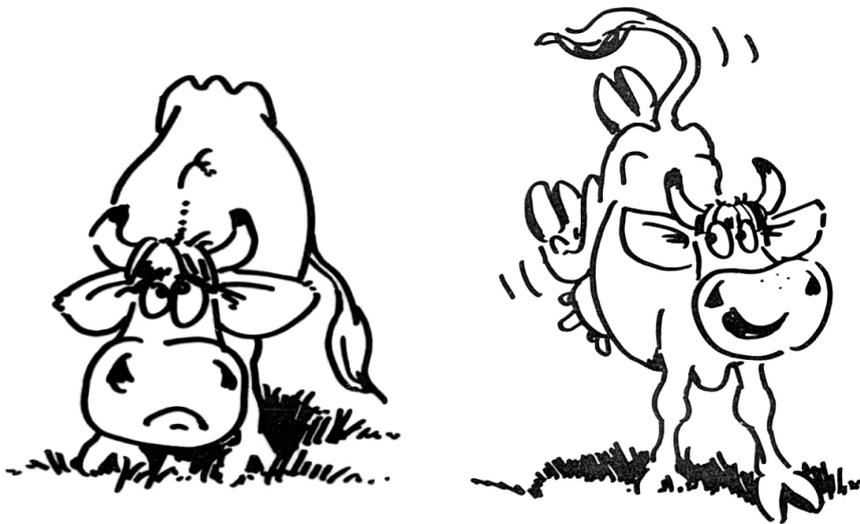


Determination of Selenium in Biological Materials by Flow Injection Hydride Generation Atomic Absorption Spectrometry (FI-HG-AAS)

Applications

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**Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2007**

Acta Universitatis Agriculturae Sueciae

2007:10

ISSN 1652-6880

ISBN 978-91-576-7309-1

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Tryck: SLU Service/Repro, Uppsala 2007



This thesis is dedicated to
Prof. Adrian Frank
on his
Eightieth Birtday

Abstract

Galgan, V. 2007. Determination of selenium in biological materials by flow injection hydride generation atomic absorption spectrometry (FI-HG-AAS) – applications. Doctor's dissertation.

ISSN 1652-6880, ISBN 978-91-576-7309-1

The selenium (Se) poor environment in the Scandinavian countries focused the interest on the development of an analytical method with high capacity, sensitivity, low limit of detection, including automated wet digestion, automated analysis and computer aided calculation.

To facilitate the choice of an appropriate analytical method, procedures for determination in biological materials were discussed. The most frequently used sample-preparation procedures and various analytical techniques were compiled.

Recent methods for determination of Se have more or less comparable sensitivity and detection limits. Thus, the choice of method is mainly influenced by economics and practical requirements such as available equipment, type of sample, length of sample series, expected Se-concentrations.

Determinations of Se were performed after automated wet digestion using a mixture of HNO₃/HClO₄ with a home-made equipment from commercially available components used by application of flow injection hydride generation atomic absorption spectrometry (FI-HG-AAS) and an electrically heated quartz tube as atomiser.

When analysing blank and Se-standard solutions after wet digestion an absorption signal at 196.0 nm was observed, which disturbed Se measurements at low concentrations. For explanation a great number of possible influencing factors were investigated, thus, the light absorption in the quartz tube depending on the gas flow rate and gas composition, and even acidity of carrier-, blank-, and standard solutions, as well as presence of HClO₄ in the solutions. When the difference in acidity between carrier-, blank-, and standard-solutions was eliminated, the gas flow stabilised resulting in disappearance of this effect. The main cause of this light absorption was found in construction of the quartz tube adapted to the light path of the Varian instrument used. The final limit of detection was in the range of 0.1-0.3 ng Se/ml measuring solution (0.3 ml injection volume) and was limited by the noise of the equipment.

Selenium determinations with FI-HG-AAS were applied to a great number of different kinds of biological materials. In routine work 2080 liver specimens of moose (*Alces alces* L.) from 14 regions of Sweden were analysed. The results indicated that the moose is useful for monitoring the amount of Se available for wild-grazing animals and confirmed that the Swedish environment is poor in Se.

Keywords: Selenium, wet digestion, flow-injection, hydride-generation, atomic absorption spectrometry, automation, quality control, biological materials, review, moose (*Alces alces* L.), monitoring.

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Galgan, V & Frank A. 1988. *Automated system for determination of selenium in biological materials*. In: Trace Element Analytical Chemistry in Medicine and Biology. (Eds. P. Brätter & P. Schramel). Walter de Gruyter & Co., Berlin, New York, Volume 5, pp 84-89.

II. Galgan, V. & Frank, A. 1993. Notes and comments on the determination of selenium in biological materials. *Norwegian Journal of Agricultural Sciences, Supplement, 11*, 57-74.

III. Galgan, V. & Frank, A. Determination of low selenium concentrations by flow injection hydride generation atomic absorption spectrometry (FI-HG-AAS) - interference by perchloric acid. (Manuscript).

IV. Galgan, V. & Frank, A. 1995. Survey of bioavailable selenium in Sweden with the moose (*Alces alces* L.) as monitoring animal. *The Science of the Total Environment* 172, 37-45.

Papers I, II and IV are reproduced by permission of the respective publisher and journals concerned.

Abbreviations

AAS	Atomic Absorption Spectrometry
BGS	Bio-Geochemical Samples
dry wt.	dry weight
EDL	Electrodeless Discharge Lamp
FI-HG-AAS	Flow Injection Hydride Generation Atomic Absorption Spectrometry
GP	Ghost Peak
GLS	Gas Liquid Separator
GSH-Px	Glutathione peroxidase
HCl	Hydrochloric acid
HClO ₃	Chloric acid
HClO ₄	Perchloric acid
HNO ₃	Nitric acid
H ₂ SO ₄	Sulphuric acid
ICP-CMA-HG	Inductively Coupled Plasma-Concomitant Mercury Analyser-Hydride Generation
LOD	Limit of Detection
LOQ	Limit of Quantitation
μ	micro, 10 ⁻⁶
NaBH ₄	Sodium tetrahydroborate
n	nano, 10 ⁻⁹
SVA	National Veterinary Institute
wet wt .	wet weight

Introduction

Background

Selenium is an essential element. Schwarz and Foltz (1957) showed that Se could prevent liver necrosis in rats deficient in vitamin E. The biochemical role of the element was elucidated in the 70s by the discovery that glutathione peroxidase is a Se-containing enzyme (Rotruck et al., 1973). Together with other defensive factors, e.g. superoxide dismutase, catalase, and vitamins E and C, this enzyme protects the organism against oxidative stress caused by free radicals (Karlmark, 1993) in general against reactive oxygen species. Several other mammalian selenium-containing proteins have now been identified (Behne & Kyriakopoulos, 2001). They can be divided into three groups: proteins containing non-specifically incorporated selenium, specific selenium-binding proteins, and specific selenocysteine-containing selenoproteins. Selenoproteins with known functions identified so far include five glutathione peroxidases, two deiodinases, several thioredoxin reductases, and selenophosphate synthetase 2. Selenium is also important for immunodefence and for the synthesis of certain antibodies (Larsen, 1993).

Sweden is a selenium deficient country. The poor natural occurrence of this element has led to nutritional Se deficiency in domestic animals in the past. Nutritional muscular dystrophy has been observed in pigs, cattle, sheep and horses fed with home-produced fodder (Norrman, 1983). Among these affected animals, sudden heart failure caused death, particularly in fast-growing lambs and calves and mulberry heart disease in pigs. Diffuse subclinical signs may also appear, such as loss of appetite, retarded growth, impaired fertility and disturbances of reproduction (Pehrson, 1993). Since the beginning of the 80s, Se supplementation of feed has been allowed in Sweden to improve the Se status in domestic animals.

Little is known about the Se status of terrestrial wild animals in Sweden. Wild animals are dependent on the occurrence of this element in nature. Its bioavailability may be influenced by several factors such as the geochemical background, aerial deposition from natural (Låg & Steinnes, 1978) and anthropogenic sources (Flueck, 1990), acidification, the buffer capacity of the soil, and the chemical form of the Se. All these factors together are decisive for the uptake of Se by plants eaten by herbivores. The factors mentioned above may cause regional differences in the bioavailability, and these might be monitored by measuring the Se concentration in the organs of herbivorous animals, preferably in the liver.

In materials of biological and environmental origin Se is often present in concentrations at trace and ultra-trace levels (i.e. < 1 mg/kg, sometimes even < 0.1 mg/kg). This demands highly sensitive analytical techniques. In recent years intensive analytical research resulted in the development and improvement of fast analytical methods and equipments for determination of Se. This is a prerequisite for increased knowledge and understanding of the biological role of this essential

element. The Se poor environment in Sweden focused the interest also at the Department of Chemistry of the National Veterinary Institute, on the development of an analytical method with high capacity, sensitivity, low limit of detection, including automated wet digestion, automated determination and computer aided calculation. The material which had to be analysed was given, i.e. biological material of animal and plant origin including different organ tissues, serum/plasma, erythrocytes, urine, milk, but also natural and processed feed, mineral feed, and so on.

