MERCURY IN HAIR: METHOD DEVELOPMENT AND APPLICATION TO POPULATION STUDIES

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ABSTRACT

As part of the Avon Longitudinal Study of Pregnancy And Childhood, the toxic metal, mercury, is studied in order to assess whether it presents a danger to the development of children in the UK.

The development and optimisation of a digestion procedure for the determination of total mercury in hair is described. The procedure chosen consists of the microwave digestion of 0.2-0.5g hair with 2 ml HNO_3 and 1 ml H_2O_2 . The effect of washing and drying of the samples is investigated, and found not to influence analytical results when hair samples are collected after shampooing. However, freeze-drying of the samples results in the loss of 30% of mercury. The distribution of mercury along the length of a strand of hair is studied. It is shown to vary in adults (up to a factor of 7) but not in children (less than 20%). In the applications of this analytical method to population studies, the frequency of fish meals, and the age of the subject are positively correlated to the mercury levels in adults' hair ($r = 0.539$ and 0.513, respectively). Breast-feeding and mercury levels in children's hair are also correlated ($r = 0.433$). The relationship between number of fillings and mercury levels in adults' hair is not significant. No correlation was found between mercury levels in children's hair and Developmental Quotient (Griffith's scales) ($r = -0.24$ to 0.18).

The optimisation of a coupled High Performance Liquid Chromatography - CV-AFS system for the study of mercury speciation is described. The use of KOH and microwave digestion for the extraction of mercury species was unsuccessful, and thus recommendations for future work are made.

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1.1- Mercury in the environment

Mercury is a heavy, silver-white metal which is liquid at normal temperature and the only metal which is liquid at 0°C. Mercury occurs in a few localised areas of the Earth's crust and rocks. The average crustal abundance of mercury is 0.08 $mg.kg^{-1}$. A number of natural processes release mercury into the environment. The major source of mercury is the natural degassing of the Earth's crust, including land areas, rivers and the ocean, and is estimated to be in the order of 25,000 to 150,000 tons per year (WHO, 1976, NRCC, 1979). Small quantities of mercury are released naturally in volcanic eruptions [1]. Although anthropogenic sources of mercury have reached about 8,000 to 10,000 tons per year since 1973, non-anthropogenic sources are still predominant. Fossil fuel may contain as much as 1 $mg.kg^{-1}$ mercury, and it is estimated that about 5,000 tons mercury per year may be emitted from burning coal, natural gas and the refining of petroleum products. As much as one third of the atmospheric mercury may be due to industrial release of organic or inorganic forms. Although mercury can occur free in nature, practically all mercury for commercial use is obtained from the ore cinnabar, HgS, which is refined by oxidation [2]. Mercury species introduced in the environment can remain unchanged or undergo a number of transformations (Figure 1).

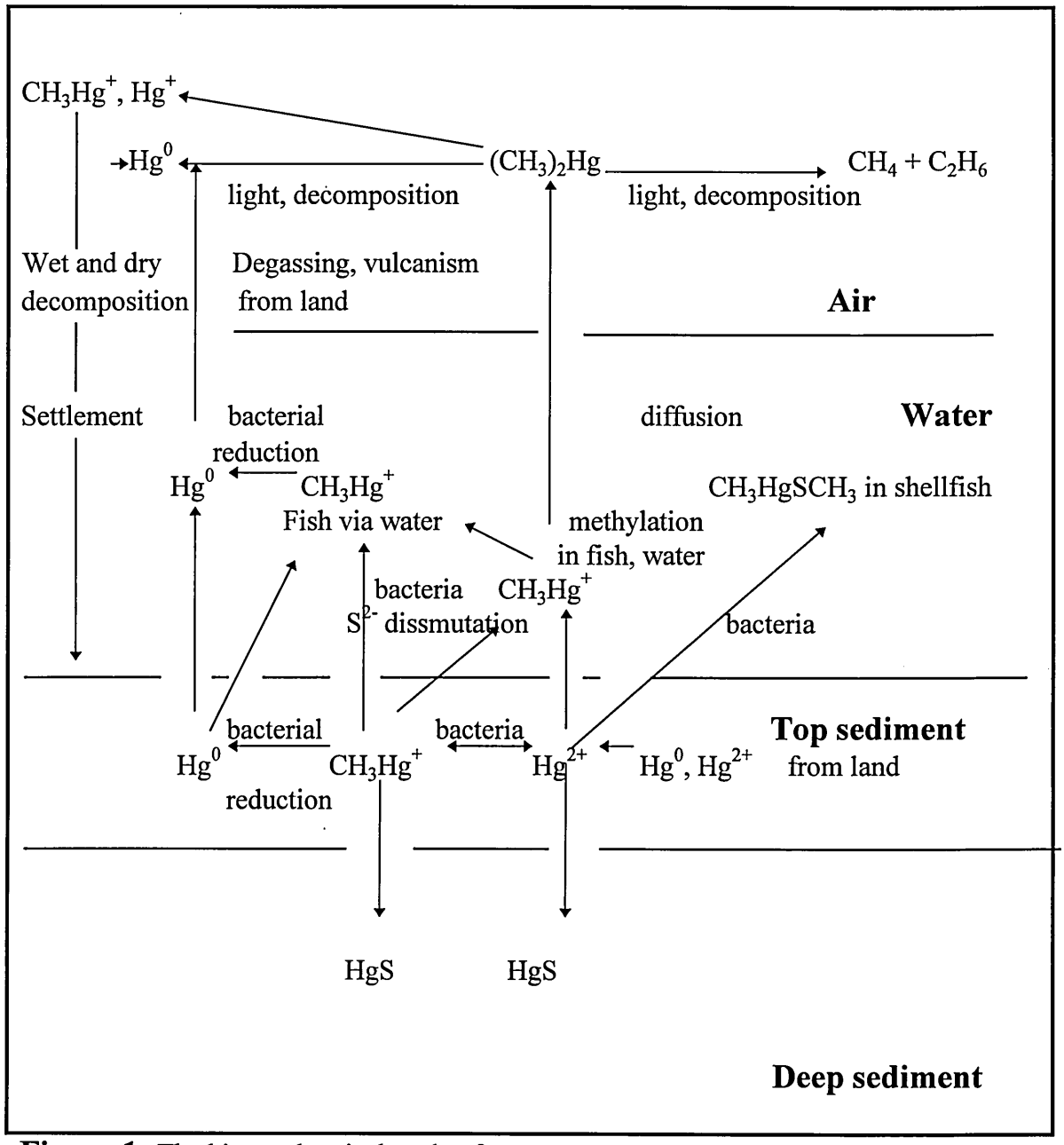


Figure 1: The biogeochemical cycle of mercury

Human activities such as mining, manufacturing, dentistry and other industrial uses account for a greater proportion of mercury in the biogeochemical cycle [3]. Although mercury has been widely used in industry, its uses are now declining, mainly because of its toxicity and the introduction of strict regulations on its authorised levels in food, water and the environment. Nevertheless, many scientific pieces of equipment depend for their working on mercury's unique combination of properties. Thermometers, barometers, gas and pressure regulators, McLeod gauges, diffusion pumps, electrical relays, standard cells and electrodes are a few examples. Industrially, mercury is used to fill fluorescent tubular lamps, high pressure street lamps, and a.c. rectifiers. Compounds of mercury also find varied uses. Mercury (II) fulmide is used as a detonator, mercury (II) oxide is a germicide and fungicide, mercury chloride forms part of a standard electrode system and many organomercury derivatives have pharmaceutical applications [3].

1.2- Toxicological effects of Hg

Different chemical forms of mercury have different toxic effects on the body. High doses of mercury (II) lead to kidney injury and perhaps death. The critical organ in methyl mercury poisoning is the brain, and penetration of the brain-blood barrier leads to sensory disturbances, tremor, ataxia, constriction of the visual fields, and impaired hearing. There is often a long latent period after exposure and the effects are usually irreversible.

Hughes [4] states that in all cases the principal reaction is with thiols, forming mercury mercaptides, and that the variations in distribution and effects are dependent upon this reaction. The lipid solubility of simple mercurials such as the methyl mercury halides,

which are 100 times more soluble in lipids than in water, would explain the rapid distribution of these compounds to all tissues. The pharmacological behaviour of metallic mercury can also be explained in terms of lipid solubility, which permits universal distribution followed by oxidation to reactive mercuric salts. Methyl mercury compounds appear to be absorbed quite rapidly through the gastro-intestinal tract, to the extent of about 90%, whereas the inorganic forms are absorbed more slowly and only to the extent of about 50% [1].

Lucas states that "The chronic effects of exposure to mercury are associated with its known ability to combine with the sulphydryl (SH) grouping found in a few amino-acids which go to make up body proteins. This process is responsible for the initial distribution to all body tissues either by attachment to plasma proteins or red cells. Mercury in combination is then enabled to penetrate various parts of tissue cells, where it may interfere with the proper functioning of membranes or of functions mediated by the cell enzymes. This ability of mercury to bind to proteins may also interfere with those liver enzymes which are normally involved in the detoxification of other foreign substances or in the potentiation of chemical drugs. The mechanisms responsible for the damage to the brain and other body organ tissues may also involve the same interaction of mercury with the sulphydryl group of enzyme proteins. There is also evidence that compounds of mercury may be a causative factor leading to long term carcinogenic, mutagenic and teratogenic effects" [1].

Other theories have been formulated to explain the toxicity of mercury [5]:

- the binding of mercury to phosphate ligands changes the cellular membrane permeability and conformational changes in macromolecules,

- mercury inhibits the uptake of iodide by the thyroid,

- or the metal acts as an antimetabolite to zinc.

The protective capability of selenium has been the subject of several studies [5] but the actual mechanism involved remains unknown.

Disasters such as Minamata (Japan, 1967) or Iraq (1971-1972) raised awareness of mercury's toxicity in many countries [6], and as a result, a lot of research has been devoted to its study.

At Minamata, Japan, in 1956 there was an outbreak of an illness characterised by gross disturbance of the central nervous system. All the persons affected had eaten fish from the bay, and cats and fish-eating birds were also affected. Investigations showed that effluent containing mercury chloride from a nearby factory had been discharged into the bay, causing contamination of water and sludge, and that fish caught from the bay contained significant amounts of mercury. By 1973, almost 1,000 Minamata victims had been identified and the number is still growing.

Fish in non-polluted water areas may contain 0.01-0.05 $mg.kg^{-1}$. Fish in heavily contaminated waters may have up to 20 $mg.kg^{-1}$ [7]. A diet consisting mainly of fish could result in the intake of high concentrations of methyl mercury. The maximum tolerable weekly intake is 0.3 mg of total mercury and 0.2 mg of methyl mercury (WHO 1989).

At Minamata, where pre-natal exposure occurred, infants born of exposed mothers showed evidence of cerebral palsy, which is characterised by mental retardation and motor disturbances. The mother may or may not show symptoms. Elimination of methyl mercury is slow. The half life in the human body for methyl mercury is about 70 days, compared with 4 to 5 days for mercury (II) [8].

In Iraq, symptoms similar to those observed in Minamata were reported in 1956, 1960, and principally in 1971-1972. Domestic animals and seed-eating birds were also affected. The illness was traced to eating seed grain treated with alkylmercury fungicides.

Acrodynia is a disease affecting infants, which is characterised by mental disturbances, insomnia, sweating, disordered sensations of the extremities and peripheral vascular phenomena. It is also known as Feer's disease or pink disease because it turns children's hands and feet a bluish pink. It occurs in children usually between the ages of 4 months and 4 years. The illness lasts for weeks or months and is often fatal [2]. Absorption of mercury is the most important cause of the disease but it was not identified before the 1950's. Calomel (mercurous chloride), a popular constituent of teething powders was

identified as the probable cause. Mercury compounds are not used any more in teething powders.

Chang and Reuhl state that "Mercury, particularly methyl mercury, should be considered one of the most hazardous and potent environmental toxicants. Its health impact on both humans and animals is not only limited to the nervous system but involves many other major organs such as liver and kidney. Because the foetal system tends to concentrate mercury via placental transfer, this mercury-trap phenomenon leads to extreme foetal risk despite low exposure to the mother" [9].

An important source of exposure to inorganic mercury in man is amalgam dental fillings [10, 11, 12, 13, 14]. Mercury vapour is slowly released from the surface of the fillings. The absorbed amount has been estimated to be about 3-18 μg per day [10]. There is a high exposure to elemental mercury vapour in dental surgeries [14, 15]. Most of the occupational exposure is to inorganic mercury, mainly elemental mercury vapour. Other sources of occupational exposure are in mining and in instrument, fluorescent tubes and chloralkali factories.

In some countries, exposure to organic mercury is still possible where organic mercury salts are used as seed dressings and fungicides [16]. Although the toxicological effects of short term high level exposure to mercury in humans have long been recognised [7], the effects of long term low level exposure are not well understood. In a bid to study the latter, it is essential that a sensitive and reliable indicator of long term exposure is used. Of the four

readily available indicators, hair, finger nails, blood and urine, only the first two meet the above criteria. By their very nature urine and blood can only be used as indicators of recent exposure. In contrast, finger nails and hair provide a historical record of exposure to a number of pollutants, including mercury. Besides, the sampling of hair is easy, and is non-invasive, which is ideal for the screening of healthy subjects. Hair conservation is practically unlimited and poses no problems, and its low density facilitates transportation.

In workers with occupational exposure to Hg^0 vapour, the level in hair may be 2-3 orders of magnitude higher than for non exposed people [7]. Mercury levels in hair were reported to be lower than 0.5 $mg.kg^{-1}$ in vegetarians, and between 0.5 and 3 $mg.kg^{-1}$ for non vegetarians with no occupational exposure, and as high as 50 $mg.kg^{-1}$ for dentists [17]. Mercury levels ranging from 0.1 to 6.9 $mg.kg^{-1}$ were reported for a population of nursing and pregnant women in Chile, with no known occupational exposure [18].

Mercury is partly eliminated by hair secretion products (hair, beard, body hair). Scientists have been conducting research since the 1960s to try and use the concentration of this element in hair to evaluate exposure [19]. A number of authors have studied exposure to mercury in various populations (c.f. section 1.5). In non-occupational exposure, the mercury levels in hair, which vary with the intake of methyl mercury, ranges between 0.1 and 10 $mg.kg^{-1}$. The different levels of mercury in hair reported in the literature are summarised in Table 1. Mercury in hair is mainly present as methyl mercury [7].

Table 1: Mercury levels reported in hair.

Population	Hg levels reported (in $mg.kg^{-1}$)	Ref.
France, group of 351 non exposed people.	Mean: 1.06 Range: 0.01-10.59	19
Chile, non-polluted area: Polluted area:	Range 0-1.6 Range 1.3-12.8	20
28 non exposed people, England	Range: 0.5-2.5	17
Non exposed people, England. Beard shavings: Head hair:	 0.1-1.5 0.35-2.7	21
Mothers and their babies, Poland Maternal hair: Maternal pubic hair Neonatal hair:	 Mean 1.88, Range 0.02-40.6 Mean 1.10, Range 0-31.8 Mean 0.1, Range 0- 0.623	22

Table 1 continued

Population	Hg levels reported (in $mg.kg^{-1}$)	Ref.
Canada 1976		23
Rural residents:	0.28-3.5	
Urban residents:	0.24-5.4	
Urban near refineries:	0.2-6.1	
Controls 1962:	0.2-4	
Controls 1976:	0.2-8	
Dental assistants:	12-45	
Minamata inhabitants:	70-730	
Fishing families on the Adriatic coast.		24
4-8 seafood meals/week:	Total Hg: 3.38, MeHg: 2.29	
3-5 seafood meals /week:	Total Hg: 3.26, MeHg: 1.86	
2-4 seafood meals/week	Total Hg: 2.44, MeHg: 1.45	
<1 seafood meal/week:	Total Hg: 1.4, MeHg: < D.L.	
China, mothers and their new-born infants.		25
Maternal hair:	Mean: 0.59 ± 0.25	
Infants hair:	Mean: 0.66 ± 0.31	

Table 1 continued

Population	Hg levels reported (in $mg.kg^{-1}$)	Ref.
Non-polluted area of Papua New Guinea. Fish eaters: Non fish eaters:	 Mean: 21.9 ± 11.2 Mean: 0.75 ± 0.4	26
Fish-eating population In the UK: In Japan:	 Mean: 1.6, Range 0.1-16.5 Mean: 3.9, Range 1.0-9.2	27

1.3- Hair

Lenihan [28] states that "Hair is a tissue of interest and value to students of the environment for several reasons:

- it records contamination from inside the body (since it is a route of excretion for many metals) and from outside, since it traps metallic vapours and dust,

- it acts as an integrating dosimeter over a period of months: human head hair grows at about 1 cm per month. Blood and urine, widely studied as indicators of exposure to toxic substances, indicate only recent exposure,

- many heavy metals are found at relatively high concentrations in hair, because of their affinity for the proteins of which hair is largely composed,

- extremely sensitive techniques are now available for the estimation of metals in hair,

- hair samples are easily collected, stored and transported without deterioration, in marked contrast to most other tissues,

- and hair is very durable".

1.3.1- The structure of hair

The body is covered in hair follicles where hair growth begins. There are roughly 100,000 follicles on the scalp and between 500,000 and 1,000,000 on the rest of the body. Follicles lie just below the skin. Hair is formed from materials in the blood and other body fluids. The visible hair is not a living tissue, the continual formation of fresh material from the follicle explains the increase in length of hair.

A typical hair consists of a root and a shaft. The shaft is made up of the cuticle, the cortex and the medulla (Figure 2). The cuticle is a thin outer layer of scales. The cortex is a hollow cylinder of keratin. The medulla is the central cavity of the hair. It is filled with cells of irregular shapes and by tiny air bubbles.The diameter of a typical hair is about 0.1 mm and the mass of a 1-cm length of a single hair is about 100μg. Living cells formed in the follicle are gradually converted into keratin. Keratin is thus composed mainly of dead cells. Many substances can penetrate the outer layers (as vapour or liquid) and become bound to keratin.

Head hair grows at about 1 cm per month. About 90% of the hair on the human head are in the growth phase at any one time, the remaining 10% are in the resting phase. Hair undergo more than one growth phase before falling out. The average life of a head hair is 1000 days.

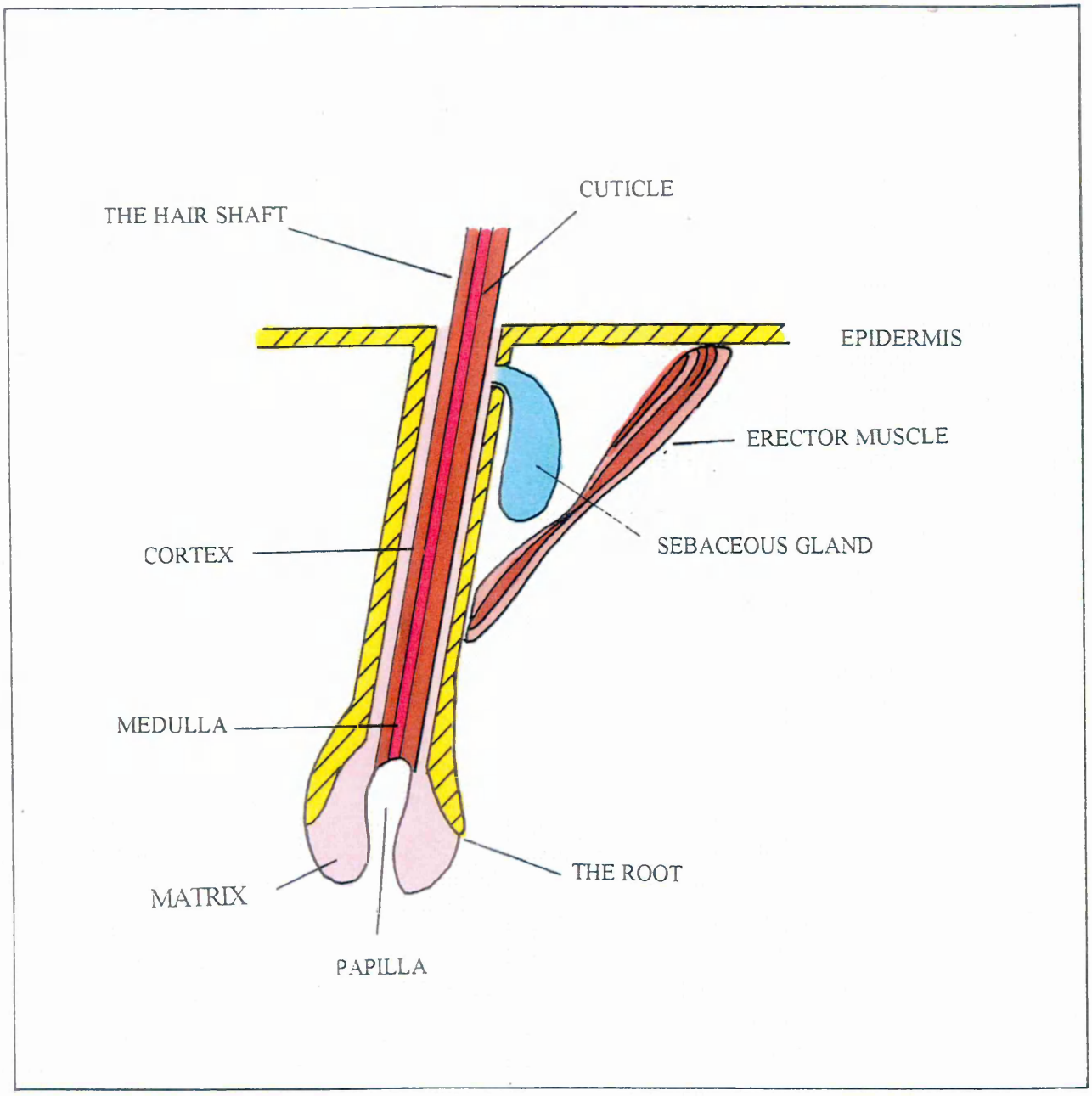


FIGURE 2 : the structure of hair

1.3.2- Metals in hair

The value of hair as a mirror of the chemical environment depends on the fact that it is composed largely of keratin, which is a mixture of proteins. Many metals readily bind to protein molecules. Metal binding is important in the operation of enzymes, many of which need metal ions to fulfil their catalytic functions. The binding of metals to hair protein does no harm but gives useful information about the medium from which the hair was formed, i.e. the blood.

Hair accumulates trace metals to a greater extent than many other tissues because of the abundance of cystine, which is double ended, with two sulphur atoms (the disulphide bridge) in between. Cystine makes up about 14% of human hair. Many metals found in hair are bound to sulphur atoms in cystine and to sulphydryl groups (SH) present in other amino-acids.

1.3.3- Analysis of hair

The analysis of a single hair is not representative because for many elements, the concentration varies greatly among hairs from the same head. This is due to the fact that a hair in the growing phase will contain trace elements recently abstracted from the blood, whereas hair in the resting phase will not.

Besides, interpretation of the results may be complicated by the presence of exogenous contamination from sweat, dust, soaps, and other cosmetic preparations. The concentration of trace elements was reported to vary depending on the collection area on the head, and

also along the length of the hair [29-33]. The differentiation between the matrix proper and extrinsic sources has proved very difficult. [30, 34, 35].

However, it should always be remembered that hair can be non-invasively sampled, and possesses the advantages cited by Lenihan. Standardisation of sample pre-treatment does permit the intercomparison of data and, in some measure, brings a constancy to the contribution from extrinsic sources.

1.4- Determination of total Hg and organomercury species in hair

A summary of the methods published for the determination of total mercury and organomercury species in hair is given in Table 2.

Table 2:

Summary	Comments	Ref.
Development of a method for the isolation of MeHg, based on volatilisation of MeHg cyanide formed in the reaction MeHg + hydrocyanic acid. MeHg cyanide captured on cysteine paper, set free with HCl, extracted in benzene and determined by GC .	Difficulties encountered: formation of persistent emulsions, and volatility of MeHg. Concentrations measured down to 1ng.g^{-1} Up to 20 samples can be analysed per day.	36

Table 2 continued

Substoichiometric extractions of inorganic Hg and MeHg were investigated using thionalide as the extracting agent. Determination of Hg by isotope dilution analysis.	Inorganic Hg at the 1 to 5 μg level and MeHg at the 0.5 to 3 μg level can be extracted.	37
Survey of hair samples in Chile using NAA for analysis.	Hair concentrates Hg about 100 fold from drinking water. Hair was washed with acetone and water following IAEA recommendations.	20
A CV-AAS based method of analysis is used and the % recovery of Hg is 73% from human hair samples when peak height evaluation is used. It is raised to 102% with peak area measurement. The so-called matrix effect is eliminated and its origin shown to be in the slower release of Hg from biological samples.	Detection limit: 1 $ng.g^{-1}$ The matrix effect appears to vary significantly between samples. Low sample throughput.	38

Table 2 continued

Summary	Comments	Ref.
Inorganic and organomercury are selectively reduced by $SnCl_2$ and $NaBH_4$, respectively. The volatilised elemental mercury is determined by CV-AAS.	Detection limits are in the 0.003 to 0.005 $ng.g^{-1}$ range. Hair was washed with laboratory glassware detergent and an ultrasonic cleaner. Samples were digested with KOH.	39
An improved technique for the determination of inorganic and total Hg by the Magos method , based on the use of a new reaction vessel, is described.	Sample throughput, precision, accuracy and sensitivity are increased, amount of sample and reagents needed is reduced. Less than 0.5 ng Hg can be detected in 200 µl of sample.	40
The acid oxidation of hair and the determination of Hg by CV-AAS have been investigated. Two novel features were shown: the difference between scalp hair Hg levels of vegetarians and non vegetarians, and the variations in scalp hair Hg levels within individual scalps.	Hair was cut at random from diverse places on the scalp and analysed without washing. Absolute limit of detection: 1 ng Hg	17

Table 2 continued

Summary	Comments	Ref
A simple, rapid, precise and accurate method for the determination of mercury is described. Biological samples were digested with nitric acid and acidified potassium permanganate, and determined by CV-AAS.	Hair samples were washed for two hours with diethyl ether and allowed to dry in air for 24 h. Hair from males is found to contain more Hg than hair from females.	41
The determination of Hg by instrumental NAA has been studied with particular emphasis on hair.	Limit of detection: 10-20 $ng.g^{-1}$. The experiments in this work suggest the disappearance of Hg salts by volatilisation during the irradiation process, which creates problems with recoveries.	21
The % recovery of mercury in the CV-AA analysis in various biological matrices is measured.	Hair, urine and fish samples give nearly quantitative recoveries. Milk and brain samples give recoveries of 90%, and liver samples 85%. Changing from peak height to peak area is not enough for some matrices.	42

Table 2 continued

Summary	Comments	Ref
The concentrations of Hg, MeHg and Se in scalps of persons from different geographical areas with different dietary habits, are described. CV-AAS, GC and RNAA were used.	Hair samples are cleaned according to the IAEA recommendations. For total mercury the samples are digested with nitric and sulphuric acids and hydrogen peroxide, and analysed by CV-AAS. For MeHg, a partly modified Westoo method is used, and GC is used for detection.	43
Parameters affecting the accuracy of MeHg determination are studied. Different isolation techniques (ion exchange, extraction, volatilisation, distillation) and final measurement via CV-AAS or GC are compared. Isolation techniques are comparable. A new separation technique based on distillation of MeHg is developed.	Absolute detection limit for standard solution: 5 pg MeHgCl and EtHgCl. The main advantage of the distillation/extraction isolation of MeHg over extraction is better recovery, due to avoidance of difficulties associated with the formation of emulsions.	44

Table 2 continued

Summary	Comments	Ref
A procedure for the determination of Hg by CV-AAS with a new reaction vessel is developed and evaluated.	This system can accommodate 20 samples per hour. The hair samples are washed following the recommended IAEA procedure, and dried in a vacuum desiccator for 24h. Samples are digested with nitric acid and oxidised with a permanganate solution. Detection limits: 0.1 $ng.g^{-1}$ and 0.44 $ng.g^{-1}$ for inorganic and organic Hg, respectively.	19
A simple modification of the Westoo extraction procedure for MeHg, and its determination by GC is presented. The cysteine clean-up step has been modified, using cysteine-impregnated paper, instead of cysteine solution. The isolated MeHg is released with sulphuric acid , extracted into benzene, and determined by GC.	Detection limit: 0.01 $ng.g^{-1}$. Avoidance of difficulties arising from emulsion formation, cleaner chromatographs, faster analysis. Hair samples are washed following the IAEA recommended procedure and decomposed using the Westoo method.	45

Table 2 continued

Summary	Comments	Ref
A sensitive atomic fluorescence system for the determination of Hg is optimised. A new gas/liquid separator is developed. The system is associated with a microwave oven for dissolving the samples.	A detection limit of 0.9 ng $Hg.l^{-1}$ is obtained (absolute amount detected: 2 pg Hg). 100 mg sample + 1 ml nitric acid are placed in sealed digestion vessels in a microwave oven. The samples are not washed before analysis.	46
This paper describes radiochemical separation procedures developed for the determination of As, Cr, Hg, Sb, and Se in biological reference materials. The methods chosen utilise ion exchange, retention on inorganic exchangers and solvent extraction techniques for the isolation of the elements of interest.	Sample dissolution is carried out in a normal oven or microwave oven, and using radioactive tracers with a mixture of nitric and sulphuric acid or nitric acid only. Hair presents some difficulty for total dissolution with nitric acid only.	47

Table 2 continued

Summary	Comments	Ref.
In order to evaluate the health risk regarding Hg toxicity due to seafood intake, an analytical protocol for Hg, MeHg and Se is prepared. Hg is determined by AAS, and MeHg by GC-EC.	Hair samples are washed 5 times (twice with acetone and 3 times with water) and left to dry in air. MeHg is determined by the partially modified Westoo method. Digestion of the samples for total Hg is carried out with nitric and sulphuric acids.	24
Relationships are established between the concentration of Hg in human scalp hair and environmental or dietary mercury exposures. The use of hair analysis for evaluating Hg intoxication is supported.	Normal range reported for mercury in hair: 2.5 to 5 $mg.kg^{-1}$.	27
FI-ICP-MS is evaluated for determining the distribution profile of trace elements along a single strand of hair.	Hair samples are solubilised with nitric acid. The recent past history of Hg exposure in individuals can be reconstructed for intervals, lasting several days to weeks. Detection limit: 0.1 $ng.ml^{-1}$	48

Table 2 continued

Summary	Comments	Ref.
A FI-ICP-AES method for the determination of Hg is described, based on the complexation of the metal ion with DPTH, and its subsequent on-line extraction into MIBK.	Detection limit: 2 $ng.ml^{-1}$. The hair samples are prepared by microwave digestion with nitric and hydrochloric acids.	49
A method for the selective determination of inorganic mercury and methyl mercury in tissues by continuous flow and CV-AAS is described.	Samples were solubilised with NaOH, cysteine and NaCl at 60°C for 30 minutes. Recoveries obtained were above 95%.	50
A two stage gold amalgamation technique is compared with a technique employing a single gold trap. When peak area is measured, and special attention paid to the gold trap orientation, the one-stage procedure provides the same results as the two-stage technique, with the analysis time reduced from 10 to 2 min.	Absolute detection limit of less than 1 pg Hg is obtained with an AFS detector.	51

Table 2 continued

Summary	Comments	Ref.
A method for the determination of mercury in hair is described. The method is based on the selective leaching of MeHg from hair with HCl, followed by determination with CV-AAS. The results are favourably compared with results obtained by the solvent extraction method.	Hair samples are washed following IAEA recommendations. Acid leaching is carried out by shaking hair samples with 2M HCl for 4h. This method is suitable for the determination of mercury levels down to 10 $ng.g^{-1}$.	52
A method for the simultaneous determination of MeHg, inorganic Hg and total Hg is developed. Biological samples are digested in KOH, then MeHg and Hg^{2+} are converted to volatile ethyl derivatives, which are preconcentrated onto a trapping column and then thermally desorbed into a GC column. Eluted Hg compounds are decomposed into Hg^0 and detected by CV-AFS.	Absolute detection limits: 0.6 pg MeHg and 0.3 pg Hg^{2+} (or 0.3 $ng.g^{-1}$ MeHg and 0.6 $ng.g^{-1}$ Hg^{2+}). Hair samples were digested with KOH at 75°C for 3h. The pH is critical for the ethylation step.	53

Table 2 continued

Summary	Comments	Ref.
CV-AAS with a laboratory-built system using an Au-Pt grid for Hg amalgamation , is applied in the determination of total mercury in human scalp hair. Two digestion procedures with nitric acid are compared: in a PTFE bomb for 1.5h at 110°C or in a sealed Pyrex ampoule for 24h at 50°C.	The absolute detection limit is 0.13 ng Hg and the limit of determination is 0.11 $\mu g.g^{-1}$ in hair. The sealed Pyrex ampoule method is preferred. Hair is washed by the IAEA recommended procedure before digestion.	18
An adaptation of a two-stage gold amalgamation preconcentration step combined with CV-AFS detection is described. It is used for the determination of total Hg in order to monitor chronic low-dose exposure.	The detection limit is 50 $pmol.l^{-1}$ (which corresponds to 50 $ng.g^{-1}$ when sample mass < 10 mg) Samples are digested in nitric and sulphuric acid.	54

Table 2 continued

Summary	Comments	Ref
The application of the combination of GC and a non-commercial plasma emission detector for organomercury speciation is presented.	MeHg and Hg^{2+} are converted into volatile species by ethylation with sodium tetraethylborate. Methanolic NaOH is used for extraction. Absolute detection limits: 15 pg MeHg, 10pg Et_2Hg and 12 pg Me_2Hg.	55
Hair samples from mothers and their new-born babies were collected and analysed by NAA, GC-EC and SRXRF.	The hair samples are washed according to the IAEA recommended procedure. MeHg is determined by GC-EC after acid leaching with HCl.	25
A new technique for the determination of MeHg and Hg^{2+} in hair samples by HPLC-PCO-CVAAS is presented.	Organomercury compounds are extracted by a buffered sodium pyrrolidinedithiocarbamate solution, separated by reverse-phase HPLC, oxidised to Hg^{2+} by UV, reduced to Hg^0 with alkaline $NaBH_4$, and determined by CV-AAS. The limit of detection is 4 $ng.g^{-1}$. Hair samples are washed with acetone prior to extraction.	56

Table 2 continued

Summary	Comments	Ref.
A simple method for simultaneous determination of inorganic and total Hg contents in human hair by NAA is developed. It is based on the selective extraction of MeHg from hair by HCl.	Hair samples are shaken for 4h with 2M HCl to separate MeHg. Inorganic Hg is measured by NAA in the remaining solid phase of the leached hair.	57

1.5- Population studies involving mercury

A summary of the papers describing population studies involving mercury and hair is given in Table 3 below.

