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Capillary electrophoresis determination of methylmercury in fish and crab meat after extraction as the dithizone sulphonate complex

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Abstract

Dithizone sulphonate (DzS) is used in place of cysteine to separate methylmercury in the final stage of a simplified Westöö extraction procedure. The intensely absorbing methylmercury DzS complex is then separated by CE using a coated capillary and determined by direct absorption measurement at 480 nm. Good quantitative performance is demonstrated by spiking experiments and analysis of DORM-1 certified reference material. Values found ranged from 218 $\mu g\ kg^{-1}$ for a tuna fish to 2.8 $\mu g\ kg^{-1}$ for a crab sample. The very stable baseline and lack of interfering peaks meant that a low detection limit of 2 $\mu g\ kg^{-1}$ could be achieved for a 10 g sample. © 1997 Elsevier Science B.V.

Keywords: Food analysis; Sample handling; Fish; Methylmercury; Organomercury compounds; Dithizone sulfonate

1. Introduction

There has been a continuing interest in the determination of trace mercury for several decades, owing in part to the high toxicity of methylmercury (MeHg) and the strong tendency towards accumulation as it passes through the food web [1]. This ''biomagnification'' is particularly large in marine ecosystems and leads directly to human exposure through the fish food chain. The biomagnification of MeHg is generally thought to be result from its lipophilic character, although the prevalence of MeHg in fish muscle rather than in fat tissue indicates its adsorption is not entirely controlled by lipid solubility [2].

Since there is such a large difference in toxicity between inorganic and organometallic forms of mercury, a great deal of effort has been put into developing methods to differentiate and determine organomercury species in biological and environmental samples. Analytical methods for the determination of mercury speciation have been recently reviewed by Puk and Weber [3]. Chromatographic methods are the most popular as they measure the MeHg directly. Of these, the majority use gas chromatography (GC) after a multi-stage solvent extraction based on the method derived by Westöö [4]. There have been a number of problems with GC, mainly attributed to irreversible interactions of organomercury compounds with the chromatographic support. These have been solved to some extent by using capillary columns and constant conditioning procedures [5,6]. There have also been a small number of studies involving high-performance liquid chromatography (HPLC) coupled to various detection systems [7–9]. These are too few at the moment to fully assess the importance of this approach.

Capillary electrophoresis (CE) is a relatively

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recent high-performance separation technique which has been applied to the determination of small ions and molecules. One advantage over chromatographic methods is the lack of a substrate with a high surface area which has caused adsorption problems with volatile inorganic species, notably involving GC, as mentioned above. CE should be especially useful for speciation investigations, particularly where isolation procedures such as the Westöö extraction results in a fairly simple matrix.

The Westöö extraction procedure for environmental samples is well established and a number of modifications have been published since its first use in the late 1960s. For fish flesh it is essentially based on a three-stage solvent extraction process. The first stage results in release of the MeHg as the halide from the fish flesh in strong acid, with extraction into toluene. In the second stage, MeHg is back extracted into an aqueous layer as the thiosulphate or cysteine complex to isolate it from the large amounts of fat in the toluene. Finally, a further extraction back into toluene is carried out for gas chromatographic analysis, which also reduces sulphur containing impurities. Since CE is based mainly on aqueous samples, the third stage may not be necessary, as long as the MeHg complex is stable and can be detected. Medina et al. [10] who were the first to investigate this approach, found that the cysteine complex was stable during electrophoretic migration and could be detected with reasonable sensitivity in the far UV. They developed a simplified two stage extraction followed by CE for the determination of organomercury species in fish flesh [10]. This technique has been further investigated with respect to sample stacking [11] and validation [12] by Carro-Díaz et al. Although good results were obtained, we considered that the chosen wavelength of 200 nm could cause problems depending on the sample type. Many inorganic and organic species absorb strongly in this region, which may result in interferences and unstable baselines, especially if the sample size is large. This ultimately could affect the measurement of samples with very low concentrations of MeHg.

