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Magnesium Absorption in Humans

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Table of contents

Acknowledgements	7
Summary.....	9
Zusammenfassung	13
Introduction	17
1. Literature review	20
1.1. Chemical properties of Mg and historical background	20
1.2. Mg in the human body.....	21
1.2.1. Body partition of Mg.....	21
1.2.2. Physiological functions of Mg	23
1.2.3. Mg homeostasis.....	24
1.2.3.1. Mg bioavailability, retention, status, and balance	24
1.2.3.2. Mg absorption.....	25
1.2.3.3. Measuring Mg apparent and true absorption	27
1.2.3.4. Mg excretion	27
1.2.3.5. Hormonal influences on Mg absorption and excretion.....	28
1.3. Mg and diet.....	30
1.3.1. Recommended dietary intake of Mg	30
1.3.2. Dietary Mg intake.....	31
1.3.3. Mg content in food sources.....	33

1.4. Mg intake and Mg status and associations with health concerns.....	36
1.4.1. Osteoporosis.....	36
1.4.2. Diabetes mellitus type II.....	37
1.4.3. Cardiovascular diseases.....	39
1.4.4. Hypertension and stroke.....	41
1.5. Factors influencing Mg absorption	43
1.5.1. Overview.....	43
1.5.2. Physical factors.....	44
1.5.3. Type of Mg salt.....	45
1.5.4. Dietary factors influencing Mg absorption.....	48
1.5.4.1. Proteins	48
1.5.4.2. Fat	49
1.5.4.3. Dietary fibre and carbohydrates.....	51
1.5.4.3.1. Insoluble fibre	51
1.5.4.3.2. Soluble fibre.....	53
1.5.4.3.2.1. Hemicellulose, pectin, and gums.....	54
1.5.4.3.2.2. Oligo(fructo)saccharides.....	55
1.5.4.3.2.3. Resistant starch.....	56
1.5.4.3.2.4. Lactose and lactulose.....	58
1.5.4.4. Minerals and trace elements.....	59
1.5.4.4.1. Calcium and phosphorus.....	60
1.5.4.4.2. Sodium and potassium	62
1.5.4.5. Vitamin D	62
1.5.4.6. Phytic acid	63
1.5.4.7. Oxalic acid	66

1.5.4.8. Polyphenols	68
1.5.4.9. Chlorophyll.....	69
1.6. Effects of dietary compounds on Mg excretion	70
1.6.1. Ethanol	70
1.6.2. Caffeine	71
1.6.3. Anions and proteins	72
1.7. Methods to study Mg absorption and metabolism	73
1.7.1. Overview.....	73
1.7.2. In vitro methods	74
1.7.3. Chemical balance technique.....	75
1.7.4. Extrinsic and intrinsic isotopic labels.....	76
1.7.5. Studies using radioactive isotopes.....	79
1.7.6. Studies using stable isotopes	81
1.7.6.1. Overview.....	81
1.7.6.2. Faecal monitoring to determine apparent Mg absorption.....	86
1.7.6.3. Faecal monitoring for estimating true Mg absorption	89
1.7.6.4. Urinary monitoring	93
1.7.6.5. Tissue retention	95
1.7.6.6. Plasma deconvolution method.....	97
1.7.7. Kinetic modelling.....	99
1.7.8. Methods to determine Mg status.....	101
1.8. Use of stable isotope labels to determine Mg absorption.....	102
1.8.1. Introduction	102
1.8.2. Quantification of Mg isotopic labels in a sample based on isotopic ratios	104
1.8.2.1. Single isotope techniques.....	104

1.8.2.2. Double isotope techniques	106
1.8.3. Quantification of Mg isotopic labels in a sample based on isotopic abundances	109
1.8.3.1. Single isotope techniques	109
1.8.3.2. Double isotope techniques	110
1.8.4. Sample preparation for Mg isotopic analysis	112
1.8.5. Mg elemental analysis	115
1.8.6. Mg isotopic analysis.....	116
1.8.7. Isotopic analysis by mass spectrometric techniques	117
1.8.7.1. Introduction	117
1.8.7.2. Analytical techniques used for Mg isotopic analysis in the past.....	119
1.8.7.3. TIMS	120
1.8.7.4. ICP-MS	122
1.8.7.5. ICP-MS compared to TIMS.....	124
References of literature review.....	126
2. Results/Manuscripts	155
2.1. Blood cell incorporation and urinary monitoring of stable isotope labels as alternative techniques to determine magnesium absorption in humans	156
2.2. Phytic acid added to wheat bread decreases apparent magnesium absorption in man.....	177
2.3. Decreased magnesium absorption from test meals based on spinach compared to test meals based on kale.....	193

2.4. Chlorophyll analysis in plant samples by HPLC using zinc-phthalocyanine as an internal standard.....	208
2.5 Chlorophyll-bound magnesium in commonly consumed vegetables and fruits	224
3. Conclusions and perspectives	238

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Summary

Background

The role of Mg in human nutrition has recently received renewed interest. Mg dietary intake has been reported to decrease during the last 100 years in Western countries, for example in the US, often to be below the recommended dietary allowances. Decreased Mg intake and/or low serum Mg have been associated with a number of major public health concerns such as diabetes mellitus type II, coronary heart disease, and osteoporosis.

For the amount of a bioavailable nutrient not only the amount consumed but also how well it is absorbed by the body is of importance. However, little is known about dietary factors influencing Mg absorption. Some dietary compounds such as phytic acid and oxalate have been shown to inhibit the absorption of other minerals and trace elements, e.g. calcium and zinc, in humans. On the other hand, chlorophyll as a dietary source of Mg has been suggested as an important source of Mg, with potentially higher absorption from chlorophyll-bound Mg as compared to inorganic Mg (similar to heme iron and non-heme iron absorption).

The lack of information regarding Mg absorption is at least partly due to the lack of suitable methods to investigate Mg metabolism in man. The chemical balance technique – monitoring dietary intake and excretion of a nutrient – is not a useful tool as absorption from single test meals cannot be determined. The use of radioisotopes of Mg is not an option as these isotopes have too short half-lives. A more recent approach to study mineral metabolism is to use stable isotope labels. Stable isotope study designs are based on measuring the change of an isotopic ratio in collected samples of stool, urine, or blood after intake of labelled test meals. Mg has 3 stable isotopes, 2 of them (^{25}Mg and ^{26}Mg) with low enough natural abundance to be used as isotopic labels. However, there is no agreement about the most suitable study design to determine Mg absorption.

Aims

The objective of this PhD programme was to develop and to evaluate different study designs based on stable isotope techniques to determine Mg absorption and to study the effect of the potential inhibitors phytic acid and oxalate on Mg absorption in man. In addition, chlorophyll-bound Mg was measured in frequently consumed plant foods to estimate its relevance to magnesium nutrition.

Study design

Sample preparation techniques were developed and optimized in order to measure Mg isotopic ratios in biological samples by thermal ionization mass spectrometry (TIMS) and inductively coupled plasma mass spectrometry (ICP-MS). Three human Mg absorption studies are the major focus of the thesis. Within the first study, different stable isotope study designs to determine Mg absorption were compared, based on a ^{25}Mg labelled test meal. In parallel, ^{26}Mg was given intravenously. Mg absorption was determined based on

- a) conventional faecal monitoring based on complete 6 d stool pools of non-absorbed isotopes of the oral label
- b) a shortened faecal collection period (3 stools), corrected for incomplete excretion of the oral ^{25}Mg label by monitoring the excretion of the nonabsorbable marker ytterbium
- c) urinary monitoring, based on the amount ratio of both Mg isotopic labels in a 24 h urine pool (collected 22-46 h after isotope administration) and a complete 6 d urine pool (days 1-6 after isotope administration)
- d) blood cell incorporation of both isotopic labels based on a blood sample drawn 14 d after test meal intake.

In the second study, the influence of phytic acid added to white wheat bread in amounts similar to those naturally occurring in wholemeal and brown bread on Mg absorption was evaluated based on the 6 d stool pool technique. In the third study, Mg absorption from an oxalate rich vegetable (spinach) was compared to Mg absorption from a vegetable with low oxalate content (kale). In a separate study, commonly consumed fruits and vegetables were analyzed for Mg and chlorophyll

contents to determine the fraction of chlorophyll-bound Mg relative to total Mg, and the amount of dietary Mg provided by chlorophyll was estimated by means of Swiss food consumption data. For this purpose, an HPLC method for quantifying chlorophylls using an internal standard was developed.

Results

Fractional (per cent) Mg absorption, based on 24 h urine samples, blood cell incorporation, and the shortened faecal collections (3 stools) collection did not differ significantly ($P>0.05$) from the conventional faecal monitoring technique (6 d faecal pool). The only method resulting in a significantly lower Mg absorption was urinary monitoring based on complete 6 d pools (ANOVA, $P=0.0003$).

In the second study, the addition of phytic acid significantly decreased Mg absorption, from $32.5\pm 6.9\%$ to $13.0\pm 6.9\%$ (1.49 mmol phytic acid/200 g bread, $P<0.0005$) and from $32.2\pm 12.0\%$ to $24.0\pm 12.9\%$ (0.75 mmol phytic acid/200 g bread, $P<0.01$). The decrease in Mg absorption was dose dependent on the amount of phytic acid added ($P<0.005$). The spinach based test meal resulted in significantly lower Mg absorption as compared to the kale based meal ($26.7\pm 12.4\%$ versus $36.5\pm 11.8\%$, $P=0.01$).

An average of 2.7% of total Mg was found to be chlorophyll-bound Mg in a variety of commonly consumed fruits and vegetables and total Mg intake was estimated to contribute $<1\%$ of total dietary Mg intake in Switzerland.

Conclusions

1. Mg absorption can be determined by different stable isotope techniques. The choice of technique depends on practical aspects such as the study population, the number of subjects available, the precision of analytical techniques, and financial aspects related to the cost of isotopes and analyses. Blood cell incorporation or 24 h urine pools (collected on day 2) are potentially useful alternative methods to the conventional faecal monitoring technique. Blood cell incorporation offers a relative simple technique, avoiding the often time consuming collection and analysis of stool and urine, provided that isotopic ratios can be measured at a precision $<0.1\%$

(external relative SD). In a young healthy study population, the technique based on the 6 day stool pools can be shortened to 3 consecutive stools by introducing a nonabsorbable rare earth element as a quantitative stool marker. Urine pools including urine collected during the first day after isotope administration do not seem to be a reliable measure of Mg absorption.

2. Addition of phytic acid decreased Mg absorption significantly in a dose dependent manner. Mg from a meal based on spinach was significantly less absorbable compared to the same test meal based on kale. This difference was attributed to the high oxalate content in spinach. However, the decrease in fractional magnesium absorption from the test meals based on spinach as well as from wholemeal bread can be assumed, at least partly, to be counterbalanced by the relatively high Mg content of these foods. These results demonstrate that Mg absorption is influenced by the composition of the diet.

3. The low dietary intake of chlorophyll-bound Mg in Switzerland did not support the suggestion that chlorophyll-bound Mg could be of importance in Mg nutrition in industrialized countries.

Zusammenfassung

Hintergrund

Die Rolle von Mg in der menschlichen Ernährung wurde in den letzten Jahren mit zunehmendem Interesse untersucht. Seit ca. 1900 ging in vielen Industrieländern, wie zum Beispiel den USA, die Mg Zufuhr über die Nahrung zurück, oft unterhalb der Ernährungsempfehlungen. Eine niedrige Zufuhr von Mg mit der Nahrung sowie niedrige Mg Serumspiegel wurden mit einer Reihe von bedeutenden Gesundheitsproblemen in Verbindung gebracht wie Diabetes mellitus Typ II, koronaren Herzkrankheiten, und Osteoporose.

Für die Menge eines vom Körper aufzunehmenden Nährstoffes ist jedoch nicht nur die verzehrte Menge, sondern auch die Menge des tatsächlich absorbierten Nährstoffes von Bedeutung. Wenig ist jedoch bekannt über den Einfluss von Nahrungsfaktoren auf die Absorption von Mg im Menschen. Einige Nahrungsinhaltsstoffe wie Phytinsäure und Oxalsäure haben einen negativen Einfluss auf die Absorption von Mineralstoffen und Spurenelementen, z. B. Calcium und Zink, gezeigt. Hingegen wurde eine höhere Mg Absorption von an Chlorophyll gebundenem Magnesium gegenüber anorganisch gebundenem Mg vorgeschlagen (ähnlich der Häm-Eisen und nicht-Häm Eisenabsorption). Chlorophyll wird ausserdem als bedeutende Quelle für Mg angesehen.

Dieser Informationsmangel bezüglich der Mg Absorption liegt nicht zuletzt an geeigneten Untersuchungsmethoden zur Bestimmung der Mg Absorption im Menschen. Mittels Bilanzierstudien - Beobachtung der Einnahme und Ausscheidung eines Mineralstoffes - kann die Mg-Absorption einer einzelnen Testmahlzeit nicht bestimmt werden. Radioisotope des Mg als Testmahlzeit- Marker weisen eine zu kurze Halbwertszeit auf. Ein neuer Ansatz beruht auf stabilen Isotopentechniken. Zwei der drei stabilen Mg Isotope weisen eine hinreichend niedrige natürliche Häufigkeit auf, um als Marker eingesetzt zu werden (^{25}Mg und ^{26}Mg). Verschiedene stabile Isotopentechniken zur Bestimmung der Mg Absorption, beruhend auf der

Messung von Isotopenverhältnisänderungen in Stuhl, Urin, oder Blut nach Gabe einer isotonenmarkierten Testmahlzeit, sind vorgeschlagen worden. Es herrscht jedoch keine Einigung über das geeignetste Studiendesign zur Bestimmung der Mg Absorption.

Zielsetzung

Ziel der Doktorarbeit war es, geeignete stabile Isotopentechniken zur Bestimmung der Magnesiumabsorption im Menschen zu entwickeln und zu evaluieren. Mit dieser Technik sollte der Einfluss von Phytinsäure und Oxalsäure auf die Magnesiumabsorption untersucht werden. Zusätzlich wurde die Bedeutung des durch Chlorophyll aufgenommenen Magnesiumanteils aus der Nahrung abgeschätzt. Hierzu wurden eine Reihe von häufig verzehrten Obst- und Gemüsesorten auf ihren Chlorophyll-Gehalt hin untersucht.

Studienaufbau

Zunächst wurden die für die Arbeit mit stabilen Mg-Isotopen notwendigen analytischen Verfahren entwickelt wie z. B. Aufschluss biologischer Proben und Separation des Mg für die Thermionenmassenspektrometrie (TIMS) und induktiv gekoppelte Plasamassenspektrometrie (ICP-MS). Drei Humanstudien bilden den Hauptteil der Doktorarbeit. In der ersten wurden verschiedene Techniken zur Bestimmung der Mg Absorption verglichen. Hierzu wurde eine einfache Testmahlzeit verwendet zu der ^{25}Mg und, parallel, ^{26}Mg intravenös gegeben wurde. Die Mg Absorption wurde bestimmt mittels

- a) dem etablierten "faecal monitoring", basierend auf einem kompletten (6 d) Stuhl-Pool nichtabsorbierter Mg Isotope der Testmahlzeit
- b) einer verkürzten (3 Stuhl) Sammlung, wobei eine Korrektur der Absorption für noch nicht ausgeschiedene orale Markerisotope mit Hilfe eines nichtabsorbierbaren Seltenerdmetalles (Ytterbium) erfolgte
- c) dem "urinary monitoring", beruhend auf Mengenverhältnismessung beider Marker in einem kompletten 6 d (Tage 1-6) und einem 24 h Urin (gesammelt 22-46 h nach Einnahme der Testmahlzeit)

d) Isotopenverhältnismessungen in Blutzellen (aus einer Blutprobe 14 d nach Testmahlzeiteinnahme).

In der zweiten Studie wurde der Einfluss der Zugabe von Phytinsäure zu Weizenweissbrot, wie sie den Gehalten in Vollkorn- und Halbweissbrot entspricht, auf die Mg Absorption untersucht. In der dritten Studie wurde der Einfluss einer oxalatreichen Testmahlzeit (Spinat) gegenüber einer oxalatarmer Testmahlzeit (Grünkohl) auf die Mg Absorption untersucht. Die etablierte "faecal monitoring" Technik, basierend auf der 6 d Stuhl-Pool Technik, wurde für die Bestimmung der Absorption angewendet. Zur Untersuchung der Bedeutung der Mg Aufnahme aus Chlorophyll in häufig konsumiertem Obst und Gemüse in der Schweiz wurde eine HPLC Methode zur Quantifizierung von Chlorophyll a und b mittels eines internen Standards entwickelt. Zusätzlich wurde der Mg Gehalt dieser Obst- und Gemüsesorten untersucht. Mit Hilfe von Verzehrdaten von Obst und Gemüse in der Schweiz wurde die prozentuale Menge an Mg, die in Form von Chlorophyll verzehrt wird, abgeschätzt.

Resultate

Die Ergebnisse der fraktionellen (prozentualen) Mg Absorption des "urinary monitoring", basierend auf einer 24 h Urinsammlung, der Inkorporation von Mg Isotopen in Blutzellen, und der verkürzten Stuhlsammlung waren nicht signifikant ($P > 0.05$) verschieden von der etablierten "faecal monitoring" Methode. Lediglich der komplette 6 d Urin-Pool lieferte signifikant niedrigere Absorptionswerte gegenüber den anderen Methoden (ANOVA, $P = 0.0003$).

In den folgenden Studien zeigte sich eine signifikante Erniedrigung der fraktionellen Mg Absorption durch Zugabe von Phytinsäure, wie sie den Gehalten in Vollkorn- und in Halbweissbrot entspricht, von $32.5 \pm 6.9\%$ auf $13.0 \pm 6.9\%$ (1.49 mmol Phytinsäure/200g, $P < 0.0005$) und von $32.2 \pm 12.0\%$ auf $24.0 \pm 12.9\%$ (0.75 mmol Phytinsäure/200g, $P < 0.01$). Die Abnahme der Mg Absorption war von der Menge an zugefügter Phytinsäure abhängig ($P < 0.005$). Die Mg Absorption aus der Spinat-haltigen Testmahlzeit gegenüber der Grünkohl-haltigen Testmahlzeit war ebenfalls signifikant erniedrigt und fiel von $36.5 \pm 11.8\%$ auf $26.7 \pm 12.4\%$ ($P = 0.01$). Die Mg

Aufnahme in Form von Chlorophyll wurde zu <1% der gesamten Mg Aufnahme in der Schweiz geschätzt.

Schlussfolgerungen

1. Verschiedene Bestimmungsmethoden zur Mg Absorption können verwendet werden, die Wahl hängt von praktischen Aspekten wie Studienpopulation, Anzahl der Probanden, Präzision der analytischen Messmethoden, und finanziellen Aspekten ab. Ein 24 h Urin und die Inkorporation von Mg in Blutzellen können sinnvolle Alternativen zur etablierten "faecal monitoring" Methode sein. Die Inkorporation von Mg Isotopen in Blutzellen bietet einen völlig neuen Ansatz, der ohne die oft zeitaufwendige Sammlung und Untersuchung von Stuhl- und Urinproben auskommt. Voraussetzung für das Anwenden dieser Methode ist eine hohe Präzision der Isotopenverhältnismessungen (<0.1% externe relative SD). In einer gesunden, jungen Studienpopulation kann die etablierte „faecal monitoring“ Methode (6 Tages Stuhl-Pool) mit Hilfe eines nichtabsorbierbaren Seltenerdmetalles auf 3 aufeinander folgende Stühle verkürzt werden. Aufgrund der Resultate dieser Studie wird vermutet, dass die Bestimmung der Mg Absorption aus Urinproben, die Urin des ersten Tages nach Gabe der Testmahlzeit enthalten, keine zuverlässige Methode zur Bestimmung der Magnesiumabsorption darstellt.

2. Zugabe von Phytinsäure zu Weissbrot führte zu einer signifikant erniedrigten Mg Absorption, die Mg Absorption war dabei von der verzehrten Menge an Phytinsäure abhängig. Die Absorption von Mg aus einer Spinat-haltigen Testmahlzeit war niedriger als die einer Grünkohl-haltigen, was am wahrscheinlichsten mit den unterschiedlich hohen Oxalsäuregehalten beider Gemüse erklärt werden kann. Im Falle von Spinat als auch von Vollkornbrot kann jedoch erwartet werden, dass die erniedrigte fraktionelle Magnesiumabsorption durch den normalerweise höheren Mg Gehalt dieser Lebensmittel, zumindest zum Teil, ausgeglichen wird. Die Ergebnisse zeigen, dass die Mg Absorption durch die Art und Zusammensetzung der Nahrung beeinflusst werden kann.

3. Die niedrige Aufnahme von Mg in Form von Chlorophyll in der Schweiz deutet darauf hin, das an Chlorophyll gebundenes Mg in der Ernährung in Industrieländern von geringer Bedeutung ist.

Introduction

Mg is an essential mineral for humans which is necessary for the activation of >300 enzyme systems, especially in energy metabolism. In addition, Mg is an important structural component of bone and soft tissue cells. Low dietary intake of Mg and low Mg serum levels have been associated with major public health problems such as non-insulin dependent diabetes mellitus (Kao *et al.*, 1999), osteoporosis (Abraham, 1991), and coronary artery diseases (Masironi *et al.*, 1979). Even though data on Mg content in food is available (Haenel, 1979; Holland *et al.*, 1994; Souci *et al.*, 1994), little is known about Mg absorption and dietary factors influencing Mg absorption. As a consequence, bioavailability of Mg cannot be estimated.

This is at least partly due to the lack of suitable methodologies to investigate Mg absorption. Chemical balance studies have been used to determine Mg absorption, e.g. by Kelsay *et al.* (1979), Kelsay and Prather (1983), but this technique does not permit determination of absorption from single test meals. The addition of radioisotopes to label meals is not a useful approach for Mg as these radioisotopes have very short half-lives. Recently, stable isotope techniques have been developed to study mineral metabolism. These techniques are based on changes of isotopic composition in urine, stool, or blood after intake of labelled test meals. Two isotopes of Mg, ^{25}Mg and ^{26}Mg , have low enough natural abundances to be used as labels (10 and 11%, respectively).

At the present time, there is no agreement which is the best technique to study Mg absorption in human subjects. The most commonly used and well established method to determine Mg absorption by stable isotope techniques is based on faecal monitoring, with complete collection of non absorbed isotope labels over a period of several days, (e.g. Schwartz *et al.*, 1978; Schwartz *et al.*, 1980; Schwartz *et al.*, 1984; Schuette *et al.*, 1993; Sabatier *et al.*, 2002). However, this technique underestimates true Mg absorption as part of the absorbed label is re-excreted

during the faecal collection period, e.g. via bile or pancreatic excretion. To correct for this fraction, a second isotope label can be administered intravenously. Based on the excretion of the iv. injected label in faeces, true absorption can be estimated (Schwartz *et al.*, 1981; Sabatier, 2001). However, the established faecal monitoring technique requires complete collection of faecal material for several days, and is thus labour intense and inconvenient for the subjects. In order to shorten the relatively long collection periods, nonabsorbable faecal markers, e.g. rare earth elements such as europium and ytterbium, can be used, and corrections for non excreted isotope label can be made based on the recovery of the nonabsorbable markers in faeces (Schuette *et al.*, 1993; Fairweather-Tait *et al.*, 1997).

An alternative isotope technique is based on urinary monitoring using the amount ratio of oral and intravenously administered isotope labels. This methodology is frequently used to determine calcium absorption (Yergey *et al.*, 1987; Yergey *et al.*, 1994) and the feasibility of this technique has been discussed recently for zinc (King *et al.*, 1997; Rauscher & Fairweather-Tait, 1997). This technique has not been evaluated for Mg.

Another alternative is to study the enrichment of an oral tracer in specific target tissues, e.g. erythrocytes, a method which has been used to determine iron absorption (e.g. Cook *et al.*, 1972; Kastenmayer *et al.*, 1994). Coudray *et al.* (1997) used erythrocyte incorporation of an oral and an intravenous label to determine Mg absorption in rats, but there is no information on the feasibility of this technique in humans.

As there are only few studies on Mg absorption reported in humans, there is little data on the influence of dietary factors on Mg absorption. Phytic acid, the major storage form of phosphorus in cereals, legumes, and oilseeds has been shown to decrease absorption of iron (e.g. Brune *et al.*, 1992), zinc (e.g. Turnlund *et al.*, 1984), and calcium (e.g. Heaney *et al.*, 1991), presumably due to the formation of poorly soluble phytic acid-mineral complexes. There is very limited information on the influence of phytic acid on Mg absorption in man.

Another potential inhibitor of Mg absorption is oxalic acid, a compound which is ubiquitous in plant cells. Spinach, an oxalic acid rich vegetable, has been shown to result in low absorbability of calcium (Heaney *et al.*, 1988), and oxalic acid has been significantly correlated with decreased zinc and Mg balances (Kelsay & Prather,

1983). This negative effect is assumed to be due to the poor solubility of oxalic acid-mineral complexes.

Enhancers of Mg absorption have also been suggested, e.g. oligosaccharides enhancing Mg absorption from the human colon (Tahiri *et al.*, 2001). Finally, it has been considered that chlorophyll might offer Mg, the central atom of chlorophyll, some protection against inhibitory factors (Hazell, 1985; Fairweather-Tait & Hurrell, 1996), as does the heme molecule for iron (Bothwell *et al.*, 1989). In order to estimate the possible importance of chlorophyll-bound Mg in relation to Mg nutrition, information of the chlorophyll content of commonly consumed vegetables and fruits is needed.

This thesis is based on 5 manuscripts and a literature review. Within the literature review, the current knowledge of Mg nutrition, with a focus on factors influencing Mg absorption and methods to determine Mg absorption, is reviewed. The first manuscript is a methodological paper which compares different stable isotope methods to determine Mg absorption in humans. The second manuscript reports on a study to investigate the effect of phytic acid from wheat bread on Mg absorption, and manuscript number 3 reports on a study to investigate the effect of spinach, an oxalate rich vegetable as compared to kale, on Mg absorption. In the fourth paper, a new HPLC method for measuring chlorophyll in plant foods is described, based on quantification by an internal standard. In the fifth manuscript, the HPLC method was used to quantify chlorophyll-bound Mg in commonly consumed vegetables and fruits, and to evaluate the importance of chlorophyll in Mg nutrition.

1. Literature review

1.1. Chemical properties of Mg and historical background

Mg is a light metal with the atomic number 12. The atomic weight of 24.32 g/mol is a mean value of the 3 existing stable isotopes ^{24}Mg , ^{25}Mg and ^{26}Mg with an abundance of 79.0, 10.0 and 11.0%, respectively (Catanzaro, 1966). Mg belongs to the second main group of the Periodic Table of chemical elements, the earth-alkali elements. It is more similar to the elements lithium and zinc than to the homologues beryllium and calcium, in particular in relation to solubility, salt formation, and isomorphous properties. The name of the element originates from the Greece district Magnesia in Thessaly/Greece, where talcum [$\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$] was discovered.

Mg is ubiquitous in nature. It is the eighth most abundant element (~1.94%), the second most common divalent ion in the oceans, the third most common on land and the most abundant intracellular ion in plants. In living organisms, it is the fourth most abundant metal (Wester, 1987). Due to its chemical reactivity, Mg in nature does not exist in free form, but in a variety of salts. It is most often found in silicates and carbonate compounds, e.g. magnesite (MgCO_3), dolomite ($\text{CaMg}[\text{CO}_3]_2$), or epsomite ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). Silicates are the most abundant Mg salts in nature due to their low solubility in water. In chemical complexes, Mg binding is partly of covalent character, but the ionic character predominates. Loss of the 2 outer (shell) electrons of Mg results in the formation of ionic Mg^{2+} , which has an ionic radius of 7.8 nm. Compared with Ca (10.6 nm), this small ionic radius and the high positive charge leads to the greater tendency of Mg to form hydrated and soluble salts. A review of the stability of some Mg complexes is given by Martin (1990). The following order of stability of Mg and its ligands has been reported: hydroxide>EDTA>citrate>oxalate>lactate>acetate.

Long before the elemental characteristics of Mg were discovered by Black in 1755, Mg salts had been used, e.g. MgSO_4 (Epsom salt) for medical treatment against constipation. The pure element was first isolated by Sir Humphrey Davy in 1808 by

electrolysis of melted Mg salts, and obtained in pure form by Bussy and Liebig in 1831 by reduction of MgCl_2 . The chemical symbol Mg was suggested in 1814 by Berzelius. Industrial production of Mg started 1866 in Germany (reviewed by Falbe & Regitz, 1995).

The importance of Mg as the central atom of chlorophyll, the molecule most important for photosynthesis and therefore for all plant life, was discovered by Willstätter and Stoll at the early years of 1900. In 1920, Denis discovered Mg in blood plasma, and Leroy (1926) demonstrated that Mg is essential for mice. The first systematic observations of Mg deficiency were made by Kruse *et al.* (1932) in rats and dogs. In man, depletion of Mg was first described by Hirschfelder & Haury (1934).

1.2. Mg in the human body

1.2.1. Body partition of Mg

The human body contains 21-28 g Mg (Seelig, 1981; Berkelhammer & Bear, 1985; Brady *et al.*, 1987). The major part (approximately 60%) is present in the bone, where about a third of it is bound to the surface of the apatite crystal or is in the hydration shell (Brautbar & Gruber, 1986; Shils, 1988). As for calcium, bone is assumed to be the most important reservoir participating in Mg homeostasis. About 20% of body Mg is present in skeletal muscles, and the remaining 20% in other tissues. Information regarding the abundance of Mg in different body compartments can be found in reports by Aikawa (1981), Walser (1967), Lentner (1981), and Classen (1984). Table 1 gives an overview of Mg concentrations in some tissues.

Mg is the second most abundant divalent cation in human cells, only potassium is found in higher concentrations. The major part of Mg is found within the cells. Less than 1% of Mg in the human body is found in the extracellular space (Wester & Dyckner, 1982; Brady *et al.*, 1987). The majority of Mg within the cells does not exist in free form. Only a minor fraction (5-10%) of intracellular Mg exists in free ionic form as Mg^{2+} . This is because Mg has the smallest ionic radius of biological relevant

cations, leading to a relatively high binding energy which results in a strong tendency to form complexes. Most intracellular Mg is bound to ribosomes (complexes of ribonucleic acid and proteins, participating in protein synthesis), nucleic acids, organic acids (e.g. citrate), proteins, or adenosine triphosphate. The binding affinities for phosphates are among the highest (Cowan, 1995).

Table 1. Abundance of Mg in human tissues.

compartment	% of total body Mg	Mg concentration (mmol/kg wet weight)
bone	60-65 ¹	45 ²
muscle	27 ¹	5.8-14 ²
blood	1 ¹	2 ³
red blood cells	0.5 ¹	2.25-3.0 ²
serum	0.3 ¹	~0.7-0.9 ²

¹: (Shiels, 1998)

²: (Classen, 1984)

³: calculated by the author based on the assumption of 6 L blood volume and 25 g total Mg in the body

Mg is transported to the different body compartments in the blood plasma, either as free ionized Mg, bound to relatively small (ultrafiltrable) complexes (e.g. citrate) or bound to proteins (albumin, globulin), which are not ultrafiltrable (Table 2). Total ultrafiltrable Mg is of interest as this fraction is influenced by renal clearance, an important mechanism in regulating Mg homeostasis. The concentration of Mg in plasma is kept relatively constant (Elin, 1994)

Table 2. Mg in blood plasma or serum

reference	free Mg (ultrafiltrable) (%)	complexed Mg (ultrafiltrable) (%)	protein bound Mg (%)
(Walser, 1967) ¹	55	13	32
(Speich <i>et al.</i> , 1981) ²	61	5	34

¹ plasma Mg

² serum Mg

1.2.2. Physiological functions of Mg

Mg is an essential cofactor to activate > 300 enzyme systems in humans (Shils, 1998), involved in carbohydrate, lipid, protein and DNA metabolism, interacting either with the substrate or the enzyme directly. Due to the high affinity of Mg^{2+} to phosphate, it is involved in all phosphorylation processes where ATP (adenosine triphosphate), primarily existing as an $[ATP^{2-}Mg^{2+}]$ complex, is involved. Mg therefore plays a predominant role in energy metabolism. Mg polarizes the ATP molecule, so that nucleophiles can be bound (reviewed by Löffler & Petrides, 2002). For example, muscle Mg^{2+} is a cofactor for phosphocreatine, needed to resynthesize ATP from phosphocreatine and ADP (adenosine diphosphate). In this respect, Mg is essential for muscle contraction because this process is based on the contraction of actin and myosin filaments, which are ATP dependent.

During glycolysis, the formation of pyruvate from glucose, 7 Mg dependent enzymes are involved, hexokinase for example is necessary for phosphorylating glucose and to activate it for further enzymatic reactions in energy metabolism (Heaton, 1990). Mg is also required in the citric acid cycle, the major energy producing mechanism in all aerobic organisms, e.g. for pyruvate- and isocitrate dehydrogenase. In addition, it is essential for gluconeogenesis from pyruvate, the pentosephosphate pathway, and in the urea cycle (reviewed by Lehninger, 1987). In lipid metabolism, Mg is necessary for the formation of phosphoglycerides in lipid synthesis, and for thiokinasis, catalyzing the first step in fatty acid degradation (Hirschfelder & Haury, 1934). Furthermore, for protein synthesis from activated amino acids and tRNA (transport- RNA) at the ribosomes, Mg^{2+} is a requisite (Flink *et al.*, 1954), and for DNA synthesis in the nucleus by DNA polymerase (responsible for chain prolongation) (reviewed by Walser, 1967). The formation of cAMP (cyclic adenosine monophosphate), an intracellular second messenger (mediating hormone action on target tissues), from $[Mg^{2+}ATP^{2-}]$, is catalyzed by the Mg^{2+} dependant adenylatcyclase. cAMP activates protein kinases, controls intracellular Ca^{2+} concentration, and binds to repressing proteins, thus regulating the transcription of nucleic acids (Lehninger, 1987). However, Mg is not always a highly specific cofactor and can be replaced to a certain extent by other divalent cations such as

manganese (Vaisius & Horgen, 1980; Niyogi *et al.*, 1981), but the concentration of these cations within the cells is usually low compared to Mg.

Besides the affinity of Mg to ATP, Mg forms complexes with the phospholipids of cell membranes, thus contributing to the cell structure. Similarly to the phospholipids, Mg stabilizes RNA and DNA by its high affinity to ribose phosphate of nucleotides (Durlach, 1988; Cowan, 1995), and is therefore an essential component of the structural integrity of every cell. Mg phospholipids control influx of other ions over the cell membrane, by reducing membrane fluidity and permeability of calcium and sodium. In addition, the ATP dependent sodium-, potassium-, and calcium ion pumps are Mg dependent.

1.2.3. Mg homeostasis

Homeostasis is the ability to maintain a dynamic equilibrium of a compound in the body and its tissues. Mg homeostasis is determined by the body requirement, absorption and excretion of Mg. Homeostasis of Mg has been evaluated in a variety of human studies, which focussed on different aspects of Mg homeostasis, such as absorption, excretion, or retention.

1.2.3.1. Mg bioavailability, retention, status, and balance

The terms bioavailability, retention, balance, and status are used to describe and characterize Mg metabolism. Bioavailability can be defined as the extent to which a nutrient can be absorbed and used for its normal physiological functions or stored. Bioavailability of Mg cannot be assessed directly as there is no specific target tissue for Mg such as erythrocytes are for iron. Therefore, other parameters such as retention, absorption, and urinary excretion are used as a measure for Mg bioavailability.

Retention is the amount of the nutrient retained by the body, usually measured as the difference between intake of a nutrient and the amount of faecal and urinary excretion. The term "balance" has been used in chemical balance studies, zero balance indicates that the same amount mineral is absorbed and excreted. A

negative balance indicates that the body loses the respective nutrient, and at a positive balance, the body accumulates the nutrient. Mg chemical balance studies are based on dietary, faecal, and urinary monitoring (reviewed by Brink & Beynen, 1992). The term status is used to describe whether the supply of a nutrient of a person is adequate or not. Similar as for bioavailability, there is no direct measure of Mg status as there is no target tissue for Mg (see chapter 1.7.8. Methods to determine Mg status).

1.2.3.2. Mg absorption

Mg is primarily absorbed in the small intestine, especially in the more distal segments (ileum) (Graham *et al.*, 1960; Brannan *et al.*, 1976; Danielson *et al.*, 1979; reviewed by Hardwick *et al.*, 1990a; and Kayne & Lee, 1993). No absorption in the colon could be demonstrated in these early studies. However, there is some evidence that, at least in infants, Mg is absorbed in the colon (Anast & Gardner, 1981; Classen, 1984), as indicated by intoxications with rectal enemas of Mg. It would seem likely however that there is a mechanism for Mg absorption in the colon as recently soluble non-digestible carbohydrates have been reported to increase Mg absorption in the colon of rats (reviewed by Kayne & Lee, 1993) and humans (Tahiri *et al.* 2001).

Mg intestinal absorption in humans starts approximately 1 h after oral intake, as indicated by increased plasma Mg in studies using radioactive ^{28}Mg . Because of this short time, it has been suggested that absorption occurs also in the more proximal parts of the small intestine. Mg absorption reaches a plateau after 2-2.5 h up to 4-5 h and then declines. At 6 h, Mg absorption is about 80% complete (reviewed by Hardwick *et al.*, 1990a).

The mechanism of Mg absorption through the enterocytes of the mucosa into the bloodstream is not entirely understood. While no metabolic energy is needed for Mg uptake as the Mg concentration in the intestinal cells is lower than in the intestinal tract after meal intake, cellular extrusion of Mg is energy dependent. Two major mechanisms of Mg absorption in the human intestine have been proposed, a saturable and a non saturable process (reviewed by Kayne & Lee, 1993). Radioactive ^{28}Mg has been used as a tracer to study the mechanism of absorption

in humans. Brannan *et al.* (1976) observed that Mg absorption in the ileum was saturated above 10 mmol in vivo intestinal Mg perfusion while absorption in the jejunum still increased, suggesting the presence of different absorption mechanisms in the intestine. Roth & Werner (1979) and Fine *et al.* (1991) described a curvilinear relation between the ingested Mg dose and Mg absorption, a combination of a hyperbolic function at low Mg content (< 5 mmol) and a linear function at higher Mg content (>5 mmol). The authors suggested a combination of a saturable (transcellular) mechanism and passive (paracellular) diffusion, dominating at higher Mg concentrations to explain these findings. It has been suggested that the existence of an inherited (genetic) disorder for Mg absorption indicated active transport across the human epithelium of the intestine, as this disorder would effect specific active carriers (Milla *et al.*, 1979; Yamamoto *et al.*, 1985). The nature of the saturable process is not well understood. A saturable mechanism could be based on active (carrier mediated) transcellular transport or facilitated diffusion, e.g. mediated by passive carrier proteins, as the permeability of the membranes is otherwise low (reviewed by Kayne & Lee, 1993). Several transport mechanism have been proposed, e.g. a proton driven dependent luminal Mg²⁺ carrier or channel (Schweigel & Martens, 2000).

However, no active transport has yet been demonstrated in humans directly. Earlier in vivo and in vitro studies tried to demonstrate the presence of active carriers participating in Mg absorption by administering substances usually inhibiting enzyme activity and thus active carriers, with contradictory results. For example, 2,4-dinitrophenol did not inhibit Mg absorption in the large and small intestine of rats (Aldor & Moore, 1970) and rabbits (Aikawa & Reardon, 1965). On the other hand, in segments of the small intestine (in vitro) of the guinea pig, Mg absorption could be reduced by cyanide, ioadacetate and fluoracetate (Ross & Care, 1962). Therefore, the role of carrier mechanisms for active Mg transport might depend on the species, and the importance for human Mg absorption remains unclear.

As the daily intake of Mg is usually in the range of 12-17 mmol (see chapter 1.3.2. Dietary Mg intake), and the saturable absorption process is saturated at about 5-10 mmol, it can be concluded that the majority or at least a large fraction of Mg is absorbed based on this saturable process.

1.2.3.3. Measuring Mg apparent and true absorption

Apparent Mg absorption (AA), as determined in balance studies, can be defined as the difference between intake (D_o) and faecal excretion (F_o) of Mg within a certain time interval.

$$AA = D_o - F_o \quad (1)$$

Fractional apparent absorption is expressed as the fraction of absorbed mineral of the dose ingested, usually in per cent:

$$AA(\%) = \frac{D_o - F_o}{D_o} \cdot 100 \quad (2)$$

with $AA(\%)$ being fractional apparent absorption. Apparent Mg absorption as determined by chemical balance studies does not differentiate whether the Mg in faeces is non absorbed Mg from food or Mg of endogenous origin, e.g. excreted in the gastrointestinal tract via bile or pancreas.

True absorption can be defined as Mg that enters the human body from the gastrointestinal tract. It is not altered by endogenous Mg losses and is therefore higher as compared to apparent absorption. True absorption can only be estimated by isotope techniques, in which the oral label administered can be distinguished from Mg of endogenous origin, which is of natural isotope composition. However, part of the absorbed isotope label is usually re-excreted in the faeces during the time of faecal monitoring, resulting in an underestimation of absorption. One method to correct for this absorbed and re-excreted fraction is by using a second isotope label, which differs from the first in its isotope composition, which is administered simultaneously by iv. injection with the oral label. By determining the fraction of the iv. label found in the faeces, the fraction of the oral label that has been absorbed and re-excreted in the faeces can be estimated, and true absorption can be determined.

1.2.3.4. Mg excretion

Because there is no known regulation for Mg intestinal absorption, and part of the absorbed Mg is presumably absorbed by passive diffusion, Mg excretion is the major pathway of regulating Mg homeostasis. Excretion can be defined as the sum of all pathways by which a nutrient leaves the body, e.g. through urine, endogenous

faecal losses, sweat, etc. The major organ for regulating Mg excretion is the kidney. About 70-80% of plasma Mg are ultrafiltrable (see Table 2) and can be removed when the blood is filtered by the kidney. However, only a minor part of this ultrafiltrable Mg is excreted in the urine. In humans, about 20-25% of this ultrafiltrable Mg is reabsorbed by the proximal tubule, 50-60% in the loop of Henle, and 5% in the terminal segments while the remainder (5-20%) is excreted in urine (reviewed by de Rouffignac, 1992; and Quamme, 1993). Based on rat studies, it is assumed that the majority of this re-absorbed Mg is due to passive absorption by the kidney tubule (reviewed by de Rouffignac & Quamme, 1994), even though active transport mechanism have been suggested (reviewed by Quamme & Cole, 2001). Total urinary Mg excretion is in the range of 100-150 mg/day (Wacker, 1980; Aikawa, 1981; Shils, 1998) for an intake of about 300-400 mg Mg.

Other routes of Mg excretion play a less important role. Endogenous faecal Mg losses in humans have been estimated to be in the range of 30 mg/day (Slatopolsky, 1984), 7 to 34 (median 11) mg/day (Avioli & Berman, 1966), and 34-60 mg/day (median 47) (Sabatier, 2001), based on radioactive (^{28}Mg) or, in the latter study, stable isotope techniques. Sweat losses under normal thermal conditions (room temperature) have been reported to be in the range of 15-25 mg/ day (reviewed by Seelig, 1964; Durlach, 1988).

1.2.3.5. Hormonal influences on Mg absorption and excretion

Hormones are known to control the homeostasis of calcium, while no influence has been shown for iron and zinc homeostasis. Similarly, no specific hormone has been identified in the regulation of Mg homeostasis in humans, as reviewed by Ebel & Gunther (1980), de Rouffignac *et al.* (1993), and de Rouffignac and Quamme (1994). Nevertheless, several hormones have been reported to influence Mg absorption in the small intestine or to influence urinary excretion. Among these hormones are parathyroid hormone (PTH), calcitonin, insulin, glucagon, antidiuretic hormone (ADH), isoproterenol (a β -adrenergic agonist), and glucocorticoids/mineralcorticoids. However, it is not known whether these hormone concentrations are influenced by Mg status, which would be the case for a real hormonal control.

PTH probably has the strongest effect on Mg absorption and excretion. PTH is an important hormone regulating Ca homeostasis. As for Ca, PTH has been reported to increase Mg absorption, partly mediated by calcitriol (Hardwick *et al.*, 1991; Rude, 1993); to increase Mg reabsorption by the kidney (Massry & Coburn, 1973; Parfitt, 1976), and to mobilize bone Mg (Heaton, 1981) in humans. For calcitonin, a PTH antagonist, the effect seems to depend on the species. While calcitonin showed to increase Mg reabsorption in the rat kidney (Quamme, 1980; de Rouffignac *et al.*, 1993), a transient magnesuria was reported after administration of pharmacological calcitonin doses in man (Singer *et al.*, 1969; Paillard *et al.*, 1972). In addition to PTH, insulin has been reported to enhance Mg reabsorption by the kidney, based on in vitro experiments using microperfused mouse nephrons (Mandon *et al.*, 1993). Insulin, a hormone necessary to stimulate glucose uptake by the cell, has been shown to increase Mg uptake by the cell (Lostroh & Krahl, 1974; Durlach & Rayssiguier, 1983). In rats with induced diabetes, urinary excretion of Mg was increased, probably due to glucosuria (Ebel & Gunther, 1980), and hypomagnesemia has been associated with diabetes mellitus (Jackson & Meier, 1968) in man. Glucagon, an insulin antagonist, has also been reported to increase Mg reabsorption by the rat kidney and in humans (de Rouffignac & Quamme, 1994). Other hormones which have been reported to increase Mg reabsorption in in-vitro experiments with perfused mice nephrons include ADH, a hormone important to increase water absorption in the kidney (Wittner *et al.*, 1988) and isoproterenol (Bailly *et al.*, 1990). Hormones decreasing Mg reabsorption in the human kidney have been reported to include thyroid hormones and corticoids (Hanna & Macintyre, 1960; Massry *et al.*, 1967; Zumkley, 1981; Classen, 1990). However, in healthy human subjects, no studies have clearly shown an effect of the discussed hormones in physiological doses, and it can be assumed from rat studies that there is no "single" hormone responsible in regulating Mg reabsorption, which does occur, at least to a large extent, passively.

1.3. Mg and diet

1.3.1. Recommended dietary intake of Mg

Recommended daily dietary intake of Mg has been proposed for different age and gender groups in different countries. However, these recommendations are somewhat imprecise as they are based on methods which have a number of limitations. Mg recommendations are typically estimated based on balance studies at zero balance. However, Mg balance may vary, depending on the composition of the diet (Reinhold *et al.*, 1976; Kelsay *et al.*, 1979; Kelsay & Prather, 1983). Alternatively, requirements could also be estimated based on status measurements of the mineral in combination with a food survey of dietary intake. As there is no general agreement on how to define Mg status and deficiency (Elin, 1991a; Sauberlich, 1999), this technique cannot be accurately used to determine Mg requirements.

More than a dozen countries have established recommended daily intakes of Mg (RDI's) for adults, ranging from 250-500 mg/day and person (reviewed by Truswell & Chambers, 1983). Mg dietary recommendations are summarized in Table 3. The most commonly used recommendation is the recommended dietary allowance (RDA), published by the US National Academy of Sciences. RDA is the daily dietary intake which should meet the nutrient requirement of 97.5% of the population in the respective population group. It is derived from the estimated average requirement (EAR) plus addition of 2 SD's of the EAR. The RDA values are currently replaced by the dietary reference intake values (DRI's) (Institute of Medicine, 1997). RDI's for Mg are also used in the UK dietary reference values (DRV's) published by the Department of Health (1991). In Switzerland, the DACH (an abbreviation for the countries Germany (D), Austria (A), and Switzerland (CH)) dietary reference values published by the German (DGE)- and Austrian Nutrition Society (ÖGE), the Swiss Association for Nutrition (SVE) and the Swiss Society for Nutrition Research (SGE) (Deutsche Gesellschaft für Ernährung *et al.*, 2000), are used. Differences between

the recommended daily intake values can be explained by the inaccuracy to estimate the average requirements of Mg for a population.