Table 3:

Title of Paper	Population studied and comments	Ref
Scalp hair as a monitor of population exposure to environmental pollutants.	310 Canadian with varied types of environmental exposure. Rural and urban controls exhibited low levels of mercury, whereas people residing near lead and gold refineries, and other industries have shown high elemental concentrations in their hair.	23
Pregnancy outcome in female dentists.	1,615 female dentists in the UK. Clinical dental practice is thought to be associated with an increased frequency of spontaneous abortions, especially late abortions, and premature deliveries.	58
Radioactivation analysis of hair as a means of biological monitoring of the environment.	382 Japanese, men, women and school-children. Hair samples from men revealed higher concentrations of mercury than hair samples of women. Frequent fish eating and a habit of smoking tobacco were found to increase the Hg concentration in hair.	59

Table 3 continued

Title of Paper	Population studied and comments	Ref
Mercury in neonatal scalp hair.	141 Polish mothers and their new-born babies. A statistically significant inverse correlation was found between the Hg concentration in the hair of a new-born and its birthweight. Excessive exposure to Hg in the early postnatal period negatively influences psychosomatic development of the exposed infant.	22
Examination of blood levels of mercurials in practising dentists using CV-AAS.	205 practising dentists in the USA. Significant enzymatic conversion of inorganic to organic Hg compounds does not occur *in vivo*. Hg vapour can be released from dental amalgams by vigorous and prolonged chewing.	60
Quality of community drinking water and the occurrence of spontaneous abortion.	286 women having a spontaneous abortion and 1391 women having livebirths in the USA. An increase in the frequency of spontaneous abortions was associated with detectable levels of Hg. High doses of MeHg were associated with frequent abortions amongst women undergoing Hg treatment for syphilis.	61

Table 3 continued

Title of Paper	Population studied and comments	Ref
Methylmercury: significance of intrauterine and postnatal exposures.	204 babies and mothers in Faroe islands. MeHg has a particularly long elimination half-time in infants, and Hg can be transferred through breastfeeding. An average maternal hair Hg concentration of 15 $\mu g.g^{-1}$ was associated with decreased test performance.	62
Association of high hair mercury content with increased wall thickness growth in carotid arteries: a 4-year follow-up study in over 1000 men.	1,019 men in Finland. Accumulation of mercury in the body is associated with accelerated atherosclerotic progression in men.	63
Milestone development in infants exposed to MeHg from human milk.	1022 births during a 21 month period in 1986-1987. Early milestone development is clearly associated with breastfeeding , which is also related to increased hair Hg levels. This is contrary to what would be expected from possible neurotoxic effects of Hg.	64

Table 3 continued

Title of Paper	Population studied and comments	Ref
NAA, GC(EC), and SRXRF study of Hg species in head hair of mothers and their new-born infants.	27 Chinese mothers and their new-born infants. Significant positive correlation between Hg content in maternal and infant head hair is found. This study suggests that women must avoid any Hg exposure during the gestation period and during nursing.	25
High hair and urinary Hg levels of fish-eaters in the non-polluted environment of Papua New Guinea.	134 fish eating and 13 non fish eating subjects investigated. In fish the average percentage of inorganic mercury is less than 20% of total mercury.	26
Dietary Hg intake and human exposure in an Adriatic population.	33 households with 92 family members in a very isolated island off the coast of Croatia. Hg levels in adults' hair did not reach critical levels of 50 $mg.kg^{-1}$ at which toxic effects could be expected. The obtained data do not suggest that fish consumption in adult population should be discouraged, even if taken on a daily basis	65
Exposure of Canadian aboriginal peoples to MeHg.	34,571 individuals were screened over 20 years. Blood, hair and umbilical cord blood samples were collected. Although very high levels of mercury were reported, no definite diagnosis of MeHg poisoning was made.	66

Table 3 continued

Title of Paper	Population studied and comments	Ref
Hg exposure in humans through food consumption from the Everglades of Florida.	350 people, living in the Everglades. The limitation of the consumption of several fish species caught from the Everglades is recommended. The death of at least one Florida Panther has been attributed to Hg poisoning. Age, sex and race were significantly associated with total hair Hg levels.	67
Total Hg conc. in human hair from 13 countries in relation to fish consumption and location.	559 hair samples from 39 locations in 13 countries were analysed. A significant correlation between fish consumption and hair mercury levels is reported.	68
Mercury toxicity in the pregnant woman, foetus and new-born infant. A review.	Minamata and Iraq cases are studied. It is recommended that women of child bearing age should not eat more than 350g fish per week.	69
Human milk as a source of methylmercury exposure in infants	583 children in a birth cohort in the Faroe Islands. Hg in hair increases with nursing period: human milk is an important source of MeHg. The elimination of this compound during the first year of life is slow or absent. The prudence of prolonged nursing (beyond 6 months) may need to be considered.	70

Table 3 continued

Title of Paper	Population studied and comments	Ref
Mercury in the umbilical cord: implications for risk assessment for Minamata disease.	50 births from the Faroe Islands. The umbilical cord blood mercury levels are correlated with maternal hair mercury levels.	71
Duplication diet study on mercury intake by fish consumers in the UK.	Hair samples were analysed for mercury in 942 persons living in either one of two coastal areas of the UK who reported above average fish consumption. People in the UK are unlikely to be adversely affected by the presence of MeHg in the fish they consume.	72

1.6- Analytical methods: a review

Combinations of a broad spectrum of sample preparation procedures with many different analysis techniques have resulted in hundreds of publications on mercury determination in biological materials during the last decades. Previous reviews on this subject include Manning (1970, atomic absorption) [73], Lindstedt and Skerfving (1972) [74], Smith (1972) [75], Uthe and Armstrong (1974) [76], Chilov (1975) [77], Ure (1975, atomic absorption and fluorescence) [78], Zmijevska (1977, activation analysis) [79], Nitschke *et al.* (1989) [80], Shimomura (1989) [81], and Schutz *et al.* (1994) [7], and the reader is referred to these for detailed background information to this work.

1.6.1- Determination of total mercury

1.6.1.1- Atomic Absorption Spectrometry (AAS)

The method of determination of mercury by atomic absorption was introduced in the 1930s by Woodson (1939) to control air mercury levels in the workplace. This method is based on the absorption by ground state atoms at 253.7 nm from a mercury discharge lamp. The main mercury resonance line at 185 nm can also be used, and it is claimed to give 10 times better sensitivity. However, it is strongly absorbed by oxygen, and as a result it is of little practical value.

The detection limit depends on the technique for transferring mercury from the sample into ground state atoms in the gas phase, and the various techniques are described below:

1.6.1.1.1- Mercury vaporisation by heat

This technique involves the adsorption of mercury from sample digests on cadmium-impregnated asbestos filters, followed by thermal release. Mercury compounds have the property to decompose quantitatively into ground-state atoms on heating. The detection limits reported were generally in the range 1-10 ng. g^{-1} in biological samples.

1.6.1.1.2- Mercury vaporisation by Cold-Vapour (CV) technique

CV-AAS utilises the unique property of Hg^0 to easily escape from aqueous solutions into the gas phase through aeration. It is by far the most common technique for the determination of mercury in biological samples. The CV liberation of mercury from sample solutions is also used with other analytical techniques such as AFS, ICP-OES and ICP-MS.

The reduction of the mercury to Hg^0 is usually achieved by addition of an excess of Sn^{2+} to the solution. Ionic mercury is reduced by Sn^{2+} in acid or alkaline solutions.

Another frequently used reducing agent is sodium borohydride $NaBH_4$. It is a stronger reductant than Sn^{2+}. This method has been widely used for the determination of mercury in hair [17-19, 40, 41, 51]. Detection limits of 1-4 ng.g^{-1} [38, 56] are commonly reported. Absolute detection limits of 100 pg were also reported [17, 46].

Interferences in the determination of mercury by CV-AAS could result from the composition of the sample solution, which affects the release rate of Hg^0, or from gaseous compounds which absorb at 254 nm, or from light scattering particles in the gas cell.

The sensitivity of the CV-AAS technique may be further improved by preconcentration of the liberated mercury through amalgamation, followed by thermal release and AAS. A gold filter is most often used for the amalgamation [51]. The detection limits for these techniques is of the order of 10-100 $ng.l^{-1}$, but values as low as 0.1 $ng.l^{-1}$ have been reported [82]. An absolute detection limit of 130 pg has been achieved [18].

CV-AAS is rapid, inexpensive, suitable for routine analysis, and in combination with a preconcentration stage using a gold trap, very low detection limits are possible.

1.6.1.1.3- Flame Atomisation (F-AAS)

Flame AAS suffers from several disadvantages, such as inefficient sample uptake through the spray chamber and background effects from the sample matrix and the flame. This results in a rather poor detection limit, which is, at best, 300 $\mu g.l^{-1}$.

1.6.1.1.4- Graphite Furnace (GF-AAS)

This is a highly sensitive method, but in the case of mercury, the extreme volatility often leads to severe losses during the drying-ashing steps. The preatomisation losses can be

minimised by addition of a suitable matrix modifier, which forms thermostable mercury compounds and thus acts as a mercury trap. Detection limits of 5 $\mu g.l^{-1}$ for blood, and 10 $ng.g^{-1}$ for fish tissue, have been reported [7].

There are several disadvantages associated with the use of AAS as the detection method. These result from the limited linear calibration range, and spectral interference arising from non-specific background absorption of volatile organic species.

1.6.1.2- Atomic Fluorescence Spectrometry (AFS)

The sensitivity of AFS is directly proportional to the excitation energy, and can thus be enhanced by increasing this energy. Compared to AAS, simpler and more energy rich excitation sources can be used, since the line width of the resonance lines is of minor importance. The electronics are also simpler in AFS. The great advance for AFS in mercury analysis is associated with the CV atomisation technique. The aeration gas is usually argon, as aeration with air causes quenching of excited mercury atoms by oxygen molecules. For the determination of mercury in hair [46, 51, 53, 54], detection limits of 0.9 $ng.l^{-1}$ [46], or absolute detection limits of 2 pg [46], less than 1 pg [51], 0.6 pg MeHg and 1.3 pg Hg^{2+} [53] have been reported.

Generally, lower detection limits seem to be obtainable by CV-AFS, and the linear range exceeds that of AAS by at least one order of magnitude.

1.6.1.3- Atomic Emission Spectrometry (AES)

Methods have been published for the determination of mercury by AES after ionisation in a gas plasma, usually argon. Besides Inductively Coupled Plasma, other sources that have been used include direct current and microwave induced plasmas. The advantages of AES include multi-element capability, a linear dynamic range of 4-6 orders of magnitude, and low detection limits of 0.1 $ng.g^{-1}$ dry weight [7].

1.6.1.4- Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

The determination of mercury by this technique seems to be free from interferences. However, several users of ICP-MS have experienced severe memory effects from samples with high mercury levels. An advantage of using ICP-MS is that it is possible to distinguish between isotopes. The most abundant isotope, ^{202}Hg (30% abundance) is normally used for analysis.

Detection limits of 200 $ng.l^{-1}$ in serum have been reported [7]. Instrument manufacturers have claimed detection limits of 1 $ng.l^{-1}$ when using flow injection and cold vapour generation. The use of flow injection with ICP-AES was reported for the successful determination of total mercury in hair [49], with a detection limit of 2 $\mu g.l^{-1}$. The use of ICP-MS allows the determination of trace element levels along single strands of hair [48], and a detection limit of 0.2 $\mu g.l^{-1}$ was reported.

The presence of sodium is claimed to suppress the mercury signal [7].

1.6.1.5- Neutron Activation Analysis (NAA)

The irradiation with thermal neutrons of stable isotopes of mercury and many other elements makes possible their conversion into radioactive daughter isotopes, that can be identified by gamma spectrometry. NAA is a well established multi-element technique and it is known to be very accurate.

The radioactive isotopes used for analysis are ^{197}Hg and ^{203}Hg. Because of interferences with other elements, the determination of mercury in biological samples at low levels is usually carried out with a radiochemical technique (RNAA), which involves the chemical separation of mercury from other interfering elements. This separation is usually carried out after irradiation. Several methods of separation have been reported, and these include:

- distillation and electrolytic deposition on a gold foil, with reported detection limits of 10 $ng.g^{-1}$,

- dry ashing of the irradiated sample and separation by selective adsorption onto a strip of selenium-impregnated filter paper,

- anion-exchange, with detection limits of 0.05 $ng.g^{-1}$, and

- separation by solvent extraction.

The development of higher resolution detectors has allowed the use of non destructive NAA, or instrumental NAA (INAA), where chemical separation is no longer necessary. The counting of the irradiated samples after varying decay periods enables the analyst to determine mercury in the presence of other radionuclides with different half times. The calculated detection limit for mercury in blood is 3.5 $\mu g.l^{-1}$ [7].

This method has been applied to the determination of total mercury in human hair [20, 21, 23, 59]. Inorganic mercury at the 1 to 5 μg level, and MeHg at the 0.5 to 3 μg level can be extracted and detected [37].

The advantages of NAA in analytical practice include good sensitivity, precision and accuracy. A major disadvantage is that a neutron source is not always available.

1.6.1.6- Other radiometric methods

1.6.1.6.1- Substoichiometric isotope dilution analysis (SIDA)

Described by Ruzicka and Lamm (1969), this method involves the burning of biological samples in oxygen, then extraction with dithizone and back extraction in an aqueous phase. A known amount of ^{203}Hg and dithizone are added. The mercury dithizonate is extracted into carbon tetrachloride and the activity is measured. Detection limits of 2 $ng.g^{-1}$ dry weight for biological samples were obtained. Among other complexing agents for SIDA analysis of mercury are thionalide, and DBBT.

1.6.1.6.2- Isotope Exchange

This method is based on a stoichiometric exchange between stable and radioactive isotopes. Interferences from other elements are limited, and detection limits in the low $ng.g^{-1}$ range seem to be obtainable.

1.6.1.7- X-ray Spectrometry

X-Ray Fluorescence (XRF) and Particle-Induced X-ray Emission (PIXE) are rapid, non destructive and multi-elemental methods. They also allow direct analysis of the samples without preparation. However, they are not very sensitive, and thus require a pre-concentration stage. These methods have usually been applied to tissues such as hair, nails and teeth, which normally contain much higher concentrations of mercury than soft tissues. XRF has been applied to investigations of single hair strands. Longitudinal studies in human hair have been carried out after the mercury poisoning in Iraq in 1971 [83].

1.6.1.8- Electrochemical methods

1.6.1.8.1- Anodic Stripping Voltammetry (ASV)

This is one of the most sensitive electrochemical methods used. It can be applied using a thin film electrode (TFE). Identification of the metal deposited is achieved by measuring the potential between the working electrode and a reference. Silver and chloride are known

to cause interferences. A detection limit just below 1 $\mu g.l^{-1}$ was reported for aqueous solutions.

1.6.1.8.2-Potentiometric Stripping Analysis (PSA)

This is a more recent development. A detection limit of 45 $ng.l^{-1}$ was reported. This required rather long analysis times.

Electrochemical methods have the advantage of requiring relatively low cost instrumentation, however, the need for a good knowledge of redox systems may be a disadvantage [7]. Other disadvantages include a limited sample throughput capability and ease of contamination of the electrodes.

1.6.2- Sample preparation

A considerable amount of work has been dedicated to the preparation of biological materials for the determination of mercury, and consequently many publications are available. A review by A. Schutz *et al.* [7] covers a number of important aspects, and a summary is given here.

1.6.2.1- Prevention of contamination and loss

Because mercury is present almost everywhere, sampling and sample handling are important aspects of any determination of mercury in biological samples. Great care must

be taken to avoid contamination of the samples. All containers and reagents must be screened for mercury contamination.

However, contamination is not the only problem, especially during the storage of samples. The loss of mercury also needs to be taken into account. In hair, inorganic and organic mercury are often strongly bound to the matrix and thus stabilised. Decomposition of MeHg in hair was found to be insignificant after years of storage.

The storage of aqueous samples containing low levels of mercury ($\mu g.l^{-1}$) is more difficult as considerable losses have been reported. Furthermore, mercury ions and compounds were reported to adsorb onto surfaces of glass and polymer materials. Many preservatives have been proposed to help prevent losses of mercury in aqueous samples: cysteine, gold in dilute nitric acid, hydrochloric acid, nitric acid with potassium dichromate and many others. The data on the preserving capacity of the different additives is sometimes contradictory.

Aqueous alkyl and methyl mercury samples should be protected from daylight as they decompose at UV irradiation. It is important to note that the use of preservatives does not guarantee the specificity of the mercury compounds: decomposition of MeHg was reported when cysteine, dilute nitric acid, potassium dichromate and hydrochloric acid, or dilute acids with hydrogen peroxide were used.

Pre-treatment of the glassware with acid and a dichromate solution was reported to reduce losses. Some contradictory results were obtained as regards the best container for

methylmercury standards, so it seems more appropriate to avoid storing low concentrated standards of MeHg.

1.6.2.2- Sampling

Hair samples are obtained by clipping. If recent exposure is of interest, hair should be obtained from close to the scalp. If one wants to assess earlier exposure, strands of hair should be obtained. The hair may then be cut into pieces, one cm representing approximately one month of growth.

1.6.2.3- Washing of the samples

The removal of surface contamination from hair samples is one of the major problems encountered in hair analysis if the surface contamination is to be distinguished from the trace elements incorporated in the body.

Various washing procedures were described in the literature and it is difficult to conclude which procedure is best suited for mercury in hair. Most washing procedures use acetone/ether, hexane/ethanol, a detergent or EDTA. These washing procedures all involve the soaking of hair for 10-30 minutes in the first solvent, with shaking, decantation and then soaking in the second solvent. The washes may be repeated several times. The samples are then rinsed repeatedly with distilled water and dried in a clean environment for several hours, either at room temperature or in an oven at 50-60°C.

Losses of mercury during drying, even at low temperatures have been reported for biological samples [7].

1.6.2.4- Digestion of the samples

Wet digestion has usually been the method of choice for hair samples. The digestion of hair samples can be carried out with a single mineral acid, but complete decomposition of organic matter can only be achieved if a strong oxidising agent such as perchloric acid or hydrogen peroxide is used. An increase in temperature during the digestion also improves the efficiency of the oxidising agents.

However, this is to be carried out with care as losses of mercury by volatilisation can occur very easily. The choice of digestion vessels can play an important role in this: closed vessels allow the heating of the samples while at the same time preventing losses by volatilisation.

1.7- Methods used in this work

1.7.1- Microwave digestion

The development of radar during World War II stimulated the rapid growth of microwave technology. The first microwave heating applications soon followed, and these included heating food with microwave energy. Investigation of industrial applications of microwave heating also began in the 1940's. Microwave heating involves direct absorption of energy (Figure 3) by the material to be heated. Because of the unique and stringent requirements of sample preparation, domestic microwave ovens were inadequate for the chemist's needs.

Until very recently, the proper hardware that would allow the analyst to fully use the properties of microwave heating was not available.

1.7.1.1- Theoretical concepts

Microwaves are electromagnetic energy. Microwave energy is a non-ionising radiation that causes molecular motion by migration of ions and rotation of dipoles, but does not cause changes in molecular structure. Microwave energy has a frequency range from 300 to 300,000 MHz. The frequency most commonly used in home microwave units is 2450 MHz. The typical energy output in a microwave system is 600-700 W.

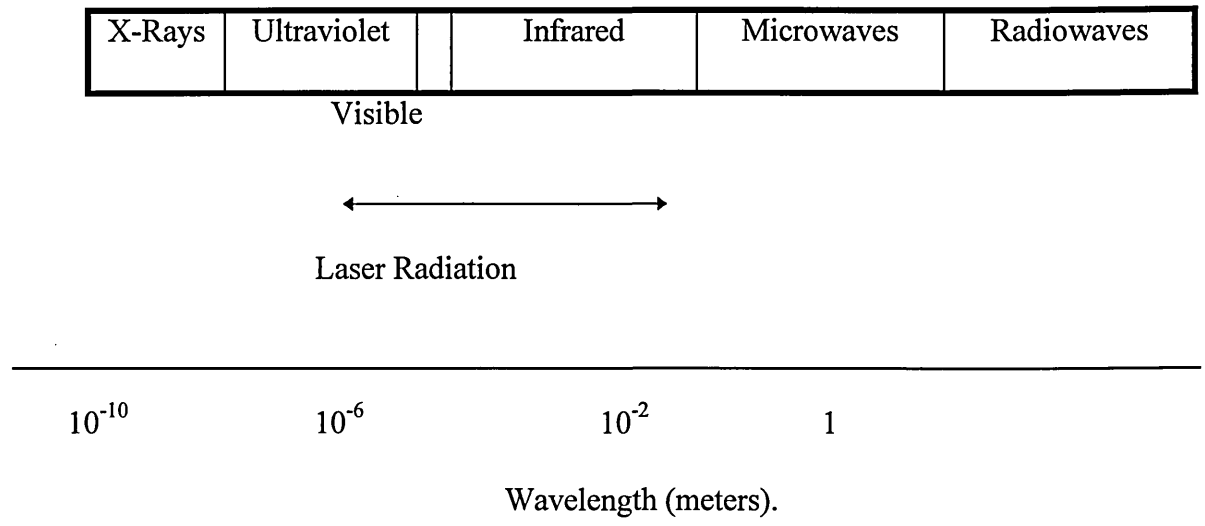


Figure 3: The electromagnetic spectrum

The typical time required to complete a wet digestion by conductive heating ranges from 1 to 2 hours. Sometimes it can be much longer. Dissolutions by microwave heating can be completed between 1-15 minutes. Vessels used in conductive heating are usually poor

conductors of heat, so it takes time to heat the vessel and transfer that heat to the solutions. Also, because of thermal gradients, only a small portion of the fluid is at the temperature of the heating source. On the other hand, microwaves heat all of the sample fluid simultaneously without heating the vessel. Therefore, the solution reaches its boiling point very rapidly.

1.7.1.2- Microwave instrumentation

The typical microwave instrument used for heating analytical samples consists of six major components: the microwave generator (the magnetron), the wave guide, the microwave cavity, the mode stirrer, a circulator and a turntable.

Microwave energy is produced by the magnetron, propagated down the wave guide, and injected directly into the microwave cavity where the mode stirrer distributes the incoming energy in various directions (Figure 4). The percentage of the incoming energy absorbed depends on the sample size and rate of energy dissipation.

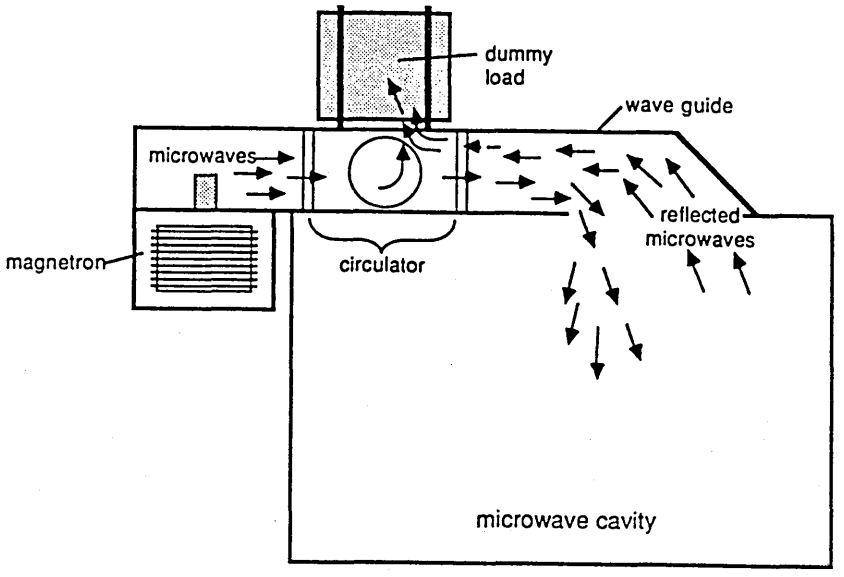


Figure 4: Schematic diagram of a microwave digestion oven [84]

1.7.1.3- Digestion vessels

Containers are constructed from materials that do not absorb microwaves, but let them pass through to the solution inside. Teflon and Polystyrene are excellent materials. Glass and fibreglass can also be used. Teflon PTFA is ideal for vessel construction for almost all acid dissolutions, because it is resistant to most acids, and has a melting point of 306°C. However, phosphoric and concentrated sulphuric acids should not be used in PTFA vessels without temperature control as their boiling point is higher than the melting point of PTFA. Recently, silica vessels have become available for use with microwave digestion, thus facilitating the use of high boiling point acids.

Closed-vessel systems for acid dissolutions have a number of advantages. Firstly, the pressure raises the boiling point of acids, achieving higher temperatures, which in turn reduces the time required for the digestion. Secondly, the possibility of losing volatile elements is virtually eliminated by the closed system. Thirdly, less acid is required as no evaporation occurs. Fourthly, the potentially hazardous fumes produced are contained within the vessel, and the possibility of airborne contamination is greatly reduced, and finally, closed-vessel digestion in a microwave requires very little time. The uniformity of heating multiple vessels can be greatly increased by rotating the vessels on a turntable [84]. The use of carousels greatly increases the sample throughput.

1.7.2- Cold Vapour Atomic Fluorescence Spectrometry

1.7.2.1- The Cold Vapour Technique

Cold Vapour utilises the unique property of Hg^0 to escape easily from aqueous solutions into the gas phase through aeration. Stannous chloride is used to reduce the mercury in solution to its elemental form Hg^0. A gas (usually argon) is bubbled through the solution, liberating mercury from the aqueous phase to the gas phase, which is then carried to the detector. This is by far the most common technique for the determination of mercury in biological samples [7]. Typically the limit of detection for the cold vapour technique is in the lower $ng.l^{-1}$ range when associated with AFS.

1.7.2.2- Atomic Fluorescence Spectrometry

1.7.2.2.1- Introduction

Atomic fluorescence spectrometry may be defined as the measurement of radiation from discrete atoms that are being excited by the absorption of radiation from a given source which is not seen by the detector. The fluorescence of atoms in flames was first reported by Nichols in 1923. The first analytical method was developed by Winefordner and his co-workers in1964. In atomic absorption spectrophotometry, the subsequent history of the energy absorbed by the atoms is of little concern. Much of the energy is lost by collisional deactivation within the flame gases. However, some of the energy imparted to the atoms is re-emitted in all directions and this phenomenon is the basis of atomic fluorescence [85].

1.7.2.2.2- Theoretical considerations

The basic atomic fluorescence arrangements consist of an intense source focused on to an atomic population in a flame or atom reservoir. Fluorescence radiation, which is emitted in all directions, then passes to a detector in the same plane, usually positioned at right angles to the incident light (Figure 5). The source may be either an atomic line source or a continuum, and serves to excite atoms by the absorption of radiation at a given wavelength. The atoms are then deactivated, partly by collisions and partly by emission of fluorescence radiation, in all directions.

The wavelength of the fluorescence radiation is generally the same or longer than the incident radiation. The wavelength of the emitted radiation is characteristic of the

absorbing atoms, and the intensity of the emission may be used as a measure of their concentration.

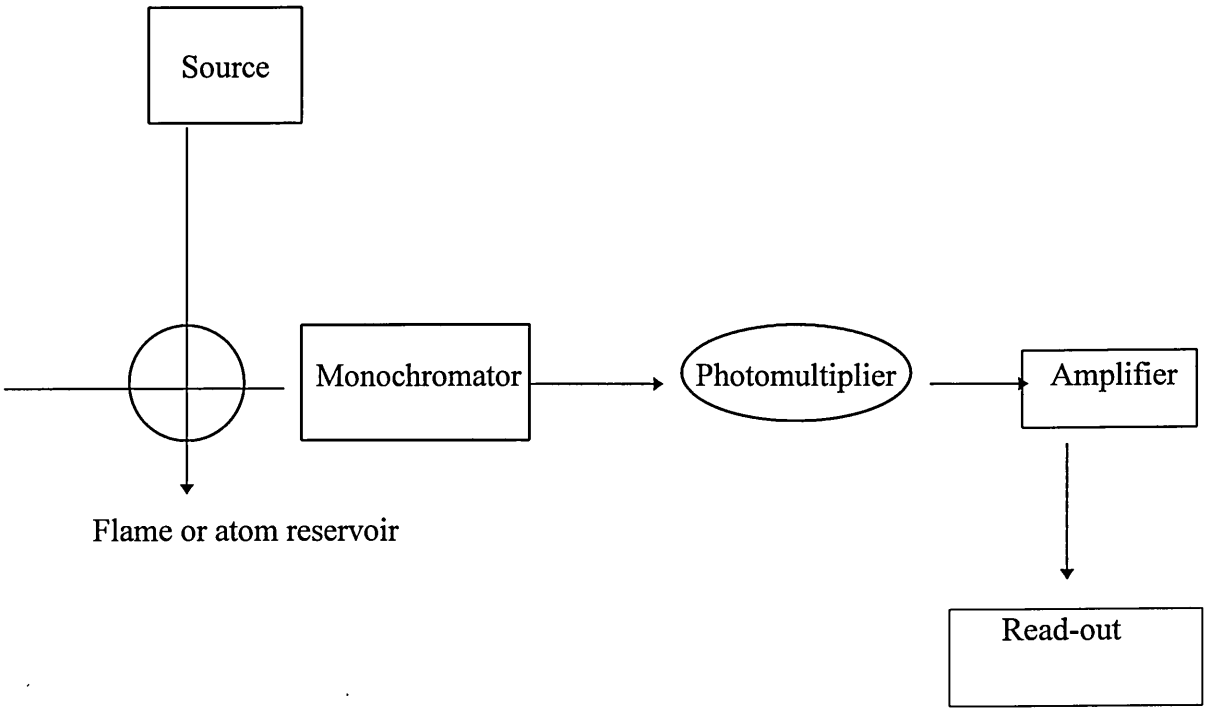


Figure 5: Schematic representation of atomic fluorescence

At low concentrations, the intensity of the emission is governed by the following relationship:

$$I_f = K\theta I_o C$$

where:

- I_f is the intensity of the fluorescent radiation
- K is a constant
- I_o is the intensity of the source at the absorption line wavelength
- θ is the quantum efficiency for the fluorescence process, it may be defined as the ratio of the number of atoms which fluoresce from the excited state to the number of atoms which undergo excitation to the same excited state from the ground state in unit time.

1.7.2.2.3- Equipment considerations

Microwave lamps (E.D.L.s) are the most commonly used sources. Vapour discharge lamp sources can be used, but they are only available for a relatively limited number of elements (Cd, Ga, Hg, In, Tl and Zn). Their advantages are that they are intense, stable and use inexpensive power supply units. Low pressure mercury vapour lamps are most frequently used in filter fluorometers. The arc discharge is diffuse and of little intensity, but very stable. The most intense mercury line is at 253.7 nm.