We have also been studying the CE separation and determination of organomercury species and recently developed a method based on the formation of water soluble dithizone sulphonate (DzS) complexes [13]. The inorganic and organometallic DzS species are very stable and intensely absorbing in the visible range. DzS, if anything, forms even more stable complexes than cysteine, so there was a possibility that it could be substituted for the cysteine in the second stage and the resulting complexes analysed by CE. The authors considered that the use of DzS would give two advantages. Firstly, the DzS complexes are more intensely absorbing. Secondly, and perhaps more importantly, there is less interference in the visible region from inorganic and organic fragments resulting from the extraction. Baseline noise should also be much less for the same reason. Preliminary results for extracted fish flesh did indeed show high sensitivity for MeHg with very stable baselines. This paper presents the results of a more thorough investigation of the quantitative performance of the DzS extraction method for the determination of MeHg in fish and crab meat by CE.

2. Experimental

2.1. Instrumentation and separation conditions

All experiments were carried out on a Dionex CES 1 CE system (Dionex, Sunnyvale, CA, USA) equipped with a reversed polarity power supply. Sample injections were performed hydrostatically at the cathodic side from a height of 100 mm for a period of 30 s, (calculated sample volume 142 nl). A constant voltage of -25 kV was used throughout. The buffer was 10 m*M* sodium acetate adjusted to pH 4.5 with acetic acid and containing 5 μg ml^{-1} DzS. Detection was carried out by on-column spectrophotometric measurements at 480 nm. Fused-silica capillaries of 65 cm$\times$100 μm I.D. were supplied by Dionex (Dionex UK, Camberley, UK) Data were recorded by a Dionex ACI computer interface, and processed using Dionex AI450 automated chromatography software, sampling at a rate of up to 50 Hz.

2.2. Samples and solutions

MeHg, ethylmercury and phenylmercury as the chloride salts were obtained from Shell UK. 1000 mg l^{-1} standard solutions of were prepared by dissolving the appropriate amount in Milli-Q water, with dissolution aided by sonication. All mercury

standards were stored in the dark and fresh solutions prepared monthly. Sodium acetate and acetic acid were of AristaR grade obtained from BDH (Poole, Dorset, UK). Acrylamide, potassium persulphate, N,N,N′,N′-tetramethylethylenediamine (TEMED) and γ-methacryloxypropyltrimethoxylsilane were obtained from Sigma (St. Louis, MO, USA). Dithizone sulphonate was manufactured in-laboratory, using a modified method based on that developed by Tanaka et al. [14]. All water used was 18 MΩ cm^{-1} obtained from a Milli-Q high purity water system (Millipore, Bedford, MA, USA). A variety of samples of tinned tuna, salmon and crab meat samples were obtained from three national supermarkets, samples of locally caught fresh cod and haddock samples were also obtained. In addition a certified reference material, DORM-1, freeze dried dog fish muscle, was also analyzed.

2.3. Capillary coating

The capillaries were coated with a polyacrylamide layer using a procedure based on that developed by Hjertén [15] and described in detail in a previous publication [13].

2.4. Sample preparation

2.4.1. Freeze dried samples

Excess liquid was drained from a 185 g tin of tuna, which was freeze dried and ground to a fine powder using a mortar and pestle.

2.4.2. Wet tinned samples

Excess liquid was drained from the tin, the contents then emptied into a beaker and thoroughly mixed using a spatula. An aliquot of this was then used for the extraction.

2.4.3. Fresh fish

A sample of fish muscle (ca. 30 g) was skinned, an aliquot of this was then used for the extraction.

2.5. Extraction procedure

The extraction procedure was based on the classical method of Westöö [4], except that only a two-stage extraction was involved and DzS was used in place of cysteine to back extract the MeHg from the toluene.

