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**TRACE ELEMENT STATUS OF SOIL AND ORGANICALLY
GROWN HERBAGE IN RELATION TO ANIMAL
REQUIREMENTS**

**SPORSTOFFER I JORD OG ØKOLOGISK DYRKET
GROVFÔR VURDERT I HENHOLD TIL DYRENES BEHOV**

ESPEN GOVASMARCK

Institutt for Plante- og Miljøvitenskap
Universitetet for Miljø- og Biovitenskap
Postboks 5003, N-1432 Ås

Department of Plant- and Environmental Sciences
Norwegian University of Life Sciences
P.O.Box 5003, N-1432 Ås

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Preface and acknowledgements

This thesis deals with trace elements in soil- and organically grown herbage in relation to animal requirements for these elements. Trace elements in organic farming, a method for analysing selenium in biological materials and the effect of nitrogen application on the selenate uptake in wheat are discussed. The first paper describes the zinc, manganese and iron concentrations in organically grown herbage and factors affecting these concentrations. The second paper deals with the “on-farm” situation of copper, molybdenum and cobalt. The third paper describes the herbage selenium concentration and the animal blood concentrations of selenium and vitamin E. These three papers describe soil and herbage trace element concentrations on 14 organic sheep and 14 organic dairy farms in four different regions in Norway. The fourth paper presents a method developed for determining trace amounts of selenium in a variety of biological materials, without the use of perchloric acid. The fifth paper focuses on the effect of the interaction between selenate and nitrogen on the uptake and concentration of selenium in wheat plants and selenium losses in leachate water.

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ABSTRACT

To obtain a general picture of the herbage zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), molybdenum (Mo), cobalt (Co) and selenium (Se) concentrations on organic livestock farms, we analysed soil (2001) and herbage (2001 and 2002) samples from 28 farms from four regions in Norway. We analysed animal blood plasma Cu, B₁₂ (Co), α - and γ -tocopherol (vitamin E) and whole blood Se to investigate if the farms feeding practice met the dietary need of Cu, Co, Se and vitamin E in animals.

The first cut herbage median (10th-90th percentile) Zn, Fe, Mn, Cu, Mo, Co and Se concentrations were 19 (14-34), 50 (36-88), 34 (22-86), 5.3 (3.9-6.8), 1.5 (0.6-4.8), <0.05 (<0.05-0.08) and <0.01 (<0.01-0.03) mg kg⁻¹ DM, respectively. The herbage trace element concentration was generally higher in the second cut. The second cut herbage median (10th-90th percentile) Zn, Fe, Mn, Cu, Mo, Co and Se concentrations were 21 (16-37), 84 (52-171), 66 (36-205), 7.0 (5.7-9.3), 3.3 (1.6-10.1), 0.06 (<0.05-0.15) and 0.02 (<0.01-0.06) mg kg⁻¹ DM, respectively. The plasma Cu and B₁₂ (except one sheep herd) concentration were within the suggested normal range set by the Norwegian Veterinary Institute. Whole blood Se concentrations were 0.10 (0.04-0.15) μ g g⁻¹ in dairy cattle and 0.14 (0.03-0.26) μ g g⁻¹ in sheep. Vitamin E concentrations were 4.2 (2.7-8.4) mg L⁻¹ in dairy cattle and 1.3 (0.9-2.4) mg L⁻¹ in sheep.

The results of mixed model analyses of herbage Zn, Fe, Mn, Cu and Mo indicated that soil pH, soil texture, botanical composition and phenological stage at harvest mostly influenced the herbage trace element concentrations within regions. There was a poor relationship between soil and herbage trace element concentrations, except for Zn. None of the soil and plant variables explained the variation in the herbage Se or Co concentration, but the number of samples was too low to draw clear conclusions on these two elements. There were some differences in soil and herbage trace element concentrations between regions.

It was generally concluded that Zn, Fe, Mn, Cu and Mo did not limit plant growth. The herbage concentrations of Fe, Mn, Cu and Mo were sufficient to meet the dietary needs of ruminants. The herbage Zn concentration was insufficient to meet the dietary needs of dairy cattle. The herbage Co and Se concentrations and the Cu/Mo ratio were not alone balanced to meet the dietary needs of ruminants. The on-farm feeding practises fulfilled the dietary needs of Cu and Co. Selenium contents were generally insufficient on dairy farms under prevailing feeding regimes, whereas the vitamin E was insufficient on sheep farms. It is therefore highly recommended to use trace element mixtures and/or concentrates fortified with Cu, Co, Se and vitamin E on Norwegian organic livestock farms.

Most open vessel digestion procedures of biological material utilize a mixture of acids that include perchloric acid. There have been many accidents associated with the use of perchloric acid where serious injury has resulted. Therefore, a microwave digestion procedure of biological material, avoiding the use of perchloric acid while maintaining accurate selenium recoveries, was developed. Biological material was digested in two steps using nitric acid followed by hydrogen peroxide. Following the addition of phosphoric acid, remaining nitric acid and hydrogen peroxide were removed by evaporation, and Se-oxides were reduced to selenite using hydrochloric acid. Samples were adjusted to a buffered pH of 1.75 and reacted with 2,3-diaminonaphthalene. The resulting piaszelenol complex was extracted into cyclohexane. A normal phase HPLC method, using an amino phase column and a cyclohexane/ethyl acetate mobile phase, was used to separate the piaszelenol complex from

any remaining impurities before fluorescence detection on a HPLC-FLD. The relationship between peak height and selenium concentration was linear between 0 and 2 mg L⁻¹. The mass detection limit of the complete procedure was 0.29 ng of selenium. Recoveries of Se were within the certified range for the material analysed.

A pot experiment was used to investigate the relationship between ammonium-nitrate and selenate in the wheat uptake and leaching water loss of Se. Ammonium-nitrate was applied by two methods, (i) entire dose at sowing (ii) in split application as 75 % at sowing and 25 % at stem elongation. Selenate was applied at sowing, tillering, stem elongation, head emergence and at milking growth stage. Split N application increased the protein content and Se concentration in grain, but decreased the Se concentration in leaf and straw. The highest Se concentration in the plant was achieved when the soil N potentially was highest. The Se leaching losses increased with response uptake by plants, being highest at highest Se uptake by plants, but decreasing with split N application.

Conclusions of the work:

- Supplement of Cu, Co, Se and vitamin E are recommended to both dairy cattle and sheep and Zn to dairy cattle in organic husbandry in Norway.
- It is possible to determine Se in biological material without use of perchloric acid.
- Applying selenate and ammonium-nitrate together after tillering increases the wheat grain Se concentration and total Se uptake, split N application having the lowest leaching losses of Se.

SAMMENDRAG

For å undersøke og vurdere innholdet av sink (Zn), jern (Fe), mangan (Mn), kopper (Cu), molybden (Mo), kobolt (Co) og selen (Se) i engavlinga (grovfôr) på økologiske husdyrgårder, ble jord (2001) og grovfôr (2001 og 2002) analysert fra 28 gårder fordelt på 4 regioner i Norge. Det var ukjent om gardbrukerens foringspraksis tilfredstilte dyrenes behov av Cu, Co, Se og vitamin E. Blod fra dyrene ble innsamlet (våren 2002) og analysert for innhold av plasma Cu, B₁₂ (Co) og α - og γ -tokoferol (vitamin E) og innhold av Se i fullblod.

Grovfôrets median (10-90 persentil) innhold av Zn, Fe, Mn, Cu, Mo, Co and Se fra førsteslåttan var henholdsvis 19 (14-34), 50 (36-88), 34 (22-86), 5,3 (3,9-6,8), 1,5 (0,6-4,8), <0,05 (<0,05-0,08) and <0,01 (<0,01-0,03) mg kg⁻¹ tørrstoff (TS). Grovfôrets innhold av de sporstoffer var generelt høyest i andreslåttan. Grovfôrets median (10-90 persentil) innhold fra andreslåttan av henholdsvis Zn, Fe, Mn, Cu, Mo, Co og Se var 21 (16-37), 84 (52-171), 66 (36-205), 7,0 (5,7-9,3), 3,3 (1,6-10,1), 0,06 (<0,05-0,15) and 0,02 (<0,01-0,06) mg kg⁻¹ TS. Konsentrasjonen av Cu og B₁₂ (unntagen en sauegård) i blodplasma var innenfor normverdiene foreslått av Norges Veterinærinstitutt. Selen i fullblod var 0,10 (0,04-0,15) μ g g⁻¹ hos melkeku og 0,14 (0,03-0,26) μ g g⁻¹ hos sau. Vitamin E konsentrasjonen var 4,2 (2,7-8,4) mg L⁻¹ hos melkeku og 1,3 (0,9-2,4) mg L⁻¹ hos sau.

Faktorer som påvirket innholdet av sporstoffer i grovfôret ble analysert statistisk med mixed model. Resultatene indikerte at grovfôrets innhold av Zn, Fe, Mn, Cu og Mo innenfor regionene var påvirket av jord pH, jord tekstur, botanisk sammensetning og plantenes fenologisk utviklingstrinn ved høsting. Det var liten sammenheng mellom innhold av sporstoffer i jord og planter, unntagen for Zn. Det var ingen sammenheng mellom grovfôrets innhold av Se eller Co sett opp mot de målte parametrene, men antall prøver er for lite til å trekke klare slutninger. Det var variasjoner i innhold av sporstoffer i både jord og planter mellom regionene.

Det ble generelt konkludert med at Zn, Fe, Mn, Cu og Mo ikke begrenset planteveksten. Grovfôrets innhold av Fe, Mn, Cu og Mo var tilstrekkelig for å møte dyrenes behov. Grovfôrets innhold av Zn var ikke tilstrekkelig for melkeku. Grovfôrets innhold av Co og Se samt forholdet mellom Cu og Mo var lavt. Foringspraksisene på gårdene tilfredstilte dyrenes Cu og Co krav. Den totale forrasjonen til melkeku inneholdt ikke tilstrekkelig Se, mens det på sauegårdene var for lite vitamin E. Det anbefales å tilføre grovfôrrasjonen ekstra Cu, Co, Se og vitamin E på norske økologiske gårder.

Mange metoder for bestemmelse av Se i biologisk materiale bruker en blanding av syrer, der perklorsyre er en av syrene, til oppslutning av prøven. Perklorsyre har gjennom tiden vært årsaken til flere alvorlige ulykker. Derfor ble en oppslutningsprosedyre utviklet som omfattet bruk av mikrobølgeovn, har god presisjon og uten bruk av perklorsyre. Biologisk materiale blir først oppsluttet i salpetersyre og deretter i hydrogenperoksid. Fosforsyre blir tilsatt før fordampingen av resterende mengder salpetersyre og hydrogenperoksid. Saltsyre reduserer Se-oksiden til selenitt. Substratet blir deretter pH justert til 1.75 og tilført 2,3-diamonaphtalene. Det blir da dannet et piaszelenol kompleks som ekstraheres inn i sykloheksan. For bestemmelse av mengde Se i prøven ble det brukt en HPLC-FLD utstyrt men aminkolonne der den mobile fasen var en blanding av sykloheksan og etylacetat.

Forholdet mellom utslaget på kromatogrammet og selenkonsentrasjonen var lineær mellom 0 to 2 mg L⁻¹. Deteksjonsgrensen for metoden er 0,29 ng Se. Gjenfunnet Se i analysert standard referansemateriale var innenfor de oppgitte verdiene for referansematerialet.

Et pottforsøk for å undersøke opptaket av selenat i hvete ved tilførsel av ammoniumnitrat ble gjennomført. Etter høsting av hveteplantene ble det undersøkt hvordan gjødslingsintervallet påvirket utvaskingen av Se fra jord. Ammoniumnitrat ble tilført på to måter; (i) alt tilført ved såing (ii) delt gjødsling der 75 % ble tilført ved såing og 25 % ved stengelstrekking. Selenat ble tilført ved såing, busking, stengelstrekking, aks-skyting og ved kornkjerneutvikling. Delt N gjødsling økte kornets proteininnhold og selenkonsentrasjon, men reduserte Se konsentrasjonen i både blad og stengel. Høyest Se konsentrasjonen i planten ble oppnådd når det var mest N tilgjengelig i jorda. Utvasking av Se økte med økende planteopptak men ble redusert med delt N gjødsling.

Konklusjoner fra arbeidet:

- Det er anbefalt å gi supplement av Cu, Co, Se og vitamin E til både ku og sau og sink til ku på økologiske gårder i Norge.
- Det er mulig å bestemme Se i biologisk materiale uten bruk av perklorsyre.
- Gjødsling med selenat og ammoniumnitrat etter busking øker Se-konsentrasjonen i hvetekorn og det totale selen opptaket, og reduserer utvaskingen av Se.

Preface and acknowledgement	
Abstract	
Sammendrag	
List of papers	
Background and justification	10
Objectives of the present investigations	12
Methodological considerations and approaches.....	13
Farm survey.....	13
Soil analyses.....	13
Plant analyses.....	14
Animal blood analysis.....	15
Interpretation of trace elements	15
Marginal band	16
Determination of Se in biological material.....	17
Selenium in spring wheat and leaching water as influenced by selenium and nitrogen application	18
Results and discussion.....	19
The trace element concentrations on Norwegian organic farms.....	19
Soil	19
Herbage	20
Animal blood.....	21
Factors affecting trace element concentrations	21
Soil	21
Plant species	22
Determination of Se in biological material.....	23
Selenium in spring wheat and leaching water as influenced by selenium and nitrogen application	23
Recommendations	24
Agricultural practise.....	24
Soil analysis versus plant analysis	24
Suggestions for further research.....	25
References	25

List of papers

I:

Govasmark, E., Steen, A., Bakken, A.K., Strøm, T. and Hansen, S. 2005. Factors affecting the concentration of Zn, Fe and Mn in herbage from organic farms and in relation to dietary requirements of ruminants. Acta Agric. Scand. B Soil Plant Sci. (*in press*).

II:

Govasmark, E., Steen, A., Bakken, A.K., Strøm, T., Hansen, S. and Bernhoft, A. 2005. Copper, molybdenum and cobalt in herbage and ruminants from organic farms in Norway. Acta. Agric. Scand. A Animal Sci. (*in press*).

III:

Govasmark, E., Steen, A., Strøm, T., Hansen, S., Singh, B.R. and Bernhoft, A. 2005. Status of selenium and vitamin E on Norwegian organic sheep and dairy cattle farms. Acta. Agric. Scand. A Animal Sci. (*in press*).

IV:

Govasmark, E. and Grimmet, M.G. 2005. A microwave method for determination of selenium in organic tissues. J. AOAC Int. (*Submitted*).

V:

Govasmark, E., Singh, B.R., MacLeod, J.A. and Grimmet, M.G. 2005. Selenium in spring wheat and leaching water as influenced by selenium and nitrogen application. Can. J. Plant Sci. (*Submitted*).

Background and justification

The work presented here was undertaken to contribute to further development of organic livestock farming with emphasis on sound nutrition and welfare of ruminants, and to increase the knowledge on some essential trace elements in the soil-plant-animal system on farms.

Leys represent more than 75 % of the organically grown area in Norway, and approximately 3 % of the total farmland (Debio 2004). The Norwegian government aims to have 10 % of the total farmland converted to organic agriculture within 2010. Organically grown herbage as feed to ruminants will most probably remain the main crop grown in organic farming after 2010. Since ruminant feed in organic farming is mainly based on roughage, and the safeguarding of animal welfare is given high priority in Norwegian livestock farming, it was considered necessary to have reliable information on the mineral composition of herbage feed. Knowledge on the nutritional quality of organic roughage should then be used to improve ruminant feeding and feed supplements by farmers, veterinarians, the agronomical extension service, and feed supplement producers.

Trace element deficiency in plants reduces not only the yield, but also the nutritional quality of the feed. An unbalanced trace element composition in the diet to ruminants is unhealthy and has a negative influence on animal welfare (Underwood & Suttle 1999).

The first aim of the experimental work in this study was therefore to investigate the herbage zinc (Zn), iron (Fe), manganese (Mn), copper (Cu), molybdenum (Mo), cobalt (Co) and selenium (Se) concentrations in organically grown herbage on Norwegian sheep and dairy farms. It was well recognized at the start of the project that feeding problems related to herbage Cu, Mo, Co and Se concentrations occurred in Norway. Grain yield losses related to Zn and Mn deficiency were also observed. However, all these reports were based on results obtained in conventional agricultural systems. It was also reported that the transition from conventional to organic farming leads to lower yields, higher soil pH, alteration in the mineral concentration in soil and plants and changes in the botanical composition of leys. All these factors have great influence on the herbage trace element concentration. Limited information existed on the herbage trace element concentration in organic farming, and it was unclear if the findings reported from the herbage in conventional farming were valid for organic farming.

The second aim was to find factors that explained most of the variation in the herbage trace element concentrations. To do this, we related the recorded herbage trace element concentrations to region, climate, edaphic factors, plant characteristics and farming practise. Knowing which factors affected the herbage trace element concentrations, it would be easier to give farmers advice on feeding strategies if they had an unfavourable herbage trace element composition.

The final use of determining herbage trace element concentrations was to relate the results to suggested concentrations in plants having normal growth and to the dietary needs of sheep and dairy cattle. If a certain herbage trace element concentration was very low, it would be possible to emphasise this, so that an advance warning could be given, and farmers could find fertiliser sources that comply with the organic standards. It is prohibited to use inorganic fertilisers in organic farming as a standard procedure, but inorganic trace elements are allowed as a feed supplement to ruminants (Debio 2003). It should therefore be possible to

adjust the trace element supplementation to ruminants through concentrates or mineral mixtures, when the herbage trace element concentrations are known. Whole blood was sampled on each farm to investigate the animal concentration of Cu, Co, Se and vitamin E. Nutritional problems related to these constituents was expected. Because many organic farmers supplement the feed with concentrates or mineral mixtures, the blood samples were used to investigate if the total feed trace element concentration was sufficient to meet the dietary need of ruminants. Corrections of the feeding regime could therefore be made, if non-optimal blood trace element concentration was found.

Fodder produced in Norway generally has a low Se concentration (Wu 1988) and preliminary results from the first harvest year agreed with this. Despite problems with Se deficiency in Norwegian organic livestock farming, Se also has other interesting aspects. Selenium is only essential for mammals. Selenium is one of the mineral elements with the smallest ratio of essential to toxic intake. This is one of the reasons why many researchers throughout the world are working to establish the levels of Se intake that would be beneficial without running any risk of inducing toxicity. Since Se fed in organic forms has proved to be better absorbed than the inorganic forms (Ortman & Pehrson 1999), the goal was to find organic Se sources acceptable in organic farming that could be used as a fertiliser to increase the plant Se concentration.

To be able to measure trace Se concentration in biological samples, we had to have a safe and reliable method. One of the acids commonly used in the oxidation of organic materials for total Se determinations is perchloric acid. Perchloric acid is prohibited in many laboratories due to the risk of use, storage and handling. A mixture of perchloric acid and acetic anhydride exploded in a Los Angeles factory in 1947, killing 15 people, injuring 400, and causing \$2 million in damage. On a smaller scale, Robinson (1985) reported a detonation of 3 g of a perchlorate salt of a rhodium-polyamine complex undergoing an evaporation step in a rotary evaporator. A violent explosion destroyed the evaporator, smashed a lab jack, cracked the bench top, and chipped walls over 15 feet away. Fortunately, this happened in an empty laboratory. Literature surveys reveal that descriptions of explosions in laboratories using perchloric acid have been reported over a period of more than a century. Many methods utilize the oxidation power of perchloric acid and a number of laboratory routine analytical methods have been adapted (Hawkes & Kutnink 1996; Watkinson 1966; Ihnat 1974). It is clear, however, that no one should attempt to use perchloric acid that is not fully conversant with the chemistry of the material, who has not made a careful appraisal of operating conditions and techniques, and who exhibits an unsafe attitude about his/her work. Our goal was therefore to develop a method for determination of Se in organic materials, which could be used as a routine procedure without the use of perchloric acid.

It was not possible to increase the wheat Se concentration by applying fresh or composted lobster waste as a fertiliser. Organic Se fertilisers do not increase the grain Se concentration. Applying inorganic Se is thus the most efficient strategy to increase the plant Se concentration, which then can be used as an organic Se source to humans or animals. The chemical form of Se is of major importance, and selenate is the most efficient way of increasing the cereal and forage Se concentrations (Gupta & MacLeod 1994; Gupta et al. 1993; Singh 1991). The growth stage of the cereal is the second most important factor, and adding selenate in the period from tillering to heading is the most efficient way of increasing the Se concentration in the grain (Singh 1994). It is also known that plant uptake of Se is depressed by sulphate because these two anions are subjected to the same uptake mechanisms. It is also known that the redistribution of S from the vegetative plant parts to the grain was

influenced by nitrogen (N) availability to the plant during vegetative growth (Eriksen et al. 2001). If this is the case with N and Se is, to my knowledge, not known. Inorganic N was used to eliminate all the factors affecting the plant availability of organically bound N, and the N treatment was the same as used by farmers to increase the wheat protein concentration.

Leaching of Se after the growing period was included to investigate the potential impact the different treatments had on the losses of Se. Selenium toxicity can cause serious deformities in wildlife (Adams et al. 2003; Hamilton 2003). Volatilization and high concentrations of mercury (Hg) in fish and sediments are problems in many lakes throughout the world (Lutter & Irwin 2002; Jackson 1991) and the concentration of Hg in fish is reported to be influenced by Se and the redox conditions of the environment (Jin et al. 1997). Since the chemical properties of nitrate and selenate are very similar they will compete for the soil binding sites.

Objectives of the present investigations

The present investigations were planned in order to:

1. investigate the herbage concentrations of Cu, Fe, Zn, Mn, Co, Mo and Se and factors affecting their concentration at Norwegian organic sheep and dairy farms in relation to the dietary needs of ruminants (Papers 1, 2, 3).
2. develop or validate a reliable and safe method for measuring Se concentrations in biological materials (Paper 4).
3. investigate the relationship between Se and N applied at different growth stages on the Se concentration in spring wheat (Paper 5).

Methodological considerations and approaches

Farm survey

The trace element status of Zn, Fe, Mn (paper 1), Cu, Mo, Co (paper 2) and Se and vitamin E (paper 3) on Norwegian organic sheep and dairy farms was investigated in a farm survey. Of 526 certified (Debio, 2001) organic sheep and dairy farms in Norway, seven farms within each of the two regions called Coast (1) and Mountain (2), having a high density of sheep farms (farm 1–14), and East (3) and Middle (4), having a high density of dairy farms (farm 15–28) were selected (Fig. 1). All farms had maintained organic plant production for more than three years and organic animal production for at least one year. On each farm, three leys that would last for two years were selected. On each farm, we recorded factors that we thought influenced the soil and herbage trace element concentrations. Soil and herbage samples were taken from three permanently marked subplots. Five animal blood samples were pooled from each farm. Soil was sampled (0-20 cm) after the first harvest in 2001, herbage was sampled at each cut in 2001 and 2002, while animal blood was sampled late in the indoor season (April-May) in 2002.

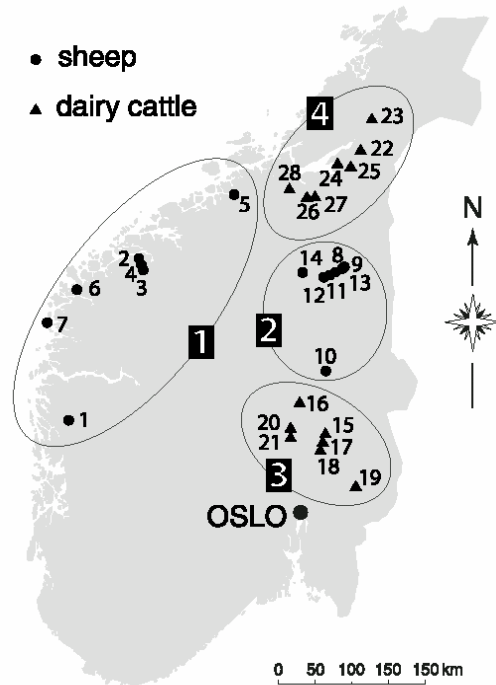


Fig. 1. Map of southern Norway indicating the location of 27 farms in the Coast (1), Mountain (2), East (3), and Middle (4) regions.

The herbage fodder for dairy cows before and during the blood-sampling period consisted of 60 to 80 % grass silage and 20 to 40 % hay, except on farm 25 which only had hay. All dairy farms, except farm numbers 19, 25 and 26, used supplements in their feeding. Fodder for sheep mainly consisted of hay, except farm number 12, which had silage. All sheep farms, except farm numbers 4, 5 and 8, used supplements in their feeding. None of the sheep farms which used feed supplements, used vitamin E deliberately, but unintentionally through the use of mineral mixtures or concentrates.

The penetrometer resistance was used for assessing the in situ soil strength, one of the extrinsic factors affecting plant growth and crop productivity. Based on the penetration resistance measurements, only one farm (Farm 19) in the East region could have soil structural problems. The farmer had used heavy machinery during the harvest of round bale silage.

Soil analyses

A soil chemical mineral analysis is a rapid and inexpensive method for obtaining information on mineral availability in soils, and is supposed to indicate the potential mineral uptake by plant roots. Soil chemical analyses make use of a whole range of extraction methods involving different forms of dilute acids, salts or complexing agents, as well as water.

Depending on the method used, quite different amounts of plant available minerals are extracted (Alva 1993; Ajwa & Tabatabai 1993; Garcia et al. 1997; Grigg 1953; McLaren et al. 1986).

Diethylenetriaminepentaacetic acid (DTPA) was used to extract the plant available fraction of soil Fe, Cu, Mn and Zn (Lindsay & Norvell 1978), and Mo was extracted with oxalic acid (Grigg 1953). Cobalt was extracted with 0.5 M acetic acid and Se with 0.016M KH_2PO_4 . Zinc, Fe, Mn, Cu, Mo and Co in the soil extracts were analysed by inductively coupled plasma atomic emission spectrometry (ICP-AES) and Se by ICP-mass spectrometry (ICP-MS).

Diethylenetriaminepentaacetic acid (DTPA) is a chelating agent that combines with free metal ions in solution, forming soluble complexes and thereby reducing the activities of the free metal ions in solution (Lindsay & Norvell 1978). In response, metal ions desorb from soil surfaces or dissolve from labile solid phases to replenish the free metal ion solution. This method is commonly used for the extraction of Fe, Cu, Mn and Zn (Liang & Karamanos 1993). Acid ammonium oxalate forms stable complexes with molybdate, in which molybdate absorbed on the soil colloids and clay is presumably replaced by the oxalate ions, the exchange being made irreversible by formation of the strong molybdenum-oxalic acid complexes. Acid ammonium oxalate is commonly used to extract soil Mo (Gupta 1993). DTPA and oxalic acid extracting procedures were used to determine the trace element concentration in soil solution and the readily available pool of the nutrients in the soil. Weak extracting chemical solutions such as weak acids or ionic solution mainly the intensity of supply reflecting their easily available pool in soils (Øien et al. 1988; McLaren et al. 1986), and was used to extract Co and Se.