A method for automated wet digestion in an open system using a mixture of $\text{HNO}_3/\text{HClO}_4$ already existed at the department (Frank, 1976) and has been proven to be efficient. The choice was natural to develop even an application for digested samples for Se determination. Flow Injection Hydride Generation Atomic Absorption Spectrometry has been applied to the analysis of Se.

Objectives

The overall objective of this thesis was the development of an analytical method for determination of Se in biological materials with high capacity, sensitivity, low limit of detection, including automated wet digestion, automated determination and computer aided calculation.

Specific objectives were:

- the development of an analytical method for determination of Se in biological materials (Paper I), the validation of the method and to test it in routine work (Paper I and II).
- to present in a short review aspects of procedures for determination of Se in biological materials in order to facilitate the choice of an appropriate analytical method (Paper II).
- to improve the limit of detection and quantitation of the method for determination of low Se concentrations in biological materials (Paper III).
- to survey the bioavailability of selenium with the moose (*Alces alces L.*) as monitoring animal and to test the application of the method in a long series of samples (Paper IV).

Methods

Reviews

Reviews, summarizing the problems and perspectives in Se analysis, are reported in Paper II. Two monographs compiling most aspects about hydride generation atomic absorption spectrometry have been published recently (Dedina & Tsalev, 1995; Welz & Sperling, 1999). Aspects concerning sampling and storage, sample preparation and determination methods for Se analysis are discussed in detail in Paper II.

Decomposition

The main principles of decomposition methods are as follows: wet decomposition in open and closed systems, dry decomposition by fusion and muffle furnace ashing, combustion methods in oxygen at normal pressure and at increased pressure and decomposition in oxygen plasmas.

Determination methods

The most frequently used techniques in routine analysis of Se are fluorimetry and atomic absorption spectrometry, the latter in combination with electrothermal atomic absorption spectrometry or hydride generation. Increased use of instruments for plasma atomic emission spectrometry, in combination with hydride technique as well as with mass spectrometry opened a new field of application for single and simultaneous multielemental determinations of hydride-forming elements. Other methods of analytical importance are chromatographic and electrochemical techniques, X-ray fluorescence, proton induced X-ray emission, and neutron activation analysis. Recently, increased interest is focused on methods for speciation of different Se-compounds in biological materials by combined (off-line) or hyphenated techniques (on-line).

Hydride generation atomic absorption technique

The hydride generation atomic absorption technique was used for determination of hydride-forming elements since the 1970s. These elements, for example arsenic, bismuth and selenium form volatile hydrogen compounds in the presence of a suitable reducing agent in acid solution. The gaseous compounds are transferred from the sample solution to a nitrogen or argon gas phase, and swept into a heated tube for atomisation and determination. The theoretical background, mechanisms, interactions and disturbances, as well as applications are surveyed in the literature (Dedina & Tsalev, 1995; Welz & Sperling, 1999). Selenium hydrogen is usually produced by reduction of Se^{IV} with sodium tetrahydroborate (NaBH₄) in hydrochloric acid solution. Hydride generation can be carried out in batch- and continuous-systems. The use of equipments with continuous flow systems, maintained by peristaltic pump, handling sample and reagents simultaneously, has been an important improvement.

Flow injection

In later developments flow injection systems were also adopted in hydride generation. In a continuously flowing carrier stream discrete sample volumes are injected and mixed with the reagent solution. The analyte-containing solution is transported to the detector and results a reproducible transient analytical signal (Ruzicka & Hansen, 1975). Flow injection systems differ from continuous flow hydride generation systems in that instead of the sample solution a carrier solution (e.g. water or dilute acid) is pumped continuously and a small volume of sample solution (e.g. 0.5 ml) is injected at regular intervals (e.g. every 30 s). A time-dependent signal is generated whose form depends on the dispersion of the measurement solution in the carrier solution. The major difference between FI and continuous flow systems is that it is not necessary with FI systems to wait until equilibrium has been obtained before measurement (Welz & Sperling, 1999). The great advantage of the FI-HG system is the use of low sample volumes, to make repeated determinations, high sampling frequency, low reagent consumption, and the ease of automation.

Flow injection hydride generation atomic absorption spectrometry

Flow injection hydride generation atomic absorption spectrometry was used for the first time by Åström (1982) for determination of the hydride-forming element bismuth. Several studies describe the combination of flow injection technique with hydride-generation AAS (Chan, 1985; Yamamoto, Yasuda & Yamamoto, 1985; Wang & Fang, 1986; Fang et al., 1986; Galgan & Frank, 1988 (Paper I), Negretti de Brätter, Brätter & Tomiak, 1990; Pettersson, 1990; Welz & Schubert-Jacobs, 1991; Chan & Sadana, 1992). The first commercial system was introduced at the end of the 1980s by Perkin-Elmer (PE). Later publications in this area have been compiled by Dedina & Tsalev(1995) and Welz & Sperling (1999).

Results and discussions

Automated wet digestion

Automated wet digestion of biological materials for subsequent Se determination is described in Papers I, II, and III.

Many analytical methods used in determination of total Se content in biological materials assume the complete destruction of the organic constituents. However, acid-resistant organoselenium compounds such as selenomethionine, selenocysteine and trimethylselenonium ion demand agents with high oxidation potential for complete destruction (Verlinden, 1982). Further, HNO₃ alone or in mixture with H₂SO₄ is insufficient to decompose materials with high fat content. Selenium readily forms volatile species and can be lost during an uncontrolled decomposition, for example by charring. In many cases HClO₄ is required to retain Se in solution. Mixtures of HNO₃/HClO₄ or HNO₃/HClO₃/HClO₄ enable complete

destruction of biological materials at temperatures < 250 °C without losing Se. But HClO₄ must be handled with greatest care and following strict regulations; special equipment is necessary such as special hoods and scrupulous precautions have to be taken. For detailed information see Paper II and the literature cited in the paper.

At the Department of Chemistry, SVA, decomposition of samples is performed in open systems by automated wet digestion. The available sample amount is usually sufficient. Thus, 5 g organ tissue, wet weight and maximum amount of fat 500 mg, (1 g organic tissue, dry wt.) is suitable for wet digestion with 15 ml oxidising acid mixture, HNO₃/HClO₄ : 7/3 vol. per vol. (Frank 1976, 1983) For Se determination in the routine usually 1 g tissue, wet wt. is taken for wet digestion. For digestion of small amounts a semi-micro accessory was developed (Frank, 1988).

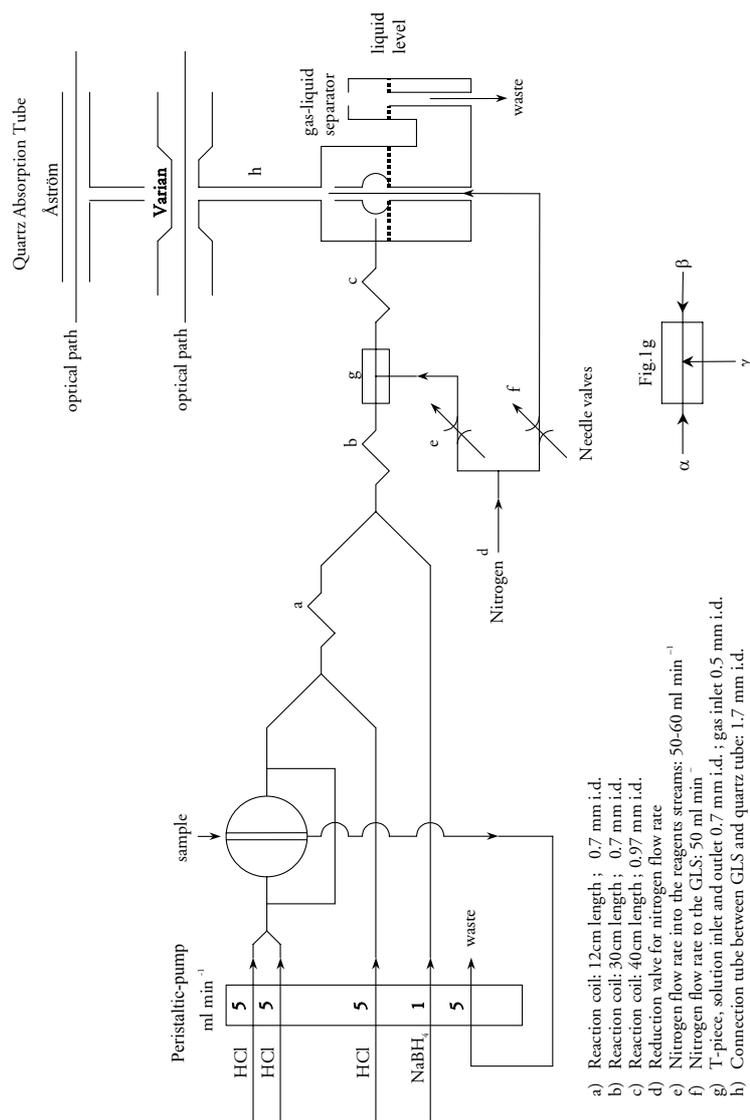
Sample digestion for subsequent Se determination

A mixture of the oxidising acids was added to the samples; separate addition of acids must be avoided. Digestion was performed in tubes of borosilicate or quartz glass in an electrically heated block of aluminium connected to a microprocessor, which controls the programming of time and temperature (Tecator Digestion System, model 40, Höganäs, Sweden). For most samples the standard program (Table 1) was used (Frank, 1988) and the digestion was performed during the night. The temperature was stopped at 180 °C in the morning. Tubes showing dark colour at visual inspection were removed from the block for repeated digestion. Tubes with clear solution were digested at 225 °C not longer than 30 min. to prevent loss of Se.