To avoid background noise, a monochromator can be used. Ideally, the monochromator should have a high resolution, large aperture and low level of stray light. The optics should be arranged so that the excited region of the atom reservoir completely fills the monochromator aperture. As a radiation detector, a photomultiplier with low dark current and high sensitivity is required.

Atomic fluorescence detection limits are highly dependent on the instrumentation used. Increasing the source intensity results in improvements to atomic fluorescence detection limits. The absence of self-absorption results in a very wide linear range of the calibration curves.

Atomic fluorescence spectrometry is a useful analytical technique, particularly for the determination of trace element contaminants in the environment and industry, because of the low detection limits (in the low $pg.g^{-1}$ range) and high specificity. Low detection limits are obtained because of the difference in directions at which the exciting and fluorescence radiation are viewed, allowing the atomic fluorescence signal from the analyte to be contrasted with, essentially, a zero background signal. It is always easier to measure a small signal directly than a small difference between two large signals. High specificity is achieved because the spectra obtained are relatively simple.

1.8- Background to this study: potential risk to human foetal development

The proposed study takes advantage of the biological samples collected and stored by the Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC). This study is concerned with all mothers who had a pregnancy with an expected date of delivery between April 1st 1991 and December 31st 1992, and who were resident in the three Bristol-based health districts of Avon. The area covered includes the city of Bristol, and surrounding rural areas. It also includes several towns, including the coastal towns of Weston-Super-Mare, Clevedon and Portishead, and the industrial surrounds of Avonmouth. A map of the area where this study was carried out is given below (Figure 6).

Mothers complete questionnaires during pregnancy relating to various aspects of their social and environmental background, including their occupational history. They complete detailed dietary frequency questionnaires, and record their medical history and history of previous pregnancies. Dental fillings during pregnancy are also recorded. Biological samples are collected from the mother during pregnancy and at the time of delivery the placenta is fixed in formalin and stored. A piece of umbilical cord is cut and stored at -20°C, and cord blood is taken and aliquoted.

The mother and child will be followed up until the child is seven years of age. Further details of the study are available from Professor Jean Golding, Institute of Child Health, University of Bristol, who directs the ALSPAC study.

With changing patterns of diet and increasing intake of fish, the overall consumption of mercury is likely to have increased among mothers and young children. The proposed study will assess foetal exposure. Increased concern about the health effects of mercury and its compounds in humans exposed to very low environmental concentrations of mercury is due to sub-clinical effects whose symptoms are difficult to detect and measure.

Clinical and epidemiological evidence indicates that pre-natal life is more sensitive to the toxic effects of mercury than adult life [18].

Lappe [86] lists 17 elements, including mercury, which pose or potentially pose risks to human foetal development, particularly during the vulnerable period of embriogenesis. Accumulating evidence is implicating mercury as causing foetal damage. Occupational exposure and levels in local drinking water have been linked to miscarriage [58, 61]. Around Minamata Bay, 6% of the children born were severely mentally retarded. Further studies on children exposed *in utero* have also shown mild motor and sensory disturbances not detectable in early infancy [9]. Exposure during infancy can also result in adverse effects on the developing brain.

Although mercury is no longer available in teething preparations, the infant may be exposed via the mother's milk or the weaning diet which appears from preliminary ALSPAC data to be increasingly concentrating on fish rather than meat preparations.

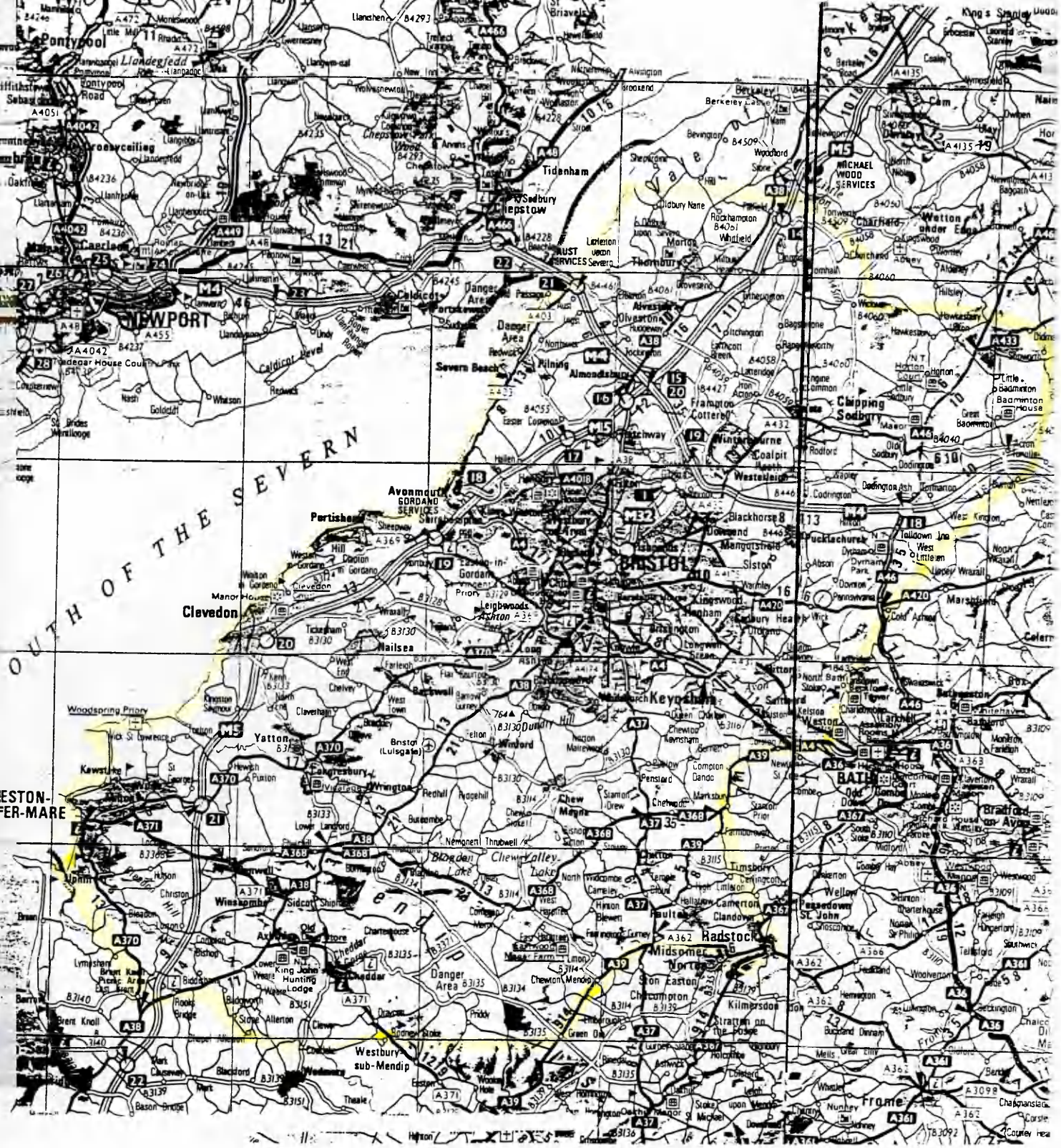


Figure 6: Map of the area where the ALSPAC study was carried out.

Maternal levels will, however, have strongly influenced the child's body burden at the time of birth. The local environment is also an important factor.

Maternal mercury exposure may occur from a variety of sources including occupational exposure, air pollution, including exposure from crematoria chimneys, possibly accumulation from dental amalgam fillings [87] , from the local water supply and from the diet [88, 89]. High levels are found in fish, mushrooms and occasionally in offal and in cereals [88]. The most effective way of nutritionally protecting against absorption is said to involve an adequate intake of zinc and selenium [90].

1.9- Aim of this work

The aim of this work is to develop a reliable method for the determination of total mercury in biological samples (mainly hair), that is suitable for the handling of very large numbers of samples. Some questions that remain unanswered in the literature, such as the influence of washing and the difference between exogenous and endogenous mercury levels are to be considered. This method is then to be applied to the needs of the ALSPAC study to provide reliable information on the influence of low level mercury exposure on the development of children in Britain. Due to the increasing concerns about the toxicity of different species of mercury, a speciation method is also to be developed.

2.1- Sample preparation

2.1.1- Collection and storage

Hair samples were collected after washing from a hairdresser's in Sheffield. They were cut with normal hairdresser's scissors, and then stored in glass containers at room temperature.

Hair samples for the ALSPAC study were collected by the mothers. They were stored in individual polythene bags at room temperature.

Each sample carries a 9-digit identification code and is therefore anonymous. The code can only be broken by the project manager at the University of Bristol.

2.1.2- Washing of the hair samples

In the preliminary study, the adult hair samples were washed as follows: the hair was covered with acetone and left to stand for 10 minutes with occasional shaking, then the acetone was discarded and the procedure repeated with double-distilled water, acetone and finally rinsed several times with double distilled water. This is the procedure recommended by the International Atomic Energy Agency (IAEA). The samples were then dried overnight in a desiccator at room temperature.

After the influence of washing and drying had been assessed, hair samples collected just after shampooing were not washed before analysis, as no difference was noted between the washed and non-washed samples.

Samples from children in the ALSPAC study were not washed, following the conclusion reached above. They were kept in polythene bags away from external contamination and dust until they were to be analysed.

2.2- Instrumentation

2.2.1- Settings on the Merlin Plus mercury detection system

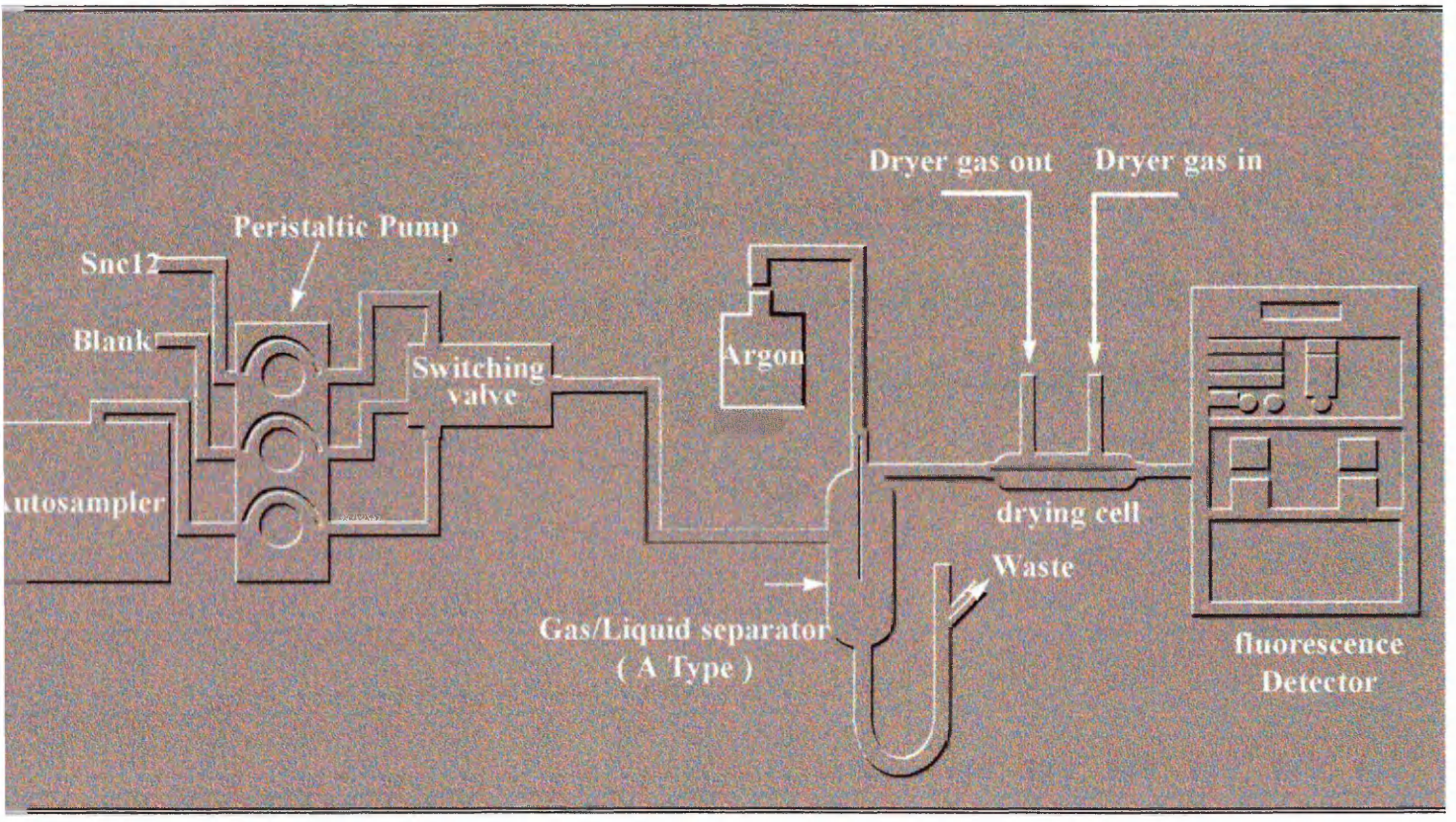
All the analyses were carried out using a Merlin Plus Mercury detection system (PS Analytical Ltd, England). The system consisted of a 386 SX computer, a printer, a random access autosampler, a peristaltic pump and a Merlin Plus mercury fluorescence detector. A PSA TouchStone software controlled the whole analysis process.

The settings used on the PSA Merlin Plus system are summarised in Table 4.

A schematic diagram of the Merlin system is given in Figure 7.

Table 4: Settings on the Merlin instrument

Parameter	Setting
Drying gas flow	2.5 l/min
Reaction gas	300 ml/min
Shield gas	300 ml/min
Detector range.	Determined by the highest standard used.



URE 7 : Schematic diagram of the PSA merlin plus sytem

The analysis program on the TouchStone software is summarised in Table 5.

Table 5: Details of the analysis program on the TouchStone software

Parameter	Setting
Delay time	5 s
Rise time	10 s
Analysis time	10 s
Recovery time	80 s

2.2.2- Regular maintenance to the system

The Merlin detector was fitted with a plug-timer, which switched it on 5 hours before any analysis was carried out in order to give it time to warm up and for the signals to stabilise. The peristaltic pump tubings were replaced every other day. Before any analysis was carried out the flow rates in the tubings were checked. The sample probes were replaced every week.

The glass gas/liquid separator was cleaned overnight with aqua-regia once a week and thoroughly rinsed with double distilled water.

The drying tube (hygroscopic Nafion membrane) was rinsed with methanol once a month, or when the detector started drifting into negative values when on standby. The membrane was replaced by a new one after a few months.

At the end of the day, the system was cleaned by pumping water in all the tubings for approximately 10 minutes.

2.3- Total mercury determination

2.3.1- Digestion procedure

About 0.1 g of hair was accurately weighed into a Teflon digestion flask. Two ml pure Aristar nitric acid and 1 ml Aristar hydrogen peroxide were added to the sample and the digestion flask was closed and positioned into the digestion carousel.

The microwave digestion system used was a closed MLS-1200 MEGA from Milestone, USA. The programme similar to that recommended by the manufacturer was used on the microwave digestion system:

- 2 min at 250 W + 2 min at 0 W + 5 min at 250 W + 5 min at 400 W + 5 min at 650 W

- followed by 10 minutes ventilation.

2.3.2- Reagents

All the acids used, hydrochloric, sulphuric and nitric, (supplied by Merck, GB) were of Aristar grade. Stannous chloride, hydroxyl ammonium chloride (Merck, GB) and hydrogen peroxide (Aldrich, GB) were of Aristar grade and "low in mercury". The other chemicals were of Analar grade.

Ultrapure water, obtained from a Millipore Milli-Q50 still was used throughout.

Stannous chloride (reducing agent) and Hydroxyl ammonium chloride (blank) were used as reagents for the determination of total mercury. They were prepared fresh every day as follows:

- Stannous chloride: 10 g $SnCl_2$ was dissolved in a 500 ml flask with 50 ml Aristar hydrochloric acid and the volume was made up with water.

- Hydroxyl ammonium chloride: 10 g $H_2NOH.HCl$ was dissolved in a 1000 ml flask with water.

2.3.3- Standard solutions

The commercially available mercury standard was a 1000 $mg.L^{-1}$ standard. It was used to prepare a 1 $mg.L^{-1}$ standard by diluting 0.1 ml in a 100 ml flask with water, and the working standards were prepared by dilution from the 1 $mg.L^{-1}$ standard with 4% Aristar nitric acid added as stabiliser. All the standards were freshly prepared daily.

2.3.4- Certified Reference Materials

Two Certified Reference Materials were used throughout this work. The first one was a Seronorm Trace Elements Whole Blood CRM (Nycomed), reference number 205052.

The Human Hair Certified Reference Material reference number CRM 397 that was used, was obtained from the Commission of the European Communities, Community Bureau of References, Brussels, Belgium.

Chapter 3: Optimisation of the Determination of Total Mercury

3.1- Factors affecting instrumental performance

3.1.1- Effect of moisture carry-over on the performance of the system

It has been shown in the literature [93, 94] that the presence of moisture is a problem associated with the method used in this work. It results in a gradual loss of sensitivity and leads to drift in the baseline. Before the complete PSA kit was available to us, a chemical desiccant (silica gel) was used as a moisture trap, but it was effective for only a short period of time, as it usually got saturated with water after about an hour. Then, when the complete PSA Merlin system was available, a more advanced method of getting rid of the moisture was included in the system, between the gas/liquid separator and the detector.

The dryer consists of two concentric tubes, the outer made of PTFE, whereas the inner one is an hygroscopic Nafion membrane. When the wet gas goes through the inner tube, the moisture is removed and passes through the Nafion membrane, to the PTFE tube where a constant dryer gas flows in the opposite direction than the wet gas. This dryer gas (argon in this case) removes the moisture from the outer surface of the hygroscopic membrane (Figure 8).

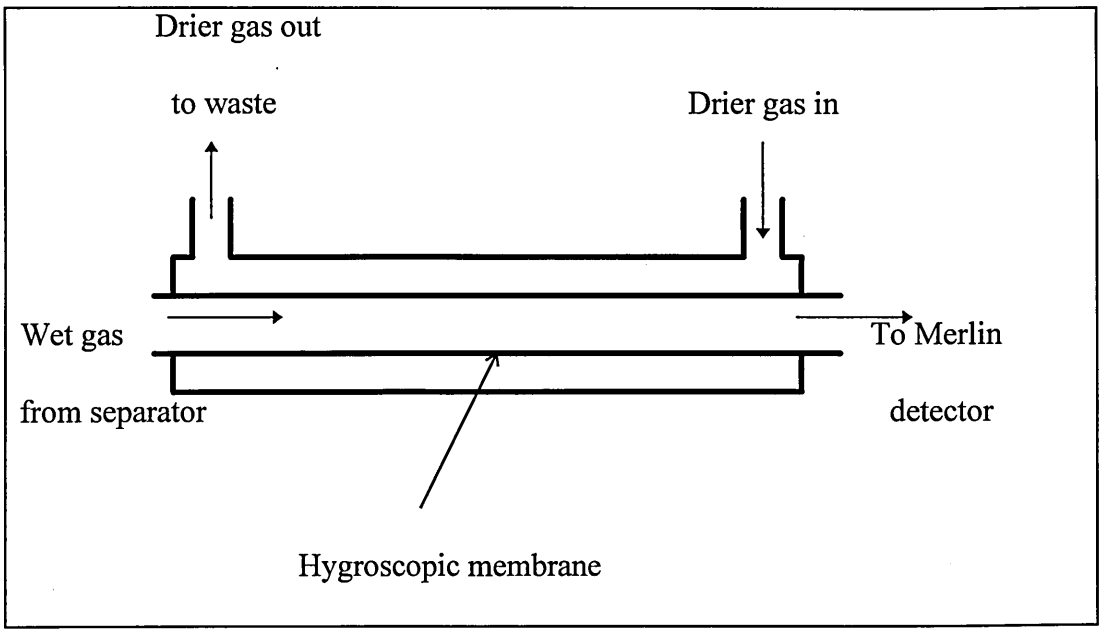


Figure 8: The Perma Pure drying cell

The three figures below show the variation of peak height with run number when no moisture trap is used (Figure 9), when a physical moisture trap is used (Figure 10) and when the Perma Pure drying cell is used (Figure 11). The use of the drying tube improves the long term stability and enhances the sensitivity of the method.

It was noticed that a Nafion membrane was effective for a few weeks, but needed changing regularly. When the baseline started drifting into negative values it was necessary to change to a new membrane.

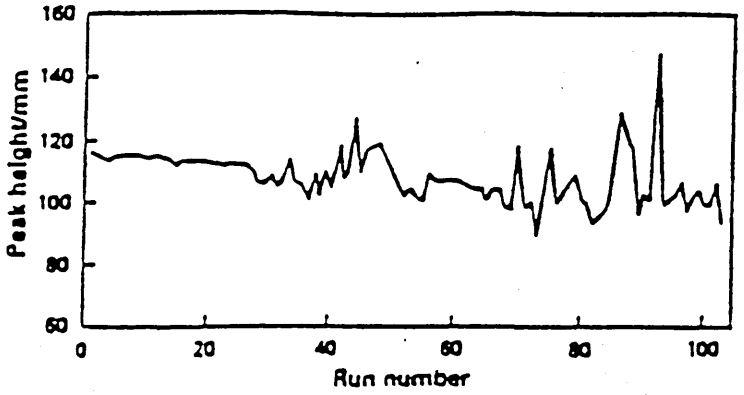


Figure 9: Variation of peak height with run number with no moisture trap

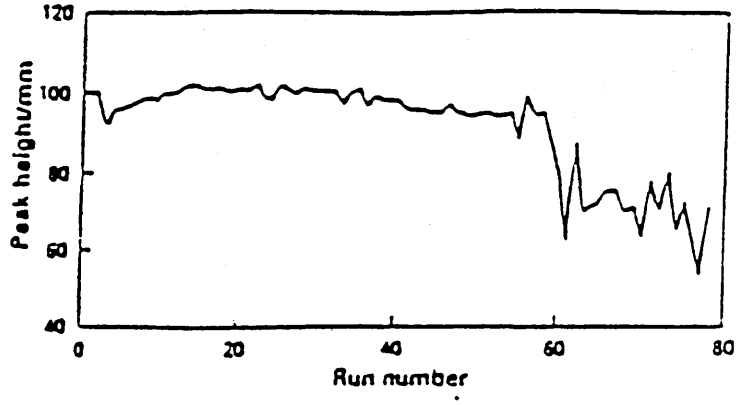


Figure 10: Variation of peak height with run number with a physical moisture trap

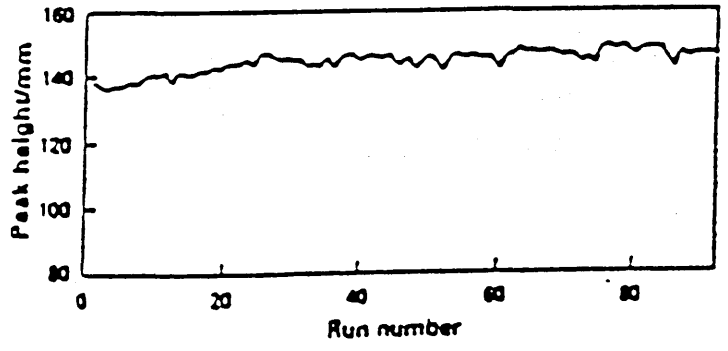


Figure 11: Variation of peak height with run number with the Perma Pure drying cell

The drying cell seems to be the best way of overcoming the problems caused by the moisture carry-over in the instrument.

3.1.2- Study of the drift of the instrument

At one time during the study, it was noticed that very high recoveries (up to 200%) were obtained for samples analysed with methods which were expected to give recovery values of less than 60%. Hair reference material samples were digested and analysed, and the average recovery obtained for 8 samples was 128%, although it was only 56% with the same procedure a few weeks before.

Initially, contamination was thought to be the cause of the problem. In order to check this assumption, blanks were analysed. The nitric acid that was used to wash the digestion flasks was not discarded but analysed. The results showed no mercury was left in the flasks. Contamination of the digestion flasks was not the cause for the high recoveries. Then, 5 blanks and 5 standards were digested and analysed. The concentration expected for the standards was 4 $ng.ml^{-1}$. The table below gives the results for the blanks and the standards. It shows clearly that the concentration measured is much higher than the concentration expected.

Table 6: Digestion and analysis of blanks and standards

Blank number	Hg ($ng.ml^{-1}$)	Standard number	Hg ($ng.ml^{-1}$)
1	0.000	6	6.584
2	0.000	7	6.598
3	0.000	8	6.653
4	0.000	9	6.724
5	0.000	10	6.269

The very high values obtained for the standards showed once again the very high recovery problem. However the values for the blanks ruled out the assumption that some contamination of the digestion flasks or the glassware had taken place.

It was then assumed that some sort of contamination or accumulation of mercury in the instrument was taking place. However, the analysis of blanks just after concentrated standards gave expected low levels of mercury, which showed that there was no accumulation of mercury in the instrument.

The highest mercury standard, 1000 $\mu g.ml^{-1}$, available commercially, was also changed, but no improvement was noticed.

Finally it was assumed that a drift in the instrument could be the cause of the problem. In order to check this assumption, a calibration curve was plotted, then 8 samples and 2 blanks were analysed, and finally the calibration curve was run again and the signal intensity given

for each standard was compared, before and after the analyses. Tables 7 and 8 give the values obtained for the 8 samples, which were digested 4 $ng.ml^{-1}$ standards, and the comparison of the signal intensity for the standards before and after the analyses.

Table 7: Analysis of 8 samples and 2 blanks

Sample	Hg ($ng.ml^{-1}$)
Digested standard 1	4.012
Digested standard 2	3.950
Digested standard 3	3.750
Digested standard 4	3.852
Digested standard 5	3.759
Digested standard 6	5.666
Digested standard 7	6.325
Digested standard 8	6.360
Blank 1	0.000
Blank 2	0.000

Table 8: Signal intensity for the standards before the analyses and after

Standard conc. ($ng.ml^{-1}$)	Signal intensity before the analyses	Signal intensity after the analyses
0	3.984	0.000
2	511.0	831.4
4	1000	1747
8	1930	3544

The results in Tables 7 and 8 show clearly that there is drift in the instrumental response. In Table 7, there is an obvious change after sample 5, and whereas the value given was close to the 4 $ng.ml^{-1}$ expected for the first 5 samples, very high recoveries were obtained for the last 3 samples.

Table 8 also shows an important difference between the signal intensity for the same standards analysed again after about half an hour. However, the linearity of the instrument was not affected (Figure 12). The drift in the instrument was thus the cause of the very high recoveries obtained.

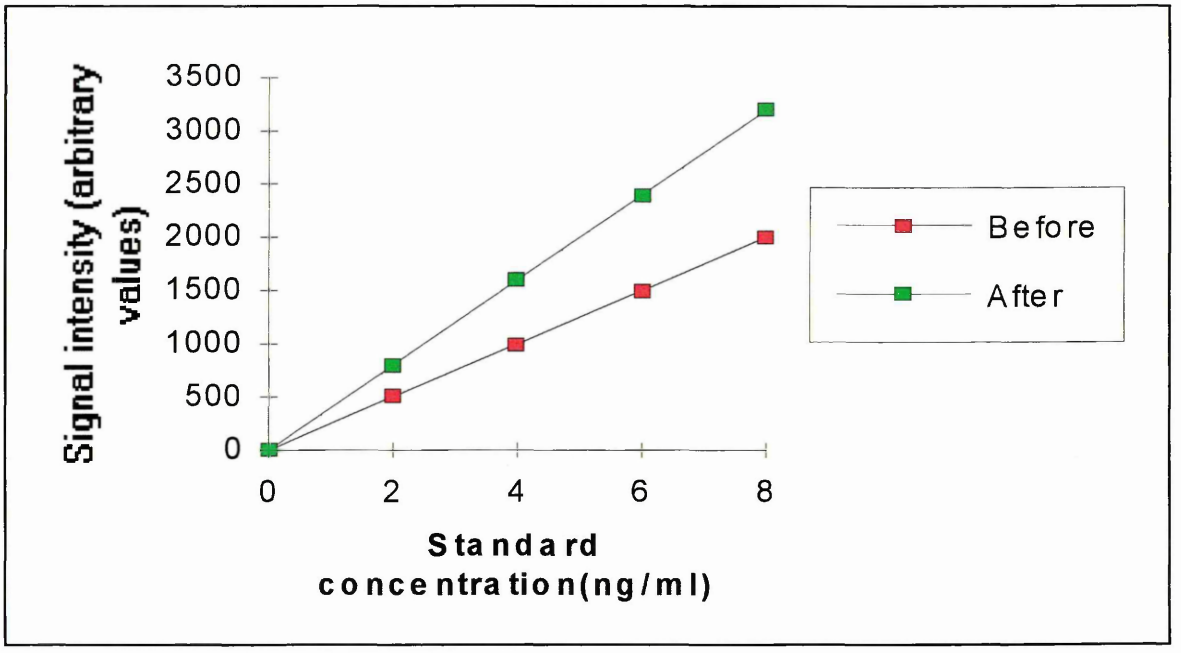


Figure 12: Influence of the drift of the instrument on the calibration curve

The next step was to try and monitor the drift of the instrument in order to control it. For this purpose, the same 5 $ng.ml^{-1}$ was analysed 25 times, and the signal intensity recorded for each analysis. The results are summarised in Figure 13. It is shown that a drift does take place in the instrument. However, this is not a daily occurrence.

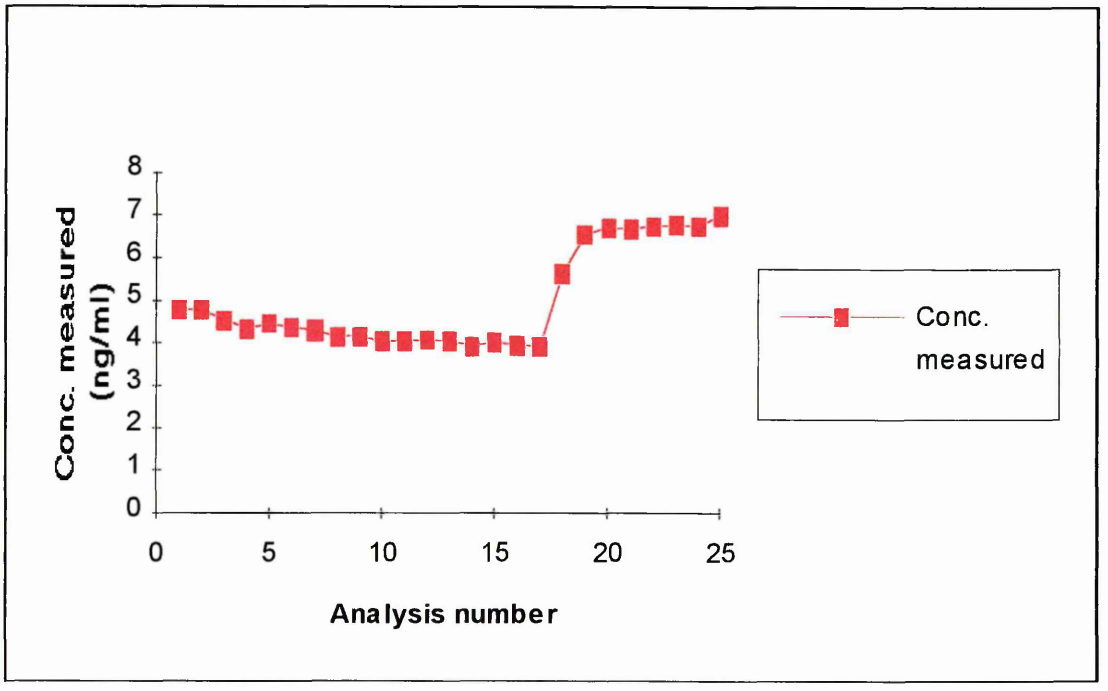


Figure 13: Variation of instrumental response with time

In order to assess the instrument response over a longer period of time, a batch of 25 samples made of the same standard (10 $ng.ml^{-1}$) was analysed 3 times and the different signal intensities for each standard were plotted on the same graph. The results of this experiment are shown in Figure 14. It is clear from both figures that drift does occur. However, the occurrence cannot be predicted, and some days no drift occured.

