



# The fate of phytic acid and vitamin E during processing of rye and wheat

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# Preface

This PhD thesis is intended to fulfil the requirements for obtaining a PhD degree at the Faculty of Life Sciences, University of Copenhagen. The presented work has been carried out at Quality & Technology, Department of Food Science under the supervision of Associate Professor Åse Hansen to whom I am grateful for inviting me to do this PhD. This study was partly financed by three industrial partners: Jørn Ussing Larsen (Aurion A/S), Susanne Danielsen (Valsemøllen A/S), Ole Kirk Østergaard (Lantmännen A/S), a local baker Thorleif Kristensen (Musik-Conditoriet) and by SOAR and DFFE. I am very grateful for their support and supply of samples. Special thanks go to laboratory technician Vibeke Sørensen (Valsemøllen A/S) for her kind help. I would also like to thank Agnete Dal Thomsen and Tina Lindeløv at Schulstad A/S for “lending” me the bakery. Søren K. Rasmussen is acknowledged for his kind help with the phytic acid HPLC measurements at Risø. I have been a part of the organic research school SOAR under Henning Høgh Jensen. This has given me a great opportunity to meet people from around the world, whom I never would have met otherwise, and scientifically it has been a big challenge to convert my work into “real” organic stuff. Thanks to Henning and Sofie for taking me under their wings.

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Family and friends! Thanks for being so patient with me and for helping me in every possible way during this PhD

Merete Møller Nielsen  
November, 2007

# Summary

Cereals and cereal products are important sources of the recommended daily intake of minerals and vitamins through our diet. This study concerns the two cereal micronutrients phytic acid and vitamin E and their degradation through cereal processing. In cereal products minerals are bound by phytic acid for which reason phytic acid is historically considered to have a negative effect on health. Phytic acid however, also functions as an antioxidant by chelating iron ions and therefore a certain amount of phytic acid is desirable. The most important antioxidant in cereals is vitamin E, which besides having an essential health effect is able to prevent oxidation of unsaturated fatty acids in cereals and thereby prolong the shelf life of the cereal products.

As phytic acid and vitamin E were studied in different cereals and in different environments, this project was divided into two parts:

## **PART 1: PHYTIC ACID (PAPER I-III)**

A method for determination of phytase activity was developed, optimised and validated. Phytase is an enzyme that hydrolyses the phosphate groups from phytic acid and thus reduces its ability to bind the minerals. The method was especially used for determination of pH and temperature optima for phytase activity under industrial conditions for rye bread making. Nuclear magnetic resonance (NMR) spectroscopy was used to elucidate the degradation pattern of phytic acid by phytase in a model system and data was evaluated using multivariate curve resolution (MCR). The hydrolysis of the six phosphate groups on phytic acid was clearly determined to be a stepwise degradation which could be elegantly followed all the way to the last phosphate group hydrolysed. NMR spectroscopy and MCR proved to be very effective tools for measuring phytic acid degradation. In addition, the phytic acid degradation was measured during industrial production of three types of whole meal rye breads. The results showed that the degradation of phytic acid in rye bread with up to 50% of

added whole rye grains was sufficient for releasing the required minerals. But the results also proved that the degradation products of phytic acid, lower inositol phosphates, which still is capable of complex bind the minerals, are still present in a significant level in rye breads with added whole grains.

## **PART 2: VITAMIN E (PAPER IV-VI)**

A normal phase-high pressure liquid chromatography (NP-HPLC) method for determination of all eight natural occurring vitamin E isomers, four tocopherols and four tocotrienols, was developed, optimised and validated. The importance of obtaining a method by which all eight isomers are detected is underlined by the fact that the different vitamin E isomers display different biological activity and that the vitamin E profile differs from cereal to cereal. Barley, for example, contains all eight isomers, whereas wheat only contains four isomers of vitamin E. The method was applied for determination of the distribution of vitamin E in the wheat grains. The results showed that the tocopherols primarily were located in the germ, whereas the tocotrienols were primarily located in the bran and endosperm. The loss of vitamin E after milling of grain to flour was also examined. The grains were milled on an industrial roller mill or on a stone mill, which is often used by organic millers. The result showed no difference between the milling methods with regards to vitamin E content in whole wheat flour. Organic wheat flour milled on a stone mill had twice the content of roller-milled wheat flour, which is due to the different recipe used. Roller-milled wheat flour was whiter, due to the lack of bran fractions, which is preferred by some consumers, whereas the stone-milled wheat flour, which consisted of more bran and germ, were darker. The content of vitamin E was highest in the stone-milled flour, but the content of vitamin E decreased for both milling methods by approx. 30% during a storage period of approx. 10 months. Current practice in Denmark is to state nine month as the shelf life for flour, but the 30% loss of vitamin E occurred within three months; thereafter the content was stable. So in order to maintain as high a content of vitamin E as possible in the breads, the flour should be used as fresh as possible. The total content of vitamin E in whole grain breads showed to be sufficient for the recommended daily intake of vitamin E.

The results from this study suggest that it should be recommended that consumers eat whole grain products rather than refined products due to the benefits of the micronutrients present in the bran and germ fractions.

# Sammendrag

Korn og kornprodukter er vigtige kilder til den daglige mængde mineraler og vitaminer vi naturligt bør indtage gennem kosten. Dette studie omhandler de to mikronæringsstoffer fytinsyre og E-vitamin og deres nedbrydning under forarbejdning. Mineraler bindes i kornprodukter af fytinsyre, som dermed historisk har været anset for at have en negativ sundhedsmæssigt effekt. Fytinsyre kan dog også fungere som antioxidant ved at binde jernioner, så derfor kan en vis mængde fytinsyre være ønskeligt. Den vigtigste antioxidant i korn er E-vitamin, som udover at have en essentiel sundhedsmæssig effekt, kan hindre oxidation af umættede fedtsyrer i kornet og dermed forlænge holdbarheden.

Da fytinsyre og E-vitamin er undersøgt i forskellige typer korn og under forskellige betingelser, er projektet inddelt i to dele.

## **DEL 1: FYTINSYRE (PAPER I-III)**

Der blev udviklet, optimeret og indkørt en metode til måling af fytase, som er et enzym, der hydrolyserer fosfatgrupperne på fytinsyre og således reducerer dets evne til at binde mineraler. Metoden blev især anvendt til bestemmelse af pH- og temperatur optima for fytase aktivitet under de industrielle betingelser, der er til stede under rugbrødsbagning. Kerne-magnetisk resonans (NMR) spektroskopi blev anvendt til at klarlægge fytinsyrens nedbrydning ved brug af fytase i et modelsystem og data blev evalueret ved brug af multivariat kurve resolution (MCR). Nedbrydningen af fytinsyrens seks fosfatgrupper blev klart påvist at være en trinvis nedbrydning og kunne følges helt ned til sidste fosfatgruppe. NMR spektroskopi og MCR viste sig at være særdeles nyttige redskaber til måling af fytinsyrenedbrydning. Derudover blev fytinsyrenedbrydningen fulgt via HPLC under industriel fremstilling af tre typer fuldkornsrugbrød. Resultatet af denne undersøgelse viste, at fytinsyrenedbrydningen i rugbrød tilsat op til 50% hele kerner, var fyldestgørende nok til, at den nødvendige mængde mineraler blev frigivet. Men resultaterne viste ligeledes, at en signifikant

mængde nedbrydningsprodukter fra fytinsyre, kaldet lavere inositol fosfater der stadig er i stand til at binde mineraler, stadig er til stede i fuldkornsbrød.

## **DEL 2: E-vitamin**

Der blev udviklet, optimeret og indkørt en væskekromatografisk (NP-HPLC) metode til måling af alle otte naturlige former for E-vitamin, fire tokoferoler og fire tokotrienoler. Vigtigheden af denne optimerede metode, hvor alle otte former kan detekteres er, at de otte E-vitamin former udviser forskellig biologisk aktivitet og at E-vitaminprofilen er forskellig fra kornart til kornart. Eksempelvis indeholder byg alle otte former, hvorimod hvede kun indeholder fire former for E-vitamin. Metoden blev ligeledes anvendt til at undersøge sammensætningen og fordelingen af E-vitamin i hvedekerner. Resultaterne viste, at tokoferolerne primært var lokaliseret i kimen, hvorimod tokotrienolerne primært var lokaliseret i kliddelene, men også i endospermen. Det blev ligeledes undersøgt om tabet af E-vitamin indholdet ved formaling af korn til mel var afhængigt af formalingsmetoden. Melet blev formalet på enten en industriel valsemølle eller på en stenkværn, som ofte anvendes af økologiske møllere. Resultatet viste, at der ikke var forskel på de to metoder med hensyn til E-vitamin indholdet i grahamsmel. Økologisk hvedemel formalet på stenkværn indeholdt derimod væsentlig mere E-vitamin end valsestolsformalet hvedemel, men det skyldtes en forskel i den anvendte recept. Valsestolsformalet mel var mere hvidt, fordi klid og kim blev fjernet, hvilket er ønsket af nogle forbrugere, hvorimod stenkværnet mel, der indeholdt flere skaldele og kim, var mørkere. Indholdet af E-vitamin var højere i stenkværnet mel og faldt for begge formalingsmetoder med ca. 30% i løbet af lagringsperioden på ca. 10 måneder. I dag anvendes 9 måneder som holdbarhedsperiode for mel i Danmark, men faldet på 30% i E-vitamin indhold var størst i løbet af de tre første måneder, hvorefter E-vitamin indholdet var stabilt. Så for at bibeholde så meget E-vitamin som muligt i de bagte brød, bør melet anvendes så frisk som muligt. Det samlede indhold af E-vitamin i grove brød lå i det anbefalede område for indtag af E-vitamin.

Resultaterne af dette studie viser, at det bør anbefales at forbrugerne at spiser fuldkornsprodukter pga de mange positive effekter af mikronæringsstoffer, som er til stede i klid- og kindelene af korn.

# List of Publications

- Paper I:** Nielsen, M.M., Damstrup, M.L. & Hansen Å. (2007) An optimised micro-titer plate method for characterisation of endogenous rye phytase under industrial rye bread making conditions. *European Food Research and Technology* (accepted)
- Paper II:** Nielsen, M.M., Viereck, N. & Engelsen, S.B. (2007) Phytic acid degradation by phytase - as viewed by <sup>31</sup>P NMR and multivariate curve resolution. In *Magnetic Resonance in Food Science: From Molecules to Man* (Eds. I. Farhat, P.S. Belton and G.A. Webb), The Royal Society of Chemistry, UK, ISBN: 978-0-85404-340-8, pp. 214-222
- Paper III:** Nielsen, M.M., Damstrup, M.L., Thomsen, A.D., Rasmussen, S.K. & Hansen Å. (2006) Phytase activity and degradation of phytic acid during rye bread making. *European Food Research and Technology* 225 (2), pp. 173-181
- Paper IV:** Nielsen, M.M. & Hansen Å. (2007) Rapid High-Performance Liquid Chromatography determination of tocopherols and tocotrienols in cereals. *Cereal Chemistry* (accepted)
- Paper V:** Nielsen, M.M. & Hansen Å. (2007) Content and distribution of vitamin E in industrial wheat roller mill and stone mill streams. *Journal of Agriculture and Food Chemistry* (submitted)
- Paper VI:** Nielsen, M.M. & Hansen Å. (2007) Stability of Vitamin E in wheat flour and whole wheat flour during storage. *Cereal Chemistry* (submitted)



# List of abbreviations

AACC	American Association of Cereal Chemists
$\alpha$ -TE	Alpha-tocopherol equivalents
DS	Damaged Starch
DF	Dietary Fibre
AX	Arabinoxylan
WE	Water-Extractable
WU	Water-Unextractable
IP <sub>6</sub>	<i>myo</i> -inositol hexaphosphate
IP <sub>5</sub>	<i>myo</i> -inositol pentaphosphate
IP <sub>4</sub>	<i>myo</i> -inositol tetraphosphate
IP <sub>3</sub>	<i>myo</i> -inositol triphosphate
IP <sub>2</sub>	<i>myo</i> -inositol diphosphate
IP <sub>1</sub>	<i>myo</i> -inositol monophosphate
IP/Ins:	<i>myo</i> -inositol
P <sub>i</sub>	Inorganic phosphate
$\alpha$ -T	Alpha-Tocopherol
$\beta$ -T	Beta-Tocopherol
$\gamma$ -T	Gamma-Tocopherol
$\delta$ -T	Delta-Tocopherol
$\alpha$ -T3	Alpha-Tocotrienol
$\beta$ -T3	Beta-Tocotrienol
$\gamma$ -T3	Gamma-Tocotrienol
$\delta$ -T3	Delta-Tocotrienol
WWF	Whole Wheat Flour
WRF	Whole Rye Flour
WF	Wheat Flour
RF	Rye Flour
WGM	Wheat Germ
DB00	Dark rye bread without added whole rye grains
SB30	Dark rye bread with 30% added whole rye grains
KB50	Dark rye bread with 50% added whole rye grains
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
MCR	Multivariate Curve Resolution
HR-MAS	High Resolution-Magic Angle Spinning
NP-HPLC	Normal-Phase High Performance Liquid Chromatography
LOX	Lipoxygenase

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## PAPER I-VI



# 1. Introduction

In recent years there has been an increasing focus on the positive influence that whole grain products have on many lifestyle diseases such as diabetes, cancer, cardiovascular diseases and obesity and in 2005 the European project "Healthgrain" was funded and initiated. This project include 43 partners from 15 different countries with the common goal to improve the well-being, and reducing the risk of diseases related to the metabolic syndrome among European consumers by increasing the intake of whole grain products ([www.healthgrain.org](http://www.healthgrain.org), 2007). One strategy to reach the goal is to identify sources of variation in bioactive compounds and to develop "omics" technologies and biotechnology for accelerated breeding of improved cultivars ([www.healthgrain.org](http://www.healthgrain.org), 2007). Another strategy is to develop technology and processing tools for nutritionally optimised cereal foods and new food ingredients from whole grains ([www.healthgrain.org](http://www.healthgrain.org), 2007). Since 1999 the Food and Drug Administration in the United States has allowed labelling of health claims if a product contain more than 51% whole grain, and the European parliament is at present time considering allowing the same type of labelling. This will inevitably increase the intake of whole grain products and make it clear for the consumers, which whole grain products have health benefits.

