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Methods of analysis used for the determination of selenium in milk and infant formulae: a review

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Selenium (Se) occurs naturally in a range of oxidation states as volatile species or analogues of organic sulphur compounds. Over a narrow concentration range (< 1 mg/day), selenium is an essential element for growth of animals and man. However at higher concentrations (>3 mg/day) it possesses toxicological properties. Low levels of the element in most foods including milk and infant formulae require a sampling procedure to be followed and the use of certified reference material. Accurate quantitative analysis is dependent on the initial digestion stage of the food matrix and the reduction of Se(VI) to Se(IV) in the final determination. In view of the difficulty in obtaining some reagents (e.g. 2,3-diaminonapthalene), alternative procedures to the classical fluorometric technique need to be evaluated. Methods for the determination of Se are described detailing detection limits, sensitivity, interferences and matrix-related problems.

INTRODUCTION

Selenium was first discovered by Jons Jacob Berzelius in 1818 (Reilly, 1993). Interest in its biological role did not develop until 1958, when selenium toxicity was shown to be responsible for muscular dystrophy in lambs and calves (Combs & Combs, 1986). It was the role of selenium in glutathione peroxidase; preceded by recognition of its essentiality in the prevention of liver necrosis in vitamin E-deficient rats (Schwartz & Foltz, 1957), that finally brought attention to the nutritional importance of this element in 1973 (Rotruck *et al.*, 1973) although our understanding of this function is still incomplete.

Selenium deficiency is now recognised as the major aetiological factor in Keshan disease, an often fatal dilated (congestive) cardiomyopathy, and Kaschin Beck disease (osteoarthritis deformans endemica) or 'enlarged joints'. These selenium deficiency syndromes occur predominately in young children and women (particularly in China) whose dietary intake of selenium is low (<3 μg/day) and blood levels in affected individuals of less than 20 μg/ml (Diplock, 1987). Daily supplementation with selenite, providing 1 mg of selenium, has been effective in preventing new cases (Litov, 1991). On the other hand, in certain parts of the world (China, Venezuela and the USA) where there are very high environmental levels of selenium, dietary intakes may reach 5 mg/day and blood levels of 3200 μg/ml which show clear signs of selenium toxicity, i.e. loss of hair, roughening of nails, nausea and fatigue (Reilly, 1993).

Selenium exists naturally in several oxidation states (-2, 0, +2, +4, +6), some of these chemical forms being volatile and analogues of organic sulphur compounds (IPCS, 1987). These forms, which include selenate (+6), selenite (+4), selenide (-2), alkyl-selenium and selenoamino acids (selenomethionine, selenocysteine) possess distinct physical and chemical properties which are fundamental to the analytical technique employed.

The selenium concentration in a sample is a major consideration in deciding which technique to use. Equally, sample inhomogeneity, matrix complexity, elemental volatility, presence of interfering species, demands for reliability, availability of specialist equipment, time and cost requirements also contribute to this decision.

Since the early 1980s selenium in milk and infant formulae has been routinely determined by a variety of methods (Olson, 1969; Cumming *et al.*, 1992*a*; Arnaud *et al.*, 1993; McGuire *et al.*, 1993*a*; Van Dael *et al.*, 1993). The most commonly used techniques depend on wet oxidation (digestion) for total destruction of organic matter, to release the selenium (Olson, 1969; Janghorbani *et al.*, 1982; Subramanian & Meranger, 1982; Essick & Lisk, 1987; Li *et al.*, 1989; Van Dael *et al.*, 1991; Tingii *et al.*, 1992; Shen *et al.*, 1993).

Central to many methods is the formation of the piazselenol which is extracted in an organic solvent and the fluorescence determined (Watkinson, 1966). Modification of this classical fluorimetric technique has led to the application of more sophisticated methods,

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i.e. gas chromatography (McCarthy *et al.*, 1981) and high-performance liquid chromatography (Oshima & Kawamura, 1990).

Hydride generation of selenium (Noda *et al.*, 1981; Kumpulainen *et al.*, 1983; Tingii *et al.*, 1992), has been coupled with newer techniques such as inductively coupled plasma mass spectrometry (Tracy & Moller, 1990). Neutron activation analysis is a very sensitive method when coupled with chemical separation of selenium (Ebert *et al.*, 1984; McOrist *et al.*, 1987; Lavi & Alfassi, 1990; Cumming *et al.*, 1992*a*), but has not been universally available due to cost and equipment needs.

Since 1981, fluorometric methods of analysis for the determination of selenium in milk have been reported in just over half the studies reviewed. The remainder included atomic absorption spectrometry (AAS) and various other methods (e.g. neutron activation analysis (NAA) and gas chromatography (GC)). This review aims to provide a general discussion of sample collection, preparation and treatment with an overview of the most commonly used methods for selenium analysis of milk and related materials to date, including examination of some of the problems relating to them.

SAMPLE COLLECTION, PROCESSING AND STORAGE

Current methods of analysis used for the determination of selenium in milk include fluorimetry, hydride generation atomic absorption spectrometry, GC with electron capture, isotope dilution mass spectrometry and neutron activation analysis (Table 1).

However, any analytical method under consideration requires verification, usually against samples of known certified selenium content. For milk, the standard reference material (SRM) non-fat milk powder (National Institute of Standard and Technology SRM 1549) has been adopted in conjunction with suitable analytical quality control measures (Van Dael *et al.*, 1992; McGuire *et al.*, 1993*a*; Branch & Crews, 1994). Other available certified reference materials (CRMs) for milk

Table 1. Studies examining the selenium content of infant formula and milk of different orgins from 1992 to 1994[a]

Substrate	Country	Study	Analysis	Reference
Human breast milk 1 week post-partum 1 month post-partum	Japan $n = 3$	Fluorimetric determination of selenium in breast milk versus wet ashing	Wet ashing $HNO_3/HClO_4$ at 160°C for 2·5–3 h HCl reduction 2,3-DAN Single test tube assay	Kawamoto *et al.* (1994)
Whole breast milk 5 days, 3 and 6 months post-partum Maternal blood serum 6 month gestation 3 and 6 months post-partum	Niamey Nigeria $n = 197$	Longitudinal study Iron supplementation during pregnancy versus selenium content in serum/ breast milk	Electrothermal AAS SRM non-fat milk powder NIST 1549	Arnaud *et al.* (1993)
Human breast milk 4, 8, 12 weeks post-partum Infant plasma Se Intrinsic Se formula selenite-supplemented formula, maternal supplements SeMet, SeY 4, 8, 12 weeks post-partum	Illinois, USA $n = 31$ $n = 28$	Longitudinal study Selenium status of infants versus supplementation of infant formula and maternal diets	GC electron capture SRM non-fat milk powder NIST 1549	McGuire *et al.* (1993*a*); McCarthy *et al.* (1981)
Material plasma and rbc selenium Human breast milk 4, 8, 12 weeks post-partum Non-lactating mothers' serum	Ilinois USA $n = 31$ $n = 22$	Longitudinal study Selenium status of lactating mothers versus different forms of selenium taken as supplements	GC electron capture	McGuire[a] *et al.* (1993*a*) McCarthy *et al.* (1981)