Table 3. Recommended dietary intake of Mg for adults

data source	male (mg/d)	female (mg/d)
DRI ¹	400	310
DRV ²	300	300
WHO-RNI ³	200-300	200-300
D.A.CH ⁴	400	310
RDI ⁵	320	270

¹ Dietary reference intake, Institute of Medicine (1997) (US, mean for age 19-30 y)

² Dietary reference values, Department of Health (1991), (UK, mean for age 19-50 y (women), 19-50 y and older, (men))

³ Recommended nutrient intake, World Health Organization (1974), (mean for age 19-50 y (women), 19-50 y and older (men))

⁴ recommended intake by Deutsche Gesellschaft für Ernährung, ÖGE, SGE and SVE (2000), (mean for age 19-25 y)

⁵ recommended dietary intake by National Health and Medical Research Council (2002), Australia (mean for age 19-64 y (men) and 19-54 y (women))

1.3.2. Dietary Mg intake

There are several ways to estimate Mg intake in a population. The least accurate but easiest way is based on food consumption (disappearance) data. For this estimate, the exported and stored amount of a food product in a region or country is subtracted from that produced, imports are added, and the result is divided by the population. The contribution of a food product to intake of a nutrient is then calculated using food data bases, e.g. the German Nutrient Food Code Data Base (Bundeslebensmittelschlüssel, BLS), a compilation of nutrients in about 11000 foods. Examples of such estimates of Mg intake include the data published by the American Society for Experimental Biology (1995), the Vierter Schweizerischer Ernährungsbericht (Bundesamt für Gesundheit, 1998), and the Deutsche

Ernährungsbericht (Deutsche Gesellschaft für Ernährung, 1996). However, this method tends to overestimate dietary intake because not all sold food is consumed. Probably the best way to evaluate food intake of a specific population is by surveys of food intake, for example by diet history (Wälti *et al.*, 2002), food records (Singh *et al.*, 1997), or 24 h recall (Humphries *et al.*, 1999). Typical eating habits are determined by diet history method by later interviews, while the 24 h recall aims to estimate precisely the amount and type of food consumed during this time period. With the food record method, the consumed amount food is determined by weighing or estimating the amount food and therefore has to be planned prospectively. Food surveys tend to result in lower intake of food products or nutrients compared to the method based on food consumption (Table 4). The advantage of food surveys is that food intake in different population groups can be evaluated, for example Mg intake data published by Institute of Medicine (1997). A review of literature data on daily Mg intake in several countries was published by Hendrix *et al.* (1995).

Table 4. Mg intake in some industrialized countries

source of data	Mg intake men/women (mg/d)	method of assessment
(Institute of Medicine, 1997)	323/228	24 h recall, Continuing Survey of Food Intakes by Individuals in the US, 1994, age > 9 y (n=4094)
(Pennington & Wilson, 1990)	294/189	dietary record (4 times/y), US Total Diet Study, 1982-1989, age 25-30 y (n>10000, not exactly specified)
(Gregory <i>et al.</i> , 1990)	323/237	dietary record (1 week), Dietary and Nutritional Survey of British Adults, 1986-1987, all age groups (n=2197)
(Deutsche Gesellschaft für Ernährung, 1996)	354/283	dietary record (1 week), National Food Survey, 1985-1989, age 25-51 (n=23000)
(Bundesamt für Gesundheit, 1998)	406	food consumption, 1994-1995
(Deutsche Gesellschaft für Ernährung, 1996)	416	food consumption, 1994
American Society for Experimental Biology (1995)	350	food consumption, 1990

A decline in Mg intake during the last 100 y in the USA, from about 400 mg/d during the period 1909-1913 to about 350 mg/d in 1980 has been reported (reviewed by the Food and Nutrition Board, Commission of Life Sciences, National Research Council 1989). This may be due to increased consumption of refined cereal products as up to 80% of Mg is lost during milling (reviewed by Davidsson, 1999). As can be seen in Table 4, some estimates of Mg intake based on food surveys are below some of the recommended Mg intake values (Table 3).

1.3.3. Mg content in food sources

As Mg is an essential mineral for all plants and animals, it is found in all plant and animal foods. High amounts of Mg, up to 460 mg/100 g, are found in nuts. Grains, leafy vegetables and coffee beans contain up to 250 mg/100 g Mg, while fruits, meat, fish, and milk based products are in general relatively low in Mg. Information about the Mg content in foods is available in food data bases, for example Haenel (1979), compiling data from the former German Democratic Republic, Souci *et al.* (1994, Germany), Holland *et al.* (1994, UK), and the United States Department of Agriculture (1991). Additional information about the Mg content in foods is provided by (Pennington & Wilson, 1990), based on the US Total Diet Study. For Switzerland, no data base is available at present, but should be available by 2003. Table 5 gives an overview of the Mg content in selected foods, based on Souci *et al.* (1994).

Table 5. Mg content in foods in order of their Mg content (Souci *et al.*, 1994)

food source	Mg content (range) mg/100 g edible portion
cocoa powder	414 (370-457)
cashew nut	267
coffee, roasted beans	201 (162-240)
black tea, leaves	184
peanut	158
rice, unpolished	157 (148-166)
lamb's lettuce	140 (130-203)
oat, whole grain	129 (87-176)

walnut	129 (92-144)
wheat, whole grain	125 (31-175)
maize, whole grain	120
wheat whole-meal bread	92
spinach, raw	58 (39-88)
sweet chestnut	45 (33-55)
coconut	39
banana	36 (31-42)
cheese, emmental, 45% fat	35 (21-48)
pea, pod and seed, green	33 (19-43)
herring	31 (26-36)
mackerel	30 (25-40)
pork, muscles only	27 (25-29)
beans, white, dry	25 (16-31)
white wheat bread	24
beef, fillet	24 (14-27)
potato	20 (17-32)
olive	19
mango	18
carrot	18 (15-24)
cauliflower	17
strawberry	15 (11-20)
mushroom	14 (11-16)
chicken egg	12 (11-13)
green pepper	12
quark, fresh cheese, from skimmilk	12 (6-19)
milk, cow, 3.5% fat	12 (9-16)
cherry	11 (10-14)
grape	10 (6-15)
lettuce	8 (6-13)
apple	7 (3-9)
honey	6
butter	3 (2-4)
drinking water*	0.5-3*

* data based on (Olhaberry *et al.*, 1983)

As Mg is found widespread in foods, there is no "major food source" (Table 6). The contribution of these sources to the total Mg intake differs depending on different dietary habits. The high contribution of the vegetable group to total dietary intake of Mg in Switzerland can be explained by the high consumption of nuts and legumes, which contribute about 24% to total Mg intake, but was reported to contribute less than 1% to total Mg intake in Germany.

Table 6. Contribution of different food categories to Mg intake

food category	Germany, Ernährungsbericht (Deutsche Gesellschaft für Ernährung, 1996) 1994	Switzerland, 4. Schweizerischer Ernährungsbericht (Bundesamt für Gesundheit, 1998) 1994-1995	UK, National Food Survey, (National Food Survey Committee, 2001) 2000	US Total Diet Study, (Pennington & Young, 1991)³ 1982-89
cereals and cereal products %	16	13	31	16
potatoes %	9	6	5	no data ⁴
fruits %	11	7	8	6
vegetables % (including legumes, nuts)	7 ²	36	11	20 ⁴
dairy products %	12	16 ¹	16	13
meat and fish %	10	10	13	17
beverages, drinks %	25	8	9	14

¹ without butter

² including mineral water, fruit- and vegetable juices

³ mean of age group 30-35 y

⁴ vegetables include potatoes

1.4. Mg intake and Mg status and associations with health concerns

1.4.1. Osteoporosis

The role of Mg in nutrition in relation to osteoporosis was recently reviewed by Rude (2001) and Schaafsma (2001). Osteoporosis is usually detected by either reduced bone mass, for example measured by x-ray spectroscopy, or reduced bone health, which is often determined by the size and shape of the bone crystals (Cohen & Kitzes, 1981). Often, x-ray spectroscopy reveals unnoticed bone traumas (Reginster *et al.*, 1989). Decreased concentration of Mg in bone, as determined by biopsy, has been reported in several studies with postmenopausal osteoporotic women (Orloff *et al.*, 1979; Cohen & Kitzes, 1981; Manicourt *et al.*, 1981) and in osteoporotic senile subjects (Milachowski *et al.*, 1981; Cohen *et al.*, 1983b). The reason for this finding is not well understood. Several human studies (Yano *et al.*, 1985; Freudenheim *et al.*, 1986; Houtkooper *et al.*, 1995; Tucker *et al.*, 1999) have investigated the influence of dietary Mg intake on bone mineral density or Mg content, with contradictory results. Low dietary Mg intake could be significantly correlated to low mineral bone Mg content or bone density in some, but not all of these studies, depending on the subject group examined, level of dietary Mg intake, and the specific bone investigated. Whether low dietary Mg intake alone is responsible for decreased bone mineral density or decreased Mg content is difficult to evaluate, as diets low in Mg are often low in other nutrients such as calcium.

In addition, the relationship between Mg status and postmenopausal and senile osteoporosis was investigated in some studies (Cohen & Kitzes, 1981; Cohen *et al.*, 1983a; Reginster *et al.*, 1989). These studies were limited due to limited number of subjects included and disagreement about how to determine Mg status. Some but not all parameters investigated such as red blood cell Mg (Reginster *et al.* 1989) or decreased bone Mg content (Cohen & Kitzes, 1981) could be significantly correlated to the incidence of osteoporosis in these studies.

Oral Mg supplementation (>250 mg/d) over 1 y or longer periods significantly increased bone density in several studies with postmenopausal women (reviewed by Abraham, 1991; Stendig-Lindberg *et al.*, 1993) and gluten-sensitive enteropathic subjects (Rude & Olerich, 1996) as measured by x-ray absorptiometry. However, the latter study did not include a control group and Stendig-Lindberg failed to show a significant further increase of bone density after 1 y, as only 10 of the 31 subjects completed the study.

The mechanisms by which Mg could improve bone health are not well understood. Mg represents 0.4-0.5% of the bone mass (Robinson & Weatherell, 1990). The essentiality of Mg for bone health and for the mobilisation of Ca was shown by Shils (1969). Mg has been reported to limit hydroxyapatite crystals in size (Eaton-Evans, 1994; Sojka & Weaver, 1995), to prevent calcium leakage of bone matrix by contributing to a higher extracellular pH as compared to blood (Driessens & Verbeeck, 1988), and to stimulate calcitonin, which inhibits osteoclast activity (Ferment & Touitou, 1988) and therefore decomposition of the bone matrix. Furthermore, Mg deficiency, as determined by low serum Mg concentration, red blood cell Mg and lymphocyte Mg, has been correlated with low PTH plasma levels, which could result in hypocalcemia (Ferment & Touitou, 1988; Rude & Olerich, 1996), a potential risk factor for osteoporosis.

In conclusion, despite the limited data available, there is a growing number of indications that low dietary Mg intake and status as determined by decreased Mg concentration in bone could be a risk factor for osteoporosis.

1.4.2. Diabetes mellitus type II

Diabetes mellitus is characterized by either impaired production of insulin (non insulin dependent diabetes, type II), or by complete inability to produce insulin (insulin dependent diabetes, type I). Diabetes mellitus is the most common pathological condition in which secondary Mg deficiency, usually determined by decreased plasma Mg concentrations, occurs (reviewed by Durlach, 1988; Garland, 1992). This prevalence of hypomagnesemia as an indicator for low Mg status in

diabetic subjects (type I and II) has been reported to be in the range of 25-39% (1993; Nadler & Rude, 1995), as defined by serum Mg concentrations below 0.71 mmol/L (Nadler & Rude, 1995). Low Mg status and low dietary Mg intake have therefore been discussed as risk factors for the development of diabetes mellitus type II. Reduced Mg serum or plasma levels (Nadler *et al.*, 1992; Kao *et al.*, 1999), reduced ionized serum Mg (Resnick *et al.*, 1993; Yokota *et al.*, 2001), reduced red blood cell Mg (Nadler *et al.*, 1992), and reduced intracellular Mg in muscle tissue (Sjogren *et al.*, 1988; Resnick *et al.*, 1993; Rude, 1996) as indicators of Mg status have been significantly correlated with the incidence of diabetes mellitus type II in humans. Oral Mg supplementation has been shown to improve insulin response and diabetes control (Paolisso *et al.*, 1989; Paolisso *et al.*, 1992). However, not all studies report a correlation between decreased Mg status and diabetes mellitus type II as based on concentrations of Mg in serum (Vanroelen *et al.*, 1985; Raz & Haivivi, 1989; Raz, 1989), ionized serum Mg (Mikhail & Ehsanipoor, 1999), red blood cells (Vanroelen *et al.*, 1985), or leukocytes (Raz & Haivivi, 1989).

The reasons for decreased Mg concentrations in these compartments remain unclear. In principle, lower Mg intake, decreased Mg absorption, altered Mg compartmentalization or higher urinary Mg excretion could be the cause. Data on Mg intake are not consistent. Recent studies (Kao *et al.*, 1999; Wälti *et al.*, 2002) do not support the hypothesis that diabetes type II subjects have low dietary Mg intake. Humphries *et al.* (1999) found low dietary Mg intake to be significantly correlated to prediabetic conditions such as insulin resistance, in men but not in women, and other potential relevant nutrient intake such as other minerals or dietary fibre were not considered. Colditz *et al.* (1992) and Meyer *et al.* (2000) found high Mg dietary intake to be significantly correlated with reduced risk of developing diabetes type II. However, the intake of other nutrients which could, in theory, have an influence on developing diabetes such as calcium, potassium, or dietary fibre, was not considered. When Colditz included calcium and potassium in a model, dietary Mg intake was no longer correlated with the risk of diabetes. In contrast to dietary Mg intake, only one study concerning Mg absorption in diabetic subjects is available (Wälti *et al.*, in preparation). No significant difference in absorption was found between subjects with type II diabetes (n=11) and non-diabetic subjects (n=10). Mg homeostasis is to a certain extent controlled by the kidney (Elin, 1994). Increased

urinary Mg was found in subjects with diabetes type II (reviewed by Durlach, 1988; Sjogren *et al.*, 1988). It was shown in humans that decreased insulin levels may lead to impaired Mg uptake by the cell (reviewed by Hwang *et al.*, 1993; Paolisso & Barbagallo, 1997) and that decreased insulin levels are associated with decreased Mg reabsorption by the kidney, as indicated in animal studies (reviewed by de Rouffignac *et al.*, 1993). In addition, hyperglycemia, occurring in less well controlled diabetic subjects, has been shown to induce hypermagnesuria via osmotic diuresis (Durlach, 1988; Garland, 1992).

The mechanisms by which impaired Mg status could contribute to the development of diabetes type II are not well understood. It has been suggested that Mg influences the responsiveness of peripheral tissue to insulin and also to affect insulin secretion by the pancreas in humans (Moles & McMullen, 1982; Durlach & Rayssiguier, 1983).

In conclusion, it has not been shown that low dietary Mg intake or decreased Mg status as determined by low Mg plasma concentration is causally associated to increased risk to develop diabetes mellitus type II. Low Mg status could be assumed to be the consequence rather than the cause of diabetes type II.

1.4.3. Cardiovascular diseases

Cardiovascular disease, especially ischemic heart disease, has been discussed in relation to low dietary intake and low Mg serum levels in humans (reviews by Karppanen, 1981; Seelig, 2001). In ischemic heart disease, the heart muscle is not appropriately supplied with arterial blood and suffers from lack of oxygen. In several studies, a significant correlation between the hardness of drinking water (i.e. the concentration of Ca and Mg) and the incidence of myocardial infarction (Masironi *et al.*, 1979; Rubenowitz *et al.*, 1996) or, more generally, ischemic heart disease (reviews by Karppanen, 1981; Marx & Neutra, 1997; Seelig, 2001) has been found. However, not all studies reported a significant correlation between ischemic heart disease and Mg hardness in drinking water (reviewed by Hammer & Heyden, 1980). The authors commented that, using multivariate analysis, water hardness did not

have a significant influence on the incidence of cardiovascular disease, and that "failure to consider factors of population density, water hardness and environmental exposure simultaneously in relation to CVD could explain some of the inconsistent results". Many studies did not consider other confounding risk factors such as calcium, contributing to water hardness, and water hardness was based on average values rather than the mineral concentration of individual waterworks, or was analyzed at a different time than the medical diagnosis (Marx & Neutra, 1997).

Based on these considerations between cardiovascular disease and water hardness, low dietary Mg intake has been discussed as a risk factor for the development of cardiovascular diseases. A significant correlation between dietary Mg intake and the prevalence of cardiovascular diseases was found in a study including about 15000 subjects (Ma *et al.*, 1995) or 400 individuals with a high risk for coronary heart disease (Singh, 1990). The intake of other nutrients was not considered. No significant effect of Mg dietary intake on the risk of developing cardiovascular disease was found in prospective studies (Elwood *et al.*, 1996; Liao *et al.*, 1998; Ford, 1999) based on about 16000 subjects after adjusting for age, smoking habits, energy intake and alcohol consumption, but not other nutrient intake.

Subjects with ischemic heart disease had a low Mg status as measured by significantly increased Mg retention measured by a loading test (Jeppesen, 1986; Rasmussen *et al.*, 1988) and decreased red blood cell Mg (Lasserre *et al.*, 1994). Furthermore, significantly decreased serum Mg and increased risk for cardiovascular disease was found by Liao *et al.* (1998) while no significant decrease of serum Mg in correlation to cardiovascular death was found by Reunanen *et al.* (1996) and Sasaki *et al.* (Sasaki *et al.*, 2000). However, serum Mg is not considered a good indicator of Mg status (see chapter 1.7.8. Methods to determine Mg status). In contrast to these results, a positive effect, a significant reduction in the death rate of postinfarct intravenously administered Mg to reduce mortality of acute myocardial infarct has been shown in >4000 patients in double blind, randomized controlled trials (Teo & Yusuf, 1993).

There can be several reasons why a beneficial effect of increased Mg intake or supplementation on reduced risk or treatment of cardiovascular disease has been demonstrated (reviews by Hennekens *et al.*, 1996; Rude, 1996; Saris *et al.*, 2000);

reduced platelet aggregation, induced vasodilatation, stabilization of cell membranes and protection of myocardial cells from catecholamine induced myocardial necrosis have been suggested.

In conclusion, the present data suggests that infusion of Mg could decrease the death rate related to acute myocardial infarct. Low Mg status as detected by loading tests or decreased serum or red blood cell Mg seems to be related to the incidence of cardiovascular diseases, as the majority of studies suggest. However, whether this is due to reduced dietary Mg intake alone or other confounding dietary compounds has not yet been demonstrated.

1.4.4. Hypertension and stroke

High blood pressure has been discussed in relation to decreased dietary Mg intake (reviews by Whelton & Klag, 1989; Mizushima *et al.*, 1998; Saris *et al.*, 2000; Seelig, 2001), although results are contradictory. Some studies reported a significant correlation between low dietary Mg intake and high blood pressure, others did not. Some studies showed a decrease in either the systolic or diastolic blood pressure, some only in one gender. Many of these studies were based on small numbers of subjects, and confounding factors such as intake of other nutrients or other risk factors were not adjusted for. Especially adjustment for dietary fibre might be of importance as it has been demonstrated that low intake of dietary fibre was significantly correlated with increased risk of developing hypertension in 31000 US males (Ascherio *et al.*, 1992). A recent meta-analysis (Burgess *et al.*, 1999) concluded that the available data do not indicate that increased dietary Mg intake is associated with a decreased risk of developing hypertension or decreases blood pressure, nor does it justify Mg medical treatment of patients with supplements. Furthermore, Peacock *et al.* (1999) found no significant correlation between dietary Mg intake and the incidence of hypertension in about 8000 US subjects after adjustment for age, race, and a number of other risk factors such as dietary intake of calcium and dietary fibre.

Besides total dietary Mg intake, soft water has been discussed in relation to increased blood pressure in a number of studies (reviewed by Seelig, 2001). However, these studies often did not distinguish between calcium and Mg content in water, and it remains to be shown whether the health benefit from hard water is causally associated with higher Mg intake. Because the contribution of drinking water to total Mg intake can be assumed to be in the range of 10-25% (see chapter 1.3.2. Dietary Mg intake), it would take relative large differences in the Mg content of the water to increase daily dietary Mg intake significantly.

In addition to dietary Mg intake, Mg status was investigated for an association with the incidence of hypertension. Studies of low serum or plasma Mg and the relation to hypertension are inconsistent as some studies demonstrated a significant correlation, others did not, some found a correlation only in one gender (Shils, 1998; Seelig, 2001). Resnick *et al.* (1984) found a significant correlation of low erythrocyte Mg and hypertension in 26 subjects. Oral Mg therapy with doses about 10 mmol-20 mmol, in most studies 15 mmol Mg/d showed to be effective in lowering blood pressure in hypertensive subjects in some but not all studies, especially not in placebo controlled double blind studies (Cappuccio *et al.*, 1985; Henderson *et al.*, 1986; reviews by Shils, 1998; Saris *et al.*, 2000). Lind *et al.* (1991) suggested that Mg supplementation can decrease blood pressure in subjects with low Mg intake and status, as measured by low urinary excretion, but not in unselected hypertensive subjects in general.

Hypertension is a risk factor for stroke. A significant correlation between the risk of stroke and low dietary Mg intake was found in >40000 US men (Ascherio *et al.*, 1998). However, there are no other studies confirming these findings.

The mechanism of Mg on the development of hypertension and the risk of stroke is not clear. The potential benefit of Mg on blood pressure has been reviewed by Rude (1996), and include decreased intracellular calcium concentration in vascular smooth muscle and platelets, and increased production of the vasodilator prostacyclin. In conclusion, the indication for a beneficial effect of increased Mg intake is not very strong as the intake of other potential relevant nutrients was not considered, and controlled intervention studies do, in the majority, not suggest a positive effect on blood pressure, even if given at pharmacological doses (at about the amount of the daily dietary Mg intake).

1.5. Factors influencing Mg absorption

1.5.1. Overview

A number of factors have been discussed and investigated in relation to Mg absorption, including physical factors such as Mg load, solubility of the salt and particle size, but mainly include the influence of other dietary compounds on Mg absorption.

Mg is typically consumed as a complex meal, not as a single compound as in supplements, although in the US, 14% of all men and 17% of all women took Mg supplements, with a median Mg intake of 102 and 100 mg/d (Institute of Medicine, 1997). In Europe, Mg intake in form of supplements can be assumed to be lower (Elmstahl *et al.*, 1996; Schellhorn *et al.*, 1998). Because meals are mixed in the stomach, other compounds consumed together with the meal may potentially influence Mg absorption, either positively or negatively.

Among the discussed dietary factors potentially inhibiting Mg absorption are organic acids such as phytic acid, oxalic acid, and polyphenols, which can form poorly soluble complexes with Mg in the small intestine. In addition, inorganic substances such as phosphate or phosphate-mineral complexes have been proposed to reduce Mg absorption as have other minerals and trace elements, such as calcium and zinc, due to competitive effects at the site of intestinal absorption (reviewed by Hardwick *et al.*, 1990b; Spencer *et al.*, 1994). Dietary fibre has also been reported to decrease Mg absorption (Drews *et al.*, 1979), although more recent studies have reported that soluble fibre such as inulin enhances Mg colonic absorption (Tahiri *et al.*, 2001). Similar as for calcium, vitamin D has been reported to positively stimulate absorption of Mg (reviewed by Hardwick *et al.*, 1991). Within the following chapters, the impact of potential enhancers and inhibitors occurring in food on Mg absorption is reviewed.

1.5.2. Physical factors

Diseases associated with reduced gastrointestinal passage time (reviewed by Walser, 1967) and damaged mucosal tissue reduce the time of interaction between diet and mucosa, increasing the risk of impaired Mg absorption. Diarrhoea, caused for example by ulcerative colitis, laxative abuse, and villous adenoma, steatorrhea, regional enteritis, gluten enteropathy, tropical sprue, and intestinal resections, especially of the small intestine, are some examples of factors that could decrease absorption of Mg (reviewed by Elin, 1988), and probably of other nutrients as well.

Physical factors influenced by the diet include pH, the amount Mg consumed, volume and viscosity of the meal, and gastrointestinal passage time. Decreased pH of the diet could increase Mg absorption because Mg salts are more soluble at acid pH, and the formation of poorly soluble $Mg(OH)_2$ or MgO is assumed to be prevented (Lindberg *et al.*, 1990), although this effect has not been demonstrated. In addition, similar as for other trace elements and minerals, a high oral Mg load has been shown to decrease Mg fractional absorption in a number of studies (Graham *et al.*, 1960; Roth & Werner, 1979; Lakshmanan *et al.*, 1984; Fine *et al.*, 1991, Table 7). For an average intake of about 200-400 mg Mg/d (see Table 4, chapter 1.3.2. Dietary Mg intake), Mg absorption can be assumed to be in the range of 20-50%.

Table 7. Oral Mg load and Mg absorption

study		Mg load		
		low	medium	high
Graham <i>et al.</i> (1960)	intake (mg/d)	23	243	550
	% Mg apparent absorption	75.8 (n=13)	44.3 (n=13)	23.7 (n=13)
Roth and Werner (1979)	intake (mg/d)	32	102	304
	% Mg apparent absorption	48 (n=23)	29 (n=23)	20 (n=23)
Fine <i>et al.</i> (1991)	intake (mg/d)	36	273	974
	% Mg apparent absorption	65 (n=8)	21 (n=8)	11 (n=8)

Viscosity has been discussed to influence mineral absorption. Liquid meals have been shown to have a shorter gastrointestinal passage time than solid meals in some (Hammer *et al.*, 1993; Hebden *et al.*, 1998; Brinch *et al.*, 1999) but not all studies (Bennink *et al.*, 1999). Viscosity might therefore influence Mg absorption per se, however, liquid meals often have a less complex matrix and contain smaller quantities of potential inhibitors to mineral absorption, such as phytate and oxalate. Relative high Mg absorption (59%) from mineral water has recently been reported (Verhas *et al.*, 2002), indicating that water is a good source of Mg. Sabatier *et al.* (2002) found slightly but significantly higher Mg absorption from mineral water served with a simple test meal (toast, butter, jam) than from mineral water alone, suggesting that prolonged gastrointestinal passage time or stimulated gastric acid secretion stimulated Mg absorption.

1.5.3. Type of Mg salt

Physical properties, especially the solubility of the type of salt used for supplementation or fortification have been discussed in relation to Mg absorption. Mg compounds used for fortification have been reviewed by Davidsson (1999), and the bioavailability of some frequently used pharmaceutical salts of Mg and their applications were reviewed by Ranade and Somberg (2001).

Due to its low cost and non-hygroscopic properties, magnesium oxide (MgO) is a frequently used supplement. However, the poor solubility of MgO (see Table 8) was assumed to be responsible for low Mg intestinal absorption in a number of studies. Based on urinary excretion 4 h post load, a relative high oral dose of MgO (610 mg) was significantly less well absorbed compared to the same amount of Mg as Mg citrate in 17 healthy human subjects (Lindberg *et al.*, 1990). Similar increased urinary excretion was found in a study with 16 healthy subjects after ingestion of 250 mg Mg/d from MgO as compared to chloride, lactate, and aspartate, the latter compounds did not differ significantly in bioavailability (Firoz & Graber, 2001). The bioavailability of 600 mg Mg as MgO compared to another poorly soluble Mg

compound (Mg hydroxide carbonate), resulted in no significant difference in urinary excretion in 16 healthy subjects (Tobolski *et al.*, 1997).

Table 8. Solubility of some Mg salts (Weast, 1989)

type of salt	solubility in water at 20 °C (g/L)
Mg orthosilicate	insoluble
Mg metasilicate	insoluble
Mg orthophosphate	insoluble
Mg pyrophosphate	insoluble
Mg hydrogenphosphate (heptahydrate)	3
Mg oxide	0.006
Mg hydroxide	0.009
Mg carbonate	0.1
Mg hydroxide carbonate	insoluble
Mg oxalate	0.7
Mg lactate	33
Mg citrate	200
Mg sulfate	260
Mg chloride	543
Mg nitrate	1250
Mg acetate	very soluble

Mg absorption determined by faecal monitoring did not reveal a significant difference of intake of 50 or 100 mg Mg as ^{26}MgO compared to the same amount Mg as ^{26}Mg diglycinate in 10 subjects with Crohn's disease and enteritis (23.2 versus 24.3%), or in 12 subjects with ileal resections (22.6 versus 23.5%) (Schuette *et al.*, 1993; Schuette *et al.*, 1994). It is suggested that a high oral load of MgO could result in incomplete dissolution of Mg in the acid pH of the stomach, leading to low Mg absorption in the intestine. In addition, the contradictory results of MgO absorption might also be explained by different particle sizes of the investigated

administered salts, even though the influence of particle size on Mg absorption has not been investigated in humans or rats. In a 4 x 4 latin square design with 4 cows, smaller particle size of MgO (238 μm as compared to 426 μm) was associated with a significant increase in urinary Mg absorption when supplemented at 4% (w/w) in the diet, indicating higher Mg absorption (Xin *et al.*, 1989).

There is poor evidence that besides poorly soluble Mg compounds, there are significant differences in Mg absorption due to the type of salt. No significant difference in Mg absorption was found after intake of 390 mg Mg as a Mg chloride solution, slow releasing Mg chloride tablets with enteric coating, and Mg gluconate in 12 healthy subjects (White *et al.*, 1992), based on 24 h urine-, leukocyte- and plasma Mg. In another study with 8 healthy adults, however, fractional Mg absorption (based on faecal excretion) from 328 mg Mg as slow releasing Mg tablets was significantly lower as compared to 325 mg Mg acetate and 355 mg Mg consumed with almonds (Fine *et al.*, 1991), which was attributed to the incomplete release of the Mg from the tablets before reaching the small intestine.

Other Mg salts have been investigated in animal studies. Mg hydroxide carbonate added to bread in amounts similar to Mg concentrations as in defatted soy flour added to bread had the same Mg bioavailability in rats based on serum and femur Mg (Winterringer & Ranhotra, 1983). It is known, that, besides the low solubility in water, Mg carbonate compounds have good solubility in diluted acids as in the stomach (Weast, 1989). In another rat study, Mg chloride and hydroxide-carbonate had significantly higher absorption compared to Mg sulphate, hydrogen phosphate, and silicate, and, in addition, Mg hydroxide-carbonate also as compared to oxide (Cook, 1973) when added at the same concentration to the diet (20 or 40 mg/100 g). These results would be in line with the solubility of the salts. Adding lower amounts of 4 mg/100 g of Mg as lactate, citrate, acetate, sulphate, oxide, chloride, phosphate and carbonate resulted in no significant difference in Mg absorption (Ranhotra *et al.*, 1976).

In conclusion, the present data indicates that poorly soluble Mg compounds such as MgO, Mg silicate and Mg phosphate are poorer absorbable compared to more soluble Mg salts, especially when consumed in high doses.

1.5.4. Dietary factors influencing Mg absorption

1.5.4.1. Proteins

The effect of proteins on Mg absorption was reviewed by Brink and Beynen (1992). Most human studies suggest a positive effect of proteins on Mg absorption. In an early human study by McCance et al. (1942), dietary intake of 145-200 g protein/d as compared to the same diet containing 45-70 g protein/d during 14 d periods in a crossover study with 4 healthy adults increased fractional apparent Mg significantly from 32% to 40%. Schwartz et al. (1973) found significantly decreased faecal Mg excretion in 12 adolescent boys consuming 265 compared to 125 mg protein/kg and day in an otherwise identical diet during 30 d periods in a randomized crossover study, indicating increased Mg absorption at high protein intake. Even a lower difference in protein intake was found to influence Mg absorption. Hunt and Schofield (1969) reported higher Mg apparent absorption in a balance study in subjects consuming 30 g as compared to 20 g protein/d (n= 5 subjects for each trial) and 48 g as compared to 34 g protein/d (n=4 subjects for each trial) during 30 d periods, apparent Mg absorption was 46.1% versus 28.3% and 57.7% versus 42.4%. However, no information about statistical significance was given. In support of these findings, Lakshmanan et al. (1984) found that the amount protein consumed in self selected diets by 34 healthy adults correlated negatively with Mg excretion in faeces. However, the effect was only significant in adults <35 years, and dietary sources of the proteins was not evaluated.

Three studies showed no effect of proteins on Mg absorption, but they all had methodological imperfections. In the first study, a modest dietary increase from 65 to 94 g protein/d during 2 x 28 d balance periods in 9 healthy adults had no effect on apparent Mg absorption (Mahalko *et al.*, 1983) in a non-randomized controlled study. However, the Mg content in the diet was not standardized, and the increase in protein might have been too low to effect Mg absorption. In a second randomized controlled study by Hunt et al. (1995) high as compared to low meat consumption (20% energy versus 10% energy from protein in the diet) during 7 wk in 14 healthy women did not influence apparent Mg absorption significantly. The high meat diet

however contained more Mg (268 versus 214 mg/d). In addition, no effect of increased protein intake (2 g/kg as compared to 1 g/kg body mass) on apparent fractional Mg absorption was found in a third study by Kitano *et al.* (1988) in 6 healthy adults during 12 d periods. However, Mg intake (mg) in the high protein group was about 1/3 higher compared to the low protein diet. Also a negative impact of protein on Mg absorption was reported (Van Dokkum *et al.* (1986) in a balance study, but the protein rich diets contained a much higher amount Mg.

In summary, a number of studies indicate a relationship between increased protein intake and increased apparent Mg absorption. The reason for this finding is not clear. Verbeek *et al.* (1993) found that a high as compared to a low casein diet (balanced for Mg, calcium, and phosphorus) increased apparent but not true Mg absorption in rats during a 14 d and a 28 d period, suggesting that high protein intake decreases endogenous Mg losses. Other possible mechanisms for increased apparent Mg absorption were discussed by the same authors. It was suggested that higher Mg solubility in the ileum due to higher protein content could be the cause as phosphopeptides were discussed to prevent the precipitation of calcium-magnesium-phosphate complexes and therefore to increase solubility and absorbability of Mg in the small intestine. However, the mechanism remains speculative.

1.5.4.2. Fat

Free fatty acids or those formed during enzymatic digestion of triglycerides in the small intestine can form insoluble soaps with Mg. Medium chain triglycerides (MCT) have been suggested to form more soluble Mg soaps (reviewed by Brink & Beynen, 1992), and it has therefore been suggested that MCT increase Mg absorption compared to long chain triglycerides (LCT). MCT are triglycerides containing C8-C12 fatty acids and occur in nature in lipids such as coconut oil, palm oil and butter. In a rat study (n=12 per group) standardized for Mg intake, in which MCT were given together with olive and sunflower oil during 1 month periods, Mg absorption increased significantly as compared to olive oil alone (Aliaga *et al.*, 1991).

There are not many human studies investigating the effect of MCT on Mg absorption, absorption has not yet been investigated in healthy adults. In 19 patients

with resections of the small intestine, no difference in Mg absorption was found when MCT replaced 50% of the LCT during 4 d periods in a randomized crossover study (Haderslev *et al.*, 2000). However, absorption of Mg was very low, 5.4% with MCT versus 2.9% from LCT rich diets, preventing the finding of significant differences. In a study with 28 healthy very low birth weight infants, Mg absorption increased significantly from 69 to 78% when a MCT instead of a LCT rich diet was given during a 3 day balance period standardized for Mg intake (Sulkers *et al.*, 1992). Similar results were obtained in a study including 34 low birth weight infants divided into 3 groups consuming a diet with 40% or 80% fat as MCT's and similar Mg content in the diet compared to a control diet without MCT's; a significant increase in Mg absorption from 58 to 85% was found in the group receiving the 80% MCT diet (Tantibhedhyangkul & Hashim, 1978).

In addition to qualitative differences in the fat composition, an increased fat intake per se has been reported to increase Mg absorption. Brink and Beynen (1992) suggested that due to formation of insoluble soaps the opposite would have been expected. However, fatty acids can also form insoluble soaps with calcium, and high calcium intake has been discussed to decrease Mg absorption even though studies in humans suggest that it is unlikely that a high calcium intake interferes significantly with Mg absorption (see chapter 1.5.4.4.1 Calcium and phosphorus).

In most rat balance studies (reviewed by Brink & Beynen, 1992), fat per se does not appear to influence Mg absorption. Tadayyon and Lutwak (1969) found significantly decreased Mg absorption from 55.2% to 43.5%, from 55.2% to 42.4% and from 55.2% to 40.7% when 5% fat (w/w) as the triglycerides triolein, tripalmitin, and tristearin were added to a fat free diet during a 1 wk period in each 35 rats. However, these triglycerides are not well absorbed, which might result in the formation of insoluble Mg soaps.

Human studies investigating fat intake and Mg absorption are scant and contradictory. Van Dokkum *et al.* (1983) reduced dietary fat intake (cream, margarine, beef) in otherwise similar diets during a 1 month period from 42 to 22% (energy) in a group of 10 healthy subjects and found no significant effect on Mg absorption. In addition, Ricketts *et al.* (1985) also found no significant effect when decreasing dietary fat (no information about dietary fat content given) in US diets consumed by 20 healthy subjects during 2 x 28 d periods in a crossover study.

However, only preliminary results were presented in abstract form and no data about intake of Mg was presented. On the other hand, in a study by Kies et al. (1992), 20 subjects were given a diet high (40% of energy) and low (30% of energy) in dietary fat during a 2 x 28 d period; Mg absorption was lower in the low fat diet (10 versus 27%), even though Mg intake was also lower in this group. No statistical information about the significance for this result was given. Differences in the diets besides the fat or the type of fat ingested might explain the contradictory results.

Thus, although the replacement of MCT for LCT has been shown to increase Mg absorption in rats and infants, increasing the amount dietary fat per se, especially fat which is well absorbed, has usually resulted in no significant effect on human Mg absorption. However, only a few studies, most with methodological imperfections, have tried to investigate the effect of dietary fat on Mg absorption in humans.

1.5.4.3. Dietary fibre and carbohydrates

Dietary fibre includes all substances that are not digested by human enzymes in the gastrointestinal tract. Dietary fibre is usually consumed in cereals, e.g. in bran, wholemeal bread or breakfast cereals, but also in vegetables, legumes and fruits. Dietary fibre can be classified into (water) insoluble fibre such as cellulose and lignin and soluble fibre such as hemicellulose, pectin, or oligosaccharides. In the Western world, the majority (about 65-75%) of the fibre intake of about 12-25 g/d (Lanza et al. 1987(reviewed by Dreher, 1987; Schlotke & Sieber, 1998) consumed is insoluble fibre (Story *et al.*, 1985).

1.5.4.3.1. Insoluble fibre

The effect of insoluble dietary fibre per se on Mg absorption is not clear. Foods rich in dietary fibre are usually also rich in Mg, and high Mg load can decrease fractional Mg absorption (see chapter 1.5.2. Physical factors). In addition, foods rich in dietary fibre, such as cereals and legumes, often are also rich in phytate, a potential inhibitor to Mg absorption. Many studies investigating the effect of fibre on Mg absorption were not standardized for Mg and/or phytate content (Reinhold *et al.*, 1976; Ismail-Beigi *et al.*, 1977; Reinhold *et al.*, 1980; Andersson *et al.*, 1983;

Schweizer *et al.*, 1983; Schwartz *et al.*, 1986; Hallfrisch *et al.*, 1987; reviewed by Torre *et al.*, 1991; and Brink & Beynen, 1992).

Camire and Clydesdale (1981) investigated the effect of wheat bran, cellulose and lignin on Mg binding ability *in vitro* at different pH, lignin showed the highest binding abilities. The amount Mg bound to these compounds increased with increasing pH, indicating that the tested compounds are potentially able to bind free Mg in the small intestine, thus reducing Mg absorption.

However, no human study could clearly demonstrate an effect of fibre on Mg absorption. A significant increase in faecal Mg was found by Slavin *et al.* (1980), when 16 g cellulose/d were added to a diet consumed by 7 healthy adults during 2 x 30 d periods but the high fibre rich diet contained more (9%) Mg. Mc Hale *et al.* (1979) studied the effect of 10 and 20 g cellulose added to a diet with similar Mg content on Mg urinary and faecal excretion in 6 adolescent healthy subjects during 3 x 6 d periods. A tendency towards higher faecal Mg excretion was found when cellulose was added, but results were not significantly different. Behall *et al.* (1987) studied the effect of different types of fibre (cellulose, Na-carboxymethylcellulose, 7.5 g per 1000 kcal) added to a basal diet on Mg absorption in 11 men during 5 x 4 wk periods. No significant difference in faecal Mg excretion was found, even though faecal Mg excretion increased from 62.4% to 78.3% of intake in the diets containing fibre. Again, Mg concentration in the diet was not standardized, but was higher from the basal diet (480 mg versus 370 and 410 mg/d for the cellulose and Na-carboxycellulose diets, respectively). Drews *et al.* (1979) found no significant effect on faecal Mg excretion in 8 adolescent male subjects fed a basal diet containing 14.2 g added cellulose during a 4 d crossover study (diets were standardized for Mg content).

In conclusion, there is no consistent evidence that dietary fibre *per se*, in the absence of phytate, decreases Mg absorption. Many studies suffer from the drawback that the Mg or phytate content in the diet was not standardized. However, because of the usually high amount of Mg in fibre rich foods, it is unlikely that total Mg absorbed would be decreased in a fibre rich diet.

1.5.4.3.2. Soluble fibre

Nondigestible "soluble" carbohydrates are dietary fibre typically found in the cell walls of plants, and are therefore frequently consumed in vegetables, especially in roots or tubers (e.g. potatoes). Many soluble dietary fibres are characterized by the ability to increase viscosity of aqueous solutions. Nondigestible soluble carbohydrates include hemicellulose, resistant starch, fructo-oligosaccharides and inulin, pectin and gums, lactulose and related sugars and sugar alcohols, and, if not digested (due to lactase deficiency) lactose (reviewed by Greger, 1999). Especially fructooligosaccharides have been investigated with growing interest during the last years and have been used as prebiotics (substances which could stimulate the growth of beneficial bacteria in the human gut) in functional foods.

Soluble carbohydrates are, at least partly, fermented by the intestinal microflora, mainly by bifidobacteria in the caecum and colon (Roberfroid & Delzenne, 1998; Cummings *et al.*, 2001; Scholz-Ahrens *et al.*, 2001). The bacteria produce short chain carboxylic acids (acetate, proprionate, butyrate) and lactate, decreasing the pH in the large intestine. This acidic fermentation has been reported to be associated with enhanced absorption of calcium and Mg (Lutz & Scharrer, 1991; Younes *et al.*, 1996).

The mechanisms for enhanced absorption however are unclear. One explanation is that the lower luminal pH increases the concentration of soluble ionised minerals, accelerating passive diffusion. Another explanation would be the existence of an exchange mechanism, e.g. $Mg^{2+}/2H^{+}$, allowing an influx of Mg^{2+} into mucosal cells against $2H^{+}$ coming from uptake of undissociated short chain fatty acids (reviewed by Roberfroid & Slavin, 2000). Short chain fatty acids have also been speculated to increase directly the resorptive surface of the gut's absorptive area (Scholz-Ahrens & Schrezenmeir, 2002).

1.5.4.3.2.1. Hemicellulose, pectin, and gums

Hemicellulose is a partly water soluble group of polysaccharides occurring in the cell wall of plants. In comparison to cellulose, they have a smaller molecular mass and are of a more branched chemical structure. The amount of hemicellulose in most vegetables and fruits is in the range of 1–2% (Souci *et al.*, 1994). Few studies indicated reduced Mg absorption due to intake of hemicellulose. Drews *et al.* (1979) found significantly increased faecal Mg excretion in 8 adolescent male subjects fed a basal diet containing 14.2 g added hemicellulose in a 4 d crossover study. Similar results were found by Taper *et al.* (1988); in a randomized cross over study, 22 healthy male subjects consumed a control diet and the same diet with 20, 30, or 40 g/1500 kcal added soy polysaccharide (mostly consisting of hemicellulose) during 4 x 11 d periods. The diet with the highest amount fibre decreased fractional Mg absorption from 31 to 25%, but no information about statistical significance of this result was given, and the fibre rich diet contained somewhat (5%) more Mg than the basal diet.

Pectin consists mainly of galacturonic-acid units, the carboxyl group is partly esterified with methanol, the molecular weight of pectin ranges from 10000 to 500000 (reviewed by Falbe & Regitz, 1995). Pectin is typically found in the cytosole and the cell membrane of plants, pectin concentration in fruits and vegetables is around 1% (w/w) (Truswell & Beynen, 1992). Several rat studies indicated bacterial fermentation of pectin and also of gums such as gum arab and guar gum (Tulung *et al.*, 1987; Demigné *et al.*, 1989; Seal & Mathers, 1989). Demigné *et al.* (1989) found that addition of 10% pectin in the diet over a 21 d period significantly increased calcium and Mg flux from the caecum to the blood, as determined by blood measurements and removal of the caecum.

No human study could demonstrate an effect of pectin on calcium or Mg absorption. No statistical significant difference was found for Mg absorption when feeding 14 healthy adults 9 g citrus pectin/d and 2550 kcal during 5 wk periods (Stasse-Wolthuis *et al.*, 1980) versus 16 subjects receiving the low-fibre control diet. Similar results were found by Drews *et al.* (1979) studying the effect of 14 g pectin/d in 8 adolescent boys during a 4 d period versus a basal fibre diet. In a third study with 6

ileostomy patients, 15 g citrus pectin during 3 d in the diet did not change apparent Mg absorption (Sandberg *et al.*, 1983).

In summary, hemicellulose, pectin and gums demonstrated have not been demonstrated a positive effect on Mg absorption in humans. As these compounds have been suggested to bind minerals in vitro (Camire & Clydesdale, 1981), it can be assumed that this effect might counterbalance the potential positive effects on Mg absorption in the large intestine.

1.5.4.3.2.2. Oligo(fructo)saccharides

Oligosaccharides include carbohydrates of 2-60 monosaccharide units, despite the IUPAC nomenclature that defines oligo as <10 monomers. Oligofructosaccharides are mainly composed of fructose. If the polymerization degree is between 2 and 60, these compounds are classified as inulin, if it is <8, these compounds are called oligofructosaccharides (or oligofructose) (Roberfroid & Slavin, 2000). Wheat, onions, and bananas are the most important sources of oligofructose and inulin in Western diets (US). Western European intake was estimated to be in the range of 3.2-11.3 g/d for adults (reviewed by Roberfroid & Slavin, 2000).

A number of rat studies indicated a positive effect of these compounds on Mg absorption. However, in these studies, usually very high amounts of oligofructosaccharides were fed. Wolf *et al.* (1998) found a significant increase in Mg absorption from 70% to 77% in rats given 0, 1, 3, or 5% (w/w, based on dry matter) oligofructosaccharides in the diet over 7d periods. Similar results were found when oligofructosaccharides (5% w/w) in the diet of rats were added over 8 d periods (Ohta *et al.*, 1995) containing the same amount Mg. However, Mg intake was significantly lower (10%) in the diet containing oligosaccharides which might have contributed to the significant difference. In another rat study, inulin increased Mg absorption significantly from 35 to 69% (Lopez *et al.*, 2000).

The first study showing an effect of inulin on calcium absorption in humans was reported by Coudray *et al.* (Coudray *et al.*, 1997a); inulin increased calcium absorption significantly from 21% to 34% in 9 healthy humans consuming up to 40 g/d inulin over a 28 d period as compared to the same diet without inulin during a

second 28 d period, but had no significant effect on Mg, iron, and zinc balance or absorption. The authors suggested that a longer adaptation period might be necessary to show an effect on mineral absorption because of the time it takes to stimulate bacterial growth and fermentation.

A recent study by Tahiri et al. (2001) showed that ingestion of 10 g/d short chain oligofructosaccharides in 11 post menopausal women over 2 x 5 wk periods enhanced apparent absorption of ²⁵Mg slightly but significantly from 30.2 to 33.9% in a randomized, double blind crossover study.

In conclusion, intake of oligofructosaccharides has been shown to improve Mg absorption in rats. In humans, the results are less conclusive, which might be related to longer periods needed to change bacterial flora. In addition, relatively high amounts, in the range of the average daily intake of oligofructose (about 10 g/d) were needed to increase Mg absorption.