When there is contradiction between soil and plant trace element concentration, it is important to find the main reasons for it. Such contradiction indicates that the soil extraction methods used fail to take into account the effect of a soil regulating the plant availability of a trace element. In order to correct for the soils regulation of a mineral nutrient, its result must be corrected so that it is in accord with results of plant analyses. Factors regulating the soil available pool of trace elements are those which are expected to influence on the ion exchange, chemisorption and precipitation of inorganic ions, mineral weathering, and oxidation-reduction reactions in the soil. Therefore, three 100 cm^3 soil cores were, at a depth of 10 to 14 cm, taken just outside the subplot for analyses of water holding capacity and soil porosity. The water retention characteristics were determined at 0.01 MPa (pF 2) (g water/100 ml soil) by the pressure plate method (Page et al. 1982) and the pore volume (ml/100 ml) according to the method of Page et al. (1982). The particle size distribution of the composite sample was analysed by the pipet method (Page et al. 1982). The pH was measured in a 1:5 soil: water suspension and organic carbon by combustion in a LECO CHN-1000 apparatus (Page et al. 1982). An estimate of the content of organic matter was obtained by multiplying the content of organic carbon by 1.72 (Riley 1996). Soil was classified according to FAO (1994).

Plant analyses

Plant analysis is an approach to determine the nutrient status of the plant and reflects the nutrient availability of a soil. This is based on the concept that the concentration of a particular nutrient in the plant is greater the higher the soil concentration is. The concept is sound, since nutrients in the plant must have been available in the soil. Assessing the trace

element status of the soil for plant growth has its drawbacks, since plant concentration not only depends on the soil trace element availability but also on plant species and phenological stage. In any case, plant analysis is a suitable approach when evaluating the herbage trace element concentration in relation to the dietary need of ruminants. Commonly used techniques for total plant cation determination is dry ashing, whereas wet decomposition is commonly used for the total determination of plant Se (Mills 1996).

Official methods according to the Association of Analytical Chemists (Pedersen & Lysnes 2002) were used for the determination of total herbage Zn, Fe, Mn, Cu, Mo and Co concentrations by dry ashing. Zinc, Fe, Mn, Cu, Mo and Co was determined in the digested solution by ICP-AES. For Se determination, herbage samples were digested in nitric and perchloric acids on a heating block. Selenium was reduced with HCl, and diluted to 25 ml with water. Herbage Se determination was performed by hydride generation atomic absorption spectrophotometry (HG-AAS).

The total herbage yield was recorded on all subplots at harvest time in 2001 and 2002. One sample was collected for determination of botanical composition, one sample for the analysis of mineral composition, and one sample of red clover (*Trifolium pratense*), if present, was collected from the plots in 2001 for mineral composition. At the time of the first cut of leys in both years, the phenological stage of development of timothy was determined according to the procedure of Moore et al. (1991).

Animal blood analysis

Element concentration in blood is often sampled to reflect the status of the transport pool of an element, which is believed to be one step closer to dysfunction than measuring the storage pools in the animal (Underwood & Suttle 1999). The blood sample can be arranged to consist of only the blood plasma or the blood serum. Blood plasma is the liquid in which blood cells are suspended. Blood serum is the same as blood plasma except that clotting factors such as fibrin have been removed. Using whole blood or blood plasma requires an anticoagulant added to the sample. Since many minerals can be stored and thus utilised by the animal on mineral deficient diets, blood should be sampled a long time (at least three weeks) after the animal has started feeding on the diet being investigated.

Plasma Cu concentration was determined by flame atomic absorption spectrophotometry at the Norwegian Veterinary Institute and plasma vitamin B₁₂ concentration as an indicator of the Co status was determined with the Dualcount Solid Phase No Boil radioassay kit at the Norwegian School of Veterinary Sciences. Whole blood Se concentration was determined by sodium borohydride reduction and atomic absorption spectrophotometry (AAS). Using whole blood instead of blood serum or plasma for Se analyses is preferred because the Se concentration is much higher in the erythrocytes (red blood cells) than in blood serum or plasma. Plasma vitamin E concentration was determined as α - and γ -tocopherol concentration by high performance liquid chromatography (HPLC).

Interpretation of trace elements

Trace element concentrations in soil, herbage or blood have to be evaluated with respect to what one aims to explain. The soil-extracted trace element concentrations were evaluated according to recommended levels for normal growth by the method used. The suggested minimum extractable concentration is very often a defined soil concentration. The soil

extraction concentration of the single trace element was also expected to be related to the herbage trace element concentration.

Herbage trace element concentrations were evaluated according to suggested normal levels in plants under normal growth conditions. Although Zn, Fe, Mn, Cu and Mo have been acknowledged as essential trace elements for a long time, there are no definite statements in the literature about what concentrations in different plant species should be considered as adequate, marginal or deficient (Whitehead 2000; Marschner 1995; Mengel & Kirkby 1987). Another problem when evaluating the trace element concentration with respect to plant growth was that the herbage concentration depends on the herbage composition (Whitehead 2000; Ylärinta 1995), phenological stage of maturity (Mengel & Kirkby 1987), and plant growth at the time of determination (Ylärinta et al. 1979). To be able to evaluate the herbage trace element concentration it was necessary to choose a level, knowing that the level was not the full truth. Cobalt and Se are not essential trace elements for plant growth, and thus no critical plant growth concentrations are listed.

There is also no definite statement in the literature about what trace element blood concentrations that should be considered as adequate, marginal or deficient. Nevertheless, herbage and whole blood concentrations are widely used as indicators of the trace element supply to animals and the trace element status of the animals (Underwood & Suttle 1999). Different authors recommend different nutritional standards, but since suggestions have to rely on recommended feed concentrations or whole blood values, the results will to some extent depend on the standard used. The different soil, herbage or blood trace element and vitamin E concentrations used for evaluating the trace element concentrations are presented in Table 1.

Table 1. Critical levels of trace elements in soil and herbage for plant growth, dietary requirements for animals and animal blood concentration for animal welfare.

	Soil (mg kg ⁻¹ DM)	Herbage (mg kg ⁻¹ DM)		Blood/Plasma
		Plant req.	Animal req.	
Fe	4.5 ^a	50 ^c	50 ^e	
Zn	0.6 ^a	20 ^c	20 ^e	
Mn	1.0 ^a	20 ^c	20 ^e	
Cu	0.2 ^a	4 ^d	3 ^f	11-20 µmol L ⁻¹ plasma ⁱ
Mo	0.14 ^b	0.1 ^d		
Co			0.05-0.11 ^{efg}	150-300 pmol L ⁻¹ plasma ^j
Se			Sheep; 0.03-0.05 ^e Dairy; 0.02-0.04 ^e	0.05-0.10 µg L ⁻¹ whole blood ⁱ
Cu/Mo			6-10 ^h	
Vit. E				2.0 mg L ⁻¹ plasma ⁱ

^a Lindsay and Norvell 1978; ^b Grigg 1953; ^c Marschner 1995; ^d Whitehead 2000; ^e Underwood and Suttle 1999; ^f Puls 1994; ^g Ulvund 1995; ^h Frøslie 1990; ⁱ National Veterinary Institute, Norway; ^j Ulvund 1990

Marginal band

A basic or minimum requirement for any trace element can be conceived as one for which all the dietary conditions affecting that trace element are optimum (Underwood & Suttle 1999). Since these exact conditions rarely apply, there can be no single requirement but rather a

series of requirements, depending on the extent to which conditioning factors are present in a particular grazing or ratio. By the same reasoning, there must be a series of maximum safe dietary levels, depending on the extent to which other minerals or compounds are affecting the absorption, retention and excretion of a mineral consumed in excess of need. These assumptions have led to the use of the marginal band (Fig. 2). A marginal band is the concentration between an expected adequate concentration and concentrations representing expected deficiency and toxicity levels. Response in animals to supplements is possible when trace element concentrations are within the marginal bands, and most certainly when they are above the toxicity or below the deficiency level. Evaluating the herbage trace element concentrations and animal tissue concentrations in relation to the dietary need of ruminants or normal tissue concentrations can thus be used to evaluate and suggest improvements of mineral feeding regimes.

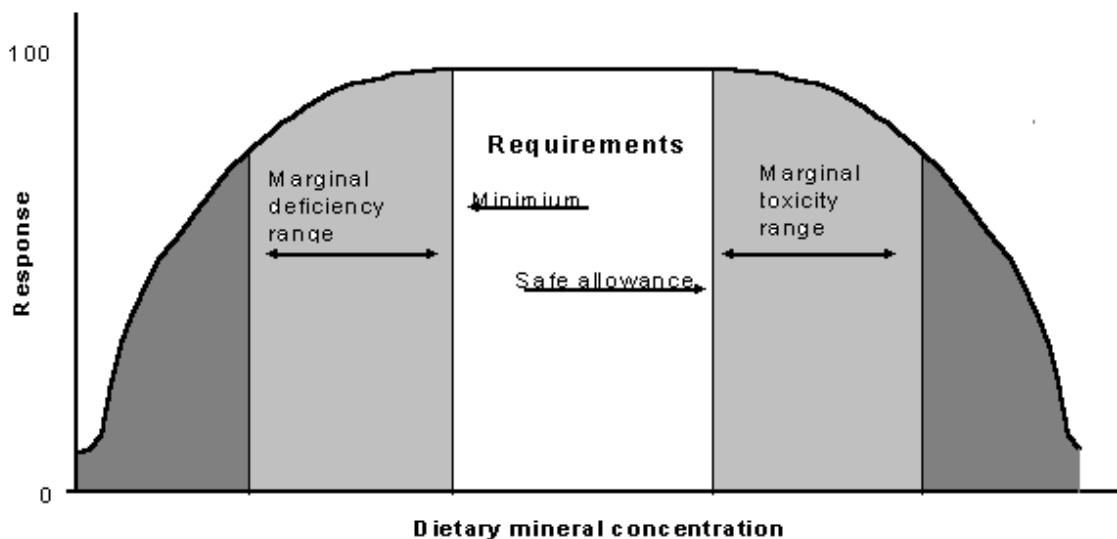


Fig. 2. Schematic dose-response relationship between mineral supply and animal production showing marginal bands between adequate and inadequate or toxic concentrations. Requirements are variously set within the central adequate band from minimum requirements to safe allowance, depending on the extent to which absorbability and other variables are taken into consideration. Rewritten from Underwood and Suttle (1999)

Determination of Se in biological material

We developed a method that was based on the earlier methods of Hawkes and Kutnink (1996) and Vézina and Bleau (1988), and modified for microwave digestion. Biological material was digested in closed Teflon vessels in two steps using nitric acid followed by 30 % hydrogen peroxide at 205 °C. A number of acids and other chemicals were tried out to digest the biological material. Sulphuric acid, phosphoric acid, an aqua regia solution, Fentons reagent added Cu or Fe, either hydrogen peroxide or nitric acid and in different amounts and concentrations. None of the solvents was by itself sufficient to decompose the organic material, or gave an explosive reaction, thus making it unsafe to use. Nitric acid and hydrogen peroxide in two steps, was the only procedure, which safely decomposed the organic material and therefore was suitable as a standard laboratory procedure. The temperature was set at 205 °C, because it gave the most effective decomposition. The next step was reducing the Se compounds in the matrix to selenite. This step did not succeed before we removed the

remaining nitric acid and hydrogen peroxide. The method on which this procedure is based does not have a step involving removal of nitric acid, but since the method involves perchloric acid at temperatures above 150 °C, most probably nitric acid (boiling point (bp) 120.5 °C) was anyhow evaporated in the procedure. Nitric acid together with hydrochloric acid is a strong oxidant, decomposing the creosol red and a dark layer on the sample in the tube could be seen. Therefore, remaining nitric acid and hydrogen peroxide (bp approximately 110 °C) were removed by evaporation. To avoid that the sample matrix did not dry out during evaporation, phosphoric acid was added (bp 213 °C). A solution pH of 1.75 is recommended since the maximum fluorescent signal of the Se-2,3-diaminonaphthalene (Se-DAN) complex is observed in the pH range 1 to 3, and because the optimum for the piaszelenol formation is between pH 1 and 2 (Rodriguez et al. 1999). The glycine buffer was adjusted to pH 1.75 and added to minimize the tube-to-tube variation in the final pH. Samples were therefore adjusted to a buffered pH of 1.75 and reacted with 2,3-diaminonaphthalene. The resulting piaszelenol complex was extracted into cyclohexane (Fig. 3) and transferred to an amber vial. A normal phase HPLC method using an amino phase column and a cyclohexane/ethyl acetate mobile phase was used to separate the piaszelenol complex from the remaining impurities before fluorescence detection on a HPLC. The mobile phase composition (80 % cyclohexane, 20 % ethyl acetate) was adjusted slightly from the Hawkes and Kutnik (1996) method (90 % cyclohexane, 10 % ethyl acetate) to give slightly more retention. Higher ethyl acetate contents were tested but did not significantly improve the resolution of the piaszelenol peak and only increased the overall run length. Hawkes and Kutnik (1996) used a silica column in contrast to the amino phase column utilized in the proposed method, which might explain the difference. Analysing a collection of botanical, food and marine standard reference material validated the analytical procedure.

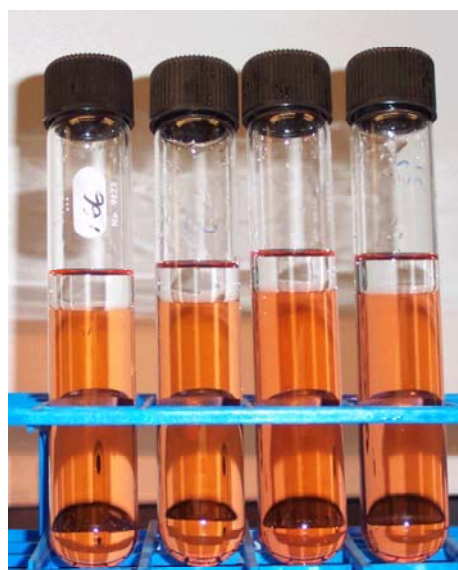


Fig. 3. The cyclohexane phase with the piaszelenol at the top of the tube, and the remaining pH adjusted solution with creosol red in the amber orange layer.

Selenium in spring wheat and leaching water as influenced by selenium and nitrogen application

The pot experiment study was conducted on a fine sandy loam (Humic-Ferric Podzol) by using spring wheat (*Triticum aestivum* L "Helena"). Total N and total S contents in the soil were 1.0 g kg⁻¹ and <0.04 g kg⁻¹ soil, respectively. Soil was sampled from the upper 20 cm, air dried and sieved (<5 mm). Each pot contained 3.5 kg soil. Eight seeds were sown in each pot and thinned to 6 plants after 10 days. The temperature ranged from 20 °C (day) to 16 °C (night), with a 14 hour day at an irradiance of 475 μmol m⁻²s⁻¹.

Nitrogen and selenate were added as NH₄NO₃ and Na₂SeO₄ in aqueous solutions. Except for the control, 0.01 mg Se kg⁻¹ soil was added in all treatments. Selenate was added at different growth stages; at sowing (0), tiller (2) at 26 days, stem elongation (3) at 59 days, head

emergence (5) at 72 days, and milk development in kernel (7) at 90 days according to the Zadoks scale (Zadoks et al. 1974). Each Se treatment was imposed with two nitrogen treatments. N1; 105 mg N kg⁻¹ soil were applied at sowing and N2; 70 mg N kg⁻¹ applied at sowing and 35 mg N kg⁻¹ at stem elongation. All plants were harvested at maturity after 101 days. A basal dose of phosphorous (P), potassium (K), sulphur (S), calcium (Ca), magnesium (Mg), Zn, Mo and B was added to each pot.

Length of the stem and the number of stems and heads were counted before grain, straw, leaf and the rest of the spike (Head) were separated. Kernels were counted and the weight of 100 kernels determined. Total Se concentrations were determined by the method described in the chapter "Determination of Se in biological material" (Paper 5). Total nitrogen in wheat kernels was determined by combustion gas analysis using a LECO CNS-1000 analyzer and multiplied by 6.25 for the conversion to total protein.

All pots were water saturated prior to the leaching experiment. Three litres of demineralised water was drained through the soil. The leachate was acidified (NH₃O) and stored cold prior to analysis. Fifty ml of the lechate water were used for Se determination, by the method described above

Results and discussion

The trace element concentrations on Norwegian organic farms

Soil

The Middle region (Fig. 1) had the highest extractable soil Cu but lowest soil Zn concentration, the East region had the highest soil Mo concentration and the Coast region had the lowest Mn and Co soil extractable concentration. No differences in soil extractable Fe was found between the regions. Zinc was the only trace element which had a significant relationship between soil and herbage concentrations (Table 4 (Paper 1); Table 1 (Paper 2)). These results indicate that the extracting procedure used did not reflect the available pool of soil Fe, Mn, Cu and Mo. The correlation between soil and herbage Se concentration was significant in both cuts, but the graphical presentation of this relationship illustrates some problems (Fig. 4). The relationship was dependent on region and harvest time, and the visual problems are seen for soil Se concentrations <0.03 mg Se kg⁻¹ and the herbage Se concentration. Extracting soil Se with 0.016M K₂HPO₄ requires considerably more investigations before it can be recommended as a procedure for determining the plant available Se fraction.

According to soil trace element extraction methods used in the present study, it was indicated that soil Fe and Cu were not in the deficient range, Mn could be considered as deficient on some farms, whereas Zn and Mo were found to be deficient (Fig. 2 (Paper 1)). This was not supported by the herbage trace element analysis, which indicated that only Zn could be deficient on dairy farms. Cobalt and Se are not essential trace elements for plant growth, and thus not growth limiting.

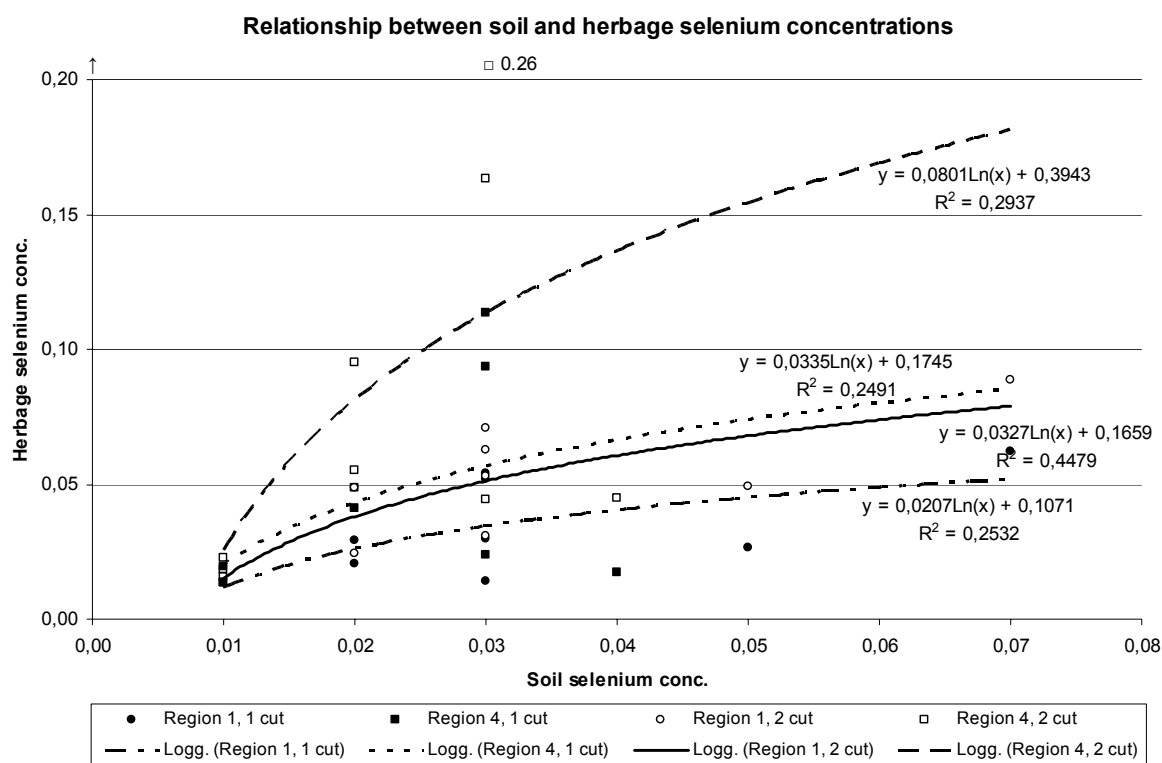


Fig. 4. The relationship between extractable soil (mg kg^{-1} DM) and herbage (mg kg^{-1} DM) Se concentration within region 1 (Coast) and 4 (Middle) for the 1st and 2nd cut. Regression line is presented for each line.

Herbage

The herbage Mo concentration was highest in the East region whereas no regional differences in the herbage Cu, Fe and Co concentrations were found. The lowest herbage Zn and Mn concentrations were found in the Middle region. The highest herbage Se concentration was found in the Coast and Middle region. The herbage trace element concentrations, except for Zn, were all above the critical growth limiting concentration, and were thus expected not to limit plant growth (Fig. 2 (Paper 1); Fig. 2 (Paper 2)). The herbage Cu:Mo ratio, which is important for ruminants, was generally low (Fig. 5). The clover Cu:Mo ratio was higher than the total herbage Cu:Mo ratio ($P < 0.05$) (data not shown). The herbage Cu:Mo ratio decreased with increasing herbage Mo concentration (Fig. 2B (Paper 2)). The Cu:Mo ratio is important because Mo can exert a depressive effect on Cu absorption and availability in animals but the interrelation only exerts on Cu in the presence of sulphate (Underwood & Suttle 1999). Sulphide is formed by ruminal micro-organisms from dietary sulphate or organic S compounds. The sulphide then reacts with Mo to form thiomolybdate, which in turn combines with Cu to form an insoluble copper thiomolybdate, thereby limiting the absorption of dietary Cu. These interrelationships will only occur when the herbage levels of S are at least 4 g kg^{-1} DM combined with a herbage Mo concentration above 3 mg kg^{-1} DM (George Fisher, pers. comm. 2004).

Ninety-six percent of all herbage samples in the first and 67 % in the second cut had less than 2 mg S kg^{-1} DM herbage in the present investigation (Strøm et al. 2005). The maximum

herbage S concentration was 3.10 mg kg⁻¹ DM, indicating that Mo induced Cu deficiency presumably is not a problem, as indicated by the low herbage Cu:Mo ratio.

According to the dietary trace element needs of ruminants, primary Zn, Co and Se deficiency can be expected because of low herbage concentrations, whereas secondary Cu deficiency can be expected because of high herbage Mo concentration (Fig. 2 (Paper 1); Fig. 3 and Fig. 5 (Paper 2); Fig. 2 (Paper 3)).

Animal blood

Results from the present investigation show that despite the low herbage Cu:Mo ratio and Co concentrations, the blood plasma Cu and Co (B₁₂) concentrations were within the suggested standards for blood plasma Cu and Co concentration. There were no higher animal blood Cu or Co concentrations on farms where trace element enriched supplements were used. This demonstrated that the low Cu:Mo relationship did not induce Cu deficiency and that the herbage Co concentration was sufficient to meet the animal dietary need. If the supplement of S increases in organic farming (total feed S above 4 g kg⁻¹ DM), this might induce Cu deficiency, requiring increased feed Cu concentration. In contrast, the Se feeding practise was generally insufficient to meet the needs of dairy cattle in the East region, but it was found to be sufficient for sheep. The six farms having the lowest animal blood Se concentration in the present study did not use Se enriched feed supplements (Fig. 3 (Paper 3)). The vitamin E fed through the herbage and supplement was sufficient to meet the dietary need of the dairy cattle herds but insufficient for the sheep herds (Fig. 3 (Paper 3)). Hay was the most common feed for sheep, whereas silage was mostly used for dairy cattle. Based on these observations it was concluded that today's feeding practice for sheep and dairy cattle herds on organic farms was sufficient regarding Cu and Co, but Se should be supplemented to all animals and vitamin E should be supplemented to animal diets consisting mainly of hay.

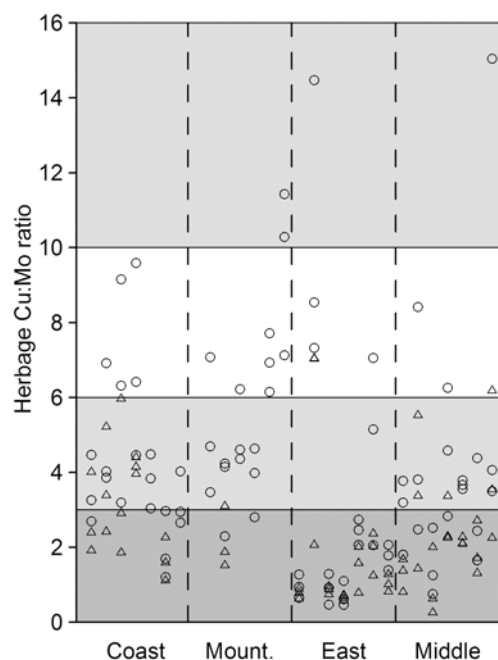


Fig. 5. Herbage Cu:Mo ratio in first (o) and second cut (Δ) from 3 leys on each of 27 farms according to region. Light area indicates recommended ratio, light shaded bands indicate marginal ratio, and dark shaded area indicates risk for secondary Cu deficiency.

Factors affecting trace element concentrations

Soil

Soil physical measurements did not explain much of the variation in the soil extractable trace element concentration, whereas the soil texture did explain some (Table 4 (Paper 1); Table 2 (Paper 2); Results (Paper 3)). Since only extractable soil Zn was found to be correlated with its herbage concentration, the relationship between soil and herbage trace element concentrations did not provide precise information for predicting the herbage trace element concentration. In general, soil pH was the soil factor that explained most of the variation in the total soil extractable trace element concentrations. The soil extractable Fe and Mn

concentrations decreased with increasing soil pH, whereas no effect was observed for other trace elements. Soil pH was also the only soil factor having significant influence on both soil extractable and herbage concentrations in both cuts, as shown for Mn. All the other soil parameters had only significant influence on one, two or none of the trace element concentrations. The lack of correlation between soil extractable and herbage concentrations verified that there was a need for measuring soil physical and textural properties to explain the variation in herbage trace element concentrations in this study.

Plant species

Different plant species can absorb widely different amounts of micronutrients from the same soil (Yläranta & Sillanpää 1984). In the present study the Cu, Zn, Mo and Co concentrations in red clover were higher than in grass herbage. The mixed model analysis showed that the presence of clover increased the herbage Cu, but it decreased the Mn concentration, while forbs increased the herbage Zn and Cu concentrations (Table 4 (Paper 1); Table 2 (Paper 2); Results (Paper 3)). Red clover is reported to have a lower Cu/Mo ratio than grasses, and herbage diets with high red clover content have a potential for inducing secondary Cu deficiency in animals (Bakken 2000). Contrary results were obtained in the present investigation, because the grass herbage Cu:Mo ratio was lower than in red clover. The mixed model analysis also showed that the herbage Cu concentration increased with increasing clover proportion whereas the herbage Mo concentration was unaffected.