Table 1. — *contd.*

Substrate	Country	Study	Analysis	Reference
Sheep's milk	Belgium local farm Pooled milk $n = 4$	Selenium content of sheep's milk: distribution in protein fractions	Skim/centrifugation 1500 × g, 30 min, 4°C precipitation Dialysis ion exchange chromatography SDS-PAGE $HNO_3/HClO_4$ digestion Hydride AAS Non-fat milk powder NIST 1549	Van Dael *et al.* (1993); Welz & Melcher (1984)
Cow's milk *in vitro*	Belgium Local farm	An *in vitro* method for selenium availability from cow's milk	Skim/centrifugation 4000 × g, 30 min, 4°C Simulated gastrointestinal digestion: enzymic $HNO_3/HClO_4$ digestion Flow injection hydride generation AAS	Shen *et al.* (1993)
Human milk Soya formula selenite supplementation	Utah, USA $n = 31$	Selenium status of term infants fed human or selenite-supplemented soya infant formula	GC electron capture MF graphite furnace AAS	Johnson *et al.* (1993); McCarthy *et al.* (1981)
Human milk	USA $n = 12$	Utilisation of two different selenium forms during lactation	Stable isotope tracers $HNO_3/H_2O_2/HPO_4$ digestion GCMS	Moser-Veillon *et al.* (1992); Wolf & & Lacroix (1988); Welz & Melcher (1984)
Bovine milk	USA, Houston, Los Angeles, Washington $n = 3$	Selenium of selected foods of significance to dietary intake	–40°C before analysis $HNO_3/HPO_4/H_2O_2$ digestion IDMS	Wolf *et al.* (1992); Reimer & Veillon (1981)
Bovine milk	Scotland $n = 6$	Selenium content of wheat for breadmaking	Freeze-dry samples $HNO_3/HClO_4$ digestion HG AAS	Barclay & Macpherson (1992)
Goat's milk	Belgium Local farm pooled $n = 10$	Selenium content of goat's milk: distribution in protein fractions	Skim/centrifugation 4°C 1500 × g, 30 min $HNO_3/HClO_4$ digestion Hydride generation AAS NIST 1549	Van Dael *et al.* (1992); Welz & Melcher (1984)
Human milk 6–12 weeks post-partum mid-morning milk = av Se Test weigh infants Maternal blood	Brisbane, Australia $n = 20$	Longitudinal study Selenium and human lactation in Australia: milk/blood selenium levels in lactating women versus selenium intake of breast fed infants	Freeze-dry samples Neutron activation analysis High-resolution γ-spectrometry	Cumming *et al.* (1992*a*)
Whole/slim bovine milk	Brisbane, Australia local stores $n = 4–10$	Selenium in foods spectrofluorimetry versus hydride generation AAS	$HNO_3/HClO_4$ digestion Hydride generation AAS NIST 1549	Tingii *et al.* (1992); Koh & Benson (1983)
Maternal rbc and plasma Human breast milk 6–12 weeks post-partum Fore/hind milk 24 h intake	Brisbane, Australia $n = 20$	Selenium intake of breast fed infants	Neutron activation analysis High-resolution γ-spectrometry	Cumming *et al.* (1992*a*); McOrist *et al.* (1987)

[a]Status studies conducted in same geographical area unless otherwise stated. See text for explanation of abbreviations. Other abbreviations: SeMet, selenomethionine; SeY, yeast supplemented with sodium selenite; and rbc, red blood cells.

powder available from the Community Bureau of Reference (BCR) are B063, B150 and B151 (Laboratory of the Government Chemist, 1994).

Several considerations must be borne in mind during the collection and treatment of samples prior to analysis. Firstly, the sample collected must be representative of the material under scrutiny. This is straightforward for dried milks and powdered infant formulae. Bulk samples from the manufacturer can be reduced in size by quartering, the combined segments being thoroughly mixed and the quartering repeated until a suitably sized laboratory sample is achieved by the combination of the opposite quarter segments. Commercially available fluid milks and formulae should be thoroughly mixed by inversion, pouring back and forth into beakers or homogenising with top or bottom drive blenders (Egan *et al.*, 1991). Representative samples may also be extracted using a 'thief'. Methods and techniques for sampling milk and milk products are described in BSI (1985) or ISO (1985).

Representative sampling of milks direct from the species is more problematic. Recent studies using human milk report wide variability in terms of sample numbers, i.e. 10–200 (Table 1).

For longitudinal studies infants tend to be fed on demand. 10 ml milk being manually expressed at the start and end of a mid-morning feed from the first breast offered (usually pooled) from 0 to 12 weeks post-partum (Cumming *et al.*, 1992*b*; McGuire *et al.*, 1993*a*). Other human milk studies require complete breast expression via a battery operated pump into acid washed plastic vials or polypropylene bottles with minimal trace element contamination from the skin (breast cleaned with deionised water) (Mannan & Picciano, 1987; Debski *et al.*, 1989; Litov *et al.*, 1989).

Following collection, samples must be protected from contamination or loss of selenium during analysis. In particular losses can occur during drying due to the volatile nature of some selenium compounds. Whilst few losses have been reported in milk samples, losses in water samples have occurred as a result of adsorption on to container walls and microorganism activity. These may be minimised by preventing formation of insoluble hydroxides and maintaining an acidic environment. Selenate has been found to be more stable than selenite at low temperatures (Dilli & Sutikno, 1984*a*).

Sample storage methods include transport on dry ice and storage at −14, −20 or −70°C until analysis (Mannan & Picciano, 1987; Cumming *et al.*, 1992*a*; Arnaud *et al.*, 1993). Prior to analysis whole milk is defatted/skimmed by centrifugation at 1500 × *g*, 30 min, 4°C (Van Dael *et al.*, 1992; Van Dael *et al.*, 1993) or 10000 × *g*, 90 min, 4°C depending on the milk species (Debski *et al.*, 1987; Mannan & Picciano, 1987; Milner *et al.*, 1987).

SAMPLE DECOMPOSITION

Quantitative analysis of selenium in aqueous solution requires complete destruction of the composite organic matrix, particularly if a chemical separation step is involved prior to the analyte determination (Yang & Yang, 1990).