1.5.4.3.2.3. Resistant starch

Resistant starch is not or only partly broken down into digestible sugars by human enzymes (alpha amylase) in the gastrointestinal tract. The reason is not completely understood, but protection of the starch through undisrupted cell walls, presence of amylose-lipid complexes, or the formation of an extensive network of intra-and interhelical hydrogen bonds may play a role (Englyst & Kingman, 1990).

The 4 major groups of resistant starch are physically inaccessible resistant starch (granules) (RS₁), occurring for example in partly milled grains and seeds, raw starch (RS₂) occurring e.g. in raw potatoes and bananas, retrograded resistant starch (RS₃) occurring in thermally treated products such as in bread, corn flakes, and cooked potatoes, and chemically modified resistant starch (RS₄) (reviewed by Englyst *et al.*, 1992). Consumption of resistant starch has been reported to be in the range of 4 g/d in Europe (Dysseler & Hoffem, 1994), which amounts therefore for about half of the nondigestible soluble carbohydrates consumed.

Especially RS₂ has been reported to positively stimulate Mg absorption in a number of rat studies, no studies in human subjects are available at present. RS₂ seems more fermentable in the large intestine than is RS₃ or RS₁ (Schulz *et al.*, 1993).

Demigné et al. (1989) found that adding 10% amylose rich starch (which is incompletely broken down in the small intestine) to the diet during a 21 d period significantly increased fluxes of calcium and Mg from caecum to blood as determined by blood measurements and removal of the caecum. Younes *et al.* (2001) found a significant increase in Mg and calcium caecal solubility and absorption in rats fed a diet containing 10% inulin, 15% resistant starch (RS₂), or a blend of 5% inulin plus 7.5% (w/w) resistant starch versus a fibre free diet. The diet containing inulin and resistant starch had the highest increase in soluble calcium and Mg concentrations and absorption in caecum, a synergistic effect was suggested by the authors. RS₂ increased Mg absorption in rats over a period of 13 d when added at 25% (w/w) in the diet (Schulz *et al.*, 1993) while RS₃ (17% in the diet, n=12) had no effect. Lopez *et al.* (2001) showed that ingestion of 20% (w/w) raw potato starch and high amylose corn starch (both RS₂) in the diet over a period of 21 d increased significantly apparent absorption of calcium, Mg, zinc, iron, and copper. No effect of resistant starch on Mg absorption was found in a study with 8 rats and 8 pigs fed a diet containing 6% RS₂ or RS₃, respectively, as compared to the control diet (de Schrijver *et al.*, 1999) over a 5 wk (rats) or 1 wk (pigs) period. However, the number of animals was low, and a nonsignificant increase in apparent Mg absorption during the RS₂ diet was seen in the rats from 36.1% to 45.0%. It could be assumed that only diets containing relatively high amounts of resistant starch showed a significant effect on Mg absorption.

The influence of resistant starch on true Mg absorption was recently evaluated in rats. In a study by Heijnen et al. (1996), RS₂ increased apparent but not true Mg absorption significantly in rats fed a diet containing 12% RS₂ (w/w) compared to a diet containing 12% RS₃ (w/w) over a 4 wk period, from 62% to 68%. True Mg absorption was estimated by using a ²⁸Mg tracer. Therefore, decreased endogenous Mg losses were suggested as an important factor. RS₂ might reduce intestinal fluid secretion, depress turnover of epithelial cells or reduce Mg influx in the lumen because of higher concentration of soluble Mg in the lumen.

In conclusion, raw resistant starch (RS₂) increased Mg absorption in rats in the majority of studies. Whether there is also an increase in true absorption is not clear.

1.5.4.3.2.4. Lactose and lactulose

Lactose, which cannot be digested by the adult rat, enhanced Mg absorption in a number of rat studies (Behling & Greger, 1990; reviewed by Brink & Beynen, 1992; Heijnen *et al.*, 1993; Yanahira *et al.*, 1997). However, there is no agreement about the effect of lactose on human Mg absorption at present, studies investigating the effect of lactose on human Mg absorption are scant. Significantly increased Mg absorption (40% and 48%) was found in 6 healthy infants given 7% (w/w) lactose in a diet standardized for Mg content during at least 11 d, compared to a diet containing similar amounts of carbohydrates as sucrose and polyose (Ziegler & Fomon, 1983). In 10 preterm infants, reduction of lactose in the diet from 100% (of sugars) to 50% did not decrease Mg absorption significantly when given during a 3 d period (Wirth *et al.*, 1990). No data about the lactose intake was presented. Moya *et al.* (1999) compared Mg absorption in 2 x 10 term infants fed either a lactose free or a lactose containing formula during 72 h balance periods and found no significant difference in Mg absorption. No data about the lactose intake was presented.

Hardly any information is available on the influence of lactose on Mg absorption in adults. Urinary excretion as an indicator for Mg absorption was monitored by Brink *et al.* (1993) in a double blind crossover study in 24 lactose tolerant, healthy adults receiving 46 g lactose/d in the diet as compared to a lactose free diet during 1 wk periods. No differences in urinary Mg excretion were found, presumably indicating no differences in Mg absorption.

The mechanism for a potential increase in Mg absorption is not clear. An early study by Kobayashi *et al.* (1975) found that apparent Mg absorption in 15 term infants was lower from a lactose free milk (n=4) than from a proprietary milk (n=8) and the same milk containing lactase (n=3) during 8-9 d periods. Apparent Mg absorption was 39.3%, 57.7% and 80.9%, respectively; no Mg intake data and information about statistical significance were presented. It was suggested that the hydrolyzed monosaccharides, rather than lactose, were responsible for increased Mg absorption in the intestine.

Lactulose, a chemically similar compound to lactose has been shown to increase Mg absorption in rats, no human studies have been done. Lactulose is a synthetic disaccharide (consisting of galactose and fructose) which cannot be digested by the rat or human. This carbohydrate is used in medicine to improve gut peristaltic action (reviewed by Falbe & Regitz, 1995). Demigné et al. (1989) found that feeding 10% lactulose (w/w) in the diet compared to a lactulose free control diet in rats during a 21 d study period significantly increased intestinal flow of Mg from the caecum into the blood as determined by blood measurements and removal of the caecum. Heijnen et al. (1993) found significantly increased Mg absorption when 14% lactulose was added to the diet of rats during 21-23 d periods. Significantly increased Mg absorption was also found in 6 dogs fed either 0,1, or 3 g lactulose/MJ during 2 wk periods in a 3 x 3 latin square design (Beynen *et al.*, 2001).

In conclusion, lactose showed to enhance Mg absorption in some, but not all studies with infants while the effect has not been demonstrated in adults. Rat studies with lactulose suggest a positive effect on Mg absorption.

In conclusion, it can be assumed that many nondigestible soluble carbohydrates which induce a decrease in pH in the caecum and colon due to increased bacteria activity could, in principle, increase Mg colonic absorption. However, in human adults this has only been demonstrated with oligofructose.

1.5.4.4. Minerals and trace elements

Many minerals and all trace elements are consumed in considerably lower amounts in the diet than Mg. Thus, it is unlikely that trace elements such as iron and zinc could significantly affect Mg absorption. Minerals consumed in the average Western diet in amounts equal or higher than Mg are calcium, potassium, sodium, phosphorus, chloride, and sulphur (Gregory *et al.*, 1990; Schlotke & Sieber, 1998).

Increasing dietary zinc from about 11.5 to 14.7 mg/d had no effect on Mg bioavailability in humans, based on urinary and faecal excretion (Greger, 1979). Pharmacological doses of 142 mg/d of zinc supplements however decreased Mg apparent absorption and balance in humans significantly (Spencer *et al.*, 1994). It

was suggested that both minerals competed with the same transport mechanism in the small intestine, even though it has been reported that Mg is primarily absorbed in the distal and zinc in the proximal parts of the intestine.

Adding calcium, magnesium, phosphorus, iron, copper, and manganese in excess to a human diet decreased absorption of the other minerals in an early study by De and Basu (De & Basu, 1949). However, no information about the statistical significance of these results were presented, and only 3-4 subjects were studied in controlled cross over trials.

1.5.4.4.1. Calcium and phosphorus

The effect of calcium on Mg absorption has been subject to some discussion (reviews by Hardwick *et al.*, 1991; Brink & Beynen, 1992). In almost all of about 2 dozen rat studies, high levels of dietary calcium decreased Mg absorption or retention. Most of these diets however were marginal in Mg and contained relatively high amounts of calcium and phosphorus.

Mg concentration in the human diet is typically less marginal and calcium and phosphorus concentrations are lower than in the rat diets. The majority of studies in humans did not indicate an effect of an intake of up to 2000 mg calcium/d on Mg absorption (Leichsenring *et al.* (1951; Spencer *et al.*, 1980; Andon *et al.*, 1996). In a more recent stable isotope study, 800 mg/d versus 1800 mg calcium/d did not alter Mg absorption or urinary or faecal excretion or balance in 5 adolescent girls during a 2 x 14 d period (Sojka *et al.*, 1997). Studies showing a negative effect of calcium on Mg absorption were based on diets containing marginal amounts of Mg (Giles *et al.*, 1990), diets containing high amounts of phosphorus (up to 5.5 g/d), data was based on only 1 subject (Heaton *et al.*, 1964), the study was only presented briefly in abstract form (Kim & Linkswiler, 1979), or were based on perfusion (Norman *et al.* (1981). One of the few studies indicating an effect of calcium on Mg absorption was the early balance study by De and Basu, (1949), addition of calcium in the diet between 300 and 1000 mg/d decreased Mg absorption significantly in 4 healthy subjects at Mg concentrations typically ingested in the diet (370 mg/d).

Studies investigating both the effect of calcium and phosphate on Mg absorption might explain the contradictory results. Several studies suggested that that phosphorus and calcium together could affect Mg absorption. As for calcium, there is no consensus on the influence of phosphorus alone on Mg absorption (Hardwick *et al.*, 1991; Brink & Beynen, 1992). Phosphorus is usually consumed in form of phosphate (Anderson, 1998), and Mg phosphates are poorly soluble at the pH of the small intestine, but more soluble at the pH of the stomach (Weast, 1989). Thus, a high phosphorus intake could, in theory, contribute to poor Mg absorption. In a rat study, Brink *et al.* (1992a) showed that intake of calcium and phosphorus, but not calcium alone, decreased apparent Mg absorption significantly, indicating that an insoluble Mg-calcium-phosphate complex was formed. Similarly, pronounced symptoms of Mg deficiency such as slow growth and soft tissue calcification were observed in guinea pigs supplemented with high amounts of calcium and phosphorus (up to 1.7 and 2.5% of the diet) to a diet marginal in Mg (0.005%), this effect was more pronounced with a high phosphorus intake (O'Dell *et al.*, 1960) but diminished when Mg was added to the diet at 0.3%.

However, no significant effect on Mg absorption has yet been shown in healthy human subjects. Heaton *et al.* (1964) showed that increasing the dietary phosphorus content from 1250 mg/d to 3890 mg/d during 6 d balance periods decreased Mg absorption significantly in 10 subjects with disorders of calcium metabolism (indicated by renal stones or metabolic bone disease). It could be speculated whether inability of calcium absorption contributed to the findings. Decreased apparent Mg absorption from both 43% to 34% was found by Greger *et al.* (1981) in 9 healthy subjects consuming a standardized diet containing 780 mg calcium and 843 mg/d phosphorus compared to a diet containing 2382 mg calcium and 2443 mg phosphorus/d and a diet containing 780 mg calcium and 2443 mg phosphorus/d during 12 days, no statement of the statistical significance was made. No influence of supplemental phosphorus on apparent Mg absorption was found by Leichsenring *et al.* (1951) between a diet containing 1500 mg calcium, 260 mg Mg, 800 mg phosphorus and a diet containing additional 600 mg phosphorus during a 4 wk period in 3 subjects each. However, the limited number of subjects and the low amount of supplemented phosphorus limited the interpretation of the results.

In conclusion, there are indications that high amounts of phosphorus in the diet, especially together with high dietary calcium intake, might decrease Mg absorption. There is no consensus whether high calcium or phosphorus intake alone can alter Mg absorption.

1.5.4.4.2. Sodium and potassium

Studies with rat gut sacs in vitro (Ross, 1961) or rat ileum in vivo (Behar, 1975) suggested that sodium ions might enhance magnesium transport through the epithelial membrane, but rat balance studies in which sodium was added to the diet at up to 3.1% (w/w) could not demonstrate a systematic effect on apparent Mg absorption. No human studies have investigated the influence of sodium on Mg absorption.

Dietary intake of potassium at 0.1-1.9% of the diet (w/w) showed no effect on Mg absorption in the majority of rat studies (Brink & Beynen, 1992). In humans, Fisler et al. (1984) found no significant change in Mg faecal excretion in a fasting group supplemented with 2300 mg potassium/d. In a study with sheep, it was suggested that high amounts of potassium in the diet depolarize the apical membrane of the epithelial cells of the mucosa, reducing the driving force for Mg uptake (Schonewille *et al.*, 1999).

However, there is no indication that sodium or potassium does influence Mg absorption in man.

1.5.4.5. Vitamin D

The majority of the vitamin D required is formed under the skin, with exception of the 3-6 winter months for people living at latitudes between 40-60 °C and higher (reviewed by DRI, 1997). Compounds with vitamin D activity, especially the biological active 1,25-dihydroxycholecalciferol have been shown to increase calcium absorption in the small intestine due to its influence on calbindin, a mucosal membrane protein (reviewed by Kumar, 1990).

The effect of vitamin D on Mg absorption however is not well established (reviews by Hardwick *et al.*, 1991; Brink & Beynen, 1992) and results are contradictory. No effect of dietary vitamin D in healthy humans has been demonstrated, and no correlation between 1,25- dihydroxycholecalciferol and Mg concentration in plasma has been found (Wilz *et al.*, 1979). Even pharmacological doses of up to 8 mg/d vitamin D₃ did not change Mg absorption significantly in 7 patients with osteomalacia and osteoporosis Heaton *et al.* (1964). Perfusing 2 µg/d 1-25- dihydroxycholecalciferol however increased jejunal Mg absorption (Schmulen *et al.*, 1980; Krejs *et al.*, 1983). Only the study by Hodgkinson *et al.* (1979) showed a positive significant effect of supplementation of 1-2 µg/d 1,25- dihydroxycholecalciferol and other compounds with vitamin D activity in amounts as consumed usually with diets during 2 wk periods on Mg absorption in 32 subjects. However, these subjects had osteomalacia and hypoparathyroidism, and it might be speculated that they were vitamin D deficient and not in normal metabolic balance.

Actually, it has been suggested that Mg absorption is partly vitamin D dependent as Mg absorption was found to be reduced in vitamin D deficient rats and did increase with vitamin D repletion (reviewed by Hardwick *et al.*, 1991). Hodgkinson *et al.* (1979) proposed that increased intestinal Mg absorption might be due to a vitamin D stimulated calcium absorption transport system, as this system might also have weak affinity for magnesium. Another hypothesis was that the reduced intestinal calcium concentration due to vitamin D stimulated absorption decreased competition for absorption between calcium and Mg. However, the reason remains speculative.

In summary, there is only poor indication that vitamin D could increase Mg absorption in healthy subjects. There are no studies on the effect of vitamin D in amounts as typically consumed in the diet in healthy subjects. In addition, because the majority of vitamin D is formed in the human skin with UV light, vitamin D intake does probably not play an important role in Mg nutrition.

1.5.4.6. Phytic acid

One of the most potent inhibitors of mineral absorption is phytic acid (myo-inositol hexakisphosphate). Phytic acid is widely distributed in nature, all plant cells contain phytic acid (Harland & Morris, 1995). In cereals, legumes, and oilseeds, it is the

major storage form of phosphorus (Harland & Oberleas, 1987). Foods based on wholemeal flour or bran contain high concentrations of phytic acid, typically in the range of 0.5% - 1.4% for wholemeal bread and up to 3.5% for bran, depending on the plant species (Reddy *et al.*, 1989). In low extraction rate (white) cereal flours, the concentration of phytic acid is lower, as most phytic acid is removed with the outer layers of the grains. The daily dietary intake of phytic acid has been reported to be in the range of 180-800 mg/day in industrialized countries (reviewed by Plaami, 1997).

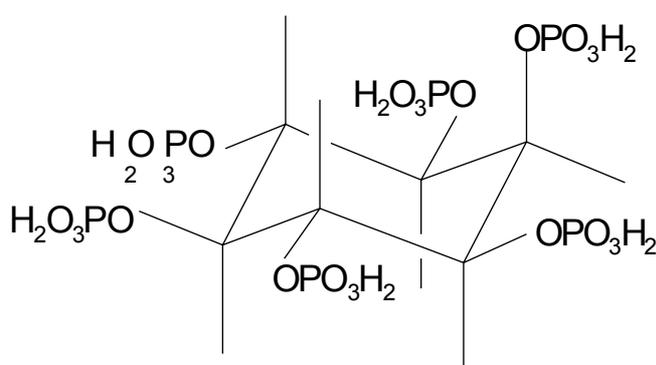


Fig. 1: Chemical structure of phytic acid

The ability to form strong complexes with phytic acid varies between the minerals/trace elements. When titrated with sodium phytate at pH 7.4, the following order of stability of the complexes was suggested: Cu>Zn>Mn>Fe>Ca (Vohra *et al.*, 1965). Based on calorimetric experiments at pH 3.6, the following order of stability was suggested (Evans & Martin, 1988): Cu>Zn>Mn>Mg>Co>Ni. In general, it is assumed that Mg does not form very strong complexes (Holleman & Wiberg, 1985).

In addition to the in-vitro investigations, phytate has been shown to decrease absorption of many of the nutritionally relevant minerals and trace elements in humans, including iron (e.g. McCance *et al.*, 1943; Hallberg, 1987; Hurrell *et al.*, 1992), zinc (e.g. Turnlund *et al.*, 1984; Navert *et al.*, 1985; Sandstrom *et al.*, 1987), calcium (e.g. Reinhold *et al.*, 1973; Heaney *et al.*, 1991), and manganese (Davidsson *et al.*, 1995).

Studies in rats indicated reduced Mg apparent absorption when phytic acid was fed at 0.6% (Matsui *et al.*, 2001), 1% (Roberts & Yudkin, 1960), or at 1.2% mass in the diet (Pallauf *et al.*, 1998), or in soybean protein at a molar ratio of phytic acid: Mg of ca. 0.5 (Brink *et al.*, 1991). However, not all rat studies found a negative effect of phytic acid on Mg absorption (Lopez *et al.*, 2000).

Magnesium balance in humans was significantly lower when 3.5 g phytate/d in wholemeal bread was given compared to white bread in a study with 2 men over a 20 day period (Reinhold *et al.*, 1976). This finding was based on increased faecal excretion, suggesting either decreased apparent Mg absorption or increased endogenous Mg losses as the cause. However, other components in the bread such as fibre could have contributed to the observed decrease in absorption. Bran compared to dephytinized bran containing either 0.2 g or 2 g phytate/d reduced apparent Mg absorption significantly from 40 to 35% (Morris *et al.*, 1988) when consumed by 10 men during 2 x 15 d periods. In an early study by McCance and Widdowson (1942), phytate added to white wheat bread (phytic acid consumption about 0.6 g/d) also indicated a significant reduction in magnesium absorption from 47% to 29% in 8 healthy volunteers when consumed during 14 d periods or longer.

It is assumed that insoluble complexes are formed in the gastrointestinal tract which are not absorbable. For Mg, it is assumed that Mg-phytate or protein-Mg-phytate complexes are formed which are insoluble >pH 6, (Cheryan, 1983; Nolan *et al.*, 1987; Champagne, 1988). Such complexes are assumed to be formed both with food Mg and endogenous Mg excreted in the gastrointestinal tract. Brink *et al.* (1992b) suggested higher endogenous losses rather than reduced true absorption as the major cause of decreased apparent magnesium absorption in a rat study fed a diet containing 0.4% phytic acid during 30 d compared to a control group. Matsui *et al.* (2001) on the other hand investigated the influence of a diet containing 0 versus 0.6% phytic acid on endogenous Mg losses in rats based on stable isotope infusing of ²⁵Mg and found no significant difference in endogenous Mg between the 2 groups.

Whether and to what extent phytic acid influences mineral absorption depends on the degradation in the gastrointestinal tract. This can be caused by natural phytase in cereals and grains, or by bacteria ingested or by bacteria present in the intestine. Rat studies suggested that the majority of phytate is broken down by dietary

phytase (Williams & Taylor, 1985; Rimbach *et al.*, 1995) rather than by endogenous phytase activity in the small intestine. This degradation of phytic acid can also occur during food processing and preparation, leading to dephosphorylation of phytic acid and the formation of different inositol phosphates. With decreasing number of phosphate groups of phytic acid, the mineral chelating ability decreases, as shown for zinc (Sandstrom & Sandberg, 1992) and iron (Sandberg *et al.*, 1999). However, as most diets contain no free phytase because of inactivation by heat, it is not likely that phytate is broken down in the human intestine.

In conclusion, phytate has been shown to inhibit absorption of several minerals including iron, zinc, and calcium. Several human balance studies have suggested a negative effect on Mg absorption in humans.

1.5.4.7. Oxalic acid

Oxalate is ubiquitous in plants, especially in the leafy parts. High amounts are found in leafy vegetables such as spinach (over 1 g/100 g, Tabekhia, 1980) and rhubarb. However, there are also high amounts of oxalate in fruits, grains, nuts, tea, coffee, and cacao (Souci *et al.*, 1994). Daily dietary oxalate intake in industrialized countries such as the UK are in the range of 70-150 mg/d (Zarembski & Hodgkinson, 1962), similar oxalate intakes for the UK have been reported by Anderson *et al.* (1971). Intake in India has shown to vary much more, depending on the income group examined, or whether rural or urban areas were analyzed, from 78-2045 mg/d, reviewed by Hodgkinson (1977b).

Oxalic acid can form poorly soluble complexes with some minerals at physiological pH, especially with divalent ions. For example, zinc, calcium, and Mg oxalates have low solubility in water at room temperature: 7.9, 6.7, and 700 mg/L, respectively. Even though it is known that all 3 oxalate components are soluble in dilute acid (Weast, 1989), it is not known to what extent they dissolve in the stomach to form one pool of oxalate.

Oxalates in plants can be classified as insoluble (calcium bound) and water soluble (potassium-, sodium bound, or free) oxalates. For spinach, around 15-20% of total oxalates have been reported to be soluble (Toma & Tabekhia, 1979; Souci *et al.*,

1994), but higher concentrations of soluble oxalic acid have been reported for spinach, up to 93% (reviewed by Hodgkinson, 1977a). Fruits usually contain about equal amounts soluble and insoluble oxalates (reviewed by Hodgkinson, 1977a). Soluble oxalates are assumed to have a negative impact on mineral absorption due to the ability to bind free minerals in the small intestine, thus reducing their absorption. Despite the low solubility, it has been suggested that the oxalate complex might be absorbed intact, as indicated in a rat study with double labelled (^{45}Ca , ^{14}C) calcium oxalate (Hanes *et al.*, 1999), although absorption of the complex was only 2%.

Information regarding the influence of oxalate on mineral, especially Mg absorption, is scant. It has been demonstrated that Ca from vegetables containing oxalate is less well absorbed than from milk in 13 healthy subjects (Heaney *et al.* 1991). In the same study, a significantly lower calcium absorption (5%) from high oxalate spinach (about 480 mg oxalate/dose) than from low-oxalate kale (42 % absorption, Heaney & Weaver, 1990) in 11 healthy subjects was reported.

In a study with Mg deficient rats by Kikunaga *et al.* (1995), Mg absorption from a diet containing raw spinach differed significantly from absorption from the same diet containing boiled spinach (about 50% of the oxalate was removed by boiling) or the control (same diet without spinach), absorption was 88.9% (control) versus 80.2% as determined by faecal monitoring during a 8 d pair fed trial with standardized Mg intake. On the other hand, supplementation of the control diet with 0.8% oxalate (as oxalic acid) had no significant effect on Mg absorption, suggesting that other factors present in spinach reduced Mg absorption or different behaviour of added oxalic acid as compared to native oxalic acid.

Mg has been shown to decrease absorption of oxalic acid in humans (Berg *et al.*, 1986; Hanson *et al.*, 1989), indicating that Mg oxalate complexes are formed which are not or only poorly absorbed. Kelsay and Prather (1983) observed decreased Mg and zinc balance in 12 human subjects when spinach was added to the diet during a 4 wk period (440 mg oxalate/d) compared to the same diet containing cauliflower (175 mg oxalate/d) in the same subjects. However, the Mg and zinc intake was significantly higher in the group receiving the spinach rich diet. Furthermore, balance of the same diet measured after 3 wk was not statistically significant.

Schwartz (1984) investigated Mg absorption from bran muffins containing spinach and found an apparent absorption of 48% in 4 healthy adults. Absorption was not significantly lower compared to muffins containing lettuce, bran, or turnip greens, but significantly lower than from collard greens, 54%. However, No information about the consumed amount oxalate was given. In addition, no information was given about the amount of spinach added to the muffins but as 40-50 mg Mg came from the spinach, it can be assumed that about 50-100 g of spinach were served (Holland *et al.*, 1994; Souci *et al.*, 1994). The amount oxalate consumed with the spinach based meal might have been too low to detect an effect on Mg absorption in only 4 subjects as compared to the other vegetable based meals.

In summary, there are a limited number of studies suggesting that oxalate has a negative impact on mineral absorption, including Mg absorption.

1.5.4.8. Polyphenols

Polyphenols include a wide variety of aromatic substances in plants which contain at least 2 hydroxyl groups, e.g. flavones, anthocyanidines, lignins, and tannins. Only few information regarding the dietary intake of polyphenols are available, as a huge variety of chemical compounds are included and quantification is difficult. The major dietary sources of polyphenols include fruits and beverages, a total polyphenol intake of about 1 g/d has been estimated for the Western diet (Scalbert & Williamson, 2000). The total polyphenol content in fruits, vegetables, and grains has been reported to be in the range of 0.04-0.6%. Similar values were reported by Gillooly *et al.* (1983). This is relatively low compared to other potential mineral inhibitors, e.g. phytate, especially when calculating molar ratios.

Polyphenols have chelating properties and have been related to decreased absorption of iron in humans (Hurrell *et al.*, 1999; reviewed by Zijp *et al.*, 2000), and zinc in rats (Coudray *et al.*, 1998). However, no information is available on their influence on calcium or Mg absorption in humans. Calcium and Mg are present in far greater quantities in the diet than zinc or iron, thus, molar ratios of polyphenols:minerals will be much lower. Because of the relative high amounts Mg

consumed with the diet and the lower stability of the Mg complexes as compared to iron and zinc, a potential negative effect can be assumed to be much lower.

1.5.4.9. Chlorophyll

Magnesium is the central atom of the major green plant pigments chlorophyll *a* and *b*. In plant leaves, up to 57% of total magnesium is bound to chlorophyll (Dorenstouter *et al.*, 1985). Green vegetables, legumes and fruits have been estimated to contribute to 18% of magnesium intake in Germany (DGE, 1996) and 21% (De Rham, 1991) or 43% (Schlotke & Sieber, 1998) in Switzerland. Chlorophyll bound magnesium may be of interest because the porphyrin structure of chlorophyll possesses similar chemical properties to the heme structure of hemoglobin, which is known to have a much higher iron bioavailability than non-heme iron (Weintraub *et al.*, 1968; Bothwell *et al.*, 1989), and, because it is absorbed intact, the heme molecule protects the iron from other absorption inhibiting compounds such as phytic acid. A similar effect for chlorophyll has been suggested (Hazell, 1985; Fairweather-Tait & Hurrell, 1996).

However, in vitro experiments at acidic pH suggest rapid, irreversible degradation of chlorophylls to their corresponding pheophytins, in which the magnesium is exchanged by two protons (Willstätter, 1907; Mackinney & Joslyn, 1940). However, these experiments were not designed to simulate in vivo conditions, e.g. free chlorophylls were used while the major part of the chlorophylls in nature is associated in a non-covalent way with the light harvesting complex. In addition to these in vitro studies, a human study comparing spinach intrinsically and extrinsically labelled with stable magnesium isotopes indicated no difference in absorption between the labels (Schwartz *et al.*, 1984).

1.6. Effects of dietary compounds on Mg excretion

The kidney is the major organ regulating Mg plasma concentration, which is kept constant within a relatively small concentration range (Elin, 1994). The kidney is therefore an important organ for Mg homeostasis in man. An increase in plasma is usually associated with decreased reabsorption by the kidney and thus loss of Mg in the urine. It can be expected that all factors, including absorption, increasing plasma/serum Mg concentration increase urinary Mg excretion. In many human studies, increased Mg absorption was associated with increased urinary excretion (reviewed by Brink & Beynen, 1992). Furthermore, the increase in urinary Mg excretion is expected to depend on the Mg status. This is used in the oral Mg load test (see chapter 1.2.3.1. Mg bioavailability, retention, and status), in which increased Mg in urine is used as an indicator for a good Mg status (Elin, 1987; Elin, 1991b). Thus, each food compound or diet resulting in increased Mg absorption can potentially increase urinary excretion, and the degree of urinary excretion can depend on Mg status.

1.6.1. Ethanol

Alcohol consumption in industrialized countries such as the UK (reviewed by Poppitt, 1998) or Switzerland (Schlotke & Sieber, 1998) are in the range of 6-8% of total energy intake. Ethanol has a diuretic effect and in alcoholic subjects, Mg depletion, determined e.g. by muscle biopsy or decreased exchangeable body Mg, has been observed (reviewed by Rivlin, 1994). Significantly increased urinary Mg losses were observed in healthy human subjects after administration of 30 ml ethanol orally and 20 ml iv. after an overnight fast (Kalbfleisch et al. 1963) and 1 ml/kg orally after an overnight fast (McCollister et al. 1963), and the effect of hypermagnesuria, but not plasma peak Mg concentration, was shown to depend on the oral ethanol dose given together with a test meal (Laitinen *et al.*, 1992). Thus,

consumption of high amounts of ethanol as ingested by alcoholics is a risk factor for Mg depletion.

1.6.2. Caffeine

Caffeine is a methylxanthine with diuretic effects, occurring e.g. in cocoa beans, tea, and mate leaves and guarana seeds. Caffeine consumption in the US was reported to be in the range of 2.6 mg/kg per day and person (reviewed by Arnaud, 1998). Studies on the effect of caffeine on Mg excretion are rare. In 12 healthy adult females consuming 240 ml decaffeinated coffee after a 10 h fasting period with either 0, 150, or 300 mg added caffeine on 3 different days, addition of caffeine increased the volume of urine and the concentration of Mg in urine. Thus, the amount of Mg excreted was increased significantly, about 50% after 300 mg added caffeine (Massey & Wise, 1984). The effect of added caffeine on increased Mg excretion showed to persist at least 2 h after caffeine intake after an overnight fast (Massey & Opryszek, 1990). Kynast-Gales *et al.* (1994) showed that the negative effect of caffeine on Mg excretion was not counterbalanced by later renal compensatory conservation in 18 healthy subjects receiving 3 mg caffeine/kg lean body mass caffeine at 7 and 10 a.m. on one day. Based on a study with 37 healthy women, Bergmann *et al.* (1990) suggested that the increased Mg urinary excretion is mainly due to decreased reabsorption by the kidney. Despite the increase in urinary excretion, the effect of caffeine on Mg balance is not clear. In a rat study by Yeh *et al.* (1986), subcutaneous injection of caffeine (2.5 and 10 mg/100g body weight/d over a 2 wk period) increased Mg excretion significantly, but not Mg balance. This was mainly due to increased Mg intestinal absorption, which was significantly higher in the group injected with 10 mg/100g body weight. However, especially coffee and cacao as methylxanthine containing beverages are relatively rich in Mg (Souci *et al.*, 1994), and thus it can be assumed that the negative effect on Mg excretion is, at least partly, counterbalanced by increased dietary Mg intake.

The effect of theobromine and theophylline, other methylxanthines present in coffee, cacao, and tea on Mg excretion has not been investigated, but both compounds have diuretic effects similar to caffeine (Mutschler, 1996).

While there is evidence that caffeine increases Mg excretion due to its diuretic effect, the effect of dietary caffeine intake on Mg balance in humans remains unclear.

1.6.3. Anions and proteins

A small number of studies suggested that increased intake of proteins and various anions can increase Mg urinary excretion.

Increased dietary protein intake has been reported to increase urinary excretion of calcium in rats (Whiting & Draper, 1981) and in man (Zemel *et al.*, 1981), which was attributed to decreased reabsorption by the kidney due to complexation by sulphate ions. Another, additional explanation was an increased calcium loss from the bone matrix, caused by acid amino acids such as sulphur amino acids (Kerstetter & Allen, 1994), even though this theory has been discussed controversial (Heaney, 2002).

As Mg is also bound to the bone matrix, especially to the surface of the apatite crystal (see chapter 1.2.1. Mg in the human body) it can be assumed that there is a similar effect on Mg. In fact, a number of studies, although not all, found increased Mg excretion due to higher dietary protein intake in humans (reviewed by Brink & Beynen, 1992). It was suggested that sulphate, excreted after increased dietary protein intake, and other anions such as chloride increase urinary Mg excretion. However, proteins have also been reported to stimulate Mg absorption (see chapter 1.5.4.1 Proteins) and whether proteins influence primarily Mg absorption or excretion is not clear.

In rat studies, supplementation with sulphate, bisulphate and chloride without proteins decreased retention of Mg in bone (Kaup & Greger, 1990; Greger *et al.*, 1991), the effect was correlated with decreased pH in urine. However, relative high doses, about 0.4 mol/kg diet were fed. Thus, it is speculative to predict a negative effect of proteins and anions on Mg excretion in humans.

1.7. Methods to study Mg absorption and metabolism

1.7.1. Overview

Mg bioavailability from a Mg supplement or from the diet cannot be measured directly because there is no target tissue for Mg. This is in contrast to iron, where the majority of the element is incorporated into a target tissue, i.e. erythrocytes, which can be used to monitor iron bioavailability (Van Dokkum *et al.*, 1996). As a consequence, absorption, excretion, or retention are monitored as indicators of Mg bioavailability.

Apparent absorption is probably the most widely used indicator for Mg bioavailability. There are a variety of methods to estimate Mg absorption. In vitro dialysability studies have been used as screening methods to compare the influence of potential absorption enhancers or inhibitors or different concentrations of these compounds on Mg dialysability (Walter *et al.*, 1998). For simulation of the large intestine, more complicated models including intestinal bacteria flora have been developed (Venema *et al.*, 2000). Furthermore, animal gut sacs or other segments from the rat intestine have been used to estimate Mg absorption in vitro. These in vitro methods are less time consuming than in vivo methods and relatively cheap. However, they are only a screening tool to predict the direction of a response, not the magnitude and cannot replace measurements of Mg absorption in vivo. Mg absorption is active and passive (see chapter 1.2.3.2. Mg absorption), many in vitro techniques cannot simulate parameters in the intact living organism. As a consequence, in vivo studies are usually essential. Animal studies are often done with rats as these studies are relatively cheap compared to human studies and more detailed and invasive protocols can be employed. However, there are numerous differences between the metabolism of rats and man regarding mineral absorption, for example the intestinal bacterial flora (Williams & Taylor, 1985). To investigate Mg absorption in humans, chemical balance studies and isotope studies have been used. The isotope studies use radioactive labels and, more recently, stable isotope labels and are based on the monitoring of isotopes in faeces, urine, or blood. Within

the following chapters, methods to estimate Mg absorption are reviewed, with a special focus on stable isotope methods.

1.7.2. In vitro methods

In vitro methods can only give limited information about Mg absorption in vivo because of the complex absorption mechanisms and dietary interactions.

In vitro methods are often used for simple screening purposes and are usually based on dialysis. Semipermeable membranes such as cellulose with defined molecular weight cut-offs are used to simulate passive diffusion through the intestinal cell membranes. Methods based on dialysis have been used frequently to compare iron and in vitro dialysability of different meals and food compounds (Miller *et al.*, 1981; Hurrell *et al.*, 1988), but also for predicting in vitro dialysability of calcium, zinc, manganese, and Mg (Walter *et al.*, 1998). For example, Walter *et al.* found that Mg dialysability increased significantly from a maize, soybean and corn starch meal containing 120 mg/100g Mg when citric acid was added at 1% to the meal. This difference was attributed to the increased solubility of Mg in these meals due to decreased pH and to ligand competition between phytic acid and citric acid. Good dialysability of Mg citrate and aspartate (82%, 85%, and 53%) as compared to the poorer soluble Mg oxide were also found by Hilleke *et al.* (1998).

However, studies based on dialysability have several limitations as active transport mechanisms are not considered. The in vitro methods therefore usually investigated only the influence of dietary components on the solubility of a mineral. If the mineral is insoluble it would not be expected to be absorbed. Any dietary influence on the active transport mechanism will not be evaluated. In addition, the bacterial flora in the large intestine cannot be simulated. Bacterial flora is thought to play a role in colonic absorption of Mg when indigestible soluble carbohydrates are fermented (see chapter 1.5.4.3.2. Soluble fibre). To study the effect of the intestinal bacterial flora in the large intestine, models including bacterial flora have been developed (reviewed by Venema *et al.*, 2000). To study active transport through the epithelial membrane in vitro, intestinal segments, e.g. rat gut sacs (Aldor & Moore, 1970;

Hardwick *et al.*, 1990a; Grimm *et al.*, 1992) are more adequate, however, bacterial microflora in these sacs is removed as well.

1.7.3. Chemical balance technique

Chemical balance studies have been performed to study apparent absorption of a variety of minerals and trace elements including iron (Wienk *et al.*, 1999), zinc (reviewed by Sandstead, 1985), copper (Pace *et al.* 2001), and calcium (review by Ernst *et al.*, 1995).

Mainly because of a lack of alternative techniques, a number of balance studies investigating Mg absorption (>30) have been carried out in humans (reviewed by Brink & Beynen, 1992). Chemical balance studies for Mg as well as the other minerals are based on measuring mineral intake and faecal and urinary excretion, usually over a period of several days or weeks. Endogenous faecal Mg losses and losses in sweat are usually not considered. For practical reasons (standardization of mineral intake), the subjects usually receive a controlled diet. Apparent absorption is finally calculated by subtracting the amount mineral excreted in the faeces from mineral intake.

Balance studies have a number of limitations (reviews by Ernst *et al.*, 1995; van Dokkum *et al.*, 1996b; and Mellon & Fairweather-Tait, 1997). A disadvantage is the strict diet which has to be followed for several days or even weeks. A short balance period may yield inaccurate absorption results because meals given during the balance period might mix with preceding meals in the intestine, an effect which might vary between subjects due to varying gastrointestinal passage time. As a consequence, mineral intake and mineral excretion in faeces cannot be strictly related. A method to decrease this risk is the administration of faecal markers such as food dyes, which are given before the test meals to indicate which stools should be pooled. Longer study periods (several weeks) on the other hand increase the risk of dietary adaptations and altering mineral metabolism, which is usually not wanted, especially when the dietary period should be repeated.

A major disadvantage of the chemical balance technique is that it is difficult to determine Mg absorption, and also mineral absorption in general from single meals or food compounds. This could, in theory, only be done indirectly by multifactorial regression which would require a large number of subjects. In addition, endogenous faecal Mg losses through bile, pancreas, etc. and thus true absorption cannot be determined because there is no possibility to distinguish between endogenous Mg and Mg from the diet. Furthermore, these studies are often inaccurate due to several potential errors, e.g. quantification of Mg intake, compliance of the subjects to the diet, complete collection of faeces. When 2 factors influencing Mg absorption should be compared, the study population either has to be divided into 2 groups which are then studied in parallel, or the balance period has to be repeated, which decreases statistical strength or increases the study time. For these reasons, balance studies are not the method of choice to study the influence of dietary compounds on Mg absorption.

1.7.4. Extrinsic and intrinsic isotopic labels

Isotopes of an element are atoms which differ only in their number of neutrons and thus behave chemically identically. When determining Mg absorption based on isotope techniques, the basic assumption is made that the added isotope label served with the meal (extrinsic tag) behaves identically to native dietary Mg, i.e. that it exchanges completely with the native Mg in the food or in the gastrointestinal tract (common pool theory), and is therefore absorbed identically to dietary Mg (Sandstrom *et al.*, 1993; Fairweather-Tait & Fox, 1996). This exchange of added label and dietary mineral depends both on the mineral and food matrix. Intrinsic labelling (incorporation of a label in the food matrix), e.g. by feeding an animal or a plant or injecting a label is much more time consuming and expensive than extrinsic labelling.

There is some evidence that, for a number of minerals, including Mg, for many, but not for all diets, the added label is absorbed similarly to a label which is incorporated in the matrix. No significant differences in absorption between extrinsic and intrinsic

labels was reported for calcium from wholemeal wheat bread (Weaver *et al.*, 1992) and from milk in adults (Martin *et al.*, 1989; Nickel *et al.*, 1996).

Similarly, labelling of a variety of foods such as grains, beans and eggs has been successfully done with extrinsic radioiron tags (Bjorn-Rasmussen *et al.*, 1972; Cook *et al.*, 1972; Bjorn-Rasmussen *et al.*, 1973; reviewed by Hallberg, 1974; reviewed by Hallberg, 1981; reviewed by Consaul & Lee, 1983).

Extrinsic labelling showed also to be a valid approach for other trace elements. Manganese absorption did not differ significantly in 6 young women fed extrinsically and intrinsically labelled chicken liver and mangold (Davidsson *et al.*, 1988; Davidsson *et al.*, 1991). Similar results were reported for copper absorption from goose breast meat, goose liver, and peanut and sunflower butter in 7 healthy adults (Johnson *et al.*, 1988).

However, extrinsic labels cannot always be used. It is known that heme iron and non heme iron do not mix (Bothwell *et al.*, 1989), thus, it is not possible to determine heme iron absorption by an extrinsic label. In addition, differences in absorption between the labelling techniques have been reported for insoluble iron such as iron phosphate and elemental iron (Consaul & Lee, 1983).

While similar results have been reported for intrinsic and extrinsic selenium labels (as $^{76}\text{SeO}_3^{2-}$) to determine absorption from poultry meat fed to 4 young men (Christensen *et al.*, 1983), and from egg white fed to 4 healthy subjects, absorption was different from egg yolk (Sirichakwal *et al.*, 1985). This effect was attributed to different behaviour of selenite and organically bound selenium (Moser-Veillon *et al.*, 1992).

A similar effect of proteins has been suggested for zinc absorption. While no difference in absorption between extrinsic and intrinsic zinc labels from a corn meal in rats (Evans & Johnson, 1977), from infant formula in 11 infants (Serfass *et al.*, 1989), from milk in 10 young women (Egan *et al.*, 1991), and from beef in 12 subjects (Gallaher *et al.*, 1988) was found, a significant difference in absorption (53 and 62%) was found between extrinsically and intrinsically labelled chicken meat fed to 10 healthy subjects (Janghorbani *et al.*, 1982). It was suggested that protein bound zinc such as in meat is more protected from other anti-nutritive complexing compounds or that absorption of the zinc-protein complex occurs at a different site.

Proteins might influence molybdenum absorption as well. Molybdenum in soy was less absorbable from an extrinsic label than intrinsic label, but absorption from kale differed not significantly in 11 young men (Turnlund *et al.*, 1999).

Besides protein complexes, other food compounds may bind to minerals. A significantly lower calcium absorption from intrinsically (2.9%) than from extrinsically (6.9%) labelled spinach was found in rats (Weaver *et al.*, 1987), suggesting incomplete exchange of added and native calcium bound to oxalates. Similar results were obtained by Weaver and Heaney (1991) in a study in 14 healthy adults, absorption of the intrinsic label from spinach was significantly lower compared to the extrinsic calcium label (2.9% versus 13%). This is in contrast to the hypothesis that calcium oxalate in spinach is soluble at low pH (2-3) as in the stomach (Weast, 1989), and complete exchange of added calcium with native calcium could be expected.

Only a few studies investigated extrinsic and intrinsic labelling techniques for Mg. Absorption of Mg was found to be similar from extrinsically and intrinsically labelled breast milk fed to very low birth weight infants (Liu *et al.*, 1989), and spinach fed to adults, suggesting at least 90% exchangeability between both labels (Schwartz *et al.*, 1984). Furthermore, similar distribution of added and native Mg in several infant food formulas in soluble, insoluble, and fat fractions was found by Lönnerdal *et al.* (1993) by using a ²⁸Mg label, suggesting the feasibility to use an extrinsic Mg label in these foods.

It is not known whether relatively strong Mg complexes such as phytate and oxalate do form a pool with free Mg. For phytate, it is assumed that at pH 2-3 (as in the stomach), phytate complexes are largely soluble, and mineral binding is weak (Nolan *et al.*, 1987; Champagne, 1988), allowing an exchange of minerals. In addition, the previous mentioned calcium studies in rats and humans indicated no difference in absorption from phytate containing bread between both labels, and the study by Schwartz *et al.* (1984) indicated no effect of oxalate on the way of labelling for Mg.

In summary, extrinsic labelling techniques are an accepted practical methodology for many, but not for all minerals in all matrices, and there is evidence that this is also a valid method to determine Mg absorption.

1.7.5. Studies using radioactive isotopes

Radioactive isotopes have been shown to be useful tools to study absorption of a number of minerals such as calcium (DeGrazia *et al.*, 1965), zinc (Arvidsson *et al.*, 1978), and iron (Cook *et al.*, 1972). Studies using radioactive ^{28}Mg in humans have been done since the end of the 50th, the earliest studies often investigated serum Mg and urinary Mg excretion in alcoholic subjects (Martin & Bauer, 1962; Mendelson *et al.*, 1965).

In principle, radioactive isotopes would be useful labels because they usually do not occur or only in negligible concentrations in nature. Thus, only very small tracer amounts would be needed to achieve a detectable signal during measurement. Radioactive tracers are therefore true tracers, reducing the risk that the tracer disturbs the metabolism, e.g. influences absorption by a high mineral load. In addition, studies with radioactive labels are relatively cheap because only very small isotope amounts are needed. Finally, detection of radioisotopes, usually by whole body counting or by scintillation counting of biological samples is relatively easy, making the method non invasive and avoids collecting urine or faecal material.

However, radioactive studies are associated with ethical problems, especially in children and women, and few laboratories are equipped with facilities to work with radioisotopes, in particular with whole body counters. Radioactive isotopes are not well suited to study absorption from several elements simultaneously as the X-ray spectra may overlap. In addition, Mg radioisotopes have relatively short half-lives (as can be seen in Table 9 below 21 h), limiting their use in absorption studies. Due to this, ^{28}Mg it has to be produced a short time before the beginning of a study and cannot be stored for a long time. Because activity of commercially available radioactive ^{28}Mg labels is often not very high (due to its fast decay), a relatively high dose (e.g. around 0.5-1.0 mmol Mg per dose of 5 μCi , Graham *et al.*, 1960, Aikawa *et al.*, 1958) is needed to obtain an active enough label for human studies, which is then not really longer a true tracer. Furthermore, true absorption cannot be determined, only net (apparent) absorption can be obtained, because parts of the absorbed tracer are re-excreted as endogenous faecal losses. To correct for these absorbed and re-excreted amount label, a second, parenterally administered

isotope label would be needed (see chapter 1.7.6.3. Faecal monitoring for estimating true absorption). Due to these problems, radioisotopes have been replaced by stable isotope methods.

Table 9. Mg radioactive isotopes and half-lives (Weast, 1989)

Mg isotope	half-life
^{20}Mg	0.1 s
^{21}Mg	122 ms
^{22}Mg	3.4 s
^{23}Mg	11.3 s
^{27}Mg	9.5 min
^{28}Mg	21.0 h
^{29}Mg	1.3 s
^{30}Mg	0.2 s
^{31}Mg	0.25 s

Table 10 gives an overview of studies using Mg radioisotopes administered to humans, studies based on simultaneous administration of radioisotopes and stable Mg isotopes are reviewed in the following chapter.