Differences in trace element concentrations between herbage species are also to some extent dependent on the soil available micro-mineral concentration (Whitehead 2000). It is therefore still unclear if a change in crop species by a farmer on a certain area would have an influence on the trace element concentration, even though some evidence for this exists. Critical deficiency or toxicity limits established for growth of the herbage consisting of a variety of plant species are also difficult to generate because of their differing demand for trace elements.

As for soil extractable trace elements, soil pH was the most influencing factor explaining the variation in the herbage trace element concentration. Increasing soil pH decreased the herbage Zn and Mn concentration, whereas the herbage Mo concentration increased. The second most influencing soil factor was soil organic matter. Increasing the soil organic matter content increased the herbage Cu and Mo and decreased the Fe concentrations (Table 4 (Paper 1); Table 2 (Paper 2)).

Cation and anion uptake are regulated differently in plants, and direct interactions between cations and anions do not necessarily occur (Marschner 1995). For instance, at low external concentrations the uptake rate of cations is not affected by the accompanying anion, and vice versa. None of the anions or cations concentrations either in soil or herbage in the present investigation were extremely high, while low concentration was more likely the situation. Several investigators have reported a relationship regarding soil mineral adsorption and/or plant uptake between several ions (Kashem & Singh 2002; Smith et al. 1987; Chiy et al. 1999; Hopper & Parker 1999). All these results are reported from controlled fertiliser experiments, and the relationship between the different ions occurs mainly at soil or plant concentrations, which are much higher than were found in the present investigation. This is the reason why the soil or plant concentration of other minerals than the one investigated was not included in the statistical mixed models used.

Determination of Se in biological material

The relationship between peak height and selenium concentrations was found to be linear between 0 and 2 mg L⁻¹ (Fig. 2, (Paper 4)). The linear response area is good enough for most biological material which is often reported to have a Se concentration within this range. The often reported Se concentration in natural soil or plant is 0.01 to 1.7 mg kg⁻¹, liver samples is 0.65 to 1.6 mg kg⁻¹, milk products is 0.06 to 0.09 mg kg⁻¹, and in human blood is 0.045 to 0.256 mg Se L⁻¹ (Gupta & Gupta 2000; Wu & Låg 1988; Singh & Mishra 1987; Gupta & Gupta 2002).

The method provides a good relationship between the peak and area within and above the linear range concentrations (Fig. 6). The curve in Figure 6 was used to determine when the column was saturated and the sample had to be diluted. When the peak and area relationship exceeded the linear response area and the line started to fatten out, the column was saturated.

The mass detection limit of the complete procedure based on the three times standard deviation of a series of blank sample analyses was found to be 0.54 ng of Se. To achieve this low detection limit it was necessary to use high purity trace analysis grade reagents, especially nitric acid. Nitric acid was also found to be the primary contaminant by Hawkes and Kutnink (1996). The procedure was validated by a collection of certified standard reference material, representing plant, meat, milk and marine sediments (Table 1, (Paper 4)). Recoveries were within the certified range for the materials analysed.

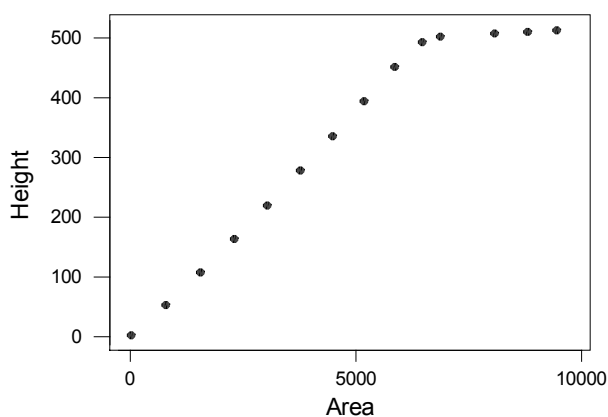


Fig. 6. Relationship between peak height and area in the data output for the proposed method. Sample Se concentrations outside the linear range indicates that the column was saturated and the sample has to be diluted.

An unattended sample digestion with a large sample capacity was devised, having low blanks, a buffered the derivatization reaction to improve reproducibility, and free of matrix interferences and systematic errors. High purity reagents are the key to low detection limits and reproducible blank samples. The method uses streamline chemical procedures without the use of perchloric acid, and should be easy to adopt in routine laboratory Se measurements.

Selenium in spring wheat and leaching water as influenced by selenium and nitrogen application

The Se and N treatments affected the concentration in different plant parts, total plant uptake and Se leaching losses (Fig. 1 A-D (Paper 5)). In the N1 treatment was the grain Se concentration highest ($P < 0.05$) with Se applied at GS 00 but higher in the N2 treatment with Se applied at growth stage (GS) 30, GS 50 and GS 70 stages. The Se concentration in the other plant parts was generally higher in the N1 treatment. The Se leaching losses increased with Se application at GS 30 and GS 50 in both N treatments with minor changes at other growth stages. The Se losses tended to be higher in the N1 treatment with selenate applied at

GS 30 and GS 50, and the loss in the N2 treatment was highest with selenate applied at GS 70.

The main reason for these differences is that ammonium-nitrate affects the soil selenate availability in several ways. Ammonium-nitrate might decrease soil Se adsorption, increase root growth, increase excretion of root exudates and increase grain protein content. The N status of the plant affects the redistribution of S (Eriksen et al. 2001), and since S and Se are incorporated into many of the same plant chemical compounds, N probably also affects the redistribution of plant Se.

The total Se uptake by plants along with leaching losses (total recovery) in the present study was higher in the N1 treatment, even when the total plant Se uptake was higher in the N2 treatment. This suggests that Se leaching losses were higher in the N1 treatment. Within the N treatments, leaching losses of Se varied considerably. Although it is difficult to understand why the highest Se leaching losses occurred in the treatment with the highest plant Se uptake, this may be related to an increased mobilization of Se in soils by increased root activity and microbial growth. The fact that leaching of Se is higher when the grain Se concentration is higher is an observation in need of further investigation. It is also interesting that applying 25 % of the ammonium-nitrate at GS 30 reduces the leaching of Se, thus reducing the environmental impact of the Se applied.

Recommendations

Agricultural practise

Based on the findings of this farm study, it is recommended that the supplementation of Cu, Co, Se and vitamin E on all organic farms and Zn on organic dairy farms should be made until alternative means to fulfil the dietary requirements of these constituents are found. Increasing the proportion of clover enhances the herbage trace element concentrations and increases the Cu:Mo ratio to better suit the recommended feed Cu:Mo ratio. The advantage of increasing the clover content of the herbage can be site specific, and hence it must be judged more carefully. Since the soil pH values at most farms are in the suggested normal range for plant growth, excessive liming should be avoided on these farms.

If the goal is to increase the grain Se concentration in both conventional and organic agriculture, selenate as a Se fertiliser should be applied in the period from tillering to heading. In conventional agriculture, selenate should be applied together with ammonium-nitrate to best utilise the applied selenate. A split application of ammonium-nitrate would increase the grain protein content and reduce the leaching losses of Se.

Soil analysis versus plant analysis

The attempt to use soil analysis to imitate the plant's ability to take up essential plant micro-minerals from the soil did not succeed, except for Zn, in the present farm investigation. The same applies to herbage uptake of Co and Se, but the number of samples is too small to make clear conclusions on these two trace elements. There was also a clear relationship between soil and clover Co concentrations. Since poor correlation between the herbage trace element concentration and soil analysis was found, it would be better to replace soil analysis with herbage analysis when the objective is to evaluate the herbage trace element concentration in relation to the animal's dietary needs. Soil analysis might also be replaced by plant analysis in

pastures to assess the trace element status of the soil, if such relationship exists. Plant analyses in the strict sense reflect the actual nutritional status of the plant. The main disadvantage of performing herbage analyses for estimating the soil trace element status is that plants have to be sampled at the same stage of maturity, and for herbage, the botanical composition should be known.

The development of wet digestion procedures of plant samples together with multiple analyses on an ICP-MS or ICP-EAS makes it more cost efficient to perform plant analysis. This is because one sample can be used to determine a number of mineral elements, instead of using soil analysis, which requires different soil extracting procedures for different elements. It is therefore my opinion that herbage trace element analysis should be preferred, especially when it is only possible to analyse only the soil or the herbage.

Suggestions for further research

There are no restrictions in the amount of trace element supplements to ruminants that can be used in organic farming, and thus no trace element deficiency problems should occur in organic livestock farming. The main focus of further investigations should therefore be to find organic sources of trace elements, either as feed supplements or soil fertilisers, which better suits the philosophy of organic farming than the use of inorganic feed supplement sources.

There are many methods for total Se determination in biological samples. Total Se determinations are often good enough for practical use and enable correction of the Se feed supplementation practise to animals. For use in research, easily applicable methods for determining different Se species in biological material should be developed. This would make it possible to increase the knowledge of Se cycling within most biological systems.

The relationship between N and Se is not fully understood and hence increased knowledge on this relationship would assist us in understanding the cycling of Se in biological systems.

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**Factors affecting the concentration of Zn, Fe and Mn in herbage
from organic farms and in relation to dietary requirements of
ruminants**

Espen Govasmark^{1,2,*}, Arvid Steen³, Anne Kjersti Bakken⁴, Turid Strøm¹ and Sissel Hansen¹

Addresses of the Authors

¹Norwegian Centre for Ecological Agriculture, NO-6630 Tingvoll, Norway,

²Agricultural University of Norway, Department of Plant and Environmental Sciences, P.O. Box 5028, NO-1432 Ås, Norway,

³Norwegian School of Veterinary Science, P.O. Box 8146 Dep, NO-0033 Oslo, Norway, and

⁴Norwegian Crop Research Institute, NO-7500 Stjørdal, Norway

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In press

Factors affecting the concentration of Zn, Fe and Mn in herbage from organic farms and in relation to dietary requirements of ruminants

Abstract

To obtain a general picture of the herbage zinc, iron and manganese concentrations and their relation to dietary requirements of ruminants on organic farms, we analysed soil and herbage samples from four regions in Norway. The soil median Zn, Fe and Mn concentrations were 0.18, 13 and 0.84 mg/L, respectively. The herbage median (10th-90th percentile) Zn, Fe and Mn concentrations (mg/kg) in herbage in the first cut were 19 (14-34), 50 (36-88), 34 (22-86) and in the second cut 21 (16-37), 84 (52-171) and 66 (36-205), respectively. The results of mixed model analysis of herbage Zn, Fe and Mn indicate that soil pH, soil texture, soil mineral concentration and botanical composition are the most influencing factors. We conclude that Zn, Fe and Mn did not limit plant growth, and that the herbage concentrations except for Zn, were sufficient to meet the dietary needs of ruminants on organic dairy farms.

Key words: animal nutrition, dairy cattle, feed, micronutrient, roughage, sheep, soil, trace element

Introduction

Agricultural soils vary widely in their total micronutrient content and in their ability to supply micronutrients in quantities sufficient for optimal crop growth (White & Zasoski, 1999). Nutrient deficiency in plants related to a low total content of zinc (Zn), iron (Fe) and manganese (Mn) in agricultural soils are rare and total soil levels are hardly indicative of plant availability. This is because availability depends on soil pH, organic matter content, adsorptive surfaces, and other physical, chemical, and biological conditions in the rhizosphere. Forage plants vary widely in their concentration of Zn, Fe and Mn as found in surveys of timothy (*Phleum pratense*) in Finland (Kahari & Nissinen, 1978), domesticated grass species grown in coastal regions of Norway (Synnes & Øpstad, 1995) and native grasses and forbs in mountain areas in Norway (Garmo et al., 1986). The variation in plant nutrient concentration has been found to be less within and between cultivated pastures than within and between native ones (Frøslie & Norheim, 1983).

A transition from conventional to organic farming practice changes the supply and availability of soil Zn, Fe and Mn and thereby the herbage concentration. Mineral fertilizers are generally not used in organic farming and livestock manure is the major source of nutrients. A study of Zn and Mn supply in conventional livestock farming in Canada (Atkinson et al., 1954) estimated that the amount supplied from manure, in general, was considerably lower than the supply from mineral fertilizers and was not sufficient to prevent deficiency of Zn and Mn in plants. Nesheim (1986) reported from a survey on 138 conventional grasslands in Nordland (North of Norway) on soils having a pH mainly in the range 5.1 to 6.0, that the herbage mineral content varied with survey year, soil type, soil nutrient content, botanical composition and stage of development. No similar survey has been conducted on organic farms in Norway. In general, changes reported after transition from conventional to organic farming are a higher soil pH, alteration in the mineral concentrations in soils and plants and changes in the botanical composition of leys (Clark et al., 1998; Pettersson et al., 1998; Gruber et al., 2001; Gustafson et al., 2003).

An adequate supply of Zn, Fe and Mn is important for the performance, health and welfare of ruminants (Underwood & Suttle, 1999). Mild mineral deficiencies are especially difficult to identify, because their effects are rarely distinguishable from those resulting from underfeeding or intestinal parasitism. An initial assessment of the actual or likely occurrence of a dietary mineral inadequacy can be made by comparing the mineral composition of the diet with appropriate standards of adequacy (Underwood & Suttle, 1999).

All feedstuffs in organic farming are to be based on certified organic sources from 2005 (Council of European Union, 1999). Organic farming has the ambition to produce high energy fodders with proper mineral concentrations utilising local resources (Debio, 2003). Since the feed in organic farming is mainly based on roughage and there is limited use of concentrates fortified with micronutrients, animals on organic farms are more dependent on nutrients in the herbage than those on conventional farms. Knowledge about herbal concentrations of Zn, Fe and Mn on organic farms can help to reveal possible imbalance, deficiency or toxicity to plants or animals.

The present study focuses on the concentrations of Zn, Fe and Mn in soil and herbage crops on organic farms in Norway. The objectives of our study were to: (I) obtain a general picture of the concentrations of Zn, Fe and Mn in soils and herbage on organic farms in different regions of Norway; (II) relate the variation of Zn, Fe and Mn in herbage to their concentrations in soils and to other plant and soil parameters; (III) and to assess if Zn, Fe and Mn concentrations in herbage meet the dietary requirements of ruminants.

Materials and methods

Sampling sites and site descriptions

Out of the total population of 526 certified (Debio, 2003) organic sheep and dairy farms in Norway, we selected two regions named Coast (1) and Mountain (2) with a high density of sheep farms (1–14) and two regions named East (3) and Middle (4) with a high density of dairy farms (15–28) (Fig. 1). The regions were selected to represent different climatic conditions (Norwegian Meteorological Institute, 2004). The number of years the farm had been approved by DEBIO before 2001 (started in 1992), the estimated chlorine depositions ($\text{mg}/\text{m}^2 \text{ yr}$) in each region which decreases with the distance from the ocean (Steinnes pers. comm.), the mean temperature sum and the mean total precipitation in the period May to August 1961 to 1990, and the soil classification of the fields are presented in Table 1. Seven farms within each region were selected randomly from farms that had maintained organic plant production for more than three years and organic animal production for at least one year. One farm in the Mountain region was later excluded from the investigation because of irregularities in data acquisition. Within each farm, three leys that contributed a large share of the total roughage used as animal fodder were selected and sampled during the growing seasons of 2001 and 2002. Only one farm in the Mountain region had two cuts each year.

Soil pH and soil physical characteristics are presented in Table 2. The herbage yield and composition, phenological stage of timothy at harvest, and the dominating plant species are presented by region in Table 3.

Soil and herbage sampling

In each of the 81 leys, soil and herbage samples were taken from three permanently marked subplots (6.0 m x 1.2 m). Subplots were parallel to each other with a maximum distance of 1 m between each of them.

In situ soil characterization and soil sampling were done as soon as possible after the first harvest of the leys in 2001. Three 100- cm^3 soil cores at a depth of 10 to 14 cm were taken just outside the subplot and kept refrigerated (4 °C) until further analyses of water holding capacity and soil porosity. About 500 ml of soil from the 0 to 20 cm layer at 10 locations within the three subplots were sampled. After drying at 35 °C and sieving through a 2 mm mesh, 250 ml soil from each subplot was pooled together to make a composite sample for further chemical and physical analyses.

The total herbage yield above a stubble height of 10 cm was recorded on all subplots at harvest time in 2001 and 2002. On most of the farms, there were two cuts per growing

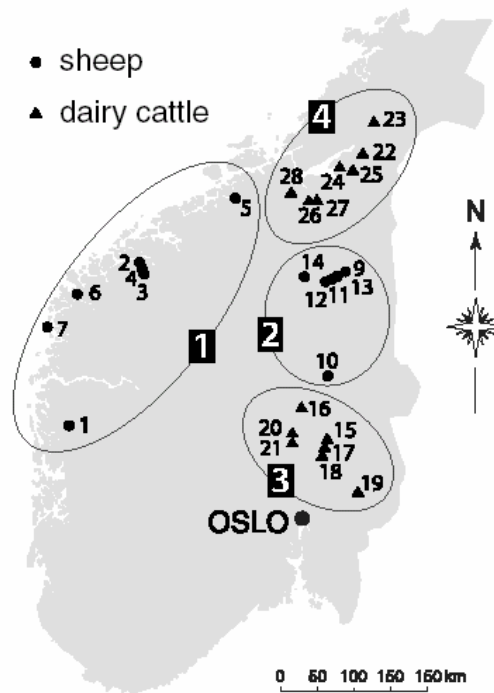


Fig. 1. Map of southern Norway indicating the locations of 27 farms in the Coast (1), Mountain (2), East (3), and Middle (4) regions.

season but some farms had only one. Three samples were taken from each subplot. The first sample of 700 g fresh weight (dried for 1.5 h at 60 °C, kept frozen at -20 °C) was collected for determination of botanical composition. The second sample of 1000 g (dried at 60 °C) for the analyses of elemental composition (stored 20 °C) for chemical element analyses (ground to a particle size of 1 mm). The third sample of red clover (*Trifolium pratense*), if present, was collected from the plots in 2001 and was processed the same way as the 1000 g sample described above.

Table 1. Location, years approved as organic before yr 2001, climate and soil group for the 27 farms in the survey.

Location			Years ¹	Cl-deposition ²	Temp-sum ³	Precipitation ⁴	Soil group
Region	Farm	Municipality	approved	(mg Cl/m ² yr)	(°C)	(mm)	(FAO-Unesco)
			4	12.4			Podzol, Regosol
1 – Coast	1	Lindås			1541	547	
	2-3-4	Sykkylven	3-9	7.5	1464	405	Regosol, Andosol, Fluvisol
	5	Halsa	6	11.9	1476	358	Regosol
	6	Eid	9	9.1	1507	395	Leptosol
	7	Flora	3	11.1	1455	475	Histosol
2 –	9	Os	9	0.35	1174	236	Cambisol
Mountain	10	Stor-Elvdal	9	0.15	1487	341	Cambisol, Podzol
	11-12-13	Tolga	4-6	0.15	1208	221	Cambisol, Podzol, Regosol
			9	0.15			
	14	Tynset			1165	212	Cambisol, Podzol
	15	Løten	9	0.51	1601	263	Podzol
3 – East	16	Ringsaker	7	0.26	1645	275	Podzol
	17-18	Stange	9	0.87	1601	239	Cambisol
	19	Sør-Odal	9	0.45	1647	255	Cambisol
	20	Gjøvik	4	0.40	1626	277	Podzol
	21	Vestre Toten	6	0.47	1595	282	Cambisol, Podzol
4 – Middle	22	Levanger	4	4.2	1485	314	Podzol, Regosol
	23	Steinkjer	8	5.0	1528	286	Cambisol
	24-25	Stjørdal	4-8	3.5	1491	303 – 368	Cambisol
	26-27	Melhus	4-7	2.2	1497	253 – 300	Cambisol, Podzol
	28	Skaun	3	3.8	1463	245	Cambisol, Podzol

¹Years approved = years approved as organic by DEBIO before 2001; ²Cl-deposition = estimated yearly chlorine deposition; ³Temp-sum = mean temperature-sum (>5 °C); ⁴Precipitation = mean precipitation May-August in the period 1961 to 1990.

Table 2. Median (10th and 90th percentile) of soil chemical and physical characteristics from 27 farms within the Coast, Mountain, East and Middle regions

	pH	¹ OM %	Clay %	² pF 2	³ Air vol.
1-Coast	6.0 (5.4-6.5)	15.1 (8.8-54.7)	4.4 (2.2-7.9)	47 (30-62)	21 (12-32)
2-Mountain	6.4 (5.9-6.8)	4.4 (2.1-7.4)	3.5 (0.4-11.1)	40 (26-44)	18 (13-29)
3-East	6.3 (5.7-6.6)	5.5 (4.1-6.7)	8.9 (4.2-17.7)	32 (25-40)	28 (11-35)
4-Middle	6.3 (6.0-6.7)	4.7 (2.3-8.2)	12.6 (3.0-26.3)	38 (31-44)	17 (12-24)

¹OM = organic matter % of DM; ²pF 2 = g water/100 ml soil at 0.1 bar pressure; ³Air vol. = air volume (ml/100ml).

Table 3. Median, 10th (10 %) and 90th (90 %) percentiles of herbage characteristics of first and second harvest from one or more subfields on 27 farms in the Coast, Mountain, East and Middle regions.

Sheep farms												Dairy farms																									
Herbage yield kg / ha DM (60°C)																																					
												East 1 st cut				East 2 nd cut				Middle 1 st cut				Middle 2 nd cut													
												Coast 1 st cut		Coast 2 nd cut		Mount. 1 st cut		Mount 2 nd cut		East 1 st cut		East 2 nd cut		Middle 1 st cut		Middle 2 nd cut											
10%												1635		1144		3548		nd		2640		1666		3080		2045											
Median												3195		1570		5223		nd		3380		2153		3700		2560											
90%												4660		1926		6370		nd		3960		2728		5315		3040											
Phenological stage of development at harvest (<i>Phleum pratense</i>) *																																					
10%												3.4		nd		3.3		nd		3.1		nd		3.1		nd											
Median												3.5		nd		3.5		nd		3.3		nd		3.3		nd											
90%												3.6		nd		3.7		nd		3.5		nd		3.4		nd											
Botanical composition (%)																																					
												Grass				Clover				Forbs				Grass				Clover				Forbs					
Region												Coast		Mount.		Coast		Mount.		Coast		Mount.		East		Middle		East		Middle		East		Middle			
Cut												1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd	1 st	2 nd
10 %												42	35	61	nd	1	2	3	nd	8	9	1	nd	56	41	60	38	10	25	5	16	1	1	1	1		
Median												57	46	80	nd	5	15	9	nd	24	26	4	nd	74	55	78	61	19	33	18	29	4	3	3	3		
90 %												80	58	90	nd	25	39	36	nd	45	47	15	nd	81	63	92	74	32	48	26	51	9	10	12	10		
Plant species																																					
Phleum pratense												Trifolium pratense				Taraxacum				Phleum pratense				Trifolium pratense				Taraxacum									
Festuca pratensis												Trifolium repens				Ranunculus acris				Festuca pratensis				Trifolium repens				Ranunculus acris									
Lolium perenne												Trifolium hybridum				Ranunculus repens				Lolium perenne				Trifolium hybridum				Ranunculus repens									
Poa pratensis																Alchemilla				Poa pratensis								Rumex									
Dactylis glomerata																Achillea millefolium				Festuca pratensis																	
Alopecurus pratensis																Leontodon				Dactylis glomerata																	
Agrostis																				Elymus repens																	
Phalaris																																					
Elymus repens																																					

* 3.0; boot stage, 3.3; spikelets fully elongated, 3.5; peduncle fully elongated, 3.7; anther emergence, nd; not determined (Moore et al., 1991)

Soil and herbage analyses

All soil profiles were classified according to FAO (1994). The water retention characteristics of the soil cores were determined at pF 2 (g water/100 ml soil) by the pressure plate method (Page et al., 1982) and the pore volume (ml/100 ml) according to the method of Page et al. (1982).

Zinc, Mn and Fe in the complete composite samples of the soil were analysed by inductively coupled plasma atomic emission spectrometry (ICP-AES) after extraction with diethylenetriaminepentaacetic acid (DTPA) (Lindsay & Norvell, 1978). The pH was measured in a 1:5 soil:water suspension and organic carbon by combustion in a LECO CHN-1000 apparatus (Page et al., 1982). An estimate of the content of organic matter was obtained by multiplying the content of organic carbon by 1.72 (Riley, 1996).

The particle size fraction of the composite sample was analysed by the pipet method procedure (Page et al., 1982). Pretreatment of soils for particle size analyses was done by removing the organic material with H₂O₂, dispersing the soil sample with 0.05 M Na₄P₂O₇, and washing with HCl and water. The soil was dried at 105 °C for 24 h for dry weight determination.

At the time of the first cut of leys in both years, the phenological stage of development of timothy was determined according to the procedure of Moore et al. (1991). Official methods of analyses of the Association of Analytical Chemists (Pedersen & Lysnes, 2002) were used for the determination of total herbage Zn, Fe and Mn. Herbage (5.0 g) and red clover (0.5 g) samples were dry ashed at 500 °C. The ashed sample of herbage was digested in 10 ml aqua-regia. The digested solutions were dissolved and evaporated twice in 5 ml concentrated HCl before being dissolved in 5 ml HCl. The sample was filtered and diluted to 100 ml with H₂O. Zn, Fe and Mn were determined in the digested solution by ICP-AES. The ashed sample of red clover was digested in 40 % aqua-regia, evaporated and then diluted to 25 ml by H₂O. Zinc in the digested solution was determined by atomic absorption spectrophotometry.

Statistical methods

The relationship between concentrations of Zn, Fe and Mn in plants and other soil and plant variables was established using mixed model analysis with the restricted maximum likelihood method for random effects in JMP release 5.0.1a, (SAS Institute Inc., Cary, NC, USA). The dependent variables were normalized by transformation: logarithms (soil Zn, soil Fe and soil Mn), reciprocals (plant Zn, plant Fe) and reciprocal logarithms (plant Mn). Differences in plant mineral concentrations between first and second cut were tested by a paired t-test.

Total yields, botanical composition and herbage Zn, Fe and Mn concentrations in first and second cuts were pooled prior to analysis because initial comparisons revealed no significant differences between years. The best regression models were selected from a maximum model by backward elimination of nonsignificant variables at 5 % level. The normality assumption was assessed by means of a plot of residuals against predicted values. The fixed independent continuous variables included in the maximal model were % clover, % forbs, yield, phenological stage of development of timothy, soil pH, % clay, % sand, % organic matter, soil micronutrient concentration, pF 2, soil air volume, and year since ploughing (field age). Region, and farm nested within region, was specified as class variables, and farm nested within region as random effects. Percentage contribution to the models of the remaining variables was given by their sequential (Type I) sum of squares as a proportion of the total sum of squares. Differences in Zn, Fe and Mn concentrations between regions were analyzed by ANOVA and multiple comparisons were tested with Tukey HSD test. Original data is

presented with medians and percentiles (ordered values divided into hundredths) because of the lack of normality.