A definition of whole grain has been discussed for a long time, and the following definition was approved and accepted by the AACC International Board of Directors in 1999 (American Association of Cereal Chemists, 1999):

*"Whole grains shall consist of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis."*

Besides dietary fibres, two important aspects of the positive health effects of whole grain cereals are the content of phytic acid and vitamin E.

Phytic acid is normally seen as an anti-nutrient factor in food, because it is capable of chelating minerals present in the flour, and thus makes the minerals unavailable for the human body, which in turn can lead to different malnutrition diseases, such as anaemia. Studies have shown, on the other hand, that phytic acid also can act as an antioxidant (Graf and Eaton, 1990) and have an anti-carcinogenic effect (Shamsuddin, 2002; Shamsuddin, 1995; Phillippy and Graf, 1997; Vucenik and Shamsuddin, 2003), and thus have a positive effect on health.

One of the aims of this study was therefore to investigate the level of phytic acid in different whole grain products commercially available in Denmark in order to determine whether or not minerals are bioavailable and thus provide nutritional benefits to humans. Rye bread was chosen in this study, because Denmark is in the middle of the rye belt and it is a local crop and is a principal whole grain product that has been consumed for more than thousands year (Jacob, 1997).

Vitamins are essential organic compounds required in the diet, because it cannot be synthesised by the human organism. Lack of vitamins in our food can lead to deficiency diseases such as osteomalacia (vitamin D) and beriberi (vitamin B<sub>1</sub>) (Bender, 2003). Deficiency of vitamin E is well known in animals, resulting in reproductive failure, liver and kidney damage and neurological abnormalities, whereas deficiency in humans is not as well defined, and is mainly a problem in premature infants with low birth weight and in people with abnormalities of lipid absorption (Bender, 2003).

Vitamin E consists of eight natural occurring isomers, four tocopherols and four tocotrienols, which display different biological activity. Knowledge about their distribution in the different botanical parts will enable the millers to produce flours for specific purposes. Vitamin E is a lipid-soluble antioxidant and as such assumed to be important for human consumption. The recommended intake of vitamin E in Denmark is 8-10  $\alpha$ -TE/day, but according to a study of the eating habits of Danes in 1995, only 40% of the registered meals fulfilled that goal (Fødevaredirektoratet, 1999). The most important dietary sources of vitamin E are cereal grains and vegetable oils (Ball, 2004; Franke et al., 2007; Piironen et al., 1986). However, since 1995 the consumption of rye bread among children has further decreased, whereas the consumption of wheat bread remains the same (Fødevaredirektoratet,

2002). Since the consumers normally want as high a content of natural occurring nutrients in their diet as possible, and since whole meal bread is a good source of nutrients, it is of great interest to produce flour and breads with the highest possible content of vitamin E and with as low as possible content of phytic acid.

Another aim of the study was therefore to determine the content, distribution and retention of the different vitamin E isomers in wheat and during storage.

Two health perspectives of whole grain products were investigated in this study, namely the degradation of phytic acid in rye breads and the degradation of vitamin E content in wheat during processing. The impact of, and knowledge about the physiological distribution, processing and storage of the two micronutrients vitamin E and phytic acid on human nutrition will provide guidance on how to maintain the best possible nutritional quality of the cereal products during processing.

The aim of this thesis is to discuss the essential findings of the six publications in relation to the established research. Chapter 2, 3 and 4, gives a general introduction to the constituents in wheat and rye, the milling processes used and the rye bread making process in a context of the experimental work described in the six papers. Chapter 5 discusses the optimised method and its use to determinate the activity of phytases influence on the degradation of phytic acid both in a model system and in different rye breads (PAPER I-III) and in chapter 6 the optimised HPLC method and the results of measuring vitamin E content in different wheat flours milled in two different ways and during storage is discussed (PAPER IV-VI). Conclusions and perspectives are given in chapter 7.





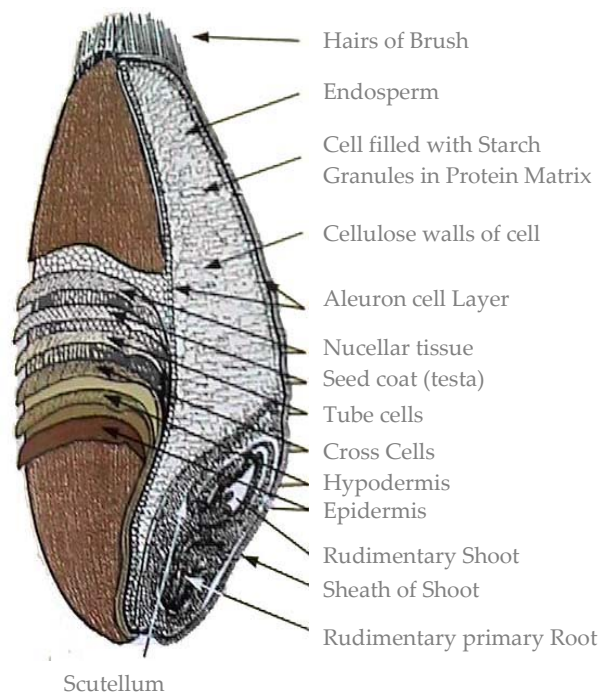
## 2. Cereals

Cereals was the basis of the agricultural revolution and have been grown in Europe for many thousands of year and has been a basic food component for humans ever since.

### 2.1 Cereal structure and composition

Botanically, wheat and rye are caryopsis, single-seeded fruits. They are composed of the same botanically parts and have many structural similarities even though they have a different size and shape. Cereal grains consist, in milling terms, of three major parts: bran, germ and endosperm. In Figure 2.1, a wheat grain is shown, and in Table 2.1 a schematic overview of the compounds present in the different cereal tissues are given.

The bran fraction consist of many layers; the pericarp, testa, nucellus and the aleurone layer and represents 14-16% of the wheat kernel and 10-12% for the rye kernel (Kent and Evers, 1994; Lorentz, 2000). The pericarp is the outer four layers of the kernel and protects the grain during development. The pericarp is characterised by a high content of dietary fibres (Hoseney, 1994c). Testa and nucellus are the two next layers and are also called the fruit coat. Testa regulates the water uptake and contains pigments that gives rye grains its



**Figure 2.1:** Wheat grain, showing the different tissues of the grain (Hoseney, 1994c)

characteristic blue-green colour and wheat an either red or white colour (Hoseney, 1994c). The aleurone layer, which is the outer layer of the endosperm, primarily consists of protein, lipids and minerals. The main function is storage of these compounds and to produce hydrolytically enzymes during germination. The aleurone cells are large and consist of lipid drops and aleurone grains, which primarily consist of phytic acid and a surrounding protein and carbohydrate coat (Reddy, 2002).

**Table 2.1:** A general schematic overview of compounds in wheat and rye tissues (Hemery et al., 2007)

	Principal outer layers				
	Pericarp	Testa	Aleurone	Endosperm	Germ
General grain (%) <sup>†</sup>	7-9	1	5-7	81-86	2-4
Proteins	–	–	**	**	**
Lipids	–	*	**	–	***
Starch	–	–	*	***	–
Dietary fibre	***	**	**	*	–
Tocopherol	–	–	*	*	***
Tocotrienol	*	*	*	*	*
Minerals	*	*	**	–	*
Phytic acid	–	–	***	–	*

<sup>†</sup> The proportions of parts in % is a average of wheat and rye (Kent and Evers, 1994).

\*, \*\*, \*\*\*: Indicates the grade of presence a tissue. –: Indicates that a compound is not found or present in low concentration in a tissue. Modified after Hemery et al. (2007).

The germ consists of scutellum and the embryonic axis and represents 2-3% of the wheat kernel and 2-4% of the rye kernel (Kent and Evers, 1994; Lorentz, 2000). Scutellum, which separates the endosperm from the embryonic axis, functions as a transfer organ between the developing seedling and its nutrient supply (endosperm). It is also a source of hydrolytic enzymes, which modifies the endosperm during the early stages of germination. The germ fraction consists of a large amount of lipid, protein and vitamin E (Hoseney, 1994c).

The endosperm represents 81–84% of the wheat kernel and 85-86% of the rye kernel (Kent and Evers, 1994; Lorentz, 2000). It consist of starch granules embedded in or surrounded by an amorphous protein matrix, which is kept together by hydrogen bindings, which for rye is weak leading to a soft endosperm (Eliasson and Larsson, 1993). The endosperm texture (or hardness) is an important quality factor of wheat for millers, because it affects the degree of damaged starch (DS), which is a performance factor of milling (Greffeuille et al., 2005; Hrusková et al., 2006; Edwards et al., 2007). Hardness corresponds to a physical parameter defined as the degree of resistance to deformation and is controlled by genetic factors (Turnbull and Rahman, 2002). A certain level (4.5%-8%) of DS is beneficial in wheat bread making due to an increase of water absorption and gassing power of the dough, however a too high level ( $\geq 8\%$ ) can result in over-hydration of the dough which in turn leads to a poorer baking performance (Dexter and Wood, 1996).

The most important macro constituents for this study are starch, dietary fibres and lipids and the content of the macro constituents are shown in Table 2.2 and discussed in the following.

**Table 2.2:** Content of macronutrients in rye and wheat in %\*

	Protein	Lipid	Starch	Dietary fibre
Rye	9-15	1-2	59-65	13-19
Wheat	8-14	1-3	53-80	9-17

\*Sources: (Hansen et al., 2002; Hansen et al., 2003; Bach Knudsen et al., 1997; Hosenev, 1994c; Kent and Evers, 1994; Shewry and Bechtel, 2001; Vinkx and Delcour, 1996; Verwimp et al., 2004).

## 2.2 Starch

Starch is the major carbohydrate reserve in plant tubers and seed endosperm where it is found as granules. Starch is composed of two types of molecules, amylose (20-30%) and amylopectin (70-80%) and constitutes of 53-80% of the total carbohydrate content in wheat and rye (Kent and Evers, 1994; Shewry and Bechtel, 2001; Verwimp et al., 2004).

During milling a certain amount of starch granules will be physically damaged dependent on the hardness of the grain. As described above, this

will have an impact on the quality of the flour. The starch content and quality of the starch affects the viscosity of the dough and the water uptake during bread making and too much DS leads also to a higher activity of the starch degrading enzyme;  $\alpha$ -amylase leading to more pasty bread, because of the high degradation of starch.

### 2.3 Dietary fibres

Non-starch polysaccharides or Dietary fibres (DF) in rye and wheat consist of arabinoxylans,  $\beta$ -glucans, cellulose and lignin (Table 2.3). DF is polymers of grain cell walls, which cannot be digested by the endogenous secretions of the human digestive tract. DF have been reported to have positive health effects on cardiovascular diseases and to promote healthy laxation and lowering the cholesterol content in the blood (Slavin et al., 1997).

The main component of wheat and rye aleurone cell walls is arabinoxylan (AX). AX are made up of a backbone  $\beta$ -1,4 linked D-xylopyranosyl residues branched with arabinofuranose and xylose residues (Vinkx and Delcour, 1996). AX is divided into two groups: water-extractable (WE) and water-unextractable (WU). Good baking quality of rye is associated with a high content of highly viscous WE-AX, and the water holding capacity is influenced by the WU-AX, which is capable of taking up 9 times its own weight in water, which in turn have a large impact on the dough and improves the bread volume (Vinkx and Delcour, 1996). The ratio between WE-AX and WU-AX changes during processing. The content of WU-AX has been reported to be 7.7% and the content of WE-AX was reported as 2.9%. After baking the content of WU-AX was then decreased to 5.0% and the WE-AX increased to 3.5% (Hansen et al., 2004).

**Table 2.3:** The content of arabinoxylan and  $\beta$ -glucan in rye and wheat\*

	Rye	Wheat
Arabinoxylan (%):		
- WE-AX	1.5 - 3.0	0.4 - 0.8
- WU-AX	4.6 - 6.1	1.1 - 1.9
$\beta$ -glucan (%)	1.4 - 2.9	0.8 - 1.4

\* (Hansen et al., 2003; Courtin and Delcour, 2002; Vinkx and Delcour, 1996)

## 2.4 Lipids

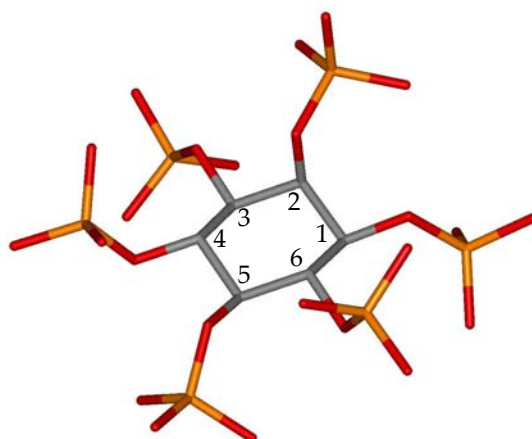
The content of lipids in cereals is between 1-3% (Salovaara and Autio, 2001; Nawar, 1996; Belitz et al., 2004; Chung, 1991), where 60-70% is found as free non-polar lipids in the germ and aleurone layer. The dominant free non-polar lipids in rye and wheat (Table 2.4) are palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) (Chung, 1991). Approximately 10% of the lipids are located in starch granules and consist primarily of polar phosphor lipids. The phosphor lipids are located in the cell membranes and in the germ. The content of the fatty acids in the different milling fractions have an impact on the shelf life of the flours and this will be discussed in chapter 6.

**Table 2.4:** Fatty acid content in rye and wheat in % (Chung, 1991; Hoseneey, 1994b)

Fatty acid	Rye	Wheat
Palmitic acid (C16:0)	15-20	15-25
Oleic acid (C18:1)	25-30	10-20
Linoleic acid (C18:2)	40-50	50-60
Linolenic acid (C18:3)	3-5	3-5

## 2.5 Phytic acid

Phytic acid ( $IP_6$ ) consist of an inositol ring with a phosphate group bound to each C-atom and the chemical name for phytic acid is *myo*-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate ( $IP_6$ ) and the structure of the molecule is illustrated in Figure 2.2.



**Figure 2.2:** The heavy atom structure of phytic acid. Only the phosphate group on C-2 is axial (PAPER II)

Many different terms for phytic acid are used in the literature. In this thesis phytic acid will be used for the free inositol hexa-phosphor acid, while phytate will be used about the salts of phytic acid. IP<sub>6</sub> will be used as a term of phytic acid and the soluble Na-phytate which is used in the model system described. The degradation products of phytic acid/phytate are called lower inositol phosphates or *myo*-inositol penta- (IP<sub>5</sub>), tetra- (IP<sub>4</sub>), tri- (IP<sub>3</sub>), di- (IP<sub>2</sub>) or mono-phosphate (IP<sub>1</sub>) and *myo*-inositol (IP/Ins).