Table 10. Mg absorption studies in humans based on radioactive Mg isotopes

author(s)	studied subjects	administered dose Mg	determination of absorption	remark
Aikawa <i>et al.</i> (1958)	12 hospitalized adults	10-25 $\mu\text{Ci } ^{28}\text{Mg}$ a) single oral dose b) 2 doses at 1 wk interval c) 3 doses at weekly intervals	faecal, urine, and blood samples measured by scintillation counter	study designed to study absorption and excretion in urine and faeces
Silver <i>et al.</i> (1960)	10 adults with hypertension and arthritis	20-104 $\mu\text{Ci } ^{28}\text{Mg}$ (single dose) orally (2 patients) and iv. (9 patients)	faecal, urine, and blood samples measured by scintillation counter	study designed to study endogenous Mg losses and Mg kinetics

Graham <i>et al.</i> (1960)	13 adults with normal gastrointestinal functions	~ 5 $\mu\text{Ci } ^{28}\text{Mg}$ (single dose) orally	faecal, urine, and plasma samples measured by scintillation counter	study designed to study intestinal peak absorption
Aikawa <i>et al.</i> (1960)	-9 healthy subjects -16 patients with various diseases	single dose iv., no information about radioactive dose	faecal, urine, and plasma measured by scintillation counter	study designed to study Mg distribution in body
Wallach <i>et al.</i> (1966)	6 uremic patients 6 control patients	50-70 μCi (single dose) iv.	blood and urine samples	study designed to study Mg kinetics in the body
Avioli and Berman (Avioli & Berman, 1966)	15 healthy adults	150-175 μCi iv., 25 μCi orally (single doses)	plasma, faecal, and urine samples measured by a scintillation counter	study designed to study Mg distribution and kinetics in body
Choné <i>et al.</i> (1968)	11 healthy subjects	2-6 $\mu\text{Ci } ^{28}\text{Mg}$ (single dose) iv.	whole body counting	study designed to study Mg distribution in body
Kniffen <i>et al.</i> (1972)	13 adult subjects	1-10 $\mu\text{Ci } ^{28}\text{Mg}$, single dose iv.	whole body counting, urinary samples by scintillation counting	study designed to investigate Mg retention
Danielson <i>et al.</i> (1979)	16 healthy adults	4-5 $\mu\text{Ci } ^{28}\text{Mg}$ (single dose) orally	in the right forearm in a lead chamber using scintillation counting	study designed to study intestinal peak absorption
Watson <i>et al.</i> (1979)	5 adult subjects	40 $\mu\text{Ci } ^{28}\text{Mg}$ (single dose) iv.	whole body counting	study designed to study Mg distribution and kinetics in body
(Verhas <i>et al.</i> , 2002)	10 adult subjects	5 $\mu\text{Ci } ^{28}\text{Mg}$ orally 2 $\mu\text{Ci } ^{28}\text{Mg}$ iv.	whole body counting, activity in the forearm	study designed to determine Mg absorption and retention

1.7.6. Studies using stable isotopes

1.7.6.1. Overview

A more recent development for studying mineral absorption is using stable isotopes. Elements in nature are often a mixture of different isotopes. Some elements are

monoisotopic, e.g. arsenic, aluminium, beryllium, cobalt, fluorine, iodine, manganese, and phosphorus. Metabolism of these elements cannot be studied using stable isotope methods as at least 2 stable isotopes of an element are needed as these methods are based on the change of the isotopic composition after administration of a label. Worldwide, there are only a few suppliers offering stable isotopes, but isotope labels enriched about 99% or higher can be purchased for many elements.

Several techniques have been used to produce enriched isotope labels. Among these are separation by mass spectrometers (e.g. cyclotrons), which is the most common technique for enrichment of Mg isotopes, centrifugation by gas-centrifuges, fractional distillation, separation based on diffusion velocity (the latter 3 techniques are preferably used for enriching lighter elements or elements forming volatile compounds such as hydrides or fluorides), and chromatography methods (gas or ion separation chromatography) (reviewed by Falbe & Regitz, 1995).

Enriched stable isotope labels have been used to study absorption of a number of trace elements and minerals such as iron, zinc, calcium, copper, molybdenum and selenium (Sandstrom, 1996). One advantage of using stable isotopes is that their administration is not associated with ethical concerns as they do not radiate. Furthermore, stable isotopes can be studied over a relatively long period of time compared to radioisotopes if detection is precise enough.

Stable isotope studies include the measurement of isotope ratios by mass spectrometry, this is the quotient of the measured abundance based on the number of atoms of two isotopes in a sample, e.g. $^{24}\text{Mg}/^{25}\text{Mg}$. However, there are some disadvantages by using stable isotopes compared to radioisotopes. Their use is relatively expensive because in comparison to radioisotopes, relatively high amounts are needed (for example, 1 mg ^{25}Mg or ^{26}Mg costs about 5-15 US dollar, and 10-20 mg/person are typically needed).

The amount label needed to yield a detectable enrichment which can be distinguished from the element of natural isotopic abundance in biological samples depends on the natural isotopic abundance of the isotope (the lower the natural abundance, the less label is needed to achieve adequate enrichment), the amount element present in the body compartment to be analyzed, and the precision of the technique to measure isotopic ratios. Therefore, the isotope with the lowest

abundance of an element is most often used as a label. Isotopic enrichment e_i in % in a sample can be defined as:

$$e_i(\%) = (R_x^{a/b} - R_s^{a/b}) / R_s^{a/b} \cdot 100 \quad (3)$$

with $R_x^{a/b}$ and $R_s^{a/b}$ being the isotopic ratio of 2 isotopes of an element in the measured sample x and the isotope standard s of natural isotopic composition, respectively.

The higher the amount of the label needed, the higher the risk to influence normal metabolism. Stable isotopes are therefore usually not considered true tracers. For Mg, the natural abundance of all 3 isotopes is relatively high (>10%), thus, either high amounts of isotopes have to be administered, or the measurement technique (i.e. measuring isotopic ratios in a sample) must have high precision.

Another disadvantage compared to the use of radioisotopes is that sample preparation for measuring stable isotope ratios is often much more complicated and often requires time consuming, blank controlled sample preparation in order to avoid sample contamination with the element of natural isotopic composition. Quantification of the isotopic label is usually time consuming and require expensive equipment. In the following chapters, methods to determine Mg absorption by means of stable isotope techniques are reviewed. These techniques are based on oral and/or intravenous injection of isotope labels and monitoring of faeces, urine, or blood. The first stable Mg isotope techniques have been evaluated with radioactive ^{28}Mg by Currie et al. (1975) in rats and by Schwartz et al. (1978) in humans. An overview of studies using Mg stable isotopes is given in Table 11, with a focus on human studies.

Table 11. Studies in which Mg stable isotopes have been used

author(s)	studied subjects	administered dose Mg	determination of absorption	remark
Currie <i>et al.</i> (1975)	2 rats	2.25 mg ²⁶ Mg orally and 0.4 μCi ²⁸ Mg iv. (single doses)	faecal und urine monitoring	study designed to test the possibility using ²⁶ Mg to determine Mg absorption
			whole body counting and neutron activation analysis	
Schwartz <i>et al.</i> (1978)	4 healthy adults	a) 50 mg ²⁶ Mg and 30 μCi ²⁸ Mg orally b) 50 mg ²⁶ Mg orally and 20 μCi ²⁸ Mg iv. (single doses)	urinary and faecal monitoring, plasma samples	study designed to compare Mg absorption by different methods and to estimate true Mg absorption
			neutron activation analysis, scintillation counting	
Schwartz and Giesecke (1979)	10 healthy subjects	50 mg ²⁶ Mg and 30 μCi ²⁸ Mg both orally (single doses)	plasma-, urine-, and faecal samples	study designed to test the possibility using ²⁶ Mg to investigate Mg metabolism
			neutron activation analysis, MS of volatile tetramethylheptanedione and scintillation counting	
Schwartz <i>et al.</i> (1980)	54 rats	3.3 mg ²⁶ Mg and 0.5 μCi ²⁸ Mg orally (single doses)	stomach, intestine, faecal and plasma samples	study designed to determine exchangeability of intrinsic and extrinsic labels in vegetables
			MS of Mg tetramethylheptanedione and scintillation counter	
Schwartz <i>et al.</i> (1984)	8 healthy subjects	50 mg ²⁶ Mg and 30 μCi ²⁸ Mg, both orally (single doses)	faecal and urinary monitoring	study designed to compare absorption from extrinsic and intrinsic labelled in spinach
			MS as Mg tetramethylheptanedione and scintillation counting	
Liu <i>et al.</i> (1989)	9 very low birth infants	ca. 1.1 mg ²⁶ Mg and 0.4 mg ²⁵ Mg orally (single doses)	urinary and faecal monitoring	study designed to compare absorption of intrinsically and extrinsically labelled breast milk
			fast atom bombardment-mass spectrometry	

Cary <i>et al.</i> (1990)	1 healthy adult	20 mg ²⁶ Mg iv. and 50 mg ²⁵ Mg orally (single doses)	serum, urine, and faecal measurements	study designed to measure oral/iv ratios in serum and urine using ICP-MS
			ICP-MS	
Schuette <i>et al.</i> (1990a)	81 adult rats	5 mg intraperitoneal (single doses)	plasma, erythrocyte, urine, and bone Mg	study designed to study Mg distribution in the body
			ICP-MS	
Schuette <i>et al.</i> (1990b)	3 infants	a) 20 mg orally (single dose) b) 20 mg orally (during 24 h) c) 60 mg orally, during 24 h each ²⁵ Mg	faecal and urinary monitoring and one blood sample	study designed to investigate Mg absorption in infants
			ICP-MS	
Schuette <i>et al.</i> (1993)	10 adult subjects with Crohn's disease and enteritis	50 mg ²⁶ Mg orally as oxide or diglycinate (single doses)	faecal monitoring	study designed to study the use of a rare earth element (Dy) as faecal markers
			neutron activation analysis	
Stegmann and Karbach (1996)	1 adult man	24 mg ²⁵ Mg orally (single doses)	plasma samples	study designed to investigate plasma curves during 50 h
			TIMS	
Knudsen <i>et al.</i> (1996)	8 healthy adults	about 65 mg ²⁵ Mg orally (in 3 doses)	urinary- and faecal monitoring	study designed to study the effect of a fibre rich diet on mineral balances
			ICP-MS	
Sojka <i>et al.</i> (1997)	5 adolescent girls	20 mg ²⁵ Mg iv. and 40 mg ²⁶ Mg orally (single doses)	urinary and faecal monitoring, blood samples	study designed to study Mg kinetics in body
			TIMS	
Coudray <i>et al.</i> (1997b)	9 rats	5 mg ²⁶ Mg orally and 0.3 mg ²⁵ Mg iv. (single doses)	faecal and urinary monitoring, plasma samples, erythrocytes	study designed to compare different methods to determine Mg absorption
			ICP-MS	
Benech <i>et al.</i> (1998)	6 healthy adults	360 mg Mg enriched in ²⁶ Mg as lactate and citrate orally plus 50 mg ²⁵ Mg iv (single doses)	faecal and urinary monitoring, plasma samples,	study designed to evaluate bioavailability of Mg citrate and Mg lactate
			ICP-MS	

Abrams <i>et al.</i> (1999)	25 boys and girls	1 mg/kg ²⁵ Mg iv. and 0.5 mg/kg ²⁶ Mg orally (single doses)	faecal and urinary monitoring	study designed to investigate absorption and excretion of Mg
			TIMS	
Feillet-Coudray <i>et al.</i> (2000a)	30 rats	1.4 mg ²⁵ Mg iv. (single doses)	plasma samples	study designed to investigate Mg pools and kinetics
			ICP-MS	
Sabatier <i>et al.</i> (2001)	6 healthy adults	70 mg ²⁶ Mg orally and 30 mg ²⁵ Mg iv. (single doses)	faecal- and urinary monitoring, plasma samples	study designed to compare different methods to determine Mg absorption and kinetics
			ICP-MS	
Tahiri <i>et al.</i> (2001)	11 healthy adults	88 mg ²⁵ Mg orally (single doses)	faecal monitoring	study designed to investigate the effect of oligo-fructosaccharides on Mg absorption
			ICP-MS	
Sabatier <i>et al.</i> (2002)	10 healthy adults	30 mg ²⁵ Mg and 30 mg ²⁶ Mg orally (each label divided into 2 doses)	faecal and urinary monitoring	study designed to study Mg absorption from mineral water with and without meal
			TIMS, ICP-MS	
Feillet-Coudray <i>et al.</i> (2002)	10 healthy adults	40 mg ²⁵ Mg iv. (single dose)	plasma samples	study designed to investigate Mg kinetics
			ICP-MS	

1.7.6.2. Faecal monitoring to determine apparent Mg absorption

Stable isotope techniques based on faecal monitoring were developed based on principles of the chemical balance technique. FM has been applied to evaluate absorption of several minerals and trace elements in humans, e.g. calcium (Davidsson *et al.*, 1996), zinc (Serfass *et al.*, 1989; Fairweather-Tait *et al.*, 1992), copper (Knudsen *et al.*, 1996), molybdenum (Turnlund *et al.*, 1999), and magnesium (Table 11). The first study using FM with Mg stable isotope administration in humans was done by Schwartz *et al.* (1978). This study is, together with administration of stable isotopes of iron, copper, and zinc (King *et al.*, 1978) the first

report of FM technique in humans. A label is mixed with the meal which is then consumed by the subject, and faecal collections are carried out until all non absorbed stable isotopes are assumed to be excreted. The collected stool samples are then usually pooled, and aliquots are prepared for measuring isotopic ratios and total Mg to detect the amount label excreted. Similar to Mg balance studies, absorption is determined by the difference between the administered oral label and the amount non absorbed label found in the faeces. Because the administered Mg label can be distinguished from endogenous Mg, measured absorption is more accurate than Mg absorption based on chemical balance techniques.

A disadvantage of FM is the need for complete collections of faecal material over several consecutive days. Incomplete stool collections due to losses of faecal material or prolonged gastrointestinal passage time of the administered label results in overestimation of absorption (Rauscher & Fairweather-Tait, 1997; Shames *et al.*, 2001). To assure long enough collection time of non absorbed isotopes, nonabsorbable food colour dyes such as brilliant blue or carmine red (Liu *et al.*, 1989; Schuette *et al.*, 1990b) can be administered orally prior to the test meal and, in addition, after 5-8 days to mark the start and the end of the faecal collection period. However, quantitative faecal markers are needed additionally if completeness of faecal material should be controlled. Radio opaque markers (Tahiri *et al.*, 2001), radioactive chromium $^{51}\text{CrCl}_3$ (Gibson *et al.*, 1988; Davidsson *et al.*, 1989), polyethylene glycol (Schwartz *et al.*, 1984), or rare earth elements (REE) (Schuette *et al.*, 1993; Fairweather-Tait *et al.*, 1997; Sabatier, 2001) have therefore been added to the labelled test meals. As these compounds are assumed to be not absorbable, recovery in the faecal pools can be used to monitor completeness of the stool collections. A prerequisite for using such markers are sufficiently similar excretion patterns to the mineral studied, sufficient low absorbability, sufficient low presence in the food, and non toxicity. Absorbability and presence of these markers in food should be low enough not to influence absorption results significantly.

These quantitative faecal markers can, in theory, also be used to correct for incomplete faecal collection of non absorbed isotope label by the recovery of these markers in the stool pool (Eqn. 4) in order to shorten the stool collection period.

$$AA(\%) = 100 \cdot \left(1 - \frac{F_0}{D_0} \cdot \frac{100}{\%FM} \right) \quad (4)$$

%FM=per cent excreted faecal marker, e.g. rare earth element

However, differences in excretion patterns between radioopaque pellets, $^{51}\text{CrCl}_3$ and ^{28}Mg were found by Schwartz *et al.* (1981). These differences were suggested to be due to microbiological sequestration of chromium. $^{51}\text{CrCl}_3$ is not completely unabsorbable (Gibson *et al.*, 1988), and for elements with a low fractional absorption, correction does not result in accurate absorption values (Davidsson *et al.*, 1989). Radio opaque pellets have the advantage that different shapes can be administered and used to label different meals simultaneously (Cummings & Wiggins, 1976).

Recently, several REE have been evaluated in order to reduce the often cumbersome faecal collection period to the first few consecutive stools (Schuette *et al.*, 1993; Fairweather-Tait *et al.*, 1997). Fourteen elements belong to the REE or lanthanides; these elements have been used to determine the transit time of different foods in man (Hutcheson *et al.*, 1979) and rats (Crooker *et al.*, 1982). Absorption of REE was suggested to be below 0.05% (reviewed by Arvela, 1979). In addition, natural occurrence in food is relatively low, in the ng/g - $\mu\text{g/g}$ range (Meloni & Genova, 1987). REE have been shown to be non toxic after oral ingestion, even though high amounts (2 g/kg) ingested resulted in mucosal lesions in the gastrointestinal tract in rats (Arvela, 1979).

Schuette *et al.* (1993) investigated the possibility of using dysprosium as a nonabsorbable marker to reduce the faecal collection period to the first 2 consecutive stools to determine absorption of zinc, copper, and Mg. Good agreement in excretion patterns was found with the exception for copper. Fairweather-Tait *et al.* (1997) suggested that 4 d stool pools were sufficient to determine iron absorption when using the recovery of dysprosium, samarium, or ytterbium to correct for not absorbed, not excreted isotope label. However, the method of faecal markers may not be used to determine absorption based on incomplete stool collections if the time when faecal material was lost is not known. At the beginning, when the major part of the isotope label is excreted, small

differences in excretion patterns between the isotope label and the REE could result in significant differences in estimated absorption. For the same reasons, it is not advisable to reduce stool collections to a too small amount of collected faecal material collected during a too short period of time. Schuette *et al.* (1993) suggested that 40% recovery of the faecal marker should suffice to determine absorption of zinc and Mg.

However, this method offers the possibility to shorten the faecal collection periods, at least in studies of young subjects with regular excretion patterns (diarrhoea or constipation might disturb the excretion patterns). The method may not be suitable for studies in which the highest degree of accuracy and precision are necessary as additional sources of error are introduced when determining the quantity of the faecal marker (Schuette *et al.*, 1993).

1.7.6.3. Faecal monitoring for estimating true Mg absorption

Based on oral administration of one stable isotope, only apparent absorption can be determined as part of the tracer is absorbed and re-excreted during the faecal collection period (Fig. 2). As a result, absorption determined by this method underestimates true absorption. True absorption based on FM can be determined by different techniques. One method was reported by English *et al.* (1979), and presented in more detail by Krebs *et al.* (1995). This method is based on measuring cumulative excretion of an orally administered label in faeces and plotting the % excretion against time (Fig. 3). At the beginning when most of the non-absorbed isotopes are excreted, the slope of the curve is relatively steep. Later, the curve reaches a final slightly positive slope. It is assumed that this final slope is due to re-excretion of absorbed oral label. Thus, by extrapolating from the final slope to time zero, true absorption can be determined from the intercept with the y-axis. The disadvantage of this method is that it requires many measurements of faecal material, and extrapolation to time zero is imprecise. Estimates based on this correction have been reported to underestimate true absorption for zinc (Shames *et al.*, 2001). It has been suggested by the authors that this is because endogenous faecal secretions are not constant with time but decrease during time, probably in proportion to the plasma zinc concentration.

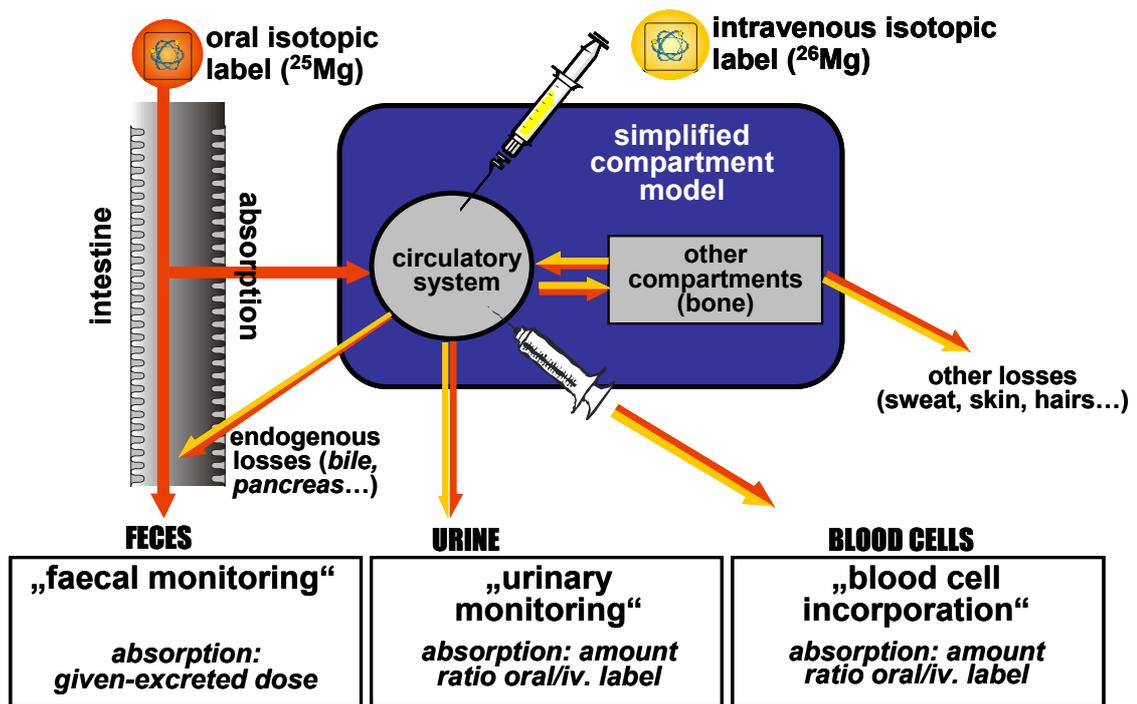


Fig. 2: Faecal excretion of stable isotopes after iv. administration of a ^{26}Mg label and oral administration of a ^{25}Mg label.

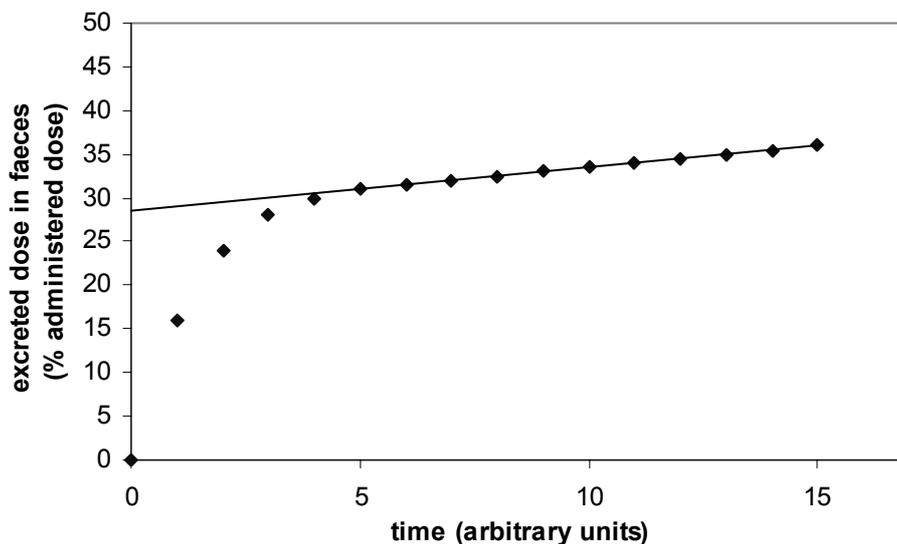


Fig. 3: Estimation of true absorption according to English et al. (1979), based on cumulative excretion of an oral isotope label. The line is assumed to represent endogenous faecal losses, extrapolated to time zero.

Another possibility to estimate true absorption based on FM is by iv. administration of a second stable isotope. This label must differ from the orally administered label in its isotopic composition. It is assumed that this iv. label is metabolized identically to the absorbed oral label and that the same fraction is excreted in the faeces. This is assumed to be the case after an initial period, the time it takes for the oral label to be absorbed. By measuring the fraction of the iv. label excreted in the faeces, as a measure for absorbed and re-excreted isotope label, true absorption can be estimated (Eqn. 5).

True absorption is calculated as apparent absorption (see eqn. 2, chapter 1.2.3.3. Measuring Mg apparent and true absorption) plus the amount absorbed and re-excreted oral label (E_0).

$$TA_{FM} = \left(\frac{D_0 - F_0 + E_0}{D_0} \right) \quad (5)$$

with TA_{FM} being true absorption based on FM, D_0 the amount oral tracer ingested, and F_0 the amount of the oral tracer in the collected stool. The fraction of the re-excreted iv. label (E_{iv}) in faeces can be described by:

$$E_{iv} = \left(\frac{F_{iv}}{D_{iv}} \right) \quad (6)$$

with D_{iv} , F_{iv} being the amount iv. label administered and excreted in faeces. As the fraction of the excreted dose of the iv. label and the absorbed oral label in faeces are assumed to be identical, the amount absorbed and re-excreted oral label can be described by:

$$E_0 = TA_{FM} \cdot D_0 \cdot \left(\frac{F_{iv}}{D_{iv}} \right) = D_0 \cdot \left(\frac{D_0 - F_0 + E_0}{D_0} \right) \cdot \left(\frac{F_{iv}}{D_{iv}} \right) \quad (7)$$

Rearranging yields:

$$E_0 = \frac{F_{iv} \cdot (D_0 - F_0)}{(D_{iv} - F_{iv})} \quad (8)$$

Substituting E_0 in eqn. (5) with the expression in eqn. (8) and rearranging yields:

$$TA_{FM} = \left(\frac{D_{iv} \cdot (D_0 - F_0)}{D_0 \cdot (D_{iv} - F_{iv})} \right) \quad (9)$$

However, this method has its limitations. First, the absorbed oral label reaches the circulatory system (blood) several hours after intake (reviewed by Hardwick *et al.* 1990). Thus, there is a delay in the peak content of the oral label in blood of several hours as compared to the iv. label. Secondly, the Mg concentration in plasma is strictly regulated by the kidney (Elin, 1994). Thus, part of the injected label might be excreted rapidly in the urine, especially if large bolus doses are administered. In addition, the iv. administered label is in a free ultrafiltrable form while serum Mg is about 55-60% in free form (see chapter 1.2.1 Body partition of Mg). This might result in different clearance patterns from plasma. As a result, a smaller fraction of the injected label would reach the intestine compared to the absorbed oral label, and the amount re-excreted isotope label would be underestimated.

Such a tendency was found when determining true zinc absorption by FM versus urinary monitoring in 4 healthy adults (Friel *et al.*, 1992). True absorption was corrected based on the method proposed by English, which has been suggested to underestimate true absorption (Shames *et al.*, 2001). The same tendency was found by (Sabatier, 2001) for estimates of true Mg absorption. However, other studies investigating zinc absorption reported higher true absorption based on FM compared to urinary monitoring (Friel *et al.*, 1992; Rauscher & Fairweather-Tait, 1997). This could, in theory, be explained by losses of faecal material. Nevertheless, determination of true absorption by the latter technique has been shown to be useful for zinc absorption (Shames *et al.*, 2001), calcium absorption (DeGrazia *et al.*, 1965) and Mg absorption (Sabatier, 2001). General disadvantages of the dual label method include the relatively high costs and the more invasive study design caused by iv. injection of the second label.

As endogenous faecal Mg secretion is in the order of 10-60 mg/d (see chapter 1.2.3.4. Mg excretion) which is only a small part of total body Mg, it could be assumed that true and apparent Mg absorption do not differ greatly. Several studies have investigated true Mg absorption based on FM. Recently, Sabatier *et al.* (2001) estimated true Mg absorption in 6 healthy adults to 48% as compared to 46% for apparent Mg absorption. Thus, the amount of absorbed re-excreted oral label in the faeces was very small in the 6-9 d faecal pools. Similar results were found by Sojka *et al.* (1997). Only 2% of the iv. administered label appeared in the faeces within 300 h after administration of 20 mg ^{25}Mg , resulting in a difference of about 1%

(absolute) between apparent- and true Mg absorption. Abrams and Wen (1999) reported an isotope enrichment of <0.5% ($^{25}\text{Mg}/^{24}\text{Mg}$ ratio) of the iv. label in a 7 d stool collection in 25 boys and girls after administration of 1 mg/kg ^{25}Mg .

1.7.6.4. Urinary monitoring

A different approach to estimate true Mg absorption is by urinary monitoring (UM). This technique is based on the administration of an oral and a second, iv. injected isotope label. This technique assumes that the relative concentration of labels in the urine reflects the relative concentration of labels in plasma. This technique has been developed to determine calcium absorption with radioactive (Garner *et al.*, 1960; Bronner & Abrams, 1998) and stable isotopes (Smith *et al.*, 1985; Yergey *et al.*, 1987). The relatively high urinary concentration of Mg, as well as similarities between Mg and calcium metabolism, indicate that UM could be a potentially useful approach to measure Mg absorption. The advantage of this technique is that absorption can, in principle, be determined from spot urine samples, and that urinary losses are not important as absorption is determined based on the ratio of the amount labels in the sample (Eqn. 10).

$$TA_{UM} = \frac{n_o}{n_{iv}} \cdot \frac{D_{iv}}{D_o} \quad (10)$$

TA_{UM} = true absorption determined by urinary monitoring

n_o, n_{iv} = amount labels in the urinary sample (in μmol)

Schwartz *et al.* (1978) were the first investigators to determine Mg absorption in humans based on UM. Four healthy subjects received 50 mg ^{26}Mg orally followed by 20 μCi ^{28}Mg iv. 3 hours later. Faecal samples collected for days 1-3 or 1-4 and urine samples for 2-24 h (spot samples collected at 4 h intervals and one 12 h interval) after dosing were sufficiently enriched to be analyzed for isotopic composition by neutron activation analysis. Absorption values based on UM tended to be lower as compared to FM but were reported to be too imprecise to be a useful method to determine Mg absorption due to low measurement precision of the isotopic ratios.

Calcium absorption is frequently measured by UM using either 24 h urine pools or a spot urine sample, collected 24 h after stable isotope administration (Lee *et al.*, 1994; van Dokkum *et al.*, 1996a), and this technique has also been proposed to determine zinc absorption (Friel *et al.*, 1992; Friel *et al.*, 1996). This technique is based on the assumption that both isotope labels are metabolized identically once the oral label has been absorbed. However, the accuracy of this method has been debated, especially for zinc (King *et al.*, 1997; Rauscher & Fairweather-Tait, 1997; Shames *et al.*, 2001). Rauscher & Fairweather-Tait (1997) reported significantly lower zinc absorption by humans based on complete 5 day urinary pools (days 1-5) versus complete 5 day faecal pools (days 1-5), and recently Sabatier *et al.* (2001) reported significantly lower Mg absorption based on 3 day urine pools (days 1-3 after isotope label administration) compared to 12 (days 1-12) day faecal pools. It has been suggested that absorption is underestimated based on urine collected during the first hours after isotope administration as the oral and the intravenous labels are metabolised differently and intestinal absorption of the oral isotope has not been completed (Yergey *et al.*, 1987; Shames *et al.*, 2001). This would result in a falsely low amount of the oral label in urine and would thus underestimate absorption.

Another potential concern of the UM technique is the administration of the intravenous magnesium label per se. An iv. bolus might result in a non-physiologically high level of free ultrafiltrable magnesium in the blood, resulting in rapid urinary excretion. Thus, the amount ratio of oral and injected isotope label in urine will underestimate absorption. To overcome this problem, Coudray *et al.* (1997b) suggested that the intravenous dose should be infused over a long period of time or divided into several smaller doses. Due to these problems, it was suggested that Mg absorption based on UM should be determined only after absorption of the oral label is complete, which is assumed to be after about 24 h. This time frame is based on reports indicating that Mg absorption in adults is 80% complete after 6 h (reviewed by Hardwick *et al.*, 1990). In a study by Abrams *et al.* (1999) however, absorption was only 64% after 8 h, 80% after 24 h, and 95% after 72 h in 10-15 y old boys and girls. These data indicate that there might be differences in Mg absorption depending on age or other study conditions such as test meal or measurement technique for absorption. In addition, the results by

Abrams were based on urinary excretion of the oral and the iv. label, in contrast to earlier methods which were based on the recovery of ^{28}Mg in plasma (Graham *et al.*, 1960) or in the forearm (Danielson *et al.*, 1979). Calculating mineral absorption from urinary samples later than 24 h hours has been done for calcium (Yergey *et al.*, 1987; Yergey *et al.*, 1994), zinc (King *et al.*, 1997), and Mg (Sabatier, 2001). It should be noted however that in absorption studies with calcium, the optimum time point for urinary collection differs between individuals (Yergey *et al.*, 1987) and also depends on the test meal composition (Griessen *et al.*, 1985).

A disadvantage of UM is that isotopic enrichment in urinary samples is usually lower than in faecal samples and urine samples require higher measurement precision. In another study by Schwartz *et al.* (1984), it was not possible to estimate Mg absorption based on the UM technique due to low measurement precision.

In conclusion, determination of absorption based on UM cannot be accurately done within the first hours after isotope administration, as metabolism of the iv. and the oral label in this early phase is not identical. These differences are assumed to be negligible after about 24 h, allowing to determine Mg absorption and to yield similar results as compared to FM.

1.7.6.5. Tissue retention

Besides determination of absorption based on the amount label in urine, absorption can, in principle, be determined from tissues, if the tissue is accessible and enrichment of the isotope labels is high enough. This technique is referred to as tissue retention technique (reviewed by Sandstrom, 1996). A major advantage of using blood cells is that they are easily accessible and, without the need for complete faecal or urine collections, blood cell incorporation of isotope labels could facilitate the determination of magnesium absorption. However, no human study has been reported until now evaluating the possibility of using blood cell incorporation based on a dual stable isotope label technique to determine Mg absorption. This technique has, so far, in humans only been used to determine iron absorption by erythrocyte incorporation of an orally administered stable or radioiron label,

assuming a mean erythrocyte incorporation of 80% (Cook *et al.*, 1972) or 90% (Walczyk *et al.*, 1997) of absorbed iron after 14 days.

In addition to radioisotopes, stable isotope techniques have been developed to measure iron absorption (Kastenmayer *et al.*, 1994; Walczyk, 1997). To determine mineral absorption by this method based on isotope dilution principles, besides isotope ratios, the total amount of a mineral in the analyzed tissue has to be determined. If estimation of the total amount mineral in the erythrocytes is not possible, a second isotope label has to be administered intravenously. For determination of iron absorption, the amount stable iron label bound to hemoglobin in the erythrocytes can be calculated based on estimates of blood volume and measurements of hemoglobin, and the incorporation factor of the iron into the erythrocytes. As the incorporation factor is not known for Mg, this method is not feasible for the determination of Mg absorption, and thus a second iv. label has to be administered. Similar to UM, it is assumed that, after absorption, the oral isotope label is metabolised in an identical way to the injected isotope label.

Although erythrocytes are not a specific target tissue for magnesium, the magnesium concentration is relatively high as compared to plasma concentration, about 2.5 mmol/L as compared to Mg in plasma (about 0.88 ± 0.05 mmol/L) (Durlach, 1988). For Mg absorption, only one rat study has been reported based on red blood cell analysis. No significant difference in absorption based on erythrocytes, plasma (both collected 48 h) or 36-48 h (urine pools) after oral and intravenous isotope administration was found (Coudray *et al.*, 1997b).

Schuetz *et al.* (1990b) determined Mg isotopic enrichment in erythrocytes 24 h after an oral dose of 20 mg and 60 mg ^{25}Mg to 3 full term infants and found enrichment of 4.2, 3.8, 2.2 and 10.1, 7.9 and 7.7%, indicating that enrichment is high enough to be measured with TIMS or ICP-MS methods. However, these doses were relatively high and blood volume of infants could be expected to be much lower as compared to adults.

1.7.6.6. Plasma deconvolution method

As for UM, a dual stable isotope approach with an oral and an iv. label can be used to measure Mg absorption by the deconvolution method based on plasma samples. The enrichment of the isotope labels in plasma are plotted against time, and the area under the curve is determined (Hart & Spencer, 1967; Gibaldi & Perrier, 1982). Fractional absorption is calculated by the following equation:

$$TA_{Dec} = AUC_o/AUC_{iv} \cdot D_{iv}/D_o \quad (11)$$

with TA_{Dec} being true absorption as determined by deconvolution and AUC_o , AUC_{iv} being the area under the (measured enrichment) curves of the oral and the iv. label. Deconvolution has been used to determine absorption of certain minerals, e.g. iron (Whittaker *et al.*, 1991), calcium (Roth & Werner, 1985; Yergey *et al.*, 1994), zinc (Shames *et al.*, 2001), and Mg (Schwartz *et al.*, 1978; Benech *et al.*, 1998; Sabatier, 2001).

This method was derived from pharmacological investigations of orally administered compounds based on measurements of the amount label at specific time intervals, e.g. every 2 or 6 h, starting from time zero. Because the plasma concentration of an absorbed mineral depends both on absorption and plasma clearance rate, absorption in % cannot be determined based on a single oral label alone. However, by using 2 oral stable isotope labels given on separate occasions, plasma curves of the concentrations of the 2 labels can be compared, and qualitative (relative) results about absorption from 2 different labels given e.g. together with different meals can be obtained.

The dual stable isotope approach based on simultaneous administration of an oral and iv. label can be used if plasma enrichment is high enough to be measured over a certain period of time, e.g. for 7 d (Sabatier *et al.* 2001), and extrapolation of the plasma curve to $t=\infty$ can be made. Plasma concentrations of administered Mg labels do not decrease linearly. The decline in the plasma curve of the Mg label is faster directly after dosing than later, about 10-20 hours after administration of an oral Mg dose, when plasma concentrations decline more linearly, (Cary *et al.*, 1990; Stegmann & Karbach, 1993; Sojka *et al.*, 1997). Similar behaviour has been

observed after iv. administration of a Mg label (Aikawa *et al.*, 1960; Avioli & Berman, 1966; Feillet-Coudray *et al.*, 2002).

This technique however has the same limitations as the UM technique as it assumes that the iv. bolus dose does not disturb normal metabolism and urinary excretion rate of Mg. As the oral dose requires a longer time before appearance in the plasma, low Mg absorption is falsely interpreted if AUC is based on the first hours after administration alone. Shames *et al.* (2001) concluded that the deconvolution method underestimates true absorption of zinc and reaches asymptotically the true absorption value after a time period of about 3 d.

Schwartz *et al.* (Schwartz *et al.*, 1978) compared absorption of 50 mg ²⁶Mg orally administered to 4 healthy adults by several techniques. The deconvolution method, based on simultaneous administration of ²⁸Mg resulted in lower absorption values compared to UM. However, this study had several limitations in comparing different absorption techniques. UM was based on spot samples collected 2-24 h after isotope dosing, and the methods were not based on the same time period and test meal. Sabatier *et al.* (2001) determined AUC based on time zero to 7d measurements extrapolated to infinity by using a computer software (SAAM II), and found no significant difference in true Mg absorption compared to UM (complete day 3-5 pool) and FM (complete 12 day pool). Similarly, no significant differences between Mg absorption based on plasma curves from 0-120 h and UM based on a 120 h (0-120 h) pool was found by Benech *et al.* (1998). However, as urine is assumed to reflect plasma Mg concentrations, it is not surprising that absorption determined by these 2 techniques did not result in significant differences.

Calcium absorption based on plasma deconvolution has been discussed controversial. Yergey *et al.* (1994) compared absorption of calcium based on deconvolution (from time zero to infinity) and UM based on either a spot urine sample (collected after 24 h) or a 24 h urine collection (0-24 h). Good agreement between the deconvolution method and 24 h urine pools, but not to the spot urine sample, was found, despite the reports suggesting that urine pools including the first 24 h may underestimate absorption of calcium (Yergey *et al.* 1987, Smith *et al.* 1996). Both 24 urine pools and deconvolution analysis resulted in non equivalent lower absorption values (as determined by a Bland and Altman plot) compared to the spot urine sample, indicating that both UM and deconvolution analysis

underestimate true absorption due to inclusion of the first 24 h after isotope administration. Similarly, no significant difference for calcium absorption was found based on 24 h (0-24 h) urine and AUC (0-30 h, Griessen *et al.* 1985) in 19 healthy adults.

From the present data it can be concluded that deconvolution analysis could be a useful tool to determine Mg absorption if the calculation of absorption is based on long enough periods, i.e. longer than the first 24 h, and extrapolated to infinity. However, only few studies have investigated this method and compared to other techniques, and the data from the calcium studies suggest that inclusion of the first hours, similarly as for the UM, might lead to an underestimation in the absorption results.

In conclusion, different techniques have been used to estimate absorption of Mg, all techniques having disadvantages and limitations. There is no consensus about the choice of technique. The choice of method depends, to a large degree, on practical considerations such as the cost of the study, study population, number of subjects available, study design, and the precision of the measurement technique available to determine isotope ratios. In practice, FM, UM, and deconvolution have been shown to result in comparable results for determination of Mg absorption.

1.7.7. Kinetic modelling

Absorption and metabolism in general can also be studied by kinetic modelling after administration of oral and or intravenous isotopes. This technique can give information about the abundance of Mg in different tissues and the extent to which a mineral is used for its specific functions after absorption. Usually, at least one tracer is administered and the distribution is monitored over a certain period of time by e.g. collecting urine, stool, or blood samples. By kinetic modelling, a simplified model is developed to obtain information about the main metabolic pathways and target tissues. To simplify the calculations, the human body or the animal studied is

divided into several compartments which are theoretical, not necessarily biological units with similar Mg exchange rates with the surrounding medium.

Estimates of Mg compartments have usually been based on radioactive or, more recently, stable isotope techniques, similar results were found. Avioli and Berman (1966) suggested a compartmental model with 3 exchangeable and a fourth, slow- or non exchangeable body compartment, using radioactive ^{28}Mg as a tracer in adults. Compartments 1 and 2 were reported to represent extracellular Mg (~17 mmol), compartment 3 (containing 80% of total exchangeable Mg, 115 mmol) was suggested to be intracellular Mg. The majority of total body Mg (>85%) was found in the fourth compartment (Mg half life >1000 h), assumed to be bone- and muscle Mg. A compartment with similar half-life (60-180 d) was found by Watson *et al.* (1979). Compartments 1, 2, and 3 had a complete turnover of 1.6, 6 and 48 h, and contributed to only 15% of total body Mg. Similarly, Sojka *et al.* (1997) reported a size of 4.8 g Mg (~20 % of total body Mg) for the exchangeable Mg compartments. Feillet-Coudray *et al.* (2002) also differentiated between 3 exchangeable (2 extracellular, 1 intracellular) compartments with 8, 4, and 101 mmol Mg, with 2, 19, and 266 h half-life in man, respectively.

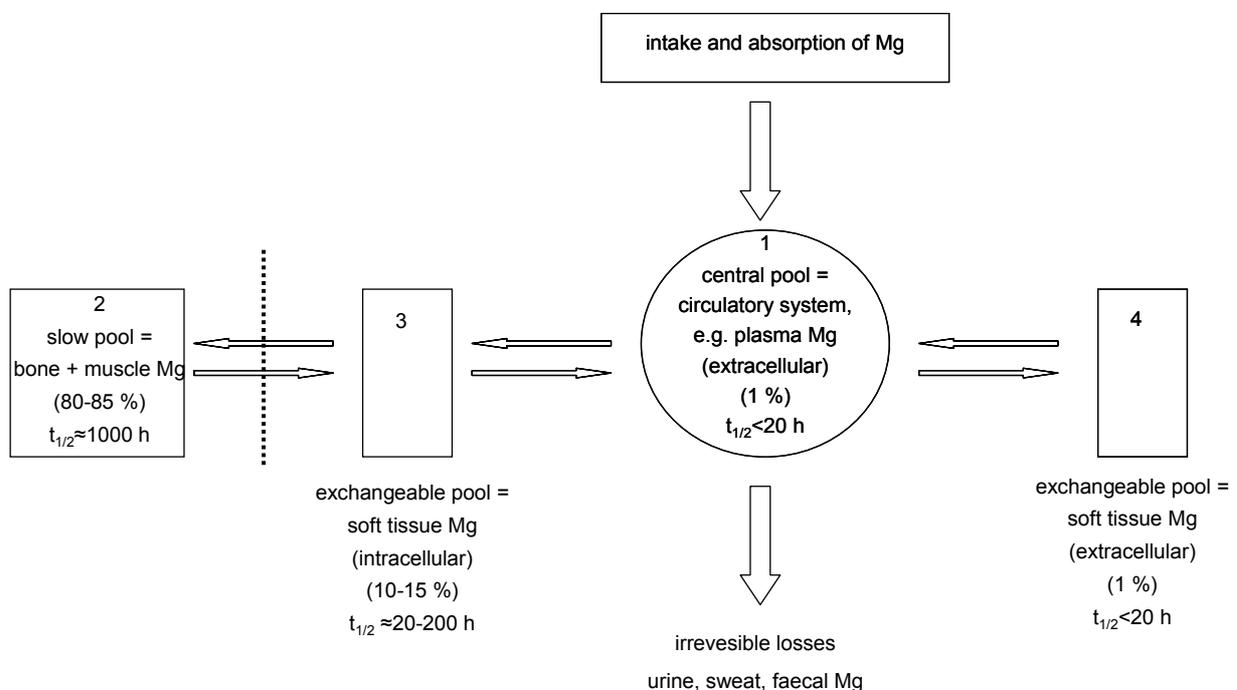


Fig. 4: Mg kinetic modelling – an example for a simple Mg compartment model, according to the literature cited in the text.

1.7.8. Methods to determine Mg status

To estimate whether individuals are well nourished with Mg, Mg status has to be assessed. As there is no general accepted method how to determine Mg status, there is a need to evaluate techniques to study Mg status.

Different methods, based on measuring Mg concentrations in different tissues, have been used to describe Mg status in humans. However, there is no general agreement which method is the most suitable. Plasma Mg concentrations are assumed to be a relatively poor indicator of Mg status, as plasma concentrations are strictly regulated by the kidney (Elin, 1994). It should be remembered that Mg in plasma represents only a very small fraction (about 1%) of body Mg. Furthermore, no correlation between plasma Mg concentration and other tissues have been reported, with exception of bone and interstitial fluid (Elin, 1987; Elin, 1991b).

As Mg is a typical intracellular ion, Mg concentration in cells have been analyzed as a measure of Mg status. However, no correlation was found between Mg concentrations in erythrocytes and certain other tissues. Mg in mononuclear blood cells was significantly correlated with muscle Mg in some, but not all studies while no correlation was found between erythrocytes and plasma. Muscle Mg represents about 43% of total fat free mass and contains about 27% of body Mg and is therefore assumed to be an appropriate tissue for assessment of Mg status (Elin, 1991b). However, because of the invasive method of sampling, it is not frequently used and has not yet been validated.

Determination of free Mg in soft tissues has been discussed as another alternative, as it is the physiologically active Mg form. However, the current methods, measuring free intracellular Mg with ion-selective electrodes, metallochrome dyes, or nuclear magnetic resonance spectroscopy have analytical limitations, especially regarding precision. While Mg selective microelectrodes reported low precision in measuring intracellular Mg concentrations, metallochrome dyes may also react with other minerals and have been reported to be inaccurate, and the ^{25}Mg signal is too insensitive to be measured by nuclear magnetic resonance spectroscopy (NMR)(Elin, 1991b).

Mg load tests, based on Mg urinary excretion or retention may be an alternative to measuring Mg concentration in different tissues. In these tests, an oral or intravenous Mg dose is given, and the amount Mg excreted in urine or Mg retention (Ryzen *et al.*, 1985; Holm *et al.*, 1987; Goto *et al.*, 1990) or the disappearance in plasma (Nicar & Pak, 1982) is measured. This technique is based on the assumption that low Mg status is associated with decreased urinary excretion of Mg and therefore increased retention. This assumption however would only be true if urinary losses are not the cause of low Mg retention, an observation which has been made for diabetic patients due to high Mg losses in the urine via osmotic diuresis (reviewed by Durlach, 1988 and Garland, 1992).