Typically wet oxidation procedures use a variety of oxidant mixtures, i.e. nitric acid–perchloric acid ($HNO_3/HClO_4$), nitric acid–hydrogen peroxide (HNO_3/H_2O_2) and nitric acid–sulphuric acid (HNO_3/H_2SO_4) in conjunction with catalytic agents such as mercury, molybdenum, vanadium and persulphate (IPCS, 1987). The preference for these wet digestion methods is based on the reduced danger of losses by volatilisation and their simplicity of operation (Adeloju *et al.*, 1984).

Conflicting views exist as to the adequacy of particular methods based on the variability of organic matrix composition, state or form of the element in solution and the method of detection employed (Analytical Methods Committee, 1979). Little attempt has been made to quantitatively assess the extent of matrix destruction. Generally, the achievement of a colourless solution and total element recovery is assumed to be indicative of complete dissolution of the organic matrix (Yang & Yang, 1990). Any residual matter retained by the oxidant mixture or possible interference properties are not accounted for. Subramanian and Meranger (1982) identified several elements (Cu^{2+}, Co^{2+}, Ni^{2+}, Hg^{2+}, Bi^{3+} and Sn^{2+}) exhibiting interference effects for selenium (IV) which were minimised by separating the selenium by coprecipitation with lanthanum hydroxide (Subramanian & Meranger, 1982). Martinie and Schilt (1976) reported compounds possessing *N*-methyl, *S*-methyl, *C*-methyl and pyridyl moieties as being most resistant to wet oxidation, and the addition of certain elements (V(V), Co(III), Co(II)) increasing catalytic activity (Martinie & Schilt, 1976). Yang and Yang (1990) combined radiotracer techniques with paper electrophoresis to assess the completeness of decomposition of rat liver samples for selenium. They concluded that selenium was almost completely released from the biological matrix into ionic form as selenate after one cycle of Sjostrand reflux (HNO_3/H_2SO_4) or pressure bomb (HNO_3/H_2SO_4, 130°C, 3 h) digestion processes (Yang & Yang, 1990).

Reported inconsistencies in data for selenium do not occur only as a result of decomposition efficiency but also by the application of inadequate analytical techniques (Adeloju *et al.*, 1984). A digestion procedure suitable for selenium determination by atomic absorption spectrometry (ashing step) may not be suitable for techniques that require the element to be in a particular state/form in solution (Analytical Methods Committee, 1979). Thus, successful analysis requires information on the identity and distribution of selenium-containing species within the decomposed sample.

Conflicting statements have been made as to the suitability of particular acid/oxidant mixtures for digesting biological materials for selenium determination.

The choice of acid is based on the following assumptions:

(1) complete conversion of native forms of selenium in the tissues to selenite–selenate;

(2) reconversion of any selenate (+6) formed during oxidation to selenite (+4); and
(3) prevention of significant loss of selenium during wet oxidation by volatilisation (Janghorbani, 1982; Ting *et al.*, 1982)

Neve *et al.* (1982) concluded nitric acid (HNO_3) alone was unsuitable for digestion of biological materials due to incomplete mineralisation of some organic Se compounds (1,1-diphenylseleno-4-*tert*-butylcyclohexane, phenyl-dimethylselenonium methylsulphonate, trimethylselenonium iodide, selenomethionine). Thus, derivatives possessing an aliphatic carbon–Se bond are more resistant to HNO_3 than those with an aromatic carbon–Se bond. Nygaard and Lowry (1982) reported that the aqueous inorganic form of selenium gave higher recoveries of selenium than the Se organo compounds using the same acid mixtures indicating the significance of speciation. The addition of perchloric ($HClO_4$) and sulphuric acids (H_2SO_4) with increased digestion time improved recoveries (Neve *et al.*, 1982). Similar studies on urine have shown that trimethylselenonium chloride and ^{75}Se are not quantitatively converted to Se(IV) with HNO_3/H_2O_2 but $HNO_3/HClO_4$, HNO_3/H_2SO_4 and $HNO_3/K_2S_2O_8$ gives a satisfactory conversion although a lower sensitivity, indicative of the high residual acid in the sample digest (Janghorbani *et al.*, 1982; Adeloja *et al.*, 1984).

Michie *et al.* (1978) proposed removal of $HNO_3/HClO_4$ from the digest to avoid interference with the formation of the Se–2,3-diaminonapthalene (Se-DAN) complex by adding H_2SO_4 (Michie *et al.*, 1978). However, sulphuric acid may induce charring, precipitation of DAN with molybdenum causing a loss in sensitivity particularly in the presence of excess organic material (Koh & Benson, 1983; Tingii *et al.*, 1992).

Procedures adopted to remove interfering substances prior to selenium measurement include coprecipitation with arsenic, tellurium or ferric hydroxide; ion exchange column chromatography; solvent extraction of Se halides, organic seleno-complexes of interfering metals, volatilisation of hydrogen selenide, precipitation of elemental selenium, thin layer chromatography, gas chromatography and addition of complexing agents EDTA (IPCS, 1987).

Sample decomposition in milk

Recent studies on selenium determination in milk adopt the nitric acid–perchloric acid oxidant mixture for decomposition of the organic matrix with HCl as a reductant (Essick & Lisk, 1987; Mannan & Picciano, 1987; Shen *et al.*, 1993; Van Dael *et al.*, 1993; Kawamoto *et al.*, 1994). Kumpulainen and Koivistoinen (1981) reported use of nitric acid–perchloric acid–sulphuric acid ($HNO_3/HClO_4/H_2SO_4$) with Ni(II) ions added as a matrix modifier to prevent selenium losses during ashing (Kumpulainen & Koivistoinen, 1981).

Nitric acid–perchloric acid is generally the preferred mixture as it gives optimum recoveries of selenium particularly when HNO_3 is the predominant acid (Janghorbani *et al.*, 1982; Ting *et al.*, 1982; Egan *et al.*, 1991). Perchloric acid facilitates the oxidation of resistant fatty material and organoselenium compounds providing satisfactory conversion to Se(IV) (Subramanian & Meranger, 1982). Losses and/or interference can be minimised by not taking the digestion to complete dryness (Tingii *et al.*, 1992). Losses incurred from drying temperature/time variation are species dependent since different matrices exhibit different volatilities (Koh & Benson, 1983). Sulphuric acid may be added to prevent drying out although, as mentioned, it enhances the risk of charring and loss of selenium through volatilisation (Haddad & Smythe, 1974).

The requirements and conditions for the reduction of Se(VI) to Se(IV) are controversial. If adopted, HCl is the usual reducing agent at ambient or elevated temperature (Nygyaard & Lowry, 1982). Overnight standing induces poor precision and low recovery and losses in selenium have been reported at this stage (Lalonde *et al.*, 1982).