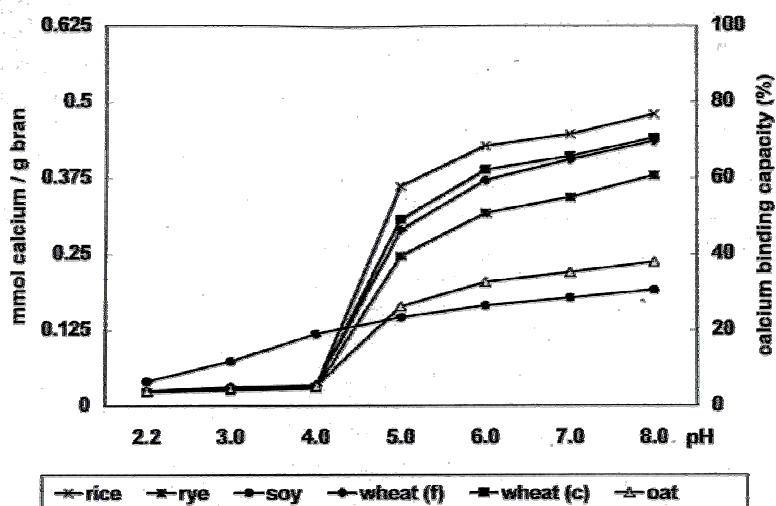
### **2.5.1 Content of phytic acid/phytate in cereals**

Phytic acid is naturally occurring in many plants, fruits and vegetables in form of phytate (Reddy, 2002). In rye grains the content of phytic acid is 0.4 – 1.6% (Lásztity & Lásztity, 1990; Graf, 1986; Ravindran *et al.*, 1994; Reddy *et al.*, 2002), while the content is 0.4 - 2.6% in wheat (García-Esteba *et al.*, 1999; Reddy, 2002; Ravindran *et al.*, 1999). Phytic acid is primarily located in the bran fractions with up to 87% in the aleurone layer and only 2% in the endosperm (Reddy *et al.*, 1989). Fretzdorff og Weipert (1986) found that especially the outer layer of the rye bran was rich in phytic acid (Fretzdorff and Weipert, 1986). This is in accordance with Anjum and co-workers (2002), who found that the content of phytic acid was in average 3 times higher in the bran from wheat than in whole wheat flour and 3 times higher than in wheat flour (Anjum *et al.*, 2002).

### **2.5.2 Phytic acid-mineral complexes**

Phytic acid is negatively charged in a large pH region, which makes phytic acid efficiently capable of chelating positive charged groups such as divalent metal ions like Ca<sup>2+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup> and Mg<sup>2+</sup>. Besides divalent ions also proteins and carbohydrates can be complex bound (Lásztity and Lásztity, 1990). Studies have shown that factors such as pH, binding capacity of the metal ion and the number of phosphate groups bond to the inositol molecule has an influence on how strong and how many minerals will be bound (Siener *et al.*, 2001; Persson *et al.*, 1991).

The binding capacity of calcium to IP<sub>6</sub> at different pH-values in different types of cereals and soy have been examined, and as seen in Figure 2.3, calcium initiate the binding to IP<sub>6</sub> at pH 4 and increases rapidly until pH 5. Hereafter the increase is less steep and at pH 8 the binding capacities reach a maximum. For rye 74% of the calcium present in the grains are complex bound to phytic acid at pH 8 (Siener *et al.*, 2001). At pH 4.5, which is relevant in rye doughs, 30% of the calcium will be bound in complexes.



**Figure 2.3:** Binding capacity of calcium in bran fractions from rice, rye, soy, wheat and oat at different pH conditions (Siener et al., 2001)

In Table 2.5 the binding strength of cobber, zinc and cadmium to phytic acid are compared, and it is seen that 3 to 6 mole of metal ions are bound to IP<sub>6</sub> depended on the number of phosphate groups present.

**Table 2.5:** Number of metal ions (n<sub>M</sub>) bound pr. inositol molecule at pH 5-6 (Persson et al., 1991)

Inositol-phosphate	n <sub>Cu<sup>2+</sup></sub>	n <sub>Zn<sup>2+</sup></sub>	n <sub>Cd<sup>2+</sup></sub>
IP <sub>6</sub>	5.8	4.9	5.3
IP <sub>5</sub>	5.7	4.8	5.1
IP <sub>4</sub>	3.3	3.0	3.3
IP <sub>3</sub>	3.1	3.0	2.4

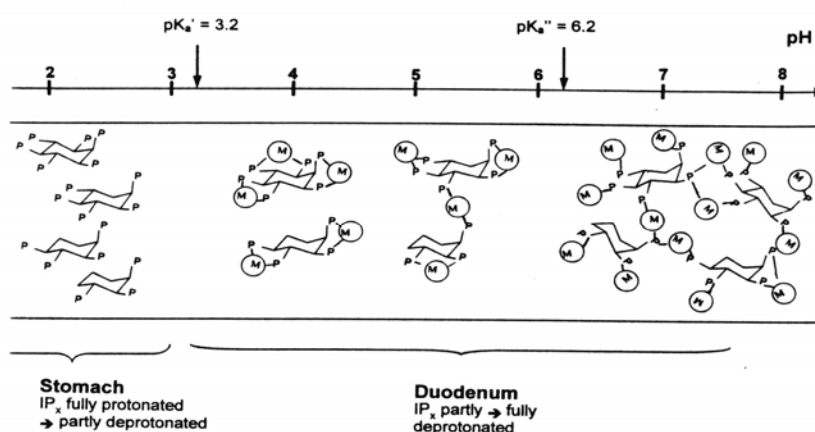
It is also observed, that the highest number of metal ions is bound to IP<sub>5</sub> and IP<sub>6</sub>, and that less metal ions is bound to the lower inositol phosphates. In agreement with this findings it has been showed that cleavage of phosphate groups results in a weaker binding strength and the solubility is increased (Weaver and Kannan, 2002). The strong binding capacity of minerals gives rise to complexes that are difficult to decompose. At physiological pH the complexes have a very low solubility (Pallauf and Rimbach, 1997). According to Tangkongchitr et al. (1982) 75% of phytate is insoluble at pH 6-7, while only 15% is insoluble at pH 5. Especially penta- and hexa-substituted phytate salts are insoluble (Tangkongchitr et al., 1982).

### 2.5.3 Nutritional aspects of IP<sub>6</sub>-mineral complexes

Brune et al. (1992) and Sandberg et al. (1989) found that all inositol-phosphates in bread binds iron and especially IP<sub>6</sub>-IP<sub>3</sub> inhibits the iron absorption (Brune et al., 1992; Sandberg et al., 1989). Appearance of IP<sub>6</sub>-IP<sub>5</sub> in food makes the bioavailability of minerals appreciably reduced and therefore phytic acid is seen as an anti-nutrient in food and unwanted in large amounts.

The increasing pH through the human stomach and intestine leads to strong complexes (Figure 2.4), which are indigestible and therefore a degradation of the complexes is required in the foodstuff prior to digestion.

It is therefore advantageous if phytate in our daily food is present as IP<sub>3</sub> or lower inositol phosphates. One of the ways to obtain this goal is to optimize the conditions for the endogenous enzymes present naturally in our cereals.



**Figure 2.4:** A model of the pH range and possible interactions of IP<sub>6</sub>-IP<sub>3</sub> and mineral ions in the human stomach and intestine system. P: phosphate groups, M: mineral ions (Türk, 1999).

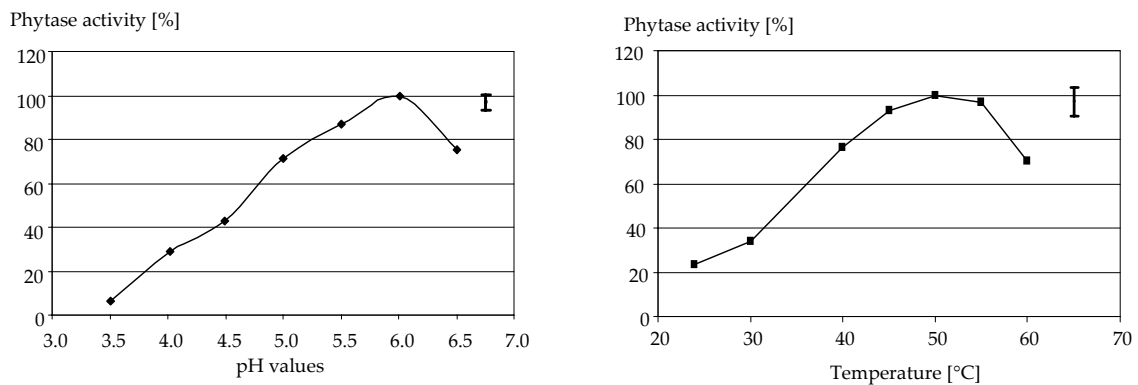
### 2.5.4 Phytase

Phytases are enzymes that catalyses the degradation of phytate to lower inositol phosphates and free inorganic phosphorous is formed depending on extend of the enzyme activity (Casey and Walsh, 2004). There are two international classified phytases: 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). The enzymes are named after the position of the first phosphor ester bond of the phytate to be hydrolysed. 3-phytase seems to be of microbial origin, while 6-phytase is synthesised by plants (Centeno et al., 2001). The degradation of IP<sub>6</sub> to IP<sub>5</sub> is specific for phytase catalytically effect,



whereas the degradation to lower inositol phosphates can be caused by other phosphatase enzymes than phytase (Nayini and Markakis, 1983). The enzymatic activity produces available minerals that are considered important for upgrading the nutritional quality of phytate-rich foods and feeds (De Angelis et al., 2003). Phytase function specifically on phytic acid by cleaving the ester bond between the inositol ring and phosphate group, and thereby lower inositol phosphates and inorganic phosphate are formed (Pallauf and Rimbach, 1997; Reddy, 2002).

The pH- and temperature optimum was examined (PAPER I) and found to be 6.0 as pH optimum and 45-55°C as temperature optimum (Figure 2.5) in accordance with former studies (Greiner et al., 1998; Peers, 1957).



**Figure 2.5:** Rye phytase activity as a function of pH and temperature (PAPER I)

For microbial phytases two pH optima has been reported; 2.5 and 5.0-5.5 and temperature optimum at 40-55°C (Brugger et al., 2004; Cho et al., 2003; Pandey et al., 2001). Phytase exhibit different levels of activity in different cereals. In rye the activity has been reported to be approx. five times higher than in wheat and approx. 10 times higher than barley (Bartnik et al., 1987; Fretzdorff and Weipert, 1986; Fretzdorff and Brümmer, 1992; Greiner et al., 1998; Greiner and Egli, 2003).

If rye grains are fractionated into the different botanical parts, it has been shown that the activity of phytase is highest in the outer layers of the bran (epidermis, testa and nucellar tissue) followed by the aleurone cells. The activity of phytase in the endosperm is 4-6 times lower than the activity in the bran fractions (Fretzdorff and Weipert, 1986).

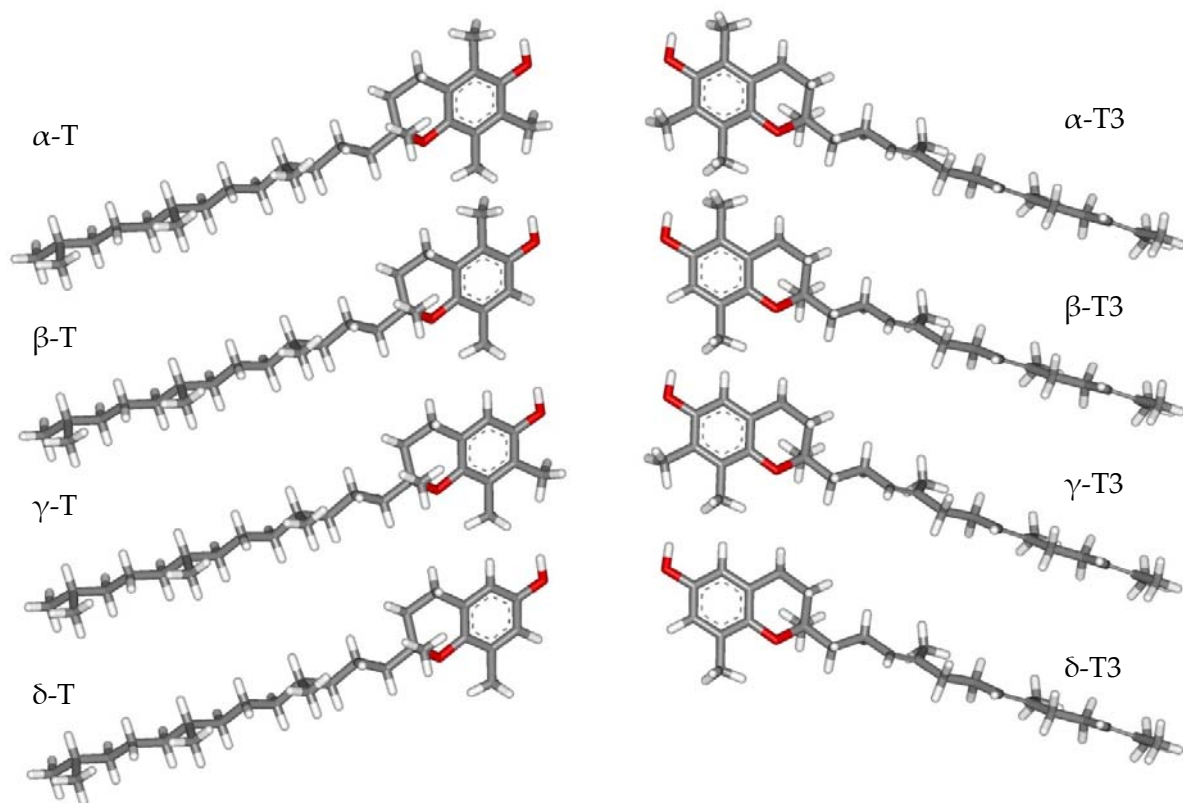
## 2.6 Vitamin E

Vitamin E was first discovered and characterised as a fat-soluble nutritional factor during reproductive studies with rats in 1922 by Evans and Bishop, and it was called “the antisterility factor”. In 1936 Bishop designated the vitamin to vitamin E or alpha-tocopherol, which in Greek words means birth (*tocos*) bringing (*feroin*) (Eitenmiller and Lee, 2004c). Vitamins are necessary for growth and metabolism in the human body, and since humans are incapable of synthesizing vitamins, the diet must supply vitamins in sufficient amounts to maintain good health. Vitamins are classified according to solubility; thus A, D, E and K are fat soluble and B and C is water soluble.

