Iron Studies in Man using Instrumental Neutron Activation Analysis and Enriched Stable Activable Isotopes

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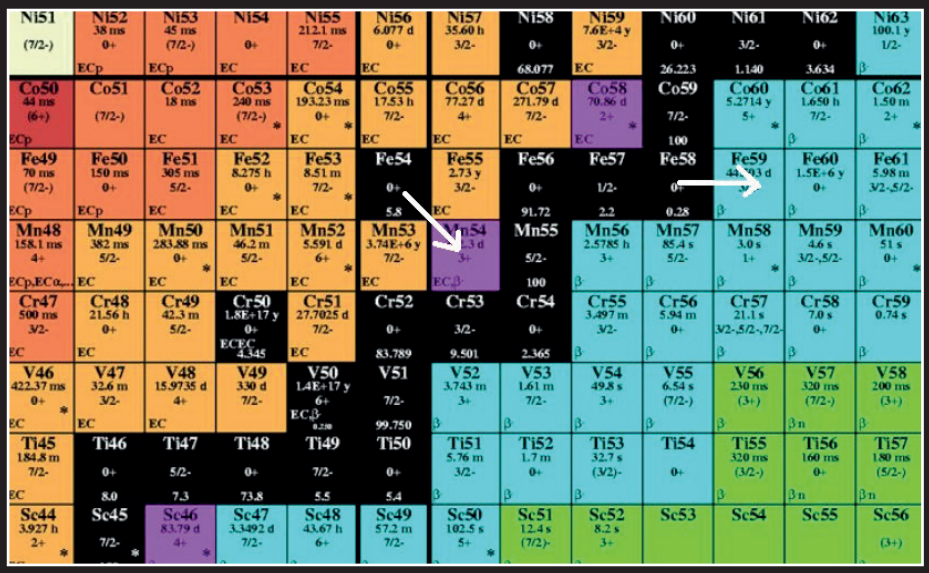

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Radiation and Isotopes for Health

Iron Studies in Man using Instrumental Neutron Activation Analysis and Enriched Stable Activable Isotopes

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Door

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dedicated to my parents

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Table of Contents

1 Introduction

1.1 Iron

Trace elements are elements, other than O, C, H and N, which play an important role in keeping the body working effectively and therefore vital for maintaining health. They are present in small amounts and they are classified according to the WHO into three groups: essential trace elements, probably essential trace elements and potentially toxic elements [1].

Iron (Fe) is an essential trace element for oxidation-reduction reactions, catalysis, bioenergetics and DNA synthesis [2-4]. Iron is absorbed in the duodenum and upper jejunum, transported in duodenal cells by a protein called divalent metal transporter 1 (DMT1) and then released via ferroprotein into the blood stream (see Figure 1). In the blood stream iron is bound by a transport glycoprotein named transferrin, which delivers iron to all other tissues. DMT1 is also used for the uptake of other trace metals; some essential like manganese, copper, cobalt, zinc and some potentially toxic like cadmium and lead.

In food iron is found in two different forms: haem iron (Fe^{2+} ferrous iron) from animal products, and non-haem iron (Fe^{3+} ferric iron) from plant products [3]. The body absorbs haem iron better than non-haem iron. The composition of food may also affect iron absorption: some food rich in citrate and ascorbate (citrus fruits for example) can form complexes with iron that enhance absorption, while tannin in tea can decrease absorption.

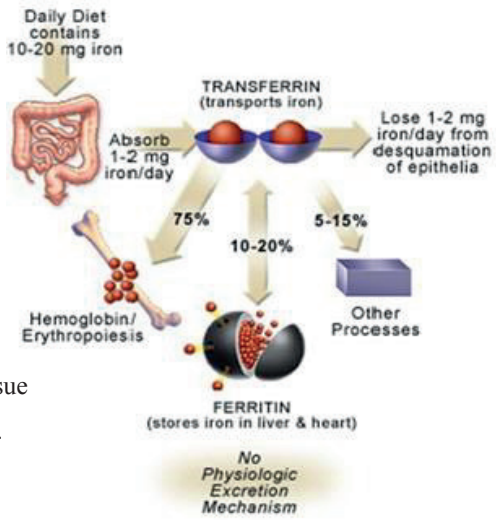
The adult body contains about 4 grams of iron [5]. The greatest portion (about 2-3 g in humans) is distributed in the haemoglobin of red blood cells, developing erythroid cells (bone marrow) and serves in oxygen transport. About 0.5 to 1 g of iron is stored in ferritin and hemosiderin.

Due to the absence of a defined pathway to excrete excess iron, regulation of iron homeostasis is highly required to avoid iron deficiency and iron overload. Iron homeostasis is regulated by intestinal iron absorption and intracellular iron handling. Both processes are under genetic control as well as influenced by ‘exogenous’ factors such as hypoxia and inflammation.

Although a deficiency of iron is harmful, an excess of iron is highly toxic related to the ability of iron to generate reactive oxygen species via the Fenton reaction. A chronic overload

of iron causes damage to organs increasing the risk of liver cancer, heart failure, vascular diseases and diabetes mellitus.

Transferrin transports Fe to the tissues: Increased need for Fe results in increase of transferrin



Ferritin reflects the tissue stockpile of Fe; decreases in case of anemia

Figure 1 Schematic representation of the Iron pathway in man.

1.2 Iron related disorders

Iron related disorders leading either to exhausted or to overloaded iron stores are extremely common in all parts of the world. The knowledge about these disorders has expanded significantly after the discovery of hepcidin, the regulator hormone of iron homeostasis [6,7]. Increased iron requirements, limited external supply, and increased blood loss may lead to iron deficiency (ID) and anaemia. In chronic inflammation, the excess of hepcidin decreases iron absorption and prevents iron recycling, resulting in iron restricted erythropoiesis, despite normal iron stores (functional iron deficiency), and finally anaemia of chronic disease (ACD). Low hepcidin expression may lead to iron overload [8]. Because of the various functions of iron in the body, iron plays a role in other disorders than anaemia and hemochromatosis. These include amongst others inflammatory and infectious diseases as well as (neuro) degenerative disorders (oxidative stress and iron overload).

Deficiency of iron causes both mortality and disability worldwide. In developed countries most cases of iron deficiency can be attributed to a loss of iron while in underdeveloped nations a deficiency in the intake of micronutrients is the main cause of anaemia. In case of malnutrition a deficiency of other micronutrients such as zinc is very likely, and such a deficiency may therefore be helpful in the differential diagnosis of iron deficiency anaemia (see chapter 9).

1.3 Methods for measuring iron and the use of iron isotopes

Since 1940 the spectrophotometer has been used in many studies on the measurement of trace elements including iron in blood . Different reagents have been used in this technique [9-15]. Later on other techniques have been developed and applied to measure iron including atomic absorption spectrometry (AAS) [16-24], inductively coupled plasma mass spectrometry (ICP-MS) [25-27], inductively coupled plasma atomic emission spectrometry (ICP-AES) [28], inductively coupled plasma optical emission spectrometry (ICP-OES) [29,30], X-ray fluorescence spectrometry (XRF) [31], instrumental neutron activation analysis (INAA) and radiochemical neutron activation analysis (RNAA) [32-36].

Most of these techniques measure total iron. In some clinical experiments, however, especially when absorption and distribution are studied, iron isotopes are used. In early research on iron metabolism the iron radioisotopes ^{55}Fe and ^{59}Fe were applied [37,38]. The advantages of radioisotopes in research is that only small amounts of the iron label are required and instrumentation for measurement of iron radioisotopes in biological samples is available in many research facilities. Moreover, measuring the gamma-rays emitting ^{59}Fe retained in the body after ingestion can be performed without sampling if a whole body counter is available . However, hazards resulting from the ionizing radiation reduce the use of radioisotopes in humans especially in infants, children, and woman of child-bearing status. Therefore techniques have been developed using enriched stable isotopes and these are generally considered safe and versatile to study mineral absorption, bioavailability and metabolism.

During the past decades, there has been a significant growth in the interest of nutritionists in the use of this technique and concerted efforts have been directed towards the practical implementation [39-45]. The use of a stable isotope of an element as a tracer in metabolic research started in 1935, when Schoenheimer and Rittenberg used deuterium to study intermediary metabolism in laboratory animals and humans [46]. The first use of an iron

stable isotope as a tracer in human metabolism was published in 1963. The plasma clearance of stable ^{58}Fe was compared to that of radioactive ^{59}Fe using neutron activation analysis (NAA) [47]. Approaches to asses bioavailability of iron using enriched stable iron isotopes include faecal isotope recovery, plasma isotope appearance and erythrocyte iron incorporation. The last one is now considered the method of choice; within 12-14 days about 80% of the absorbed iron will incorporate into the erythrocytes [48].The primary drawbacks of the use of enriched stable isotopes are the high costs of their production, the complexity and costs of analysis, and lack of access to suitable measurement techniques.

There are two basic techniques for measuring stable isotopes: mass spectrometry (MS) and instrumental neutron activation analysis (INAA). Among the mass spectrometry methods, thermal ionization mass spectrometry (TIMS) and multiple collector inductively coupled plasma mass spectrometry (MC-ICP-MS) are the methods mostly used [48]. The choice of a method depends on the availability of the instrument, the isotopes of an element to be measured, and the analytical sensitivity and precision required. Both INAA and MC-ICP-MS techniques have their pro's and con's. MC-ICP-MS is a very sensitive technique and available in advanced research facilities. However there are technical limitations by the need of processing small samples that have been brought into a solution; MC-ICP-MS is not quantitative in terms of the total mass of iron (which implies the need for additional ICP-MS) and, for some elements spectroscopic interferences and non-spectroscopic interferences limit the applicability[49] .

INAA is not as widely applied as mass spectrometry because of its requirement of access to a nuclear analytical facility, including a research reactor. It has, however, several attractive analytical advantages. Since it is a non-destructive method, there is no need to convert and/or dilute a sample into a suitable solution prior to analysis –with inherent risks of contamination or element loss. This is especially attractive when using biological material containing low amounts of trace elements such as tissues, nails and bones. Moreover, its specificity and selectivity for iron is close to 100%. There are no unresolvable gamma-ray spectrum interferences and the contribution from interfering nuclear reactions is statistically insignificant when analysing human biota.

1.4 Studies on iron metabolism

Despite progress made during the last years in the field of iron metabolism, little is known about the extent to which the gastro-intestinal tract is able to increase or decrease its capacity

to absorb iron in various pathological conditions [50-53]. This is due to the lack of adequate tests to measure iron absorption accurately and reliably especially in various in vivo situations. The use of ^{59}Fe is restricted because of its side effects and the application of enriched stable iron (^{54}Fe, ^{56}Fe, ^{57}Fe and/or ^{58}Fe) has not been introduced in clinical studies yet apart from some nutritional ones. Most of these studies were done in either healthy subjects or children, and data in adults with various disorders are scarce. Furthermore in many studies iron is measured in plasma, while the iron concentration in erythrocytes might be a more accurate marker for iron deficiency. So there is a need for a safe and standardized method to study iron metabolism in both normal and pathological conditions.

Such a method should ideally be able to give information on the various parts of iron metabolism. This implies the ability to measure iron in other biological materials than plasma such as faeces, urine, and tissues, but also in food and supplements. Because the materials in which iron has to be measured may differ considerably in size (from meals to small tissue samples) and the distribution of iron in the samples may vary greatly, a technique, that is able to overcome these problems, should be welcomed.

In this respect INAA is a technique that meets many of these needs (see feasibility experiments described in chapters 6 and 8). Another advantage of INAA concerns commutability. Many isotopic (certified) reference materials used for measuring iron isotopes by mass spectrometric methods are synthetic. They do not represent the biological matrix and this may raise commutability problems. Such commutability problems are almost absent in INAA due to its nuclear physics character, but may have more impact to the trueness of MS measurements due to the isobaric interferences.

The use of INAA for measuring trace element levels in human biota was pioneered in the 1970s by e.g. Versieck and Cornelis [54-56]. In the following years, the technique never reached a mature position in the clinical field, partly due to the need for access to a research reactor, partly due to the fact that many INAA laboratories concentrated their attention to methodological development rather than to collaborative applications. In the meantime new techniques, such as ICP-MS, not necessitating a reactor facility, emerged and proved to be useful for most research questions. But INAA has matured with metrology now being fully established [57], with more sensitive detectors available and expansion of the technique towards the analysis of very large test portions –up to kilogram size- which avoids the need for sample size reduction and care for the representativeness of small test portions.

So, it may be stated that the current methodological opportunities of INAA have not fully been evaluated for clinical research, which hypothesis is the main basis of the research, described in this thesis.

The applicability of INAA was additionally evaluated for trace elements in patients with severe iron deficiency in Sudan. In this country, several ICP instruments (e.g. ICP-OES, ICP-MS) have been implemented as part of special development programmes. In developing countries, however, the performance of these instruments is seriously affected by e.g. not optimal laboratory and environmental conditions and frequent power interruptions. As a result, the practice has shown that often these instruments work only well for 1-2 years and once instrumental problems occur after this period, there is no budget for maintenance, spare parts and repair; nor are company experts nearby. There have been thoughts for realizing Sudan's first nuclear research reactor, which would provide also INAA capability in the country. Instrumentation for neutron activation analysis such as gamma-ray spectrometers is less vulnerable for external factors as mentioned in the above, needs hardly to none maintenance and can be used 10-15 years without severe problems or costly repair.

1.5 Objective of PhD research

The main aim of this thesis is to evaluate the applicability of INAA as a research tool to study parts of iron metabolism in men. Iron is not only measured in blood and urine, but also in erythrocytes as well as in food making use of the advantage of INAA to measure in all sorts and quantities of material without complicated preparation steps. These experiments and studies pave the way for true mass balance studies in which the metabolic fate of one or more elements can be studied within one individual. Since the use of enriched stable isotopes is a safe and promising way to study the metabolism of various elements in both normal and pathological conditions, a pharmaceutical formula of ^{58}Fe had to be developed and its applicability tested in a clinical setting. The use of this isotope and its measurement by INAA will not only increase our knowledge of the iron handling by the gastro-intestinal tract in pathological conditions resulting in a better and more adequate medical treatment, but may also be of value for the preparation and use of adequate food and supplements in case of illness. The position of INAA in this kind of research was further evaluated by making a direct comparison with MC-ICP-MS ,the currently most used method for measuring stable Fe isotopes .

1.6 Thesis outline

Chapter 2 This chapter is an introduction to iron metabolism and iron related disorders; it describes the distribution of iron in the body, its absorption and regulation in iron homeostasis. The different iron related disorders, their current diagnostic steps and treatment modalities are also described in this chapter. Since some of the disorders may result from dietary deficiencies, other elements may have synergistic or antagonistic effects to iron. In this respect, the relation of especially Cu and Zn to iron metabolism is mentioned.

Chapter 3 gives an overview of isotopic techniques to assess iron bioavailability in humans, both with (isotopically enriched) stable and radioactive isotopes, outlining the advantages and disadvantages of their use in human studies. The three primary methodological approaches for isotopic studies of the iron bioavailability in humans are presented: faecal recovery of the ingested isotope, plasma appearance of the orally ingested isotope and red blood cell incorporation of the iron isotope. The basic concepts of administration of isotopically enriched stable isotopes are described, including their chemical form, dose selection and distinctions between intrinsic versus extrinsic labelling.

Chapter 4 describes in detail the basic principles of (instrumental) neutron activation analysis (INAA) and inorganic mass spectrometry, the two analytical techniques for measuring stable iron isotopes. Special attention is given to MC-ICP-MS since this form of mass spectrometry has been tested in this research project because of its supreme precision in mass ratio measurements. Several analytical characteristics of INAA and MC-ICP-MS have been evaluated for measurement of iron and isotopic ratios, like specificity, selectivity, degree of accuracy, uncertainty of measurement and minimum detectable amounts.

Chapter 5 presents an experimental assessment of the practicability of INAA and MC-ICP-MS for the measurement of $^{58}Fe/^{54}Fe$ isotopic ratio in blood. A clinical experiment was conducted in which ^{58}Fe was given in enriched form via an oral supplement to both anaemic and hemochromatosis patients as well as to healthy controls. This required the preparation of a ^{58}Fe-labeled $FeSO_4$ pharmaceutical supplement. Once the blood samples have been collected, INAA and MC-ICP-MS set entirely different requirements to the preparation of the test portion, being much more critical in MC-ICP-MS than in INAA because of the various chemical processing steps and its inherent sensitivity to commutability.

Chapter 6. In this chapter the results of a number of experiments are described where total iron is measured with INAA not only in blood, but also in urine and faeces before and after iron supplementation both orally and intravenously in patients with iron deficiency anaemia. It has been studied if INAA is able to detect small changes in iron concentrations in the various biomaterials and whether it is therefore an adequate instrument in case mass balance studies with iron are applied. The experiments form the preparatory step of the application of ^{58}Fe in clinical studies such as mass balance studies.

Chapter 7 describes the measurement by INAA of the iron concentrations in total blood, plasma and erythrocytes of patients with iron deficiency anaemia and hemochromatosis as well as samples taken from healthy controls. The measurement results are compared to literature values which are mostly derived from studies with techniques other than INAA.

Chapter 8. Trace element analysis is usually performed on samples in the range of tens to hundreds of mg. Analysis of larger samples can be preferable e.g., when a representative 100 mg sample is difficult to obtain, when the distribution of the trace element in a sample is uncertain or when a sample must remain intact. Instrumental neutron activation analysis has all the potentials to analyse, even with adequate accuracy, large samples in the kilogram range. Such sample sizes become relevant if e.g. dietary intake collected over several days (and faecal excretion) has to be analysed as incomplete homogenization is circumvented by large sample INAA.

The feasibility of measuring Fe in the complete dietary intake, collected via the double portion approach using large sample neutron activation analysis is described in this chapter.

Chapter 9 describes a study in which both zinc and iron were measured in blood samples of 22 anaemic patients from Sudan and 17 anaemic patients from the Netherlands. Iron was measured in the blood samples of the Sudanese patients with INAA as well as with ICP-OES –being the available analytical technique in Sudan. Zinc and iron in blood of the Dutch patients were measured with INAA. The simultaneous measurement of zinc was done since in the Netherlands the majority of cases of iron deficiency is the result of blood loss with a normal nutritional status while in Sudan a lack of zinc in food contributes to the anaemia. Zinc may therefore be an indicator of nutritional deficiency in case of iron deficiency anaemia.

Chapter 10 gives an overview of all experiments and studies performed. It highlights the most significant results, and discusses the position of INAA when used to study the metabolism of trace elements and metals in both normal and pathological clinical conditions. Recommendations and suggestions for future research are given.

To make sure that each chapter can be read separately, some information is repeated throughout the thesis.

1.7 References

[1] A. Mehri and R. F. Marjan. Trace elements in human nutrition: A review. Int. J Invest; (2013), 2(3): 115-128.

[2] R. Crichton. Inorganic Biochemistry of iron Metabolism.. Second Edition. John Wiley & Sons, LTD, 2001.

[3] O. Han. Molecular mechanism of intestinal iron absorption. Metallomics, (2011), 3, 103-109.

[4] R. Crichton. Iron Metabolism. Wiley. (2009). ISBN: 978-0-470-01028-0.

[5] M. E. Conrad, and J. N. Umbreit. Pathways of iron Absorption. Blood Cells, Molecules, and Diseases. (2002), 29 (3): 336-355.

[6] E. Rossi. Hepcidin - the Iron Regulatory Hormone. Clin. Biochem. Rev. (2005), (26): 47-49..

[7] G. Rishi, D. F. Wallace and V. N. Subramaniam. Hepcidin: regulation of the master iron regulator. Biosci. Rep. (2015) / 35 / art:e00192 / doi 10.1042/BSR20150014.

[8] J. F. Collins, M. Wessling-Resnick, and M. D. Knutson. Hepcidin Regulation of Iron Transport[1–3]. J. Nutr. (2008), 138: 2284–2288.

[9] W. N. M. Ramsay, The determination of iron in blood plasma or serum, Biochem J. 53(2) (1953) 227-231.

[10] W. N. M. Ramsay, The determination of iron in blood plasma or serum, Clin. Chim. Acta. 2 (1957) 214-220.

[11] D'A. Kok and F. Wild, Serum iron determination, J. Clin. Path. 13 (1960) 241-245.

[12] H. V. Connerty and A. R. Briggs, New method for the determination of whole-blood iron and hemoglobin. Clin. Chem. 8 (1962) 151-7.

[13] D. J. Johnson and H. L. Williams. Rapid spectrophotometric assay of serum iron, Chlin. Chim. Acta, 189(2) (1990) 199-203.

[14] A. Jafarian-Dehkordi, L. Saghaie , N. Movahedi, A new spectrophotometric method for direct determination of iron (III) in serum, DARU Journal of Pharmaceutical Sciences, Vol. 16(2) (2008), 76-82.

[15] U. Abu Mohsen, K. Abu Elaish, M. Issa, Determination of Iron in Blood Serum by Spectrophotometric and Atomic Absorption Methods as a Comparative Study, Cukurova Med.l J.,38 (3) (2013) 358-364.

[16] D. O. Rodgerson and R. E. Helfer, Determination of iron in serum or plasma by atomic absorption spectrophotometry, Clin. Chem. 12(6) (1966) 338-349.

[17] H. T. Delves, G. Shepherd and P. Vinter, Determination of Eleven Metals in Small Samples of Blood by Sequential Solvent Extraction and Atomic-absorption Spectrophotometry, Analyst 96 (1971) 260-273.

[18] J. Lotterle, Measurement of the hemoglobin content of fresh blood by atomic-absorption-spectrophotometric iron determination, Z Rechtsmed 85(4) (1980) 283-8.

[19] S. Lugowski, D. C. Smith, and J. C. van Loon, The determination of Al, Cr, Co, Fe, and Ni in whole blood by electrothermal atomic absorption spectrophotometry, J. Biomed.l Mat. Res., 21 (1987) 657-671.

[20] Z. A. de Benzo, R. Fraile , C. Gomez and N, Carrion. Determination of serum iron by aas: deproteinization versus direct analysis. Clin. Chim. Acta, 197 (1991)141-148.

[21] F. Shah , T. Gul Kazi , H. I. Afridi , J. A. Baig , S. Khan , N. F. Kolachi , S. K. Wadhwa , A. Shah, Environmental exposure of lead and iron deficit anemia in children age ranged 1–5 years: A cross sectional study. Sci. Tot. Env. 408 (2010) 5325–5330.

[22] S. F. A. Ali, S. U. Mehdi, M. Asif, S. N. U. Hassan and E. H. Mirza, Detection of Iron and Manganese Concentrations in Human Biological Fluid with Flame Atomic Absorption Spectroscopy (FAAS), International Conference on Bioscience, Biochemistry and Pharmaceutical Sciences (ICBBPS'2012) Penang, Malaysia.

[23] J. Kaluza, D. Madej, A. Brzozowska, The effect of iron and zinc supplementation and discontinuation of this practice on iron and zinc level in tissues in rats fed deficient diets, J. Trace Elem.Med.Biol., 27 (2013) 334–338.

[24] S. S. Najim, Determination of iron in human serum samples of thalassaemia patients by flame atomic absorption. J. Basrah Res. (Sciences), 40(1) (2014), 102-109.

[25] C. S. Mun˜iz, J. M. M. Gayo´n, J. I. G. Alonso and A. S. Medel, Accurat determination of iron, copper and zinc in human serum by isotope dilution analysis using double focusing ICP-MS, J. Anal. At. Spectrom., 14 (1999) 1505–1510.

[26] C. P. Case, L. Ellis, J. C. Turner, and B. Fairman, Development of a routine method for the determination of trace metals in whole blood by magnetic sector inductively coupled plasma mass spectrometry with particular relevance to patients with total hip and knee arthroplasty, Clin. Chem. 47(2) (2001) 275–280.

[27] G. Li, J. D. Brockman, S.W. Lin, C. C. Abnet, L. A. Schell and J. D. Robertson, Measurement of the trace elements Cu, Zn, Fe, and Mg and the ultra-trace Elements Cd, Co, Mn, and Pb in limited quantity human plasma and serum samples by inductively coupled plasma-mass spectrometry, Am. J. Anal. Chem., 3 (2012) 646-650.

[28] P. Mamatha. Analyis of human serum with various cataracts by Inductively Coupled Plasma Atomic Emission Spectrometry by using different digession proceduers, Int. J. Pharm. Res. Dev., 3(6) (2011) 42 – 49.

[29] C. Prohaska, K. Pomazal and I. Steffan, Determination of Ca, Mg, Fe, Cu, and Zn in blood fractions and whole blood of human by ICP-OES, Fres. J. Anal Chem, 367 (2000) 479-484.

[30] I. O. Olabbanji, E. A. Oluyemi, F. O. Fatoye and J. C. Ngila, Elemental composition of blood and hair of mentally-ill patients using ICP-OES techniques, Int. J. Biol. Chem. Sci., 5 (2) (2011) 663-679.

[31] E. A. H. Caraballo and L. M. M. Parra, Direct analysis of blood serum by total reflection X-ray fluoresence spectrometry and application of an artificial neural network approach for cancer diagnosis, Spectrochim. Acta Part B 58 (2003) 2205-2213.

[32] K. Fritze and R. Ropertson, Instrumental and radiochemical neutron activation analysis techniques for protein bound trace metals in human serum, J. Radioanal. Chem. 1 (1968) 463-473.

[33] N. I. Ward and D. E. Ryan, Multi-element Analysis of blood for trace metals by neutron activation analysis, Anal. Chim. Acta, 105 (1979) 185-197.

[34] K. Kasperek, J. Kiem, G. V. Iyengar and L. E. Feinendegen. Concentration Differences between serum and plasma of the elements cobalt, iron, mercury, rubidium, selenium and zinc determined by Neutron Activation Analysis, Sci.Tot.Env., 17 (1981)133-143.

[35] W. Maenhaut, L. Dereu and U. Tomza. The determination of trace elements in commercial human serum albumin solutions by Proton-Induced X-ray Emission Spectrometry and Neutron Activation Analysis. Anal. Chim. Acta, 136 (1982) 301-309.

[36] L. Xilei, D. Van Renterghem, R. Cornelis and L. mees. Radiochemical Neutron Activation Analysis for thirteen trace metals in human blood serum by using inorganic iron exchangers. Anal. Chim. Acta, 211 (1988) 231-241.

[37] P. F. Hahen, W. F. Bale, E. O. Lawrence, and G. H. Whipple Radioactive Iron and Its Metabolism in Man. J. Exp. Med., (1939)69, 739-753.

[38] P. F. Hahen, J. F. Ross, W F. Bale , and G. H. Whipple. The Utilization of Iron and The Rapidity of Hemoglobin Formation in Anemia Due to Blood Loss. J. Exp. Med., (1940) 71, 731-736.

[39] S. J. Fairweatter-Tait, and J. Dainty. Use of Stable Isotopes to Assess the Bioavailability of Tace Elements: A review. Food Add. Contam., (2002),19 (10): 939-947.

[40] J. R. Turnlund. Bioavailability of Dietary Minerals to Human: The Stable Isotope Approach. Critical Review in Food Sci. Nutr., (1991),30 (4) 387-396.

[41] J. R. Turnlund. Mineral Bioavailability and Metabolism Determined by Sable Isotope Tracers. J. Anim. Sci, (2006), 84, E73-E78.

[42] J. C. King, W. L.Raynolds, and S. Margen. Absorption of Stable Isotopes of Iron, Copper, and Zink During Oral Contraceptive Use. Am. J Clinical Nutr., (1978), 31, 1198-1203.

[43] M. Janghorbani, and V. R. Young. Use of Stable Isotopes to Detremine Bioavailability of Minerals in Human Diets Using the Method of Fecal Monitoring. Am. J. Clin Nutr., (1980), 33: 2021-2030.

[44] U. Ulusoy, and J. E.Whitley, Determination of Intestinal Uptake of Iron and Zinc Using Stable Isotopic Tracers and Rare Earth Markers. Nutr. Res., (1999),19 (5): 675-688.

[45] M. Janghorbani, B. TG. Ting, and S. J. Fomon, Erythrocyte Incorporation of Ingested Stable Isotope of Iron (^{58}Fe). Am. J Hematol., (1986), 21: 277-288.

[46] R. Schoenheimer and D. Rittenberg. Deuterium as an indicator in the study of intermediary metabolism. Science, (1935) Aug 16;82(2120):156-7.

[47] J.T. Lowman and W. Krivit. New in vivo tracer method with the use of nonradioactive isotopes and activation analysis. J. Lab Clin. Med, (1963), Jun;61:1042-8.

[48] Assessment of iron bioavailability in human using stable iron isotopes techniques. IAEA human heath series No. 21. International Atomic Energy Agency, Viena, 2012.

[49] K. E. Jarvis, A. L. Gray and R. S. Houk Handbook of Inductively Coupled Plasma Spectrometry. (2003), ISBN 0-9544891-0-1.

[50] A. von Drygalski and J. W. Adamson. Iron Metabolism in Man. J. Par. Ent. Nutr., 37 (5), September (2013) 599-606.

[51] T. Ganz , E. Nemeth. Hepcidin and iron homeostasis. Biochim. Biophys.Acta 1823 (2012) 1434–1443.

[52] C. Camaschella and P. Strati. Recent advances in iron metabolism and related disorders. Int. Emerg. Med (2010) 5:393–400.

[53] M. W. Hentze, M. U. Muckenthaler, B. Galy, and C. Camaschella. Two to Tango: Regulation of Mammalian Iron Metabolism. Cell 142, July 9, 2010 ©2010 Elsevier Inc.

[54] J. Versieck, J. Hoste, F. Barbier, H. Steyaert, J. De Rudder and H. Michels. Determination of Chromium and Cobalt in Human Serum by Neutron Activation Analysis. Clin. Chem., (1978), 24(2): 303-308.

[55] R. Cornelis, A Speecke and J. Hoste. A multi-element serum standard for neutron activation analysis. Anal. Chim.Acta, (1973), 68: 1-10.

[56] J. Versieck. Neutron Activation Analysis for the Determination of Trace Elements in Biological Materials. Biol Trace Elem Res (1994), 407-413.

[57] R. R. Greenberg, P. Bode, Elisabete A. De Nadai Fernandes. Neutron activation analysis: A primary method of measurement. Spectrochim. Acta B 66 (2011) 193–24.

2 Iron Metabolism and Iron Related Disorders

2.1 Introduction

Iron is element 26 in the periodic table and belongs to a group of elements known as transition metals. It is the second most abundant metal on earth after aluminium and essential for almost all organisms living in an oxygen-rich environment [1]. It plays a crucial role in various biochemical activities such as oxygen sensing and transport, drug metabolism, steroid synthesis, cellular respiration, electron transfer, DNA synthesis and catalysis [2-5]. The biological functions of iron are based on its chemical properties. It exists in two redox states ferrous (Fe^{2+}) and ferric (Fe^{3+}), and can convert from one to another by donation or acceptance of electrons, respectively. The bioavailability of iron is generally limited, because under aerobic conditions, Fe^{2+} is oxidized in solution to Fe^{3+}, which is almost insoluble at physiological pH [6,7].

Iron participates in the oxidation-reduction reaction known as the Fenton reaction [6,8].

$Fe^{3+} + O^{-} \rightarrow Fe^{2+} + O_{2;}$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$

Iron levels must be well maintained within cells, because an excess of iron is highly toxic related to the ability of iron to generate reactive oxygen species via the Fenton reaction. Oxygen radicals, such as hydroxyl (OH^{-}) and superoxide (O_2^{-}), are aggressive compounds because they react rapidly and with high affinity with almost every molecule found in living cells thereby attacking and damaging cellular components. A multitude of antioxidant mechanisms have evolved to protect cells against “oxidative stress”. A compromise in the antioxidant defence may ultimately lead to cell death and tissue degeneration.

Mammals obtain iron from the diet. Inorganic, non-heme iron is present in a wide variety of food, while heme iron, which is more efficiently absorbed, mainly originates from haemoglobin and myoglobin in animal meat [3].

2.2 Distribution of iron in the body

The iron balance is determined by the rate of iron loss and the effectiveness of an adaptive change in the rate of iron absorption from the diet [7]. There is no regulating mechanism for iron excretion from the body. Therefore the balance is maintained by the meticulous regulation of iron absorption in the duodenum [7]. Although the total body iron content is about 4 g [9], only 0.5-2 mg enters the body through the proximal small intestine and the same amount leaves the body through blood loss and the shedding of skin and mucosal cells. Daily dietary iron requirements are about 8 mg for adult men and 18 mg for adult women with menstrual iron losses [10]. The greatest portion of total iron (about 2-3 g in human) is distributed in the haemoglobin of red blood cells and developing erythroid cells (bone marrow) and serves in oxygen transport. The macrophages content of iron is up to 600 mg, while the myoglobin of muscles contains ~300 mg; excess body iron (~1 g) is stored in the liver. Around 0.1 % of the total body iron is bound to transferrin, the plasma iron carrier that is the exclusive source of iron for erythropoiesis. The lifespan of erythrocytes is about 110-120 days, so that each day the oldest 0.9% of erythrocytes are degraded by macrophages and their iron content is returned to plasma transferrin [8,10]. The recycling of erythrocytes generates a stream of 20-25 mg of iron per day. The pathways involved in iron homeostasis are shown in chapter 1, figure 1.

2.3 Iron Containing Proteins

The regulation of iron metabolism involves the interaction of a number of specific proteins. Some of these proteins contain iron for its metabolic functions others for the supply, transport and storage of iron. These include:

2.3.1. Hemoproteins

The compounds of this group, haemoglobin and myoglobin, all contain the iron(II)-protoporphyrin complex heme as specific prosthetic group. Their function involves the transport of oxygen and cell respiration [2,11]. Oxygen binding is mediated by the heme moieties. Other haemoproteins include various cytochromes and enzymes, such as oxygenases, peroxidases, nitric oxide (NO) synthases, or guanylate cyclase. The heme moiety also plays a role in electron transfer reactions (e.g. in cytochromes), as a substrate activator

(e.g. in cytochrome oxidase, cytochrome P450, catalase) or as an NO sensor (in guanylate cyclase) [2].

2.3.2 Proteins responsible for storage and transport of iron

2.3.2.1 Ferritin and Hemosiderin

Organs involved in iron storage are the liver, spleen and bone marrow. Storage proteins include ferritin (the major storage form of iron) and hemosiderin (the water-insoluble form of iron) [1-3,11]. Ferritin is a large, water-soluble, crystallizable protein, which can store up to 4500 atoms of iron within its spherical cavity [12]. The iron in ferritin is trivalent, but is incorporated and released in the bivalent form. A normal adult male with a plasma ferritin of 50-100 ng/mL has iron stores of approximately 500-1000 mg. An increase in plasma ferritin may be the result of iron overload but can also indicate inflammation, since ferritin acts as an acute phase reactant being a member of a group of proteins, that orchestrate cellular defense against oxidative stress. Unlike ferritin, hemosiderin is insoluble in water. Around 70% of the total body iron is in heme compounds (haemoglobin and myoglobin), 29% is stored as ferritin and hemosiderin, < 1% is incorporated into heme-containing enzymes (e.g. cytochromes, catalase, peroxidase) and < 0.2% is found circulating in the plasma bound to transferrin [13].

2.3.2.2 Transferrin

Transferrin, the iron-transporting plasma protein, provides most of the iron for the physiological needs of iron-requiring cells, and is normally the only known source of iron for haemoglobin synthesis [11].

Of the approximate 3 grams of body iron in the adult male, approximately 3 mg or 0.1% circulates in the plasma as an exchangeable pool. Essentially all circulating plasma iron is bound to transferrin. This chelation serves three purposes: it renders iron soluble under physiologic conditions, it prevents iron-mediated free radical toxicity, and it facilitates transport into cells [1,2].

