Prediction of the bioavailability of minerals and trace elements in foods

The influence of dietary fibre, phytic acid and other food components on the in vitro availability of calcium, magnesium, iron, copper and zinc

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aan mijn ouders

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Part I

Introduction

CHAPTER 1

Scope and objectives of the study

Scope

The dietary pattern of people in developing countries is quite different from that of people in Western countries (1). In developing countries, the consumption of unrefined carbohydrates and fats is high (ca. 82%) and the intake of refined carbohydrates and fats is low (ca. 8%). In contrast, in Western countries the consumption of refined carbohydrates and fats is high (ca. 58%) and the intake of unrefined carbohydrates and fats is low (ca. 32%). This difference in dietary pattern is strongly related to the higher prosperity in Western countries (1).

Refining of food products generally results in removal of fibre-rich components. Therefore, the shift from unrefined to refined food products has led to a substantial decrease in the consumption of dietary fibre.
Because dietary fibre has no direct nutritional value, it has long been believed to be a useless food component. People believed that refining increased the quality of food products. Only recently, Burkitt and Trowell (2,3,4,5,6,7,8,9) recognized dietary fibre as a food component with important physiological properties. In their "fibre hypothesis", they postulate that the consumption of unrefined, high-fibre carbohydrates protects against many Western diseases such as ischaemic heart disease, diverticular disease, colon cancer, diabetes mellitus, appendicitis, gallstones, constipation, haemorrhoids, hiatus hernia and obesity. The protective effects of dietary fibre on some diseases are now well documented, while the effects on other diseases are still being investigated (10,11,12).
The protective health effect of fibre-rich foods is an important motive for stimulating the consumption of dietary fibre. In many Western countries this has led to governmental recommendations or guidelines concerning fibre intake (13,14,15,16).

An increased consumption of unrefined food products, however, may also have adverse implications for health. Unrefined food products not only have higher contents of dietary fibre, but also higher contents of phytic acid. High dietary levels of phytic acid and dietary fibre have been shown to decrease the degradation of starch, proteins and lipids (10,11,17,18,19,20,21). Although this may have some positive consequences for human nutrition (management of diabetes and hyperlipidaemia), this decreased degradation is undesirable in animal nutrition, as regards feed efficiency, growth rate and production of manure.

The consumption of fibre-rich foods is also associated with a decreased bioavailability of minerals and trace elements (22). Some scientists attribute this negative effect to dietary fibre (10,11,23,24,25), others to phytic acid (26,27,28,29). The relative contribution of dietary fibre and phytic acid to the decreased bioavailability of minerals and trace elements is still a matter of dispute.

Minerals and trace elements play essential roles in biological and biochemical processes in animals and man. A deficiency, an overdose or an infavourable ratio between minerals or trace elements will exert a negative effect on health (30,31,32,33,34,35,36). In general, it is not the ingested dose of minerals and trace elements that is important to maintain balance, but rather the amount that is bioavailable (available for biological and biochemical processes in the organism). Several food components may influence this bioavailability positively or negatively by influencing the availability for absorption in the small intestine.

The bioavailability of minerals and trace elements can be determined in vivo and in vitro. A major advantage of in vivo experiments is the fact that the experiment can be carried out with the target species (e.g. man or pig). Drawbacks of in vivo experiments are the long duration of the experiments required to obtain reliable results and the high costs. Moreover, the variation from species to species and from individual to individual is often substantial and thus hampers the interpretation of results. In vitro experiments, on the other hand, have as advantages their speed of analysis, low costs and potentially well controlled experimental conditions. However, in vitro systems are, by necessity, an oversimplification of the real conditions in a biological system such as man or pig. The correlation of in vitro results with results from in vivo experiments, which is essential, is often not demonstrated.

Because of the advantages and drawbacks of the in vitro and in vivo methods described above, there is a great need in the science and practice of human nutrition and animal nutrition for an in vitro method that predicts the bioavailability of minerals and trace elements in vivo.

Objectives

The objectives of the study presented in this thesis are:

- to develop an in vitro method for the prediction of the bioavailability of calcium, magnesium, iron, copper and zinc;
- to investigate the effects of dietary fibre, phytic acid and other food components on the in vitro availability of calcium, magnesium, iron, copper and zinc.

CHAPTER 2

Experimental approach of the study

The objectives described in Chapter 1 ask for a multidisciplinary approach. The experimental procedures can be divided into four main activities, each including several subactivities:

A. Investigation of methods for analysis of dietary fibre and phytic acid.

- Comparison of methods of analysis for dietary fibre.
  In general, it is rather difficult to evaluate the merits of methods of analysis for dietary fibre because it is not quite clear which fibre components are analysed in each of these methods. Therefore, several methods for the analysis of dietary fibre were compared with respect to the amount and the composition of the dietary fibre analysed.
- Development of a method for the analysis of phytic acid.
  Many methods for the determination of phytic acid are not specific (interference from lower inositol phosphates, metal ions, etc.). Therefore, a specific liquid chromatographic method for the analysis of phytic acid was developed.

B. Development of in vitro methods for the prediction of the bioavailability of minerals and trace elements.

- Development of an in vitro method based on equilibrium dialysis.
  Miller (37) as well as Hazell and Johnson (38,39) have described an in vitro method based on equilibrium dialysis for the estimation of the bioavailability of iron. Promising correlations between in vitro and in vivo availability of iron and zinc have been reported (38,39,40,41,42,43,44). The in vitro method based on equilibrium dialysis was designed, modified and used for the determination of the bioavailability of calcium, magnesium, iron, copper and zinc.
- Development of an in vitro method based on continuous dialysis.
  In an in vitro method based on equilibrium dialysis, degradation products of peptic and pancreatic digestion may influence the dialysis of minerals and trace elements. Therefore, an in vitro method was developed in which dialysable components (including minerals and trace elements) are continuously removed, corresponding to the situation in vivo.
- Comparison of in vitro methods.
  The in vitro method based on equilibrium dialysis and the in vitro method based on continuous dialysis were compared with respect to the availability of calcium, magnesium, iron, copper and zinc from food products.

C. Validation of the use of in vitro methods for the prediction of the bioavailability of calcium, magnesium, iron, copper and zinc.

- The availability of calcium, magnesium, iron, copper and zinc was determined with the in vitro method based on equilibrium dialysis, the in vitro method based on continuous dialysis and in an in vivo experiment with piglets. The in vitro results were compared with in vivo results.

  This study was combined with an in vivo experiment carried out at the TNO department of Animal Nutrition and Physiology. Pig feed may contain a substantial proportion of *Vicia faba* beans. Because these beans have high contents of tannins, one of the purposes of the in vivo experiment was to investigate the influence of tannins on the bioavailability of minerals and trace elements for piglets.

D. Investigation, description and prediction of the influence of phytic acid, dietary fibre and other food components on the in vitro availability of calcium, magnesium, iron, copper and zinc.

- Investigation of the influence of food components on the in vitro availability of calcium, magnesium, iron, copper and zinc.

  A large number of food products containing phytic acid and/or dietary fibre (cereal products, nuts, vegetables and fruits) were selected and analysed for their contents of phytic acid, dietary fibre components and other food components that may influence the bioavailability. The bioavailability of calcium, magnesium, iron, copper and zinc was determined in vitro. The relation between the contents of certain food components and the in vitro availability of minerals and trace elements was studied.
- Development of a mathematical model for the description and prediction of the influence of food components on the in vitro availability of calcium, magnesium, iron, copper and zinc.

  The relative influences of food components on the bioavailability of minerals and trace elements are difficult to assess because several food components affect the bioavailability simultaneously. Therefore, a mathematical model was developed that describes the influence of food components on the in vitro availability of calcium, magnesium, iron, copper and zinc. In addition, it was investigated whether the in vitro availability of these minerals and trace elements could be predicted by means of this mathematical model.

The results of these activities are described in this thesis. Part II concerns dietary fibre. Brief literature reviews concerning definitions and terminology, and methods for the analysis of dietary fibre are presented in Chapter 3 and 4 respectively. In Chapter 5, several methods for the analysis of dietary fibre are compared. Part III concerns phytic acid. After a brief review of the literature on methods of analysis (Chapter 6), an improved method for the analysis of phytic acid is described in Chapter 7. Part IV concerns in vitro methods for the prediction of the bioavailability of minerals and trace elements. In Chapter 8, a brief literature review is presented concerning in vitro methods for the prediction of the bioavailability of minerals and trace elements in foods and their correlation with in vivo results. A continuous in vitro method for the estimation of the bioavailability of minerals and trace elements is described in Chapter 9. The validation of the use of two in vitro methods for prediction of the bioavailability of minerals and trace elements is discussed in Chapter 10. Part V concerns the influence of dietary components on the bioavailability of calcium, magnesium, iron, copper and zinc. A brief survey of the literature on this topic is presented in Chapter 11. In Chapter 12, the development and use of a mathematical model to investigate the relation between the in vitro availability and the composition of food products is discussed. Part VI concerns a discussion of the results (Chapter 13) and a summary of the conclusions (Chapter 14).

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Part II

Dietary fibre

CHAPTER 3

Definitions and terminology of dietary fibre (Review)

Introduction

Historically, dietary fibre has been seen as inert and indigestible material with negative effects on the nutritional value of human food and animal feed. Today, substantial evidence exists that dietary fibre affects digestive physiology in a positive way. This has led to an increased interest in dietary fibre.

In the past years several definitions and terminologies of dietary fibre have been developed. The definitions of dietary fibre can be divided into three types (1):

- botanical definitions
- physiological definitions
- chemical definitions

The definitions and related terminologies of dietary fibre are reviewed below.

Botanical definitions of dietary fibre

The botanical term "fibre" (plant cell wall material) came into usage in relation to the characterization of animal forage in the nineteenth century. A crude method of analysis was used to measure a residue called "crude fibre". Crude fibre thus analysed consists largely of cellulose and lignin. In 1929, McCance and Lawrence (2) reported the defectiveness of the term "crude fibre" and suggested the terms "available" and "unavailable" carbohydrates. In their concept, unavailable carbohydrates consist of cellulose and hemicelluloses.

Physiological definitions of dietary fibre

In 1935, Williams and Olmstedt (3) introduced the physiological term "indigestible residue" for the vegetable material not attacked by digestive enzymes in the mammalian gut. In 1953, Hipsley (4) was the first to use the term "dietary fibre" and defined it as including lignin, cellulose and hemicelluloses. In 1972, Trowell (5,6) suggested a new definition of dietary fibre to be based on physiological considerations. Dietary fibre was defined as "the remnants of plant cells that are resistant to hydrolysis by the alimentary enzymes of man". The definition constituted cellulose, hemicellulose, lignin and uronic acids. Because the definition was restricted to the undigested remnants of plant cell walls, Trowell et al. (7) extended the definition to include "all plant polysaccharides and lignin that are resistant to hydrolysis by the digestive enzymes of man". This new definition constituted not only cellulose, hemicellulose, lignin and uronic acids, but also indigestible storage polysaccharides, algal polysaccharides and mucilages. According to Southgate (8), the inclusion of these polysaccharides is justified because they have a similar structure and a similar physiological behaviour as the cell wall polysaccharides. Furthermore, it is rather difficult to distinguish cell wall polysaccharides and other polysaccharides analytically.

In 1974, Kritchevsky et al. (9,10) introduced the term "non-nutritive fibre" (NNF) with two subclasses: synthetic non-nutritive fibre (highly refined materials) and natural non-nutritive fibre (the fibrous complex naturally found in cell walls of edible plants).

Spiller and Amen (11,12) suggested the terms "non-purified plant fibre" (NPPF) for any fibrous material in its natural state with all cell wall components present (polysaccharides, lignin, cutins, minerals, etc.), "purified plant fibre" (PPF) for the sum of the polymeric fibrous components (lignin, cellulose, hemicellulose and pectins), and "non-nutritive synthetic fibre" (NNSF) for fibres not usually consumed by man. Schaller (13), however, stated that if fibre is defined as the fraction not broken down by digestive enzymes, terms like "purified" and "non-purified" are redundant. In 1976, Trowell (14) suggested that the term "dietary fibre" should be restricted to the structural polymers, cellulose, other matrix polysaccharides, and lignin of the plant cell wall, and that a new term, "dietary fibre complex", should be employed to include all of the structural polymers of dietary fibre together with all associated chemical substances naturally associated with the structural polymers.

Several authors have stated that the term "dietary fibre" is incorrect because there is no question of fibrous constituents in the food (15,16,17,18,19). Instead of the terms "dietary fibre" and "dietary fibre matrix", Spiller et al. (15,16) suggested the terms "plantix" and "complantix".

As the definition of Trowell et al. (7) is restricted to plant polysaccharides, it was suggested that the definition should be extended to consider the total diet (20,21). Godding (21,22) suggested a modified definition: "edible fibre: polysaccharides, related polymers, and lignin, which are resistant to hydrolysis by the digestive enzymes of man".

Hellendoorn (17,18) suggested the term "indigestible residue" for all unassimilated food residues (including proteins and lipids) that leave the small intestine unabsorbed and enter the large intestine where they are liable to attack and to conversion by colonic bacteria. Schaller (23) also thought that all indigestible remnants should be included in the concept of dietary fibre. Saunders and Betschart (24) believed that indigestible protein should be considered part of the dietary fibre composite because it may represent a considerable percentage of the total protein and can contribute significantly to the physiological effects of dietary fibre. Southgate et al. (25), however, believed that inclusion of indigestible proteins, lipids, waxes, cutins and inorganic constituents in the definition of Trowell et al. (7) is not justifiable. Lignin, however, should be included because of the presence of lignocellulose complexes.

Chemical definitions of dietary fibre

The chemical definition of dietary fibre, "non-starch polysaccharides" (NSP), was proposed by Cummings in 1981 (26). This definition probably includes dextrins, inulin and glycogen present in liver, pharmaceutical polysaccharides and certain food additives (27). Englyst et al. (19,28,29) reported that NSP is the best chemical index of plant cell walls and related materials in the diet. As a consequence, lignin and resistant starch should not be included in the definition of dietary fibre. If starch resisting digestion in the small intestine and lignin are proved to be important food ingredients, they should be measured separately (28,29). Björck et al. (30) recommended resistant starch formed during technological treatment to be regarded as dietary fibre rather than as dietary starch. Asp et al. (31) stated that, from a nutritional point of view, lignin as well as resistant starch should be included in the definition of dietary fibre. In a reaction, Englyst (32) explained that the original physiological definition of dietary fibre is still supported, but that this is no basis for analytical measurement. Trowell (33) suggested that the debate on resistant starch should be broadened and include resistant protein and resistant fat as well. Schweizer (34) stated that no exact definition of dietary fibre is needed because the operational definition "plant polysaccharides and lignin which are resistant to hydrolysis by the digestive enzymes of man" is sufficient.

Conclusion

Many definitions and terminologies have been proposed for dietary fibre. Although the term "dietary fibre" is now widely accepted, there is still quite some confusion about its definition. This is related to different points of view: analytical or physiological.

CHAPTER 4

Methods for the analysis of dietary fibre (Review)

Introduction

Discussions about a correct definition of dietary fibre have paralleled discussions about suitable methods for the analysis of dietary fibre. The first measurements of fibre were performed in animal feeds, but when it was discovered that the ingestion of particular components of the fibre fraction might be related to the management of certain diseases, the need arose to specifically measure those fibre components also in human foods.

The different types of methods for the analysis of dietary fibre developed in the past are discussed below.

Survey of methods for analysis of dietary fibre

The first known measurements of fibre were performed by Einhof in 1806 (35,36). He obtained fibre values by macerating feeds and subsequently extracting these with hot water. In the early nineteenth century, crude fibre methods for plant foods were developed based on determination of the residue remaining after sequential extraction by ether, acid and alkali. The crude fibre method (Weende method) was adopted by the Association of Official Agricultural Chemists (AOAC) in 1887 (37). For a long time the crude fibre method was favoured because it involves simple reagents and is reasonably reproducible. However, in the crude fibre method major fractions of the plant fibre are lost due to their solubility in acid or alkali (hemicellulose 80-90%; cellulose 20-50%; lignin 50-90%) (23,36,38). Williams and Olmsted (3) have pointed out the limitations of the crude fibre method and designed a fibre procedure in which starch and protein are removed enzymatically.

In 1936, McCance et al. (39) developed a method for the estimation of unavailable carbohydrates "by difference". The amount of fibre was estimated to be that part of the food which is left in the fat-free alcohol-insoluble residue after subtraction of available carbohydrates and protein. The method is very inaccurate because errors from all analyses are included (19).

In the period 1963-1968, Van Soest and co-workers (38,40,41,42,43,44,45) developed two gravimetric methods for analysis of hemicellulose, cellulose and lignin in animal feeds. One of these is an acid-detergent fibre (ADF) method, in which all constituents except cellulose and lignin are solubilized. The other one is a neutral-detergent fibre (NDF) method, in which all constituents except cellulose, hemicellulose and lignin are solubilized. Cellulose is determined by removing the lignin from the ADF with potassium permanganate. Hemicellulose is calculated as the difference between NDF and ADF. The ADF and NDF procedures can also be used for human foods (36,46). Van Soest's method approximates the value for dietary fibre more closely than the crude fibre method, but soluble fibres are also lost in his method (25,46,47). The value for NDF is often seen as the amount of insoluble dietary fibre (47,48). According to Selvendran and Dupont (49), the main problems encountered in the detergent procedures are the loss of lignin and detergent-soluble components, and contamination of the NDF residue with (modified?) starch, leading to an overestimation of NDF. Several modifications of the NDF procedure have been suggested in which the starch is degraded by α-amylase (50,51,52) or pancreatin (53).

Southgate (25) has developed a combined enzymatic and chemical procedure to analyse unavailable carbohydrates in foods, based on the method of McCance et al. (39). The method was first described in 1969, and modifications have been published since (54,55,56). The method was claimed to provide a measure of total dietary fibre as the sum of non-cellulose polysaccharides, cellulose and lignin. After hydrolysis the hexose and pentose contents of the polysaccharides are determined by colorimetry. The method is rather simple and no sophisticated instrumentation is needed. Selvendran and Dupont (49) mention coprecipitation effects (common to all methods using methanol- or ethanol-insoluble residues), incomplete pectin solubilization, inaccurate lignin values, incomplete removal of starch, and inaccuracies in the estimation of sugars by colorimetric methods (different sugars give a different colour yield; interference of different sugars) as disadvantages of the Southgate method.

In 1975, Hellendoorn et al. (57) proposed a simple in vitro digestion procedure to measure dietary fibre, using the digestive enzymes pepsin and pancreatin. Hellendoorn claimed that his method gives a better indication of the indigestible residue because it approximates human physiological conditions (17). The original method measures only the insoluble indigestible residue. In subsequent papers, however, Hellendoorn recommended the measurement of soluble pectins (17,18). According to Selvendran and Dupont (49) and Southgate and Englyst (19), incomplete disruption of tissue structure can lead to incomplete degradation of starch or proteins in the Hellendoorn procedure. Honig and Rackis (58) improved the Hellendoorn method by isolating the soluble and the insoluble indigestible fractions to determine the total indigestible residue.

In 1979, Theander and Aman (59) introduced a method for the measurement of total dietary fibre as the sum of soluble and insoluble dietary fibre. In their method starch is hydrolysed by sequential treatments with Termamyl (heat-stable α-amylase) and

amyloglucosidase. Soluble dietary fibre is recovered by freeze-drying the dialysed extract. This was done to minimize the loss of aqueous alcohol-insoluble polysaccharides. The neutral sugar composition of the insoluble and the soluble residue is determined by gas chromatography (GC) after derivatization to alditol acetates. Uronic acids are analysed by a rapid decarboxylation method. According to the authors, this decarboxylation method is not influenced by neutral sugars or other constituents, in contrast with colorimetric methods. Lignin is determined gravimetrically. The method of Theander and Aman was slightly modified by Theander and Westerlund (60). In this modification, soluble dietary fibre was isolated by precipitation with absolute ethanol. Very recently, the method was further improved to increase the speed of analysis (61). Englyst et al. (62) have suggested that Termamyl contains an appreciable amount of hemicellulase activity. Since Theander and Westerlund (60) recommend addition of Termamyl after the temperature of the suspension has reached 80-90 °C, the hemicellulases are probably inactivated (63).
Schweizer and Würsch (20) developed a method closely resembling that of Theander and Aman (59). Protein is digested with pepsin prior to the enzymatic degradation of starch with pancreatin and amyloglucosidase. Soluble dietary fibre is precipitated with ethanol. Neutral sugars in the insoluble and the soluble residue are determined as their aldonitrile acetates by GC. Uronic acids are determined by a modification of the carbazol method. Lignin is determined gravimetrically. According to Selvendran (49), the procedure of Schweizer and Würsch (20) for removal of protein and starch is very effective because enzymatic degradation of proteins facilitates subsequent starch hydrolysis.

In 1981, Englyst (64) described a method for the determination of dietary fibre as non-starch polysaccharides (NSP). Total NSP is determined as the sum of soluble and insoluble fractions. After gelatinization, starch is removed with amyloglucosidase. Soluble NSP are precipitated with ethanol. Neutral sugars are determined in the soluble residue and the insoluble residue by GC after derivatization to alditol acetates. Uronic acids are determined colorimetrically. In a subsequent paper, Englyst et al. (62) described procedures for the measurement of total NSP and insoluble NSP. Soluble NSP are calculated as the difference between total and insoluble NSP. In that paper Englyst et al. also described a procedure for the determination of resistant starch. They recommended the use of the more specific enzymes pancreatic α-amylase and pullulanase instead of amyloglucosidase for the hydrolysis of starch. According to Selvendran and Dupont (49), pancreatic α-amylase is used in excessive amounts. The reason for this could be the pH of the enzyme medium. Englyst et al. incubate in acetate buffer at pH 5.2, whereas mammalian α-amylase is unstable at a pH below 6.9-7.0. Englyst and Cummings (65) further modified the procedure for the determination of NSP. Resistant starch is now solubilized by DMSO prior to enzymatic starch degradation. In addition, *N*-methylimidazole is used to catalyse the formation of alditol acetates. Englyst and Hudson (66) described a colorimetric procedure for the determination of NSP, which produced accurate quantitative results comparable to the results of the gas chromatographic procedure. In 1987, Englyst et al. (67,68) published results of collaborative trials in which their gas chromatographic procedure and their colorimetric procedure were tested. In 1988, Englyst and Cummings (69) developed an improved method for the determination of NSP by GC and colorimetry. This modification includes a new enzyme preparation for removing starch (pancreatin instead of pancreatic α-amylase) and a new GC column for a faster measurement of neutral sugars.

In 1983, an enzymatic gravimetric procedure for determination of insoluble dietary fibre and soluble dietary fibre was described by Asp et al. (70). This procedure is partly based on the procedure of Hellendoorn (57). After gelatinization of starch in the presence of Termamyl to remove bulk starch, residual starch and protein are removed by pepsin and pancreatin. According to the authors, all starch is removed but some protein remains in the fibre residues. In 1984, Prosky et al. (71) reported an interlaboratory study for the determination of total dietary fibre by an enzymatic gravimetric method. This method is mainly based on the method of Asp et al. (70). After gelatinization of starch in the presence of Termamyl, protein and residual starch are removed with protease and amyloglucosidase respectively. Soluble dietary fibre is precipitated with ethanol. After determination of residual protein and ash in the fibre residue, the amount of total dietary fibre is calculated. In this determination, resistant starch is included in the dietary fibre. According to Englyst et al. (28,29,72), however, this is only part of the starch escaping digestion in the small intestine. To reduce the coefficient of variation, the method was modified and tested again in an interlaboratory study (73). In the method of Prosky et al. (73) filtration of the ethanol-treated suspension through a Celite bed can cause problems (63). In 1986, the enzymatic gravimetric procedure of Prosky et al. (73) was adopted by the AOAC (74,75). In 1988, Prosky et al. (76) presented an enzymatic gravimetric procedure for the determination of insoluble, soluble and total dietary fibre.

In the past few years, determination of the neutral sugar composition of dietary fibre by high-performance liquid chromatography (HPLC) has attracted attention. An important advantage of HPLC over GC is that for HPLC no derivatization is required. Slavin and Marlett (77) evaluated two HPLC methods with refractive index (RI) detection for the measurement of neutral sugars in neutral-detergent fibre (NDF). They concluded that a normal-phase column gives no adequate separation of the sugars, but a heavy-metal column is two to three times more sensitive. In 1989, Garleb et al. (78) concluded that, compared to previous HPLC techniques, the use of anion-exchange chromatography coupled with pulsed amperometric detection results in superior separation and detection of the neutral monosaccharides. A similar conclusion is reached by Martens and Frankenberger (79) who compared high-performance anion-exchange chromatography with pulsed amperometric detection and HPLC with RI detection.

Conclusion

Many different methods for the analysis of dietary fibre have been developed. The gravimetric AOAC procedure and the procedures in which the neutral sugar composition of the fibre is analysed by GC are now the most popular ones. HPLC procedures with pulsed amperometric detection are gaining popularity because of their speed of analysis.

As methods for the analysis of dietary fibre are based on a definition of dietary fibre it is important that consensus is reached regarding the type of components that should be included in the definition of dietary fibre. This definition should preferably be based on chemically defined components. These dietary fibre components can then be determined with specific analytical methods.

Comparison of different methods for analysis of dietary fibre

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Abstract

Several methods are available for analysis of dietary fibre. To increase insight into the relative merits of these methods, the acid-detergent fibre (ADF) and neutral-detergent fibre (NDF) methods of Van Soest et al., the Hellendoorn method, the method of Prosky et al. (AOAC method) and the Englyst method were compared with respect to the amount and the NSP composition of dietary fibre in four food products.

Our results show that the ADF/NDF detergent methods are inaccurate for the determination of cellulose, hemicellulose and lignin, and that the NDF and Hellendoorn methods are less suited for the determination of insoluble dietary fibre.

There is a discrepancy between the amount and the NSP composition of the dietary fibre determined by the AOAC and Englyst methods. This is either due to overestimation of the amount of dietary fibre in the AOAC method (coprecipitation of oligosaccharides or Maillard reaction products) or to underestimation of the amount of dietary fibre in the Englyst method (loss of polysaccharides during hydrolysis or derivatization), or both. Differences in isolation methods lead to differences in amounts of soluble and insoluble dietary fibre found by different methods. For both the Englyst and the AOAC methods, calculation of the amount of soluble dietary fibre from the difference between total and insoluble dietary fibre is preferable, because large errors may occur in determining the soluble portion.

We have shown that although different methods can yield comparable dietary fibre values, the NSP composition can vary greatly. Therefore we recommend using those methods that determine specifically well-defined components of the dietary fibre. The chromatographic Englyst method is preferred for this reason and because it gives insight into the type of polysaccharides present.

Introduction

For several years, dietary fibre has received considerable attention because of its possible beneficial effects for human health. In 1974, Trowell defined dietary fibre as "that part of plant material in our diet which is resistant to digestion by secretions of the human digestive tract" (6). As this definition did not include polysaccharides present in some food additives, e.g. gums and pectins, Trowell et al. extended the definition to include "all the polysaccharides and lignin that are undigested by endogenous secretions of the human digestive tract" (7).
Since this definition (6), definitions of dietary fibre have been the subject of much discussion (9,14,15,21,28,31,32,33,62), paralleled by discussion about suitable methods for determination of dietary fibre. The main problem was that the definitions of dietary fibre were more or less based on its physiological properties, which cannot be analysed chemically. At present, there is still confusion as to the type of components that should be included in the term "dietary fibre" and hence about the most appropriate method of analysis.
Several methods had previously been developed for the analysis of crude fibre, unavailable carbohydrates or dietary fibre. The first known measurements of fibre were performed by Einhof in 1806 (35,36). Since then, a number of gravimetric, colorimetric and chromatographic methods have been developed. The Weende method determined only crude fibre and was adopted by the Association of Official Agricultural Chemists (AOAC) in 1887 (37). In the period 1963-1981, Van Soest et al. developed and improved the acid-detergent fibre (ADF) and the neutral-detergent fibre (NDF) methods for determination of hemicellulose, cellulose and lignin (40,41,42,43,44,46). In 1969, Southgate introduced an enzymatic method for determination of soluble and insoluble dietary fibre (54,55,56), and Hellendoorn et al. published an enzymatic gravimetric method for insoluble dietary fibre in 1975 (24).
Theander et al. developed a gas chromatographic method for determination of soluble and insoluble dietary fibre (59,60,61), and Englyst et al. developed a method with stepwise acid hydrolysis and gas chromatographic and/or colorimetric quantification of total, insoluble and soluble non-starch polysaccharides (NSP) (28,62,65, 66,67,68,69). In 1983, Asp et al. published a gravimetric method for determining total, insoluble and soluble dietary fibre (70). The determination of total dietary fibre was given official final action by the Association of Official Analytical Chemists (AOAC) in 1986 (71,73,74,75). In 1988, Prosky et al. presented an enzymatic gravimetric method for determining insoluble, soluble and total dietary fibre in food based on the method of Asp et al.(76).

Methods are usually compared with respect to the amount of dietary fibre determined. However, this comparison gives no information about the type of dietary fibre that is determined with these methods.
The purpose of our study was to improve insight into the relative merits of methods for determination of dietary fibre with respect to the amount and composition of the dietary fibre determined.

We compared the gravimetric methods of Van Soest et al. (ADF, NDF), Hellendoorn et al., and Prosky et al. (AOAC) and the chromatographic Englyst method with respect to the amount of dietary fibre and the composition of its non-starch polysaccharides (NSP). The Hellendoorn method was selected because it was developed in our laboratories and was used widely until a few years ago. The ADF and NDF methods were selected because they have been very popular for several decades and are still used in many laboratories. The AOAC and the Englyst methods were selected because these are believed to be important methods for determination of dietary fibre at this time.

Four food products were chosen as representatives of fibre-containing foods: whole meal, dried apples, untoasted soya bran and toasted soya bran. Whole meal and dried apples were chosen because these products are important sources of dietary fibre. The two samples of soya bran were chosen because these products are very rich in dietary fibre.

Materials and methods

Materials

Whole meal and dried apples were bought in local stores. Untoasted and toasted soya bran were kindly supplied by a Dutch manufacturer. All samples were milled at 1 mm and stored at -25 °C. All reagents used were of analytical grade.

Methods

Acid-detergent fibre (ADF) was determined according to Van Soest and Wine (44). A 1 g sample was hydrolysed with 0.5 M sulphuric acid for 1 h at 100 °C. After filtration, the residue was washed with hot water, acetone and petroleum ether, dried and weighed. A correction was made for the amount of ash in the residue, and the amount of ADF was calculated.

The lignin was solubilized in potassium permanganate, and values for the amount of lignin and cellulose were obtained.