### 2.6.1 Structure of vitamin E

Natural occurring vitamin E consists of 4 tocopherols ( $\alpha$ -,  $\beta$ -,  $\delta$ - and  $\gamma$ -T) and the 4 corresponding tocotrienols ( $\alpha$ -,  $\beta$ -,  $\delta$ - and  $\gamma$ -T3) (Kamal-Eldin and Appelqvist, 1996). They are designated according to the number and position of substituent methyl groups on the chromanol ring as seen in Figure 2.6. The tocotrienols differs from the tocopherols in having three double bonds at position 3', 7' and 11' in the phytyl side chain. The tocopherols consist of three asymmetric carbons (2', 4' and 8') and therefore each of the tocopherol can exist as one of eight possible stereo isomers. The RS system of asymmetric configuration is used to specify the chirality of vitamin E compounds. In nature, tocopherol always exist in their RRR-form, the tocotrienols which possess only one centre of asymmetry at position 2, only exist in the 2R,3'-trans,7'-trans configuration. Synthetic tocopherol is normally a racemic mixture of equal parts of each stereoisomer (2RS, 4'RS, 8'RS- $\alpha$ -tocopherol) (Kamal-Eldin and Appelqvist, 1996). A study of supplementation of synthetic vitamin E (*all-rac*- $\alpha$ -tocopheryl acetate) to cow feed showed that 84-89% of the measured stereo isomers were the native RRR- $\alpha$ -tocopherol (Slots et al., 2007)

Since vitamin E is a fat-soluble vitamin it is present in the lipid environment of the grains. The vitamin E, and mostly  $\alpha$ -T, is specifically and actively incorporated within biological membranes, where it stabilises the lipoprotein structure. The tocopherol molecule is anchored in the phospholipid bilayer by means of its phytyl side chain with its polar head group located very close to the lipid-water interface.



**Figure 2.6:** Structure of tocopherols (T) and tocotrienols (T3). The three thin bonds in the tocotrienols molecules are double bonds.

### 2.6.2 Content of vitamin E in cereals

Previously the only method to estimate vitamin E content in foods was by comparing the activity in bioassays with an international standard. This activity was expressed in IU/mg (Table 2.6), but in 1980, the designation of vitamin E changed to an expression of equivalents, based on biological activity (Ball, 2004). This means that all vitamin E activity is expressed relative to the naturally occurring and most active form of vitamin E: RRR- $\alpha$ -tocopherol. The total vitamin E activity in food is therefore reported as  $\alpha$ -tocopherol equivalents ( $\alpha$ -TE):  $\alpha$ -TE = ( $\alpha$ -T\*1.0) + ( $\beta$ -T\*0.5) + ( $\gamma$ -T\*0.1) + ( $\delta$ -T\*0.03) + ( $\alpha$ -T3\*0.5) + ( $\beta$ -T3\*0.05) + ( $\gamma$ -T3\*0.01) (Eitenmiller and Lee, 2004b).

**Table 2.6:** Biological activity of the different vitamin E isomers

Vitamin E	Biological activity	
	IU/mg	Compared to <i>RRR</i> - $\alpha$ -T (%)
$\alpha$ -tocopherol	1.49	100
$\beta$ -tocopherol	0.75	50
$\gamma$ -tocopherol	0.15	10
$\delta$ -tocopherol	0.05	3
$\alpha$ -tocotrienol	0.75	50
$\beta$ -tocotrienol	0.08	5
$\gamma$ -tocotrienol	0.01	1
$\delta$ -tocotrienol	not known	not known

Alpha-T is the most abundant of the eight isomers, and as seen in Table 2.7, the content is highest in the wheat germ. The content of the tocopherols are in the germ are 15-30 times higher than in wheat flour. Whereas the content of tocotrienols are 4-7 times higher in wheat bran compared with wheat flour (Ko et al., 2003). It is also observed from the table that barley consist of all eight isomers, whereas wheat and rye only contains the alpha- and beta isomers.

**Table 2.7:** Content of different vitamin E isomers in different cereal grains and wheat germ and bran \*

	Vitamin content [ $\mu$ g/g d.m.]								average
	$\alpha$ -T	$\beta$ -T	$\gamma$ -T	$\delta$ -T	$\alpha$ -T3	$\beta$ -T3	$\gamma$ -T3	$\delta$ -T3	
Barley grains	2.0-9.8	0.4-0.9	0.3-0.9	0.1-0.9	11.0-33.2	3.0-7.1	5.4	0.9	11.4
Wheat grains	8.9-15.9	3.8-9.5			3.5-6.4	18.6-42.5			17.2
Oat grains	5.0-14.9	0.9-3.0	0.4-0.9	-	11.0-56.4	0.9-5.4	-	-	18.6
Rye grains	8.9-16.0	2.7-4.0			12.4-15.0	8.0-9.8			18.7
Wheat germ	110.8-619.0	47.0-139.0	6.2	0.1-0.9	2.7-5.0	9.0-26.9		0.1	344.8
Wheat bran	14.3-16.0	7.1-10.0			11.0-13.4	50.1-54.0			25.7

\* (Franke et al., 2007; Grella, 1996; Håkansson et al., 1987; Ko et al., 2003; Panfili et al., 2003; Peterson and Qureshi, 1993; Piironen et al., 1986; Sheppard et al., 1992)

### **2.6.3 Health impacts of vitamin E**

Vitamin E functions as a radical scavenging antioxidant both in vitro and in vivo. It has been proven that  $\alpha$ -T has a positive effect on different diseases such as cardiovascular diseases, cancer and cataracts.

We can only guess the importance of knowing the exactly composition of vitamin E in our cereal products, but several studies has shown that the antioxidative effect of tocotrienols is higher than that of the tocopherols – both in humans (Nesaretnem et al., 1995; Nesaretnem et al., 2007) and in food matrices (Wagner et al., 2001).  $\alpha$ -T3 exhibit functions, that are not shared by  $\alpha$ -T (Sen et al., 2006). The tocotrienols has been shown to possess powerful neuroprotective, antioxidant, anti-cancer and cholesterol lowering properties that often differ from the properties of the tocopherols (Sen et al., 2007).  $\delta$ -T3 has been shown to be capable of induce breast cancer cells to undergo cell death, called apoptosis (Kline et al., 2004).  $\gamma$ -T3 has been showed to provide significant protection against oxidative stress-induced apoptosis in astrocytes, while  $\alpha$ -T was much less effective (Sylvester, 2007).

However, at the bottom line all these diverse biological activities are not well-described nor well established but they do indicate that the biological activities of the different vitamin E isomers is quite different, wherefore the individual concentrations need to be determined for a thorough description of the products.



### 3. Milling methods

Wheat flour has been produced for thousands of years, ever since the first people began to settle in permanent communities. The purpose of milling the grains to flour is to transform the whole grains into forms suitable for consumption. The primitive people used stones in shapes of mortar to pound the grain and later saddle stones, lever mills and rotary mills were developed and used, as shown in Figure 3.1 (Bass, 1988). Stone milling has been used in many ways, first operated by humans and later by horse-, water- or wind power. In the beginning of the 19<sup>th</sup> century steam power was introduced in Denmark and was strong competition to water- and wind-driven mills. In the late 19<sup>th</sup> century with the introduction of electrically driven roller mills most of the old water and windmills were outdone and replaced (Bass, 1988; Valsemøllen A/S, 1999).

Historically, white wheat flour was only available for the rich people, because the milling process was very timeconsuming. Stone mills were/are not able to remove the germ and all bran from the flour, so they had to sift it through a silk filter over and over again until the flour was white. This long process made the flour very expensive and therefore only available for wealthy people and consequently very attractive to poor people (Katz and Weaver, 2003). With the introduction of roller mills in the late 1800's the miller was able to produce the flour and thereby the breads at a price which was achievable for common people. But this evolution in flour making led to poor nutrition and many health problems, because all the nutritional beneficial parts of the grains were separated from the flour and used as feed for animals (Katz and Weaver, 2003).

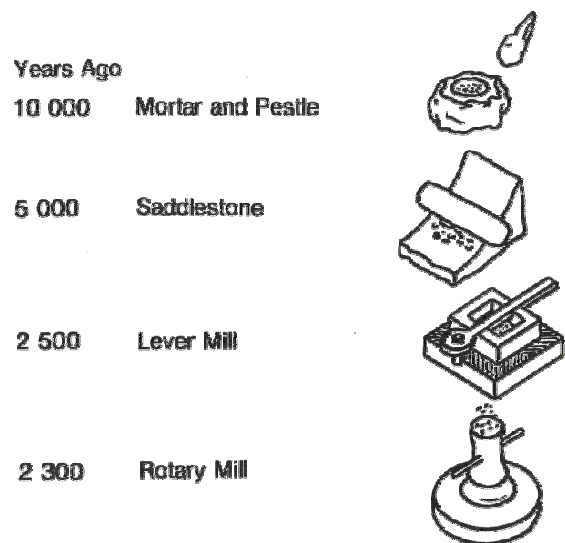


Figure 3.1: Ancients mills (Bass, 1988)

The overall aim of wheat milling was and is to separate the floury endosperm from the bran and to reduce the endosperm particles into flour. This is commercially done on either stone mills or roller mills and both methods have been used in this study. In PAPER V and PAPER VI the two methods are compared with respect to content, distribution and stability of vitamin E during milling and storage. A short description of the two milling methods will be given in the following.

### 3.1 Disc milling

The stone mill (Figure 3.2) is one of the oldest type of mills used for grinding wheat and is known from water and windmills. Disc mills have the advantage of being smaller and they can therefore be located decentralized compared to the big and very expensive roller mills. Among bakers and smaller bakeries which often produce organic products, there is an increasing interest in using freshly produced flour from their private mills, because this is seen as a quality parameter for flavour, aroma and maintenance of vitamins.

The grinding action for disc milling occurs between two mill stones, called discs, and the mills grind the kernels according to the disc mill principle, which is the use of the forces of compression, shear and abrasion (Posner, 2005).

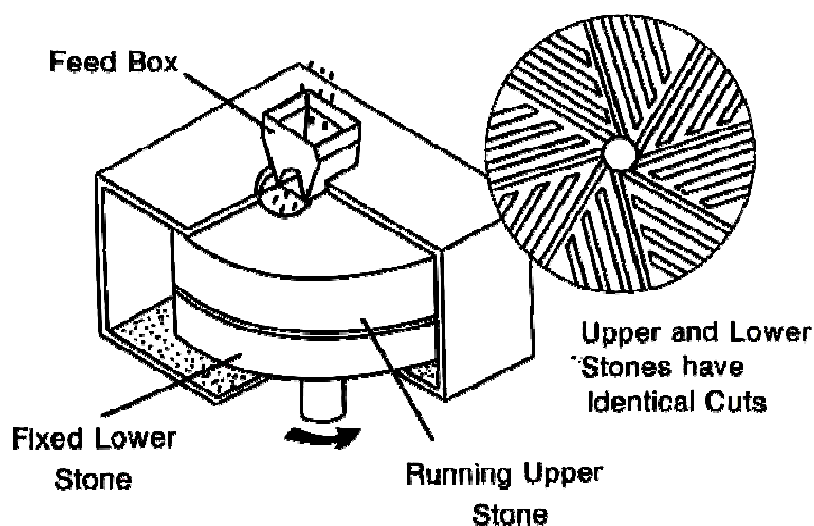
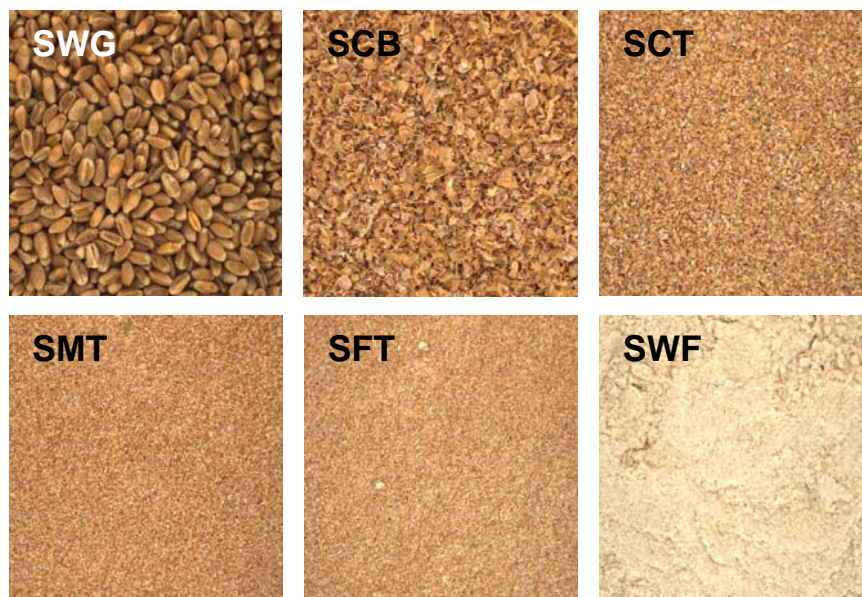


Figure 3.2: Stone mills (Bass, 1988)



The grains are fed at the centre of the top stone, which is rotated, while the bottom stone is stationary. The kernels are broken and ground by the edge of the flutings in the stones, and the material is discharged by the rotating stone and outlet. The separation of bran and flour is carried out using sifters (Bass, 1988; Posner, 2005).

Flour products from disc mills contain a larger share of the outer layers and parts of the vitamin E-rich germ compared to flour from roller mill in which the germ and bran can be sorted. In Figure 3.3 the five different fractions obtained from the disc mill at Aurion A/S during this study (PAPER V) are visualised. There are three tailing fractions (SCT, SMT and SFT). These fractions are fine bran fractions and have very little endosperm left. It is also seen that the flour fraction (SWF) is rather dark due to the relatively high content of bran (1.2% ash) left in the flour. The coarse bran fraction (SCB) is normally further grinded into a fine bran fraction.