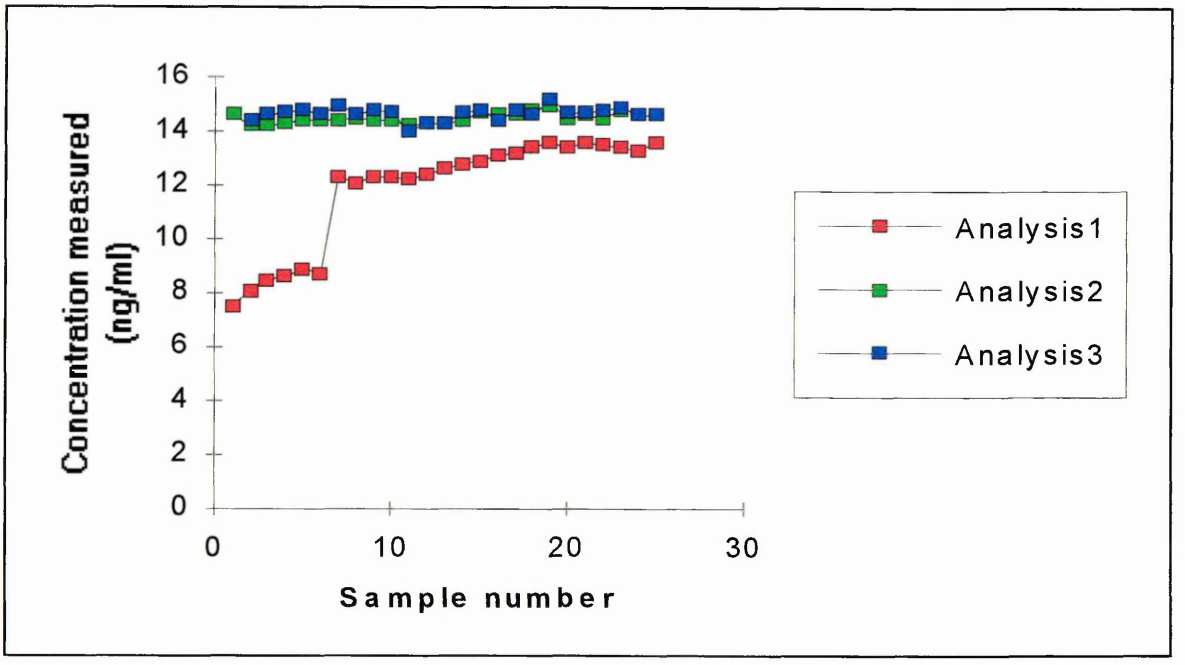


Figure 14: Response of the instrument over time

It is not possible to prevent the instrument from drifting. However it is possible to check whether the instrument has drifted or not. In order to check the drift, a standard is analysed every 5 or 10 sample and the signal intensity given on the analysis is compared with the signal intensity given for the same standard when the calibration curve was plotted.

A function in the TouchStone software allows the checking of the standard to be programmed, so it is automatically carried out every 5 or 10 sample. When the instrument

has drifted beyond a predetermined level, the computer aborts the analysis program. A new calibration curve must be plotted before the analyses can be continued.

Alternatively, it is possible to reduce the chances of the instrument drifting by switching on the detector a few hours before the analyses are carried out. The manufacturer recommends that the instrument should be left to warm up for about half an hour. However, this is not always sufficient. In our case, the instrument was always switched on using a timer at 2 a.m., and left to warm up until at least 9 a.m. before use.

3.1.3- Accuracy and precision of the instrument

The detection limit of the method was calculated from the standard deviation on 10 measurements of a 50 $ng.l^{-1}$ standard. The detection limit calculated was 10 $ng.l^{-1}$. The coefficient of variation of the method was 6.6% at 50 $ng.l^{-1}$. It was only 2% at $ng.ml^{-1}$ levels.

Values of 12.1 ± 0.2 $\mu g.g^{-1}$ of mercury were obtained for hair Certified Reference Materials (certified value 12.3 ± 0.5 $\mu g.g^{-1}$) over a period of 5 working days, on 25 samples.

3.2- Digestion procedures

3.2.1- Preliminary investigation of different digestion procedures

Three different techniques were investigated. Methods based on these techniques are summarised in Table 9.

Table 9: Summary of digestion procedures investigated

Method	Type of digestion procedure	Digestion procedure	Microwave program
A	Open microwave digestion	0.1 g hair, 0.25 ml $K_2Cr_2O_7$, and 2 ml HNO_3 were placed in a quartz digestion flask, followed by microwave digestion	5 min at 25% power
B	Bromination	0.1 g hair + 0.1 ml $K_2Cr_2O_7$ + 2 ml H_2SO_4 + 1 ml conc. fuming HNO_3. Heating for 3h at 80°C, then cooling, + 0.5 ml bromine, leave for 30 min. Add 0.2 ml $H_2NOH.HCl$ + enough saturated $KMnO_4$ to get an excess of $KMnO_4$. Heating for 10 min at 80°C, and finally 0.5 ml saturated oxalic acid was added to decolorise the solution	None

Table 9 continued

Method	Type of digestion procedure	Digestion procedure	Microwave program
C	Closed microwave digestion	0.1 g hair was placed in a Teflon digestion bomb with 0.25 ml $K_2Cr_2O_7$ and 2 ml HNO_3.	2 min at 250W, 2 min at 0W, 5 min at 250W, 5 min at 400W, 5 min at 650W, and 10 min ventilation

After digestion procedure A, the digestion flask was put in an ice bath and left to cool for 10 min. The digest was then transferred into a 25 ml flask, and the volume made up to mark with ultra pure water.

After digestion procedure B, the sample tubes were carefully agitated to remove the gas formed, then the digests were transferred into 25 ml flasks, and the volume made up with ultra pure water.

After digestion procedure C, the microwave carousel and the bombs were left to cool in a water bath for 1 hour before they were carefully opened, and the digests transferred into 25 ml flasks. The Teflon bombs were rinsed thoroughly with ultra pure water, and the content transferred to the flasks, and the volume made up to 25 ml.

3.2.2- Further investigation of the digestion procedures

Variations on method C were further investigated and the modifications are summarised in Table 10.

Table 10: Summary of the various modifications to method C

Method	Procedure	Microwave program
1	0.05 g hair + 0.25 ml $K_2Cr_2O_7$ + 2 ml HNO_3 + 2 ml $KMnO_4$	Same as for method C
2	0.05 g hair + 0.25 ml $K_2Cr_2O_7$ + 2 ml HNO_3 + 2 ml $KMnO_4$. The samples were then made up to 25 ml and exposed to UV light overnight.	Same as for method C
3	0.05 g hair + 2 ml HNO_3 + 1 ml H_2O_2	Same as for method C

3.3.- Results and discussion

3.3.1- Optimisation of the Merlin system

3.3.1.1- Optimisation of the analysis times

The analysis program in the TouchStone software is divided into four sections: delay, rise, analysis, and memory times. During the delay stage, the computer measures the baseline of the signal. It is also the time the sample takes to reach the detector from the autosampler. During the rise time, the mercury in the sample has started to reach the detector. During the

analysis time, the signal intensity has reached its maximum and it is measured. During the memory time, the signal returns to baseline.

It is very important that the analysis time is carefully monitored because the amount of sample is limited in most cases. The sample probes are automatically removed from the sample after the analysis time, so delay, rise and analysis times are critical for sample volume. The different times investigated are summarised in Table 11 below.

Table 11: Optimisation of the analysis program

Delay (s)	Rise (s)	Analysis (s)	Memory (s)	Comments
5	5	20	30	The signal does not return to baseline: memory too short.
5	5	20	50	The plateau during analysis is too long: analysis too long.
5	5	10	50	The signal does not reach its maximum: rise too short.

Table 11 continued

Delay (s)	Rise (s)	Analysis (s)	Memory (s)	Comments
5	10	10	50	Good signal for low concentrations (under 2 $ng.ml^{-1}$) but memory too short for more concentrated samples: no return to baseline.
5	10	10	80	Good signal for higher concentrations (up to 20 $ng.ml^{-1}$) .

The last program described in Table 11 keeps the sample intake to a minimum (25 seconds), while still allowing the signal intensity to reach its maximum, and the longer memory time allows the signal to return to the baseline before the next sample is analysed. Each sample is analysed in less than 2 minutes, and up to 30 samples can be analysed per hour. Sample preparation rather than analysis time is thus the limiting factor of controlling sample throughput.

3.3.1.2- The concentration of HCl in the reducing solution

The stannous chloride solution used as the reducing agent to reduce Hg^{2+} to Hg^{0} can be either alkaline or acidic. The maufacturer recommends the use of an acidic solution with 2% (w/v) stannous chloride. The concentrations of HCl investigated were 5%, 10% and 15% (v/v). The peak areas obtained for the analysis of a 4 $ng.L^{-1}$ standard are summarised in Table 12.

Table 12: Influence of %HCl on signal

%HCl (v/v)	Peak Area measured (n=3)
5%	859 ± 72
10%	1017 ± 94
15%	887 ± 54

The optimum peak area is obtained for a 10% (v/v) HCl concentration in the reducing solution.

3.3.2- Digestion procedures

3.3.2.1- Preliminary experiments

The recoveries obtained using the three methods, A, B, and C, with digested standards, spiked hair samples and certified reference materials (CRM) respectively are summarised in Table 13.

Table 13: Recovery of mercury obtained during the preliminary study

		Method A	Method B	Method C
Digested standards recoveries	Number of samples	3	4	7
	Expected conc. (in ng/ml)	5	5	5
	Recovery ± SD	105 ± 16 %	80 ± 13 %	82 ± 0.2 %
Spikes recoveries on hair samples	Number of samples	4	4	15
	Expected conc. (in ng/ml)	5	5	5
	Recovery ± SD	35 ± 9 %	80 ± 13 %	51 ± 12 %

Table 13 continued

		Method A	Method B	Method C
CRM recoveries	Material used	blood	blood	hair
	Number of samples	6	5	8
	Expected conc.	5 $ng.ml^{-1}$	5 $ng.ml^{-1}$	12.3 $\mu g.g^{-1}$
	Recovery ± SD	34 ± 14 %	36 ± 12 %	56 ± 3 %

The low recoveries obtained for the CRM were first thought to be due to losses of mercury during sample digestion. As a result methods A and B were not investigated any further since they were both carried out in open systems. Furthermore, they were both time-consuming procedures. It is noteworthy that high recoveries were obtained for the standards but not for the spiked samples and reference materials, suggesting that the presence of a matrix might have an effect on the recovery of mercury. This effect of the matrix on the recovery of mercury had been previously reported [19, 38].

3.2.2.2- Matrix effect

In order to demonstrate the effect of the hair matrix on the mercury signal, two calibration curves were produced from the analysis of inorganic standards in an acid matrix and the same standards in a hair matrix. A comparison of the two calibration curves is shown in Figure 15.

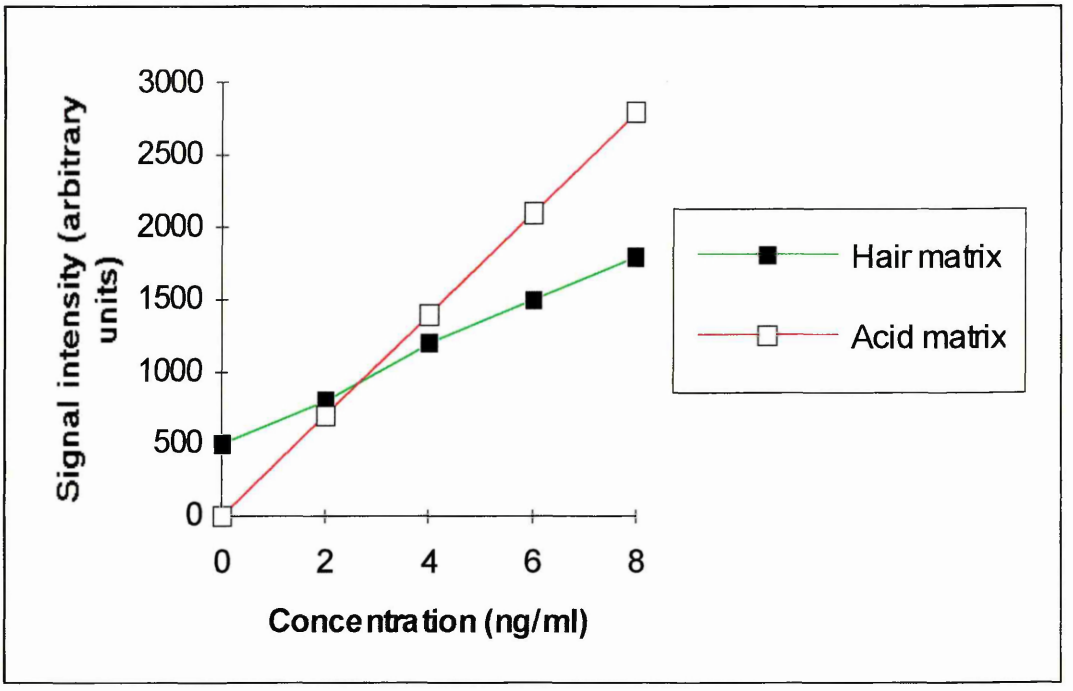


Figure 15: Comparison of the slopes of the calibration curves obtained from an acid matrix and a hair matrix.

From this figure, it can be seen that the hair matrix is having an effect on the signal intensity obtained. This could explain the low recoveries obtained for method C for the spiked and the CRM samples. It has been claimed that some digestion procedures tried are not able to release all the mercury from the matrix constituent to which it is bound to its free Hg^{2+} state [38]. As a result, matrix matching has been extensively used to overcome this problem [19]. The following experiment was carried out to test the possibility of obtaining good recoveries when using matrix matched standards: spiked hair samples were analysed against both

calibration curves. Recoveries of up to 84 ± 4% were obtained with the matrix matched standards.

However, the similarity of the matrix effect obtained with different types of hair, has not yet been shown, thus matrix matching of standards with only one type of hair when several types of hair have to be analysed would be a likely source of error. As the preparation of matrix matched standards for each hair type would not be possible when the routine analysis of a number of samples is to be carried out, it was considered important to try and develop a digestion method that would decompose the matrix completely and thus eliminate the matrix effect, and yield quantitative recoveries.

3.3.2.3- Further investigations of the digestion procedures

Method C was investigated further on the basis of the higher mercury recovery values obtained with the Certified Reference Material (N° 397).

Table 14: Recoveries obtained with modifications to method C

	Method 1	Method 2	Method 3
Recoveries on Hg^{2+} spikes	39 ± 9%	102 ± 12%	94 ± 3 % for 20, 40 and 60 ng/ml spikes
Recoveries on Hg^{2+}, and MeHg standards	95 ± 3% 77 ± 3%	91 ± 9% 78 ± 8%	94 ± 2% 94 ± 3%
Recoveries on hair CRM	76 ± 7 %	83 ± 8%	100 ± 4 %

It was assumed that the low recoveries obtained were not due to losses by volatilisation but mainly to incomplete decomposition of the mercury species. This implies that the mercury is still present in the digest but not in a form that gives a detector response.

The atomic fluorescence detector responses to the presence of Hg°, so it is essential that the mercury species in the digest are in a form that can be reduced readily by stannous chloride. Consequently, for Hg species that are not readily reduced, a stronger oxidising agent is needed, in order to ensure complete decomposition.

The first oxidising agent tried was potassium permanganate. At first it seemed very promising (Figure 16) but its limit was rapidly found (Figure 17). The maximum recovery of mercury at optimum $KMnO_4$ volume (5ml) was 41%. Pineau *et al.* [19] reported using $KMnO_4$ as an oxidising agent, but they still needed matrix matched standards to achieve

good recoveries. This is consistent with our results. It shows that $KMnO_4$ is not strong enough an oxidising agent to break all the bonds with which mercury is attached to hair constituents. As mercury is present in hair mainly as methyl mercury , these are Hg-C bonds. UV light is known to destroy these bonds. Therefore, after digestion using $KMnO_4$ the digests were exposed to UV light.

This gave good recoveries for inorganic spikes on hair samples: recoveries of 100% were obtained for the maximum volume of $KMnO_4$ (9 ml) (Figure 18). These results show that it is possible to get good recoveries with $KMnO_4$ and UV light when inorganic standards are used. UV light seems to reduce significantly the hair matrix effect. However, a similar increase in the recoveries obtained for methyl mercury standards was not observed. This would indicate that, although UV light reduces the matrix effect, it does not break all the Hg-C bonds, as previously thought. The low recoveries obtained for hair CRM samples also suggests that UV light and $KMnO_4$ are not sufficient to release all the mercury from its bound form to its free Hg^{2+} form. Other digestion procedures using stronger oxidising agents, were therefore investigated.

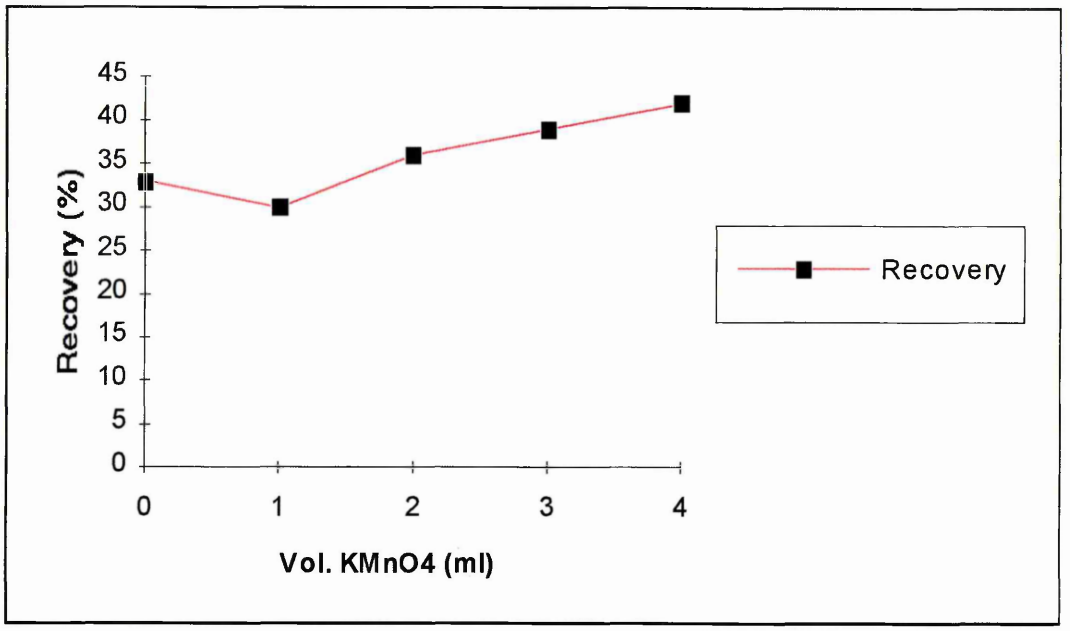


Figure 16: The effect of the addition of $KMnO_4$ on spike recovery

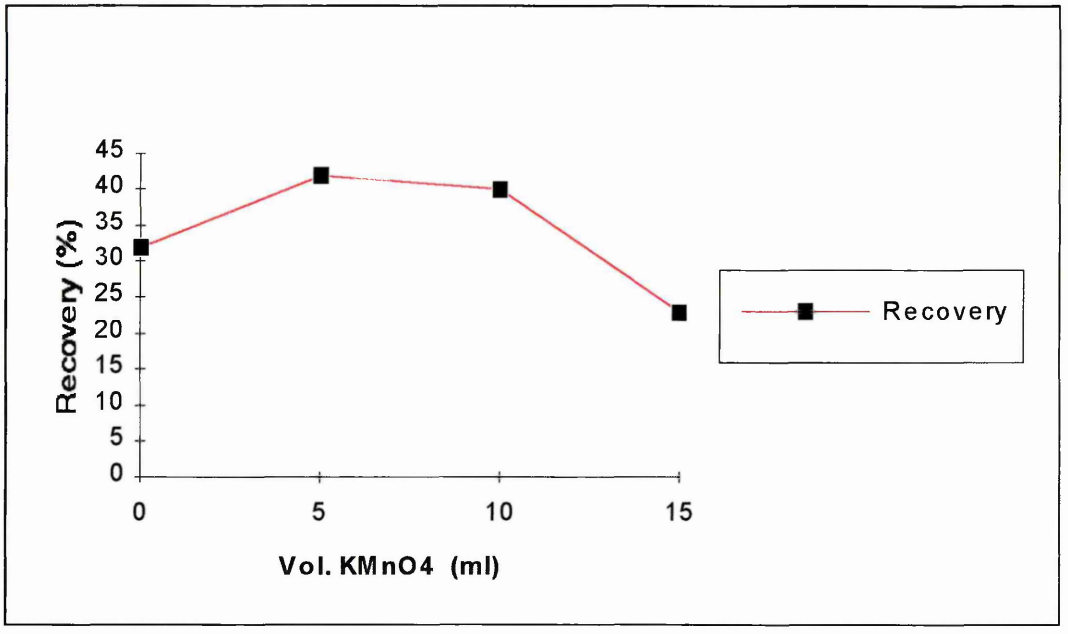


Figure 17: Influence of the volume of $KMnO_4$ added on the recovery of mercury spikes

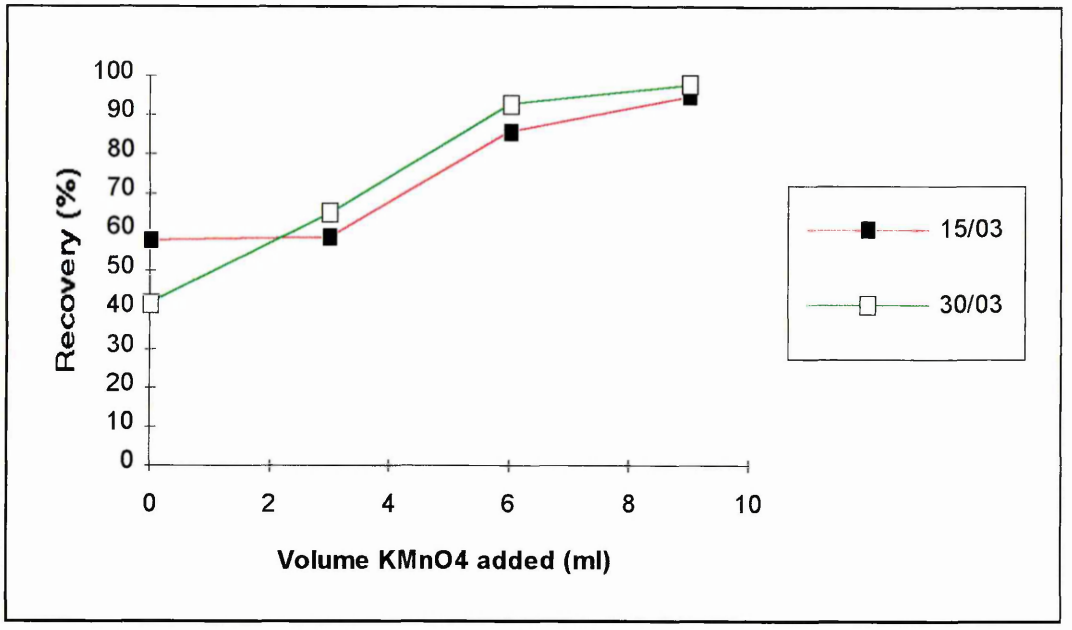


Figure 18: Influence of the volume of $KMnO_4$ added on the recovery of mercury after UV irradiation overnight

Hydrogen peroxide, which is a stronger oxidising agent was tried. Its use was recommended by Schutz *et al.* [7]. However they recommend that it is added to the nitric acid digest. In order to reduce handling of the samples, H_2O_2 was added before digestion at the same time as nitric acid.

Recoveries of mercury in the three types of samples investigated were 94% or above (Table 14). Method 3 was then the procedure chosen to digest hair samples for the determination of total mercury by CV-AFS. This method is simple and with a high sample throughput, about 25 samples an hour can be digested. The cooling period of the digestion flasks is about 1h. However, it was necessary to investigate the influence of other parameters in order to optimise this method.

3.3.3- Optimisation of the procedure chosen

The parameters investigated were the effect of the presence of $K_2Cr_2O_7$, the amount of hair used with the same amount of reagents, the volume of hydrogen peroxide added during digestion and the stability of the hair CRM.

Potassium dichromate is often added as a stabiliser for mercury [7] and it was thought that its presence may prevent the reduction of Hg^{2+} to Hg° before analysis. It was necessary to investigate the influence of the stabiliser on the recoveries for mercury in CRM hair samples. In order to assess the importance of $K_2Cr_2O_7$, two batches of hair CRM were digested using method 3, with and without $K_2Cr_2O_7$ added. The results of the experiment are summarised in Table 15.

Table 15: Recovery of Hg in the presence of $K_2Cr_2O_7$

	Method 4 with $K_2Cr_2O_7$	Method 4 without $K_2Cr_2O_7$
Number of samples	4	4
Av. conc. ($\mu g.g^{-1}$)	11.9	11.9
RSD	2.8 %	4.3%
Average recovery	97 %	97%

The results in Table 15 indicate that the presence of potassium dichromate does not affect the recoveries (comparison of the mean of two samples: $\underline{t = 0}$ for a critical value of 2.45 for P=0.05 and 6 degrees of freedom, c.f. Appendix 1). In this case it is not useful, and in order

to simplify the procedure it can be omitted. However, if the samples are stored between digestion and analysis, the addition of potassium dichromate is recommended. In this study, samples were kept in a fridge overnight, and analysed on average the day after they were digested. This seems sufficient to ensure the stability of mercury in the samples.

The effect of increasing the weight of hair but keeping the amount of nitric acid and hydrogen peroxide constant was investigated. Two batches of samples of 0.05 g and 0.1 g hair CRM were digested using 2 ml HNO_3 and 1 ml H_2O_2. The results of the analyses are summarised in Table 16.

Table 16: Recovery of mercury using different weights of hair

	Batch 1	Batch 2
Number of samples	4	4
Weight used	0.05 g	0.1 g
Average recovery ± SD	91.3 ± 1.1 %	83.6 ± 2.5 %

The recoveries are influenced by the weight of sample used (comparison of the mean of two samples: $\underline{t = 5.6}$ for a critical value of 2.45, for P = 0.05 with 6 degrees of freedom) and are better when 0.05 g CRM hair is used. This shows that the amount of reagents is critical. If there is not enough nitric acid and hydrogen peroxide, it will not be possible to decompose the matrix fully and the recoveries obtained will be low. However, the recovery obtained for batch 1 in Table 16 is lower than usual. This finding needed further investigation. All

the results obtained for the same hair CRM bottle were reported since the day it was opened, and the recoveries were compared. The results are shown in Table 17.

Table 17: Recoveries obtained with time

Date	Number of samples	Hg ($\mu g.g^{-1}$)	RSD	Average recovery
30/01/95 Day 1	8	12.18	1.5%	99.0%
02/02/95 Day 3	8	12.21	1.9%	99.3%
07/02/95 Day 8	8	11.91	0.2%	96.8%
10/02/95 Day 11	4	11.23	1.2%	91.3%

The comparison of the mean of two samples gave:

- between day 1 and day 3: $t = -0.35$, for a critical value of 2.14 for $P = 0.05$ and 16 degrees of freedom: there is no significant difference in the recovery values,
- between day 3 and day 8: $t = 3.7$, for a critical value of 2.14: there is a significant difference
- between day 8 and day 11: $t = 13.2$, for a critical value of 2.23 for $P = 0.05$ and 10 degrees of freedom: there is a significant difference between the recoveries obtained on these two days.

The results show a decrease with time in the recoveries obtained for the same samples. A possible explanation is that the CRM takes up water vapour with time. The loss of mercury by volatilisation could also be a cause. The study in this case is too short to draw definite conclusions.

However, it was interesting to investigate the influence of drying of the CRM on the recoveries. Two batches of 4 samples each were prepared. Each sample was made of around 0.05 g CRM weighed accurately. In batch A, the samples were not dried, and were digested with 2 ml nitric acid and 1 ml hydrogen peroxide, and then analysed. In batch B, the samples were placed in an oven at 80°C overnight. This is the procedure recommended by the supplier of the CRM to correct for moisture. However, the supplier recommend this method for all the elements they certify, except mercury. The results obtained are given in Table 18 below.

Table 18: Influence of drying on the recovery of mercury in hair CRM samples

	Batch A (non dried)	Batch B (dried)
Number of samples	4	4
Average concentration ± SD ($\mu g.g^{-1}$)	10.28 ± 0.1	10.61 ± 0.78
Average recovery	84%	86%

The results of the comparison of the mean of two samples showed that the difference was not statistically significant (t=0.84 for a critical value of 2.45, with P=0.05, and 6 degrees of freedom). Thus, the presence of moisture cannot completely explain the lowering recovery values. It is likely that losses due to volatilisation of mercury could be also partly responsible.

The effect of increasing the volume of hydrogen peroxide added before digestion to the same amount of hair samples was investigated. Mercury was determined in both hair reference material and real hair. The results obtained are summarised in Table 19.

Table 19: The influence of the volume of hydrogen peroxide added for digestion

Volume (ml) H_2O_2 added	Recovery (%) obtained for CRMs	Average Hg conc. ($\mu g.g^{-1}$) for real hair samples
0	88 ± 3	1.38 ± 0.06
0.25	92 ± 5	1.39 ± 0.21
0.5	89 ± 3	1.45 ± 0.28
0.75	80 ± 1	1.51 ± 0.16
1	80 ± 1	1.65 ± 0.13
1.5	81 ± 1	1.57 ± 0.17

The results shown in Table 19 indicate that the effect of hydrogen peroxide is different on CRMs compared to real hair samples. The higher recoveries are obtained for CRMs with small volumes of hydrogen peroxide: 0-0.5 ml. The subsequent decrease may be explained

by an excess of hydrogen peroxide oxidising the stannous chloride, which is then no longer available to reduce mercury.

However for real hair samples, the highest results are obtained with 1 ml hydrogen peroxide. This may be explained by the fact that CRMs are probably spiked with mercury (the Hg concentration in CRMs is at least one order of magnitude higher than normal Hg values in real hair) and thus the hair matrix does not need as much oxidising to release the mercury in CRMs as it does in real hair samples, where Hg is tightly bound to the hair matrix. In the light of these observations, the digestion procedure proposed used 1 ml hydrogen peroxide with 2 ml nitric acid for 0.05 g hair.

Matrix effect experiment

The optimised digestion method was used to investigate whether the observed matrix effect had been eliminated. In addition, as mercury is present in hair mainly as methyl mercury, standards and spikes containing methyl mercury were also examined. The calibration curves are presented in Figures 19 and 20. In both cases, the normal hair matrix still had an effect on inorganic spikes recoveries. However, the difference in slopes was much smaller than that shown in Figure 15. It is noteworthy that the matrix effect is much smaller with CRM samples (Figure 20). This effect is not significant when normal hair samples were spiked with methyl mercury (Figure 19). Examination of the calibration curve obtained with the methyl mercury standards also shows that the digestion procedure used destroys all the methyl mercury and allows complete recoveries.