Table 1. Standard temperature and time program for wet digestion of biological materials and subsequent Se determination. *Dark coloured samples are removed from the block.

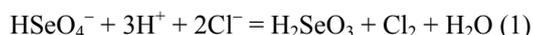
Temperature (°C)	Ramp (hour)	Time (hour)
50	:15	1:00
70	:15	1:45
100	:15	1:45
120	:15	1:15
138	1:15	1:00
150	1:30	1:30
160	:45	:45
180	:15	:45*
225	:30	:30

Fig. 1. Flow injection manifold for selenium determination by hydride generation and atomic absorption spectrometry (FI-HG-AAS). The design of the quartz absorption tubes of Varian and Åström was as follows: **Varian - Quartz tube** according to Sturman (1985): 4.5 mm i.d. for the 60 mm central part, 14.0 mm i.d. for the outer parts, total length ca. 170 mm, fused at the centre with a 1.4 mm i.d. quartz tube for gas inlet. **Åström - Quartz tube** according to Åström (1982): 6 mm i.d., length 170 mm, fused at the centre with a 1.3 mm i.d. quartz tube for gas inlet.



Reduction of Se

After cooling to ambient temperature 4-10ml (depending on tube volume) of 2.4 M (mol/l) HCl was added to each tube and Se was reduced from Se^{VI} to Se^{IV} by heating 30 minutes at 110°C (Norheim, 1986) according to equation ((1), Pettersson, 1990).



At ambient temperature the solutions were diluted to a suitable volume of 5, 10, 25 or 50 ml with 2.4 M HCl in the same tubes.

Determination of Se

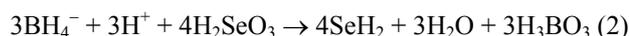
For determination of Se a method with the FI-HG-AAS technique was developed as described in Papers I, II, III.

Equipment

The system used was combined from commercially available components with the exception of the electrically heated oven and its power supply. During measurements the analogue signal from the detector (AAS) was displayed by a recorder. The signal was converted simultaneously to digital form and the Se concentration in the sample solution was calculated by the FIA-star (Tecator AB, Höganäs, Sweden). Up to six standard concentration values could be entered. A linear or non-linear calibration graph could be chosen. The evaluation of the non-linear calibration graph is based upon the Lagrange interpolation theorem, which means that the bend of the calibration graph is taken into account. The results could be printed out or transferred to a computer for calculation of the results, as well as for collection and storage of data. However, use of a signal amplifier was necessary to raise the level from the FIA-star into the I/U-port of the computer. By using a AA-spectrometer från Varian (Model AA-1475), FIA-star with auto sampler, chemifold, gas-liquid separator (GLS, Varian), needle valves for regulation of gas flow, and a computer, the FI-HG-AAS system was totally automated. The set-up did not require the continuous attention of an operator. The system worked successfully by using a Varian as well as a Perkin-Elmer atomic absorption spectrometer. The flow injection manifold for selenium determination by FI-HG-AAS is presented in Figure 1.

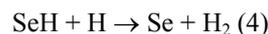
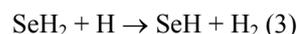
Hydride generation

Seleniumhydrogen (SeH₂) was generated by mixing NaBH₄ solution in the FI-system with HCl as the carrier (according to equation (2), Welz & Sperling, 1999). Nitrogen as purge gas was introduced to the FI- system and SeH₂ liberated from solution in the GLS. Operating conditions for the FI-system were 10s for sample injection time, 25s between two injections (rinse time 15s), 0.3 ml (Paper III) or 0.5 ml (Paper I and II) injection volume and peak height evaluation of the signal.



Atomic absorption spectrometry

Seleniumhydrogen was transported from the GLS with nitrogen as purge gas to a quartz tube in an electrically heated oven constructed according to Åström (1982). Atomisation was performed in the electrically heated quartz tube (equation (3) and (4), Dedina & Tsalev, 1995) at a temperature with maximum selenium absorption signal.



Selenium was determined at 196.0 nm using a hollow cathode lamp (HCL) for the Varian-instrument and an electrodeless discharge lamp (EDL) for the Perkin-Elmer (PE) (AAS , Model 403) instrument.

Optimisation of the system

In both commercial and home-made equipment the systems are optimised to achieve maximum signal intensity and stability. Owing to the interactive character of the different instrumental and chemical parameters, the optimisation becomes intricate. Thus, the systems are optimised in respect to reagent concentrations, carrier flow rate as well as gas flow rate. The length of reaction coil, different designs of gas-liquid separator and dimensions of quartz tube atomiser (cuvette) have to be studied as well. For the chemical reaction in the present dynamic system optimum concentrations and relationship between HCl and NaBH₄ solutions, as well as flow rate of both, had to be found. In addition, carrier flow rate and gas flow rate were important parameters for optimisation of the system.

Increased flow rate of the carrier resulted in increased sensitivity (Chan, 1985; Pettersson, 1990; observations of the author). The simultaneously increased back pressure of the system can be controlled by stable tubing connections (observations of the author).

Control of gas flow rate and its fine adjustment with needle valves or mass-flow controller is important for optimisation (Chan, 1985; Negretti de Brätter, Brätter & Tomiak, 1990). A special problem concerning purge gas flow rate is described in Paper III and will be discussed below.

The reaction of hydride generation is fast, but needs a definite time for reaction, and for this reason, length and inner diameter of the reaction coil plays also a role in optimisation (Chan, 1985; Narsito, Agterdenbos & Santosa, 1990; observations of the author) .

Different designs of gas-liquid separators are described in the literature. A stable gas flow into the quartz tube, minimized transport of liquid droplets and low dead volume are important parameters (Sturman, 1985; Welz & Schubert-Jacobs, 1991; Welz & Sperling, 1999).

Dimensions of the quartz tube atomiser should be adapted to the AAS instrument in regard to the mechanical and optical conditions (Sturman 1985; Welz &

Schubert-Jacobs, 1991). To achieve sufficient Se atomisation in the quartz tube some amount of oxygen in the carrier gas is necessary, as well as an atomisation temperature above 700 °C (Agterdenbos et al., 1986). Generally, temperatures of 800 - 900 °C are used.

Quartz tube atomiser

Quartz tube atomisers are most often used in connection with hydride generation atomic absorption spectrometry (Dedina & Tsalev, 1995). Normally, they are T-shaped with an optical bar having an inlet in the centre. Traditionally, they are divided in two groups: Flame-in-tube atomisers and externally heated ones. Flame-in-tube atomisers need not to be heated. The optical bar of the externally heated atomisers has to be heated either electrically or by flame to a temperature above 800°C. The hydrides are atomised at the beginning of the hot zone (Dedina & Tsalev, 1995). The hydride atomisation in quartz tubes is widely accepted to proceed via a hydrogen radical mechanism, which was confirmed by a number of studies (see monograph (Dedina & Tsalev, 1995) and the references therein). It appears to be identical in both atomiser types. The hydrides are atomised by consecutive reactions with hydrogen radicals, which are formed by the reaction of an excess of hydrogen with oxygen. Oxygen is either added at special inlet for the flame-in-tube atomisers, or it is present in sufficient quantities as a contaminant in the gas and/or it is stripped from solutions. The atomisation proceeds only in a small part of the atomiser volume.

Interferences

Interferences in the hydride-generation technique can take place during hydride generation, transport, and in the atomiser.

Chemical interferences are well documented in the determination of Se with HG-AAS (Meyer et al., 1979; Welz & Sperling, 1999). Interferences may be caused by other hydride-forming elements (As, Sb, Sn, etc) and certain metals (Co, Cu, Ni, etc). These effects are less pronounced in FI-HG-AAS than in HG-AAS because of the relatively low concentration of NaBH₄ and rapid transfer of SeH₂ from the sample solution to the gas phase, and thus, from contact with interfering elements (Chan, 1985; Wang & Fang, 1986; Pettersson 1990; Welz & Schubert-Jacobs 1991). In biological materials disturbances from interfering elements are usually not serious with the exception of those from copper. The liver from some Cu accumulating animals inter alia sheep at chronic copper poisoning and mute swan, can contain Cu up to 1000 mg/kg wet wt. or more. In our system, when the sample solution contained > 1 µg Cu/ml, the solution had to be diluted and/or Fe(III)-solution was added to reduce signal depression (Wang & Fang, 1986).

Other interferences discussed in the literature are caused by strong acids, nitrose gases, boric acid and interferences in the atomiser, caused by other hydride forming elements which are also vaporized during the reaction. All of them cause lower sensitivity in Se determination (Dedina & Tsalev, 1995; Welz & Sperling, 1999)

Molecular gases like oxygen, carbon monoxide and carbon dioxide absorb at the Se 196.0 nm line (Dedina & Tsalev, 1995).