**Figure 3.3:** Videometer (Videometer A/S, Denmark) images of the fractions from stone milling. SWG: whole wheat grains, SCB: coarse bran, SCT: coarse tailing, SMT: tailings, SFT: fine tailing and SWF: wheat flour (PAPER V)

### 3.2 Roller milling

Roller milling is the principal commercial milling method, as it has very high capacity. Roller milling is done on break rolls, sizing rolls and reduction rolls. The principle of the break rolls is that the kernels move between two large metal rollers (see Figure 3.4). The rollers are of two different sizes and move at different speeds.

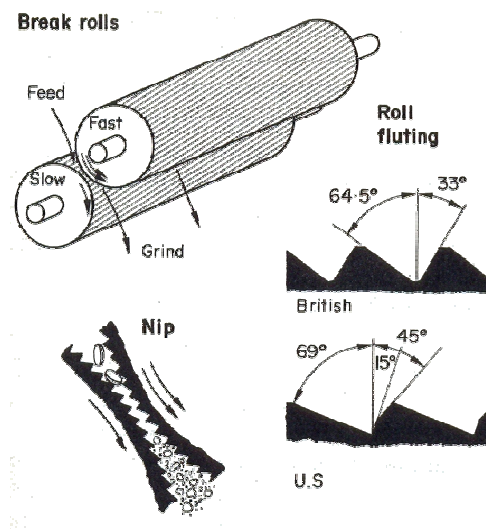
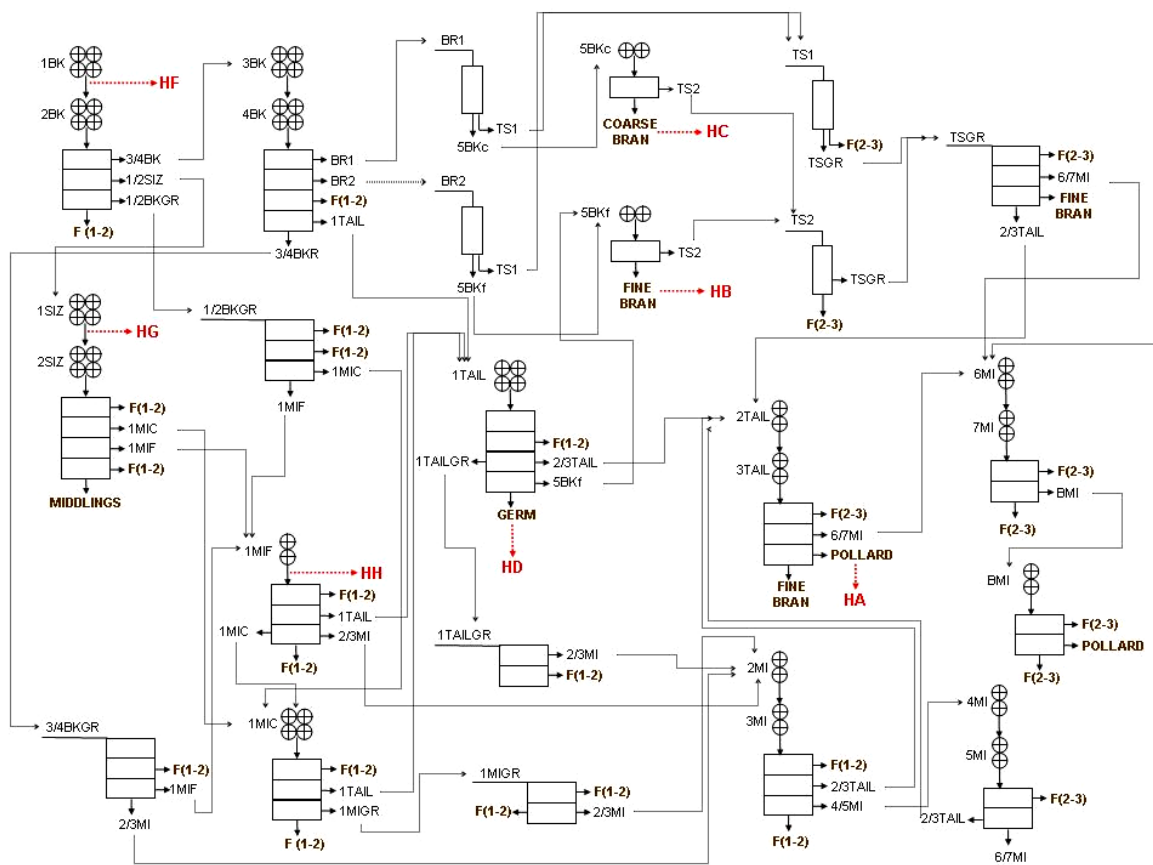


Figure 3.4: Break rolls (Kent and Evers, 1994)

The objective of the breaking system is to break open the wheat grain, remove the endosperm and germ from the bran coat, and gradually grind the practically pure endosperm into flour. The sizing system has the objective to detach the bran pieces attached to the large middlings and produce clean middlings. This is done on smooth rolls which also run at different speeds. The purpose of the reduction system is to reduce the middlings to flour (Posner, 2005). As in disc milling, the separation of bran and flour is carried out using sifters (Bass, 1988; Posner, 2005).

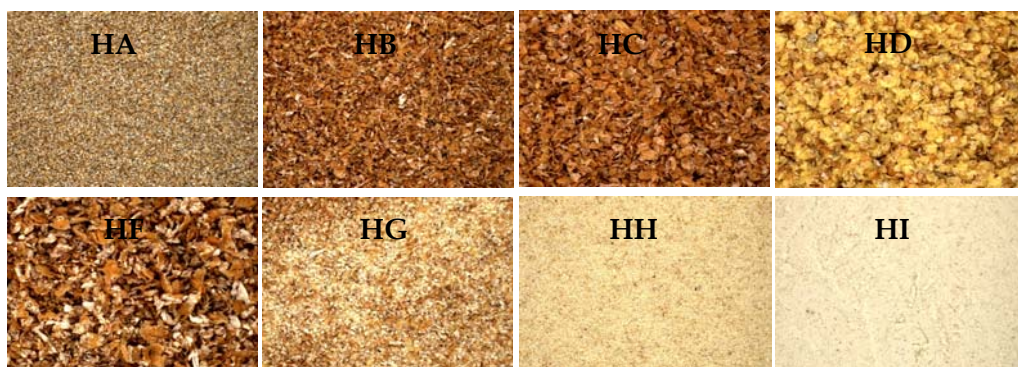
The roller mill system used at Valsemøllen A/S, the industrial mill which provided samples used in PAPER V, consists of four breaking rolls (BK), two sizing rolls (SIZ) and eight middlings rolls (MI).

The flow chart in Figure 3.5 illustrates the wheat flour production at Valsemøllen A/S, which is a rather complicated process involving many different process steps. The red arrows and letters in the flow chart refer to the fractions analysed in PAPER V.



**Figure 3.5:** Flow chart for production of wheat flour at Valsemøllen A/S. The red arrows and letters in the flow chart refer to the fractions analysed in PAPER V

In Figure 3.6 examples of fractions obtained from Valsemøllen A/S (used in PAPER V) are shown. By visual inspection it is seen that the fractions differ in content of remaining endosperm (white particles) in the bran fractions.

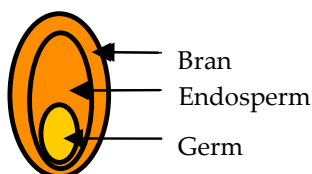


**Figure 3.6:** Videometer (Videometer A/S, Denmark) images of the fractions from roller milling. HA: tailings, HB: fine bran, HC: coarse bran, HD: germ, HF: after 1. break roll, HG: after sizing rolls, HH: after middling roll and HI: wheat flour (PAPER V)

### 3.3 Milling products

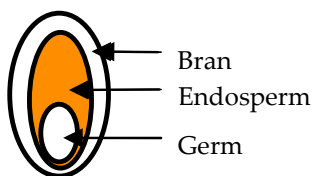
It is possible to produce many different flour products during milling. Especially roller milling provides the possibility to produce practically any desired flour product. During this study milling products of different extraction rate have been used in different experiments. Flours are characterised by the rate of extraction. The extraction rate is the proportion of flour, derived by milling, from a known quantity of grain. The extraction rate is used to define various types of flours. White flour has an extraction rate of 75-78%, which means that the germ and approx. 30% of the outer layers have been removed. Whole grain flour has an extraction rate of 100%, meaning that all parts of the grain are retained in the flour. Ash content is the residual inorganic material (minerals) in the flours. The gradient of ash content increases from the centre to the outer layers of the kernel, so often the ash content in the flour is used as an indicator of how much of the outer layers of the kernel has been ground to flour. In wheat flour the ash content is < 0.7%, whereas the ash content of whole wheat flour is about 1.7-2.0%.

Whole wheat flour (WWF) was used in PAPER IV, V and VI while whole rye flour (WRF) was used in PAPER I and III:



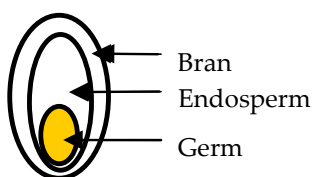
WWF/WRF has an extraction rate of 100%, which means that all parts of the grains are retained. The ash content for WWF was 1.7 (PAPER V)

Wheat flour (WF) was used in PAPER IV, V and VI and Rye flour (RF) was used in PAPER I and III:



WF/RF has an extraction rate of 70-75%, which means that the germ and bran are removed. The ash content in WF was 0.6 (roller-milled) and 1.2 (stone-milling) (PAPER V).

Wheat germ (WGM) was used in PAPER V and VI:



WGM is a germ fraction obtained from roller milling. This fraction is only possible to produce using roller mill. The ash content was 3.6 (PAPER V).

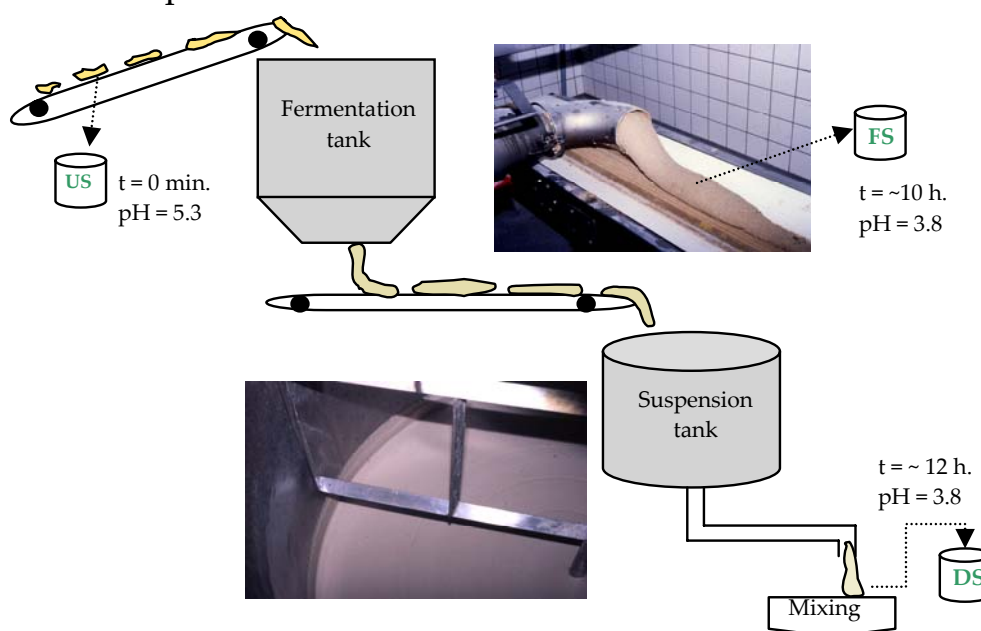
## 4. Rye bread making

This chapter describes the different steps of industrial rye bread making with a special view to elucidate the time, pH and temperature conditions present in industrial sourdough making and the baking process used in PAPER III.

Making bread is a complex process involving many critical steps which may affect the fate of phytic acid. The rye breads examined in this study were made by the use of rye sourdough and WRF added 0%, 30% or 50% whole rye grains, respectively (PAPER III). The whole grains were made by soaking the squeezed rye grains in hot water with added vinegar.

### 4.1 Sourdough making

In Figure 4.1 a schematic overview of the sourdough process used in PAPER III is shown. The small boxes with green letters refer to the abbreviations used for the sample outtakes in PAPER III.

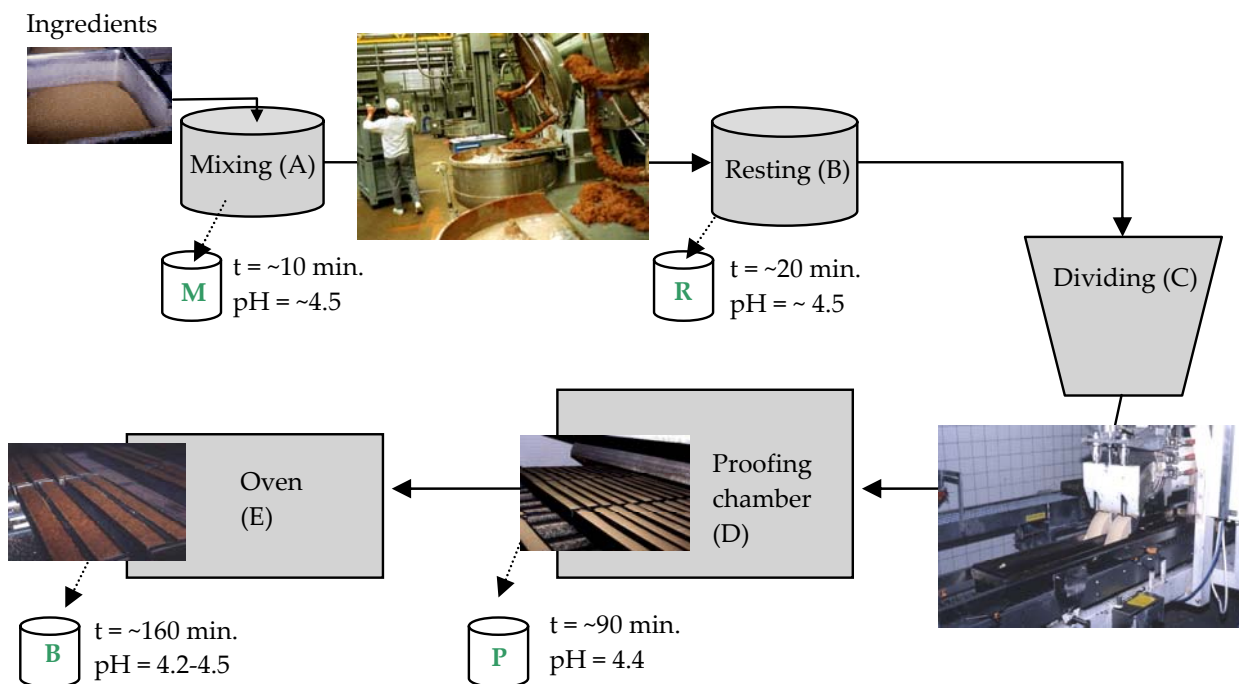


**Figure 4.1:** Industrial sourdough production, with pH and time conditions. US: unfermented sourdough, FS: fermented sourdough and DS: diluted sourdough

Overall, the sourdough process involves two important steps: fermentation and suspension. The first step in sour dough making is to refresh the mature sourdough with rye flour and water. The sourdough is then stored in fermentation tanks for 10-12 hours where pH decreases from approx. 5.3 to approx. 3.8 and the temperature increases from 25°C to 30°C. These changes are primarily due to the increased activity of lactic acid bacteria. The fermented sourdough is then transported to a suspension tank, where water is added and the sourdough is stored for additional 10-12 hours. This step is included to make the sourdough easy flowing and thus transportable in the bakery.