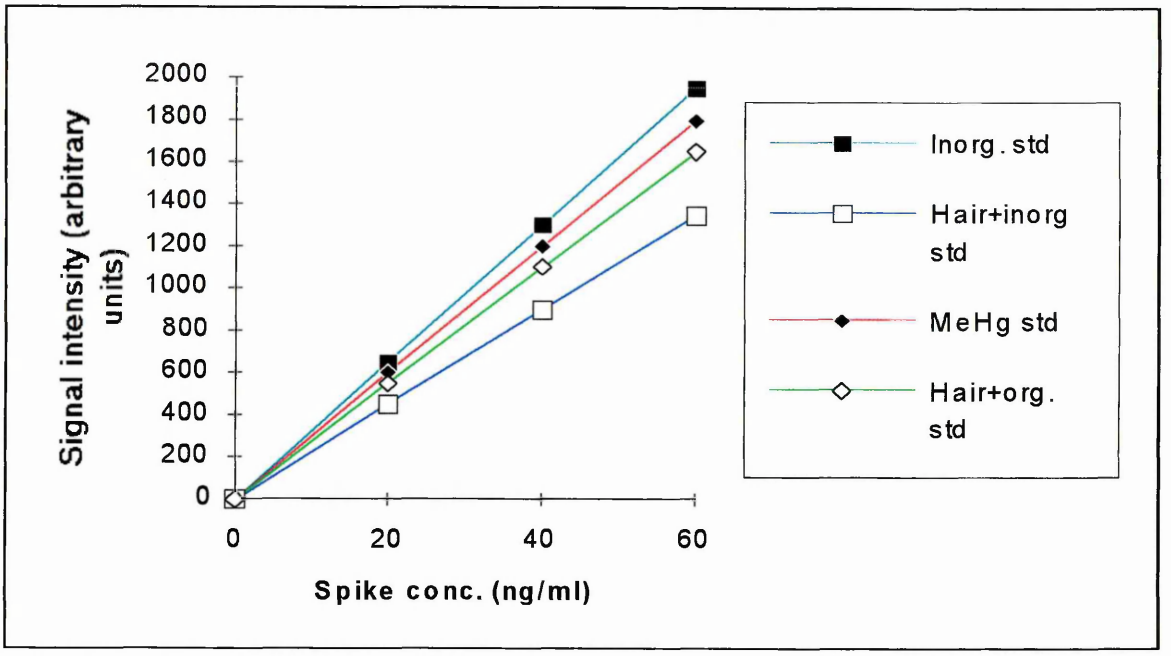


Figure 19: Calibration curves obtained from the analysis of inorganic and organic standards, and hair spiked with inorganic and organic standards

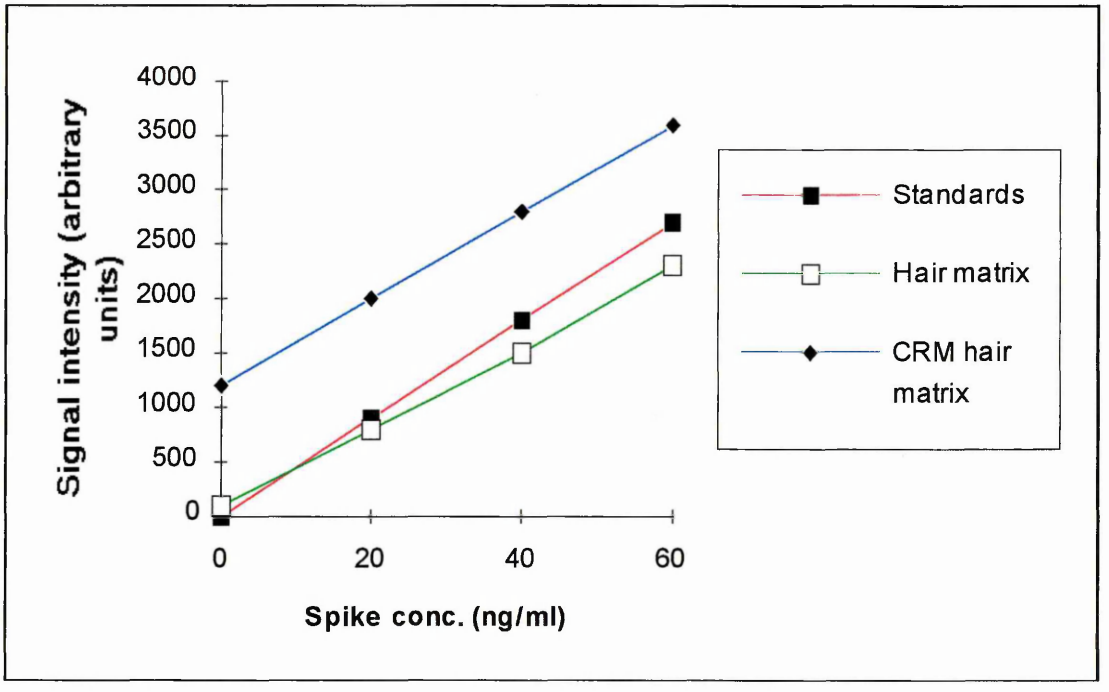


Figure 20: Standard addition of mercury to hair and CRM samples

3.3.4- The influence of washing and drying

There is a great amount of confusion in the literature about the effect of washing hair samples before analysis. The influence of washing and drying of hair samples before mercury determination was therefore investigated. Two batches of hair samples were collected for this purpose. Batch A was collected at a hairdresser's just after shampooing. The hair samples were homogenised by cutting into 0.5 cm strands and mixed. Batch B was collected from volunteers at the University, during the day, and without any special pretreatment, and homogenised.

Batches A and B were then both divided into 3 groups, each of which underwent a different washing and drying pretreatment. Procedure 1 was the analysis of the hair samples without any prior washing or drying. Procedure 2 was the analysis of the hair samples after washing in water and acetone and drying in an oven at 80°C for about 1.5h. Procedure 3 was the analysis of the hair samples after washing in water and acetone and drying in a freeze-drier overnight. The results are recorded in Table 20.

Table 20: The influence of washing and drying on the determination of mercury in hair samples

	Batch A	Batch B
Procedure 1 (no pretreatment)	Mean: 351 ± 35 $ng.g^{-1}$	Mean: 501 ± 31 $ng.g^{-1}$
Procedure 2 (washed and dried in oven)	Mean: 351 ± 86 $ng.g^{-1}$	Mean: 470 ± 21 $ng.g^{-1}$
Procedure 3 (washed and dried in freeze-drier)	Mean: 248 ± 81 $ng.g^{-1}$	Mean: 415 ± 46 $ng.g^{-1}$

The comparison of the mean of two samples showed that procedures 1 and 2 for batch A did not give statistically different results, but there was a statistical difference between procedure 3 and procedures 1 and 2 for batch A, and a statistical difference between every procedure for batch B. This shows that the freeze-drying of hair samples before the determination of mercury should be avoided as this leads to mercury losses.

On the other hand, when samples were shampooed before collection, the washing procedure did not make any difference in the mercury concentration. When samples were collected "dirty", washing did make a difference. It can be assumed that during shampooing, any mercury present in the hair due to exposure to air or other outside contamination is removed.

Thus if hair can be collected just after shampooing, washing of the samples with water and acetone is not necessary, thus simplifying sample pretreatment. This finding is important in relation to samples from babies, for whom it can be said that, because they spend very little time outside, their hair samples do not need to be washed before analysis. It has been reported in the literature that whenever possible, any pretreatment of hair samples should be avoided before analysis [92]. Those results are in agreement with the findings of this investigation.

3.3.5- Mercury repartition along the length of a strand of hair

It has been shown in the literature [48, 29-33] that the concentration of mercury in hair varies along the length of the hair sample in adults, but this has not been shown for babies.

However, the variation of the mercury concentration along the length of adults' hair was investigated in order to confirm the published findings. Long hair samples from adults were collected, cut into 3 cm subsamples, then digested and analysed. The results obtained confirm the fact that Hg concentration varies dramatically along the length of adults'hair (Figure 21).

In order to prove the viability of mercury measurement in children's hair as an indicator of exposure, it was necessary to make sure that the sampling position did not affect the result dramatically, as the sampling on the children taking part in the study was made by the mothers and could not be controlled. A batch of 14 hair samples from different children

was studied. The hair samples were cut into 2-3 cm subsamples, digested and analysed. The results are plotted in Figure 22.

Out of the 14 samples, 11 had a relative variation of mercury concentration along the length of the hair inferior to 20%. This represents almost 80% of the group studied. It was thus accepted that in young children, the concentration of Hg in hair does not vary significantly along the length of hair. This proves that the measurement of mercury in children's hair is a reliable indicator of exposure even though sampling position cannot be controlled.

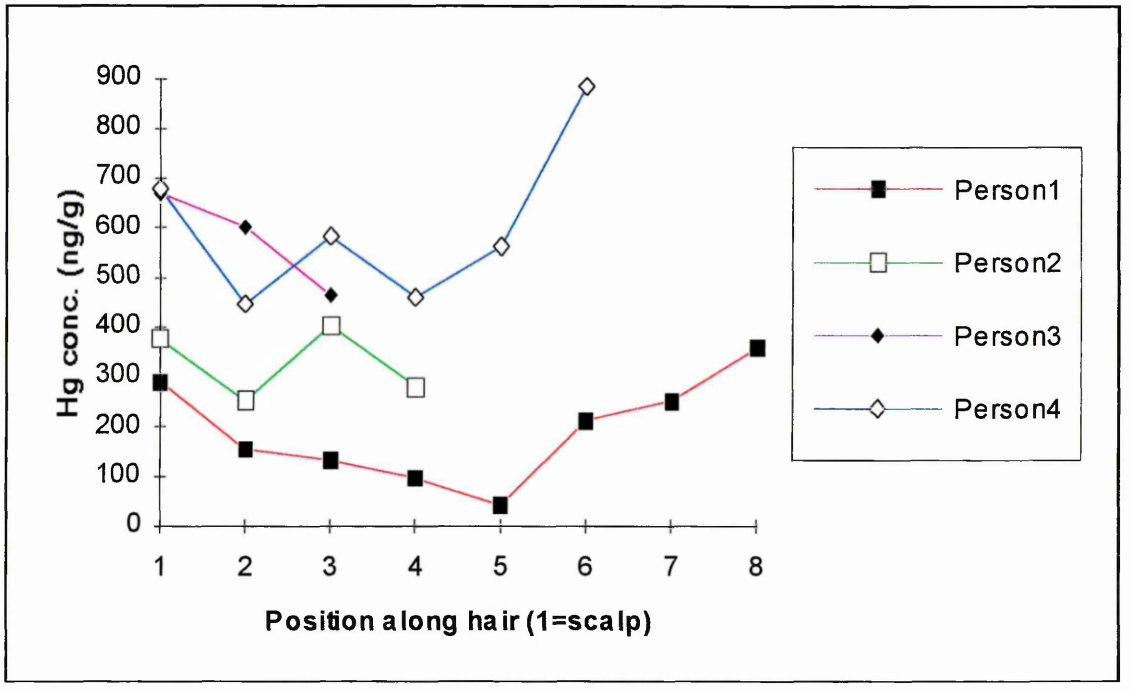


Figure 21: The variation of Hg concentration along the length of adults' hair

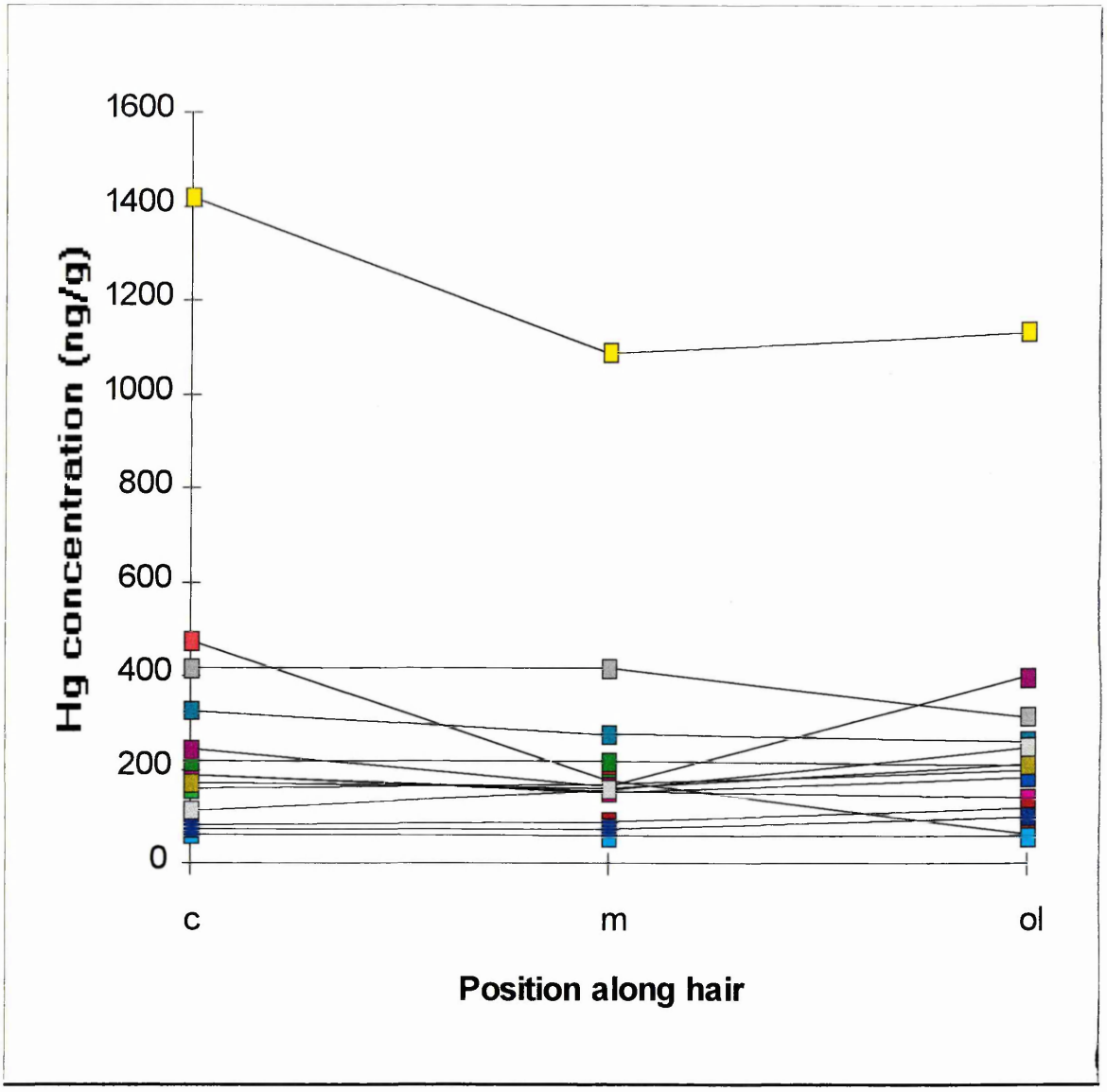


Figure 22: Analysis of Hg concentration along the length of children's hair, C: cut end, M: middle sample, O: old end. (The hair used were approximately 7-9 cm long)

3.4- Conclusion

In this section a method based on microwave digestion of human hair followed by the determination of mercury by atomic fluorescence spectrometry has been developed and optimised. The method is accurate, fast, simple and sensitive. The microwave digestion procedure requires only one manipulation step, and the process is complete in twenty seven minutes. The use of a 2:1 digestion mixture of nitric acid and hydrogen peroxide eliminates the need for matrix matching the samples and standards. Atomic fluorescence spectrometry is a sensitive mercury detection technique, and as a result preconcentration is not necessary. Up to forty samples could be processed and analysed in a working day using the recommended method. However, the Merlin system shows weaknesses, such as its drift, that require stringent control. The method of analysis was applied to determine whether the concentration of mercury varies along the length of a strand of hair. It was showed that it does in adults but not in babies. This enables the use of hair as a reliable indicator of a child's exposure even though sampling cannot be controlled.

Chapter 4: Human population studies

4.1- Preliminary study: at the hairdresser's

In order to check the usefulness of population studies, and to familiarise ourselves with the principle, a small-scale population study was carried out in Sheffield. Twenty people were selected at random at a hairdresser's, a sample of their hair was collected after washing, and a questionnaire was filled in by the volunteers. The questionnaire was devised to target especially these factors known to affect mercury levels in humans, such as diet and number of amalgam fillings. A copy of the questionnaire is given in Table 21.

Table 21: Questions asked to the volunteers for the first population study

1- Age
2- Occupation
3- Are you a Vegetarian?
4- Are you a Vegan?
5- How often do you eat fish or seafood?
6- Number of fillings
7- Did you have any fillings done recently?
8- How many pieces of fresh fruit do you eat everyday?
9- What area of Sheffield do you live in?
10- What sort of shampoo do you use?

After collection, the hair samples were homogenised, separated into 3 aliquots (where possible) and analysed separately using the method described in Chapter 3 for the determination of total mercury in human hair.

The study involved 19 women and one man, aged between 14 and 55. However, only 15 samples and their questionnaires could be used, as some questionnaires were not filled in accurately, or some samples were too small to allow their analyses. The mercury levels were then correlated with some of the information given on the questionnaires.

The group was divided into subgroups by age and number of fillings respectively:

- Age: -group 1: <25 years old
  -group 2: between 25 and 34 years old
  - group 3: > 34 years old

- Fillings: -group A: less than 5 fillings
  -group B: between 5 and 9 fillings
  -group C: more than 9 fillings

All the information obtained for the 15 volunteers is summarised in Table 22.

Table 22: Mercury levels obtained and summary of the information on volunteers

Subject	Age group	Filling group	Number of fish meals/week	Hg conc. ($ng.g^{-1}$)
1	1	B	0	269
2	2	C	1	591
3	1	A	0	335
5	2	A	2	889
7	2	B	2	800
8	2	A	0.5	532
9	2	B	3	594
10	3	B	0.25	531
12	3	C	2	828
13	3	C	1	988
14	2	A	0.5	223
15	3	B	2	270
16	1	A	1	439
17	2	B	1.5	725
19	1	A	1	128

A plot of the histogram of the results is shown in Figure 23.

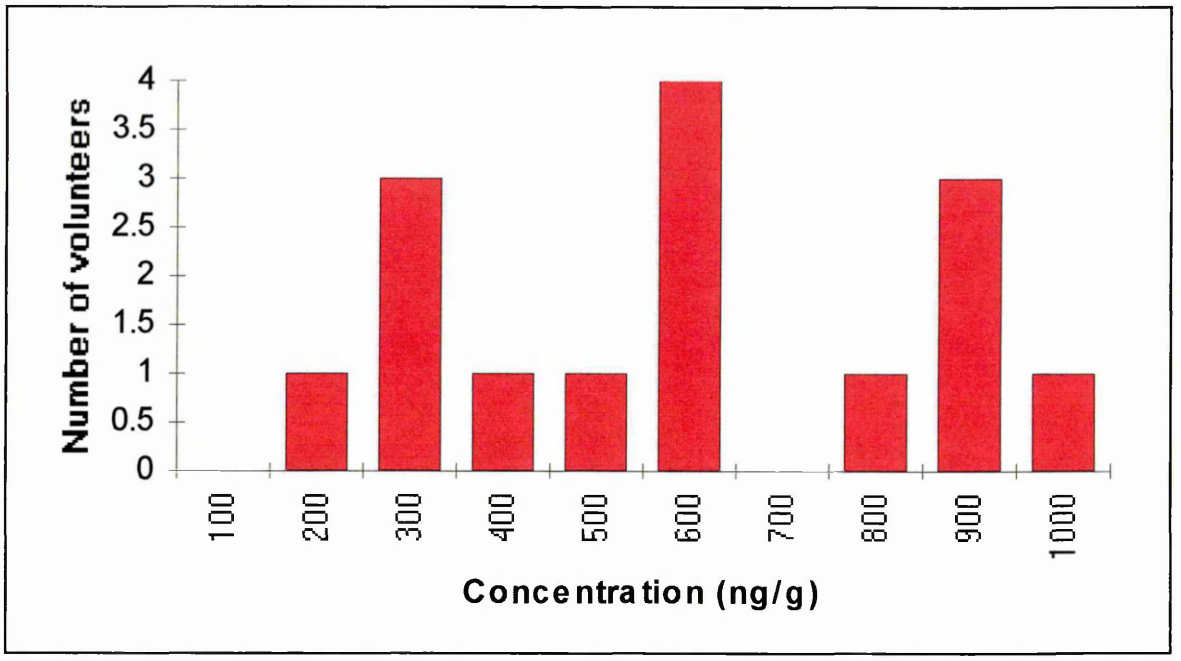


Figure 23: Histogram of the distribution of mercury concentrations in the samples of the group studied

This histogram reveals that the distributions of mercury concentration in the hair of the group studied is not normally distributed. This implies that a non-parametric method, such as Spearman's method (c.f. Appendix 3), has to be used to investigate the existence of a correlation between the mercury concentration in hair, and other parameters mentioned in this study, such as the number of fillings, the age and the number of fish meals per week.

4.1.1- Correlation between age and mercury concentration in hair

The geometric mean of the mercury concentration for each age group is shown in Table 23. The geometric mean is the inverse logarithm transformation of the mean of the logarithm values.

Table 23: Mean mercury concentration for each age group

Age group	Mean of the log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)
1	2.426	267	128	439
2	2.762	578	223	889
3	2.767	585	270	988

A plot of the log values of the mercury concentrations in the hair samples against age group is given in Figure 24.

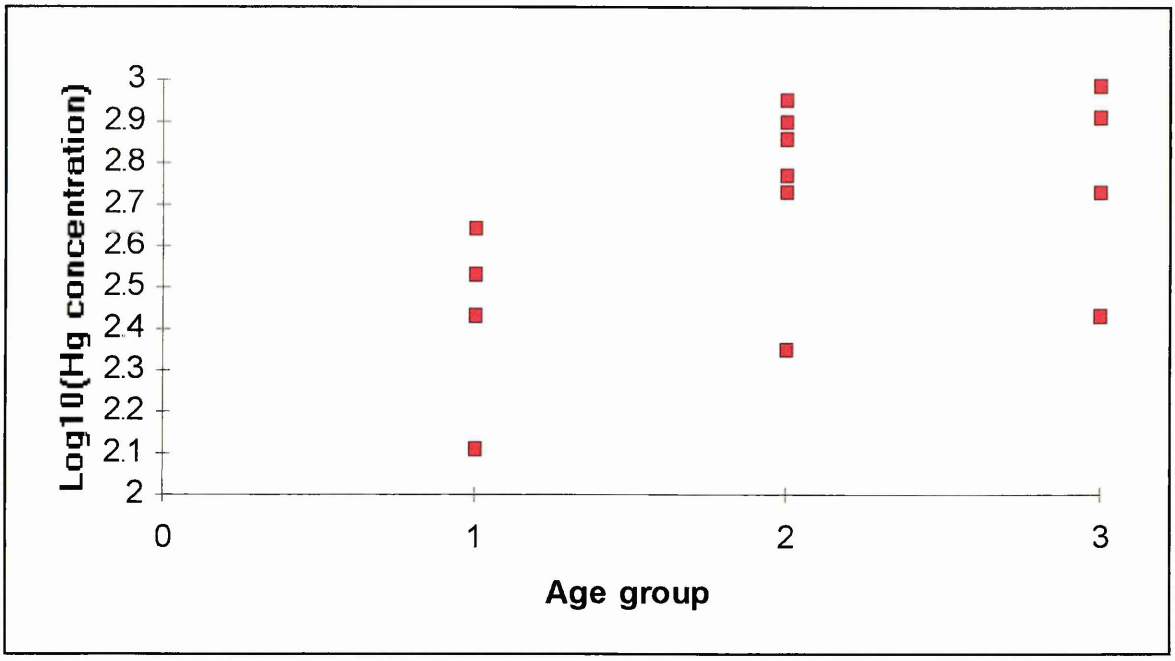


Figure 24: Log_{10} (Hg concentration in hair) vs age group

The product moment correlation coefficient, ρ, between the age and the mercury concentration in hair, was calculated using the Spearman's method.

The value of ρ was: $\rho = 0.5071$ (95% Confidence Interval -0.007 to 0.809). The confidence interval includes zero, so the correlation is considered non significant, however, the indication of a trend exists ($P = 0.054$).

Because the population considered is so small, it is difficult to get significant correlations. If a larger population was to be studied, it is possible that this trend would become a significant correlation.

4.1.2- Correlation between number of fillings and mercury concentration in hair

The geometric means of the mercury concentration in hair for each filling group are given in Table 24. The logarithm values of mercury concentration in hair are plotted against the filling group (Figure 25).

Table 24: Mean mercury concentration for each filling group

Filling group	Mean of the log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)
A	2.55	355	128	889
B	2.69	490	269	800
C	2.89	776	591	988

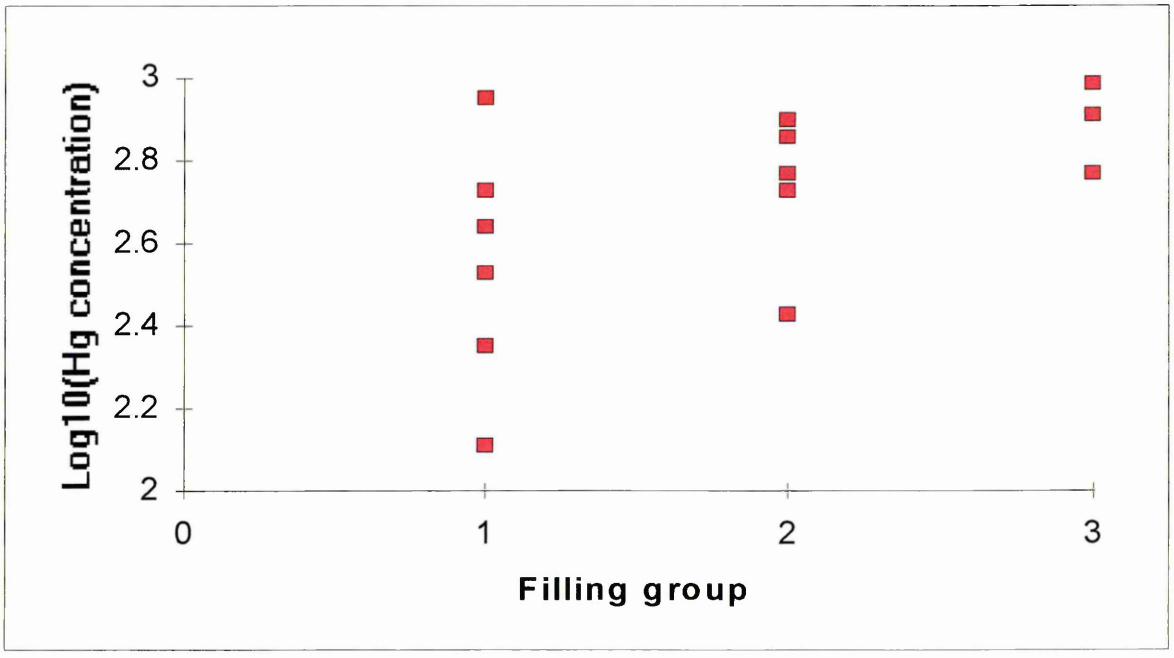


Figure 25: Log(Hg concentration) vs filling group (1=A, 2=B, 3=C)

The value of ρ obtained for the correlation coefficient was: ρ = 0.5003 (95% Confidence Interval -0.016 to 0.806)

The results are similar to the ones obtained for the age groups. The correlation is considered non significant because zero is included in the correlation coefficient, but a trend is present (P= 0.058): it is likely that there is a correlation between the number of fillings and the mercury concentration in hair. If a larger population was studied, the correlation would possibly be found to be significant.

4.1.3- Correlation between number of fish meals per week and mercury levels in hair

The results are summarised per number of fish meal in Table 25, and the logarithms of the mercury concentrations in hair are plotted in Figure 26.

Table 25: Mean mercury concentrations for different numbers of fish meals per week

Number of fish meals per week	Mean of the log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)
0	2.48	302	269	335
0.25	2.73	531	531	-
0.5	2.54	347	223	532
1	2.63	427	128	988

Table 25 continued

Number of fish meals per week	Mean of the log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)
1.5	2.86	725	725	-
2	2.80	627	270	889
3	2.77	594	594	-

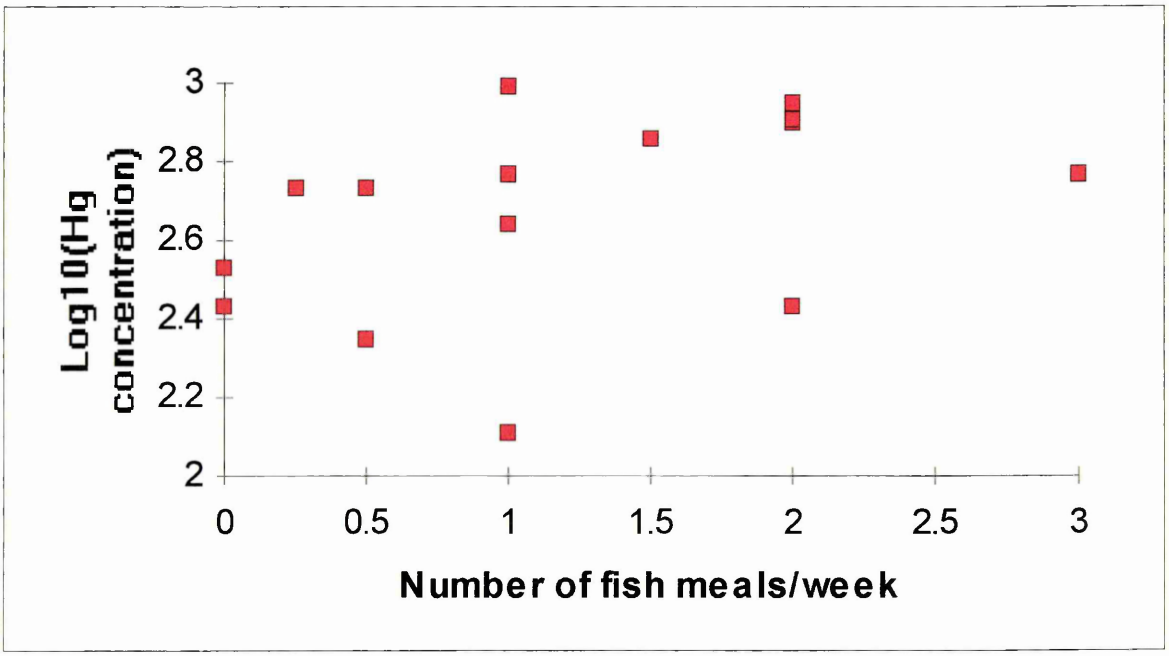


Figure 26: Log_{10} (mercury concentration in hair) vs frequency of fish meals

Spearman's calculations were carried out. The results were: $\rho = 0.5393$ (95% Confidence Interval 0.037 to 0.824)

In this case, although the correlation is relatively low, it is statistically significant: zero is not included in the confidence interval. The concentration of mercury in hair is thus significantly

correlated to the amount of fish eaten: fish seems to be the most important source of mercury exposure to people in the UK.

4.1.4- Summary

Despite the small number of cases involved, some interesting trends could be determined, such as the positive correlation between the mercury concentration in hair and the age, and the number of fillings.

The frequency of fish meals was found to be significantly correlated to the mercury levels in hair. This was in accordance with results already published [59, 67, 68].

It would have been interesting to study the influence of other factors, such as the shampoo used and the area in which the volunteers lived, on the concentration of mercury in their hair. However, because the population considered was so small, it was not possible to define different subgroups for each shampoo and each area of Sheffield.

4.2- Correlation study

Thousands of cord samples were routinely analysed for mercury as part of the ALSPAC study. After about 1000 samples were analysed, about 10% (with the highest concentration of mercury) of the samples were selected: 107 reference numbers were noted, and the existence of matching blood and hair samples was investigated.

The cord samples were collected at birth. The mothers' blood samples were collected during the first term of pregnancy. The babies' hair samples were collected when the children were eight months old.

Unfortunately, because of the number of samples stored in Bristol and the complexity of looking for one particular sample amongst thousands of others, only 20 cord samples could be matched with blood samples, and 26 with hair samples.

The hair and blood samples were all analysed for total mercury and the existence of a correlation between these concentrations was investigated.

The histograms of the frequency of mercury concentrations were plotted, in cord (Figure 27), in hair (Figure 28), and in blood (Figure 29).