Neutral detergent fibre (NDF) was determined according to Van Soest and Wine (43) with the modification according to Pikaar et al. (53). The sample was boiled in water for 1 min at 100 °C to gelatinize the starch. After cooling, the starch in the sample was hydrolysed with pancreatin for 1 h at 37 °C, and the sample was treated for 1 h at 100 °C with NDF reagent. After filtration, the residue was washed with hot water, acetone and petroleum ether, dried and weighed. A correction was made for the amount of ash in the residue, and the amount of NDF was calculated.

The NDF reagent (pH 7) consisted of 30 g sodium lauryl sulphate, 18.61 g sodium hydrogen-EDTA, 6.81 g sodium tetraborate, 4.56 g disodium hydrogen phosphate and 10 ml 2-ethoxyethanol in 800 ml distilled water.

Dietary fibre was determined according to Hellendoorn et al. (57) by boiling a 0.5 g sample in water for 1 h to gelatinize the starch. After cooling, the sample was incubated with pepsin and hydrochloric acid for 18 h at pH 1 and 40 °C to hydrolyse the protein. The sample was neutralized and then incubated with pancreatin and sodium lauryl sulphate for 1 h at pH 7 and 40 °C to hydrolyse the starch. The pH was adjusted to 4.5, and the sample was filtered and washed with water and acetone. The residue was dried and weighed. No corrections were made for the amount of ash and for residual protein.

Dietary fibre was determined by the AOAC method according to Prosky et al. (76). A 0.5-1 g sample was incubated at pH 6.0 for 15 min at 100 °C with α-amylase (Termamyl; NOVO Biolabs, Copenhagen, Denmark) and allowed to cool. The pH was adjusted to 7.5 and the sample was incubated with protease for 30 min at 60 °C to hydrolyse the protein. After cooling, the pH was adjusted to 4.5 and the sample was incubated with amyloglucosidase for 30 min at 60 °C to hydrolyse the starch dextrins. From this point, the methods for total dietary fibre and soluble/insoluble dietary fibre proceed differently.

For determination of total dietary fibre, the soluble portion was precipitated overnight with 80 % (v/v) ethanol at room temperature. After filtration, the residue was washed successively with ethanol and acetone, dried and weighed. A correction was made for ash and protein, and the amount of total dietary fibre was calculated.

For separate determination of insoluble and soluble fibre, the samples were filtered and the residue was washed successively with ethanol and acetone, dried and weighed. A correction was made for ash and protein, and the amount of insoluble dietary fibre was calculated. The soluble fibre in the filtrate was precipitated overnight with 80 % (v/v) ethanol, collected by filtration, and washed with ethanol and acetone. The residue was dried and weighed. A correction was made for ash and protein, and the amount of soluble dietary fibre was calculated.

Dietary fibre was determined according to Englyst by a condensed version of Englyst and Cummings (69), which was supplied to participants of the Dietary Fibre Collaborative Trial, Part IV (joint UK Ministry of Agriculture, Fisheries and Food (MAFF)/EC Trial) organized by R. Wood in 1990. In this method, a 300 mg sample was boiled in DMSO for 30 min to disperse the resistant starch. Then the sample was incubated with α-amylase (Termamyl; NOVO Biolabs, Copenhagen, Denmark) for 10 min at 100 °C, cooled to 50 °C, and incubated with pancreatin and pullulanase at pH 5.2 for 30 min at 50 °C and then for 10 min at 100 °C to hydrolyse the starch and protein.

For determination of total dietary fibre, the soluble portion was precipitated with 80% (v/v) ethanol for 0.5 h at 0 °C, and centrifuged. The residue was washed with ethanol, dried with acetone, and subjected to stepwise acid hydrolysis. To disperse the cellulose, the residue was treated with 12 M sulphuric acid for 1 h at 35 °C. To hydrolyse the non-starch polysaccharides, the solution was diluted with water to 2 M sulphuric acid and incubated for 1 h at 100 °C. Neutral sugars in the hydrolysate were determined by gas chromatography after derivatization to alditol acetates. The neutral

sugars were calibrated and calculated as described in the MAFF/EC trial method. Inositol was used as internal standard. The amount of uronic acids in the hydrolysate was determined spectrophotometrically by the dimethylphenol method (69).
For determination of insoluble dietary fibre, precipitation with ethanol was replaced with a treatment with pH 7 phosphate buffer for 30 min at 100 °C. Then, the determination proceeded as described above.
The amount of soluble dietary fibre in the supernatant was determined after buffer treatment and centrifugation in the method for insoluble dietary fibre. The soluble fibre in the supernatant was precipitated by 80% (v/v) ethanol for 30 min at 0 °C. The method continued as described above for determination of total dietary fibre.

For determination of the NSP composition of dietary fibre residues isolated in the gravimetric ADF, NDF, Hellendoorn and AOAC methods, the residues were hydrolysed and derivatized as described for the Englyst method.

Cellulose was measured using the method of Updegraff (80). Lignin and hemicelluloses were removed by extraction with an acetic acid/nitric acid reagent. The remaining cellulose was then dissolved in 67% sulphuric acid, anthrone reagent was added, and the cellulose was determined colorimetrically.

The gas chromatographic determinations were performed on a Carlo Erba HRGC 5300 mega series equipped with an autosampler, a flame ionization detector and an integration computer. A Supelco SP-2330 wide-bore capillary column (30 m x 0.75 mm) was used. GC conditions were as follows: injection temperature 270 °C; column temperature 220 °C; detector temperature 270 °C; carrier gas hydrogen; column pressure 0.35 kPa; split flow 15 ml/min; injection volume 1 μl.

Statistics

All analyses were performed in duplicate unless stated otherwise. The statistical significance of the results was determined by analysis of variance with a two-tailed Student's *t*-test. A significance level of 5% was used.

Results and Discussion

As stated above, to obtain insight into the relative merits of methods for determination of dietary fibre, the gravimetric methods of Van Soest et al. (ADF and NDF), Hellendoorn et al., and Prosky et al. (AOAC) and the chromatographic Englyst method were compared with respect to the amount of dietary fibre and the NSP composition of the dietary fibre found in four food products.

For insight into dietary fibre composition, the amounts of cellulose, hemicellulose, uronic acids and lignin were determined in whole meal, dried apples, untoasted soya bran and toasted soya bran. Cellulose and lignin were determined according to

Table 1: Amounts of cellulose, hemicellulose, uronic acids and lignin in four products (g / 100 g d.m.)*

Product	Cellulose	Hemi-cellulose	Uronic acids	Lignin
Whole meal	1.9	7.9	0.3	1.0
Dried apples	5.4	3.1	3.2	0.4
Untoasted soya bran	42.6	18.2	11.3	1.9
Toasted soya bran	30.1	12.6	6.8	1.5

* Cellulose and lignin were determined according to the ADF method. Hemicellulose was calculated as the difference between NDF an ADF residues. Uronic acids were determined colorimetrically according to the Englyst method.

the ADF method. Hemicellulose was calculated as the difference between NDF and ADF residue. Uronic acids were determined colorimetrically according to the Englyst method. The results are shown in Table 1.
The dietary fibre of whole meal largely consists of hemicellulose and contains almost no uronic acids. The dietary fibre of dried apples consists mainly of cellulose, uronic acids and hemicellulose. High levels of cellulose, hemicellulose and uronic acids, and a small amount of lignin were found in the two samples of soya bran. Because there is a substantial difference in the composition of untoasted and toasted soya bran, it is assumed that the source of these products is different.

Table 2 shows the amounts of dietary fibre in whole meal, dried apples, untoasted soya bran and toasted soya bran as determined with the ADF, NDF, Hellendoorn, AOAC, and Englyst methods. The AOAC and Englyst methods determine total, insoluble, and soluble dietary fibre. For each product, dietary fibre values with different superscripts are significantly different ($P < 0.05$).

Tables 3-6 list the NSP composition of the dietary fibre residues as isolated by the ADF, NDF, Hellendoorn and AOAC methods, as well as the amount of NSP measured by the Englyst method, for whole meal, dried apples, untoasted soya bran and toasted soya bran, respectively. The methods do not differ significantly for every product tested. Overall statistical analysis of data obtained by the NDF, ADF, Hellendoorn, AOAC-total, AOAC-insoluble, AOAC-soluble, Englyst-total, Englyst-insoluble and Englyst-soluble methods, however, showed the following significant differences in NSP composition. The amounts of uronic acids, rhamnose, and arabinose are different for all methods. The amounts of fucose, xylose, galactose and glucose are similar for the NDF and Hellendoorn methods. The amounts of xylose and mannose are similar for the NDF and AOAC-insoluble methods. The amount of xylose is similar for the NDF and Englyst-total, Hellendoorn and AOAC-insoluble, Hellendoorn and Englyst-total, Hellendoorn and Englyst-insoluble, and AOAC-insoluble and Englyst-insoluble methods. The amounts of galactose and glucose are similar for the AOAC-total and the Englyst-total methods. The total amount of NSP is similar for the NDF and Englyst-insoluble and the Hellendoorn and Englyst-insoluble methods.

Table 2: Dietary fibre content in four products determined with five different methods (mean* and standard deviation in g/100 g d.m.)**

Dietary fibre method	Whole meal	Dried apples	Untoasted soya bran	Toasted soya bran
NDF	$10.8 \pm 0.2^{a,e}$	$8.9 \pm 0.6^{a,e}$	$62.6 \pm 1.5^{a,e}$	$44.1 \pm 0.5^{a,e}$
ADF	2.9 ± 0.2^{b}	5.8 ± 0.1^{b}	44.5 ± 0.4^{b}	31.5 ± 0.2^{b}
Hellendoorn	$9.3 \pm 0.3^{a,e}$	$9.1 \pm 0.1^{a,e}$	$66.5 \pm 1.2^{a,e}$	$46.8 \pm 0.1^{a,e}$
AOAC-total	12.0 ± 0.4^{c}	13.0 ± 0.5^{c}	76.1 ± 2.2^{c}	53.9 ± 2.3^{c}
AOAC-insoluble	10.1 ± 0.6^{a}	9.8 ± 0.1^{a}	69.3 ± 1.2^{a}	49.0 ± 0.8^{a}
AOAC-soluble	1.1 ± 0.4^{d}	2.8 ± 0.6^{d}	8.7 ± 0.6^{d}	6.4 ± 0.5^{d}
Englyst-total	8.9 ± 0.1^{e}	9.9 ± 0.5^{e}	63.7 ± 0.8^{e}	44.8 ± 0.2^{e}
Englyst-insoluble	7.2 ± 0.1^{f}	7.2 ± 0.1^{f}	53.8 ± 0.7^{f}	38.2 ± 0.5^{f}
Englyst-soluble	3.3 ± 0.1^{g}	4.5 ± 0.6^{g}	9.6 ± 0.5^{d}	7.6 ± 0.2^{d}

* Mean of four determinations

** For each product, dietary fibre values with a different superscript are significantly different ($P < 0.05$).

We conclude that, although different methods for determination of dietary fibre can give comparable values, the NSP composition of dietary fibre residues can differ greatly. Thus, it can be risky to use gravimetric methods for determination of dietary fibre without knowing which components are actually measured.

ADF and NDF methods

Cellulose and lignin are considered to be the major constituents of ADF. The amount of cellulose can be determined in the ADF residue after lignin is removed with potassium permanganate (44). Tables 3 to 6 show that the ADF residue contains not only glucose but also other polysaccharidic components (mainly xylose and uronic acids, average amount 19 %). Although non-crystalline regions of cellulose may include small amounts of sugars other than glucose, the amounts of non-glucose polysaccharidic components found in the ADF residues indicate the presence of non-cellulose polysaccharides. According to Englyst et al., the ADF residue may contain up to 10% of non-cellulose polysaccharides (62). Tables 3 to 6 also show that when the ADF is treated with permanganate to remove lignin, small but significant amounts of the NSP are also removed in most products (average 7 %). The ADF residue from which the lignin has been removed (ADF-lignin) still contains some non-cellulose polysaccharides. These results indicate that the ADF method is not suited for accurate determinations of the amounts of cellulose and lignin.
Table 7 shows the amount of cellulose in the four products determined with the ADF method, as calculated from the amount of glucose in the ADF residue, and as determined with the Updegraff method. Except for the dried apples, there is a good agreement between the amount of glucose in the ADF residue and the results of the Updegraff method. In the ADF method, the amount of cellulose is overestimated: for all products there is a discrepancy between the amount of cellulose determined by the

Table 3: NSP composition of dietary fibre in whole meal isolated by different methods and analysed by the gas chromatographic Englyst method (g / 100 g d.m.)

	NDF	ADF	ADF-lignin	Hellendoorn	AOAC-total
Uronic acids	0.24	0.05	0.08	0.28	0.47
Rhamnose	0.08	0.03	0.03	0.09	0.15
Fucose	0.00	0.00	0.00	0.00	0.00
Arabinose	1.77	0.00	0.00	1.38	2.37
Xylose	3.21	0.15	0.14	2.70	4.10
Mannose	0.13	0.06	0.05	0.14	0.35
Galactose	0.13	0.00	0.00	0.14	0.38
Glucose	2.29	1.49	1.33	2.18	2.57
Total NSP	7.85	1.79	1.62	6.90	10.37

	AOAC-insoluble	AOAC-soluble	Englyst-total	Englyst-insoluble	Englyst-soluble
Uronic acids	0.31	0.10	0.31	0.25	0.08
Rhamnose	0.09	0.05	0.13	0.13	0.12
Fucose	0.00	0.00	0.00	0.00	0.00
Arabinose	1.93	0.29	2.07	1.63	0.45
Xylose	3.52	0.44	3.54	2.84	0.68
Mannose	0.14	0.14	0.19	0.14	0.09
Galactose	0.15	0.14	0.43	0.15	0.28
Glucose	2.23	0.10	2.29	2.04	1.64
Total NSP	8.38	1.25	8.95	7.16	3.34

Table 4: NSP composition of the dietary fibre in dried apples isolated by different methods and analysed by the gas chromatographic Englyst method (g / 100 g d.m.)

	NDF	ADF	ADF-lignin	Hellendoorn	AOAC-total
Uronic acids	0.47	0.19	0.21	1.34	3.89
Rhamnose	0.15	0.04	0.04	0.18	0.31
Fucose	0.15	0.00	0.00	0.14	0.17
Arabinose	0.47	0.00	0.00	0.21	0.88
Xylose	0.93	0.24	0.15	0.97	1.04
Mannose	0.21	0.18	0.13	0.24	0.47
Galactose	0.53	0.04	0.01	0.54	0.82
Glucose	5.09	4.84	4.28	5.06	4.92
Total NSP	8.00	5.51	4.81	8.66	12.50

	AOAC-insoluble	AOAC-soluble	Englyst-total	Englyst-insoluble	Englyst-soluble
Uronic acids	1.00	2.42	3.20	0.64	2.45
Rhamnose	0.17	0.10	0.25	0.21	0.17
Fucose	0.15	0.01	0.13	0.11	0.00
Arabinose	0.67	0.18	0.74	0.46	0.32
Xylose	1.00	0.07	0.76	0.75	0.10
Mannose	0.24	0.18	0.22	0.21	0.21
Galactose	0.63	0.17	0.72	0.49	0.28
Glucose	4.71	0.08	3.92	4.37	0.99
Total NSP	8.55	3.21	9.93	7.23	4.49

Table 5: NSP composition of the dietary fibre in untoasted soya bran isolated by different methods and analysed by the gas chromatographic Englyst method (g / 100 g d.m.)

	NDF	ADF	ADF-lignin	Hellendoorn	AOAC-total
Uronic acids	2.79	3.10	2.37	6.35	10.49
Rhamnose	1.00	0.44	0.43	1.22	1.55
Fucose	0.19	0.00	0.00	0.21	0.34
Arabinose	3.43	0.01	0.07	2.46	4.59
Xylose	7.17	3.63	3.34	7.29	7.19
Mannose	4.93	1.31	1.02	3.56	8.12
Galactose	1.33	0.06	0.05	1.22	2.95
Glucose	28.34	31.95	30.74	28.35	25.17
Total NSP	49.16	40.49	38.01	50.67	60.38

	AOAC-insoluble	AOAC-soluble	Englyst-total	Englyst-insoluble	Englyst-soluble
Uronic acids	7.82	2.84	11.31	5.49	4.72
Rhamnose	1.38	0.19	1.31	1.06	0.37
Fucose	0.34	0.02	0.34	0.28	0.06
Arabinose	4.36	0.19	4.77	4.18	0.56
Xylose	7.04	0.06	7.30	7.30	0.17
Mannose	5.18	2.84	7.63	5.72	1.87
Galactose	1.98	1.02	3.04	1.95	1.17
Glucose	24.89	0.12	27.98	27.81	0.67
Total NSP	52.98	7.26	63.68	53.78	9.58

Table 6: NSP composition of the dietary fibre in toasted soya bran isolated with different methods and analysed by the gas chromatographic Englyst method (g / 100 g d.m.)

	NDF	ADF	ADF-lignin	Hellendoorn	AOAC-total
Uronic acids	2.24	2.11	1.52	4.10	7.80
Rhamnose	0.79	0.25	0.24	0.86	1.11
Fucose	0.16	0.00	0.00	0.17	0.27
Arabinose	2.25	0.02	0.04	1.77	3.32
Xylose	5.20	2.57	2.55	5.24	4.93
Mannose	2.26	0.80	0.72	2.09	4.07
Galactose	0.98	0.08	0.07	1.16	2.57
Glucose	22.09	22.39	22.76	22.39	19.64
Total NSP	35.97	28.22	27.90	37.78	43.70

	AOAC-insoluble	AOAC-soluble	Englyst-total	Englyst-insoluble	Englyst-soluble
Uronic acids	5.26	2.42	7.42	3.92	3.43
Rhamnose	0.97	0.17	0.90	0.71	0.30
Fucose	0.27	0.02	0.27	0.24	0.04
Arabinose	2.96	0.23	3.45	2.90	0.61
Xylose	4.85	0.07	5.27	5.25	0.12
Mannose	2.12	1.66	3.77	2.60	1.23
Galactose	1.83	0.66	2.47	1.74	1.22
Glucose	19.02	0.12	21.26	20.80	0.64
Total NSP	37.26	5.38	44.83	38.16	7.59

Table 7: The amount of cellulose in four products determined with the ADF method, calculated from the amount of glucose in the ADF residue and determined with the Updegraff method (g / 100 g d.m.)

Product	Cellulose determined with the ADF method	Cellulose as glucose in the ADF residue	Cellulose determined with the Updegraff method
Whole meal	1.9	1.5	1.5
Dried apples	5.4	4.9	0.7
Untoasted soya bran	42.6	30.7	27.2
Toasted soya bran	30.1	22.7	20.1

ADF method and the amount calculated from the glucose in the ADF residue or determined by the Updegraff method ($P < 0.05$).

NDF is considered to be composed of hemicellulose, cellulose and lignin. The amount of hemicellulose can be calculated as the difference between NDF and ADF (43). Tables 3 to 6 show that the NDF residue contains considerable amounts of uronic acids. The ADF residue also contains uronic acids (generally not to the same extent as the NDF residue ($P < 0.05$)) and xylose. Therefore, we find the NDF/ADF methods inaccurate for the determination of the amount of hemicellulose.

NDF and Hellendoorn methods

The NDF and Hellendoorn methods are believed to measure insoluble dietary fibre (18,46,47,55,57). Table 2 shows that there is no difference in dietary fibre content determined by the NDF, Hellendoorn and AOAC-insoluble methods for all products examined. Neither were differences observed between the amounts of dietary fibre determined by the NDF, Hellendoorn and Englyst-total methods. The NSP composition given in Tables 3 to 6 shows that smaller amounts of NSP are determined by the NDF and Hellendoorn methods than by the AOAC-insoluble and the Englyst-total methods ($P < 0.05$). For most products, a higher amount of glucose is determined by the NDF and Hellendoorn methods than by the AOAC-insoluble and Englyst-total methods ($P < 0.05$). In addition, the NSP composition of the NDF and Hellendoorn residues is different: the NDF residue usually contains more arabinose and less uronic acids and rhamnose than the Hellendoorn residue ($P < 0.05$).

According to Selvendran and Dupont, the main problems encountered with the NDF method are losses of lignin and detergent-soluble components and contamination of the NDF residue with (modified) starch, leading to overestimation of NDF (49). Englyst et al. also mention incomplete starch removal by the NDF method (62). Although the problem of incomplete starch removal is partly overcome in the modified detergent methods (49,53,62), our results show higher glucose values in the NDF residue, indicating incomplete starch removal. We conclude that, although the amount of NDF compares well with the amount of insoluble dietary fibre determined in the AOAC method, it is not only the insoluble dietary fibre that is determined.

Hellendoorn claims to measure the insoluble indigestible residue (18,57). Incomplete disruption of tissue structures, however, can lead to incomplete degradation of starch and protein in the Hellendoorn method (19,49), and no correction is made for ash and residual protein. The higher glucose values found in the Hellendoorn residue compared with the AOAC-insoluble residue (Tables 3 to 6) point to incomplete starch removal. Furthermore, during the hydrolysis of protein with pepsin/hydrochloric acid, some hydrolysis of polysaccharides may occur (81). This means that, although the amount of dietary fibre determined by the Hellendoorn method compares well with the amount determined in the AOAC-insoluble method, the composition of the Hellendoorn residue is less representative of insoluble dietary fibre than the composition of the AOAC-insoluble residue.

AOAC and Englyst methods

The AOAC method is considered to include NSP, lignin and resistant starch in the dietary fibre, whereas the Englyst method only includes NSP (69,76). Table 2 shows that the amounts of dietary fibre determined with the AOAC-total method are indeed higher than those analysed with the Englyst-total method ($P < 0.05$). The discrepancy, however, seems too large to be explained by the amounts of lignin and/or resistant starch only.

Reistad and Frohlich have found that Asp's method gives dietary fibre values that are 1.1 to 1.4 times higher than those obtained with the method of Englyst. This difference could not be explained by the amount of lignin present (82). Deelstra et al. and Mongeau and Brassard also conclude that the difference between the amounts of dietary fibre determined by the AOAC and Englyst methods cannot be explained by lignin only (83,84). Coprecipitation of oligosaccharides and Maillard reaction products can be a problem in methods in which the ethanol-insoluble residue is isolated (such as the AOAC method), especially for products rich in starch or protein (49,85).

The total composition of the two samples of soya bran was determined (protein, starch, fat, dietary fibre, and ash). The amount of components not included in either of these determinations (free sugars, oligosaccharides, phenolic compounds) was estimated to be 3 to 4%. When the dietary fibre values determined by the AOAC method were used, the total composition slightly exceeded 100% (107% and 105% for untoasted and toasted soya bran, respectively). When the dietary fibre values determined by the Englyst method were used, the total composition was less than 100% (96 % for both samples). Although the total composition of only two samples was investigated, the results suggest that the AOAC method slightly overestimates the dietary fibre content. On the basis of just the amount of NSP determined in the Englyst method, the amount of dietary fibre is slightly underestimated, partly because resistant starch and lignin are not included in this method.

Tables 3 to 6 show that the AOAC-total residue usually contains larger amounts of uronic acids, rhamnose, fucose, arabinose, xylose, mannose and total NSP, and similar amounts of galactose and glucose compared to residues obtained with the

Englyst method ($P < 0.05$). Reistad and Frohlich reported that the Asp method gave higher dietary fibre values than the Englyst method. However, the NSP content of the residue of the gravimetric Asp method (determined according to Englyst) was equal to the NSP content determined by the Englyst method. The NSP composition differed between the two methods. Reistad and Frohlich conclude that the difference in dietary fibre values between both methods is caused by components other than NSP, or by an underestimation of the NSP content in the Englyst method (82). A loss of NSP in the Englyst method could occur during hydrolysis or derivatization of the polysaccharides. In our experiments, the results were corrected for the estimated losses during hydrolysis and for possible losses during derivatization as described in the MAFF/EC trial method. It is not clear, however, to what extent these corrections cover the actual losses. Selvendran et al. report that no acid hydrolysis method will cleave all sugar-sugar linkages and give a quantitative yield of each monosaccharide; at the same time all hydrolysis methods cause some degradation of mono- or polysaccharides. Moreover, uronic acids linked to neutral sugars yield acid-resistant aldobiuronic acids, and the tendency of pectins and pectic acids to precipitate in acid can result in incomplete hydrolysis of the associated neutral sugars (63).

Precipitation of oligosaccharides can cause the amount of NSP in the AOAC method to be overestimated. Marlett and Navis have compared the AOAC and Theander methods (85). They indicate that, because the AOAC method does not call for the removal of oligosaccharides, they can coprecipitate with 80% ethanol or lead to an increase in lignin content through the formation of Maillard reaction products (85). In the AOAC method, coprecipitation of phosphate is another problem. Errors could be large, especially in products with a low dietary fibre content. This problem has been partly overcome by using dilute phosphate buffer but, according to Selvendran et al., the method needs further improvement, particularly for products low in dietary fibre (63).

Our results indicate that either some NSP is lost in the Englyst method or the amount of NSP is overestimated in the AOAC method, or both.

The sum of soluble and insoluble dietary fibre determined with the AOAC method compares well with the amount of total dietary fibre found by the method. For the Englyst method this holds for both samples of soya bran, but for the whole meal and the dried apples the sum of soluble and insoluble dietary fibre is significantly higher than the total amount of dietary fibre ($P < 0.05$). In his method, Englyst calculates the amount of soluble dietary fibre as the difference between total and insoluble dietary fibre (69). In our experiments we actually determined the soluble portion to compare the results with the AOAC method, in which soluble dietary fibre has to be determined (76). The cause of the discrepancy between the Englyst-total and the Englyst-insoluble and Englyst-soluble methods could be the different environment from which the soluble dietary fibre is precipitated. It can be seen from the NSP composition that the difference between the sum of soluble and insoluble dietary fibre and total dietary fibre is mainly caused by glucose. Therefore, either not all of the cellulose is determined in the Englyst-total method or the soluble dietary fibre is contaminated with oligosaccharides. The first possibility is not very likely because no difference between the sum of soluble plus insoluble dietary fibre and total dietary fibre was found for the two soya bran products, which contain the highest levels of cellulose. Englyst et al. suggest that incomplete precipitation of soluble dietary fibre can be avoided by calculating soluble fibre as the difference between total and insoluble dietary fibre (62). Our results, however, suggest that overestimation of the amount of soluble dietary fibre is the reason for calculating it as the difference between total and insoluble dietary fibre.

The AOAC method can be improved if the amount of soluble dietary fibre is calculated rather than determined. Large errors may occur in the determination of the (often small) amounts of soluble dietary fibre.

Distribution of NSP over insoluble and soluble dietary fibre differs between the AOAC and the Englyst method (Tables 3 to 6). Generally, in the AOAC method, a larger part of the dietary fibre is found to be insoluble dietary fibre, whereas in the Englyst method, a larger part of the dietary fibre is found to be soluble dietary fibre ($P < 0.05$). Tables 3 to 6 show that there are also some differences between the composition of insoluble and soluble dietary fibre as determined by the AOAC and Englyst methods ($P < 0.05$).

Reistad and Frohlich have found that differences in the amounts of soluble and insoluble dietary fibre are due to differences in the isolation methods (82). Marlett et al. reported that the amount of soluble dietary fibre varies from method to method and that DMSO treatment (as used in the Englyst method) does not affect the distribution between soluble and insoluble dietary fibre (86). According to Englyst and Cummings, the reason for the higher proportion of soluble dietary fibre obtained by the Englyst method is due to the pH used for extraction (72).

Our results show that differences in reaction conditions under which soluble and insoluble dietary fibre are separated have great influence on the amounts of insoluble and soluble dietary fibre measured.

Conclusions

We have shown that, although different methods for analysis of dietary fibre can give comparable values, the NSP composition of dietary fibre residues can differ greatly. For this reason, it can be risky to use a gravimetric method for analysis of dietary fibre without knowing which components are actually measured.

From the results presented here, we conclude that the ADF and NDF methods are not suited for accurate determinations of cellulose, hemicellulose, and lignin. The NDF and Hellendoorn methods are less suited for the determination of insoluble dietary fibre.

Our results indicate that the dietary fibre content is slightly overestimated when determined by the AOAC method and slightly underestimated when analysed by the Englyst method. Nonetheless, we prefer the Englyst method because it determines well defined components of the dietary fibre. Englyst's gas chromatographic determination has advantages over the colorimetric determination because estimation of neutral sugars by colorimetric methods is not very reliable due to interferences and different colour yields of the different sugars. Moreover, gas chromatographic determination of the neutral sugars yields insight into the types of polysaccharides present.

For determination of dietary fibre, modern methods like the AOAC and Englyst methods should be preferred to older methods like the ADF/NDF and Hellendoorn methods. Both the AOAC method and the colorimetric Englyst method are well suited for routine analysis of dietary fibre because the methods are relatively rapid and no sophisticated equipment is needed.

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Part III

Phytic acid

CHAPTER 6

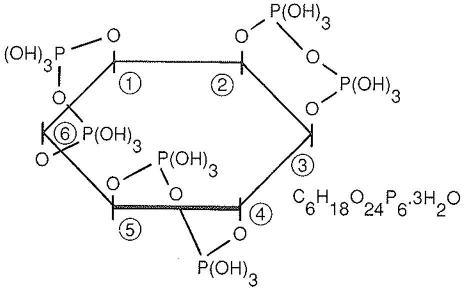
Methods for the analysis of phytic acid (Review)

Introduction

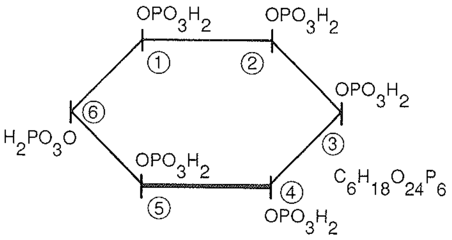
Phytic acid (myo-inositol-1,2,3,4,5,6-hexakis(dihydrogen phosphate)) is a constituent of a wide range of plant seeds. Phytic acid is predominantly present as the (combined) potassium, calcium and magnesium salt and acts as a storage form of phosphorus for germinating seeds.

In 1897, Winterstein proposed the name "inosite-phosphoric acid" for the phosphorus-containing compound present in plant seeds because the compound yielded phosphoric acid and inosite (inositol) upon hydrolysis (1). Posternak studied the chemical and physical properties of the phosphorus-containing compound extensively. He suggested the name "phytin" (2,3,4,5,6).

In 1908, Neuberg proposed a structure for phytic acid (7). A different structure was proposed in 1914 by Anderson (8). The main difference between both structures concerns the conformation of the phosphate groups within the molecule. The Anderson structure has been confirmed in the past few years by various spectroscopic techniques and is now generally accepted (9).



Structure for phytic acid
as proposed by Neuberg (7)



Structure for phytic acid
as proposed by Anderson (8)

Several methods have been developed for the analysis of phytic acid. However, few are found to be specific. A major problem when analysing phytic acid is that no specific reagent or characteristic absorption spectrum has been found that can be used for quantification.