The procedure for wet fish samples is a follows: 20 ml water and 10 ml HCl were added to a beaker, a weighed sample of fish flesh was added to the beaker and the contents homogenised for 5 min using an homogeniser. The contents of the beaker were transferred to a 100 ml conical flask and 40 ml of toluene was added. The flask was then placed in a Gallencamp flask shaker and shaken for 5 min. The contents of the flask were then transferred to a glass centrifuge tube, which was sealed and centrifuged at 3000 rpm for 5 min. An aliquot of the supernatant was then transferred to a conical flask containing 1 ml of 500 ppm DzS. The size of the aliquot taken depended on the degree of stable emulsion that was formed. The flask was shaken for 2 min, the aqueous layer was then removed by pipette and transferred to a 2 ml glass centrifuge tube and centrifuged at 3000 rpm for 5 min; 0.5 ml of the dye solution was removed and analyzed by CE. The procedure for freeze dried samples was essentially the same, differing only in that the homogeniser was not used.

3. Results and discussion

3.1. CE method

The CE method for determination of MeHg was developed previously [13] using aqueous standards. The procedure was based on the separation of mercury species as the pre-formed highly absorbing dithizone sulphonate complexes. The complexes were stable enough to survive the electrophoretic migration and determination was achieved by direct absorption measurement at 480 nm. A small amount of DzS was added to the running buffer to reduce interference from metal impurities in the reagents. A pH of 5 was found to be optimum and coated columns were necessary as the difference between the electroosmotic flow and electrophoretic mobilities of the complexes was too great for adequate separation to be achieved. The organometallic species were well separated from each other, from inorganic mercury and from the dye impurities resulting from the synthesis. The organomercury species were also well separated from other inor-

ganic metal DzS complexes, though few survived the electrophoretic migration without dissociating. A typical separation using aqueous standards is shown in Fig. 1. Although other organomercury species are not expected to be present in the animals, it is important to show that a clear separation of different organometallic forms is possible. The method was found to be quantitive and very sensitive, due not only to the highly absorbing DzS complexes, but also the extremely low and stable absorbance background. The detection limit for MeHg using aqueous standards was 5 μg l^{-1}, which is more than adequate for the investigation of marine animals. All results were calculated from calibrations obtained by adding DzS to aqueous organomercury standards.

3.2. Extraction efficiency

All solvent extraction processes involve losses, whether due to less than 100% partition efficiency or reduction in volume due to emulsion formation etc. It is important therefore to obtain a reliable overall extraction efficiency and to assess the reproducibility. The extraction efficiency was determined by adding 1 μg MeHg to a solution containing 20 ml Milli-Q water and 10 ml HCl. 40 ml of toluene was added to this and placed in a flask shaker for 5 min. The mixture was then transferred to a 100 ml glass centrifuge tube, sealed and centrifuged at 3000 rpm for 5 min. An aliquot of the toluene layer was transferred to a conical flask containing 1 ml of DzS and placed in the flask shaker for 2 min. The mixture was then centrifuged at 3000 rpm for a further 5 min and 0.5 ml of the DzS layer was analyzed. This procedure was replicated fifteen times giving a mean of 82.5% recovery with a standard deviation of 1.63. It was considered that the extraction efficiency could have been improved by extracting the toluene layer a second time. However, this significantly reduced the reproducibility of the technique without substantially increasing the efficiency and it was decided that one extraction was sufficient as good reproducibility was the more important.

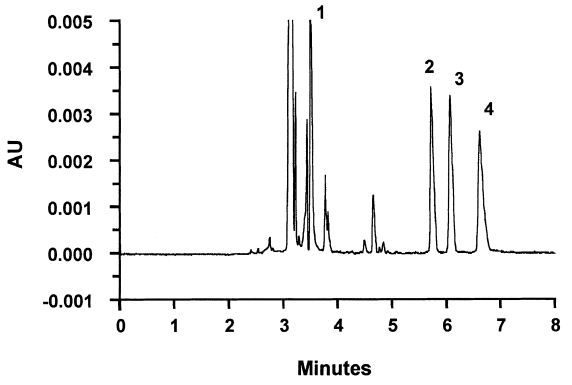

Fig. 1. Electropherogram showing the separation of inorganic and organomercury species (1 μg ml^{-1} each) as the DzS complexes. Separation conditions as in Section 2.1. Key: 1=inorganic mercury, 2=methylmercury, 3=ethylmercury, 4=phenylmercury.