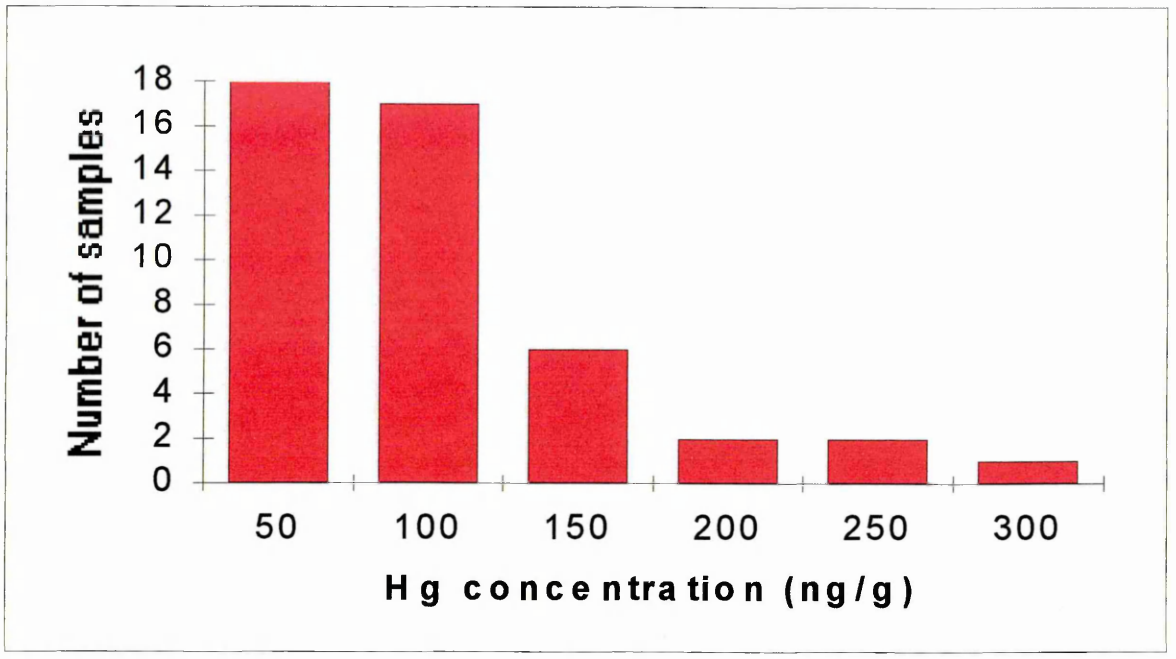


Figure 27: Histogram of the frequency distribution of mercury in cord samples

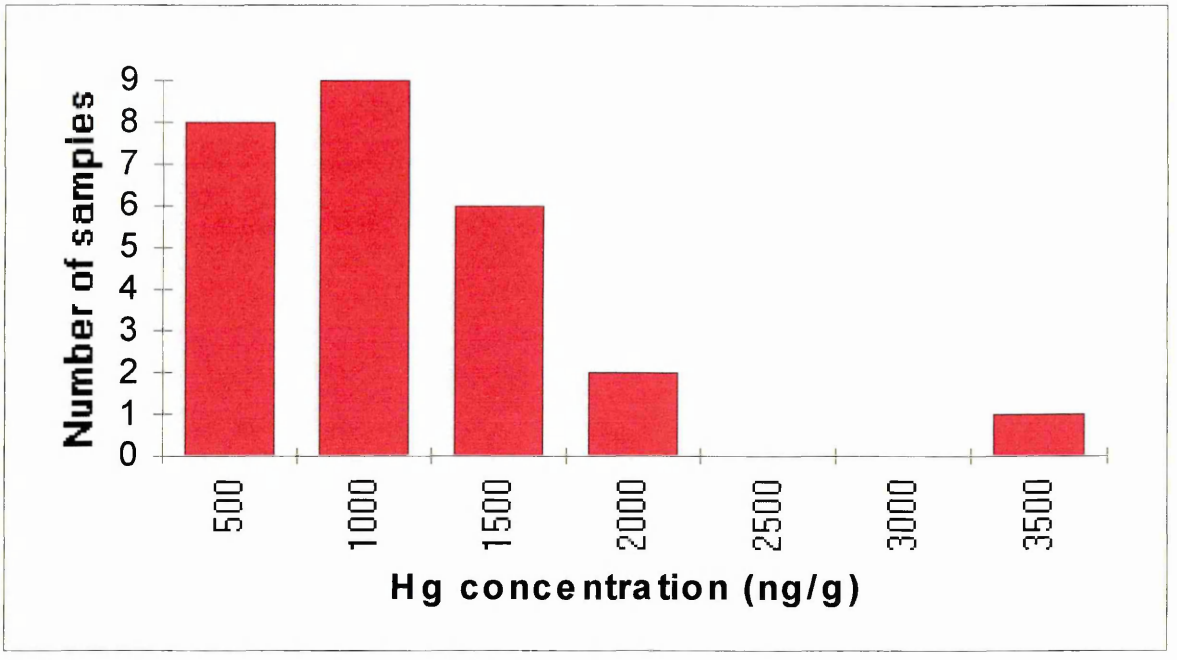


Figure 28: Histogram of the frequency distribution of mercury in hair

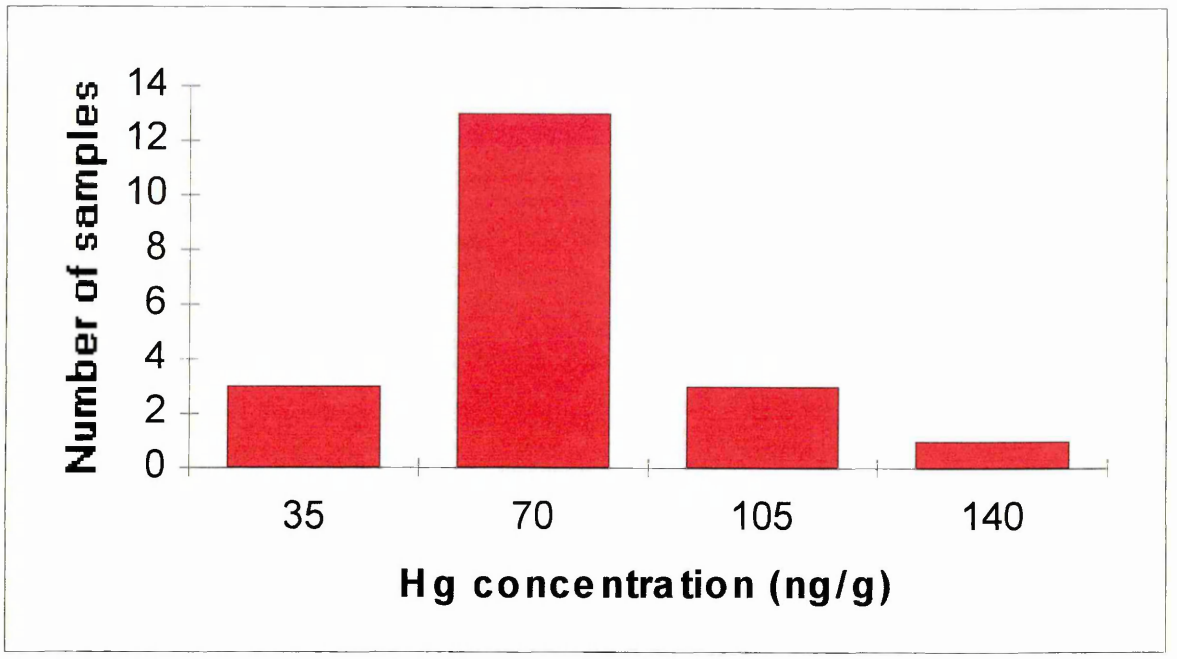


Figure 29: Histogram of the distribution of mercury levels in blood

As can be seen from Figures 27, 28 and 29, the frequency distribution of the mercury concentrations is positively skew. It is then possible to use a logarithm transformation for each value in x and y, in order to normalise the data, and thus, Pearson's method can be used.

The existence of a correlation between the mercury concentrations in cord samples and the mother's blood at the beginning of the pregnancy, and the cord samples and the baby's hair at 8 months old was investigated.

4.2.1- Correlation between cord and blood samples

Logarithm transformations were used throughout. The geometric means for mercury levels in cord and blood are given in Table 26. The logarithms of the mercury values in mothers'

blood samples were plotted against the logarithms of the mercury concentrations in cord samples (Figure 30).

Table 26: Geometric means for mercury levels in cord and blood samples

	Mean of the log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)
Cord samples	1.8046	64	37	207
Blood samples	1.7118	52	32	109

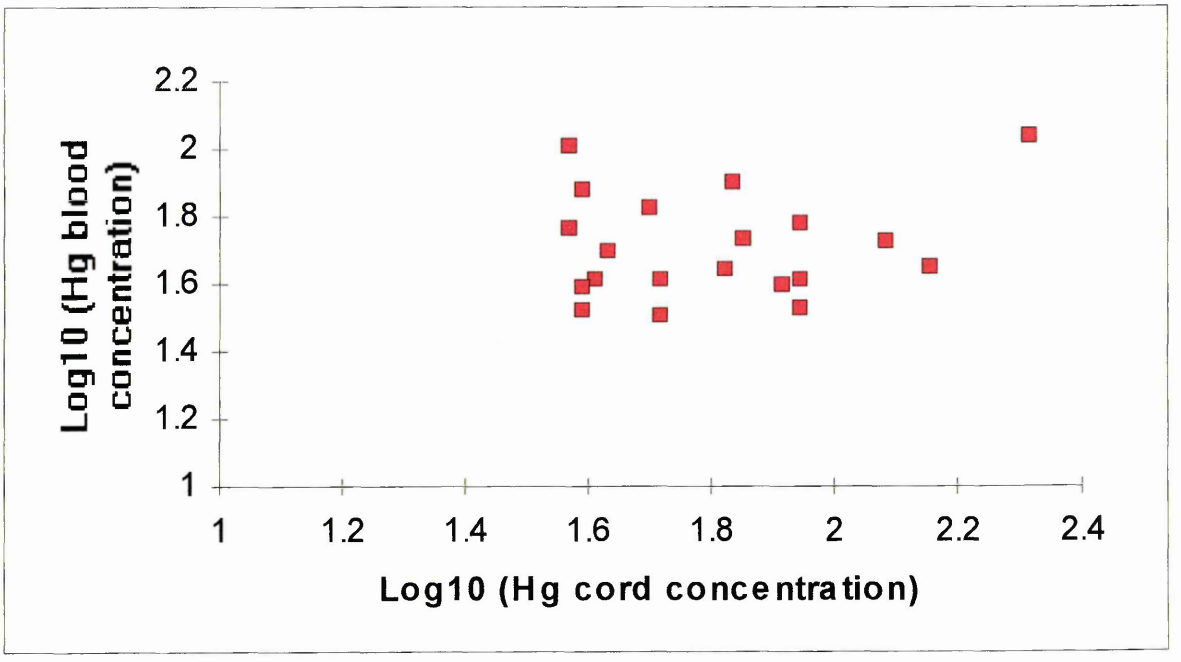


Figure 30: Log_{10} (Hg concentration in blood) Vs Log_{10} (Hg concentration in cord)

The product-moment correlation coefficient, r, was calculated for the logarithm transformed values with the Pearson's method. The details of the calculations are given in Appendix 4.

The results were: r = 0.154 (95% Confidence Interval -0.310 to 0.558)

r is very low and zero is included in the confidence interval, so there is no significant correlation between the mercury levels in cord samples at birth and the mothers' blood during the first term of pregnancy (P = 0.516).

4.2.2- Correlation between cord and hair samples

The geometric means for the mercury levels in hair and cord are given in Table 27. The logarithm transformed values for mercury concentrations in hair were plotted against the values for cord samples (Figure 31). Pearson's method was used to calculate the correlation coefficient between these two variables.

Table 27: Geometric means for mercury levels in hair and cord samples

	Mean of the log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)
Cord samples	1.8360	69	35	252
Hair samples	2.8387	690	149	3124

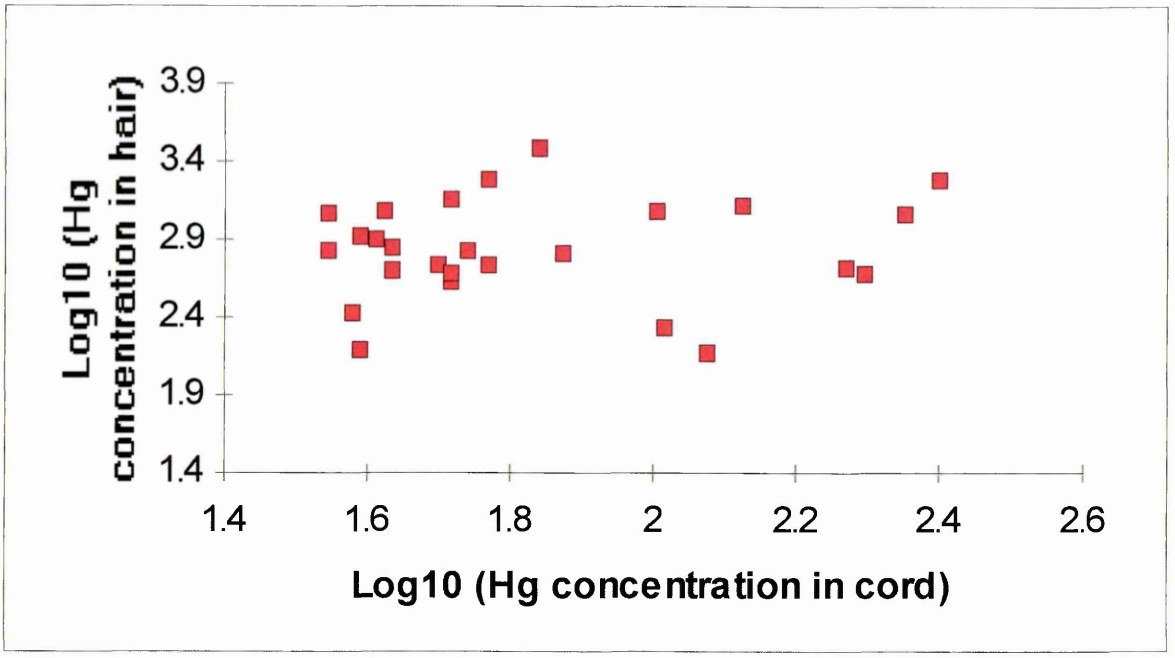


Figure 31: Log_{10} (Hg concentration in hair) Vs Log_{10} (Hg concentration in cord)

The calculations for r can be found in Appendix 5. The results were: r = 0.114 (95% Confidence Interval -0.286 to 0.480)

With r so small, and zero being included in the confidence interval, there does not seem to be a significant correlation between the levels of mercury in cord and in the child's hair at eight months old (P = 0.58).

4.2.3- Summary

No correlation was found between the mercury levels in cord samples and the mothers' blood during the first term of pregnancy, and cord samples and the babies's hair at 8 months old.

In both cases, the results were in accordance with Keenan and Cooke's work (submitted for publication), where the correlation of antimony concentrations in the same matrices (cord, hair and blood) was investigated, and found to be non significant.

This suggests that the mercury burden of a child's body after birth is more influenced by external factors, such as the environment and the diet, than by the body burden of the mother at birth. On the other hand, blood may be a poor sample to estimate body burden, as it is representative of a limited time period only, and it does not accumulate mercury.

4.3- Child/mother/partner study

Hair samples collected as part of the ALSPAC study, from children, their mother and the mother's partner, were analysed for total mercury using the method described in Chapter 3. In order to investigate further the conclusions obtained in section 4.1, the existence of a correlation between the mercury concentrations of each family member was investigated. A total of 24 families were included in this study. The results are summarised in Table 28.

Table 28: Hg concentration in child's, mother's and partner's hair

Family identification number	Hg conc. in child's hair ($ng.g^{-1}$)	Hg conc. in mother's hair ($ng.g^{-1}$)	Hg conc. in partner's hair ($ng.g^{-1}$)
1	472	263	181
2	204	200	130
3	49	153	193
4	174	333	598
5	136	106	102
6	46	95	31
7	62	74	201
8	117	72	73
9	102	77	65
10	86	486	1
11	129	225	151
12	-	116	-
13	169	593	608
14	182	199	447

Table 28 continued

Family identification number	Hg conc. in child's hair ($ng.g^{-1}$)	Hg conc. in mother's hair ($ng.g^{-1}$)	Hg conc. in partner's hair ($ng.g^{-1}$)
15	136	247	115
16	-	394	225
17	210	40	190
18	246	203	402
19	-	298	200
20	107	-	-
21	266	117	221
22	50	20	-
23	-	41	158
24	-	109	-

The mean mercury concentration in children's hair was 161 ± 100 $ng.g^{-1}$. The mean concentration in mothers' hair was 109 ± 150 $ng.g^{-1}$. The mean concentration in partners' hair was 215 ± 170 $ng.g^{-1}$. It is noteworthy that the mercury concentration in the men's hair is significantly higher than in the women (comparison of the mean of two samples: $t = 2.17$, for a critical value of 2.02, for $P = 0.05$ and 41 degrees of freedom), as reported in the literature [41, 59, 67]. All the concentrations were plotted onto the same graph (Figure 32).

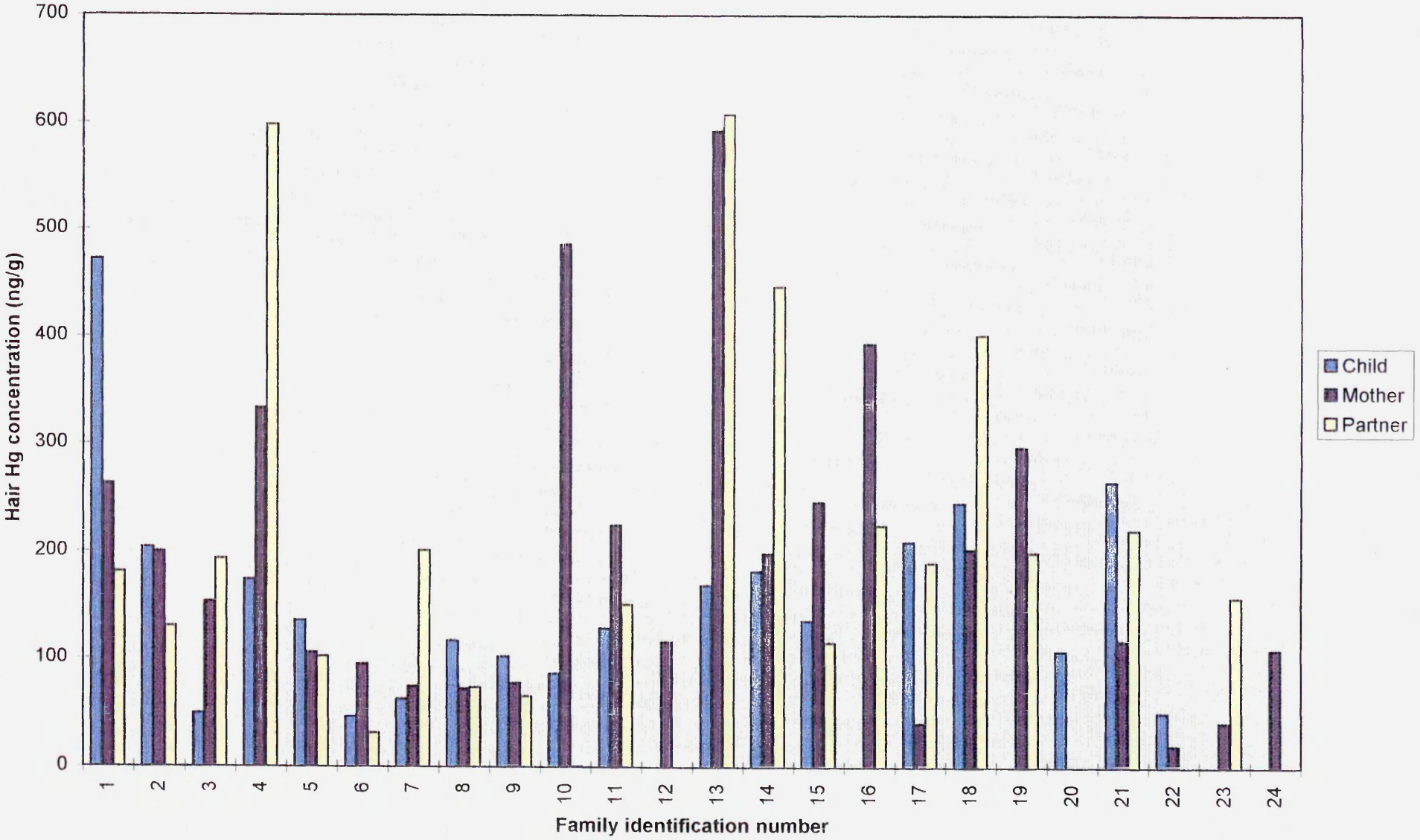


Figure 32: Mercury Concentration in families' hair

The frequency of the distribution of mercury levels in hair was plotted, in order to choose the best statistical method for the determination of correlations, for the child (Figure 33), the mother (Figure 34), and the partner (Figure 35).

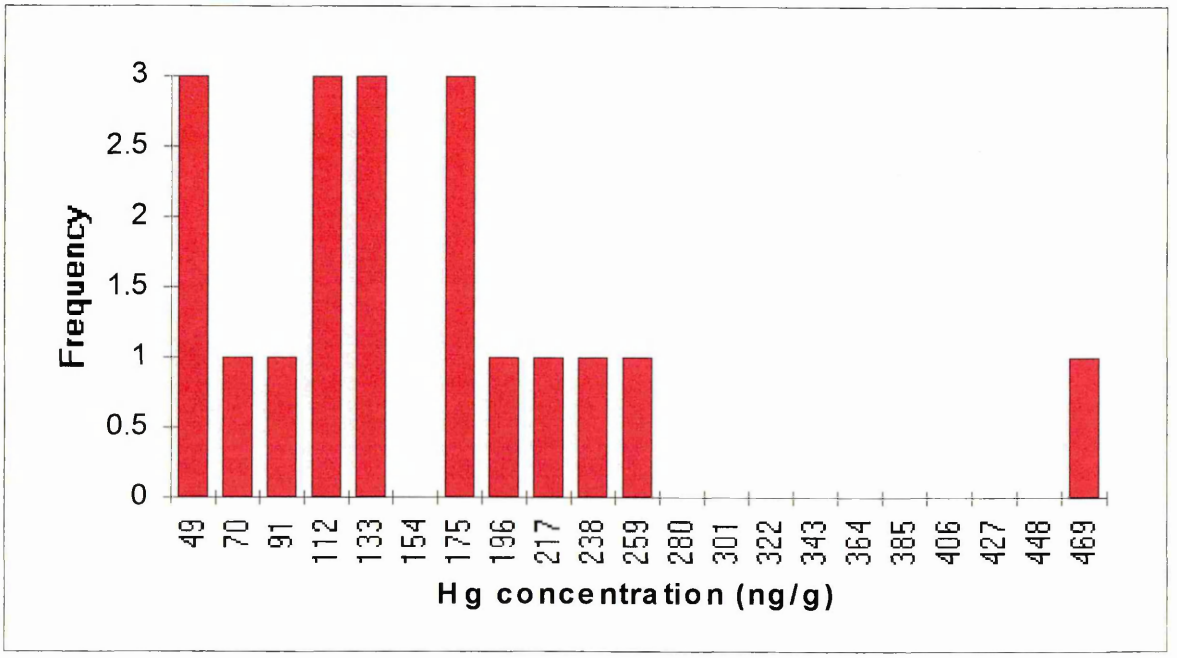


Figure 33: Frequency histogram of the mercury levels in child' hair

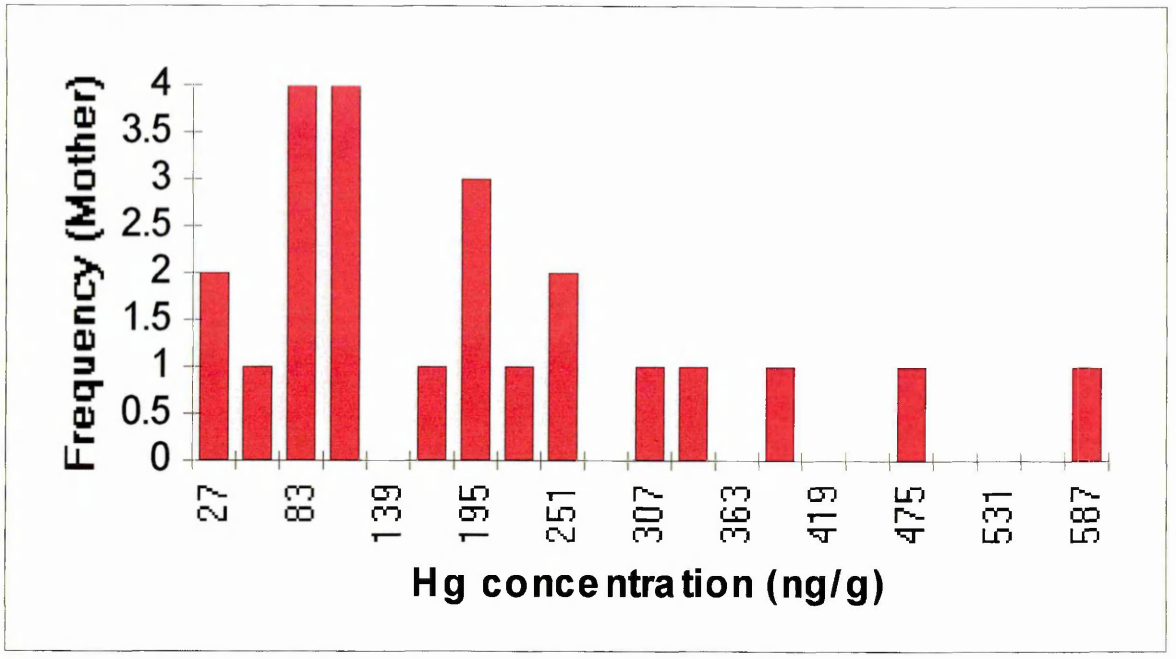


Figure 34: Frequency histogram of the mercury levels in mother's hair

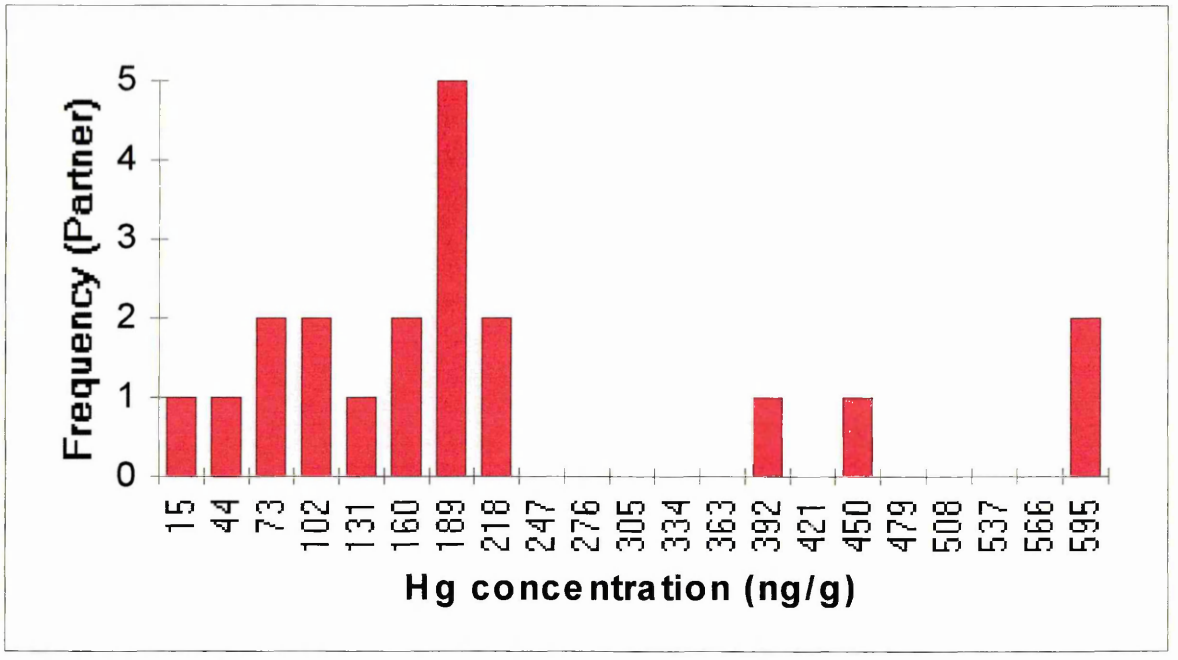


Figure 35: Frequency histogram for the mercury levels in partner's hair

The histograms plotted above clearly show that the frequency of mercury concentration in positively skew in all cases. Pearson's method can be used with the logarithm transformed data.

4.3.1- Correlation coefficients

- *Correlation coefficient between the child and the mother*

The logarithm transformations of the child's and the mother's hair mercury concentration were plotted against one another (Figure 36), and the geometric means for the mercury values are given in Table 29.

Table 29: Geometric means for the hair mercury concentrations in mother and child

	Mean of the log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)
Child	2.164	146	46	472
Mother	2.157	143	20	593

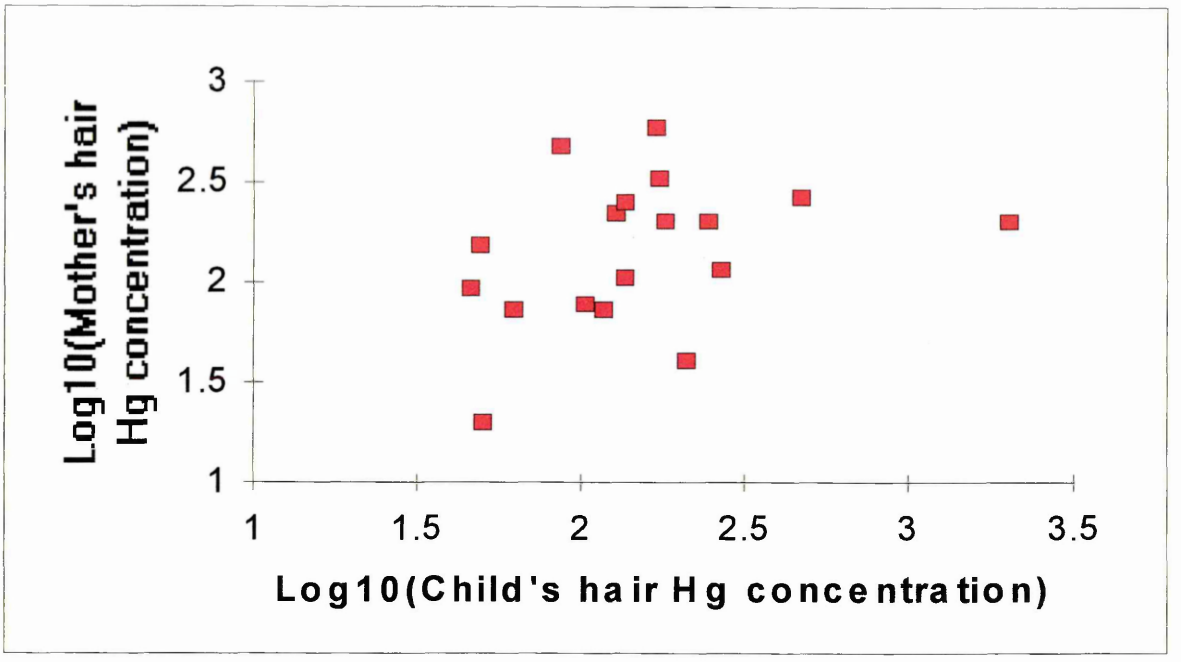


Figure 36: Log_{10} Child's Hg concentration Vs Log_{10} Mother's Hg concentration

Pearson's calculations were performed with the Minitab software and the results were:

r = 0.382 (95% Confidence Interval -0.103 to 0.721)

As zero is included in the confidence interval, there is no significant correlation between the mother's and the child's hair mercury concentrations (P = 0.234).

- *Correlation coefficient between the child and the partner*

The mean for the partner's hair mercury concentrations is summarised in Table 30. A plot of the logarithm transformations of the child's values against the logarithm transformations of the partner's values is shown in Figure 37.

Table 30: Geometric mean of the mercury concentrations in partner's hair

	Mean of the log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)
Partner	2.131	135	1	608

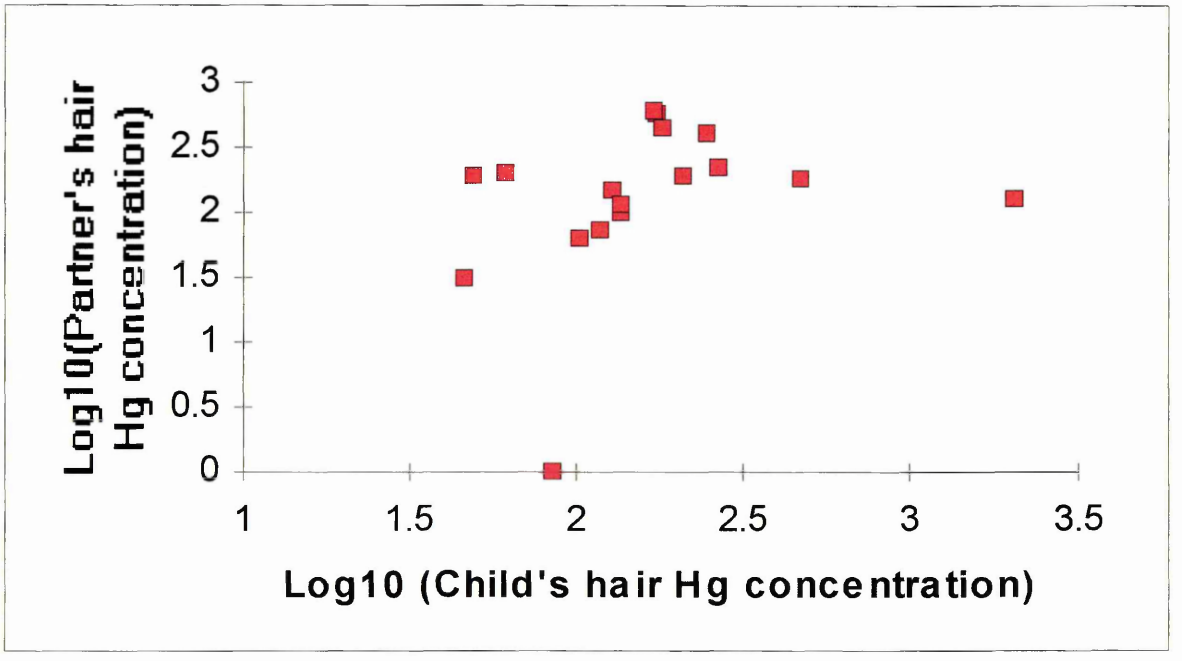


Figure 37: Log_{10} Child's Hg concentration Vs Log_{10} Partner's Hg concentration

The calculations using Pearson's method gave: r = 0.412 (95% Confidence Interval -0.086 to 0.745).