METHODS OF DETERMINATION

Fluorimetry

Spectrofluorimetry is a well-established method used for the determination of selenium in foods and biological materials (Olson, 1969). Few studies document quantitative analyses of milks and infant formulae (Table 2). Hojo (1986) determined the selenium content of various baby foods by a fluorimetric method according to Watkinson (1966). Fluorimetry has been applied to several longitudinal studies investigating the selenium status of milk or formula fed infants using modified versions (Table 2) of well-established methods (Higashi *et al.*, 1983; Levander *et al.*, 1987; Litov *et al.*, 1989).

Following wet digestion, the selenium is converted to Se(IV) by boiling with hydrochloric acid, and determined by measurement of the fluorescence of the piazselenol formed on reaction with DAN or 3,3'-diaminobenzidine (DAB) (Watkinson, 1966; Olson., 1969). DAN is the preferred reagent as it has a greater sensitivity to fluorescence than DAB being extractable into organic solvent (Watkinson, 1966). Izquierdo (1981) reported a fluorimetric method using 4,5-dimercaptopyrimidine (DPP) with sensitivity more than twice that of methods using DAB without complex extraction into the organic phase (Izquierdo *et al.*, 1981). However, some opposition exists as to the use of DAN because of its potentially carcinogenic nature (Horler, 1989). This was further recognised in March 1994 with the implementation of shipping restrictions banning the entry of DAN into the UK (Sigma Chemical Co., 1994).

The formation of the Se-DAN complex is strongly influenced by pH. The optimum pH for complexation can range from 1·1 to 2·4 with values of 1·8–2·0 being most common (Analytical Methods Committee, 1979;

Moreno-Dominguez *et al.*, 1983). Fluctuations in pH may occur as a result of incubation temperature and time variation. Heating the reaction mixture for 30 min at 60°C altered the fluorescence response from pH 1·4 to 2, the response being acid independent from pH 2·0 to 3·5 (Michie *et al.*, 1978). Tingii *et al.* (1992) obtained the first pink colour after dropwise addition of 1 M HCl at pH 3, post-digestion with nitric and perchloric acids achieving Se-DAN complex formation after 20 min at 60°C.

For the fluorescence reaction with DPP Izquierdo *et al.* (1981) reported colour development in an acid

Table 2. Fluorimetric methods of selenium analysis in milk

Author	Watkinson (1966)	Hoffman (1968)	Olson (1969)[a]	Analytial Methods Committee (1979)[b]	Koh & Benson (1983)	Tingii *et al.* (1992)[c]	Kawamoto *et al.* (1994)
Destruction of organic matter	Sample 1 ml	Sample <1 g	Sample <1 g	Sample[d]	Sample <2 ml[e]	Sample 0·25–1 g	Sample 0·5 ml
Acid mixture[g]	HNO_3/ $HClO_4$	HNO_3 6 ml $HClO_4$ 2 ml H_2SO_4 5 ml	HNO_3	HNO_3/ $HClO_4$ (5 + 1) 30 ml	HNO_3/ $HClO_4$ 4 ml	10 ml HNO_3/ 2 ml $HClO_4$	HNO_3/ $HClO_4$ (2 + 1)
	HNO_3 stand overnight 5 ml		stand > 4 h 10 ml	Stand overnight	Stand 1–2 h, 210°C		
	Slow heat minimum charring $HClO_4$	Slow heat minimum foaming: heat vigorously	Mild to vigorous heating	Mild heating foaming Boiling,[f] vol <50%	Heat to fumes overnight	Reflux but not to complete dryness-risk of explosive metal perchlorates	100°C increased to 160°C
	2 ml boil		$HClO_4$ 2 ml	10 ml			
	Digestion time 3 h	Add HNO_3*, white fumes	H_2O_2 2 ml	HNO_3/ H_2SO_4 (1 + 1)	Cool		2·5–3·0 h
Reduction Se(VI) to Se(IV)	2 ml H_2O 30 min evaporation HCl 2 ml	HCl (30%) 1 ml	1 ml HCl	4M HCl 5 ml	HCl (35%) 0·5 ml	HCl 1 ml	6M HCl
	overnight		100°C 30 min	100°C 5 min	100–150°C 30 min	100°C 15–20 min	20–30 min
Fluorescence reaction	0·04 M EDTA 20 ml 0·02% cresol red 7M NH_4OH pH 1 (+ $HClO_4$)	0·02 M EDTA 10 ml NH_4OH 25 ml	NH_2OH/ EDTA 5 ml cresol indicator neutral yellow—NH_2OH, HCl — pink	H_2O 20 ml formic acid 5 ml NH_4OH 10 ml EDTA pH 1·8	0·0025 M EDTA 16 ml	Masking agent: EDTA/ $HONH_2HCl$ methyl orange M HCl pH 3	pH 4·2 citrate buffer 0·5 ml masking agent 0·2 ml
	0·1% DAN 5 ml	DAN 5 ml	0·1% DAN 5 ml	DAN 5 ml	1 ml DAN	DAN 5 ml pH 2	0·1% DAN 0·5 ml
	Dark: 50°C/20 min	Boil 2 min Leave 1–2 h	Yellow light: 50°C/30 min	Dark: 50°C/ 30 min	60°C/30 min	Dark: 60°C/20 min	50°C/15 min
Extraction	Cyclohexane 10 ml 0·1 M HCl	Cyclohexane 6 ml	Decalin decahydro-napthalene	Decalin or cyclohexane 10 ml	Cyclohexane 5 ml	Cyclohexane 5 ml	Cyclohexane 3 ml
	Centrifuge 2000 rpm/ 2 min	Centrifuge moderate speed/5 min	Centrifuge moderate speed/ 2 min		Screw-cap culture tube[e]		
Detection Wavelength	Organic layer 606 nm filter	Organic layer	Organic layer	Organic layer	Organic layer	Organic layer	Organic layer
Excitation		365 nm	369 nm	369 nm	364 nm	382 nm	377 nm
Emission		525 nm	522 nm	525 nm	523 nm	522 nm	520 nm

*HNO_3 (sp.gr. 1·42). $HClO_4$ (70%). H_2SO_4 (SP.GR. 1·84) prevents charring.
[a]Modification of Watkinson (1966).
[b]Analytical Method Committee report by the metallic impurities in organic matter sub-committee (1979).
[c]Modification of Koh & Benson (1983).
[d]Depends on: composition of material and expected Se content?
[e]Charring, volatiles lost i.e. SeCl, SeO_2, # combine EDTA/DAN stages.
[f]Fuming due to $HClO_4$. Prevent charring with 1 ml HNO_3. Fumes = setrioxide oxidation.
[g]Acid concentrations as for Hoffman *et al.* (1968).

medium only, 1 M HCl. Koh and Benson (1983) eliminated this step allowing complex formation to go to completion since the rate of Se-DAN formation was not at optimum pH.