Alternatively to Mg loading, a stable or radioactive label can be administered, and blood samples can be taken at timed intervals to estimate Mg body compartment sizes by kinetic modelling. This technique has been used to estimate Mg status in alcoholic patients (Mendelson *et al.*, 1965; Wallach & Dimich, 1969). In a rat study (Feillet-Coudray *et al.*, 2000a), ^{25}Mg was used to determine the mass of exchangeable Mg pools, which showed to be positively correlated to measurements of Mg in plasma, but not erythrocyte and tibia. In a human study however (Feillet-Coudray *et al.*, 2000b), 8 wk supplementation with 366 mg/d Mg failed to result in a significant change in the mass of exchangeable body Mg, but did increase plasma ionized Mg and urinary Mg significantly. It was suggested that full Mg stores at the beginning of the study could have been the cause.

1.8. Use of stable isotope labels to determine Mg absorption

1.8.1. Introduction

To determine the absorption of a mineral or trace element by the human body accurately, the respective element in the test meal has to be labelled isotopically. The absorption and/or utilization of the element in the test meal is determined after test meal administration by the appearance of the label either in urine, faeces, or in

body tissues such as erythrocytes. When using radioisotopes for isotopic labelling, the amount of tracer can be determined directly via the radiation of the isotope. When stable isotopes are used, quantification can only be performed indirectly. The stable isotopes in the label cannot be distinguished from the stable isotopes in the sample as they are physically and chemically identical. Instead, the amount of isotopic label in a sample has to be quantified by the induced changes in the isotopic abundances and, thus, the isotope ratios in the sample. Isotopic abundances are highly constant in nature while the label is highly enriched in one of the isotopes. The more label is present in the sample, the stronger the isotopic abundances are altered.

The underlying mathematical concept is known as the ‘isotope dilution’ principle. The same principle is used in isotope dilution mass spectrometry for quantification of an element at highest accuracy and precision. The number of stable isotope labels in isotope dilution that can be used in parallel are determined by the number of stable isotopes that exist for a given element. For an element having n stable isotopes, $(n-1)$ isotopic labels are available. Accordingly, isotope dilution concepts are not applicable to monoisotopic elements, i.e. elements for which only one stable isotopes exists in nature such as phosphorus and manganese. For magnesium with its three stable isotopes ^{24}Mg , ^{25}Mg , and ^{26}Mg , two isotopic labels can be used in parallel.

Calculations can be based either on isotope ratios (De Bièvre & Peiser, 1997) or isotopic abundances (Kastenmayer *et al.*, 1994; Walczyk, 1997; Sabatier, 2001). However, the isotopic abundances of an element in a sample and thus its isotopic composition cannot be determined directly by using mass spectrometric techniques. They have to be calculated from the measured isotope ratios R which are defined as the ratio of the amount n (in moles) or the ratio of the isotopic abundances $^x a$ of the two isotopes, with x being the mass number of the isotope.

Accordingly, the $^{25}\text{Mg}/^{24}\text{Mg}$ ($^{25/24}R$) and $^{26}\text{Mg}/^{24}\text{Mg}$ ($^{26/24}R$) isotope ratios in the sample are defined as:

$$^{25/24}R = \frac{^{25}a}{^{24}a} = \frac{^{25}n}{^{24}n} \quad (12)$$

$$^{26/24}R = \frac{^{26}a}{^{24}a} = \frac{^{26}n}{^{24}n} \quad (13)$$

The abundances of all stable isotopes sum up to 1 when expressed as fractions or to 100% when expressed as percentages:

$$1 = ({}^{24}a + {}^{25}a + {}^{26}a) \quad (14)$$

Replacing the Mg isotopic abundances ${}^{25}a$ and ${}^{26}a$ in eqn. (14) with the measured isotope ratios ${}^{25/24}R$ and ${}^{26/24}R$ (see eqns. 12 and 13) yields:

$$1 = ({}^{24}a + {}^{24}a \cdot {}^{25/24}R + {}^{24}a \cdot {}^{26/24}R) \quad (15)$$

or, when rewritten:

$$1 = {}^{24}a \cdot (1 + {}^{25/24}R + {}^{26/24}R) \quad (16)$$

By solving eqn. (16) for ${}^{24}a$, the isotopic abundance of ${}^{24}\text{Mg}$ in the sample can be calculated from the measured isotope ratios:

$${}^{24}a = \frac{1}{1 + {}^{25/24}R + {}^{26/24}R} \quad (17)$$

With the calculated ${}^{24}\text{Mg}$ isotopic abundance in the sample, the isotopic abundances of ${}^{25}\text{Mg}$ and ${}^{26}\text{Mg}$ can be obtained using eqn. (12) and (13).

$${}^{25}a = {}^{24}a \cdot {}^{25/24}R \quad (18)$$

$${}^{26}a = {}^{24}a \cdot {}^{26/24}R \quad (19)$$

1.8.2. Quantification of Mg isotopic labels in a sample based on isotopic ratios

1.8.2.1. Single isotope techniques

To calculate the amount of Mg in a sample coming from a ${}^{26}\text{Mg}$ isotopic label, the isotope ratio ${}^{26/24}R_{iso}$ of the pure isotope label, the natural magnesium isotope ratio ${}^{26/24}R_{nat}$ and the isotopic ratio in the sample ${}^{26/24}R_{sample}$ containing the isotopic label have to be measured.

$${}^{26/24}R_{iso} = \frac{{}^{26}n_{iso}}{{}^{24}n_{iso}} \quad (20)$$

$${}^{26/24}R_{sample} = \frac{{}^{26}n_{sample}}{{}^{24}n_{sample}} \quad (21)$$

$${}^{26/24}R_{nat} = \frac{{}^{26}n_{nat}}{{}^{24}n_{nat}} \quad (22)$$

with ${}^x n_{iso}$, ${}^x n_{sample}$, and ${}^x n_{nat}$ being the amount of the isotope x coming from the label, the sample, and natural Mg.

The ratio ${}^{26/24}R$ in the sample containing the isotopic label can be expressed as the sum of the isotope amount (in moles) coming from the isotopic label and natural Mg:

$${}^{26/24}R_{sample} = \frac{{}^{26}n_{nat} + {}^{26}n_{iso}}{{}^{24}n_{nat} + {}^{24}n_{iso}} \quad (23)$$

The amount of ${}^{26}\text{Mg}$ in eqn. (23) can be expressed using eqns. (20) and (22):

$${}^{26/24}R_{sample} = \frac{{}^{26/24}R_{nat} \cdot {}^{24}n_{nat} + {}^{26/24}R_{iso} \cdot {}^{24}n_{iso}}{{}^{24}n_{nat} + {}^{24}n_{iso}} \quad (24)$$

When multiplied with the denominator, eqn. (24) can be rearranged to obtain the amount ratio of isotope label and natural magnesium $\frac{{}^{24}n_{nat}}{{}^{24}n_{iso}}$ in the sample :

$$\frac{{}^{24}n_{nat}}{{}^{24}n_{iso}} = \frac{{}^{26/24}R_{iso} - {}^{26/24}R_{sample}}{{}^{26/24}R_{sample} - {}^{26/24}R_{nat}} \quad (25)$$

The unknown amount of ${}^{24}\text{Mg}$ isotope in the sample coming from the isotopic label and natural magnesium, respectively, can be expressed by eqns. (26) and (27):

$${}^{24}n_{nat} = {}^{24}a_{nat} \cdot n_{nat} = \frac{n_{nat}}{1 + {}^{25/24}R_{nat} + {}^{26/24}R_{nat}} \quad (26)$$

$${}^{24}n_{iso} = {}^{24}a_{iso} \cdot n_{iso} = \frac{n_{iso}}{1 + {}^{25/24}R_{iso} + {}^{26/24}R_{iso}} \quad (27)$$

with n_{iso} and n_{nat} being the total Mg amount coming from the isotopic label and natural Mg, respectively. Combining eqns. (25-27) yields the amount of natural magnesium in the sample.

$$n_{nat} = n_{iso} \cdot \frac{{}^{26/24}R_{iso} - {}^{26/24}R_{sample}}{{}^{26/24}R_{sample} - {}^{26/24}R_{nat}} \cdot \frac{1 + {}^{25/24}R_{nat} + {}^{26/24}R_{nat}}{1 + {}^{25/24}R_{iso} + {}^{26/24}R_{iso}} \quad (28)$$

The total amount Mg in the sample n_{sample} is the sum of the Mg amount coming from the isotopic label n_{iso} and natural Mg n_{nat} .

$$n_{sample} = n_{nat} + n_{iso} \quad (29)$$

Eqns. (28) and (29) can be combined and yield:

$$n_{sample} = n_{iso} \cdot \frac{{}^{26/24}R_{iso} - {}^{26/24}R_{sample}}{{}^{26/24}R_{sample} - {}^{26/24}R_{nat}} \cdot \frac{1 + {}^{25/24}R_{nat} + {}^{26/24}R_{nat}}{1 + {}^{25/24}R_{iso} + {}^{26/24}R_{iso}} + n_{iso} \quad (30)$$

By rearranging eqn. (30), the amount of isotopic label in the sample n_{iso} can be obtained:

$$n_{iso} = n_{sample} \cdot \left(1 + \frac{{}^{26/24}R_{iso} - {}^{26/24}R_{sample}}{{}^{26/24}R_{sample} - {}^{26/24}R_{nat}} \cdot \frac{1 + {}^{25/24}R_{nat} + {}^{26/24}R_{nat}}{1 + {}^{25/24}R_{iso} + {}^{26/24}R_{iso}} \right)^{-1} \quad (31)$$

To determine the total amount Mg in the sample n_{sample} , a number of techniques can be used such as atomic absorption spectroscopy, ICP-MS or isotope dilution mass spectrometry.

1.8.2.2. Double isotope techniques

When two isotope labels have been used in parallel, the amount of both labels in the sample can be calculated following the same principles as described for single isotope techniques. In a sample containing two isotopic labels ($n_{iso,1}$ $n_{iso,2}$), the isotope ratios are defined as:

$${}^{26/24}R_{iso,1} = \frac{{}^{26}n_{iso,1}}{{}^{24}n_{iso,1}} \quad (32)$$

$${}^{26/24}R_{iso,2} = \frac{{}^{26}n_{iso,2}}{{}^{24}n_{iso,2}} \quad (33)$$

$${}^{26/24}R_{nat} = \frac{{}^{26}n_{nat}}{{}^{24}n_{nat}} \quad (34)$$

$${}^{26/24}R_{sample} = \frac{{}^{26}n_{sample}}{{}^{24}n_{sample}} \quad (35)$$

with ${}^x n_{iso,1}$, ${}^x n_{iso,2}$, ${}^x n_{sample}$, and ${}^x n_{nat}$ being the amount of Mg isotope x coming from the labels, the sample, and natural Mg, respectively.

Accordingly, the isotope ratio ${}^{26/24}R$ in the sample can be expressed as:

$${}^{26/24}R_{sample} = \frac{{}^{26}n_{nat} + {}^{26}n_{iso,1} + {}^{26}n_{iso,2}}{{}^{24}n_{nat} + {}^{24}n_{iso,1} + {}^{24}n_{iso,2}} \quad (36)$$

Substituting the ${}^{26}\text{Mg}$ amount coming from both labels and natural Mg in eqn. (36) using eqns. (32-35) yields:

$${}^{26/24}R_{sample} = \frac{{}^{26/24}R_{nat} \cdot {}^{24}n_{nat} + {}^{26/24}R_{iso,1} \cdot {}^{24}n_{iso,1} + {}^{26/24}R_{iso,2} \cdot {}^{24}n_{iso,2}}{{}^{24}n_{nat} + {}^{24}n_{iso,1} + {}^{24}n_{iso,2}} \quad (37)$$

Multiplication with the denominator and assembling yields:

$${}^{24}n_{nat} = \frac{{}^{24}n_{iso,2} \cdot ({}^{26/24}R_{iso,2} - {}^{26/24}R_{sample}) - {}^{24}n_{iso,1} \cdot ({}^{26/24}R_{sample} - {}^{26/24}R_{iso,1})}{{}^{26/24}R_{sample} - {}^{26/24}R_{nat}} \quad (38)$$

The unknown amount of ${}^{24}\text{Mg}$ in the sample coming from the isotopic labels ${}^{24}n_{iso,1}$ and ${}^{24}n_{iso,2}$ and natural magnesium ${}^{24}n_{nat}$, respectively, can be expressed by eqns. (26) and (27) and (39) based on isotope ratios, e.g.:

$${}^{24}n_{iso,2} = {}^{24}a_{iso,2} \cdot n_{iso,2} = \frac{n_{iso,2}}{1 + {}^{25/24}R_{iso,2} + {}^{26/24}R_{iso,2}} \quad (39)$$

This allows rewriting of eqn. (38) as:

$$n_{nat} = n_{iso,2} \cdot A - n_{iso,1} \cdot B \quad (40)$$

with A and B being defined as:

$$A = \frac{({}^{26/24}R_{iso,2} - {}^{26/24}R_{sample}) \cdot (1 + {}^{25/24}R_{nat} + {}^{26/24}R_{nat})}{({}^{26/24}R_{sample} - {}^{26/24}R_{nat}) \cdot (1 + {}^{25/24}R_{iso,2} + {}^{26/24}R_{iso,2})} \quad (41)$$

$$B = \frac{(^{26/24}R_{sample} - ^{26/24}R_{iso,1}) \cdot (1 + ^{25/24}R_{nat} + ^{26/24}R_{nat})}{(^{26/24}R_{sample} - ^{26/24}R_{nat}) \cdot (1 + ^{25/24}R_{iso,1} + ^{26/24}R_{iso,1})} \quad (42)$$

A similar equation can be derived based on the second measured isotope ratio $^{25/24}R$:

$$n_{nat} = n_{iso,2} \cdot C - n_{iso,1} \cdot D \quad (43)$$

with C and D being defined as:

$$C = \frac{(^{25/24}R_{iso,2} - ^{25/24}R_{sample}) \cdot (1 + ^{25/24}R_{nat} + ^{26/24}R_{nat})}{(^{25/24}R_{sample} - ^{25/24}R_{nat}) \cdot (1 + ^{25/24}R_{iso,2} + ^{26/24}R_{iso,2})} \quad (44)$$

$$D = \frac{(^{25/24}R_{sample} - ^{25/24}R_{iso,1}) \cdot (1 + ^{25/24}R_{nat} + ^{26/24}R_{nat})}{(^{25/24}R_{sample} - ^{25/24}R_{nat}) \cdot (1 + ^{25/24}R_{iso,1} + ^{26/24}R_{iso,1})} \quad (45)$$

The total amount of magnesium in the sample is the sum of the magnesium amounts coming from the labels and natural Mg:

$$n_{sample} = n_{nat} + n_{iso,1} + n_{iso,2} \quad (46)$$

Eqn. (46) is used to substitute n_{nat} in eqn. (40) and (43):

$$n_{sample} - n_{iso,2} - n_{iso,1} = n_{iso,2} \cdot A - n_{iso,1} \cdot B \quad (47)$$

$$n_{sample} - n_{iso,2} - n_{iso,1} = n_{iso,2} \cdot C - n_{iso,1} \cdot D \quad (48)$$

Solving eqn. (47) and (48) for $n_{iso,1}$ yields:

$$n_{iso,1} = \frac{n_{iso,2} \cdot (A + 1) - n_{sample}}{(B - 1)} \quad (49)$$

$$n_{iso,1} = \frac{n_{iso,2} \cdot (C + 1) - n_{sample}}{(D - 1)} \quad (50)$$

Equations (49) and (50) can be equated and solved for the amount of isotopic label $n_{iso,2}$:

$$n_{iso,2} = n_{sample} \cdot \frac{D - B}{AD + D + C - A - CB - B} \quad (51)$$

which can be used, in turn, to calculate the amount of the second isotopic label using either eqn. (49) or (50).

1.8.3. Quantification of Mg isotopic labels in a sample based on isotopic abundances

1.8.3.1. Single isotope techniques

Alternatively, calculations can be based on isotopic abundances. The total amount of Mg in the sample n_{sample} (in moles) is the sum of the molar Mg amount of natural isotopic composition n_{nat} and the amount of isotopic label n_{iso} :

$$n_{sample} = n_{nat} + n_{iso} \quad (52)$$

The total amount of an a Mg isotope x in the sample ${}^x n_{sample}$ is given by its isotopic abundance in nature ${}^x n_{nat}$ and in the label ${}^x n_{iso}$

$${}^{24} n_{sample} = {}^{24} a_{nat} \cdot n_{nat} + {}^{24} a_{iso} \cdot n_{iso} \quad (53)$$

$${}^{25} n_{sample} = {}^{25} a_{nat} \cdot n_{nat} + {}^{25} a_{iso} \cdot n_{iso} \quad (54)$$

$${}^{26} n_{sample} = {}^{26} a_{nat} \cdot n_{nat} + {}^{26} a_{iso} \cdot n_{iso} \quad (55)$$

The ${}^{26/24}R$ isotopic ratio in the sample can therefore be expressed as:

$${}^{26/24} R_{sample} = \frac{{}^{26} a_{nat} \cdot n_{nat} + {}^{26} a_{iso} \cdot n_{iso}}{{}^{24} a_{nat} \cdot n_{nat} + {}^{24} a_{iso} \cdot n_{iso}} \quad (56)$$

Rearranging of eqn. (56) yields n_{nat} :

$$n_{nat} = n_{iso} \cdot \frac{{}^{26} a_{iso} - {}^{26/24} R_{sample} \cdot {}^{24} a_{iso}}{{}^{26/24} R_{sample} \cdot {}^{24} a_{nat} - {}^{26} a_{nat}} \quad (57)$$

which can be used to replace n_{nat} in eqn. (52):

$$n_{sample} = n_{iso} + n_{iso} \cdot \frac{{}^{26} a_{iso} - {}^{26/24} R_{sample} \cdot {}^{24} a_{iso}}{{}^{26/24} R_{sample} \cdot {}^{24} a_{nat} - {}^{26} a_{nat}} \quad (58)$$

By transformation of eqn. (58) the total amount of isotopic label n_{iso} in the sample can be obtained:

$$n_{iso} = n_{sample} \cdot \left(1 + \frac{{}^{26}a_{iso} - {}^{26/24}R_{sample} \cdot {}^{24}a_{iso}}{{}^{26/24}R_{sample} \cdot {}^{24}a_{nat} - {}^{26}a_{nat}} \right)^{-1} \quad (59)$$

1.8.3.2. Double isotope techniques

Similar equations as given for the use of a single isotope label can be set up for 2 administered labels present in a sample. The amount of an isotope in the sample is the sum of the isotope amounts coming from both the isotopic labels and natural Mg. The respective amount of isotope is defined by its isotopic abundance and the element amount:

$${}^{24}n_{sample} = {}^{24}a_{nat} \cdot n_{nat} + {}^{24}a_{iso,1} \cdot n_{iso,1} + {}^{24}a_{iso,2} \cdot n_{iso,2} \quad (60)$$

$${}^{25}n_{sample} = {}^{25}a_{nat} \cdot n_{nat} + {}^{25}a_{iso,1} \cdot n_{iso,1} + {}^{25}a_{iso,2} \cdot n_{iso,2} \quad (61)$$

$${}^{26}n_{sample} = {}^{26}a_{nat} \cdot n_{nat} + {}^{26}a_{iso,1} \cdot n_{iso,1} + {}^{26}a_{iso,2} \cdot n_{iso,2} \quad (62)$$

The isotopic ratios ${}^{26/24}R$ and ${}^{25/24}R$ can therefore be expressed as:

$${}^{26/24}R_{sample} = \frac{{}^{26}a_{nat} \cdot n_{nat} + {}^{26}a_{iso,1} \cdot n_{iso,1} + {}^{26}a_{iso,2} \cdot n_{iso,2}}{{}^{24}a_{nat} \cdot n_{nat} + {}^{24}a_{iso,1} \cdot n_{iso,1} + {}^{24}a_{iso,2} \cdot n_{iso,2}} \quad (63)$$

$${}^{25/24}R_{sample} = \frac{{}^{25}a_{nat} \cdot n_{nat} + {}^{25}a_{iso,1} \cdot n_{iso,1} + {}^{25}a_{iso,2} \cdot n_{iso,2}}{{}^{24}a_{nat} \cdot n_{nat} + {}^{24}a_{iso,1} \cdot n_{iso,1} + {}^{24}a_{iso,2} \cdot n_{iso,2}} \quad (64)$$

Solving eqns. (63) and (64) for the total amount Mg coming from the label $n_{iso,1}$ yields:

$$n_{iso,1} = n_{nat} \cdot M + n_{iso,2} \cdot N \quad (65)$$

$$n_{iso,1} = n_{nat} \cdot O + n_{iso,2} \cdot P \quad (66)$$

with M , N , O , and P being defined as:

$$M = \frac{{}^{26}a_{nat} - {}^{26/24}R_{sample} \cdot {}^{24}a_{nat}}{{}^{26/24}R_{sample} \cdot {}^{24}a_{iso,1} - {}^{26}a_{iso,1}} \quad (67)$$

$$N = \frac{{}^{26}a_{iso,2} - {}^{26/24}R_{sample} \cdot {}^{24}a_{iso,2}}{{}^{26/24}R_{sample} \cdot {}^{24}a_{iso,1} - {}^{26}a_{iso,1}} \quad (68)$$

$$O = \frac{{}^{25}a_{nat} - {}^{25/24}R_{sample} \cdot {}^{24}a_{nat}}{{}^{25/24}R_{sample} \cdot {}^{24}a_{iso,1} - {}^{25}a_{iso,1}} \quad (69)$$

$$P = \frac{{}^{25}a_{iso,2} - {}^{25/24}R_{sample} \cdot {}^{24}a_{iso,2}}{{}^{25/24}R_{sample} \cdot {}^{24}a_{iso,1} - {}^{25}a_{iso,1}} \quad (70)$$

Eqn. (65) and (66) can be equated and solved for $n_{iso,2}$:

$$n_{iso,2} = n_{nat} \cdot \frac{M - O}{P - N} \quad (71)$$

By replacing $n_{iso,2}$ in eqn. (66) with eqn. (71), $n_{iso,1}$ can be obtained:

$$n_{iso,1} = n_{nat} \cdot \frac{MP - ON}{P - N} \quad (72)$$

In addition, the Mg amount in the sample is the sum of the Mg amounts coming from natural Mg and the two isotopic labels:

$$n_{sample} = n_{nat} + n_{iso,1} + n_{iso,2} \quad (73)$$

Eqns. (71) and (72) are solved for n_{nat} :

$$n_{nat} = n_{iso,1} \cdot Q \quad (74)$$

$$n_{nat} = n_{iso,2} \cdot R \quad (75)$$

with Q and R being:

$$Q = \frac{(P - N)}{(MP - ON)} \quad (76)$$

$$R = \frac{(P - N)}{(M - O)} \quad (77)$$

Eqns. (74) and (75) can be used to replace the expression of n_{nat} eqn. (73), resulting in the following 2 eqns:

$$n_{sample} = n_{iso,1}Q + n_{iso,1} + n_{iso,2} \quad (78)$$

$$n_{sample} = n_{iso,2}R + n_{iso,1} + n_{iso,2} \quad (79)$$

Both eqns. (78) and (79) are solved for $n_{iso,2}$ and equated:

$$n_{sample} - n_{iso,1} \cdot Q - n_{iso,1} = \frac{n_{sample} - n_{iso,1}}{1 + R} \quad (80)$$

Resolving eqn. (80) for the Mg amount in the isotopic label $n_{iso,1}$ yields:

$$n_{iso,1} = \frac{n_{sample} \cdot R}{Q + QR + R} \quad (81)$$

Determination of absorption based on urine, plasma, or tissue samples after oral and iv. administration of two different labels of an element is based on the amount ratio of the 2 labels and in these samples ($n_{iso,1}/n_{iso,2}$). This can be obtained by rearranging and equating eqn. (71) and (72), yielding:

$$\frac{n_{iso,1}}{n_{iso,2}} = \frac{MP - ON}{M - O} \quad (82)$$

1.8.4. Sample preparation for Mg isotopic analysis

Sample preparation for Mg stable isotope analysis depends on the choice of method for isotopic analysis (i.e. the mass spectrometer) as well as the sample matrix. This is most often faeces, urine, or blood. Usually, the sample is mineralized to destroy the organic matrix of the sample, and, if required, Mg is separated from inorganic matrix constituents such as calcium, sodium, and potassium (Fig 5).

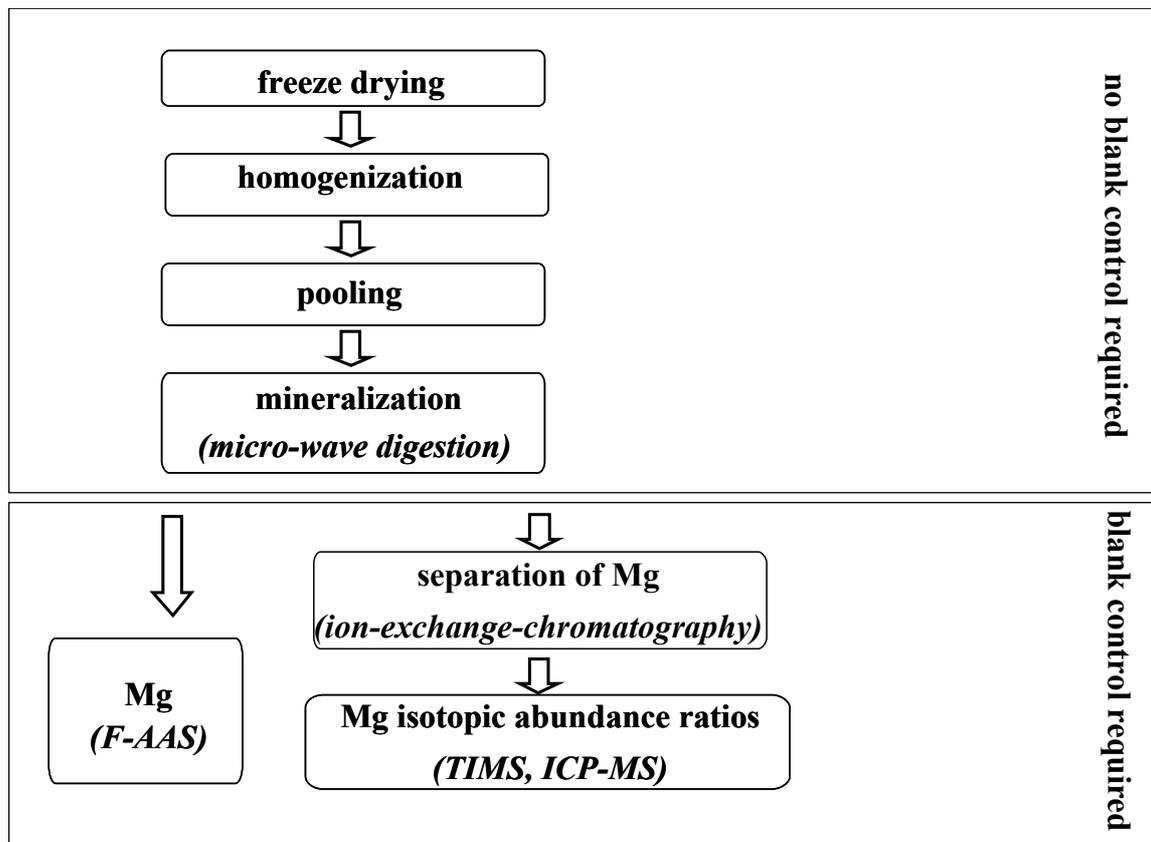


Fig. 5: Preparation of faecal samples for magnesium isotopic and elemental analysis by F-AAS (flame atomic absorption spectrometry)

Faecal samples are usually collected, freeze dried and homogenized separately (Fairweather-Tait *et al.*, 1997; Sabatier, 2001). Unless Mg excretion does not have to be followed over time, e.g. in kinetic studies, all faecal samples containing non absorbed isotopic label are pooled and an aliquot is taken for further sample preparation (Sojka *et al.*, 1997; Sabatier, 2001). The organic sample matrix is destroyed, e.g. by microwave digestion using strong oxidizing agents such as a mixture of conc. HNO₃ and H₂O₂ (30%) (reviewed by Ruiz *et al.*, 1995; Stegmann *et al.*, 1996). Alternatively, samples can be dry ashed in a muffle furnace at 500-600°C (Turnlund & Keyes, 1990), followed by dissolution of the ashes in HCl or other strong acids and removal of undissolved matter.

Urine samples are usually collected in pools over several hours up to 24 hours. Collected urine is acidified by adding conc. HCl at 0.5-1% of the urine mass to prevent precipitation during storage (Sabatier, 2001). An aliquot is commonly taken and kept frozen until further processing. Mineralization is done usually by microwave digestion similar as described for faecal material.

Blood samples are commonly taken to study the appearance and fate of the isotopic label in plasma in kinetic studies. To determine Mg concentrations or isotope ratios in plasma samples, it is advisable to separate the plasma as soon as possible from the blood cells, e.g. by centrifugation, to prevent any lysis of erythrocytes as Mg concentrations in erythrocytes are 3-4 times higher when compared to plasma (Durlach, 1988). Contamination control is essential due to the low Mg concentration in plasma, special acid washed glassware and needles are required for blood drawing (Crews *et al.*, 1994). Contamination control has to be less rigorous if Mg absorption is determined from the amount ratio of an iv. and an orally administered isotopic label. Sample contamination by natural Mg alters the isotopic composition of the sample but does not alter the amount ratio of the labels which bears the information on Mg absorption.

For measuring Mg isotope ratios by ICP-MS at moderate precision and accuracy, mineralization alone might be sufficient for sample preparation to improve sample-throughput (Sabatier, 2001). For high precision isotopic analysis using TIMS or multicollector ICP-MS, however, Mg has to be separated from calcium, potassium, sodium and other elements in the sample. In TIMS, as an example, concomitant elements may disturb the thermal ionization process (Sandstrom *et al.*, 1993; Kastenmayer, 1996). For Mg separation from other minerals, cation exchange chromatography (Turnlund & Keyes, 1990; Vieira *et al.*, 1994) and Mg extraction, e.g. as the volatile Mg-2,2',6,6' tetramethyl-3,5-heptanedione)₂ complex (Schwartz *et al.*, 1980; Schwartz *et al.*, 1984) or bound to dicyclohexano-18-crown-6 ether (Nishizawa *et al.*, 1996) as well as 8-hydroxyquinoline precipitation (Vieira *et al.*, 1994) have been used successfully in the past. However reagents are often impure and may increase the risk of contamination. Precipitation procedures, on the other hand, may result in co-precipitation of other minerals from the sample, e.g. calcium. The most frequently used technique for Mg separation from biological samples is cation-exchange chromatography. Procedures can be automated, result usually in

reproducible recovery rates and allow adequate Mg separation from other minerals while sample contamination by natural Mg can be minimized effectively (Turnlund & Keyes, 1990; Vieira *et al.*, 1994).

Because sample contamination with natural Mg has to be minimized onwards from the step where the sample is divided for isotopic and elemental analysis, highly purified chemicals are required for sample preparation such as purified water and acids especially cleaned by sub-boiling distillation (reviewed by Crews *et al.*, 1994). Again, these precautions are not necessary if samples are used to determine absorption based on the amount ratio of the oral label and the iv. label in the sample which is not altered by the influx of Mg of natural isotopic composition.

Even when taking the most rigorous precautions, sample contamination during sample preparation cannot be prevented completely. To monitor the amount of natural Mg introduced during sample processing, control blanks have to be run in parallel to the samples. This is often done by treating an aliquot of pure isotopic label of known isotopic composition identical to the samples. The amount of natural Mg introduced during sample processing can then be calculated from the induced changes in the isotopic composition of the isotopic label following isotope dilution principles.

1.8.5. Mg elemental analysis

Unless Mg absorption is determined based on the simultaneous administration of an iv. and an oral label, the total Mg amount (in moles) in the sample has to be known for quantifying the isotopic label. In addition, measuring the total amount of an element is required to determine Mg retention. Because Mg concentrations are relatively high in faeces (about 100 mg/100g) and urine (100-150 mg/L) analytical sensitivity is of less relevance for choosing the most appropriate technique. Precision and accuracy are decisive as they affect directly the analytical error in the absorption value.

Mg elemental analysis is often done by flame atomic absorption spectroscopy (F-AAS) as this method is widely available and offers a high sample throughput at an external relative precision (1 SD) of less than 3% (Christian, 1994; Pelly, 1994;

Gottelt *et al.*, 1996; Ibanez & Cifuentes, 2001). For AAS analysis, in general, separation of the element of interest from matrix elements is mostly unnecessary and mineralization of the organic matrix is sufficient for sample preparation.

However, ionization processes and precipitations of the element of interest may occur in presence of other elements which may affect the accuracy and precision of the analysis. For alkaline earth elements such as Mg, precipitation as its pyrophosphate ($Mg_2P_2O_7$) in the atomizer is probably the main source of error in F-AAS when using an air/acetylene flame. This can be reduced effectively by adding a surplus of lanthanum ions to the solution (2000-5000 ppm La) which bind preferably to phosphates in the sample (Christian, 1994). Matrix effects can be further reduced by using standard addition techniques. However, reference materials similar in matrix have to be processed in parallel to verify the accuracy of the analysis in either case.

Isotope dilution mass spectrometry is used increasingly for Mg elemental analysis too. Although more time consuming, data are superior in accuracy and precision when compared to data generated by F-AAS. Mg elemental analysis by ICP-MS with its high sensitivity can be advantageous when sample concentrations are low, e.g. in plasma, or when the sample amount is a limiting factor, e.g. when blood samples have to be taken from babies or infants.

1.8.6. Mg isotopic analysis

When using Mg stable isotope labels to study Mg metabolism *in vivo*, isotope doses have to be kept as low possible. The Mg content of the test meal should not be altered significantly to ensure that the isotopic label mimics closely natural Mg in the test meal. Element absorption is usually dose dependent, i.e. the more element is present in a test meal the lower is the fraction absorbed. When giving Mg stable isotopes intravenously, dose minimization is even more important. A high Mg dose may disturb Mg homeostasis in blood and, thus, Mg metabolism. Finally, isotope doses determine largely the costs of a study as they are relatively expensive.

Any reduction in isotope dose results automatically in a lower isotopic enrichment in the sample. Accordingly, the achievable precision in isotopic analysis determines in

how far isotope doses can be reduced. When using stable isotopes as labels, it is not necessary to determine isotope ratios at the highest possible absolute accuracy. Systematic errors in isotopic analysis cancel out when applying isotope dilution principles to quantify the label in the sample. High relative accuracy in isotopic analysis is, therefore, sufficient, i.e. the bias in the measured isotope ratio has to be proportional to its value and has to be constant within and between analytical runs.

Oral doses of Mg stable isotope labels used in human absorption are usually between 10 and 50 mg. However, doses as high as 360 mg have been used in the past (see Table 11). Doses for intravenous use are in the order of 20 mg. At these oral and iv. doses, changes in the respective isotope ratio relative to its natural value are in the order of >10 % in stool samples pooled over 2-4 days and usually <5% in urine and serum/plasma samples taken 12 h post dosing (Cary *et al.*, 1990; Abrams & Wen, 1999). Isotopic enrichment in blood cells after oral administration of 20 mg ²⁵Mg during 24 h were 2-5% in infants, and can be expected to be significantly lower in adults (Schuette *et al.*, 1990b). Thus, precision requirements are higher to determine absorption in urine and blood when compared to faecal monitoring techniques.

1.8.7. Isotopic analysis by mass spectrometric techniques

1.8.7.1. Introduction

Mass spectrometry allows determination of the isotopic composition of an element based on the physical separation and quantitative detection of its isotopes. This requires, in general, ionization of the element and acceleration of the generated ions into the mass analyzer as a focussed ion beam under high vacuum conditions. In the analyzer, ions are separated or filtered based on their mass to charge ratio. A number of ion sources have been developed in the past, each having their specific advantages and disadvantages. The most prominent ion sources used in inorganic mass spectrometry are based on thermal ionization (TIMS) and ionization of the sample in an inductively coupled plasma (ICP-MS), respectively.

A number of devices are available for mass analysis using different physical principles. In quadrupole based analyzers, only those ions having the selected mass

to charge ratio follow a stable flight pass through the device (Platzner *et al.*, 1997). Isotope ratios are measured by switching the quadrupole back and forth to select ions of the selected mass to charge ratio (peak hopping). Quadrupole instruments are less expensive but do not allow simultaneous ion intensity measurements as ions are filtered and not separated. Simultaneous ion beam detection becomes possible by using a magnetic sector field for ion separation. Ions are accelerated linearly into the sector field and deflected differently due to their mass to charge ratio (Platzner *et al.*, 1997). Magnetic sector field analyzers are preferred for high precision isotope ratio measurements as they generate flat top peaks in the mass spectrum contrary to quadrupole based mass spectrometers showing bell shaped peaks.

Conventional quadrupole mass filters and magnetic sector field analyzers do not allow differentiation between different ions having the same nominal mass to charge ratio, e.g. $^{26}\text{Mg}^+$ and $^{12}\text{C}^{14}\text{N}^+$. This fundamental source of bias in mass spectrometry, in particular ICP-MS, can be controlled either by destruction of interfering molecular ions in a collision cell where they collide with residual gas atoms or molecules or by coupling the magnetic sector field to an electrostatic analyzer (Becker & Dietze, 2000). By selecting ions of the same energy in an electrostatic field, peak width is reduced and allows for resolution of the small differences in the mass to charge ratio of the interfering ions.

The choice of the detector system depends largely on the mass analyzer, the ion intensities and the targeted precision in isotopic analysis. For high ion currents, Faraday cup detection is the method of choice offering the highest precision for measuring ion intensities. Separated ion beams can be measured simultaneously in magnetic sector field instruments by using a multicollector array of Faraday cups. This may further improve the precision of the measurement as fluctuations in signal intensity do not affect the measurement when compared to dynamic measurements based on peak hopping. When the sample amount is limited, Faraday cups can be replaced by more sensitive secondary ion multipliers such as dynode multipliers or Daly detectors. For measuring extreme isotope ratios, Faraday cups and secondary electron multipliers can be used in parallel (reviewed by Crews *et al.*, 1994; Platzner *et al.*, 1997).

For either mass spectrometric device, mass discrimination effects limit the achievable accuracy and precision in isotope ratio measurements as they alter the measured isotope ratio systematically. Mass discrimination effects are proportional to the relative mass difference of the ions being the stronger the larger the differences are. Mass discrimination occurs at multiple stages during the measurement process. In TIMS, lighter isotopes evaporate more quickly from the sample and are ionized preferentially. In ICP-MS, isotopic fractionation effects are the strongest during sampling from the plasma and during ion transfer from atmospheric pressure to high vacuum conditions. When using electron multipliers, discrimination can also occur because of the tendency of heavier ions to produce more secondary electrons than lighter ions when hitting the detector system (reviewed by Crews *et al.*, 1994).

1.8.7.2. Analytical techniques used for Mg isotopic analysis in the past

A variety of techniques have been used successfully over the past decades to determine Mg isotope ratios in biological samples. Impressive improvements in measurement precision have been achieved, allowing to administer doses at physiologically meaningful levels and to reduce costs for stable isotope studies in humans. In earlier studies, neutron activation analysis (NAA), fast atom bombardment (FAB-MS) and GC/MS have been used predominantly due to their wider availability. However, they are used rarely nowadays as the achievable precision in isotopic analysis using these techniques is, in general, inferior to TIMS or ICP-MS.

In NAA, the biological sample is bombarded with neutrons in a nuclear reactor, causing some of the isotopes to capture a neutron to become a radioisotope which can be detected by its radiation. However, neutron capture cross sections differ significantly between elements and isotopes which excludes a number of isotopes from analysis making it not generally applicable. In addition, this method is relatively limited in precision. Achievable precisions expressed as the RSD are in the order of 1-5% depending on the element and the purity of the sample (Fairweather-Tait, 1996). ^{26}Mg labels in urine and faeces have been quantified by NAA in rats (Currie *et al.*, 1975) and in humans (Schwartz *et al.*, 1978; Schwartz & Giesecke, 1979)

after ashing of the sample and extraction of Mg with fluorhydrocarbons. These authors reported a RSD (triplicate analysis) of 26% for a ^{26}Mg isotopic enrichment (expressed as the change in the respective isotope ratio relative to its natural value) of 8.9% in the sample. This measurement precision is certainly too low to make NAA a suitable technique to determine Mg isotope ratios in stable isotope studies in biomedical research.

FAB-MS is commonly used to identify non volatile organic compounds, e.g. peptides or polysaccharides, but is also used for isotopic analysis. In FAB-MS, samples are usually bombarded with argon or xenon atoms for ionization. FAB-MS has been used to determine Mg isotope ratios in urine and faeces from infants after dry ashing and precipitation of Mg as oxalate (Liu *et al.*, 1989). No data concerning the measurement precision was reported but can be assumed to be in the percent range as reported for other elements (Eagles & Mellon, 1996).

Although not designed for this purpose, GC/MS instruments can be used for isotopic analysis too. In GC/MS, volatile compounds are separated by gas chromatography and introduced into a mass spectrometer for compound identification rather than for isotopic analysis. However, Schwartz and Giesecke (1979) developed a GC/MS technique based on a volatile Mg chelate. However, RSD of the measurement for triplicate analysis was relatively low, about 0.9-3.8% for an enrichment of ^{26}Mg (expressed as the change in the respective isotope ratio relative to its natural value) of about 8.1% or higher, measurement precision decreased to 21% RSD for 4% enrichment.

1.8.7.3. TIMS

TIMS has been used since the 1950s to determine isotope ratios, mostly in isotope geology and nuclear chemistry. The application of TIMS to measure isotope ratios in isotopically enriched biological samples started in the 1970s (Rabinowitz *et al.*, 1973). For most elements in biomedical research, TIMS is still accepted as the standard method for isotopic analysis due to its high precision and accuracy (Hachey *et al.*, 1987).

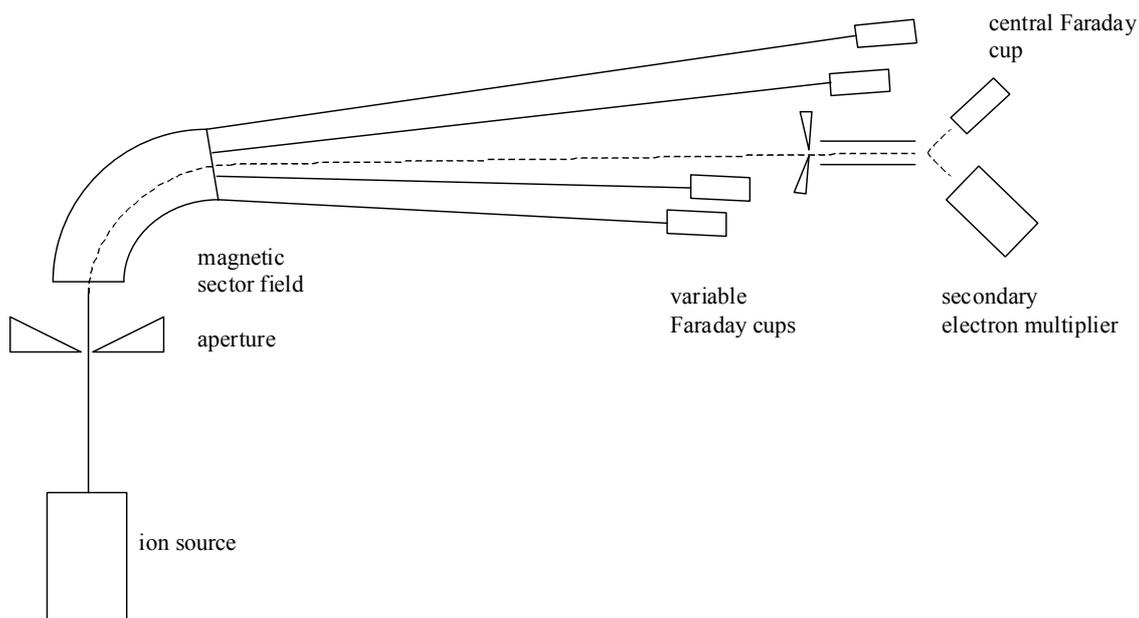


Fig. 6: Scheme of a TIMS instrument equipped with a magnetic sectorfield for ion separation and a Faraday cup multicollector device for ion detection.

In TIMS, the sample is loaded usually in ng- μ g amounts on a metal filament that can be heated electrothermally. The filament consists of a purified refractory metal with a high melting point such as rhenium, tungsten or tantalum. For TIMS measurements, the element of interest usually has to be separated from the organic matrix and concomitant elements as they may disturb the ionization process by reducing the ionization efficiency and destabilization of the ion current. A stable and constant ion current is important, especially for dynamic measurements using a single ion collector and peak hopping. For the same reason, reproducible loading of the sample on the filaments is important.

Often, the mineral is loaded together with other compounds to achieve a more constant ion beam and to enhance ion yield, e.g. silica gel, boric acid, phosphoric acid or aluminium. The optimum loading technique depends strongly on the element (reviewed by Heuman, 1988; Turnlund & Keyes, 1990). Isotopic analysis by TIMS is commonly based on the generation of single charged positive ions but negative ions can be generated for a number of elements too by choosing a different filament preparation technique. For Mg, it is recommended to use purified silica gel with phosphoric acid (Turnlund & Keyes, 1990; Stegmann & Karbach, 1993) or boric acid

(Stegmann *et al.*, 1996) to enhance and stabilize ion emission. Contrary to common practice, the latter authors loaded mineralized human blood, urine, and faecal samples without further purification on a rhenium filament (direct loading technique). In any case, the sample is dried on the filament after loading in solution.

Single, double, or triple filament configurations have been used in which each filament can be heated independently. The ionization filament(s) remain often unloaded, as for Mg. The advantage of using a multiple filament configuration is that for some elements optimum evaporation and ionization temperatures differ and the evaporation process can be separated from the ionization process. The filaments are heated stepwise under high vacuum conditions for ion generation, usually to a temperature between 800-2100 °C (1400-2100 °C for Mg). Optimum filament temperatures depend strongly on the element and the chosen filament preparation technique. Attempts have been made to describe the process of thermal ionization theoretically but is still not completely understood (Platzner *et al.*, 1997).

A number of studies investigating Mg absorption used TIMS for Mg isotopic analysis (Table 11). Between run precisions (1 RSD) of better than 0.1% for both the $^{25}\text{Mg}/^{24}\text{Mg}$ and the $^{26}\text{Mg}/^{24}\text{Mg}$ isotope ratio have been reported for an aqueous Mg standard using a magnetic sector field instrument equipped with a multicollector device (Vieira *et al.*, 1994). Measurement of biological samples, however, yields poorer precisions. $^{26}\text{Mg}/^{24}\text{Mg}$ and $^{25}\text{Mg}/^{24}\text{Mg}$ isotope ratios were measured at about 0.5% between run precision (1RSD) (Turnlund *et al.* 1990). Abrams *et al.* (1999) measured Mg isotope ratios at about 0.2% precision for urine samples, nor information was given whether these was between run precision or within run precision.

1.8.7.4. ICP-MS

ICP-MS is a relatively recent development compared to the MS techniques described previously. The first report about coupling an inductively coupled plasma ion source to a mass spectrometer is about 20 years old (Houk *et al.*, 1980). ICP-MS was designed originally as a tool for quantitative multi-element analysis (reviewed by Stuewer & Jakubowski, 1998). However, over the past decade, ICP-

MS has established itself as a tool for isotopic analysis too, in particular in biomedical research (Crews *et al.*, 1994; Crews *et al.*, 1996; Yergey, 1996).

The major parts of an ICP-MS instrument are the nebulizer, the ion source (plasma), the mass analyzer and the ion detector (Fig.7). Liquid samples are introduced e.g. by pneumatic or ultrasonic nebulization, the latter usually having a higher sampling efficacy of about 10% (Crews *et al.*, 1996). The droplets are aspirated into an argon or helium plasma operating at 4000-8000 K at atmospheric pressure for sample evaporation, atomization and ionization at high efficacy. The ions are then accelerated into a high vacuum and focused by electrostatic lenses into the mass analyzer. The ionization efficacy is much less affected by the sample matrix when compared to TIMS. Accordingly, mineralized samples can be introduced directly into the plasma.

