracy and precision in order to diminish systematic errors. It is therefore necessary to exhaustively monitor blank values to ensure that no contamination is affecting this ratio [50] and to regularly

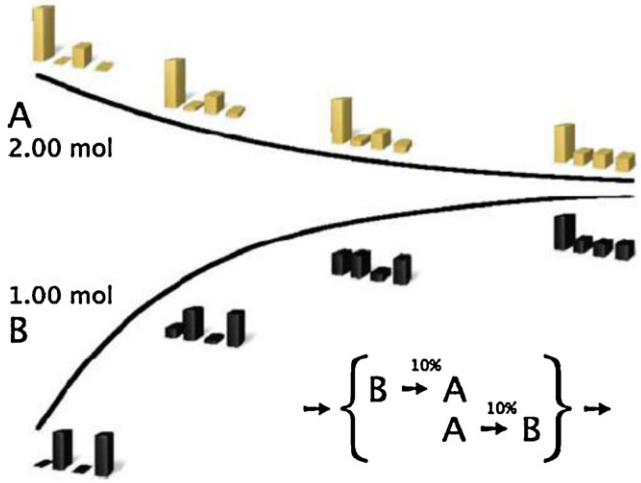

Fig. 1. Consequences of interconversion of two compounds A and B on their isotope patterns.
Source: [52].

evaluate detector mass bias and dead time. To conclude, a lack of peak symmetry may affect the accuracy of "R_m" [51].

A major limitation of M-IDA is concentration differences between species. When iHg and MeHg concentrations are very different (ratio MeHg/iHg < 0.05), the minority species must be preconcentrated prior to injection into the separation device which adds a further step to sample preparation and thus increases uncertainty. D-IDA methodologies are able to provide both accurate and precise results for iHg and MeHg with similar relative concentrations, a condition that holds for most biotissues [42].

It should be noted that IDA has been widely applied for over ten years but some points of this method are not yet fully understood. IDA is based on the hypothesis that spikes act identically to natural endogenous species but this behaviour has not been proven despite numerous studies conducted in this area [37]. Furthermore, Meija et al. [52] recently raised the possibility of inter-isotope reactions that may occur between spikes and analytes during interconversion reactions. Over time, these reactions erase the dissimilarity of isotope patterns between the analytes involved and can result in identical isotopic signatures regardless of the initial amounts or interconversion rate constants, as illustrated in Fig. 1. They conclude that due to the ability of multiple-spiking isotope dilution to correct for any interconversion, less effort is made to minimise them while vigilance should be maintained.

To conclude, in numerous studies IDA has shown its ability to determine Hg species levels with a high degree of precision and accuracy. Applications related to seafood products will be discussed in the following part. However, the analyst must remain critical regarding the final results and check their consistency, for example by applying total mercury analysis or by comparing the results obtained with those reported in the literature.

3.4. Evaluation of Hg speciation analysis in seafood using isotope dilution techniques

Many experiments using different stable isotope tracers have been carried out and methodologies have been developed over time. Table 1 shows some applications of Hg speciation analysis in seafood since 2000.

A slight preference for simple IDA rather than multiple IDA can be noted. However, the most recent applications involve species-specific labelling with several Hg isotopes, allowing for discrimination between species and investigation of M and D processes [17,25,28,39,41,42,44,45,50,74,77,79].

The developed analytical methodologies using isotope tracers generally follow the common model presented in Fig. 2.

The discussion below will focus on selected applications from Table 1, dealing with the optimisation of sample preparation for seafood analysis [35,39,41,44,82].

3.4.1. Evaluation of the lyophilisation/storage step

Applications of total Hg analysis and Hg speciation analysis in seafood are generally conducted on freeze-dried samples [83–86]. This pre-processing aims to homogenise samples and facilitate their transport and storage. Only two studies in the literature have examined the behaviour of freeze-dried samples compared to fresh samples. Yu and Yan [82] studied the influence of various parameters including fresh and lyophilised materials on iHg and MeHg stability in environmental and biological samples, not using isotopic tracers. Analysis demonstrated that MeHg in fresh and dried fish muscle and CRMs showed good stability over time and against thermal cycling. However, if fresh shellfish was repeatedly frozen and thawed, losses of MeHg could occur. This study concluded that these two textures are equivalent in terms of species conservation [82].