The determination of phytic acid in any material requires an initial extraction step. Dilute hydrochloric acid and trichloroacetic acid are the most commonly used extractants.

The methods available for the determination of phytic acid can be classified into four categories:

- iron precipitation methods
- ion-exchange chromatographic methods
- reversed-phase chromatographic methods
- phosphorus-31 NMR methods

The developments in these methods are reviewed below.

Methods for the analysis of phytic acid based on iron precipitation

In 1914, Heubner and Stadler (10) observed that phytic acid forms an insoluble stable complex with ferric ions in dilute acid. They used this as a basis for the quantitative analysis of phytic acid by titrating phytate-containing extracts with a ferric chloride solution. Ammonium thiocyanate was used as an indicator. However, formation of a white ferric phytate precipitate during titration makes it difficult to establish the end-point of titration (11,12,13,14). Another drawback of this method is that a large amount of sample is required to obtain a reasonable titration. Analysis is even more complicated if the extracts are markedly coloured from their own (15). Harris and Mosher (16) modified the method of Heubner and Stadler by titrating beyond the end-point, removing the ferric phytate precipitate by filtration, and determining the excess of iron colorimetrically.

Various adaptations of the original method of Heubner and Stadler (10) have been published since. These can be classified into direct methods and indirect methods. In the direct methods, the insoluble ferric phytate precipitate is removed and in the precipitate the content of phosphorus or iron is determined (13,17,18,19,20,21,22). The indirect methods are based on the determination of the residual iron in solution after precipitation of ferric phytate from a known concentration of ferric salt in acid solution (14,23,24,25). The accuracy of this method depends on the efficiency of precipitation of ferric phytate having a known iron/phosphorus ratio. Samotus and Schwimmer (24) stated that high phytic acid values obtained with the indirect method can be attributed to the presence of reducing substances such as ascorbic acid and chlorogenic acid. Reduction of Fe(III) to Fe(II) by these agents can yield high phytic acid values. Addition of hydrogen peroxide prior to precipitation with ferric chloride was suggested to overcome this problem. In general, the indirect methods are more convenient and reproducible than the direct methods. According to Makower (21), however, indirect methods for the determination of phytic acid are subject to large errors for samples with a low phytic acid content because the results are based on a small difference between two relatively large values.

Although the methods for the analysis of phytic acid based on precipitation with ferric chloride have been used extensively for several decades, they are not very accurate. Many inositol phosphate esters are precipitated under the conditions commonly used in these methods and thus interfere with the determination of phytic acid (15,26,27,28,29). High inorganic phosphate contents also interfere with phytic acid determination (30). According to Anderson (31), the phytate content is underestimated in extracts containing large amounts of iron (either by adding too much ferric chloride or due to a natural high iron content) because the equilibrium is shifted, and soluble ferric phytate complexes are formed. Moreover, the methods using iron determination are based on the assumption that the precipitate is tetra-iron(III) phytate. As this will not always be the case, these methods should be regarded with caution (32).

Methods for the analysis of phytic acid based on ion-exchange chromatography

Smith and Clark (33) and Cosgrove (34) used anion-exchange chromatography for the separation of inositol phosphates using a stepwise gradient with hydrochloric acid. In 1977, Harland and Oberleas (35) developed an anion-exchange procedure for the determination of phytic acid. The method involved concentration of the phytic acid in a hydrochloric acid extract on small anion-exchange columns. Inorganic phosphate was stripped off the column with 0.05 M sodium chloride, and phytic acid was eluted with 0.7 M sodium chloride. The final eluate was digested with sulphuric acid and nitric acid and the liberated phosphate was determined colorimetrically. The method is simple and reproducible and the phytic acid values obtained are slightly lower than with the iron precipitation methods. Latta and Eskin (36) modified the procedure of Harland and Oberleas. In their procedure, the phytic acid in the purified extract is determined directly by reaction with the Wade reagent (ferric chloride/sulphosalicylic acid solution). The colour reaction is measured spectrophotometrically. There is no interference from orthophosphate, ascorbic acid or chlorogenic acid (36,37). According to Vaintraub and Lapteva (37), phytic acid can be determined directly in the crude extract with the method of Latta and Eskin without prior anion-exchange purification. However, this method is expected to be inaccurate because of interferences of impurities.

Ellis and Morris (38) reported that phytic acid values obtained by the iron precipitation method are about 40% higher than those obtained with ion-exchange chromatography. They attributed this to interfering substances present in the acid extracts. Ellis and Morris (39) improved the ion-exchange procedure by treating the sample with ethylenediaminetetraacetate (EDTA) and sodium hydroxide prior to ion-exchange chromatography to eliminate interfering metal ions. Ellis and Morris found a close agreement between phytic acid values obtained with their modified ion-exchange procedure and those found with the iron precipitation procedure. The modified procedure of Ellis and Morris was studied in a collaborative study (40). It was concluded that the ion-exchange method for the analysis of phytic acid is rapid, simple, reproducible, accurate and sensitive at low levels and that it compares well with the iron precipitation method. Unfortunately, large variations in anion-exchange resins lead to inaccurate phytate determinations (41). The small ion-exchange columns have also been used for the serial separation of inositol mono-, di-, tri-, tetra-, penta- and hexaphophates (42,43).

In 1985, Phillippy and Johnston (44) developed an ion chromatographic method for the determination of phytic acid in which the anion-exchange mode previously used for prepurification was now used for analytical separation. Phytic acid was detected spectrophotometrically after post-column derivatization with a solution of ferric nitrate in perchloric acid. Phillippy and Johnston (44) compared the results of their method with results obtained with the method of Ellis and Morris (39) and found that their ion chromatographic values were lower. They concluded that their own method is more specific for phytic acid than the Ellis and Morris procedure. Cilliers and van Niekerk (45) also published an ion chromatographic procedure with post-column colorimetric detection for the determination of phytic acid. They used a solution of ferric chloride in sulphosalicylic acid (Wade reagent) for post-column derivatization.
Several authors have used anion-exchange chromatography for the separation of lower inositol phosphates (inositol mono-, di-, tri-, tetra- and pentaphosphate) (28,45,46,47).

Methods for the analysis of phytic acid based on reversed-phase chromatographic methods

In 1980, Tangendjaja et al. (48) proposed an HPLC method for the separation of inositol and phytic acid using a reversed-phase octadecyl column with refractive index detection. Sodium acetate was used as mobile phase. However, phytic acid eluted in the solvent front (49,50). Camire and Clydesdale (51) modified the procedure of Tangendjaja et al. by inserting a clean-up step to obtain a better resolution of phytic acid. They precipitated the phytic acid with iron chloride and converted it to sodium phytate before injecting it onto a reversed-phase octadecyl column. The phytic acid, however, showed little or no retention on the column. Graf and Dzintis (52,53) used anion-exchange chromatography as a purification step prior to reversed-phase chromatography on an octadecyl column. However, phytic acid still eluted in the solvent front. According to Minear et al. (50), the general problem with HPLC determination of phytic acid is the failure of the polar compounds to interact with the non-polar column.
Lee and Abendroth (49) developed an HPLC method in which the phytic acid was eluted significantly later than the solvent front. They used anion exchange for clean-up in combination with HPLC using a tetrabutylammonium formate ion pair on an octadecyl column. Phytic acid was detected by refractive index measurements.

In 1986, Sandberg and Ahderinne (54) developed a method for the determination of inositol tri-, tetra-, penta- and hexaphosphates using ion-pair octadecyl reversed-phase HPLC after prepurification by anion-exchange chromatography. The inositol phosphates were well separated in this procedure. Other authors have also used ion-pair reversed-phase liquid chromatography for the separation of inositol phosphates (55,56,57).

Methods for the analysis of phytic acid based on phosphorus-31 NMR

In 1980, O'Neill et al. (58) developed a direct quantitative method for the analysis of phytic acid using phosphorus-31 Fourier transform nuclear magnetic resonance (NMR) spectrometry. EDTA was added as a competitive chelating agent for cations so that phytic acid gave sharp, well-resolved NMR signals. The spectrum of phytic acid was found to be strongly depending on pH. Mazzola et al. (59) modified the method of O'Neill et al. to improve its convenience and accuracy. Furthermore, interference from paramagnetic ions was eliminated by addition of excess quantities of EDTA. Mazzola et al. (59) compared the results of their NMR method with those of the ion chromatographic method described by Phillippy and Johnston (44) and found a good agreement between the two methods.

Analysis of phytic acid by NMR is specific, and discriminates against both lower inositol phosphates and inorganic phosphates (29,58,59,60).

Conclusion

A variety of methods have been developed for the analysis of phytic acid. However, due to interferences of lower inositol phosphates, inorganic phosphates and metal ions, only few methods are specific for phytic acid. At present, methods based on anion-exchange chromatography with post-column derivatization and methods based on phosphorus-31 NMR show the best results. A drawback of the phosphorus-31 NMR methods is that quite expensive equipment is needed.

Improved determination of phytate by ion-exchange chromatography

Klaas D. Bos , Cornelis Verbeek, C.H. Peter van Eeden, Pier Slump and Mechteldis G.E. Wolters

Abstract

An improved method for the ion chromatographic determination of phytic acid is presented. The method is based on a recently published chromatographic method combined with a treatment of the samples with EDTA. Separation and quantitation of phytic acid were performed on an anion-exchange column. Phytic acid was detected at 300 nm after reaction with a ferric salt in an in-line post-column derivatization.

The improved method described here was compared with a recently published HPLC method without EDTA treatment and with a method using small single-use ion-exchange columns.

It was found that the HPLC method without EDTA treatment of the samples results in too low values for phytic acid, whereas the method using small single-use ion-exchange columns gives too high values. The differences between the results of the three methods were statistically significant ($P < 0.01$).

Introduction

Phytate is the trivial name for inositol hexaphosphate and is present in many grains and seeds. It is a potential source of phosphorus which can be liberated during the germinating stage of the seeds. Phytate is a polydentate ligand capable of binding metal ions; divalent and trivalent ions especially are firmly bound. These bound metal ions are nutritionally only partly available. Moreover, the analytical determination of phytate may be disturbed by these strong metal-ligand interactions. These interactions render the determination of phytate complicated in many samples.

An early method for the determination of phytate was based on the precipitation of a phytate-iron complex (25). This method has been the standard for more than a decade, yielding satisfactory results for grains and seeds with higher phytate contents. The procedure is less suited for products having a low phytate content.

An improved method, using ion-exchange column separation, was published more recently (35). This method includes the following steps. An acidic extract of the sample is applied to a small column filled with an anion-exchange resin, and the column is rinsed with a buffer solution of low ionic strength. The phytate is then eluted from the column with a salt solution of higher ionic strength and, finally, the phytate content of the eluate is determined colorimetrically.

This procedure was further improved by Ellis and Morris (39), who treated the extracts with EDTA to eliminate the interference of metal ions in the phytate determination. This method was recently tested in a collaborative study (40). Unfortunately, optimal results are obtained only with special batches of the ion-exchange resin. Another disadvantage of the procedure of Ellis and Morris is that it is rather laborious. Automated procedures with high performance liquid chromatography (HPLC) are much more attractive.

Phillippy and Johnston (44) recently described a promising HPLC method. The phytate content in a series of samples was determined, and the results were compared with those obtained with the procedure of Ellis and Morris (39). The phytate values obtained with the HPLC method were lower than those obtained with the Ellis and Morris procedure. The cause of this difference might be that the procedure of Phillippy and Johnston does not include an EDTA treatment of the extracts.

Recently, Cilliers and van Niekerk (45) also published a liquid chromatographic procedure for the determination of phytic acid. Some aspects of their method have been incorporated in our improved analytical procedure. This new method is essentially the chromatographic procedure described by Phillippy and Johnston (44) and by Cilliers and van Niekerk (45), combined with a treatment of the sample extracts with EDTA to eliminate the interference of metal ions.

A different approach was published by Sandberg and Ahderinne (54). They developed an HPLC method using a reversed-phase silica column.

In the present study three analytical procedures for the determination of phytate are compared and the differences discussed.

Materials and methods

Materials

Phytic acid was obtained from Sigma (St. Louis, MO, USA). The batch was found to contain 15 % moisture. Phytase from wheat was obtained from Sigma. The other reagents used were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Food samples were purchased in food stores in Zeist. The grains and the chyme sample were obtained from the Institute for Livestock Feeding and Nutrition Research (Lelystad, The Netherlands).

The small pre-packed columns with ion-exchange resin were of the type "Baker 10", No. 7091 (Baker, Phillipsburg, NJ, USA). They were used with the "Baker 10" extraction system, No. 7018-0. Membrane filters with 0.45 µm pore size were supplied by Millipore Corporation (Bedford, UK).

Liquid chromatography system

The chromatograph consisted of two modified LDC-Milton Roy minipumps from Biotronik (Munich, Germany). One pump was used for the eluent and one for the reagent. The single-headed pumps were provided with pulse damping devices as are commonly used in automated amino acid analysers. The anion-exchange column used was a Dionex HPIC AS-3 (0.50 cm i.d. x 25 cm). A Dionex HPIC AG-3 guard column was used. The injector was from Pharmacia (Uppsala, Sweden). The injection volume was 50 µl. The reaction coil was of polytetrafluoroethylene tubing with an internal diameter of 0.3 mm and a length of 3 m. It was kept in an oil bath at 50 °C. A variable UV-vis detector (300 nm) (ABI Analytical, Kratos Division, Ramsey, NJ, USA) was used.

Methods

Extraction procedure

About 2 g of each sample was accurately weighed and shaken with 20 ml of 0.8 M HCl for 2 h at room temperature. The slurry was centrifuged at 1800 *g* for 10 min. The supernatant was separated by decantation and filtered over a paper filter. The phytate content of this filtrate was determined by HPLC (Method A). For methods B and C part of the filtrate was mixed with a solution of EDTA and NaOH (see below). The mixture was stored at 4 °C until analysed.

Method A

The sample extract (1.0 ml) was diluted with water (10 ml). After filtration through a Millipore filter (0.45 µm), 50 µl of this mixture was injected onto the column of the liquid chromatography system. Only eluent C was used.

Method B (the new method)

In a 10-ml volumetric flask 1.0 ml of the sample extract was mixed with respectively 0.25 ml 2.8 M NaOH, 0.75 ml of a solution of sodium acetate (167 g/l), and 1.0 ml EDTA solution (40 g/l, adjusted to pH 6.0 with 2.8 M NaOH). The mixture was allowed to stand at room temperature for 15 min. The volumetric flask was made up to volume with water. The pH of the resulting mixture was about 6. If necessary, this solution can be diluted further with a solution of sodium acetate (15 g/l, pH 6). After filtration through a Millipore filter (0.45 µm), 50 µl of the solution was injected onto the column of the liquid chromatography system.

Method C

The Baker 10 anion-exchange columns were rinsed sequentially before use with 3 ml methanol, 3 ml ammonia (25 %), 3 ml water, 3 ml 0.3 M HCl and 3 ml water.

The sample extract (1.0 ml) was mixed with 1 ml of a solution containing 0.11 M EDTA and 0.75 M NaOH. The resulting mixture had a pH of about 6. Of the mixture, 1.0 ml was diluted with 30 ml of water and applied to the Baker 10 column. The column was rinsed twice with 5 ml water and twice with 5 ml 0.3 M NaCl. Then the column was eluted twice with 2 ml 0.8 M NaCl. The eluent obtained was transferred to a 10-ml volumetric flask and the flask was made up to volume with deionized water. The phytic acid was determined by a colorimetric method as described by Latta and Eskin (36). One ml of the reagent (a solution of 0.03 % iron(III) chloride hexahydrate and 0.3 % sulphosalicylic acid in water) was transferred into a disposable cuvette. Depending on the concentration of the phytic acid solution, a volume between 0.1 and 2.0 ml of the sample solution was added to the reagent in the cuvette. Water was added to make a total volume of 3.0 ml and the liquid was mixed. After standing for about 10 min, the colour was read at 500 nm on a Beckman spectrophotometer. The phytic acid concentration was calculated using a standard curve.

Liquid chromatography conditions

Eluent A was an aqueous solution of sodium nitrate and EDTA. 2.5 g of sodium nitrate was dissolved in 1 litre water containing 0.5 ml of an EDTA solution (40 g/l) and 0.2 ml of a pentachlorophenol solution (500 mg in 100 ml ethanol). The pH was adjusted to 6.0 with 2.8 M NaOH.

Eluent B was an aqueous solution of 0.1 M sodium nitrate containing 0.2 ml pentachlorophenol solution (500 mg in 100 ml ethanol) per litre. The pH was adjusted to 3.5 with eluent C.

Eluent C was an aqueous solution of nitric acid (9 ml/l).

The elution programme was: eluent A for 5 min, eluent B for 5 min, eluent C for 13 min and eluent A for 7 min. The flow rate of the eluent was 1 ml/min.

The post-column reagent was a solution of 2.2 g of iron(III) perchlorate nonahydrate and 12.8 ml of 70 % perchloric acid in 1.0 litre of water. The reagent was stored in a brown flask. A flow of 0.5 ml/min was applied.

Results and discussion

The phytate contents in several samples of foodstuffs and feeds were analysed by the following three methods:

- a chromatographic method very similar to the method of Phillippy and Johnston (44) and to that of Cilliers and van Niekerk (45) (Method A);
- a new method which is a chromatographic method combined with an EDTA treatment of the extracts (Method B);
- a colorimetric determination after concentration of the phytic acid on a small ion-exchange column, a procedure very analogous to that of Ellis and Morris (39) (Method C).

Table 1: Phytic acid content of samples determined with methods A, B and C (mean values and S.E.M. of four analyses)*

Sample	Phytic acid (g/100g dry matter)					
	method A		method B		method C	
	mean	SEM	mean	SEM	mean	SEM
Soya bean meal	0.965^{a}	0.029	1.442^{b}	0.005	1.590^{c}	0.012
Maize	0.583^{a}	0.014	0.735^{b}	0.008	0.651^{c}	0.011
Wheat	0.667^{a}	0.013	0.785^{b}	0.004	0.824^{b}	0.006
Barley	0.693^{a}	0.014	0.822^{b}	0.007	0.845^{b}	0.019
Chyme	0.527^{a}	0.004	0.693^{b}	0.001	0.837^{c}	0.007
Wheat bran	3.963^{a}	0.016	4.315^{b}	0.016	3.978^{a}	0.042
Roasted coffee	0.099^{a}	0.003	0.108^{a}	0.003	0.179^{b}	0.009

* Mean values with a different superscript differ significantly ($p < 0.01$)

The results are summarized in Table 1. The results of the three methods do not differ significantly for every sample tested. Overall, however, the results of the three methods were found to be significantly different ($P < 0.01$).

The procedure described by Phillippy and Johnston (44) (method A) resulted in lower phytate values than did the procedure of Ellis and Morris (39) (method C). The procedure of Phillippy and Johnston (44) may result in too low values for phytate because the extracts have not been treated with EDTA. EDTA functions as a metal scavenger, eliminating the interference of metal ions with the phytate determination as was shown by Ellis and Morris (39). On the other hand, the procedure of Ellis and Morris (39) may result in too high values for phytate because in their procedure lower inositol phosphates are only partly separated from inositol hexaphosphate and raise the phytate values obtained. Seeds and grains usually contain few if any lower inositol phosphates, but in bread or in chyme samples high levels of these lower inositol phosphates may be present (61). Polycarboxylic acids may also interfere with the phytate determination according to the method of Ellis and Morris (39) (see below). Our improved chromatographic method (method B) generally results in higher phytate values than the procedure of Phillippy and Johnston (44) (method A). Our values are lower than the values obtained with the method of Ellis and Morris (39) (method C).

Extraction procedure

The samples were extracted with 0.8 M HCl (38). For comparison, some extractions with a 3 % solution of trichloroacetic acid (TCA) were performed (45). However, analysis of these extracts resulted in lower phytate values. This may be explained as follows. TCA is often used for the denaturation of proteins. Phytate has a high affinity to proteins with a high polarity. Denaturation of proteins during the extraction with TCA will probably result in occlusion of phytic acid, which will be lost in the filtration step.

The HCl extracts were treated with EDTA at a pH of about 6. At this pH the metal ions are more strongly bound by EDTA than they are by phytate. The processing of some samples, with a high content of calcium carbonate, resulted in a pH much higher than 6. This problem was solved by adding only 0.1 ml instead of 0.25 ml of 2.8 M NaOH to the acidic sample extract.

Colorimetric method (Method C)

Ellis and Morris (39) used the anion-exchange resin AG 1-X8 and obtained satisfactory results when properly selected batches of resin were used (41). We found that columns for single use gave invariably good results. The ion-exchange columns were first rinsed with a solution of 0.3 M NaCl to remove free phosphate and other interfering substances. The inositol hexaphosphate was eluted from the column with a solution of 0.8 M NaCl. The phytate in the eluate was determined according to Latta and Eskin (36) by using the Wade reagent, a solution of ferric sulphosalicylate.

The linearity of the method was tested with solutions of pure phytic acid. A linear response was observed for a quantity of phytic acid between 5 and 5000 μg. A linear calibration curve of $y = 0.713 - 0.311\,x$ with a correlation coefficient of 0.998 ($n = 6$) was obtained for extinction at 500 nm against the phytic acid concentration applied.

Experiments showed that gallic acid, chlorogenic acid and inositol penta-phosphate interfere with phytic acid in Method C. One mg of gallic acid, chlorogenic acid and inositol pentaphosphate gave a response comparable with 0.07, 0.04 and 0.27 mg of phytic acid respectively. Many other substances such as oxalic acid or salicylic acid appeared not to interfere with phytic acid in Method C.

Liquid chromatographic methods (Methods A and B)

For a study on the degradation of phytic acid by phytase in our laboratory, many determinations of phytic acid were necessary. The work published by Cilliers and van Niekerk (45), who used a Waters IC-PAK A column, looked very promising. We adopted their procedure and obtained very good results with the first two columns of that type. Several thousands of analyses were carried out successfully. Unfortunately, the third and fourth IC-PAK A columns from Waters had different properties. With these columns the analytical procedure of Cilliers and van Niekerk (45) gave irreproducible results. Then we adopted the procedure of Phillippy and Johnston (44) who used a Dionex HPIC AS-3 column. This type of column has now been in use in our laboratory for more than 3 years. Until now, all of the columns of this type have shown good comparable results. A chromatogram of the analysis of phytic acid with method A is presented in Figure 1.

The improved HPLC method (method B) is a specific method for the determination of phytic acid. A chromatogram is presented in Figure 2. Only very acidic substances will elute together with phytic acid from the anion-exchange column. Only acids with strong coordinative properties will give complexes with the ferric ions of the reagent at this low pH, resulting in an increase of the absorption at 300 nm. Several compounds that might interfere with the phytic acid peak were injected. These include

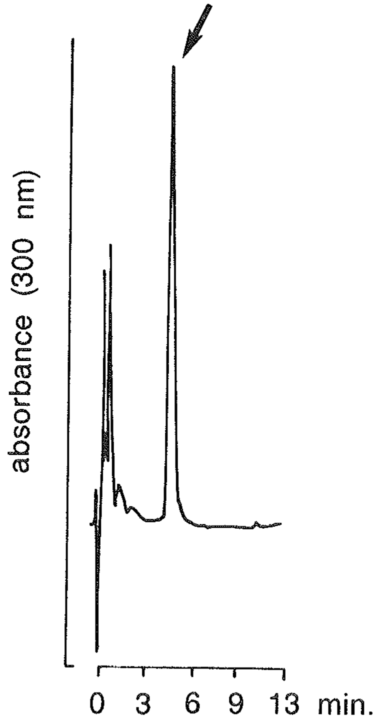


Figure 1. Chromatogram of the analysis of phytic acid in wheat with method A

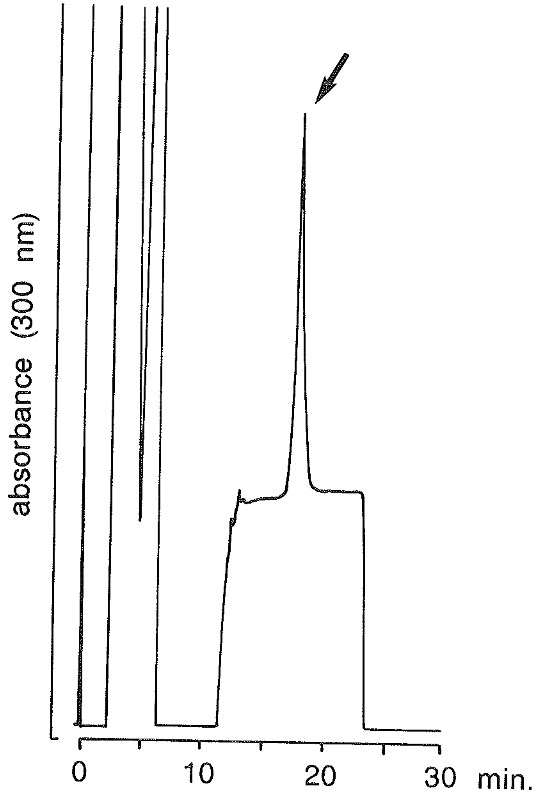


Figure 2. Chromatogram of the analysis of phytic acid in barley with method B

salicyclic acid, gallic acid, chlorogenic acid, ferulic acid, citric acid, and oxalic acid. None of these compounds gave a peak in the chromatogram and so they do not interfere with phytic acid.

The anion-exchange column used (Dionex AS-3) has a low capacity and is easily overloaded. A linear response was observed when the injected quantity of phytic acid was between 0.25 and 15 µg. A linear calibration curve of $y = -1.909 + 41.68\,x$ and a correlation coefficient of 0.9994 ($n = 12$), were obtained for the peak area against the phytic acid concentration injected. The life span of the Dionex AS-3 column, protected by a guard column, is more than 2000 runs.

The acidic sample extracts were treated with EDTA. A concentration of 4000 mg of EDTA /l in the resulting mixture was found to be optimal. Concentrations of 2000 mg of EDTA /l or less resulted in too low phytate peaks. Probably these concentrations of EDTA were too low to inhibit the interference of metal ions with the phytate. Concentrations of 6000 mg of EDTA /l or higher disturbed the chromatography as a result of too high an ion concentration.

The use of a heated reaction coil resulted in higher peaks in the chromatogram. Maximum peak heights were obtained when a 3 m long coil (internal diameter 0.3 mm) heated to 50 °C was used.

The improved HPLC method can easily be modified for the determination of lower inositol phosphates. The pH of the eluting buffer must be higher and the ionic strength must be lower than was used for the analysis of phytic acid.

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Part IV

Prediction of the bioavailability of minerals and trace elements in foods

CHAPTER 8

In vitro methods for the prediction of the bioavailability of minerals and trace elements in foods and their correlation with in vivo results (Review)

Introduction

Bioavailability, i.e. the availability of components for biological and biochemical processes in the organism, is a biological concept. Strictly speaking, it should be determined by measurements in vivo. Human in vivo studies, however, are time-consuming and expensive, and sometimes quite variable results are obtained. Laboratory animal in vivo studies are less expensive, but limited by uncertainties with regard to differences in metabolism between animal and man. In vitro methods offer an appealing alternative to human and animal in vivo studies in that they can be simple, rapid and comparatively inexpensive. It is, however, not possible to simulate all important physiological conditions in vitro, so the correlation with in vivo experiments is sometimes poor.

In the past few years, interest in the bioavailability of minerals and trace elements has increased because of environmental concerns in animal nutrition and health concerns in human nutrition. This has prompted the development of several types of in vitro methods for the prediction of the bioavailability of minerals and trace elements.

The in vitro methods can roughly be classified into three types:

- methods based on determination of soluble components under simulated physiological conditions
- methods based on determination of dialysable components under simulated physiological conditions
- methods based on determination of the molar ratio between a complexing agent and a mineral in the diet.

Most studies on mineral bioavailability have concerned iron, but some studies on zinc, copper, selenium, calcium and phosphorus have also been performed. The interest in the bioavailability of other minerals or trace elements has been minimal.

The development of in vitro methods and their usefulness in predicting the bioavailability of minerals and trace elements in vivo are reviewed below.

In vitro methods based on the determination of soluble components under simulated physiological conditions

In the period 1933-1938, several authors suggested that ionizable iron determined by extracting foods with a solution of α,α'-dipyridyl represented available iron (1,2,3,4,5,6). This method did not take into account the conditions in the stomach and the small intestine, which may influence the availability of iron in vivo. This was recognized in 1960 by Sanford (7), who introduced saline extraction and peptic digestion into the α,α'-dipyridyl method.

In 1969, Jacobs and Greenman (8) proposed a method for available iron based on the estimation of iron solubilized from foods by treatment with pepsin-hydrochloric acid at pH 1.5-2.0 for 90 min, simulating gastric digestion. They believed that the amount of iron soluble in the stomach would be available for absorption. Mg-Mg Thwin et al. (9) and Narasinga Rao and Prabhavathi (10) stated that most of the dietary iron is absorbed from the small intestine and not from the stomach. Therefore, they concluded that solubility under simulated conditions of the stomach does not determine the true availability of iron.

In 1980, Lock and Bender (11) studied the solubility of iron after incubation with human gastric juice for 90 min and the solubility of iron after incubation with pepsin-hydrochloric acid, simulating gastric juice, for 90 min at a pH between 1.4 and 2.8 as potential indicators for iron bioavailability. They concluded that the proportion of soluble iron liberated by incubation with human gastric juice correlates well with in vivo absorption of iron in man. Iron liberated by the pepsin-hydrochloric acid system did not correspond with available iron in vivo. Narasinga Rao and Prabhavathi (10) also concluded that there is no correlation between the proportion of soluble iron or ionizable iron measured under conditions simulating those prevailing in the stomach and iron absorption in vivo. Forbes et al. (12) reported a good correlation between solubility of iron and in vivo absorption for compounds having a high bioavailability of iron, and a poor correlation for compounds having a low bioavailability of iron.

In 1978, Narasinga Rao and Prabhavathi (10) described a method based on the release of ionizable iron after digestion with pepsin-hydrochloric acid at pH 1.35 for 90 min, and subsequent adjustment of the pH to 7.5, followed by 90 min incubation, simulating conditions in the stomach and small intestine respectively. They believed that bioavailability had to be determined under conditions prevailing in the small intestine and concluded that soluble iron released at pH 7.5 gives a reasonable correlation, and ionizable iron released at pH 7.5 gives a good correlation with in vivo absorption of iron in man. Others have used soluble or ionizable iron determined according to Narasinga Rao and Prabhavathi (with minor modifications) to estimate the bioavailability of iron (11,13,14,15). Some reported a poor correlation between both soluble and ionizable iron and in vivo absorption of iron in man (11,13), whereas a good correlation between ionizable iron and in vivo absorption was reported also (15).