2.4 Iron absorption

Absorption may be defined as the movement of iron from the intestinal lumen across the epithelial cells of the digestive tract into the circulation, while bioavailability generally refers to the proportion of iron in a given food or diet that the body can actually utilize [14]. Iron is present in the human diet in two forms: heme and non-heme iron. Heme iron refers to all

forms of iron from animal sources in which the iron is tightly bound within the porphyrin ring structure as is found in both myoglobin and haemoglobin. Non-heme iron (inorganic iron) refers to all other forms of iron present in a wide variety of foods [3].

Iron absorption occurs throughout the small intestine but mostly in the duodenum [15]. The uptake of iron from the lumen of the intestine into the mucosal cells represents the first step in the process of iron absorption. In order to be taken up by the enterocytes of the duodenum, dietary iron must be modified into an acceptable form.

Both inorganic iron and heme-iron enter the absorptive cell non-competitively. Non-heme iron bound to components of food is liberated in the gastrointestinal tract [15]. Once released from food components, most non-heme iron is present in the ferric form remaining fairly soluble as long as the pH of the environment is acidic (stomach). This oxidised Fe^{3+} form which is not bioavailable must first be reduced to the Fe^{2+} form before it is transported across the intestinal epithelium. The responsible ferrireductase enzyme is a membrane bound hemoprotein called Dcytb expressed in the brush border of the duodenum [3]. Fe^{2+} is then transported into the cell by a transporter called divalent metal transporter 1 (DMT1) [1-3] which also traffics other metal ions such as zinc, copper, cobalt and lead by a proton coupled mechanism [1].

Before heme iron can be utilized, heme must be released from dietary haemoglobin and myoglobin by proteolytic activity in the lumen of the stomach and small intestine. Heme iron is absorbed into the enterocyte by a different yet unidentified receptor. Specific transporters exist for heme on the apical surface of enterocytes and efforts are being made to characterize this heme transporter. Heme carrier protein 1 (HCP1) was identified and proposed as a protein involved in dietary heme uptake in duodenal enterocytes [16]. HCP1 is primarily a H+-coupled folate transporter suggesting that absorption of heme-iron is affected by folate availability.

Several dietary components may bind with non-heme iron to either enhance or inhibit non-heme iron's bioavailability. Citric acid, some amino acids and ascorbic acid are known to promote non-heme iron absorption [17]. While citric acid enhances non-heme iron absorption by chelating iron to keep it in solution, ascorbic acid and some amino acids (e.g., cysteine) promote iron absorption by reducing ferric iron to the more soluble and absorbable ferrous form. The bioavailability of heme-iron exceeds that of non-heme iron since it is less affected by other components of the diet [17].

Following intracellular traffic within the enterocyte, Fe^{2+} is exported to the blood circulation by the basolateral transporter ferroportin 1. Ferroportin is also expressed in macrophages and plays a crucial role in the export of iron from macrophages to the circulation. The ferroportin-mediated transport of Fe^{2+} across the cell membrane is followed by a reoxidation to Fe^{3+} and binding to transferrin

2.5 Regulation of iron homeostasis

Since excretion of iron is not under control and unpredictable, iron homeostasis is regulated by intestinal iron absorption and intracellular iron handling. Both processes are under genetic control as well as influenced by 'exogenous' factors such as hypoxia and inflammation. Erythroid and most non-erythroid cells take up iron from circulating transferrin using the binding capacity of the cell surface transferrin receptor 1 (TfR1). Within cells iron is stored in the form of ferritin. The expression of TfR1 and ferritin is controlled at the posttranscriptional level by the IRE/IRP system. The mRNAs encoding TfR1 and ferritin contain "iron responsive elements"(IRE's) in their untranslated regions. These regions can interact with "iron regulatory proteins" (IRP 1 and 2) from the cytosol, which process is influenced by the intracellular iron concentration. Under iron deficiency conditions IRPs actively bind multiple IREs localizations in TfR-1 mRNA, determining mRNA stabilization and increased translation of the protein, and simultaneously decrease the translation of ferritin mRNA, thereby maximizing the uptake and availability of iron in the cell. When the iron body levels are high, decreased IRE binding facilitates efficient translation of ferritin mRNA and decrease the stability of TfR-1 mRNA, leading to iron sequestration over uptake [1,3, 18-20].

Intestinal iron absorption is regulated by signalling to precursor enterocytes in the crypts of the duodenal epithelium. These cells mature and migrate along the crypt-villus axis. Signals sensed in the crypts program mature enterocytes to absorb dietary iron from the lumen in response to body iron demands. Macrophages may also respond to similar signals to regulate the release of iron for erythropoiesis. A genetic influence on this system is illustrated by the iron overload disorder hemochromatosis with mutations in the HFE-gene. An important signalling hormone is hepcidin, a peptide produced by the liver. It was discovered in the year 2000, and appears to be the master regulator of iron homeostasis in humans and other mammals [18-20]. Hepcidin inhibits iron transport across the gut mucosa, thereby preventing excess iron absorption and maintaining normal iron levels within the body. It is produced in response to the iron content of the blood and interacts with villous enterocytes by controlling

the expression of ferroportin 1 at their basolateral membranes. Ferroportin 1 molecules present in macrophages and liver are also targets for hepcidin (Figure 1) [20].

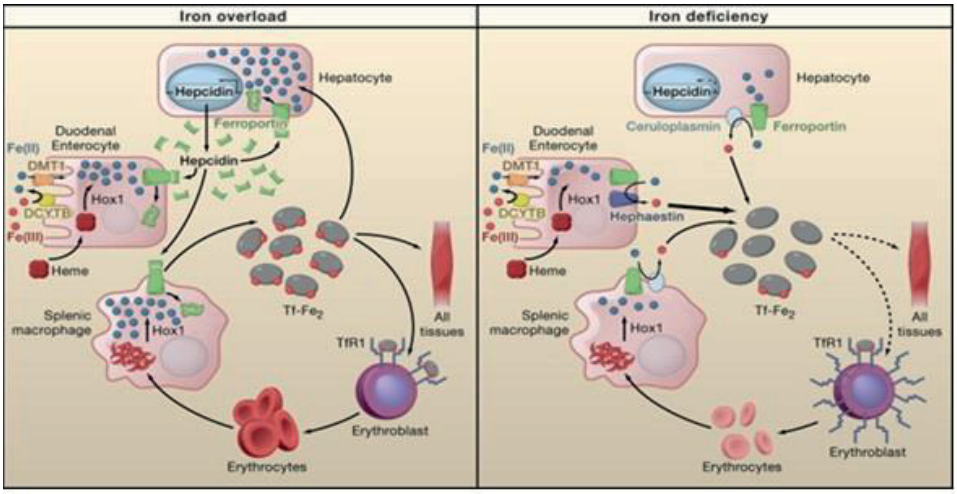


Figure 1. Regulation of Systemic Iron Homeostasis [20].

Cells involved in systemic iron regulation are shown. Divalent metal transporter 1 (DMT1) at the apical membrane of enterocytes takes up iron from the lumen of the duodenum after DCYTB reduces Fe(III) to Fe(II). Ferroportin at the basolateral membrane cooperates with hephaestin that oxidizes Fe(II) to Fe(III). Iron-loaded (diferric) transferrin (Tf-Fe2), indicated by red dots, supplies iron to all cells by binding to the transferrin receptor 1 (TfR1) and subsequent endocytosis. TfR1 is highly expressed on haemoglobin-synthesizing erythroblasts. Hepatocytes sense transferrin saturation/iron stores and release hepcidin accordingly. Red cell iron is recycled by macrophages via ferroportin and the ferroxidase ceruloplasmin. In iron overload (left), high hepcidin levels inhibit ferroportin-mediated iron export by triggering internalization and degradation of the complex to reduce transferrin saturation. Hepcidin expression is high. In iron deficiency (right), iron is released by ferroportin into the circulation. Haemoglobin-derived heme is catabolized in macrophages by hemoxygenase-1 (HOX1). Hepcidin expression is low.

2.6 Iron Related Disorders

Iron related disorders leading either to exhausted or to overloaded iron stores are extremely common in all parts of the world. The knowledge about these disorders has expanded over the past few years after the discovery of hepcidin. Increased iron requirements, limited external supply, and increased blood loss may lead to iron deficiency (ID) and iron deficiency anaemia. In chronic inflammation, the excess of hepcidin decreases iron absorption and prevents iron recycling, resulting in hypoferraemia and iron restricted erythropoiesis, despite normal iron stores (functional iron deficiency), and finally anaemia of chronic disease (ACD). Low hepcidin expression may lead to iron overload [20]. Because of the various functions of iron in the body, iron may play a role in other disorders than anaemia and hemochromatosis.

These include among others inflammatory and infectious diseases as well as (neuro) degenerative disorders (oxidative stress and iron overload)

2.6.1 Iron overload

Accumulation of iron in the body from any cause is known as iron overload.. The most important causes are hereditary hemochromatosis (HH), a genetic disorder, and chronic iron overload resulting from repeated blood transfusion. This is the case in patients who receive multiple transfusions for anaemia's caused by various conditions such as major thalassemia, aplastic anaemia, sickle cell disease and myelodysplastic syndrome.

2.6.1.1 Hereditary Hemochromatosis (HH)

Hereditary hemochromatosis (HH) is a common autosomal recessive disorder that results in iron overload with a high prevalence in Caucasians. It is characterized by abnormal iron absorption from the diet resulting in progressive iron overload causing tissue damage of several organs [21].

Four types of hereditary hemochromatosis are distinguished, each caused by a mutation of a gene involved in iron metabolism. The most common ones are HH type I, with a mutation in the HFE gene and type II with mutations of the hemojuvelin and hepcidin genes. Mutations in the transferrin receptor 2 gene lead to HH type III, whereas those of the ferroportin gene lead to HH type IV (Table 1) [22-26]. All these syndromes are characterised by iron overload. As transferrin becomes saturated in iron overload states, non-transferrin bound iron appears. Part of this iron is highly reactive (labile plasma iron) leading to free radical formation. The latter are responsible for the parenchymal cell injury associated with iron overload syndromes

Table 1. Hereditary iron overload disorders:

Type	gene	Human chromosome	Mechanism
HFE-hemochromatosis (Type I)	HFE	6p21	Decreased hepcidin production
Juvenile hemochromatosis (Type II), A	HJV	1q21	Decreased hepcidin production
Juvenile hemochromatosis (Type II), B	HAMP	19q13	Decreased hepcidin production
Transferrin receptor 2 hemochromatosis (Type III)	TFR2	7q21	Decreased hepcidin production
Ferroportin disease (Type IV)	SLC40A1	2q32	Resistance to hepcidin

Four main categories of pathophysiological mechanisms of HH are now well recognised: (1) the increased absorption of dietary iron in the upper intestine, (2) decreased expression of the

iron-regulatory hormone hepcidin, (3) the altered function of HFE protein, and (4) tissue injury and fibrogenesis induced by iron [25].

About 32 mutations of the HFE gene have been described until now. The two most common mutations of HFE are C282Y and H63D. Most patients with hereditary hemochromatosis type 1 are homozygous for the C282Y mutation of the HFE gene [27,28]. Those who inherit the defective gene from only one parent are carriers for the disease but usually do not develop it; however, they still may have higher than average iron absorption.

The diagnosis of iron overload requires sequential steps. Clinical evaluation, biochemical testing, assessment of total body iron, and molecular tests concur to reach the correct diagnosis. Several comprehensive diagnostic and therapeutic algorithms have been recently proposed [25].

Bloodletting (phlebotomy) is the standard treatment for all forms of hemochromatosis [25,25]. The rationale for blood removal is that iron depletion will reduce tissue injury. This may prevent or diminish some complications of hemochromatosis. It may decrease dyspnoea, pigmentation, fatigue, arthralgia and hepatomegaly, or improve control of diabetes mellitus and left ventricular diastolic function. However, the course of hepatic cirrhosis, and increased risk of primary liver cancer is usually not changed. For most patients with hemochromatosis and iron overload, standard therapy starts with the weekly removal of blood to bring the ferritin level into the low reference range (20-50 ng/ml), followed by a life-long maintenance phlebotomy schedule for maintaining ferritin levels at approximately 50 ng/ml. Phlebotomy can be stopped at the point at which iron stores are depleted, and the patient should be assessed for whether they require maintenance phlebotomy. For reasons that are unclear, not all patients with HH reaccumulate iron and, accordingly, they may not need a maintenance phlebotomy regimen [26,29].

Management of iron overload and treatment of iron toxicity by chelation in patients with acquired iron overload (eg, transfusion- dependent anaemia) have been demonstrated to reduce iron burden and improve survival. According to recent consensus guidelines, patients with serial serum ferritin levels exceeding 1000 ng/ml and a total infused red blood cell volume of 120 mL per kg of body weight or more should be treated with chelation therapy. Serum ferritin levels should be monitored every 3 months during chelation therapy to ensure that treatment adequately reduces iron levels.

2.6.2 Iron Deficiency (ID) & Iron Deficient Anaemia (IDA)

The most common dietary deficiency worldwide is that of iron, affecting almost 1.2 billion persons, in particular women and children [30]. Anaemia is a disorder defined as a decreased number of red blood cells, which can be caused by a number of different conditions. Iron deficient anaemia (IDA) is a specific type of anaemia that in the Western world is almost always caused by blood loss. Very rarely, decreased absorption of iron from foods plays a role. In developing countries IDA affects more than 500 million people primarily due to deficiencies of iron in their diet.

Iron deficiency (ID) can result in anaemia, a continuous process evolving three stages. The first is depletion of storage iron, characterized by low serum ferritin levels. The second stage of ID is a state of iron-deficient erythropoiesis. In this stage, there is a shortage of iron available to the erythroid precursors in the bone marrow for haemoglobin (Hb) synthesis. This second stage may be characterized by abnormalities in particular iron parameters, including low transferrin saturation and elevations in free erythrocyte zinc protoporphyrin (ZnPP). The third and most severe degree of ID involves overt microcytic anaemia [30,31]. IDA is characterized by a significant reduction in haemoglobin level and a decrease in mean corpuscular volume (MCV). A normal Hb level does not exclude ID, because individuals with normal body iron stores must lose a large portion of body iron before the Hb falls below the laboratory definition of anaemia [32].

Additional laboratory findings in IDA include elevated total iron-binding capacity (TIBC), low transferrin saturation and low serum iron levels. Apart from iron staining of bone marrow material serum ferritin is the best laboratory test for the diagnosis of iron deficiency as it may decrease before a fall in serum iron level is detected. Patients should be considered to suffer from IDA when they present with low Hb (<11 g/dL(<7 mmol/L)), serum iron<7μmol/L, transferrin saturation <20% and ferritin concentrations <30 ng/mL without signs of inflammation [33].

2.6.3 Anaemia of chronic disease (ACD)

This is the most common anaemia in hospitalized persons with chronic illness, e.g. chronic infection, chronic immune activation, or malignancy, also referred to as anaemia of inflammatory response [32]. It is a condition in which there is impaired utilization of iron, without either a deficiency or an excess of iron. The syndrome is likely the result of the body's upregulation of hepcidin, the key role player in iron metabolism. In response to inflammatory

cytokines the liver produces more hepcidin. Hepcidin in turn causes increased internalisation of ferroportin molecules on cell membranes which prevents release of iron from it's stores. Inflammatory cytokines also appear to affect other important elements of iron metabolism, including decreasing ferroportin expression, and probably directly blunting erythropoiesis by decreasing the ability of the bone marrow to respond to erythropoietin.

Patients should be considered to have anaemia of chronic disease (ACD), when they have: (1) evidence of chronic inflammation (eg, high CRP level); (2) Hb concentration of <11 g/dl (<7 mmol/L and (3) a low transferrin saturation (<20%),. Ferritin levels are mostly increased but can be normal [25].

2.6.4 Treatment of Iron Deficiency

Oral iron has been used to treat iron deficiency for centuries. However a rather long period of treatment is necessary to obtain an adequate rise in haemoglobin level. Furthermore there are some side-effects such as nausea, epigastric discomfort and obstipation. These symptoms vary in proportion to the concentration of ionizable iron in the upper gastrointestinal tract Although food reduces absorption of medicinal iron, symptoms may be alleviated by taking the iron with food. Ferrous sulphate and ferrous gluconate are the preferred forms of oral iron because of low cost and high bioavailability[33].

Parenteral iron therapy is another option for treatment of iron deficiency and indicated in situations such as intolerance and contraindications or inadequate response to oral iron. Parenteral iron is a useful treatment in cases where there is a short time to surgery, severe anaemia, especially if accompanied by significant ongoing bleeding, and in combination with the use of erythropoiesis stimulating agents [33,34]. The administration of intravenous iron enables a fivefold erythropoietic response in normal individuals with significant blood loss. Haemoglobin starts to rise after a few days and the percentage of responding patients is higher than when iron is given orally.

Various pharmaceutical formulas are used for intravenous or sometimes intramuscular therapy. The most common preparation is iron dextran. The other two forms are iron gluconate and iron sucrose. Iron dextran can be used to administer a large dose of iron on a single occasion, but can produce serious and sometimes fatal allergic reactions. Anaphylaxis occurs rarely if ever with iron gluconate or iron sucrose [33].

2.7 Iron and other trace elements

Dietary deficiencies may not only involve iron but other trace elements as well. The clinical outcome of a combined deficiency is not always clear depending on whether an element is synergistic or antagonistic to iron [35]. Some of the elements can even have both synergistic and antagonistic effects. As an example, copper is considered synergistic due to its requirement in ferroxidase activity. However, excessive intake of copper competes with iron for absorption. Antagonism of iron may occur either by inhibiting absorption, compartmental displacement or interference with cellular iron enzymes. Such synergistic and antagonistic effects are also important in case of an overload with several elements and the danger of toxicity.

Two elements are especially of interest, since both are clearly linked with iron homeostasis: copper and zinc. Copper is needed to absorb iron [36], and it is a component of several metalloenzymes required for oxidative metabolism, including cytochrome oxidase, ferroxidases, amino oxidases, superoxide dismutase, ascorbic acid oxidase and tyrosinase. Interactions between copper and iron homeostasis have been known since the nineteenth century when anaemia in humans was first described due to copper limitation, reflected primarily in reduced erythrocyte numbers and lower haemoglobin per cell [37]. However, the mechanism remains unknown. Intestinal and liver iron concentrations are usually higher following copper deficiency. The iron and copper homeostases are also linked by the inability to export Fe in the absence of Cu to the systemic circulation. However, Cu deficiency does not affect ferroportin expression [38]. Ferroportin activity is tightly controlled by the ceruloplasmin-homologue hephaestin [39]. Hephaestin is an integral transmembrane ferroxidase, which co-migrates with ferroportin to the basal membrane, in response to increased intracellular iron levels, in which they form a complex. Exported ferrous iron requires oxidation to ferric iron, which is accomplished by the Cu-dependent ferroxidase activity of hephaestin [40].

Zinc is the second most ubiquitous transition element in biological systems after iron. The first recognition of a role for zinc in biochemistry was the discovery in 1939 that it was an essential component of carbonic anhydrase, a key enzyme in erythrocytes that catalyses the formation of bicarbonate [1]. Later over two-hundred zinc-enzymes catalysing all sorts of reactions have been found. Some of these enzymes are involved in pathways of major importance in iron metabolism, such as δ-aminolaevulinate dehydratase (also known as

porphobilinogen synthase), which catalyses the condensation of two molecules of δ-aminolaevulinate to yield porphobilinogen in the haem biosynthetic pathway [1].

Zinc protoporphyrin (ZnPP) is a normal metabolite that is formed in trace amounts during haem biosynthesis. However, in iron deficiency or in impaired iron utilization, zinc becomes an alternative substrate for ferrochelatase and elevated levels of zinc protoporphyrin are formed. This zinc-for-iron substitution is one of the first biochemical responses to iron depletion, and erythrocyte zinc protoporphyrin is therefore a sensitive index of bone-marrow iron status. In addition, zinc protoporphyrin may regulate haem catabolism by acting as a competitive inhibitor of haem oxygenase, the key enzyme of the haem degradation pathway [41].

Combined supplementation with iron and zinc is one of the strategies, which can be used to improve the iron and zinc status of a population. However, studies performed in humans have shown an inhibitory effect of zinc on iron absorption so the combined supplementation with both minerals might be less efficacious than single supplementation with iron in reducing the prevalence of anaemia and in improving iron status. Many questions remain concerning the best strategy for an adequate supplementation of the two elements in case of deficiencies.

2.8 Conclusions

In recent years, substantial progress has been made in our knowledge of iron metabolism. Some of the key players have been identified and it has become clear that the intestinal absorption of this element is crucial for maintaining homeostasis. Absorption can be upregulated in case of iron deficiency and is over activated in hemochromatosis. Genetic, local and environmental factors all play a role to keep the iron balance intact. Of special importance in iron metabolism is hepcidin, a humoral factor produced by the liver. This protein is able to regulate the iron absorption capacity of the gastro-intestinal tract. In case of inflammation an increase of hepcidin results in a lower uptake of iron by the gut as well as in a decrease in the release of iron from storages. A functional iron deficiency is the result, indicating that the body wants to protect itself against the presence of too much iron. This may be related to the capability of iron to increase oxidative stress and thereby promoting tissue damage. On the other hand iron is vital in many biochemical activities such as oxygen transport and cellular respiration. It is therefore crucial for the body to find the right equilibrium between lack and overload.

2.9 References

[1] R. Crichton, Inorganic Biochemistry of iron Metabolism, Second Edition. John Wiley & Sons, LTD, (2001).

[2] R. Crichton. Iron Metabolism. Wiley. (2009). ISBN: 978-0-470-01028-0.

[3] O. Han. Molecular mechanism of intestinal iron absorption. Metallomics, (2011), 3, 103-109.

[4] S. K. S. Srai, Adrian Bomford and Harry J. Iron Transport Across Cell Membranes: Molecular Understanding of Duodenal and Placenta iron Uptake. Best Pract.Res. Clin. Haem. (2002), 15 (2): 243-259.

[5] P. Ponka, Cellular iron Metabolism. Kidney Int. (1999), 55, Suppl. 69, S2-S11.

[6] J. M. McCord, Iron, Free Radicals, and Oxidative Injury. Seminars in Hematology, (1998) Vol. 35, No. 1, 5-12,.

[7] J. Wang and K. Pantopoulos. Regulation of Cellular Iron Metabolism. Biochem. J, (2011), 434, 365-381.

[8] G. Papanikolaou and K. Pantopoulos. Iron metabolism and toxicity. Tox. Appl. Pharm. (2005) 202, 199– 211.

[9] M. E. Conrad, and J. N Umbreit. Pathways of iron Absorption. Blood Cells, Mol. Dis. (2002), 29 (3): 336-355.

[10] T. Ganz and E. Nemeth. Hepcidin and Iron Homeostasis. Biochim Biophys.Acta. (2012) 1823, 1434-1443.

[11] P. Aisen, M. Wessling- Resnick and E. A. Leibold. Iron Metabolism. Curr.Opi.Chem.Biol., (1999), 3, 200-206.

[12] I. G. Macara, T. G. Hoy and P. Harrison. The Formation of Ferritin from Apoferritin. Biochem. J, (1972) 126, 151-162.

[13] T. D. Johnson-Wimbley and D. Y. Graham. Diagnosis and management of iron deficiency anemia in the 21st Century. Therap.Adv.Gastr., (2011) 4(3) 177-184.

[14] S. J. Fairweather-Tait. Bioavailability of Nutrients and Other Bioactive Components from Dietary Supplements: Iron. J.Nutr., (2001), 1383S-1386S.

[15] H. Gunshin, B. Mackenzie, U. V. Berger, Y. Gunshin, M. F. Romero, W. F. Boron, S. Nussberge, J. L. Gollan and M. A. Hediger. Cloning and Characterization of A mammalian Proton-coupled Metal- iron Transporter. Nature, (1997), 388, 482-488.

[16] M. Shayeghi, G. O. Latunde-Dada, J. S. Oakhill, A. H. Laftah, K. Takeuchi, N. halliday, Y. Khan, A. Warley, F. E. McCann, R. C. Hider, C. D. Vulpe, R. J. Simpson, and A. T. McKie. Identification of An Intestinal Heme Transporter. Cell, (2005), 122, 789-801.

[17] J. D. Cook, S. A Dassenko, and S. R. Lynch. Assessment of the role of nonheme-iron availability in iron balance. Am J.Clin. Nutri., (199l), 54:7l7-722.

[18] A. von Drygalski and J. W. Adamson. Iron metabolism in man. J. Par. Ent. Nutr., (2013), 37 (5): 599-606.

[19] C. Camaschella and P. Strati. Recent advance in iron metabolism and related disorders. Int. Emerg. Med., (2010), 5: 393-400.

[20] M. W. Hentze, M. U. Muckenthaler, B. Galy, and C. Camaschella. Two to Tango: Regulation of Mammalian Iron Metabolism. Cell, (2010), 142(1): 24–38.

[21] J. Carlson and S. Olsson. Hereditary Hemochromatosis and Iron Metabolism. J. Int. Fed. Cli. Chem. Lab. Med, (2002), 13(2), htt://www.ifcc.org/ejifcc/vol13no2/1301200103htm..

[22] C. N. Roy and N. C. Andrews. Recent Advances in Disorders of Iron Metabolism: Mutations Mechanisms and Modifiers. Hum.Mol. Gen., (2001), 10 (20), 2181-2186.

[23] G. D. McLaren and V. R. Gordeuk. Hereditary Hemochromatosis: Insights from the Hemochromatosis and Iron Overload Screening (HEIRS) study. Hematology, (2009), 195-206.

[24] M. Franchini. Hereditary Iron Overload: Update on Pathophysiology, Diagnosis, and Treatment. Am. J. Hem., (2006),81, 202–209.

[25] M. Munoz, J. A. Garcia-Erce and A. F. Remacha. Disorders of Iron metabolism. Part II: Iron Deficiency and Iron Overload. J. Clin. Pathol., (2011), 64: 287-296.

[26] B. R. Bacon, P. C. Adams, K. V. Kowdley, L. W. Powell and A. S. Tavill., Diagnosis and Management of Hemochromatosis: 2011 Practice guideline by the American Association for the Study of liver diseases. Hepatology, (2011), 54 (1), 328-343.

[27] A. Waheed, S. Parkkila, X. Y. Zhou, S. Tomatsu, Z. Tsuchihashi, J. N. Fede, R. C. Schatzman, R. S. Britton, B. R. Bacon, and W. S. Sly. Hereditary Hemochromatosis: Effects of C282Y and H63D Mutation on Association with β2- Microglobulin, Intracellular Processing, and Cell Surface Expression of the HFE Protein in COS-7 Cells. Proc. Natl. Acad. Sci. USA, (1997), 94: 12384-12389.

[28] T. Hohler, S. Leininger, H. H. Kohler, P. Schirmacher and P.R. Galle. Heterozygosity for The Hemochromatosis Gene in Liver Diseases – Prevalence and Effects on Liver Histology. Liver, (2000), 20: 482-486.

[29] A. S. Tavill. Diagnosis and Management of Hemochromatosis. Hepatology, (2001), 33 (5): 1321-1328.

[30] A. Koulaouzidis, E. Said, R. Cottier and A. A. Saeed. Soluble transferrin Receptors and iron Deficiency, a Step Beyond Ferritin. A Systemic Review. J Gastr. Liver Dis., (2009), 18, (3), 345-352.

[31] M. Sanad and A. F Gharib. Urinary Hepcidin Level as an Early Predictor of Iron Deficiency in Children: A Case Study. Ital. J. Ped., (2011), 37: 37. http://www.ijpoonline.net/content/37/1/37.

[32] W. Reinisch, M. Staun, S. Bhandari, and M. Munoz. State of the Iron: How to Diagnose and Efficiently Treat Iron deficiency Anemia in Inflammatory Bowel Disease. J. Crohn's Colitis, (2013), 7, 429-440.

[33] J. D. Cook. Diagnosis and Management of Iron- deficiency Anaemia. Best Pract.Res. Clin. Haem., (2005), 18, (2): 319-332.

[34] E. C. Shan Chew and J. C. Mei Lam. Diagnosis and Management of Iron Deficiency Anaemia in Children- A Clinical Update. Proc. Singapore Healthcare, (2012), 21 (4): 278-285.

[35] D. L. Watts, The Nutritional Relationships of Iron., J.Orthomol. Med., (1988), 3 (3): 110-116.

[36] A. A. Jones, R. A. Disilvestro, M. Coleman, and T. L. Wagner. Copper Supplementation of Adult Men: Effects on Blood Copper Enzyme Activities and Indicators of Cardiovascular Disease Risk. Metabolism, (1997), 46 (12): 1380-1383.

[37] P. L. Fox., The Copper-Iron Chronicles: The Story of an Intimate Relationship. Biometals, (2003),16: 9-40.

[38] J. R. Prohaska and M. Broderius., Copper Deficiency Has Minimal Impact on Ferroportin Expression or Function. Biometals, (2012), 25: 633-642.

[39] P. N. Ranganathan, Y. Lu, L. Jiang, C. Kim and J. F Collins., Serum Ceruloplasmin Protein Expression and Activity Increases in Iron-deficient Rats and is Further Enhanced by Higher Dietary Copper. Blood, (2011), 118 (11): 3146-3153.

[40] N. Scheers., Regulatory Effect of Cu, Zn, and Ca on Fe Absorption: The Intricate Ply between Nutrient Transpoters. Nutrients, (2013) 5: 957-970.

[41] R. F. Labbe, H. J. Vreman and D. K. Stevenson. Zinc Protoporphyrin: A metabolite With a Mission. Clin. Chem., (1999), 45 (12):,2060-2072.

3 Use of Iron Isotopes to Study Iron Metabolism

3.1 Introduction

Enriched stable isotopes are now generally regarded as the most accurate, safe and versatile method of studying mineral absorption, bioavailability and metabolism. Bioavailability refers to the fraction of an element in a given food or diet that the body can utilize for its normal physiological functions [1,2]. In case of studying iron metabolism the first iron isotope used was the radionuclide ^{59}Fe. It was used in animal studies in 1939 [3] and in humans in 1942 [4]. However, hazards resulting from the ionizing radiation reduces its use in humans especially in infants, children, and woman of child-bearing status.

During the past three decades, there has been significant growth in the use of enriched stable isotopes of trace elements as metabolic tracer and concerted efforts have been made in the development and application of methods based on stable isotopes as tracers. The enriched stable isotopes provide a means of labelling minerals in humans with no exposure to radioactivity. In addition, multiple isotope experiments can be conducted. Thus, mineral absorption, utilization, and interactions can be studied for several minerals simultaneously [2,5,6]. Its primary drawbacks, however, are the high costs of production and analysis, lack of access to suitable instrumentation and demanding preparation of samples. The costs of enriched stable isotopes depend on the natural abundance, ease of enrichment, enrichment level, as well as the country of origin, supplier, and quantity ordered.

Until now, studies of iron absorption using stable Fe isotopes have been mainly performed in the field of food research focusing on infants and children [5,7-10]. Studies in a clinical setting such as in pregnant women, elderly men and patients are scarce [11-16]. In this project we focus on the use of INAA to study iron metabolism in patients with iron-related disorders and make a first step to introduce the enriched stable ^{58}Fe isotope in clinical medicine.

3.2 Stable iron isotopes

Iron has four naturally occurring stable isotopes and isotopically enriched iron is available for any of these isotopes. The composition of the ^{58}Fe enriched stable isotope used in our study as compared to the natural composition of iron is shown in Table 1. The isotopes ^{58}Fe and ^{57}Fe are most commonly used in enriched form as tracer in human nutrition research due to their

low natural abundances. This enables the use of smaller amounts of this isotope in experiments to measure absorption. Enriched stable isotopes are usually provided as iron metal and are converted to ferrous sulfate before oral administration. Both the supply and costs of these isotopes have remained relatively constant or have decreased in recent years. Because dosing of iron enriched stable isotopes is dependent on enriching the circulating body iron pool, the dose administered is usually dependent on the subject's weight, and increases in proportion to weight and hemoglobin concentration [11,17].

Table 1. Natural Stable Isotope Composition of Iron amd enriched isotopic composition, as used in this project.

Stable isotope	Natural abundance (atom, %) [18]	Available (atom, %)	Enrichment
^{54}Fe	5.845 +/- 0.023	<0.01	
^{56}Fe	91.754 +/- 0.024	1.0	
^{57}Fe	2.1191 +/- 0.0065	6.7	
^{58}Fe	0.2819 +/- 0.027	92.3	

3.3 Preparation and administration of the enriched ^{58}Fe containing oral iron supplement

The isotope was purchased as a metal from Isoflex Inc®. A total of 73 mg of ^{58}Fe was mixed with 6.86 mL 0.5 M H_2SO_4 and heated to 53° C until all iron was dissolved. Fe^{3+} was reduced to Fe^{2+} by adding 219 mg of ascorbic acid and the solution was diluted with deionized water to give an Fe concentration of 1 mg/mL. The deaerated solution was then distributed into vials each containing 5 ml of the iron sulfate drink. The vials were stored at 15° C and all used within three weeks after preparation.

3.3.1 Dosage considerations

When designing a stable isotope study the basic requirement is to achieve a sufficient isotope enrichment in the body tissue/fluid of interest so that the added tracers can be quantified with the necessary degree of precision. The amount of isotope added depends on four major factors [11]: (1) The amount of expected absorption and retention. This varies widely with different elements. Fe absorption, for example, can vary from < 1% to almost 100% depending on the Fe status of the subject and the meal composition. (2) Relative distribution between different

body tissues and fluids. The total body content of different minerals (and thus their constituent stable isotopes) varies widely as does their distribution between the different body compartments and fluids. For example, while some 60% of the body's 2.3 g of Zn is in the muscle only 0.1 % is in the blood plasma. In comparison about 60% of the body's 4 g of Fe is in the red blood cell hemoglobin. It is essential to know the amount of mineral naturally present in urine, stool and blood if these pools are to be enriched. (3) The natural abundance of the isotope administered. The higher the natural abundance, the greater the quantity of isotope that should be administered to detect a difference. Therefore the preference for isotopes with a low natural presence. (4) The degree of isotope enrichment required.

3.3.2 Labeling methods

The labeling with ^{58}Fe in our study is an example of extrinsic labeling. In extrinsic labeling, the stable isotope label is added directly onto a supplement or mixed with the test meal being studied. It is not incorporated into the food as would be the native iron found in the food. As it may not be located in the same compartments of the plant or bound to the same substances that it would be naturally, there is a risk that the bioavailability of the extrinsic label will not mimic the bioavailability of the iron found naturally in the test substance. In intrinsic labeling, the stable iron isotope is incorporated into the food during the growth process to produce a labelled food which resembles the native food to the greatest extent possible [19-21]. For a plant source, this would involve growing the food with an enriched source of iron. Animal tissue may likewise be intrinsically labelled by adding the isotope to the diet, administering by gavage, or injecting it either intravenously, intraperitoneally or intramuscularly. The advantage of this approach is that the label is then located in the same biological compartments and in the same form as the naturally occurring element. Multiple isotopes of a single mineral can be administered to study a number of variables simultaneously [19].

3.4 Methods to assess iron bioavailability

Iron absorption after administration of a single or multiple oral stable isotopes, can be estimated using one of three approaches;

1) Faecal recovery method.
2) Plasma appearance method, or
3) Erythrocytes iron incorporation method.

3.4.1 Faecal recovery method

Absorption of iron can be determined from the difference between the oral isotope dose and faecal excretion [22,23]. This technique is referred to as faecal monitoring and it measures the luminal disappearance of the isotope, generating apparent absorption data. The advantage of this technique is that there is only a limited effect of intestinal re-excretion of Fe. If urinary isotope excretion is also measured, retention of the trace element can be calculated. The need to measure urinary isotope appearance depends on the trace element under study. Faecal monitoring is a difficult technique, which is unpopular with both volunteers and scientists and should only be carried out in experienced laboratories. It is prone to several sources of error, the main ones being incomplete collection (which leads to an overestimate of absorption) and poor homogenization of sample material [17]. Furthermore it is not very precise and time consuming (collection during several days).