The chelation/extraction step involves the addition of ethylenediaminetetraacetic acid (EDTA), DAN, and cyclohexane under specified conditions: The incubated mixture must be cooled to ambient temperature prior to solvent addition to minimise solvent evaporation (Watkinson, 1966; Hoffman *et al.*, 1968; Olson, 1969). Koh proposed the use of screw cap culture tubes to counteract evaporation, and separate addition of EDTA and DAN which eliminated EDTA crystallisation effects (Koh & Benson, 1983).

Diffuse or amber light is recommended at this stage since exposure of DAN to ultraviolet or daylight causes decomposition with the slow formation of brown DAN polymer precipitates (Olson, 1969). Conflicting information exists on the stability of the Se-DAN complex. In contrast to Koh, Michie *et al.* (1978) found a 10% decrease in fluorescence response for standards stored for 12 h. Traces of water in the solvent extract have been implicated as a source of Se-DAN complex destabilisation (Grant, 1981).

The choice of oxidant mixture for organic decomposition has a direct bearing on the sensitivity of the fluorescence signal. HNO_3/H_2SO_4 is reported as an effective decomposition mixture, but the presence of sulphuric acid caused formation of crystallised DAN which interfered with the fluorescence signal. Glass wool filtering and added $HClO_4$ failed to rectify this (Watkinson, 1966; Tingii *et al.*, 1992). Coprecipitation of Se with lanthanum prior to piazselenol formation may prevent interference by DAN precipitation (Maher, 1982). Fluorescent impurities may be removed by washing the extract with 0·1 M HCl depending on the efficiency of the purification procedure (Analytical Methods Committee, 1979).

Hubert *et al.* (1987) reported fluorimetric analysis of milk powder as, part of an interlaboratory study, by sample wet decomposition in a closed system which prevented iron(III) contamination: a known source of interference (Hubert *et al.*, 1987).

Water has been implicated as a cause of fluorescence interference warranting its removal by centrifugation, filtration and extract storage in a desiccator with silica gel (Grant, 1981; Koh & Benson, 1983).

Atomic absorption spectrometry (AAS)

Hydride generation is most commonly used in conjunction with AAS for selenium determination in milk and infant formulae. Whilst reports often document excellent sensitivity by isolation of selenium from other elements in biological materials and fluids (Veber *et al.*, 1994), hydride generation is subject to interferences when applied to complex matrices such as infant formula (Reimer & Veillon, 1981; Subramanian & Meranger, 1982). This has been confirmed by interlaboratory studies using biological materials, highlighting problems in accuracy and precision (Ihnat & Thompson, 1980; Kumpulainen & Koivistoinen, 1981).

Different matrix modifiers affect oxidation states, causing variations in thermal stability. Typical modifiers used include copper and magnesium, nickel, nickel and magnesium, palladium, palladium and ascorbic acid and palladium and magnesium for isoformation, converting the analyte into a phase of higher thermostability (Koops *et al.*, 1989). Some workers propose use of nickel as a matrix modifier to prevent selenium losses during ashing by premature volatilisation. However this is only applicable to serum, plasma and milk selenium and the amount present affects the reproducibility (Kumpulainen *et al.*, 1983, 1985, 1987). It cannot be applied to infant formulae due to spectral interferences at the 196·0 nm resonance line caused by high levels of iron and phosphorous which are intrinsic to the formula (Fernandez *et al.*, 1981). Similar interferences have been observed with whole blood and red blood cells which are high in iron, but were eliminated when rhodium was added as a matrix modifier (Alfthan & Kumpulainen, 1982).

For human milk analysis, replacement of Cu(II) matrix modifier with Ni(II) markedly lowered the selenium recovery from 98·4 to 82·1% (Kumpulainen *et al.*, 1983). Use of palladium nitrate (Arrunda *et al.*, 1994; Oilunkaniemi *et al.*, 1994) and magnesium nitrate as a universal matrix modifier has been reported used in conjunction with stabilised temperature platform furnace atomic absorption spectrometry for selenium analysis in biological samples, milk and fruit juices. Spectral interferences from phosphorous and iron were observed (Maage & Julshanin, 1991) but corrected using the Zeeman background correction. This has also been reported elsewhere (Hatano *et al.*, 1985; Lewis *et al.*, 1985; Pettersson *et al.*, 1986). This method was found to be less precise than either spectrofluorimetry or HGAAS (Koops *et al.*, 1989).

Kumpulainen *et al.* (1983) eliminated interfering cations by chelating with EDTA, separating the selenium with an ammonium pyrrolidine dithiocarbamate-methyl-isobutyl ketone (APDC-MIBK) extraction system. They demonstrated that the interference limit for Fe(II) ion, present as sulphate, is three times higher in infant formula (1600 limit ion/Se, wt/wt) than water (500 limit ion/Se, wt/wt) and Fe(III)ion, present as chloride, is four times higher (3300 limit ion/Se, wt/wt) in the infant formula matrix than in water. Phosphorous did not show any interference since the PO_4^{3-} ions cannot be extracted into the MIBK. Improved extraction recovery of human milk (98·4%) was reported; the chelation of Se with APDC blocking competition from cations in the system (Kumpulainen *et al.*, 1983).

Noda *et al.* (1981) noted that nitrate ions interferred with selenium reduction during commercial milk powder analysis. No interference was observed with sodium, potassium, calcium, magnesium and phosphorous which are abundant in this matrix. Interference was also observed with the addition of potassium iodide, producing a reduction in peak height. The addi-

tion of HCl gave maximum absorbance, which reduced if sulphuric acid was used. $HClO_4$ did not impair the system (Noda *et al.*, 1981).

Tingii *et al.* (1992) compared spectrofluorimetry and HGAAS for selenium determination in a range of food materials including homogenised whole and skim bovine milk. High concentrations of nitric acid were found to suppress the absorption signals, due to the presence of nitrate, causing a reduction in reproducibility and sensitivity. Optimum recoveries were achieved with $HNO_3/HClO_4$ or $HNO_3/HClO_4/H_2SO_4$ mixtures in open vessels during digestion which increased oxidising power and minimised digestion time (Tingii *et al.*, 1992). This is in agreement with Kumpulainen *et al.* (1983, 1985) and Welz and Melcher (1984) who reported that the accuracy of selenium determination in human body fluids is critically dependent on the sample decomposition method used, recommending similar acid mixtures heated to a final temperature of 310°C in long-necked flasks.

Such harsh treatment is necessary as the majority of selenium in milk exists as organoselenium compounds (Van Dael *et al.*, 1993) and for analysis, the selenium must be present in its tetravalent ionic form as selenite to allow its complete reduction to gaseous hydrogen selenide (H_2Se) (Neve *et al.*, 1980). No interferences from copper or iron were observed, as confirmed by a later interlaboratory trial involving nine laboratories (Welz *et al.*, 1987).