The solubility of calcium after simulation of the conditions prevailing in the stomach and its solubility after simulation of the conditions in the stomach and small intestine as parameters for the bioavailability of calcium have also been studied. Both methods showed to correlate poorly with in vivo absorption of calcium in rats or in man (15,16,17,18,19).

The solubility of zinc after simulation of conditions in the stomach and in the small intestine has been studied by Zemel (15) and by Hunt et al. (20). Zemel reported a good correlation between soluble zinc and in vivo absorption in man, whereas Hunt et al. reported a poor correlation between soluble zinc and in vivo absorption in rats.

In vitro methods based on the determination of dialysable components under simulated physiological conditions

In 1981, Camire and Clydesdale (21) studied the binding of calcium, magnesium, zinc and iron to dietary fibre components using ultrafiltration or dialysis at a pH varying between 5 and 7, with membrane filters excluding the passage of molecules larger than 20,000 Da. They stated that metals that do not pass the membrane, are bound by the fibre components.

Fischer et al. (22) studied the influence of dietary zinc on the absorption of copper using everted duodenal segments of rats. In this way they tried to simulate the absorption process in the human small intestine.

In 1981, Miller et al. (23) introduced an in vitro method for the estimation of the bioavailability of iron using the proportion of iron diffusing across a semipermeable membrane as an index of iron bioavailability. They stated that their method differs from the methods described before (5,8,10,11) in two respects. First, pH adjustment from gastric to intestinal levels (crucial in simulating gastrointestinal digestion) is gradual and reproducible. Second, only low-molecular-weight soluble iron compounds are used in the estimation of available iron. The in vitro method of Miller et al. (23) consists of a pepsin-hydrochloric acid digestion performed at pH 2.0 for 120 min, followed by an adjustment of the pH to 7.5 by means of a 30 min dialysis with sodium hydrogen carbonate (at a concentration corresponding with the titratable acidity). Then, pancreatin and bile acids are added and the dialysis is continued for an additional 120 min. Miller et al. used a dialysis membrane with a molecular weight cut-off of 6000-8000. The authors conclude that their method correlates well with in vivo results and that it gives an excellent measure of relative iron availability. According to Hazell and Johnson (24), the pH adjustment proposed by Miller et al. (23) closely parallels the events occurring when food leaves the stomach and enters the duodenum. The use of diffusible-iron measurements in the estimation of available iron permits the discrimination between low- and high-molecular-weight soluble iron compounds. This is not possible in methods based on centrifugation to separate soluble and insoluble iron compounds.

Schricker et al. (25) have compared the in vitro availability of iron determined according to Miller et al. (23) with the in vivo availability of iron determined in rats and in man. They concluded that there is a significant correlation between the in vitro and human in vivo studies. There is also a significant correlation between the rat and the human in vivo methods, but correlations between the in vitro method and the rat in vivo method are less significant or, in some cases, not significant at all.

Others have used the proportion of dialysable iron determined according to Miller et al. (23) (with minor modifications) to estimate the bioavailability of iron from foods. All reported good correlations between results of in vitro and human in vivo experiments (12,24,26,27). Forbes et al. (12), however, strongly recommend standardization of the in vitro method. According to Miller et al. (23) and Hazell and Johnson (24), in vitro measurements of nutrient availability should be considered a relative rather than an absolute predictor of availability.

The bioavailability of calcium has been estimated in vitro by Walsh et al. (28) using ultrafiltration after peptic digestion at pH 1.9 (30 min) and pancreatic digestion at pH 7.0 (125 min). They concluded that an in vitro system is no substitute for bioassays, but that it may provide preliminary information useful to subsequent in vivo studies.

Hunt et al. (20) used the proportion of dialysable zinc as an estimate for zinc bioavailibility. After peptic and pancreatic digestion, the digests were dialysed against water for 48 h at 5 °C. The dialysis tubing had a molecular weight cut-off of 1000. The authors obtained a poor correlation between in vitro and rat in vivo experiments. They attributed this to the fact that important physiological factors could not be simulated in vitro. Sandström et al. (29,30,31) used a modification of the in vitro method of Miller et al. (23) to study zinc availability from foods. The proportion of dialysable zinc was determined after peptic digestion at pH 1 and after peptic and tryptic digestion at pH 8. The authors conclude that dialysable zinc at pH 1 gives a poor correlation, while dialysable zinc at pH 8 gives a good correlation with in vivo absorption in man.

In vitro method based on the determination of the molar ratio between a complexing agent and a mineral in the diet

In 1975, Oberleas (32) proposed a method to estimate the bioavailability of zinc which is quite different from the methods simulating digestion in vivo described above. As phytate is a very strong chelator for zinc, Oberleas suggested that the bioavailability of zinc might depend upon the phytate/zinc molar ratio of the diet. He proposed the phytate/zinc molar ratio to be used as an indicator of zinc availability.

Davies and Olpin (33), and Morris and Ellis (34,35,36) investigated the proposal of Oberleas (32). Their results supported Oberleas' notion that the main determinants of zinc availability in phytate-containing foods are the relative amounts of phytate and zinc. Several groups have used the phytate/zinc molar ratio to study the bioavailability of zinc in human foods (37,38,39,40,41,42,43,).

As calcium has been shown to decrease zinc absorption in the presence of phytate, it was suggested that the dietary phytate x calcium/zinc molar ratio may provide an even more useful measure of zinc bioavailability than the phytate/zinc molar ratio alone (44,45). It has been suggested that ratios equal to or greater than 200 may induce marginal zinc deficiency in man (45). The phytate x calcium/zinc molar ratio was used to provide an index of zinc bioavailability in man (41,42,43,46). Fordyce et al. (46) investigated the usefulness of the phytate x calcium/zinc molar ratio as a predictor of zinc bioavailability to rats. They concluded that the phytate x calcium/zinc ratio is a better predictor of zinc bioavailability than the phytate/zinc ratio. However, in some cases the phytate x calcium/zinc molar ratio is not useful since some processing procedures apparently alter the binding of minerals and trace elements to phytic acid and to other food components.

Conclusion

Several in vitro methods for estimation of the bioavailability of minerals and trace elements have been developed. The most promising in vitro methods are based on the determination of the amount of dialysable minerals and trace elements under simulated physiological conditions. These in vitro methods show reasonable correlations with in vivo bioavailability. However, the prediction of bioavailability of minerals and trace elements with in vitro methods is only relative because it is not possible to simulate all important physiological conditions prevailing in the stomach and the small intestine.

For prediction of Zn bioavailability the phytate x calcium/zinc molar ratio of a diet seems promising.

CHAPTER 9

A continuous in vitro method for estimation of the bioavailability of minerals and trace elements in foods: application to breads varying in phytic acid content

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Abstract

A continuous in vitro method for the estimation of the bioavailability of minerals and trace elements is presented. This in vitro method is believed to be more representative of in vivo physiological conditions than in vitro methods based on equilibrium dialysis, because dialysable components are continuously removed from the pancreatic digestion mixture.

The continuous in vitro method is compared with the equilibrium in vitro method with respect to the dialysability of Ca, Mg, Fe, Cu and Zn from eight different types of bread (varying in phytic acid content). The results show a pronounced effect of continuous removal of dialysable components from the pancreatic digestion mixture on the dialysability of minerals and trace elements. Furthermore, removal of dialysable components influences the effect of phytic acid on the bioavailability of minerals and trace elements. For these two reasons the importance of removal of dialysable components in vitro for the estimation of bioavailability in vivo needs further investigation.

The bioavailability of minerals and trace elements from bread samples is not related to the phytic acid content only. Therefore, the effect of phytic acid on the bioavailability of minerals and trace elements cannot be studied separately from the effects of other components on bioavailability.

Introduction

A good mineral balance is of importance to animal and man. Deficiency, overdose, or imbalances between inorganic nutrients have a negative effect on health (47,48,49).

Peptic digestion

25 g of dry food sample was suspended in 200 ml Milli Q water (Millipore Co.) in a plastic bottle. After setting the pH to 2.1 with HCl, 7.5 ml of pepsin suspension was added. The pH was adjusted to 2.00 ± 0.03, the weight of the sample was brought to 250 g with Milli Q water and the sample was incubated in a shaking water bath at 37 °C for 2 h. The pH was adjusted to 2.00 every 30 min.

pH adjustment for pancreatic digestion

The titratable acidity was determined as described by Hazell and Johnson (24).
The suspension after peptic digestion was divided into 5 portions of 20 g each, which were transferred into plastic bottles. Segments of dialysis tubing (MW cut-off 12,000-14,000, diameter 28.6 mm, Spectra/Por, Spectrum, Houston, TX, USA) containing an amount of 6 % $NaHCO_3$ equivalent to the titratable acidity, filled up to 25 ml with Milli Q water, were placed in each bottle. The bottles were incubated in a shaking water bath for 30 min at 37 °C. For one bottle the incubation was stopped at this moment ($t = 0$).

Pancreatic digestion with equilibrium dialysis

To each of the four remaining bottles 5 ml pancreatin/bile extract mixture was added, and the samples were incubated in a shaking water bath at 37 °C for 0.5, 1, 2.5 or 4 h ($t = 0.5$, $t = 1$, $t = 2.5$ and $t = 4$) respectively. Depending on the buffering capacity of the food samples, the resulting pH after dialysis against $NaHCO_3$ and addition of the pancreatin/bile extract mixture varied between 6.7 and 7.0. At the end of the pancreatic digestion the pH was measured; during pancreatic digestion the pH remained fairly constant.
In the dialysates the concentrations of Ca, Mg, Fe, Cu and Zn were determined. A blank was run in each experiment to correct for small amounts of dialysable minerals and trace elements from the reagents.

The method is based on the formation of an equilibrium across a semipermeable membrane. In general, an equilibrium is reached after 2.5 h. Consequently, the amount of dialysed Ca, Mg, Fe, Cu and Zn is calculated as the mean value at $t = 2.5$ and $t = 4$. As the volumes at both sides of the membrane are equal, the amounts of dialysed Ca, Mg, Fe, Cu and Zn represent only half of the amounts dialysable. For this reason the amounts of dialysable minerals and trace elements are calculated as twice the amount dialysed. The dialysability is expressed as a percentage of the amounts of Ca, Mg, Fe, Cu and Zn present in the food sample. The dialysability is calculated according to the following equation:

$$\text{dialysability (\%)} = \frac{2 \times D}{W \times A} \times 100$$

where: D = amount of mineral dialysed, calculated as the mean of the values at $t = 2.5$ and $t = 4$ (in mg)
W = dry weight of the food sample used for pancreatic digestion (in g)
A = concentration of mineral present in dry food sample (in mg/g).

In vitro method with continuous dialysis using a hollow fibre

The in vitro method with continuous dialysis is partly based on the in vitro methods described by Miller et al. (23) and Hazell and Johnson (24). The simulation of the small intestine is different. Furthermore, samples and reagents are used twice as concentrated as described for the method with equilibrium dialysis because otherwise the levels of minerals and trace elements in the (continuous) dialysate are sometimes too low to allow accurate determination.

The continuous in vitro method consists of three parts: peptic digestion, pH adjustment and pancreatic digestion with continuous dialysis.

Peptic digestion

50 g dry food sample was suspended in 175 ml Milli Q water (Millipore Co.) in a plastic bottle. After adjustment of the pH to 2.1 with HCl, 15 ml pepsin suspension was added. The pH was adjusted to 2.00 ± 0.03, the weight of the sample was brought to 250 g with Milli Q water and the sample was incubated in a shaking water bath at 37 °C for 2 h. The pH was adjusted to 2.00 every 30 min.

pH adjustment for pancreatic digestion

Titratable acidity was determined as described by Hazell and Johnson (24). After peptic digestion, 20 g suspension was transferred into a reaction vessel. A segment of dialysis tubing (MW cut-off 12,000-14,000, diameter 28.6 mm, Spectra/Por, Spectrum, Houston, TX, USA) containing an amount of 6 % $NaHCO_3$ equivalent to the titratable acidity, filled up to 5 ml with Milli Q water, was placed into the reaction vessel. The reaction vessel was incubated for 30 min at 37 °C in a shaking water bath, and the contents of the dialysis tubing were added to the reaction vessel. The dialysis tubings were rinsed with 5 ml Milli Q water which was also added to the reaction vessel.

Pancreatic digestion with continuous dialysis

5 ml pancreatin/bile extract mixture was added to the reaction vessel and the mixture was incubated for 4 h at 37 °C. During this pancreatic digestion the mixture was led through a hollow-fibre system. Every 30 min the dialysate was collected. As in the in vitro method with equilibrium dialysis, the resulting pH after addition of $NaHCO_3$ and pancreatin/bile extract mixture varied between 6.7 and 7.0. During pancreatic digestion the pH in the reaction vessel decreased by 0.1 - 0.4 units (depending on the type of sample).

The concentrations of Ca, Mg, Fe, Cu and Zn in the dialysates were determined. Every 4 or 5 experiments a blank experiment was carried out to correct for small amounts of dialysable minerals and trace elements from the reagents.

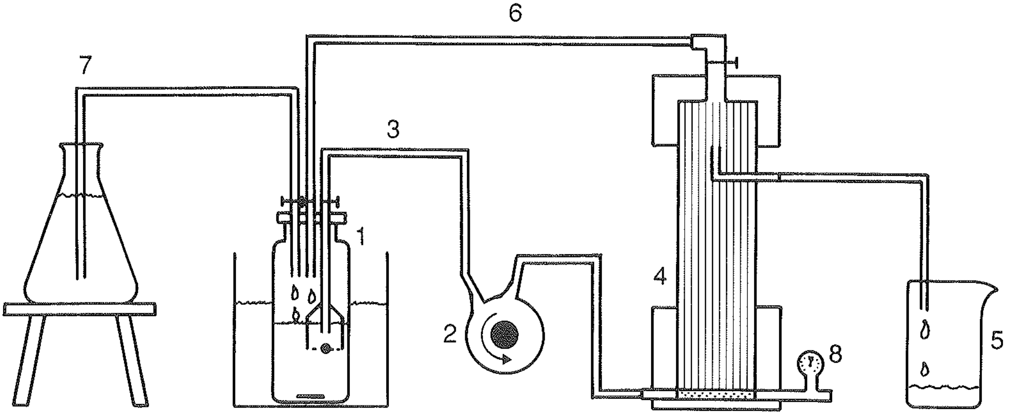

Figure 1: Schematic representation of hollow-fibre system for continuous dialysis

The hollow-fibre system is represented schematically in Figure 1. The reaction vessel (1) is placed in a water bath of 41 °C (the temperature inside the reaction vessel is 37 °C). In this reaction vessel pancreatic digestion is taking place. The suspension is pumped via a peristaltic pump (2) through the suction tube (3) into the hollow fibre (4) (Amicon, MW cut-off 10,000, Type H1P3-20, Amicon Division, W.R. Grace & Co., Danvers, MA, USA). Components in the suspension that can pass the hollow-fibre membrane are dialysed and collected in a plastic bottle (5). The dialysis flow rate is 2 ml/min. That part of the suspension that cannot pass the hollow-fibre membrane is pumped back into the reaction vessel via the recycle tube (6). In the reaction vessel these components can be digested further. The recycle flow rate is 50 ml/min. The volume in the reaction vessel is kept constant by a siphon (7). The siphon vessel is filled with Milli Q water (pH 7.0).
The suction tube has a plastic cap on the end. This cap has some holes on the top to maintain a constant pressure inside and outside the cap. A fine filter cloth is stretched over the plastic cap to prevent large food particles from entering the hollow-fibre system. A magnetic stirrer is put inside the plastic cap to prevent clogging of the filter cloth. A filter is placed in front of the hollow-fibre membrane (4) to prevent clogging of the membrane. The pressure on the hollow-fibre membrane can be measured by a manometer (8). This pressure may not exceed 170 kPa.
After each experiment the hollow-fibre system is cleaned by pumping successively 0.1 M HCl and water through the system.

The amount of dialysed material (= dialysability) is expressed as a percentage of the total amount present in the food sample. The dialysability is calculated according to the following equation:

$$\text{dialysability (\%)} = \frac{D}{W \times A} \times 100$$

where: D = total amount of mineral dialysed in 4 h (in mg)
W = dry weight of food sample used for pancreatic digestion (in g)
A = concentration of mineral present in dry food sample (in mg/g)

Investigation of interactions between the hollow-fibre membrane and Ca, Mg, Fe, Cu and Zn

The possibility of interactions between the hollow-fibre membrane and Ca, Mg, Fe, Cu and Zn was investigated by dialysing pure salts. $CaCl_2$ was dissolved in Milli Q water (pH 7.0). $MgSO_4$ was dissolved in 0.1 M phosphate buffer (pH 7.0). $FeSO_4$ was dissolved in 0.1 M phosphate buffer (pH 7.0). A precipitate was formed immediately. The supernatant was filtered and the Fe content determined. As the amounts of $CuSO_4$ and $ZnCl_2$ soluble in phosphate buffer of pH 7.0 were too small to be dialysed and analysed accurately, these salts were dissolved in 0.1 M Tris buffer (pH 7.0).
The clear solutions were put in the reaction vessel of the hollow-fibre system and dialysed for 4 h. Other reagents were not added. The siphon vessel contained Milli Q water during the experiment with Ca, 0.1 M phosphate buffer (pH 7.0) during the experiments with Mg and Fe, and 0.1 M Tris buffer (pH 7.0) during the experiments with Cu and Zn. Ca, Mg, Fe, Cu and Zn were determined in the dialysate and in the reaction vessel after dialysis.

Analytical methods

Phytic acid was determined after extraction with dilute HCl by ion chromatography with post-column derivatization and UV detection as described by Bos et al. (60).
Ca, Mg and Zn were determined with flame atomic absorption spectroscopy. Fe and Cu were determined with graphite-furnace atomic absorption spectroscopy.

Results and discussion

To estimate the bioavailability of minerals and trace elements a continuous in vitro method was developed as an alternative to the equilibrium in vitro method developed by Miller et al. (23). In the equilibrium in vitro method components that pass the membrane are not removed, in contrast with the situation in vivo. As we expect these dialysable components to influence the equilibrium dialysis of minerals and trace elements, we studied the influence of continuous removal of dialysable components from the pancreatic digestion mixture on the dialysability of Ca, Mg, Fe, Cu and Zn.

Investigation of interactions between the hollow-fibre membrane and Ca, Mg, Fe, Cu and Zn

The hollow-fibre membranes are made of polysulphone and should be inert. Still, we investigated whether Ca, Mg, Fe, Cu or Zn bind to the hollow-fibre membrane, because this might lead to errors. Possible interactions were investigated by dialysis of pure salts.

It was found that the Ca from $CaCl_2$ solubilized in water (pH 7.0), the Mg from $MgSO_4$ solubilized in phosphate buffer (pH 7.0), the Cu from $CuSO_4$ and the Zn from $ZnCl_2$ solubilized in Tris buffer (pH 7.0) were completely recovered.

Dialysis experiments with $FeSO_4$ were hampered because the phosphate buffer appeared to be severely contaminated with Fe. When only this phosphate buffer was dialysed in the hollow-fibre system, the Fe from this buffer was completely recovered.

No evidence was found for binding of Ca, Mg, Fe, Cu or Zn to the hollow-fibre membrane at pH 7. This, in combination with the inert character of the polysulphone membrane, leads to the conclusion that dialysability measurements are not likely to be disturbed by binding of minerals or trace elements to the hollow-fibre membrane.

Repeatability of the continuous in vitro method

The repeatability of the continuous in vitro method was tested with a sample of whole wheat meal. The dialysability of Ca, Mg, Fe, Cu and Zn was tested in triplicate. The results (mean value and standard deviation, in %) were: Ca, 35 ± 4; Mg, 57 ± 3; Fe, 21 ± 3; Cu, 76 ± 5; Zn, 24 ± 6.

It is concluded that the repeatability of the determination of the dialysability of Ca, Mg, Fe, Cu and Zn with the continuous in vitro method is good.

Influence of pH during pancreatic digestion on dialysability

The solubility of minerals and trace elements decreases and the binding of minerals and trace elements by dietary fibre and phytic acid increases with increasing pH (14,61,62,63,64,65,66). Therefore, it is expected that the pH influences the bioavailability of minerals and trace elements. We investigated the influence of pH during pancreatic digestion on the dialysability of Ca, Mg, Fe, Cu and Zn from a sample of rye bread. Dialysability was measured with both the in vitro method with continuous dialysis and the in vitro method with equilibrium dialysis. The dialysability with the equilibrium in vitro method was studied at pH 6.2, 6.6, 6.9, 7.1 and 7.4, while the dialysability with the continuous in vitro method was studied at pH 6.6 and 7.1. The results are shown in Table 1. For the in vitro method with equilibrium dialysis there is a marked decrease in the dialysability of Ca and a slight decrease in dialysability of Fe with increasing pH. The dialysability of Mg, Cu and Zn was not affected by pH. For the in vitro method with continuous dialysis there is a decrease in the dialysability of Ca, Mg, Fe and Cu with increasing pH. The dialysability of Zn is not affected.

Table 1: Influence of pH on dialysability of Ca, Mg, Fe, Cu and Zn from rye bread (in % of the amount present)

Element	pH	In vitro method with equilibrium dialysis	In vitro method with continuous dialysis
Ca	6.2	74	-
	6.6	68	73
	6.9	50	-
	7.1	44	33
	7.4	42	-
Mg	6.2	68	-
	6.6	69	66
	6.9	68	-
	7.1	68	55
	7.4	69	-
Fe	6.2	22	-
	6.6	21	30
	6.9	22	-
	7.1	18	23
	7.4	18	-
Cu	6.2	38	-
	6.6	40	95
	6.9	39	-
	7.1	38	59
	7.4	41	-
Zn	6.2	46	-
	6.6	48	50
	6.9	47	-
	7.1	46	48
	7.4	49	-

As the pH influence may depend on the type of sample, we investigated the influence of pH on the dialysability of Ca, Mg, Fe, Cu and Zn from carrots for the in vitro method with equilibrium dialysis (data not shown). For carrots the dialysability of Ca, Mg, Fe and Zn was influenced by pH. Only the dialysability of Cu was not influenced. This demonstrates that the pH influence is dependent on the type of food sample.
These results clearly show that there is a great influence of pH during pancreatic digestion on the dialysability of minerals and trace elements. The actual influence depends on the type of food sample and on the in vitro method used. Miller et al. (23) found no or only a very small influence of pH during pancreatic digestion on the dialysability of Fe as estimated with the in vitro method with equilibrium dialysis. This may be explained by the type of samples studied by Miller (complex meals).

Dialysability of Ca, Mg, Fe, Cu and Zn from several types of bread: comparison between equilibrium dialysis and continuous dialysis

The dialysability of Ca, Mg, Fe, Cu and Zn from eight different types of bread was determined with both the equilibrium in vitro method and the continuous in vitro method presented in this paper. Figure 2 shows the results of the continuous dialysis of Ca, Mg, Fe, Cu and Zn from wholemeal wheat bread. The dialysis was almost complete after 3 h. The dialysability was calculated as the total amount of Ca, Mg, Fe, Cu or Zn dialysed in 4 h.

In the first hour, the amount of dialysed minerals and trace elements is very high. This may partly be due to the high initial concentration of minerals and trace elements and partly to the pH. At $t = 0$, the pH varies between 5 and 5.5 and the pancreatin/bile extract mixture is added. In the next 30 min the pH rises to 7. As is shown in Table 1, a lower pH generally results in a higher dialysability. A similar pH course was found in the equilibrium in vitro method. The pH course observed in vitro agrees well with the in vivo situation: the pH in the duodenum is lower than in the jejunum, where the pH is about 7 (67). In the duodenum a large part of the minerals and trace elements is absorbed (68,69,70,71,72), but it is not clear whether the higher initial concentration and the lower pH are the only important factors here.

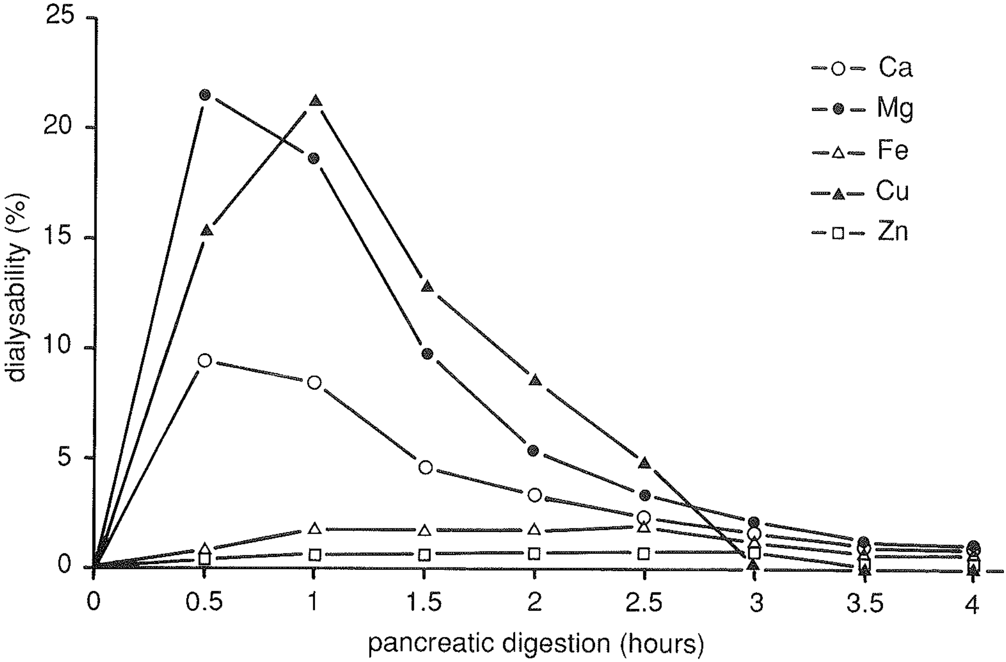

Figure 2: Continuous dialysis of Ca, Mg, Fe, Cu and Zn from wholemeal wheat bread

Table 2: Dialysability of Ca, Mg, Fe, Cu and Zn (in % from the present content) from eight different types of bread determined with the equilibrium in vitro method and the continuous in vitro method*

Type of bread	In vitro method	Dialysability				
		Ca	Mg	Fe	Cu	Zn
White bread	equilibrium	12	52	36	50	27
	continuous	53	76	46	64	27
Brown bread	equilibrium	22	56	14	44	11
	continuous	30	64	27	67	15
Wholemeal wheat bread	equilibrium	17	51	7	41	5
	continuous	34	66	11	63	7
Rye bread	equilibrium	33	66	11	25	4
	continuous	43	63	13	57	10
Brown bread with sunflower seeds	equilibrium	12	41	7	61	7
	continuous	40	59	29	71	16
White bread with hazelnuts	equilibrium	16	50	6	37	38
	continuous	31	62	9	60	44
Sour-dough-fermented brown bread	equilibrium	32	63	9	47	13
	continuous	35	68	12	72	12
Sour-dough-fermented brown bread with sunflower seeds	equilibrium	15	45	13	63	14
	continuous	42	61	25	78	17

*Mean of duplicate analyses

As a consequence of the continuous removal of dialysable components in the continuous in vitro method, the pH during the 4-hour pancreatic digestion decreases slightly. This is in agreement with the situation found in in vivo experiments with pigs: the pH in the ileum is slightly lower than in the jejunum (67). In the equilibrium in vitro method, on the other hand, the pH remains rather constant during pancreatic digestion.

The dialysability of Ca, Mg, Fe, Cu and Zn from eight different types of bread as determined with the in vitro method with equilibrium dialysis and the in vitro method with continuous dialysis is presented in Table 2. In general, the dialysability of Ca, Mg, Fe and Cu determined with the continuous in vitro method is higher than determined with the equilibrium in vitro method (up to a factor 3 to 4). For Zn both methods yield comparable dialysabilities. Linear regression analysis showed a positive linear correlation between the dialysabilities of Fe, Cu and Zn as determined with the two in vitro methods (r = 0.84, r = 0.89 and r = 0.96 respectively). For Mg and Ca no correlation was found.

Table 3: Contents of phytic acid (g/kg d.m.), Ca, Mg, Fe, Cu and Zn (mg/kg d.m.) in various types of bread

Type of bread	Phytic acid	Ca	Mg	Fe	Cu	Zn
White bread	0.1	360	320	17	2	11
Brown bread	2.9	470	890	32	4	20
Wholemeal wheat bread	4.3	880	1020	44	4	27
Rye bread	2.0	430	1100	35	4	38
Brown bread with sunflower seeds	8.2	570	1670	47	6	27
White bread with hazelnuts	1.6	620	480	22	4	12
Sour-dough-fermented brown bread	0.5	360	760	34	3	17
Sour-dough-fermented brown bread with sunflower seeds	4.0	470	1380	40	7	29

These results show that removal of dialysable components from the pancreatic digestion mixture has a marked influence on the dialysability of minerals and trace elements. Whether removal of dialysable components leads to better estimates of bioavailability in vivo is currently being investigated.

The influence of phytic acid on the dialysability of Ca, Mg, Fe, Cu and Zn.

The breads were chosen such as to contain variable amounts of phytic acid, because phytic acid is known to have a strong negative influence on the bioavailability of some minerals and trace elements. The contents of phytic acid, Ca, Mg, Fe, Cu and Zn in the breads are presented in Table 3. Table 2 shows the dialysability of Ca, Mg, Fe, Cu and Zn from the breads.

In the range of white bread, brown bread and wholemeal wheat bread the phytic acid content increases, whereas the dialysability of Fe and Zn as determined with the equilibrium in vitro method decreases dramatically. There is a smaller decrease in the dialysability of Cu. For the continuous in vitro method the dialysability of Ca, Fe and Zn decreases with increasing phytic acid content. There is a smaller decrease in dialysability of Mg. These results agree with other studies where in vitro and in vivo experiments have shown a strong negative influence of phytic acid on the bioavailability of Ca, Fe and Zn, and a less marked influence on the bioavailability of Mg and Cu (56,57).

Addition of sunflower seeds to brown bread leads to a 3-fold increase in phytic acid content. This leads to a decrease in the dialysability of Ca, Mg, Fe and Zn as determined with the equilibrium in vitro method. The dialysability of Cu increased. Apart from a small increase in Ca dialysability, the continuous in vitro method results in similar dialysabilities for brown bread and brown bread with sunflower seeds. This shows that phytic acid is not the only component influencing the bioavailability of minerals and trace elements from bread samples. Therefore, the effect of phytic acid cannot be studied separately from the effects of other components on the bioavailability of minerals and trace elements.

Addition of hazelnuts to white bread leads to an increase in phytic acid content. At the same time the dialysability of Fe and Cu as determined with the equilibrium in vitro method decreases. When determined with the continuous in vitro method the dialysability of Ca, Mg and Fe decreases. Despite the rise in phytic acid content, both in vitro methods show an increase in Zn dialysability.