The results show that there is no significant correlation between the child's hair mercury concentration and the partner's hair mercury concentration (P = 0.200).

- *Correlation coefficient between mother and partner*

The results of Pearson's calculations were: r = -0.016 (95% Confidence Interval -0.455 to 0.43).

As zero is included in the confidence interval, and r is very low, there is no correlation between the mercury concentration in the mother's and her partner's hair (P = 0.948).

- *Summary*

This study of correlation coefficients between the mercury levels in a child's hair, his mother's and her partner's hair, shows no correlation. It is likely that the child's diet may be different from his parents'. Also, the number of fillings and the exposure to mercury through work all contribute to the mercury levels in hair, and may differ between the mother and her partner.

It is thus very difficult to find a correlation between mercury levels in the hair of people from a same family, as so many external parameters intervene.

4.3.2- Study of some parameters influencing mercury levels

During the ALSPAC study, many questionnaires were filled in by the volunteers. The relationship between some of the information in these questionnaires and the mercury levels measured in hair was investigated.

A few parameters known to influence mercury levels, such as age, fish consumption, number of fillings, and breastfeeding of the child, were considered.

4.3.2.1- For the child

- *Current feeding practice*

Mothers were asked how they were currently feeding their baby. The answers are summarised in Table 31. Logarithm transforms of the mercury values are used again, as the histogram distribution of the mercury in hair was showed to be positively skew earlier.

Table 31: Mean of mercury levels in child's hair related to current feeding

	Mean of log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)	Number of cases
Breast	2.248	179	117	266	6
Bottle	1.918	83	46	210	8
Breast + bottle	2.318	208	107	472	4

The comparison of the mean of two samples was carried out (Appendix 1):

- between breast and bottle: $t = 2.92$ for a critical value of 2.18 for $P = 0.05$ and 12 degrees of freedom,
- between breast and bottle + breast: $t = 0.55$ for a critical value of 2.31 for $P = 0.05$ and 8 degrees of freedom,
- and between bottle and breast + bottle: $t = 2.58$ for a critical value of 2.23 for $P = 0.05$ and 10 degrees of freedom.

The results indicate that there is no significant difference in the mercury levels found in groups 1 and 3 (breast alone, and breast and bottle together). In contrast, group 2 (bottle alone) had significantly different mercury levels than groups 1 and 3. This proves that breast-feeding is the most important source of mercury exposure for babies. This is well in agreement with the literature [62, 64, 70].

- *Duration of breast-feeding*

Mothers were asked how long they had breast-fed their babies, and their answers were correlated with the mercury levels found in the babies' hair. Results are summarised in Table 32.

Table 32: Mercury levels in child's hair and the duration of breast-feeding

	Mean of log values	Geometric mean ($ng.g^{-1}$)	Minimum value ($ng.g^{-1}$)	Maximum value ($ng.g^{-1}$)	Number of cases
Never	1.969	93	46	136	3
< 1 month	1.938	87	50	210	3
1- <3 months	2.113	130	49	472	7
3- <6 months	2.316	207	174	246	2
6+ months	2.243	175	102	266	4

The correlation coefficient was calculated: $r = 0.433$,

The correlation coefficient, r, is low, so the correlation between the duration of breast-feeding and mercury levels in the child's hair is considered non significant. However, a trend does exist between the two variables ($P = 0.064$), which confirms the results obtained of the current feeding practice, as well as the literature [70].

- *Development scores*

Ten tests were carried out on the children, to determine their development scores. Their scores were then related to the mercury levels in the children's hair. The correlation coefficients are given for each test:

- Test 1: r = 0.05, test 2: r = 0.18, test 3: r = -0.12, test 4: r = -0.03, test 5: r = -0.24, test 6: r = -0.10, test 7: r = 0.09, test 8: r = 0.09, test 9: r = -0.11, test 10: r = 0.07.

The correlation coefficients are very low for all the tests, so there is no correlation between the mercury levels in children's hair and their development scores at 6 months of age. This is in disagreement with Sikorski *et al.* [22], and Grandjean *et al.* [62], who reported a negative influence of mercury on the development of children. It is also in disagreement with Grandjean *et al.*[64], who reported early milestone development associated with longer breast-feeding and higher mercury levels. The influence of mercury on the development of children depends on the level of mercury measured in the hair samples. Grandjean states that a maternal hair mercury level of 15 $\mu g.g^{-1}$ is associated with decreased test performance [62], however, in our study, the mercury levels measured in hair were all inferior to 1 $\mu g.g^{-1}$. It is likely that the difference in exposure, and thus the difference of hair mercury concentrations explains the different statements about the influence of mercury on child development.

Children considered in this study are therefore not at risk from mercury, as mercury levels measured are at least one order of magnitude lower than mercury levels reported to be associated with decreased performances. This is in agreement with Sherlock's work [72], where people in the UK are considered unlikely to be affected by mercury.

4.3.2.2- For the mother

The influence of the frequency of fish eating was considered. No significant correlation or trend could be found. This is not in agreement with our results in Section 4.1, where a trend was present, even though a significant correlation could not be detected. The fact that the data in 4.1 was obtained from a Sheffield population, and this data was obtained from people in Bristol may explain this difference.

Another parameter studied for the mother was her age. The logarithm transforms of hair mercury concentrations were plotted against the age of the mother (Figure 38).

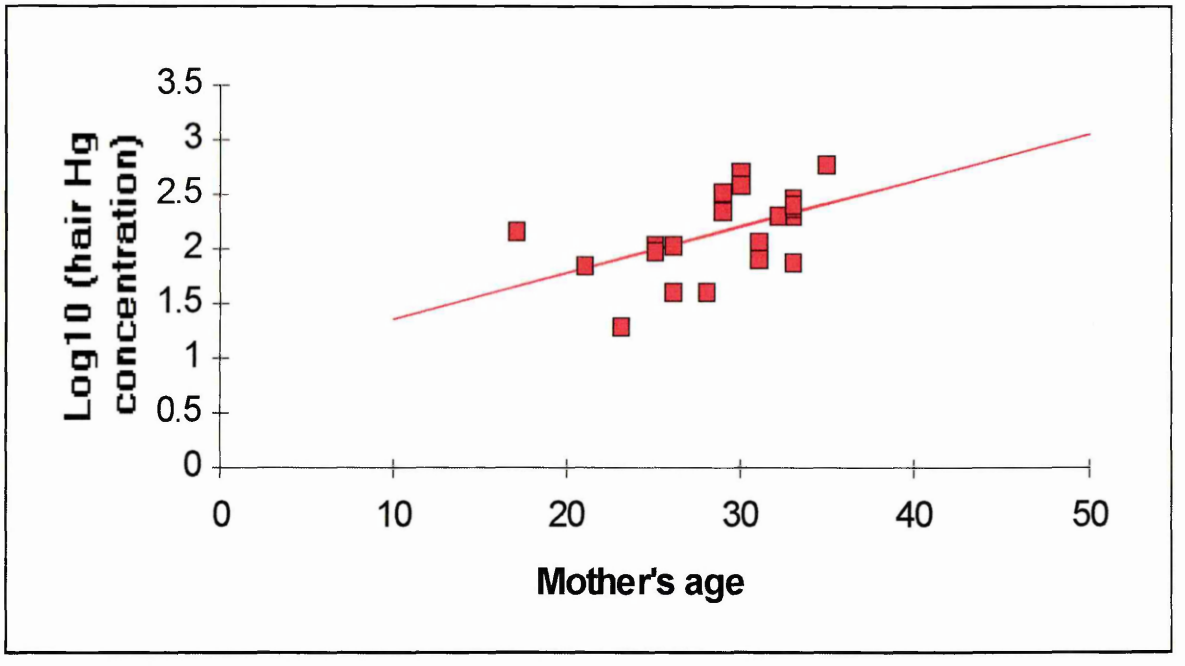


Figure 38: Mother's age vs Log_{10} hair mercury concentration

The calculations for the correlation coefficient between the age and the hair mercury concentrations for the mothers gave r = 0.513.

The correlation coefficient is low, but there is a significant positive correlation between the age of the mothers and the mercury levels in their hair (P = 0.014).

4.3.2.3- For the partner

The age of the partner was also studied in order to try and find a correlation with the hair mercury levels. The logarithm transformations of the mercury concentrations are plotted against the age in Figure 39.

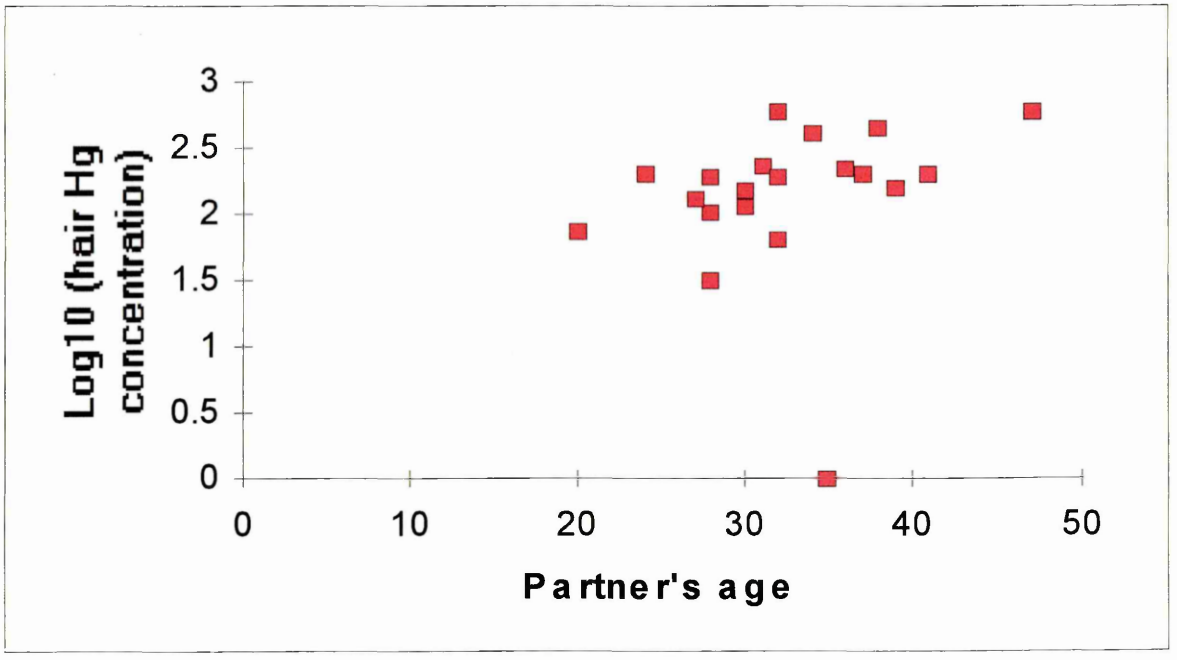


Figure 39: Partner's age vs Log_{10} hair mercury concentration

The calculations for the correlation coefficient gave r = -0.257.

These results indicate that there is no significant correlation between the age of the partners and their mercury levels in hair (P = 0.288). However, the graph plotted above (Figure 39) is very similar to the graph plotted for the mothers (Figure 38). It would thus be expected that a significant correlation would be found. It is possible that the lowest point (where Log_{10} hair Hg concentration = 0) acts as an outlier and prevents the calculation of a correlation coefficient. Indeed, the same graph can be plotted without the outlier, and the graph shows clearly a correlation between the age of the partners and their mercury levels (Figure 40).

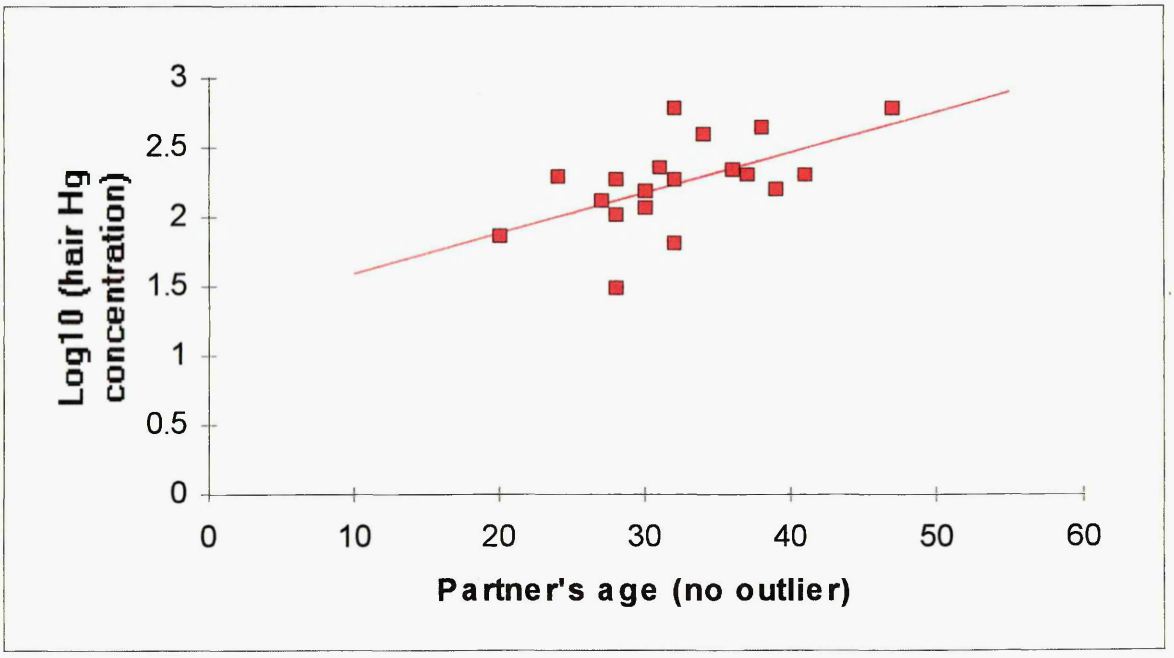


Figure 40: Partner's age vs Log_{10} hair mercury concentration (without outlier)

Chapter 5: An outlook on future work:

Speciation of mercury in hair

5.1-Introduction

5.1.1- Methylation of mercury in the environment

Methylation is an important process in the cycle of mercury in the environment. Methylated species are the main forms of mercury in the biota (fish, and subsequently humans). Methylation occurs mainly in sediments and in fish. A number of factors, such as temperature, pH, organic enrichment, oxygen content and depth, determine the extent of methylation [97].

5.1.1.1- Mechanisms of methylation

Two mechanisms have been identified for methylation: a microbial methylation, and an abiotic methylation.

- *Microbial methylation*

Methylcobalamine is one of three naturally occurring methylating agents, with S-adenosylmethionine and N- methyltetrahydrofolate, and is thought to be the most important one [97]. Methylcobalamine is formed from vitamin B_{12}, present in most living organisms, by methane-producing bacteria. Microbial methylation can be enzymatic or non-enzymatic. The role of bacteria, such as Clostridium and Pseudomonas, is crucial for methylation.

• *Abiotic methylation*

The two main abiotic processes are transmethylation and photochemical reaction. These methylation processes are non-enzymatic, and involve a reagent which is produced biotically. During the transmethylation process, one methyl group is transferred from one metal to another. In the photochemical process, methyl is produced photochemically, e.g. by exposure of acetate, methanol, ethanol, or aliphatic amino-acids to intense UV light.

The process of demethylation can be chemical or involve micro-organisms.

5.1.1.2- Factors influencing methylation and demethylation

As mentioned before, a number of factors influence methylating processes.

• *The effect of oxygen*

Methylation and demethylation can occur aerobically and anaerobically. In sediments, the best conditions seem to be anaerobic [97].

• *The effect of temperature*

Methylation increases with temperature, and different methylating rates have been noted following the seasons. This could be linked to the effect of temperature on bacterial population.

• *The effect of pH*

Methylation seems to be more important under slightly acidic conditions.

• *Other factors*

The concentration of mercury affects methylation: the formation of methyl mercury increases when the concentration of mercury is increased. The sulphides concentration also affects methylation. Finally, methylation also depends on depth: it occurs mainly on sediment surface.

5.1.2- Review of analytical methods

5.1.2.1- The separation between inorganic and organic mercury

5.1.2.1.1- Step-wise reduction

Both inorganic and organic mercury species have been determined after selective reduction [7]. The method developed by Magos (1971) [96], and later modified for higher sensitivity, involves the use of stannous chloride to reduce inorganic mercury, followed by aeration, then organic mercury was decomposed with alkaline Cd and determined by AAS. Stannous chloride was also used to reduce inorganic mercury, followed by sodium borohydride to reduce organic mercury, after digestion of the hair with KOH [47].

5.1.2.1.2- Solvent extraction

Solvent extraction was used to separate inorganic and organic mercury in fish tissues. Magos [96] used back-extraction into an aqueous solution containing cysteine, and CV-AAS was then used for the measurement. He reported detection limits of 10 $ng.g^{-1}$. Extraction of mercury species from digested hair samples using benzene, thionalide and toluene + cysteine + benzene have been reported [37, 44, 45, 52].

5.1.2.1.3- Steam distillation

Organic and inorganic mercury were separated by steam distillation by Mitani (1976) in tissue samples. Horvat et al. (1988) also used this procedure for a variety of biological samples.

5.1.2.1.4- Ethylation

Hair samples were digested with KOH, then ethylated with $NaBEt_4$ and separated by GC. After separation, mercury was then converted to its elemental form and detected by AAS [53].

5.1.2.1.5- Ion-exchange chromatography

An ion-exchange column was used to separate MeHg and inorganic mercury in biological samples by May *et al.* [82]. Inorganic mercury was adsorbed on the column, while MeHg was eluted and measured. Inorganic mercury was later eluted with nitric acid. CV-AAS was used as the measurement method.

5.1.2.2- The use of coupled (or hyphenated) techniques

5.1.2.2.1- Gas chromatography

Gas chromatography is the method of choice for the determination of organic mercury in biological samples. In conventional GC, an electron capture detector (ECD) is used, the compounds are thus determined as halides. AAS, AFS and OES detectors offer better selectivity. For positive identification of compounds, mass spectrometry detectors have been used.

Gas Chromatography has been used for the speciation of mercury in hair [36, 45], and it has also been associated with MIP-PED [55], and CV-AFS [53]. GC-AES offers a wide dynamic calibration range, the possibility of simultaneous multi-element detection, and high elemental sensitivity.

This is a more recent development for the determination of organic mercury. High Performance Liquid Chromatographic systems have been coupled to CV-AFS [97, 98], CV-ICP-AES and ICP-MS [7].

5.1.3- Theory of High Performance Liquid Chromatography (HPLC)

5.1.3.1- General considerations

Only about 20% of known compounds lend themselves to analysis by gas chromatography either because they are not sufficiently volatile or because they are thermally unstable. High Performance Liquid Chromatography (HPLC) is not limited by sample volatility or thermal stability.

HPLC is able to separate macromolecules and ionic species, labile natural products, polymeric materials and a wide variety of other high molecular weight polyfunctional substances. Chromatographic separation in HPLC is the result of selective partitioning between sample molecules between the stationary and mobile phases.

HPLC offers a reasonable choice of stationary phases and solvents, which permit an adequate variety of these selective interactions and so facilitates most separations.

5.1.3.2- HPLC instrumentation

The general instrumentation for HPLC incorporates the following components: a solvent reservoir for the mobile phase, a pump to deliver the mobile phase to the column, a sample valve or loop to inject the sample into the flowing mobile phase, a separation column with the appropriate packing to achieve desired HPLC separation, and a detector with a data handling device (e.g. an integrator).

5.1.3.3- Columns

Columns are made of heavy wall, glass-lined metal tubing or stainless steel tubing to withstand high pressures and the chemical action of the mobile phase. Straight columns are preferred and are usually operated in the vertical position.

Most columns range in length from 10 to 30 cm, whereas short, fast columns are 3 to 8 cm. For exclusion chromatography, columns are 50 to 100 cm long. Many HPLC separations are performed on columns with an internal diameter of 4 to 5 mm. Such columns provide a good compromise between efficiency, sample capacity and the amount of solvent and packing required.

Column packing feature particles that are uniformly sized and mechanically stable. Particle diameter lies in the range 3-5 μm. Packing may be either silica or polymer based.

5.1.3.4- Detectors

The sensitive universal detector for HPLC has not been designed yet, unless one accepts mass spectrometry in this role. Thus it is necessary to select a detector on the basis of the problem at hand. For this project, a mercury fluorescence detector is used.

5.2- Instrumentation

5.2.1- Instrumental settings

The equipment used is an adaptation of the Merlin Plus system described in Chapter 3. A schematic representation of the instrument is given in Figure 41. It is made up of a mobile phase reservoir, three pumps, an injection loop (100 µl loop), two T-pieces, a reaction loop placed in a hot water bath, a gas/liquid separator, a fluorescent detector and finally an integrator/chart recorder. The temperature of the water bath was 85°C.

The gas flows used for the system were the same as used for the determination of total mercury and summarised in Table 4. The reagents flow rates are summarised in Table 33.

Table 33: Flow rates used for the speciation of mercury

Solution	Flow rate
Mobile phase	1.7 ml/min
Oxidising solution	3 ml/min
Reducing agent	4 ml/min

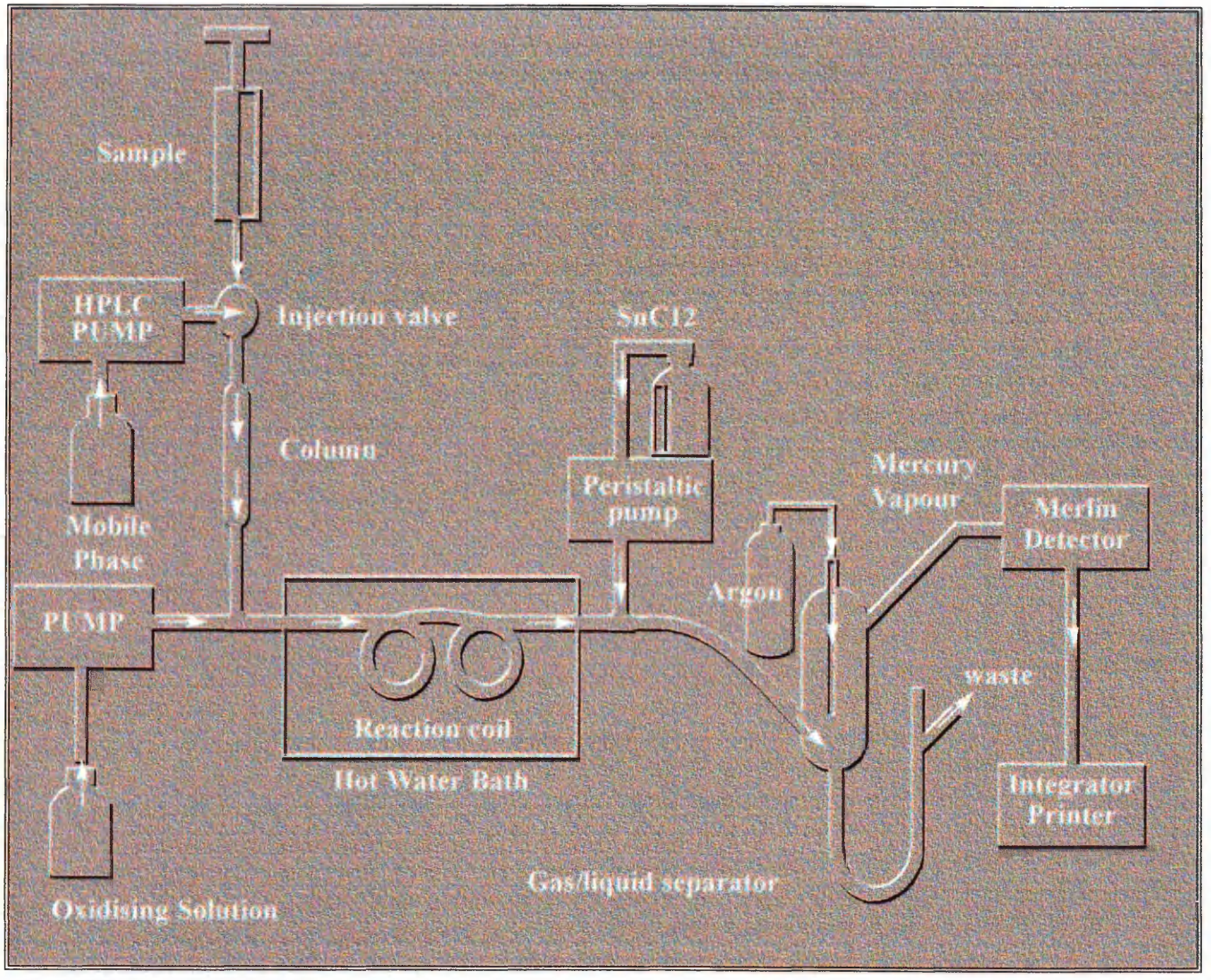


FIGURE 41: schematic diagram of the speciation system

5.2.2- Standard solutions

The inorganic mercury standards were prepared as described in section 2.2.3. The 1000 $mg.l^{-1}$ methyl mercury standard was prepared by dissolving 0.0125 g solid methyl mercury in a 10 ml flask with HPLC grade methanol.

The solution is protected from light and stored at 4°C , and can be used for up to a month after preparation. Standards were prepared using glass pipettes only.

A 10 $mg.l^{-1}$ standard was prepared by diluting 1 ml of the 1000 $mg.l^{-1}$ standard in a 100 ml flask with distilled water. This standard was stored in the dark at 4°C , for a week.

An intermediate 1 $mg.l^{-1}$ standard was prepared daily by diluting 1 ml of the 10 $mg.l^{-1}$ standard in a 10 ml flask with water. Working standards were prepared from the 1 $mg.l^{-1}$ standard.

Mixed standards of the same concentration of MeHg and Hg^{2+} were prepared by diluting the same volume of the 1 $mg.l^{-1}$ MeHg standard and of the 1 $mg.l^{-1}$ Hg^{2+} standard in a 100 ml flask with water.

5.2.3- Reagents

The mobile phase used was made up of 1.5% HPLC grade methanol in water with 0.01% (v/v) 2- mercaptoethanol ($HS.CH_2.CH_2.OH$).

The oxidising solution was prepared by dissolving 50 g di-potassium peroxodisulphate (potassium persulfate) and 3.6 g cupric sulphate with 40 ml pure Aristar sulphuric acid in a 1000 ml flask with water. The flask was placed in a sonic bath for 2h to speed up the dissolution of potassium persulfate.

The reducing agent was a stannous chloride solution prepared as described in section 2.2.3.

The acidified water used to remove the mercury present on the outside of the hair was prepared as follows: 100 µl Aristar hydrochloric acid was added to 200 ml Milli-Q water, and the pH was checked to be 2-2.5.

All the solutions were prepared fresh daily.

5.2.4- Maintenance of the system

All the maintenance details given in section 2.2.5 still apply. All glassware were cleaned overnight with a solution of bromine and dichloromethane, and thoroughly rinsed with distilled water.

The injection valve was cleaned by injecting distilled water several times at the end of each day.

The column was reconditioned regularly by pumping pure HPLC grade methanol through it, for about 1h every night, and overnight once a fortnight.

5.3- Experimental

To collect and measure the mercury adsorbed on the surface of the hair, approximately 0.2 g hair was shaken for 30 min on a mechanical shaker with 2 ml acidified water.

To extract all the mercury species from the hair, 0.2 g hair or 0.05 g CRM hair were placed in a Teflon microwave digestion flask with 5 ml KOH, and digested in the Milestone microwave digester following program 3 (c.f. chapter 3). The digests were left to cool in a water bath for one hour.

After digestion, the pH of the samples was about 14. The pH was rectified to the recommended range (2-6) with the addition of dilute hydrochloric acid, and the volume was made up to 25 ml with ultra pure water.

The samples were then injected through the system.

5.4- Results and discussion

5.4.1- Optimisation of the HPLC system

The method used was inspired from M. Gairin-Calvo's work [97], where a method for the speciation of mercury in soils and sediments by HPLC is investigated.

M. Gairin-Calvo's method uses the PSA Merlin Plus Mercury detection system described in section 3.1, to which an HPLC pump and column, as well as a hot water bath and a reaction coil, have been added. The set-up of the system in this work is similar, and shown on Figure 41.

In this section, the possibility of separating MeHg from Hg^{2+} in hair is assessed, and the analytical conditions are optimised.

The different parameters optimised in this work were: the length of the reaction coil, the strength of the oxidising solution, the flow rate of the mobile phase, and the concentration of methanol and mercapto-ethanol in the mobile phase.

5.4.1.1- Optimisation of the length of the reaction coil

The reaction coil is a critical part of the HPLC system. It is situated just after the first T-piece where the oxidising solution and sample solution meet. The oxidising solution is used to break down the mercury- carbon bond so that Hg^{2+} is released.

The amount of time both solutions stay in contact determine the yield of the oxidation, and thus the amount of MeHg converted into Hg^{2+}. Obviously, the ideal situation is when both solutions stay in contact for long enough to allow all the methyl mercury to be oxidised to Hg^{2+}.

However, the impact of the coil length on inorganic standards must also be assessed, if both species are to be determined simultaneously. The results of the study of the coil length influence on the signal intensity are plotted in Figure 42.

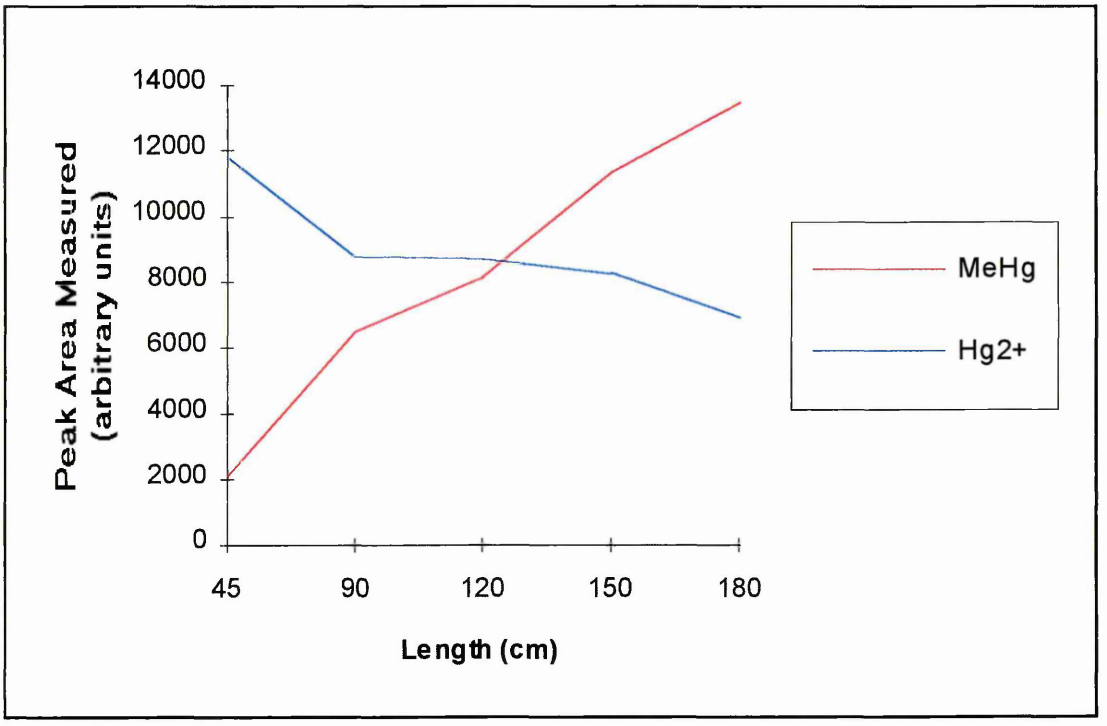


Figure 42: Influence of length of reaction coil on peak area measurement for MeHg and Hg^{2+} (10 $\mu g.l^{-1}$ standards were used)

In the case of methyl mercury, it can be seen in Figure 42, that the longer the reaction coil, the better the yield of oxidation. In contrast, the increase in the length of the reaction coil has an opposite effect on inorganic mercury. A likely explanation for the difference in behaviour is that, as the coil length increases, the presence of excess oxidising solution prevents the conversion of Hg^{2+} to Hg° by stannous chloride. Some of the mercury then stays in its Hg^{2+} form and is not detectable, which would explain the decrease in peak area measurement for inorganic mercury.