Interference by perchloric acid

Interference by perchloric acid on the determination of low selenium concentrations by FI-HG-AAS is presented and discussed in Paper III.

In routine measurements for Se determinations, performed with the FI-HG-AAS system described above, 2.4 M HCl was used as reagent blank, the same concentration as that of the carrier solution, and corresponded to the zero point of the calibration curve. During the routine work an absorbance signal was observed caused by the digested reagent blank, however, its cause was not understood.

Calibration curves (Fig. 2) were calculated from the absorbance signals of diluted Se-standard and digested Se-standard solutions. The latter had another slope and an appearance of sigmoid shape than that of the diluted standards. To find the cause for the aberration below 0.5 ng Se/ml from the usual form of calibration curve passing through the origin or having an intercept, investigations started when Se concentrations below 1 ng/ml measuring solution had to be determined, for example infusion solutions, cereal- and grass samples.

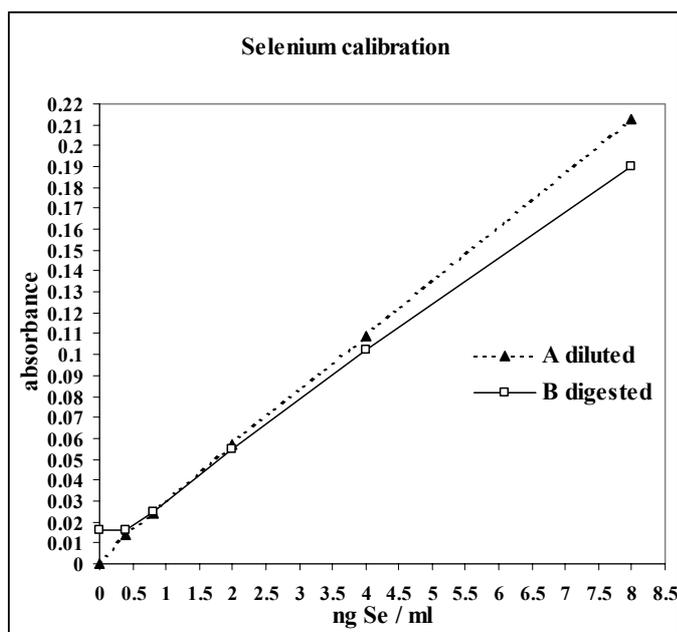


Fig. 2. Selenium (Se) calibration curves based on:

A: standard solutions from 0.0 – 8.0 ng Se/ml in 2.4 M HCl, 0.0 ng Se/ml was used as reagent blank.

B: digested Se-standard solutions from 0.0 – 8.0 ng Se/ml containing HClO₄ and 2.4 M HCl, 0.0 ng Se/ml was used as reagent blank.

Both series of solutions were analysed for Se-concentrations by the FI-HG-AAS-system

For curve A a linear regression ($y = 0.026x + 0.003$; $R^2 = 0.9996$) was calculated. Thus, factors suspected to influence the analysis were systematically investigated in a tedious process as described in the experimental part of Paper III. The changes in absorbance of the light beam in the quartz tube were monitored by time-resolved signals on a chart recorder. When running the digested reagent blanks an unspecific absorption peak was detected. However, studies of interacting factors described in the literature for hydride generation methods did not give any acceptable explanation for the peak that eventually was called 'ghost peak' (GP). Studies of time-resolved signals of the digested reagent blank and that of digested Se-standard solutions at low concentrations displayed that the GP was not identical with and clearly separated from the real Se-peaks. In time-resolved signals with the shape of a double peak the GP appeared as first peak before the Se-signal, the second peak.

The further efforts were directed to search for the cause of occurrence and control of the GP. When HClO_4 of the same concentration as in the blank was added to the carrier the GP disappeared. Unfortunately the absorbance signals became very noisy excluding reliable measurements. To achieve complete destruction of organic biological materials use of HClO_4 is necessary. Obviously, acidity had some influence on the occurrence of GP. Thus, by increasing the HCl concentration in the carrier to 6.2 M HCl and diluting digested reagent blank solution with 5 M HCl the GP could be controlled and eventually even disappeared in the background noise.

Still the cause of occurrence of the GP was not understood. Previously we noted increasing UV light absorbance when heating the quartz tube under constant N_2 gas flow rate for equilibration of temperature before measurements. Increased gas flow rate reduced the light absorbance; decreased gas flow, in opposite increased the absorbance signal. In the flow-system H_2 continuously was generated in steady state condition. Atomisation of SeH_2 was performed in a gas atmosphere containing both N_2 and H_2 . Nitrogen diluted with H_2 at the same total gas flow rate showed reduced absorbance due to higher thermal conductivity properties of H_2 than that of N_2 , which had a cooling effect on the electrically heated quartz tube. Lower temperature of the quartz tube decreased the absorbance. Summarised, the light absorption in the heated tube was influenced by the gas flow rate and by the dilution of N_2 gas by H_2 .

Another important factor was the effect of acidity in the carrier solution influencing the N_2 gas flow rate under unchanged initial gas flow rate. Consequently, the temperature and absorbance increased due to reduced gas flow rate. In routine measurements, HClO_4 -containing blank/samples, when injected into a carrier solution of 2.4 M HCl, caused sudden reduced purge gas flow rate. This resulted in increased temperature of the quartz tube and increased absorbance, until the blank/samples had passed the system. This effect was even more clearly demonstrated when He was used as purge gas.

The quartz tube of Varian, constructed particularly for Varian AAS instruments, was used for the experiments described above. The following investigations were

performed to see if a GP would occur when analysing a digested reagent blank with another type of quartz tube. Therefore a quartz tube according to Åström (1982) (Fig. 1) was tested in an AAS-instrument of Perkin-Elmer using an EDL radiation source. The change of tube and instrument displayed an unexpected effect namely the absence of the first peak, the GP. Digested reagent blank and Se-standard solutions were measured with 2.4 M HCl as carrier solution and 1.5% NaBH₄ solution using the same FI-system (Fig.1). Under these conditions a digested reagent blank gave no detectable signal. However, Se-standards had the same sensitivity as compared with measurements performed with the quartz tube and AAS from Varian. Consequently, the different constructions of the two quartz tubes were decisive on the appearance or absence of a double peak analysing a reagent blank containing HClO₄. The experiments described above indicated that the electrically heated quartz tube from Varian was more sensitive for temperature changes than the electrically heated quartz tube constructed according to Åström (1982).

Sturman (1985) discussed the design of three different quartz tubes developed for analysing hydride forming elements with a Varian AAS-instrument. The quartz tubes were heated with a fuel-lean air-acetylene flame. He found that a T-shaped tube with 7 mm i.d. eliminated the problems of the swirling of the gas stream found with a larger tube with an internal diameter of 15 mm. But the percentage light transmission through the tube was unsatisfactory. A third quartz tube (Fig. 1 quartz absorption tube, Varian) was designed to minimize obstruction of the light beam and focus the light beam in the centre of the smaller part of the quartz tube resulting in improved percentage transmission.

Summarising the results: evolution of H₂ gas was under steady state condition. Consequently, the temperature of the quartz tube remained unchanged. However, injecting blank/samples with high concentration of HClO₄ into the carrier solution caused instantaneous violent H₂ development decreasing temporarily the purge gas flow volume considerably. This caused temperature and absorbance increase-decrease in the quartz tube of Varian and in the UV light path, explaining the occurrence of the absorbance peak.

The absorbance peak was caused by the difference in acid strength between blank/sample solutions containing HClO₄ and carrier solution. Increasing the HCl concentration of the carrier solution and optimising the HCl concentration in blank/sample solutions resulted in control and even disappearance of the peak. The final detection limit achieved was in the range of 0.1-0.3 ng Se/ml measuring solution (0.3 ml injection volume) and was limited by the noise of the equipment.

The higher HCl concentration in carrier and sample solutions and the concentration of the NaBH₄ solution affected the curvature of the Se-calibration curve as shown in Figure 3. The GP disappeared in the base-line noise at high HCl concentrations in the carrier solutions (5 M and 6.2 M) and at NaBH₄ concentrations ≥ 1.5% (Paper III). When analysing Se-standard solutions (1 to 24 ng/ml) diluted with 5 M HCl and 6.2 M HCl in the carrier solution and different NaBH₄ concentrations the curvature of the calibration curve was highest at the

highest concentration (2 %) of the NaBH₄ solution and the sensitivity was lowest at higher concentrations of Se-standards. The decrease is mainly due to a decrease in SeH₂ concentration, caused by its acid dissociation (Welz & Sperling, 1999).