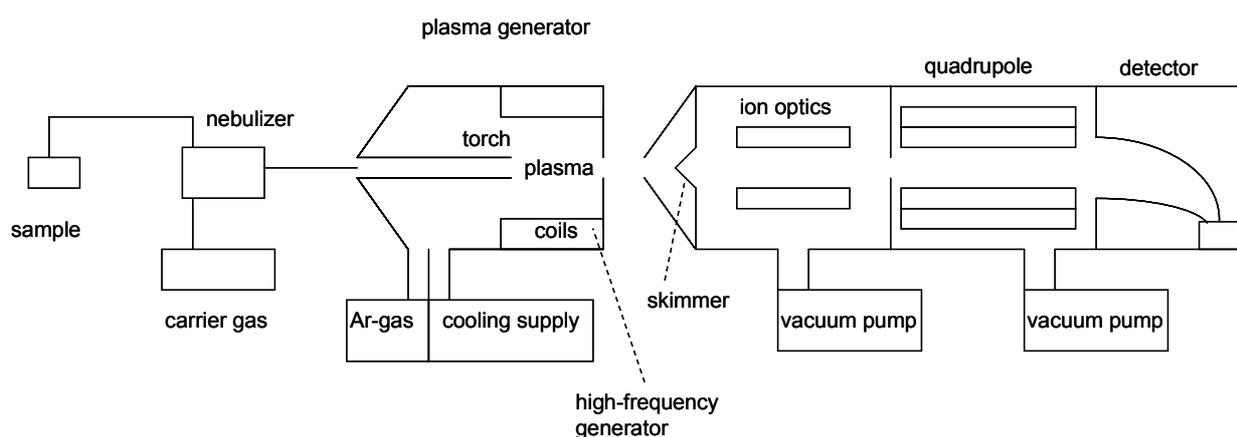


Fig. 7. Scheme for a quadrupole ICP-MS

Quadrupoles are most commonly used for mass analysis in ICP-MS due to their relative low costs. However, for most elements the achievable accuracy and precision in isotopic analysis is limited by the occurrence of isobaric interferences, especially when the analyte is present in a complex matrix. For high precision isotopic analysis, isobaric interferences have to be reduced efficiently by using either a double-focussing mass analyzer or a collision cell in combination with sample desolvation and element separation from the matrix prior to isotopic analysis.

A few studies have investigated Mg absorption or metabolism using ICP-MS for Mg isotopic analysis (see Table 11). One of the first applications of ICP-MS was by Schuette et al. (1988). By using a quadrupole based instrument the authors achieved a remarkable between run precision (RSD) of 0.1-1% for the relevant Mg isotope ratios. A similar between run precision has been reported for faecal, urine, and plasma samples at <0.3% for the $^{25}\text{Mg}/^{24}\text{Mg}$ and the $^{26}\text{Mg}/^{24}\text{Mg}$ isotope ratio, respectively (Sabatier, 2001). Benech et al. (1998) reported a between run (interday) precision between 0.01 and 0.15% for both $^{25}\text{Mg}/^{24}\text{Mg}$ and $^{26}\text{Mg}/^{24}\text{Mg}$ isotope ratios.

In these studies, isotopic ratios were measured by quadrupole ICP-MS using peak hopping. Vanhackle et al. (1996) achieved an within run precision of 0.04% for the $^{25}\text{Mg}/^{26}\text{Mg}$ isotopic ratio when using ICP-MS with a magnetic sector-field mass analyser and a single ion detector. There are no reports at present in the literature about multicollector ICP-MS for measuring Mg isotope ratios in biological samples. However, Mg in a meteorite was measured with a high resolution ICP-MS multicollector instrument with a between run precision of about 0.06 ‰ ($^{25}\text{Mg}/^{24}\text{Mg}$) and 0.12 ‰ ($^{26}\text{Mg}/^{24}\text{Mg}$) (Galy *et al.*, 2001).

1.8.7.5. ICP-MS compared to TIMS

TIMS is still widely accepted for most elements as the reference method to determine isotope ratios because of its high accuracy and precision. However, ICP-MS became over the past decade a powerful alternative due to its specific advantages that can balance its disadvantages. For Mg isotopic analysis at moderate precision by quadrupole ICP-MS, as an example, separation of Mg from other elements is not necessarily required, as shown for plasma and stool samples (Sabatier, 2001). Even simple sample dilution turned out to be sufficient for urine samples, reducing the time of sample preparation significantly and making it an ideal tool for routine analysis. Furthermore, measurements can be automatized more efficiently using autosampling devices. However, if high precision and accuracy is targeted, e.g. when using multicollector ICP-MS, sample preparation procedures nearly as time consuming as for TIMS measurements are usually required (Walczyk, 2001). Sample throughput is further reduced by the need to monitor

intermittently mass discrimination by bracketing the sample with a standard of known isotopic composition. Mass discrimination effects are strong in ICP-MS and vary significantly within and between days, depending on instrument conditions. In TIMS, on the contrary, mass discrimination is much smaller and can be controlled efficiently by reproducible filament loading and heating.

Under routine conditions, sample throughput in multicollector ICP-MS is still significantly higher and precisions in isotopic analysis can be achieved which surpass those achievable by TIMS, especially when internal normalization techniques cannot be applied to correct for mass discrimination effects in the ion source. Multicollector ICP-MS can be considered, therefore, a complimentary tool to TIMS and the favourable tool for applications that require a high sample through-put such as in nutritional studies employing stable isotope labels to study mineral and trace element metabolism.

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2. Results/Manuscripts

2.1. Blood cell incorporation and urinary monitoring of stable magnesium isotope labels as alternative techniques to determine magnesium absorption in humans

Running title: Techniques to determine magnesium absorption

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Magnesium absorption: Stable isotopes: Faecal monitoring: Urinary monitoring:
Blood cell incorporation

Abstract

Magnesium absorption in humans is usually determined by faecal monitoring of orally ingested stable isotope labels. We have evaluated urinary monitoring and blood cell incorporation of stable isotope labels as alternative techniques. Ten healthy adult subjects were given 2.2 mmol ^{25}Mg in water, together with wheat bread, followed 15 min later by intravenous injection of 0.6 mmol ^{26}Mg (day 1). Brilliant blue and ytterbium (given on day 0 and day 1, respectively) were used as qualitative and quantitative faecal markers. Urine was collected in 24 h pools for 6 days after test meal intake. Complete collections of faeces were made until excretion of the second brilliant blue marker (given on day 7). Magnesium isotopic ratios were determined by thermal ionisation mass spectrometry (TIMS) or inductively coupled plasma mass spectrometry (ICP-MS). Absorption was determined based on: a) 6 day urine pools b) 24 h urine pools (collected 22-46 h after test meal intake) c) blood drawn on day 14 d) complete 6 day faecal pools e) faecal pools based on the first 3 consecutive stools after excretion of the first brilliant blue marker. Mean magnesium absorption was 42-44% by all techniques, except for data based on 6 day urine pools (33%, $P=0.0003$, ANOVA). The results indicate that magnesium absorption can be determined from a 24 h urine pool or from blood drawn 14 days after test meal intake, as alternatives to the more time consuming and labour intense faecal monitoring method. The choice of methodology depends on practical and financial aspects.

Introduction

Inadequate intake of magnesium and low serum magnesium levels have been discussed in relation to major public health problems such as osteoporosis (Abraham, 1991) coronary artery diseases (Masironi *et al.*, 1979; Karppanen, 1981), and non-insulin-dependent diabetes mellitus (Kao *et al.*, 1999). Although information about dietary intake of magnesium is available in many industrialised countries, very little is known about magnesium bioavailability and the influence of diet composition on magnesium absorption. The lack of information is, at least partly, due to the lack of suitable methodologies to investigate magnesium metabolism in man. The chemical balance technique is not a useful tool as absorption from single meals cannot be determined, and although radioactive isotopes have been shown to be useful to study the absorption of minerals such as calcium (DeGrazia *et al.*, 1965) and zinc (Arvidsson *et al.*, 1978), magnesium radioisotopes have too short half-lives (below 21.3 h) to be useful.

Stable isotopes techniques however can be used as magnesium has 3 stable isotopes, 2 of them with low enough natural abundance to be employed as enriched labels (^{25}Mg and ^{26}Mg , 10.0 and 11.0% natural abundance)(Catanzaro, 1966). The technique most often used to measure mineral and trace element absorption is based on faecal monitoring. This technique, based on the analysis of non-absorbed stable isotope label excreted in faeces, has been applied to measure magnesium absorption in humans (Schwartz *et al.*, 1978; Schwartz *et al.*, 1984) and to evaluate mineral and trace element absorption in humans, e.g. the bioavailability of calcium (Davidsson *et al.*, 1996), zinc (Serfass *et al.*, 1989; Fairweather-Tait *et al.*, 1992), copper (Knudsen *et al.*, 1996), and molybdenum (Turnlund *et al.*, 1999). A second isotope label can be injected intravenously so as to estimate the absorbed and re-excreted isotope label during the faecal collection period. True absorption can then be estimated by correcting for excretion of the intravenous label in faeces. A major drawback of faecal monitoring however is the need for complete faecal collections over several consecutive days. Non-absorbable rare earth elements as quantitative faecal markers have therefore been introduced, allowing the possibility of a reduced collection period (Schuette *et al.*, 1993; Fairweather-Tait *et al.*, 1997).

Alternative techniques, based on urinary monitoring after administration of an oral and a second, injected isotopic label have been developed to determine calcium

absorption with radioactive (Garner *et al.*, 1960; Bronner, 1962) and stable isotopes (Smith *et al.*, 1985; Yergey *et al.*, 1987), and this technique has also been proposed for the determination of zinc absorption (Friel *et al.*, 1992). The accuracy of this method however has been debated (King *et al.*, 1997; Rauscher & Fairweather-Tait, 1997; Shames *et al.*, 2001). Nevertheless, the relatively high urinary concentration of magnesium, as well as similarities between magnesium and calcium metabolism, indicate that the urinary monitoring technique could be a useful approach to measure magnesium absorption.

Techniques based on erythrocyte incorporation of isotope labels are routinely used to measure iron absorption either with radioisotope labels (Cook *et al.*, 1972) or stable isotope labels (Kastenmayer *et al.*, 1994), as most of newly absorbed iron is incorporated into this tissue. Although erythrocytes are not a specific target tissue for magnesium, their magnesium concentration is relatively high, ~2.5 mmol/L (Durlach, 1988). Coudray *et al.* (1997) reported erythrocyte incorporation of magnesium stable isotope labels to be a useful method to determine magnesium absorption in rats.

The aim of this study was to determine magnesium absorption in adult humans by magnesium double stable isotope techniques based on 6 day or 24 h urine pools and by incorporation into blood cells, and to compare these values with data based on 6 day faecal pools. In addition, absorption data based on 3-stool pools corrected for the recovery of ytterbium (a nonabsorbable faecal marker) were evaluated.

Materials and Methods

Subjects

Ten apparently healthy, free living subjects (5 men, 5 women, BMI: 21.2±3.7 kg/m²; age: 35.0±10.0 y) were recruited for the study. No lactating or pregnant women were included and no medication was allowed, except for oral contraceptives. Intake of mineral/vitamin supplements was not permitted 2 weeks before and during the study. The subjects were informed orally and in written form about the aims and the procedure of the study and written informed consent was obtained. The study protocol was reviewed and approved by the Ethical Committee of the Swiss Federal Institute of Technology, Zurich.

Isotopic labels

Highly enriched ^{25}MgO (^{24}Mg : $1.04\pm 0.01\%$; ^{25}Mg : $98.73\pm 0.01\%$; ^{26}Mg : $0.23\pm 0.01\%$) and ^{26}MgO (^{24}Mg : $0.39\pm 0.01\%$; ^{25}Mg : $0.11\pm 0.01\%$; ^{26}Mg : $99.51\pm 0.01\%$) were purchased from Chemgas, Paris, France. ^{25}MgO (29.3 mmol) were dissolved in 2.5 ml 4 mol/L HCl and diluted to 100 ml with water to be served as an orally ingested label. The pH of the solution was adjusted to 4-5 by addition of an aqueous NaHCO_3 (Merck, Darmstadt, Germany) solution.

Doses for intravenous administration were prepared at the Cantonal Pharmacy, Zurich. Sterile water and sterile materials were used for their preparation. ^{26}MgO (9.5 mmol) was dissolved in 2.5 ml 4 mol/L HCl, diluted with water to 50 ml and adjusted to pH 6 as described above. The sterile filtered solution was divided into individual doses of 3.2 ml (~ 0.6 mmol ^{26}Mg), transferred into glass vials, capped and sealed. The magnesium concentration of the ^{25}Mg and ^{26}Mg isotopic label in solution was determined by isotope dilution using TIMS against a commercial magnesium standard of natural isotopic composition (Titrisol, Merck).

Unless otherwise noted, all chemicals were of analytical grade, and all acids were further purified by surface distillation. Only 18 M Ω water (Milli Q water system, Millipore, Zurich, Switzerland) was used for laboratory work and test meal preparation.

Test meal

The standardised breakfast consisted of wheat bread rolls, prepared from 75 g white flour, using a standard recipe. A relatively long fermentation (5 h) was used to degrade phytic acid, a potential inhibitor of magnesium absorption. Bread rolls were prepared in bulk and stored frozen. Water (200 g) was served as a drink, to which 2.20 (range: 2.03-2.31) mmol ^{25}Mg and 25.4 (range: 25.3-26.5) μmol ytterbium (as $\text{YbCl}_3 \cdot 6\text{H}_2\text{O}$, Aldrich, Buchs, Switzerland) were added in solution. The amount intake of isotope labels and ytterbium was determined by weighing.

Study design

Urine and faecal spot samples were collected on the day before test meal administration to determine baseline magnesium isotopic ratios. A gelatine capsule containing ~100 mg brilliant blue (Warner Jenkinson Europe, King`s Lynn, UK) was administered orally to mark the start of the faecal collection period. Venous blood samples (10 ml) were drawn into heparinised glass tubes (Vacutainer Systems, Plymouth, UK) for plasma magnesium analysis.

The test meal was served after an overnight fast. Subjects were instructed to eat half of the bread before drinking the labelled water in order to slow down gastrointestinal passage of the isotopic label. Fifteen min after test meal intake, 0.60 (range: 0.57-0.65) mmol ²⁶Mg were administered intravenously. A sterile injection system consisting of a 2-way catheter and a septum injection port was used. The isotope solution was transferred quantitatively by flushing the system with physiological saline (10 ml). The amount of injected isotopic label was determined by weighing the syringe before and after injection. No food or drink was allowed for 3 h following breakfast. Standardised meals were provided for lunch (lasagne) and dinner (pizza) on day 1. Water (2 L) was provided as the only beverage for day 1. No additional food was allowed on day 1. From day 2 onwards, diet was unrestricted. On day 7, a second brilliant blue capsule was administered to mark the endpoint of the faecal collection period. On day 14, a venous blood sample (10 ml) was drawn into a heparinised glass tube for magnesium isotopic analysis.

All stool samples were collected separately, starting immediately after test meal administration. Faeces was collected until the second brilliant blue marker appeared. Urine was collected in parallel in 24 h pools until the end of day 6.

Urine, stool and blood sampling

Urine samples were collected in pre-weighed polyethylene containers (Semadeni, Ostermündingen, Switzerland). Each 24 h pool was weighed, a 50 g aliquot was removed and acidified with 0.5 g 10 mol/L HCl before storage (–25 °C). Six day urine pools were prepared by combining 1% of each 24 h pool by weight.

Stools were collected in pre-weighed polypropylene containers (Semadeni), freeze dried (Modulyo, Edwards, North Bergen, NJ, USA), weighed, and ground in a mortar. Two stool pools were prepared:

- a) 6 day stool pools: all stools collected from the appearance of the first brilliant blue marker until but not including the stool dyed by the second brilliant blue marker (given on day 7). For preparation of the pools, 5% of each stool were taken by weight, combined, and thoroughly mixed.
- b) 3-stool pools: the first 3 consecutive stools after the appearance of the first brilliant blue marker, including the first dyed stool, were pooled by combining 5% of each stool by weight.

Venous blood samples (10 ml) were centrifuged at 20 °C at 500 x g (Omnifuge 2.0 RS, Heraeus, Zurich, Switzerland) to separate plasma from blood cells. Blood cells were washed 3 times with small portions (2-3 ml) of physiological saline. Plasma and washed blood cells were stored in acid washed polyethylene vials (-25 °C) for later analysis.

Sample mineralization

Freeze-dried bread (1 g), acidified urine (3 ml), blood plasma (1 ml) and blood cells (1 ml) were mineralised by microwave digestion (MLS 1200, MLS GmbH, Leutkirch, Germany), using a mixture of 14 mol/L HNO₃ and 8.8 mol/L H₂O₂ (Merck). Faecal samples were dry ashed in covered pyrex glass beakers in a muffle furnace (M 110, Heraeus) at 550 °C for 12 h after addition of 5 ml 14 mol/L HNO₃. Ashes were dissolved in 10 ml 5 mol/L HCl. Beakers were discarded after use.

Sample preparation for isotopic analysis

All samples were analysed in duplicate. Magnesium was separated from the mineralised samples by cation-exchange chromatography using a strongly acidic ion-exchange resin (AG 50W X-8, 200-400 mesh, Bio-Rad, Hercules, CA, USA). Aliquots of the mineralised stool, urine- and blood cell samples, containing ~8.2 µmol magnesium (faecal samples) or 2.5 µmol magnesium (urinary and blood samples), were evaporated to dryness and redissolved in 1 ml 0.7 mol/L HCl and

transferred onto the top of a column (Bio-Rad, 1 cm inner diameter), filled with the ion-exchange resin to a height of 7 cm. The column was rinsed with 56 ml 0.7 mol/L HCl, followed by 24 ml 0.9 mol/L HCl to elute sodium and potassium. Magnesium was eluted with 12 ml 1.4 mol/L HCl. The solution was evaporated to dryness and redissolved in 50 μ l water. Magnesium recovery was evaluated with a diluted magnesium standard solution (Merck, Titrisol) and was found to be $94.8 \pm 1.8\%$ ($n=10$). Resins were regenerated with 30 ml 6 mol/L HCl and renewed after the fifth run. Isotopic and elemental analysis was performed under blank control. Only acid washed teflon and polyethylene labware was used for sample processing. Aliquots of the ^{26}Mg isotopic label were processed in parallel with each batch for blank monitoring from ion exchange chromatography onwards. Sample contamination due to natural magnesium was found to be 10.3 ± 4.1 nmol ($n=6$) for combined sample preparation and filament loading, which is $< 0.5\%$ of the magnesium separated.

Isotopic analysis by TIMS

About 20 nmol separated magnesium from faecal, blood, and urinary samples, respectively, was loaded onto the metal surface of the evaporation filament of a double-rhenium filament ion source. Magnesium was coated with 5-10 μ g silicagel 100, 0.8 μ mol boric acid and 30 nmol aluminium as AlCl_3 (all chemicals from Merck). Compounds were loaded in aqueous solution and dried at 0.8 A after each step. Finally, the evaporation filament was heated to dull red heat (1.6 A) for 30 s. The ionisation filament remained unloaded. Isotopic ratios were determined with a single-focussing magnetic sector field instrument (MAT 262, Finnigan MAT, Bremen, Germany), equipped with a Faraday cup multicollector device for simultaneous ion beam detection. The evaporation filament was heated to 1230°C , using a standardised procedure. The ionisation filament was heated gradually to $1250\text{-}1350^\circ\text{C}$ until a stable Mg^+ ion beam of $1\text{-}2 \times 10^{-11}\text{A}$ was obtained. Each measurement consisted of 30 consecutive isotopic ratio measurements.

Reproducibility (5 independent runs) was $\pm 0.2\%$ (relative SD) for the $^{24}\text{Mg}/^{25}\text{Mg}$ isotopic ratio and $\pm 0.4\%$ for the $^{24}\text{Mg}/^{26}\text{Mg}$ isotopic ratio.

Isotopic analysis by ICP-MS

All ICP-MS (inductively coupled plasma mass spectrometry) measurements were carried out using a magnetic sector ICP-MS (IsoProbe, Micromass, Manchester, UK) equipped with a multi-collector system of nine Faraday cups. The sample introduction system consisted of a Micromist nebulizer and a Cinnabar spray chamber (both Glass Expansion, Romainmotier, Switzerland). The instrument utilises a hexapole collision cell for collisional focussing and interference reduction. Hydrogen (1 ml/min) and helium (7.5 ml/min) were used as collision gasses.

Further studies using the high resolution mode of the instrument revealed an interference at mass 26. This interference remained stable even with 100 µg/g NaCl added to the measurement solution. No matrix effects on the measured isotopic ratios were observed. The interference on mass 26 was therefore corrected by the external blank subtraction.

The measurement sequence for each enriched sample was NBS980 (standard reference material 980, National Institute of Standards and Technology, Gaithersburg, MD, USA), baseline sample, enriched sample, baseline sample and finally again NBS980. Drifts in mass discrimination were corrected by assuming a linear drift between the two measurements of the isotopic reference material. Reproducibility (6 independent runs) was ±0.01% (relative SD) for the $^{24}\text{Mg}/^{25}\text{Mg}$ isotopic ratio and ±0.02% for the $^{24}\text{Mg}/^{26}\text{Mg}$ isotopic ratio.

More details about the applied ICP-MS procedure can be obtained from Klingbeil *et al.* (2001).

Magnesium, ytterbium, and phytic acid

Total magnesium content of stool pools, plasma, and bread rolls was determined after dilution of the mineralised samples by flame atomic absorption spectroscopy (F-AAS, SpectrAA 400, Varian, Mulgrave, Australia), using standard procedures. A commercial magnesium standard (Titrisol, Merck) was used for internal calibration (standard addition technique) to minimise matrix effects. Certified reference materials (Wheat Flour 1567 a, National bureau of standards, Gaithersburg, MD, USA and Seronorm Trace Elements Serum, Nycomed, Oslo, Norway) were analysed in parallel for quality control. Ytterbium was measured in the mineralised

and diluted faecal samples by electrothermal atomic absorption spectroscopy (ET-AAS) using an external calibration technique against an ytterbium standard solution (Titrisol, Merck).

For phytic acid analysis, wheat bread rolls were freeze dried and ground in a mortar. Phytic acid was extracted from a ~1 g aliquot with 0.5 mol/L HCl. The obtained solution was purified by anion exchange chromatography, evaporated to dryness, and redissolved in water prior to HPLC reversed phase chromatography using a refractory index detector (Sandberg & Ahderinne, 1986).

Calculations

Molar amounts and ratios of the ^{25}Mg and ^{26}Mg isotopic label in the samples were calculated based on double isotope dilution principles (Walczyk *et al.*, 1997; Sabatier *et al.*, 2002). Magnesium absorption was calculated by 5 different techniques based on the molar amounts and molar amount ratios of the isotope labels:

Technique 1: Fractional true absorption (TA) based on 6 day pools or on 24 h (collected 22-46 h after test meal intake) urine pools, respectively:

$$\text{TA}(\%) = \frac{n_{25}}{n_{26}} \cdot \frac{D_{iv}}{D_0} \cdot 100 \quad (1)$$

where n_{25}/n_{26} is the amount ratio of isotopic labels in urine, D_{iv} the amount isotope label injected and D_0 the amount oral isotope label (μmol).

Technique 2: True fractional absorption (%) based on a blood cell sample drawn 14 days after isotope label administration. Absorption is calculated using eqn. (1), with n_{25}/n_{26} equivalent to the amount ratio of the isotopic labels in the blood cell fraction.

Technique 3: Fractional apparent absorption (AA) in % was calculated based on 6 day stool pools as the difference between the oral dose given and the amount of oral label excreted (μmol) in faeces (F_0).

$$\text{AA}(\%) = \frac{D_0 - F_0}{D_0} \cdot 100 \quad (2)$$

Technique 4: True absorption (%) was derived from AA by correcting for the amount of oral isotopic label that was absorbed and rapidly excreted, based on the amount

(μmol) of the intravenous label F_{iv} recovered in the 6 day and the 3-stool pools, respectively.

$$\text{TA}(\%) = 100 \cdot \left(\frac{(D_0 - F_0)/D_0}{1 - F_{\text{iv}}/D_{\text{iv}}} \right) \quad (3)$$

Technique 5: Fractional apparent absorption AA was based on the first 3 consecutive stools after appearance of the first brilliant blue marker, including the first dyed stool. Incomplete recovery of the oral isotopic label was corrected for by using the fraction of rare earth element recovered in faeces (%REE).

$$\text{AA}(\%) = 100 \cdot \left(1 - \frac{F_0}{D_0} \cdot \frac{100}{\% \text{REE}} \right) \quad (4)$$

Statistics

Calculations were performed using commercial spreadsheet software (Excel 97, Microsoft, Chicago, Illinois, and SPSS 10.0, SPSS inc., Chicago, Illinois). Analysis of variance (ANOVA) was made using a general linear model, followed by either a Bonferroni test or Student's paired, 2-tailed t-test to determine differences between methods. Statistical significance was considered for $P < 0.05$. Correlation between methods was evaluated by Pearson correlation coefficients. Normal distribution of absorption values was verified by skewness and Kolmogorov-Smirnoff test. Homogeneity of variance between the methods was verified with Levene's test. Absorption values are presented as arithmetic mean \pm SD.

Results

One subject was excluded from the 3-stool pool evaluation due to gastrointestinal problems, and one subject was excluded from the double isotope evaluations due to incomplete intravenous injection. Plasma concentrations of magnesium were 1.02 ± 0.09 (range 0.92-1.22, $n=10$) mmol/L, reported normal range: 0.75-0.96 mmol/L (Lowenstein & Stanton, 1986). Native magnesium content of the wheat bread was 1.91 mmol per serving. Phytic acid content was below the detection limit ($< 0.5 \mu\text{mol}$ phytic acid/100 g).

Fractional magnesium absorption

Mean magnesium absorption from the wheat bread consumed with water varied from 32.9% to 44.2% (Table 1) based on the different techniques. Apparent absorption values differed significantly from true absorption values (6 day stool pools: $P=0.001$; 3-stool pools: $P=0.01$). True absorption based on the 6 day urine pools was significantly lower than true absorption calculated using the other techniques (Bonferroni, $P=0.002$). There were no significant differences between true absorption values based on the 24 h urine pools (22-46 h), blood cell incorporation, and the 6 day faecal pools or 3-stool pools.

Brilliant blue appeared in faeces on day 1 or 2 in all subjects. Completeness of faecal collection was evaluated by the recovery of ytterbium; $98.7\pm 5.6\%$ ($n=10$, range 91.3-107.9%) was recovered in the 6 day stool pools. Excretion of the ^{25}Mg label and ytterbium, measured in the 3-stool pools, was significantly correlated (Fig. 1). Ytterbium recovery in the 3-stool pools, excreted within 3.0 ± 0.9 days after intake of the isotope labels, was $80.5\pm 18.5\%$ ($n=9$, range 55.2-101.0%). Apparent and true absorption determined by faecal monitoring based on the 6 day stool pools and the 3-stool pools were significantly correlated ($r=0.76$, $P<0.05$; $r=0.83$, $P<0.05$) as was true absorption based on 6 day urine- and 24 h urine pools ($r=0.73$, $P<0.05$). Magnesium absorption based on the blood cell incorporation technique was significantly correlated only to absorption based on the 24 h urine pools ($r=0.78$, $P<0.05$).

Table 1

Fractional magnesium absorption based on different techniques. Mean isotopic enrichments (%) over baseline values (\pm SD) in the sample material are given for the $^{24}\text{Mg}/^{25}\text{Mg}$ ($\Delta(^{24}\text{Mg}/^{25}\text{Mg})$) and the $^{24}\text{Mg}/^{26}\text{Mg}$ ($\Delta(^{24}\text{Mg}/^{26}\text{Mg})$) isotope ratio, respectively.

subject	faecal monitoring				urinary monitoring			blood cell incorporation true absorption
	apparent absorption		true absorption		true absorption			
	6 d pool	3-stool pool	6 d pool	3-stool pool	6 d pool	24 h pool*	d 14	
1	39.7	34.2	51.1	42.8	26.2	37.9	34.7	
2	52.7	47.1	63.6	52.8	43.8	50.0	51.4	
3	44.4	43.8	47.8	50.1	43.3	55.5	49.4	
4	36.3	36.0	40.5	37.2	28.3	34.0	38.8	
5	29.9	36.7	32.4	39.0	24.5	45.0	45.8	
6	36.9	41.0	48.5	41.6	35.2	51.2	48.7	
7	32.5	39.3	38.9	40.2	29.9	39.0	30.0	
8	30.1	29.2	ND	ND	ND	ND	ND	
9	38.1	ND	48.8	ND	33.4	39.1	44.9	
10	22.8	34.3	25.7	36.7	31.8	36.9	37.9	
Mean \pm SD	36.3 \pm 8.4 ^A	38.0 \pm 5.5 ^A	44.2 \pm 11.2 ^B	42.6 \pm 5.9 ^B	32.9 \pm 6.9 ^C	43.2 \pm 7.6 ^B	42.4 \pm 7.4 ^B	
$\Delta(^{24}\text{Mg}/^{25}\text{Mg})$	15.2 \pm 3.1	28.2 \pm 12.4	15.2 \pm 3.1	28.2 \pm 3.1	5.6 \pm 1.0	6.4 \pm 1.1	1.7 \pm 0.2	
$\Delta(^{24}\text{Mg}/^{26}\text{Mg})$	0.84 \pm 0.50	0.95 \pm 0.50	0.84 \pm 0.50	0.95 \pm 0.50	4.4 \pm 0.5	3.9 \pm 0.5	1.0 \pm 0.2	

ND: not determined

* based on urine collected 22-46 hours after test meal intake

^{A, B, C} differences between mean absorption values with identical superscripts are statistically non-significant (P>0.05)

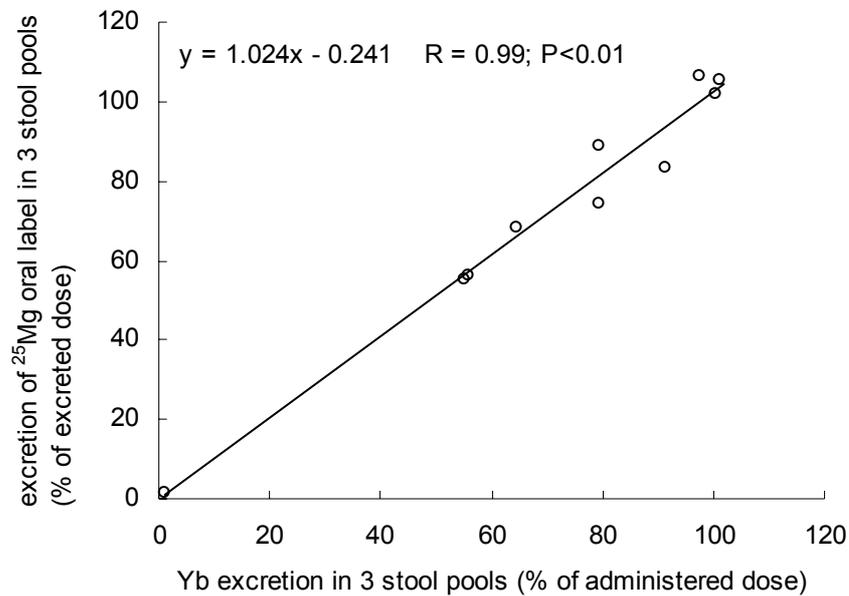


Fig. 1

Correlation between fractional excretion of the oral isotopic label (²⁵Mg) and the quantitative fecal marker (ytterbium) in 3-stool pools. Data based on 10 subjects.

Magnesium isotopic variation in sample material

Baseline magnesium isotopic ratios in blood cells of 10 subjects were higher by 2.1 ± 0.3 ‰ (²⁵Mg/²⁴Mg) and 3.6 ± 0.8 ‰ (²⁶Mg/²⁴Mg) than the isotopic reference material (standard reference material 980), ($P = 0.00001$, $n = 10$).

Discussion

Our results demonstrate for the first time that the amount ratio of orally ingested and intravenously administered magnesium stable isotope labels in blood cells are potentially useful techniques to determine magnesium absorption in humans. Similar results were found for the 24 (22-46) h urine pools. In addition, we have shown that the shorter 3-stool pool method, corrected for incomplete faecal collections by excretion of ytterbium, resulted in similar Mg absorption as the 6 day faecal

monitoring method. It would therefore seem possible that any of these alternative methods could be used to monitor dietary factors influencing magnesium absorption, at least in a healthy young study population. The only method which resulted in significantly lower mean absorption data than the 6 day faecal collections was that based on the 6 day urine pools (Table 1).

Blood cells are easily accessible and, without the need for complete faecal or urine collections, blood cell incorporation of isotope labels would greatly reduce time and labour needed to determine magnesium absorption. The method assumes that, after absorption, the isotope label added as an extrinsic tag to a meal is metabolised in an identical way to an isotope label injected intravenously. Although injecting the isotope label intravenously as a bolus 15 min after consumption of a simple test meal appeared satisfactory in our studies, timing and duration of the infusion needs further evaluation with more complex meals. Iron absorption is routinely determined using erythrocyte incorporation of a single orally administered isotope label, assuming a mean erythrocyte incorporation of 80% after 14 days (Cook *et al.*, 1972). In our studies, about 1% of the absorbed oral dose was recovered in blood cells. Although further studies are needed to validate blood cell incorporation as a method for determination of magnesium absorption, this approach is supported by an earlier study in rats which reported no significant difference in absorption based on erythrocytes, plasma, and urine samples collected 48 h (erythrocytes and plasma) or 36-48 h (urine) after oral and intravenous isotope label administration (Coudray *et al.*, 1997). However, this technique would require precise measurements of magnesium isotopic ratios of about 0.1% external RSD.

True absorption was also determined in the present study based on the amount ratio of an oral and an intravenous isotope label excreted in urine. Calcium absorption is frequently measured by urine monitoring using either 24 h urine pools or a spot urine sample collected 24 h after stable isotope label administration (Lee *et al.*, 1994; van Dokkum *et al.*, 1996). In the present study, urine monitoring to determine magnesium absorption gave conflicting results. True magnesium absorption based on 6 day urine pools was significantly lower compared to all other techniques of true absorption, including the 24 h urine pools (Table 1). Rauscher & Fairweather-Tait (1997) have reported significantly ($P < 0.05$) lower zinc absorption by humans based on 5 day urinary pools versus 5 day faecal pools, and recently

Sabatier (2001) reported significantly lower magnesium absorption based on 3 day (days 1-3) urine pools compared to 12 day faecal pools. It has been suggested that absorption is underestimated based on urine collected during the first hours after isotope label administration because oral and intravenous labels are metabolised differently and absorption of the oral isotope label might not yet be complete (Yergey *et al.*, 1987; Shames *et al.*, 2001). Including the first 24 h pools in our 6 day urine pools could thus explain the lower absorption value generated by the 6 day pools. In addition, a potential concern is the administration of the intravenous magnesium label. Serum magnesium levels are strictly regulated by the kidney (Elin, 1994). Thus, if the intravenous bolus results in a non-physiologically high level of free magnesium in the blood, excess magnesium may be rapidly excreted in urine, and the amount ratio of oral and injected isotope label in urine will underestimate absorption. To overcome this problem, Coudray *et al.* (1997) suggested that the intravenous dose should be infused over a long period of time or divided into several smaller doses. Our findings indicate that a single intravenous bolus administered 15 min after the oral isotope label is suitable to determine magnesium absorption provided urine collection is initiated later than about 20 h after isotope label administration. Similar observations were made for calcium (Yergey *et al.*, 1987; Yergey *et al.*, 1994), zinc (King *et al.*, 1997), and magnesium (Sabatier, 2001). It is assumed that after about 20 h, absorption based on the amount ratio of both isotope labels in urine corresponds to true absorption as determined by 6 day faecal collections. It should be noted however that in absorption studies with calcium, the optimum time point for urine collection may differ between subjects (Yergey *et al.*, 1987) and also depends on the test meal composition (Griessen *et al.*, 1985).

The conventional method for measuring magnesium absorption, based on faecal monitoring of all non-absorbed isotope labels, is time consuming and laborious. To determine true absorption by this technique, a second intravenous isotope label is necessary to estimate the amount of absorbed oral isotope label that is re-excreted during the faecal collection period. This makes the method as invasive and expensive as urinary monitoring or blood cell incorporation of isotope labels. For screening purposes however, determination of apparent absorption by faecal monitoring seems suitable. Although true magnesium absorption is significantly

underestimated by this method (Table 1), the technique is less invasive and cheaper as administration of an intravenous isotope label is not needed.

A major technical problem with faecal monitoring is the incomplete recovery of non-absorbed oral isotope label, either due to incomplete faecal collection by the subjects or due to prolonged gastrointestinal passage, resulting in an overestimate of absorption (Shames *et al.*, 2001). This source of error was minimised in our study by the use of brilliant blue to determine the starting point and the end point of the faecal collection period, and by ytterbium as a non-absorbable faecal marker. Our data indicate that faecal collections could be shortened from 6 days to 3 consecutive stools after excretion of the first dose of brilliant blue by correcting for incomplete recovery of isotopic label using a rare earth element. In the 3-stool pools, excretion of the oral magnesium isotope label and ytterbium was significantly correlated (Fig. 1), and apparent and true absorption determined by both techniques did not differ significantly (Table 1). Gastrointestinal passage time of the oral magnesium isotope label and ytterbium were relatively fast and the minimum recovery was 55% in the 3-stool pools. Schuette *et al.* (1993) suggested that at least 40% of the rare earth element should be excreted in order to correct for incomplete collection, using dysprosium as a faecal marker. Although it can be assumed that many of the rare earth elements exhibit similar excretion patterns (Fairweather-Tait *et al.*, 1997), we chose ytterbium over dysprosium or samarium because it could be detected with higher sensitivity by ET-AAS. In order to apply a correction by using a non absorbable faecal marker, excretion patterns of magnesium and the rare earth elements have to be similar. Based on data from the present study, the shorter stool collection technique is well suited for studies in young, healthy adults.

Natural isotopic abundances of an element can be altered, in principle, by physiological processes (Galimov, 1985). Using TIMS, no such effects were observed for magnesium in faecal and urine baseline samples relative to a magnesium standard of non biological origin (Titrisol, Merck), but the more precise multicollector ICP-MS measurements revealed significant differences in magnesium isotopic composition between blood cells and an isotopic reference material. This problem was overcome by measuring the enriched sample together with the baseline sample for each subject.

In conclusion, our study showed that the amount ratio of orally administered and intravenously injected magnesium isotope labels in blood cells 14 days after isotope label administration or in 24 h urine pools, collected 22-46 h after isotope label administration, could be useful alternative methods to determine magnesium absorption from food. If isotopic ratios can be measured at high precision ($<0.1\%$), blood cell incorporation offers a relatively simple technique avoiding faeces and urine collections. In addition, in a young healthy study population, the 6 day faecal monitoring technique can be shortened to 3 consecutive stool pools by including ytterbium as a non absorbable faecal marker.

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2.2. Phytic acid added to white wheat bread decreases apparent magnesium absorption in man

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Running head: Phytic acid decreases magnesium absorption

Abstract

Background. Phytic acid has been reported to impair absorption of minerals and trace elements such as calcium, zinc, and iron in humans. Limited information is available on the effect of phytic acid on magnesium absorption.

Objective. To evaluate the effect on magnesium absorption in humans of added phytic acid to white wheat bread in amounts similar to those naturally present in brown bread and wholemeal bread.

Design. Two stable isotope studies were performed, each in 8-9 healthy adults. Magnesium absorption from 200 g phytic acid-free bread prepared from 150 g white wheat flour with added phytic acid (1.49 mmol, study 1; or 0.75 mmol, study 2) was compared to no added phytic acid. Test meals containing phytic acid and the phytic acid free control meals were served on days 1 and 3, following a randomized order, with each subject acting as his/her own control. Each test meal was labeled with 0.7 mmol ²⁵Mg or 1.1 mmol ²⁶Mg. Total magnesium content was 3.6 mmol in all test meals. Apparent magnesium absorption was based on fecal monitoring.

Results. Adding 1.49 mmol and 0.75 mmol phytic acid decreased fractional apparent magnesium absorption from 32.5±6.9% to 13.0±6.9% ($P<0.0005$) and from 32.2±12.0% to 24.0±12.9% ($P<0.01$), respectively. The decrease in magnesium absorption depended on the amount phytic acid added ($P<0.005$).

Conclusion. Magnesium absorption from white wheat bread decreased significantly by adding phytic acid at concentrations similar to those naturally present in brown and wholemeal bread. The inhibition of absorption depended on the amount phytic acid added.

Key words: Magnesium absorption, phytic acid, wheat bread, stable isotopes, fecal monitoring

Introduction

Phytic acid, myo-inositol hexakisphosphate, is widely distributed in nature as it is the major storage form of phosphorus in cereals, legumes, and oilseeds (Harland & Oberleas, 1987). It is typically found in the outer (aleurone) layers of cereal grains and in the endosperm of legumes and oilseeds. Wholemeal cereal flour or bran-based products contain high concentrations of phytic acid, typically in the range of 0.5-1.4% for wholemeal bread and up to 3.5% for bran (Reddy *et al.*, 1989). About 200-800 mg phytic acid/d are consumed in industrialized countries, compared to about 2 g/d in developing countries (Plaami, 1997). In Western diets, wholemeal cereal products are the major source of phytic acid.

Phytic acid has been shown to decrease absorption of many of the nutritionally relevant minerals and trace elements in humans, including iron (McCance *et al.*, 1943; Hallberg *et al.*, 1987), zinc (Turnlund *et al.*, 1984; Navert *et al.*, 1985), calcium (Reinhold *et al.*, 1973; Heaney *et al.*, 1991), and manganese (Davidsson *et al.*, 1995). It is generally assumed that insoluble, non absorbable complexes of phytic acid and minerals are formed in the small intestine. However, information is scarce on the effects of phytic acid on magnesium absorption. It has been reported, based on chemical balance studies, that negative magnesium balances correlate with dietary phytic acid intake (in 2 human subjects) (Reinhold *et al.*, 1976), and that magnesium absorption was significantly impaired when phytic acid was added to white wheat bread (McCance & Widdowson, 1942). In addition, balance studies have indicated significantly increased magnesium absorption after dephytinization of bran muffins (Morris *et al.*, 1988). However, chemical balance technique does not permit determination of absorption from single test meals and thus the influence of a potential enhancer or inhibitor on absorption cannot be measured directly.

The aims of the present study were to evaluate the effect on magnesium absorption of adding phytic acid to white wheat bread at concentrations similar to that naturally present in brown and wholemeal wheat bread. Magnesium absorption was evaluated by a stable isotope technique based on extrinsic labeling of the meals and fecal monitoring of the excreted label.

Subjects and methods

Subjects

Apparently healthy, free-living subjects (20 men and women) were recruited. No lactating or pregnant women were included in the study. No medication was allowed during the study except for oral contraceptives. Intake of mineral/vitamin supplements was not permitted 2 weeks before the start of the study and during the entire study. The subjects were informed about the aims and the procedures of the study orally and in written form and written informed consent was obtained. The participants were instructed not to change their dietary habits or life-style during the study. The study protocol was reviewed and approved by the Ethical Committee at the Swiss Federal Institute of Technology, Zurich.

Isotopic labels

Highly enriched ^{25}MgO ($1.04\pm 0.01\%$ ^{24}Mg , $98.73\pm 0.01\%$ ^{25}Mg and $0.23\pm 0.01\%$ ^{26}Mg) and ^{26}MgO ($0.39\pm 0.01\%$ ^{24}Mg , $0.11\pm 0.01\%$ ^{25}Mg and $99.50\pm 0.01\%$ ^{26}Mg) labels were purchased from Chemotrade, Düsseldorf, Germany. The enriched ^{25}Mg label (28 mmol as ^{25}MgO) and ^{26}Mg label (43 mmol as ^{26}MgO) were dissolved in 10 ml 4 mol/L HCl and diluted to 100 ml with water. Solid NaHCO_3 (Merck, Darmstadt, Germany) was added to adjust to pH 6. The concentration of the ^{25}Mg and ^{26}Mg isotope label in solution was determined by isotope dilution mass spectrometry against a commercial magnesium standard of natural isotopic composition (Titrisol, Merck). Unless otherwise specified, all chemicals were of analytical grade, and acids were further purified by surface distillation. Only 18 M Ω water (Milli Q water system, Millipore, Zurich, Switzerland) was used for laboratory work and test meal preparation.

Test meals

The composition of the meals is shown in Table 1. All test meals were based on phytic acid-free wheat bread rolls. The rolls were prepared in batches by mixing 1 kg white wheat flour (Migros, Zurich, Switzerland) with water (600 g), salt (10 g), white

sugar (32 g) and dry yeast (15 g). The dough was left to ferment for 5 h at room temperature. Bread rolls were prepared from dough, baked for 15 min at 200 °C, and stored frozen (-25°C).

Individual servings were weighed and frozen (-25 °C) immediately after preparation. The test meals were standardized for their magnesium content by adding a solution of MgCl₂ (Merck). Phytic acid in its dodecasodium form (Sigma, Buchs, Switzerland) was dissolved in water to a concentration of 73.5 mmol/L, and aliquots were added to the meals 1 h before administration. Weighed aliquots of the isotope label solution were pipetted onto the test meals 1 h before (wheat bread).

The rare earth elements ytterbium and europium were used as nonabsorbable fecal markers. Both compounds (in chloride form, Aldrich, Buchs, Switzerland), were added to 300 ml 18 MΩ water given at breakfast and lunch.

Study design

The day before isotope administration, a fecal sample was collected to determine baseline magnesium isotope ratios. In addition, brilliant blue (100 mg, Warner Jenkinson Europe, King`s Lynn, UK), a dye used as a fecal marker, was given in a gelatin capsule to indicate the start of the fecal pooling. After an overnight fast, a venous blood sample (10 ml) was drawn into a heparinized glass tube (Vacutainer Systems, Plymouth, UK) for determination of magnesium concentration in plasma. Plasma was separated from blood by centrifugation (Omnifuge 2.0 RS, Heraeus, Zurich, Switzerland) at 20°C and ~500 x g (5 min), and stored in acid washed plastic vials at -25 °C.

Each subject acted as his/her own control, receiving both the phytic acid and the phytic acid free meal. Test meals A and B within each study (Table 1) were randomly allocated to be served on day 1 or day 3.

Study 1 and study 2: Test meal A consisted of 4 wheat bread rolls (200 g bread) prepared from 150 g flour, to which the phytic acid solution and the ²⁵Mg label solution were added. Test meal B (without added phytic acid) consisted of 4 wheat bread rolls with added ²⁶Mg label. In addition, 600 ml 18 MΩ water was served to which the rare earth elements were added. The test meals were divided into 2 identical portions served at 7.30-8.30 and again at 12.00-13.00 on the same day.

No food or drinks were allowed between breakfast and lunch on days 1 and 3 and for 3 h following lunch. Standardized meals (pizza) were provided for dinner, and white wheat crisp bread as an afternoon and evening snack. Water (18 MΩ, 2 L) was the only permitted beverage on days 1 and 3. No additional food or drinks were allowed on days 1 and 3. Diet was unrestricted at all other times.

Pre-weighed polypropylene containers (Semadeni, Ostermündingen, Switzerland) were provided for stool collections. The subjects collected all stools separately, starting immediately after intake of the first test meal on day 1. On day 8, a second brilliant blue capsule was given. Collections were continued until excretion of the second brilliant blue marker. Stool samples were stored frozen (−25 °C) until processed.

Preparation of fecal pools and mineralization

Each individual stool sample was freeze-dried (Modulyo, Edwards, North Bergen, NJ, USA) and ground to a powder in a mortar. All stools, from the first stool dyed by the first brilliant blue marker until, but not including, the stools dyed by the second marker, were included in the fecal pool. After a drying step in a drying chamber (Binder, Tuttlingen, Germany) for 20 h at 65 °C to standardize humidity, and cooling at room temperature for 4 h, all individual stools were weighed and all stools included in one pool were milled individually in a mill (ZM1, Retsch, Haan, Germany), equipped with a sieve of 1 mm pores, starting with the first (most enriched) samples. Each milled stool was transferred back into its original container, dried again for 20 h at 65 °C, cooled for 4 h at room temperature and re-weighed to determine losses. All milled stools included in a single pool were combined in a 2 L polyethylene container (Semadeni) and mixed for 90 min using a rotator (Micro Motor, UG 70/20, Basel, Switzerland).