3.4.2 Plasma appearance method

Assessing bioavailability can also be made from measurements of the appearance of oral doses of isotopically enriched sources of the element in plasma [2,17,24]. A one-compartment model can be used to describe the plasma disappearance of certain minerals, in particular, iron. By taking regular blood samples over 6 to 8 hours after the administration of an orally labelled dose, it is possible to calculate the quantity of iron that is absorbed into the body. This technique is derived from pharmacokinetics where the plasma appearance/disappearance of many drugs can be described in this way. The equation governing this type of kinetics is:

$$C = \frac{K1}{K1-k2} * \frac{A}{V} * \left(e^{-k2t} - e^{-k1t}\right)$$

where C is the measured plasma concentration of the stable isotope labelled oral dose, A is the amount of the oral dose that has been absorbed, V is the plasma volume (estimated), K_1 is the elimination rate constant from the gut, K_2 is the elimination rate constant from the plasma and t is the elapsed time from the consumption of the oral dose.

The increase in plasma mineral levels, usually expressed as the area under the curve above fasting level, is thought to give an indication of the absorption of the mineral.

The doses used in these studies to produce detectable increases of plasma concentrations often exceed the usual range of dietary intake. For zinc and iron amounts from 5 mg up to 100 mg

have been applied, mostly in the form of zinc or iron sulphate. Plasma Fe tolerance curve seems to be a promising method, although, interfering factors should be taken into account, such as gastrointestinal transit time and rate of entry of Fe from the gastrointestinal mucosal cell into the bloodstream

3.4.3 Erythrocytes iron incorporation method

The method of choice for assessing the bioavailability of iron supplements is haemoglobin incorporation,. Since the majority of newly absorbed iron is incorporated into reticulocytes (immature red blood cells), the proportion of an oral dose of isotopically labelled iron that is found in blood haemoglobin 14 d after the last oral dose can be used to determine bioavailability [25-27]. The stable isotope methodologies are based on original studies using radioisotopes. Some countries are reluctant to permit the use of radioisotopes for nutrition research, particularly in women and children where unnecessary radiation exposure is not ethically acceptable. Thus the haemoglobin incorporation technique has been adapted for use with stable isotopes in infants, either as a single or double isotope technique. Percentage iron absorption is calculated on the basis of blood volume (estimated from height and weight) and red cell incorporation of the absorbed dose assuming that 80-90% of the absorbed iron is incorporated into haemoglobin [11,17].

The dose of isotope required for the test depends on the anticipated absorption and the detection system whereby the limit of detection is three times the SD, and limit of quantification ten times the SD. Adapting the method for use in adults is more difficult because of the large doses of isotopes that have to be given for measureable enrichment of red blood cells (approximately ten times the quantity required for infants). This is costly and requires a multiple-dosing protocol.

3.5 References

[1] S. J. Fairweather-Tait. Bioavailability of Nutrients and Other Bioactive Components from Dietary Supplements: Iron. The journal of Nutrition, (2001), 1383S-1386S.

[2] S. J. Fairweather-Tait and J. Dainty. Use of Stable Isotopes to Assess the Bioavailability of Trace Elements: A review. Food Additives and Contaminants, (2002), 19 (10): 939-947.

[3] P F. Hahen, W F. Bale, W F. Hettig , R A. Kamed, and G H. Whipple. Radioactive iron and Its Excretion in Urine, Bile, and Feces. J. Exp. Med., (1939) 70: 443-451.

[4] W. M. Balfour, P. F. Hahn, W. F. Bale, W. T. Pommerenke and G. H. Whipple. Radioactive iron absorption in clinical conditions: Normal, pregnancy, anaemia and hemochromatosis. J. Exp. Med., (1942), 15- 30.

[5] S. A Abrams, Using Stable Isotopes to Assess Mineral Absorption and Utilization by Children. Am. J. Clin Nutr, (1999),70, 955-964.

[6] J. R. Turnlund. Zinc, copper, and iron nutrition studied with enriched stable isotopes. Biol. Trace Elem.Res., (1987), 12 (1): 247–257.

[7] Z. Chen, I. J. Griffin, L. M. Plumlee, and S. A. Abrams. High Resolution Inductively Coupled Plasma Mass Spectrometry Allows Rapid Assessment of Iron Absorption in Infants and Children. J. Nutr. (2005), 135 (7), 1790-1795.

[8] M. C. McDonald, S. A Abrams and R. J. Schanler. Iron Absorption and Red Blood Cell Incorporation in Premature Infants Fed an Iron-Fortified Infant Formula. Ped. Res., (1998) 44, 507–511.

[9] J. A Widness, K. A Lombard, E. E. Ziegler, R. E Serfass, S. J Carlson, K. J Johnson and J. E Miller. Erythrocyte Incorporation and Absorption of ^{58}Fe in Premature Infants Treated with Erythropoietin. Ped. Res., (1997) 41, 416–423.

[10] S. J. Fomon, M. Janghorbani, B. T. G. Ting, E. E. Ziegler, R. R. Rogers, S. E. Nelson, L. S. Ostedgaard and B. B. Edwards. Erythrocyte Incorporation of Ingested 58-Iron by Infants. Ped. Res., (1988), 24 (1): 20-24.

[11] N. Lowe and M. Jackson. Advances in Isotope Methods for the Analysis of Trace Elements in Man. CRC Press 2001. ISBN 1420036718, 9781420036718.

[12] P. G. Whittaker, J. F. R. Barrett and T. Lind. The erythrocyte incorporation of absorbed non-haem iron in pregnant women. Brit. J. Nutr. (2001), 86, 323–329.

[13] P. G. Whittaker and T. Lind. Iron absorption during normal human pregnancy: a study using stable isotopes. Brit. J. Nutr. (1991), 65, 451-463.

[14] J. R. Turnlund, R. D. Reager, and F. Costa. Iron and copper absorption in young and elderly men. Nutr. Res., (1988), 8, 333-343.

[15] P. Krayenbuehl, T. Walczyk, R. Schoenberg, F. von Blanckenburg, and G. Schulthess. Hereditary hemochromatosis is reflected in the iron isotope composition of blood. Blood, (2005) 105: 3812-3816.

[16] J. Chen, S. Shahnavas, N. Singh, W. Ong and T. Walczyk. Stable iron isotope tracing reveals significant brain iron uptake in adult rats. Metallomics, (2013), 5, 167-173.

[17] Assessment of iron bioavailability in human using stable iron isotopes techniques. IAEA human heath series No. 21. International Atomic Energy Agency, Vienna, 2012.

[18] M. Berglund and M. E. Wieser. IUPAC. Pure & Appl. Chem, 83 (2) (2011) 397-410.

[19] J. R. Turnlund. Mineral bioavailability and metabolism determined by using stable isotope tracers. J. Anim. Sci., (2006), 84 (E. Suppl.):E73–E78.

[20] M. A. Grusak. Intrinsic stable isotope labelling of plants for nutritional investigations in human. Nutr. Biochem., (1997), 8: 164-171.

[21] C. M. Weaver. Intrinsic mineral labelling of edible plants: methods and uses. Crit Rev Food Sci Nutr., (1985), 23 (1): 75-101.

[22] M. Janghorbani, B. T. G. Ting and V. R. Young. Accurate analysis of stable Isotopes ^{68}Zn, ^{70}Zn, AND ^{58}Fe in human feces with neutron activation analysis. Clin. Chim. Acta, (1980), 108: 9-24.

[23] M. Janghorbani, B. T. G. Ting and Vernon R. Young. Absorption of Iron in Young Men Studied by Monitoring Excretion of a Stable Iron Isotope (^{58}Fe)in Feces. J. Nutr., (1980), 110: 2190-2197.

[24] B. Sandstrom , S. Fairweatter-Tait, R. Hurrel, and W. Van Dokkum, Methods for Studying Mineral and Trace Elements Absorptin in Humans Using Stable Isotopes. Nutr. Res. Rev. (1993), 6: 71-95.

[25] P. V. M. Kalpalathika, A. Mahoney, P. Whittaker and D. Hendricks. Incorporation of Absorbed Iron from Different Dietary Sources into Hemoglobin. Nutr. Res., (1991), 11: 185-195.

[26] M. Janghorbani, B. T Ting and S. J. Fomon. Erythrocyte incorporation of ingested stable isotope of iron (^{58}Fe). Am J Hematol, (1986), 21(3): 277-88.

[27] S. H. Zlotkin, D M Lay, J. Kjarsgaard and T. Longley. Determination of iron absorption using erythrocyte iron incorporation of two stable isotopes of iron (^{57}Fe and ^{58}Fe) in very low birthweight premature infants. J Pediatr Gastroenterol Nutr., (1995), 21(2): 190-199.

4 Analytical Techniques for Measuring Total Iron and Iron Stable Isotopes

4.1 Introduction

Applications of enriched stable isotopes of iron (Fe) require that suitable isotope-specific analytical methods for accurate measurement are available. Two such methods are: activation analysis (with neutron activation analysis being the most common form) and inorganic mass spectrometry. In addition, the total amount of iron can also be measured by several other analytical techniques, such as those based on atomic absorption spectrometry (AAS), providing varieties like : flame (F-AAS), electro-thermal (ET-AAS, GF-AAS) , with generation of hydrides (HG-AAS) and cold vapours of mercury (CV-AAS) and, more rarely atomic fluorescence spectrometry (AFS); and on basis of plasma spectrometry, such as inductively coupled plasma-optical emission spectrometry (ICP-OES), also known as inductively coupled plasma-optical absorption spectrometry (ICP-AES), and inductively coupled plasma-mass spectrometry (ICP-MS). All of these techniques are relative techniques, and do not allow absolute measurements, therefore calibrations like external calibration or standard addition must be performed before. Neutron activation analysis and mass spectrometry each have their own analytical characteristics that define their scope of application and limitations for isotope-specific clinical medicine applications. These will be discussed in the following paragraphs.

4.2 Analytical Techniques for Measuring Stable Isotopes

4.2.1 Neutron Activation Analysis NAA

Neutron activation analysis is based on the capture of a neutron by the nucleus of a stable isotope of a chemical element, such as iron, which subsequently is converted into a radioactive isotope with suitable radioactive emissions (Table 1) [1]. The intensity of the radioactive emissions is proportional to the number of nuclei of the stable isotope undergoing the nuclear reaction. The most suitable (and strongest) source of neutrons for this application is a research reactor. NAA is not suitable for the measurement of every stable isotope, since the nuclear transformation must result in eventual production of a radioactive nuclide capable

of emitting suitable radiation that can selectively and specifically be measured, e.g., using a high resolution gamma-ray spectrometer.

When Fe is irradiated with neutrons, radioactive ^{59}Fe is formed from both the natural stable ^{58}Fe as well as ^{54}Mn and ^{56}Mn from the natural ^{54}Fe and ^{56}Fe isotopes (see Table 1) . Neutron activation of isotopes in biological materials may result in the production of high activity from radionuclides produced from matrix components such as C1, Na and K. However, these radionuclides all have much shorter half-lives than the long half-life activation products of Fe (^{59}Fe, ^{54}Mn) (Fig. 1), and their interferences are negligible after a decay period of about 10 days. [2].

Table 1: Nuclear properties of ^{58}Fe, ^{56}Fe, ^{54}Fe stable isotopes and their main γ-lines [1]:

	Nuclide	Relative isotopic abundance	Nuclear Reaction	Half live of the product	Cross section σ (in b, 10^{-24} cm^{-2})	Activation products (decay/sec per 1 µg element) after one hour irradiation $ø_{th}$ =thermal neutron flux $ø_{epi}$=epithermal neutron flux $ø_f$= fast neutron flux	Main γ-lines and between ( ): gamma-ray emission probabilities (%)
Slaw reactor neutrons						$ø_{th}=10^{13}$, $ø_{epi}= 2.10^{11}$ $cm^{-2}sec^{-1}$	
	^{58}Fe	0.28	(n, γ)^{59}Fe	44.6 d	1.15±0.02 b	0.256	1099.3(56), 1291.6(44)
Fast reactor neutrons						$ø_f=10^{12}$ $cm^{-2}sec^{-1}$	
	^{54}Fe	5.8	(n,p)^{54}Mn	312.5 d	82.5±5 mb	4.77E-3	834.8(100)
	^{56}Fe	91.7	(n,p)^{56}Mn	2.6 h	1.07±0.08 mb	2.49	846.6(99), 1811.2(30), 2112.6(15.5)

The two long half-life activation products of Fe thus open the opportunity for tracer experiments by e.g. the use of enriched stable ^{58}Fe in nutritional supplements, and neutron activation of e.g., blood, urine and faeces.

. The measured ^{54}Mn activity indicates the pathway of the natural present Fe, and the measured ^{59}Fe activity, together with the earlier determined activity ratio of ^{59}Fe/^{54}Mn for Fe of natural isotopic composition, allow to estimate the fraction originating from the

supplemented iron and thus the efficacy of the supplementation. Figure 1 shows the Gamma-ray spectra of ^{59}Fe and ^{54}Mn.

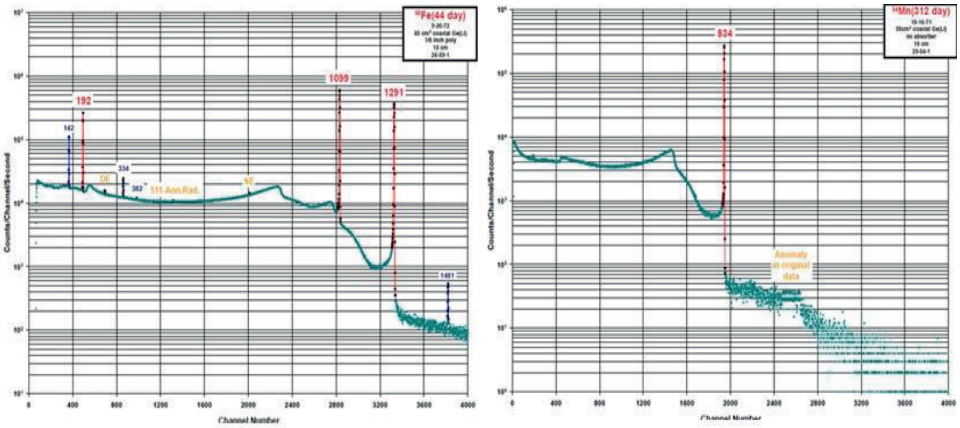


Fig. 1 Gamma-ray spectra of ^{59}Fe and ^{54}Mn [3].

4.2.2 Inorganic Mass Spectrometry

This technique measurement of (enriched) stable isotopes. It is based on the use of a magnetic sector which separates masses in a magnetic field [8,9]. Several types of mass spectrometry have been applied for measurement of stable isotopes of iron, sometimes by hybridization with another analytical technique. Examples are thermal ionization mass spectrometry (TIMS), ICP-MS and MC-ICP-MS. Although TIMS has a high ionization efficiency [4,5,9] which allows very precise measurements of mole fraction ratios of elements that can be ionized thermally, it has some limitations [10,11] which include:

- not all elements are easily ionized, which restricts applications to elements with low ionization potentials;
- ionization is not equally efficient for all elements.
- elementally pure solutions are required to avoid isobaric interferences, which require extensive preparation; and
- accurate mass fractionation correction is limited to elements with 3 or more isotopes of which at least 2 are stable. Mono-isotopic elements cannot be analyzed.

In 1983, the inductively coupled plasma mass spectrometry (ICP-MS) became commercially available. ICP-MS makes use of a mass analyzer to separate positive ions, extracted from an inductively coupled argon plasma. After mass separation, ions with the selected mass can enter the detector, which normally is an electron multiplier. In almost all ICP-MS instruments a quadrupole is used as a mass filter [8], now available in combination with different techniques; e.g.; quadrupole with collision cell technique, quadrupole with dynamic reaction cell technique and quadrupole – Ked mode (kinetic energy discrimination mode). It allows ions with a mass difference of at least 0.5 amu to be separated. Double focusing magnetic sector instruments were become more and more available nowadays. The technique is widely acknowledged for its capability of accurate quantitative multi-element analysis at very low levels. Conventional ICP-MS analysis uses a quadrupole analyser, which only allows single-collector analysis. Due to the inherent instability of the plasma, this limits the precision of ICP-MS for isotopic ratio measurement, and thus for the applications in the frame of the study described in this thesis.

Since 15 years, multiple collector-ICP mass spectrometry (MC-ICP-MS) has been described as a much better mass resolution technique for mole fraction ratio measurements, such as for of iron and other essential metals in bio-fluids [11-19]. As such, the capabilities of MC-ICP-MS and INAA should be compared.

4.2.2.1 Measurement of iron isotopic ratio(s) using MC-ICP-MS

MC-ICP-MS was first used by geochemist and more recently its capability for biomedical applications have also been discovered. As a hybrid mass spectrometer, MC-ICP-MS combines an inductively coupled plasma source, an energy filter, a magnetic sector analyzer, and multiple collectors for the measurement of ions (Fig. 2) It is used in this project as a technique to compare INAA with in the measurement of stable iron mole fraction ratios in blood of patients with iron related disorders.

The advantages of the MC-ICP-MS are the stable mass bias with little drift within and between runs. The disadvantages of the MC-ICP-MS can be concluded from the fact that, the accuracy of stable mole fraction ratio determination of iron and other transition metals depends on (1) the quantitative removal of molecular interferences (e.g. Ar-oxides, Ar-nitrides, Ar-and metal hydrides); (2) accurate corrections for even smallest amounts of isobaric elemental interferences (e.g. $^{54}Cr+$ on $^{54}Fe+$, $^{58}Ni+$ on $^{58}Fe+$, etc.); and (3) precise correction of the instrumental mass bias which is usually found in a few % per atomic mass

unit in ICP-MS and that strongly depends on the instrumental set-up, the matrix, and the degree of purity in which elements are presented [20].

In general all MS methods for biological materials require some degree of chemical separation, while the nature of the matrix and extent varies with the specific technique.

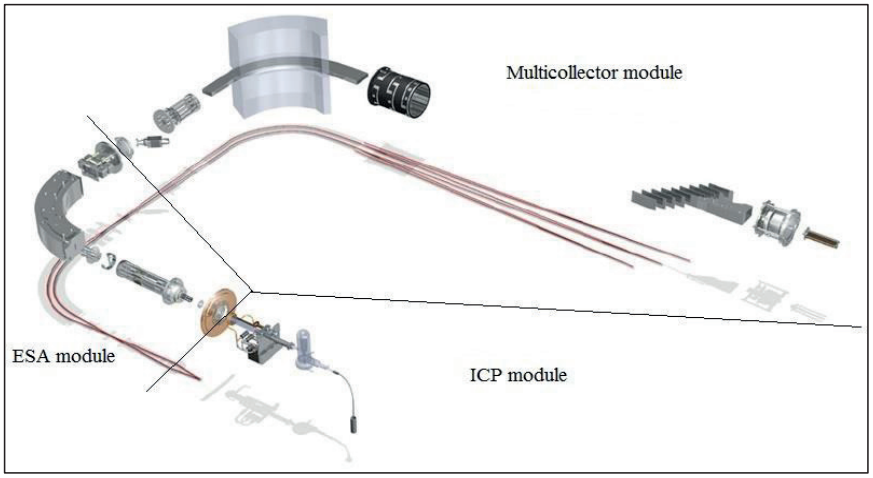


Fig. 2. Schematic drawing of Neptune MC-ICP-MS [21].

4.3 A comparison of the practical aspects of INAA and (MC)-ICP-MS

In projects like described in this thesis, both the total amount of Fe and the ratio of the amounts of Fe isotopes has to be measured. Whereas this is done simultaneously in NAA, it requires both ICP-MS – for the Fe amounts- and MC-ICP-MS –for the Fe isotopic ratios. The pros and cons of NAA and (MC)ICP-MS can be evaluated on the basis of several criteria like the metrological analytical characteristics, the nature of the material analysed, the elements to be measured and the practical aspects in using these techniques.

4.3.1 Preparation of the test portion

Measuring iron mole fraction ratios in blood by (MC)-ICP-MS needs two pre-treatment steps, first digestion with acids and secondly chromatographic separation of iron from its sample matrix and all potential interfering elements/isotopes such as from Cr and Ni. INAA is a non-destructive method, there is no need to convert or dilute a sample into a suitable solution prior

to analysis, which is especially attractive using solid biological material such as tissues, nails and bones. Only freeze drying is needed for liquid samples such as blood.

In general MC-ICP-MS is well suited for the direct analysis of solutions while in INAA of solutions is not allowed in most reactors because of radiological safety requirements.

4.3.2 Sample amount

In most analytical techniques the sample size is about 100 mg. Analysis of larger samples can be preferable e.g., when a representative 100 mg sample is difficult to obtain, the trace element distribution is of interest, or when the sample must remain intact. Instrumental neutron activation analysis has all the potentials to analyse, even with adequate accuracy, large samples in the kilogram range, when specially designed irradiation positions and carefully planned measuring conditions are available. The methodology is well established [22,23]. This is a unique characteristic for NAA and it is used in this project to measure iron in the total dietary intake.

4.3.3 Interferences

4.3.3.1 Interferences in MC-ICP-MS

There are two types of interferences, which can occur in MC-ICP-MS, spectroscopic interferences and non-spectroscopic interferences or matrix effects.

4.3.3.1.1 Spectroscopic interferences

The important spectroscopic interference that can be happen in MC-ICP-MS is the interference due to isobaric overlap which occurs when two elements have isotopes with the same mass. Other examples of the spectroscopic interferences are[8]:

(1) The interference due to poly-atomic ions which represents a serious interference problem more than the interference due to isobaric overlap and therefore the main significant limiting factor. Polyatomic species consist of atoms originating from the reaction between plasma gas and the solvent and the reagents used to prepare the sample solution, e.g., ArO^+. Argon, hydrogen and oxygen are the main species present in the plasma while the major elements present in the solvent or acids are e.g. N, S and Cl. This problem is significant in ICP-MS but less problematic in MC-ICP-MS due to its much higher mass resolution.

(2) Interference due to refractory oxides ions, which type of interference occurs because of incomplete dissociation of the sample matrix or from recombination in the plasma tail and always occurs as an interference as an integral value of 16 mass units above the interfering

element, the result is an interference 16 (MO^+), 32 (MO_2^+) or 48 (MO_3^+) mass units above the M^+ peak.

(3) The last possible type of the spectral interferences that can occur is the interference due to double charged ions, which form when the 2nd ionization potential of the element is less than the first ionization potential of Ar. It is typical of alkaline earths, a few transition metals, and some rare earth elements.

Table 2 shows the possible spectral interferences for iron stable isotopes in ICP-MS analysis [24]

Table 2: Iron isotope interferences in ICP-MS analysis.

Isotope	Natural abundance	Spectral interference
	atomic %	
^{54}Fe	5.845	$^{40}Ar^{14}N^+$, $^{53}Cr^1H^+$, $^{108}Cd^{++}$, $^{108}Pd^{++}$, $^{54}Cr^+$
^{56}Fe	91.754	$^{40}Ar^{16}O^+$, $^{40}Ca^{16}O^+$, $^{55}Mn^1H^+$, $^{112}Cd^{++}$, $^{112}Sn^{++}$
^{57}Fe	2.119	$^{40}Ar^{16}O^1H^+$, $^{40}Ca^{16}O^1H^+$, $^{56}Fe^1H^+$, $^{114}Cd^{++}$, $^{114}Sn^{++}$
^{58}Fe	0.282	$^{40}Ar^{18}O^+$, $^{40}Ca^{18}O^+$, $^{42}Ca^{16}O^+$, $^{57}Fe^1H^+$, $^{116}Cd^{++}$, $^{116}Sn^{++}$, $^{58}Ni^+$

4.3.3.1.2 Non- spectroscopic interferences (matrix effects)

The non-spectroscopic interferences are caused by matrix components [20,24-26], e.g. deposits of solids on the cones, memory effects, different viscosities of samples and standards, shifts in the ion/atom equilibrium due to changes in the plasma conditions and scattering effects of ions with matrix atoms. Memory problems are less severe, because the analysis conducted with clean solution of separated element In general and as a rule, non-spectral interferences cause suppression of the signal, but in certain cases they can cause signal amplification. Matrix effects can be automatically corrected for when additional internal standard or isotope dilution are used for standardization [27].

4.3.3.2 Interferences in INAA

For INAA, there are several different types of interferences in the measurement of Fe isotopes. Primary interferences occur when the desired product radionuclide can be produced by more than one nuclear reaction [28]. For example, Fe radionuclides can also be formed not only by neutron activation of Fe, but also due to the presence of Cobalt: :

Main reaction: ^{58}Fe (n, γ) ^{59}Fe

Interference reaction: ^{59}Co (n, p) ^{59}Fe

Secondary interferences occur when the desired product radionuclide is produced by the decay of another product nuclide during the irradiation and subsequent neutron activation of the decay product.

A third type of interference can occur in the gamma spectroscopy. Two or more radionuclides may emit gamma rays of (approximately) the same energy , and hence, one may interfere in the detection of the other. This can be resolved by using other gamma rays that are emitted from that same radionuclide for quantification; or, if the radionuclides have strongly different half-lives, by measuring the sample after different decay times.

Table 3. Overview of principal (bold) and interfering nuclear reactions for Fe measurement by NAA, and induced activity under stipulated conditions[1].

Radionuclide of interest	Element	Reaction	Induced activity (Bq/μg) 1 h irradiation	Comments
^{59}Fe	**Fe**	**^{58}Fe (n, γ) ^{59}Fe**	0.25	Thermal neutrons
	Co	^{59}Co (n, p) ^{59}Fe	$9.4.10^{-3}$	14 MeV neutrons
	Ni	^{62}Ni (n, α) ^{59}Fe	$8.8.10^{-6}$	Fast reactor neutrons
^{56}Mn	**Fe**	**^{56}Fe (n, p) ^{56}Mn**	2.5	Fast reactor neutrons
	Mn	^{55}Mn (n, γ) ^{56}Mn	$3.5.10^{5}$	Thermal neutrons
	Co	^{58}Co (n, α) ^{56}Mn	0.38	Fast reactor neutrons
^{54}Mn	**Fe**	**^{54}Fe (n, p) ^{54}Fe**	$4.8.10^{-3}$	Thermal neutrons
	Mn	^{55}Mn (n,2n) ^{54}Mn	$2.6.10^{-4}$	Fast reactor neutrons

The primary interference is the most important in this study, because iron measurement will be via ^{59}Fe and ^{54}Mn and these radionuclides can be produced by other different ways rather than the main reactions. An overview of these reactions and estimates of the activity induced is given in Table 3.

All the above interferences can be corrected for if estimates for the amounts of Co, Ni and Mn are available.

[1] Reference [1]: Assumed thermal neutron flux : 10^{13} $cm^{-2}s^{-1}$;
assumed epithermal+ fast neutron flux: $2.10^{11}cm^{-2}s^{-1}$.

4.3.4 Calibration

All phenomena in INAA (nuclear reaction, decay, emission of radiation) relate to properties of the atomic nucleus [28]. Hence, the determination of an element via measurement of its induced radioactivity is independent of its chemical state or physical form; there is no difference if an element is bound to an inorganic compound or an organic compound, or if it is present as a pure metal, provided the degrees of gamma-ray self-attenuation in sample and calibrator are equivalent and can be quantified. Chemical matrix effects, known to be significant sources of error in some other types of instrumental chemical analysis, are insignificant in INAA or can be quantified and corrected for. This characteristic implies that the calibrators (standards) in INAA do not have to be identical in composition than the measurands which eases the analyses of a phenomenal variety of sample types.

Calibration in ICP-MS is done by the 'Comparative Method' in which the measurement of an element is based upon comparison of the signal of the unknown with the signal of the same element in a standard in the same physical and chemical form as the sample of interest. Calibration usually takes place prior to actual analysis of the unknown sample [28]. Analysts face the practical problem that differences in chemical composition of sample and standard ("matrix-dependency") may have significant impact to the degree of trueness of the results in (MC)-ICP-MS. Three calibration methods can be used in ICP-MS [29]: (i) general external calibration, sufficient in most cases. (ii) Standard addition is applied in cases that matrix induced signal suppression or enhancement is expected or turned out to be insufficiently corrected for by the internal standard. (iii) Isotope dilution is applied only exceptionally.

4.3.5 Detection limit

The detection limit of an element or limit of detection (LOD) is defined as the smallest quantity of an analyte that can produce a signal that is significantly higher than the blank with some certainty [30]. The methodological detection limit (MDL) is sometimes defined as "The constituent concentration that, when processed through the complete method, produces a signal with a 99% probability that it is different from the blank" [31]. However, this definition can only be interpreted if also the concept 'blank' is unambiguously defined. The concept of MDL is different from the concept 'instrument detection limit'(IDL). Instrument detection limits provide the analyst with information on the lowest levels that can actually be measured in real samples, which are sometimes quoted in the manuals of instruments. They are useful for comparing the instrumental detection capability of different methods.

Detection limits and quantitation limits[2] in measurements of radioactivity are commonly estimated following the concepts developed by Lloyd Currie [32]. In INAA this detection limit is related to the ability of detecting a gamma-ray peak in the presence of interference from natural radioactivity and activities, induced by neutron activation [33]; and therefore commonly calculated from the background signal resulting from all activities in a real sample. Detection limits in INAA depend on irradiation and counting conditions, sample composition and also strongly vary for the chemical elements. Such values can also be established for a 'blank', which results in the lowest detection limits under the selected conditions; however, the detection limits in real samples will always be higher. In this study for iron the blank (empty capsule) revealed in an absolute detectable amount for Fe of 0.13 μg. While for blood samples it was 0.4 μg. Radiochemical INAA can be used to eliminate spectral interferences by isolating the radionuclide of interest, thus improving detection limits in the actual matrix and the precision. Light elements (such as H, C, N, O) and some elements with high atomic number (T1, Pb and Bi) cannot be measured by INAA.

The instrumental detection limits in ICP-MS differ from those commonly reported in NAA. The reported instrumental limits of detection for ICP-MS are usually calculated as equivalent concentrations corresponding to 3 times the standard deviation on the results of these measurements. It is based on a large number of measurements (e.g. 20) of the background level for a blank solution measured under reproducible circumstances. For the MC-ICP-MS used in this study, the minimum detectable amount of Fe was a few hundred ppb, it varies from day to day with machine settings and with chosen resolution of the instrument combined with the integration time per ratio measured.

Unlike INAA, the detection limits of the ICP-MS not strongly scatter throughout the periodic table. The sensitivity of ICP-MS increases with increasing mass number and is lower for elements with a high first ionisation potential [34].

The methodological detection limit (MDL) or Practical Quantitation Limit (PQL), is generally higher (2-10 times) than the IDL and depends on many factors, including laboratory and instrument background levels, sample matrix, sample collection and preparation methods, and operator skill level.

[2] However named, like detection limit, determination limit, quantitation limit, reporting limit, minimum dateable amount, minimum detectable activity, etc.

4.3.6 Accuracy and Trueness

Measurement accuracy is defined as closeness of agreement between a measured quantity value and a true quantity value of a measurand [35]. The concept 'measurement accuracy' is not a quantity and is not given a numerical quantity value as it combines the concepts of accuracy and precision. A measurement is said to be more accurate when it offers a smaller measurement error. Measurement trueness is defined as 'the closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value [35].

INAA can be executed with a high degree of trueness (and therefore also at a high degree of accuracy) since all potential sources of error are known and procedures have been optimised and developed to avoid, minimize or quantify and correct for systematic errors. It has been the main consideration to designate INAA of having the capability to meet the requirements of a primary (ratio) method of measurement. [36].

Although accurate results can also be produced by ICP-MS, the accuracy for mole fraction ratio measurements is limited because of several effects [37]. The mass discrimination effect - which occurs due to the space charge effect- plays an important role in mass spectrometry. In the step of ion extraction, the light ions are deflected more than the heavy ions. Therefore the ratio of lighter to heavier isotope can be smaller than the true value e.g.; $^{54}Fe/^{58}Fe_{measured} < ^{54}Fe/^{58}Fe_{true}$.

An important factor that limits the accuracy of the mole fraction ratio measurement by MC-ICP-MS is the occurrence of a multitude of different isobaric interferences with the analyte ions such as isobaric atomic or molecular ions; e.g analysis of ^{58}Fe in solution by MC-ICP-MS is only possible if the ^{58}Ni has been separated [34].

Other limitations on mole fraction ratio measurement are the instrumental background, contamination on the sample introduction system and skimmer cone, plasma instabilities, drift of ion intensities and matrix effects.

Mole fraction ratio measurements with single-collector double focusing sector field ICP-MS instruments are limited by using single collector ion detection system or the poor peak shapes produced by mass analyser. MC-ICP-MS instruments were built to overcome these limitations, they combine the Ar ICP-source of conventional ICP-MS instruments with the magnetic sector analyser and multiple-Faraday cup array of the TIMS The magnetic sector

mass analysers of MC-ICP-MS instruments are similar to those used in TIMS, with the ultimate aim of achieving the flat-topped peaks necessary for high-precision mole fraction ratio measurements. While the use of a detector array with multiple Faraday cups allows the simultaneous collection of the separated isotopes and this cancels out the effect of a "noisy" signal on the mole fraction ratio measurement. Moreover, the Faraday cups are independently adjustable, such that the collector can be adjusted to permit isotopic analyses of a wide range of elements having isotopes with different mass.

4.3.7 Uncertainty of measurement

Uncertainty is defined as "non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information use"[35]. All sources that contribute to its uncertainty in INAA are known and can be quantified [36]. The degree of accuracy of the physical data is accounted for and the contributions to the uncertainty are estimated in the various steps in the analysis process like weighting, calibration, counting statistics, neutron flux monitoring and geometry.

MC- ICP-MS can measure isotope ratios with great precision because the ion beams from different isotopes are measured simultaneously with different detectors. Accurate isotope ratio results require that the measurement system be fully understood. Accounting for all sources of uncertainty in such analyses, according to JCGM Evaluation of measurement data — Guide to the expression of uncertainty in measurement (GUM) [37], requires understanding the characteristics of the ion beam, and the instrument response. Modern multi-collector mass spectrometers record multiple electronic integrations of ion beam signals for a set time period, for example 5 seconds, and ratios of these signals on two detectors are calculated. The standard uncertainty on the mean of these ratios, a measure of the repeatability, is an important part of the combined standard uncertainty. [38]

4.3.8 Blanks and Contamination

Contamination in INAA may occur during the sample (test portion) preparation for irradiation such as the manipulation of solid samples and packing in pure plastic (polyethylene) or quartz vials. Therefore, the contamination control directs mainly to this stage. During irradiation, contamination of the test portion may occur due to recoil of radionuclides from neutron-activated impurities in the encapsulation material, such as by ^{51}Cr from Cr impurities in polyethylene. After irradiation, any contamination due to manipulation does not interfere with the measurements unless the contamination results from radionuclides. This is a major

advantage of INAA, making the technique very suitable for analysis of samples with low (trace) element content, e.g. biological tissue.

In MC- ICP-MS the risk of contamination may be higher. Reagents and water must be of high purity; losses due to incomplete dissolution are possible. Memory effect should be considered when measuring by ICP-MS and can be reduced or eliminated by rinsing the sample introduction system with (diluted) acid; often HNO3. Blanks must be prepared and measured for each analysis.

4.3.9 Practical Aspects

INAA is not as widely applied as mass spectrometry because of its requirement of access to a nuclear analytical facility, and the availability of the alternative techniques, as outlined in the Introduction of this Chapter. The decreasing number of INAA users also results from insufficient awareness within the applied field regarding the opportunities of this technique. For many elements the turnaround of INAA is relatively long and the daily sample throughput is much lower than what ICP-MS can offer. Moreover, the cost of INAA is often considered to be higher than other analytical methods. However, the cost of analysing 20 whole blood samples for Fe with INAA at the facilities of the Delft University of Technology is equivalent to the costs of analysing the same samples for isotope Fe ratio measurement using MC-ICP-MS at the VU University, Amsterdam, if the costs of the sample digestion procedure and its validation, and the costs of chromatography separation (to avoid the matrix interferences) are accounted for.