Sour-dough fermentation of bread results in a significant reduction in phytic acid content. This has been reported before by van Lonkhuijsen and van Gelderen (73). Although the equilibrium in vitro method shows a small increase in Ca dialysability for sour-dough-fermented brown bread and a small increase in Fe and Zn dialysability for sour-dough-fermented brown bread with sunflower seeds, neither the equilibrium in vitro method nor the continuous in vitro method show a clear positive influence of the decrease in phytic acid content on the dialysability of Ca, Mg, Fe, Cu and Zn. In general, reduction of the phytic acid content by sour-dough fermentation is believed to increase the bioavailability of minerals and trace elements because the phytic acid content is reduced. However, the phosphate produced from the phytic acid during sour-dough fermentation may also have a negative effect on the bioavailability of minerals and trace elements (74,75,76,77). Moreover, some lower inositol phosphates may influence the bioavailability of minerals and trace elements (14, 55,78).

It should be noted that although the relative dialysability of minerals and trace elements is lower for certain breads, the absolute bioavailability may be higher due to the higher absolute content of minerals and trace elements present in these breads (Table 2).

Our results show that for white bread, brown bread and wholemeal wheat bread there is a marked negative effect of phytic acid on the dialysability of Ca, Fe and Zn. Results for brown bread with sunflower seeds, white bread with hazelnuts and the two types of sour-dough-fermented bread, however, show that the bioavailability of minerals and trace elements from bread samples is not related to the phytic acid content only. Therefore, it is concluded that the effect of phytic acid on the bioavailability of minerals and trace elements cannot be studied separately from the effects of other components on this bioavailability. Removal of dialysable components as performed in the continuous in vitro method appears to have a marked effect on the influence of phytic acid on the bioavailability of minerals and trace elements. This once more stresses the importance of a better understanding of the effect of removal of dialysable components.

Conclusions

The in vitro method with continuous dialysis for the estimation of the bioavailability of minerals and trace elements presented here takes continuous removal of dialysable components into account.

Experiments showed that there is no interaction between the hollow-fibre membrane used in the continuous in vitro method and Ca, Mg, Fe, Cu or Zn ions. Therefore, dialysability measurements are not likely to be disturbed by binding of minerals or trace elements to the hollow-fibre membrane.

There is a large influence of the pH during pancreatic digestion on the dialysability of Ca, Mg, Fe, Cu and Zn determined with both in vitro methods. The actual influence depends on the type of sample and on the in vitro method used.

For most types of bread the continuous in vitro method leads to higher dialysabilities of Ca, Mg, Fe and Cu than the equilibrium in vitro method. The dialysability of Zn is for most breads comparable for the two methods. It is concluded that removal of dialysable components has a marked influence on the dialysability of some minerals and trace elements. However, the importance of this finding for the estimation of bioavailability in vivo needs further investigation.

Phytic acid has a negative effect on the bioavailability of Ca, Fe and Zn. However, the effect of phytic acid cannot be studied separately from effects of other components on the bioavailability of minerals and trace elements from breads. It is shown that removal of dialysable components in vitro influences the effect of phytic acid on the bioavailability of minerals and trace elements.

CHAPTER 10

Validation of the use of in vitro methods for the prediction of the bioavailability of minerals and trace elements in pigs

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Abstract

The use of an in vitro method based on equilibrium dialysis and an in vitro method based on continuous dialysis for the prediction of the bioavailability of Ca, Mg, Fe, Cu and Zn is validated in an in vivo experiment with piglets. In addition, the influence of tannins on the bioavailability of these minerals and trace elements in pig diets is investigated by combining results of in vivo availability, in vitro availability and the availability calculated with a mathematical model.

There is a discrepancy between the absolute values of the bioavailability of Ca, Mg, Fe, Cu and Zn determined in piglets, and the availability of these minerals and trace elements determined in vitro. However, both the equilibrium in vitro method and the continuous in vitro method represent the effects of differences in the composition of the pig diets on the bioavailability of minerals and trace elements in vivo well. Therefore, it is concluded that the in vitro methods can be used for a relative prediction of the bioavailability of minerals and trace elements.

By combining the results of the in vitro experiments, the in vivo experiment and the mathematical model predictions, it is shown that tannins decrease the availability of Fe and Cu, while they do not seem to affect the availability of Ca, Mg and Zn.

Introduction

A good mineral balance is of importance to animal and man. Deficiency, overdosis or imbalances between minerals or trace elements will have a negative effect on health. In general, however, it is not the ingested dose of a mineral that is important to maintain balance, but rather the amount that is bioavailable (available for biological and biochemical processes in the organism).

The best way to study the bioavailability of minerals and trace elements for animals and man is an in vivo experiment with the target object. Unfortunately, in vivo studies with large animals or test persons are time-consuming and expensive, and often quite variable results are obtained which are difficult to interpret. The rat is often used as a model for man (12,20,25,55,58). These experiments, however, are limited by uncertainties with regard to differences in metabolism between rat and man. Recently, Reddy and Cook (59) have shown that rat studies cannot be used for assessment of the quantitative importance of dietary factors in iron nutrition.

In vitro methods for the estimation of the bioavailability of minerals and trace elements offer an appealing alternative to human and animal in vivo experiments because they are relatively simple, rapid and inexpensive. In addition, experimental conditions can be well controlled. Although several types of in vitro methods for the estimation of the bioavailability of minerals and trace elements have been developed, correlation with in vivo availability is generally poor (10,11,12,13,17,18).

In 1981, Miller et al. (23) introduced an in vitro method using the dialysability of Fe under simulated conditions of the stomach and the small intestine as an index for its bioavailability. This method has been the basis for several in vitro methods for the estimation of the bioavailability of Fe and Zn. Promising correlations between in vitro dialysability and in vivo bioavailability have been reported (12,24,25,27,31). Wolters et al. (79) developed an in vitro method with continuous dialysis. Because dialysable components are continuously removed from the pancreatic digestion mixture, this in vitro method was considered to be more representative of in vivo physiological conditions than in vitro methods based on equilibrium dialysis.

Recently, a mathematical modelling approach was presented (80). The resulting mathematical model was shown to describe and predict the in vitro availability of minerals and trace elements, as determined with the equilibrium in vitro method, quite well on the basis of the concentrations of food components influencing this availability. The mathematical model proved also valuable for the investigation of the relative influences of food components on the availability of minerals and trace elements (80). Although it has been suggested that tannins reduce the bioavailability of minerals and trace elements (54,81,82,83,84), the influence of tannins was not included in that study.

The objectives of the study presented in this paper are:

1. to validate the use of the in vitro method based on equilibrium dialysis and the in vitro method based on continuous dialysis for prediction of the bioavailability of Ca, Mg, Fe, Cu and Zn in pigs;
2. to investigate the influence of tannins on the bioavailability of Ca, Mg, Fe, Cu and Zn.

The bioavailability of Ca, Mg, Fe, Cu and Zn was determined in an in vivo experiment with piglets. The in vitro availability of these minerals and trace elements in the pig diets was determined with both the equilibrium method and the continuous method and compared with in vivo availability. Pig diets were studied composed such as to differ in tannin content only. To investigate the influence of tannins on the availability of Ca, Mg, Fe, Cu and Zn, results of in vivo availability, in vitro availability and the availability calculated with the mathematical model are compared.

Materials and methods

Pig diets

Five different pig diets were investigated. The diets I to V contain 20 g of *Vicia faba* hulls per 100 g feed, consisting of an increasing proportion of hulls with a high content of condensed tannins (cv. Alfred) and a decreasing proportion of hulls with a low content of condensed tannins (cv. Toret). This results in an increasing level of condensed tannins from diet I to V. The composition of the diets is presented in Table 1. The contents of Ca, Mg, Fe, Cu, Zn and P in the pig diets were adjusted to the physiological requirements of the animals according to the National Research Council (85). Chromium oxide (Cr_2O_3) was added to the pig diets as a transit time marker.

In vitro methods for the estimation of the bioavailability of minerals and trace elements

The in vitro method with equilibrium dialysis and the in vitro method with continuous dialysis are described below. As the two methods have been described in detail before (79), only a summary will be presented here.

In vitro method with equilibrium dialysis
The in vitro method with equilibrium dialysis was performed according to Miller et al. (23) and Hazell and Johnson (24,26) with slight modifications. To simulate peptic digestion, a sample was suspended in Milli Q water, pepsin suspension was added, the pH was adjusted to 2.00, and the mixture was incubated for 2 h at 37 °C in a shaking water bath. Every 30 min the pH was adjusted to 2.00.
After the peptic digestion, the suspension was divided into 5 portions, which were transferred into plastic bottles. In these portions the pH was adjusted to 7 by means of the following dialysis procedure: segments of dialysis tubing (MW cut-off 12,000 - 14,000, diameter 28.6 mm, Specta/Por, Spectrum, Houston, TX, USA) containing an amount of $NaHCO_3$ equivalent to the titratable acidity, filled up to 25 ml with Milli Q water, were placed in each bottle and dialysis was performed for 30 min at 37 °C. For one of the five portions the incubation was stopped at this moment ($t = 0$).
To each of the remaining portions a pancreatin/bile extract mixture was added and the pancreatic digestion was performed in a shaking water bath of 37 °C for 0.5, 1, 2.5 or 4 h respectively ($t = 0.5$, $t = 1$, $t = 2.5$, $t = 4$). In the dialysates the concentrations of Ca, Mg, Fe, Cu and Zn were determined by atomic absorption spectroscopy (AAS).
The amount of dialysed minerals and trace elements is calculated as the mean value of $t = 2.5$ and $t = 4$. Because the volumes at both sides of the dialysis membrane are equal, the amount of dialysed minerals and trace elements represents only half the amount dialysable. Therefore the dialysability is calculated as twice the amount

Table 1: Composition of the pig rations I to V (g/100 g)

Ingredients	Ration I	Ration II	Ration III	Ration IV	Ration V
Maize starch	18.45	18.45	18.45	18.45	18.45
Casein	9.00	9.00	9.00	9.00	9.00
Dextrose	15.00	15.00	15.00	15.00	5.00
Vicia faba cotyledons (cv. Alfred)	25.00	25.00	25.00	25.00	25.00
Vicia faba hulls (cv. Toret)	20.00	15.00	10.00	5.00	0.00
Vicia faba hulls (cv. Alfred)	0.00	5.00	10.00	15.00	20.00
Sunflower oil	5.05	5.05	5.05	5.05	5.05
Molasses	2.00	2.00	2.00	2.00	2.00
Chalk	1.20	1.20	1.20	1.20	1.20
$Ca_3(PO_4)_2$	1.50	1.50	1.50	1.50	1.50
NaCl	0.50	0.50	0.50	0.50	0.50
$KHCO_3$	0.40	0.40	0.40	0.40	0.40
$NaHCO_3$	0.50	0.50	0.50	0.50	0.50
DL-methionine	0.13	0.13	0.13	0.13	0.13
L-threonine	0.01	0.01	0.01	0.01	0.01
Premix*	1.00	1.00	1.00	1.00	1.00
Cr_2O_3	0.25	0.25	0.25	0.25	0.25

* The premix provides per kg feed: 9000 I.E. vit. A, 1800 I.E. vit. D-3, 40 mg vit. E, 5 mg riboflavin, 39 mg niacin, 12 mg *d*-panthothenic acid, 1000 mg choline chloride, 0.04 mg vit. B-12, 2 mg vit. thiamin, 3 mg vit. B-6, 0.1 mg biotin, 1 mg folin, 3 mg vit. K, 50 mg vit. C, 200 mg $ZnSO_4 . H_2O$, 15 mg MnO_2, 400 mg $FeSO_4 . 7H_2O$, 2.5 mg $CoSO_4 . 5H_2O$, 0.5 mg KI, 40 mg $CuSO_4 . 5H_2O$, Tylosin.

dialysed. The dialysability is expressed as a percentage of the amount of minerals and trace elements present in the sample.

In vitro method with continuous dialysis

The main difference between the continuous in vitro method and the equilibrium in vitro method concerns the simulation of the small intestine. In the continuous in vitro method, dialysable components (including minerals and trace elements) are continuously removed, using a hollow-fibre system.

A peptic digestion was performed by suspending a sample in Milli Q water, adding pepsin suspension, adjusting the pH to 2.00, and incubating the mixture for 2 h at 37 °C in a shaking water bath. Every 30 min the pH was adjusted to 2.00.

The peptic digestion mixture was transferred to a reaction vessel. The pH of this mixture was increased to 7 by means of the following dialysis procedure: a segment of dialysis tubing (MW cut-off 12,000 - 14,000, diameter 28.6 mm, Specta/Por, Spectrum, Houston, TX, USA) containing an amount of $NaHCO_3$ equivalent to the titratable acidity, filled up to 5 ml with Milli Q water, was placed into the reaction vessel. Dialysis was performed for 30 min at 37 °C in a shaking water bath. After this, the contents of the dialysis tubing were added to the reaction vessel.

Pancreatic digestion was performed by adding a pancreatin/bile extract mixture to the reaction vessel and incubating the mixture for 4 h at 37 °C. During this pancreatic digestion the mixture was led through a hollow-fibre system (MW cut-off 10,000, Type H1P3-20, Amicon Division, W.R. Grace & Co., Danvers, MA, USA). Every 30

min the dialysate was collected. In the dialysates the concentrations of Ca, Mg, Fe, Cu and Zn were determined by atomic absorption spectroscopy (AAS).
The dialysability is calculated as the total amount of minerals and trace elements dialysed in 4 h. The dialysability is expressed as a percentage of the total amounts of minerals and trace elements present in the sample.

Mathematical model

A mathematical model was developed that describes and predicts the in vitro availability (determined with the equilibrium in vitro method) of Ca, Mg, Fe and Zn from cereal products, fruits, vegetables and nuts well. As this mathematical model has been described elsewhere (80), we will suffice here with a brief description.
The mathematical model is based on a combination of Langmuir isotherms, each Langmuir isotherm describing the influence of one food component on the in vitro availability of minerals and trace elements.
The equation for the Langmuir isotherm is: $L = K.C/(1 + K.C)$
where: K is indicative of the extent to which a food component is able to influence the availability of minerals and trace elements and C is the concentration of a food component.

Because the influence of a food component on the availability of minerals and trace elements depends on the influences of all the other components, the Langmuir isotherm of each food component was corrected for all these influences. This resulted in the following generic mathematical model:

$$A = \frac{100 + 100.L_1/(1\text{-}L_1) + 100.L_3/(1\text{-}L_3) + \dots + 100.\ L_m/(1\text{-}L_m)}{1 + L_1/(1\text{-}L_1) + L_2/(1\text{-}L_2) + L_3/(1\text{-}L_3) + L_4/(1\text{-}L_4) + \dots + L_n/(1\text{-}L_n)}$$

where: A is the availability of minerals and trace elements;
$L_1, L_3, \dots, L_m$ represent the Langmuir isotherms of the food components with a positive influence on mineral availability;
$L_1, L_2, L_3, L_4, \dots, L_n$ represent the Langmuir isotherms of all food components with an influence on mineral availability (both positive and negative).

For each mineral and trace element, the generic mathematical model was fitted to experimental data concerning the in vitro availability of minerals and trace elements and the composition of more than 50 food products. This resulted in a specific mathematical model for each mineral and trace element under investigation. A specific mathematical model is characterized by the mineral or trace element under consideration, a set of food components with an influence on the availability of this mineral or trace element, a set of corresponding direction specifiers (indicating whether the food component has a positive or a negative influence on availability) and a set of corresponding K values. The specific mathematical models for Ca, Mg, Fe, Cu and Zn developed in a previous study (80) are used in this study to calculate the availability of these minerals and trace elements from the five pig diets.

In vivo experiment

Thirty piglets were selected on the basis of body weight and lineage, and divided into five groups. Each group of six pigs received one of the diets described in Table 1. After a period of one week, in which the animals were allowed to adapt to the diets, a one-week experimental period was started. By then the pigs were six weeks of age.
The diets were supplied to the pigs in the form of pellets. At the start of the experimental period, the amount of feed to be given was calculated for each pig individually on the basis of body weight, using the following formula:

$$\text{amount of feed per day (kg)} = \frac{419 \times G^{3/4} \times 0.7 \times 2.7}{\text{NE}}$$

where:
419 = maintenance requirement for energy in pigs (in KJ ME/day)
$G^{3/4}$ = metabolic weight of the pig
2.7 = feeding level relative to maintenance requirement for energy
NE = net energy of the diet
0.7 = factor for conversion of ME (metabolizable energy) to NE (net energy)

During the experimental period the feeding level was kept constant. The pigs were fed twice a day, at 08.00 and at 16.00 h. The animals were allowed to take water ad libitum via drinking nipples. The intake of minerals and trace elements from the drinking water was negligible in comparison to the intake from the diets.
The pigs were individually housed in metabolic cages. The faeces were collected quantitatively by means of ileostomy bags. Faeces samples were freeze-dried prior to analysis of the concentrations of Ca, Mg, Fe, Cu and Zn.

Analytical methods

Phytic acid was determined after extraction with dilute HCl by ion chromatography with post-column derivatization and UV detection as described by Bos et al. (60).
Dietary fibre was determined as non-starch polysaccharides (NSP) with a condensed version of the gas chromatographic procedure described by Englyst and Cummings (86) which was supplied to participants of the Dietary Fibre Collaborative Trial, Part IV in 1990 (joint UK Ministry of Agriculture, Fisheries and Food (MAFF)/EC Trial).
The concentration of tannins was determined with the vanillin-H_2SO_4 method as described by Kuhla and Ebmeier (87), using catechin as a reference agent.
L-ascorbic acid was calculated from the composition of the pig diets in Table 1.
Citric acid was determined enzymatically according to Boehringer (88).

Ca, Mg and Zn were determined with flame atomic absorption spectroscopy. Fe and Cu were determined with graphite-furnace atomic absorption spectroscopy.

Results and discussion

Figures 1 to 5 show the availability of Ca, Mg, Fe, Cu and Zn, respectively, in the five pig diets, as determined with the equilibrium in vitro method and the continuous in vitro method, as calculated with the mathematical model and as determined in vivo in piglets. Earlier studies have shown that phytic acid, non-starch polysaccharides (particularly arabinose-containing components), citric acid and ascorbic acid influence the availability of Ca, Mg, Fe, Cu and Zn (80). Table 2 shows the concentrations of these food components in the pig diets. As the present study pays special attention to the influence of tannins on the bioavailability of minerals and trace elements, the concentration of tannins is presented in Table 2 as well. Because tannins were not included in our previous study concerning the development of the mathematical model (80), the potential effects of tannins on the availability of minerals and trace elements are not included in the mathematical calculations of the bioavailability of minerals and trace elements in the pig diets as presented in Figures 1-5.
The validation of the use of the two in vitro methods for the prediction of the bioavailability of Ca, Mg, Fe, Cu and Zn, and the influence of tannins on the bioavailability of these minerals and trace elements are discussed below.

Validation of the use of in vitro methods for prediction of the bioavailability of Ca, Mg, Fe, Cu and Zn in pig diets

Figures 1 to 5 show that the level of the in vitro availability of minerals and trace elements differs from the level of the availability determined in vivo. Both in vitro methods show lower availabilities for Ca, Fe and Zn, and higher availabilities for Mg and Cu. The cause of this discrepancy is likely the fact that not all physiological conditions that are important in determining the bioavailability of minerals and trace elements can be simulated in vitro. For example, the in vitro methods estimate the amount of minerals and trace elements that is available for absorption in the small intestine, while in the in vivo experiment the actual amount of minerals and trace elements that is absorbed in the piglets is determined. Active absorption processes that are present in vivo, as well as the fact that piglets are able to adjust the degree of absorption of minerals and trace elements to their physiological need, cannot be simulated in vitro.

Apart from the absolute values of availability, the relative effects of differences in food composition on availability are of interest. Figures 1, 2 and 4 show that for Ca, Mg and Cu both the equilibrium in vitro method and the continuous in vitro method correspond quite well with the in vivo method. The equilibrium in vitro method, the continuous in vitro method as well as the in vivo experiment show no differences in the availability of Ca and Mg from rations I to V (Figures 1 and 2). For the availability

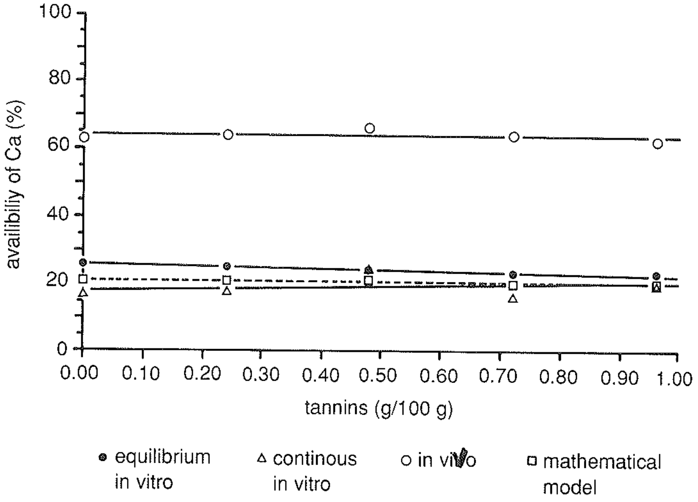

Figure 1: In vitro, in vivo and calculated availability of Ca in five pig diets

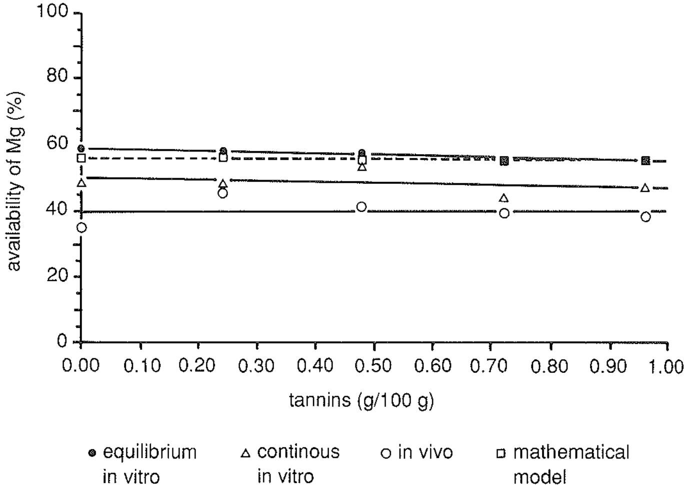

Figure 2: In vitro, in vivo and calculated availability of Mg in five pig diets

of Cu, the in vitro methods as well as the in vivo experiment show a decrease in the availability of Cu from ration I to V (Figure 4).

To our knowledge, a comparison of in vivo availability and in vitro availability of Ca, Mg and Cu (determined with an in vitro method based on dialysis) has not been reported before.

The in vivo availability of Fe in pigs decreases from ration I to ration V (Figure 3). Although the in vitro methods show the same tendency for the availability of Fe, the

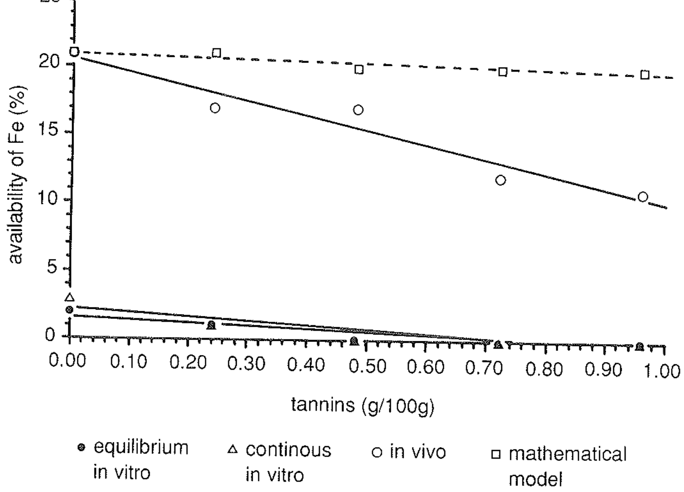

Figure 3: In vitro, in vivo and calculated availability of Fe in five pig diets

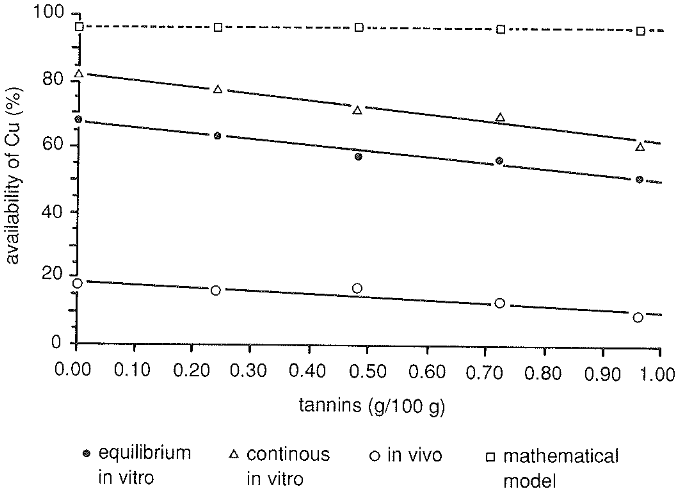

Figure 4: In vitro, in vivo and calculated availability of Cu in five pig diets

level of in vitro availability is too low to draw conclusions about a correspondence between in vitro and in vivo availability.

For Zn, the equilibrium in vitro method gives similar availabilities for diets I to V, while the continuous in vitro method shows a tendency towards a decrease in the availability of Zn from ration I to V (Figure 5). Unfortunately, the bioavailability of Zn in vivo showed rather variable results among the piglets in the groups. Therefore, values for the in vivo availability of Zn in Figure 5 are difficult to interpret. The cause of this variation in results may be the fact that the pigs were held in Zn-containing

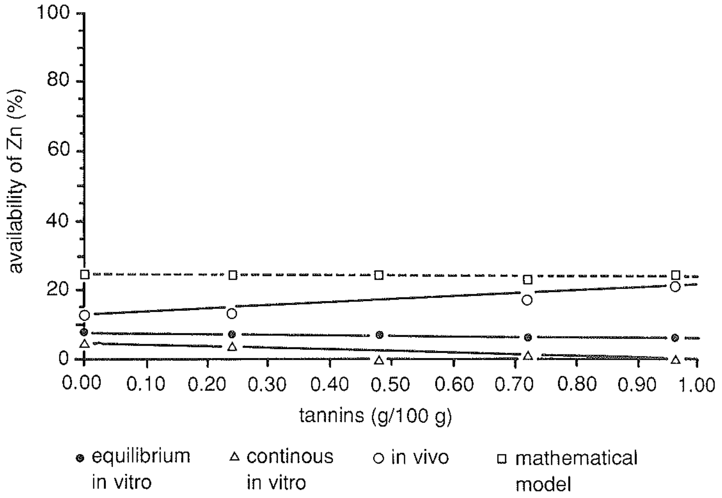

Figure 5: In vitro, in vivo and calculated availability of Zn in five pig diets

metal cages. Some animals may have licked these cages, thus increasing their Zn intake and disturbing the absorption measurements. Therefore, conclusions about the relation between the in vitro availability and the in vivo availability of Zn are not justifiable.

Our results are not conclusive with respect to the relative relation between the in vitro availability and the in vivo availability of Fe and Zn. For vitro methods based on equilibrium dialysis, others have reported good correlations between in vitro availability and in vivo absorption of Fe and Zn in man (12,24,25,26,27,31).

Based on our results, we conclude that the results of in vitro methods cannot be used for a direct prediction of the bioavailability of minerals and trace elements. Miller et al. (23) and Hazell and Johnson (24), who studied the availability of Fe with an in vitro method based on equilibrium dialysis, stated that in vitro measurements of nutrient availability should be considered as a relative rather than an absolute predictor of bioavailability. This statement is supported by our results. An estimate of the absolute bioavailability might be obtained by correcting in vitro values for the discrepancy between in vitro and in vivo availability. Such a correction factor, however, should be determined separately for each mineral and trace element and for each group of food products, and be validated carefully against standardized in vivo data.

Previously we have reported a marked influence of removal of dialysable components on the vitro availability of minerals and trace elements. In general, the continuous in vitro method resulted in higher availabilities of Ca, Mg, Fe and Cu in different types of bread than the equilibrium in vitro method (79). Both methods gave comparable values for the availability of Zn. Our present results with pig diets show quite

Table 2: Concentrations of phytic acid (g/100 g), non-starch polysaccharides (NSP) (g/100 g), tannins (g/100 g), ascorbic acid (mg/100 g) and citric acid (g/100 g) in five pig rations

Food component	Ration I	Ration II	Ration III	Ration IV	Ration V
Phytic acid	0.47	0.48	0.49	0.50	0.49
Total NSP	13.32	12.75	12.17	11.60	11.03
uronic acids	2.05	2.02	1.98	1.94	1.90
rhamnose	0.18	0.17	0.16	0.16	0.15
arabinose	0.18	0.19	0.20	0.21	0.22
xylose	1.81	1.65	1.50	1.34	1.18
mannose	0.03	0.03	0.02	0.02	0.02
galactose	0.17	0.16	0.16	0.15	0.15
glucose	8.88	8.50	8.13	7.75	7.38
Tannins*	0.00	0.24	0.48	0.72	0.96
Ascorbic acid	5.0	5.0	5.0	5.0	5.0
Citric acid	0.1	0.1	0.1	0.1	0.1

* The concentration of tannins is expressed as catechin equivalents

comparable values for the in vitro availability of Ca, Fe and Zn, higher values for the availability of Cu and lower values for the availability of Mg for the continuous in vitro method relative to the equilibrium in vitro method.

In the experiments with pig diets presented here, the continuous in vitro method gives a slightly better relative estimate of the bioavailability of Cu than the equilibrium in vitro method (correlation coefficients of the linear relation between in vitro and in vivo availability of 0.90 and 0.84 for the continuous in vitro method and the equilibrium in vitro method respectively). Data for the other elements do not allow calculation of a meaningful correlation coefficient. This indicates that continuous removal of dialysable components might be a relevant factor for in vitro prediction of the bioavailability of minerals and trace elements. This aspect, however, needs further investigation.

The influence of tannins on the bioavailability of Ca, Mg, Fe, Cu and Zn

In a previous study, a mathematical model was developed that was shown to describe and predict the in vitro availability of minerals and trace elements quite well on the basis of the concentrations of food components influencing this availability (80). This mathematical model is used here to calculate the availability of Ca, Mg, Fe, Cu and Zn in pig diets, on the basis of the composition of these diets. Because in our previous study tannins were not included, the mathematical model calculations presented in Figures 1-5 do not include the potential effects of tannins.

Comparison of the mathematical model predictions of the availability of minerals and trace elements with the availability determined in vitro shows a good correspondence between the absolute values for Ca and Mg (Figures 1 and 2), and a reasonable agreement for Fe, Cu and Zn (Figures 3 and 5). This is in agreement with previous results (80).