Results

Zinc in herbage and soil

The herbage Zn concentration was lowest in the Middle region on both cuts (Fig. 2). Herbage Zn concentration in each region was higher in the second cut for each year. The herbage Zn concentration was generally higher than the suggested critical growth limiting Zn level of 20 mg Zn/kg dry matter (DM) in leaves (Marschner, 1995) and 20 mg Zn/kg DM for the dietary requirements for ruminants (Underwood & Suttle, 1999) in the Coast and Mountain regions. Half of the herbage samples in the East and all samples in the Middle region had lower Zn concentration than suggested levels. The herbage Zn concentration was approximately 40 % of the red clover Zn concentration (data not shown), and the red clover Zn concentration was higher than the suggested dietary needs. The most significant influencing parameters explaining the herbage Zn concentration in all regions were soil pH, forbs and extractable Zn in soil (Table 4).

The soil Zn concentration was generally lower than the suggested critical level of 0.6 mg Zn/kg DM for plant growth (Lindsay & Norvell, 1978) and among regions, the lowest soil Zn concentration was found in the Middle region (Fig. 2). The extractable soil Zn was positively correlated to soil organic matter, sand and clay content (Table 4).

Iron in herbage and soil

The herbage Fe concentration showed only small differences in the second cut between regions, and was higher in the second cut than in the first cut (Fig. 2). More than 50 % of the herbage samples had higher herbage Fe concentration than the suggested deficiency level of 50 mg Fe/kg DM in leaves (Marschner, 1995) and the suggested level of 50 mg Fe/kg DM for the dietary needs of sheep and dairy cattle (Underwood & Suttle, 1999). The herbage Fe concentration on all farms was explained by the soil sand and organic matter (OM) content (Table 4).

The soil extractable Fe concentration was higher than the suggested critical level of 4.5 mg Fe/kg DM for plant growth (Lindsay & Norvell, 1978) and no differences were found among regions (Fig. 2). The soil extractable Fe concentration was negatively correlated to soil pH and positively to pF 2 (Table 4).

Manganese in herbage and soil

The herbage Mn concentration was lower in the Middle region than on the Coast for both cuts (Fig. 2). The Mn concentration was significantly higher in the second cut in all regions than in the first cut. The herbage Mn concentration was generally higher than the recommended critical deficiency level in leaves of 20 mg Mn/kg DM for normal plant growth (Marschner, 1995) and 20 mg Mn/kg DM for the dietary requirements for sheep and dairy cattle (Underwood & Suttle, 1999). The herbage Mn concentration was mainly explained by the negative correlation of soil pH (Table 4).

The lowest soil Mn concentration was found in the Coast region, where most of the soil samples had soil Mn concentration lower than the suggested critical level of 1.0 mg Mn/kg DM for plant growth (Lindsay & Norvell, 1978). The corresponding proportion of deficient samples was 50 % in the other regions (Fig. 2). The soil Mn concentration on all farms was positively correlated to soil clay content and negatively to soil pH (Table 4).

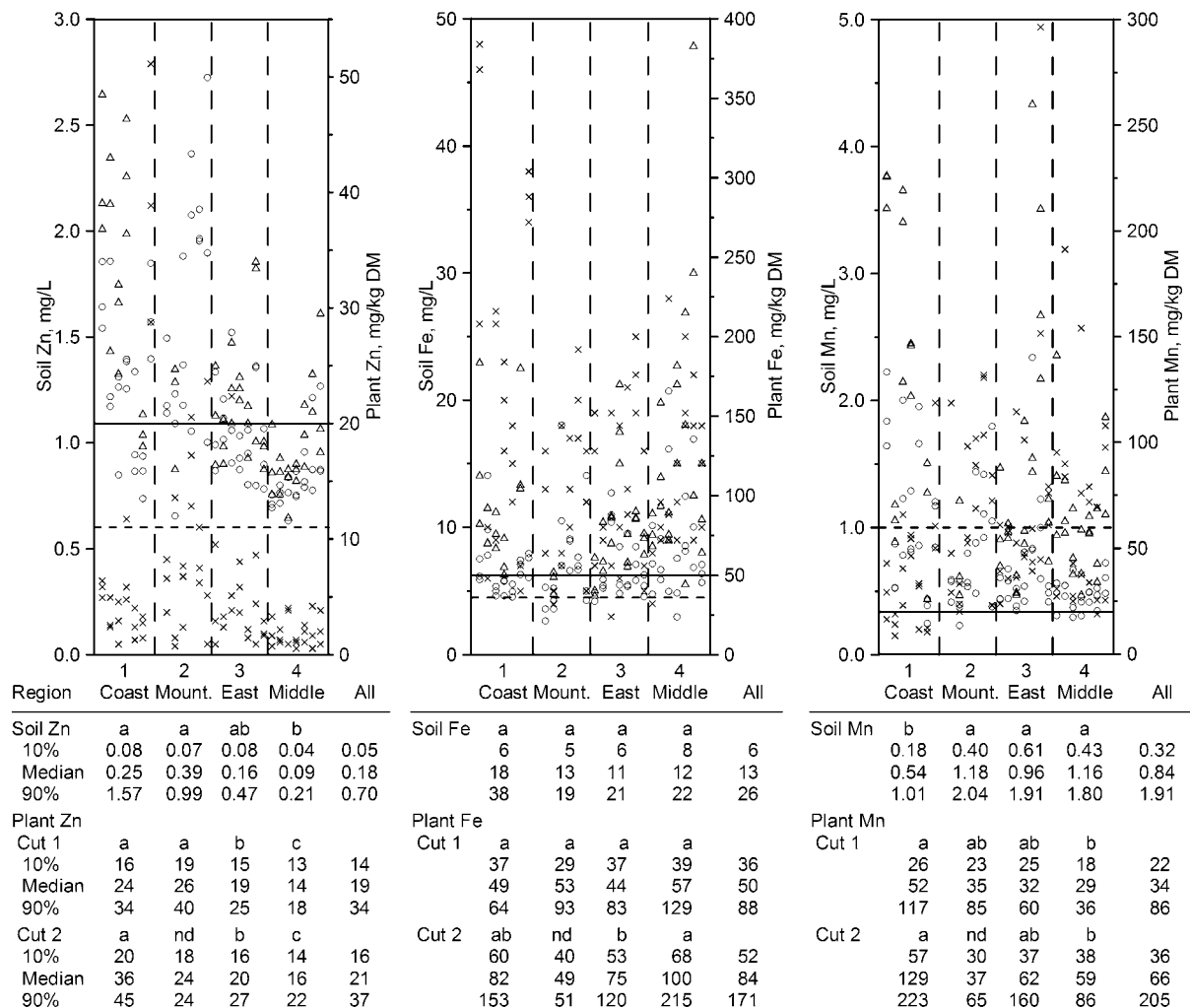


Fig. 2. Soil (x) Zn, Fe and Mn, mg/L, and first (o) and second (Δ) cut of plant Zn, Fe and Mn, mg/kg DM, from 3 leys on each of the 27 farms within the Coast, Mountain, East and Middle regions. Horizontal lines indicate recommended critical levels in soils (----, left axis) and plants (—, right axis) for plant growth, and for the dietary requirement for ruminants (—, right axis), overlapping plant critical concentration. Original data are presented with medians and 10th (10 %) and 90th (90 %) percentiles. Differences in transformed Zn, Fe and Mn concentrations between regions were analyzed by ANOVA and multiple comparisons were tested with Tukey HSD test, regions not connected with same letters (a, b, c) are different ($P < 0.05$). nd = not determined because of few samples.

Table 4. Estimated positive or negative regression coefficients (β) and R^2 from mixed model analysis of soil and herbage Zn, Fe and Mn concentrations¹. Percentage contribution (Cont) to the model of the variables is given by their sequential (Type I) sum of squares as a proportion of the total sum of squares.

Parameter	Zinc						Iron						Manganese					
	Soil		Herbage				Soil		Herbage				Soil		Herbage			
	β	Cont	β	Cont	β	Cont	β	Cont	β	Cont	β	Cont	β	Cont	β	Cont	β	Cont
pH			-***	8.6	-***	9.2	-***	20.0					-***	10.3	-***	19.7	-***	39.5
OM	****	13.1								-*	3.2							
Clay	+	0.5											+	2.4	+	0.1		
Sand	**	5.8	+	0.9					-*	4.0					****	3.2		
pF 2					-*	1.6	***	2.2										
Soil air vol															-*	2.0		
Field age																	+	3.7
Clover	nd	nd					nd	nd					nd	nd	**	2.8		
Forbs	nd	nd	**	2.2	+	1.9	nd	nd					nd	nd				
Yield	nd	nd					nd	nd					nd	nd			-*	4.2
Soil conc.	nd	nd	****	7.4	***	3.8	nd	nd					nd	nd				
R^2		0.50		0.77		0.85		0.72		0.50		0.65		0.59		0.77		0.72

¹The variables tested in the model were soil pH, organic matter % (OM), clay %, sand %, pF 2 (g water/100 ml soil), soil air volume (ml/100ml), field age, clover %, forbs %, yield (kg DM/ha), development of timothy at harvest, and for the first and second cut, soil concentration of the mineral (mg/L). *, **, **** Significant at 0.05, 0.01 and 0.001 levels of probability, respectively. nd = not determined in the model

Discussion

Zinc

The median herbage Zn concentration in our investigation was lower than the median concentration found in grasses from the mountain area in southern Norway (Garmo et al., 1986) and timothy in Finland (Kahari & Nissinen, 1978). The herbage Zn concentration in the Coast region in our study compared fairly well to that reported by Synnes and Øpstad (1995) for the coastal region in Norway. The herbage Zn concentration on the Coast was lower for the first cut than the mean concentration found in timothy from cultivated pastures by Frøslie and Norheim (1983) at the coastal area of Norway. The herbage Zn concentration in the Mountain, East and Middle regions was lower for both cuts compared to Frøslie and Norheim (1983).

In our investigation, the red clover Zn concentration was higher than in the total herbage fraction containing more grass species than forbs and clovers. This is in accordance with Metson et al. (1979) and Whitehead (2000). The higher Zn concentration in the second cut is probably due to a higher proportion of clover in the herbage and an earlier development stage at harvest of the herbage. This is in accordance with Whitehead and Jones (1969) who found a rapid decline in the Zn concentration in clover during the first month of growth, whereas Metson et al. (1979) only found a small change in grass and clover Zn concentration with increasing development stage at harvest. A decline in the herbage Zn concentration during senescence is reported to be more likely when the supply of Zn is adequate than when it is deficient (Longnecker & Robson, 1993). The soil Zn concentration adequate for plant growth was reviewed by Brennan et al. (1993). They found the critical concentration of DTPA extractable Zn to be 0.1 to 1.0 mg/kg DM soil. The critical value for plant growth also depends on the soil type (Brennan & Gartrell, 1990). They reported a critical DTPA concentration for clover to be 0.13 mg Zn/kg for sand and 0.55 mg Zn/kg in clay soils. It was not possible to evaluate the soil Zn concentration in relation to deficiency for plant growth in our investigation. However, the East and Middle regions had the highest soil clay content and the lowest herbage Zn concentration, thus showing a possible relationship to soil type. There was also a good relation between our soil Zn concentrations and the total Zn concentration found in overbank sediments (alluvial soils) (Ottesen et al., 2000). The soil extractable Zn concentration showed positive, and soil pH negative influence on plant Zn concentration, as was reported by Lindsay and Norvell (1978). The generally higher soil pH reported on organic farms in relation to conventional farms (Clark et al., 1998) has a negative effect on the herbage Zn concentration, and might explain the lower herbage Zn concentration found in our investigation compared to other studies.

The pH of the extracting solution used for soil Zn measurements does not have any influence on the amount of extractable Zn in the method used (Lindsay & Norvell, 1978). Zinc tends to form inner-sphere bounds with organic matter, but Zn often has a hydration shell in water and remains loosely bound (McBride, 1994). Zinc bound in organic matter is not necessarily plant available even if it is extractable with DTPA. This could explain the positive influence we found of soil organic matter on soil Zn but not on herbage Zn concentration (Table 4).

Plant species differ in their sensitivity to Zn deficiency (Brennan et al., 1993; Marschner, 1995; Lombnaes & Singh, 2003b). Genc et al. (2002) suggested that the critical deficiency concentration of barley plants (*Hordeum vulgare*) is 20 mg Zn/kg DM but information on the critical concentration in grasses is limited. Grain yields are depressed to a relatively greater extent by Zn deficiency than the total plant dry matter production, and reported critical Zn concentrations in clovers for dry matter production are 12 to 14 mg Zn/kg DM (Brennan et al., 1993). All herbage samples in our investigation had Zn concentrations higher than 12 mg Zn/kg DM, and Zn was not a limiting micronutrient for plant growth.

Herbage Zn concentrations below 10 mg Zn/kg DM indicate that there may be positive benefits from Zn supplementation to livestock, and concentrations between 10 and 20 mg Zn/kg DM (marginal band) indicate the possibility of future benefits if the Zn status does not improve (Underwood & Suttle,

1999). Half of the herbage Zn samples (Fig. 2) from the East and almost all samples from the Middle had concentrations below 20 mg Zn/kg DM, thus indicating benefits from Zn supplementation in organic dairy farming.

Iron

The median herbage Fe concentration was lower in our investigation than found in grasses in mountain areas of central southern Norway (Garmo et al., 1986) and in orchardgrass (*Dactylis glomerata*) hay in Scotland (Reid et al., 1967) but the same Fe concentration as in our investigation was found in timothy in Finland (Kahari & Nissinen, 1978).

The soil Fe concentration in our investigation was pH dependent, which is in agreement with Mengel and Kirkby (1987). The herbage Fe concentration was therefore expected to be pH dependent, but this was not found. Our result is in agreement with Fystro and Bakken (2003) who did not find any effect of liming on plant Fe concentration.

The higher herbage Fe concentration in the second cut may be due to a higher proportion of clover in the herbage. The clover Fe concentration in our investigation was not analysed, but comparisons of grasses and legumes have generally shown higher variation and concentration in forbs than in grasses (Garmo et al., 1986), whereas no appreciable difference was found between grass and clover from a mixed sward in New Zealand (Wheeler, 1998). The higher herbage Fe concentration in the second cut may also be due to an earlier stage of development at harvest. Whitehead and Jones (1969) found that the Fe concentration in clover decreased in the first month of growth, and thereafter had fluctuating concentrations with advanced maturity. Metson et al. (1979) found no seasonal variation in pastures, but they suggested that the increased Fe concentration with increased maturity was due to soil contamination in the growth period. Samples in our investigation were not washed before analysis and the main variation in the herbage Fe concentration might be due to contaminations. This probably explains why the herbage Fe concentration only was mainly correlated with the farm location.

Some of our herbage samples had Fe concentrations within a marginal band of 30 to 50 mg Fe/kg DM, which indicate the possibility of positive response to Fe feed supplements (Underwood & Suttle 1999). Iron supplementation in ruminants feeding on organic farms can not be recommended based on our results.

Manganese

The median herbage Mn concentration found in grasses and forbs in our study was lower than that found in the mountain area of southern Norway (Garmo et al., 1986), in timothy in Finland (Kahari & Nissinen, 1978), and in orchardgrass hay and pasture herbage in Scotland (Reid et al., 1967). The herbage Mn concentration in the first cut on the Coast was at the same level as found by Synnes and Øpstad (1995) in grasses harvested within one week after heading of timothy in the same area as our Coast.

In our investigation, soil pH was the overall most important factor for the herbage Mn concentration, and a further increase in the soil pH on the farms in our investigation would lower the herbage Mn concentration even further. The soil pH on farms in the survey could explain the lower herbage Mn concentration we found compared to the other investigations mentioned above. Manganese has its lowest soil solubility at pH above 7.0 (Marschner, 1995), and raising the soil pH above 7.0 on farms in our investigation promotes Mn deficiency for plant growth and thus necessitates Mn supplementation in ruminant feed.

The herbage Mn concentration was higher in the second cut in all regions and can probably be explained by harvest at an earlier stage of development. Manganese concentrations in grass and clover increase during the first 4 to 10 weeks of growth, before the concentration declines (Reid et al., 1967; Metson et al., 1979). The concentration of Mn in red clover was not investigated, but the Mn

concentration in grasses is often found to be higher than in forbs (Garmo et al., 1986) and red clover (Metson et al., 1979). In our investigation, the herbage Mn concentration was negatively influenced by the proportion of clover in the first cut. We found that increased yield in the second cut decreased the herbage Mn concentration, and this could possibly be a dilution effect because of higher yield.

In our investigation, the soil Mn concentration was only lower than the recommended critical deficiency level on the Coast, but the herbage Mn concentration was the same as in the other regions. The suggested critical soil deficiency level for plant growth was by Sillanpää (1982) increased from 1 to not less than 2-3 mg Mn/L soil. This suggestion was uncertain, because the method had been used in a relatively short time. Because of the low soil Mn and high herbage Mn concentrations, the increased suggested level can not be supported by the data in our investigation. The available soil Mn was reviewed by Reisenauer (1988), who summarized that most scientists have had little success in relating soil analyses to plant uptake of Mn in either greenhouse or field experiments. Reisenauer (1988) also reported that sample preparation and soil condition is crucial for the soil extractable Mn, but that results obtained contradicted each other. The effect of sample preparation was investigated by Warden (1991), who suggested that soil Mn analyses should be done immediately in moist rather than in air-dried samples to not underestimate the plant available soil Mn. Our samples were stored as air-dried soil, and soil Mn concentrations were thus probably underestimated. This is supported by the high levels of Mn in our herbage. High concentration of Mn in solution suppresses the uptake of Fe and high concentration of phosphorus enhances the uptake of Mn in sorghum (*Sorghum vulgare*) (Kuo & Mikkelsen, 1981). These relationships were of no relevance in our investigation due to high concentration of both Mn and Fe in the herbage, and a study in barley and oat (*Avena stiva*) in hydroponics reported that observed antagonistic relationships between Mn, Fe, Zn and Cu only are valid when plant growth is limited by Mn (Lombnaes & Singh, 2003a).

Almost all herbage samples in our investigation had Mn concentrations above the marginal band of 10 to 20 mg Mn/kg DM (Underwood & Suttle 1999). Manganese supplementation in ruminants feeding on organic farms can not be recommended based on our results.

Conclusions

Half of the herbage Zn samples from the East and almost all samples from the Middle regions had concentrations below 20 mg Zn/kg DM, thus indicating benefits from Zn supplementation in organic dairy farming. Iron and Mn supplementation in ruminant feeding on organic farms is not necessary based on our results.

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**Copper, molybdenum and cobalt in herbage and ruminants from
organic farms in Norway**

**Espen Govasmark^{1,2}, Arvid Steen³, Anne Kjersti Bakken⁴, Turid Strøm¹, Sissel Hansen¹
and Aksel Bernhoff⁵**

Addresses of the authors

¹Norwegian Centre for Ecological Agriculture, NO-6630 Tingvoll, Norway

²Norwegian University of Life Sciences, Department of Plant and Environmental Sciences,
P.O. Box 5028, NO-1432 Ås, Norway

³Norwegian School of Veterinary Science, P.O. Box 8146 Dep., NO-0033 Oslo, Norway

⁴The Norwegian Crop Research Institute, NO-7500 Stjørdal, Norway, Norway

⁵National Veterinary Institute, P.O. Box 8156 Dep., NO-0033 Oslo, Norway

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In press

Copper, Molybdenum and Cobalt in Herbage and Ruminants from Organic Farms in Norway

Abstract

To evaluate the animal nutritional status of copper, molybdenum and cobalt on Norwegian organic farms, soil, herbage and animal blood samples were collected from 27 dairy and sheep farms and analysed for Cu, Mo and Co. The herbage median (10th-90th percentile) Cu, Mo, and Co concentrations (mg/kg DM) and the Cu:Mo ratio in the first cut were 5.3 (3.9-6.8), 1.5 (0.6-4.8), <0.05 (<0.05-0.08) and 3.8 (1.1-8.3) and in the second cut 7.0 (5.7-9.3), 3.3 (1.6-10.1), 0.06 (<0.05-0.15) and 2.0 (0.8-5.2), respectively. The results of mixed model analyses of herbage Cu and Mo indicated that soil pH, soil organic matter content, herbage botanical composition, yield and phenological stage of timothy at harvest mostly influenced the herbage micronutrient concentration. We conclude that plant growth was not limited by the supply of Cu, Mo or Co, but the herbage mineral nutrient concentration alone was not balanced to meet the dietary needs of ruminants. Supplements of mineral nutrient mixtures and/or concentrates fortified with Cu and Co are required to ensure sufficient supply for ruminants.

Keywords: Co, Cu, dairy cattle, molybdenosis, Mo, roughage, sheep, soil, vitamin-B₁₂

Introduction

Ruminants depend on an adequate supply of copper (Cu), molybdenum (Mo) and cobalt (Co) for mammalian functions. An initial assessment of the actual or likely occurrence of a dietary mineral inadequacy can be made by comparing the mineral composition of the diet with appropriate standards of adequacy (Underwood & Suttle, 1999). Interpretation of mineral nutrient levels as an indication of nutrient deprivation is facilitated by the use of a marginal band between values consistent with health and those consistent with poor health. With regard to the needs of ruminants, there has been concern about low and/or variable concentrations of Cu, Mo and Co in roughage harvested in several regions in Norway (Frøslie & Norheim, 1983; Synnes & Øpstad, 1995; Ulvund, 1995; Sivertsen & Plassen, 2004). Although no direct nutritional disorders related to sub- or supraoptimal supply of Mo have been reported, the content of the micronutrient in the ration is of interest because Mo affects the absorption of Cu. Low and high Cu:Mo ratios induce Cu deficiency and toxicity, respectively, in sheep (Underwood & Suttle, 1999).

A transition from conventional to organic farming practice may change the supply and availability of soil Cu, Mo and Co and thereby their herbage concentration and the Cu:Mo ratio. Changes reported after transition to organic farming include a higher soil pH, alteration of the mineral concentrations in soils and plants, and of the botanical composition of leys (Clark et al., 1998; Pettersson et al., 1998; Gruber et al., 2001). All of the reported changes can influence the plant availability of microminerals (Marschner, 1995).

Organic farming aims to produce high energy fodders with proper mineral composition utilising local resources (Debio, 2003). As of 2005, the entire feed ration in organic farming has to be from organic sources (Council of European Union, 1999). Ruminant feed in organic farming is mainly based on roughage with a limited use of concentrates fortified with micronutrients. Ruminants are thus more dependent on nutrients in the herbage than in conventional farming. Knowledge about the herbage concentration of Cu, Mo and Co on organic farms may be a helpful tool to reveal possible imbalances, deficiency or toxicity to animals.

The investigation on Cu, Mo and Co in soil, herbage, sheep and cattle presented here is complementary to another published study on the status of other trace elements (zinc, iron, manganese) in organic farming in Norway (Govasmark et al., 2005). Both of these investigations are based on a survey performed on organic farms, and the overall aim of the project and methods used are described in Govasmark et al. (2005).

The objective of the present study was to investigate the soil and herbage Cu, Mo and Co concentrations, and to evaluate the herbage supply of these elements for sheep and dairy cattle on Norwegian organic farms. We also determined blood plasma Co, as vitamin B₁₂, and blood plasma Cu concentration in selected animals.

Material and methods

Soil and herbage samples were collected from 13 organic sheep and 14 organic dairy farms located in the four regions of Norway; Coast (1), Mountain (2), East (3) and Middle (4) (Fig. 1). Details of years of organic production on each farm, rainfall, temperature, chloride deposition, yields, botanical composition of the herbage, phenological stage of development at harvest of timothy (*Phleum pratense*) (mean stage by count, MSC), dominating plant species as well as soil chemical and physical properties within regions, were presented in the previous paper (Govasmark et al. 2005). Cobalt and Mo concentrations in red clover (*Trifolium pratense*) from the same fields as in our investigation were analysed in 2001, and

results are published by Bakken et al. (2004). All investigated farms started to feed the animals with concentrates and/or mineral mixtures fortified with Cu and Co, because of increased knowledge on micro-mineral nutrients in feed to animals during the recent years.

Soil analyses

Soil Cu was extracted with diethylenetriaminepentaacetic acid (DTPA) adjusted to pH 7.30 (Lindsay & Norvell, 1978), Mo by using ammonium oxalate and oxalic acid adjusted to pH 3.3 (Grigg, 1953) and Co with 0.5M acetic acid (McLaren et al., 1986). Copper, Mo and Co in the soil extracts were analysed by inductively coupled plasma-atomic emission spectrometry (ICP-AES).

Plant analyses

Herbage samples, consisting of grass, clovers and forbs, from 1st and 2nd cuts in 2001 and 2002 from three different leys on each farm were analysed for their concentration of Cu, Mo and Co according to official methods of the Association of Analytical Chemists (Pedersen & Lysnes, 2002). Dried herbage (5.0 g) was dry ashed at 500 °C and digested in 10 ml aqua-regia (HCl:HNO₃ in 3:1 ratio). The digested solutions were evaporated and dissolved in concentrated HCl twice before a final dissolution in 5 ml HCl, filtration and dilution to 100 ml with H₂O. The herbage sulphur (S) concentration was determined according to Rodhuskin et al. (1999). Dried herbage sample was digested in a mixture of nitric acid and hydrogen peroxide in closed Teflon vessels. The concentrations of Cu, Mo, Co and S in the digested solutions were determined by ICP-AES. The detection limit for herbage Co analyses was 0.05 mg Co/kg DM. At the time of the first cuts in both years, the mean stage by count at harvest of timothy (MSC) was determined according to Moore et al. (1991), and at all sampling occasions the botanical composition of the gross yield was determined by sorting and later drying of subsamples.