The turn-around times are different though. With MC-ICP-MS the measurement of e.g. 20 elements in 5 samples takes less than 1 hour whereas for multi-element determination, a full protocol with INAA may require, depending on the elements asked for, 15 weeks although the measurement of the induced activity in such 5 samples requires e.g. only 5 hours or less. The choice of the INAA protocol depends on the sample matrix and the type of element (related short or long half-life radionuclides) and potential interfering radionuclides therefore the necessary decay time prior to measurement.

In general MC-ICP-MS (often in combination with ICP-MS) is more used worldwide than INAA. There are about 125 INAA labs worldwide (based on the information from the IAEA Research Reactor Data Base) [39].

4.4. A summary of the characteristics of INAA and (MC)-ICP-MS

4.4.1 Instrumental neutron activation analysis

INAA has the following attractive features for the type of research described in this thesis:

(1) High specificity and selectivity for Fe based on the individual characteristics of the induced radionuclides, e.g; the specificity and selectivity for iron close to 100%.

(2) Relative freedom from matrix and interference effects.

(3) The method is non-destructive. There is no need to convert the sample into a solution prior to analysis.

(4) Almost absence of an analytical blank. The method is relatively free from contamination.

(5) High degree of trueness and acceptable precision. The method is physically and chemically fully understood; so a complete uncertainty therefore, all contributions to uncertainty of measurement can be quantitatively evaluated.

(6) Little to none dependency on commutability of calibrators; method validation applicable to a wide variety of sample types.

(7) Large samples can be analysed without homogenization, which is relevant for dietary intake studies.

4.4.2 (MC)-ICP-MS

(1) Specificity and selectivity for Fe and Fe isotopes affected by isobaric interferences, requiring (for MC-ICP-MS) tedious quantitative removal of Cr and Ni

(2) Very high sensitivity (ICP-MS) and precision (MC-ICP-MS)

(3) Sample need to be brought into a solution prior to analysis.

(4) Blank requires ample attention, especially related to sample preparation and purification.

(5) Acceptable degree of trueness in ICP-MS and very high degree of trueness in MC-ICP-MS. Degree of trueness in ICP-MS depends on quantification of the recovery if samples have to be brought into a solution. The major contributions to uncertainty of measurement can be quantitatively evaluated.

(6) Dependency on commutability of calibrators in ICP-MS ; method validation has to be very specific for the type of samples. Commutability in MC-ICP-MS can be circumvented by isolation of the measurand.

4.5. References

[1] G. Erdtmann, Neutron activation Tables, Verlag Chemie, Weinheim (1976). ISBN 3-527-25693-8.

[2] M. Blaauw. The k_0-Consistent IRI gamma-ray catalogue for INAA. (1996), ISBN 90-73861-32-2.

[3] http://www4vip.inl.gov/gammaray/catalogs/pdf/gecat.pdf.

[4] P. E Johnson. Methodology for isotope analysis in biological materials (a review). J. Micronutr. Anal. 6 (1989) 59-83.

[5] Assessment of iron bioavailability in humans using stable iron isotope techniques. IAEA human health series publications No. 21, (2012), ISBN 978-92-0-126510-4.

[6] A. Yergey. Analytical instruments for stable isotopic tracer in mineral metabolism. J. Nutr. 126 (1) (1996) 355s-361S.

[7] F. A. Mellon and S. J. Fairweather-Tait. Stable isotope method for studying nutrient mineral metabolism in humans. Endeavour, (1997); 21(1): 12-18.

[8] K. E. Jarvis, A. L. Gray and R. S. Houk. Hand book of Inductively Coupled Plasma Spectrometry. (2003), ISBN 0-9544891-0-1.

[9] The use of ICP-MS for human biomonitoring (Biomonitoring Methods). (1999), Published Online: 31 Jan. 2012.

[10] J. D. Inglis, Y. Kinugawa and K. O´Brien. Improving iron isotope analysis for stable isotope analysis using ID-TIMS. FASEB J. 21 (6) A1115.

[11] F. Vanhaeke and M. Costas-Rodriquez. What's up doc?-High-precision isotopic analysis of essential metals in biofluids for medical diagnosis. Spectr. Europe, (2015) vol. 27 (3), 11-14.

[12] N. Dauphas, A. Pourmand and F. Teng. Routine isotopic analysis of iron by HR-MC-ICPMS: How precise and how accurate?. Chem. Geol. 267 (2009) 175–184.

[13] F. von Blanckenburg, M. Oelze, D. G. Schmid, K. van Zuilen, H. Gschwind, A. J. Slade, S. Stitah, D. Kaufmann and P. Swart. An iron stable isotope comparison between human erythrocytes and plasma. Metallomics, (2014), 6, 2052-2061.

[14] O. Takeshi , S. Atsuko , K. Ichitaro, C. Momoko and H. Takafumi. Isotopic analysis of Fe in human red blood cells by Multiple Collector-ICP-Mass Spectrometry. Anal. Sci. 20, (2004), 617-621.

[15] L. Van Heghe, J. Delanghe, H. Van Vlierberghe and F. Vanhaecke. The relationship between the iron isotopic composition of human whole blood and iron status parameters. Metallomics, 2013, 5, 1503-1509.

[16] T. Walczyk and F. von Blanckenburg. Natural iron isotope variations in human blood. Science 295 (2002), 2065-2066.

[17] L. Van Heghe, E. Engstrom, I. Rodushkin, C. Cloquet and F. Vanhaecke. Isotopic analysis of the metabolically relevant transition metals Cu, Fe and Zn in human blood from vegetarians and omnivores using multi-collector ICP-mass spectrometry. J. Anal. At. Spectrom., (2012), 27, 1327–1334.

[18] R. Schoenberg, F. von Blanckenburg. An assessment of the accuracy of stable Fe isotope ratio measurements on samples with organic and inorganic matrices by high-resolution multicollector ICP-MS. Int.J. Mass Spectr.242 (2005) 257–272.

[19] K. Hotz, H. Augsburger and T. Walczyk. Isotopic signatures of iron in body tissues as a potential biomarker for iron metabolism. J. Anal. At. Spectrom., (2011), 26, 1347-1353.

[20] M. Rehkamper, M. Schonbachler and C. H. Stirling. Multiple collector ICP-MS: Introduction to Instrumentation, measurement techniques and analytical capabilities. J. Geost. Geoanal. (2000), 25 (1), 23-40.

[21] http://lmv.univ-bpclermont.fr/mc-icpms-neptune/

[22] R. M. W. Overwater. The physics of big sample instrumental neutron activation analysis. PhD thesis Delft University of Technology (1994), ISBN: 90-407-1048-1.

[23] P. Bode. "Activation Analysis of Large Samples" in: Encyclopedia of Analytical Chemistry, eds R.A. Meyers, John Wiley: Chichester. DOI: 10.1002/9780470027318.a9021. Published 29 September 2008.

[24] Z. Chen, I. J. Griffin, L. M. Plumlee, and S. A. Abrams. High Resolution Inductively Coupled Plasma Mass Spectrometry allows rapid assessment of iron absorption in infants and children. J. Nutr. (2005) 135(7):1790-1795.

[25] R. F. J. Dams, J. Goossens and L. Moens. Spectral and non-spectral interferences in Inductively coupled Plasma Mass- Spectrometry. Mikrochim . Acta 119, (1995), 277-286.

[26] E. Bjorn and W. Frech. Non-spectral interference effects in inductively coupled plasma mass spectrometry using direct injection high efficiency and microconcentric nebulisation. J.Anal. At Spectrom, (2001), 16, 4-11.

[27] A. J. R. Kent, B. Jacobsen, D. W. Peate, T. E. Waight and J. A. Baker. Isotope Dilution MC-ICP-MS Rare Earth Element Analysis of Geochemical Reference Materials NIST SRM 610, NIST SRM 612, NIST SRM 614, BHVO-2G, 2, BCR-2G, JB-2, WS-E, W-2, AGV-1 and AGV-2. J. Geost. Geoanal. (2004), 28 (3), 417-429.

[28] S. J. Parry. Handbook of Neutron Activation Analysis. (2003), ISBN 0-9544891-1-X.

[29] L. Moens, H. Vanhoe, F. Vanhaecke, J. Goossens, M. Campbell and R. Dams. Application of Inductively coupled Plasma Mass Spectrometry to the Certification of Reference Materials from the Community Bureau of Reference. J. Anal. Atom.Spectr., (1994), 9: 187-191.

[30] R. C May, H. Chu, J. G. Ibrahim, M. G Hudgens, A. C. Lees and D. M. Margolis. Change-point model to estimate the limit of detection. Stat. Med. (2013), 32(28), 4995-5007.

[31] APHA (American Public Health Association). 1998. Standard methods for the examination of water and wastewater, 20th edition, L.S. Clesceri, A.E. Greenberg, and A.D. Eaton. Washington, DC: American Public Health Association.

[32] L.A Currie, "Limits for Qualitative Detection and Quantitative Determination: Application to Radiochemistry". Anal. Chem. 40 (1968) 586-593.

[33] P. Bode. Instrumental and Organizational aspects of a neutron activation analysis laboratory. PhD thesis Delft University of Technology (1996), ISBN 90-73861-42-X.

[34] L. Mones and R. Dams. NAA and ICP-MS: A comparison between two methods for trace and ultra-trace element analysis. J. Radioanal.Nucl. Chem., Articles, 192, No. 1 (1995) 29-38

[35] JCGM 200: (2012), International vocabulary of metrology – Basic and general concepts and associated terms (VIM) 3rd edition.

[36] R. R. Greenberg, P. Bode, E. A. De Nadai Fernandes. Neutron activation analysis: A primary method of measurement. Spectrochim.Acta B 66 (2011) 193–241.

[37] JCGM 100: (2008) GUM 1995 with minor corrections. Evaluation of measurement data — Guide to the expression of uncertainty in measurement.

[38] R. Williams. Uncertainty in Measurement of Isotope Ratios by Multi-Collector Mass Spectrometry. IAEA-CN-184/168.
https://www.iaea.org/safeguards/symposium/2010/Documents/PapersRepository/168.pdf

[39] IAEA Research Reactor Data Base http://nucleus.iaea.org/RRDB.

5 Practicability of Measuring the Enriched Stable Isotope ^{58}Fe in Blood Samples of Patients With Iron Related Disorders Using INAA and MC-ICP-MS

5.1 Introduction

The absorption and distribution of iron in men can be studied with the use of iron (Fe) isotopes. Since ^{59}Fe has the side effect of radiation, enriched stable iron isotopes are an attractive alternative. However, they can only be measured with an isotope-specific technique either mass spectrometry (MS) or instrumental neutron activation analysis (INAA). A direct comparison of INAA and MC-ICP-MS with the focus on the analytical and practical aspects in a clinical setting to measure iron isotopes has not been performed yet. Since INAA has the ability to measure low concentrations of elements in various biological materials without complicated preparation steps, we are convinced that it can contribute to clinical research. However its practicability in clinical medicine needs to be established among others by comparing it with the standard technique for measuring on atomic level, MS.

Analytical aspects include e.g. the degree of trueness and (instrumental and methodological) detection limits while availability of instruments and facilities, preparation of material, time consumption and costs determine the practical aspects. In INAA, the detection limit is based on the noise level in the actually measured sample. The MC-ICP-MS instruments are designed to combine the benefits of other mass spectrometric techniques such as the ordinary ICP-MS (plasma based ionisation) and TIMS (simultaneous detection of isotopes). The use of a detector array with multiple Faraday cups which permits the simultaneous collection of the separated isotopes eliminates the effect of a “noisy” signal on the isotope ratio measurements. The magnetic sector mass analysers of MC-ICP-MS are similar to those used in TIMS which achieve the flat-topped peaks necessary for high precision [1].

MC-ICP-MS has been used to measure the natural iron stable isotopes in human blood [2-6], animal tissues [7], synthetic Fe [8] and in samples with organic and inorganic matrices [9].

INAA can be executed with a high degree of trueness (and therefore also at a high degree of accuracy). Since the concept 'measurement accuracy' is combines the concepts of accuracy and precision, therefore INAA classified as a high precise technique. All potential sources of error are known and procedures have been optimised and developed to avoid, minimize or quantify and correct for systematic errors. It has been the main consideration to designate INAA of having the capability to meet the requirements of a primary (ratio) method of measurement [10].

Data on the use of INAA to measure Fe isotopes in clinical research are scarce. The aim of this experiment is to compare INAA and ICP-MS in the measurement of enriched stable ^{58}Fe in blood samples of people with either iron deficiency anaemia or haemochromatosis. For this purpose, we had to prepare a pharmaceutical formula of iron sulphate containing the enriched stable isotope ^{58}Fe. The labelling with ^{58}Fe in this study is an example of extrinsic labelling, in which an enriched stable isotope is added directly onto a supplement or mixed with a test meal. A liquid pharmaceutical containing 5 mg of iron sulphate consisting of 92.3% ^{58}Fe was given together with a glass of orange juice to patients and healthy controls. Before and two weeks after administration blood samples were taken in which the $^{58}Fe/^{54}Fe$ ratio was measured using both INAA and MC-ICP-MS.

5.2 Materials and methods

5.2.1 Preparation of the enriched ^{58}Fe containing oral iron supplement

The ^{58} Fe enriched oral supplement was prepared at the department of pharmacy of the St. Antonius Hospital, Nieuwegein, The Netherlands. The enriched iron was purchased from Isoflex, San Francisco, USA and contained 92.3% ^{58} Fe (Table 1). A total of 73 mg of ^{58}Fe was mixed with 6.86 ml 0.5 M H_2SO_4 and heated to 53° C until all iron was dissolved. Fe^{3+} was reduced to Fe^{2+} by adding 219 mg of ascorbic acid and the solution was diluted with deionized water to a final Fe concentration of 1 mg/mL. The solution was deaerated and distributed into vials each containing 5 mL of the iron sulfate solution. The vials were stored at 15° C and all were used within three weeks after preparation.

Table 1. Enriched Stable Isotope Composition of Iron used in this experiment:

Stable isotope	Natural abundance (atom. %)	Available Enrichment (atom. %)
^{54}Fe	5.845	<0.01
^{56}Fe	91.754	1.0
^{57}Fe	2.119	6.7
^{58}Fe	0.282	92.3

5.2.2 Patients and administrating Fe supplement

After approval by the medical ethical committee, eleven individuals that were under medical treatment at the Meander Medical Centre, Amersfoort, the Netherlands, participated in this study: Four persons with iron deficiency anaemia (Hb < 7 mmol/L (<11g/dL), serum iron< 7μmol/L and MCV< 75fL), three with hereditary hemochromatosis (C282Y mutation) under treatment with blood-letting and four healthy subjects (hospital workers/volunteers with normal Hb and iron status). The medical history of each person was recorded, as well as eating pattern and smoking status. Some of the demographic data as well as haemoglobin concentration and iron parameters are presented in Table 2. Following overnight fasting (8-9 hour), all subjects attended the hospital where 10 ml blood was collected from each of them and separated in two tubes. One sample allowed for the measurement of the person's full blood count and serum ferritin, transferrin and haemoglobin (Hb) in the hospital. The second sample served for measuring the iron isotopic ratios before the start of the supplementing.

Each person was given 5 mL of the supplement containing 5 mg of enriched 58Fe (92.3%) for oral intake. To obtain a better absorption, the supplement was mixed with 100 mL orange juice. Intake of food, tea or coffee was not allowed for the next 2 h. After two weeks (to allow the incorporation of iron into the erythrocytes) [11] another 10 mL blood was collected. All blood samples were frozen and sent to the Reactor Institute, Delft, the Netherlands for analyses.

Table 2. Demographic data, haemoglobin (Hb) concentration and iron parameters of the individuals participating in the study:

No	diagnosis	sex	Age (years)	Weight (kg)	Length (m)	Hb (mmol/L)	Serum Fe (µmol/L	Ferritin (µg/L)	Trf. (g/L)	Trf. Sat. (%)
1	IDA	F	64	61	1.70	5.9	5	130	2.4	9
2	IDA	M	52	85	1.89	6.8	4	37	2.5	10
3	IDA	F	52	112	1.52	6.8	6	10	3.0	8
4	IDA	M	67	98	1.79	6.2	4	21	2.8	6
5	HH (homozygous)	M	66	107	1.88	8.4	21	312	1.8	49
6	HH (homozygous)	F	42	58	1.67	9.0	24	181	1.8	56
7	HH (homozygous)	M	46	105	1.90	9.2	24	41	2.2	46
8	Contr.	M	64	88	1.82	10,2	20	208	2.0	42
9	Contr.	F	28	64	1.68	9.1	16	24	3.9	17
10	Contr.	M	27	80	1.83	9.5	22	70	2.6	36
11	Contr.	F	27	63	1.62	8.7	19	27	2.7	29

F: female, M: male, IDA: iron deficiency anaemia, HH: hereditary hemochromatosis, Contr.: control

5.2.3 INAA, sample preparation, irradiation and measurement

The blood samples were freeze-dried in an EZ- dry freeze drier (MNL-036-A) from FTS System Inc., Stone Ridge, New York, USA. About 200 mg of each dried sample was weighed and packed in high purity polyethylene capsules. The irradiation took place in the 2 MW nuclear research reactor of the Reactor Institute Delft, Delft University of Technology, The Netherlands. During the irradiation zinc was used as neutron flux monitor [12]. Each batch of samples irradiated contained also a similarly prepared blank (empty capsule) and a

sample of the Standard Reference Material "Bovine liver" (NIST 1577c); both serving for internal quality control. The irradiation duration was 10 hours at a thermal neutron flux of ~ $4.5*10^{12}$ cm $^{-2}$ s^{-1}. Samples were allowed to decay for two weeks. thereby effectively eliminating all activity from the short half-life radionuclides such as ^{24}Na in order to improve the detection limits. The induced radioactivity was measured during 3 hours using a well type Ge detector from ORTIC, USA, with an absolute photopeak efficiency of 11% for the 1332 keV photopeak of ^{60}Co and 13% for the 1099 keV photopeak of ^{59}Fe. The gamma-ray spectra were analyzed using the APOLLO software [12].

The principle of measuring the $^{59}Fe/^{54}Mn$ mole fraction ratios by INAA as follows:

- When irradiated with neutrons, radioactive ^{59}Fe is formed from both the natural stable ^{58}Fe (present for 0.3 % in natural iron) and the supplemented isotopically enriched ^{58}Fe. However, radioactive ^{54}Mn is formed from the natural Fe isotope (present for 6 % in natural iron). We firstly measure the $^{59}Fe/^{54}Mn$ activity ratio from natural iron only.
- After supplementing isotopically enriched stable ^{58}Fe (now present for 92.3 % in iron), blood, urine and faeces samples are collected for neutron activation. The measured ^{54}Mn activity indicates the pathway of the natural present Fe, and the measured ^{59}Fe activity, together with the earlier determined activity ratio, makes possible to estimate which the fraction originating from the supplemented iron; and thus the efficacy of the supplementation

(n, gamma) beta, gamma-Ray

^{58}Fe ----------> ^{59}Fe (T1/2=45 days) ----------> ^{59}Co

(n, P) gamma -Ray

^{54}Fe ----------> ^{54}Mn (T1/2 = 312 days)---------- ^{54}Cr

5.2.4 MC-ICP-MS

5.2.4.1 Sample preparation

Sample preparation was performed in the Reactor Institute Delft (RID) of the Delft University of Technology following a procedure provided by Van Heghe et.al [13]. It consists of two main steps: sample digestion and isolation of iron fractions using a chromatographic

purification. All acids used were of ultrapure quality prepared in a clean polyethylene volumetric flask with ultrapure water from Milli-Q system (Advantage A10). All apparatus used (bottles, vials, pipet tips etc.)was cleaned very well first with diluted nitric acid and secondly by Milli-Q water. For the digestion 7 mL of nitric acid (HNO_3) (14M) was added to 2 mL of the whole blood sample. This mixture was heated to 110 °C for 14 hours in a closed vessel. After cooling, the digested sample was dried down at 90°C. The residue was redissolved in 1 mL of (HCl (8M) + 0.001% H_2O_2). One hour before the separation 50 µL of H_2O_2 was added to make sure that Fe is present in its highest oxidation state.

The chromatographic separation was performed using a Bio-Rad Poly-Prep column (Figure 1) as follows:

1. The Bio-Rad Poly-Prep column was filled with 2 ml of the strong anion exchange resin AG 1-X8 (100-200 mesh) from Bio-Rad, Hercules, California, USA.
2. Before loading the digested sample, all the Fe was removed from the resin with 10 mL of 5 M HNO_3, followed by 5 mL of 0.3 M HCl, followed by 4 mL of H_2O, followed by 5 mL of 0.3 M HCl and followed 4 mL of H_2O.
3. After cleaning, the resin was conditioned with 10 mL of (8 M HCl + 0.001% H_2O_2).
4. Then, all the digested sample was loaded onto the Bio-Rad Poly-Prep column.
5. The matrix elements eluted with 16 mL of (8 M HCl + 0.001% H_2O_2).
6. Fe eluted with 4 mL H_2O and 10 mL of 0.165 M HCl.
7. Fe elute was dried at 90°C and the organic components oxidised by adding 0.100 mL concentrated HNO_3.
8. The samples were dried down and re-dissolved in 3 mL 0.3 M HNO3.
9. The last two steps were repeated two times to obtain total removal of organic components. The final solution was diluted with the Milli-Q water to 5mg/L for isotope ratio analysis.

Figure 1 Bio-Rad Poly-Prep column filled with 2 ml of the strong anion exchange resin AG 1-X8 (100-200 mesh).

The validation of the isolation procedure (column calibration) was achieved by passing a digested sample of NIST Standard Reference Material 1577c "Bovine Liver", (containing 1 mg of Fe) through the column. The eluted elements were collected in 1 mL fractions. First the matrix element were eluted and then Fe fractions. The iron fractions was measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) to check for the presence of other matrix elements. Perkin Elmer Optima 4300DV (ICP-OES) was used. The instrumentation setting and parameters of the ICP-OES are shown in Table 3.

Table 3. instrumentation parameters and settings (ICP-OES):

Parameter	Settings
Power:	1300 W
Plasma gas flow:	15 L/min
Auxiliary gas flow:	0.2 L/min
Nebulizer	0.8 L/min
View distance	15 mm
Sample flow rate:	1.5 ml/min
Wash rate	1.5 ml/min
Wash time	30 sec
Wavelength	238.204, 239.562 nm

Analytical validation:

A series of Fe standard solution used for calibration was prepared by diluting the stock solution of 1000 mg/L Fe (Iron ICP standard). The calibration of iron was found to be linear with correlation coefficients above 0.99.

The ICP-OES results are shown in Table 4 and Figure 2. It was found that the solution contained no other elements such as Na, Ni, Cr and Co. The column efficiency for isolation of Fe was 90%.

Table 4: Results of the Fe elute for Bovine Liver (mg/L) measured with ICP-OES:

Elution Step	Ca	Fe	Na	Ni	Cr	Co
1	0.2691	0.0015	0.0503	<0.0005	<0.0004	<0.0002
2	0.1338	57.1406	<0.0005	<0.0005	<0.0004	<0.0002
3	0.1025	20.6122	<0.0005	<0.0005	<0.0004	<0.0002
4	0.0697	20.5971	<0.0005	<0.0005	<0.0004	<0.0002
5	0.0349	14.1503	<0.0005	<0.0005	<0.0004	<0.0002
6	0.0417	7.0365	<0.0005	<0.0005	<0.0004	<0.0002
7	0.0072	3.4139	<0.0005	<0.0005	<0.0004	<0.0002
8	0.0052	1.8520	<0.0005	<0.0005	<0.0004	<0.0002
9	0.0248	1.2498	<0.0005	<0.0005	<0.0004	<0.0002
10	0.0566	0.9196	<0.0005	<0.0005	<0.0004	<0.0002
11	0.0383	0.6304	0.0018	<0.0005	<0.0004	<0.0002
12	0.0339	0.4293	<0.0005	<0.0005	<0.0004	<0.0002
13	0.0051	0.2431	<0.0005	0.0002	<0.0004	<0.0002
14	0.0030	0.2043	<0.0005	0.0001	<0.0004	<0.0002

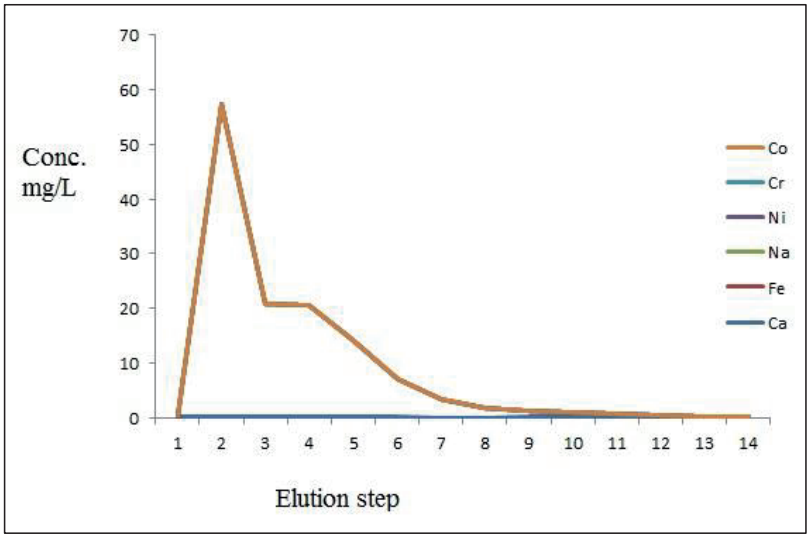


Figure 2. Result of ICP-OES of Fe elute (mg/L) of Bovine Liver using 2 mL of the strong anion exchange resin AG 1-X8 (100-200 mesh).

5.2.4.2 MC-ICP-MS analysis

MC-ICP-MS analysis was performed at the Faculty of Earth and Life Sciences of the VU University, Amsterdam, the Netherlands. The instrument used for the $^{58}Fe/^{54}Fe$ mole fraction ratio determination was a Thermo Finnigan MC-ICP-MS (Neptune), operating in high resolution mode (R>9000 5%-95%) using typical operating conditions adjusted on a per day basis for optimum sensitivity and peak shape. The sample introduction system used was a Scott type-double pass spray chamber combination equipped with a PFI-ST auto-aspirating nebulizer. Instrumentation setting and parameters are shown in Table 5.

Samples were analysed using IRMM-014 Fe (from EU Institute for Reference Materials and Measurements. Geel Belgium) as bracketing standard [14]. An in-house Fe solution as used as a (long term) internal laboratory standard. It was prepared by diluting 1000 mg/L ICP iron standard (Merck, ICP standard solution) in 1 % HNO_3 (prepared from sub-boiling double distilled suprapure HNO_3, Merck).

Table 5. Instrumentation parameters and settings (MC-ICP-MS):

Parameter	Setting
Sample uptake rate	100μL.min^{-1}
Sample gas flow rate	0.9 L.min^{-1}
Effective sample uptake rate	~ 85μL.min^{-1}
RF power	1200 W
Plasma gas flow rate	14 L.min^{-1}
Auxiliary gas flow rate	0.9 L.min^{-1}
Number of ratios analysed	90
Integration time	4.196 sec.

5.3 Results and discussion

The INAA calibration was verified by measuring the ratio of the iron mass fractions for iron of natural isotopic composition in human whole blood (n=12) and the Standard Reference Material bovine liver (NIST 1577c) (n=3). Calculated on the basis of the 1099 keV peak of ^{59}Fe and the 835 keV of ^{54}Mn the ratio should ideally be 1.00. The experiments resulted in an average value peak area ration of 1.04±0.04 for the human whole blood and 1.02±0.03 for the SRM (NIST 1577c) which values are in statistical agreement with the ideal value of the peak area ratios. The small bias could be attributed to small differences in the neutron energy distribution in the irradiation facility at the time of calibration and at the time of this experiment.

The natural mole fraction ratio of ^{58}Fe/^{54}Fe is 0.0482±0.0005. Any increase in this ratio is due to the absorbed fraction of enriched ^{58}Fe administered. The percent enrichment or (Δ% excess) is a term used in iron absorption studies to express measurement of enrichment relative to base line ratio (at time = 0) [11]; it is calculated according the following equation:

$$\Delta\%\ \text{excess} = \frac{58\text{Fe}: 54\text{Fe (enriched)} - 58\text{Fe}: 54\text{Fe(baseline)}}{58\text{Fe}: 54\text{Fe(baseline)}}\ X\ 100$$

As shown in Table 6. the Δ% excess is high in all anaemic patients compared to the hemochromatosis and the healthy group which means that the blood is significantly enriched in ^{58}Fe and therefore reflects their ability to absorb iron and incorporate it into blood within 2 weeks.

For the control group the t=0 was not determined because unintentionally the blood was not collected before intake of enriched ^{58}Fe, hence the natural ratio was assumed.

Table 6. INAA results of ^{58}Fe/^{54}Fe mole fraction ratio before and after intake of enriched ^{58}Fe

Patient No.	Iron disorder	^{58}Fe/^{54}Fe mole fraction ratio (before intake)	^{58}Fe/^{54}Fe mole fraction ratio (after intake)	The absorbed fraction of ^{58}Fe	Δ% excess
1	IDA	0.0480	0.0860	0.0380	79.20
2	IDA	0.0470	0.0619	0.0149	31.70
3	IDA	0.0481	0.0680	0.0199	41.40
4	IDA	0.0480	0.0580	0.0100	20.80
5	HH (homoz)	0.0472	0.0489	0.0010	3.60
6	HH (heteroz)	0.0481	0.0482	0.0001	0.21
7	HH (homoz)	0.0470	0.0480	0.0010	2.13
8	Contr.	0.0482	0.0482	0.0000	0.00
9	Contr.	0.0482	0.0532	0.0050	10.37
10	Contr.	0.0482	0.0488	0.0006	1.24
11	Contr.	0.0482	0.0531	0.0049	10.17

IDA: iron deficiency anaemia, HH: hereditary hemochromatosis, Contr.: control

In contrast to what expected an increase of iron absorption was not seen in the hemochromatosis patients. The exact reason is not known. They were all treated with blood-lettings and their hepcidin was not measured, so an effect of their blood-lettings on their iron homeostasis can not be excluded. A specific inability of hemochromatosis patients to absorb the ^{58}Fe isotope seems unlikely. Two of them were known to be fond of meat and since we did not strictly advised them to totally refrain from meat a competitive effect of dietary iron is another possibility. Since the absence of an increased iron absorption was also measured with MC-ICP-MS, this finding can not be attributed to INAA as measurement technique (see table 7). Further studies are necessary to elucidate this finding. The two female controls also showed some increased.

The degree of accuracy (trueness and precision) of stable mole fraction ratio measurements of iron and other transition metals by MC-ICP-MS depends on; (1) the quantitative removal of molecular interferences (e.g. Ar-oxides, Ar-nitrides, Ar-and metal hydroxides); (2) possible corrections for even the smallest amounts of isobaric elemental interferences (e.g. $^{54}Cr^{+}$ on $^{54}Fe^{+}$, $^{58}Ni^{+}$ on $^{58}Fe^{+}$, etc.); and (3) precise correction of the instrumental mass bias.

The isobaric interferences by Cr and Ni gave quite some problems in the interpretation of the results.

An essential precondition in any (chemical) test method is that the calibrator and, if applicable, trueness control material, behave functionally the same during the analysis procedure as the real samples to be analysed [15-17]. This requirement has led to the definition of the concept of commutability: " property of a reference material, demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in this material, obtained according to two given measurement procedures, and the relation obtained among the measurement results for other specified materials" [18]. Commutability problems may occur due to, e.g. differences in liberation of chemical elements from the matrix of materials (such as in case of analyses requiring dissolution), particle size differences or isotopic effects. Commutability problems are often observed in clinical measurements, which led Franzini defining commutability as "the equivalence of the mathematical relationships between the results of different measurement procedures for a reference material (RM) and for representative samples from healthy and diseased individuals" [19]. It may be obvious that non-commutable calibrators such as certified

reference materials increase variation between analytical methods, which may lead to incorrect diagnosis and even incorrect treatment of patients.

Since the RM using with MC-ICP-MS is a synthetic inorganic material and the blood is a biological material, the chromatography separation of iron to its pure organic fractions helps to overcome the commutability problem.

In our experiment using MC-ICP-MS, the internal standard gave a good result for $^{58}Fe/^{54}Fe$ mole fraction ratio of 0.048 in the first run, reflecting the natural mole fraction ratio. However, the result of this ratio in blood samples was vacillating. For some samples the $^{58}Fe/^{54}Fe$ mole fraction ratio was higher than after intake of the enriched ^{58}Fe. This could be attributed to the presence of small amounts of Cr and Ni in all samples although a chromatography separation was done. The quality of the separation was initially verified using ICP-OES in Delft, and no remaining Cr and Ni could be quantified. However, an additional analysis at the VU in Amsterdam using ICP-MS –with which much lower detection limits can be attained than with ICP-OES- confirmed the presence of these elements at the μg/kg level. As such, and applying correction for the Cr and Ni interference, the MC-ICP-MS results were in agreement with the INAA results.

The results (see Table 7) show higher values for the mole fraction ratio in blood of anaemic patients after intake than before intake. In hemochromatosis group no difference between before and after intake and the healthy group slightly absorbed iron depending on their body needs); this result was comparable to the INAA result (Table 7).

The comparable results of $^{58}Fe/^{54}Fe$ mole fraction ratios of the 7 patients (1-7) after intake of enriched ^{58}Fe using INAA and MC-ICP-MS (after correction (2^{nd} run)) are shown in Figure 3.

Although accurate results can also be produced by MC-ICP-MS, the accuracy for mole fraction ratio measurements can be influenced by several effects [20]. The mass discrimination effect -which can occur due to the space charge effect- plays an important role in mass spectrometry. In the step of ion extraction (during the analysis), the light ions are deflected more than the heavy ions. Therefore the ratio of lighter to heavier isotope is smaller than the true value eg; $^{54}Fe/^{58}Fe_{measured} < ^{54}Fe/^{58}Fe_{true}$ and vice versa, this can be overcome by measuring the ratio difference between sample and RM.

Table 7. Results of the $^{58}Fe/^{54}Fe$ mole fraction ratios before and after intake of enriched ^{58}Fe using MC-ICP-MS compared to INAA (1-4 anaemic, 5-7 Hemochromatosis, 8-11 controls).

Patient No.	**$^{58}Fe/^{54}Fe$ mole fraction ratio (before intake)**			**$^{58}Fe/^{54}Fe$ mole fraction ratio (after intake)**		
	1st run No correction for Cr and Ni	2ed run With correction for Cr and Ni	INAA	1st run No correction for Cr and Ni	2ed run With correction for Cr and Ni	INAA
1	0.0470	0.0470	0.0480	0.0820	0.0820	0.0860
2	0.0440	0.0470	0.0470	0.0620	0.0620	0.0619
3	0.0480	0.0480	0.0481	0.0470	0.0650	0.0680
4	0.0477	0.0477	0.0480	0.0440	0.0570	0.0580
5	0.0477	0.0480	0.0472	0.0480	0.0490	0.0489
6	0.0478	0.0480	0.0481	0.0480	0.0480	0.0482
7	0.0478	0.0480	0.0470	0.0480	0.0510	0.0480
8	-	-	-	0.0480	0.0480	0.0482
9	-	-	-	0.0480	0.0510	0.0532
10	-	-	-	0.0480	0.0480	0.0488
11	-	-	-	0.0476	0.0510	0.0531

Other limitation on isotope ratio measurement are the matrix effects which can be overcome with chromatographic purification, but as this study demonstrates, the quality of the separation needs to be closely monitored to obtain the required level of separation.