3.3. Fish and crab meat results

The types and properties of samples analyzed are summarized in Table 1. Tinned fish samples, where possible, were obtained in brine owing to the higher solubility of MeHg in vegetable oils. It was thought that oil may leach out some of the MeHg making it necessary to analyze the oil portion as well. All samples were extracted from the wet fish sample, except tuna number four which was freeze dried prior to extraction.

During the extraction certain samples caused the formation of persistent emulsions between the aqueous and organic phases. In the worst cases an emulsion also developed between the toluene and dye, requiring filtration of the dye through a 0.45 μm PTFE membrane. This in itself did not cause a serious problem to the extraction procedure, although it is worth noting that there is a significant correlation between the formation of this emulsion and the higher relative standard deviations (R.S.D.s) obtained. With the freeze dried samples very little emulsion formed resulting in improved precision.

The effect of the matrix on the extraction was assessed by spiking 10 g portions of a tuna sample

Table 1
Origin and characteristics of samples analysed

Sample	Origin of can	Matrix in can
Tuna fish 1	Philippines	Spring water
Tuna fish 2	Thailand	Brine
Tuna fish 3	Spain	Brine
Tuna fish 4	Philippines	Vegetable oil
Crab	Thailand	Brine
Salmon	Alaska	–

Table 2
Recovery results for a tuna sample spiked with 2.5 μg of MeHg

Sample spiked	Mass of sample (g)	Spike (μg kg^{-1})	Found (μg kg^{-1})	Sample (μg kg^{-1})	Amount recovered (μg kg^{-1})	Recovery[a] (%)
Tuna 3	10.20	245	435	193	242	98.7
Tuna 3	10.04	249	455	193	262	105.2
Tuna 3	10.12	247	444	193	251	101.6

[a] 82.5% extraction efficiency taken into account. Mean Recovery=102±3.2%.

(No. 3) with 2.5 μg of MeHg. The results displayed in Table 2 show good quantitative recoveries were obtained.

A certified reference material was also investigated to assess the overall accuracy of the technique. DORM-1 is freeze dried dogfish muscle with a MeHg value of 731±60 μg kg^{-1}. Four extractions were performed using 0.5 g of DORM-1 for each. The results gave an average of 714±12 μg kg^{-1} (1.7% R.S.D.).

Although slightly lower than the certified mean value, the concentration found was well within the certified range.

The fish and crab results are summarized in Table 3. From the data it can be seen that as expected the tuna contains the highest concentration of MeHg, although the levels are within the US Food and Drug Administration action level of 1000 μg kg^{-1} (wet mass) for Hg in fish and the 500 μg kg^{-1} limit set in Canada [16]. The concentration found in the freeze dried tuna is surprisingly low considering this relates to approximately 45 μg kg^{-1} wet mass. The low result is possibly due to losses of MeHg to the oil in the can, but unfortunately the oil was discarded. A more likely source of loss is the freeze drying process causing volatilization of the MeHg. Losses of up to 60% have previously been reported during freeze drying [17]. The levels found in the cod and haddock are lower, consistent with their position in the food chain. Fig. 2 shows the electropherograms illustrating the high efficiency and good peak shapes well separated from impurity peaks. The impurity peaks are almost exclusively derived from the DzS, with few if any resulting from the extractions. These impurities are constant for a particular batch of DzS and have a fixed relationship to the mercury species in terms of migration time. Interestingly, they actual-