## 4.2 Rye bread making

In Figure 4.2 the rye bread making process is illustrated schematically including pictures that visualise the process.



**Figure 4.2:** Industrial rye bread production with time and pH conditions. M: dough after mixing, R: dough after resting, P: dough after proofing and B: baked bread

The first step is simple mixing (A) where all ingredients (e.g. flour, sourdough, water and soaked grains) are mixed together in a large kneading trough. The mixing is continued for 10 minutes and pH in the mixed dough is approx. 4.5. After the mixing (A), the dough is resting (B) for another 10

minutes in the kneading trough before it is transferred to the dividing chamber (C). Then the dough is poured into forms and the next step is proofing (D). During this stage the dough is moved through a proofing chamber with a relative humidity of 85% and 37°C for 70 minutes. After the proofing the breads are baked (E) for 70 minutes. In the first minutes admission of steam is provided to maintain a moist surface and avoid cracks in the crust. The temperature in the first part of the oven is high (240-265°C) to make sure the incrustation appears quickly. Then the temperature is decreased to 150-200°C. During the baking process the centrum temperature in the dough is raised from 37°C to 98°C.



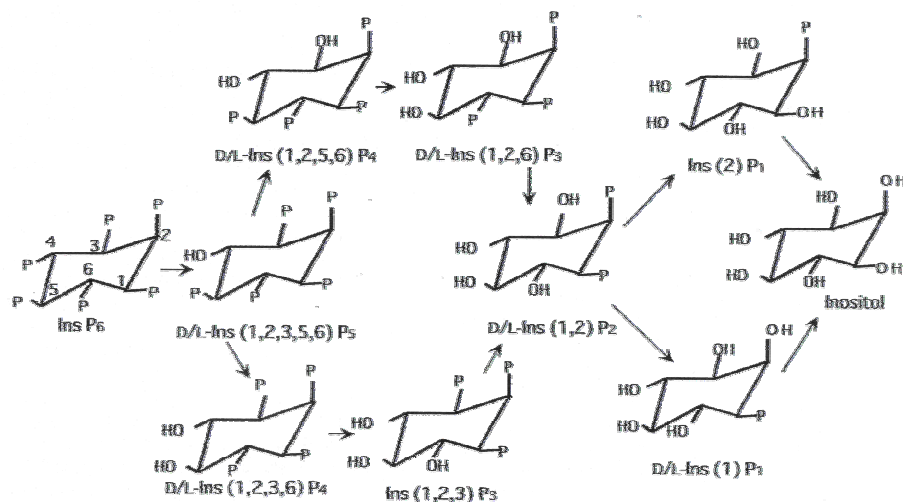


## 5. Phytic Acid in Rye Grains and Bread

In this chapter the degradation of phytic acid by phytase in a model system will be evaluated using high-resolution NMR and chemometrics (PAPER II). Next the development of an optimised method for phytase measurement is outlined (PAPER I) and used to evaluate the degradation of phytic acid during rye bread making (PAPER III).

### 5.1 Phytic acid degradation by phytase – a model system

The degradation of phytic acid by phytase is reported as a stepwise process (Frølich et al., 1986; Nakano et al., 2000), but the further degradation to lower inositol phosphates has been a subject of discussion. Nakano and co-workers suggested a possible dephosphorylation pathway of IP<sub>6</sub> by wheat bran phytase, as shown in Figure 5.1 (Nakano et al., 2000).



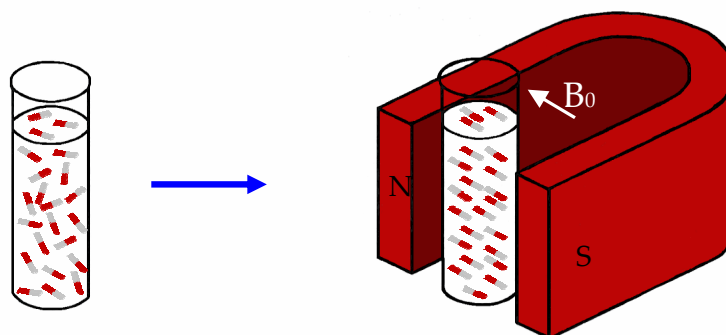
**Figure 5.1:** Possible dephosphorylation pathway of IP<sub>6</sub> by wheat bran phytase (Nakano et al., 2000)

Figure 5.1 illustrate the complex degradation process. In the study we set out to investigate if it is possible to predict the degradation pattern of phytate in a model system where phytase was added to an aqueous solution of Na-phytate. The degradation was monitored *in situ* by using high-resolution NMR spectroscopy and multivariate data analysis (chemometrics).

### 5.1.1 Nuclear magnetic resonance (NMR) spectroscopy

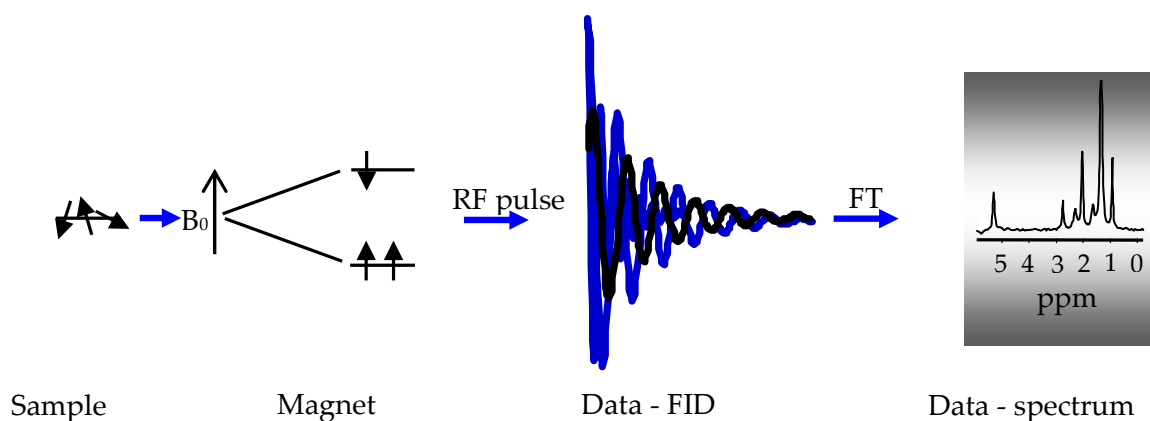
Nuclear Magnetic Resonance (NMR) spectroscopy is divided into high-field (HF)- and low-field (LF)-NMR, where LF-NMR, for example, is used for profiling solid/liquid states in foods, whereas HF-NMR is mostly used for determination of structure. NMR spectroscopy measures the nuclear spin in contrast to optical spectroscopic methods which measure electronic transitions, molecular vibrations or molecular rotations. Only nuclei with a magnetic moment can be measured by NMR. The most widely used nuclei with a magnetic moment are  $^1\text{H}$ ,  $^{31}\text{P}$ ,  $^{15}\text{N}$  and  $^{13}\text{C}$  (Viereck et al., 2005a). Since the degradation of IP<sub>6</sub> leads to release of inorganic phosphate and “new” configurations of the lower inositol phosphates,  $^{31}\text{P}$  NMR spectroscopy was chosen to monitor the different populations of inositol phosphate.

In HF-NMR the sample is placed in a strong static magnetic field where the nuclei are aligned and radiated (Figure 5.2).



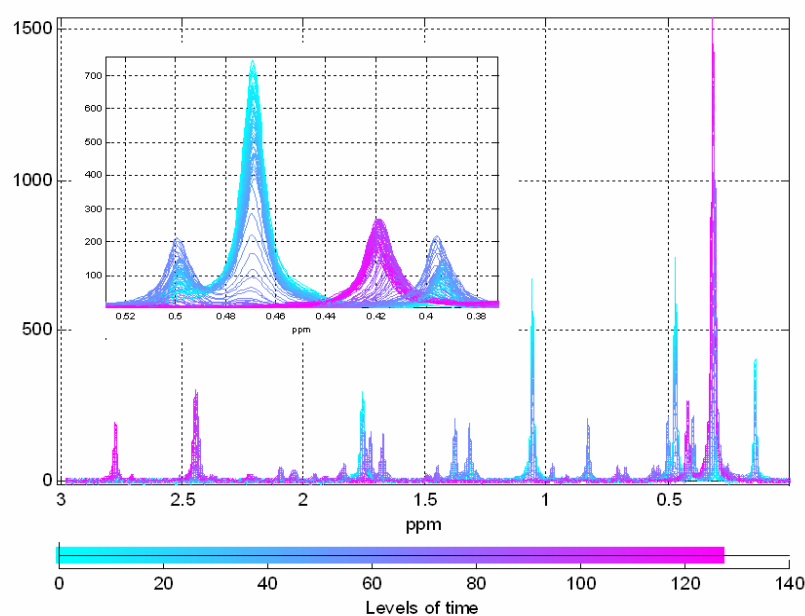
**Figure 5.2:** Simplified principle of NMR.  $B_0$ : direction of the magnetic field. When brought in a strong magnetic field the protons act as small bar magnets and align parallel and antiparallel to  $B_0$ .

By radiation the nucleus is brought out of equilibrium and it is the relaxation of the nucleus that gives the signals in the NMR spectrum (Figure 5.3).



**Figure 5.3:** Simplified schematic overview of the different steps during measurements by HF-NMR. RF pulse: radiofrequency pulse, FID: free induction decay, FT: Fourier Transformation

Figure 5.4 shows the superimposed NMR spectra from the reaction of phytase activity in which the degradation of IP<sub>6</sub> in a model system with added phytase was monitored using <sup>31</sup>P NMR spectroscopy (PAPER II).



**Figure 5.4:** Superimposed NMR spectra of degradation of IP<sub>6</sub> by phytase over a period of 14 hours (PAPER II)

As observed in Figure 5.4 the NMR spectra are quite complicated. However, before addition of phytase (light blue) only four <sup>31</sup>P peaks were present (0.14, 0.47, 1.06 and 1.76 ppm) with a intensity ratios of 1:2:2:1 resulting from the six P atoms in the phytic acid molecule in which 2 pairs of phosphate atoms are degenerated due to the symmetry of the phytic acid molecule (Frølich et al., 1986). After addition of phytase the pattern and intensity of the peaks

change as a consequence of the stepwise degradation pattern of IP<sub>6</sub> to lower inositol phosphates. These changes in the <sup>31</sup>P NMR spectra over time are illustrated in the zoomed area in Figure 5.4. From the figure it is apparent that three peaks decrease (including IP<sub>6</sub> at 0.47 ppm), while one increases. This pattern of changes continues as IP<sub>5</sub> degrades to IP<sub>4</sub> and IP<sub>4</sub> to IP<sub>3</sub> and so on until only IP<sub>1</sub> signals or inositol and inorganic phosphorus remain. The enzymatic process becomes more and more complex and after 14 hours of analysis only four peaks remain (0.38, 0.42, 2.45 and 2.77 ppm; shown by the magenta line in the spectra). The four peaks at the end correspond to the three isomers of IP<sub>1</sub> that remain after the hydrolysis and the inorganic phosphate (0.38 ppm).

In order to be able to assign the many different peaks obtained during the experiment to an actual structure or distribution of structures the spectroscopic results were analysed with multivariate data analysis (chemometrics).

### 5.1.2 Multivariate data analysis

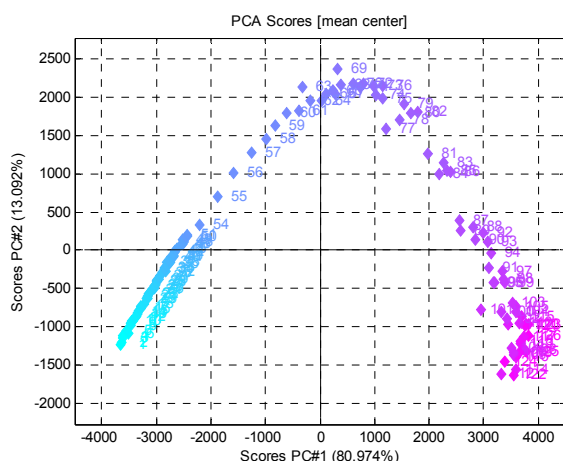
Chemometrics is popularly referred to as “statistics without tears”, because it facilitates handling of large data sets and deals efficiently with real-world multivariate data, by taking advantage of the co-linearity of spectral data and providing the possibility of projecting the data into a few dimensions via a graphical representation (Micklander et al., 2003).