Aliquots of the freeze dried pooled stool samples (1.0-1.6 g), the freeze dried wheat bread (0.25 g), and plasma (1 g), were mineralized in a microwave digestion system (MLS 1200, MLS GmbH, Leutkirch, Germany) in a mixture of 14 mol/L HNO₃ and 8.8 mol/L H₂O₂ (Merck). All samples were mineralized in duplicate.

Separation of magnesium

Magnesium was separated from the mineralized stool samples by cation-exchange chromatography using a strongly acidic ion-exchange resin (AG 50W X-8, 200-400 mesh, Bio-Rad, Hercules, CA, USA). Aliquots containing about 30 μmol magnesium were evaporated to dryness, redissolved in 1 ml 0.7 mol/L HCl and transferred onto the top of a column (Bio-Rad, 1 cm inner diameter), filled with the ion-exchange resin to a height of 7 cm. The column was rinsed with 56 ml 0.7 mol/L HCl, followed by 24 ml 0.9 mol/L HCl to elute sodium and potassium. Magnesium was eluted with 12 ml 1.4 mol/L HCl. The solution was evaporated to dryness and redissolved in 50 μl water. Magnesium recovery, evaluated with a diluted magnesium standard solution (Titrisol, Merck) was found to be $94.8 \pm 1.8\%$ ($n=10$). Resins were regenerated with 30 ml 6 mol/L HCl and replaced after the fifth run. Only acid washed teflon and polyethylene labware was used for sample processing. Aliquots of the ^{26}Mg isotope label were processed in parallel with each batch for blank monitoring from ion exchange chromatography onwards. Sample contamination due to natural magnesium was found to be 10.8 ± 7.0 nmol ($n=9$) for combined sample preparation and filament loading, which was $< 0.4\%$ of the amount magnesium separated.

Isotopic analysis by thermal ionization mass spectrometry (TIMS)

About 20 nmol separated magnesium from fecal samples was loaded onto the metal surface of the evaporation filament of a double-rhenium filament ion source. Magnesium was coated with 5-10 μg silicagel 100, 0.8 μmol boric acid and 30 nmol aluminum as AlCl_3 (all chemicals from Merck). Compounds were loaded in aqueous solution and dried at 0.8 A after each step. Finally, the evaporation filament was heated to dull red heat (1.6 A) for 30 s. The ionization filament remained unloaded. Isotope ratios were determined with a single-focusing magnetic sector field instrument (MAT 262, Finnigan MAT, Bremen, Germany), equipped with a Faraday cup multicollector device for simultaneous ion beam detection. The evaporation filament was heated to 1230 $^\circ\text{C}$, using a standardized procedure. The ionization filament was heated gradually to 1250-1350 $^\circ\text{C}$ until a stable Mg^+ ion beam of $1\text{-}2 \times 10^{-11}\text{A}$ was obtained. Each measurement consisted of 30 consecutive isotope ratio measurements.

Reproducibility (5 independent runs) was $\pm 0.2\%$ (relative SD) for the $^{24}\text{Mg}/^{25}\text{Mg}$ isotope ratio and $\pm 0.4\%$ for the $^{24}\text{Mg}/^{26}\text{Mg}$ isotope ratio.

Atomic absorption spectroscopy

Quantitative magnesium analysis of the mineralized and diluted plasma-, flour-, and fecal samples was performed by flame atomic absorption spectroscopy (F-AAS, SpectrAA 400, Varian, Mulgrave, Australia). Plasma samples were measured by external calibration using a commercial magnesium standard (Titrisol, Merck). All other samples were measured by an internal calibration technique (standard addition) to minimize matrix effects. In addition, all measured solutions contained $\text{La}(\text{NO}_3)_3$ at 5000 mg La/L to suppress matrix effects. Certified reference materials (Seronorm Trace Elements Serum, Nycomed, Oslo, Norway, and wheat flour 1567 a, National Bureau of Standards, Gaithersburg, MD, USA) were analyzed in parallel to monitor accuracy of analysis. Ytterbium and europium were measured in the mineralized and diluted fecal samples by electrothermal atomic absorption spectroscopy (ET-AAS) by external calibration, using an ytterbium/europium standard solution (Titrisol, Merck), following standard procedures.

Phytic acid

Wheat bread rolls were freeze dried and ground in a mortar. Phytic acid was extracted from a 1 g aliquot with 0.5 mol/L HCl. The obtained solution was purified by anion exchange chromatography, evaporated to dryness, and redissolved in water prior to HPLC reversed phase chromatography (Sandberg & Ahderinne, 1986).

Methods and calculations

Molar amounts and ratios of the ^{25}Mg and ^{26}Mg isotope labels in the samples were calculated based on double isotope dilution principles (Walczyk *et al.*, 1997; Sabatier *et al.*, 2002). Fractional apparent magnesium absorption (AA%) was calculated as the difference between the oral dose (D_o) and the amount oral label excreted in feces (F_o) in μmol .

$$AA(\%) = \frac{D_o - F_o}{D_o} \cdot 100$$

The recovery of the rare earth elements ytterbium and europium was used to control completeness of stool collections.

Statistics

Calculations were made using commercial software (Excel 97, Microsoft, Chicago, Illinois, US, and SPSS 10.0, SPSS inc., Chicago, Illinois, US). Results are presented as arithmetic means \pm SD. Normal distribution of absorption values was verified by skewness and Kolmogorov-Smirnoff test. Paired student's t-tests (2-tailed) were used to compare magnesium absorption from the 2 different test meals within each study. The dose effect of phytic acid on magnesium absorption was tested with an unpaired student's t-test using the absorption ratio (with/without added phytic acid) from both studies. P values <0.05 are referred to as statistically significant.

Results

Subjects and test meals

Mean age and mean BMI of the subjects were 27 \pm 12 y and 22.1 \pm 3.8 kg/m² (n=9, study 1); and 24 \pm 2 y and 21.7 \pm 1.0 kg/m² (n=8, study 2). Mean plasma magnesium concentrations were 0.77 (range 0.67-0.85) mmol/L, and 0.84 (range 0.77-0.90) mmol/L, for studies 1 and 2, respectively (reported normal range 0.75-0.96 mmol/L, Lowenstein & Stanton, 1986). Two subjects had slightly lower plasma magnesium concentrations (0.67 and 0.73 mmol/L).

Phytic acid could not be detected in the bread rolls (limit of detection <0.5 μ mol/100 g).

The magnesium content of the unlabeled bread was 0.96 \pm 0.03 mmol/100 g (n=3). The magnesium and phytic acid content of the test meals are summarized in Table 1.

Table 1.

Magnesium content (Mg), added isotope labels, added phytate (PA) content and molar ratios phytate/magnesium (PA/Mg) of the test meals ⁺

	study 1		study 2	
	test meals A	test meals B	test meals A	test meals B
total Mg (mmol)	3.63±0.04	3.64±0.05	3.63±0.04	3.65±0.03
added Mg label				
²⁵ Mg (mmol)	0.65±0.02	-	0.65±0.02	-
²⁶ Mg (mmol)	-	1.12±0.01	-	1.12±0.01
PA (mmol)	1.488±0.022	nd ⁺⁺	0.746±0.002	nd ⁺⁺
molar ratio PA/Mg	0.41	nd ⁺⁺	0.21	nd ⁺⁺
fecal markers				
ytterbium (nmol)	31.38±0.58	-	31.38±0.58	-
europium (nmol)	-	33.17±0.26	-	33.17±0.26

⁺test meals A and B in study 1 and 2 consisted of 200 g phytic acid-free white wheat bread prepared from 150 g flour. Test meals A and B in each study were divided into 2 identical portions served at breakfast and lunch on the test day.

⁺⁺: not detectable, limit of detection for phytic acid <0.5 µmol/100 g

Magnesium absorption

Addition of 1.49 mmol phytic acid to 200 g bread decreased apparent magnesium absorption significantly, from 32.5±6.9% to 13.0±6.9% (P<0.0005, **Fig 1**). One subject was excluded from the evaluation due to low recovery of ytterbium (< 85%). For all other subjects, mean ytterbium recovery was 99.5% (range: 86.4-107.1) and mean europium recovery was 101.7% (range: 89.5-116.3). Addition of 0.75 mmol phytic acid to 200 g bread decreased magnesium absorption significantly, from 32.2±12.0% to 24.0±12.9% (P<0.01, **Fig. 2**). Two subjects were excluded from the evaluation due to low ytterbium recovery. For all other subjects, mean ytterbium recovery was 105.4% (range: 97.5-115.6) and europium recovery was 96.9% (range: 90.2-120.6). The decrease in magnesium absorption was dose dependent (P<0.005).

Mean loss of fecal material during stool pool preparation, determined by weighing the pools before and after milling, was $1.9 \pm 1.1\%$. Measured mean isotopic enrichment of the stool pools, expressed as the isotopic shift of a ratio, was calculated from the difference of the measured isotope ratio of the sample and the measured natural isotope ratio of the standard (Titrisol, Merck) divided by the measured isotope ratio of the standard, and was $5.2 \pm 1.9\%$ ($^{24}\text{Mg}/^{25}\text{Mg}$) and $8.4 \pm 2.7\%$ ($^{24}\text{Mg}/^{26}\text{Mg}$).

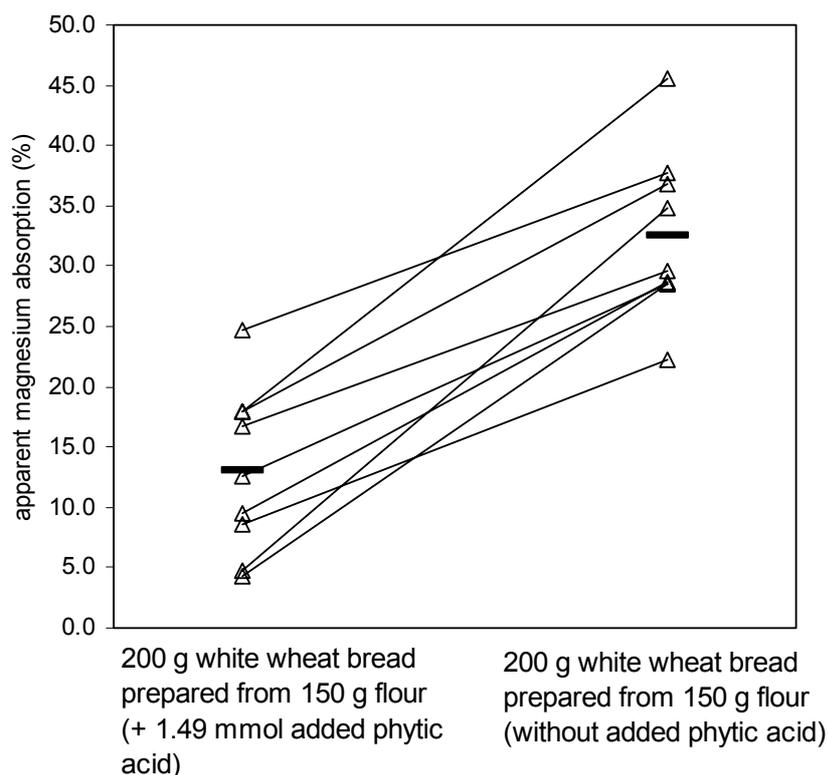


Figure 1.

Apparent magnesium absorption from 200 g white wheat bread prepared from 150 g flour with 1.49 mmol added phytic acid versus 200 g white wheat bread (no phytate added) in 9 subjects. Each triangle represents one subject, the mean is indicated by the dash.

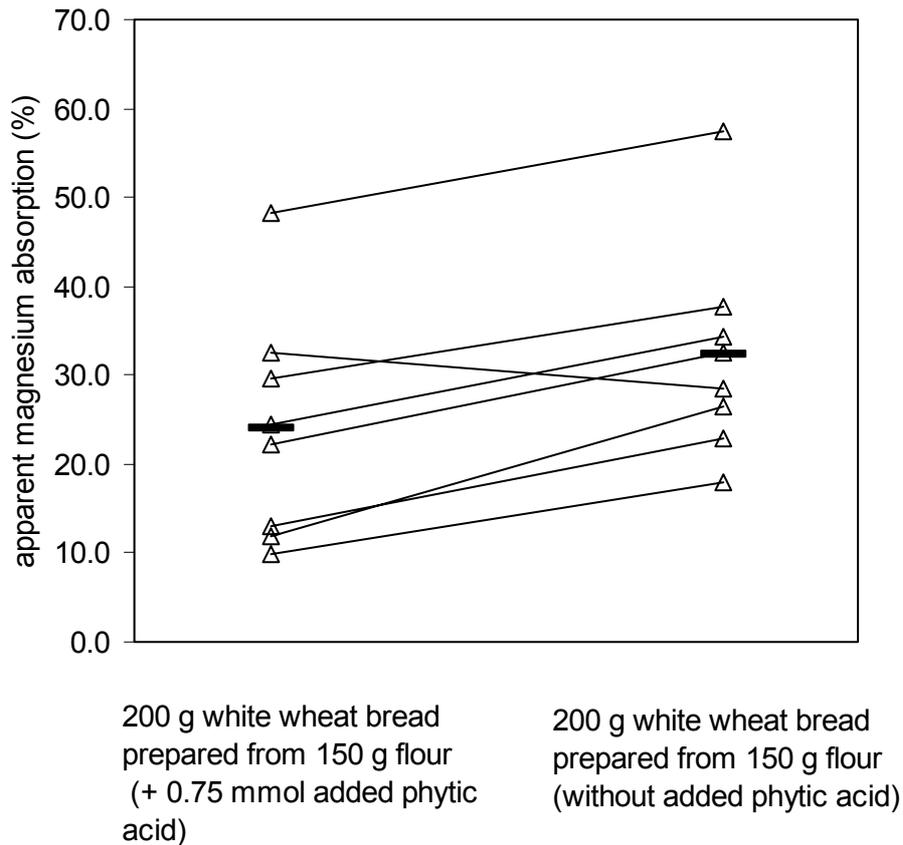


Figure 2.

Apparent magnesium absorption from 200 g white wheat bread prepared from 150 g flour with 0.75 mmol added phytic acid versus 200 g white wheat bread (no phytate added) in 8 subjects. Each triangle represents one subject, the mean is indicated by the dash.

Discussion

Mean fractional apparent magnesium absorption was decreased by about 25% when phytic acid was added to phytic acid-free white wheat bread to a concentration that could be expected to be present in brown wheat bread (0.75 mmol/200 g), and by about 60% when added at a concentration that could be expected to be present in wholemeal wheat bread (1.49 mmol/200 g). The magnitude of inhibition of magnesium absorption depended on the amount of phytic acid added ($P < 0.005$). Although this is the first time that the inhibition of magnesium absorption by phytic acid has been demonstrated in single meals, such an effect was indicated by

balance studies in humans evaluating bran and dephytinized bran (Morris *et al.*, 1988) or phytic acid added to white bread (McCance & Widdowson, 1942). In these studies, a somewhat lower inhibitory effect of phytic acid on fractional apparent magnesium absorption was found (13% and 38%), at similar molar ratios of phytic acid:magnesium (0.2 and 0.4) as in the present study. This lower inhibitory effect might have been due to the control diets, which were not completely free of phytic acid, or because of adaptations in the subjects due to decreased bioavailability of magnesium from the diet.

As with iron, zinc, and calcium, it is assumed that magnesium-phytic acid or protein-magnesium-phytic acid complexes are formed in the intestine which are insoluble >pH 6, (Cheryan, 1983; Nolan *et al.*, 1987; Champagne, 1988) and thus not absorbable, although the stability of the magnesium-phytic acid complex is weaker as compared to iron, copper and zinc complexes with phytic acid (Vohra *et al.*, 1965; Evans & Martin, 1988). Similar molar ratios of phytic acid:mineral of about 0.2 as in the present study have been proposed to significantly decrease absorption of calcium in a balance study (Morris and Ellis 1985). When Hallberg (1989) added phytic acid to phytic acid-free wheat bread rolls, absorption of a radioactive iron label decreased by about 20 and 40% respectively, at phytic acid:iron molar ratios of 0.2:1 and 0.5:1. When compared to iron, a higher fraction of magnesium body losses are endogenous losses secreted into the gastrointestinal tract, and phytic acid could be expected to form complexes with both food magnesium and endogenous magnesium. However, based on rat studies, it is not certain whether the re-absorption of endogenous magnesium is inhibited by phytic acid (Brink *et al.*, 1992; Matsui *et al.*, 2001).

In the present study, the test meals were identical except for their phytic acid content. We chose not to use brown and wholemeal bread or to dephytinize wholemeal bread so as to avoid differences between the test meals and the control meals due to the fermentation. The phytic acid concentrations and the phytic acid:magnesium molar ratios in the test meals were similar to those reported for brown bread and wholemeal bread. In brown bread, the phytic acid concentration is reported to be <0.1-0.4 mmol/100 g and the molar ratio of phytic acid:magnesium is in the range <0.1-0.3; in wholemeal bread the corresponding values are 0.7-1.6 mmol/100 g and the molar ratio between 0.2-0.5 (Reddy *et al.*, 1989). Although it

could be argued that native phytic acid may not behave identically to added phytic acid, adding phytic acid is considered to be a suitable approach to simulate native phytic acid in bread as at pH 2-3 (as in the stomach), phytic acid complexes are largely soluble and mineral binding is weak (Nolan *et al.*, 1987; Champagne, 1988), allowing an exchange of minerals bound to phytic acid. Adding phytic acid to investigate its influence on mineral absorption in humans has been used for magnesium (McCance & Widdowson, 1942), zinc (Hallberg *et al.*, 1989), and iron (Hallberg *et al.*, 1989). In addition, the magnitude of the inhibitory effect of different concentrations of added phytic acid on iron absorption in the study by Hallberg *et al.* (1989) was similar to that reported for different concentrations of native phytic acid obtained by phytic acid degradation (Hurrell *et al.*, 1992).

Despite the expected lower fractional magnesium absorption from brown and wholemeal breads as compared to phytic acid-free white bread, the absolute amount of magnesium absorbed from these foods would still be expected to be slightly higher than from white bread as the magnesium content of brown bread (3.1 mmol/100 g) and of wholemeal bread (2.2 mmol/100 g) is usually much higher than in white bread (1.0 mmol/100 g) (Holland *et al.*, 1994). This assumes however that other components present in brown and wholemeal bread, such as dietary fiber, calcium, and trace elements, do not influence magnesium absorption or modify to any great extent the effect of phytic acid on magnesium absorption.

In conclusion, these results demonstrate that magnesium absorption from white wheat bread was significantly decreased by phytic acid added at concentrations similar to those naturally present in brown and wholemeal bread, and that this decrease depended on the amount phytic acid added.

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2.3. Decreased magnesium absorption from test meals based on spinach compared to test meals based on kale

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Reprints will not be available

Running head: Magnesium absorption from spinach versus kale

Abstract

Background. Spinach, an oxalate rich vegetable, has been reported to inhibit calcium absorption in humans. No information is available on the effect of oxalate rich foods on magnesium absorption in humans.

Objective. To evaluate the effect of an oxalate rich vegetable (spinach) on magnesium absorption as compared to kale, a vegetable with a low oxalate content.

Design. A stable isotope study was performed with 9 healthy adults. Magnesium absorption from 300 g spinach (6.6 mmol oxalate) consumed with 100 g phytate-free white bread (prepared from 75 g white wheat flour) was compared to phytate-free white bread served with 300 g kale (0.1 mmol oxalate). The test meals were divided into 2 identical portions, served at breakfast and lunch on the same day. Each test meal was labelled with 0.7 mmol ²⁵Mg or 1.1 mmol ²⁶Mg and standardized for total magnesium content (5.8-5.9 mmol). Test meals were served on days 1 and 3 in a cross-over design. Each subject acted as his/her own control. Apparent magnesium absorption was determined based on faecal monitoring of the excreted stable isotope labels.

Results. Apparent magnesium absorption was significantly lower from the meal served with spinach than the meals served with kale (26.7±10.4% versus 36.5±11.8%, P=0.01).

Conclusion. Fractional apparent magnesium absorption was decreased from the meal served with spinach. This effect is attributed to its relatively high oxalate content.

Key words: Magnesium absorption, spinach, oxalate, stable isotopes, faecal monitoring.

Introduction

Oxalic acid and its salts are ubiquitous in plant cells. Relatively large amounts are found in leafy vegetables such as spinach (Tabekhia, 1980), but also in fruits, grains, nuts, tea, coffee, and cocoa (Zarembski & Hodgkinson, 1962; Souci *et al.*, 1994). Daily oxalate intakes in the UK have been reported to be in the range of 70-150 mg/d (Zarembski & Hodgkinson, 1962; Anderson *et al.*, 1971; Hodgkinson, 1977b).

Although oxalic acid forms insoluble complexes at physiological pH with divalent cations such as calcium, zinc, and magnesium (Weast, 1989), there have been few investigations concerning the influence of oxalate on mineral absorption in man. Based on studies with spinach, oxalic acid appears to be a major inhibitor of calcium absorption. Fractional apparent calcium absorption in human subjects from spinach (5%) was significantly lower than from kale (42%), a vegetable with a low oxalate content (Heaney *et al.*, 1988; Heaney & Weaver, 1990). Spinach added to human diets was similarly reported to result in negative magnesium and zinc balances (Kelsay & Prather, 1983) and to decrease magnesium absorption by magnesium deficient rats (Kigunaga *et al.*, 1995). Additionally, it has also been shown that magnesium inhibits oxalate absorption in man (Berg *et al.*, 1986; Hanson *et al.*, 1989) indicating that magnesium, like calcium, forms insoluble, non absorbable complexes with oxalic acid in the gastrointestinal tract.

The aim of the present study was to compare the influence of an oxalate-rich vegetable (spinach) and a vegetable with a low oxalate content (kale) on magnesium absorption in human subjects. Magnesium absorption was measured by a stable isotope technique based on extrinsic labelling of the meals and faecal monitoring of the excreted isotope label.

Subjects and methods

Subjects

Apparently healthy, free-living subjects (10 men and women, age 23 ± 1 y, BMI 22.4 ± 3.1 kg/m²) were recruited. No lactating or pregnant women were included in the study. No medication was allowed during the study except for oral contraceptives. Intake of mineral/vitamin supplements was not permitted 2 weeks

before the start of the study and during the entire study. The subjects were informed about the aims and the procedures of the study orally and in written form and written informed consent was obtained. The participants were instructed not to change their dietary habits or life-style during the study. The study protocol was reviewed and approved by the Ethical Committee at the Swiss Federal Institute of Technology, Zurich.

Isotopic labels

Highly enriched ^{25}MgO ($1.04\pm 0.01\%$ ^{24}Mg , $98.73\pm 0.01\%$ ^{25}Mg and $0.23\pm 0.01\%$ ^{26}Mg) and ^{26}MgO ($0.39\pm 0.01\%$ ^{24}Mg , $0.11\pm 0.01\%$ ^{25}Mg and $99.50\pm 0.01\%$ ^{26}Mg) labels were purchased from Chemotrade, Duesseldorf, Germany. The enriched ^{25}Mg label (28 mmol as ^{25}MgO) and ^{26}Mg label (43 mmol as ^{26}MgO) were dissolved in 10 ml 4 mol/L HCl and diluted to 100 ml with water. Solid NaHCO_3 (Merck, Darmstadt, Germany) was added to adjust to pH 6. The concentration of the ^{25}Mg and ^{26}Mg isotope label in solution was determined by isotope dilution mass spectrometry against a commercial magnesium standard of natural isotopic composition (Titrisol, Merck). Unless otherwise specified, all chemicals were of analytical grade, and acids were further purified by surface distillation. Only 18 M Ω water (Milli Q water system, Millipore, Zurich, Switzerland) was used for laboratory work and test meal preparation.

Test meals

The composition of the test meal is shown in Table 1. Kale and spinach were purchased frozen at a local supermarket, cooked and pureed to prepare one batch of each vegetable. Individual servings were weighed and frozen ($-25\text{ }^\circ\text{C}$) immediately after preparation. The test meals were standardized for their magnesium content by adding a solution of MgCl_2 (Merck). Weighed aliquots of the isotope label solution were pipetted immediately before administration.

The phytate-free wheat bread rolls were prepared in batches by mixing 1 kg white wheat flour (Migros, Zurich, Switzerland) with water (600 g), salt (10 g), white sugar (32 g) and dry yeast (15 g). The dough was left to ferment for 5 h at room temperature. Bread rolls were prepared from dough, baked for 15 min at $200\text{ }^\circ\text{C}$, and stored frozen ($-25\text{ }^\circ\text{C}$).

The rare earth elements ytterbium and europium were used as nonabsorbable faecal markers. Both compounds (in chloride form, Aldrich, Buchs, Switzerland), were added to 300 ml 18 MΩ water given at breakfast and lunch.

Study design

The day before isotope administration, a faecal sample was collected to determine baseline magnesium isotope ratios. In addition, brilliant blue (100 mg, Warner Jenkinson Europe, King`s Lynn, UK), a dye used as a faecal marker, was given in a gelatine capsule to indicate the start of the faecal pooling. After an overnight fast, a venous blood sample (10 ml) was drawn into a heparinised glass tube (Vacutainer Systems, Plymouth, UK) for determination of magnesium concentration in plasma. Plasma was separated from blood by centrifugation (Omnifuge 2.0 RS, Heraeus, Zurich, Switzerland) at 20°C and ~500 x g (5 min), and stored in acid washed plastic vials at –25 °C.

Each study was of randomized cross over design, with each subject acting as his/her own control. Test meals A and B within each study were randomly allocated to be served on day 1 or day 3. Test meal A consisted of spinach (300 g), to which the ²⁵Mg label solution was added, and 2 wheat bread rolls (about 100 g) prepared from 75 g flour. Test meal B consisted of kale (300 g), to which the ²⁶Mg label was added, and 2 wheat bread rolls. Spinach and kale were heated in a micro-wave oven prior to serving. In addition, 600 ml 18 MΩ water was served, to which the rare earth elements were added. The test meals were divided into 2 identical portions served at 7.30-8.30 and again at 12.00-13.00.

No food or drinks were allowed between breakfast and lunch on days 1 and 3 and for 3 h following lunch. Standardized meals (pizza) were provided for dinner, and white wheat crisp bread as an afternoon and evening snack. Water (18 MΩ, 2 L) was the only permitted beverage on days 1 and 3. No additional food or drinks were allowed on days 1 and 3. Diet was unrestricted at all other times.

Pre-weighed polypropylene containers (Semadeni, Ostermündingen, Switzerland) were provided for stool collections. The subjects collected all stools separately, starting immediately after intake of the first test meal on day 1. On day 8, a second brilliant blue capsule was given. Collections were continued until excretion of the

second brilliant blue marker. Stool samples were stored frozen ($-25\text{ }^{\circ}\text{C}$) until processed.

Preparation of faecal pools and mineralization

Each individual stool sample was freeze-dried (Modulyo, Edwards, North Bergen, NJ, USA) and ground to a powder in a mortar. All stools, from the first stool dyed by the first brilliant blue marker until, but not including, the stools dyed by the second marker, were included in the faecal pool. After a drying step in a drying chamber (Binder, Tuttlingen, Germany) for 20 h at $65\text{ }^{\circ}\text{C}$ to standardize humidity, and cooling at room temperature for 4 h, all individual stools were weighed and all stools included in one pool were milled individually in a mill (ZM1, Retsch, Haan, Germany), equipped with a sieve of 1 mm pores, starting with the first (most enriched) samples. Each milled stool was transferred back into its original container, dried again for 20 h at $65\text{ }^{\circ}\text{C}$, cooled for 4 h at room temperature and re-weighed to determine losses. All milled stools included in a single pool were combined in a 2 L polyethylene container (Semadeni) and mixed for 90 min using a rotator (Micro Motor, UG 70/20, Basel, Switzerland).

Aliquots of the freeze dried pooled stool samples (1.0-1.6 g) and wheat bread (0.25 g), kale and spinach (1 g), plasma (1 g), were mineralized in a microwave digestion system (MLS 1200, MLS GmbH, Leutkirch, Germany) in a mixture of 14 mol/L HNO_3 and 8.8 mol/L H_2O_2 (Merck). All samples were mineralized in duplicate.

Separation of magnesium

Magnesium was separated from the mineralized stool samples by cation-exchange chromatography using a strongly acidic ion-exchange resin (AG 50W X-8, 200-400 mesh, Bio-Rad, Hercules, CA, USA). Aliquots containing about 30 μmol magnesium were evaporated to dryness, redissolved in 1 ml 0.7 mol/L HCl and transferred onto the top of a column (Bio-Rad, 1 cm inner diameter), filled with the ion-exchange resin to a height of 7 cm. The column was rinsed with 56 ml 0.7 mol/L HCl, followed by 24 ml 0.9 mol/L HCl to elute sodium and potassium. Magnesium was eluted with 12 ml 1.4 mol/L HCl. The solution was evaporated to dryness and redissolved in 50 μl water. Magnesium recovery, evaluated with a diluted magnesium standard

solution (Titrisol, Merck) was found to be $94.8 \pm 1.8\%$ ($n=10$). Resins were regenerated with 30 ml 6 mol/L HCl and replaced after the fifth run. Only acid washed teflon and polyethylene labware was used for sample processing. Aliquots of the ^{26}Mg isotope label were processed in parallel with each batch for blank monitoring from ion exchange chromatography onwards. Sample contamination due to natural magnesium was found to be 10.8 ± 7.0 nmol ($n=9$) for combined sample preparation and filament loading, which was $< 0.4\%$ of the amount magnesium separated.

Isotopic analysis by thermal ionization mass spectrometry (TIMS)

About 20 nmol separated magnesium from faecal samples was loaded onto the metal surface of the evaporation filament of a double-rhenium filament ion source. Magnesium was coated with 5-10 μg silicagel 100, 0.8 μmol boric acid and 30 nmol aluminium as AlCl_3 (all chemicals from Merck). Compounds were loaded in aqueous solution and dried at 0.8 A after each step. Finally, the evaporation filament was heated to dull red heat (1.6 A) for 30 s. The ionization filament remained unloaded. Isotope ratios were determined with a single-focussing magnetic sector field instrument (MAT 262, Finnigan MAT, Bremen, Germany), equipped with a Faraday cup multicollector device for simultaneous ion beam detection. The evaporation filament was heated to 1230 $^\circ\text{C}$, using a standardized procedure. The ionization filament was heated gradually to 1250-1350 $^\circ\text{C}$ until a stable Mg^+ ion beam of $1\text{-}2 \times 10^{-11}$ A was obtained. Each measurement consisted of 30 consecutive isotope ratio measurements.

Reproducibility (5 independent runs) was $\pm 0.2\%$ (relative SD) for the $^{24}\text{Mg}/^{25}\text{Mg}$ isotope ratio and $\pm 0.4\%$ for the $^{24}\text{Mg}/^{26}\text{Mg}$ isotope ratio.

Atomic absorption spectroscopy

Quantitative magnesium analysis of the mineralized and diluted plasma-, flour-, faecal-, and vegetable samples was performed by flame atomic absorption spectroscopy (F-AAS, SpectrAA 400, Varian, Mulgrave, Australia). Plasma samples were measured by external calibration using a commercial magnesium standard (Titrisol, Merck). All other samples were measured by an internal calibration

technique (standard addition) to minimize matrix effects. In addition, all measured solutions contained $\text{La}(\text{NO}_3)_3$ at 5000 mg/L to suppress matrix effects. Certified reference materials (Seronorm Trace Elements Serum, Nycomed, Oslo, Norway, and wheat flour 1567 a, National Bureau of Standards, Gaithersburg, MD, USA) were analyzed in parallel to monitor accuracy of analysis. Ytterbium and europium were measured in the mineralized and diluted faecal samples by electrothermal atomic absorption spectroscopy (ET-AAS) by external calibration, using an ytterbium/europium standard solution (Titrisol, Merck), following standard procedures.

Oxalate and phytate analysis

Total and soluble oxalate content was determined by an enzymatic method (No. 1856120, Roche Diagnostics, Mannheim, Germany) after extraction of oxalate in the pureed vegetables with 2 mol/L HCl (total oxalate) or water (soluble oxalate).

For phytate analysis, wheat bread rolls were freeze dried and ground in a mortar. Phytic acid was extracted from a 1 g aliquot with 0.5 mol/L HCl. The extract was purified by anion exchange chromatography, evaporated to dryness, and redissolved in water prior to HPLC reversed phase chromatography (Sandberg & Ahderinne, 1986).

Methods and calculations

Molar amounts and ratios of the ^{25}Mg and ^{26}Mg isotope labels in the samples were calculated based on double isotope dilution principles (Walczyk *et al.*, 1997; Sabatier *et al.*, 2002). Fractional apparent magnesium absorption (AA%) was calculated as the difference between the oral dose (D_o) and the amount oral label excreted in faeces (F_o) in μmol .

$$\text{AA}(\%) = \frac{D_o - F_o}{D_o} \cdot 100$$

The recovery of the rare earth elements europium and ytterbium was used to control completeness of stool collections.

Statistics

Calculations were made using commercial software (Excel 97, Microsoft, Chicago, Illinois, US; and SPSS 10.0, SPSS inc., Chicago, Illinois, US). Results are presented as arithmetic means \pm SD. Normal distribution of absorption values was verified by skewness and Kolmogorov-Smirnoff test. Paired student's t-tests (2-tailed) were used to compare magnesium absorption from the 2 different test meals. P-values below 0.05 were referred to as statistically significant.

Results

Subjects and test meals

Mean age and mean BMI of the subjects were 23 ± 1 y and 22.6 ± 3.2 kg/m² (n=9). Mean plasma magnesium concentration was 0.80 (range 0.73-0.89) mmol/L (reported normal range 0.75-0.96 mmol/L, Lowenstein & Stanton, 1986). Two subjects had slightly lower plasma magnesium concentration (both 0.73 mmol/L).

The magnesium and oxalate content of the test meals are presented in Table 1. The native magnesium content of the bread, spinach, and kale was 0.96 ± 0.03 , 1.12 ± 0.09 , and 0.89 ± 0.01 mmol/100 g, respectively (n=3). Of total oxalates, $87.5\pm 2.8\%$ were found to be soluble in spinach (n=3) and $51.8\pm 11.7\%$ in kale (n=3).

Table 1.

Magnesium content (Mg), added isotope labels, oxalic acid (OA) content, and molar ratios of oxalic acid/magnesium (OA/Mg) of the test meals ⁺

	spinach meal	kale meal
total Mg (mmol)	5.94±0.01	5.77±0.05
added Mg label		
²⁵ Mg (mmol)	0.66±0.01	-
²⁶ Mg (mmol)	-	1.19±0.02
OA (mmol)	6.6±0.2	0.11±0.01
molar ratio OA/Mg	1.1	<0.02
fecal markers		
ytterbium (nmol)	31.38±0.58	-
europium (nmol)	-	33.17±0.26

⁺test meals consisted of 100 g phytate-free white wheat bread prepared from 75 g flour together with 300 g of either spinach or kale. Test meals were divided into 2 identical portions served at breakfast and lunch on the test day.

Magnesium absorption

Magnesium absorption from the test meal served with spinach (26.7±10.4%) was significantly lower than from the test meal served with kale (36.5±11.8%, P=0.01, **Fig.1**). One subject was excluded from the evaluation due to low recovery of ytterbium (<85%). For all other subjects, mean ytterbium recovery was 98.8% (range: 90.6-115.4) and mean europium recovery was 95.0% (range: 90.0-99.1).

Mean loss of faecal material during stool pool preparation, determined by weighing the pools before and after milling, was 1.1±0.6%. Measured mean isotopic enrichment of the stool pools, expressed as the isotopic shift of a ratio, was calculated from the difference of the measured isotope ratio of the sample and the measured natural isotope ratio of the standard (Titrisol, Merck) divided by the measured isotope ratio of the standard, and was 5.6±1.8% (²⁴Mg/²⁵Mg) and 8.1±2.4% (²⁴Mg/²⁶Mg).

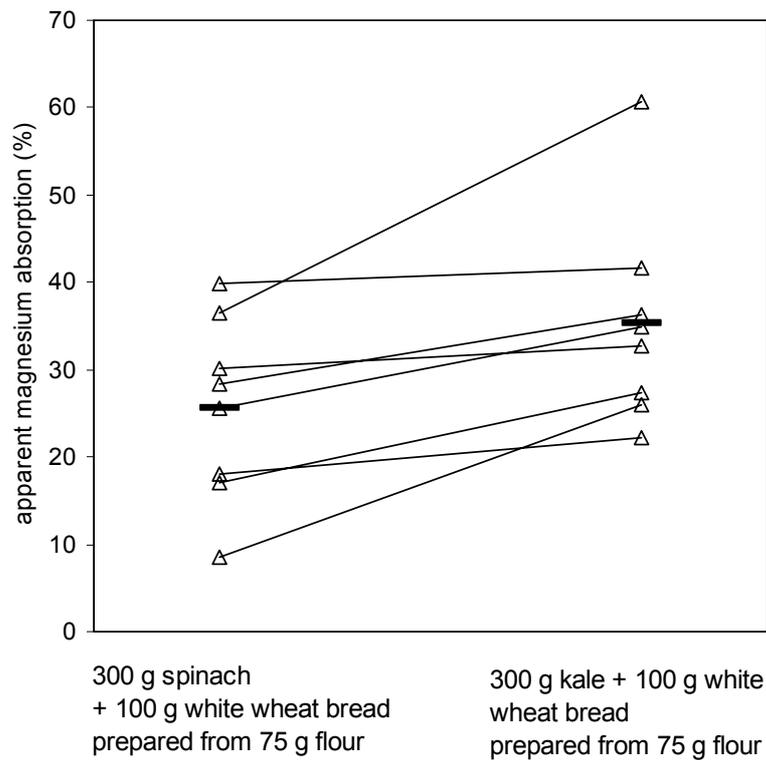


Figure 1.

Apparent magnesium absorption from a meal based on 300 g spinach (6.6 mmol oxalate) and 100 g white wheat bread versus a meal based on 300 g kale (0.11 mmol oxalate) and 100 g white wheat bread in 9 subjects. Each triangle represents one subject, mean absorption is indicated by the dash.

Discussion

The mean fractional apparent magnesium absorption from a meal based on white bread and 300 g spinach was about 30% lower than from the same meal with 300 g kale. The difference in fractional absorption is assumed to be due to the different oxalate content which was 6.6 mmol in the meal based on spinach compared to 0.1 mmol in the meal based on kale. The present study is, to our knowledge, the first report showing a negative effect of an oxalate rich vegetable on magnesium

absorption from a single meal. The lower absorption from the spinach based meal compared to the kale based meal is analogous to, although less pronounced, the reported 90% decrease in calcium absorption from spinach compared to kale (Heaney *et al.*, 1988; Heaney & Weaver, 1990). The greater inhibitory effect of spinach on calcium absorption could be due to the much lower solubility of calcium oxalate as compared to magnesium oxalate (Weast, 1989).

Adding 100 g spinach on alternate days to a fibre rich diet has previously been reported to decrease magnesium balance (Kelsay & Prather, 1983). An earlier stable isotope study in 4 adult human volunteers resulted in contradictory data. While no significant effect of spinach containing bran muffins was found compared to lettuce or turnip greens containing bran muffins on magnesium absorption, absorption was significantly higher from bran muffins and collard (54% versus 48%). Collard greens are botanically similar to kale (Schwartz *et al.*, 1984). Although the quantity of spinach in this study was not reported, it can be estimated from the reported magnesium content to be in the range of 50-100 g, which is at least 3 times less than in the present study. However, such an amount may be too small to detect changes in magnesium absorption with a small number of subjects.

Oxalates in plants are present as water soluble (sodium-, potassium-, free-) and water insoluble forms (calcium-, magnesium-) (Hodgkinson, 1977a; Souci *et al.*, 1994). It is not clear to what extent the insoluble oxalates dissolve in the gastric juice (pH 2-3) to form a common pool, however, as the pH rises in the small intestine, insoluble, non absorbable calcium and magnesium oxalates can be expected to be formed. The soluble oxalates could thus have a more pronounced negative impact on mineral absorption due to their ability to bind free minerals in the intestine. In the present study, a relatively high fraction (88%) of total oxalates in spinach were soluble, which can be assumed to have contributed to the observed significant reduction in magnesium absorption. Literature data on the percentage of soluble oxalates relative to total oxalates in spinach vary from 15-20% (Toma & Tabekhia, 1979; Souci *et al.*, 1994) up to 93% (Hodgkinson, 1977a).

In a study with magnesium deficient rats, magnesium absorption was reported to be decreased by spinach but not by an equivalent amount of added oxalic acid (Kigunaga *et al.*, 1995). Therefore it can be speculated that other components of spinach and/or kale could influence magnesium absorption, for example phenolic

compounds, which have been reported to have a strong negative effect on iron absorption (Gillooly *et al.*, 1983; Brune *et al.*, 1989). However, the amount of total phenolic compounds however has been reported to be similar in spinach and kale (Lucarini *et al.*, 2000). Another possibility would be the soluble fibre content. Kale is reported to contain about 2% fibre as pentosans and hexosans, which is about double the amount compared to spinach (Souci *et al.*, 1994). These partly soluble hemicelluloses are non digestible and would be expected to be fermented by bacteria in the large intestine, similarly to fructo-oligosaccharides, which were reported to increase fractional magnesium absorption in humans significantly from 30 to 34% when consumed in amounts of 10 g/d (Tahiri *et al.*, 2001). The enhancing effect is attributed to increased colonic absorption of magnesium. Earlier balance studies in human subjects however have reported a significant negative effect of a mixture of galactans, pentosans, and hexosans (14 g/d) on magnesium absorption (Drews *et al.*, 1979). Therefore, it does not appear likely that the observed 30% decrease in mean fractional magnesium absorption in test meals based on spinach compared to the kale based test meals in the present study was due to differences in the content of dietary fibre.

The present findings however do not suggest that spinach is a poor source of dietary magnesium. The observed decrease in fractional apparent magnesium absorption can be assumed to be, at least partly, counterbalanced by the usually 1/3 higher magnesium content of spinach as compared to kale (Holland *et al.*, 1994; Souci *et al.*, 1994).

In conclusion, the results from the present study demonstrate that mean fractional apparent magnesium absorption from a test meal based on spinach was about 30% lower than from a test meal based on kale. It is suggested that this reduction in magnesium absorption is due to the higher oxalate content of spinach.

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2.4. Chlorophyll analysis in plant samples by HPLC using zinc-phthalocyanine as an internal standard

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Abstract

Chlorophyll analysis at high precision and accuracy is limited by the lack of suitable, commercially available internal standards for HPLC analysis. Here we present a new HPLC technique, using the commercially available dye zinc-phthalocyanine as an internal standard to quantify chlorophyll *a* and *b* in vegetable foods and to detect products of chlorophyll degradation. Pigments were extracted with N, N-dimethylformamide from the vegetables and purified by solid phase extraction. Chlorophyll *a*, *a'*, *b*, *b'*, corresponding pheophytins, and zinc-phthalocyanine were separated by HPLC using a C₁₈ reverse-phase column and fluorescence detection. The technique was thoroughly evaluated and finally applied to a selection of vegetable foods.

Introduction

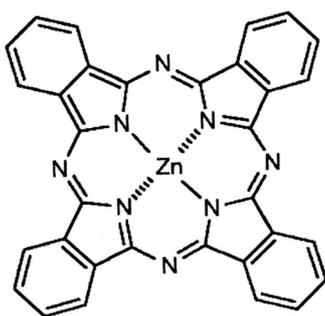
Quantitative analysis of chlorophylls and their degradation products is performed in a number of scientific disciplines, e.g. in oceanography for monitoring phytoplankton growth in seawater¹. In food science, chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*) and their copper complexes are of interest as food dyes (E 140, E 141). In addition, chlorophylls and their degradation products are used as markers of food processing as chlorophylls are sensitive to light, pH, temperature, oxygen and enzymatic degradation^{2,3}.

Spectrophotometry^{4,5} and fluorometry^{6,7} are the techniques most commonly used for chlorophyll quantification. Fluorescence methods are more sensitive and selective than spectrophotometric techniques for chlorophyll but of limited accuracy when applied to complex sample matrices because of unpredictable quenching effects⁸. Furthermore, chlorophylls have to be present in non-aqueous solutions as fluorescence activity decreases with increasing amounts of water⁹. For both spectroscopic techniques, chlorophylls are usually separated from the sample matrix to remove spectral interferences^{10,11}. HPLC has established itself as the most powerful tool for this purpose^{12,13}.

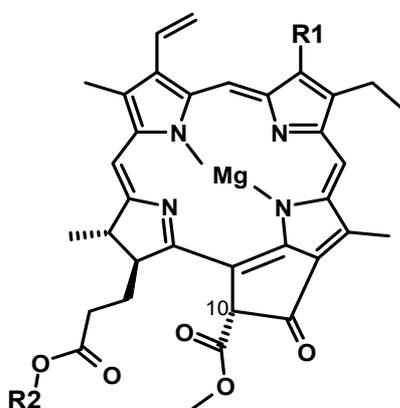
Pigment quantification is usually done by external calibration or via compound specific absorption coefficients. Accuracy and precision for these techniques is

limited by variable analyte losses that may occur during sample preparation. This limitation can be overcome by using an internal standard. Provided that there is no discrimination between analyte and internal standard during sample preparation, analyte losses do not affect the accuracy of the analysis. Despite these advantages, internal standards are rarely used for chlorophyll analysis because of the lack of chemically similar compounds that are stable, pure and commercially available. Bessiere and Montiel¹⁴ used fluoranthene as an internal standard to determine chl *a* and chl *b* in phytoplankton by HPLC and fluorescence detection. However, fluoranthene differs chemically from the chlorophyll pigments which increases the risk of differences in pigment degradation during extraction and purification. The same is true for sudan II¹⁵ and β -apo-8'-carotenal¹⁶. Some potentially useful internal fluorescence standards have been discussed by Mantoura and Repeta¹⁷ but standards were either relatively expensive such as mesoporphyrin IX dimethylester¹⁸, co-eluted with the pigments (etioporphyrin or deuteroporphyrin IX dimethylester) or were not commercially available (zinc-pyropheophorbide *a*). As a consequence, laboratories are usually obliged to synthesize their own internal standards or use external calibration methods.

After a literature search, several compounds were tested for their usefulness as internal fluorescence standards. Here we show that zinc-phthalocyanine, a commercially available dye used in food industry and cancer research, can be used as an internal HPLC standard for routine analysis of chl *a* and chl *b* in plant foods. Zinc-phthalocyanine (Fig.1) shows structural and spectroscopic similarities to the chlorophylls, is relatively cheap, stable, and can be purchased at sufficient purity.



A



B

R1=CH₃: chlorophyll *a*

R1=CHO: chlorophyll *b*

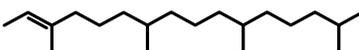
R2=phytyl: 

Fig. 1

Structure of zinc-phthalocyanine (A), chlorophyll *a*, chlorophyll *b* (B)

Experimental section

Standards and chemicals

Chl *a* and chl *b* (Fluka, Buchs, Switzerland, dye content > 95%), and zinc-phthalocyanine (Aldrich, Buchs, Switzerland, dye content > 97%) were purchased in solid form. Reagents and solvents were of analytical grade unless otherwise specified. Chlorophyll standards were prepared by dissolving 5 mg chl *a* and 5 mg chl *b* in 50 g acetone (Merck), respectively, and 30 mg zinc-phthalocyanine in 100 g N,N-dimethylformamide (DMF) (Fluka). Standards were prepared gravimetrically by weighing reagents to ± 0.05 mg and dissolution in cooled solvents (5°C). Pheophytin standards were obtained from the chl *a* and chl *b* standards by acidifying 20 ml aliquots with 0.5 ml 1 mol/L HCl. Standard solutions were stored in the dark under argon at -25 °C. In addition to zinc-phthalocyanine, other compounds were tested for their usefulness as internal fluorescence standards, including anthracene, perylene, naphthalene, decacyclene, cobalt-(II)-phthalocyanine, copper-(II)-phthalocyanine nickel-(II)-2,11,20,29-tetra-tert-2,3-butyl-naphthalocyanine (all Aldrich), chlorophyllin (sodium-copper salt), fluorescein (sodium salt) and hemin (all Sigma).