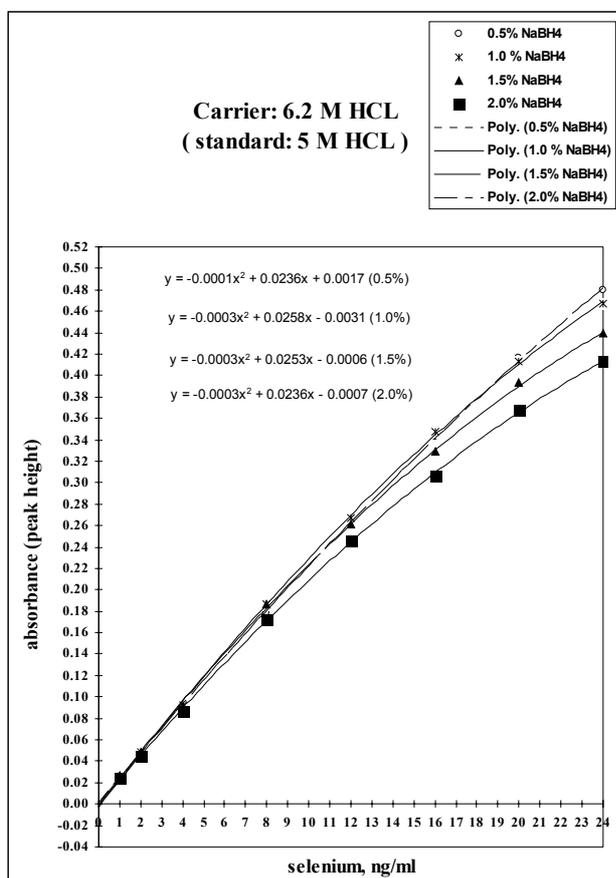


Fig. 3. Selenium calibration curves calculated from the absorbance signals of Se-standard solutions analysed with the FI-HG-AAS system. The Se-standard solutions were diluted with 5 M HCl. The carrier solutions was 6.2 M HCl and the concentration of the NaBH₄ was 0.5, 1.0, 1.5 and 2.0 %.

Validation data

Limit of detection (LOD) and quantitation (LOQ)

According to the International Union of Pure and Applied Chemistry (IUPAC) recommendations, LOD is the mean concentration of the blank plus three times its standard deviation. The LOD is the concentration at which we can decide with a reasonable certainty whether an element is present for a given analytical procedure or not. Quantitation is generally agreed to begin at a concentration equal to 10

standard deviations of the blank. This is called the limit of quantitation (LOQ), that means $LOQ = 3.3 LOD$ (Thomsen, Schatzlein & Mercurio, 2003).

Measurements of digested reagent blank- and Se-standard solutions, diluted with 2.4 M HCl and using 2.4 M HCl as carrier, resulted in a LOD of 0.8 ng Se/ml calculated by using absorbance values. Digested reagent blank- and Se-standard solutions, diluted with 5 M HCl and using 6.2 M HCl as carrier, resulted in a LOD of 0.3 ng Se/ml calculated similarly as above (Fig 4.). A further improvement of LOD, 0.1-0.2 ng Se/ml, was obtained when recorder signals were used for calculations and the LOD was limited by the noise of the HCL-lamp, the detector and the absorption of the atomiser atmosphere.

A control chart with signals in absorbance units ($n=20$) of digested reagent blanks and digested (Se)-standard solutions (0.8 ng/ml) is shown in Figure 4. Solutions were diluted with 5 M HCl and the carrier solution was 6.2 M. A blue line for the LOD ($mean + 3 SD$) of the digested reagent blanks and a red line ($mean - 3 SD$) of the Se-standard solutions are shown in the figure. The means and their confidence level of about 90 % were clearly separated from each other. Consequently, Se was detectable (LOD) at a concentration of 0.3 ng/ml. The LOQ was 1 ng Se/ml.

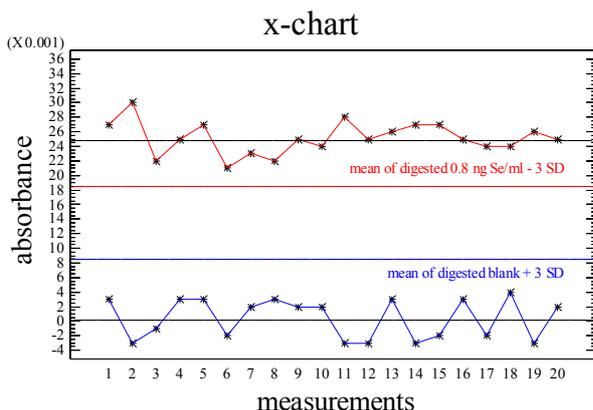


Fig. 4. A control chart with signals in absorbance units ($n=20$) of digested reagent blanks and digested Se-standard solutions (0.8 ng/ml) is shown in the figure. Solutions were diluted with 5 M HCl and the carrier solution was 6.2 M. A blue line for the LOD ($mean + 3 SD$) of the digested reagent blanks and a red line ($mean - 3 SD$) of the Se-standard solutions are shown in the figure.

Certified reference materials

The accuracy of the method was checked by analysing several different certified reference materials and a reference material (Bovine muscle, NIST RM 8414). The materials were analysed as control samples during a long period of time. The results are presented in Table 2.

Table 2. Selenium concentration in certified reference materials, certified and measured values. ^aRM = reference material ; (mean ± 95 % confidence interval). The materials were analysed as control samples during a long period of time

Reference material	n	Certified	Measured (mean ± SD)	Measured (mean ± 2 SD)	Unit (dry weight)	Recovery %
Bovine liver (NIST 1577b)	47	0.73 ± 0.06	0.75 ± 0.036	0.75 ± 0.072	mg/kg	103
Bovine muscle (NIST RM8414) ^a	20	0.076 ± 0.010	0.076±0.006	0.076 ± 0.012	mg/kg	100
Non-fat milk powder (NIST 1549)	25	0.11 ± 0.01	0.11 ± 0.006	0.11 ± 0.012	mg/kg	100
Bovine muscle (BCR 184)	8	0.183 ± 0.012	0.173 ± 0.005	0.173 ± 0.010	mg/kg	95
Bovine liver (BCR 185)	20	0.446 ± 0.013	0.474 ± 0.032	0.474 ± 0.064	mg/kg	106
Rye grass (BCR 281)	13	0.028 ± 0.004	0.023 ± 0.002	0.023 ± 0.004	mg/kg	82

Comparison between two hydride generation methods for Se determination

Selenium concentration in digested liver samples from moose were analysed with two different determination methods, FI-HG-AAS and (ICP-CMA-HG) a combination of inductively coupled plasma (ICP-AES) and hydride generation. The later method is accredited by the Swedish Board for Accreditation and Conformity Assessment, Borås, Sweden and is used at the Department of Chemistry, SVA.

The calculated linear regression and confidence limits between the analytical results of the two series are presented in Figure 5.

linear regression, predicted and confidence limits (95% confidence level)

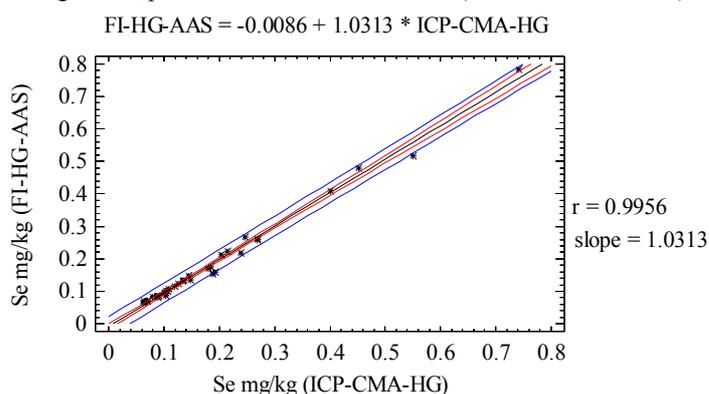


Fig. 5. Selenium concentration (mg Se/kg wet wt.) in digested liver samples from moose, comparison of the methods FI-HG-AAS and ICP-CMA-HG. The same digested sample solution was used in the methods (n=34).

Applications

Moose

An application of the FI-HG-AAS determination method is presented in Paper IV, a survey of bioavailable selenium in Sweden with the moose (*Alces alces* L.) as monitoring animal.

It has been shown earlier that the moose, a wild ruminant is a suitable animal for monitoring the bioavailability of some elements occurring in the natural environment. (Frank & Petersson, 1984). The moose is widespread in Sweden, and is hunted according to an organized system, which facilitates collection of organ tissues. The moose is relatively stationary and its migration is limited (Stålfelt, 1992). Age determination of the animals is feasible and is also necessary, as the concentration of some elements is age-dependent. The aim of this investigation was to obtain time-related basic data for the bioavailability of Se in Sweden using the moose as monitoring animal. This was done by determining the Se concentration in the liver of moose from different regions of the country. It was considered that such reference material could be used in the future for comparison to document environmental changes with possible consequences for the wild Swedish fauna.

Liver tissues were collected from moose from the regular hunting seasons 1981 and 1982. The material (from about 4 300 animals) was stored at - 20 °C at the National Veterinary Institute (SVA). From this material, 2 080 specimens from 12 counties representing 14 regions were analysed for selenium. The counties included were Norrbotten, Västernorrland (northern region), Uppsala, Kopparberg, Gävleborg, Jämtland (central region), north and south Kalmar (eastern region), Halland, north and south Älvsborg (western region), and Blekinge, Kristianstad and Malmöhus (southern region). The analysis was performed by FI-HG-AAS. The Se concentration in the liver in the entire material was:

Median = 0.15; mean \pm SD = 0.25 \pm 0.29;(range: 0.03 - 3.1) mg Se/kg liver wet wt.