Figures 3 and 4 show that both the in vitro methods and the in vivo experiment with piglets reveal a clear tendency for a reduced availability of Fe and Cu with an increasing content of condensed tannins in the diets. In a previous study it was shown that arabinose-containing components have a negative effect on the availability of Fe and that NSP in general have a negative effect on the availability of Cu (80). Although there is a small difference in the content and composition of NSP between the pig diets I to V, calculations with the mathematical model show that the difference in NSP and arabinose content is far too small to cause the decrease in availability of Fe and Cu from diet I to V. Therefore, we conclude that the negative influence on the bioavailability of Fe and Cu must be attributed to the tannins. These results are in agreement with results of Narasinga Rao and Prabhavathi (81), Christian and Seshadri (82), Brune and Rossander (54), Kies and Umoren (83) and Siegenberg et al. (84), who reported a negative effect of tannins on the availability of Fe and Cu. Figures 1 and 2 show that tannins have no influence on the availability of Ca and Mg. Because of reasons described above, the influence of tannins on the bioavailability of Zn in vivo is not clear. However, there is a good correspondence between the availability of Zn determined with the equilibrium in vitro methods and that calculated with the mathematical model (Figure 5). Because the mathematical model was fitted to experimental in vitro data obtained with the equilibrium in vitro methods and because tannins were not included in the mathematical model, this suggests that tannins have no influence on the availability of Zn.

As tannins are important for the availability of Fe and Cu, the mathematical model for these elements must be extended with a factor for tannins. Although the number of pig diets is too small to determine the exact K values for tannins, mathematical model calculations show that for (estimated) K values of 1.2×10^3 and 7.0×10^2 for Fe and Cu, respectively, the mathematically calculated availability of these elements shows a similar negative tendency in availability as determined in vitro and in vivo. This indicates that, after incorporation of tannins, the mathematical model may be used for a relative prediction of the bioavailability of Fe and Cu in pig diets. Although our present results do not allow for a rigorous validation of the use of the mathematical model for the prediction of the bioavailability of minerals and trace elements, we conclude that the mathematical modelling approach is promising.

Conclusions

There is a discrepancy between the absolute values of the bioavailability of Ca, Mg, Fe, Cu and Zn determined in piglets, and the availability of these minerals and trace elements as determined in vitro. However, both the equilibrium in vitro method and the continuous in vitro method represent well the effects of differences in the composition of the pig diets on the bioavailability of minerals and trace elements in vivo. Therefore, it is concluded that the in vitro methods can be used for a relative prediction of the bioavailability of minerals and trace elements.

Due to the continuous removal of dialysable components from the pancreatic digestion mixture, the continuous in vitro method gives a slightly better relative estimate of the bioavailability of Cu in vivo than the equilibrium in vitro method (correlation coefficients of 0.90 and 0.84 respectively). The importance of continuous removal of dialysable components for the prediction of the bioavailability of minerals and trace elements needs further investigation.

By combining the results of the in vitro experiments, the in vivo experiment and the mathematical model predictions, it is shown that tannins decrease the bioavailability of Fe and Cu, while they do not seem to affect the bioavailability of Ca, Mg and Zn.

Although the experimental design does not allow for a validation of the use of the mathematical model for the prediction of the bioavailability of minerals and trace elements, the mathematical modelling approach seems promising. Mathematical model simulations may be helpful in designing cost-effective in vivo experiments and in bridging the gap between in vitro experiments and in vivo experiments. In general, combining in vivo experiments, in vitro experiments and mathematical modelling results in a powerful research approach to gain an understanding of the mechanisms and important factors involved in digestion and absorption processes.

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Part V

The influence of dietary components on the bioavailability of calcium, magnesium, iron, copper and zinc

CHAPTER 11

Influence of dietary fibre, phytic acid, and other dietary components on the bioavailability of Ca, Mg, Fe, Cu and Zn (Review)

Introduction

Minerals and trace elements play important roles in biological and biochemical processes in animals and man. Deficiencies, overdoses or imbalances between minerals or trace elements may have a negative influence on health.

Several components of our diet can form soluble or insoluble complexes with minerals and trace elements under gastro-intestinal conditions. These dietary components are thus able to influence the bioavailability of minerals and trace elements (the availability for biological and biochemical processes in the organism) by decreasing or increasing the availability for absorption in the small intestine. Dietary fibre, phytic acid and polyphenolic components have been shown to decrease the bioavailability of some minerals and trace elements, while organic acids, lactose and animal proteins are known to increase bioavailability.

The influence of a specific food component on the bioavailability of minerals and trace elements is often difficult to assess due to the presence of many other components in the complex food products which may also affect the bioavailability of minerals and trace elements. On the other hand, results of experiments with an isolated component may not always reflect the true effect of this component when it is intrinsically present in the complex structure of food products. In addition, results of different studies are hard to compare because the binding of minerals and trace elements by food components depends on many factors such as the dietary source of the component, the specific structure of the component, the type of mineral studied, the pH, the experimental design of the study and the method of processing of the food products.

In 1989, Spivey Fox and Tao (1) published an extensive review on the effect of phytic acid on mineral bioavailability. Very recently, Torre et al. (2) published a review on the effects of dietary fibre and phytic acid on mineral bioavailability. As these publications correspond well with our objectives, it seemed somewhat redundant to prepare another review. Therefore, the section on the influence of dietary fibre components on mineral bioavailability is based on the conclusions of Torre et al. (2), while the section on the influence of phytic acid on mineral bioavailability is based on the conclusions of Spivey Fox and Tao (1) and Torre et al. (2). For more detailed information on either of these subjects, the reader is referred to these reviews.

Influence of dietary fibre components on the bioavailability of Ca, Mg, Fe, Cu and Zn

The capacity of fibre components to bind polyvalent mineral ions may result in a negative effect on the bioavailability of minerals and trace elements. Several in vitro and in vivo experiments have been carried out to elucidate this.

In vitro experiments have shown that cellulose, hemicellulose, pectin, lignin and gums are able to bind Ca, Mg, Fe, Cu and Zn ions. Cellulose may bind mineral ions by means of its free hydroxyl groups, while pectin binds the divalent ions mainly by means of the functional carboxyl groups of the D-galacturonic acid units. Therefore, pectins with a high degree of esterification bind significantly less minerals than pectins with a low degree of esterification. Hemicelluloses and gums probably bind mineral ions by means of their acid substituents and/or free hydroxyl groups. Lignin may form strong complexes with metal ions by forming multidentate ligands in which the methoxy and hydroxyl groups of lignin act as ligands. The relative strength of the interaction between dietary fibre components and cations depends on several aspects such as the pH, the precise structure of the dietary fibre component, the type and valency of the cation and the presence of other cations.
Results of in vivo experiments with laboratory animals are rather contradictory, but in general a negative influence of cellulose, hemicellulose and pectins with a low degree of esterification on the bioavailability of some minerals and trace elements is reported. In vivo experiments with test persons show no clear negative influence of cellulose, pectin, lignin or gums on the bioavailability of Ca, Mg, Fe, Cu or Zn. However, some negative effects of hemicelluloses on the bioavailability of Mg, Fe, Cu and Zn have been reported (2,3,4). Others have shown that dietary fibre from fruits and vegetables decreases the bioavailability of Ca, Mg, Cu and Zn (5,6). Due to the presence of organic acids the bioavailability of Fe increased in these studies.

The discrepancy between in vitro and in vivo results may be attributable to such factors as inadequate simulation of gastrointestinal conditions in the in vitro experiments, and the presence of other dietary components which influence the bioavailability. It has also been suggested that the discrepancy between in vitro and in vivo results could partly be due to bacterial degradation of soluble fibre components in the colon, making bound minerals and trace elements be liberated and become available for absorption (2).

Influence of phytic acid on the bioavailability of Ca, Mg, Fe, Cu and Zn

Due to the dissociation of ionizable protons, phytic acid (myo-inositol-1,2,3,4,5,6-hexakis(dihydrogen phosphate)) is able to form complexes with polyvalent cations. In foods this complex formation is complicated by interactions with proteins. Since phytic acid-mineral and phytic acid-protein-mineral complexes cannot be absorbed in the intestinal tract, and animals and man generally possess no or only a limited capacity to hydrolyse these complexes, phytic acid may have a negative influence on the bioavailability of minerals and trace elements.

In vitro and in vivo experiments have clearly shown that phytic acid strongly binds Ca, Fe and Zn and thus markedly decreases their bioavailability. Phytic acid has less marked adverse effects on the bioavailability of Mg and Cu. Fe, Cu and Zn cations, which are present in small amounts in the diet, may be coprecipitated by Ca-phytate and/or Mg-phytate complexes (7).
Phytic acid forms a complex with a cation within one phosphate group or between two phosphate groups on either the same or different molecules. The relative strength of the interaction between phytic acid and the cations depends on such factors as amount of phytic acid present, cation concentration, size and valency, pH and presence of other cations. In the pH range 5-10 protein-mineral-phytate complexes may be formed. The negatively charged protein and phytic acid are linked together by cations (7).

The phytic acid content in foods may be decreased by the action of the enzyme phytase, resulting in the formation of lower inositol phosphates. It is generally recognized that inositol pentaphosphate has a negative influence on the bioavailability of minerals and trace elements. However, results from experiments with inositol tetraphosphate and inositol triphosphate are contradictory.

Influence of other dietary components on the bioavailability of Ca, Mg, Fe, Cu and Zn

Besides dietary fibre components and phytic acid, several other dietary constituents can influence the bioavailability of minerals and trace elements. Components in the diet that may increase the bioavailability of Ca, Mg, Fe, Cu and Zn are listed in Table 1. Components that may decrease the bioavailability of Ca, Mg, Fe, Cu and Zn are summed in Table 2. Table 3 shows the mutual influence of the minerals and trace elements on their bioavailability.

Table 1: Dietary components with a positive effect on the bioavailability of some minerals and trace elements

Component	Type of study	Effect on bioavailability	References
Lactose	in vivo	increase for Ca, Mg and Fe	8,9,10
		no effect for Zn	11
	in vitro	increase for Fe	12
Ascorbic acid	in vivo	increase for Fe	10,13,14
		no effect for Ca, Cu and Zn	15,16,17
	in vitro	increase for Fe	12,18,19,20
Citric acid	in vitro	increase for Fe	18,20,21
Fructose	in vivo	increase for Ca, Mg, Fe, Cu and Zn	11,22
Animal protein	in vivo	increase for Fe and Cu	13,23,24
		decrease for Ca and Zn	25
	in vitro	increase for Fe	26,27
Amino acids	in vivo	increase for Zn due to cysteine, histidine and tryptophan	25
	in vitro	increase for Cu due to glycine, and histidine	28
Caffeine	in vivo	increase for Ca and Mg	29

Table 2: Dietary components with a negative effect on the bioavailability of some minerals and trace elements

Component	Type of study	Effect on bioavailability	References
Oxalic acid	in vivo	decrease for Ca	30,31,32
		no effect for Zn	30
	in vitro	decrease for Fe	33
Tannins	in vivo	decrease for Fe	34
	in vitro	decrease for Fe and Cu	12,35
Flavanoids	in vitro	decrease for Fe and Cu	35
Phosphate	in vivo	decrease for Fe	13,36,37
	in vitro	decrease for Fe and Zn	19,38

Table 3 Effects of calcium, magnesium, iron, copper and zinc on their mutual bioavailability

Component	Type of study	Effect on bioavailability	References
Calcium	in vivo	decrease for Zn	39,40
	in vitro	decrease for Fe, Cu and Zn	7,41,42,43
Magnesium	in vitro	decrease for Fe, Cu and Zn	7,42
Iron		no effects known	
Copper	in vivo	decrease for Zn	44,45
Zinc	in vivo	decrease for Fe and Cu	10,24,44,46 47,48

Conclusion

From this concise review of the literature it is evident that assessment of the bioavailability of minerals and trace elements is a very complex issue because a variety of dietary components may influence the bioavailability directly or indirectly. For this reason, the influence of dietary fibre, phytic acid and other dietary components on the bioavailability of minerals and trace elements should not be studied separately but in a whole, well characterized, food matrix.

Relation between the in vitro availability of Ca, Mg, Fe, Cu and Zn and the composition of food products: investigations by means of a mathematical model

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Abstract

To study the relative influence of phytic acid, dietary fibre components, oxalic acid, citric acid and ascorbic acid on the in vitro availability of Ca, Mg, Fe, Cu and Zn, a mathematical model was developed. This model is based on a combination of interdependent Langmuir isotherms. Throughout a diverse set of food products (cereal products, fruits, vegetables and nuts) the mathematical model describes and predicts the in vitro availability of Ca, Mg, Fe and Zn well.
The mathematical model proves valuable in studying the simultaneous effects of food components on the in vitro availability of minerals and trace elements in real food matrices. Therefore, the mathematical model is expected to be important in bridging the gap between in vitro simulations and in vivo experiments.

Introduction

Several components in the diet of animals and man are able to form soluble or insoluble complexes with minerals and trace elements under gastro-intestinal conditions and may thus affect the bioavailability of these minerals and trace elements positively or negatively. The bioavailability of minerals and trace elements may be influenced negatively by phytic acid, dietary fibre, oxalic acid, tannins, flavanoids and phosphate (1,2,13,30,34,36,49,50,51), and positively by lactose, fructose, citric acid, ascorbic acid, animal protein, some amino acids and caffeine (8,18,22,25,27,29, 50,52).

The influence of a specific food component on the bioavailability of minerals and trace elements is often difficult to assess due to the presence of other dietary components which influence this bioavailability simultaneously. On the other hand, results of experiments with an isolated component will not reflect the true effect of this component when it is intrinsically present in the food. Therefore, we believe that the influence of dietary components on the bioavailability of minerals and trace elements should be studied in the complete food matrix, despite the difficulties described above.

The objective of our study was to investigate the relative influence of food components on the in vitro availability of Ca, Mg, Fe, Cu and Zn in several food matrices.

One of the methodological routes to interpret the relation between food composition and in vitro availability of minerals and trace elements is the development of a mathematical model. This model must be able to describe both the influence of a single food component and the interdepending influences of several food components. Apart from describing and predicting the in vitro availability, such a model may contribute significantly to the understanding of mechanisms involved in the in vivo availability of minerals and trace elements.

Food products were selected from four product groups: cereal products, fruits, vegetables and nuts. Food components taken into account were dietary fibre components, phytic acid, oxalic acid, citric acid and ascorbic acid.

In this paper, the concentrations of food components in the selected food products, and the in vitro availability of Ca, Mg, Fe, Cu and Zn as determined in these food products are presented. The assumptions underlying the mathematical model, as well as its derivation and optimization, are worked out. Also, the results of experiments to test the predictive power of the mathematical model with respect to in vitro availability are presented. Finally, the influence of food components on the availability of minerals and trace elements, as investigated with the mathematical model, is discussed and related to other research.

Materials and methods

Food products

All cereal products, fruits, vegetables and nuts were bought in local stores. Bread samples were dried at 60 °C for 24 h. The nuts were defatted with hexane. The edible parts of fruits and vegetables were selected. All vegetables (except cucumber, tomato and lettuce) were investigated both in a raw and in a cooked form. The vegetables were cooked with water (home-cooking) and the water was discarded. The fruits and vegetables were freeze-dried. All dry samples were milled on a 0.5 mm sieve.

Estimation of the bioavailability of Ca, Mg, Fe, Cu and Zn

The bioavailability of Ca, Mg, Fe, Cu and Zn from the fruits and vegetables was estimated with an in vitro method with equilibrium dialysis. The method is a modification of the method described by Miller (19) and Hazell and Johnson (52) and has been described before (51).
The dialysability was expressed as a percentage of the amount of minerals and trace elements present in the food products.

Analytical methods

Phytic acid was determined after extraction with dilute HCl by ion chromatography with post-column derivatization and UV detection as described by Bos et al. (53).
Dietary fibre was determined as non-starch polysaccharides (NSP) with a condensed version of the gas chromatographic procedure described by Englyst and Cummings (54) supplied to participants of the Dietary fibre Collaborative Trial, Part IV in 1990 (joint UK Ministry of Agriculture, Fisheries and Food (MAFF)/EC Trial).
Total vitamin C (L-ascorbic acid plus dehydro-L-ascorbic acid) and dehydro-L-ascorbic acid were determined by HPLC as described by Speek et al. (55). L-ascorbic acid was calculated as the difference between these two values.
Citric acid was determined enzymatically according to Boehringer (56).
Oxalic acid was determined by HPLC according to Hughes et al. (57).
Ascorbic acid, citric acid and oxalic acid were determined only in the fruits and vegetables, because the other products were not expected to contain organic acids.
Ca, Mg and Zn were determined by flame atomic absorption spectroscopy. Fe and Cu were determined by graphite-furnace atomic absorption spectroscopy.

Mathematical model

The in vitro availability of minerals and trace elements was plotted against the concentration of a food component. Interpretation of these plots was strongly hampered by the presence of other food components which influence this in vitro availability simultaneously. To investigate the relation between the in vitro availability of minerals and trace elements and the concentration of food components, a mathematical model was developed and optimized.

Development of the mathematical model

The basic assumption underlying the mathematical model is that the complex equilibrium in the in vitro method for the estimation of the bioavailability of minerals and trace elements can be modelled by interdependent Langmuir isotherms.
In the in vitro method based on equilibrium dialysis, the dialysability of minerals and trace elements is believed to be an estimate of the in vivo availability. The equilibrium is the final result of the actions of all food components that affect the in vitro

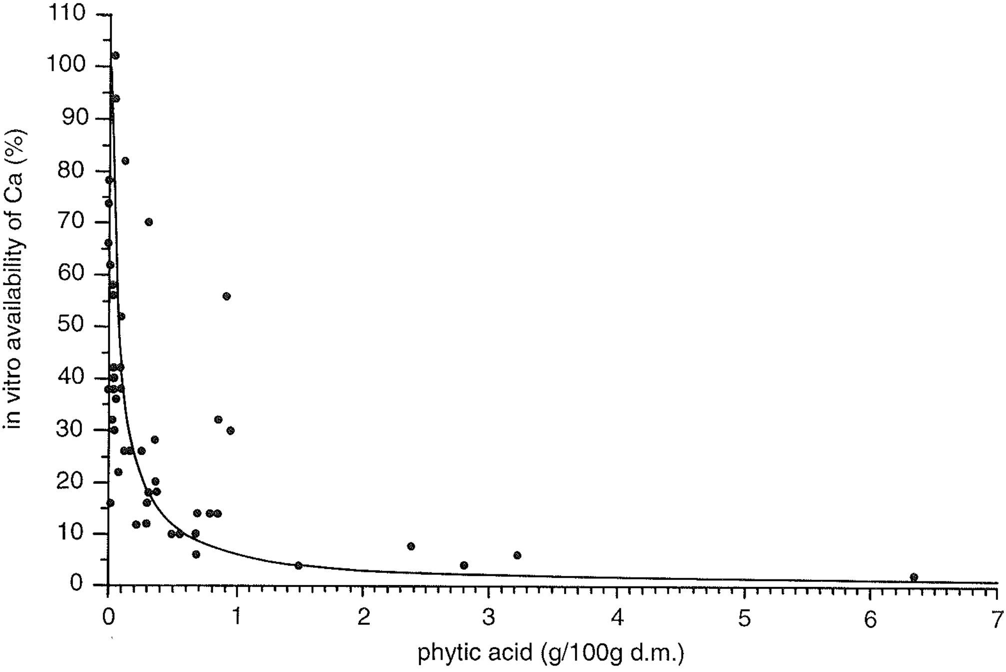

Figure 1: Relation between the in vitro availability of calcium and the concentration of phytic acid in cereal products, fruits, vegetables and nuts.

availability in a positive or negative way. It is assumed that components with a negative influence on the in vitro availability withdraw minerals and trace elements from the dialysable complexes to form insoluble or soluble, non-dialysable complexes. Similarly, it is assumed that components with a positive influence on the in vitro availability withdraw minerals and trace elements from the non-dialysable complexes to form soluble, dialysable complexes. Indeed, phytic acid, which is known to have a negative influence on availability, forms insoluble complexes with a number of minerals and trace elements under simulated intestinal conditions (43,58,59, 60,61). As a Langmuir isotherm is known to describe heterogeneous equilibria well (62,63), we adopt this isotherm as a basic element in our modelling approach. In addition, the assumption of a Langmuir isotherm is in agreement with the more pronounced plots of in vitro availability of minerals and trace elements versus the concentration of a food component as shown in Figure 1, and as presented by Hazell (50).

The Langmuir isotherm for the interaction between a food component and minerals and trace elements under heterogeneous equilibrium conditions can be derived as described below.

The adsorption process of a metal ion to a food component with a negative effect on the in vitro availability can be described by:

$$k_1 \,.\, (1 - L) \,.\, [X]$$

where: $[X]$ = the concentration of the food component;
L = the fraction of metal ions already bound to the food component $(0 < L < 1)$
$(L = 1$: all metal ions bound;
$L = 0$: no metal ions bound).

The desorption process of a metal ion from the food component-metal ion complex can be described by:

$$k_2 \,.\, L$$

Under equilibrium conditions the magnitude of the adsorption process equals that of the desorption process, and therefore:

$$k_1 \,.\, (1 - L) \,.\, [X] = k_2 \,.\, L$$

This equation can be solved for L:

$$L = k_1 \,.\, [X] \,/\, (k_2 + k_1 \,.\, [X])$$

Substituting K for k_1 / k_2 (equilibrium constant) results in:

$$L = K \,.\, [X] \,/\, (1 + K \,.\, [X]) \qquad \text{(Langmuir isotherm)}$$

In a real food matrix, several food components influence the availability of minerals and trace elements simultaneously. Therefore, the Langmuir isotherm of each food component has to be corrected for the influences of these other food components. The correction factor can be derived as described below.

We assume that the in vitro availability (A) of minerals and trace elements is 100% if no food components are present that influence the availability. If, for example, two food components x and y are present of which x has a negative influence and y has a positive influence on mineral availability, then the in vitro availability of minerals and trace elements is equal to:

$$A = 100 - N_x + N_y \tag{1}$$

where: N_x and N_y represent the absolute influence of food components x and y, respectively.

Food component x can only decrease the in vitro availability by acting on the dialysable part of the minerals and trace elements that results from the influence of the other food components (in this example food component y, responsible for a dialysable part A_y). Food component y can only increase the in vitro availability by acting on the non-dialysable part that results from the influence of the other food components (in this example food component x, responsible for a non-dialysable part $(100 - A_x)$.

From equation 1 we obtain:

$$A_x = 100 - N_x = A - N_y \qquad (2)$$
$$A_y = 100 + N_y = A + N_x \qquad (3)$$

The influence of food components x and y can be represented by:

$$N_x = A_y \cdot L_x = (A + N_x) \cdot L_x \qquad (4)$$
$$N_y = (100 - A_x) \cdot L_y = (100 - (A - N_y)) \cdot L_y \qquad (5)$$

where L_x and L_y represent the Langmuir isotherm of food components x and y, respectively.

Rewriting equations 4 and 5 leads to:

$$N_x = A \cdot L_x / (1 - L_x) \qquad (6)$$
$$N_y = (100 - A) \cdot L_y / (1 - L_y) \qquad (7)$$

Substituting equations 6 and 7 in equation 1 leads to:

$$A = 100 - A \cdot L_x/(1 - L_x) + (100 - A) \cdot L_y/(1 - L_y) \qquad (8)$$

Solving equation 8 for A leads to:

$$A = \frac{100 + 100.L_y/(1-L_y)}{1 + L_x/(1-L_x) + L_y/(1-L_y)} \qquad (9)$$

Generalizing this derivation procedure to more food components leads to the generic mathematical model that is used for description of the in vitro availability of minerals and trace elements:

$$A = \frac{100 + 100.L_1/(1-L_1) + 100.L_3/(1-L_3) + \ldots + 100.L_m/(1-L_m)}{1 + L_1/(1-L_1) + L_2/(1-L_2) + L_3/(1-L_3) + L_4/(1-L_4) + \ldots + L_n/(1-L_n)} \qquad (1$$

where: A is the availability of minerals and trace elements;
$L_1, L_3, \ldots, L_m$ represent the Langmuir isotherms of food components with a positive influence on mineral availability;
$L_1, L_2, L_3, L_4, \ldots, L_n$ represent the Langmuir isotherms of all food components influencing mineral availability (both positive and negative).

The mathematical model presented in equation 10 corrects for the simultaneous influences of food components on the availability of a mineral or trace element. However, it does not take into account the potential mutual influence of minerals and trace elements on their availability. Examples of such influences are coprecipitation effects (which may lower the availability of a specific element), and competition of minerals and trace elements for binding sites (which may increase the availability of a specific element). Because specific knowledge of these processes is lacking, no corrections are made for them. A practical reason for not taking into account these effects is the fact that the concentration of phytic acid and the concentrations of Ca, Mg, Fe, Cu and Zn in the cereal products and nuts are highly correlated (r = 0.88, 0.98, 0.60, 0.91 and 0.92 respectively). This may disturb the optimization process.

By expressing the concentrations of food components in the mathematical model as weight fractions between 0 and 1 (e.g. in g/g), the K values in the Langmuir parts become dimensionless. In agreement with the assumptions underlying the mathematical model, the K values in the Langmuir parts of the mathematical model are indicative of the extent to which a food component is able to influence the availability of minerals and trace elements. The actual influence of a food component on the availability of a mineral or trace element in a specific food matrix depends, however, on the concentration of this food component, the direction of this food component's influence (positive or negative), the (metal-specific) K value of this food component, as well as the concentrations, directions of influence and K values of other food components with a potential influence on the availability of minerals and trace elements.

Optimization procedure

To be able to use the mathematical model presented in equation 10, both structural and numerical parameters must be specified. Structural parameters specify the actual structure of the model: which food components should be taken into account and whether they contribute in a positive or in a negative way to the availability of a mineral or trace element. Numerical parameters are the K values in the Langmuir part of the mathematical model, which are specific for each food component.
A completely specified mathematical model, therefore, is characterized by a list consisting of the mineral or trace element under consideration, a set of relevant food components, a set of corresponding direction specifiers (positive or negative contributions, determining the structure of the model), and a set of corresponding K values:

<Metal ; Components ; Directions ; *K* values>   generic
<Mg ; phytic acid citrate ; negative positive ; 190 150>   specific

This specific information can be used to convert the generic mathematical model of equation 10 into a specific model for calculation of the availability of minerals and trace elements in a food matrix, provided the concentrations of the relevant food components in this matrix are known.

An optimization procedure was designed in order to obtain for each mineral or trace element the list that provides the best correspondence between calculated and measured availability throughout the complete set of food products. Typical characteristics of the optimization problem (a least-squares minimization of the difference between calculated and measured availabilities) are the fact that both a structural and a numerical optimization is required, and the fact that the number of relevant food components is not known in advance. In addition, the search space contains local minima, which implies that a robust optimization procedure is required.

The steps constituting the optimization procedure can be outlined globally as follows:

1. Obtain the model structure and a first estimate of *K* values.
   This step is performed by applying a real-coded genetic algorithm (64), facilitating the simultaneous optimization of structural parameters (direction of contribution) and numerical *K* values.
2. Eliminate food components with negligible influence on availability.
   For this step, a small program was made, revealing non-contributing food components by eliminating their contribution and comparing the resulting calculated availability with the original calculated availability throughout the set of food products.
3. Refine the numerical parameters.
   In this step, the *K* values of the pruned mathematical model with known structure are further optimized by applying a simplex algorithm (65,66). A random sample of 200 sets of initial *K* values was generated, spread well around the first (crude) estimate of the *K* values obtained in step 1. The 10 best sets of *K* values (according to the least-squares criterion) were optimized further with the simplex algorithm for 30 iterations.

By applying this optimization procedure, 10 final candidate lists are obtained for each mineral and trace element.

Modelling and optimization equipment

All modelling and optimization activities were performed using a Harlequin LISP developer, a software environment providing program development facilities in the language Common LISP.
To generate mathematical models, taking into account different numbers of food components with a positive or negative influence on the availability of minerals and

Table 1a: Concentrations of Ca, Mg, Fe, Cu and Zn in various types of cereal products and nuts

Product	Ca g/kg DM	Mg g/kg DM	Fe mg/kg DM	Cu mg/kg DM	Zn mg/kg DM
Cereal products					
Wheat flour	0.17	0.15	7	1	5
Wholemeal flour	0.37	1.08	47	4	29
Wheat bran	0.88	3.65	150	12	116
Wheat semolina	0.19	0.78	10	1	6
White bread	0.28	0.27	20	2	10
Brown bread	0.43	0.91	35	4	25
Wholemeal bread	1.33	1.17	46	4	26
Rye bread	0.37	0.94	35	5	28
Crisp bread wheat	1.18	1.07	32	3	26
Crisp bread rye	0.39	1.01	34	3	33
Rice	0.99	0.30	6	1	22
Whole-grain rice	1.26	1.32	14	1	28
Cornflakes	0.07	0.12	8	1	2
Rolled oats	0.67	1.39	40	1	37
Muesli	0.55	1.02	35	5	25
High-fibre biscuits	0.40	0.89	40	5	25
Breakfast cereals	0.38	0.78	34	4	17
Instant cereal porridge	0.52	0.82	36	4	26
Instant cereal porridge	0.51	1.11	51	6	27
Children's biscuits	3.69	0.25	87	2	9
Nuts					
Hazelnut	3.02	3.29	67	35	47
Brazil nut	5.04	11.17	77	66	126
Walnut	2.75	3.39	65	43	64
Peanut	1.00	3.86	83	14	68
Almond	4.41	4.89	70	29	86

trace elements, a mathematical model generator was developed.

The optimization experiments were conducted using the optimization toolbox ICAROS, an in-house developed optimization environment (Common LISP), facilitating the monitoring and coupling of optimization algorithms by means of an expert system.

Results and discussion

The concentrations of phytic acid, dietary fibre components, citric acid, oxalic acid, ascorbic acid, Ca, Mg, Fe, Cu and Zn in the cereal products, fruits, vegetables and nuts are presented in Tables 1-3. Table 4 shows the in vitro availability of Ca, Mg, Fe, Cu and Zn in the food products, as determined with the in vitro method with equilibrium dialysis.

The effect of cooking on the in vitro availability of minerals and trace elements from vegetables; the description and prediction of the in vitro availability of Ca, Mg, Fe,

Table 1b: Concentrations of Ca, Mg, Fe, Cu and Zn in various types of vegetables and fruits.