Chl *a* and chl *b* standards in acetone have been shown to be stable for several months when stored under inert gas at -20 °C^{11, 12}. Stability of the prepared zinc-phthalocyanine internal standard solution (IS) was monitored using HPLC, no degradation was observed over a period of 6 months.

Sample preparation

Spinach, lettuce, iceberg lettuce and endive were purchased in 50–500 g portions at a local supermarket, weighed and stored at -65 °C. For analysis, frozen plant material was crushed into small pieces (ca. 1 cm²) and a 10-20 g aliquot was weighed into a mortar. A mixture of 10 g quartz sand (Merck, fine granular, washed and calcinated) for facilitating cell rupture was added together with 10 g soluble starch (Merck) to increase viscosity. Aliquots of the IS solution containing ~ 0.6 mg

IS were added together with 3 ml aqueous phosphate buffer (pH 7, Einecs, Fluka). Mixtures were homogenized thoroughly with a pestle while adding liquid nitrogen repetitively. From the mixtures, 1-4 g were transferred into a 20 ml polyethylene vial containing 2 ml aqueous buffer (pH 7) and 13 ml DMF and soaked for 2 h at $-25\text{ }^{\circ}\text{C}$. After sonification for 5-10 min at $5\text{ }^{\circ}\text{C}$, the vial content was transferred into a 20 ml disposable syringe filled to a height of 2 cm with quartz sand and filtered through a $0.45\text{ }\mu\text{m}$ disposable filter (Chromafil A-45/25, Macherey-Nagel, Oensingen, Switzerland). DMF (5 ml) was passed 3 times through the syringe containing the sample. Fractions were combined in a 50 ml argon flushed glass vial containing 3 ml aqueous buffer (pH 7). The whole preparation was carried out in an ice-bath under dim light.

Collected extracts were further purified for HPLC analysis by solid phase extraction (SPE). SPE columns (Supelclean LC-18 SPE Tube 1 g, Supelco, Buchs, Switzerland) were preconditioned with 2 ml methanol (Merck) followed by 5 ml aqueous phosphate buffer (pH 7). A 1-10 ml aliquot of the pigment extract was diluted with the same volume of aqueous phosphate buffer (pH 7) for increasing the solvent's polarity. The column was washed after sample loading with 5 ml aqueous phosphate buffer (pH 7) and 6 ml of a methanol/phosphate buffer mixture (94:6, v/v). Pigments and IS were eluted with 6 ml pure methanol followed by 4 ml DMF at a flow rate of $\sim 1\text{ ml/min}$, allowing the resin to run dry at the end. The methanol and the DMF fractions were combined in argon flushed polyethylene vials and stored immediately at $-25\text{ }^{\circ}\text{C}$ for HPLC analysis. SPE columns were regenerated with acetone (5-10 ml) and reused up to 5 times. Prepared samples can be stored for later HPLC analysis for several weeks at $-25\text{ }^{\circ}\text{C}$. Chlorophyll solutions in DMF have been shown to be stable for at least 20 d at $5\text{ }^{\circ}\text{C}$ ^{19, 20}.

HPLC analysis

Samples were analyzed by reverse-phase HPLC using fluorescence detection. The HPLC system consisted of an injection port (Rheodyne 7125) with a $5\text{ }\mu\text{l}$ loop (Rheodyne, Cotati, CA, USA), a high pressure pump (Bischoff Modell 2200, Bischoff, Leonberg, Germany), a degasser (ERC 3511, Erma, Tokyo, Japan), a gradient/system control mixer (300 Benchtop, Autochrom, Milford, MA, USA), a

fluorescence detector (RF 511, Shimadzu, Duisburg, Germany), a column oven (CTO 10 AC, Shimadzu) and an integrator (Merck- Hitachi D 2520 GPC, Merck). A 25 cm narrow bore RP-18 column (Merck supersphere, LiChroCART 250-2, 4 μ m particles, 2 mm inner diameter) was used in combination with a 4 mm RP-18 (LiChroCART 4-4, filled with LiChrospher 100, 5 μ m particles, Merck) pre-column. Flow rate was adjusted to 0.28 ml/min and temperature was set to 31 °C. However, lower column temperatures (down to 16 °C) were also investigated on their usefulness.

Chlorophylls and their C-10 epimers were eluted in 100% methanol, methanol was replaced after 9.5 min by a mixture of 80% methanol, 15% acetone and 5% DMF within 6.5 min using a linear gradient. IS, pheophytins and their epimers were eluted from the column in the same eluent. The column was conditioned for the next run by changing the eluent back to 100% methanol within 2 min (min. 38-40).

Excitation/emission wavelengths for fluorescence detection (in nm) were 429/664 for chl *a* and chl *a'*, 456/648 for chl *b* and chl *b'*, 405/661 for phe *a* and phe *a'*, 436/655 for phe *b* and phe *b'* and 360/665 for zinc-phthalocyanine. Chlorophylls and pheophytins were identified by their retention times as determined for the prepared standard solutions. Epimers were identified by comparison with chromatograms reported in the literature²¹⁻²⁴.

Statistics and calculation

Calculations were done using commercial spreadsheet software (Excel 97, Microsoft, Chicago, Illinois, USA and SPSS 10.0, SPSS inc., Chicago, Illinois, USA). Means are expressed as arithmetic means \pm 1 SD. P-values <0.05 were referred to as statistically significant.

Prior to quantification of chl *a* and chl *b* in vegetable foods, the instrumental response factors (*RF*) for chl *a* and chl *b* against the IS, i.e. the relative fluorescence detector response for a chlorophyll compound versus the IS, had to be determined based on external calibration methods. Based on these response factors, chl *a* and chl *b* in the plant foods can be quantified based on the measured areas of the pigments and the amount of IS added prior to sample preparation.

$$RF = \frac{A_{IS}}{C_{IS}} \cdot \frac{C_{chl a, chl b}}{A_{chl a, chl b}}$$

with A_{IS} and $A_{chl a, chl b}$ being the areas of the peaks of IS and chl *a* or chl *b* in the chromatograms and C_{IS} and $C_{chl a, chl b}$ the corresponding concentrations of the external standard solutions. Pearson correlation coefficients *R* as indicators for linearity were calculated for the regression curves of each pigment.

Stability of response factors and recoveries

To make use of the zinc-phthalocyanine signal for quantification, the instrumental response factor has to be independent from the respective amount ratio. To verify whether response factors were constant, varying amounts of chl *a* and chl *b* (0.15 - 3.2 µg/g of each compound) were added to constant amounts of IS (0.3, 0.6 and 0.9 µg/g), using acetone as the solvent.

To evaluate the long-term stability of the response factors, external calibration curves (Table 1) for IS and pigments were established over a period of 6 months. These calibration curves were, in addition to the pigment mixtures described above, used to calculate response factors based on the slope of the regression curves obtained.

Table 1

Analytical parameters of regression curves for chl *a*, chl *b*, and IS (zinc-phthalocyanine). Each calibration graph consisted of 6 measured concentrations of the pigments. Concentration ranges were 0.12-3.41 µg/g for chl *a*, 0.05-2.11 µg/g for chl *b* and 0.03-0.45 µg/g for IS.

	chl <i>a</i>	chl <i>b</i>	IS
<i>intercept</i> ±	-1.15±1.28	-0.26±0.22	-0.20±0.33
<i>SD (AU)*</i>			
slope ± SD	44.24 ±0.62	18.30 ±0.17	64.56±1.16
(AU/CU ⁺)			
R	0.9997	0.9998	0.9993

*:AU=area units

+CU=concentration units ($\mu\text{g/g}$)

Independently, the amount ratio of IS and chlorophylls and therefore response factors should not be altered during sample preparation and analysis. Discrimination of pigments against IS and vice versa translates automatically into a systematic error in the calculated chlorophyll concentration. To evaluate the effect of sample preparation on the response factors, mixtures ($n=14$) of chl *a*, chl *b* and IS containing chl *a* (6.1 - 31.8 μg), chl *b* (4.9 - 27.6 μg) and zinc-phthalocyanine (15.2 - 35.6 μg) in different amount ratios were prepared from the standards. Mixtures were treated identically to vegetable samples after addition of fresh onion (10 g) at the homogenization step which served as a chlorophyll-free matrix. The percent recovery of the pigments and amount ratios before and after sample preparation and analysis were determined by external calibration.

In addition, recoveries of the pigments were also determined without matrix for the SPE procedure alone ($n=9$ independent runs), using solutions containing 41.5 μg chl *a*, 15.0 μg chl *b* and 13.9 μg IS. The recoveries were determined based on area units measured before and after SPE.

Results

Typical chromatograms obtained for a leafy vegetable (spinach) are shown in Fig. 2. Samples prepared as described showed no or only minor signs of chlorophyll degradation. Chlorophyll degradation became visible when the same extract (10 ml) was acidified with 50 μl 2 mol/L HCl and exposed for 15 h to air and light (Fig. 2). Chlorophylls were found to be degraded into pheophytins (phe *a* and phe *b*) as well as into their C-10 epimers (chl *a'*, chl *b'*, phe *a'* and phe *b'*). Fig. 2 shows that the zinc-phthalocyanine signal can be clearly resolved from the pigment signals even for samples containing multiple degradation products. However, chlorophylls and their epimerisation products could not be completely resolved. At the lower column temperature tested (16°C), chlorophylls and epimerization products could be

separated completely, but separation of zinc-phthalocyanine and phe *b* was unsatisfactory.

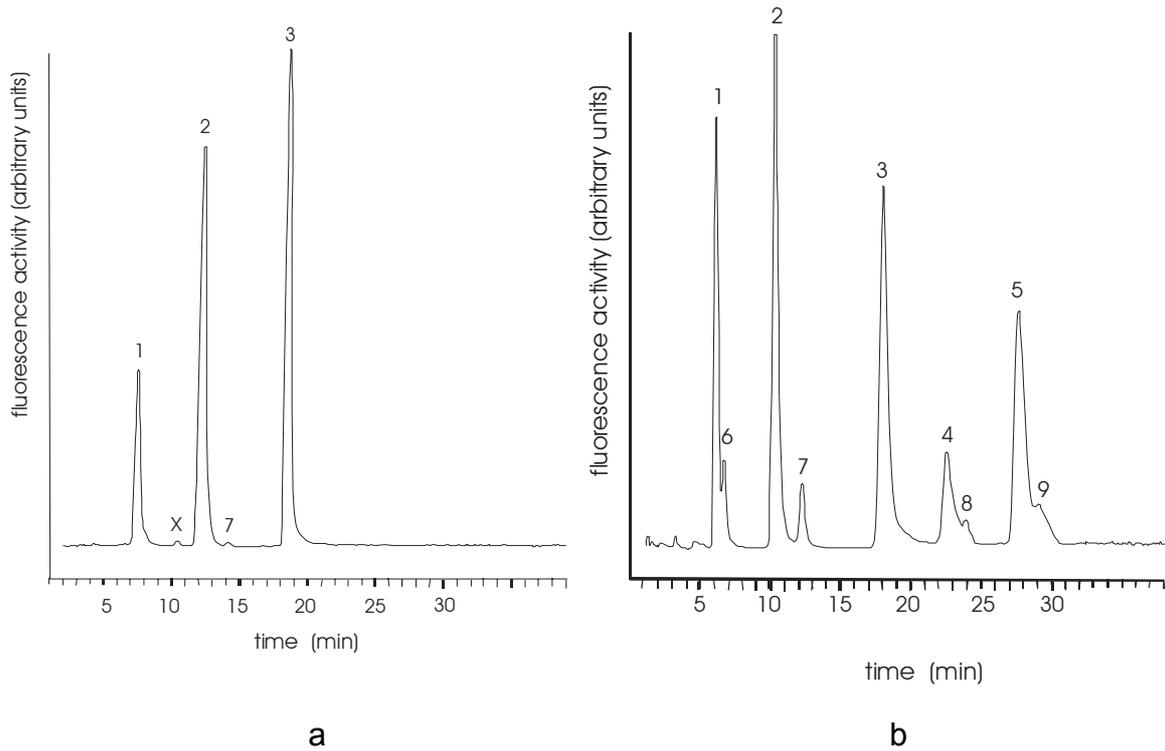


Fig. 2

Chromatogram of spinach, stored frozen until analysis by HPLC (a) and after acidifying the same pigment extract with 0.2 ml mol/L HCl per 10 ml extract and exposure to air and light for 15 h (b)

1= chlorophyll *b*, 2 = chlorophyll *a*, x, y = unknown degradation products, 3 = internal standard zinc-phthalocyanine (IS), 4 = pheophytin *b*, 5 = pheophytin *a*, 6 = chlorophyll *b'*, 7 = chlorophyll *a'*, 8= pheophytin *b'*, 9 = pheophytin *a'*.

HPLC analysis of the different mixtures of IS and chl *a* and chl *b* used to investigate the stability of the response factors showed no dependence on the amount ratio of chlorophylls to IS over a range of 0.2 to 10 (Fig. 3). Average instrumental response factors as determined for the different mixtures ($\pm 1SD$) were 1.39 ± 0.10 for chl *a* ($n=18$) and 3.53 ± 0.20 for chl *b* ($n=18$), respectively. In addition, response factors were obtained by dividing the slope of the calibration lines for chl *a* and chl *b*, respectively, through the slope of the IS calibration line, obtained within a period of 6 months. No drift in the response factors with time was observed. Average response factors were 1.46 ± 0.08 for chl *a* and 3.54 ± 0.26 for chl *b* ($n=4$, respectively), which is not significantly different from the response factors obtained from the mixtures of IS, chl *a* and chl *b* (paired Student's *t*-test). Furthermore, *RF* based on external calibration graphs ($n=6$) based on pigment solutions stored at $-25\text{ }^{\circ}\text{C}$ under argon were stable during a period of 6 months.

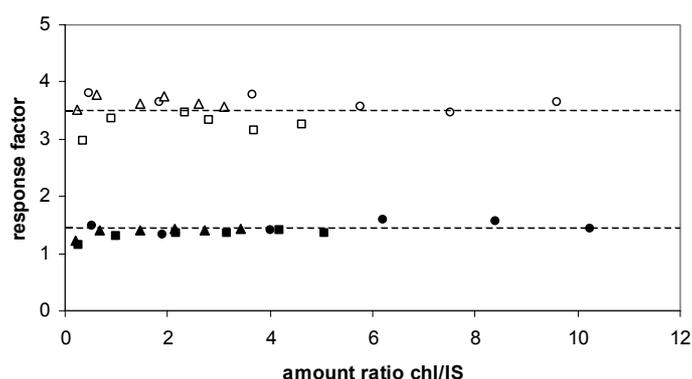


Fig. 3.

Response factors for chlorophyll *a* (chl *a*) and chlorophyll *b* (chl *b*) as determined for mixtures containing chl *a* and chl *b*, respectively, in different amount ratios to zinc-phthalocyanine as the internal standard (IS). Mixtures of chl *a* and IS varied in zinc-phthalocyanine concentrations: $0.3\text{ }\mu\text{g/g}$ (\bullet), $0.6\text{ }\mu\text{g/g}$ (\blacksquare) and $0.9\text{ }\mu\text{g/g}$ (\blacktriangle), the mixtures of chl *b* and IS were of the same zinc-phthalocyanine concentrations: $0.3\text{ }\mu\text{g/g}$ (\circ), $0.6\text{ }\mu\text{g/g}$ (\square) and $0.9\text{ }\mu\text{g/g}$ (Δ).

The amount ratio of the pigment standards after analysis relative to the amount ratio before analysis was 0.91 ± 0.10 for chl *a*/IS and 0.98 ± 0.12 for chl *b*/IS. Mean recoveries were $87.8 \pm 9.4\%$ for chl *a*, $94.8 \pm 12.8\%$ for chl *b* and $97.5 \pm 8.2\%$ for zinc-phthalocyanine for the different mixtures. Recoveries differed not significantly between chl *b* and IS (paired Student's *t*-test) but recovery of chl *a* was significantly lower when compared to zinc-phthalocyanine ($P < 0.005$) and chl *b* ($P = 0.01$). No significant discrimination of chl *a* against IS was observed when mixtures were analyzed without the preceding extraction step and without addition of fresh onion. Recoveries for the SPE/HPLC procedure alone were $98.3 \pm 5.3\%$, $94.4 \pm 1.7\%$ and $99.2 \pm 6.1\%$ for chl *a*, chl *b* and IS, respectively. Recoveries for phe *a* and phe *b*, respectively, were $94.5 \pm 12.7\%$, and $91.5 \pm 9.5\%$, which shows that the method can be used basically to monitor chlorophyll degradation, too.

Instrumental precision of the HPLC system was evaluated using two standard solutions containing chl *a* and chl *b* at different amount ratios relative to IS. Solutions were injected consecutively ($n=5$). Relative SDs were 4.1% ($\sim 6.5 \mu\text{g/g}$) and 3.2% ($\sim 1.0 \mu\text{g/g}$) for IS, 2.1% ($\sim 4.5 \mu\text{g/g}$) and 3.2% ($\sim 0.8 \mu\text{g/g}$) for chl *a* and 4.8% ($\sim 3.0 \mu\text{g/g}$) and 3.8% ($\sim 0.5 \mu\text{g/g}$) for chl *b*. Detection limits were determined by replicate analysis ($n=6$) of a highly diluted chlorophyll standard containing 98 ng/g chl *a*, 47 ng/g chl *b* and 28 ng/g IS, respectively. Detection limits defined as 3 times the SD obtained for the analysis of the diluted standard were 6.6 ng/g for chl *a*, 10.7 ng/g for chl *b* and 4.5 ng/g for zinc-phthalocyanine.

Chlorophyll contents of the different vegetables were determined in a series of independent runs and are shown in Table II. The total chlorophyll content and the chl *a*:chl *b* ratio varied between vegetables. Iceberg lettuce had the lowest total chlorophyll content but the highest chl *a*:chl *b* ratio. In the other vegetables analyzed, the chl *a*:chl *b* ratio was found to be similar. Mean recovery of IS over all runs ($n=16$) was $(100.2 \pm 12.3)\%$ as determined by external calibration. For independent analysis of the same vegetable, the relative SD varied between 2.0% for spinach to 9.3% for endive.

Table II

Chlorophyll content (fresh weight basis) of 4 fresh and immediately frozen green leafy vegetables.

vegetable	n	chl <i>a</i> ($\mu\text{g/g}$)	chl <i>b</i> ($\mu\text{g/g}$)	total chlorophyll ($\mu\text{g/g}$)	ratio chl <i>a</i> : chl <i>b</i>
spinach	5	691 \pm 20	193 \pm 7	884 \pm 18	3.60 \pm 0.21
endive	5	273 \pm 26	83 \pm 7	356 \pm 33	3.29 \pm 0.08
lettuce	3	283 \pm 18	70 \pm 5	353 \pm 24	4.04 \pm 0.06
ice-berg	3	19 \pm 1	4 +1	22 \pm 1	5.07 \pm 0.09

Discussion

Zinc-phthalocyanine was found to be sufficiently stable, could be separated well from chlorophylls and degradation products by HPLC, showed similar fluorescence properties compared to chlorophylls and pheophytins and can be purchased in sufficient purity at a relatively low price. Zinc-phthalocyanine would thus appear to be the most suitable internal standard for chlorophyll analysis of the different compounds which have been tested. Polycyclic aromatic hydrocarbons (anthracene, perylene, naphthalene, decacyclene) differ strongly from chlorophylls in chemical and fluorescence properties from chlorophylls, chlorophyllin and fluorescein are too polar, and hemin and nickel-(II)-2,11,20,29-tetra-tert-2,3-butyl-naphthalocyanine are only poorly soluble in the most suitable solvents (DMF, methanol and acetone) used for chlorophyll extraction. Other investigated phthalocyanines (cobalt-(II)-phthalocyanine and copper-(II)-phthalocyanine) were found to co-elute with the pheophytins.

Column temperature was found to be decisive to achieve complete pigment separation. Separation of the zinc-phthalocyanine signal became possible by increasing the temperature to 31°C. Separation of chlorophyll and pheophytin from their C-10 epimers was less good under these conditions compared to lower temperatures but could be achieved, in principle, by using a methanol/water mixture

for column elution before switching to pure methanol. This was not possible with our equipment which allowed to run binary gradients only.

To separate the pigments from the plant matrix for HPLC analysis, DMF extraction of the homogenized plant material was used in combination with SPE purification. Pigment separation/purification before HPLC analysis is necessary because proteins, fats, hemicelluloses or other pigments co-extracted with chlorophylls as well as their degradation products may interfere with pigment signals or may obstruct analytical equipment. DMF allows chlorophyll extraction from plant matrices at high extraction yields^{19, 25}. Other possible pigment extraction procedures include extraction with acetone and methanol or their water mixtures in combination with grinding, soaking, or sonification^{9, 25}. However, most of these techniques require large quantities of chemicals, yield suboptimal pigment extraction, do co-extract undesired compounds such as fats or facilitate pigment degradation.

For sample clean-up, liquid/liquid partition²⁶, thin-layer-chromatography TLC,²⁷ and SPE³ have been applied. SPE was chosen here because liquid/liquid extraction usually requires large quantities of chemicals and TLC increases the risk of analyte losses due to the susceptibility of the pigments to air. In addition, SPE allows parallel processing of large sample numbers and pigment enrichment.

Despite the precautions taken during sample preparation to prevent chlorophyll degradation, chl *a* recovery for the entire sample preparation procedure was lower compared to chl *b*. Because recoveries determined for the SPE procedure alone indicated no significant losses, discrimination of chl *a* against chl *b* during DMF extraction or degradation during sample preparation prior to SPE are the most probable explanations. It has been shown in the past that chl *a* is more rapidly degraded than chl *b* to the corresponding pheophytins at lower pH^{28, 29}. However, the observed chl *a* losses during sample preparation were in the order of 10%, which is not considered a major limitation for most applications. If required, empirically derived correction factors could be introduced to correct for chl *a* underestimation.

Data obtained for the four analyzed vegetables agreed well with literature data. Chlorophyll concentration is known to vary significantly between plants. Chlorophyll contents as high as 1 mg/g can be found in spinach and is less than 0.1 mg/g in broccoli and brussels sprouts^{22, 30}. As expected, spinach had the highest chlorophyll

content of the vegetables analyzed (691 $\mu\text{g/g}$). The amount ratio of chl *a*:chl *b* is also known to vary between vegetables. Typical chl *a*:chl *b* ratios are in the range of 2.8-4.7^{22, 30} which is in good agreement with our results. Precision in chlorophyll analysis differed for independent runs of the same vegetable, ranging from 2.0% for spinach to 9.3% for endive, whereas the lowest chlorophyll content was found in iceberg lettuce. These differences in reproducibility cannot be explained by the lower chlorophyll content alone. Sampling also seems to limit the achievable reproducibility. Plant material consists of stems, stalks and leafy parts which usually differ significantly in chlorophyll content. Multiple sampling of the same plant leads, accordingly, to stronger variations in the data. This suggests that sampling strategies have to be evaluated carefully to make fully use of the potential of zinc-phthalocyanine as an internal standard for chlorophyll analysis.

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2.5. Chlorophyll-bound magnesium in commonly consumed vegetables and fruits

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Abstract

Heme iron represents about 10-15 % of iron intake in industrialized countries but up to 50 % absorbed iron. Magnesium is similarly bound as the central atom of the porphyrin ring structure of the green plant pigments chlorophyll *a* and *b*, and it has been suggested that chlorophyll-bound magnesium may play an important role in magnesium nutrition. We have analyzed 22 frequently consumed fruits and vegetables for the chlorophyll content by HPLC and for magnesium with atomic absorption spectroscopy. Chlorophyll concentrations ranged from 6 µg/g (grape, fennel) to 790 µg/g (spinach) with a median of 63 µg/g. Magnesium concentrations ranged from 48 µg/g in grape to 849 µg/g in spinach with a median of 122 µg/g. In the green leafy vegetables such as spinach and various salads, chlorophyll-bound magnesium represented 2.5-10.5 % of total magnesium whereas other common green vegetables and fruits such as peas, green beans, broccoli, celery, fennel, kiwi and grapes contained <1 % chlorophyll-bound magnesium. The chlorophyll content of spinach was further decreased by about 35 % on thawing frozen spinach or on chopping fresh spinach, and these losses increased to about 50 % after boiling and steaming. Based on the present results, and from published food consumption data, we estimate that chlorophyll-bound magnesium represents <1 % of total magnesium intake in industrialized countries and is therefore of little relevance to magnesium nutrition.

Introduction

When iron is bound within the porphyrin ring of heme, it is absorbed to a much greater extent than non-heme iron. In industrialized countries, heme iron from animal foods represents about 10-15 % of mean iron intake but it provides up to 50 % of absorbed iron (Allen & Ahluwalia, 1997).

Magnesium is similarly bound to the central atom in the porphyrin ring of the major green plant pigments chlorophyll *a* and *b*. In plant leaves, up to 25 % of total magnesium can be present as chlorophyll-bound magnesium (Marschner, 1986) although this can increase up to 60 % (Dorenstouter *et al.*, 1985).

It has been suggested that chlorophyll-bound magnesium may play an important role in magnesium nutrition (Hazell, 1985; Fairweather-Tait & Hurrell, 1996). Vegetables and fruits have been reported to contribute 18% of the magnesium intake in Germany (Deutsche Gesellschaft für Ernährung, 1996), 21-43 % in Switzerland (De Rham, 1991; Schlotke & Sieber, 1998), 16 % in the UK (Spring *et al.*, 1979) and 26 % in the USA (Pennington & Young, 1991). The chlorophyll content of most common plant foods is unknown, although there is limited data on some leafy vegetables (Kaur & Manjrekar, 1975; Khachik *et al.*, 1986). In order to evaluate the importance of chlorophyll-bound magnesium intake in industrialized countries we have measured chlorophyll *a* and *b* and magnesium in a variety of commonly consumed green vegetables and fruits, and calculated the ratio of chlorophyll-bound magnesium to total magnesium. In addition, the influence of food preparation and cooking on the chlorophyll content was investigated with spinach as a model. Based on the chlorophyll-bound magnesium fraction in the analyzed plant foods, and on published food consumption data, we have evaluated the importance of chlorophyll-bound magnesium in magnesium nutrition.

Materials and Methods

Plant foods

Frequently consumed vegetables and fruits, expected to contain relatively high amounts of chlorophyll, were selected based on Swiss food consumption data (Erard & Sieber, 1991; Grüter *et al.*, 1998). Twenty-one fresh plant foods in amounts of 50-500 g were bought between July and August 2000 at local supermarkets, weighed, and immediately frozen at -70°C until analysis. Green peas were purchased already frozen. Only the edible parts of each plant food were analysed with the exception for artichoke where whole leaves were measured and cucumber which was analyzed unpeeled.

Quantification of chlorophylls

Chlorophyll *a* and *b* were measured in duplicate by HPLC as described by Bohn *et al.* (2002, prepared for publication). The chlorophylls were extracted from the untreated frozen plant material by N,N-dimethylformamide, purified by solid-phase

extraction, and chlorophyll *a* and *b* were quantified by a HPLC fluorescence technique, using zinc-phthalocyanine as an internal standard. Pigments were identified by retention times. The degradation products chlorophyll *a'* and *b'* (the C-13² epimerization products), and pheophytins (*a*, *b*, *a'*, *b'*) were used as markers for degradation and were detected by comparing retention times with literature values (Khachik *et al.*, 1986; Zapata *et al.*, 1987; Vanbreemen *et al.*, 1991; Brotas & Plante-Cuny, 1996). All reagents and solvents were of analytical grade or superior, and only 18 MΩ water (Milli Q water system, Millipore, Zurich, Switzerland) was used.

Quantification of total magnesium and chlorophyll-bound magnesium

Frozen uncooked plant material (10-20 g) was thoroughly crushed and mixed at room temperature in a mortar. An 1-3 g aliquot was weight into a teflon vial, mineralized in a micro-wave digestion system (MLS 1200 Mega, MLS GmbH, Leutkirch, Germany) in a mixture of 65 % HNO₃ and 30 % H₂O₂, and quantified by flame atomic absorption spectroscopy (SpectrAA 400, Varian, Mulgrave, Australia) using external calibration standards. All measured solutions contained La(NO₃)₃ at 5000 mg/L to suppress matrix effects. A certified reference material (wheat flour 1567 a, NBS, National Bureau of Standards, Gaithersburg, MD, USA) was analyzed in parallel to monitor accuracy of analysis. All samples were analyzed in duplicate.

Chlorophyll-bound magnesium of each plant food was calculated based on the chlorophyll *a* and *b* content determined by HPLC and the theoretical magnesium content (in % mass) of chlorophyll *a* (2.72 %) and chlorophyll *b* (2.68 %).

Food preparation and cooking procedures

As most plant foods are not consumed raw, the effect of food preparation and cooking procedures on the chlorophyll content was investigated. The influence of thawing and chopping followed by boiling and steaming was investigated with spinach, a vegetable with a high chlorophyll content.

To estimate the effect of thawing on fresh frozen vegetables, about 200 g fresh spinach was frozen (−70 °C) for 24 h and then thawed at room temperature (4 h)

before analysing (n=6). The effect of chopping on chlorophyll degradation (n=6) was investigated by cutting fresh spinach leaves into ~25 cm² pieces with a sharp knife. For achieving improved homogeneity of the samples, a large amount spinach (1 kg) was chopped, mixed, and then divided into 3 equal portions for analysis of chopped, boiled, and steamed spinach. Fresh chopped spinach (50 g) was weighed into 250 ml glass beakers (n=6), 1 g sodium chloride and 100 g tap water were added and the beaker was covered with a lid and brought to boiling on a heating plate. After boiling had started, the beaker was stirred for ~5 sec every min for 3 min, after which the heating plate was switched off. After a further 2 min, the beaker was removed from the plate, allowed to cool for 10 min at room temperature, and the boiling water was decanted. The boiled spinach and the decanted water were weighed and analysed separately for their chlorophyll content. A similar steaming procedure was followed with 5 g tap water without stirring. All samples obtained after thawing, chopping, boiling, and steaming were frozen to -70 °C and analysed for chlorophyll content as described previously.

Statistics

Calculations were done using commercial spreadsheet software (Excel 97, Microsoft, Chicago, Illinois, USA; and SPSS 10.0, SPSS inc., Chicago, Illinois, USA). Means are expressed as arithmetic means±SD. Level of significance was defined as P<0.05. Paired student's, 2-tailed t-tests were used to compare chlorophyll a and b degradation, and unpaired, 2-tailed t-tests were used to compare the different food preparation procedures. Normal distribution of absorption values was confirmed by skewness and Kolmogorov-Smirnoff test.

Results

Magnesium, chlorophyll, and chlorophyll bound-magnesium

The results are shown in Table 1. The magnesium content of the commonly consumed, uncooked vegetables and fruits varied from 48 µg/g (grape) to 849 µg/g (spinach) with a median of 122 µg/g.

Table 1.

Magnesium (Mg), chlorophyll (chl), chlorophyll-bound magnesium (chl-Mg), and chlorophyll-bound magnesium as % of total magnesium (chl-Mg/total-Mg) of commonly consumed, uncooked vegetables and fruits.

plant food	Mg ¹ (µg/g)	chl ² (µg/g)	ratio chl _a /chl _b ³	chl-Mg ⁴ (µg/g)	chl-Mg/ total-Mg (%)
artichoke	347	49	3.2	1.33	0.38
broccoli	139	21	3.5	0.58	0.41
celery	264	23	3.4	0.64	0.24
chinese cabbage	115	58	2.7	1.59	1.38
cress	271	311	2.6	8.49	3.13
cucumber	85	36	2.5	1.00	1.17
endive	90	104	3.1	2.85	3.16
fennel	81	6	5.4	0.16	0.19
grape	48	6	5.3	0.17	0.34
green bean	246	75	3.5	2.05	0.83
green pea	243	50	3.3	1.36	0.56
ice-berg lettuce	51	29	3.7	0.81	1.58
kiwi	128	15	2.7	0.41	0.32
lettuce	103	245	4.3	6.67	6.49
green paprika	82	38	2.8	1.03	1.26
parsley	112	632	3.1	17.26	15.41
prickly lettuce	101	388	3.4	10.61	10.50
leek	66	87	3.2	2.38	3.60
rocket salad	310	408	3.6	11.15	3.60
spinach	849	791	2.9	21.59	2.54
sugar pea	266	76	4.2	2.07	0.78
zucchini	225	68	2.6	1.85	0.82
Mean:	191.9	159.8	3.4	4.4	2.7
Median	121.5	63.0	3.3	1.7	1.2

¹: magnesium content as determined by atomic absorption spectroscopy

²: total chlorophyll content as determined by HPLC

³: amount ratio of chlorophyll *a*/chlorophyll *b*

⁴: calculated based on measured magnesium and chlorophyll content and the magnesium content of chlorophyll of 2.72 % (chlorophyll *a*) and 2.68 % (chlorophyll *b*) by mass.

The chlorophyll content also varied considerably. The lowest total chlorophyll concentrations were found in grape and fennel (both 6 µg/g), the highest in spinach

(791 µg/g). The median of total chlorophyll content was 63 µg/g. Chlorophyll-bound magnesium as % of total magnesium varied between 0.2 % (fennel) and 15.4 % (parsley) with a median of 1.2 %. The ratio of the amount chlorophyll *a* to chlorophyll *b* varied from 2.5-5.4 with a median of 3.3. No, or only minor indications of degradation products were detected in the analysed fresh food samples. Pheophytin *a*, *b*, and the C-13² epimerisation products pheophytin *a'*, *b'* and chlorophyll *a'* and *b'* were detected in the peas which were bought frozen.

Food preparation and cooking procedures

Chopping was the major cause of chlorophyll degradation in the food preparation procedures. Chopping of spinach resulted in 38.7±6.1 % total chlorophyll degradation in spinach (Fig. 1). Chopping together with boiling and steaming caused only small additional chlorophyll losses of about 6 % and 13 %, respectively (total losses were 44.2±5.9 and 51.5±4.4 %). Chopping and boiling resulted in significantly lower chlorophyll degradation than chopping and steaming ($P < 0.05$). The boiling and steaming water contained <1% of the original chlorophyll content (~0.6 % and ~0.3 %). Thawing of frozen fresh spinach also resulted in high chlorophyll degradation (34.6±4.2 %). Mean chlorophyll *a* degradation was significantly higher than chlorophyll *b* degradation when the results for all preparation and cooking procedures were combined (45.6±8.0 % compared to 32.8±9.8 %, $P < 0.0001$, $n=24$), and also for each of the 4 individual food preparation procedures ($P < 0.05$, $n=6$).

Pheophytin *a*, *b*, and the C-13² epimerisation products pheophytin *a'*, *b'* and chlorophyll *a'* and *b'* were detected after thawing, chopping and additional boiling and steaming of spinach.

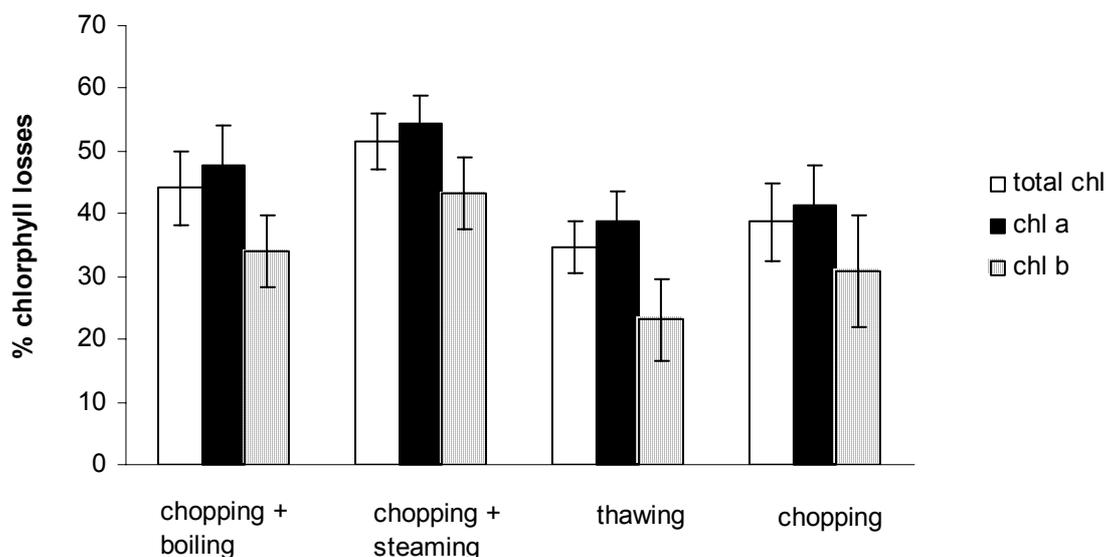


Figure 1.

Degradation of chlorophyll in spinach after several preparation and cooking procedures (n=6 for each procedure, 100 % = fresh frozen spinach).

Discussion

The magnesium content of the 22 common plant foods ranged from 48-849 $\mu\text{g/g}$. This range is similar to that reported in food data bases (Haenel, 1979; Holland *et al.*, 1994; Souci *et al.*, 1994). The chlorophyll content in the plant foods ranged from 6-791 $\mu\text{g/g}$, a far larger variation when compared to the range of the magnesium content. This is to be expected, as chlorophyll is primarily needed for photosynthesis whereas magnesium has many functions, and would therefore assumed to be more evenly distributed.

A high chlorophyll content was found in leafy vegetables such as spinach and salads. As photosynthesis takes place mainly in leaves, leafy vegetables have a much higher chlorophyll content than other vegetables or fruits. In previous studies

(Kaur & Manjrekar, 1975; Khachik *et al.*, 1986), the chlorophyll content of 5 plant foods (spinach, cabbage, brussels sprouts, kale, and broccoli) was measured. The chlorophyll content in spinach reported by these authors was found to be around 130 mg/100 g, which is somewhat higher than in our study (80 mg/100 g), while chlorophyll content of cabbage was reported to be 1.7 mg/100 g, which is slightly below the value which was obtained for chinese cabbage in the present evaluation (5.8 mg/100 g). These differences are not unexpected as magnesium and chlorophyll content would be expected to vary with growing site, agricultural practices, time point of crop, weather, soil composition, and different sub-species. The amount ratios of chlorophyll *a*/chlorophyll *b* (2.5-5.4) were similar to the previously reported ranges of 2.8-4.7 (Kaur & Manjrekar, 1975; Khachik *et al.*, 1986; Rousos *et al.*, 1986).

No chlorophyll degradation was observed during pigment extraction and purification during analysis of the untreated plant foods, as indicated by the virtual absence of pheophytins and epimerization products in the HPLC analysis. The presence of degradation products in the frozen peas however indicated chlorophyll degradation during the industrial process, probably during the initial blanching step prior to freezing (Schwartz & Vonelbe, 1983). Blanching is usually done to inactivate enzymes, including chlorophyllase, thus preventing further degradation of the chlorophylls. Detection or quantification of chlorophyll degradation products has been suggested as an indicator for food processing (Clydesdale & Francis, 1968; Ashgar *et al.*, 1978; Mangos & Berger, 1997).

The percentage of total magnesium that is chlorophyll-bound was relatively low and ranged from 0.2-15.4 %. It was highest in leafy vegetables and herbs. These results are similar to the ranges of chlorophyll-bound magnesium of total magnesium reported by Michael (1941) for maize leaves (0.3-19 %), Scott and Robson (1990) for clover leaves (6-35 %), and the range (6-25 %) given in a review by Marschner (1986) including in addition norway spruce needles. Under more extreme conditions, such as for magnesium deficient plant leaves that are in the shade, chlorophyll-bound magnesium as percentage of total magnesium can rise to nearly 60 % (Dorenstouter *et al.*, 1985).

The amount of chlorophyll-bound magnesium can be expected to further decrease after food preparation and cooking procedures. We found chlorophyll losses as a

result of thawing and chopping of about 35 %, while boiling or steaming of chopped fresh spinach lead only to small additional chlorophyll losses (Fig. 1). This can be explained by breakdown of the cell structures and the release of chlorophyllase, leading to saponification of the phytol chain of chlorophyll to form the respective chlorophyllides. During heat treatment, the chlorophyllase is inactivated and the small amount of further degradation observed in the present evaluation may have been caused by organic acids released by cell rupture or heat (Haisman & Clarke, 1975). However, a study by Khachik *et al.* (1986) indicated chlorophyll losses of 37 % and 39 % for cooked kale and brussels sprouts alone.

All food preparation and cooking procedures caused a significantly higher degradation of chlorophyll *a* than chlorophyll *b* (45.6 ± 8.0 compared to 32.8 ± 9.8 %), which is in line with earlier reports on chlorophyll degradation (Mackinney & Joslyn, 1940; Schanderl *et al.*, 1962; Siefermann-Harms & Ninnemann, 1983). However, as chlorophyll content and enzyme activities differ between plant parts and plant species, degradation pathways and rate of chlorophyll degradation may differ.

In order to evaluate the nutritional importance of chlorophyll-bound magnesium, it is necessary to evaluate whether chlorophyll-bound magnesium will be absorbed to a different extent than other food magnesium. When iron as heme iron is bound within a similar porphyrin structure as magnesium in chlorophyll, its absorption is up to 10 times higher than non-heme iron (Weintraub *et al.*, 1968; Bothwell *et al.*, 1989), because the heme molecule is absorbed intact and iron absorption is not inhibited by food components such as phytic acid. Although a similar effect has been suggested for chlorophyll (Hazell, 1985; Fairweather-Tait & Hurrell, 1996), this seems unlikely because at the acid pH of the gastric juice, a rapid and irreversible degradation of the chlorophylls to their corresponding pheophytins has been reported. During this degradation, magnesium is released from the porphyrin ring and replaced by two protons (Schanderl *et al.*, 1962; Siefermann-Harms & Ninnemann, 1983). Some indication as to the absorption of chlorophyll-bound magnesium can be obtained from the study of Schwartz *et al.* (1984) who reported no significant difference in magnesium absorption from intrinsically labelled spinach and an extrinsic isotope label. As spinach is rich in chlorophyll-bound magnesium, these results would indicate that chlorophyll-bound magnesium and non chlorophyll-bound magnesium are absorbed to a similar extent.

The importance of chlorophyll-bound magnesium to magnesium nutrition can be evaluated by estimating the percent of total magnesium intake provided by chlorophyll-bound magnesium. Fruits and vegetables have been reported to contribute between 16 and 43 % of the total magnesium intake in industrialized countries (Spring *et al.*, 1979; De Rham, 1991; Pennington & Young, 1991; Deutsche Gesellschaft für Ernährung, 1996; Schlotke & Sieber, 1998). The amount of green fruits and vegetables containing chlorophyll which are consumed can be expected to be lower. In Switzerland, green fruits and vegetables can be estimated to contribute to about 60 % of totally consumed fruits and vegetables (Erard & Sieber, 1991; Grüter *et al.*, 1998). Based on recent Swiss data (Erard & Sieber, 1991; Grüter *et al.*, 1998) we estimated that chlorophyll-bound magnesium represents below 1 % of total magnesium intake. This is based on 43 % of total magnesium intake coming from fruits and vegetables, 60 % of these fruits and vegetables containing chlorophyll and, from the present investigation, that an average of 2.7 % of total magnesium is chlorophyll-bound in green fruits and vegetables. Furthermore, cooking and food preparation will cause additional losses, especially if the plant foods are chopped. Although this is a very approximate estimate it is clear from the present investigations that chlorophyll-bound magnesium contributes to only a small and nutritionally insignificant part of total magnesium intake in industrialized countries.

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Conclusions and perspectives

In the present studies, different approaches using stable isotope techniques were found to be useful to determine Mg absorption in human subjects. However, one exception was observed for the complete 6 d urinary pools (days 1-6 after test meal intake), which resulted in statistically significantly underestimates of Mg absorption, while Mg absorption based on 24 h urine pools (collected on day 2) was not significantly different from results based on the conventional 6 day faecal monitoring technique. These findings highlight the possibility of differences in Mg metabolism between the intravenous and oral isotopic label in the early phase after administration. Further studies are needed to evaluate the possibility to optimize double isotope techniques, in particular the administered dose, time point, and duration of the injection need to be considered in order to avoid perturbing normal Mg metabolism.

In addition to the conventional faecal and urinary monitoring techniques, Mg absorption was also determined by a novel method based on simultaneous incorporation of an intravenous and an oral label into blood cells, thus avoiding time consuming and labour intense stool and urine collections and sample preparation procedures. The potential usefulness of this method needs to be investigated in more detail. However, a limitation is that this method will depend on the availability of sophisticated mass spectrometric equipment for precise isotope ratio measurements, e.g. multi collector ICP-MS or high resolution ICP-MS. In addition to measurements of isotopic ratios in biological samples with low enrichment of the isotopic label such as blood cells or saliva, these instruments would offer the possibility to decrease the administered dose of isotopic labels and thus limit the influence of stable isotope labels on total Mg content of labelled test meals and on Mg metabolism.

Mg absorption in humans was found to be significantly influenced by the dietary composition. Phytate at similar concentrations as usually occurring in whole-meal bread and brown bread decreased fractional (percent) Mg absorption significantly in a dose dependent manner as compared to phytate-free white bread. Whether even lower concentrations of phytate, for example similar to those present in white bread, influence fractional Mg absorption, needs to be investigated in future studies. In

addition to phytate, test meals based on spinach, an oxalate rich vegetable, resulted in significantly lower fractional Mg absorption as compared to test meals based on kale, a vegetable with low oxalate content. Nevertheless, spinach can be expected to be a good source of Mg, as the decrease in fractional Mg absorption from spinach is, at least partly, counterbalanced by the relatively high native Mg content. Similar assumptions can be made for whole grain cereals rich in phytic acid. The amount of Mg absorbed from wholemeal or brown bread would therefore be expected to be similar to that from phytate-free white bread.

It would be of interest to investigate whether the observed inhibitory effect of phytic acid and an oxalate rich vegetable on Mg absorption can be counteracted by other food compounds, as, for example, is known for iron and ascorbic acid. This will depend on the underlying mechanism of decreased Mg absorption, e.g. whether decreased Mg absorption is due to decreased absorption of food Mg or due to increased endogenous Mg losses. This could be the subject of future investigations.

In industrialized countries, intake of phytic acid and oxalate can be assumed to be in a range not to considerably alter bioavailability, and oxalic and phytic acid rich foods are often rich in Mg. Therefore, it is unlikely that the reported association between Mg intake and major health concerns such as osteoporose, diabetes type II, or coronary artery disease is significantly influenced by the intake of oxalate and phytic acid rich foods. In developing countries, where higher amounts of phytic acid and oxalate can be expected to be consumed in a cereal, vegetable, and fruit based diet, dietary Mg bioavailability might be of greater importance. However, the present knowledge about the correlation between Mg nutrition and major health concerns is scarce. This is mainly because there is currently no reliable indicator of Mg status, which emphasises the need to evaluate a method to determine Mg status in human subjects.

Besides the influence of phytic acid and oxalate, very little is known about dietary factors influencing Mg absorption. However, a number of other dietary factors have been suggested to influence Mg absorption, for example proteins, polyphenols, and nondigestible soluble carbohydrates such as inulin, oligofructose, and resistant starch, oligofructose has already been shown to increase Mg absorption.

Nondigestible soluble carbohydrates would therefore be promising compounds to be investigated. This could be of interest especially in the functional food sector.

Green plant foods containing chlorophyll-bound Mg have been reported to be good sources of Mg and therefore to be of importance in Mg nutrition, and have been suggested to contribute a separate Mg pool in the diet. The present study included the development of an analytical method to measure chlorophyll-bound Mg and to estimate the proportion of chlorophyll-bound Mg in common plant foods. These preliminary findings indicate that chlorophyll-bound Mg is very low and contributes only a very small part to total dietary Mg intake in industrialized countries and therefore is of limited relevance to Mg nutrition.

Curriculum Vitae

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