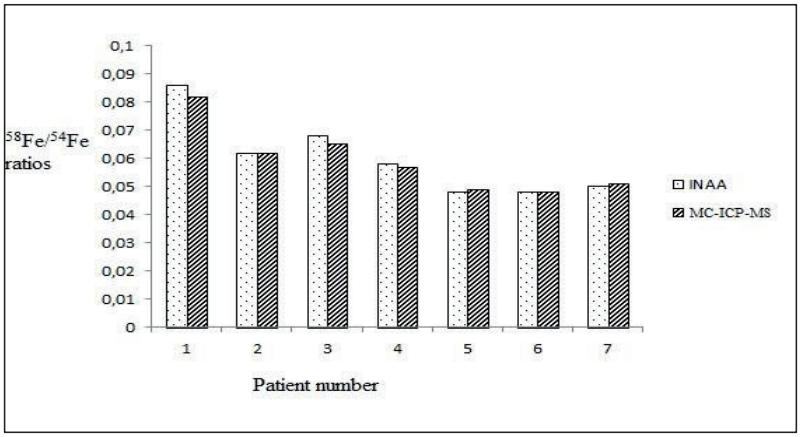


Figure. 3. Results of $^{58}Fe/^{54}Fe$ mole fraction ratios of the 7 patients after intake of enriched ^{58}Fe using INAA as well as MC-ICP-MS (only 2nd run presented).

The uncertainty of ratios determined by MC-ICP-MS depends on the measurement conditions and the measurement protocol (integration time and the number of measured ratios). It is similarly based on the identification of the contribution of each analytical parameter occurring during the measurement. However, some contributions to the uncertainty are very difficult or barely possible to estimate, such as those related to the chemical yield and the chemical matrix effects.

On the other hand, INAA is the only analytical technique of which objective evidence exists that it can meet the requirements of a primary method of analysis. All sources that contribute to its uncertainty are known and can be quantified [10]. The limitations to the degree of trueness depend on the quality of calibration. This is one of the considerations that INAA is highly appreciated in the characterization of candidate reference materials.

The question which technique has to be used is hard to answer. It mainly depends on the clinical research question. MC-ICP-MS is very sensitive and easier available than INAA. However, the preparation of material before measurement is more critical and demanding. Since both techniques are accurate and reliable other motives come into play such as costs and time consumption.

If we compare the practical aspects of the two techniques such as the duration of analysis and costs, we come to this overview:

- Duration of the analysis e.g. 20 samples

INAA:

- Sample preparation: 1 day
- Irradiation: 2 day
- Decay: 14 days
- Measurement: 3 days (each sample 3 h)
- Total: 20 days

MC-ICP-MS:

- Sample digestion: 3 day (if microwave digestion)
- Fe separation: 10 days (in a well prepared laboratory for isotope ratio measurement this time can be reduce to 2 days)
- Measurement: 2 day
- Total 15 days

- Cost for analysing e.g. 20 samples

INAA: ≈ 2340 euro

MC-ICP-MS: ≈ 2400 euro (include samples pre-treatment).

The cost of analysis is based on among others the cost of materials, machine/apparatus and contribution of personnel. From the above comparison we can conclude that the costs of both methods are approximately equal. The turnaround time of INAA is longer than MC-ICP-MS, most of this time is spent with the decay time before the actual measurement. In MC-ICP-MS the turnaround time is less than in INAA when the laboratory is totally equipped for this type of measurement. In case preparation has to be performed in another lab, as in my experiments, then more time is needed. In case of INAA precautions are necessary because of working with radioactivity.

5.4 Conclusion

In conclusion, both INAA and MC-ICP-MS are able to measure changes in iron isotope composition in blood when an enriched stable iron isotope is applied in clinical research. The critical and most time consuming part of MC-ICP-MS concerns the preparation of materials prior to measuring when not performing Fe isotopic analysis on a routine basis. In INAA most of the time is consuming as a decay time to effectively eliminating all activity from the short half-life radionuclides such as ^{24}Na.

Although the magnetic sector mass analyser of the MC-ICP-MS works for achieving a high-precision mole fraction ratio measurement the high mass resolution makes impossible to separate ions of the same nominal mass to charge ratio by such mass analyser. Another step of a chemical chromatography separation is needed to overcome this problem. It has been shown in this study that very high demands have to be set to the effectiveness of such a separation and removal of the elements Cr and Ni interfering with the Fe mole faction ratio measurement. Although the MC-ICP-MS technique is very sensitive, some contributions to the uncertainty of the measurement are very difficult or barely possible to estimate such as those related to the chemical matrix effects when the quality and efficiency of the chromatographic separation are not under control. A good chromatographic separation is not only necessary but it is an integral part of the method.

In contrast, the contributions to uncertainty in INAA are all well-known and can be quantified. In case of measuring iron isotopes, the uncertainties in INAA are substantially higher than those achievable by MC-ICP-MS but the INAA technique offers a high specificity and selectivity for iron close to 100% based on the individual characteristics of the induced radionuclides. Its greatest advantage is that the preparative steps before measurement are simple and there are hardly demands on the kind and size of materials. Its drawbacks however are the availability of a research nuclear reactor and the production of radioactive rest material.

5.5 References

[1] M. Rehkamper, M. Schonbachler and C. H. Stirling. Multiple collector ICP-MS: Introduction to Instrumentation, measurement techniques and analytical capabilities. J. Geost. Geoanal. (2000), 25 (1), 23-40.

[2] L. Van Heghe, J. Delanghe. H. Van Vlierberghe and F. Vanhaecke. The relationship between the iron isotopic composition of human whole blood and iron status parameters. Metallomics. (2013). 5. 1503-1509.

[3] F. von Blanckenburg, M. Oelze, D. G. Schmid, K. van Zuilen, H. P. Gschwind, A. J. Slade, S. Stitah, D. Kaufmann and P. Swart. An iron stable isotope comparison between human erythrocytes and plasma. Metallomics. (2014). 6. 2052-2061.

[4] F. Vanhaecke and M. Costas-Rodriquez. What's up doc?-High-precision isotopic analysis of essential metals in biofluids for medical diagnosis. Spectr. Europe. (2015) 27 (3). 11-14.

[5] T. Walczyk and F. von Blanckenburg. Natural iron isotope variations in human blood. Science 295 (2002). 2065-2066.

[6] T. Ohno, A. Shinohara, I. Kohge, M. Chiba and T. Hirata. Isotopic analysis of Fe in human red blood cells by Multiple Collector-ICP-Mass Spectrometry. Anal. Sci. 20. (2004). 617-621.

[7] K. Hotz, H. Augsburger and T. Walczyk. Isotopic signatures of iron in body tissues as a potential biomarker for iron metabolism. J. Anal. At. Spectrom. (2011) 26. 1347-1353.

[8] S. Weyer and J. B. Schwieters. High precision Fe isotope measurements with high mass resolution MC-ICPMS. Int. J.Mass Spectr. 226 (2003) 355–368.

[9] R. Schoenberg and F. von Blanckenburg. An assessment of the accuracy of stable Fe isotope ratio measurements on samples with organic and inorganic matrices by high-resolution multicollector ICP-MS. Int. J. Mass Spectr.242 (2005) 257–272.

[10] R. R. Greenberg, P. Bode and E. A. De Nadai Fernandes. Neutron activation analysis: A primary method of measurement. Spectrochim. Acta B 66 (2011) 193–241.

[11] Assessment of iron bioavailability in human using stable iron isotopes techniques. IAEA human heath series No. 21. International Atomic Energy Agency. Vienna (2012).

[12] M. Blaauw. The holistic analysis of gamma-ray spectra in Instrumental Neutron Activation Analysis. PhD dissertation. Delft University of Technology. 1993. ISBN 90-73861-16-0.

[13] L. Van Heghe, E. Engstrom, I. Rodushkin, C. Cloquet and F. Vanhaecke. Isotopic analysis of the metabolically relevant transition metals Cu. Fe and Zn in human blood from vegetarians and omnivores using multi-collector ICP-mass spectrometry. J. Anal. At. Spectrom. (2012) 27. 1327–1334.

[14] P.D.P. Taylor, R. Maeck, F. Hendrickx and P. De Bièvre. The gravimetric preparation of synthetic mixtures of iron isotopes. Int. J. Mass Spectr. Ion Proc. (1993) 128: 9 l-97.

[15] H. W. Vesper, W Gregory Miller and G. L Myers. Reference Materials and Commutability. Clin Biochem Rev. (2007). Vol 28: 139-147.

[16] D. M Bunk. Reference Materials and Reference Measurement Procedures: An Overview from a National Metrology Institute. Clin Biochem Rev. (2007). Vol 28: 131-137.

[17] W.G. Miller, G.L. Myers And R. Rej R. Why Commutability Matters. Clin Chem; (2006).52: 553-4.

[18] BIPM JCGM 200:2012 International vocabulary of metrology – Basic and general concepts and associated terms, (VIM), 3rd edition.

[19] C. Franzini, Commutability of reference materials in clinical chemistry. J Int Fed Clin Chem 5 (1993)169-173.

[20] J. Sabine Becker. Inductively coupled plasma mass spectrometry (ICP-MS) and laser ablation ICP-MS for isotope analysis of long-lived radionuclides. Int. J. Mass Spectr. 242 (2005) 183–195.

6 Monitoring Iron Status in various Human Biomaterials using Instrumental Neutron Activation Analysis

6.1 Introduction

In many clinical situations the iron status in man can be measured with standard laboratory techniques. However when iron needs to be measured in food and tissue material or when isotopes of iron are used special techniques are needed such as specific forms of mass spectrometry (MS) or instrumental neutron activation analysis (INAA). A good example of this kind of research are mass-balance measurements or net dietary balances referring to the retention of nutrients determined by dietary intake and excretion in urine and faeces. Only a few mass balance studies of iron have been performed and described in literature. This can mainly be attributed to the need of strict monitoring, sometimes even necessitating hospitalisation, during such a study period, In case of an out-patient design of such a study errors are easily made both at the site of intake as in the reliable collection of excretions. In case such a test takes several days it is often difficult to exclude bias during the test. Therefore, research on iron absorption is commonly built on one of the three approaches for estimating the iron absorption; faecal monitoring; haemoglobin incorporation or plasma turnover. Most of the mass balance studies involving iron have been done in infants [1-5].

Although the contribution of INAA in iron research is most prominent in food and tissues analyses as well as in measuring isotopes of iron, the technique should also be able to measure total iron in blood, urine and faeces. After all it would be a great advantage if all measurements within an experiment can be performed with one single technique. Since data on the use of INAA in clinical iron research are scarce, it was decided to perform a number of feasibility experiments in clinical situations where iron has to be measured in commonly used materials such as blood, urine and faeces. Iron was measured in blood, blood compartments, urine and faeces using INAA either to compare with standard laboratory techniques or to prove that INAA can measure even small changes in iron concentration after supplementation.

6.2 Total blood iron in patients with various forms of benign haematological disorders

In a first experiment total blood iron was measured using INAA in 25 patients with various forms haematological disorders, in which iron metabolism might be changed. All patients visited the out-patient department of the Meander Medical Centre, Amersfoort, The Netherlands, and gave informed consent that extra tests were performed on the blood samples taken for routine control. Nine patients were newly diagnosed with iron deficiency anaemia (IDA), having a microcytic anaemia with a low serum iron (< 7 μmol/L), haemoglobin (Hb) <7mmol/l (<11g/dL), (MCV) < 75fL and low ferritin (< 20 μg/l). Seven patients were diagnosed with the anaemia of chronic disease (ACD), characterized as an anaemia with normal levels of serum iron, folic acid and vitamin B12 but high inflammatory parameters (ESR). Five patients were already known with hereditary hemochromatosis (HH; C282Ymutation) and were currently treated with blood lettings. Two patients had polyglobulinemia secondary to obstructive sleep apnea, also treated with blood-lettings, one patient was known with sickle cell anaemia and one patient with β thalassemia. Table 1 shows the relevant blood parameters used for diagnosis and clinical follow up in these 25 patients.

A whole blood sample in an EDTA tube of each patient was transferred to the Reactor Institute Delft (RID), where it was analysed for iron concentration by instrumental neutron activation analysis (INAA). Samples were freeze-dried and about 200 mg of each dried sample was weighed and packed in high purity polyethylene capsules. During radiation in the 2 MW nuclear research reactor of the Reactor Institute Delft, Delft University of Technology, zinc was used as neutron flux monitor [6]. Samples along with similarly prepared blanks (empty capsule) and the Standard Reference Material "Bovine liver" (NIST 1577c), both serving for internal quality (trueness) control were irradiated for 10 hours at a thermal neutron flux of ~ $4.5*10^{12}$ cm^{-2} s^{-1}. Samples were allowed to decay for two weeks, thereby effectively eliminating all activity from the short half-life radionuclides such as ^{24}Na in order to improve the detection limits. The induced radioactivity was measured during 3 hours using a well type Ge detector, with an absolute photopeak efficiency of 11% for the 1332 keV photopeak of 60Co and 13% for the 1099 keV photopeak of 59Fe. The gamma-ray spectra were analyzed using the APOLLO software [6].

Table 1. routine blood count and iron parameters of the 25 patients, whose blood samples were used for INAA

Pat.	Hb mmol/L	RDW-SD fL	MCV fL/red cell	Serum Iron µmol/L	Ferritin µg/L	Trf. g/L	ESR mm/hr	Trf. Sat, (%)	diagnosis
1	5.4	47	62	2	2	3.7	-	2	IDA
2	6.2	49	76	2	2	3.2	-	3	IDA
3	6.8	44	85	9	5	3.3	-	11	IDA
4	7.1	41	80	5	3	4.2	-	5	IDA
5	6.3	45	75	5	3	3.8	-	6	IDA
6	5.5	46	80	6	4	3.8	-	7	IDA
7	6.6	70	79	3	4	2.9	-	4	IDA
8	5.4	45	75	4	9	3.1	-	5	IDA
9	4.1	45	60	2	3	3.6	-	2	IDA
10	5.6	47	89	4	473	1.4	79	12	ACD
11	6.9	44	89	4	304	1.6	59	11	ACD
12	6.1	53	96	4	545	1.6	84	11	ACD
13	7.7	51	87	6	412	0.9	29	28	ACD
14	7.0	47	90	10	408	2.1	80	20	ACD
15	4.8	61	96	18	1369	1.1	101	68	ACD
16	6.0	50	92	3	624	1.3	113	10	ACD
17	9.0	-	95	29	233	2.5	-	49	HH
18	11.4	-	86	45	126	2.5	-	76	Sec polyglobul.
19	7.4	-	96	31	1022	1.5	-	87	HH
20	8.7	-	88	34	64	1.7	-	84	HH
21	11	-	96	50	25	2.7	-	78	HH
22	9.2	-	89	28	108	2.1	-	56	HH
23	9.4	-	89	26	454	2.4	-	45	Sec polyglob.
24	5.7	-	86	-	-	-	-	-	sickle cell anemia
25	6.2	33	61	15	7	3.3	-	-	Beta-thalassemia

1. **Blood parameters (and reference values):** Hb= haemoglobin (M=8.5-10.5, F= 7.8-10 mmol/L), RDW-SD= red cell distribution width (36-50 fl), MCV= mean cellular volume (80-102), serum iron (M=10-30, F= 7-30 micromol/), Ferritin (M=24-336, F=20-300 micrg/l), Transferrin (2.0-3.6 g/L), Trf Sat = serum transferrin saturation (<50%); ESR= erythrocytes sedimentation rate (M </= 20 mm/hr, F </= 30 mm/hr).
2. **Abbreviations:** IDA: Iron deficiency anaemia, ACD: Anaemia of chronic diseases, HH: Hereditary hemochromatosis

INAA in Delft is calibrated following the single-comparator approach [7]. By irradiation of a known amount of natural iron, calibration factors can be established that relate, under specific irradiation, decay and measurement conditions, the known amount of iron to the peak areas of the 835 keV, 1090 keV and 1292 keV gamma-ray with the requirements of the ISO/IECI7025: 2005 [8].

The mass fractions were calculated on dry mass basis and converted to $mg.L^{-1}$ (Table 2), assuming that the freeze dried residue is 20% of whole blood (density is 1.06 $g.mL^{-1}$), 9% of

plasma (density is 1.026 $g.mL^{-1}$), and 37% of erythrocytes (density is 1.096% $g.mL^{-1}$) [9] Results are shown in table 2.

Table 2. Fe measured in whole blood (mg/kg & mg/L)with INAA and corresponding Hb level in 25 patients with various haematological disorders

Pat. No.	Fe ± uncertainty (1 SD), (mg/kg)	Fe (mg/L)	Hb (mmol/L)	diagnosis
1	1330 +/- 12	190	5.4	IDA
2	1700 +/- 15	280	6.2	IDA
3	1820 +/- 16	380	6.8	IDA
4	1860 +/- 15	370	7.1	IDA
5	1680 +/- 13	300	6.3	IDA
6	1570 +/- 14	300	5.5	IDA
7	1850 +/- 17	320	6.6	IDA
8	1580 +/- 16	270	5.4	IDA
9	1300 +/- 12	170	4.1	IDA
10	1610 +/- 18	250	5.6	ACD
11	1750 +/- 35	310	6.9	ACD
12	1780 +/- 16	300	6.1	ACD
13	1940 +/- 21	400	7.7	ACD
14	1820 +/- 20	330	7.0	ACD
15	1630 +/- 21	225	4.8	ACD
16	1740 +/- 16	280	6.0	ACD
17	2080 +/- 19	450	9.0	HH
18	2260 +/- 27	530	11.4	Sec polyglob.
19	1990 +/- 18	365	7.4	HH
20	2100 +/- 21	470	8.7	HH
21	2280 +/- 23	516	11	HH
22	2080 +/- 21	450	9.2	HH
23	2130 +/- 21	446	9.4	Sec polyglob.
24	1680 +/- 17	290	5.7	sickle cell anemia
25	1640 +/- 18	320	6.2	Beta-thalassemia

IDA: Ion deficiency anaemia, ACD: Anaemia of chronic diseases, HH: Hereditary hemochromatosis

Since total iron in blood was only measured with INAA and not with another (routine laboratory) test, results of INAA were correlated to haemoglobin level, since most of the iron in blood is present within haemoglobin in the erythrocytes even in cases with high ferritin as in ACD. This correlation was analysed using SPSS (IBM Statistic 20). Because the iron concentrations in blood have a skewed distribution, they were firstly converted to their

logarithms before calculating means and standard deviations. Pearson's regression test was used to examine the correlation between iron fractions and Hb. A strong positive correlation was found with (r =0.925) and (P value =0.00). This is shown in table 3 and figure 1.

Table 3. Correlations between Fe and Hb level in the 25 whole blood samples:

		Fe (mg/L)	Hb (mmol/L)
Fe (mg/L)	Pearson Correlation	1	0.925**
	Sig. (2-tailed)		0.000
	N	25	25
Hb (mmol/L)	Pearson Correlation	0.925**	1
	Sig. (2-tailed)	0.000	
	N	25	25

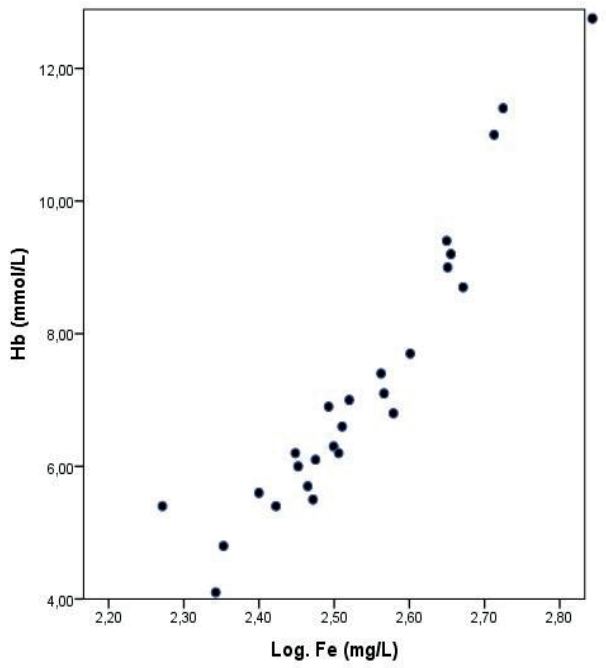


Figure 1. Comparison between Fe fractions and Hb concentrations in the 25 whole blood samples.

In conclusion total iron concentrations in blood as measured with INAA show a high correlation with haemoglobin levels in patients with various forms of benign haematological disorders affecting iron metabolism indicating that the majority of iron in blood is located within the erythrocytes despite the underlying haematological disorder.

6.3 Measurement of iron in blood compartments

Another way to get information on the iron distribution in blood is to measure iron directly in both plasma and erythrocytes. By studying these concentrations in both normal and iron deficient individuals it is possible on the basis of ratios to analyse which compartment provides the best information on shifts in iron status. Five volunteers, three with iron deficiency anaemia and two healthy volunteers with normal haemoglobin levels from the Meander Medical Centre were willing to participate in this small study and gave informed consent. Two EDTA tubes of blood were taken from each individual, one for total blood measurement and one for measurement plasma and erythrocytes after centrifugation. Samples were transferred to the department of clinical chemistry of the Reinier de Graaf Hospital, Delft, the Netherlands. From each patient one part of the sample was used for measuring haemoglobin concentration and iron parameters, the other part was used for INAA at the Reactor Institute Delft, to measure iron in whole blood, plasma and erythrocytes. INAA was performed as described in paragraph 6.2 of this chapter.

Table 4 shows the haemoglobin concentrations as well as the iron parameters in the five volunteers participating in this study. One of the healthy volunteers proved to have a complete normal iron status while the other person had somewhat low values. All of the anaemic individuals had low serum iron levels, but showed different levels of ferritin. In table 5 iron concentrations, as measured with INAA, in the various blood compartments of these five individuals are shown. The highest concentration of iron is found in the erythrocytes.

Compared to reference values in literature the healthy individuals 1 and 4, had normal iron concentrations in all blood components (whole blood 430-460 mg/L, plasma 1.2-2.1 mg/L, erythrocytes 840-930mg/L) while the anaemic patients showed low values in all compartments (whole blood 190-240 mg/L, plasma 0.2-1.9 mg/L, erythrocytes 470-600 mg/L). The unexpected high concentration of iron in plasma of patient 2 (anaemic) may be due to contamination of plasma by red blood cells during the separation.

Table 4: Haemoglobin concentration and iron parameters of the five volunteers in the second experiment

Pat. No.	Sex	Hb (mmol/L) M {8.5 -11} F {7.5 -10}	Iron (µmol/L M (10-30) F (7-30)	TIBC (µmol/L) {45 – 81}	Ferritin (µg/L) {5-204}	Trf. (g/L) {1.8 - 3.6}	Trf. Sat. (%) {20-60}	Diagnosis
1	M	8.0	14	77	70	3.52	18	normal patient without anaemia or iron problem.
2	F	4.0	3	69	36	3.2	4	anaemia with low to normal ferritin.
3	F	4.3	2	68	15	3.12	3	anaemia, and low iron status. Ferritin depot is low.
4	M	9.0	8	81	13	3.71	9	no anaemia but low iron status.
5	F	4.0	6	19	283	0.86	32	anaemia with normal iron status.

Table 5. Fe fractions in blood components of the five volunteers

Pat. No.	Sex	Plasma (mg/L) {0.6-4.8}*	Erythrocytes (mg/L) {985-1140}*	Whole blood (mg/L) {420-560}*	Diagnosis
1	M	2.1	840	430	normal patient without anaemia or iron problem.
2	F	1.9	470	190	anaemia with low to normal ferritin.
3	F	0.2	600	240	anaemia, and low iron status. Ferritin depot is low.
4	M	1.2	930	470	no anaemia but low iron status.
5	F	0.4	590	230	anaemia with normal iron status.
Average normal persons		1.7	890	450	
Average anaemic patients		0.3	550	220	

*Normal range according to literature values [10-14].

Based on the mean concentrations of iron in both normal and anaemic patients, a ratio can be calculated for each compartment: The ratios of the iron concentrations in blood components

(whole blood (WB), Plasma (P) and erythrocytes (E), of the normal subjects (N) to iron deficient patients (ID), $\frac{N}{ID}$ resulted in:

$$\frac{Fe_{WB}^{N}}{Fe_{WB}^{ID}} = \frac{450}{220} = 2.0$$

$$\frac{Fe_{P}^{N}}{Fe_{P}^{ID}} = \frac{1.7}{0.3} = 4.9$$

$$\frac{Fe_{E}^{N}}{Fe_{E}^{ID}} = \frac{890}{550} = 1.6$$

Since the highest ratio is found in plasma, this compartment is the most sensitive one in reflecting changes in iron status and therefore the best biomarker to detect an iron deficiency.

6.4 Effect of a pulse dose of orally administered Fe sulphate in healthy volunteers

Since INAA is a technique, which is able to detect small concentrations in biomaterials an experiment was performed to find out whether an orally administered high dose of iron in normal healthy individuals results in detectable changes in blood iron concentrations. Three healthy volunteers with normal iron status were given three doses of iron sulphate per day (each dose contains 325 mg ferrosulfate with 105 mg Fe2+) during three days. The test was performed at the out-patient unit of the Meander Medical Centre (MMC), Amersfoort after approval of the medical ethical committee. Blood samples were collected before and one day after the last dose. Total iron was measured in all samples at the Reactor Institute Delft using the INAA procedure described in the previous paragraphs. The results are shown in Table 6.

Table 6. Fe mass fractions (mg/kg), in blood before and 3 days after intake of 975mg $FeSO_4$ per day during 3 days. Typical relative measurement uncertainties (one standard deviation) for Fe is 1%:

Subject No.	Before intake of $FeSO_4$	After 3 days of intake $FeSO_4$
1	2230	2250
2	2160	2140
3	2200	2210

It is clear that such a pulse dose, actually an overdose, does not result in a measurable increase in whole blood iron concentration. This is probably due to the fact that these normal healthy volunteers have a normal iron status in which case only 1-2 mg of iron is absorbed per day This small uptake cannot be measured accurately with INAA and as stated earlier, whole blood is probably not the best biomarker for changes of iron within the blood pool.

6.5 Effect of an orally administered pulse dose of iron sulphate on iron concentrations in blood compartments and faeces of healthy volunteers

This experiment is an extension of the experiment described in the former paragraph and the same three healthy volunteers participated. One extra healthy volunteer was included. This time blood was collected 14 days after the last dose of iron. This time period was chosen to find out whether supplemented iron would be incorporated into red blood cells. Therefore iron was not only measured in whole blood, but also in plasma and in erythrocytes. Apart from that faeces was collected prior and during three days after the last dose. This extension was performed to find out whether INAA can be applied in faeces analysis without technical problems during irradiation. Preparation of blood components and measurement with INAA took place according the protocols described in the paragraphs above. Results of the iron measurements in the blood components before and after iron administration are shown in table 7. In accordance with the results presented in paragraph 6.4. even after 14 days no significant increase in iron concentration could be observed in the different blood compartments. Again this suggests that in case of the absence of iron deficiency even a high dose of oral iron supplementation does not result in an increased uptake in healthy individuals.

In contrast a significant increase of iron concentrations were found in the faeces collected after oral iron administration (Table 8). Faecal monitoring or recovery is one of the methods being used for measurement of chemical element absorption in humans [15,16]. It is defined as the difference between the amount of oral stable isotope ingested and the amount lost from the body in faeces. Complete faeces collection is normally obtained during 7-10 days after supplementing [17], but because of the difficulties in handling, treatment and measurement of this type of sampling, many researchers avoid this method to measure the iron absorption and direct themselves towards the haemoglobin incorporation method.

Table 7. Result of iron (mg/L) in blood components before and 2 weeks after intake of 975 mg $FeSO_4$ tablets per day during three days. Typical relative measurement uncertainties (one standard deviation) for measurement of Fe by INAA are 10% for plasma, 1% for whole blood and erythrocytes.

Patient	Plasma		Whole blood		Erythrocytes	
NO.	After	Before	After	Before	After	Before
1	1.4	1.3	450	440	890	860
2	1.6	1.6	390	360	880	890
3	1.7	1.5	500	490	840	810
4	1.4	< detection limit	480	480	890	lost

However, in anticipation of real mass balance studies and to build up experience with measuring iron with INAA in faeces, it was decided to incorporate faeces analysis in this experiment. Faeces samples were collected in special containers for stool sampling from Fecotiner (Figure 2). The container was weighed before and after sample collection. All faeces collected was stored deep-frozen at -20˚ and sent to the Reactor Institute Delft. Before sending over cultures revealed no pathological organisms and all volunteers were free of the hepatitis A,B and C virus. In Delft, samples were freeze dried, mortared and homogenized. About 200 mg of each dried sample was weighed and packed in high purity polyethylene capsules, irradiated and measured according the protocol of the blood samples.

Figure 2. Stool sampler from Fecotainer

Table 8. Measurement of Fe in faeces (mg per 100 g), before and after the oral intake of $FeSO_4$. Typical relative measurement uncertainties (one standard deviation) for Fe is 1 %.

Sample No.	Amount of Fe in faeces (mg) before intake of Fe sulphate	Amount of Fe in faeces (mg) after intake of Fe sulphate
1	27	670
2	72	245
3	30	382
4	35	500

In all individuals significant increases of iron were measurable after oral iron administration (table 8) and no problems occurred during irradiation of faecal material, indicating that INAA can be applied in mineral analysis of faeces.

6.6 Iron concentrations in urine after an intravenous pulse dose of iron isomaltoside

Urine is assumed to be a solution with high ionic strength containing gram amounts of Na^+, K^+, Cl^+, PO_4^{3-} and SO_4^{2-} , which leads to a high induced activity of ^{24}Na, ^{42}K and ^{38}Cl – albeit that these elements have a rather very short half-life- after neutron irradiation. Therefore, measurement of Fe in urine requires a decay time of 2-3 weeks to avoid interferences by these radionuclides . In general, INAA deals with dry samples and they have been used before to measure bulk and trace elements in urine after lyophilisation [18,19].

In mass balance studies large amount of urine may by collected, so subsamples have to be taken for practical reason. However, due to the inhomogeneous nature of urine, such samples may not be representative. Furthermore, freeze drying of such large amounts is complicated. In this study an attempt was made to optimise a method for measuring iron in liquid urine to make the measurement more practical.

Six iron deficient anaemic patients visiting the Meander Medical Centre, Amersfoort, were scheduled to receive iron intravenously (800-1000 mg iron isomaltoside) as part of their treatment. They were used to receive iron with regular intervals and gave informed consent for the collection of their urine and analysis. Table 9 shows the dose of iron given to each patient and their haemoglobin levels.

Before injection, and twelve hours after injection, urine was voided directly into an acid-washed polyethylene container . All subjects were instructed to minimize contamination of the sample by avoiding contact with the inside of the container or lid, and to refrain from leaving the container open to the air longer than necessary. After collection, refrigerated samples were send to the Reactor Institute Delft in iced boxes.

Table 9. Dose of Fe isomaltoside given to patients and their haemoglobin:

No	Fe (mg)	Hb (mmol/L) Normal range male: (8.5 -11) Normal range female: (7.5 -10)
1	800	7.2
2	1000	6.9
3	1000	6.7
4	800	4.7
5	800	5.3
6	800	6.5

A special quartz bottle was manufactured at RID, with a loose lid to allow release of gases resulting from radiolysis during irradiation (see Figure 3).

About 20 g of urine was added to each quartz bottle. A 3 mg Zn metal in a quartz tube was tied to the urine bottle as a neutron flux monitor. A 100 mg/L iron solution was prepared (from iron ICP standard) to serve as control sample in this experiment. The samples were irradiated for 5 hours in a thermal neutron flux of ~ 7.0 * 10^{12} $cm^{-2}s^{-1}$ in a manually loaded pool-type facility (Befa 6, HOR grid position D8) in the 2 MW nuclear research reactor of the Reactor Institute Delft (see Figure 4). After a decay time of 2 weeks the samples were transferred to plastic bottles, their masses measured and the induced activity was measured for 10 hours on the end-cap of a coaxial Ge detector (relative efficiency 25 %; see Figure 5). The detector was calibrated at end cap distance using pure Fe powder.

Four Fe standard solutions samples containing 1.0 mg of iron were irradiated separately. The solutions were, similarly as the urine samples, after irradiation transferred to plastic bottles and the volume was carefully adjusted with water to the same height as of the urine samples. The measurement results as shown in Table 10 showed a large variation. This is probably due to a geometrical mismatch between the position of the Zn flux monitor and the urine sample.

Table 10. Verification of large sample urine analysis procedure: Fe standard solution analysed by INAA

Fe in the standard solution (mg)	1st sample Fe (mg)	2ed sample Fe (mg)	3ed sample Fe (mg)	4th sample Fe (mg)
1.02	1.22	0.87	1.28	0.76

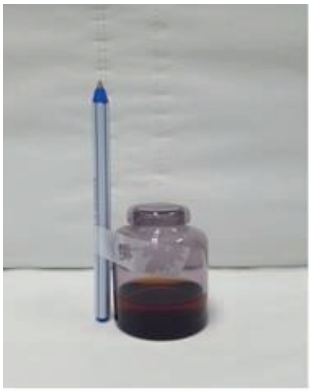


Figure. 3. A quartz bottle containing urine after irradiation

Figure. 4. Urine irradiation, 1&2: Loading the sample inside the Befa for irradiation, 3 : taking out the sample after irradiation.

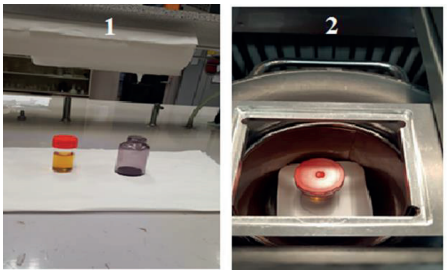


Figure 5: (1) Urine sample transferred into a plastic container (left) and the irradiated quartz vial (right), (2) Plastic bottle positioned on the end-cap of the semiconductor detector

For this feasibility study, the arithmetic mean value of the 4 results of the Fe standard solution was used. As shown in Table 11, there is a measurable increase in the amount of iron in the urine of anaemic patients after they have been injected with pharmaceutical iron.

Table 11. Fe (mg) in the 20 gram urine samples of the anaemic patients before and after Fe injection measured by INAA:

No	Fe (mg)	Fe (mg), before injection	Fe (mg), after injection
1	800	3.75	8.50
2	1000	0.54	2.89
3	1000	0.11	1.22
4	800	0.57	2.95
5	800	-	1.34
6	800	0.41	3.70

In most patients only very small amounts of iron could be measured before administration with a significant increase after administration. This indicates that a part of the injected iron gets lost in urine shortly after administration. The exact amount or percentage lost could be calculated in case the collection would have been continued during several days. Our experiment shows that with some adjustments urine in liquid form can be analysed with INAA, but the technique still needs optimization.

6.7 Conclusions

The results from the experiments described in this chapter lead to the following conclusions:

- Iron present in biological material can be measured with INAA making INAA an attractive technique for mass balance studies and the measurement of iron in tissues. In such studies researchers can rely on one single technique. However in all the experiments no direct comparison was made with other techniques to measure iron so at this stage no conclusions can be drawn on the quantitative accuracy of the method.
- In healthy individuals INAA could not show a significant increase in blood iron concentration after an orally administered pulse dose of iron sulphate in healthy individuals, probably because the intensity of absorption stays under the detection limit.
- Although most iron in blood is present in the erythrocytes changes in blood iron status can be best monitored in plasma.
- There are no real technical barriers to apply INAA for the measurement of iron in faeces and urine. However faeces collection is difficult in an out-patient setting and the necessary time to collect sometimes hard to judge. Urine in liquid form can be irradiated with special adjustments but the technique, including the design of irradiation container and neutron flux monitor needs further attention and optimization.
- A urine standard reference solution should be used in the next experiments.
- A quartz bottle with different dimensions " small diameter and big height" should be prepared for the next experiments, to be able to irradiate the sample and the standard solution at the same time.

6.8 References

[1] S. A Abrams, Using Stable Isotopes to Assess Mineral Absorption and Utilization by Children. Am J Clin Nutr, 70, 955-964, 1999.

[2] Z. Chen, I. J. Griffin, L. M. Plumlee, and S. A. Abrams. High Resolution Inductively Coupled Plasma Mass Spectrometry Allows Rapid Assessment of Iron Absorption in Infants and Children. J. Nutr., (2005), 135 (7), 1790-1795.