The highest median values obtained were 0.26, 0.28 and 0.29 mg/kg, in the counties of Gävleborg (central region) and north and south Älvsborg (western region), respectively. The lowest medians, 0.09 - 0.10 mg/kg, were found in the counties of Uppsala (central region), north and south Kalmar (eastern region), and Jämtland (central region). Intermediate median values were obtained in the other counties: 0.13 mg/kg in the county of Västernorrland (northern region), 0.15 - 0.16 mg/kg in the counties of Blekinge and Malmöhus (southern region) and Norrbotten (northern region), 0.18 mg/kg in the county of Halland (western region), and 0.23 mg/kg in the counties of Kristianstad and Kopparberg (southern and central regions, respectively). A map for selenium concentrations in the liver samples from moose are presented in Figure 6.

Reports on Se concentrations in organs from terrestrial wild animals are rare. Some data are presented in Paper IV. Levels and time trends of metals in organs from Swedish moose have been monitored for over 20 years as part of the ongoing Swedish National Environmental Monitoring Programme funded by the Swedish Environmental Protection Agency. Samples of muscle, liver and kidney have been collected from the Grimsö area in central Sweden since 1980 (Odsjö et al., 2006). Tissue samples of male calves were selected for analyses. Selenium concentrations (ng/g fresh wt.) in muscle and liver samples are presented in Figure 7. The overall geometric mean value of selenium in muscle and liver of moose from Grimsö was 50.3 and 218 ng/g (fresh weight), respectively for the period 1999-2004. No significant trends were detected in the time series of selenium concentration in muscle and liver.

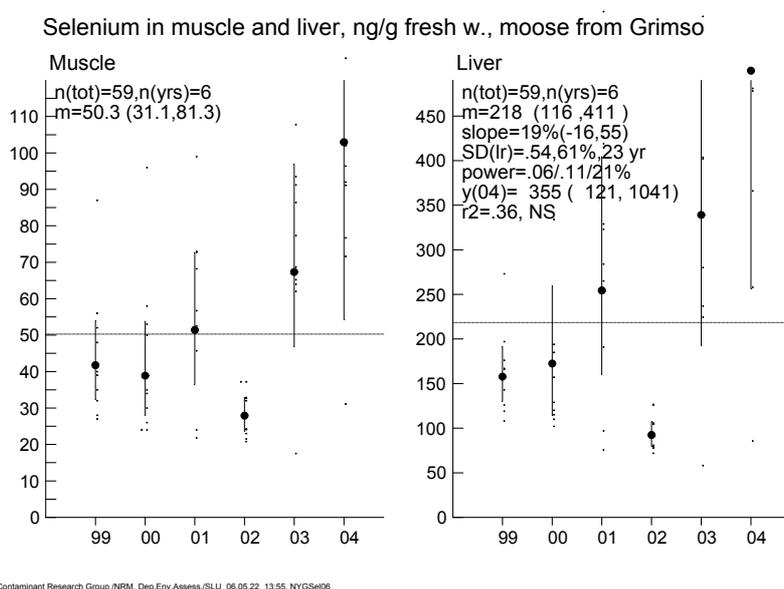


Fig. 7. Selenium concentrations (ng/g fresh wt.) in muscle and liver samples from moose collected from the Grimsö area in central Sweden. The plot displays the geometric mean concentration of each year (dots) together with the individual analyses (small dots) and the 95% confidence intervals of the geometric means. The overall geometric mean value for the time series is depicted as a horizontal, thin, dashed line.

In the present investigation, high median Se concentrations were found in moose liver samples from the western regions of Sweden. Low liver Se concentrations were found in the counties of Uppsala (central region) and north and south Kalmar (eastern region). The findings are in good accordance with the observations on bio-geochemical samples (BGS) (Fig.8). BGS are roots of certain aquatic plants and mosses suitable for monitoring elements dissolved in stream water. (Andersson et al., 1994). The Se concentration is low in the Scandinavian Precambrian rock and also in the soil. By weathering and microbial activity

insoluble Se compounds are converted into soluble forms available to plants. These compounds can also be leached or volatilised (Gissel-Nielsen, 1984; Combs & Combs, 1986). Younger sedimentary rocks, such as limestone in the province of Skåne in the south of Sweden, contain higher Se concentrations, as shown in the investigation of BGS (Fig. 8).

Nutritional Se status of moose

Available data for evaluation of the optimum Se status in wild animals are limited. Values obtained in domestic ruminants may be used for this purpose (Flueck, 1990). Concentrations in liver above 0.3 mg Se/kg wet wt. are considered sufficient (Radostits et al., 1999), and between 0.15 and 0.3 marginal. Selenium status in cattle is evaluated according to similar principles at the Department of Chemistry, SVA. Concentrations < 0.05 mg Se/kg liver are regarded as severe Se deficiency, > 0.05-0.1 as deficiency, > 0.1-0.2 as marginal/insufficient. Liver Se concentrations above this latter value are regarded as adequate (Puls, 1994; Rosenberger et al., 1994; Radostits et al., 1999). According to these criteria, 50-60 % of the animals in the counties of Uppsala (central region) and Kalmar (eastern region) had an insufficient or marginal Se status. These latter counties were found to be the most Se poor regions in the present investigation (Fig. 5). Selenium-deficiency diseases in wild animals are difficult to diagnose. Clinical signs are seldom apparent. Subclinical deficiency may exist but is difficult to diagnose as well. The most plausible way to find of ascertaining a subclinical deficiency status is by supplementation, which will result in improved reproduction, as reported in deer (Flueck, 1990). In addition, as mentioned previously, Se together with other compounds such as vitamin E are involved in the protection of the organism against oxidative damage.

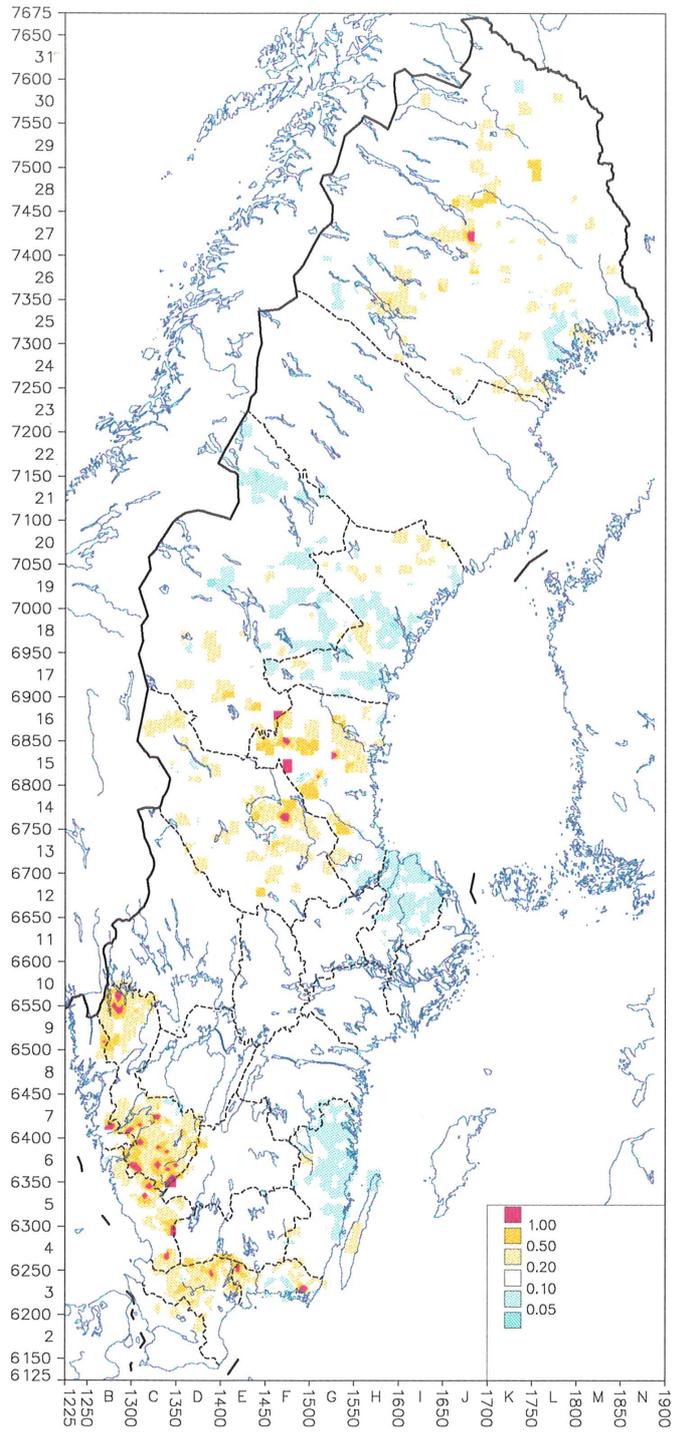


Fig. 6. Selenium concentration (mg/kg wet wt.) in livers collected in 1982 from moose in part of Sweden (n=2080).