Product	Ca g/kg DM	Mg g/kg DM	Fe mg/kg DM	Cu mg/kg DM	Zn mg/kg DM
Vegetables					
Potato raw	0.30	0.54	19	2	10
Potato cooked	0.25	0.40	15	2	6
Cauliflower raw	3.06	1.64	49	5	43
Cauliflower cooked	2.98	1.38	43	4	34
Pea raw	1.53	1.48	61	9	50
Pea cooked	1.52	1.32	50	8	47
Leek raw	4.01	1.25	65	4	30
Leek cooked	6.26	1.11	133	6	24
Rhubarb raw	8.85	1.46	24	7	69
Rhubarb cooked	8.88	1.43	31	7	66
Red cabbage raw	3.62	1.10	29	3	14
Red cabbage cooked	3.59	1.10	28	3	14
French bean raw	6.78	2.77	110	8	37
French bean cooked	6.95	2.59	109	7	34
Spinach raw	13.21	13.66	179	16	246
Spinach cooked	15.83	7.16	113	13	183
Carrot raw	3.03	0.65	27	3	15
Carrot cooked	2.97	0.64	29	3	15
Brown bean raw	1.12	1.51	74	8	30
Brown bean cooked	1.14	1.50	75	8	31
Cucumber	5.42	2.62	44	6	32
Lettuce	9.80	2.75	121	8	57
Tomato	1.22	1.16	45	7	22
Fruits					
Strawberry	1.49	1.16	29	4	11
Apricot	1.07	0.57	21	1	6
Apple	0.15	0.28	12	5	1
Banana	0.26	1.18	17	8	6
Pear	0.61	0.44	12	6	8
Peach	0.69	0.65	16	2	8
Orange	2.16	0.80	8	3	3

Cu and Zn from cereal products, fruits, vegetables and nuts; and the influence of food components on the availability of minerals and trace elements are described and discussed below.

Influence of cooking on the in vitro availability of Ca, Mg, Fe, Cu and Zn

Comparison of the results for raw and cooked vegetables (Tables 1b, 2b, 3 and 4b) reveals some differences. In some cases the content of a specific component in the cooked product is lower, and in other cases higher than in the raw product (Tables 1b, 2b and 3). A lower content of a component in the cooked product may be explained by degradation or by leaching of this component during the cooking process. A higher content of a component in the cooked product may be explained by degradation or leaching of other components.

The effects of cooking on the in vitro availability of minerals and trace elements are not clear (Table 4). In some cases the in vitro availability is decreased, in other cases it is increased by cooking, and sometimes there is no effect at all.

A high linear correlation exists between the availability of Ca, Mg and Zn in raw vegetables and in cooked vegetables (r = 0.99, 0.96 and 0.93, respectively), while the correlation for Fe and Cu is much lower (r = 0.67 and 0.59, respectively). These low correlations may be due to the concentration of ascorbic acid which differs widely between raw and cooked products. This is supported by the results presented in the last section, which show that ascorbic acid has an important positive influence on the in vitro availability of Fe, and indicate a similar effect for Cu. Our results are in agreement with results of experiments of Hazell and Johnson (18), who showed that for some vegetables boiling had a strong effect on the in vitro availability of Fe.

Table 2a: Concentrations of phytic acid (PA), total non-starch polysaccharides (NSP), uronic acids (Ura), rhamnose (Rha), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glu) in various types of cereal products and nuts (g/100 g DM).

Product	PA	NSP	Ura	Rha	Ara	Xyl	Man	Gal	Glu
Cereal products									
Wheat flour	0.08	3.74	0.36	0.01	0.93	1.21	0.16	0.48	0.57
Wholemeal flour	0.83	8.95	0.31	0.06	2.07	3.54	0.19	0.43	2.29
Wheat bran	2.81	32.28	1.69	0.11	7.59	12.32	0.58	1.23	8.71
Wheat semolina	0.09	3.95	0.38	0.01	0.92	1.26	0.19	0.50	0.66
White bread	0.05	3.38	0.27	0.02	0.73	1.04	0.16	0.43	0.70
Brown bread	0.31	8.09	0.48	0.04	1.79	2.94	0.23	0.58	2.01
Wholemeal bread	0.55	9.49	0.53	0.04	2.13	3.57	0.20	0.57	2.43
Rye bread	0.03	12.07	0.49	0.05	2.75	4.45	0.45	0.59	3.25
Crisp bread wheat	0.68	8.71	0.52	0.05	1.95	3.13	0.19	0.65	2.18
Crisp bread rye	0.21	13.00	0.51	0.05	2.99	5.04	0.44	0.62	3.32
Rice	0.17	0.81	0.04	0.00	0.06	0.06	0.06	0.13	0.31
Whole-grain rice	0.91	2.68	0.40	0.04	0.46	0.46	0.12	0.37	0.82
Cornflakes	0.02	1.41	0.28	0.01	0.19	0.20	0.05	0.25	0.40
Rolled oats	0.80	8.96	0.53	0.04	1.15	1.57	0.18	0.50	4.96
Muesli	0.69	7.77	0.19	0.08	1.73	2.41	0.19	0.36	2.80
High-fibre biscuits	0.30	9.61	0.53	0.04	2.17	3.80	0.18	0.59	2.28
Breakfast cereals	0.29	7.98	0.60	0.05	1.63	2.75	0.46	0.57	1.88
Instant porridge	0.50	9.15	0.52	0.04	1.93	3.15	0.24	0.54	2.70
Instant porridge	0.68	10.66	0.59	0.05	2.48	4.16	0.20	0.62	2.53
Children's biscuits	0.13	2.68	0.26	0.00	0.57	0.77	0.11	0.40	0.55
Nuts									
Hazelnut	1.91	15.11	3.17	0.60	2.64	0.85	0.40	1.33	5.95
Brazil nut	6.34	13.49	2.69	0.34	2.12	0.96	0.28	1.14	5.76
Walnut	2.38	9.71	2.76	0.37	1.53	0.88	0.15	0.70	3.21
Peanut	1.50	8.00	1.16	0.23	2.22	0.65	0.24	0.42	2.97
Almond	3.22	14.55	3.38	0.49	3.72	1.27	0.31	0.87	4.33

Table 2b: Concentrations of phytic acid (PA), total non-starch polysaccharides (NSP), uronic acids (Ura), rhamnose (Rha), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glu) in various types of vegetables and fruits (g/100 g DM).

Product	PA	NSP	Ura	Rha	Ara	Xyl	Man	Gal	Glu
Vegetables									
Potato raw	0.04	5.99	1.23	0.16	0.35	0.14	0.13	1.95	2.00
Potato cooked	0.03	5.99	1.26	0.17	0.36	0.15	0.13	2.10	1.80
Cauliflower raw	0.05	23.97	6.34	0.90	3.39	1.32	0.76	2.47	8.58
Cauliflower cooked	0.06	26.47	7.35	0.82	3.89	1.55	0.78	2.67	9.18
Pea raw	0.26	22.71	3.73	0.34	5.04	0.97	0.19	0.74	11.61
Pea cooked	0.36	24.07	3.89	0.38	5.40	1.07	0.19	0.76	12.29
Leek raw	0.03	25.65	8.77	0.59	0.93	1.26	0.74	4.39	8.97
Leek cooked	0.04	27.37	6.91	0.70	1.21	1.39	0.81	5.19	11.00
Rhubarb raw	0.01	26.17	9.27	0.82	0.38	2.07	0.79	1.12	11.72
Rhubarb cooked	0.01	24.11	9.04	0.74	0.34	2.07	0.75	1.12	10.05
Red cabbage raw	0.03	26.11	6.61	0.88	4.04	1.37	0.95	2.23	9.86
Red cabbage cooked	0.03	26.65	6.32	0.95	4.21	1.43	0.98	2.27	10.31
French bean raw	0.37	27.12	8.48	0.44	2.31	1.08	1.37	2.60	10.71
French bean cooked	0.36	27.47	6.75	0.47	2.49	1.14	1.40	2.71	12.35
Spinach raw	0.01	16.42	4.91	0.82	1.01	0.84	0.48	1.40	6.85
Spinach cooked	0.02	20.90	5.22	1.05	1.58	1.12	0.57	1.69	9.57
Carrot raw	0.09	22.24	8.63	0.80	1.64	0.36	0.59	3.04	7.18
Carrot cooked	0.09	22.00	8.59	0.76	1.64	0.36	0.58	3.03	7.05
Brown bean raw	0.95	16.74	2.97	0.32	5.46	1.67	0.45	1.52	3.98
Brown bean cooked	0.85	16.12	2.71	0.29	5.27	1.61	0.47	1.44	3.98
Cucumber	0.05	19.47	5.83	0.38	0.62	1.09	0.78	2.88	7.91
Lettuce	0.04	25.22	11.11	0.88	0.90	0.98	0.83	1.67	8.85
Tomato	0.31	17.69	5.54	0.39	0.79	0.92	1.58	1.11	7.35
Fruits									
Strawberry	0.13	12.14	3.76	0.24	0.61	1.58	0.26	1.05	4.56
Apricot	0.00	12.37	4.65	0.32	1.27	0.69	0.45	0.98	3.92
Apple	0.00	9.49	2.60	0.23	1.51	0.61	0.33	0.99	3.11
Banana	0.00	4.65	1.87	0.06	0.31	0.28	0.53	0.45	1.12
Pear	0.00	15.54	3.00	0.28	1.02	4.50	0.23	0.78	5.64
Peach	0.00	11.77	3.76	0.27	1.77	0.59	0.23	1.01	4.00
Orange	0.02	13.12	5.31	0.30	1.87	0.44	0.42	1.80	2.90

Use of the mathematical model to investigate the relation between the in vitro availability of Ca, Mg, Fe, Cu and Zn and the composition of cereal products, fruits, vegetables and nuts

The mathematical model described (equation 10) was used to investigate the relation between the in vitro availability of Ca, Mg, Fe, Cu and Zn and the concentrations of phytic acid, non-starch polysaccharides (NSP), ascorbic acid and citric acid in cereal products, fruits, vegetables and nuts. The mathematical model was optimized as described above, resulting in a specific mathematical model for each mineral and trace element. In all cases, the 10 lists resulting from the optimization procedure were (nearly) identical, indicating a robust optimization procedure. Values for the

Table 3: Concentrations of ascorbic acid (mg/100 g d.m.), citric acid (g/100 g d.m.) and oxalic acid (g/100 g d.m.) in various types of vegetables and fruits

Product	Ascorbic acid	Citric acid	Oxalic acid
Vegetables			
Potato raw	3	1.6	1.0
Potato cooked	33	1.4	1.1
Cauliflower raw	158	0.0	2.5
Cauliflower cooked	295	0.6	1.4
Pea raw	53	0.7	0.3
Pea cooked	1	0.8	0.3
Leek raw	3	0.0	1.8
Leek cooked	30	0.5	0.7
Rhubarb raw	2	1.2	62
Rhubarb cooked	-	1.3	68
Red cabbage raw	120	-	0.8
Red cabbage cooked	116	-	1.1
French bean raw	0	0.5	0.9
French bean cooked	13	0.6	0.7
Spinach raw	0	0.3	143
Spinach cooked	0	0.1	66
Carrot raw	0	0.5	2.1
Carrot cooked	3	0.5	1.4
Brown bean raw	0	1.0	0.2
Brown bean cooked	0	1.0	0.2
Cucumber	15	0.1	0.6
Lettuce	1	0.0	0.6
Tomato	99	2.8	1.5
Fruits			
Strawberry	455	5.4	0.5
Apricot	0	2.2	0.6
Apple	0	0.2	0.4
Banana	0	1.3	0.3
Pear	0	0.7	0.3
Peach	0	2.1	0.4
Orange	432	8.0	0.5

calculated availability of the minerals and trace elements were obtained by substituting the concentrations of food components in the specified mathematical model.

To minimize the number of variables, first the concentrations of phytic acid, total NSP, ascorbic acid and citric acid were used in the optimization of the mathematical model. When this optimization revealed a contribution of NSP to the in vitro availability, the concentration of total NSP was replaced with the concentrations of the constituent sugars of the NSP. In this way we obtained insight into the specific NSP components responsible for the influence on the in vitro availability of minerals and trace elements.

The food component oxalic acid and the food products spinach (raw and cooked) and rhubarb (raw and cooked) were not included in the optimization experiments because these vegetables contain very high concentrations of oxalic acid while the other food products contain only a small amount of oxalic acid, if any at all (Table 3). This puts a large stress on the *K* value for oxalic acid and thus disturbs a good optimization of the other food products.

The product cornflakes was discarded from the data set because in all cases the calculated availability exceeded the measured availability by far.

Table 5 shows for Ca, Mg, Fe, Cu and Zn the food components influencing the in vitro availability, the direction of their influence, the *K* values for these food components, the mean and standard deviation of the difference between the in vitro availability measured and the in vitro availability calculated with the specified mathematical model, and the linear correlation coefficient between measured and calculated in vitro availabilities.

Table 4a: Dialysability of Ca, Mg, Fe, Cu and Zn from different types of cereal products and nuts as determined with the in vitro procedure of Hazell and Johnson (in % of the amount present)

Product	Ca	Mg	Fe	Cu	Zn
Cereal products					
Wheat flour	22	64	52	44	64
Wholemeal flour	14	76	6	54	12
Wheat bran	4	36	2	38	4
Wheat semolina	52	20	24	52	50
White bread	30	86	24	38	30
Brown bread	18	70	4	44	6
Wholemeal bread	10	58	2	38	2
Rye bread	38	68	14	38	48
Crisp bread wheat	10	60	10	46	8
Crisp bread rye	12	76	8	78	14
Rice	26	84	12	82	12
Whole-grain rice	56	52	20	98	22
Cornflakes	16	52	8	0	14
Rolled oats	14	42	12	97	10
Muesli	14	36	4	52	12
High-fibre biscuits	16	38	10	40	4
Breakfast cereals	12	40	10	44	18
Instant cereal porridge	10	32	4	52	8
Instant cereal porridge	6	60	4	32	2
Children's biscuits	26	70	24	28	4
Nuts					
Hazelnut	0	20	4	54	0
Brazil nut	2	16	8	32	0
Walnut	8	26	4	22	4
Peanut	4	28	6	64	4
Almond	6	32	6	52	4

Table 4b: Dialysability of Ca, Mg, Fe, Cu and Zn from different types of vegetables and fruits as determined with the in vitro procedure of Hazell and Johnson (in % of the amount present)

Product	Ca	Mg	Fe	Cu	Zn
Vegetables					
Potato raw	94	98	42	98	104
Potato cooked	102	98	102	106	98
Cauliflower raw	30	100	44	100	82
Cauliflower cooked	36	108	64	66	86
Pea raw	26	66	16	84	88
Pea cooked	20	58	14	76	66
Leek raw	32	80	26	84	104
Leek cooked	42	74	18	56	88
Rhubarb raw	0	96	40	74	90
Rhubarb cooked	0	94	32	68	92
Red cabbage raw	56	86	30	50	92
Red cabbage cooked	58	82	34	46	92
French bean raw	18	68	12	80	70
French bean cooked	28	74	10	78	42
Spinach raw	0	38	46	30	0
Spinach cooked	0	42	34	48	6
Carrot raw	38	92	10	78	50
Carrot cooked	42	98	34	108	40
Brown bean raw	30	68	4	74	90
Brown bean cooked	32	60	4	62	74
Cucumber	70	86	50	92	100
Lettuce	40	78	6	76	80
Tomato	70	104	96	106	110
Fruits					
Strawberry	82	96	2	32	82
Apricot	78	106	22	58	104
Apple	74	94	10	34	100
Banana	50	112	0	30	70
Pear	38	80	12	80	102
Peach	66	94	10	108	100
Orange	62	92	100	8	106

The linear correlation characteristics and the mean and standard deviation of the difference between calculated and measured availability are indicators for the goodness of fit of the mathematical model. High correlation coefficients and good values for the mean and standard deviation of the difference between the measured and calculated availability were obtained for Ca, Mg, Fe and Zn. The slopes of the linear relations are close to 1 (between 0.84 and 0.97) and the abscissae are close to zero (between -1 and 4). For each of the elements Ca, Mg, Fe and Zn, a few food products (1-3) showed a relatively large deviation between calculated and measured availability. For Cu, the in vitro availability could not be described by the mathematical model. These effects might be due to food components not

Table 5: Mathematical description of the in vitro availability of minerals and trace elements in cereal products, fruits, vegetables and nuts.

Mineral/ trace element	Food component	+ or - influence on in vitro availability	*K* value	Difference (%) between calculated and measured availability (mean ± SD)	Correlation coefficient between calculated and measured availability
Calcium	phytic acid	-	9.2×10^2	1 ± 12	0.89
	arabinose	-	1.5×10^2		
	citric acid	+	2.4×10^2		
Magnesium	phytic acid	-	1.9×10^2	-1 ± 14	0.87
	citric acid	+	1.5×10^2		
Iron	phytic acid	-	2.0×10^3	4 ± 12	0.90
	arabinose	-	9.6×10^2		
	ascorbic acid	+	4.0×10^4		
Copper	NSP	-	2.0×10^1	-5 ± 27	0.54
	ascorbic acid	+	1.2×10^6		
	citric acid	+	8.1×10^2		
Zinc	phytic acid	-	3.4×10^3	0 ± 16	0.92
	citric acid	+	4.2×10^3		

taken into account in this study which may be very relevant for the availability (such as tannins and some amino acids for Cu). Also, the mutual influence of minerals and trace elements may be relevant in these cases.

We conclude that, throughout the complete set of food products, the in vitro availability of Ca, Mg, Fe and Zn can be described well with the optimized mathematical model based on the concentration of only two or three food components.

An optimization experiment taking into account only cereals and nuts showed that the in vitro availability of Mg and Zn in these products can be well described on the basis of the concentration of phytic acid only, while the in vitro availability of Ca and Fe can be described on the basis of the concentrations of phytic acid and arabinose.

Prediction of the in vitro availability of Ca, Mg, Fe and Zn

The results presented above showed that the mathematical model describes the in vitro availability of Ca, Mg, Fe and Zn well. To test the model more rigorously, prediction experiments were carried out.

Ten food products were randomly discarded from the data set and step 3 of the optimization procedure was applied to the remaining food products. The specified mathematical models resulting from this optimization were used to predict the in vitro availability for the 10 "unknown" food products. This prediction experiment was carried out in triplicate for each mineral and trace element.

No or only slight differences were found between the predicted availability and the in vitro availability calculated for these products in earlier experiments. Table 6 shows the mean and standard deviation of the difference between the predicted availability and the calculated availability, and between the predicted availability and the availability determined in vitro for Ca, Mg, Fe and Zn. On the basis of these results it is concluded that the mathematical model is able to predict well the in vitro availability of minerals and trace elements. Figure 2 shows the linear relation between the predicted availability of calcium and its availability determined in vitro.

Influence of phytic acid, non-starch polysaccharides, ascorbic acid, citric acid and oxalic acid on the availability of Ca, Mg, Fe and Zn

To obtain information on the specific contribution of a food component to the availability of a mineral or trace element, this food component was eliminated from the specified mathematical model. The difference between the calculated availabilities with and without this food component gives insight into the contribution of this food component to the availability of the mineral or trace element in a specific food product. For this purpose, we used the program mentioned in step 2 of the optimization procedure.

These experiments clearly showed the complexity of the influence of food components on the availability of minerals and trace elements. In general, the presence of a food component as such gives no information about the actual availability of a mineral or

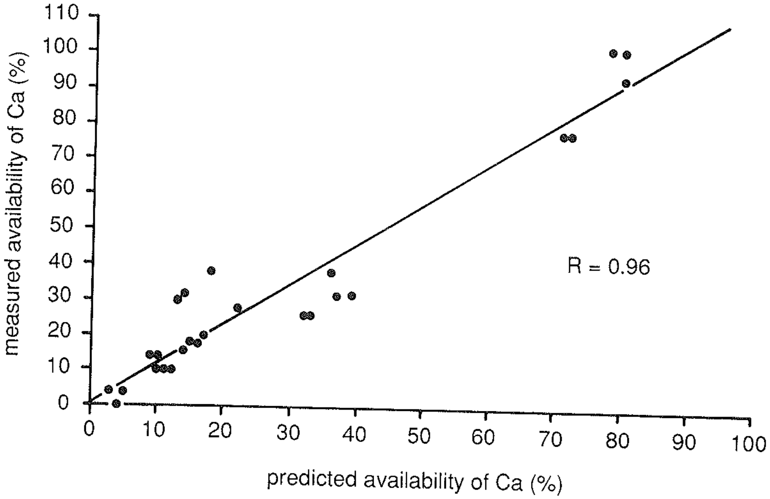

Figure 2: Linear relation between the availability of Ca · in food products predicted with the mathematical model and determined in vitro

Table 6: Prediction of the in vitro availability of Ca, Mg, Fe and Zn with the mathematical model in randomly selected food products

Mineral/ trace element	Difference (%) between calculated and predicted in vitro availability (mean ± SD)	Difference (%) between measured and predicted in vitro availability (mean ± SD)
Calcium	0 ± 3	4 ± 9
Magnesium	-1 ± 2	-4 ± 16
Iron	0 ± 3	2 ± 13
Zinc	0 ± 3	1 ± 16

trace element, because the presence of other food components determines the actual contribution of this food component to mineral availability. For example, in raw French bean, the measured in vitro availability of Zn is 70 %. Based on the concentration of phytic acid only, the calculated availability would be 7 %. Taking into account both phytic acid and citric acid results in a calculated availability of 64 %, well in line with the measured value.

In general, phytic acid has a strong negative effect on the calculated availability of Ca, Fe and Zn and a less strong effect on the calculated availability of Mg. This is in agreement with the *K* values for phytic acid, which increase in the order Mg < Ca < Fe < Zn.
The fact that phytic acid influences the availability of Ca, Fe and Zn has been reported before (1,2,13,36,49,67). Evans and Martin (61) showed that phytic acid is able to form insoluble complexes with Mg. In agreement with our results, Torre et al. (1) reported a slight negative influence of phytic acid on the bioavailability of Mg.

Arabinose-containing components have a negative influence on the calculated availability of Ca and Fe. The mechanism is not clear. Associated acidic components may play a role in this.
In cereal products, arabinose is mainly present in the form of arabinoxylans, while in fruits and vegetables the arabinose is mainly present as arabinogalactans (often associated with pectic material). We are not aware of specific studies regarding the influence of arabinoxylans and arabinogalactans on the availability of minerals and trace elements. Although studies on the influence of dietary fibre on mineral availability are often hampered by the presence of phytic acid, several authors have shown that dietary fibre components are able to bind Ca and Fe and thus influence their availability (2,5,7,21,68,69).
Although the fact that pectin binds divalent cations like Ca and Fe is well documented (69,70,71), our results did not show an effect on the availability of minerals and trace elements. This might be due to a high degree of esterification of the pectins in the food products. Another explanation might be the presence of other food components

in the real food matrices. Although pectins can bind minerals and trace elements, this effect may be overruled by the effects of other food components.

Ascorbic acid has a positive influence on the calculated availability of Fe (Table 5). This effect of ascorbic acid has been reported before (13,14,18,19,50,52).

Citric acid has a positive influence on the calculated availability of Ca, Mg and Zn. Pallauf et al. (72) reported that citric acid enhances the bioavailability of Zn in rats. No information is available on effects of citric acid on the bioavailability of Ca and Mg. Although Fernandez and Phillips (21), Hazell and Johnson (18,52) and Hazell (50) reported a positive effect of citric acid on the in vitro availability of Fe, our results do not support this. This discrepancy might be attributed to the fact that we studied the effect of the citric acid intrinsically present in the fruits and vegetables. This means that citric acid might have a positive effect on the availability of Fe, but because the positive influence of ascorbic acid is much stronger, citric acid plays only a minor role in the availability of iron from fruits and vegetables containing ascorbic acid.

For reasons mentioned above, oxalic acid was excluded from the optimization experiments. To get an indication of the influence of oxalic acid on the calculated availability of the minerals and trace elements, the availability of the food products spinach and rhubarb was predicted using the mathematical model. If oxalic acid has a strong negative influence on the availability of minerals or trace elements, this must result in a large discrepancy between the measured and the calculated in vitro availabilities (calculated » measured).
Indeed, for spinach the mathematical model overestimates the availability of Ca, Mg and Zn by far. For rhubarb, the Ca availability is largely overestimated, while for Mg and Zn the calculated and measured availabilities are well in line. Studying this phenomenon more extensively by means of the mathematical model revealed that oxalic acid also has a negative effect on Mg and Zn availability in rhubarb, but in this product the effect is counterbalanced by the presence of citric acid. This once more stresses the importance of taking the complete food matrix into account.
In vivo experiments have clearly shown the negative effect of oxalic acid on the bioavailability of Ca (30,31,32). Liebman and Doane (30) reported that oxalic acid has no negative effect on the bioavailability of Zn. Although Chawla et al. (33) reported a negative effect of oxalic acid on the in vitro availability of Fe, our results do not support this.

Conclusions

The mathematical model presented in this paper describes well the experimental data for the in vitro availability of Ca, Mg, Fe and Zn throughout the complete set of food products, taking only two or three relevant food components into account. The standard deviation of the difference between the calculated availability and the availability of these minerals and trace elements determined in vitro is between 12 and

16 %. For Cu no satisfactory model could be obtained, indicating that either relevant food components were not taken into account, or that the assumptions underlying the mathematical model do not hold for Cu.

Prediction tests show that the mathematical model predicts well the in vitro availability of Ca, Mg, Fe and Zn. The standard deviation of the difference between the predicted availability and the in vitro measured availability of these minerals and trace elements is between 9 and 16 %.

In addition, the mathematical model proves to be a valuable tool for investigating the relative influences of food components on the in vitro availability of minerals and trace elements. In general, the simultaneous effects of several food components are difficult to distinguish using in vitro and in vivo experiments. Mathematical model calculations clearly show the presence of simultaneous effects of food components on the availability of minerals and trace elements, which demonstrates the importance of studying complete food matrices.

According to the mathematical description of in vitro availability, phytic acid has a strong negative influence on the availability of Ca, Fe and Zn, and a less strong effect on the availability of Mg. Arabinose-containing components decrease the availability of Ca and Fe. Ascorbic acid increases the availability of Fe, while citric acid increases the availability of Ca, Mg and Zn. Oxalic acid has a negative influence on the availability of Ca, Mg and Zn. These influences are generally in line with results presented in the literature.

Although we did not relate our mathematical model to in vivo experiments, the procedure presented in this paper can also be applied to experimental in vivo data. In addition, mathematical model simulations may be helpful in designing cost-effective in vivo experiments and in bridging the gap between in vitro simulations and in vivo experiments.

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Part VI

Discussion and conclusions

CHAPTER 13

Discussion

The study presented in this thesis concerns the bioavailability of minerals and trace elements from food products to animals and man. In particular, the objectives of the study were:

- to develop an in vitro method for the prediction of the bioavailability of calcium, magnesium, iron, copper and zinc;
- to investigate the effects of phytic acid, dietary fibre and other food components on the in vitro availability of calcium, magnesium, iron, copper and zinc.

The nature of these closely related objectives asked for a multidisciplinairy approach. Expertise was required with respect to analytical chemistry, in vitro techniques, in vivo methods, mathematical modelling and optimization procedures.

First, methods of analysis for dietary fibre and phytic acid were investigated. Because the merits of methods for the analysis of dietary fibre were not clear, these methods were studied in detail. Because few methods for the analysis of phytic acid were specific, an improved method was developed. Second, in vitro methods for the determination of the availability of minerals and trace elements were developed. Third, an in vivo experiment was carried out to validate the use of in vitro methods for predicting the bioavailability of minerals and trace elements in vivo. Fourth, because several food components affect the availability of minerals and trace elements simultaneously, a mathematical model was developed to study the relative influence of food components on the in vitro availability.

These topics are described and discussed in this thesis. The most important points of discussion will be summarized below.

Methods for the analysis of dietary fibre and phytic acid

Dietary fibre

Several methods are available for the determination of dietary fibre. It is, however, rather difficult to evaluate the relative merits of these methods because it is not exactly clear which dietary fibre components are analysed in each of these methods. Therefore, a specific study directed at a comparison of different methods for the analysis of dietary fibre was conducted (Chapter 5).

The relative merits of the ADF, NDF, Hellendoorn, AOAC and Englyst methods for the analysis of dietary fibre (1,2,3,4,5) were investigated by comparing these methods with respect to the amount and the composition of the dietary fibre analysed. Modern methods like the AOAC and Englyst methods yield more accurate determinations of dietary fibre than older methods such as the ADF/NDF methods and Hellendoorn's method. It is shown that although different methods may yield comparable integral dietary fibre values, the composition may vary greatly, indicating the importance of detailed chemical knowledge of the types of components determined in the various methods.

The discussion on suitable methods for the analysis of dietary fibre is severely hampered by the lack of consensus about the type of components that should be included in the definition of dietary fibre. Because dietary fibre is primarily important from a physiological point of view, its definition should include all physiologically important components, such as NSP, resistant starch and lignin. On the other hand, a method for the analysis of dietary fibre should preferably be based on chemically well defined components. Therefore, the different constituents of dietary fibre, such as NSP, resistant starch and lignin, should be determined separately, each with a specific analytical method.

At present, methods for analysis of dietary fibre in which NSP are measured have to be preferred because NSP is a well defined group of dietary fibre components. Moreover, gas chromatographic and liquid chromatographic methods provide insight into the types of NSP present.

Phytic acid

Quite some methods are available for the analysis of phytic acid (inositol hexaphosphate). However, due to interferences of lower inositol phosphates and metal ions, few methods are sufficiently specific. Therefore, a study aimed at developing an improved method for the determination of phytic acid was conducted (Chapter 7).

In the literature, methods based on anion-exchange chromatography with post-column derivatization and methods based on phosporus-31 NMR spectroscopy show the best results for the determination of phytic acid. Because phosphorus-31 NMR requires quite expensive equipment, methods based on anion-exchange chromatography are the most attractive at present.

Anion-exchange chromatography with post-column derivatization allows for a good separation of inositol mono-, di-, tri-, tetra-, penta- and hexaphosphate (6). However, because phytic acid is known to form strong complexes with di- and trivalent metal ions, these metal ions may interfere with an anion-exchange chromatographic determination of phytic acid, resulting in too low values. This may especially be a problem in complex food products or animal feed, which contain high concentrations of minerals and trace elements.

We adopted an anion-exchange chromatographic method similar to the methods described by Phillippy and Johnston (7) and Cilliers and van Niekerk (6) and improved it by eliminating the interference of metal ions by means of an EDTA treatment of the sample extracts. This improvement resulted in higher values for phytic acid, revealing the importance of prior removal of metal ions.

In vitro methods for the prediction of the bioavailability of Ca, Mg, Fe, Cu and Zn

Although numerous in vitro methods for the determination of the availability of minerals and trace elements have been developed over the years, there is often a poor relation between in vitro availability and bioavailability in vivo. Therefore, an in vitro method was developed that was believed to simulate the conditions of stomach and small intestine better than most of the in vitro methods described before, and thus allows for a better prediction of the bioavailability in vivo (Chapter 9).