[3] M. C McDonald, S. A Abrams and R. J Schanler. Iron Absorption and Red Blood Cell Incorporation in Premature Infants Fed an Iron-Fortified Infant Formula. Pediatric Res., (1998) 44, 507–511.

[4] J. A Widness, K. A Lombard, Ekhard E Ziegler, Robert E Serfass, Susan J Carlson, Karen J Johnson and June E Miller. Erythrocyte Incorporation and Absorption of 58Fe in Premature Infants Treated with Erythropoietin. Pediatric Res., (1997) 41, 416–423.

[5] S. J. Fomon, M. Janghorbani, B. T. G. Ting, E. E. Ziegler, R. R. Rogers, S. E. Nelson, L. S. O. and B. B. Edwards. Erythrocyte Incorporation of Ingested 58-Iron by Infants. Pediatric Res., (1988), 24 (1): 20-24.

[6] M. Blaauw, The holistic analysis of gamma-ray spectra in Instrumental Neutron Activation Analysis, PhD dissertation, Delft University of Technology. 1993, ISBN 90-73861-16-0.

[7] A. Simonits, F. de Corte and J. Hoste. Single-Comparator Methods in Reactor Neutron Activation Analysis. J. Radioanal. Chem., 24 (1975), 31-46.

[8] Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparisons, International Organization for Standardization, ISO 13528(2005).

[9] I. G. Gokmen, G.E. Gordon and N. K. Aras, Application of Activation Analysis techniques for determination of trace elements in human blood. J. Radioanal.Nucl. Chem., 113(2) (1987) 453-459.

[10] G. V. Lyengar. Concentration of 15 trace elements in some selected adult human tissues and body fluids of clinical interest from several countries: results from a pilot study for the establishment of reference values, United Kingdom Atomic Energy Authority, 1985.

[11] V. Iyengar and J. Wolttlez. Trace elements in human clinical specimens: Evaluation of literature data to identify reference values. Clin. Chem. 34(3) (1988) 474-48.

[12] L. E. Walther , K. Winnefeld and O. Solch. Determination of iron, copper, zinc, magnesium and selenium in plasma and erythrocytes in neurosurgical patients. J. Trace Elements Med. Biol. 14 (2000) 92 – 95.

[13] S. Caroli, A. Alimonti, E. Coni, F. Petrucci, O. Senofonte and N. Violante. The assessment of reference values for elements in human biological tissues and fluids: A Systematic Review. Critical Reviews in Analytical Chemistry, 24(5& 6) (1994) 363-398.

[14] S. Godeny, I. Borbely-Kiss, E. Koltay, S. Laszlo and G. Szabo. Determination of trace and bulk elements in plasma and erythrocytes of healthy pregnant women by Pixi method. Int. J. Gynaecol. Obstet., 24 (1986) 201-207.

[15] M. Janghorbani, B. T. G. Ting and V. R. Young. Accurate analysis of stable Isotopes 68Zn, 70Zn, AND 58Fe in human feces with neutron activation analysis. Clin. Chim. Acta, (1980), 108: 9-24.

[16] M. Janghorbani, B. T. G. Ting and V. R. Young. Absorption of Iron in Young Men Studied by Monitoring Excretion of a Stable Iron Isotope (58Fe)in Feces. J. Nutr., (1980), 110: 2190-2197.

[17] Assessment of iron bioavailability in human using stable iron isotopes techniques. IAEA human heath series No. 21. International Atomic Energy Agency, Viena, 2012.

[18] R. Cornelis, A. Speecke and J. Hoste. Neutron Activation Analysis for bulk and trace elements in urine. Anal. Chim.Acta, (1975),78: 317-327.

[19] A. Avino, G. Capannesi, E. Sabbioni, G. Visco and A. Rosada. Element assessment in blood, serum and urine of two Italian healthy sub-population by INAA. CMA4CH (2010), Mediterraneum Meeting.

7 Iron Concentrations in Blood Compartments Measured by Instrumental Neutron Activation Analysis

7.1 Introduction

In 1940 the spectrophotometer was introduced by Arnold J. Beckman and his colleagues, and this initiated many studies on the measurement of trace elements including iron in blood serum using different reagents [1-7]. Later on other methods have been developed and applied to determine iron in blood including AAS [8-16], ICP-MS [17-19], ICP-AES [20], ICP-OES [21,22], XRF [23], and RNAA [24-28]. In many analyses it is sufficient to measure total iron, but in certain situations, especially in studies on resorption and bioavailability, the fate of an iron supplement must be traceable. For a long time the radionuclide ^{59}Fe has been used for this purpose, but the side-effect of radiation limits it's applicability. During the last decennium a growing interest can be observed in the use of stable iron isotopes, since they lack radioactivity and are safe for in vivo studies.

A drawback of these stable isotopes is that their concentrations in biological material can only be measured with special techniques such as INAA and advanced forms of mass spectrometry such as multi collector inductively coupled plasma mass spectrometry (MC-ICP-MS). Both techniques have pro's and con's. MC-ICP-MS is a very sensitive technique and available in advanced research facilities. However there are technical limitations by the need of processing small samples that have been brought into a solution; and, for some elements spectroscopic interferences and non-spectroscopic interferences limit the applicability[29]

Neutron activation of iron, as performed in a nuclear research reactor, results in the radionuclides ^{59}Fe by thermal neutron capture in ^{58}Fe and ^{54}Mn by fast neutron capture in ^{54}Fe and subsequent proton emission. The radionuclide ^{59}Fe decays by emission of gamma-rays of 1099 keV and 1292 keV; the radionuclide ^{54}Mn decays with emission of a gamma-ray of 835 keV. The two radionuclides are measured simultaneously and selectively with a gamma-ray spectrometer using a high resolution semiconductor detector. INAA is not as widely applied

as mass spectrometry because of its requirement of access to a nuclear analytical facility. It has, however, several attractive analytical advantages, as described in chapter 4.

The aim of this work is to introduce INAA as a method for measuring trace elements including iron in clinical medicine especially in the field of metabolism and in tissue materials. The present study was intended to assess the performance of INAA to measure iron in biological material as well as by comparing INAA result with the result obtained by the ICP-OES technique. As a first step of the introduction of the technique in clinical practice we measured iron concentrations in different blood compartments of patients with both iron deficiency and iron overload.

7.2 Methodology

Materials: Blood was collected in EDTA tubes from 26 individuals, 12 males and 14 females, age 18-65, visiting the El Gedarif Hospital, Eastern Sudan. Twenty two of these patients had anaemia, 13 based on iron deficiency microcytic anaemia with (serum iron <7 µmol/L, Hb <7 mmol/L (<11g/dL) and MCV<75 fL), 3 diagnosed as anaemia of chronic disease and 6 with a combination of iron deficiency and a chronic disorder (malaria). Four healthy controls were included having a normal haemoglobin level without clinical or laboratory signs of inflammation. Samples from each patient were measured by ICP-OES as well as by INAA. The NIST Standard Reference Material 1577c "bovine liver" was used for the quality control of the INAA results.

In a second clinical experiment, blood samples were collected in EDTA tubes from 12 subjects, 5 anaemic, 4 hemochromatosis patients and 3 healthy controls under the supervision of the Meander Medical Centre (MMC) in Amersfoort, The Netherlands. The anaemic patients were known with iron deficiency (serum iron <7 µmol/L, Hb <7mmol/l (<11g/dL), MCV<75fL) and blood samples were taken before iron supplementation. The hemochromatosis patients were already known with hereditary hemochromatosis (HH; C282Ymutation) and blood was taken from a scheduled bloodletting. Two blood samples were taken from each patient. One sample was analysed as a whole blood sample and the other was separated into plasma and erythrocytes by centrifuge 2000 x g for 10 minutes.

ICP-OES: 0.05 g of each blood sample and IAEA-A-13 animal blood (reference) were accurately weighed and digested using 3 ml of analytical grade 65% HNO_3 . The digested samples were diluted to 50 mL with double distilled water and measured using ICP-OES (Varian 725-ES) in Petroleum Laboratory, Sudan. ICP-OES operating conditions were well

optimised and carefully selected in order to maximise the sensitivity and to obtain the best precision and accuracy. Details of the operating conditions are summarised in Table 1

Table 1. Instrumentation parameters and settings (ICP-OES):

Parameter	Settings
Power:	1200 W
Plasma gas flow:	15 L/min
Auxiliary gas flow:	1.5 L/min
Nebulizer	0.75 L/min
View distance	10 mm
Sample uptake time	30 sec.
Wash rate	1.5 ml/min
Wash time	25 sec
Replicates	3 times
Wavelength (Fe)	238.204, 239.562 nm

A series of Fe standard solution used for calibration was prepared by diluting the stock solution of 1000 mg/L Fe (Iron ICP standard). The calibration of iron was found to be linear with correlation coefficients above 0.99.

INAA: Each sample, either whole blood, plasma or erythrocytes, was freeze-dried in an EZ-dry freeze drier (MNL-036-A). About 200 mg of each dried sample was weighed and packed in high purity polyethylene capsules. During radiation in the 2 MW nuclear research reactor of the Reactor Institute Delft, Delft University of Technology, zinc was used as neutron flux monitor [30]. Samples along with similarly prepared blanks (empty capsule) and the Standard Reference Material "Bovine liver" (NIST 1577c), both serving for internal quality control were irradiated for 10 hours at a thermal neutron flux of ~ $4.5*10^{12}$ cm^{-2} s^{-1}. Samples were allowed to decay for two weeks, thereby effectively eliminating all activity from the short half-life radionuclides such as ^{24}Na in order to improve the detection limits. The induced radioactivity was measured during 3 hours using a well type Ge detector, with an absolute photopeak efficiency of 11% for the 1332 keV photopeak of ^{60}Co and 13% for the 1099 keV photopeak of ^{59}Fe. The gamma-ray spectra were analyzed using the APOLLO software [30]. INAA in Delft is calibrated following the single-comparator approach [31]. By irradiation of a known amount of natural iron, calibration factors can be established that

relate, under specific irradiation, decay and measurement conditions, the known amount of iron to the peak areas of the 835 keV, 1090 keV and 1292 keV gamma-ray. Minor adjustments are made regularly as changes in the configuration of the reactor core affect the ratio of the activation rates of the two radionuclides.

Ethical aspects: both Sudanese and Dutch patients gave informed consent for the extra laboratory tests. For the Dutch patients this happened after approval of the study protocol by the local medical ethical committee of the Meander Medical Center, Amersfoort

7.3 Statistical Analysis

Statistical analyses were performed using SPSS (IBM Statistics 20). Box Whisker Plots have been applied to compare the INAA results with those of ICP-OES and in the comparison of the three groups (anaemic, hemochromatosis and controls) in the clinical experiment. The uncertainty and detection limit (DL) of each measurement is calculated by INAA software and given with the results. The trueness of the method was evaluated by applying Zeta score for the Standard Reference Material bovine liver [32].

The Zeta score was calculated using equation:

$$\zeta = \frac{|x_m - x_{ref}|}{\sqrt{u_m^2 + u_{ref}^2}}$$

x_m= the measured mass fraction of Fe in NIST SRM 1577c

x_{ref} = the certified reference value of Fe in NIST SRM 1577c

u_m = the combined standard uncertainty of the measured value

u_{ref} = the combined standard uncertainty of the reference value

7.4 Results

First of all a quality control of INAA was performed by measuring iron in a standard reference material (SRM). The analysis of the NIST SRM 1577c yielded a mean value for Fe of 194 +/- 3 $mg.kg^{-1}$ (N=3), whereas the certified value is 197.94+/- 0.31 $mg.kg^{-1}$. The recovery was 98% with a bias of 2.0%.

Zeta score was found to be 1.3 which is well within the laboratory's quality control criteria (|zeta| < 3).

Thereafter iron was measured in 26 Sudanese individuals, including four healthy controls and 22 anaemic patients, with both ICP-OES and INAA.

As shown in Figure 1, iron concentrations measured by INAA show a very good correlation (0.949) with those measured with ICP-OES.

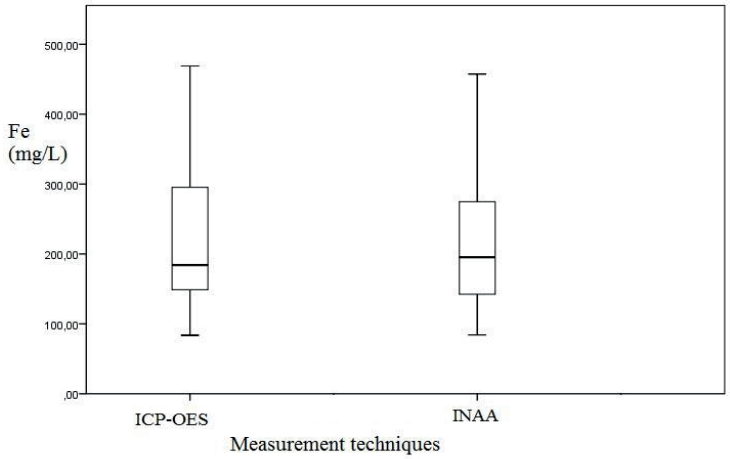


Fig 1: Box-whisker plots showing the Fe whole blood concentrations in 26 Sudanese patients measured with INAA as well as with ICP-OES

In a second experiment iron was measured with INAA in 12 Dutch individuals, five with iron deficiency, four with haemochromatosis and three healthy controls. Iron was measured in whole blood, plasma and erythrocytes. Results of iron mass fractions in whole blood, plasma and erythrocytes of the three groups are shown in Figure 2. The mass fractions were calculated on dry mass basis and converted to $mg.L^{-1}$ assuming that the freeze dried residue is 20% of whole blood (density is 1.06 $g.mL^{-1}$), 9% of plasma (density is 1.026 $g.mL^{-1}$), and 37% of erythrocytes (density is 1.096% $g.mL^{-1}$) [34].The iron concentrations are lower in anaemic patients (whole blood 306±17 $mg.L^{-1}$, plasma 0.5±0.3 $mg.L^{-1}$, erythrocytes 820±60 $mg.L^{-1}$) and higher in hemochromatosis patients (whole blood 500±30 $mg.L^{-1}$, plasma 2.5±1.5 $mg.L^{-1}$, erythrocytes 1040±60 $mg.L^{-1}$) than the control subjects (whole blood 450±10 $mg.L^{-1}$, plasma 2.0±0.6 $mg.L^{-1}$, erythrocytes 960 ±30 $mg.L^{-1}$).

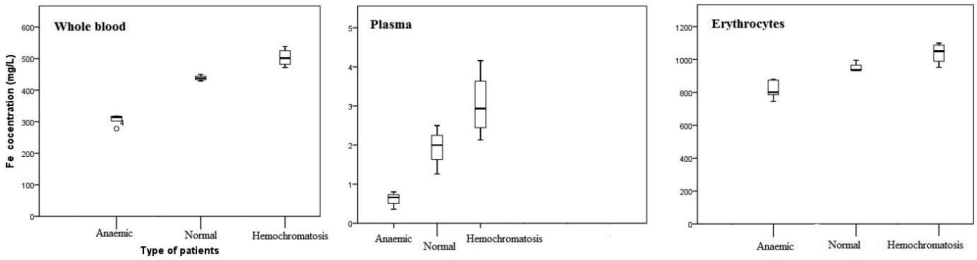


Fig. 2 Box-whisker plots showing the Iron concentration (mg.L^{-1}) in whole blood, plasma and erythrocyte samples of anaemic, normal and hemochromatosis subjects using INAA.

Table 2 shows a comparison of the measured iron concentrations in blood components of the control subjects and the literature values.

Table 2. Iron concentrations, average ±SD (mg.L^{-1}) in blood components of iron deficient anaemic, hemochromatosis and normal subjects in this work compared with literature values for the normal subjects:

Blood component	Anaemic	Hemochromatosis	Control	Literature (Control)	References
Whole blood	306±17	500±30	450±11	420-520	9, 20,34,35, 36
Plasma	0.5±0.3	2.5±1.5	2.0± 0.6	0.6-2.0	34,36,37,38
Erythrocytes	820±60	1040.95±60	960±30	950-1014	34,37,39

7.5 Discussion and conclusion

The present study demonstrates the analytical capability of INAA for measuring iron in material relevant to clinical medicine. Indicative values of iron concentrations have been established from healthy people and patients with iron disorders. It is the first step towards further studies with enriched, stable, neutron activable ^{58}Fe analysing iron resorption and metabolism.

By comparing INAA results with those of another established analytical method (ICP-OES) we were able to show that INAA produced equivalent results with a very good correlation of 0.949. The analysis of the NIST SRM 1577c bovine liver revealed a mean value for Fe of 194

± 3 mg.kg^{-1} with a bias of 2.0% from the certified value (197.94 ± 0.31 mg.kg^{-1}). The analysis showed a good recovery of 98%, while the Zeta score of 1.3 is well within the laboratory's quality control criteria. The measured iron concentrations in blood components of the control subjects are in a good agreement with literature values. So we conclude that INAA is an accurate technique to measure iron in biological materials and can therefore be used in the analysis of iron metabolism in various clinical conditions.

Although INAA is somewhat less sensitive than ICP-OES and less accessible it has characteristics, that make it very attractive for clinical research. These include the possibility of measurement without sample pre-treatment (only freeze drying). There is no need for matrix-matching calibrators, the sample is retained for further analysis, and the degree of trueness and uncertainty of measurement are both well suited for evaluations in clinical medical studies.

Since our research is directed to the measurement of trace elements in various biological materials including tissues and the application of stable isotopes in clinical medicine we decided to introduce INAA for this purpose. The present study shows its capability and, as expected, in a clinical experiment low iron concentrations in total blood, plasma and erythrocytes of patients with iron deficiency anaemia and high iron concentrations in all compartments of hemochromatosis patients. The measured iron concentrations in blood compartments of the control subjects are in good agreement with literature values.

In conclusion, the sensitivity of INAA is adequate for measuring low concentrations of iron in blood compartments and a promising technique for studies with enriched stable isotopes in clinical conditions.

7.6 References

[1] W. N. M. Ramsay, The determination of iron in blood plasma or serum, Biochem J. 53(2) (1953) 227-231.

[2] W. N. M. Ramsay, The determination of iron in blood plasma or serum, Clin. Chim. Acta. 2 (1957) 214-220.

[3] D'A. Kok and F. Wild, Serum iron determination, J. Clin. Path. 13 (1960) 241.

[4] H. V. Connerty and A. R. Briggs, New method for the determination of whole-blood iron and hemoglobin. Clin, Chem. 8 (1962) 151-7.

[5] D. J. Johnson and H. L. Williams. Rapid spectrophotometric assay of serum iron, Chlin. Chim. Acta, 189(2) (1990) 199-203.

[6] A. Jafarian-Dehkordi, L. Saghaie , N. Movahedi, A new spectrophotometric method for direct determination of iron (III) in serum, DARU, (2008),Vol. 16(2), 76-82.

[7] U. Abu Mohsen, K. Abu Elaish, M. Issa, Determination of Iron in Blood Serum by Spectrophotometric and Atomic Absorption Methods as a Comparative Study, Cukurova Med. J.,38 (3) (2013) 358-364.

[8] D. O. Rodgerson and R. E. Helfer, Determination of iron in serum or plasma by atomic absorption spectrophotometry, Clin. Chem. 12(6) (1966) 338-49.

[9] H. T. Delves, G. Shepherd and P. Vinter, Determination of Eleven Metals in Small Samples of Blood by Sequential Solvent Extraction and Atomic-absorption Spectrophotometry, Analyst 96 (1971) 260-273.

[10] J. Lotterle, Measurement of the hemoglobin content of fresh blood by atomic-absorption-spectrophotometric iron determination, Z Rechtsmed 85(4) (1980) 283-8.

[11] S. Lugowski, D. C. Smith, and J. C. van Loon, The determination of Al, Cr, Co, Fe, and Ni in whole blood by electrothermal atomic absorption spectrophotometry, J. Biomed, Mat. Res., 21 (1987) 657-671.

[12] Z. A. de Benzo, R. Fraile , C. Gomez and N, Carrion. Determination of serum iron by aas: deproteinization versus direct analysis. Clin. Chim. Acta, 197 (1991)141-148.

[13] F. Shah , T. Gul Kazi , H. I. Afridi , J. A. Baig , S. Khan , N. F. Kolachi , S. K. Wadhwa , A. Shah, Environmental exposure of lead and iron deficit anemia in children age ranged 1–5 years: A cross sectional study. Sci. Tot. Environm. 408 (2010) 5325–5330.

[14] S. F. A. Ali, S. U. Mehdi, M. Asif, S. N. U. Hassan and E. H. Mirza, Detection of Iron and Manganese Concentrations in Human Biological Fluid with Flame Atomic Absorption Spectroscopy (FAAS), International Conference on Bioscience, Biochemistry and Pharmaceutical Sciences (ICBBPS'2012) Penang, Malaysia.

[15] J. Kaluza, D. Madej, A. Brzozowska, The effect of iron and zinc supplementation and discontinuation of this practice on iron and zinc level in tissues in rats fed deficient diets, J.Trace Elem. Med.Biol., 27 (2013) 334–338.

[16] S. S. Najim, Determination of iron in human serum samples of thalassaemia patients by flame atomic absorption. J. Basrah Res. (Sciences), (2014), 40(1), 102-109.

[17] C. S. Mun˜iz, J. M. M. Gayo´n, J. I. G. Alonso and A. S. Medel, Accurat determination of iron, copper and zinc in human serum by isotope dilution analysis using double focusing ICP-MS, J. Anal. At. Spectrom., 14 (1999) 1505–1510.

[18] C. P. Case, L. Ellis, J. C. Turner, and B. Fairman, Development of a routine method for the determination of trace metals in whole blood by magnetic sector inductively coupled plasma mass spectrometry with particular relevance to patients with total hip and knee arthroplasty, Clin. Chem. 47(2) (2001) 275–280.

[19] G. Li, J. D. Brockman, S.W. Lin, C. C. Abnet, L. A. Schell and J. D. Robertson, Measurement of the trace elements Cu, Zn, Fe, and Mg and the ultra-trace Elements Cd, Co, Mn, and Pb in limited quantity human plasma and serum samples by inductively coupled plasma-mass spectrometry, Am.J.Anal.Chem., 3 (2012) 646-650.

[20] P. Mamatha. Analyis of human serum with various cataracts by Inductively Coupled Plasma Atomic Emission Spectrometry by using different digession proceduers, Int. J. Pharm. Res. Dev., 3(6) (2011) 42 – 49.

[21] C. Prohaska, K. Pomazal and I. Steffan, Determination of Ca, Mg, Fe, Cu, and Zn in blood fractions and whole blood of human by ICP-OES, Fres. J Anal Chem, 367 (2000) 479-484.

[22] I. O. Olabbanji, E. A. Oluyemi, F. O. Fatoye and J. C. Ngila, Elemental composition of blood and hair of mentally-ill patients using ICP-OES techniques, Int. J. Biol. Chem. Sci., 5 (2) (2011) 663-679.

[23] E. A. H. Caraballo and L. M. M. Parra, Direct analysis of blood serum by total reflection X-ray fluoresence spectrometry and application of an artificial neural network approach for cancer diagnosis, Spectrochim.Acta B 58 (2003) 2205-2213.

[24] K. Fritze and R. Ropertson, Instrumental and radiochemical neutron activation analysis techniques for protein bound trace metals in human serum, J. Radioanal. Chem. 1 (1968) 463-473.

[25] N. I. Ward and D. E. Ryan, Multi-element Analysis of blood for trace metals by neutron activation analysis, Analyt.Chim.Acta, 105 (1979) 185-197.

[26] K. Kasperek, J. Kiem, G. V. Iyengar and L. E. Feinendegen. Concentration Differences between serum and plasma of the elements cobalt, iron, mercury, rubidium, selenium and zinc determined by Neutron Activation Analysis, Sci.Tot.Env., 17 (1981)133-143.

[27] W. Maenhaut, L. Dereu and U. Tomza. The determination of trace elements in commercial human serum albumin solutions by Proton-Induced X-ray Emission Spectrometry and Neutron Activation Analysis. Anal. Chim.Acta, 136 (1982) 301-309.

[28] L. Xilei, D. Van Renterghem, R. Cornelis and L. mees. Radiochemical Neutron Activation Analysis for thirteen trace metals in human blood serum by using inorganic iron exchangers. Anal. Chim.Acta, 211 (1988) 231-241.

[29] K. E. Jarvis, A. L. Gray and R. Sam Houk. Hand book of Inductively Coupled Plasma Spectrometry. (2003), ISBN 0-9544891-0-1

[30] C. B. Boss and K. J. Fredeen. Concepts, Instrumentation and techniques in Inductively Coupled Plasma Optical Emission Spectrometry. 2004 PerkinElmer, Inc. 005446B.

[31] M. Blaauw, The holistic analysis of gamma-ray spectra in Instrumental Neutron Activation Analysis, PhD dissertation, Delft University of Technology. 1993, ISBN 90-73861-16-0.

[32] A. Simonits, F. de Corte and J. Hoste. Single-Comparator Methods in Reactor Neutron Activation Analysis. J. Radioanal. Chem., Vol. 24 (1975), 31-46.

[33] Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparisons, International Organization for Standardization, ISO 13528(2005).

[34] I. G. Gokmen, G.E. Gordon and N. K. Aras, Application of Activation Analysis techniques for determination of trace elements in human blood. J. Radioanal. Nucl. Chem., 113(2) (1987) 453-459.

[35] G. V. Lyengar., Concentration of 15 trace elements in some selected adult human tissues and body fluids of clinical interest from several countries: results from a pilot study for the establishment of reference values, United Kingdom Atomic Energy Authority, 1985.

[36] V. Iyengar and J. Wolttlez, Trace elements in human clinical specimens: Evaluation of literature data to identify reference values. Clin. Chem. 34(3) (1988) 474-48.

[37] L. E. Walther 1, K. Winnefeldand O. Solch. Determination of iron, copper, zinc, magnesium and selenium in plasma and erythrocytes in neurosurgical patients, J. Trace Elem. Med. Biol. 14 (2000) 92 – 95.

[38] A. Caroli , A. Alimonti, E. Coni, F. Petrucci, O. Senofonte and N. Violante. The assessment of reference values for elements in human biological tissues and fluids: A Systematic Review. Crit. Rev.Anal.Chem., 24(5& 6) (1994) 363-398.

[39] S. Godeny, I. Borbely-Kiss, E. Koltay, S. Laszlo and G. Szabo. Determination of trace and bulk elements in plasma and erythrocytes of healthy pregnant women by PIXI method. Int. J. Gynaecol. Obstet., 24 (1986) 201-207.

8 Dietary Intake Assessment of Iron Without Subsampling of Double Portion using Large Sample Neutron Activation Analysis

8.1 Introduction

Mass balance studies are used to get information on the actual bioavailability of major and trace elements present in food. In such studies it is essential to measure the amounts of an element both at the site of intake and at the sites of excretion , such as in urine and faeces. A mass balance study can easily cover a period of 5-7 days in which 8-10 kg of food and 10-14 L of drinking solutions are consumed. A double portion technique, one portion consumed by the test person, an identical other portion used for analysis, is usually used to quantify the intake of an element. The intake of an element is calculated by either adding up the amounts of that element present in each component of the food intake or by homogenizing the entire intake and analysing a representative sub-sample of e.g. 1 g or less. The latter will only give reliable data in case the element of interest is distributed homogeneously and the sub-sample is truly representative. The quality of the homogenization has to be checked by analysis of e.g. 15 small test portions.

Dietary intake collection by the double portion technique will result in a highly inhomogeneous mixture of food ingredients, including liquid ones. From experiments with food using the traditional (small sample) INAA technique we faced difficulties in preparing representative sub-samples. The common approach implies freeze drying of the food and very careful homogenization after which small samples (200 mg) are available for measurement. However, if e.g. pork liver is freeze dried the resulting product is quite a hard peace of dried liver that cannot easily be crushed in usable small parts. In another case freeze-dried peaches, could be easily crushed, but the material, still containing sugar, gets very sticky once liquid nitrogen is poured on it (Figure 1). It would therefore be highly attractive if the dietary intake collected over several days, could be analysed as received without attempting to remove the moisture fraction by drying it to constant weight.

Figure 1. Peaches and pork liver after freeze drying

These problems can be overcome by using large sample neutron activation analysis (LS-NAA) since this technique has nowadays the capability of measuring the content of an element in the entire collected amount of food (multi-kilogram size) without sub-sampling and homogenization.

Neutron irradiation of moist samples results into enhanced neutron self-absorption and neutron self-thermalization of epithermal and fast neutrons. The latter effect is not opportune for the large sample NAA at the facilities in Delft due to the high ratio of thermal to epithermal and fast neutrons in the thermal column facility (a factor of 3000). The first effect is accounted for by the calculus of the thermal neutron flux distribution –and thus also the average thermal neutron flux on basis of the neutron flux depression outside the sample, and comparison thereof with a reference condition [1].

One of the characteristics of large sample analysis is that there are no quality control ('trueness control') materials available at such a large scale. We therefore verified the validity of this calculus for the analysis of a large moist food sample has by using a powdered wheat flour, assumed to be homogeneous in trace element composition. The material was analysed as a small-dry- sample by 'normal' INAA to obtain a reference value for the Fe mass fraction.

The same powdered wheat grain material was converted into a moist porridge to test the validity of the LS-NAA software accounting for the neutron self –absorption by the moisture

in food. As such, an indication for the degree of trueness of large, moist, sample analysis can be obtained.

The feasibility of real dietary intake analyses was tested by the analysis of commercially available microwave food products –inhomogeneous in composition- after freeze drying.

8.2 Materials and methods

8.2.1 Validity assessment

These experiments were done with commercially available porridge fine wheat grain powder purchased from Nestlé (Figure 2). About 750 g dry material was used for the large sample analyses; another 750 g was prepared as a real porridge by adding 1.3 L of Millipore water. The completed porridge was then transferred to a 2 L polyethylene bottle for irradiation. A 2 L bottle only filled with Millipore water was used as a blank..

Four subsamples from the dry porridge were used for the first experiment and 10 subsamples from the finished porridge of the second experiment were prepared for analyses by traditional small sample INAA along with the Standard Reference Material NIST-1547 (Peach leaves) as a control sample.

8.2.2 Feasibility of real meal analyses

8.2.2.1 Sample collection and preparation

Five different microwave meals and bread were purchased from a supermarket in Delft, The Netherlands. Theses meals contained different types of food like chicken, beef, rice, salad, bread, pasta, and different vegetables like potato and pea (Figure 3). All types of meats contain some fats and muscles which make it difficult to prepare a complete freeze dried and homogeneous powder. On the other hand the plant origin foods such as salad and vegetables are easy to be dried.

Before freeze drying, these products were kept in a freezer at -50 °C for about 24 hours. As such, it was assumed that all moisture present such as sauces were frozen. The meals were freeze dried in an EZ freeze drier (MNL-036-A), mortared and transferred to a polyethylene bottle of 2 L volume and shaken for an even distribution of the materials inside the bottle. The total mass of the 5 meals was 738 g.

Figure 2. Porridge fine wheat grain from Nestlè

Figure 3. Five microwave meals and bread collected from a supermarket

8.2.2.2 Neutron irradiation

Neutron irradiation was performed in the Big Sample Neutron Irradiation System (BISNIS) in the thermal column at the Hoger Onderwijs Reactor (Figure 4). The samples are positioned inside a graphite cylinder insert in the irradiation container. Each sample was surrounded by eighty neutron flux monitors (zinc foils) positioned in a fixed grid in the walls of this graphite cylinder.

The dry and moist porridge as well as the real meal samples were irradiated for 6 days at a thermal neutron flux of ~ $3.0*10^{8}$ cm^{-2} s^{-1}.

Fe fractions in the small samples from the dry porridge fine wheat grain were measured using normal INAA, they were irradiated for 10 hours at a thermal neutron flux of ~ $4.5*10^{12}$ cm^{-2} s^{-1}. Zn was used as a flux monitor for both LS-INAA and normal INAA [2].

8.2.2.3 Measurement

All large samples were measured during four days, starting ca. 15 days after irradiation using a high purity Germanium (HPGe) coaxial detector, relative efficiency 96 %. The measurement facility is shown in Figure 5 and described before [1,3 6]. The distance of the sample (bottle) vertical center axis to the detector endcap is 20 cm, which is large as compared to normal NAA counting geometries. The obtained spectra were corrected taking into consideration the corresponding gamma ray background spectra and the sample's natural radioactivity [1,3]. Since in the large sample irradiation facility the ratio of thermal to epithermal neutrons is very high (~ 3000) only ^{59}Fe can be measured as an indicator for total iron. The zinc flux monitors (0.937 mg each) were measured with a well-type Germanium detector (active volume ca. 250 cm^3) with an absolute photopeak efficiency of 13% for the 1099 keV photopeak of ^{59}Fe.

The small samples from the dry porridge fine wheat grain were measured during 3 hours using the same well-type Ge detector. The gamma-ray spectra were analyzed using the APOLLO software [2].

Data processing

Spectrum analysis and interpretation was done on basis of the k_1 method [4] which is related to the k_0 method [5].

Neutron and γ self-attenuation corrections were performed on the basis of the measured values of the neutron flux depression outside the sample, from which the neutron diffusion coefficient and the neutron diffusion length of the sample could be derived [1] resulting in the neutron density distribution in the sample. The γ ray transmission coefficients were measured separately [3]. For more information about the facility see ref. [1, 3,6,7].

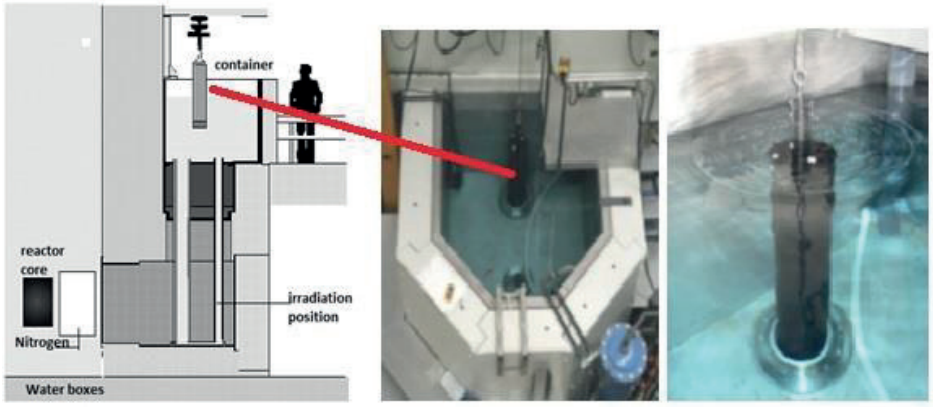


Figure 4. A cross-section of the thermal column with the irradiation container

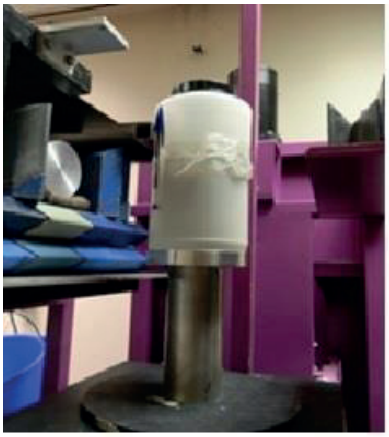


Figure 5. The 738 g sample being counted after neutron activation

Validity assessment

The Zeta (ζ) score has been used to compare the mass fractions measured in the Standard Reference Material NIST-1547 (Peach leaves) used for control in normal NAA with its certified reference values, as well for comparing the mass fractions measured in the small samples NAA (as a reference values to the large samples) with those from the large sample analysis. The score is calculated as follows:

$$\zeta = \frac{|x_m - x_{ref}|}{\sqrt{u_m^2 + u_{ref}^2}}$$

In which $x_{m,\ ref}$ is the mass fraction from the measured samples (*m*) and the reference value (*ref*), respectively and $u_{m,\ ref}$ is the combined standard uncertainty of the mass fraction from the measured sample and the reference value, respectively. Our acceptance criterion for degree of equivalence of the results was $|\zeta| < 3$.