Some years later, Point et al. [44] analysed iHg, MeHg and THg in biological CRMs including three freeze-dried (FD) and two fresh-frozen (FF) materials, by S-IDA and D-SS-IDA. For S-IDA determination, FF and FD materials were not always commutable as MeHg concentrations tend to be overestimated in FD materials due to methylation of iHg. The source of this transformation appeared to be linked to the type of acid used to adjust the pH. In D-SS-IDA determination, MeHg, iHg and THg concentrations were systematically in good agreement with certified values and the materials were commutable. Consequently, this study showed that several parameters of the analytical procedure can cause the formation of artefacts of mercury and demonstrated the value of using D-SS-IDA analysis instead of S-IDA.

To conclude, Yu and Yan [82] and Point et al. [44] evaluated the potential difference in behaviour between freeze-dried and fresh matrices but they did not study the impact of the freeze-drying process on species (e.g. degraded species or transformation reactions). To the authors' knowledge, no studies dealing with Hg species and the freeze-dried process have been published. There is thus a lack of information about this topic.

3.4.2. Evaluation of the spiking procedure/isotopic equilibrium

The isotope equilibrium must be complete and reached quickly otherwise a difference in the extraction efficiency between natural species and spikes will result, yielding errors in the measurement of "R_m". If the sample is a liquid, equilibration by gentle agitation should be sufficient. If it is a solid material, precautions should be taken to prevent preferential extraction of the spiked species over the natural species [17,27].

Clough et al. [35] assessed isotope equilibration by determining MeHg and THg concentrations in DORM-2. The spike ($Me^{199}Hg$) in a solution containing 2% of HNO_3 was equilibrated in a solution of 50:50 H_2O:MeOH (v/v) containing 0.01% of 2-mercaptoethanol to which DORM-2 was added. The mixture was agitated at 25 °C by a magnetic stirrer and aliquots were taken at different times, extracted by MAE and detected by HPLC-Q-ICP-MS. To determine whether equilibration was attained, measured "$R_m^{202/199}$" was compared to the theoretical mixed isotope ratio ($R_m^{202/199} = 1$). During the first few minutes, "$R_m^{202/199}$" rose drastically until a plateau at 6 min was reached, meaning that complete isotope equilibrium had been reached. Furthermore, calculated MeHg and THg concentrations were in good agreement with certified values, while

Table 1
Examples of mercury speciation analysis in biological matrices using isotopic tracers.