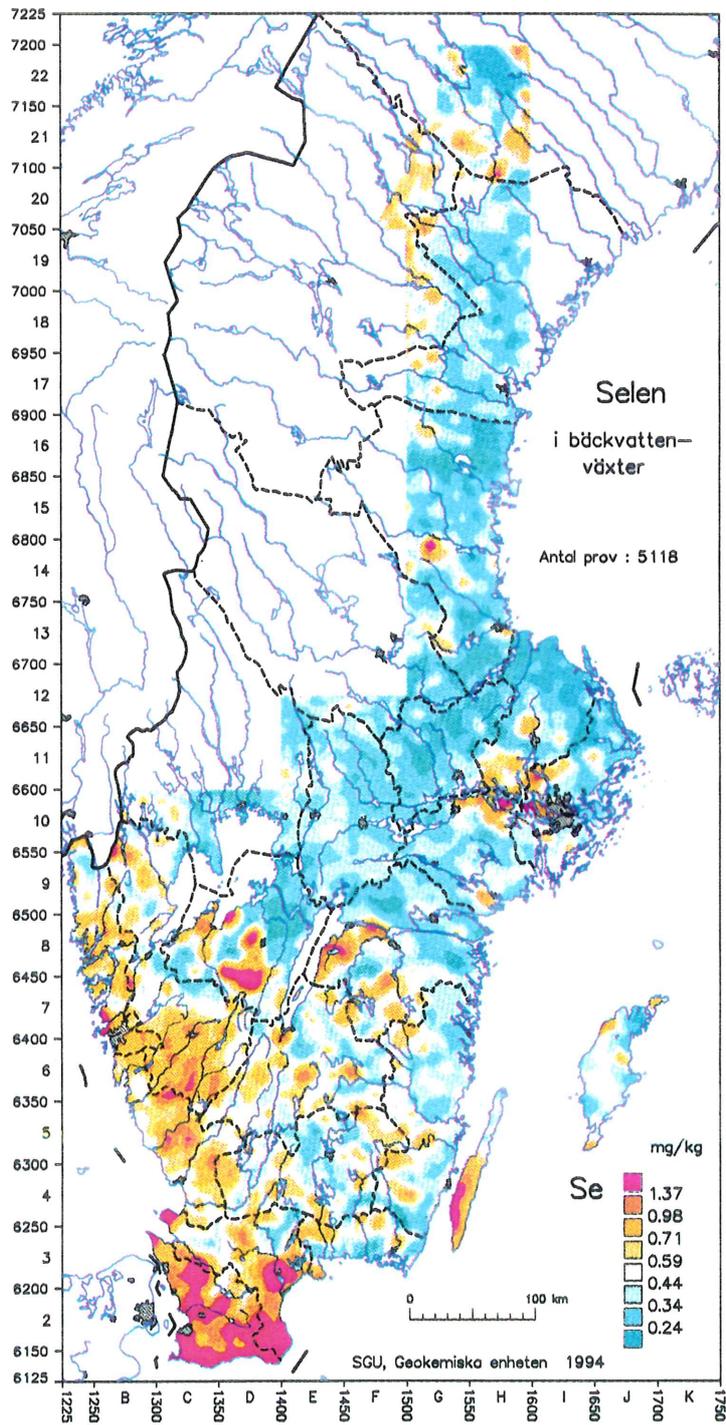


Fig. 8. Bio-geochemical map for selenium in BGS (n=5118) in part of Sweden (Reproduced with permission of the Swedish Geological Survey (SGU), Uppsala).

Nutritional Se status of cows

Selenium status of cows was evaluated with liver as indicator organ. Liver samples were collected from 4 slaughterhouses in Sweden - Luleå, Uppsala, Visby, Kristianstad - at the end of indoor feeding in spring 1987, 1989 and 1994. In addition, also liver from cattles sent to the Department of Pathology of the National Veterinary Institute (SVA) were analysed. The results are compiled for the years 1992-1993 and 1994-2006 (until August).

Supplementation of fodder with 0.1 mg Se/kg dry wt. was allowed in 1987. In 1989 a total Se-content in commercially produced fodder of 0.3 mg/kg dry wt. was allowed in Sweden and in 1994 a total Se-content of 0.5 mg/kg dry wt. was permitted. Selenium status was assessed according to the principles as mentioned above. (National Research Council, 1980; Puls, 1994; Rosenberger et al., 1994; Radostits et al., 1999). The results for the year 1987 were presented as a poster at an international meeting in Tokyo (Frank & Galgan, 1989). The results for the whole material are compiled in Figure 9. Liver Se concentrations above 0.2 mg/kg wet wt. are regarded as adequate. According to this criterion, only 13 % (1987) and 14 % (1989) of the animals from the slaughterhouse in Uppsala had an adequate Se status. The situation changed, when the Se-content in commercially produced fodder was maximised to 0.5 mg/kg dry wt. In 1994 58% of cows from the slaughterhouse in Uppsala had an adequate Se-status. The results from the three other slaughterhouses showed similar trends. High Se-concentrations, above 0.75 mg/kg, were found in the pathological material indicating acute parenteral Se-treatment.

The FI-HG-AAS method has been used for routine measurements at the Department of Chemistry, SVA, during many years and in many different biological materials. Samples with low Se-concentrations like in milk (Pehrson, 2003) and/or small sample amount as serum from children (Salih et al., 1994) were analysed without difficulties. Very high Se-concentrations were found in organ samples of seals from Swedish waters, some of them with over 200 mg/kg wet wt. (Frank et al., 1992).

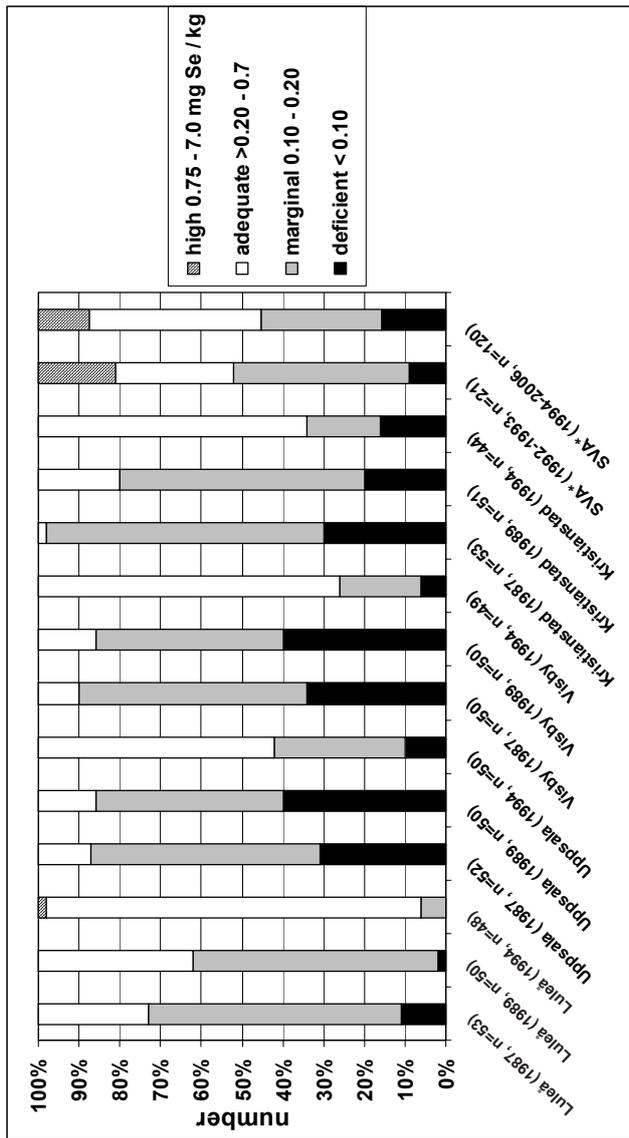


Fig. 9. Selenium status of cows assessed with liver as indicator. Number of samples are given in %. The liver samples were collected from 4 slaughterhouses in Sweden - Luleå, Uppsala, Visby, Kristianstad - at the end of indoor feeding in spring 1987, 1989 and 1994. Selenium status of cattle sent to the Department of Pathology of the National Veterinary Institute (SVA*) is shown for the years 1992-1993 and 1994-2006 (until August). Supplementation of fodder with 0.1 mg Se/kg dry wt. was allowed 1987. In 1989 a total Se-content in fodder of 0.3 mg/kg dry wt. was allowed. In 1994 the total allowed Se-content was 0.5 mg/kg dry wt. Selenium status was assessed according to information from the literature (National Research Council, 1980; Puls, 1994; Rosenberger et al., 1994; Radostits et al., 1999).

Conclusions

The great advantages of the FI-HG-AAS system are the use of low sample volumes/amounts, the possibility to make repeated determinations, high sampling frequency, low reagent consumption, and the ease of automation. The system is characterised by high sensitivity and reproducibility in Se determination and relatively low sensitivity to interferences.

- The sensitivity of the method allows determination in the $\mu\text{g}/\text{kg}$ (ng/g) range. Se concentrations at the level of a few $\mu\text{g}/\text{kg}$ in milk, serum, plasma, grass are not unusual in the Scandinavian countries.
- Combination of automated wet digestion and FI-HG-AAS for Se determination was most suitable for routine measurements.
- In all truthfulness, there is no best method of choice, which can solve all the problems in the analysis of biological materials with different matrices and a broad range of concentrations. The choice of method is influenced by several parameters, depending on prerequisites such as economics, equipments, type and quantity of available material, analyte concentration, length of series, etc.