Van Dael and co-workers (1991–1993) investigated the organoselenium compounds in different protein fractions of skimmed cow's, goat's and sheep's milk using the hydride generation AAS according to Welz and Melcher (1984). The majority of selenium was found to reside with the casein fraction. This contradicts an earlier study on cow's milk which found the whey proteins to contain the greatest proportion (Deschuytere *et al.*, 1987).

Tingii *et al.* (1992) observed no interference from Fe, Cu, Zn, Mn or Ch although above 20 μg/g chlorocomplexation of copper occurred, suppressing the absorption signal unless HCl was added. Spectrofluorimetry was recommended for milk analysis due to its greater sensitivity for Se levels occurring in milk.

Many studies exist investigating the relationship between breast milk and formula feeding on maternal and infant selenium status. In these investigations flameless graphite furnace AAS is frequently used for the selenium analyses of plasma, serum, erythrocytes and human milk (Hatano *et al.*, 1985; Kumpulainen *et al.*, 1987; Gropper *et al.*, 1990; Johnson *et al.*, 1993). As previously, Ni(II) is generally used as a matrix modifier with nitric acid, eliminating organic matrix effects and interferences in background absorption which cause apparently high Se concentrations not accounted for by the method of standard additions.

Automatic correction of background absorption has been reported using deuterium lamps or the Zeeman effect (Carnick *et al.*, 1983; Hatano *et al.*, 1985; Kumpulainen *et al.*, 1987) and the validity of such methods successfully tested by interlaboratory comparisons using pooled human serum (Kumpulainen *et al.*, 1987).

Gas chromatography with electron capture detection (GC–EC)

The determination of selenium in milks and other biological fluids by GC–EC is well established (Young & Christian, 1973; McCarthy *et al.*, 1981; Dilli & Sutikno, 1984*b*). Its success is due to the precise conditions required for piazselenol formation which are specific for the element.

In principle, GC–EC is based on the selective complexation of the selenium with an *o*-diamine derivative ligand in acidic media to form the piazselenole (Elaseer & Nickless, 1994). Historically, the term 'piazselenole' originated as an abbreviation of para-diazselenol when the suffix 'ole' was used to represent a five-membered ring with two nitrogen atoms ('diaz') in the *para* position (Dilli & Sutikno, 1984*b*). From the synthesis of the first 'parent' piazselenol in 1889, many derivatives have been prepared, 14 of which have adopted in GC. These include 5-chloro, 5-bromo, 5-nitro, 5,6-dichloro, 4,6-dibromo and 5,6-benzopiazselenol (Dilli & Sutikno, 1984*a*).

Recently, Elaseer and Nickless (1994) reported a rapid method of Se determination in sediments using 3-bromo-5-trifluoro methyl-1,2-diaminobenzene as a complexing ligand. Optimum sensitivity, shorter retention time and good chromatographic peak shape were reported. They utilised a novel procedure of high-temperature derivatisation to increase the reaction rate; sensitivity and precision were not limited and its use in biological samples was recommended (Elaseer & Nickless, 1994). This contradicts an earlier study by Dilli and Sutikno (1984*a*), on biological fluids including milks, who reported that with increasing temperature and reaction rate, more spurious peaks occurred in the chromatogram which could not be eliminated, limiting sensitivity and producing wide response variations (Dilli & Sutkino, 1984*a*).

As mentioned initially, interference in the GC method for selenium determination is minimal because of the selective nature of the reaction. The selenium must be present as Se(IV) to react with the *o*-diamine, at pH2, to form the piazselenol which is extracted into an organic phase prior to GC (McCarthy *et al.*, 1981). A possible source of interference involves the interaction of the diamine reagent with foreign ions in the milk digest, i.e. Ni(II), Fe(III), Mo(VI), C(III), Sn(IV) and V(V), at higher pH values resulting in the formation of stable compounds. Whilst such compounds have been detected at earlier stages in the reaction, no metal complexes or column disturbance have been reported in the chromatograms. However any possible effects could be eliminated by adding masking agents such as EDTA (Dilli & Sutikno, 1984*a*).

Spurious peaks have been observed in homogenised and human milk samples as a result of co-extraction of

excess reagent with the piazselenol into the organic phase (simultaneous formation of volatile species with volatilisation of the piazselenol). This has been eliminated by using toluene which has a low solubility. Hence, the free base has been indicated as the source of spurious peaks, its formation being dependent on acid concentration (Dilli & Sutikno, 1984*a*). Dilli detected some interference, following milk digestion, visible as pink or violet compounds transfered into the toluene layer. He recommended adding urea post digestion and washing the extract with perchloric acid which removed any excess reagent and resulting interference (Dilli & Sutikno, 1984*b*).

McCarthy *et al.* (1981) minimised interferences associated with biological matrices using wet digestion with nitric acid and magnesium nitrate in closed glass stoppered weighing vials, converting Se(VI) to Se(IV) with hydrochloric acid. This reduced errors associated with losses and contamination, unlike with other acid mixtures (HNO_3, H_2SO_4, $HClO_4$) (see sample decomposition). As confirmed by Dilli and Sutikno (1984), hydroxylaminosulphate and urea eliminated any spurious peaks. Blank interferences were dependent on the analytical grade of HCl used and overcome by using reagents specifically prepared for trace metal analysis (McCarthy *et al.*, 1981). The McCarthy method is fundamental to many investigations requiring selenium determination in biological fluids (blood plasma, erythrocytes, urine, human milk and infant formulae) to establish selenium status (Table 1) (Smith *et al.*, 1982; Debski *et al.*, 1987; Milner *et al.*, 1987; Smith *et al.*, 1991).

This GC–EC technique has also been applied to studies investigating the distribution of selenium in milk of various species. Debski *et al.* (1987) detected selenium in at least nine selenoprotein fractions following gel chromatography and concluded that the majority of selenium is associated with the casein protein fraction, depending on dietary intake, with <3% being associated with lipid. This has further been confirmed by Milner *et al.* (1987), who reported similar selenium values using the McCarthy method.

Gas chromatography with mass spectrometry detection (GC–MS)

As mentioned previously traditional gas chromatography methods suffer certain limitations including laborious preparative techniques necessary to prevent interference from fluorescent impurities. This has led to further advancement of GC, by coupling with alternative techniques such as isotope dilution mass spectrometry (IDMS), AAS and flame photometric detection (Reimer & Veillon, 1981; Moser-Veillon *et al.*, 1992).