#### 5.1.2.1 Principal component analysis (PCA)

One of the main advantages of applying multivariate chemometric data analysis to co-linear spectroscopic data is the possibility of carrying out an exploratory inductive investigation (Munck et al., 1998). The universal basic chemometric algorithm, principal component analysis (PCA) (Hotelling, 1933), is a most useful tool for this purpose. PCA is based on the calculation of underlying latent data structures using a two-dimensional data strategy. PCA finds the main variation in a multidimensional data set by creating new linear combinations (orthogonal) of the raw data, as is the case in most spectroscopic or instrumental techniques (Hotelling, 1933; Bechmann et al., 1999). The model to be solved is  $X = T \cdot P^t$  in which the data matrix (samples  $\times$  spectra)  $X$  is decomposed into a lower dimensional score matrix ( $T$ ) and a loading matrix ( $P$ ). In this way the information in  $X$  is projected onto a lower dimensional subspace where the loading vectors for the principal components (PC) can be regarded as pure mathematical spectra that are

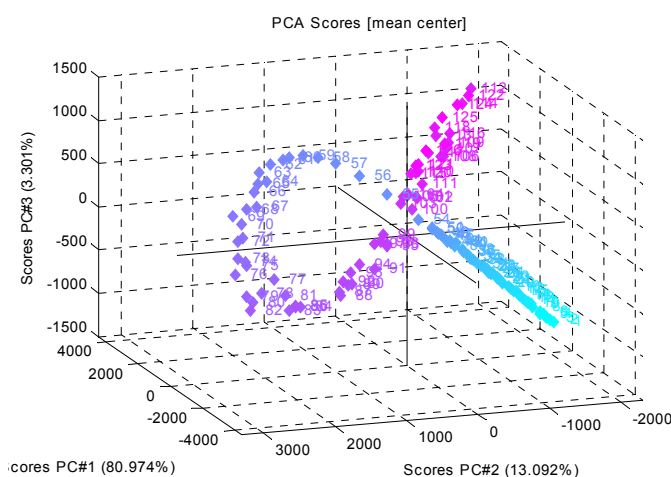
common to all the measured spectra. What makes the individual raw spectra different are the amounts (scores) of hidden spectra (loadings).

A PCA on the  $^{31}\text{P}$  NMR spectra obtained from the phytase reaction is shown in Figure 5.5. The score plot in Figure 5.5 sums up 94% of the total variation in the spectral data, and has a typical horseshoe shape characteristic to mean-centred (A to B) reaction data.



**Figure 5.5:** PCA score plot of 72 reaction time point  $^{31}\text{P}$  spectra. PC1 and PC2 explain 94% of the variation (PAPER II)

This apparent simplicity is not anticipated for the  $\text{IP}_6$  to  $\text{IP}_1$  reactions, which is already confirmed if a third PC is added in a 3D scoreplot (Figure 5.6). The additional 3% variation described by PC3 gives a complex loop pattern of the scores, indicating a more complex chemical reaction.



**Figure 5.6:** 3D PCA score plot of the mean-centred  $^{31}\text{P}$  spectra. PC1, PC2 and PC3 together explain 98% of the variation (PAPER II)

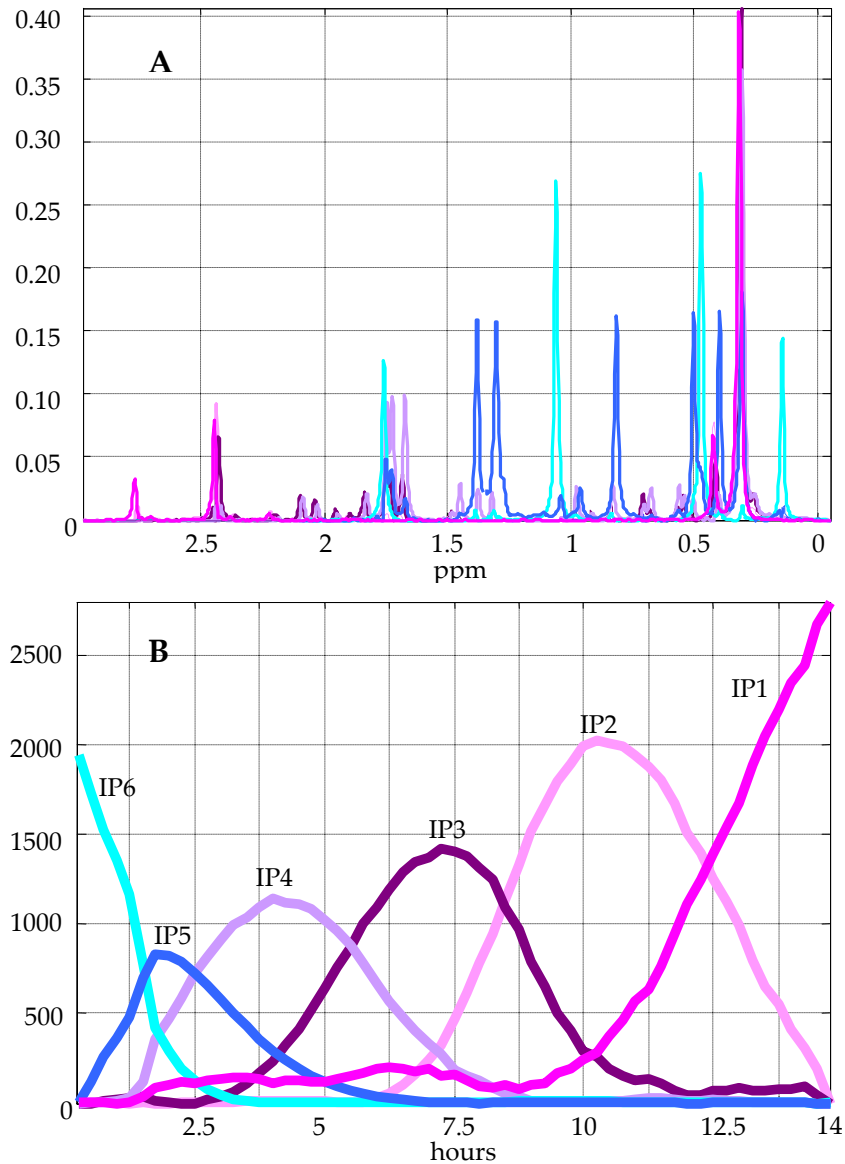
Instead of modelling the  $^{31}\text{P}$  NMR spectra by PCA, a more useful model for interpretation of the degradation pattern of phytic acid may result from the multivariate curve resolution (MCR) method which, if successful, will provide the pure NMR spectra in the loadings rather than the pure mathematical spectra of the PCA.

#### 5.1.2.2 Multivariate curve resolution (MCR)

Alternating regression, also called multivariate curve resolution (MCR) (Tauler, 1995; Winning et al., 2007), is a mathematical method for curve resolution that does not impose the orthogonality constraint as utilised in the PCA algorithm. Analogous to PCA, the input to the algorithm is a matrix  $X$  with spectral measurements of the relevant mixtures to be resolved, and the output is two matrices:  $C$  that contains the estimated concentrations of the mixture components, and  $S$  that contains the pure spectra of the mixture components. The model to be solved is  $X = C \cdot S^t$ , where  $t$  is transposed and dimensions are:  $X$  (n samples, m wavelengths),  $C$  (n samples, p pure components) and  $S$  (m wavelengths, p pure components) (Nørgaard et al., 2005).

In contrast to PCA, MCR requires a guess of  $S$  (spectral loadings) in order to initiate the algorithm, but with the presented data the algorithm proved rather stable and reproducible, regardless of the initial guess.

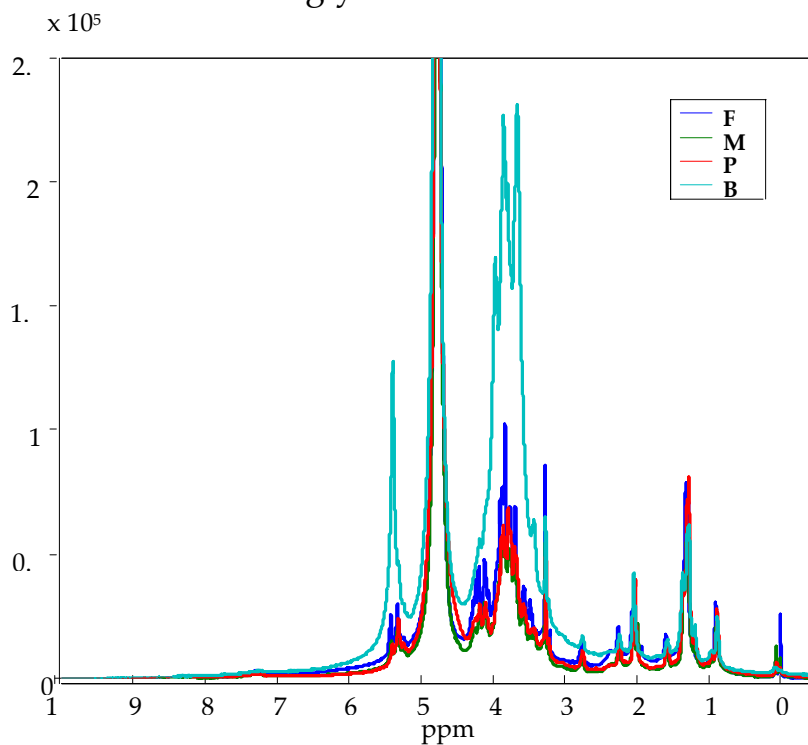
Figure 5.7 displays the result of a six-component MCR analysis of the  $^{31}\text{P}$  NMR time series of the phytic acid degradation. The cyan line in Figure 5.7 A, shows the four peaks representing  $\text{IP}_6$ .  $\text{IP}_5$  is represented by five peaks shown with the blue line. From then on the reaction becomes more complex because of the many possible configurations of phytate. In the case of  $\text{IP}_4$ , 3 peaks illustrated by the light purple lines coexist with many small peaks. These patterns are in good agreement with the rare literature found about  $^{31}\text{P}$  NMR on pure phytate model systems (Frølich et al., 1986; Frølich et al., 1988; Kemme et al., 1999). In the case of  $\text{IP}_3$  and lower inositol phosphates, it becomes very difficult to separate the peaks without the use of chemometrics; however, the use of the multivariate curve resolution (MCR) makes it possible to separate all the components.



**Figure 5.7:** (A) The resolved 'pure'  $^{31}\text{P}$  NMR spectra from the MCR model. Cyan line=IP<sub>6</sub>, blue line=IP<sub>5</sub>, light purple line=IP<sub>4</sub>, purple line=IP<sub>3</sub>, pink line=IP<sub>2</sub> and magenta line=IP<sub>1</sub>. (B) Plot of the relative intensity of the six pure profiles as a function of time. The same colours are used as in (A) (PAPER II)

Figure 5.7 (B) beautifully shows the stepwise degradation pattern of the different inositol phosphates almost as a schematic diagram in a text book. Nevertheless, the figure presents the complete degradation pattern of the phytase reaction using real multivariate experimental data. From the figure it is evident that already after 2.5 hours all IP<sub>6</sub> is degraded.

After approximately 1.5 hours the formation of IP<sub>5</sub> reaches a maximum and for the next six hours undergoes degradation to IP<sub>4</sub>, which reaches a maximum after four hours and is fully degraded after ten hours. This is in agreement with Frølich and co-workers (Frølich et al., 1986) who performed a similar NMR experiment, but without chemometrics, and found that IP<sub>4</sub> was degraded after approx. 10 hours. According to the literature, IP<sub>6</sub> should be degraded to at least IP<sub>3</sub> before the complex bound minerals are released and thus become bioavailable (Brune et al., 1992; Sandberg et al., 1999). This indicates that it takes approximately 10 hours under the conditions present in rye bread making to degrade phytate to a form where the mineral complexes are no longer very strong and the minerals thus become bioavailable for humans and animals. IP<sub>3</sub> has a “lifetime” of approximately 10 hours; for IP<sub>2</sub> it is approximately 8 hours. IP<sub>1</sub> is formed during the entire hydrolysis, but increases strongly after 10 hours.



**Figure 5.8:** HR MAS <sup>1</sup>H NMR on rye bread process samples (Viereck et al., 2005b). F: rye flour, M: dough after mixing, P: dough after proofing and B: baked bread.

In this study we have recorded spectra, which are not all assigned yet, of rye dough and rye bread samples evaluated later in this chapter. The spectra were obtained using a different type of NMR, called high-resolution magic angle spinning (HR MAS). The sampling was done on solid samples in contrast to the solution used in the model system. Moreover the method



employed for HR MAS was  $^1\text{H}$  NMR instead of  $^{31}\text{P}$  NMR. An example of the obtained spectra is shown in Figure 5.8. The perspective is to be able to quantify the state of phytic acid degradation in the bread.

By this study we have demonstrated that HF-NMR is capable of monitoring the stepwise degradation pattern of phytate in a model system and obviously it would be interesting to examine the possibilities of measuring the rye bread samples. This would create opportunities for using HF-NMR combined with chemometrics in bread quality control. This is an area which is of growing interest, because the food industry wants to produce as healthy and nutritionally adequate products as possible and not least be able to prove it.

## ***5.2 An improved spectrophotometric method for measurement of phytase activity***

Phytase activity is normally measured indirectly using a colorimetric quantification of the inorganic phosphate released by the enzymatic cleavage of phytate. This type of measurement is not only specific to the phytase enzyme, but also to other phosphatases (Stauffer, 1989). Therefore, more advanced spectroscopic and chromatographic methods have been developed to determinate the changes in phytate content and thereby indirectly phytase activity. High Performance Ion Chromatography (HPIC) (Greiner et al., 2000; Skoglund et al., 1997; Türk et al., 2000; Carlsson et al., 2001; Chen, 2004), High Performance Liquid Chromatography (HPLC) (Camire and Clydesdale, 1982; Hatzack et al., 2001; Sandberg and Ahderinne, 1986; Sandberg et al., 1989; Türk et al., 1996; Graf and Dintzis, 1982), Flow Injection Analysis (FIA) (Vieira and Nogueira, 2004) and, as mentioned in the previous chapter, Nuclear Magnetic Resonance (NMR) spectroscopy (Nakano et al., 2000; Phillippy, 1989) all have the potential to identify and quantify isomers of inositol phosphates and give a more detailed picture of the degradation of phytate. However, in many cases a simple assay based on measuring the concentration of released phosphate by an enzyme-substrate reaction is sufficient (Eeckhout and De Paepe, 1994; Engelen et al., 1994; Fretzdorff and Weipert, 1986; Heinonen and Lahti, 1981; Lolas and Markakis, 1977; Peers, 1957). The main problems with such assays are the time of analysis, the requirement of a phosphate calibration curve, and last but not least, the requirement for extract is considerable.