The ζ scores between the large sample results and the reference values from the small sample results are considered acceptable if $|\zeta| < 3$, in agreement with the quality control criterion of of the laboratory for INAA in Delft [8].

8.3 Results

The results of the dry porridge fine wheat grain analysis by large and small sample NAA ('reference' value) are shown in Table 1.

Table 1. Fe mass fraction in dry porridge sample by large sample and normal INAA

	Fe and combined standard uncertainty (1 SD) (mg/kg)	Irradiation time	Decay time	Measurement time	Detection Limit (mg/kg)
LS-NAA	74 +/- 3	6.0 (d)	14 (d)	4.0 d	6.2
Normal INAA (n=4)	67.1 +/- 1.3	10 (h)	14 (d)	3 (h)	2

The result of the prepared porridge (porridge +water) measured and the derived small sample by large and small sample INAA are shown in Table 2.

Table 2. Fe mass fraction in prepared porridge (porridge +water) by large sample and normal INAA

	Fe and combined standard uncertainty (1 SD) (mg/kg)	Irradiation time	Decay time	Measurement time	Det. Limit (mg/kg)
LSNAA	73 +/- 4	6.0 (d)	14 (d)	4.0 (d)	3.7
Normal NAA (n=10)	66.8 +/- 1.3	10 (h)	14 (d)	3 (h)	2

The results of the (small sample) analysis of the Standard Reference Material NIST-1547 (Peach leaves) used for trueness control in normal NAA for the dried samples , and for the prepared porridge samples are given in Table 3.

Table 3. Results of SRM NIST-1547 and zeta score for comparison with certified value

	Experiment	Certified value with combined standard uncertainty (1 SD), (mg/kg)	INAA result with combined standard uncertainty (1 SD) (mg/kg)	ζ
Normal INAA	Dried samples	218 +/- 7	217.4 +/- 3.5	0.08
	Prepared porridge samples		218.3 +/- 3.2	0.04

The Fe mass fractions in the large sample analysis of the large dry powder fine wheat grain and the derived moist porridge material are mutually in excellent agreement (74 +/- 3 mg/kg, and 73 +/- 4 mg/kg respectively, $\zeta = 0.18$); the same mutual agreement is for the small sample analyses (67.1 +/- 1.3 mg/kg and 66.8 +/- 1.8 mg/kg, $\zeta = 0.14$) However, the results in Tables

1 and 2 indicate a systematic difference with the small sample results of the same material, resulting in a bias for the dry material of 10.3 % and zeta score of 2.1, and for the moist material a bias of 9.3 % and ζ= 1.5.

The approximate 10 % bias could, in retrospect, be traced back towards an unexpected difference in the neutron flux distribution in the thermal column irradiation facility between the date of calibration and the date of the experiments described in this paper. The measurement procedure has meanwhile been adapted to accommodate such variations.

Feasibility of real meal analyses

The measured Fe mass fraction as found after analysis of the freeze dried (combined) 5 meals was (30 +/- 2) mg/kg with a detection limit of 6 mg/kg.

8.4 Discussion and conclusion

This study shows that LS-INAA is a useful method for non-destructive multi-element analysis of bulky food samples, up to several kilogram with adequate accuracy [2]. It is an attractive alternative for the standard approach, that has to rely on careful and laborious homogenization and the representativeness of small samples. LS-INAA can be directly applied in mass balance studies both for element analysis of food supplied for several days and faeces collected during such a test. The water content of the fresh food does not cause any problem during the irradiation such as sample swelling and exploding. Moreover, the moisture sample can give a result statistically comparable to the dried sample. These observations are also valuable in view of eventual large sample analysis of faeces, as might be collected during a multi-day mass balance study. Another advantage, as shown in this study, is that a sample can be analysed again after a reasonable decay time of 2-3 weeks.

8.5 References

[1] R. M. W. Overwater. The physics of big sample instrumental neutron activation analysis. PhD thesis Delft University of Technology (1994), ISBN: 90-407-1048-1.

[2] M. Blaauw. The holistic analysis of gamma-ray spectra in Instrumental Neutron Activation Analysis. PhD thesis Delft University of Technology (1993)

[3] M. Blaauw, O. Lakmaker, and P.van Aller. The Accuracy of Instrumental Neutron Activation Analysis of Kilogram-Size Inhomogeneous Samples. Anal. Chem. (1997), 69, 2247-2250.

[4] M. Blaauw and P. Bode. Introduction of The k_1-Concept for The Interpretation of Artificial peaks in k0-Based NAA. J. Radioanal.Nucl. Chem., Articles, Vol. 169, No. 1 (1993) 201-208.

[5] A. Simonits, F. de Corte and J. Hoste. Single-Comparator Methods in Reactor Neutron Activation Analysis. J.Radioanal.Nucl.Chem, 24 (1975), 31-46.

[6] R. M. W. Overwater, Peter Bode, Jeroen J. M. de Goeij, and J. Eduard Hoogenboom. Feasibility of Elemental Analysis of Kilogram-Size Samples by Instrumental Neutron Activation Analysis. Anal. Chem. (1996), 68, 341-348.

[7] P. Bode, R. M. W. Overwater and J. J. M. De Goeij. Large-sample neutron activation analysis: Present status and prospects. J.Radioanal.Nucl.Chem., 216, No. 1 (1997) 5-11.

[8] Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparisons, International Organization for Standardization, ISO 13528(2005).

9 Low zinc Status in Sudanese Patients With Iron Deficiency Anaemia

9.1 Introduction

Anaemia affects low, middle and high income countries and causes both mortality and disability worldwide. The World Health Organization has estimated that around 800 million children and women globally suffer from anaemia with the highest proportion of individuals affected in Africa [1]. Apart from inherited forms of anaemia such as sickle cell anaemia and thalassemia, anaemia may be acquired either by disorders or deficiencies of micronutrients such as folic acid, vitamin B12 and iron. Approximately half of the cases of anaemia are due to iron deficiency, an advanced stage of iron depletion. A lack of iron may be the result of an increased loss of iron from the body or an inadequate intake or uptake of this micronutrient. In some cases of anaemia there is a functional deficiency of iron, when iron is being stored in the body and not available for the bone marrow, the so-called anaemia of chronic disease (ACD). In food two forms of iron can be distinguished , organic and inorganic iron. The last one is present in vegetables and cereals, while organic or haem iron is present in red and organic meat. This haem-iron is more readily absorbed by the body than non-haem iron.

Sudan is both a developed and a low-income country with inadequate food intake by many. The Food and Agriculture Organization of the United Nations (FAO) states in a report of 2010 that more than 90% of the population in Sudan suffer from poverty and food insecurity [2]. Little is known about the prevalence of anaemia in Sudan and studies have mainly focused on anaemia in particular groups such as children and pregnant women [3-11].

Apart from iron other trace elements, including zinc, play a role in haemoglobin synthesis [12]. Zn is present in meat, nuts, grains, and cheese and acts as a co-factor of many enzymes in the human body [13]. Zn deficiency has been found associated with anaemia and both Zn and Fe deficiency represent two of the most important nutritional problems in developing countries. Zn deficiency is hardly present in the Dutch population, but data on the Sudanese population are scarce.

In the present study the focus is on Zn levels in anaemic patients with a comparison between an undeveloped and a developed country but Fe concentrations were also measured. For the Zn comparison all patients were included and for the comparison of Fe only iron deficiency patients were included. In Dutch patients the main cause of their iron deficiency anaemia is blood loss and they have a normal nutritional status. In contrast the Sudanese patients mainly suffer from an inappropriate nutritional intake apart from blood loss in some cases.

9.2 Materials and methods

9.2.1 Design

This study was conducted at the Reactor Institute, Delft (RID), Netherlands. Samples were Collected from Meander Medical Center, (MMC) Amersfoort, The Netherlands and Al Gadarif Hospital, Sudan, between December 1, 2013 and February 1, 2014. The Sudanese samples were freeze dried in Sudan, part of samples were analysed using the ICP-OES in Sudan, the other part transferred to the RID, the Netherlands, and analysed again by the Instrumental Neutron Activation Analysis (INAA). The MMC samples were transferred directly to the IRD and freeze dried and analysed by INAA. Figure 1 shows the design of the study.

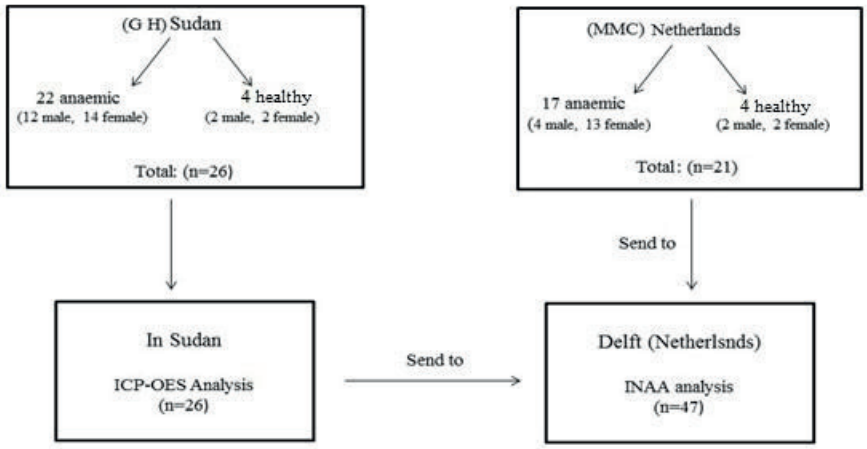


Figure 1 The design of the study

9.2.2 Patients

The Sudanese part of the study includes adults > 18 years with anaemia (Hb <11 g/dL;(<7 mmol/L), 12 male and 14 females age 18-65 and mean age 44 years visiting the Al Gadarif Hospital in Eastern Sudan (Figure 1). The Dutch part includes 21 adults with various forms of anaemia , 6 males and 15 females with age 22-85 and mean age 52 years being patients of the Meander Medical Centre, Amersfoort, The Netherlands. All subjects in the study were filling in a questionnaire including their medical history, lifestyle, type of diet and the use of medication and micronutrient supplementation. Patients using iron, vitamins, or micronutrient supplementation were excluded. Other exclusion criteria were pregnancy, a malignancy or renal insufficiency. The diagnosis of anaemia was based on their haemoglobin level (Hb < 11 g/dL (<7 mmol/L)) but the type of anaemia was further analysed by serum iron, ferritin, transferrin and MCV. Macrocytic forms of anaemia were excluded. The majority of the Sudanese patients were diagnosed with iron deficiency anaemia due to poor iron intake, while some of them were also known to suffer from malaria. Unstable malaria transmission occurs in this part of Sudan [14]. Table 1 and 2 show the type of anaemia diagnosed, age and gender of the individuals participating in this study . The study also included eight healthy controls, four in the Sudanese and four in the Dutch group.

Figure 1. Map of Sudan

Table 1: Diagnosis, gender, age and haemoglobin concentrations of Sudanese individuals participating in the study. (IDA: Iron deficiency anaemia, ACD: Anaemia of chronic disease, GIT: Gastro Intestinal Tract; Malaria: Patients known with a positive parasite staining in the blood; Poor intake: Diet mainly containing cereals and hardly any meat.

No	diagnosis	sex	age	Hb (g/dL)
1	IDA, severe malaria	M	35	6.3
2	IDA, blood loss from GIT	M	37	8.0
3	IDA, severe malaria	F	65	4.3
4	IDA, nutritional poor intake	M	65	4.1
5	ACD	M	45	4.7
6	IDA, ACD, nutritional poor intake	F	52	2.2
7	ACD	M	48	3.0
8	IDA, nutritional poor intake	M	57	4.0
9	ACD	F	60	4.3
10	IDA, blood loss - irregular menstruation	F	18	4.1
11	IDA, severe malaria	M	32	6.5
12	IDA, nutritional poor intake	F	23	5.8
13	IDA, severe malaria	M	46	4.4
14	IDA	F	25	4.3
15	IDA, nutritional poor intake	F	40	4.2
16	IDA, nutritional poor intake	F	65	8.0
17	IDA, nutritional poor intake	F	19	7.0
18	IDA, blood loss from GIT	M	55	8.4
19	IDA, nutritional poor intake	F	28	8.0
20	IDA, severe malaria	M	25	3.7
21	IDA, ACD	F	43	8.5
22	IDA, nutritional poor intake	F	32	4.0
23	Healthy	M	35	13.0
24	Healthy	M	30	13.5
25	Healthy	F	28	13.0
26	Healthy	F	43	12.0

Table 2: Diagnosis, gender, age and haemoglobin concentrations of Dutch individuals participating in the study: (IDA: Iron deficiency anaemia, ACD: Anaemia of chronic disease, HT: Beta homozygous thalassemia)

No	diagnosis	sex	age	Hb (g/dL)
1	IDA	F	82	8.7
2	IDA	F	25	9.9
3	IDA	F	23	10.4
4	IDA	F	53	10.6
5	IDA	F	24	10.0
6	IDA	F	42	8.8
7	IDA	F	43	10.5
8	IDA	F	85	8.7
9	ACD	M	73	9.0
10	ACD	M	81	10.4
11	ACD	M	85	9.6
12	ACD	F	66	10.5
13	ACD	M	74	10.6
14	ACD	F	62	7.7
15	ACD	F	85	9.6
16	HT	F	50	9.9
17	HT	F	36	9.2
18	Healthy	M	29	13.7
19	Healthy	M	58	14.0
20	Healthy	F	33	12.2
21	Healthy	F	31	12.5

9.2.3 Methods

From the Dutch patients, blood for routine haematological tests was collected in EDTA tubes while heparin tubes were used to measure iron, ferritin and transferrin in the hospital. They were centrifuged 2000 x g for 10 minutes. Five mL blood in EDTA tubes of all patients were transferred to Delft and there freeze dried to measure their iron and zinc concentrations. From all the Sudanese patients a sample was used to measure iron in Sudan using inductively coupled plasma optical emission spectrometry (ICP-OES). An EDTA tube was freeze dried in Sudan and transferred to the Reactor Institute Delft, TU Delft, The Netherlands and, together with the material of the Dutch patients, analyzed using INAA.

9.2.4 Sample preparation and measurement

***ICP-OES**:* about 0.05 g of each blood sample and IAEA-A-13, Animal Blood were accurately weighed and digested using 3 mL of analytical grade 65% HNO_3. The digested samples were diluted to 50 mL with double distilled water and measured using ICP-OES (Varian 725-ES) in Petroleum laboratories in Sudan. Details of the operating conditions are summarised in Table 3.

Table 3. Instrumentation parameters and settings (ICP-OES):

Parameter	Settings
Power:	1200 W
Plasma gas flow:	15 L/min
Auxiliary gas flow:	1.5 L/min
Nebulizer	0.75 L/min
View distance	10 mm
Sample uptake time	30 sec.
Wash rate	1.5 ml/min
Wash time	25 sec
Replicates	3 times
Wavelength (Fe)	238.204, 239.562 nm

A series of Fe standard solution used for calibration was prepared by diluting the stock solution of 1000 mg/L Fe (Iron ICP standard). The calibration of iron was found to be linear with correlation coefficients above 0.99.

INAA: About 200 mg of each dried sample was weighed and packed in high purity polyethylene capsules. Zinc was used as comparators in order to measure the neutron flux during irradiation [15]. Samples along with similarly prepared blank (empty capsule) and NIST Standard Reference Material 1577c "Bovine Liver" were irradiated for 10 hours at a thermal neutron flux of ~ $4.5*10^{12}$ cm $^{-2}$ s^{-1}.

Samples are allowed to decay for 2 weeks, thereby effectively eliminating all activity from the short half-life radionuclides such as ^{24}Na. The activity of the induced radioisotopes was measured during 3 hours using a well-type Ge detector, with an absolute photopeak efficiency of 16.5% for 835 keV photopeak of ^{54}Mn and 13% for the 1099 keV photopeak of ^{59}Fe. The gamma-ray spectra were analyzed using the APOLLO software [15].

***Statistical Analysis*:** Statistical analyses were performed using SPSS (IBM Statistic 23). Because the iron concentrations in blood have a skewed distribution, they were firstly converted to their logarithms before performing the statistical analysis. A comparison between INAA and ICP-OES results for the iron concentration in blood of the Sudanese anaemic patients was performed using the Box Whisker Plot. The same method was used to compare the Fe concentrations between Sudanese and Dutch anaemic patients. Pearson's regression test was used to examine the correlation between the iron and zinc concentration using INAA for the two groups of anaemic patients (Table 3).

9.2.5 Ethical aspects

All patients and controls (from Sudan and Netherlands) gave informed consent and the study was approved by the medical ethical committee of the Meander Medical Center, Amersfoort, The Netherlands.

9.3 Results

The median iron concentrations in freeze dried whole blood samples of the Sudanese anaemic patients were significantly lower (190±50 mg/L) compared to controls (440±20 mg/L), while the iron concentration in the blood of the Dutch anaemic patients was found to be 300±40 mg/L. All Sudanese blood samples were measured both by ICP-OES and INAA. As shown in Figure 2, iron concentrations measured by the two techniques can be considered as equivalent. It was found that the two techniques have a correlation coefficient of 0.949.

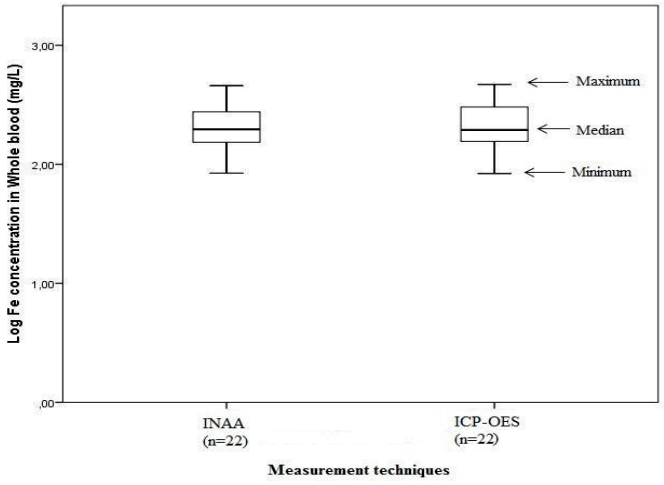


Fig. 2 Iron concentrations in blood of Sudanese anaemic patients using INAA and ICP-OES

Some of the Sudanese patients had extreme low values for Hb and iron status, reflected by the lower mean Hb value compared to Dutch patients and the larger standard deviation (figure 3a). Especially the combination of malaria and poor nutritional intake is associated with these low values. Since is to be expected that Sudanese patients with very low haemoglobin levels have lower iron concentration Sudanese and Dutch patients were matched for the haemoglobin (Hb) content (7-9 g/dL). In that case iron concentration in the Sudanese group still tend to be somewhat lower than in the Dutch group (P value 0.114) but the numbers of patients that could be matched was small (6) as can be seen from Figure 3b.

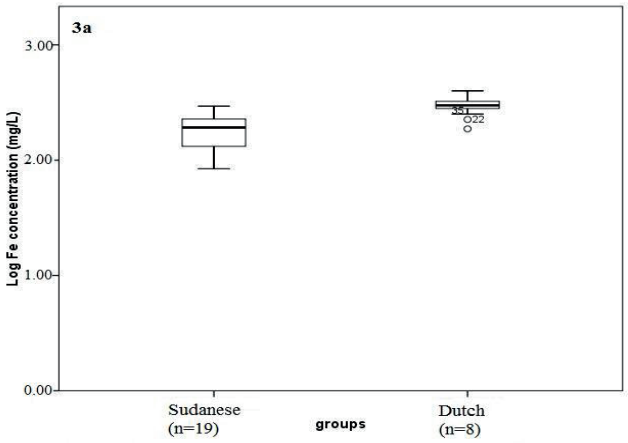


Fig. 3a Fe concentrations in blood in Sudanese and Dutch IDA patients.

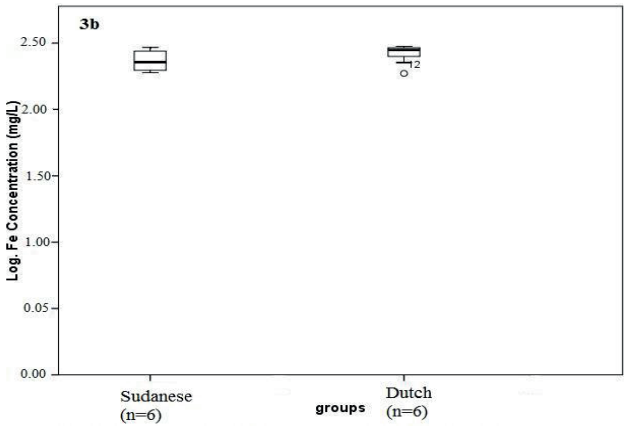


Fig. 3b Fe concentrations in Sudanese and Dutch patients matched for haemoglobin level

Table 4. Results of Pearson correlation test:

Pearson test	**(r) value**	**(p) value**
Zn concentration in blood of Dutch vs Sudanese	0.088	0.737
Fe in blood for both groups vs haemoglobin (Hb)	0.920	< 0.010
Fe in blood vs Zn (Sudanese anaemic patients)	0.654	< 0.010
Fe in blood vs Zn (Dutch anaemic patients)	0.080	0.732
Zn (Sudanese poor intake) vs Zn (Dutch IDA)	0.237	0.572

Zn concentrations were measured in all Sudanese anaemic patients (n= 22), in all Dutch anaemic patients (n = 17) and in healthy controls from both countries (n =8). Zn concentration was found to be 3±1 mg/L, 5±1 mg/L, and 5±1 mg/L in Sudanese anaemic patients, Dutch anaemic patients and healthy controls respectively. The healthy Sudanese individuals had both normal iron and zinc levels comparable to the Dutch healthy individuals. As shown in Figure 4 significant lower concentrations were found in the Sudanese anaemic patients compared to the Dutch anaemic patients. The Pearson regression test showed a negative correlation between the two groups (r= –0.088) (p value 0.737).

The zinc concentration in the blood of the Dutch patients did not differ from the concentrations in the blood of the control group. Even in the blood of the group of anaemic patients matched for their Hb level, the Sudanese patients showed a significant lower Zn concentration than the Dutch patients, (p value 0.114), (Figure 5a).

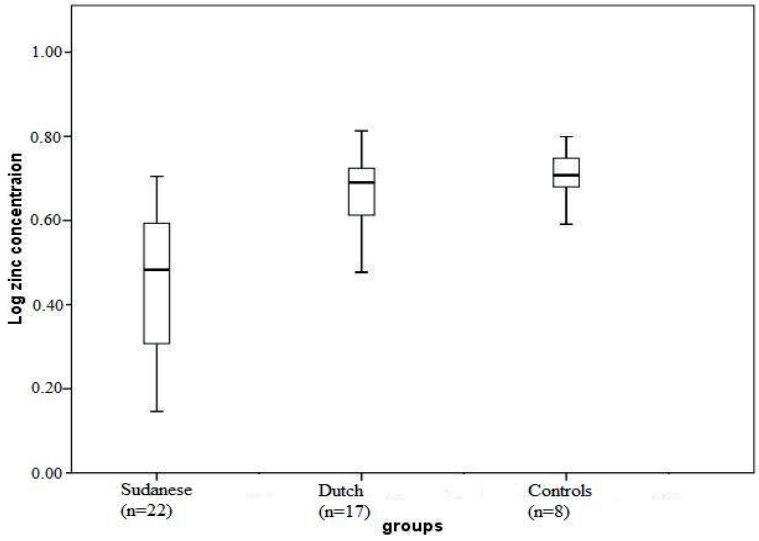


Fig. 4. Zinc concentration in blood of Sudanese and Dutch iron deficient anaemic patients compared to healthy subjects

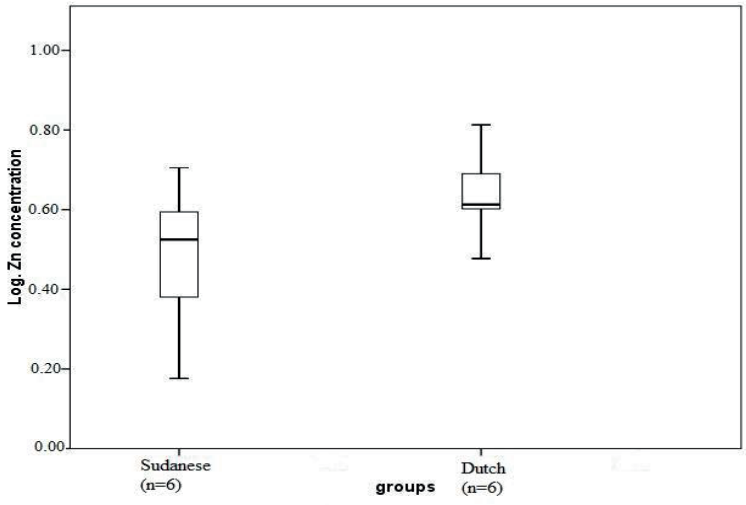


Fig. 5a: Zn concentrations in Sudanese and Dutch anaemic patients matched for haemoglobin level

A Pearson correlation between the level of haemoglobin and iron in the whole (both Dutch and Sudanese anaemic) group showed a very strong correlation between Fe and Hb level (r =

0.920) and (p value < 0.01), while the correlation between Zn and Hb level was only poor (r =0.185). A Pearson correlation was also done to examine the relations between iron and zinc for the two groups (Sudanese and Dutch). A strong correlation was found between Fe and Zinc in the whole blood of Sudanese subjects: r = 0.654, p value <0.01. However, there is no or a very weak relation between Fe and Zn in the whole blood of the Dutch group: r = 0.080, p value = 0.732 (table 4).

Finally, the Zn concentrations in Sudanese patients with registered poor intake (n=9) were compared to the levels in the blood of the Dutch IDA group (n=8), Significant lower zinc concentrations were found in the blood of the Sudanese poor intake patients compared to the Dutch IDA patients (see Figure 5b) . A Pearson correlation for the same groups showed a negative correlation for Zn in blood of the Sudanese poor nutritional intake group and of the Dutch IDA group with r= – 0.237 and p value = 0.572.

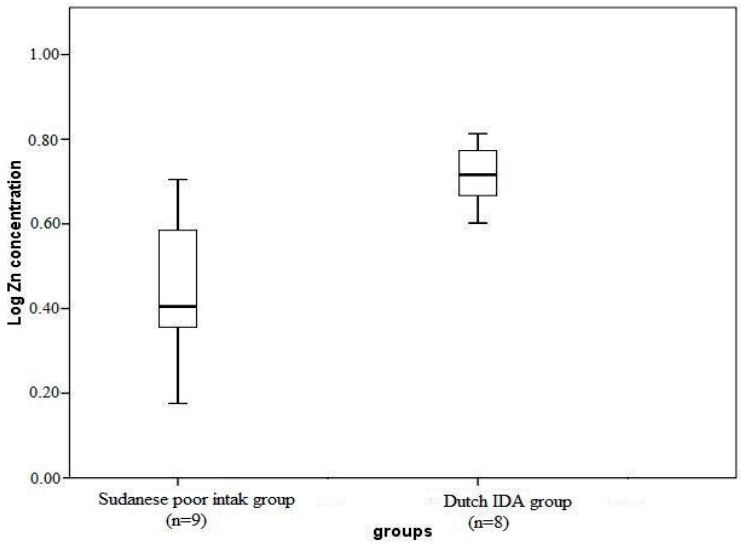


Fig. 5b: Zinc concentration in the Sudanese poor intake group and the Dutch IDA group.

9.4 Discussion and conclusion

Iron deficiency anaemia is a major public health problem especially in underdeveloped and developing countries. In most developed nations the main cause of this type of anaemia is blood loss either related to menses or to gastro-intestinal blood loss as in inflammation or cancer. Nutritional deficiencies, however, are not totally uncommon and can be found in anorectic patients, alcoholics, the elderly, low income families and in case of specific diets. In developing countries a lack of sufficient micronutrients in food is the main cause of anaemia, and can be extreme in case of additional factors such as pregnancy, menses and inflammatory diseases. Anaemia based on an insufficient dietary intake is not only characterized by a deficiency of iron but also of other trace elements such as copper and zinc. This is clearly demonstrated by the results of our study showing severe deficiencies of iron and zinc in blood of Sudanese anaemic patients. In contrast to the Dutch anaemic patients, who had a normal blood zinc level, concentrations of this element were low to very low in the blood of the majority of the Sudanese patients even those without a history of bad intake. This can be explained for the most part by a lack of red meat in their diet. On the other hand, the basic content of their food are cereals and grains, which usually contain zinc. This indicates, as has been shown in other countries, that either the soil or the bread consumed is rather deficient of zinc [16].

Zinc plays an essential role in a number of enzymatic processes in the synthesis of haemoglobin and a deficiency may therefore contribute to the extent of anaemia. This is relevant in case of supplementation because the focus should not only be on iron but also on zinc. Since zinc and iron show some chemical similarity and they do share transport pathways such as the divalent metal transporter 1 (DMT-1) in the intestinal cell, they may therefore be competitive. It was found that zinc administration with an iron aqueous solution lead to acute inhibition of iron bioavailability when both elements are given together in fasting condition [17,18]. It has also been reported that, inhibition of iron absorption by zinc depends on the ratio of these two elements in the solution[19,20]. Within the normal range of dietary intakes (ratio 1:2 to 2:1), no significant interaction was observed. Adverse effects of Zn supplementation on Fe status have been demonstrated in rat pups. Pups supplemented with high Zn supplementation showed lower haemoglobin concentration [21]. A lack of iron and zinc increases the risk of infections, as iron-deficiency anaemia dampens immunity and zinc plays a role in immune function [22]. It is reported that, even mild to moderate zinc

deficiency can impair killer-cell activity that helps the immune system fight off infections and illnesses[23-27].

In contrast to the Sudanese patients, all Dutch patients with iron deficiency anaemia had normal zinc levels. All of them had a normal diet, that did not show deficiencies of micronutrients. In some cases of iron deficiency anaemia it is not easy to attribute iron deficiency to gastro-intestinal blood loss and then to decide to perform an endoscopy. Normal zinc levels may be helpful to exclude a nutritional deficiency.

In conclusion Sudanese patients in contrast to Dutch patients with iron deficiency anaemia also have a zinc deficiency reflecting a lack of both micronutrients in their food. When supplementation is considered the focus should not only be on iron but also on other micronutrients including zinc.

9.5 References

[1]. World Health Organization, The global prevalence of anaemia in 2011. ISBN 978 92040156496 0 . WHO, Geneva (2015).

[2] N. Akhtar, R. Ahsan, M. d. Abdul Wakil, C G M Mostafa, S. Chowdhury, M A Hai. Relationship Between Zinc and Anaemia in Chronic Haemodialysis Patients. TAJ, (2003), 16 (1), 12-14.

[3] O. Loréal, T. Cavey, E. Bardou-Jacquet, P. Guggenbuhl, M. Ropert and P. Brissot. Iron, hepcidin, and the metal connection. Frontiers in Pharm. | Drug Metab.Transp. (2014) 5, 1-10.

[4] Food and Agriculture Organization of the united State (FAO), Nutrition country profiles: Sudan summary, (2010). http://www.fao.org/ag/agn/nutrition/sdn_en.stm

[5] H. H. Mahmoud, A. M. Muddathir, S. E. M. Osman, M. A. AlKhawad, Awadia K. Mohamed, ElHady E. ElMubarak, Mamoun M. Mohamed. Iron Deficiency Anemia among Children under Three years in Kassala, Eastern Sudan. Sudanese J. Publ. Health, (2014), 9 (1), 33-37

[6] M. D Hussein. and S. Mohamed. Prevalence of anaemia in preschool children in Karma Albalad area, Northern State, Sudan. East Mediterr. Health J, 20(1), (2014), 33-8.

[7] F. M. Osman. Iron deficiency anaemia in pre-school children (1-6 years) in Al Shigia area (Khartoum State): Prevalence and Aetiology. PhD thesis (1999). University of Khartoum.

[8] E. M. Elgaili, H. Y. Zaki, B. E. Abdalla, E. Mohd Elhassan. Iron status during pregnancy in Gezira central Sudan. Sudanese J. Publ. Health, (2013), 8 (1), 29-33.

[9] E. Tayrab, A. Hamid, H. M Idriss and G. Mahmoud. Serum Copper and Iron Status in Pregnant Women with Iron Deficiency Anemia. J. Physiobiochem. Metab. (2013), 2:2, 1-3.

[10] E. A. Abdelgader, T. A. Diab, A. A. Kordofani, S. E. Abdalla. Haemoglobnin level, RBCs Indices, and iron status in pregnant females in Sudan. Basic Res. J. Med. Clin. Sci. ISSN 2315-6864 Vol. 3(2), (2014), 8-13.

[11] I. Adama, A. H. Khamisb, M. I. Elbashirc. Prevalence and risk factors for anaemia in pregnant women of eastern Sudan. Transact. Royal Soc. Trop. Med.Hyg. (2005) 99, 739-743.

[12] N. Mubaraka, G. I. Gasimb, K. E. Khalafallac, N. I. Ali and I. Adama. Helicobacter pylori, anemia, iron deficiency and thrombocytopenia among pregnant women at Khartoum, Sudan. Trans. R. Soc. Trop. Med. Hyg. doi:10.1093/trstmh/tru044, (2014),

[13] M. M. Munsoor, M. O. Gibreel and M. El Saeed El Karsani. Nutritional Anemia Among Patients Referred to Hematology Laboratories in Port Sudan City. Clin. Med. J. 1, (2), (2015), 38-42.

[14] A. A. Hamad , A. D. Nugud , D. E. Arnot , H. A. Giha , A. A. Abdel-Muhsin , G. M.H. Satti ,T. G. Theander , A M. Creasey , Hamza A. Babiker and Dia-Eldin A. Elnaiem . A marked seasonality of malaria transmsission in two rural sites in eastern Sudan. Acta Tropica 83 (2002) 71–82.

[15] M. Blaauw, The holistic analysis of gamma-ray spectra in Instrumental Neutron Activation Analysis, PhD dissertation, Delft University of Technology. 1993, ISBN 90-73861-16-0.

[16] M. Nikolic, N. Nicoloc, L. Kostic, J. Pavlovic, P. Bosnic, N. Stevic, J. Savic, N. Hristov. The assessment of soil availability and wheat grain status of zinc and iron in Serbia: implications of human nutrition. Sci Total Environ 2016; 553: 141-148.

[17] M. Olivares, F. Pizarro, D. Gaitan, M. Ruz. Acute inhibition of iron absorption by zinc. Nutr. Res. 27 (2007) 279– 282.

[18] M. Olivares, F. Pizarro, M. Ruz and D. Lopez de Romana. Acute inhibition of iron bioavailability by zinc: studies in humans. Biometals (2012) 25:657–664.

[19] J.Peres, F. Bureau, D. Neuville, P. Arhan and D. Bougle. Inhibition of zinc absorption by iron depends on their ratio. J. Trace Elem. Med. Biol, 15, (2001), 237-241.

[20] M. Arredondo, R. Martinez, M. T. Nunez, M. Ruz and M. Olivares. Inhibition of iron and copper uptake by iron, copper and zinc. Biol. Res. (39), (2006), 95-102.

[21] S. L. Kelleher and B. Lonnerdal. Zinc Supplementation Reduces Iron Absorption through Age-Dependent Changes in Small Intestine Iron Transporter Expression in Suckling Rat Pups. J. Nutr. (2006), 1186-1191.

[22] S. J Fairweather- Tait. Iron-Zinc and calcium-Fe interactions in relation to Zn and Fe absorption. Proceed. Nutr. Soc.(1995), 54, 465-473.

[23] K. Schumann, T. Ettle, B. Szegner, B. Elsenhans and N. W. Solomons. On risks and benefits of iron supplementation recommendations for iron intake revisited. J.Trace Elem.Med. Biol. 21 (2007) 147–168.