Blood analyses

Blood was sampled by local veterinarians in April and May 2002 from the five cows that had calved most recently and from five pregnant adult ewes from each herd. Blood was sampled with heparin-containing evacuated plastic tubes without rubber stoppers (Venoject II, Terumo Europe, Belgium). The blood samples were centrifuged the day after sampling and the plasma was kept frozen until analysis. Plasma Cu concentration in pooled samples within herd was determined at the Norwegian Veterinary Institute. The samples were diluted and analysed by flame atomic absorption spectroscopy. The detection level was 2 µmol/L. Cobalt is a prosthetic group of cyanocobalamin, known as vitamin B₁₂. Plasma vitamin B₁₂ concentration in individual animal samples was determined with the Dualcount Solid Phase No Boil radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA) at the Norwegian School of Veterinary Science by a method specified by the manufacturer

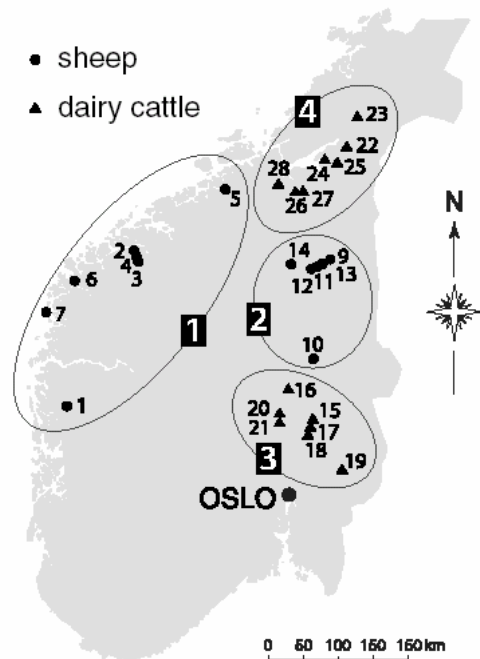


Fig. 1. Map of southern Norway indicating the locations of 27 farms in the Coast (1), Mountain (2), East (3), and Middle (4) regions.

(http://www.dpcweb.com/package_inserts/ria/pdfs/anemia/kdsp-2.pdf). Plasma samples with vitamin B₁₂ concentrations above 1771 pmol/L were not diluted and reanalysed because we were only concerned about low concentrations.

Statistics

The relationship between concentrations of Cu and Mo in plants and other plant and soil variables was established using mixed model analysis with the restricted maximum likelihood method for random effects in JMP release 5.0.1a, (SAS Institute Inc., Cary, NC, USA). Variables were normalized by a logarithmic transformation (soil Cu, soil Mo, plant Cu and plant Mo). Differences in plant mineral concentrations between first and second cut were tested by a paired t-test. Pooled weighted means for yields in 2001 and 2002 were used for first and second cut concentrations of Cu and Mo because initial comparisons revealed no significant differences between years. The best regression models for Cu and Mo were selected from a maximum model by backward elimination of nonsignificant variables at 5 % level, using Type III sum of squares. The normality assumption was assessed by means of a plot of residuals against predicted values. The fixed independent continuous variables included in the maximum model were % clover, % forbs, yield, phenological stage of development of timothy, soil pH, % clay, % sand, % organic matter, soil micronutrient concentration (mg/L), soil moisture at pF 2 (ml H₂O/100 ml soil), soil air volume (ml/100 ml soil), and the year since ploughing. Region, and farm nested within region, was specified as class variables, and farm nested within region as random effect. Percentage contribution to the models of the remaining variables was given by their sequential (Type I) sum of squares as a proportion of the total sum of squares. Differences in Cu, Mo and Co concentrations between regions were analyzed by ANOVA and multiple comparisons were tested with Tukey HSD test. Original data are presented with medians and percentiles (ordered values divided into hundredths) because of the lack of normality. The correlation between the red clover Co (mg/kg DM) and soil Co (mg/L) concentrations was analysed by linear regression. Herbage Co concentrations below the detection limit of 0.05 mg Co/kg DM were randomised uniformly between 0.00 and 0.05 mg/kg DM.

Results

Copper

The highest median (10th-90th percentile) soil Cu concentration was 0.22 (0.08-0.64) mg Cu/L in the Middle region, whereas no difference was found between the other regions (0.09 (0.03-0.29) mg Cu/L soil). According to the standard set by Lindsay and Norvell (1978), approximately 50 % of soil samples in the Middle region and 75 % in the other regions had lower soil Cu concentrations than the suggested critical value for plant growth of 0.2 mg Cu/kg soil. The soil Cu concentration was positively correlated to the clay content in the soil in all regions.

The median (10th-90th percentile) herbage Cu concentrations in the first and second cut were 5.3 (3.9-6.8) and 7.0 (5.7-9.3) mg Cu/kg DM, respectively. The herbage Cu concentration was higher in the second cut and with no difference between regions. For the first cut, only 12 % of herbage samples had lower Cu concentrations than 4 mg Cu/kg dry matter (DM) (Fig. 2A), which is considered as the critical concentration for growth limitations in grass leaves (Whitehead, 2000). The red clover Cu concentration in the first cut in year 2001 was approximately 50 % higher than in the herbage sample (Table 1). All red clover samples had a higher Cu concentration than the suggested critical level for red clover growth of 5 mg Cu/kg DM (Whitehead, 2000). Herbage Cu concentration was positively

Table 1. The herbage and red clover Cu, Mo and Co concentrations and herbage S concentrations from the first cut in year 2001 in the investigated regions. Animal blood plasma Cu and vitamin B₁₂ concentrations sampled late spring 2002. Data are presented with medians and 10th (10%) and 90th (90%) percentiles.

District	Copper			Molybdenum		Cobalt		Vitamin B ₁₂	Sulphur
	Herbage 1 st cut 2001 mg/kg DM	Red clover 1 st cut 2001 mg/kg DM	Blood plasma µmol/L	Herbage 1 st cut 2001 mg/kg DM	Red clover 1 st cut 2001 mg/kg DM	Herbage 1 st cut 2001 mg/kg DM	Red clover 1 st cut 2001 mg/kg DM	Blood plasma pmol/L	Herbage 1 st cut 2001 g/kg DM
Coast	n=20	n=7	n=6	n=21	n=7	n=21	n=7	n=6	n=21
Median	5.5	8.7	13	1.53	1.68	<0.05	0.07	1040	1.7
10%-90%	3.6-8.8	5.9-10.1	11-15	0.54-3.10	0.27-3.49	<0.05-0.10	0.05-0.11	444->1771	1.1-2.1
Mountain	n=17	n=15	n=7	n=18	n=15	n=18	n=15	n=7	n=21
Median	5.2	6.9	15	0.94	0.96	<0.05	0.05	1370	1.4
10%-90%	3.8-7.7	5.3-10.4	12-23	0.50-2.28	0.27-2.32	<0.05-0.14	0.02-0.22	190->1771	1.0-1.8
East	n=21	n=20	n=7	n=21	n=20	n=21	n=20	n=7	n=21
Median	4.8	6.9	13.	3.52	3.93	<0.05	0.11	>1771	1.2
10%-90%	3.6-6.7	5.9-8.4	12 -18	0.74-6.88	0.47-9.78	<0.05-0.11	0.05-0.23	all >1771	1.0-1.5
Middle	n=21	n=21	n=7	n=21	n=21	n=21	n=21	n=4	n=21
Median	5.0	7.5	16	1.55	1.71	0.06	0.13	>1771	1.1
10%-90%	4.0-6.4	6.4-9.1	12-20	0.76-5.45	0.76-4.12	<0.05-0.11	0.06-0.20	all >1771	0.9-1.5

correlated with the proportion of clover and forbs in all regions (Table 2). The first cut herbage Cu concentration was positively correlated with organic matter content in the soil but negatively with the herbage yield and MSC. The second cut herbage Cu concentration was, in contrast to the first cut, positively correlated with herbage yield.

All herbage Cu concentrations were above 3 mg Cu/kg DM, which is the suggested critical concentration level in the diet for primary Cu deficiency in ruminants (Puls, 1994). The plasma Cu concentrations were within the normal range of 11 to 20 $\mu\text{mol Cu/L}$ (0.7 to 1.3 mg Cu/L) given by the Norwegian Veterinary Institute (Table 1) and only one sheep herd had Cu blood concentrations within the marginal band for possible chronic Cu poisoning.

Molybdenum

The highest median (10th-90th percentile) soil Mo concentration was 0.36 (0.06-3.41) mg Mo/L in the East region, whereas no differences were found between the other regions (0.10 (0.04-0.28) mg Mo/L soil). All soil samples in the Coast region and approximately 50 % of the soil samples in the other regions had lower soil Mo concentration than the suggested critical level of 0.14 mg Mo/kg soil (Grigg, 1953). The soil content of extractable Mo was highest in the East region, and positively correlated to the clay and sand contents.

The highest herbage Mo concentration was found in the East region (median (10th-90th percentile)) in first and second cut, 3.4 (0.6-8.4) and 7.4 (1.4-12.9) mg Mo/kg DM, respectively. No difference was found between the other regions for the first and second cut (median (10th-90th percentile) 1.4 (0.6-9.8) and 2.8 (1.5-7.0) mg Mo/DM), respectively.

Similar to Cu, the herbage Mo concentration was higher in the second cut. The red clover Mo concentration (determined in the first cut in year 2001) was approximately the same as found in the herbage sample (Table 1). All herbage and red clover samples had higher Mo concentrations than the suggested critical level of 0.1 mg Mo/kg DM in grass (Whitehead, 2000) (Fig. 2B) and 0.2 mg Mo/kg DM in red clover (Bakken et al., 2004). Soil pH was positively correlated with the herbage Mo concentration, and was the factor that contributed

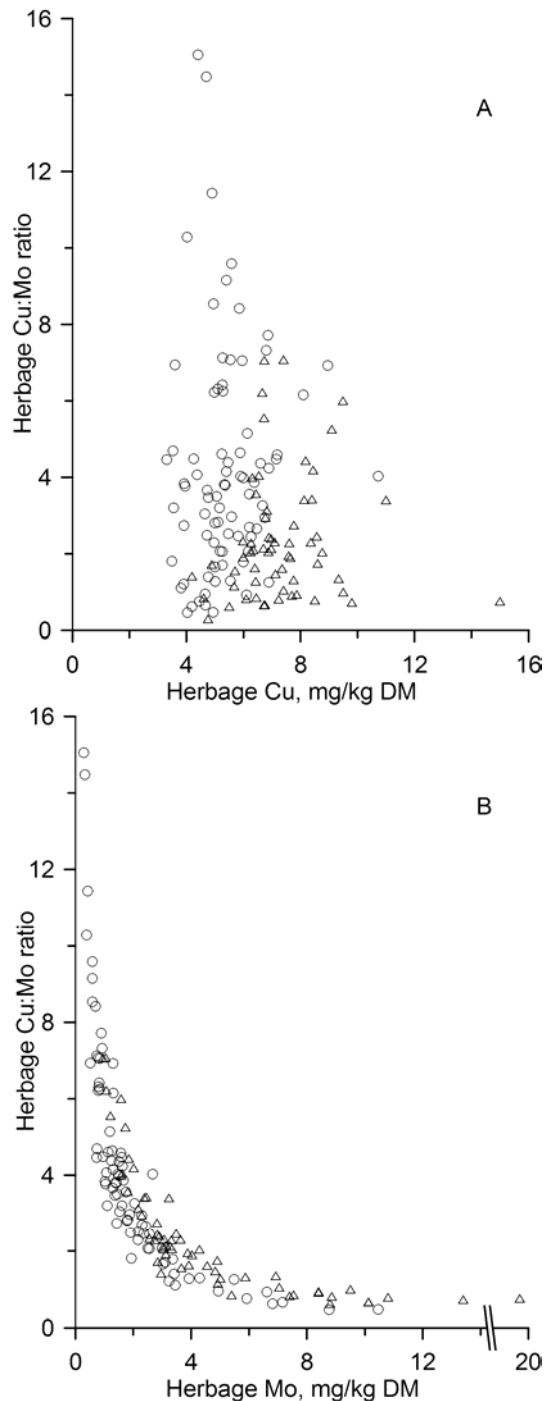


Fig. 2. Herbage Cu:Mo ratio according to concentration of Cu (A) and Mo (B) in the first and second cut from 3 leys on each of 27 farms.

most to the variation in herbage Mo concentration. In the first cut, the herbage Mo concentration was also positively correlated with soil organic matter content but negatively with DM yield (Table 2).

Table 2. Estimated positive or negative regression coefficients (β) and R^2 from mixed model analysis of soil and herbage Cu and Mo concentration¹. Percentage contribution (Cont) to the model of the variables is given by their sequential (Type I) sum of squares as a proportion of the total sum of squares.

Parameter	Copper						Molybdenum					
	Soil		Herbage				Soil		Herbage			
	β	Cont.	β	Cont.	β	Cont.	β	Cont.	β	Cont.	β	Cont.
pH									+ ***	9,4	+ ***	9,8
OM			+ ***	10,0					+ *	1,1		
Clay	+ **	4,2					+ **	0,5				
Sand							+ *	0,6				
Clover	nd	nd	+ **	1,9	+ ***	10,7	nd	nd				
Forbs	nd	nd	+ ***	9,5	+ ***	14,2	nd	nd				
Yield	nd	nd	- *	1,5	+ *	3,2	nd	nd	- *	0,9		
MSC	nd	nd	- ***	10,3		nd	nd	nd				nd
Soil conc.	nd	nd					nd	nd				
R^2		63,1		49,2		68,2		89,5		85,5		79,7

¹The variables tested in the model were soil pH, organic matter % (OM), clay %, sand %, pF 2 (g H₂O/100 ml soil), soil air volume (ml/100 ml soil), field age, clover %, forbs %, yield (kg DM/ha), MSC, and for the first and second cut, soil concentration of the mineral (mg/L).

*, **, *** Significant at 0.05, 0.01 and 0.001 levels of probability, respectively, nd = not determined in the model.

Copper:molybdenum

The lowest median (10th-90th percentile) herbage Cu:Mo ratio was in the first and second cut 1.8 (0.5-8.3) in the East region (Fig. 3). No differences were found among the other regions in any of the cuts (2.4 (0.8-6.4)). Only 20 % of the herbage samples in the first cut had the recommended Cu:Mo ratio of 6 to 10 (Frøslie, 1990), whereas most of the herbage samples had a lower ratio in the second cut. The Cu:Mo ratio decreased with increasing herbage Mo concentration (Fig 2B), but no clear pattern was seen with the herbage Cu concentration (Fig. 2A).

Cobalt

The lowest median (10th -90th percentile) soil Co concentration was 0.07 (0.03-0.12) mg Co/L in the Coast region. No differences in the soil Co concentration were found among the other regions (0.12 (0.04-0.25) mg Co/L). Approximately 80 % of the soil samples on the Coast and 50 % in the other regions had lower soil Co concentration than 0.11 mg Co/kg soil, which is considered to give a low herbage Co concentration in relation to dietary needs (Aasen, 1997). The red clover Co concentration correlated positively with the soil Co concentration ($R^2 = 0.43$, $P < 0.001$) (Fig. 4).

Approximately 60 % and 40 % of all herbage samples had lower herbage Co concentration than the detection limit of 0.05 mg Co/kg DM in the first and second cut, respectively (Fig. 5). A marginal band in the diet between 0.05 and 0.11 mg Co/kg DM is suggested (Puls, 1994; Ulvund, 1995; Underwood & Suttle, 1999). The herbage Co concentration did not differ between regions and was higher in the second than in the first cut. The red clover samples had a higher Co concentration than the herbage samples.

Cobalt is part of vitamin B₁₂, which is synthesised in the rumen. Vitamin B₁₂ in plasma showed that only three of five sampled sheep on one farm in the Mountain region had vitamin B₁₂ concentrations within or below the suggested marginal band of 150 to 300 pmol vitamin B₁₂/L (Ulvund, 1990). None of the cows had low plasma vitamin B₁₂ concentrations (Table 1).

Discussion

Copper

The positive correlation between soil Cu and clay content on the investigated farms was in accordance with the findings of Semb and Øyen (1966), who also reported that the Cu concentration in sandy soils was dependent on the parent material. In the present investigation, soil pH was neither related to soil nor herbage Cu concentrations. Fystro and Bakken (2003) reported only a 10 % lower herbage Cu concentration after surface liming to leys, and the reported increase in soil pH after transition from conventional to organic farming (Clark et al., 1998) does probably not have any effect on the herbage Cu concentration.

The median herbage Cu concentration was at the same level or higher than corresponding concentrations found in different parts of Norway by others (Frøslie & Norheim, 1983; Synnes & Øpstad, 1995; Fystro & Bakken, 2003; Johansen et al., 2003) and also higher than found in timothy in Finland (Kahari & Nissinen, 1978). According to Whitehead (2000), levels of less than 4 mg Cu/kg DM in grasses and 5-7 mg Cu/kg DM in clovers are considered critical for plant growth. The herbage Cu concentration was below the level regarded critical for plant growth (Whitehead, 2000) only in a few first cut samples. Thus, Cu is not considered as a growth limiting mineral nutrient for plant growth in our investigation. Therefore, it is unlikely that the supply of this mineral is critical for plant growth on organic livestock farms in Norway. The herbage Cu concentration was higher in the second cut, probably due to the increased proportion of clover (Metson et al., 1979). Another reason may be that the second cut was harvested at an earlier stage of development (Whitehead, 2000). The MSC was not determined in the second cut in the present investigation, but a negative correlation between the herbage Cu concentration and MSC was found in the first cut (Table 2). Forbs are reported to have a higher Cu concentration than

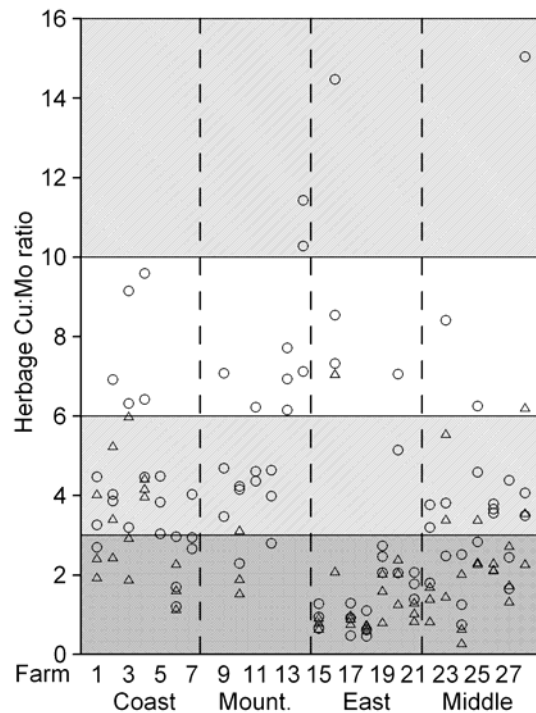


Fig. 3. Herbage Cu:Mo ratio in first (○) and second cut (△) from 3 leys on each of 27 farms according to region. Light area indicates recommended ratio, light shaded bands indicate marginal ratio, and dark shaded area indicates risk for secondary Cu deficiency.

grasses (Garmo et al., 1986), and the same was found in our investigation. An increased content of forbs in organic leys would decrease the risk of primary Cu deficiency to ruminants. The concentration of Cu in the various forbs species was not investigated in the present investigation.

Soil organic matter content was positively correlated with the herbage Cu concentrations in the first cut. Copper deficiency on soils having a high content of organic matter has been known in Norway for a long time, and many farmers used fertilisers fortified with Cu to increase the Cu concentration in their soils. No information on earlier Cu application to the fields in our study was available. A positive effect of Cu fertilizer on grass Cu concentration 46 years after application has been reported (Aasen & Myhr, 1995). Although it may be very speculative, perhaps the Cu applied to soils many years ago still is available to plants, thus explaining why organic matter had a positive influence on the herbage Cu concentration. The method used for determining plant available Cu in the soil did not provide data on the herbage Cu concentration, which is in accordance to the results reported by Lindsay and Norvell (1978), who did not find any growth response to Cu application because all their soils contained Cu concentrations above 0.2 mg Cu/ kg soil.

None of the herbage Cu concentrations were extremely low. Thus, even without supplemental feeding with mineral mixtures or concentrates fortified with this micronutrient, the risk of primary Cu deficiency on these farms is unlikely.

Molybdenum

The reported critical herbage concentration of Mo for normal plant growth is 0.1 to 0.2 mg Mo/kg DM (Whitehead, 2000). Molybdenum was not considered as a growth limiting mineral nutrient for the nitrogen fixation by red clover (Bakken et al., 2004) or grass growth in our investigation. The median herbage Mo concentration in our investigation was higher than the normal concentration of timothy and grasses of 0.1 to 0.5 mg Mo/kg DM suggested by Cheng and Ouellette (1973) and higher than earlier reported in Norway (Frøslie & Norheim, 1983; Fystro & Bakken, 2003), whereas the Mo concentration was in the same range as reported by Synnes and Øpstad (1995) on the Coast. The herbage Mo concentration was higher in the second cut, possible due to the increased proportion of clover. The latter was not verified statistically in our investigation (Table 2), but a higher Mo concentration in red clover than grasses is reported (Bakken, 1999; Bakken et al., 2004) whereas no difference was found between forbs and grasses in the mountain area of Norway (Garmo et al., 1986).

The soil pH was the prominent factor correlating to the herbage Mo concentration, and the same was reported by Fystro and Bakken (2003). They found that herbage Mo concentration increased by 40 % in grass swards when the soil pH increased by 0.9 to 1.4 pH

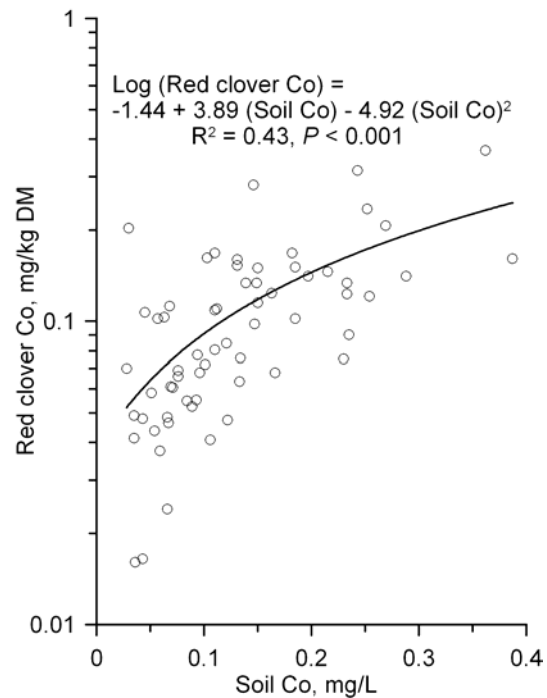


Fig. 4. Regression plot of the red clover Co concentration (log scale) in first cut 2001 against soil Co from 3 leys on each of 27 farms within the Coast, Mountain, East, and Middle regions.

units from a base line of pH 4.8 to 5.2. The higher soil pH in our investigation (6.2 ± 0.4) compared to Fystro and Bakken (2003) may be one of the reasons for the differences in the herbage Mo concentration between these two investigations. Also Grigg (1953) reported that soil pH was the most influencing factor determining Mo concentration in plants, but poor correlation between top-dressed Mo and plant concentration was reported when soil pH was above 6.0 and soil Mo below 0.14 mg Mo/kg soil. The soil Mo concentration did not correlate with the herbage Mo concentration within the regions, but the highest soil and herbage Mo concentration was found in the East region, thus suggesting a relationship between soil and herbage Mo concentration.

The large variation in the herbage Mo concentration found in our investigation was also reported in timothy in Finland (0.02-16.6 mg Mo/kg DM) (Paasikallio, 1978). This variation causes problems in mineral feeding to ruminants because of the antagonistic relationship to Cu and S in the rumen (Underwood & Suttle, 1999).

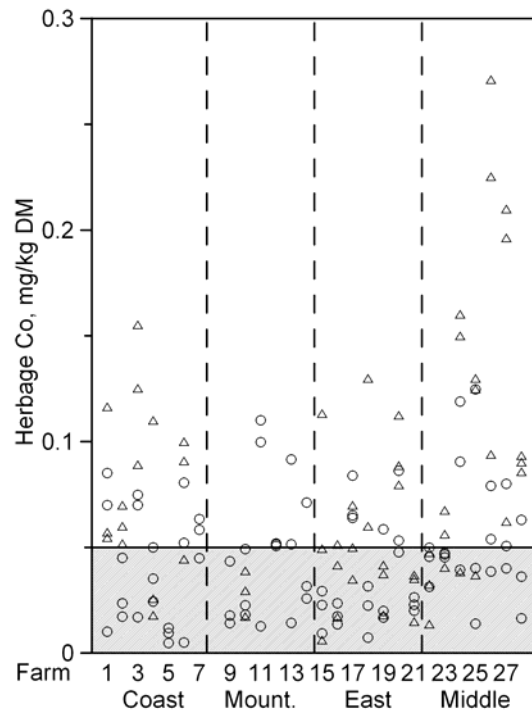


Fig. 5. The herbage Co concentrations in first (o) and second cut (?) from 3 leys on each of 27 farms within the Coast, Mountain, East, and Middle regions. Shaded area indicates herbage Co concentrations lower than the detection limit of the method used.

Copper:molybdenum

The Cu:Mo ratio found in the herbage, though variable, was generally low, 73 % below 6 (Fig. 3). This result is contradictory to Frøslie & Norheim (1983) who found that 75 % of their samples had a Cu:Mo ratio above 10. The low Cu:Mo ratio in our investigation was not caused by generally low herbage Cu concentrations, but by a combination of normal or subnormal levels of Cu and relatively high levels of Mo. Both the herbage Cu and Mo concentrations increased in the second cut, but the herbage Mo concentration increased relatively more than the herbage Cu concentration. This explains the lower Cu:Mo ratio in the second cut. The concentration of Mo in herbage increased with increasing soil pH and the Cu:Mo ratio is lower in red and white clover than grasses (Bakken, 1999) which can be reasons for the lower Cu:Mo ratio in organically grown leys. Thus we recommend avoiding high soil pH. It is important to be aware of the often reported increase in soil pH after transition from conventional to organic farming.

The addition of excess S to feed can result in the formation of insoluble copper thiomolybdates in the rumen (Underwood & Suttle, 1999). The herbage S concentration in the present investigation was low (Table 1) and the highest herbage S concentration found was 3.10 g S/kg DM. Copper deficiency related to excess of S is observed at relatively high S levels only (above 4.0 g S/kg DM; Fisher, 2004). Thus the herbage S concentration found in the present investigation may probably not suppress Cu absorption. If farmers increase the herbage S concentration, this could lead to induced Cu deficiency in animals. On the contrary, Chiy et al. (1999) suggested that the overall effect of an increased herbage S concentration

because of S fertilisation is positive on the absorption of Cu because they found a decrease in herbage Mo concentrations with increased S fertilisation.

Despite the low herbage Cu:Mo ratio, blood plasma Cu concentrations were within the normal range of 11-20 $\mu\text{mol Cu/L}$ (McCosker, 1968) in most herds. One sheep herd had plasma Cu which could be associated with chronic Cu poisoning. The reasonable explanation is that most animals in our investigation were given concentrates and mineral mixtures. The Cu:Mo ratio in concentrates is generally high, 10 to 35 (Frøslie et al., 1983), and mineral mixtures do not contain Mo.