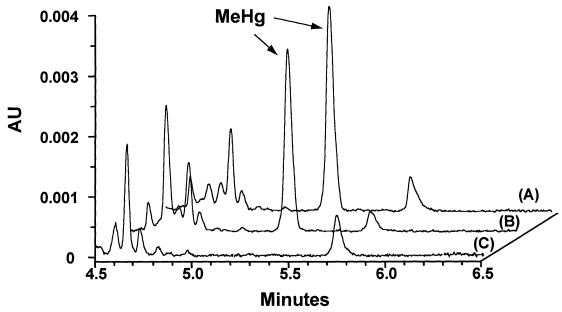

Fig. 2. Electropherograms showing the MeHg peaks obtained after extraction of cod and haddock meat. Extraction and separation conditions as in Sections 2.1, 2.5 and Table 3. Key: (A) cod, (B) haddock, (C) blank.

Table 3
MeHg concentrations found in fish and crab meat

Sample	Mean mass (g)	Mean concentration[a] (μg kg^{-1})	Standard deviation	Replicates	R.S.D. (%)
Tuna fish 1	10.33	113	3.86	4	3.4
Tuna fish 2	10.62	218	11.5	4	5.5
Tuna fish 3	10.53	193	9.5	4	4.9
Tuna fish 4	10.51	469	4.5	4	0.95
Cod	21.02	70	–	2	–
Haddock	23.47	58	–	2	–
Crab	10.26	2.8	–	2	–
Salmon	10.19	3.7	–	2	–

[a] Assuming 82.5% extraction efficiency.

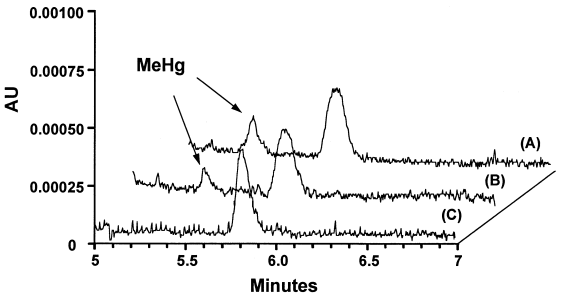

Fig. 3. Electropherograms showing the MeHg peaks obtained after extraction of salmon and crab meat. Extraction and separation conditions as in Sections 2.1, 2.5 and Table 3. Key: (A) salmon, (B) crab, (C) blank.

ly help by acting as ''markers'' for the inorganic and organomercury positions. This can be seen in Fig. 3 which shows the electropherograms for salmon and crab meat where the MeHg peaks are a constant 0.4 min from an impurity peak. The salmon MeHg concentration is surprisingly low considering its predatory nature, but may reflect its relatively short life span. MeHg was even detected in crab meat which is sometimes used as a control sample, as the organomercury is below the detection limit of the method used [18]. This is an indication of the very high sensitivity of the method, where the baseline is flat even when the sensitivity control is increased so that the clipped digitised noise is seen. The average peak-to-peak noise is 0.00004 absorbance units and defining a detection limit as twice this figure, corresponds to a minimum detectable concentration of 2 $\mu g\ kg^{-1}$ MeHg for a 10 g sample.

4. Conclusions

The results show that DzS can be substituted for cysteine in the Westöö procedure without any problems and the omission of a third extraction stage made the overall process more efficient and relatively simple. The CE method showed high sensitivity and selectivity, good linearity and excellent day-to-day reproducibility. Good quantitative results were obtained for MeHg in fish flesh indicating that the method can be used with confidence for the analysis of MeHg in biological materials of marine origin. The low detection limits are sufficient to allow the technique to be applied to wide variety of marine flora and fauna to study MeHg bioaccumulation in food chains. Other sample systems such as natural waters and sediments should also be capable of being adapted to this CE method. The lack of a substrate and the fact that conditioning the capillary is unnecessary, makes the technique potentially a more useful alternative to GC or LC methods.

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