In vitro methods based on the determination of the amount of dialysable minerals and trace elements under simulated physiological conditions according to Miller et al. (8) show reasonably good correlations between in vitro and in vivo availability. Because minerals and trace elements are absorbed in the small intestine, simulation of the conditions in the small intestine is considered the most critical step for in vitro methods aiming at predicting the bioavailability of minerals and trace elements. In vitro methods based on the method of Miller et al. use equilibrium dialysis of minerals and trace elements across a semipermeable membrane as a model for the passage across the intestinal wall. In contrast with the situation in vivo, however, this is a static process in which components that pass this membrane are not removed.

We developed a dynamic in vitro method in which dialysable components (including minerals and trace elements) are continuously removed from the pancreatic digestion mixture by means of a hollow-fibre system. It is hypothesized that this in vitro method leads to a better estimation of bioavailability because in vivo physiological conditions are approached more closely. A direct comparison of the in vitro method based on continuous dialysis and the in vitro method based on equilibrium dialysis revealed a pronounced effect of the continuous removal of dialysable components on the dialysability of minerals and trace elements. Generally, the dialysability determined was higher for the continuous in vitro method than for the equilibrium in vitro method.

Validation of the use of in vitro methods for the prediction of the bioavailability of Ca, Mg, Fe, Cu and Zn

The in vitro method based on equilibrium dialysis and the in vitro method based on continuous dialysis were validated in an in vivo experiment with piglets for their use in predicting the bioavailability of minerals and trace elements (Chapter 10).

There is a discrepancy between the absolute values of the bioavailability of Ca, Mg, Fe, Cu and Zn determined in piglets and the availability of these minerals and trace elements determined in vitro. However, both the equilibrium in vitro method and the continuous in vitro method reflect adequately the effects of differences in the composition of the pig diets on the bioavailability of minerals and trace elements in vivo. Therefore, it is concluded that the in vitro methods can be used for a relative prediction of the bioavailability of minerals and trace elements. An absolute prediction might be obtained by introducing a correction factor allowing for calculation of the bioavailability in vivo on the basis of the availability determined in vitro. Such a correction factor has to be determined by comparing values for in vitro and in vivo availability, and has to be validated carefully. A different correction factor will be necessary for each mineral and trace element and for each group of food products.

The in vitro method based on continuous dialysis corresponds slightly better with the bioavailability of minerals and trace elements in vivo than the in vitro method based on equilibrium dialysis. However, the importance of the continuous removal of dialysable components for the prediction of the bioavailability of minerals and trace elements in vivo needs further investigation.

The influence of food components on the in vitro availability of Ca, Mg, Fe, Cu and Zn

Several components in the diet of animals and man can form soluble or insoluble complexes with minerals and trace elements under gastro-intestinal conditions, and may thus influence the bioavailability of these minerals and trace elements. The relative influence of a specific food component on the bioavailability of minerals and trace elements is, however, difficult to assess due to the presence of other dietary components which influence this bioavailability simultaneously. We investigated the relative influence of several food components on the in vitro availability of Ca, Mg, Fe, Cu and Zn in food products with a mathematical model (Chapter 12).

The influence of food components on the bioavailability of minerals and trace elements should preferably be studied in a complete food matrix rather than with an isolated food component. However, in vitro and in vivo experiments aimed at investigating the availability of minerals and trace elements from a complete food matrix are often a black box: no insight is obtained into the relative influence of the various food components that simultaneously affect the availability of minerals and trace elements.

To describe the influence of dietary fibre, phytic acid, citric acid, ascorbic acid and oxalic acid on the in vitro availability of Ca, Mg, Fe, Cu and Zn, a mathematical model was developed based on chemical theoretical assumptions. After optimization of both the model structure and the numerical model parameters, this mathematical model was shown to describe and predict adequately the in vitro availability of Ca, Mg, Fe and Zn throughout a set of 55 food products on the basis of the concentrations of only two or three food components with a negative or positive influence on in vitro availability. In addition, the mathematical model proved to be a valuable tool for gaining insight into the relative influence of the different food components on the in vitro availability of minerals and trace elements. According to the mathematical description of the in vitro availability, phytic acid has a strong negative influence on the availability of Ca, Fe, and Zn, and a less pronounced influence on the availability of Mg; arabinose-containing components decrease the availability of Ca and Fe; oxalic acid has a negative influence on the availability of Ca, Mg and Zn; and tannins decrease the availability of Fe and Cu. Ascorbic acid increases the availability of Fe, while citric acid increases the availability of Ca, Mg and Zn. It is demonstrated that food components with a positive influence on the availability of minerals and trace elements can counteract the effects of food components with a negative influence. Without an insight into the relative influence of food components obtained with the mathematical model, these counterbalancing effects may well lead to misinterpretations.

Although the mathematical model was used here to describe and predict the in vitro availability of minerals and trace elements, it is expected that, by applying the same optimization procedure, it is also possible to use the mathematical model for describing and predicting the in vivo availability of minerals and trace elements. In addition, mathematical model simulations may be helpful in designing more cost-effective in vivo experiments and in bridging the gap between in vitro and in vivo experiments. In general, combining in vivo experiments, in vitro experiments and mathematical modelling will result in a powerful research approach to obtain a better understanding of the mechanisms and important factors involved in digestion and absorption processes.

CHAPTER 14

Conclusions

Although different methods for the analysis of dietary fibre may yield comparable integral values, the composition of the dietary fibre analysed may vary widely. At present, gas chromatographic or liquid chromatographic methods determining the amount of NSP are to be preferred because well defined components of the dietary fibre are analysed and insight is obtained into the types of polysaccharides present.

Incorporation of an EDTA treatment in the anion-exchange chromatographic determination of phytic acid eliminates the interference of metal ions, and thus allows for a more specific determination of phytic acid.

Due to the removal of dialysable components from the pancreatic digestion mixture, the continuous in vitro method presented in this thesis generally results in a higher dialysability of minerals and trace elements than the in vitro method with equilibrium dialysis. The importance of continuous removal of dialysable components for the prediction of the bioavailability in vivo needs further investigation.

Although there is a discrepancy between the absolute values of the bioavailability of minerals and trace elements determined in piglets and the availability determined in vitro, both the continuous in vitro method and the equilibrium in vitro method reflect adequately the effects of differences in food composition on the bioavailability of minerals and trace elements. Therefore, the in vitro methods can be used for a relative prediction of the bioavailability of minerals and trace elements, and are thus very useful in providing preliminary or additional information in support of in vivo experiments, or in the ranking of foodstuffs.

Throughout a set of different food products, the in vitro availability of Ca, Mg, Fe and Zn can be well described and predicted by a mathematical model based on chemical theoretical assumptions. In addition, the mathematical model provides insight into the relative influence of food components on the availability of minerals and trace elements.

According to the mathematical description of the in vitro availability of minerals and trace elements from different food products, phytic acid has a strong negative influence on the availability of Ca, Fe and Zn, and a less pronounced negative influence on the availability of Mg; arabinose-containing components decrease the availability of Ca and Fe; oxalic acid has a negative influence on the availability of Ca, Mg and Zn; and tannins decrease the availability of Fe and Cu. Ascorbic acid increases the availabilty of Fe, while citric acid increases the availability of Ca, Mg and Zn.

Because in vitro methods are rapid and relatively inexpensive, and mathematical models provide insight into the relative influence of food components, the combination of in vivo experiments, in vitro experiments and mathematical modelling is a powerful approach to obtain a better understanding of the mechanisms and important factors involved in the bioavailability of minerals and trace elements.

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Summary

Minerals and trace elements play essential roles in numerous biochemical and physiological processes in animals and man. A deficiency, an overdose or imbalances between minerals or trace elements will exert a negative effect on health. Generally, it is not the ingested dose of minerals and trace elements that is important to maintain balance, but rather the amount that is bioavailable (available for biological and biochemical processes in the organism). Several food components are able to form soluble or insoluble complexes with minerals and trace elements under gastro-intestinal conditions. These food components thereby increase or decrease the availability for absorption in the small intestine, and thus the bioavailability of minerals and trace elements. Due to the complexity of food products, however, the relative contributions of food components to the bioavailability of minerals and trace elements are often not clear.

Although in vivo experiments are the best way to study the bioavailability of minerals and trace elements, in vitro methods offer an appealing alternative because they are relatively simple, rapid and inexpensive. Therefore, there is a great need in human nutrition and animal nutrition for an in vitro method which predicts the bioavailability of minerals and trace elements in vivo.

Part I describes the scope and the objectives (Chapter 1), and the experimental approach (Chapter 2) of the research presented in this thesis. The objectives were twofold:

- to develop an in vitro method for the prediction of the bioavailability of calcium, magnesium, iron, copper and zinc
- to investigate the effects of phytic acid, dietary fibre and other food components on the in vitro availability of calcium, magnesium, iron, copper and zinc.

To meet these objectives a multidisciplinary approach was followed.

Part II discusses several aspects of dietary fibre.

In Chapter 3 a literature review of the definitions and terminology of dietary fibre is presented. Although the term "dietary fibre" is now widely accepted, there is still some confusion concerning its definition.

Chapter 4 presents a literature review of methods for the analysis of dietary fibre. As such methods are based on a definition of dietary fibre, it is important that there is consensus regarding the type of components that should be included in the definition. An advantage of a definition based on chemically defined components is that it allows for a specific analytical determination of dietary fibre components.

In Chapter 5 a comparison of different methods for the analysis of dietary fibre is presented. The ADF, NDF, Hellendoorn, AOAC and Englyst methods are compared

with respect to the amount and the composition of the dietary fibre analysed. Although different methods for the analysis of dietary fibre can yield comparable integral values, the NSP (non-starch polysaccharides) composition of the dietary fibre residues may differ greatly. It is shown that modern methods such as the AOAC and Englyst methods give more accurate determinations of dietary fibre than older methods such as the ADF/NDF method and Hellendoorn's method. Methods in which the NSP are determined (such as the Englyst method) are to be preferred because a well defined group of dietary fibre components are determined. Moreover, the gas chromatographic procedures give insight into the types of NSP present.

Part III concerns the analysis of phytic acid (myoinositol hexakis(dihydrogen)-phosphate).
A literature review of methods for the analysis of phytic acid is presented in Chapter 6. According to the literature, few methods for the analysis of phytic acid are specific because of interferences from lower inositol phosphates, inorganic phosphates and metal ions. Currently, methods based on anion-exchange chromatography with post-column derivatization and methods based on phosphorus-31 NMR show the best results.
In Chapter 7 an improved method for the determination of phytic acid is presented. The method is based on an anion-exchange chromatographic procedure with post-column derivatization in which EDTA treatment of the samples is incorporated to eliminate interferences from metal ions. The improved method is compared with a HPLC method without EDTA treatment and with a method using small single-use ion-exchange columns. It is shown that elimination of the interference of metal ions by means of EDTA treatment in the anion-exchange chromatographic procedure allows for a more specific determination of phytic acid.

Part IV deals with in vitro methods for the prediction of the bioavailability of Ca, Mg, Fe, Cu and Zn.
Chapter 8 presents a literature review of in vitro methods for the estimation of the bioavailability and its correlation with bioavailability in vivo. According to the literature, the most promising in vitro methods are based on equilibrium dialysis under simulated physiological conditions, in which the dialysability of minerals and trace elements is taken as a measure for their bioavailability.
In Chapter 9 a continuous in vitro method for the estimation of the bioavailability of Ca, Mg, Fe, Cu and Zn is described. In this in vitro method dialysable components (including minerals and trace elements) are continuously removed from the pancreatic digestion mixture. The in vitro method based on continuous dialysis is compared with the in vitro method based on equilibrium dialysis with respect to the dialysability of Ca, Mg, Fe, Cu and Zn from eight types of bread. The continuous in vitro method generally results in higher dialysabilities of minerals and trace elements than the equilibrium in vitro method. The relevance of this effect for prediction of the bioavailability in vivo needs further investigation.
The in vitro method with continuous dialysis and the in vitro method with equilibrium dialysis were validated for their usefulness as predictors of the bioavailability of Ca,

Mg, Fe, Cu and Zn in an in vivo experiment with piglets. This study is presented in Chapter 10. It is shown that although both in vitro methods show a discrepancy between the absolute values of the availability determined in vitro and the bioavailability determined in vivo, they reflect quite well the effects of differences in food composition on the bioavailability in vivo. Therefore it is concluded that the in vitro methods can be used for a relative prediction of the bioavailability of minerals and trace elements and thus may be very useful in providing preliminary or additional information to in vivo experiments.

Part V discusses the influence of food components on the availability of minerals and trace elements.
Chapter 11 is a literature review of the influence of dietary fibre, phytic acid and other food components on the availability of Ca, Mg, Fe, Cu and Zn. This concise review shows that the bioavailability of minerals and trace elements is a very complex issue because it may be influenced by a variety of dietary components. Therefore, the influence of dietary fibre, phytic acid and other dietary components on the bioavailability of minerals and trace elements should not be studied separately but in the whole food matrix.
Chapter 12 describes a mathematical model to investigate the relative influences of food components on the in vitro availability of Ca, Mg, Fe, Cu and Zn. The mathematical model is based on chemical theoretical assumptions and optimized with respect to the model structure and the numerical model parameters. It is shown that the mathematical model describes and predicts the in vitro availability of Ca, Mg, Fe and Zn quite well on the basis of the concentrations of two or three food components. The mathematical model proves also valuable for obtaining insight into the relative influences of food components on the in vitro availability of minerals and trace elements. According to the mathematical description of the in vitro availability, phytic acid has a strong negative effect on the availability of Ca, Fe and Zn, and a less pronounced effect on the availability of Mg. Arabinose-containing components decrease the availability of Ca and Fe. Oxalic acid has a negative influence on the availability of Ca, Mg and Zn. Ascorbic acid increases the availability of Fe, while citric acid has a positive influence on the availability of Ca, Mg and Zn. It is shown that food components with a positive influence on availability can counteract the effects of food components with a negative influence.
Because in vitro methods are rapid and relatively inexpensive, and mathematical models provide insight into the relative influences of food components, the combination of in vivo experiments, in vitro experiments and mathematical modelling is a powerful approach to obtain a better understanding of the mechanisms and important factors involved in the bioavailability of minerals and trace elements.

The studies presented in this thesis investigated the bioavailability of minerals and trace elements from different points of view. In Part VI the most relevant points of discussion (Chapter 13) and conclusions (Chapter 14) are presented.

Samenvatting

Mineralen en spoorelementen spelen een belangrijke rol in biologische en biochemische processen in het lichaam van mens en dier. Een tekort, een overschot of een verkeerde balans tussen mineralen of spoorelementen heeft dan ook negatieve gevolgen voor de gezondheid. In het algemeen is het niet de geconsumeerde hoeveelheid van mineralen en spoorelementen die van belang is voor een goede balans, maar de mate waarin de mineralen en spoorelementen biologisch beschikbaar zijn (beschikbaar voor biologische en biochemische processen in het lichaam). Onder de omstandigheden die heersen in de maag en dunne darm kunnen verschillende componenten uit de voeding oplosbare of onoplosbare complexen vormen met mineralen en spoorelementen. Deze voedingscomponenten verhogen of verlagen hierdoor de beschikbaarheid voor opname vanuit de dunne darm, en hiermee de biologische beschikbaarheid van mineralen en spoorelementen. Vanwege de complexe samenstelling van veel voedingsmiddelen is de relatieve invloed van verschillende voedingscomponenten op de biologische beschikbaarheid van mineralen en spoorelementen echter moeilijk vast te stellen.
Hoewel de beschikbaarheid van mineralen en spoorelementen voor mens en dier het best bestudeerd kan worden met behulp van in vivo experimenten, vormen in vitro methoden een aantrekkelijk alternatief omdat ze relatief eenvoudig, snel en goedkoop zijn. Daarom bestaat er bij de bestudering van de voeding van mens en dier een grote behoefte aan in vitro methoden waarmee de biologische beschikbaarheid van mineralen en spoorelementen in vivo voorspeld kan worden.

Deel I beschrijft de achtergronden en de doelstelling (hoofdstuk 1) en de experimentele opzet (hoofdstuk 2) van het onderzoek dat beschreven is in dit proefschrift. De doelstelling van het onderzoek was tweeledig:

- het ontwikkelen van een in vitro methode waarmee de biologische beschikbaarheid van calcium, magnesium, ijzer, koper en zink voorspeld kan worden
- het bestuderen van de invloed van voedingsvezel, fytinezuur en andere voedingscomponenten op de in vitro beschikbaarheid van calcium, magnesium, ijzer, koper en zink.

Om deze doelstellingen te realiseren werd gekozen voor een multidisciplinaire opzet van het onderzoek.

In deel II wordt ingegaan op een aantal aspecten van voedingsvezel.
In hoofdstuk 3 wordt een literatuuroverzicht gegeven over de definities en de terminologie van voedingsvezel. Hoewel de term "voedingsvezel" (dietary fibre) inmiddels algemeen geaccepteerd is, bestaat er nog steeds verwarring en onenigheid over de juiste definitie van voedingsvezel.

Hoofdstuk 4 bevat een literatuuroverzicht over analysemethoden voor voedingsvezel. Aangezien de analysemethoden in het algemeen gebaseerd zijn op een definitie van voedingsvezel, is het van groot belang dat overeenstemming bereikt wordt over het type componenten dat tot de voedingsvezel behoort. Een definitie die gebaseerd is op chemisch duidelijk omschreven componenten heeft als voordeel dat een specieke analytische bepaling van voedingsvezelcomponenten mogelijk is.
In hoofdstuk 5 wordt een vergelijking van verschillende analysemethoden voor voedingsvezel beschreven. De ADF-, NDF-, Hellendoorn-, AOAC- en Englyst-methoden worden vergeleken met betrekking tot de hoeveelheid en de samenstelling van de voedingsvezel die ermee bepaald wordt. Hoewel verschillende analysemethoden eenzelfde integrale waarde kunnen opleveren voor de hoeveelheid voedingsvezel die bepaald wordt, kunnen er grote verschillen bestaan in de samenstelling van de niet-zetmeel-polysachariden (NSP) in de voedingsvezel. Moderne analysemethoden, zoals de AOAC- en Englyst-methoden, geven een nauwkeuriger analyse van voedingsvezel dan oudere analysemethoden zoals de ADF/NDF- en Hellendoorn-methoden. Op dit moment moet de voorkeur worden gegeven aan analysemethoden die het gehalte aan NSP bepalen (zoals de Englyst-methode) omdat in deze methoden een chemisch duidelijk omschreven groep van componenten wordt bepaald. Bovendien geven de gaschromatografische methoden inzicht in het type NSP dat aanwezig is.

In deel III wordt de analyse van fytinezuur (myo-inositolhexafosfaat) besproken.
Hoofdstuk 6 bevat een literatuuroverzicht over analysemethoden voor fytinezuur. Vanwege interferenties door lagere inositolfosfaten, anorganisch fosfaat en metaalionen bestaan er volgens de literatuur maar weinig analysemethoden die specifiek zijn voor fytinezuur. Momenteel worden de beste resultaten bereikt met analysemethoden die gebaseerd zijn op anionenwisselaarchromatografie met nakolomsderivatisering en met methoden gebaseerd op fosfor-31 NMR.
In hoofdstuk 7 wordt een verbeterde methode voor de analyse van fytinezuur gepresenteerd. De methode is gebaseerd op anionenwisselaarchromatografie met nakolomsderivatisering gecombineerd met een EDTA-behandeling van de monsters om interferentie door metaalionen te elimineren. De verbeterde methode wordt vergeleken met een HPLC-methode zonder EDTA-behandeling en met een methode die gebruik maakt van kleine ionenwisselaarkolommetjes. Eliminatie van de interferentie door metaalionen door middel van een EDTA-behandeling van de monsters in de anionenwisselaarchromatografische procedure blijkt een specifiekere analyse van fytinezuur mogelijk te maken.

In deel IV worden in vitro methoden ter voorspelling van de biologische beschikbaarheid van calcium (Ca), magnesium (Mg), ijzer (Fe), koper (Cu) en zink (Zn) besproken.
Hoofdstuk 8 bevat een literatuuroverzicht over in vitro methoden ter bepaling van de beschikbaarheid van mineralen en spoorelementen en hun correlatie met de biologische beschikbaarheid in vivo. Volgens de literatuur zijn de meest veelbelovende in vitro methoden gebaseerd op een evenwichtsdialyse onder gesimuleerde

omstandigheden van de maag en dunne darm, waarbij de dialyseerbaarheid van mineralen en spoorelementen als maat wordt genomen voor de biologische beschikbaarheid.
In hoofdstuk 9 wordt een continue in vitro methode voor de bepaling van de beschikbaarheid van Ca, Mg, Fe, Cu en Zn gepresenteerd. In deze in vitro methode worden dialyseerbare componenten (inclusief mineralen en spoorelementen) continu verwijderd uit het verteringsmengsel. De in vitro methode met continue dialyse wordt vergeleken met een in vitro methode gebaseerd op evenwichtsdialyse met betrekking tot de dialyseerbaarheid van Ca, Mg, Fe, Cu and Zn uit acht verschillende typen brood. De continue in vitro methode geeft in het algemeen hogere waarden voor de dialyseerbaarheid dan de in vitro methode met evenwichtsdialyse. Het belang van dit effect voor het voorspellen van de biologische beschikbaarheid met behulp van in vitro methoden dient echter nader onderzocht te worden.
De waarde van het gebruik van de in vitro methode met continue dialyse en de in vitro methode met evenwichtsdialyse als voorspellers van de biologische beschikbaarheid van Ca, Mg, Fe, Cu and Zn werd vastgesteld met behulp van een in vivo experiment met jonge varkens. Dit onderzoek wordt beschreven in hoofdstuk 10. Hoewel beide in vitro methoden een verschil laten zien tussen de beschikbaarheid bepaald in vitro en de biologische beschikbaarheid in vivo, geven ze de effecten van verschillen in voedingssamenstelling op de biologische beschikbaarheid van mineralen en spoorelementen goed weer. Er wordt dan ook geconcludeerd dat de in vitro methoden geschikt zijn om de relatieve biologische beschikbaarheid van mineralen en spoorelementen te voorspellen. De in vitro methoden kunnen daarom zeer nuttig zijn voor het verschaffen van voorlopige of aanvullende informatie voor in vivo experimenten die gericht zijn op het bestuderen van de biologische beschikbaarheid van mineralen en spoorelementen.

In deel V wordt ingegaan op de invloed van voedingscomponenten op de biologische beschikbaarheid van Ca, Mg, Fe, Cu en Zn.
In hoofdstuk 11 wordt een literatuuroverzicht gepresenteerd over de invloed van voedingsvezel, fytinezuur en andere voedingscomponenten op de biologische beschikbaarheid van Ca, Mg, Fe, Cu en Zn. Hieruit blijkt dat de biologische beschikbaarheid van mineralen en spoorelementen zeer complex is omdat een scala aan voedingscomponenten deze beschikbaarheid kunnen beïnvloeden. Om een goed beeld te krijgen van de invloed van voedingscomponenten op de biologische beschikbaarheid van mineralen en spoorelementen moet deze dan ook bestudeerd worden in de complete voedselmatrix en niet met een geïsoleerde voedingscomponent.
In hoofdstuk 12 wordt een mathematisch model gepresenteerd om de relatieve invloed van voedingscomponenten op de in vitro beschikbaarheid van Ca, Mg, Fe, Cu en Zn te bestuderen. Het mathematische model is gebaseerd op chemisch theoretische grondslagen en werd geoptimaliseerd met betrekking tot zowel de structuur als de numerieke parameters van het model. Het mathematische model blijkt de in vitro beschikbaarheid van Ca, Mg, Fe en Zn goed te beschrijven en te voorspellen op basis van de concentraties van twee tot drie voedingscomponenten.

Het mathematische model blijkt bovendien zeer waardevol voor het verkrijgen van inzicht in de relatieve invloed van voedingscomponenten op de in vitro beschikbaarheid van deze mineralen en spoorelementen. Volgens de mathematische beschrijving van de in vitro beschikbaarheid heeft fytinezuur een sterke negatieve invloed op de beschikbaarheid van Ca, Fe en Zn, en een minder sterke invloed op de beschikbaarheid van Mg. Arabinose-bevattende componenten leiden tot een verlaagde beschikbaarheid van Ca en Fe. Oxaalzuur heeft een negatieve invloed op de beschikbaarheid van Ca, Mg en Zn. Ascorbinezuur verhoogt de beschikbaarheid van Fe, terwijl citroenzuur een positieve invloed heeft op de beschikbaarheid van Ca, Mg en Zn. Voedingscomponenten met een positieve invloed op de beschikbaarheid blijken de effecten van voedingscomponenten met een negatieve invloed op de beschikbaarheid te kunnen opheffen.
Omdat in vitro methoden relatief snel en goedkoop zijn, en omdat een mathematisch model inzicht geeft in de relatieve invloed van voedingscomponenten, vormt de combinatie van in vivo experimenten, in vitro experimenten en het gebruik van mathematische modellen een veelbelovende benadering voor het vergroten van het inzicht in de mechanismen en belangrijke factoren van de biologische beschikbaarheid van mineralen en spoorelementen.

In het onderzoek dat beschreven is in dit proefschrift is de biologische beschikbaarheid van mineralen en spoorelementen vanuit verschillende disciplines bestudeerd. In deel VI worden de meest relevante discussiepunten (hoofdstuk 13) en conclusies (hoofdstuk 14) van het gehele onderzoek gepresenteerd.

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Hilda.

Curriculum vitae

Hilda Wolters werd op 22 november 1962 geboren te Montfort (L). In 1981 behaalde zij het diploma Gymnasium-β aan het Bisschoppelijk College "Schöndeln" te Roermond. In datzelfde jaar begon zij met de studie Scheikunde aan de Katholieke Universiteit te Nijmegen. Als hoofdvakken koos zij voor Analytische Chemie aan de Katholieke Universiteit Nijmegen (prof. G. Kateman) en voor Levensmiddelenchemie aan de Landbouwuniversiteit Wageningen (prof. W. Pilnik). Op 15 december 1986 sloot zij de studie af met het behalen van het doctoraalexamen (cum laude).

Op 1 december 1986 trad zij in dienst bij de afdeling Algemene Levensmiddelenchemie van het Instituut CIVO-Analyse TNO te Zeist (dr. P. Slump). In deze afdeling onderzocht zij verschillende analytisch-chemische aspecten van de voeding van mens en dier. Sinds de reorganisatie van TNO-Voeding in 1990 maakt zij deel uit van de (nieuw opgerichte) afdeling Biochemie en Fysische Chemie van het TNO-Instituut voor Biotechnologie en Chemie te Zeist (dr. R.J. Hamer). In deze afdeling onderzoekt zij de functionele eigenschappen van koolhydraten.

In de periode 1988-1992 werkte zij, gedeeltelijk als onderdeel van en gedeeltelijk naast haar reguliere werkzaamheden, aan een promotie-onderzoek. De resultaten van dit onderzoek worden beschreven in dit proefschrift.

Stellingen

1. De relevantie van voedingsvezel in voedingsmiddelen is voornamelijk fysiologisch van aard. Eenduidige kwantificering van voedingsvezel daarentegen vereist een definitie op basis van chemische criteria. Dit leidt vaak tot verwarring.
(dit proefschrift)

2. Voor de analyse van voedingsvezel verdienen gaschromatografische en vloeistofchromatografische analysemethoden waarbij de hoeveelheid niet-zetmeel-polysachariden (NSP) bepaald wordt, de voorkeur boven gravimetrische analysemethoden omdat enerzijds een groep duidelijk omschreven polysachariden wordt bepaald en anderzijds inzicht wordt verkregen in de samenstelling van de NSP.
(dit proefschrift)

3. EDTA-behandeling van een voedingsmiddelenextract voorkomt metaal:fytaat-interacties en elimineert hiermee de interferentie van metaalionen bij de ion-chromatografische analyse van fytinezuur.
(dit proefschrift)

4. Hoge gehalten aan mineralen en spoorelementen in voedingsmiddelen vormen geen garantie voor een hoge biologische beschikbaarheid.
(dit proefschrift)

5. In vitro methoden zijn waardevol voor een relatieve voorspelling van de biologische beschikbaarheid van mineralen en spoorelementen uit voedingsmiddelen.
(dit proefschrift)

6. Aangezien de invloed van een voedingscomponent op de biologische beschikbaarheid van mineralen en spoorelementen afhankelijk is van de aanwezigheid van andere voedingscomponenten die de biologische beschikbaarheid tegelijkertijd kunnen beïnvloeden, dient de invloed van een voedingscomponent bij voorkeur bestudeerd te worden in een complete, chemisch goed gekarakteriseerde voedselmatrix.
(dit proefschrift)

7. Een benadering gebaseerd op mathematische modellen is een waardevol hulpmiddel bij het verkrijgen van inzicht in de relatieve invloed van voedingscomponenten op de beschikbaarheid van mineralen en spoorelementen.
(dit proefschrift)

8. Het gecombineerd toepassen van in vitro technieken, mathematische modellen en in vivo experimenten is een veelbelovende benadering voor het vergroten van inzicht in de mechanismen en belangrijke factoren betrokken bij de biologische beschikbaarheid van mineralen en spoorelementen.
   (dit proefschrift)

9. Het feit dat onze mening over voedingsvezel binnen 25 jaar drastisch is veranderd (eerst werd voedingsvezel beschouwd als een waardeloos, ongewenst voedingsbestanddeel, nu als een gewenste voedingscomponent met een beschermende werking tegen ziekten) dient een stimulans te zijn voor een voortdurende herbezinning op hetgeen we denken te weten.

10. Toch is het zo dat, als wij zelf beter worden, ook de "omgeving" verbetert. Het wordt tijd eindelijk eens op te houden de schuld apathisch af te wentelen op de omgeving.
    (F.M. Dostojevski, Woorden als spiegels)

11. Het acute probleem bij een voedselintolerantie is de intolerantie van het lichaam ten aanzien van het voedsel. Het chronische probleem is echter de intolerantie van de medemens ten aanzien van de patiënt.

12. Mensen zijn niet wit, mensen zijn niet zwart, mensen zijn mooi.
    (Uitspraak van een bejaarde uitbater van een Antilliaans cafetaria)

13. Het in rechts-extremistische kringen gepropageerde belang van landsgrenzen leidt tot een vervaging van morele en ethische grenzen.

14. Een advertentie voor een onderzoeker waarin ervaring met WP wordt gevraagd zegt meer over de capaciteiten van de opsteller van deze advertentie dan over de te verwachten capaciteiten van de reflectant.

15. Soms is het voldoende als je iets start.

16. De negende symfonie van Beethoven is een goed bewijs van hetgeen mensen kunnen bereiken door samen te werken.

Stellingen behorende bij het proefschrift
Prediction of the bioavailability of minerals and trace elements in foods
M.G.E. Wolters
Wageningen, 2 november 1992.