Cobalt

Cobalt is not essential for higher plants, but it is required for N_2 fixation by the rhizobia in the nodules of leguminous plants (O'Hara et al., 1988; O'Hara, 2001). According to Mengel and Kirkby (1987), soils must contain at least 0.1 mg Co/kg soil to meet, in most instances, the Co demand of the *Rhizobium*-legume symbiosis. Bakken et al. (2004) suggested from the analyses of red clover samples that the supply of soil Co might not be sufficient to sustain the demands of the N-fixating symbiosis in all soils, but it was not possible to draw any conclusion about a true casual relationship. Approximately 80 % of the soil samples in the Coast region and 50 % in the other regions had lower soil Co concentrations than 0.11 mg Co/kg soil, which is considered to give a low herbage Co concentration in relation to dietary needs (Aasen, 1997).

The herbage Co concentration was generally lower than reported in timothy in Finland (Kahari & Nissinen, 1978), and herbage in Norway (Synnes & Øpstad, 1995; Fystro & Bakken, 2003). A marginal band in the diet between 0.05 and 0.11 mg Co/kg DM is suggested (Puls, 1994; Ulvund, 1995; Underwood & Suttle, 1999). Most cases of Co deprivation in Norway have been in lambs grazing on fertilised and limed cultivated pastures in coastal areas. The main problem is encountered in Rogaland (southwestern Norway), but sporadic cases of Co deficiency have also been reported in coastal districts from Agder in the south to Troms in the north (Ulvund, 1995). We found low herbage Co concentrations in all regions. Our plasma vitamin B_{12} concentrations, however, did not indicate Co deficiency in the animals, except sheep on one farm in the Mountain region (three of five sampled sheep) which had vitamin B_{12} concentrations within or below the suggested marginal band of 150 to 300 pmol vitamin B_{12}/L (Ulvund, 1990). None of the cows had low plasma vitamin B_{12} concentrations. The reasonable explanation is the use of concentrates and mineral nutrient mixtures containing Co.

Feeding recommendation

The content of Cu in the herbage on the investigated farms may be high enough to avoid primary Cu deficiency in sheep and dairy cows. The low Cu:Mo ratio in herbage from many of the sites, most of them with a high soil pH, might however, dispose for secondary Cu deficiency. All investigated herbage samples were low in Co. Even though organic farming aims to only use local resources, it was most probably the use of mineral nutrient mixtures and concentrates fortified with Cu and Co which explained why plasma Cu and vitamin B_{12} contents in the animals were still within the normal range. It seems that home-grown feed does not fulfil ruminants' needs of Cu and Co and that mineral nutrition mixture or concentrates fortified with these nutrients have to be supplied.

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Status of selenium and vitamin E on Norwegian organic sheep and dairy cattle farms

Espen Govasmark^{1,2}, Arvid Steen², Turid Strøm¹, Sissel Hansen¹, Bal Ram Singh³ and Aksel Bernhoft⁴

Addresses of the authors

¹Norwegian Centre for Ecological Agriculture, NO-6630 Tingvoll, Norway

²Norwegian School of Veterinary Science, P.O. Box 8146 Dep., NO-0033 Oslo, Norway

³Department of Plant and Environmental Sciences, Norwegian University of Life Sciences P.O. Box 5003, NO-1432 Ås, Norway

⁴National Veterinary Institute, P.O. Box 8156 Dep., NO-0033 Oslo, Norway.

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Abstract

Herbage selenium (Se) concentration is generally low in Norway. It is unknown, if feeding practises on Norwegian organic farms fulfil the dietary need of Se and vitamin E to sheep and dairy cattle. Therefore, we analysed Se in soil and herbage, and Se and vitamin E in animal blood in the indoor feeding season at 14 organic dairy and 14 organic sheep farms. The herbage Se concentration was low. Approximately 50 and 35 % of all samples in the first and second cut, respectively, had Se concentrations below the detection limit of 0.01 mg/kg dry matter (DM). The median (10th, 90th percentile) Se concentrations were <0.01 (<0.01, 0.03) and 0.02 (<0.01, 0.06) mg/kg DM in the first and second cuts, respectively. Whole blood Se concentrations were 0.10 (0.04, 0.15) µg/g in dairy cattle and 0.14 (0.03, 0.26) µg/g in sheep. Vitamin E concentrations were 4.2 (2.7, 8.4) mg/L in dairy cattle and 1.3 (0.9, 2.4) mg/L in sheep. None of the soil or plant variables explained the variation in herbage Se concentration, although Se in soil and plant tended to be correlated. Herbage Se concentration was inadequate to meet the dietary Se requirements. Vitamin E requirement was only met in dairy herds. We recommend Se and vitamin E supplementation to ruminants on organic farms.

Keywords: feed, mineral nutrition, organic farming, roughage, Se, soil

Introduction

The total selenium (Se) concentration in soils is reported from 0.1 to 0.4 mg/kg in the inland regions to 0.5 to 1.4 mg/kg soil in the coastal regions of Norway (Wu & Låg, 1988). However, the total soil Se concentration is not a good indicator of the herbage Se concentration (Johnsson, 1991). The herbage Se concentration is generally low on conventional farms in Norway (Frøslie et al., 1980) and most probably it may also be low on organic farms (Mikkelsen & Hansen, 1967; Frøslie, 1990). Selenium and vitamin E are two important contributors of the antioxidant function of cells. Deficiencies have been associated with nutritional myodegeneration, reproductive disorders, mastitis because of lowered disease resistance, and ill thrift (Weiss et al., 1997).

In order to meet animal Se requirements, concentrates and mineral nutrient mixtures in Norway have been enriched with Se since 1980 (Frøslie, 1990). Selenium deficiency diseases in Norwegian ruminants have thus been prevented by the use of these Se enriched supplements and prophylactic medication by injection. In recent years, Se has also been added to some commercial fertilizer to increase the plant Se concentration in conventional agriculture (Tveitnes et al., 1996).

Organic farming aims to produce high-energy fodders with optimum mineral composition utilising local resources (Debio, 2003). It is the ambition of the Council of European Union that all feeds in organic farming must come from organic sources after 2005 (Council of European Union, 1999). The feed in organic farming is primarily based on roughage and aims to limit the use of concentrates or mineral mixtures fortified with micronutrients in the feeding scheme.

Fresh herbage is rich in vitamin E, but natural vitamin E is subject to degradation during storage. There is very limited information on the vitamin E concentration in harvested or stored herbage in Norway. Bernhoft et al. (2002) measured the concentration of vitamin E (α - and γ -tocopherol) in ensilaged herbage stored in round bales and silos. They found three and four times higher vitamin E concentration in silo stored silage than in silage in the middle and the top, respectively, of stored round bales. About half of the round bales contained silage with lower vitamin E concentration than their recommended critical concentration of 30 mg vitamin E/kg DM. Nadeau et al. (2004) reported that vitamin E concentration can be preserved during harvest and ensiling, but that there may be a greater risk of vitamin E loss in round-baled silage than in silage stored in silos.

The objectives of the present study were to: (i) investigate the herbage Se concentration on organic farms, (ii) provide information about the antioxidant status of Norwegian organic dairy cattle and sheep, and (iii) evaluate if the current vitamin E and Se feeding practice meet the dietary needs on organic farms.

Material and methods

This study used the same experimental materials as previously used by Govasmark et al. (2005). It involved three meadows, having 3 subplots, on each of 14 sheep and 14 dairy cattle organic farms in Norway (Fig. 1) representing four regions, i.e. Coast (1), Mountain (2), East (3) and Middle (4). A brief description of rainfall (mm), chloride deposition (mg Cl/m² yr), yield (kg dry matter /ha), botanical composition of the herbage, phenological stage at harvest of timothy (*Phleum pratense*) (mean stage by count, MSC) (Moore et al., 1991), and soil chemical and physical properties in each region are presented in Table 1. Detailed information of all parameters and the farm position, temperature (°C), dominating plant species, and soil classification group were presented in the previous paper by Govasmark et al. (2005). Farm 8

and 9 are in the same municipal and has the same climatic conditions. In addition to herbage, Se concentration was also determined in soils collected from 17 random fields in the Coast and Middle region, which all had herbage Se concentrations higher than the detection limit of 0.01 mg Se/kg DM. Farm 8 (sheep) is only represented with blood Se and vitamin E analyses, and from farm 1 (sheep), blood analyses are lacking.

The herbage fodder for dairy cows before and during the blood-sampling period consisted of 60 to 80% grass silage and 20 to 40 % hay, except on farm 25 which only had hay. All dairy farms, except farm number 19, 25 and 26, used Se enriched supplements in their feeding.

Fodder for sheep consisted of mainly hay, except farm number 12 which had silage. All sheep farms, except farm number 4, 5 and 8, used Se enriched supplements in their feeding. None of the sheep farms used vitamin E deliberately, but used vitamin E indirectly through the use of mineral mixtures or concentrates. Concentrations of herbage Se, given diets adequate in vitamin E, were assessed against a marginal band of values between deficiency and adequacy (Underwood & Suttle, 1999). Responsiveness to supplement is possible with concentrations within the marginal band and probable below. Concentrations of Se and vitamin E in blood were assessed against a marginal band defined by the National Veterinary Institute, Norway.

Soil analyses

In situ soil characterization and soil sampling were done as soon as possible after the first harvest of the leys in 2001. All soil profiles were classified according to FAO (1994). The water retention characteristics of the soil cores were determined at 0.01 MPa (pF 2) (g water/100 ml soil) by the pressure plate method (Page et al., 1982) and the pore volume (ml/100 ml) according to the method of Page et al. (1982). The pH was measured in a 1:5 soil:water suspension and organic carbon by combustion in a LECO CHN-1000 apparatus (Page et al., 1982). The particle size fraction of the composite sample was analysed by the pipet method procedure (Page et al., 1982). More details of these procedures are described in Govasmark et al. (2005). For Se determination, 20 g air dried soil with 50 ml 0.016 M KH_2PO_4 was shaken for one hour at room temperature (20 °C). The suspension was filtrated (Munkell, quality 00H), and diluted (1:10, v/v) with 0.5% HNO_3 . Selenium concentration in the digested solution was analysed by inductively coupled plasma-mass spectrometry (ICP-MS)

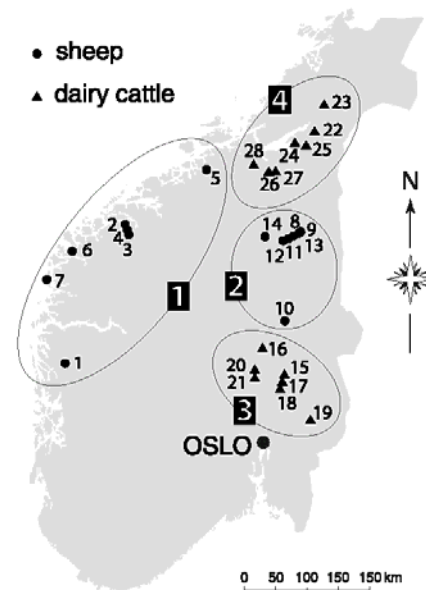


Fig. 1. Map of southern Norway indicating the farm locations in the four regions Coast (1), Mountain (2), East (3) and Middle (4).

Herbage analyses

At the time of the first cut of leys in both years, the phenological stage of development of timothy was determined according to the procedure of Moore et al. (1991). The total herbage yield above a stubble height of 10 cm was recorded on all subplots at harvest time in 2001 and 2002. Two samples were taken from each subplot. The first sample of 700 g fresh weight was collected for determination of botanical composition and the second sample of 1000 g for the chemical element analyses. For more details, see Govasmark et al. (2005).

Table 1. Climate, soil chemical and physical characteristics and herbage characteristics from the 28 farms within the Coast, Mountain, East and Middle regions.

	1-Coast	2-Mountain	3-East	4-Middle
Climate (mean (min-max))				
¹ Cl deposition	9.6 (7.5-12.4)	0.26 (0.15-0.51)	0.47 (0.26-0.87)	3.5 (2.2-5.0)
² Precipitation	427 (358-547)	241 (212-341)	261 (239-282)	296 (245-368)
Soil (median (90th-10th%))				
pH	6.0 (5.4-6.5)	6.4 (5.9-6.8)	6.3 (5.7-6.6)	6.3 (6.0-6.7)
³ OM %	15.1 (8.8-54.7)	4.4 (2.1-7.4)	5.5 (4.1-6.7)	4.7 (2.3-8.2)
Clay %	4.4 (2.2-7.9)	3.5 (0.4-11.1)	8.9 (4.2-17.7)	12.6 (3.0-26.3)
⁴ pF 2	47 (30-62)	40 (26-44)	32 (25-40)	38 (31-44)
⁵ Air vol.	21 (12-32)	18 (13-29)	28 (11-35)	17 (12-24)
Herbage (median 1 st cut / median 2 nd cut)				
Yield (kg/ha DM)	3195 / 1570	5223 / nd	3380 / 2153	3700 / 2560
⁶ MSC	3.5 / nd	3.5 / nd	3.3 / nd	3.3 / nd
Grass %	57 / 46	80 / nd	74 / 55	78 / 76
Clover %	5 / 15	9 / nd	19 / 33	18 / 29
Forbs %	24 / 26	4 / nd	4 / 3	3 / 3

¹Cl-deposition = estimated yearly chlorine deposition (mg Cl/m² yr); ²Precipitation = mean precipitation May-August in the period 1961 to 1990m (mm); ³OM = organic matter % of DM; ⁴pF 2 = g water/100 ml soil at 0.01 MPa pressure; ⁵Air vol. = air volume (ml/100ml); ⁶MSC = 3.3; spikelets fully elongated, 3.5; peduncle fully elongated (Moore et al., 1991); nd = not determined.

For Se determination, 1 g herbage sample (DM) was digested in 16 ml of a mixture of nitric and perchloric acids (3+1 v/v) on a programmed heating block (Tecator, Sweden). Selenium was reduced to Se⁴⁺ with hydrochloric acid. The digest was diluted to 25 ml with deionised water and determined by hydride generation atomic absorption spectrometry (Varian VGA-76) with sodium borohydride. The detection limit was 0.01 mg/kg DM. The analysis followed an accredited method (Norwegian Accreditation, NS-EN ISO/IEC 17025) and was carried out at the National Veterinary Institute, Norway.

Blood analyses

Blood samples were taken by local veterinarians in April and May 2002 from the five most recently calved cows and five pregnant adult ewes from each herd. The blood was collected in heparinized tubes (10 ml). Whole blood samples and centrifuged plasma samples,

respectively, were pooled with equal amounts from each individual within herd and kept frozen until analysis.

For Se determination, 2 g of whole blood wet weight was analysed by using the same method as for the herbage samples (see above) at the National Veterinary Institute, Norway. The detection limit was 0.01 µg/g wet weight.

Vitamin E (α - and γ -tocopherol) in plasma was determined using high-pressure liquid chromatography (HPLC). The principle was as follows: proteins were trapped by ethanol, containing 1 % ascorbic acid to avoid tocopherol oxidation. The tocopherols were extracted in hexane, which was removed by evaporation and the residue dissolved in ethanol. The vitamin E isomers were determined using a C18 column and a fluorescence detector. Tocol was internal standard used quantitatively, and α -tocopherol was external standard. The recoveries of internal and external standards were within 80-110 %. The detection limit was 0.1 mg/L. The analysis was carried out at the National Veterinary Institute, Norway.

Statistical analysis

Herbage and soil Se concentrations were normalized by logarithmic transformation. Herbage Se concentrations below the detection limit were randomised uniformly between 0.00 and 0.01 mg/kg DM. All tests were done on all data (also randomized) but also on herbage samples which all had Se concentrations above the detection limit, to confirm the results. Pooled means, weighted for yields, in 2001 and 2002 were used for the first and second cut concentrations because initial pairwise *t*-test comparisons revealed no significant differences between years. Differences in plant Se concentrations between first and second cut were also tested by a paired *t*-test. Differences in Se concentrations between regions were analysed by ANOVA and multiple comparisons among treatment means were made by the Tukey HSD test. The relationship between concentrations of herbage Se and various soil (except soil Se concentration) and plant variables was established using mixed model analysis with the restricted maximum likelihood method for random effects in JMP release 5.0.1a, (SAS Institute Inc., 2002, Cary, NC, USA). The best regression models for Se were selected from a maximum model by backward elimination of non-significant variables at 5 % level of probability. The normality assumption was assessed by means of a plot of residuals against predicted values. The fixed independent continuous variables included in the maximum model were % clover, % forbs, yield, phenological stage of development of timothy (MSC), soil pH, clay, sand, organic matter contents (%), moisture content at 0.01 MPa (pF 2), soil air volume, and year since ploughing (field age). Region and farm nested within a region were specified as class variables and farm nested within region as random effect. The present model was also run for the Coast and Middle district using only samples with herbage concentrations above 0.01 mg Se/kg DM, but not using field age, MSC, soil air volume and year since ploughing in the model. The correlation (Pearson) between herbage Se and soil Se concentrations was only established in the Coast and Middle regions, because other regions had low number of samples.

Results and discussion

Soil and plant

The herbage Se concentration was low in all regions and approximately 50 and 35 % of all herbage samples had Se concentrations below the detection limit of 0.01 mg Se/kg in the first and second cut, respectively. The median (10th, 90th percentile) herbage Se concentrations in first and second cuts were <0.01 (<0.01, 0.03) and 0.02 (<0.01, 0.06) mg/kg DM, respectively

(Fig. 2). The herbage Se concentration was higher in the second cut than in first cut ($P < 0.01$). The herbage Se concentrations in the present study was generally within or slightly lower than Underwood and Suttle's (1999) marginal band for dietary needs of 0.03 to 0.05 mg Se/kg DM for sheep and 0.02 to 0.04 mg Se/kg DM for dairy cows. These low marginal bands are derived from factorial modelling and assumptions that diets are adequate in vitamin E. All herbage Se concentrations were below more pragmatically recommended nutritional requirements of 0.1 mg Se/kg DM for sheep (National Research Council, 1985) and 0.3 mg Se/kg DM for cattle (National Research Council, 2001). Among the regions, Coast and Middle regions showed the highest herbage Se concentrations. This may be assigned to natural marine influx of Se to soils as was observed in other investigations (Låg & Steinnes, 1974; Mosher & Duce, 1987; Johnsson, 1989).

None of the measured soil or plant variables in the mixed model were associated with the herbage Se concentration, but there was a tendency of increased plant Se concentrations with increased soil organic matter and moisture content at 0.01 MPa using only observations in the Coast and Middle region with herbage Se concentration above 0.01 mg/kg DM. The reason why none of the measured parameters were associated with the herbage Se concentration was likely the low herbage Se concentrations in most samples. Most of the observations in Mountain and East regions had Se concentrations below the detection limit of 0.01 mg Se/kg DM, which made it impossible to explain the variation with regard to soil and plant factors measured and only possible to relate the Se concentration to the geographical position of the farm. Most forages grown on soils with low Se do not differ markedly in their Se concentration, and no differences between grass and clover are also reported from Se fertilised meadows (Garmo et al., 1986; Gupta & MacLeod, 1994). Borowska and Lyszczarz (2002) reported that grass grown on mineral soils with total Se concentration of 0.2 mg/kg, low in organic matter (0.8 %) and high soil pH (7) in Poland contained Se ranging from 0.12 to 0.31 mg/kg DM. High soil pH and low organic matter content in Polish soils may explain the higher levels of Se in grasses as compared to plants grown in Norway even at higher soil

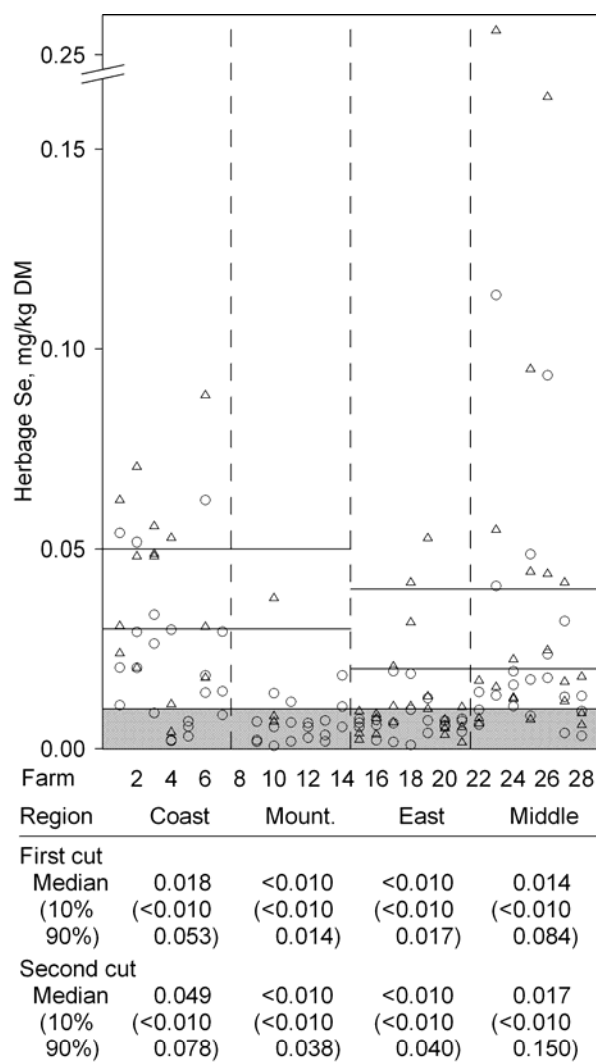


Fig. 2. Pooled herbage Se concentration from 2001 and 2002 in the first (o) and second cut (Δ) from three meadows on each of the 27 farms within the Coast, Mountain, East, and Middle regions. Values in the shaded area were below the detection limit and all these values are uniformly randomised. Area between horizontal lines (—) indicate the marginal band for the dietary needs of sheep (Coast and Mountain) and dairy cattle (East and Middle).

Se concentration (Wu & Låg, 1988) because at higher soil pH, selenate is the dominant Se form which is weakly bound to soils and is more available to plants (McBride, 1994). Johnsson (1991) investigated the Se uptake in spring wheat (*Triticum aestivum*) and winter rape (*Brassica napus*) and found that the effect of soil pH on the Se uptake decreased with increasing contents of clay and organic matter in the soil without Se application and that the initial Se in untreated soils had little effect on the Se uptake by plants. Singh and Mishra (1987) did not find significant correlation between the plant Se concentration and soil pH, organic matter, cation exchange capacity, CaCO₃, elevation, rainfall or temperature in the Himalayan grasslands of India.

Median (10th, 90th percentile) soil Se concentration of the 17 samples was 0.03 (0.01, 0.05) mg/kg (data not shown). Correlation between normalised herbage Se concentrations in the first and second cut and soil Se concentrations were 0.46 ($P = 0.061$) and 0.58 ($P < 0.001$), respectively. The correlation values between our 17 herbage Se and soil extractable Se samples was higher than reported by Wang and Sippola (1990) based on their 128 samples.

Blood selenium and vitamin E

Suggested marginal band between deficiency and adequacy for sheep and cattle is 0.05 to 0.10 µg Se/g whole blood (National Veterinary Institute, Norway). The median (10th, 90th percentile) whole blood Se concentration in dairy cattle was 0.10 (0.04, 0.15) µg/g. Dairy cattle whole blood Se concentrations were marginal or deficient in half of the herds, and the three lowest blood Se concentrations were on farms 19, 25 and 26 which did not use any Se enriched supplements in their feed ration (Fig. 3). The generally low blood Se concentrations reflected the low herbage Se concentrations, and that the Se-supplementation from mineral nutrient mixtures and concentrates was generally insufficient and should be increased on many of the investigated farms to meet the needs of the dairy cattle. Pehrson et al. (1997) found very low activity of glutathione peroxidase in the erythrocytes, equivalent to 0.02 to 0.03 µg Se/g whole blood, of dairy cattle in Estonia, which received no or only minor amounts of Se-supplemented mineral mixtures. Bernhoft et al. (2004) found that the blood Se status of 3 to 7 week-old beef calves was associated with the use of mineral mixtures supplied to their mothers during pregnancy, and not to the Se supplied in the concentrates, illustrating the importance of mineral mixture as the

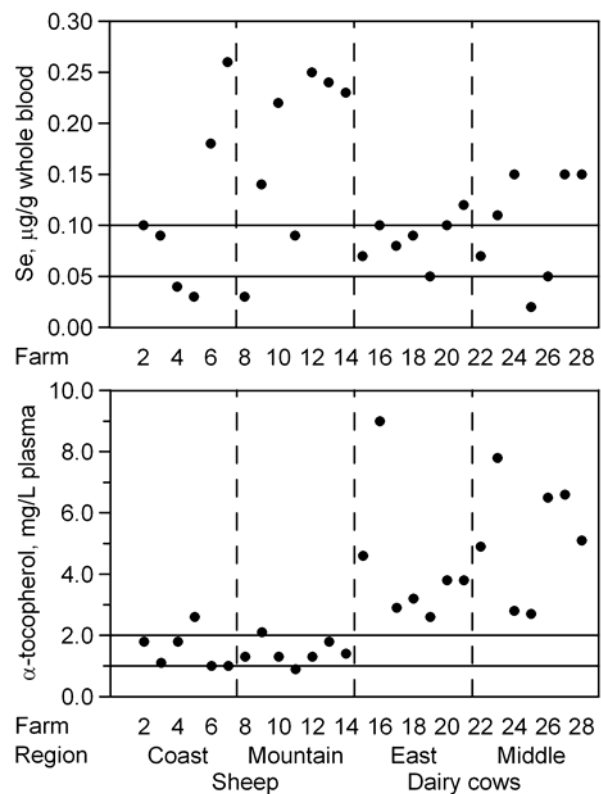


Fig. 3. Whole blood Se and plasma α-tocopherol (vitamin E) concentrations in pooled herd samples from 5 pregnant sheep (Coast and Mountain) and 5 recently calved cows (East and Middle) from the 27 herds. Area between horizontal lines (—) indicate the marginal band for the dietary needs of sheep and dairy cows. Farm 4, 5, 8, 19, 25 and 26 did not use Se enriched supplements.

source of Se in extensive beef production. Calves suckling mothers that received recommended daily doses of mineral mixture (100 g) had 0.11 µg Se/g whole blood (SD 0.03) in contrast to 0.03 µg Se/g (SD 0.00) in calves suckling mothers that received no supplement of mineral mixture.

The median (10th, 90th percentile) whole blood Se concentration in sheep was 0.14 (0.03, 0.26) µg/g. Sheep whole blood Se concentrations were marginal or deficient close to half of the herds, but higher than those found in the dairy cows (Fig. 3). The three lowest sheep blood Se concentrations were found on farms 4, 5 and 8, which did not use Se enriched supplements in their feed ration. The relatively high blood Se concentrations found on sheep farms using Se enriched supplements, also showed high Se herbage concentration. This suggests that their current feeding practise is sufficient to meet the Se needs of sheep.

The median (10th, 90th percentile) plasma vitamin E (α -tocopherol) concentrations in dairy cows and sheep were 4.2 (2.7, 8.4) and 1.3 (0.9, 2.4) mg/L, respectively. γ -tocopherol was not detected in any of the plasma samples (<0.1 mg/L). All dairy cow herds had plasma α -tocopherol concentration higher than 2.0 mg/L (Fig. 3), the minimum level considered adequate (National Veterinary Institute, Norway). The indoor feed ration to the dairy cows consisted mainly of silage but also considerable amounts of hay, which is a poor vitamin E source. The plasma vitamin E concentrations indicated that the silage or/and the vitamin E supplements, which all herds received, had sufficient vitamin E concentration to meet the needs of dairy cattle at all our organic farms.