Reference materials/biological tissues	ID procedure	Extraction/derivatisation	Separation – detection	Figure of merit	Reference
DORM-2, BCR-463, TORT-2	US spiking mode, $^{200}Hg(II)$	Solid sampling	ETV–ICP-MS	LOD (MeHg) = 2 ng g^{-1} LOD (THg) = 6 ng g^{-1} RSD_r[a] = 5–14%	[53]
IAEA-436, DOLT-3	S-IDA, ^{198}Hg	0.1% (v/v) 2-mercaptoethanol or 0.15% (w/v) KCl or 0.1% (v/v) HCl	ICP-MS	–	[54]
Zooplankton, aquatic samples	S-IDA, ^{199}iHg	2:1 HNO_3/HCl (v/v)	CV–ICP-MS	LOD ($Me^{201}Hg$) = 0.6 ng L^{-1} LOD (^{200}iHg) = 1.4 ng L^{-1} RSD_r = 0.6–1.4%	[55]
DORM-2, NIST-1566b, KRISS tuna CRM	S-IDA, ^{199}iHg	TMAH/$SnCl_2$	CV–ICP-MS	LOD = 0.018 ng g^{-1}	[56]
IAEA-407, plankton (FF[b])	S-IDA, ^{199}iHg	HCl + US/propylation	GC–ICP-MS	LOD = 0.05 pmol L^{-1} RSD_r = 5%	[57]
Trout (FF), cuttlefish (FF)	S-IDA, ^{200}iHg	Distillation with H_2SO_4 + HCl	HPLC–ICP-MS	LOD (MeHg) = 15 pg g^{-1} RSD_r = 5%	[58]
Freshwater fish (FF)	S-IDA, ^{200}Hg	Solid sampling	ETV–ICP-MS	LOD = 6 ng g^{-1} RSD_r = 10%	[59]
DOLT-2, BCR-463, TORT-2	S-IDA, ^{201}iHg	HCl + US/ethylation + SPME	GC–MIP-AES or GC–ICP-MS	CV_r = 6%	[60]
NIST-1946 (FF)	S-IDA, ^{201}iHg	HNO_3/$HClO_4$ + MAE/$SnCl_2$ reduction	CV–ICP-MS	RSD_r = 0.62%	[61]
BCR-463, CCQM-P39	S-IDA, ^{202}Hg	HNO_3/H_2O_2 (v/v) + MAE	ICP-MS	RSD_r = 0.5%	[62]
BCR-463, BCR-464	S-IDA, ^{202}Hg	25% (m/v) TMAH + MAE	HPLC–ICP-MS	LOD (MeHg) = 0.5 μg g^{-1} RSD_r = 6%	[63]
DOLT-2	S-IDA, ^{202}iHg	HCl/ethylation, propylation or butylation	GC–ICP-MS	LOD (MeHg) = 100–200 fg LOD (iHg) = 500–600 fg RSD_r (MeHg) = 0.3–4.2% RSD_r (iHg) = 0.1–7.0%	[21]
DORM-2, DOLT-2, DOLT-3	S-IDA, $Me^{198}Hg$	25% (m/v) KOH/MeOH/propylation + SPME	GC–ICP-MS	LOD = 2.1 ng g^{-1} RSD_r = 0.65%	[64]
DORM-2	S-IDA, $Me^{198}Hg$	25% (m/v) KOH/MeOH/propylation + SPME	GC–MS	LOD = 37 ng g^{-1} RSD_r = 2.1%	[65]
DORM-2	S-IDA, $Me^{199}Hg$	50:50 (v/v) H_2O:CH_3OH + 0.01% 2-mercaptoethanol	HPLC–ICP-MS	RSD_r = 11%	[35]
DORM-2, BCR-464	S-IDA, $Me^{199}Hg$	50:50 (v/v) H_2O:CH_3OH + 0.01% 2-mercaptoethanol	HPLC–CV–MC-ICP-MS	RSD_r = 0.45%	[66]
BCR-463, DORM-1	S-IDA, $Me^{201}Hg$	25% (m/v) TMAH + MAE/ethylation	GC–ICP-MS	LOD = 20–30 fg RSD_r = 0.3%	[25]
CRM-710	S-IDA, $Me^{201}Hg$	25% (m/v) TMAH + MAE/ethylation	GC–ICP-MS	LOD = 0.11 μg kg^{-1} RSD_r = 1.7–2.8	[67]
BCR-464, CRM-477	S-IDA, $Me^{201}Hg$	25% (m/v) TMAH + MAE or (3/1, v/v) AcOH/MeOH + MAE/ethylation	GC–EI-MS	–	[68]
DORM-2, TORT-2, SRM-1566b, zoobenthos, zooplankton	S-IDA, $Me^{201}Hg$	4 M HNO_3/ethylation	GC–ICP-MS	LOD = 1 ng g^{-1} RSD_r = 2.3–7.5%	[69]
CCQM-P39	S-IDA, $Me^{202}Hg$	HCl or TMAH/BuMgCl	GC–ICP-MS	RSD_r = 1.4%	[70]
BCR-463, DORM-2, mussels, prawns, tuna, plaice, pollock, shark	S-IDA, $Me^{202}Hg$	25% (m/v) TMAH/ethylation, propylation	GC–ICP-MS	LOD (MeHg) = 1.4 ng g^{-1}	[71]
BCR-464, CCQM-P39	S-IDA, $Me^{202}Hg$	HCl/propylation + SPME	GC–EI-MS	LOD = 28 ng g^{-1}	[72]
DORM-2, molluscs, crustaceans, fish, prepared fish meals	S-IDA, $Me^{202}Hg$	25% (m/v) TMAH + MAE/propylation	GC–ICP-MS	LOD = 0.3 ng g^{-1} CV_R = 5%	[73]