In principle, the IDMS method measures the concentration of an element by the change in its isotopic composition caused by the addition of an enriched isotope (^{76}Se spike) to the sample (Ducros & Favier, 1992). This concept has several advantages over more traditional techniques. In particular, quantitative or reproducible recovery of the element is not required once spike equilibration occurs. Any possible interferences affect the internal standard (^{76}Se) and the natural Se equally as they are chemically identical as reported by Reimer and Veillon (1981). They observed a 10% loss of selenium in biological samples due to incomplete extraction by a single toluene extract. However quantification of the analyte was possible because of the similar reactions of ^{76}Se and natural Se. Toluene was also used to extract non digested lipids from the sample prior to derivatisation, which removed any interfering peaks (Reamer & Veillon, 1981), as is in agreement with Dilli and Sutikno (1984*b*).

It is well established that the addition of a capillary column has increased the efficiency of the chromatographic separation and speed of analysis. Bleeding of the column is decreased, which is an important parameter in any GC-type analysis (Ducros *et al.* 1988). The availability and expense of GC–MS equipment has however limited the application of this method to research rather than routine selenium analyses.

Mangels *et al.* (1990) modified these techniques (Reimer & Veillon, 1981) to examine a range of biological materials (blood plasma, erythrocytes, urine, milk) for utilisation of selenomethionine and selenite during human lactation. Samples were digested using nitric acid/orthophosphoric acid/hydrogen peroxide in a microwave digestion system producing values in agreement with those using standard nitric/perchloric digestion (Hoffman *et al.*, 1968; Koh, 1980) then chelated using 4-trifluoromethyl-*o*-phenylene diamine (NPD) (Moser-Veillon *et al.*, 1992) and analysed for ^{74}Se, ^{76}Se and natural selenium.

A comprehensive study by Lewis *et al.* (1985) determined the selenium level in various types and blends of infant formulae by GC–IDMS. Losses associated with volatility and matrix interference were minimised with acid digestion (Reimer & Veillon, 1981) and the use of the stable isotope ^{82}Se as an internal standard (Lewis *et al.* 1985). This procedure has since been adopted by several researchers to investigate selenium dietary intake and status of lactating women and breast fed infants via milk, plasma, erythrocytes and standard reference materials analysis (Mannan & Picciano, 1987; Moser *et al.*, 1988).

Moser-Veillon *et al.* (1992) simultaneously evaluated absorption, retention and appearance of selenium in milk and blood of two different chemical forms of selenium (selenite and selenomethionine) during lactation using stable isotope tracers. This method coupled GC with atomic absorption spectrometry, the samples being digested (Reimer & Veillon, 1981) and the selenium reacted with NPD to form the corresponding volatile NPD-Se derivative. This compound has also been used with GC–EC (McCarthy *et al.*, 1981).

GC–AAS is extremely specific being able to detect the metal moiety of the volatile chelate, and any possible interferences are eliminated through the destruction of organic compounds in the atomiser. Potential interferences are limited to irregular molecular band struc-

ture or non specific light scattering. Phosphoric acid digestion further eliminates interference and risks associated with perchloric acid digestion (Moser-Veillon *et al.*, 1992).

Recently a sensitive and selective GC-flame photometric detection method has been reported for the determination of selenocystamine, selenocysteine and selenomethionine standards. Isopropyl chloroformate and HCl–methanol were used as derivatising agents which eliminated spurious peak formation and oxidation of seleoxide and selenone derivatives was prevented with diethyl ether as an extraction solvent. Good sensitivity was reported with standards but further development is required for application of this technique to biological materials (Kataoka *et al.*, 1994).

High-performance liquid chromatography (HPLC)

Methods using HPLC with fluorimetric detection have recently been developed for the selective determination of selenium in biological fluids, although not specifically for milk analysis. Fluorimetry, based on the formation of piazselenol from selenium(IV) and DAN (see the fluorimetry section) is widely used due to its low detection limit and good sensitivity (Nakagawa *et al.*, 1989). Fluctuations occur in blank values, as shown by thin-layer chromatography, caused by interferences from various separated chemical species within the Se-DAN complex.

Shibata *et al.* (1984) reported an HPLC method for separation of this Se-DAN complex using an optimised fluorescence detection system in conjunction with C_{18} reverse-phase liquid chromatography. The optimum signal to noise ratio was achieved using acetonitrile as the eluting solvent. They identified several chemical species associated with chelation and sample extraction prior to chromatographic determination, which were attributed to DAN derivatives (Shibata *et al.*, 1984) further limiting the suitability of this reagent in newer methods of selenium analysis (Sigma Chemical Co., 1994).

Oshima and Kawamura (1990) have described a similar HPLC procedure with fluorimetric detection based on the chelation of Se(IV) with 2,3-DAN forming 4,5-benzopiazselenol. To investigate speciation selenite and selenocystine (Se(IV)) were determined in a range of foods via nitric acid digestion, total selenium determination following treatment with HCl and selenate and selenomethionine by difference between Se(IV) and total Se values. Method reproducibility was limited as indicated by the high degree of variability between data.

Other piazselenols and Se-diethylithiocarbamates can be separated from reagents by reversed-phase chromatography and determined by UV detection in the nanogram and picogram range (Moser-Veillon *et al.*, 1992). Alternatively, Nakagawa *et al.* (1989) describe an HPLC–fluorimetric method for Se(IV) determination in a range of certified standards using precolumn reaction of SE(IV) with penicillamine to produce stable selenotrisulphide which undergoes derivitisation to a fluorophore by reaction with 7-fluornitrobenz-2,1,3-oxadiazole. A linear relationship over 10–2000 ppb is reported with a detection limit of 5ppb. Proposed determination of total selenium involved sample digestion and reduction of Se(VI) to Se(IV). Fluorescence intensity was pH dependent above pH 8·0 and the reaction optimised in acid solution. The addition of EDTA to penicillamine solution minimised ion interference with selenotrisulphide formation reaction preventing the formation of precipitates of copper chelating with penicillamine (Nakagawa *et al.*, 1989). Ion exchange (separation of free and complex ions) and reversed phase (ion pairing) HPLC techniques are applicable to selenium speciation. Laborada and Loos Vollebregt (1991) describe an anion exchange procedure using inductively coupled plasma–atomic emission spectrometry as an element specific detector for selenium standard speciation in $(CH_3)_3$ Se^+, SeO_3^{2-} and SeO_4^{2-}. Use of a cross-flow nebuliser and thermospray vapouriser improved resolution, detection limits and sensitivity in conjunction with increasing methanol/water mobile phase gradients.

Similarly, Pederson and Larsen (1994) use anion HPLC with an organic polymeric anion exchange column for separation of selenomethionine, selenocystine, selenite and selenate with detection via online coupling of flame atomic absorption spectrometry or inductively coupled plasma–mass spectrometry. Detection limits of 1 ppm were achieved for all four species, thus limiting this method to certain biological materials only.