[24] V. Kumar and V. P. Choudhry. Iron Deficiency and Infection. Ind.J. Pediatr. 77, (2010), 789-793.

[25] C. P. Wong, N. A. Rinaldi and E. Ho. Zinc deficiency enhanced inflammatory response by increasing immune cell activation and inducing IL6 promoter demethylation. Mol. Nutr. Food Res. 59, (2015), 991–999.

[26] H. Haase and L. Rink. The immune system and the impact of zinc during aging. Imm. Ageing 2009, 6:9. doi:10.1186/1742-4933-6-9.

[27] C. L. Keen. Zinc Deficiency and immune function. Annu. Rev. Nutr. (1990), 10, 415-31.

10 General Discussion

Iron is an essential trace element involved in many processes in the human body. Some disorders like iron deficiency anaemia and haemochromatosis directly result from changes in iron status, while on the other hand iron metabolism changes during illness. Since these adjustments in the iron handling of the body may have consequences for the clinical outcome and treatment of patients, a reliable and accurate test to measure iron concentrations and to study iron metabolism in normal and pathological conditions is required. Such a technique should not only be able to measure the total concentration of iron, but also isotopic ratios, because these are indispensable tools to study the absorption and distribution of iron. Ideally the technique should also provide adequate information when biological materials other than fluids (blood, urine), such as tissues and faeces, are analysed. Another advantageous property of the technique would be the ability to measure other elements involved in iron metabolism during the same experiment. Based on its specific characteristics Instrumental Neutron Activation Analysis (INAA) should meet these criteria. The goal of the research described in this thesis was to investigate and evaluate the use of INAA as a research tool to study parts of iron metabolism in men. INAA was applied to measure iron not only in blood and urine, but also in faeces, red blood cells and even in food and INAA outcomes were compared to those of other techniques such as inductively coupled plasma optical emission spectrometry (ICP-OES) for measuring total iron and multi-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) for measurement of iron isotopes.

10.1 Overview

After an introduction into iron metabolism and the description of currently available techniques to measure total iron and iron isotopes, the capability of INAA to measure accurately the iron stable isotopes ratio in biological material has been described. Since the methodology of using enriched stable iron for distinguishing supplemented iron from naturally present iron is based on the measurement of two neutron activation products of iron, ^{59}Fe and ^{54}Mn, the quality of the calibration was verified by measuring the ratio of the iron mass fractions for iron of natural isotopic composition in human whole blood (n=12) and the Standard Reference Material bovine liver (NIST 1577c) (n=3). Calculated on the basis of the 1099 keV peak of ^{59}Fe and the 835 keV of ^{54}Mn, this ratio should ideally be 1.00. The experiments resulted in an average value of 1.04±0.04 for the human whole blood, and

1.02±0.03) for the SRM (NIST 1577c) which values are in statistical agreement with the ideal value of the peak area ratios. The small bias may be attributed to small differences in the neutron energy distribution in the irradiation facility at the time of calibration and at the time of this experiment.

A clinical experiment was performed using the enriched ^{58}Fe stable isotope administered to patients suffering from anaemia, hemochromatosis as well as to healthy persons as a control group (Chapter 5). For this experiment a pharmaceutical formula of iron sulphate containing the enriched stable isotope ^{58}Fe had to be prepared. Blood samples were collected before and two weeks after administration of ^{58}Fe [1]. This experiment showed a $^{58}Fe/^{54}Fe$ ratio in blood of the anaemic patients as expected with a significant increase in ^{58}Fe after two weeks, while there was no change in the hemochromatosis group. A clear explanation for this lack of response in the hemochromatosis patients can not be given at this stage. The healthy volunteers showed no or only a slight increase. These data reflect the state of "iron hunger" in the deficient patients, which seems to exceed that of the hemochromatosis patients. The results may be influenced by dietary concentrations of iron, since there was no real control on the iron intake with food during the test apart from general recommendations. INAA proved to have enough sensitivity to measure the small change in ^{58}Fe concentrations in blood after supplementation. MC-ICP-MS was used as a comparison technique. Although an anion-exchange chromatography separation for Fe was done, still some 58Ni and 54Cr was detected in all samples using ICP-MS. After a correction for ^{58}Ni and ^{54}Cr , the $^{58}Fe/^{54}Fe$ ratio values were equivalent to the values that can be derived from the 59Fe/54Mn activity ratios as found by INAA.

The next step included a number of feasibility experiments relevant for clinical practice. Firstly, iron was measured with INAA in patients with various haematological disorders such as iron deficiency anaemia (IDA), anaemia due to chronic disease (ACD), thalassemia (TH) and hereditary haemochromatosis (HH). The relationship between the INAA results and standard measurements such as haemoglobin (Hb), transferrin, transferrin saturation and ferritin was analyzed. A strong positive correlation was found between iron and Hb (r =0.925; P value =0.00). It was also found that plasma is a better biomarker for changes in iron status than whole blood and erythrocytes. The plasma Fe ratio of the normal subjects (N) to iron deficient patients (ID), $\frac{N}{ID}$ was found to be 4.9, while this ratio in whole blood and erythrocytes was 2 and 1.6 respectively. A next experiment concerned the detectability of

changes in iron concentrations in blood, faeces and urine after various forms of supplementation. After the intake of three doses of the pharmaceutical $FeSO_4$ (975 mg total) orally during three days by healthy volunteers, no increase in iron concentrations in whole blood was found while a significant increase of Fe was demonstrable in faeces. Although whole blood may not be the best biomarker for changes in iron concentrations the uptake of iron in healthy individuals is too small to be detectable and most iron disappears with faeces. In another experiment iron was given intravenously (800-1000 mg iron isomaltoside) to patients with iron deficiency anaemia and urine was collected before and after iron administration. A significant excretion of iron could be demonstrated in all patients 12 hours after supplementation. Because this was the first experiment using liquid samples to be irradiated special adjustments had to be made and some optimisation of the technique is still needed.

In chapter 7 it has been described how iron concentrations were measured with INAA not only in whole blood, but also in plasma and erythrocytes of patients with iron deficiency anaemia, haemochromatosis and in healthy controls. As expected, iron concentrations are lower in anaemic patients (whole blood 306±17 $mg.L^{-1}$, plasma 0.5±0.3 $mg.L^{-1}$, erythrocytes 820±60 $mg.L^{-1}$) and higher in hemochromatosis patients (whole blood 500±30 $mg.L^{-1}$, plasma 2.5±1.5 $mg.L^{-1}$, erythrocytes 1040±60 $mg.L^{-1}$) than in the control subjects (whole blood 450±10 $mg.L^{-1}$, plasma 2.0±0.6 $mg.L^{-1}$, erythrocytes 960 ±30 $mg.L^{-1}$). The measured iron concentrations in blood components of the control subjects were in a good agreement with literature values. The conclusion of this experiment is that the sensitivity of INAA is adequate for measuring low concentrations of iron in blood and some of its components and thus offers an outlook for studies with enriched stable isotopes in clinical conditions.

In real life food is the main source of iron for the human body. Future mass balance studies may require the collection –via the double portion approach- of the dietary intake over a number of days, thus resulting in an amount of material that may be up to the kilogram range. Since the amount may be difficult and laborious to homogenize for obtaining a small, representative test portion, the methodology of large sample INAA was tested for measuring Fe in a large portion of food. Fe was measured both in an amount of approximately 1 kg freeze-dried food as well as in a moist product thereof (containing water). The latter was done to assess the quality of the results if a collected double portion would be analysed without any additional processing. Because of the absence of a quality control (‘trueness control’) of materials of such a large size, a (commercially available) porridge fine wheat grain was used

as a reference sample (assumed to be homogeneous in the Fe content). The real amount of iron in the fine wheat grain was measured using routine small sample INAA. The water content of the fresh food did not cause any problem during the irradiation such as swelling or excessive gas formation due to radiolysis. The results obtained for the moist sample were statistically equivalent to those found for the dried sample. In the analysis of the porridge a bias was observed between the large sample results and the small sample results of 10.3% (dry sample) and 9.3% (moisture sample) in comparison which could be attributed to an unforeseen calibration problem. The applicability of this large sample method was further illustrated by measurement of Fe in a real commercial microwave meal. It is concluded that LS-INAA represents an attractive approach for measurement of elements in mass balance studies. It can be directly applied without sample preparation for the input part (food) as well as for faeces (output) collected during 5-7 day in mass balance experiments.

Anaemia is a major health problem all over the world and approximately half of the causes of anaemia are due to iron (Fe) deficiency [3]. In developed countries most cases of iron deficiency can be attributed to a loss of iron while in underdeveloped nations a deficiency in the intake of micronutrients is the main cause of anaemia. In case of malnutrition a deficiency of other micronutrients such as zinc is very likely, and such a deficiency may therefore be helpful in the differential diagnosis of iron deficiency anaemia. The opportunity occurred for measuring both iron and zinc in blood of 26 individuals (22 with iron deficiency and 4 healthy persons) visiting the Al Gadarif Hospital in Sudan representing sub-Saharan Africa. Measurement results were compared with those of 21 individuals (17 anaemic and 4 healthy) visiting the Meander Medical Center, Amersfoort, the Netherlands representing western Europe. Iron and zinc were measured both with INAA in the Netherlands while iron levels of the Sudanese patients were also measured with ICP-OES in Sudan. In the blood of the Sudanese patients very low concentrations of both iron and zinc were measured, while the Dutch patients had low iron, but normal zinc levels in their blood. Even when matched for haemoglobin level, the Sudanese patients showed lower iron and zinc concentrations in blood. A significant positive correlation was observed between Fe and Zn in blood of the Sudanese subjects ($r = 0.654$). On the other hand there is no or a very week relation between Fe and Zn in blood of the Dutch group ($r = 0.08$). The low level of Fe and Zn for the Sudanese group is due to the poor dietary intake of these two nutrients in the local daily food containing mostly cereals. The combination of low zinc and iron levels do therefore suggest a nutritional cause of iron deficiency.

10.2 Final Remarks

The overall conclusion is, that the research described in this thesis demonstrates that INAA meets all the requirements as a research tool to study iron metabolism in man, both in normal and pathological situations utilizing isotopically enriched, neutron activable Fe isotopes. There are no commutability problems of the calibrator, the reference material and the real samples. INAA has high specificity and selectivity, close to 100 %, based on the individual characteristics of the induced radionuclide. There is no need to convert the sample into a solution prior to analysis and even large samples with inhomogeneous distribution of elements can be used. This applies to both dry and moist materials. This is especially of interest, because in clinical studies errors in collection and correct handling of material prior to delivery to the laboratory are easily made, The preparation of test portions, possible contamination and chemical steps prior to measurement are far more critical in MC-ICP-MS although the sensitivity and precision of this technique is superior to INAA and even shifts in isotope composition of an element can be measured. Because of the time consumption, the need of the availability of a research nuclear reactor, and the costs generated, INAA should be mainly considered for research purposes rather than for measurements related to e.g. diagnosis. In some situations, when a large series of the same material should be analyzed for a programmed set of elements, a more routine use can be considered. Although a number of the advantages of INAA in measuring iron also applies for other elements, the isotopic properties of those elements necessitate specific adjustments.

10.3 Future Research

The foundations for future research in iron metabolism using isotopically enriched, neutron activable Fe isotopes and INAA are now laid. Real mass balance studies using the isotopically enriched ^{58}Fe isotope are within reach since the supplemented Fe can be distinguished from the naturally present Fe. A baseline iron compartmental model, hypothesis driven, may be tested, both on a ‘macroscale’ – comparing the intake with e.g. the excretion and internal storage- and on a ‘microscale’ – comparing e.g. the various interactions in the blood compartments-. Some technical problems such as adequate measuring of iron in liquid samples as in urine have to be dealt with. Specific parts of iron metabolism can now be studied in more detail such as the absorption of iron in the gastro-intestinal tract in the presence of sepsis or inflammation as well as in disorders of this tract such as in celiac disease without the side effect of radiation intrinsically connected to the use of ^{59}Fe.

The microbiota, the ecological community of commensal, symbiotic and pathogenic microorganisms present in our gut, is considered to play an essential role in a number of disorders like diabetes mellitus, rheumatoid arthritis and multiple sclerosis and maybe in cancer. Iron orally administered may have a deleterious effect on this system and may promote the absorption of toxic elements. Element analysis with INAA of faeces, gut mucosa and blood may supply useful information for researchers of this system.

The metals Mn and Cu and non-metal Se are essential trace elements. They are found along with Fe in the period 4 of the periodic table, and the biological activities of the four elements are strongly associated with the presence of unpaired electrons that allow their participation in redox reactions [4]. The interaction of these trace elements with iron can be studied in more detail when enriched stable isotopes are used. Another element with a strong relationship to iron is zinc. It influences iron absorption and in case of iron deficiency a zinc ion is incorporated into protoporphyrin IX, the immediate precursor of heme. Enriched zinc isotopes are at disposal and await interest of clinical researchers.

In conclusion both INAA and the application of enriched stable isotopes, which can be adequately measured with INAA, have the potential to deliver a valuable contribution in research on the role of metals and trace elements in health and disease.

10.4 References

[1] Assessment of iron bioavailability in human using stable iron isotopes techniques. IAEA human heath series No. 21. International Atomic Energy Agency, Vienna, 2012.

[2] ISO 15194:2002. In vitro diagnostic medical devices– Measurement of quantities in samples of biological origin – Description of reference materials. Geneva, Switzerland: ISO; 2002.

[3] World Health Organization, The global prevalence of anaemia in 2011. ISBN 978 92040156496 0 . WHO, Geneva (2015).

[4] C. G. Fraga. Relevance, essentiality and toxicity of trace elements in human health. Mol. Asp. Med.. (2005), 26: 235–244.

Summary

The context for the research described in this thesis is to evaluate the applicability of INAA as a research tool to study parts of iron metabolism in man. Research described in this thesis was done in a collaboration between TU Delft and the Meander Medical Centre Amersfoort and was funded by the Netherlands Fellowship Programme (NFP). Other partner institutions in this project were the Al Gedarif Hospital, Sudan and the Reinier de Graaf Gasthuis, Delft, The Netherlands.

In Chapter 1 a short introduction is given on the role of iron in human metabolism and on the most prominent iron-related disorders. Research on such disorders requires methods not only for measuring the amount of iron in the body but also for tracing iron such as in case of supplementation. Tracing can be done with radioactive iron isotopes and/or enriched stable iron isotopes. The latter approach, preferred for several reasons, requires isotopic techniques such as ICP-MS and NAA. It is concluded that the current methodological opportunities of INAA have not fully been evaluated for clinical research. It is not unlikely that the use of enriched stable isotopes and measurement by INAA will not only increase our knowledge of the iron handling by the gastro-intestinal tract in pathological conditions resulting in a more effective medical treatment, but may also be of value for the preparation and use of adequate food and supplements in case of illness. These considerations form the basis of the objective of this research project, the evaluation of the applicability of INAA as a research tool to study parts of iron metabolism in man. . Iron is not only measured in blood and urine, but also in erythrocytes as well as in food making use of the advantage of INAA to measure in all sorts and quantities of material without complicated preparation steps. Apart from that a comparison was mas made between INAA and ICP-MS with regard to the practical aspects of studying iron metabolism in a clinical setting.

At the end of the chapter the various research questions are identified, which are dealt with in the other chapters of the thesis.

More details on iron metabolism and iron related disorders are given in chapter 2 . This chapter also discusses the current diagnostic steps and treatment modalities of iron related disorders. The synergetic and antagonistic effects of copper and zinc to iron homeostasis are described. It is concluded that in recent years, substantial progress has been made in our

knowledge of iron metabolism. Some of the key players have been identified and it has become clear that the intestinal absorption of this element is crucial for maintaining homeostasis. However, many questions remain concerning the best strategy for an adequate supplementation not only of iron but also of copper and zinc in case of iron deficiency.

Chapter 3 provides an overview of the use of iron isotopes to study iron bioavailability in humans. This can be done by either the faecal recovery method, the plasma appearance method or the erythrocytes iron incorporation method. The basic concepts of administration of isotopically enriched stable isotopes are described in this chapter, including their chemical form, dose selection and distinction between intrinsic versus extrinsic labelling.

Chapter 4 provides an overview of the methodological principles of the basic two analytical methods for measuring iron stable isotopes, INAA and ICP-MS. Analytical characteristics including specificity, selectivity, degree of accuracy, uncertainty of measurement and minimum detectable amounts have been evaluated together with practical aspects in using these techniques such as the different requirements to the preparation of the test portion and to its size, method-related interferences during measurement, calibration approaches, contamination and turnaround time and costs. It is concluded that INAA has a number of properties making it the preferred method to measure iron in some clinical research settings.

Chapter 5 presents a practical comparison of INAA and MC-ICP-MS for the measurement of the enriched stable isotope ^{58}Fe in blood samples of patients with iron related disorders. A time-consuming anion- exchange chromatography was done to avoid in the ICP-MS measurement ^{58}Ni and ^{54}Cr interferences with ^{58}Fe and ^{54}Fe respectively. Excellent agreement was observed between the ICP-MS and NAA measurement results for the ratio of ^{58}Fe and ^{54}Fe, the latter isotope measured using ^{54}Mn in INAA. Both INAA and MC-ICP-MS are able to measure changes in iron isotope composition in blood when an enriched stable iron isotope is applied in clinical research. The critical and most time consuming part of MC-ICP-MS concerns the preparation of materials prior to measuring. Whereas ICP-MS has a better sensitivity for measurement of iron isotopes, the advantages of INAA to ICP-MS lie with the less time consuming sample preparation step, and the much less significant dependency on commutability of calibrators and chemical matrix effects , and absence of a chemical yield assessment.

Chapter 6 summarises a number of feasibility experiments relevant for clinical practice. Total Fe was measured in blood and blood compartments, faeces and urine using INAA, before and

after iron supplementation orally and intravenously in patients suffering from iron deficiency anaemia and in healthy volunteers. In addition, other iron-related indicators such as ferritin, transferrin, haemoglobin were measured. The iron mass fractions in blood were matching the haemoglobin levels. Also the effect of a pulse dose of orally administered Fe-sulphate to healthy volunteers was studied. It was observed that such a pulse dose, actually an overdose, does not result in a measurable increase in whole blood iron concentration; however a significant increase was measurable in the faecal excretion. The feasibility of measuring iron in urine after an intravenous pulse dose of iron isomaltoside to anaemic patients was also studied . To this end, a new method for measuring large quantities of liquid urine by INAA was developed. The results of this experiment learned that in the urine of most patients only very small amounts of iron could be measured before administration with a significant increase after administration. This indicates that a part of the injected iron gets lost in urine shortly after administration.

These feasibility studies show that INAA is able to detect small changes in iron concentrations in the various biomaterials and is therefore an adequate instrument in case mass balance studies with iron are applied.

Chapter 7 presents the measurement of iron using INAA and ICP-OES in whole blood and blood compartments (plasma and erythrocytes) in anaemic, hemochromatosis and healthy persons. The results obtained by the two techniques are in excellent agreement and also in a good agreement with literature values. The present study shows INAA's capability to measure iron in these compartments and, as expected, low iron concentrations in total blood, plasma and erythrocytes were found in patients with iron deficiency anaemia and high iron concentrations in all compartments in hemochromatosis patients.

Chapter 8 demonstrates how large sample INAA can be used for measuring the total iron content of daily (or even multi-daily) dietary intake without the need for homogenization and sub-sampling associated with the use of conventional analytical techniques. This was demonstrated by analysis of a commercially available microwave meal. In the absence of large amounts of certified reference materials for trueness control, the degree of trueness was assessed by large and small sample analysis of a wheat powder, assumed to be homogeneous for its iron content. Moreover, this was tested for samples with and without drying prior to irradiation. It was found that iron can be measured accurately and with acceptable trueness in kilogram amounts of food even if it is fresh and moist, and that freeze drying may not be

necessary. It is concluded that LS-INAA represents an attractive approach for measurement of element content of dietary intake collected by double portion technique. It can be directly applied without sample preparation for the input part (food) as well as for faeces (output) collected during 5-7 day in mass balance experiments.

Chapter 9 describes a study in which both zinc and iron were measured in blood of Sudanese and Dutch anaemic patients using INAA. ICP-OES was used as a comparison technique. Zn levels were found to be in the normal range in the blood of all Dutch anaemic patients but low to very low in the blood of the majority of the Sudanese patients. This can be attributed to the fact that in most cases iron deficiency in Dutch patients is due to blood loss, while in Sudanese patients it is due to a lack of zinc in food. This can be explained for the most part by a lack of red meat in the Sudanese diet. On the other hand, the basic content of their food are cereals, which usually contain zinc. This may indicate , as has been shown in other countries, that either the soil or the bread consumed is rather deficient of zinc.

Normal zinc levels may therefore be helpful to exclude a nutritional deficiency in the analysis of iron deficiency anaemia.

Chapter 10 provides an overview of all studies done, highlights the most significant results, and some provides suggestions for future research.

Samenvatting

Het doel van het in dit proefschrift beschreven onderzoek is het vaststellen van hoe INAA kan bijdragen in onderzoek naar een deel van de ijzer stofwisseling bij de mens. Het onderzoek is uitgevoerd in een samenwerking van de TU Delft en het Meander Medisch Centrum in Amersfoort, en is financieel ondersteund door het Nederlands Fellowship Programma (NFP). Andere instellingen die bij dit onderzoek betrokken waren het Al Gedarif Hospital in Sudan, en het Reinier de Graaf Medisch Centrum in Delft.

Een korte inleiding over de rol van ijzer in de stofwisseling bij de mens, en de met ijzer gerelateerde aandoeningen wordt gegeven in Hoofdstuk 1. Voor onderzoek van deze ijzer gerelateerde aandoeningen zijn niet alleen metingen nodig van de hoeveelheid ijzer in het lichaam maar moet ijzer ook kunnen worden gevolgd als het via voeding of een supplement wordt toegediend. Dit volgen van ijzer geschiedt door gebruik te maken van zowel radioactieve als isotopisch verrijkte stabiele isotopen. Die laatste categorie heeft, om verschillende redenen, de algemene voorkeur, maar vereist meettechnieken die isotoop-specifiek zijn, zoals ICP-MS en NAA. Het is gebleken dat de huidige analytische mogelijkheden van INAA voor gebruik in klinisch onderzoek nauwelijks zijn onderzocht. Het is aannemelijk dat het gebruik van isotopisch verrijkte stabiele isotopen en INAA leidt tot een verruiming van de kennis van het gedrag van ijzer in het maag-darm kanaal, en daardoor tot een meer effectieve medische behandeling betrekking hebbende op voeding en het toepassen van supplementen. Die overweging leidt tot het doel van het onderzoek, het vaststellen van hoe INAA kan bijdragen in onderzoek naar een deel van de ijzer stofwisseling bij de mens. Hiervoor moet onder meer worden nagegaan wat de verschillen zijn in het gebruik van INAA en ICP-MS voor metingen die voortkomen uit vragen in klinisch onderzoek. Verder moet onderzocht worden of met INAA ijzer gemeten kan worden niet alleen in bloed en urine, maar ook in de rode bloedlichaampjes en in de voeding. Daarbij zou optimaal gebruik gemaakt kunnen worden van de eigenschap van INAA dat er geen ingewikkelde monster voorbereiding nodig is, en dat de techniek kan worden gebruikt voor verschillende monstermassa's en type materialen. Aan het eind van Hoofdstuk 1wordt een overzicht gegeven van de verschillende onderdelen van het onderzoek dat in het proefschrift wordt beschreven.

In Hoofdstuk 2 wordt dieper ingegaan op de ijzer stofwisseling en de aan ijzer gerelateerde aandoeningen. De momenteel toegepaste diagnostiek en behandelingswijzen worden beschreven. Ook wordt aangegeven dat de zelfregulatie van het lichaam voor ijzer versterkt of verzwakt kan worden door de elementen koper en zink. Er is de laatste jaren veel vooruitgang geboekt in de kennis van de ijzer stofwisseling, veel van de belangrijkste factoren die daarbij een rol spelen zijn gevonden, en het is nu bekend dat de opname van ijzer door de darm van wezenlijk belang is voor de zelfregulatie door het lichaam. Desalniettemin blijven er vragen over m.b.t. de juiste wijze van suppletie van ijzer, maar ook van koper en zink, bij ijzer gerelateerde aandoeningen.

In Hoofdstuk 3 wordt een overzicht gegeven van de ijzerisotopen die gebruikt kunnen worden voor onderzoek naar bijvoorbeeld de biologische beschikbaarheid van ijzer. Dat kan door het meten van de hoeveelheid ijzer in de feces, in het bloedplasma of in de rode bloedlichaampjes. Verder worden de basis beginselen van het toedienen van verrijkte stabiele isotopen beschreven, zoals de chemische vorm, de keuze van de toegediende hoeveelheid en het verschil tussen intrinsieke en extrinsieke labeling.

De beginselen van de twee analytische technieken voor isotoop-specifieke metingen, INAA en ICP-MS worden beschreven in Hoofdstuk 4. De analytische kenmerken voor het meten van ijzer zijn naast elkaar gezet, zoals specificiteit, selectiviteit, mate van nauwkeurigheid, meetonzekerheid en de kleinst mogelijke hoeveelheid die gemeten kan worden. Daarnaast zijn er een aantal praktische aspecten van de twee technieken onderzocht, zoals de voorbereiding van het genomen monster tot de uiteindelijke test-portie en de grootte daarvan, storingen tijdens de meting, kalibraties, besmetting, analyse tijd en kosten. Op basis van een aantal overwegingen is vastgesteld dat INAA de voorkeur verdient voor de meting van ijzer bij bepaald klinisch onderzoek.

In Hoofdstuk 5 wordt beschreven hoe INAA en MC-ICP-MS beiden zijn gebruikt om isotopisch verrijkt ^{58}Fe te meten in bloed van patiënten met ijzer-gerelateerde aandoeningen. Een tijdrovende anion-uitwisselings chromatogafie moest worden toegepast om de storing door ^{58}Ni en ^{54}Cr in de meting van respectievelijk ^{58}Fe en ^{54}Fe door ICP-MS te minimaliseren. De resultaten van INAA en ICP-MS metingen zijn verwerkt tot de isotoop verhouding van ^{58}Fe en ^{54}Fe, en stemden bijzonder goed overeen (bij INAA door ^{58}Fe op basis van het gemeten ^{59}Fe en ^{54}Fe op basis van het gemeten ^{54}Mn). De kritische en tijdrovende stap bij de MC-ICP-MS metingen is de bereiding van de test-portie. De gevoeligheid van ICP-MS is

beter dan van INAA voor de meting van ijzer, maar de voordelen van INAA zijn de veel eenvoudigere en snellere test-portie bereiding, de veel mindere afhankelijkheid van 'commutability' van kalibratiestandaarden en chemische matrix effecten en het ontbreken van de noodzaak tot meting van de chemische opbrengst.

In Hoofdstuk 6 wordt beschreven hoe een aantal experimenten zijn uitgevoerd om de haalbaarheid van INAA vast te stellen voor metingen, zoals deze bij klinisch onderzoek voorkomen. Het totaal gehalte aan ijzer is gemeten met INAA in bloed en bloed bestanddelen voor en na toediening van een ijzer houdend supplement dat zowel oraal als intraveneus is toegediend aan patiënten met ijzer-gerelateerde aandoeningen, en gezonde vrijwilligers. Daarnaast zijn er door het Meander Medisch Centrum nog een aantal andere indicatoren voor het ijzer gehalte gemeten, zoals ferritine, transferrine en hemoglobine. De massa fractie van ijzer in bloed is in overeenstemming met het hemoglobine gehalte. Ook is nagegaan of een pulse-dosering met aan gezonde vrijwilligers oraal toegediend ijzersulfaat –in feite een overdosis- kan worden waargenomen. Gebleken is dat dit niet leidt tot een verhoging van het ijzer gehalte in het bloed; wel is een verhoging in het ijzergehalte in de feces gemeten. Op zelfde wijze is onderzocht of ijzerverlies in de urine optreedt na een intraveneuze puls dosering met ijzer isomaltoside bij patiënten lijdend aan bloedarmoede (anemie) . Daarvoor is een nieuwe methode ontwikkeld voor het meten met INAA van ijzer in een relatief grote hoeveelheid urine. Dit onderzoek heeft uitgewezen dat in de urine van de meeste patiënten zeer kleine hoeveelheden ijzer aanwezig zijn voor de toediening, en dat die hoeveelheid aanzienlijk toenam na de toediening. Deze haalbaarheidsmetingen hebben aangetoond dat het mogelijk is om met INAA kleine veranderingen in de ijzer gehalten te meten in verschillende biomaterialen en dat de techniek geschikt is voor gebruik bij een onderzoek naar de massa balans van ijzer.

De meting van ijzer met INAA en ICP-OES in bloed en bloedbestanddelen (plasma en rode bloedlichaampjes) bij patiënten lijdend aan bloedarmoede en ijzerstapeling, en bij gezonde vrijwilligers wordt beschreven in Hoofdstuk 7. De resultaten van de twee technieken komen uitstekend met elkaar overeen. Dit onderzoek onderstreept INAA's kwaliteiten voor meting van zeer lage ijzergehalten in bloed en bloedbestanddelen bij patiënten met een ijzer tekort, en van veel hogere ijzergehalten bij patiënten lijdend aan ijzerstapeling.

In Hoofdstuk 8 wordt beschreven hoe INAA kan worden toegepast voor meting van ijzer in grote hoeveelheden materiaal, zoals aan de orde is indien de dagelijkse (of meerdaagse)

inname vanuit de voeding moet worden vastgesteld door middel van de duplicaat portie methode. Het is daarbij niet nodig om die duplicaat portie te homogeniseren om daarvan een representatief klein deelmonster te nemen. Dit is aangetoond door analyse van een commercieel verkrijgbaar magnetron maaltijd, die in zijn geheel werd gemeten. Er bestaan geen gecertifieerde referentiematerialen van die afmetingen om de juistheid te controleren. Daarom is de juistheid geverifieerd door analyse van een groot en een klein monster (met normaal INAA) van tarwebloem waarvan is aangenomen dat het ijzer daar homogeen in aanwezig is. Bovendien is er zowel een groot monster droog tarwebloem als een groot monster pap van dit tarwebloem geanalyseerd. Vastgesteld is dat ijzer nauwkeurig gemeten kan worden en dat de juistheid adequaat is in zowel droge als vocht bevattende monsters van voeding, zodat (vries)drogen niet nodig is. Dit onderzoek leidt tot de conclusie dat het analyseren van grote monsters met INAA aantrekkelijke kansen biedt voor meting van de (meer)daagse element inname door voeding bij toepassing van de duplicaat portie methode. Er is geen noodzaak om enige verdere voorbereiding aan het duplicaat te doen. Het onderzoek toont ook aan dat grote monster INAA evenzogoed kan worden uitgevoerd aan de totaal hoeveelheid feces die tijdens zo'n meerdaags duplicaat portie methode is verzameld.

In Hoofdstuk 9 wordt een onderzoek beschreven waarbij zink en ijzer zijn gemeten met INAA en ICP-OES in bloed van patiënten met bloedarmoede ut Sudan en Nederland. Het zink gehalte in het bloed van de patiënten uit Nederland lag binnen het gebied van de normaalwaarden, maar het zink gehalte in het bloed van de patiënten uit Sudan was (zeer) laag. De bloedarmoede bij de patiënten uit Nederland komt voornamelijk door bloedverlies maar bij de patiënten uit Sudan door een tekort aan ijzer in de voeding. Dit kan voor een deel verklaard worden door het ontbreken van rood vlees in het dagelijks dieet, maar kennelijk bevat het hoofdbestanddeel van dit dieet, granen, geen ijzer en nauwelijks zink. Daarom kan niet worden uitgesloten dat, wat ook in andere landen is aangetoond, dat de bodem in een deel van Sudan erg zink arm is. Bij de analyse van een ijzergebreksanemie kan een laag zink gehalte wijzen op een nutritionele oorzaak van het ijzergebrek. In Hoofdstuk 10 worden nog eens de belangrijkste resultaten van dit onderzoek op een rijtje gezet, en worden aanbevelingen gedaan voor verder onderzoek.

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Curriculum Vitae

Tayser Ismail Yagob Mohamed was born on 1st January 1977 in Elgadarif, Sudan. She studied Science at the University of Khartoum, Sudan and later she obtained a Master degree in Chemistry from the same university with a thesis titled "Determination of Some Trace Elements in Elsaraf Dam area (Sudan) using AAS & XRF".

She started her work career in 2004 as a researcher at Sudan Atomic Energy Commission (SAEC) at Institute of Chemistry and Nuclear Physics until our days. She has being actively participating in the execution of technical cooperation projects supported by the IAEA and Arab Atomic Energy Agency in the field of nuclear analytical chemistry. Her contribution was very instrumental in the progress of those projects which was highly appreciated by both local and international partners.

In 2012 she started her PhD research project at Department of Radiation Science & Technology (RST) of the Delft University of Technology, where her main research area was trace elements, neutron activation analysis and use of enriched stable isotopes. The results of her research are presented in this booklet.

List of publication

Journal articles

T. Yagob, A. van de Wiel, P. Bode and H. T. Wolterbeek. Iron deficient anaemia in Sudan may lack nutritional zinc status. Inter. J. Vit. Nutr. Res. Submitted.

T. Yagob, P. Bode, A. van de Wiel and H. T. Wolterbeek. Dietary intake assessment of iron without subsampling of double portion using Large Sample Neutron Activation Analysis. Inter. J. Vit. Nutr. Res. Submitted.

T. Yagob, P. Bode, A van de Wiel, B. van der Wagt, P. Krystek, H. T Wolterbeek. Measurement of the enriched stable isotope ^{58}Fe in blood samples of patients with iron related disorders using INAA and MC-ICP-MS. To be submitted.

T. Yagob, A. van de Wiel, P. Bode and H. T. Wolterbeek. Monitoring iron status in various human biomaterials using instrumental Neutron Activation Analysis. To be submitted.

Journal abstract

T. Yagob, A. van de Wiel, R. Kraaijenhagen, P. Bode and H. T. Wolterbeek (2015). Iron concentrations in blood compartments in anaemic and hemochromatosis patients using neutron activation analysis (NAA). J. Trace Elem. Med. Biol., 32 S1-S42.

T. Yagob1, A. van de Wiel, R. Kraaijenhagen, P. Bode and H. T. Wolterbeek (2013). Stable neutron-activable isotopes to study the efficacy of Fe supplementation in individuals with iron disorders. J. Trace Elem. Med. Biol., 27 S1-S50.

Conferences

T. Yagob, A. van de Wiel, R. Kraaijenhagen, P. Bode and H. T. Wolterbeek. Iron concentrations in blood compartments in anaemic and hemochromatosis patients using neutron activation analysis (NAA). ISTERH 2015, conference on trace elements research on health and diseases. Dubrovnik, Croatia, 18-22 Okt. 2015.

T. Yagob, P. Bode, A. van de Wiel and H. T. Wolterbeek. Measurement of Iron in Food using Large Sample NAA. 14th International Conference on Modern Trends in Activation Analysis

& 11th International Conference on Nuclear Analytical Methods in the Life Sciences. Delft, The Netherlands, 23 - 28 August 2015.

T. Yagob, A. van de Wiel, R. Kraaijenhagen, P. Bode and H. T. Wolterbeek. Stable neutron-activable isotopes to study the efficacy of Fe supplementation in individuals with iron disorders. ISTERH 2013, conference on trace elements research on health and diseases. Tokyo, Japan, 18-22 Nov. 2013.

T. Yagob and S. A. Khalid. Element Content of Four Medicinal Plants Commonly Used in the Sudanese Traditional Medicine. The 5th African Conference on Research Reactors Utilization "Role of Research Reactors in Nutrition, Environment And Health". Cairo, Egypt, 2-3 December 2007.

T. Yagob and Tag Elsir A. Ahmed. Determination of Some Trace Elements in Elsaraf Dam area (Sudan) using AAS & XRF. Eighth Arab Conference on the Peaceful Uses of Atomic Energy. Amman, Jordan, 3-7 December 2006.

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