Almost all sheep, on the other hand, had plasma α -tocopherol concentrations within a suggested marginal band from 1.0 to 2.0 mg/L (National Veterinary Institute, Norway) (Fig. 3). All sheep, except on farm 12, were given varying amounts of hay as a part of their diet. Hay generally contains one fifth of the level of vitamin E found in fresh forage, but since vitamin E is subject to degradation during storage and the levels can vary so much within one stock of feed, is it virtually impossible to predict levels in stored hay or silage very precisely (Puls, 1994). Feeding with both large quantities of hay and silage and obviously too little mineral and vitamin mixture supplementations was probably the reason why the sheep were deficient in vitamin E on our organic farms. Jukola et al. (1996) also found that dry dairy cows fed hay and varying amounts of mineral and vitamin mixture supplementations had lower mean serum vitamin E concentrations, 1.5 mg/L (SD 0.35), than comparable cows fed silage, 4.5 mg/L (SD 2.24). Bernhoft et al. (2004) found that the mean herd concentration of blood plasma vitamin E (α -tocopherol) in 3 to 7 week-old beef calves in herds that received baled silage or straw from barley or oats was 0.9 mg/L (range 0.3 to 1.8). The roughage, the main vitamin E source, had been stored nearly a year and the authors concluded that an additional supply of vitamin E seems to be necessary at times to ensure adequate vitamin E status in the animals.

Conclusion

Our investigation on Norwegian organic farms showed that the herbage Se concentration was insufficient to meet the dietary Se requirements of ruminants. Herbage Se concentrations could not be predicted from soil Se concentrations. Although dietary vitamin E requirement was met in organically raised dairy herds, the same requirement was not met in organically raised sheep herds. Lowest whole blood Se concentrations were found in stocks on farms that did not use Se enriched supplements in their feeding. Based on these finding, we recommend Se and vitamin E supplementation to ruminants on all organic farms till alternative means to fulfil the dietary requirements of these feed constituents are found.

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A microwave digestion method for determination of selenium in organic tissues using liquid chromatography

Espen Govasmark^{1,2}, Mark G Grimmett³

Addresses of the authors

¹University of Life Sciences, Department of Plant- and Environmental Sciences, P.O. Box 5003, N-1432 Ås, Norway

²Norwegian Centre for Ecological Agriculture, N-6630 Tingvoll, Norway,

³Agriculture and Agri-Food Canada, Crops and Livestock Research Centre, 440 University Avenue, Charlottetown, P.E.I. C1A4N6, Canada.

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Abstract

An existing laboratory procedure for selenium analysis using open vessel wet digestion and liquid chromatographic fluorescence determination was modified for use with microwave digestion. The proposed microwave digestion method eliminated the hazards associated with the use of HClO_4 while maintaining excellent recoveries of selenium. Various digestion parameters including time, temperature, and type and quantity of acid were investigated and a two-step $\text{HNO}_3/\text{H}_2\text{O}_2$ digestion procedure was developed. Digested samples were derivatized with 2,3-diaminonaphthelene and the resultant piarselenol complex was measured fluoremetrically using a liquid chromatograph. Measured values were in agreement with 9 different certified reference materials. The detection limit for this method was 0.54 ng Se/gram of tissue (3σ), the calibration curve remained linear ($R^2=0.9968$) up to 2 ppm Se.

Introduction

Selenium (Se) is of great importance to human and animal health as an essential element (1,2). Physiologically, Se has a dual role as an essential nutrient at low concentrations and as a toxic substance at higher concentrations (3). However, there is a narrow range between deficiency and toxicity for Se (4, 5) and this dictates the need for accurate and reliable methods of determination. Various analytical methods have been developed and applied to Se analysis and some of them have good detection limits, i.e. 50-1000 pg at present (6-9).

The choice of digestion procedure can strongly influence analytical recoveries of Se. Many selenium compounds are volatile and can be lost during the process of converting the organically bound selenium into its ionic forms (10). Classically, sample digestion has been performed by the use of open-vessel digestion on hotplates or heating blocks. Important variables in any wet digestion procedure are the amount of sample and the volume of acid used (11). Most open-vessel wet digestion procedures require the use of a combination of acid(s) and oxidant(s), of which the most commonly used are nitric-, sulphuric- and perchloric acid, and hydrogen peroxide (12). Perchloric acid is a very effective oxidizing agent and is commonly used in sample digestion procedures for Se determination (6, 9, 13, 14), but its use raises the argued problem of explosion risk and health hazards. There are many documented cases of fatal accidents associated with the use of perchloric acid or the repair of equipment exposed to perchloric acid such as fume hoods (15). In order to reduce these risks, certain precautions are to be taken when using perchloric acid but even when well-established guidelines are in place, the risk of an accident always exists - especially when large numbers of samples are handled. Consequently, most laboratories prohibit the use of perchloric acid. Other digestion methodologies employ the use of charring during sample digestion. Charring is utilized in many open vessel digestion procedures and requires large samples (1 g) and large amounts of oxidising agents (6, 13). During the charring process volatile elements can be lost if insufficient quantities of acid are utilized. It is necessary to maintain oxidizing conditions at all stages of the decomposition process (10).

An alternative to open vessel digestion is the wet oxidization of organic material in a closed vessel(s) within a microwave oven. By combining the use of a closed digestion vessel and the resultant pressure created during the digestion process, it makes it possible to have digestion temperatures higher than the boiling point of the oxidising agent. Microwave digestion of biological material using closed vessels is a commonly used practise in laboratories (16-18). Microwave digestion has the advantages of reduced digestion times and reduced losses of volatile elements and usually requires less complicated acid mixtures – nitric acid may be all that is required for some matrices (16). Microwave digestion is the method of choice for the mineralization of organic and biological matrices without the use of hazardous perchloric acid (4). Deaker and Maher (19) reported the complete recovery of five selenium species (selenite, selenate, selenomethionine, selenocystine, selenocystamine) added to marine tissues when using microwave digestion for sample preparation.

Among analysis methods, fluorimetric analysis is commonly used for the determination of Se. There are many different possible fluorophores that can be used for the measurement of selenium but 2,3-diaminonaphthalene (DAN) gives some of the best detection limits (20-22). However, DAN only forms piarselenol derivatives with selenite (20-22). This can be useful if the determination of selenite separate from selenate is desired but if total Se is to be measured then selenate must be reduced to selenite prior to its derivatization with DAN. This reduction can be accomplished by the addition of hydrochloric acid to the sample followed by heating (8, 9).

Along with excellent sensitivity, the use of a fluorescent derivative also has the advantage of reduced method interferences since the final piarselenol derivative is extracted into an organic solvent prior to analysis (21, 23). A liquid chromatograph equipped with a

fluorescence detector is usually used for the determination of the piaszelenol derivative. A reversed-phase or normal phase method is often used to separate the piaszelenol peak from other organic solvent extractible impurities present in the sample (9, 21). Other techniques for the analysis of selenium include the use of a liquid chromatograph in-line with an ICP-MS. While the ICP-MS offers excellent detection limits for selenium it is also subject to interferences associated with the formation of monoatomic and polyatomic ions in the region of the plasma. The major interferences are the formation of the dimmers $^{38}\text{Ar}^{40}\text{Ar}$ and $^{40}\text{Ar}_2$, which overlap the two most abundant Se isotopes (5).

A reliable and robust analytical method for Se analysis must possess accuracy, sensitivity, repeatability, have minimal interferences and be suitable for a wide range of biological sample matrices with large differences in Se concentrations. A method that fulfils these criteria should also have a large sample size to increase the possibility of having a homogenised sample (24) and also be able to measure samples with very low Se concentrations. The analytical method should also provide a wide area of linear response in its calibration curve and be dynamic in the amount of sample injected when samples with concentrations outside the linear response area are encountered.

The aim of this study was to obtain a practical and safe laboratory method for Se determination in a broad spectrum of organic materials using a microwave with closed auto-venting vessels and a liquid chromatograph equipped with a fluorescence detector.

Material and methods

Sample preparation

Biological materials were digested in closed auto-venting Omni vessels in a MARS 5 Laboratory Microwave (CEM Corp, Matthews, NC, USA) in two steps. One gram of sample was weighed analytically into the digestion vessel and followed by the addition of 15 ml of concentrated ultra high purity nitric acid (Anachemia Science, Quebec, Canada). The digestion program consisted of a 15 minute ramp to 205 °C followed by a hold of 20 minutes at 205 °C. Samples were allowed to cool and remaining pressure was carefully vented prior to the addition of 5 ml of 30 % hydrogen peroxide (Fisher Scientific, Ontario, Canada). Caution: take appropriate measures when handling and storing strong oxidizers such as nitric acid and hydrogen peroxide. The microwave digestion program was repeated. Following sample cooling, digested samples were transferred to acid washed 250 ml glass digestion tubes. During the transfer step, digestion vessels were rinsed with deionized water (diH_2O). To facilitate the removal of the remaining nitric acid and hydrogen peroxide, 4 ml of concentrated ortho-phosphoric acid (Fisher Scientific, Ontario, Canada) was added to each sample and samples were heated in a digestion block at 145 °C (in a fume hood) until all nitric acid and hydrogen peroxide was evaporated. Tubes were removed from digestion block and allowed to cool. Four ml of concentrated hydrochloric acid (Fisher Scientific, Ontario, Canada) was added to each sample and samples were placed back in the 145 °C heating block for 5 minutes to reduce selenate to selenite. Using a small amount of diH_2O , samples were carefully transferred to, acid washed, 50 ml glass test tubes with Teflon lined caps and the level of liquid was matched in all test tubes with diH_2O . Capped test tubes were placed in a heating block at 90 °C (water bath could also be used) for 20 minutes to complete the Se reduction step. Repeated analysis showed that samples prepared to this point were stable for several days at room temperature.

DAN-HCl Preparation

The purchased 2,3-diaminonaphthalene (DAN) (Sigma-Aldrich, Ontario, Canada) was further purified and converted to the hydrochloride form (described below) in a manner similar to that used by Hawkes and Kutnink (9). Caution: DAN is a suspected carcinogen – follow supplier's instructions and material safety data sheet for safe use, handling, and disposal. In a fume hood while wearing personal protective equipment, DAN was dissolved in boiling, concentrated hydrochloric acid (HCl). The solution was boiled and stirred on a hotplate in a beaker covered with a watchglass for 90 minutes (2.0 g DAN in 100 ml HCl). Following the dissolution step, 1.5 g of activated carbon (Darco-60, Fisher Scientific, Ontario, Canada) was added to the hot solution and heated and stirred for a further 5 minutes. The hot solution was then filtered under vacuum, through a fritted glass funnel containing a 5 mm layer of diatomaceous earth (Sigma-Aldrich, Ontario, Canada). Prior to filtering, the diatomaceous earth layer was rinsed with diH₂O and dried under vacuum. The filtered solution was cooled on an ice bath, covered, and placed in a refrigerator for 2 hours to allow the 2,3-diaminonaphthalene-HCl (DAN-HCl) to crystallize. The crystallized DAN-HCl was separated from the solution by vacuum filtration, dried, and placed in a clean amber vial. The DAN-HCl used in the derivatization step was prepared in small batches (100 ml) as a 0.1 % solution of DAN-HCl in 0.1 M HCl. During its preparation, the 0.1 % DAN-HCl was kept in low light and mixed in a flask wrapped in aluminium foil. Following dissolution the 0.1 % DAN-HCl solution was transferred to an amber bottle and stored in a dark cool place.

Sample pH adjustment

Prior to derivatization with DAN-HCl, the pH of each sample must be adjusted to optimize this reaction. pH adjustment was accomplished using an approach similar to Hawkes and Kutnink (9) where the pH of each sample was monitored using the pH indicator cresol red. In a fume hood, samples were placed in an ice bath and 2.5 ml of concentrated ultra pure ammonium hydroxide (NH₄OH) containing 2 M glycine and 0.09 M tetra-sodium EDTA (Na₄EDTA) was added in 1 ml, 1 ml and 0.5 ml aliquots respectively. Samples were mixed between aliquot additions to prevent violent reactions with the strong base. Four drops of 0.2 % cresol red (in water) was added to each sample. The pH of each sample was then adjusted to its final endpoint with concentrated ultra-pure NH₄OH until the solution turned an amber-orange colour (pH 1.5-2.0). Four more drops of 0.2 % cresol red solution was added to each sample to insure proper endpoint colour. Finally, 4.5 ml of 2 M glycine, pH 1.75 (pH adjusted with HCl) was added to each sample. At this point, samples were stable for a several days and multiple batches may be prepared prior to continuing to the derivatization step.

Derivatization and Extraction of Piazselenol

From this point, all steps must be done under reduced light conditions and whenever possible samples should be kept in the dark. Prior to derivatization, equal amounts of DAN-HCl and cyclohexane were shaken in a separatory funnel for 15 seconds and allowed to separate. This was done to remove any impurities from the DAN-HCl solution prior to derivatization. After draining off the DAN-HCl layer from the separatory funnel, 3 ml of the DAN-HCl solution was added to each sample. The samples were capped, shaken, and placed in a 50 °C water bath for 10 minutes, shaken again, and placed back in the water bath for a further 10 minutes. Samples were then cooled in a room temperature water bath for 10 minutes. The piazselenol complex was extracted from the sample by adding 4 ml of cyclohexane to each test tube and shaking it for 15 minutes on an oscillatory shaker. The cyclohexane layer was allowed to separate and 1.75 ml of the cyclohexane layer was transferred into 2 ml amber glass

autosampler vial, capped, and stored in a light proof box until analysis on the liquid chromatograph.

Instrumentation and Measurement

Quantitation of Se (piaszelenol) was done using an Agilent 1100 series liquid chromatograph equipped with a 1100 series fluorescence detector (Agilent Technologies, Delaware, USA). The extraneous components in the sample were separated from the piazselenol peak using an Agilent Hypersil NH₂ column (4.6 mm x 200 mm) heated to 40 °C. A guard column containing a NH₂ cartridge (Security Guard Column, Phenomenex, California, USA) was attached to analytical column and changed when system backpressures became elevated. The mobile phase employed for the separation was 80 % cyclohexane and 20 % ethyl acetate (isocratic) at a flow rate of 1 ml/min. A 100 µl sample was injected using an 1100 series autosampler at 10 °C (Agilent Technologies, Delaware, USA). The fluorescence detector was configured with an excitation wavelength of 378 nm and emission wavelength 530 nm. The piazselenol eluted after 4.6 minutes, and the total run took 16 minutes (some unidentified peaks eluted late in the run for some samples). During sample analysis the room containing the liquid chromatograph was kept in low light and the autosampler windows were covered with aluminium foil to reduce stray light from reaching the samples. The method was calibrated using standard solutions prepared from certified Se standard (SCP Science, Quebec, Canada) and validated using certified reference materials from The National Research Council (Ottawa, Canada) and The National Institute of Standards and Technology (Gaithersburg, Maryland, USA). The chromatograms were produced using Agilent Chemstation software (Rev A.08.03) and samples were quantified based on peak height. Statistical calculations and figures were produced using Microsoft Excel (MS Excel 2002, SP-2).

Results and Discussion

Method of calibration

Following column flushing and equilibration to the mobile phase (80 % cyclohexane/ 20 % ethyl acetate) according to column manufacturers instructions (required an intermediate solvent to switch from shipping solvent to cyclohexane/ethyl acetate mobile phase) standards were run to determine the retention time for the piazselenol peak. The piazselenol peak was found to elute just after the system dead volume ($t = 0$) at 4.6 minutes following injection (Fig. 1). The mobile phase composition was adjusted slightly from the Hawkes and Kutnink (9) method (90 % cyclohexane, 10 % ethyl acetate) to give slightly more retention. The Hawkes and Kutnink (9) method utilized a silica column in contrast to the amino phase column utilized in the proposed method. Higher ethyl acetate contents were experimented with but did not significantly improve the resolution of the piazselenol peak and only increased the overall run length. Although the piazselenol peak eluted relatively early in the sample run there were some late eluting, highly retained compounds (for some samples) that required the run length to be extended to 16 minutes (Fig. 1). No ghost peaks were observed in successive blank runs following sample injection and confirmed that a run time of 16 minutes was sufficient time to flush the chromatography system between injections.

When high-purity reagents were used in the method and glassware was acid-washed (3 M nitric acid) and carefully rinsed with diH₂O, method blanks were very low (Fig. 1). This also contributed to the methods low detection limit. The absolute detection limit of the proposed method was 0.54 ng Se/g of sample, which is very similar to 0.48 ng Se/g of sample reported by Hawkes and Kutnink (9). Like Hawkes and Kutnink (9) nitric acid was also found

to be the primary contaminant in the proposed method. Since a larger volume of nitric acid was used in the proposed method (15 ml versus 2.5 ml) (9) its contribution to the blank was greater. There were even differences found between trace grades of nitric acid from different reagent manufacturers. An early blank contamination problem was remedied by switching to a different supplier for nitric acid. Vezina et al. (25) also reported the need for high purity reagents to achieve low blanks and low detection limits and that the analytical column could be eliminated from the HPLC system under specific assay conditions to produce even more accurate, precise and sensitive selenium determinations. Low blank values were also the result of the DAN purification step employed prior to the derivatization. Tamari (22) also reported

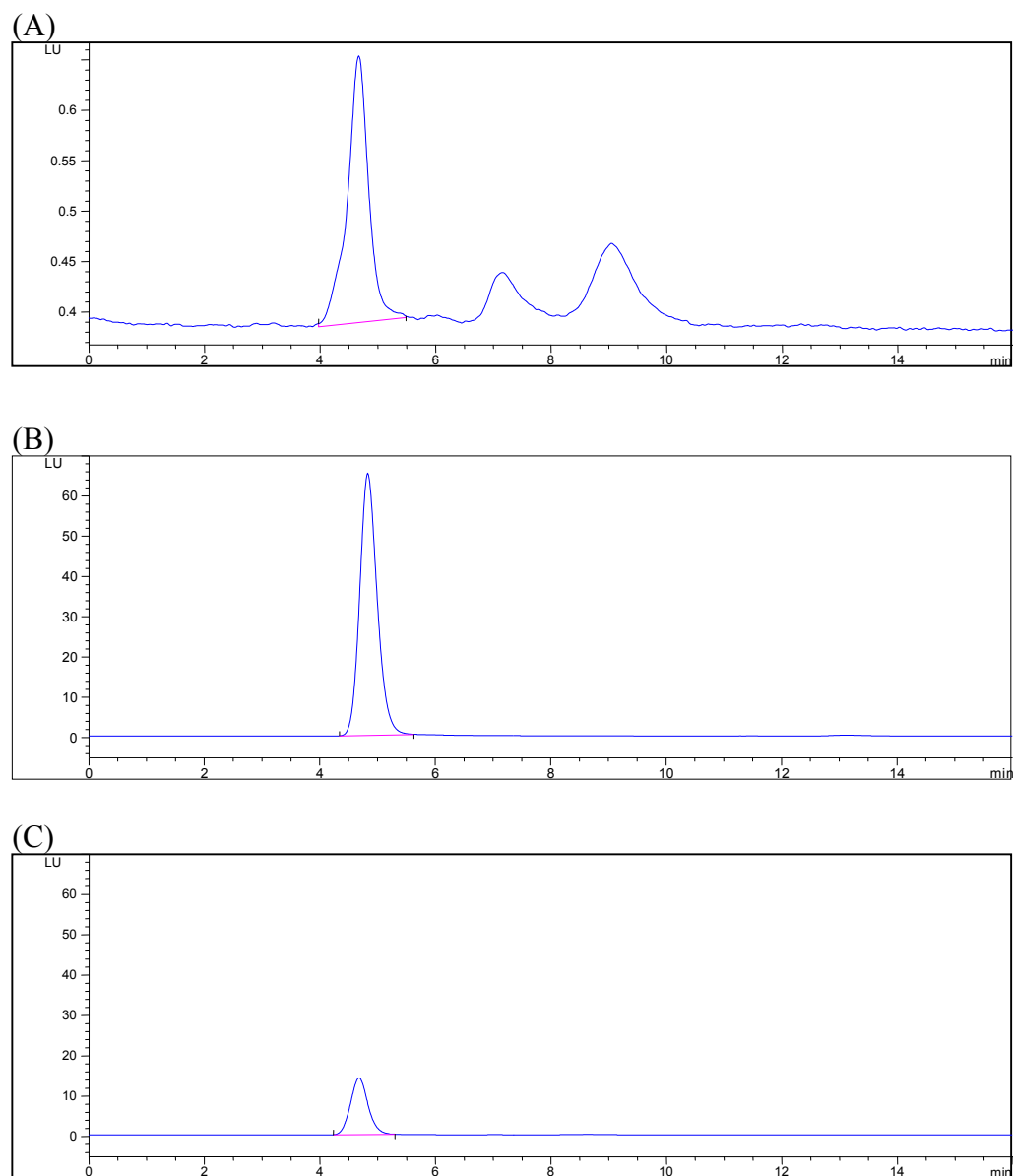


Figure 1. Representative chromatograms for: (A) a blank; (B) 0.5 ppm Se Standard; and (C) NIST* Peach Leaves SRM 1547 (0.120 ppm) samples run through the entire method procedure. Note scale differences between the relative fluorescence measurements (y-axis) for (A), (B) and (C) chromatograms. (*NIST = National Institute of Standards and Technology, Gaithersburg, MD, USA).

lower blanks when using a series of purification steps prior to derivatization. Despite the fact that the detection limit is slightly higher in the proposed method it is still sufficient to accurately quantitate most biological material. The often reported Se concentration in natural soil or plant is 0.01 to 1.7 mg/kg, liver samples is 0.65 to 1.6 mg/kg, milk products is 0.06 to 0.09 mg/kg, and in human blood is 0.045 to 0.256 mg Se/L (2, 26-28).

The calibration curve produced for the method is shown in Figure 2. A linear response was observed up to 2 mg kg⁻¹ Se and plotted values were well in agreement with the regression line (R-squared = 0.9968). This R² value is very similar to that reported by Vezina et al (25) and Hawkes and Kutnink (9). At concentrations higher than 2 mg kg⁻¹ the detector became saturated and lost linearity. However, when higher standards were diluted to fall within the linear range of the calibration curve and injected again, the dilution-corrected value was in agreement with the standard. This indicated that the reagents such as the DAN-HCl were not the limiting factor but rather the detector itself was.

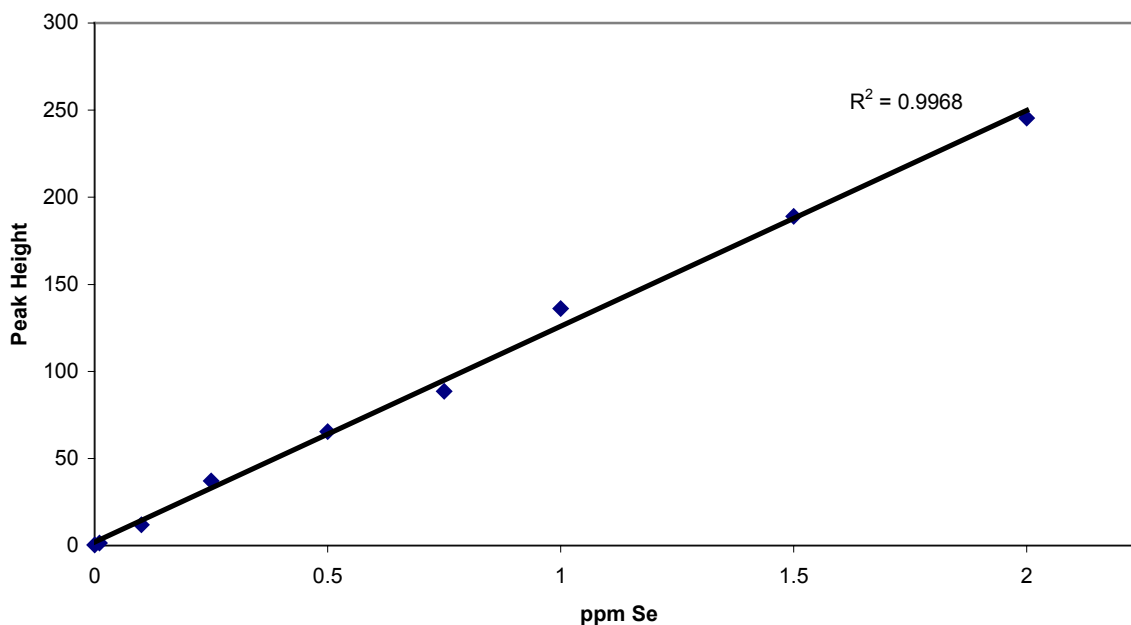


Figure 2. A typical calibration curve for the proposed method. Freshly prepared standards from a certified stock solution were run through the entire procedure to create the linear calibration curve shown above.

Method Validation

The method was validated with a suite of certified reference materials that represented plant, animal and marine sources (Table 1). The measured values were in excellent agreement with the certified values and indicated that this method was acceptable for the quantification of selenium in these types of organic materials. The very good recovery values also indicated that the oxidation process with nitric acid/hydrogen peroxide was efficient in decomposing the organic matrix, and that no losses of Se occurred during sample digestion. Therefore, the oxidizing agents used in the proposed method were effective in replacing perchloric acid. The substitution of perchloric acid with hydrogen peroxide in digestion of organic plant material using microwave digestion has also been reported successfully by others (18). It should also be noted that marine sediment sample required an additional centrifugation step following the

cyclohexane extraction to give quick separation of the cyclohexane layer before transfer to the autosampler vial. Although the proposed method did not employ hydrofluoric acid for the dissolution of the sediment, it was still effective in recovering the selenium present in the marine sediment sample (Table 1).

Procedure and Instrumentation

In addition to the advantages of avoiding the use of perchloric acid, the method also allowed larger sample sizes to be utilized during the digestion step. Traditionally sample size in microwave digestion closed vessel systems has been limited in order to avoid excessive pressures during digestion. The products of the decomposition reaction have been found to limit the decomposition reaction and result in incomplete digestion if not removed from the system (29). Thus, the utilization of a vessel which vents at lower pressures (< 400 psi) permitted the use of sample sizes up to 1 g. The excellent recoveries of selenium for standards and certified reference materials demonstrated that no selenium was lost during vessel venting in the digestion process. The larger sample size also increased the probability of having a homogenous representative sample and helped produce mean sample concentrations with low variability for the certified reference materials (Table 1).

Table 1. Certified and measured values for various botanical, food, and marine standard reference materials used to validate method. In all cases 1.0000 g of sample was digested and the injection volume was adjusted to ensure that the piaszelenol peak remained within the linear portion of the calibration curve.