NMIJ CRM 7402-a	S-IDA, $Me^{202}Hg$	25% (m/v) KOH/MeOH + US or HCl + US/propylation, phenylation	GC–ICP-MS	RSD_r = 1.6%	[74]
SRM 1947, 1566b, TORT-2, oyster, mullet	S-IDA, $Me^{201}Hg$	1.5% (w/v) pancreatin solution/phenylation + SPME	GC–ICP-MS	LOD (MeHg, EtHg) = 0.3 μg kg^{-1} RSD_r < 15%	[40]
SRM 1566b, 2976, 2977and 1974a, b (FF)	S-IDA/D-IDA, $Me^{202}Hg$, ^{201}IHg	25% (m/v) TMAH + MAE/ethylation	GC–ICP-MS	LOD = 5 pg g^{-1} RSD_r = 1.3–4.0%	[44]
DOLT-2, TORT-1	D-IDA, $Me^{198}Hg$, ^{201}IHg	25% (m/v) TMAH + US + L-cysteine	HPLC–ICP-MS	LOD (MeHg) = 1 pg LOD (IHg) = 5 pg RSD_r = 5%	[75]
BCR-464	D-IDA, $Me^{200}Hg$, ^{199}IHg	a) 25% (m/v) TMAH + US b) 25% (m/v) KOH/MeOH + US c) 25% (m/v) TMAH + MAE d) HCl + US e) HNO_3 + MAE f) HAc + MAE g) L-cysteine extraction h) Protease XIV, enzymatic extraction	HPLC-ICP-MS	LOD (MeHg) = 0.78 μg L^{-1} LOD (IHg) = 0.46 μg L^{-1}	[39]
BCR-464, DOLT-3, NIST-1946 (FF)	D-IDA, $Me^{200}Hg$, ^{199}IHg	HCl + MAE	HPLC–ICP-MS	LOD (MeHg) = 16 μg kg^{-1} LOD (IHg) = 9 μg kg^{-1}	[41]
Bleak, asp, carp, perch, roach, pikeperch	D-IDA, $Me^{201}Hg$, ^{199}IHg	25% (m/v) TMAH + MAE/ethylation	GC–ICP-MS		[76]
Mussel tissue, golden grey mullet, anchovy, zooplankton, sea urchin, oyster tissue, BCR-710	D-IDA, $Me^{201}Hg$, ^{199}IHg	25% (m/v) TMAH + MAE/propylation, ethylation	GC–ICP-MS	LOD (MeHg) = 20 pg L^{-1} LOD (IHg) = 120 pg L^{-1}	[42]
Aquatic invertebrates, small fish	D-IDA, $Me^{201}Hg$, ^{199}IHg	HNO_3 at 50 °C/ethylation	GC–ICP-MS	LOD (MeHg) = 72 pg g^{-1} LOD (IHg) = 540 pg g^{-1} RSD_r = 0.72–5.1%	[77]
BCR-464, DOLT-4	D-IDA, $Me^{201}Hg$, ^{199}IHg	25% (m/v) TMAH + MAE/propylation, ethylation	GC–EI-MS, GC–ICP-MS	LOD (MeHg) = 8.8 ng g^{-1} LOD (IHg) = 9.2 ng g^{-1} CVr = 2–7%	[28]
BCR-464, DOLT-4, TORT-2, seafood	D-IDA, $Me^{201}Hg$, ^{199}IHg	25% (m/v) TMAH/propylation	GC–ICP-MS	LOD (MeHg) = 1.2 μg kg^{-1} LOD (THg) = 1.4 μg kg^{-1}	[78]
DORM-2, BCR-414	D-IDA, $Me^{201}Hg$, ^{201}IHg	25% (m/v) KOH/MeOH + US/ethylation	GC–ICP-MS	LOD (MeHg) = 2.8 ng LOD (IHg) = 4.6 ng	[79]
SRM 1947, SRM 1946, SRM 1974a, 1974b	D-IDA, $Me^{202}Hg$, ^{201}IHg	a) 25% (m/v) TMAH + MAE/ethylation b) HCl + MAE/phenylation + SPME	a) GC–ICP-MS b) GC–MS		[80]
SRM 1974a, 1566b	D-IDA, $Me^{202}Hg$, ^{201}IHg, $MeCys^{202}Hg$, $^{201}CysIHg$	25% (m/v) TMAH + MAE or US/ethylation, propylation or ethylation	GC–ICP-MS	LOD (MeHg) = 9 pg LOD (IHg) = 22 pg	[45]
DORM-2, DOLT-2	D-IDA, ^{196}Hg, ^{204}Hg	BrCl	MC-CV-ICP-MS		[81]

[a] Relative standard deviation in repeatability conditions.
[b] Fresh-frozen.