Selenium speciation has also been achieved via direct interfacing of paired ion reversed phase HPLC followed by on line interfacing with direct current plasma emission. Selenite and selenate standards (20 ppm) were separated using tetrabutylammonium hydrogen sulphate (TBAHS) and UV detection at 205 nm. However selenium analysis of animal feed samples incurred interference at this wavelength from other UV-absorbing species in the elution regions of the selenium species, mobile phase components and baseline disturbance. Such effects were overcome using flow injection analysis (FIA) with TBAHS mobile phase and direct current plasma emission (DCP) detection at 196 nm (Van Loon & Barefoot, 1992). Many of these speciation techniques are developmental, hence no specific studies detailing speciation in milk are available, probably due to the complexity of the matrix.

Neutron activation analysis (NAA)

NAA produces several radionuclides of selenium following sample irradiation, e.g. ^{75}Se ($t_{1/2}$ = 120 days), ^{81}Se ($t_{1/2}$ = 18·6 min) and ^{77m}Se ($t_{1/2}$ = 17·5 s) (IPCS, 1987). The long-lived ^{75}Se radionuclide isotope has been most frequently adopted for non-destructive selenium determination by NAA in biological materials, milks and infant formulae since minimum sample treatment is required prior to counting (Ebert *et al.*, 1984; Lavi & Alfassi, 1990; Bratter *et al.*, 1991; Cumming *et al.*, 1992*a*). However, problems relating to expense, limited sample numbers, long delay and a long counting period are common (McOrist *et al.*, 1987).

Selenium is identified through the ^{75}Se stable isotope, via its emission of γ-rays with energies of 136keV and 265keV, respectively. Methods using destructive analysis are limited due to spectral interferences caused by decay of other radioisotopes present in the matrix. Typically these are ^{24}Na ($t_{1/2}$ = 15 h), ^{42}K ($t_{1/2}$ = 35 h), ^{82}Br ($t_{1/2}$ = 35·4 h) and ^{64}Cu ($t_{1/2}$ = 12·7 h) (Hani *et al.*, 1986). Lavi and Alfassi (1990) identified similar interfering elements.

Alternatively, chemical separation techniques (before or after irradiation) have been used to eliminate these interfering elements (long decay period), thus shortening analysis time and lowering the detection limit (Ebert *et al.*, 1984; Hani *et al.*, 1986).

In principle, Se(IV) forms stable complexes with pyrrolidine dithiocarbamate (PDC) derivatives (usually nickel) during extraction. Consequently, the major interfering elements do not complex with the PDC extracting agents and can be subsequently removed (Hani *et al.*, 1986). This technique has been developed for analysis of human blood serum and market milk; selenium was preconcentrated by dissolving into its respective pyrrolidine dithiocarbamate chelate and precipitated with a nickel carrier prior to irradiation (Lavi & Alfassi, 1990). Hani *et al.* (1986) adopted a three-stage process which used acid digestion, sample extraction with lead diethylthiocarbamate to remove interfering elements, followed by extraction with sodium diethylthiocarbamate in acidic chloroform and back extraction with nitric acid.

Ultrafiltration has also been used as an alternative separation technique to remove interfering species (McOrist *et al.*, 1987), e.g. sodium chloride and potassium as reported in biological fluids during a study of selenium dietary intake of breast fed infants (Cumming *et al.*, 1992*a*).

During ultrafiltration, the selenium becomes concentrated and desalted as it is bound to the higher molecular weight proteins. Up to 90% Na removal was achieved using this method and ^{77m}Se peak interference by ^{19}O was eliminated by sample drying to completeness. In comparison, polyacrylamide gel filtration produced very poor separation and high data variability (McOrist *et al.*, 1987).

Recent analysis of selenium in milk by NAA identified complex spectra with similar gamma ray energies which were difficult to resolve. This was thought to be indicative of the complex nature of the sample matrix and further developmental work is required to reduce data variability (Garg *et al.*, 1993).

Other methods

Several other techniques have been used to quantitatively determine selenium though not specifically in milk. Recently, inductively coupled plasma (ICP) with atomic emission (AES) detection (Suddendorf & Cook 1984; Schelkoph & Milne, 1988) or mass ectrometric (MS) detection (Emmett, 1988; Buckley *et al.*, 1992) has become a highly sensitive, powerful tool for Se analysis in conjunction with hydride vapour generation (Tracy & Moller, 1990; Haldimann & Zimmerli, 1994), replacing earlier, less sensitive ICP optical emission methods, i.e. detection limit 100 ng/ml (Rose, 1983). Interference from transition metals, molecular ions and HCl was minimised by hydride generation producing detection limits of 1·3 ng/ml (Tracy & Moller, 1990).

ICPMS with hydride generation has been used in multielement studies on milk and infant formula to determine Na, Ca, K, Mg, Mn, P, Cr, Fe, Al but no data on selenium is reported (Suddendorf & Cook 1984; Emmett, 1988). Pretty *et al.* (1993) determined Se(IV) in urine using ICPES and ICPMS coupled with on-line anodic stripping voltametry which eliminated polyatomic interferences caused by chlorine in the sample matrix (Pretty *et al.*, 1993). Differential pulse cathode stripping voltametry with ion exchange chromatography has been used for routine analysis and selenium determination in blood and milk samples (Adeloju & Bond, 1983). Other developmental methods include polarimetry using a dropping mercury electrode (Dunhu *et al.*, 1989) and kinetic catalytic spectrometry (Gokmen & Abdelqader, 1994).

Wet digestion followed by fluorimetric measurement has until recently formed the method of choice for selenium determinations in milk. Irrespective of the final methods used, two essential criteria need to be satisfied. Firstly, the initial complete digestion of the sample with nitric and perchloric acid is essential to maximise the release of selenium from the food matrix as well as insuring minimum charring. Secondly, the reduction of Se(VI) to Se(IV) is critical in the final determination and is dependent on the concentration of HCl used.

Consideration of the sample matrix is equally important as most techniques suffer interferences from concomitant elements. These effects have been minimised using appropriate acid mixtures and solvents during digestion and extraction, water removal, masking agents, chelating agents, coprecipitants, chemical separation, background correction–Zeeman effect, appropriate solvents and detection systems.

Atomic absorption spectrometry with hydride generation and GC with electron capture are particularly suitable for milk analysis (intrinsically low Se content of 0·02 μg/g), achieving good sensitivity and limits of detection.

Further studies are needed to accurately test and quantify selenium in milk and infant formulae as existing information is limited. This is of paramount importance since milk fluids form the sole source of nutrition in infants. In addition, speciation studies are required in order to characterise the various chemical forms of the element and provide information as to their bioavailability and overall significance.

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