Reference Material	Source	N	Injection Volume	Certified Se Concentration* $\mu\text{g g}^{-1}$	Mean Se Concentration Measured** $\mu\text{g g}^{-1}$
Pine Needles SRM 1570a	NIST ^a	9	100	0.099 +/- 0.004	0.112 +/- 0.003
Non-Fat Milk Powder SRM 1549	NIST	6	100	0.11 +/- 0.01	0.12 +/- 0.01
Spinach Leaves SRM 1570a	NIST	10	100	0.117 +/- 0.009	0.119 +/- 0.002
Peach Leaves SRM 1547	NIST	10	100	0.120 +/- 0.009	0.123 +/- 0.002
Hard Red Spring Wheat Flour RM8437	NIST	10	100	0.56 +/- 0.04	0.55 +/- 0.01
Bovine Liver 1577b	NIST	8	100	0.75 +/- 0.02	0.75 +/- 0.02
Dogfish Muscle DORM-2	NRC ^b	9	100	1.40 +/- 0.09	1.53 +/- 0.04
Lobster Hepatopancreas TORT-2	NRC	10	25	5.63 +/- 0.67	5.75 +/- 0.11
Marine Sediment HISS-1	NRC	10	100	0.050 +/- 0.007	0.042 +/- 0.001

*Uncertainty range represents 95% confidence limits, see NIST or NRC Certificates of Analysis for details. **Uncertainty range represents the 95% confidence limit.

^a National Institute of Standards and Technology, Gaithersburg, MD, USA, ^b National Research Council of Canada, Ottawa, ON, Canada

Homogenization of the collected material is a step that constitutes a very great potential source of error (24). Hawkes and Kutnink (9) stated that a main limitation of their method was the recovery of selenium in some samples decreased as sample mass approached 0.4 g dry

material. The proposed method overcomes this by using venting microwave digestion vessels and sample sizes of 1 g.

Following decomposition of the sample in oxidizing conditions, a reduction of selenate (Se(VI)) to selenite (Se(IV)) was necessary to form the piaszelenol complex. Chloride in hot non-oxidizing acid medium reduces Se(VI) to Se(IV) but not further (30). However, for this reaction to occur efficiently, the sample must be free of nitric acid and hydrogen peroxide. Residual nitric acid or hydrogen peroxide was also later found to decompose the creosol red during the pH adjustment step and was an indication of a compromised sample that should be run again. Vezina et al (31) indicated that the use of hydrochloric acid in the reduction step also eliminated residual nitric acid that would interfere with DAN derivatization.

The pH adjustment of the sample prior to derivatization with DAN-HCl was also a very important step. Glycine was used in the reagents to help buffer the pH adjustment process and also to stabilize sample pH once adjusted. The pH adjusted glycine buffer (pH 1.75) was added to minimize the tube-to-tube variation in the final pH. Rodriguez et al. (32) recommended a solution pH between 1 and 2 for optimum piaszelenol formation. Alfthan (7) studied the effects of pH on sample derivatization and found a slight increase in fluorescence between pH 1.0 and 2.4 but ultimately selected a pH range of 1.5 to 2.0 for sample analysis. Most fluorescence methods for selenium analysis that use DAN as their fluorophore utilize a pH of greater than 1.0. Rodriguez et al (32) found that when the sample was too acidic DAN was degraded.

The sensitivity of the proposed method was easily adjusted by either adjusting the final volume of cyclohexane used to extract the piaszelenol derivative or by adjusting the injection volume of the autosampler on the liquid chromatograph. Both techniques were found to work well and were verified with certified reference materials. The maximum injection volume of the autosampler used during the proposed method development was increased by the addition of an autosampler multi-draw kit but ultimately was not required for most samples. An injection volume of 100 μ l was found to work well for most materials analyzed. Samples high in Se, such as lobster hepatopancreas, were effectively quantified by simply reducing the sample injection volume on the liquid chromatograph until the piaszelenol peak fell within the linear portion of the calibration curve and final results were corrected for the adjustment in sample injection volume (Table 1). Other liquid chromatograph adjustments included keeping the autosampler temperature at 10 °C -cyclohexane freezes at 6 °C. The autosampler needle height was also adjusted so that a sample was drawn from the middle of the autosampler vial. This alleviated the problems associated with accidental transfer of small quantities the acidic aqueous layer from the sample. Injection of sample containing even minute quantities of the acidic layer was unfortunately found to have detrimental effects on the analytical column used on the liquid chromatograph.

Overall, microwave digestion followed by fluometric measurement is a reliable method for the determination of selenium in organic samples. Renard and Tompkins (33) recently compared selenium determination for selenium enriched yeast and selenomethionine by fluometric measurement, ICP-MS, and instrumental neutron activation analysis and found that all three methods produced results within 5% of expected values for standard samples. The proposed method has also been shown to be reliable and produced measured values for selenium that were in agreement with several certified reference materials.

Conclusions

Important factors that should be taken into account when choosing a method for routine laboratory measurements are the duration of the digestion procedure, safety aspects associated with the reagents and methods, the cost of instrumentation and reagents, and the volume of

reagents used. The proposed digestion utilized nitric acid and hydrogen peroxide – a safer alternative to perchloric acid. The duration of the procedure and the detection limit were comparable with other highly sensitive methods of Se analysis and gave reliable and reproducible results when compared to certified values for standard reference materials. The proposed method provided a reliable and sensitive method for the determination of selenium in organic tissues and is an excellent option for laboratories, which may not have access to more expensive instrumentation such as ICP-MS.

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**Selenium in spring wheat and leaching water as influenced by
selenium and nitrogen application**

Espen Govasmark^{1,2}, Bal Ram. Singh², John MacLeod³, and Mark G. Grimmett³.

Addresses of the authors

¹The Norwegian Centre for Ecological Agriculture, N-6630 Tingvoll, Norway.

²Department of Plant and Environmental Sciences, Norwegian University of Life Sciences, P.O. Box 5003 , N-1432 Ås, Norway.

³Crops and Livestock Research Centre, Agriculture and Agri-Food Canada, 440 University Avenue, Charlottetown, Prince Edward Island, C1A 4N6, Canada.

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Selenium in spring wheat and leaching water as influenced by selenium and nitrogen application

ABSTRACT

This paper shows a relationship between spring wheat selenium uptake, leaching water selenium losses and the timing of application of ammonium-nitrate (N) and sodium selenate. Ammonium-nitrate was applied by two methods, (i) whole amount at sowing (ii) in split application as 75 % at sowing and 25 % at stem elongation. Selenate was applied at sowing, tillering, stem elongation, head emergence and at milking growth stage. Split N application increased the protein content and Se concentration in grain, but decreased the Se concentration in leaf and straw. The highest Se concentration in plant was achieved at the split N application. Selenium leaching losses increased with increasing selenium uptake by wheat. In contrast, selenium leaching losses were lower with split N application. Applying selenate and ammonium-nitrate together after tillering increased the grain Se concentration and total Se uptake, and reduced the leaching losses of Se.

Key words: Grain Se, growth stages, leaching losses, N-fertiliser, spring wheat

INTRODUCTION

In many countries of the world, including Norway, the selenium (Se) concentration in soils is insufficient to provide adequate levels of Se for food and fodder crops. The regional distribution of Se concentrations in cereals varies depending on Se concentration in soils and soil parameters such as pH and clay and sulphate contents. Cereal Se content is also governed by the ability of plants to absorb and translocate Se to above ground plant parts (Gissel-Nielsen et al. 1984). In addition, Se form and time of application affect the Se concentration in cereals. The selenate form of Se is several times more effective in increasing the cereal Se concentration than the selenite form (Gupta et al. 1993; Singh 1991). The timing of Se application at different growth stages can also influence the Se concentration and efficiency of uptake (Singh 1994). Selenium is not an essential nutrient for cereals but its uptake and redistribution from vegetative parts to grains is dependent on the Se availability in soils and the sink strength in the grain filling period (Marschner 1995).

Wheat in Norway is priced according to its protein concentration and thus top dressing of N is a common practise to raise the protein content. Increasing the protein concentration in grains potentially increases the sink strength for Se (Marschner 1995). Nitrogen (N) uptake and protein concentration in grain increased almost linearly with increasing N fertilization, and applying 25 % of the N at heading stage increased the protein concentration further, without any further increase in grain yield (Riley et al. 1996). It is also reported that Se can substitute for sulphur (S) in amino acids at high Se concentrations, and thus Se is incorporated into proteins (Läuchli 1993). Since interactions between N and S affected the uptake, distribution and redistribution of S compounds and the yield of all plant parts in barley (Eriksen et al. 2001; Eriksen & Mortensen 2002), nitrogen fertilization may indirectly also influence the uptake of Se in cereals.

Selenium concentration in ground water and lakes has been associated with Se fertilization (White et al. 1991; MacLeod et al. 1998). Excess soil Se can cause serious deformities in wildlife (Adams et al. 2003; Hamilton 2003) and thus leaching losses of applied Se should be kept at a minimum. Soil nitrate and selenate behave similarly with selenate being slightly better adsorbed than nitrate (McBride 1994). Applying N would eventually decrease the adsorption of soil selenate, making it more plant available but also more prone to leaching.

The objectives of the present study were (1) to investigate the effect of ammonium-nitrate application on protein and Se concentration in wheat and (2) to assess the potential loss of Se in drainage water after application of ammonium-nitrate and selenate at different growth stages to spring wheat.

MATERIALS AND METHODS

A greenhouse study was conducted using fine sandy loam, collected from Agriculture and Agri-Food Canada, Harrington, Prince Edward Island, with spring wheat (*Triticum aestivum* L. "Helena") as a test crop. Soil was sampled from the upper 20 cm, air dried and sieved (< 5 mm). The soil had a pH of 5.9 (Soil:water ratio of 1:2.5) and organic carbon content of 1.9 %. It was classified as a Humic-Ferric Podzol in the Canadian Classification System (Carter 1987). Total N and S contents in the soil were 1.0 and < 0.04 g kg⁻¹ soil, respectively. The processed soil (3.5 kg) was filled into circular polyethylene pots (diameter: 22cm, volume: 4.8 L). Three holes (6 mm diameter) were drilled in the bottom to ensure free drainage. Eight seeds in each pot were sown which after 10 days were thinned to 6 plants. Pots were arranged

randomly in a growth chamber. The temperature ranged from 20 °C during day (14 hour) to 16 °C at night with an irradiance rate of 475 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Nitrogen and selenate water solutions were prepared from NH_4NO_3 and Na_2SeO_4 forms, respectively. The experiment was a 6 x 2 factorial with 6 Se and 2 N. The Se treatments were: control (no Se added) and 0.01 mg Se kg^{-1} soil added at the following growth stages: at sowing (GS 00), tillering (GS 20) at 26 days, stem elongation (GS 30) at 59 days, head emergence (GS 50) at 72 days, and milking stage (GS 70) at 90 days according to the Zadoks scale (Zadoks et al. 1974). The two nitrogen treatments were: N1; all N (105 mg N kg^{-1} soil) applied at sowing and N2; split N application with 70 mg N kg^{-1} applied at sowing and 35 mg N kg^{-1} at stem elongation (GS 30). A basal dose of all other nutrients in water solution (pH 6.0) containing 20, 50, 10, 15, 8, 6, 0.25 and 0.3 mg kg^{-1} soil of phosphorus, potassium, S, calcium, magnesium, zinc, molybdenum and boron, respectively, was added to each pot. When N or Se was applied, the same amount of water (demineralized water (pH 5.9)) was applied to the other pots. The amount of water applied in the period from one phenological stage to the next one is presented in Table 1. Pots were watered regularly during the whole growing period by keeping the water content above 26 % (pot weight at 4.4 kg).

Table 1. Average millilitres of water/pot required at various growth stages (GS) to maintain >26 % water content.

Growth stage* (GS)	Day	Water used in period between GS	
		Total (ml)	Day (ml)
GS 00	0	1400	67
GS 20	26	2200	69
GS 30	59	1200	86
GS 50	72	1500	94
GS 70	90	700	39
Harvest	101		

*(Zadoks et al. 1974)

Plants were harvested at maturity (101 days) and stem length and number of stems and heads were counted prior to separating them into grain, straw, leaf and the rest of the spike (Head). All plant parts were dried at 60 °C, weighed, ground to pass a 1 mm sieve and stored for chemical analyses. The weight of 100 kernels for each treatment was also determined.

After harvesting the crop, pots were water saturated for one day and then leached with 3 litres of deionized water. Leachate was collected until drainage ceased and then acidified with HNO_3 and stored in refrigerated conditions (4 °C) until Se determination.

Soil organic carbon and total N and S in soil and total N in grain were determined by combustion gas analysis using a LECO CNS-1000 analyzer (LECO Corporation, St. Joseph, Michigan, USA). The total N concentration was multiplied by a factor of 6.25 for conversion to total protein.

The Se concentration in all plant parts was determined by the recently developed method of Govasmark and Grimmett (manuscript in preparation). In brief, 1 g sample of each plant part was digested in closed Teflon® microwave vessels using ultra-pure HNO_3 and 30 % hydrogen peroxide. Selenium in the digested solution was reduced to selenite by concentrated HCl, derivatized with 2,3-diaminonaphthalene, and then extracted into cyclohexane. The sample was then analyzed for Se on a high-performance liquid chromatograph equipped with a fluorescence detector.

The experiment included 4 replicate observations in separate pots for each factor combination. Statistical analysis was conducted by JMP release 5.0.1a, (SAS Institute Inc., Cary, NC, USA) at a significance level of 0.05. For comparison of means of the Se concentrations in different plant parts, plant yields, and Se in leachate between the N treatments N1 and N2, Student's t-test was performed. Total recovery of Se was calculated by

subtracting the control mean Se concentration from the treatment mean Se concentration and dividing by the amount of Se applied.

RESULTS

The total grain protein content of all treatments increased significantly from 11.1 ± 1.2 % in N1 to 13.8 ± 1.7 % in N2 treatments ($P < 0.001$) (Table 2). Although the total dry matter (DM) yield, grain yield and 100 kernel weights between treatments differed at different growth stages, the effects of neither N nor Se application were found to be consistently significant. The average number of kernels per head of all Se treatments decreased from 35.6 ± 4.7 in N1 to 32.9 ± 4.1 in N2 ($P = 0.042$). Differences in kernel numbers among Se treatments were significant only at GS 7. No significant differences in leaf weight, stem number, stem length, stem weight, head length, and head weight were found neither between N nor Se treatments (data not shown).

Table 2. Mean \pm standard deviation for plant biomass yield and various grain characteristics of spring wheat for all treatment combinations.

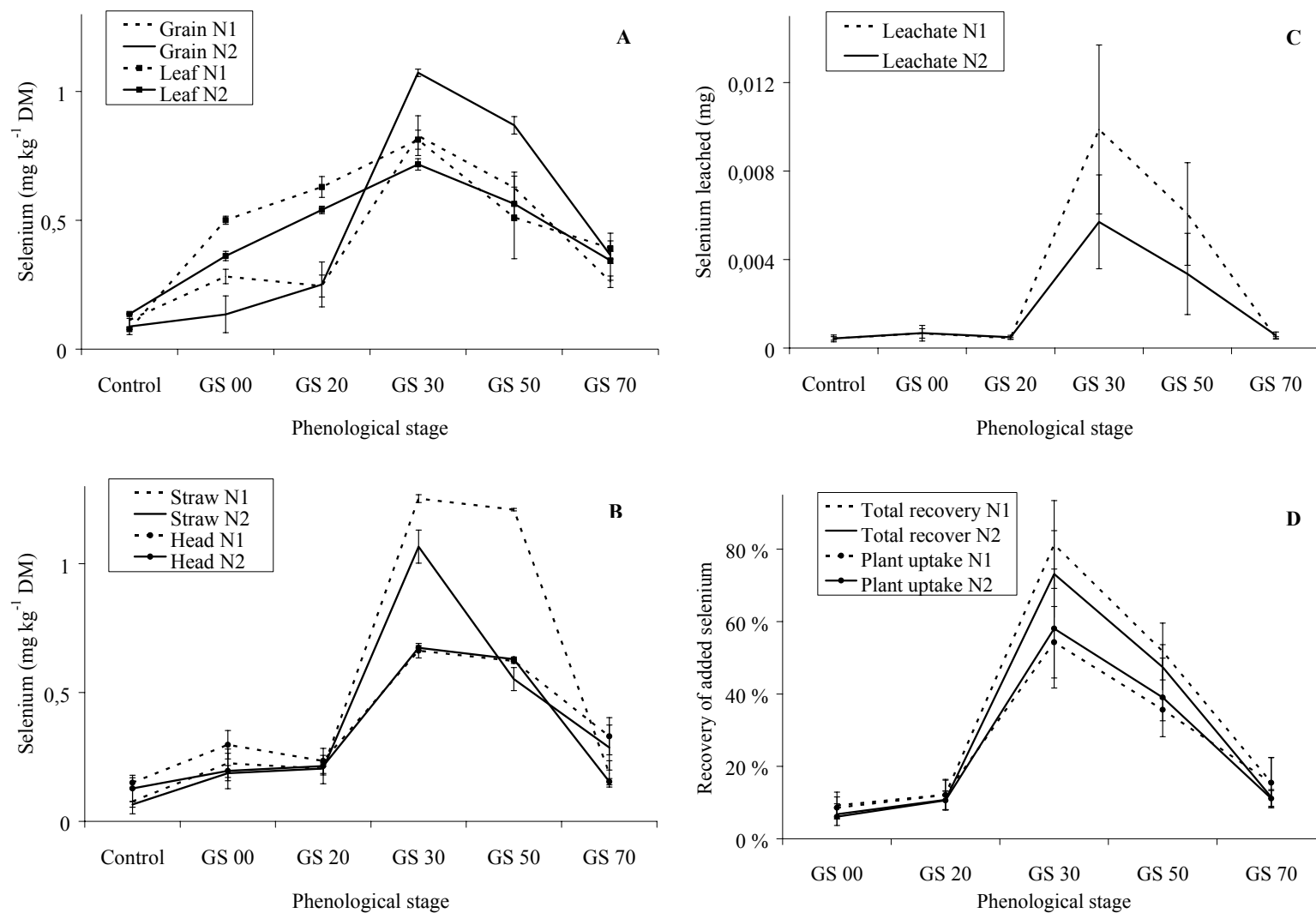
		Application of 0.01 mg Se kg ⁻¹ soil					
		Control	GS 00	GS 20	GS 30	GS 50	GS 70
Total Plant Yield (g/pot)	N1 ^a	18.7 \pm 5.7 c	16.5 \pm 5.1 c	20.3 \pm 3.8 abc	27.1 \pm 7.4 a	19.3 \pm 1.9 bc	26.7 \pm 8.0 ab
	N2 ^b	18.5 \pm 5.7 c	20.3 \pm 4.8 abc	20.0 \pm 4.6 abc	22.8 \pm 5.4 abc	21.7 \pm 4.7 abc	17.9 \pm 1.3 c
Grain Protein (%)	N1	11.1 \pm 1.9 def	11.4 \pm 1.4 def	10.7 \pm 1.1 ef	10.5 \pm 0.3 f	11.7 \pm 0.7 cdef	11.1 \pm 1.4 def
	N2	13.0 \pm 2.3 bc	12.5 \pm 2.3 bcd	11.7 \pm 1.1 cde	13.5 \pm 1.7 bc	14.6 \pm 1.3 ab	16.0 \pm 0.5 a
Grain Yield (g/pot)	N1	8.2 \pm 2.1 bc	7.4 \pm 2.2 c	8.8 \pm 1.6 abc	11.1 \pm 3.0 ab	8.7 \pm 0.8 abc	11.7 \pm 3.0 a
	N2	7.9 \pm 2.5 c	8.2 \pm 2.0 bc	8.1 \pm 1.6 bc	9.9 \pm 1.8 abc	9.5 \pm 2.2 abc	7.9 \pm 0.6 c
100 Kernel Weight (g)	N1	4.8 \pm 0.2 ab	4.5 \pm 0.3 abc	4.9 \pm 0.3 a	4.9 \pm 0.3 a	4.4 \pm 0.1 bc	4.9 \pm 0.4 ab
	N2	4.5 \pm 0.5 abc	4.6 \pm 0.5 abc	4.6 \pm 0.4 abc	4.6 \pm 0.4 abc	4.5 \pm 0.4 abc	4.2 \pm 0.3 c

^aN1=105 mg N kg⁻¹ soil applied at GS 00. ^bN2=70 and 35 mg N kg⁻¹ soil applied at GS 00 and GS 30 respectively. Means of the treatment with the same letter are not significantly different at $P < 0.05$.

The Se concentration in grain, leaf, straw and head increased with Se application in both N treatments, having the highest Se concentration at growth stage 30 (Fig. 1 A-B). The grain Se concentration was highest ($P < 0.05$) in the N1 treatment of N with Se applied at GS 00. The grain Se concentration was or tended to be higher in the N2 treatment of N with Se applied at GS 30, GS 50 and GS 70 stages. On the other hand, leaf Se concentration was or tended to be higher in the N1 treatment of N when Se applied at GS 00, GS 20, GS 30 and GS 70 stages. The straw Se concentration was higher in the N1 treatment of N when Se was applied at GS 30 and GS 50. The head Se concentration tended to be higher in the N1 treatment of N with Se applied at GS 00 or at GS 70.

The Se leaching losses increased with Se application at GS 30 and GS 50 in both N treatments of N with minor changes at other growth stages (Fig. 1 C). The Se losses tended to be higher in the N1 treatment with selenate applied at GS 30 and GS 50 and the loss was in

Figure 1. Selenium concentration in different plant parts (A-B), leachate water (C) and plant uptake and recovery of applied Se (D) as it relates to nitrogen application method and timing of 0.01 mg Se kg⁻¹ soil selenium application.



Error bars represent standard deviation. Note scale difference between figures. N1=105 mg N kg⁻¹ soil applied at GS 00. N2=70 and 35 mg N kg⁻¹ soil applied at GS 00 and GS 30 respectively.

the N2 treatment highest with selenate applied at GS 70 (Fig. 1 D). The highest Se uptake by plants and Se loss in leachate water occurred at GS 30 in both N treatments. The total Se recovery (plant uptake and leaching loss) tended to be higher in the N1 treatment of N whereas total plant Se uptake tended to be higher in the N2 treatment of N at GS 30 and GS 50.

DISCUSSION

The grain Se concentration in the control treatment did not reach the level required for human food (Gupta & Gupta 2002), implying that the soil Se was not able to provide sufficient Se to increase the grain Se concentration above $0.1 \text{ mg kg}^{-1} \text{ DM}$. The grain, leaf, straw and head Se concentration increased with selenate supplementation, showing that selenate is a good Se source to increase the total plant Se concentration. These results bear similarity to those reported in the literature (Stephen et al. 1989; Singh 1994). Delaying the Se application to growth stages of GS 30 to GS 50 is a better strategy to increase the grain Se concentration to desired level.

The main intention of applying Se to wheat for human consumption is to increase the grain Se concentration. Grain may get its Se either from root absorption Se after anthesis or through translocation of Se accumulated in vegetative tissue before or during anthesis. The total plant Se uptake was highest in treatments which most recently had received most N and thus, potentially had the highest soil N concentration. The N1 treatment had potentially higher soil N concentration at GS 00 and GS 20, whereas the N2 treatments had higher soil N concentration at GS 30 and GS 50 growth stages. Ammonium-nitrate application may affect selenate availability in several ways. (I) Nitrate applied together with selenate may decrease the soil Se adsorption because they have very similar soil adsorption mechanisms (McBride 1994), and thus making selenate more plant available. (II) Nitrogen is known to increase root growth, making roots finer and thus increasing the total root surface area (Marschner et al. 1986). (III) Enhanced root growth increases the excretion of root exudates resulting into increased number of rhizosphere micro-organisms in the rhizosphere. In root exudates of Indian mustard (*Brassica juncea* L.) heat-labile compounds were found which increased the Se accumulation into plant tissues (de Souza et al. 1999). (IV) Increasing N content in the plant enhances the protein concentration and thus raises the sink strength for Se (Marschner 1995). Selenate assimilation leads to the synthesis of selenocysteine and selenomethionine in wheat which are readily incorporated into proteins (Läuchli 1993).

The other source of grain Se is the Se which is re-translocated from vegetative plant parts to the grain. Plant protein bound Se behaves similar to protein bound S, and is not easily transported within the plant (Marschner 1995). However, the mobility of S in plant parts depends on the phenological stage of the plant part. For example, it is reported that up to 75 % of the S taken up at a later stage of leaf expansion in barley was re-exported to developing leaves (Adiputra & Anderson 1992). Similar observations were made by Eriksen et al. (2001). They also reported that the redistribution of S was dependent on the N status of the plant tissue. Straw developed after GS 30 and the N2 treatment was expected to have a higher Se concentration, but opposite was true as straw Se concentration was higher in the N1 treatment. This may suggest a relatively higher proportion of Se taken up at GS 30 and GS 50 in the N2 treatment was redistributed to grain as compared to N1 treatment. The straw Se concentration decreased from GS 30 to GS 50 because of lower total plant Se uptake, but probably also due to a higher re-translocation to the grain at GS 50 than at GS 30.

The grain protein represents a sink for Se in the plant, and the sink strength has been reported to influence the nutrient accumulation in oat (*Avena sativa*) plants (Peterson &

Rendig 2001). Higher protein concentration in the N2 treatment of the present study represents a stronger sink for Se deposition in leaf and straw, which could lead to increased re-translocation of Se to grain.

The total Se uptake by plants along with leaching losses (total recovery) in the present study was higher in the N1 treatment, even when the total plant Se uptake was higher in the N2 treatment. This suggests that Se leaching losses were higher in the N1 treatment. Within the N treatments, leaching losses of Se varied considerably. Although it is difficult to understand why the highest Se leaching losses occurred in the treatment with the highest plant Se uptake, it may be related to an increased mobilization of Se in soils by increased root activity and microbial growth (Fig. 1). Alternatively selenium leaching losses may be related to the time elapsed between the Se application and the leaching procedure at the end of the experiment. A longer reaction time between the soil and applied selenium may result in decreased Se solubility. The lower Se leaching observed following the last two applications of Se (GS 50 and GS 70) may be related to the lower amounts of water applied to these treatments following Se application (Table 1). It is possible that 3 litres of leaching water was insufficient to elute the selenium from the GS 50 and GS 70 applications. This observation would require further investigation into the leaching dynamics of sodium selenate in order to better explain the mechanisms at work. It is also interesting that applying 25 % of the ammonium-nitrate at GS 30 reduces the leaching of Se, and thus reducing the environmental impact of Se applied.

The main finding of the present investigation is that splitting of N fertilisation into two doses; one at sowing and another at tillering growth stage enhance the protein content and thereby the Se concentration in grain but it reduces the leaching Se losses. Furthermore, Se application at later growth stages (GS 30-70) is better than Se applied at sowing or early growth stages (< 20) in raising the Se content in grain.

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