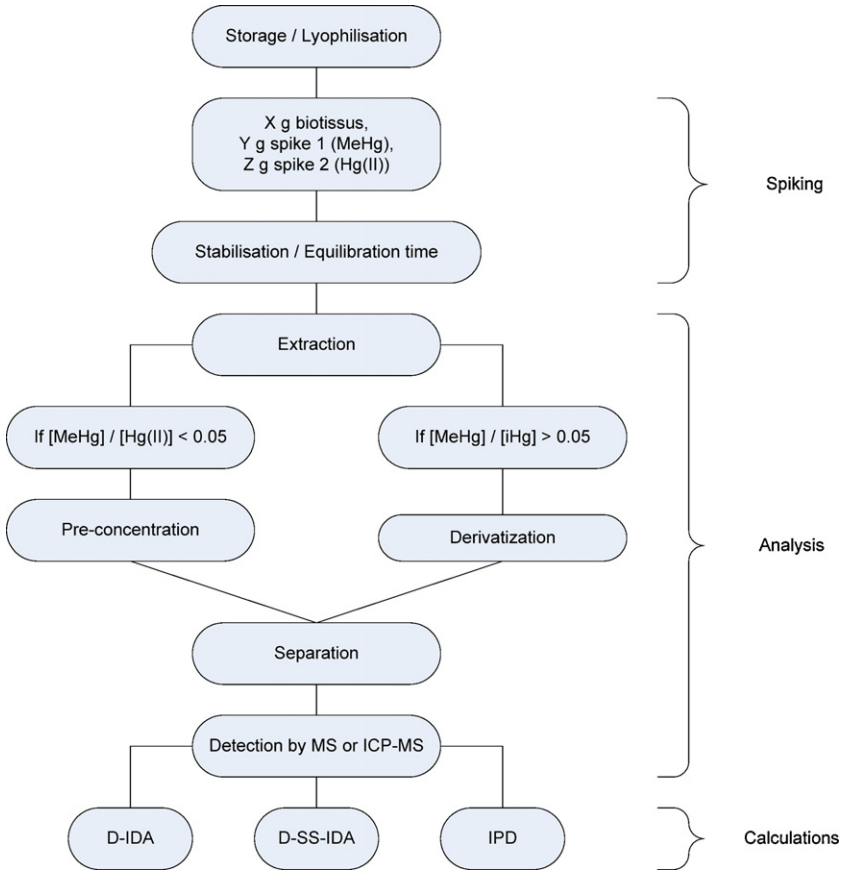

Fig. 2. Schematic flow diagram of isotope dilution protocol for mercury speciation analysis in solid samples.

extraction recovery was 53%, which demonstrated that complete extraction of analytes is not necessary for accurate quantification by IDA, when spiking takes place in a solution.

Later, Point et al. [45] were interested in the influence of a complexing ligand on isotope equilibrium. ^{201}iHg, Me^{202}Hg and their cysteine-complexed analogues (^{201}Hg (Cys)$_2$ and Me^{202}HgCys) were compared in terms of reactivity and inter-species transformations induced on a FF and a FD biological CRM. MeHg and iHg concentrations obtained were in good agreement with certified values, except for iHg concentrations for CRMs spiked with cysteine-complexed solutions in which a systematic over-estimation was observed. For all experiments, negligible methylation rates were noted. For CRMs spiked with labile spike solutions, D rates of 1.8% for the FD material and 16% for the FF material were obtained while for standard reference materials (SRMs) spiked with cysteine-complexed spike solutions: D rates of 7.5% for SRM 1566b and 33% for SRM 1974a were observed. They concluded that with the FF and FD matrices, directly spiking without extraction solvent can give accurate results if samples and spikes are homogenised and if an equilibration time of 15 min is applied. Furthermore, for iHg species, cysteine-complexed species have an influence on isotope equilibrium and transformations.

These studies have shown that a complete isotopic equilibrium can be reached whether samples are in a solid or a liquid medium. Point et al. [45] have shown the importance of the spikes chosen, because they can produce M and D reactions. Equilibrium can be achieved relatively quickly, and once established, it allows accurate quantification, corrected for transformations, despite low extraction rates.

3.4.3. Evaluation of the extraction/derivation steps

As it is difficult to completely avoid species transformations and non-quantitative extraction, it is important to optimise the analytical procedure to minimise such phenomena and/or to correct for their effects. It has been shown that extraction and derivation steps are precursors of M and D reactions and are consequently regularly reviewed [18,24,28,39,41].