In order to optimise this parameter, one must thus choose a coil length that allows a good oxidation for methyl mercury without being too detrimental to the determination of inorganic mercury. The point at which both curves meet seems to be a reasonable compromise. The coil length chosen was therefore 120 cm.

5.4.1.2- Optimisation of the strength of the oxidising solution

In M. Gairin-Calvo's work, a solution of 25 g potassium persulfate + 1.8 g cupric sulphate + 20 ml sulphuric acid per litre is used. This oxidising solution has also been used elsewhere [98].

This solution was called strength 1, and the effect of using solutions of strengths 0.5, 1,1.5, 2, 2.5 and 3 were investigated. The solution of strength 0.5 was defined as a solution with 0.5 times the concentration of each compound in solution 1, and so on. The results are shown in Figure 43.

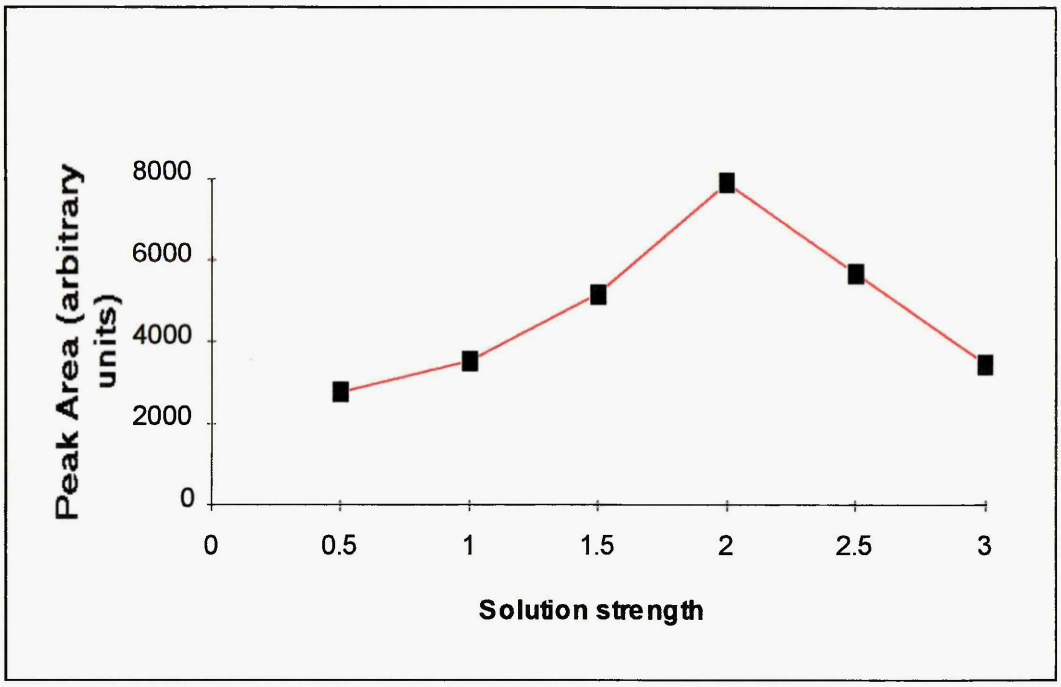


Figure 43: Influence of the strength of oxidising solution on peak area measurement (10 $\mu g.l^{-1}$ standards were used)

The strength of the oxidising solution has a dramatic effect on the peak area measured for the same standard (Figure 43). At first, as it was expected, the stronger the oxidising solution, the higher the peak area. However, after a strength of 2, the peak area starts decreasing.

The excess oxidising solution probably uses all the stannous chloride, and then there is not enough reducing agent to reduce all the mercury, which explains the decrease in peak area. The optimum strength for the oxidising solution is then 2, which corresponds to a solution of 50 $g.l^{-1}$ potassium persulfate + 3.6 $g.l^{-1}$ cupric sulphate + 4 % (v/v) sulphuric acid .

5.4.1.3- Optimisation of the composition of the mobile phase

The concentration of methanol in the mobile phase determines the resolution of the MeHg and inorganic mercury peaks. Concentrations of 0 to 10% (v/v) were tried, and the best separation was obtained for a 1.5% (v/v) methanol in water solution.

The concentration of mercapto-ethanol is critical, as the absence of it prevents the detection of any peaks but too high a concentration (about 1%) destroys the column. No difference was found in the quality of the separation when experiments were carried out with 0.01, 0.02 and 0.05% (v/v). Consequently, the concentration of mercapto-ethanol chosen was 0.01%.

5.4.2- Example of a calibration curve obtained with this system

An example is given of a typical calibration curve with the optimised parameters discussed previously. Standards of 10, 20, and 40 $\mu g.l^{-1}$ MeHg and Hg^{2+} are used. The mean retention times R_t and peak area P_A for each standard are given in Table 34. The calibration curve is plotted in Figure 44. A typical chromatogram is shown in Figure 45.

Table 34: Mean peak area and retention times for each standard

Standard ($\mu g.l^{-1}$)	R_t MeHg ± SD (s)	P_A MeHg ± SD (x 1000)	R_t Hg^{2+} ± SD (s)	P_A Hg^{2+} ± SD (x 1000)
40	12.47 ± 0.12	46125 ± 2096	16.43 ± 0.32	38261 ± 2859
20	12.67 ± 0.09	21455 ± 574	17.23 ± 0.43	20651± 2903
10	12.65 ± 0.2	10485 ± 910	16.67 ± 0.05	9245 ± 2351

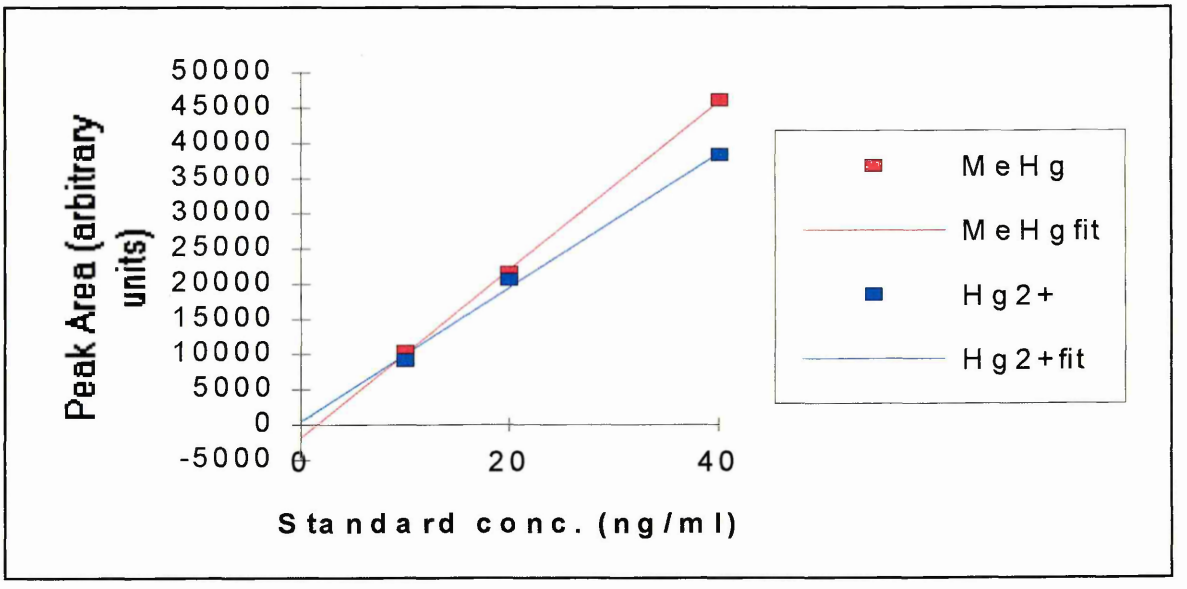


Figure 44: A typical calibration curve with the speciation system

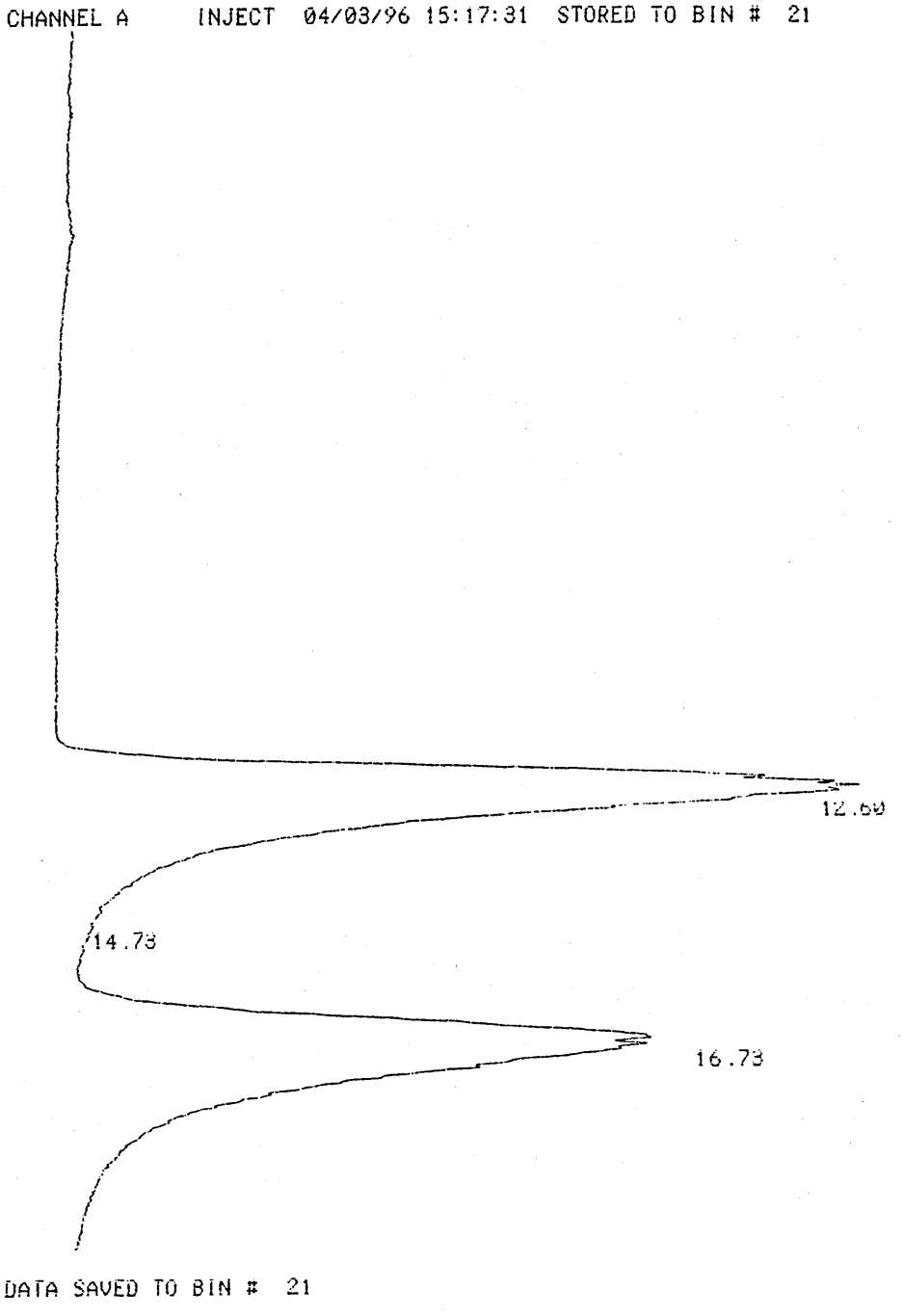


Figure 45: A typical chromatogram

5.4.3- Extraction procedure

It is very important to find a method that allows the complete recovery of mercury from hair samples, while keeping the integrity of the chemical species intact. However, two main problems arise. First, the HPLC column cannot withstand extreme pHs, and secondly, one must bear in mind that this method is meant to be applied to population studies, and hence the sample preparation must be straightforward and rapid.

5.4.3.1- Acid leaching of the mercury present on the surface of hair

It is necessary to investigate whether it is possible to differentiate between external contamination on the hair and the mercury bound in the hair internally.

Hair was mechanically shaken for 30 min with 5 ml of acidified water. No decomposition of the hair could be observed after this step. The pH of the water was controlled to allow direct injection into the HPLC system.

When the water from the first wash was injected, a peak for mercury could be detected. After a second wash of the same hair sample, no mercury was detected. This procedure is thus sufficient to remove the mercury present on the outer surface of the hair, probably due to external contamination from air pollution, but not strong enough to actually break the hair matrix. This is crucial if one wants to distinguish between the intrinsic and extrinsic sources of mercury in the hair.

5.4.3.2- Determination of mercury species bound to the hair matrix

Different methods for the separation of mercury species described in the literature were considered [24, 43-45, 52, 53, 56]. However, the need for a relatively short and straight-forward method that can be applied to large numbers of samples makes the most complicated methods unpractical.

The simplest method described [53] involves the digestion of biological materials with KOH, and the conversion of mercury species to volatile ethyl derivatives, for determination by GC. As the compounds do not need derivatization before analysis with HPLC, the only step to be carried out to release the mercury species from the hair matrix is the digestion of the samples with KOH.

•*The effect of KOH on the system*

First, the influence of KOH on the determination of mercury by HPLC-CV-AFS had to be investigated. Mixed inorganic and organic mercury standards were prepared in water and in KOH (20% v/v), analysed and compared.

The standards in KOH did not give rise to peaks. It seemed likely that the pH of the solution had an influence on the detection. Two possibilities arose: the pH had an influence on the reduction of mercury by stannous chloride, or the column did not tolerate the basic solutions.

The column was disconnected and solutions of inorganic mercury of different pHs were analysed with the remaining of the system. No difference could be noticed in the detection of acidic, neutral or basic solutions. Therefore the pH is not a critical parameter for the determination of mercury by CV-AFS.

When a solution in KOH was injected, no peak could be detected. Subsequent injections of standards in water did not give rise to peaks either, which indicates that the basic solution deteriorates the column and prevents further determinations.

However, the column could be reconditioned by pumping pure HPLC grade methanol through it for 30 minutes. The column could then be re-used for the separation of inorganic and organic mercury.

In order to determine whether the pH was the problem, or whether the presence of potassium interfered with the determination, the standards prepared in KOH were neutralised with dilute hydrochloric acid to different extent. Standard solutions of pHs ranging from 2 to 6 were then available. These standards were then analysed, and gave rise to good separation of the mercury species. This clearly shows that the presence of potassium does not present a problem for the speciation of mercury, but the pH of the standard solutions and samples is critical for the column separation but not for CV-AFS.

It is thus recommended that all solutions analysed with the HPLC-CV-AFS system should have a pH in the range 2-6. This unfortunately is a setback, as the neutralisation of each sample takes time and makes the analysis of large number of samples less straightforward.

•*Extraction of mercury species from hair samples*

This procedure did not give rise to peaks that were quantitatively representative. As the pH was rectified, and it was shown that standards with rectified pHs showed good peaks, it was then assumed that the digestion procedure was not appropriate for the release of all the mercury species from the hair matrix.

As the successful use of KOH has been reported in the literature [53], with the sample being placed in an oven at 75°C for 3 hours, it can then be assumed that the problem comes from the digestion method. It is likely that the program used on the microwave was not appropriate for this application. The mercury species are likely to be still bound to the hair matrix.

It would be necessary to optimise the microwave program, to make it efficient enough to ensure the total release of mercury without actually being so strong that it would destroy the organic species.

5.4- Summary

In this chapter, the development and optimisation of a method for the speciation of mercury species, using high performance liquid chromatography coupled with cold vapour atomic fluorescence spectrometry, was described.

The system comprises a column for the separation of different species, an oxidation step for the transformation of organic mercury species into Hg^{2+}, followed by a reduction step, for the reduction of Hg^{2+} to Hg^{0}. Hg^{0} is then detected by fluorescence spectrometry. The pH is critical for the column separation, and is optimum in the range 2-6.

This method has been used for the determination of mercury species in hair. Hair is digested with KOH in a microwave digester. This step has not been successful, as all the mercury species were not released from the hair matrix, and requires more investigation before it can be applied to population studies.

6.0- Conclusion

In this study a method based on microwave digestion of human hair followed by the determination of mercury by cold vapour atomic fluorescence spectrometry has been developed and optimised. The method is accurate, simple, fast, and sensitive. The microwave digestion procedure requires only one manipulation step, and the process is complete in twenty seven minutes. The use of a 2:1 digestion mixture of nitric acid and hydrogen peroxide eliminates the need for matrix matching the standards and samples. Atomic fluorescence spectrometry is a sensitive mercury detection technique, and as a results, preconcentration is not necessary. A throughput of 40 samples per day allows the analysis of large numbers of samples required for population studies.

However, the Merlin Plus Mercury system presents some problems, such as the instrumental drift and the moisture carry-over, that need careful monitoring, so that optimum working conditions can be achieved.

It was showed that mercury is not distributed evenly along the length of a strand of hair in adults, but that it is in children. Therefore, hair can be used as a reliable indicator of a child's exposure to mercury.

The washing and drying of hair before analysis has been the subject of much debate. It was found that the collection of hair samples after shampooing of the head was suitable, as shampooing removes external contamination and dust, but does not affect the hair matrix. Mercury losses were noted after drying in a freeze-drier.

Population studies linked the concentration of mercury in hair to age and fish consumption for adults, and to breast-feeding for babies. Although significant correlations could not be found between the mercury levels in adults' hair and number of fillings, definite trends were present. There was no correlation between the mercury levels in the hairs of different family members. Development scores for children were not correlated with mercury levels in their hair. Therefore, mercury does not present a danger to the development of children in the UK nowadays. This does not mean that mercury is safe, but that the levels found in children in the UK are very low and do not cause any problems. However, it is really important to carry on monitoring mercury, and be aware of the risks if the levels were to increase.

The importance of the differentiation between mercury species is now widely accepted. In this study, a simple method for the speciation of mercury using high performance liquid chromatography on line with cold vapour atomic fluorescence spectrometry is described, and optimised. The extraction of mercury species from the hair matrix using potassium hydroxide and microwave digestion has not allowed the quantitative recovery of mercury from hair samples, and more work is required to ensure the development of a reliable speciation method.

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PRESENTATION OF THIS WORK

Parts of this work have been presented at different venues:

1- Poster at the Pre-Doctoral Symposium, University of Hull, July 1995

2- Poster at the Pre-Doctoral Symposium, The University of Sheffield, September 1995

3- Oral presentation at the Analytical Chemistry Society, Sheffield Hallam University, May 1996

APPENDICES

APPENDIX 1

Comparing the mean of two samples involves calculating the probability that the observed difference between the means of two samples arises solely as a result of random errors [91].

We assume, as the null hypothesis, that the two methods give the same result. If the probability that the difference occurs by chance is low, then the null hypothesis has to be rejected. It is usually rejected if the probability of the difference occurring by chance is less than 5%, and the difference is then said to be significant at the 0.05 level, or at P=0.05, P standing for probability. If the two samples have standard deviations that are not significantly different, then a pooled estimate of standard deviation can be calculated:

$$s^2 = \{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2\}/(n_1 + n_2 - 2)$$

t is then given by:

$$t = \frac{X_1 - X_2}{s\{(1/n_1 + 1/n_2)^{1/2}\}}$$

where: s is the pooled estimate of the standard deviation

s_1 and s_2 are the standard deviations for methods 1 and 2 respectively

n_1 and n_2 are the sample sizes in methods 1 and 2 respectively

X_1 and X_2 are the arithmetic mean values for methods 1 and 2

$n_1 + n_2 - 2$ is the number of degrees of freedom of t

and t is a quantity used in significance testing of means

If the value of $| t |$ obtained experimentally is greater than the critical value of t for a confidence interval of 95% ($P = 0.05$) (c.f. Appendix 2), then the null hypothesis is to be rejected, i.e. there is a significant difference between the results obtained by method 1 and method 2.

APPENDIX 2

The *t*- distribution

The critical values of $|t|$ given in Table 35 are appropriate for two tailed tests.

Table 35: Critical values of $|t|$

Value of t for a confidence interval of	90%	95%	98%	99%
Critical value of $\|t\|$ for P values of	0.10	0.05	0.02	0.01
Number of degrees of freedom				
1	6.31	12.71	31.82	63.66
2	2.92	4.30	6.96	9.92
3	2.35	3.18	4.54	5.84
4	2.13	2.78	3.75	4.60
5	2.02	2.57	3.36	4.03
6	1.94	2.45	3.14	3.71
7	1.89	2.36	3.00	3.50
8	1.86	2.31	2.90	3.36
9	1.83	2.26	2.82	3.25
10	1.81	2.23	2.76	3.17
12	1.78	2.18	2.68	3.05
14	1.76	2.14	2.62	2.98

Table 35 continued

Value of t for a confidence interval of	90%	95%	98%	99%
Critical value of $\lvert t \rvert$ for P values of	0.10	0.05	0.02	0.01
Number of degrees of freedom				
16	1.75	2.12	2.58	2.92
18	1.73	2.10	2.55	2.88
20	1.72	2.09	2.53	2.85
30	1.70	2.04	2.46	2.75
50	1.68	2.01	2.40	2.68
∞	1.64	1.96	2.33	2.58

APPENDIX 3

Introduction to statistics for population studies

Type of data

Statistics means "numerical data". It is useful to distinguish between three types of data: qualitative, discreete quantitative and continuous quantitative.

- **Qualitative data** arise when individuals may fall into separate classes, without numerical relationship with one another, e.g. sex, colour of eyes.

- **Quantitative data** are numerical

  - if the values of the measurements are integers, the data are said to be **discreete**, e.g. number of people in a household.

  - if the values of the measurements can take any number in a range, the data are said to be **continuous**, e.g. weight, height.

Frequency distributions

When data are qualitative, the simplest way to deal with them is to count the number of cases in each category. The count of individuals having a particular quality is called the **frequency** of that quality. The proportion of individuals having the quality is the **relative frequency**. The set of frequencies of all the possible categories is called the **frequency distribution** of the variable. The most common way of depicting a frequency distribution

is by a **histogram** (Figure 46): this is a diagram where the class intervals are on an axis and rectangles with heights or areas proportional to the frequencies erected on them.

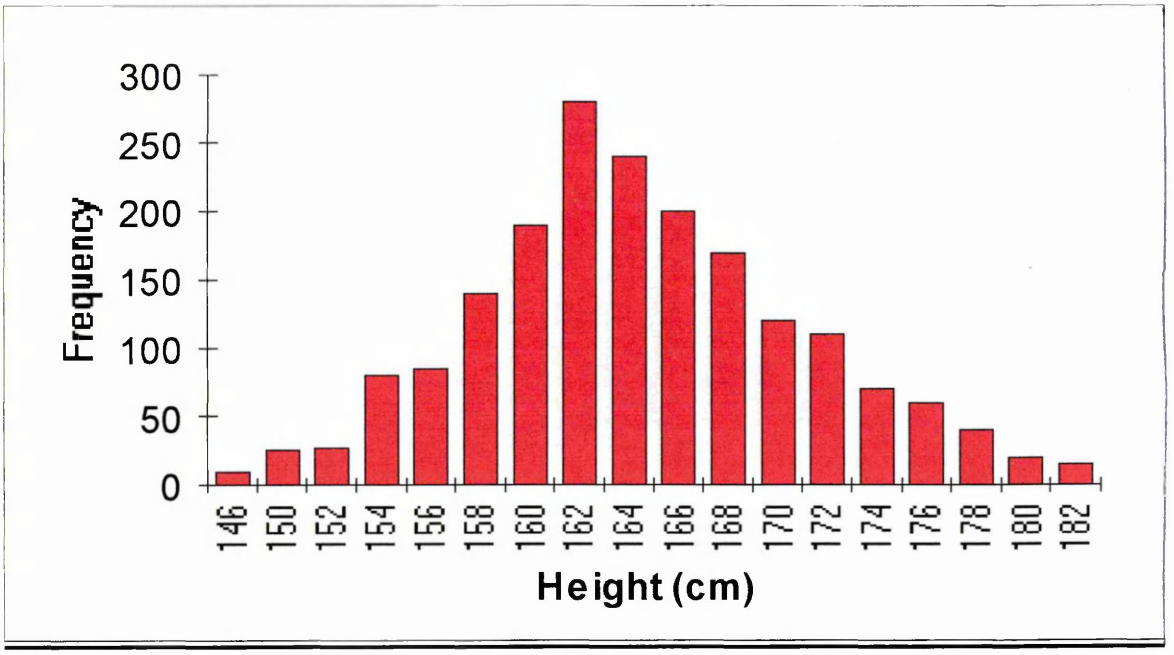
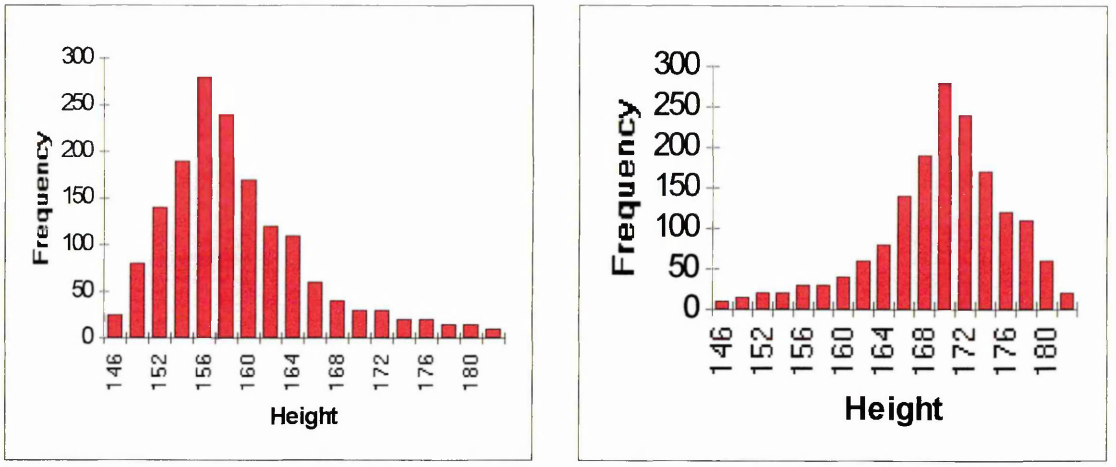


Figure 46: A typical histogram

The distribution can be roughly symmetrical about its central value, or **skew**, that is, the distance from the central value to the extreme is much greater on one side than it is on the other. The parts of the histogram near the extremes are called the **tails**. The distribution can be skew to the right, or positively skew, or skew to the left, or negatively skew (Figure 47).



a) b)

Figure 47: Skew distributions: a) positively skew, b) negatively skew

The Binomial distribution

It is the distribution followed by the number of successes in n independent trials when the probability of any single trial being a success is p. It is in fact a family of distributions, the members of which are defined by the values of n and p. Examples of different binomial distributions are given in Figure 48.

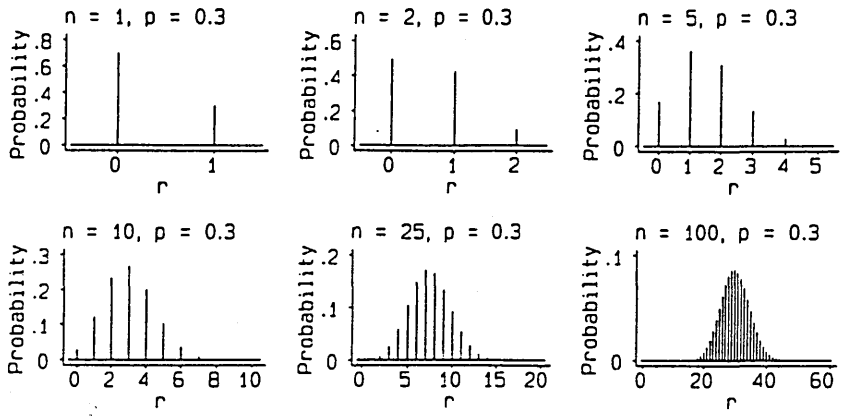


Figure 48: Examples of binomial distributions with different n, $p = 0.3$ [95]

The Normal distribution

The Normal distribution, also known as the Gaissian distribution, may be regarded as the fundamental probability distribution of statistics. The Normal distribution is the form to which the binomial distribution tends as its parameter n, increases (Figure 48).

Confidence intervals

The estimate of the mean of a variable for a sample is a single value, called a point estimate. There is no reason to suppose that the population mean will be exactly equal to the point estimate, the sample mean. It is likely to be close to it, however. Limits which are likely to include the population mean can be found, and the population mean is estimated to lie somewhere in the interval between these limits.

The 95% confidence interval, noted 95%C.I., is often calculated. It means that there is a 95% probability that the limits calculated from a random sample will include the population value.

Correlation coefficients

Before correlations can be studied between two sets of data, say, x and y, it is necessary to decide which method to use. There are two categories of methods: **parametric** methods and **non-parametric** methods.

Parametric methods- Pearson's method

Parametric methods are used when both variables x and y come from a Normal distribution. One of the most common parametric methods used for the calculation of correlation coefficients is Pearson's method:

A correlation exists if experimental points fit a straight line. In order to estimate how well experimental points fit a straight line, it is necessary to calculate the **product-moment correlation coefficient r**, often referred as simply the correlation coefficient.

The value of r is given by:

$$r = \frac{\Sigma\{(x_i - X)(y_i - Y)\}}{\{[\Sigma(x_i - X)^2][\Sigma(y_i - Y)^2]\}^{1/2}}$$

r can take values in the range $-1 \leq r \leq +1$. A value of $r = -1$ describes perfect negative correlation, and $r = +1$ describes perfect positive correlation. In the equation above, X and Y represent the means of x and y values [91]

The data for the calculation of r are usually presented in a table, as shown below. However, it is most useful to use a computer to perform all the calculations.

Table 36: Presentation of the data for the calculation of r

x_i	y_i	$x_i - X$	$(x_i - X)^2$	$y_i - Y$	$(y_i - Y)^2$	$(x_i - X)(y_i - Y)$
x_1	y_1					
...	...					
Σx_i	Σy_i	$\Sigma (x_i - X)$	$\Sigma(x_i - X)^2$	$\Sigma (y_i - Y)$	$\Sigma(y_i - Y)^2$	$\Sigma(x_i - X)(y_i - Y)$

When a low value is obtained for r, it is recommended [91] that a proper statistical test should be used to see whether the correlation coefficient is indeed significant, bearing in

mind the number of pairs of points used in the calculation. It is possible to do this by calculating the confidence interval for r. Another method of doing so is to calculate a *t*-value. The *t*-value is given by the equation:

$$t = \frac{|r|\sqrt{(n-2)}}{\sqrt{(1-r^2)}}$$

The calculated value of *t* is compared with the tabulated value at the desired significance level, using a *two-tailed* test and (n - 2) degrees of freedom (Appendix 1). The null hypothesis is this case is that there is no correlation between x and y. If the calculated value of *t* is greater than the tabulated value, the null hypothesis is rejected, that is, a significant correlation does exist.

Logarithm transformations

When the distributions of both sets of data are skewed, the data can be made Normal by using logarithm transfer: i.e. calculate the $\log_{10}$ of every value. When the data are made Normal, Pearson's method can be used again.

Non-parametric methods

Non-parametric methods are used when either x or y are not Normal and logarithm transfer is not possible, e.g. if x or y is qualitative. Rank order methods, such as

Spearman's or Kendall's, are used when data can be ordered. Both are better used with a statistical software, such as Minitab or spss. Spearman's method is simpler, and allows the easy calculation of confidence intervals. In Spearman's approach, the observations are ranked, then the product moment correlation of the ranks, ρ (rho) or r_s , is calculated (rather than of the observations themselves).

After the ranks for the two variables are found, the calculation of ρ are performed on a statistical software (Minitab) using an adaptation of Pearson's formulae for the product moment correlation coefficient:

$$\rho = \frac{\text{sum of products about mean of rank}}{(\text{sum of squares of ranks for first variable} \times \text{sum of squares of ranks for second variable})^{1/2}}$$

When the correlation coefficient is small, it is necessary to find out whether it is statistically significant, i.e. there is a correlation, but it is weak, or not, i.e. there is no correlation between the two variables considered. In this aim, a statistical *t*-test can be carried out. The software allows the calculation of a second value, called P, which represents the probability that there is no correlation. When P is smaller than 0.05, there is a significant correlation. If P is bigger than 0.05, there is no significant correlation.