Sammanfattning

Avhandlingens mål var att utveckla en analysmetod för bestämning av selen i biologiskt material där höga krav ställdes på känslighet och noggrannhet. Dessutom skulle metoden ha hög kapacitet, vara automatiserad och datoriserad för att kunna användas i rutinarbete.

Inledningsvis beskrivs ett automatiserat och datoriserat system för bestämning av selen i biologiskt material. Biologiskt material våtuppluts med en blandning av oxiderande syror. Den totala selenkoncentrationen bestäms med flödesinjektion hydrid-generering atom absorptions spektrometri (FI-HG-AAS). Automatiserad våtuppslutning av provmaterialet (serum/plasma, erythrocyter, urin, mjölk, olika vävnader såsom lever, muskel, njure, etc., foderprover m.m.) enligt en förutbestämt temperaturgradient genomförs med oxiderande syror (salpetersyra och perklorosyra) i borsilikatglasrör placerade i ett aluminiumblock med elektrisk uppvärmning och automatisk kontroll av tid och temperatur. Efter våtuppslutning tillsätts saltsyra och allt selen i provet reduceras från Se (VI) till Se (IV). Efter volymjustering tillförs en viss mängd provlösning till ett flödesinjektionssystem där natrium borhydrid reducerar i en saltsurlösning Se (IV) till Se (II) (hydridgenerering) varvid selenväte bildas. Denna gasformiga produkt (SeH_2) transporteras med kvävgas som bärare via en gas/vätske separator till en elektriskt upphettad kvartskvett som är placerad i strålgången av en atom absorptions spektrofotometer (Varian, model AA-1475). Ljusabsorptionen registreras vid 196.0 nm. Utrustningen är försedd med en provpåsättare. Analysförloppet registreras av en analogskrivare. Systemet är kopplat till en dator försedd med printer. De elektriska signalerna bearbetas i datorn eller manuellt. Valideringsdata för metoden redovisas.

En litteraturoversikt redovisar olika analyssystem för bestämning av selen i biologiskt material. Provinsamling och lagring, provberedning, olika analysmetoder och deras för och nackdelar beskrivs. Även erfarenheter från egna analyssystemet diskuteras.

Analysmetodens prestanda förbättrades framför allt för att kunna analysera låga selenkoncentrationer. Selenhalter lägre än 0.010 mg/kg kan t.ex. förekomma i höprover som skördas på selenfattiga svenska jordar eller i mjölk och blodprover från tamboskap som betar på selenfattiga betesmarker eller/och inte får selen tillskott. Behovet att förbättra metodens detektionsgräns resp. bestämningsgräns uppstod speciellt vid analys av torra prover. Våtuppslutning med salpetersyra och perklorosyra begränsar i det beskrivna systemet invägningen av torrt (t.ex. vegetabiliskt) material till max 1g. Det visade sig att våtuppslutna blanklösningar som innehåller perklorosyra orsakade en absorptionsignal som motsvarade en detektionsgräns (medelvärde av blank + 3 SD) av 0.8 ng Se/ml mätlösning motsvarande 0.020 mg Se/kg prov (vid 1 g invägt prov och 25 ml slutvolym).

Absorptionssignalen från lösningar som innehöll perklorosyra förhindrade förbättringen av detektionsgränsen. Perklorosyra är nödvändig för fullständig uppslutning av samtliga Se-föreningar. Sökandet efter orsaken till denna signal var omfattande. Med hjälp av en analog skrivare kunde mätsignalen från AAS instrumentet upplösas varvid en absorptionssignal av två maxima (dubbeltopp) med kort tidsavstånd avslöjades. Det andra maximum var identisk med Se's absorptionssignal medan den första inte kunde förklaras. I fortsättningen redovisas en omfattande undersökning av olika parametrar som med kännedom från tidigare studier i litteraturen skulle kunna orsaka denna ospecifika absorptionssignal. Efter successiva studier av gasflöden med olika sammansättningar och flödes hastigheter samt studier av surhetsgradens inverkan i de olika reagenserna kunde signalen elimineras genom optimering av natriumborhydrid och saltsyrakoncentrationer i flödessystemet. Blanksignalen motsvarade därefter instrumentets baslinjebrus och ligger nu mellan 0.1-0.3 ng Se/ml mätlösning.

Applikationen av metoden visas genom en inventering av biotillgängligt selen med älg som indikator djur. På detta sätt kunde även analysmetodens användbarhet i en lång analysserie testas.

Under höstjakterna 1981 och 1982 insamlades älgorgan (från ca 4300 djur) från hela Sverige och förvarades nedfryst vid SVA. Av detta referensmaterial uttogs sammanlagt 2080 leverprover från 14 regioner, tillhörande 12 län och analyserades med avseende på selen. Selenkoncentrationen i lever beräknat för hela undersökta materialet var

median = 0.15; medelvärde \pm SD = 0.25 ± 0.29 (range 0.03 – 3.1) mg/kg våtvikt.

De högsta medianvärdena för de enskilda regionerna var 0.26, 0.28 resp. 0.29 mg Se/kg lever från Gävleborgslän, samt norra och södra Älvsborgslän. Lägsta medianvärden, 0.09 – 0.10 mg/kg, hade Kalmar-, Uppsala- och Jämtlandslän. Selenkoncentrationer under 0.1 mg/kg i lever från tamboskap betraktas som brist. Det finns regioner i det undersökta älgmaterialet där frekvensen av sådana låga selenhalter uppgår till 63 % av materialet.

Andra applikationer omfattar vävnadsanalys av organ från ett stort antal vilda och tama djurarter, samt blod och mjölk. Analys av olika typer av foder och växtlighet utfördes. Metoden har fått stor betydelse för analys vid större projekt. Av sådana undersökningar nämns studier av slakthusprodukter från kalv, ko, svin.

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Acknowledgements

The investigations in the present work have been performed at the previous Department of Clinical Chemistry, now Department of Biomedical Sciences and Veterinary Public Health, Faculty of Veterinary Medicine and Animal Sciences and at the Department of Chemistry, National Veterinary Institute (SVA), Uppsala, Sweden. Financial support was provided by the National Veterinary Institute, and by grants from the National Swedish Environmental Protection Board and the “Stiftelsen Bengt Lundqvists Minne”. The completion of this work was made possible by the generosity of the National Veterinary Institute.

I wish to express my sincere gratitude to:

Professors Göran Hugosson and Lars Edqvist former General Directors of SVA, Prof Lars Edqvist also former Head of the Department of Clinical Chemistry at the Veterinary Faculty of the Swedish University of Agricultural Sciences (SLU) for accepting me as a PhD student, and Professor Anders Engvall, General Director of SVA, Håkan Palin, Head of HR department, SVA, and Professor Ulf Bondesson, Head of the Department of Chemistry, SVA, for their support and interest in the studies.

Professor Bondesson for giving me the possibility to complete and compile this thesis.

Professor Bernt Jones, my supervisor, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences (SLU) for support, enlightening discussions and never loosing interest during all this years.

Professor Adrian Frank, my supervisor in chemistry, the former Head of the Department of Chemistry, SVA, for never giving up. The thesis would not have been accomplished without his support, engagement and encouragement.

Professor Bo Karlberg, Department of Analytical Chemistry, Stockholm University, for introducing me in flow-injection technique, for practical support and many fruitful theoretical discussions.

Dr. Roland Pettersson, Senior Lecturer at the Department of Physical and Analytical Chemistry, Analytical Chemistry, Uppsala University, for interest, advice and valuable discussions at the end of my studies.

Assoc. Prof. Jean Pettersson, Department of Physical and Analytical Chemistry, Uppsala University for enlightening discussions at the beginning of my studies.

Dr. Olle Selinus, Senior Geologist at the Geological Survey of Sweden (SGU) for discussions, cooperation and support with geochemical maps.

I want to thank friends and colleagues supporting me during the long time of research and routine work and facilitating my work in different ways at the Department of Chemistry, SVA and the previous "Department of Clinical Chemistry" at the Faculty of Veterinary Medicine and Animal Sciences, SLU.

Especially thanks to my friends and colleagues at the Department of Chemistry, SVA:

Dr. Lars R. Peterson for collegial support and excellent teamwork.
Inger Hägglund, Birgitta Eberhardsson, Gabor Sellei, and Ann Meder for practical help and Lotta Thorelius for administrative support.

I want to express my gratitude to:

Adrian Frank for developing a computer program to register and calculate analytical data.

Sören Andersson for enthusiastic technical support in construction of different advanced electric devices.

Bengt Ekberg for excellent photographic support and

Gunnel Erne and Agneta Lind for excellent library service
Lennart Ivarsson for always-helpful support in laboratory work.

Mrs. Dr. Elisabeth Pape, a very appreciated colleague at the Research Institute for Child Nutrition in Dortmund, Germany, for introducing me to searching in literature, for many fruitful discussions about nutrition, supporting me with brand new scientific literature and monographs after my leave from Germany to Sweden, for her all-round interest and always enlightening discussions and long distance calls.

All my love to
my dear family, my mother Johanna, my sister Marion, and my aunt Helene.
my best friend Tatjana Jorke.

In Memory of my dear father late Oswald Galgan.