Reyes et al. [39] evaluated eight different extraction protocols of mercury species on the BCR-464: three alkaline extractions (TMAH + MAE, TMAH + sonication (US) and KOH/MeOH + US), three acid extractions (HCl + US, HNO_3 + MAE and CH_3COOH + MAE), an extraction using L-cysteine hydrochloride (bath at 60 °C) and an enzymatic digestion with protease XIV (hybridization). On each occasion, the sample was spiked with ^{199}iHg and Me^{200}Hg and equilibrated 1 h before extraction, then analysed by HPLC-ICP-MS. For all the procedures, samples were spiked with ^{199}iHg and Me^{200}Hg and equilibrated 1 h before extraction. Data were processed by external calibration (EC) and M-IDA. With EC determination, only MeHg and THg concentrations determined after alkaline extraction using sonication were in good agreement with certified values because of the non-quantitative extraction of the other techniques. With M-IDA quantification, these problems were automatically corrected and the MeHg concentrations obtained for all extraction procedures were in good agreement with the reference values, except for acid extraction using HNO_3 where the MeHg concentration was too high. All the procedures showed similar percentages of M (3–6%) and D (0.8–6%), except for two procedures which led to more transformation reactions; i.e. HNO_3 and MAE, with 18% of M, and CH_3COOH and MAE with 27% of D. This study highlighted that isotope dilution is able to ignore non-quantitative

extraction and also showed that inter-species transformations may be purely related to the extraction technique used.

Later on, Reyes et al. [41] optimised an acid MAE (HCl/NaCl) procedure for the determination of iHg and MeHg in three CRMs and six freeze-dried fish tissue samples. Data were processed by external calibration; ID applied after extraction (DSAE) and ID applied before extraction (DSBE). Irrespective of which of the 3 techniques was used, MeHg and iHg concentrations of the 3 CRMs were in good agreement with references values, except for the iHg concentration in NIST-1946. For real fish samples, good agreement was observed between THg concentrations obtained by mercury speciation analysis and THg analysis, although the MeHg values were significantly lower in some of the analysed samples when calculated by DSAE compared to results obtained by EC and DSBE. These differences were probably due to an incomplete isotopic equilibration. Negligible D rates were noted for all CRMs and fish samples. M reactions were observed with higher rates obtained using DSBE than DSAE, showing that M reactions occurred during the extraction step. Furthermore, percentages of M were higher in real tissues than in CRMs because of differences in particle sizes and protein denaturation, showing the matrix-dependent nature of M reactions. Except for DOLT-3, iHg concentrations in the studied matrices were largely lower than MeHg concentrations, consequently M reactions did not affect quantification by external calibration. In samples where iHg concentrations were higher, traditional quantification by EC could be biased.

Castillo et al. [28] determined iHg and MeHg concentrations in BCR-464 and DOLT-4 by GC–EI-MS after optimisation of the extraction and derivation steps. For the first stage, MAE methods using TMAH and different temperatures and extraction times were compared. MeHg and iHg concentrations obtained were in good agreement with certified values but significant demethylation rates were observed after a short irradiation time. To determine the source of these transformations, ^{199}iHg and Me^{201}Hg were added before and after extraction. For both experiments, there was no significant difference between the results obtained, showing that transformation reactions were not induced by the extraction but by the derivatisation process. Ethylation and propylation were then evaluated in a second stage. It was observed that D decreased faster with the increase of microwave irradiation when propylation was used. The authors concluded that in the presence of non-irradiated TMAH, D reactions occur during the derivatisation step, even if they are reduced by the use of propylation.

These results demonstrate that transformation reactions are dependent on many parameters, including the extraction and derivation steps, matrix, species form and concentrations, solvent used. Consequently, it is not possible to develop an optimised preparation procedure suitable for all types of matrices, hence the need to apply multiple spiking methodologies for the determination of MeHg and iHg in biological samples.

4. Conclusion

Mercury speciation analysis in seafood is influenced by the nature of the matrix and by the analytical method used. Consequently, the main difficulty is to preserve the initial distribution of Hg species in the sample because of losses and/or cross-species transformations that may occur. Nowadays, IDA is often used nowadays in place of external calibration, because the addition of at least two isotopically enriched Hg species in the sample makes it possible to quantify these potential transformations and to correct its Hg species concentrations from its. Indeed, IDA allows researchers to identify critical steps of the method (e.g. lyophilisation/storage, spiking procedure/isotopic equilibrium and extraction/derivation) by acting on the time at which spikes are added to the sample.

The application of a validated method based on the use of certified references materials is not a sufficient criterion to ensure the accuracy of results as inter-species transformations are matrix dependent. IDA is therefore an essential tool, offering accurate results with small uncertainties.

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