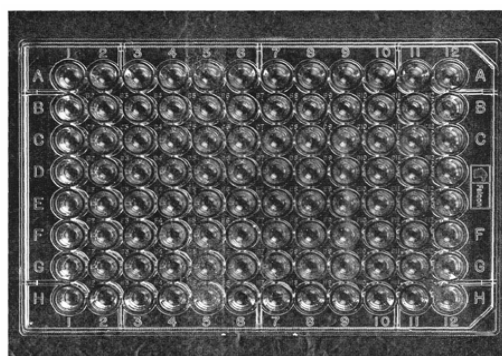
We therefore set out to develop a rapid and simple method to measure rye phytase (PAPER I). The method was optimised by modifying the original method described by Eeckhout & De Paepe (1994) and the main parameters that were examined and altered are shown in Table 5.1.

**Table 5.1:** Comparison of the parameters used in the original and optimised method

Method	Eeckhout and De Paepe, 1994	Optimised method
<b>Preparation of extract</b>		
Sample weight (g)	0.100 - 0.200	0.200
Substrate	1.6 mM Na-phytate	6 mM Na-phytate
Buffer	0.25 M Na-acetate pH 5.5	0.25 M Na-acetate pH 5.5
Volume (mL)	50	50
Temperature (°C)	37	37
Shaking time (min)	15	0
Extraction time (min)	70	45
Filtration	Yes	Yes
<b>Assay</b>		
Extraction volume used (µL)	1000	100
Volume of colour reagent (µL)	1000	100
Phosphate calibration series	Yes	No
Apparatus	Spectrophotometer	Micro-titer-Plate-Reader
Colour developing time (min)	Not specified	10
Unit	U kg <sup>-1</sup>	nkatal g <sup>-1</sup>

As seen in Table 5.1, the main difference of the two methods is the use of micro-titer plates (Figure 5.9) instead of cuvettes, the time of analysis and the amount of extract required for the assay.

Using micro-titer plates, only a small amount of extract is required and facilitates high-throughput measurements. The method thus provides a better sampling and a higher capacity than other assays reported. Compared to the



**Figure 5.9:** A micro-titer plate with 96-wells

original method, the time of analysis was reduced by 45 min. Other parameters like substrate concentration and amount of flour were also optimised (PAPER I).

### ***5.3 The degradation of phytic acid during rye bread baking***

The optimised method for phytase measurement was applied to study the phytase activity at a range of pH values and temperatures relevant to the rye breadmaking process. The degradation of phytic acid during rye bread making was investigated for three bread types: dark rye bread without added whole rye grains (DB00), dark rye bread with 30% added whole rye grains (SB30) and dark rye bread with 50% added whole rye grains (KB50).

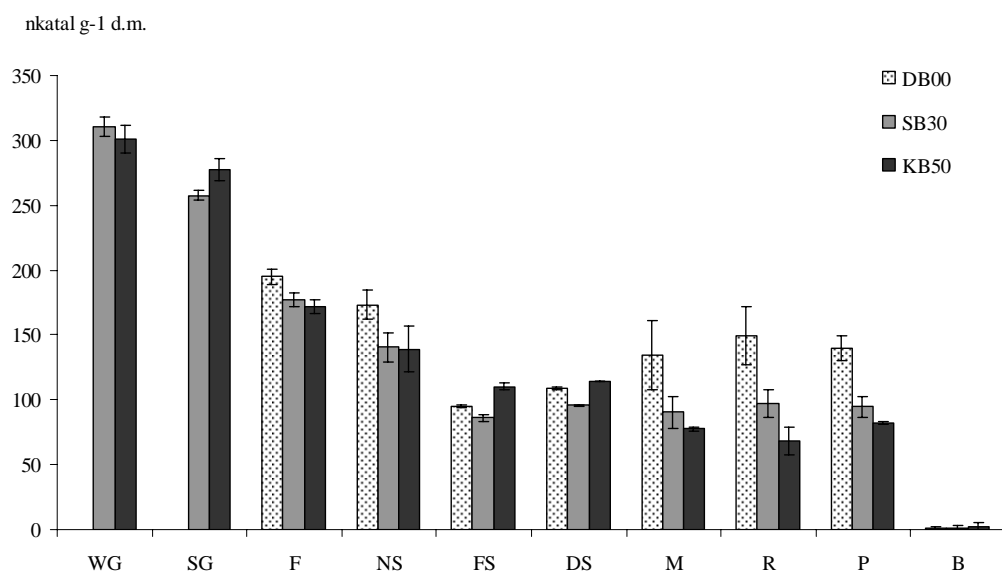
#### **5.3.1 pH and phytase activity**

Phytase activity is very sensitive to pH and the measured pH optimum for endogenous phytase in rye was 6.0 (PAPER I) (Greiner et al., 1998; Peers, 1957), which was higher than the pH measured in the rye doughs (PAPER III).

Activity measurements of phytase during the seven different process steps (Figure 5.10) revealed that a relatively high phytase activity was present throughout the process. According to the measurements of activity of phytase as a function of pH, the activity was expected to be approx. 45% lower in the doughs than the activity at optimum pH. In the fermented sourdough (FS), the phytase activity was expected to be 20% of the optimum activity, but since pH was 5.1-5.2 in the unfermented sourdough corresponding to 80% of optimum phytase activity, the first hours of the sourdough fermentation support high endogenous phytase activity to hydrolyse phytate.

In the whole grains (WG) pH was approximately 7.0 and in the rye flour (F) pH was 6.5. pH was a little higher in the bread dough without addition of rye grains (pH=4.6) compared to the doughs with rye grains (pH=4.3) due to the addition of vinegar in the soaking water, but after proofing the difference in pH was no longer statistically different.

The phytase activity in the samples from the three different bread preparations is shown in Figure 5.10. The phytase activity was high in WG (pH 7) and slightly lower in the soaked grains (pH 4.8-5.4). The lower activity in the soaked grains (SG) might be due to the lower pH, but also due to a partial inhibition of the phytase due to the addition of 70°C hot water. The phytase activity was 10-15% lower in the unfermented sourdough compared to the corresponding flour, which is due to a lowering of pH by the addition of fermented sourdough with a low pH value.



**Figure 5.10:** Phytase activity during rye bread making (PAPER III). WG: whole grain, SG: soaked grains, F: rye flour, NS: unfermented sourdough, FS: fermented sourdough, DS: diluted sourdough, M: dough after mixing, R: dough after resting, P: dough after resting and B: baked bread (PAPER III)

In the fermented sourdough the activity was halved compared to the flour, but the relatively high phytase activity in the fermented sourdough (despite a low pH) might be due to some activity of microbial phytases from the sourdough yeast (Leenhardt et al., 2005; Reale et al., 2004). Microbial phytases has a lower pH optimum than cereal phytases (Pandey et al., 2001; Peers, 1957; Sandberg et al., 1996; Türk et al., 1996); pH optimum has been reported to be 4.5 for yeast phytases (Nakamura et al., 2000; Nayini and Markakis, 1984). The presence of microbial phytase was confirmed in a HPLC determination of the different inositol phosphates (data not shown).

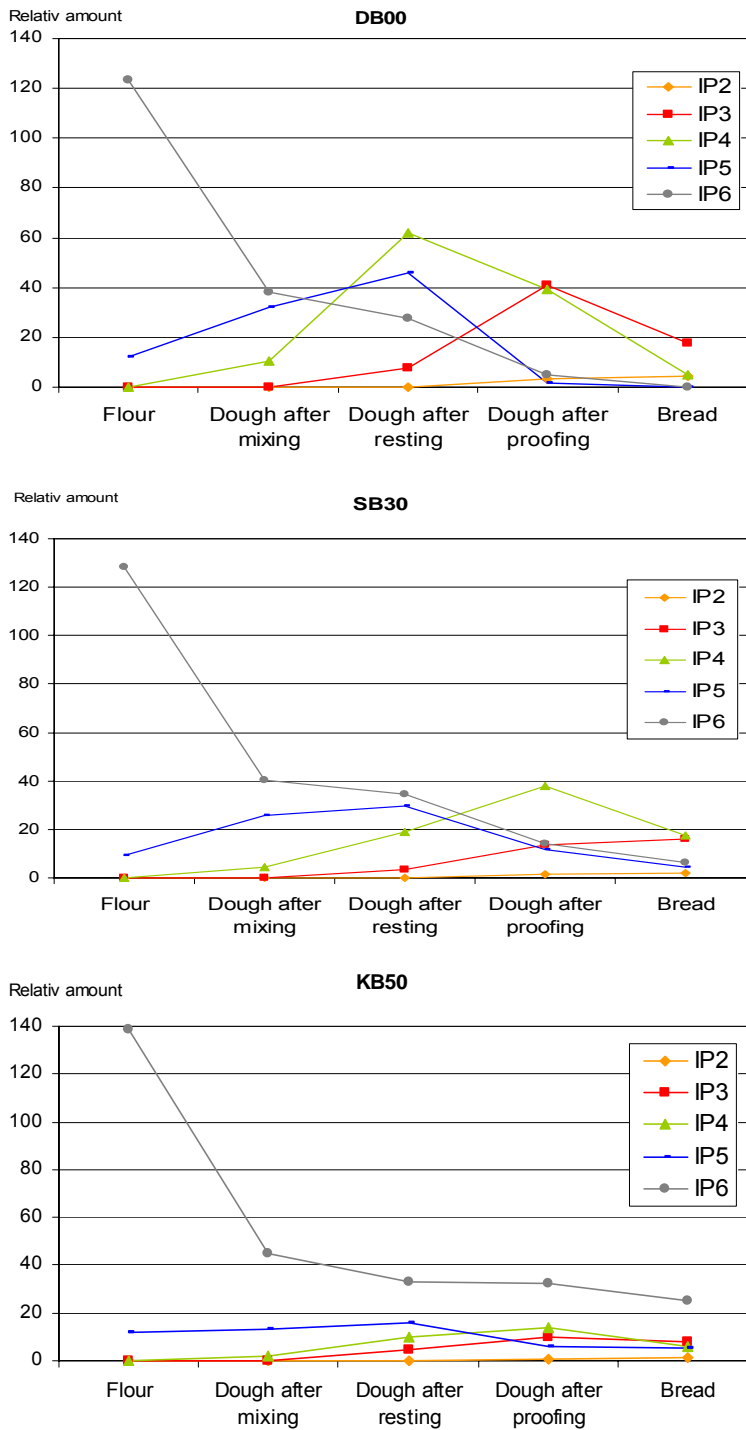
The phytase activity was highest in DB00 in all dough stages, followed by SB30 and KB50. The higher activity cannot be explained by differences in pH alone, but also by the fact that phytase is not in contact with the substrate (inositol phosphate). The phytase activity was stable during the dough processes for all three bread types.

In conclusion, the phytase showed high activity level during the entire rye bread production, regardless of the addition of whole grains. The question was therefore if the phytase activity was sufficient to degrade the phytic acid under these conditions.

### **5.3.2 Degradation of phytate in rye breads**

The content of IP<sub>6</sub> and the lower inositol phosphates in the three bread types during processing is shown in Figure 5.11. The IP<sub>6</sub> content was high in the flour despite a high activity of phytase; the enzyme first shows activity when it comes into a solution. This was confirmed by the content of IP<sub>6</sub> in the soaked grains where the activity was almost halved compared to the content in the whole grains. After mixing of the bread dough, the content of phytic acid was reduced by up to 60% compared to the content in the flour. A further degradation of IP<sub>6</sub> occurred in the doughs after resting for 10 minutes, after proofing as well as in the baked bread. In agreement with the phytase activity, where the activity was highest in DB00, the content of IP<sub>6</sub> was lowest in DB00. In DB00, 99% of the IP<sub>6</sub> from the flour was degraded to lower inositol phosphates, and in SB30 and KB50 containing whole grains 94% and 82% of IP<sub>6</sub>, respectively, was degraded.

As previously mentioned, phytate should be degraded to at least IP<sub>3</sub> before the complex bound minerals become bioavailable (Brune et al., 1992; Sandberg et al., 1999). The degradation of phytate to lower inositol phosphate was further examined and analysed at Risø A/S using HPLC (Hatzack et al., 2001). The degradation pattern was investigated in order to determine whether the degradation was due to cereal or microbial phytases.



**Figure 5.11:** Phytate degradation of the three bread types as a function of the processing step (PAPER III). DB00: dark rye bread without added whole grains, SB30: dark rye bread with 30% added whole grains and KB50: dark rye bread with 50% added whole grains.

The same degradation pattern is observed in Figure 5.11 for all three bread types, where IP<sub>6</sub> is dephosphorylated to IP<sub>5</sub>. The sequential dephosphorylation continues via IP<sub>5</sub> over IP<sub>4</sub> to IP<sub>3</sub> and subsequently IP<sub>2</sub>, as

evidenced in the model system. In DB00, IP<sub>5</sub> and IP<sub>4</sub> reach their maximum in dough after resting.

Subsequently, IP<sub>3</sub> reaches a maximum in dough after proofing to leave only a little amount of IP<sub>3</sub> and a negligible amount of IP<sub>5</sub> and IP<sub>4</sub> in the bread. As phytic acid is degraded to at least IP<sub>3</sub> in the bread, most minerals should be bioavailable for human digestion in rye bread types made from WRF.

For the bread type SB30 containing 30% grains, IP<sub>5</sub> reached a maximum in dough after resting, as was also the case for DB00, while IP<sub>4</sub> peaks in dough after proofing. This later degradation in the grain bread compared to DB00 show that phytase has more difficult access to the phytic acid in SB30, because of the grain structure. The content of IP<sub>3</sub> increased in the dough after proofing and did not decreased before baking. This means that almost no IP<sub>2</sub> is present in the baked bread, because there is no time for further degradation before the enzyme is inactivated by the heat. The content of IP<sub>5</sub> was very low in SB30 bread and the content of IP<sub>4</sub> was at the same level as IP<sub>3</sub>. The SB30 bread had the highest content of IP<sub>4</sub> of all three bread types. Together with the fact that 6% of IP<sub>6</sub> remained in the bread it is to be expected that some minerals remain unavailable for human digestion in this type of rye bread.

In the bread with 50% grains (KB50), IP<sub>5</sub> reaches its maximum in dough after resting and IP<sub>4</sub> reaches a maximum in the dough after proofing. IP<sub>3</sub> increases in dough after proofing and does not decrease before baking. This means that there is still a pool of IP<sub>5</sub> and IP<sub>4</sub> in this bread type and together with the fact that 18% IP<sub>6</sub> remain some minerals are not bioavailable for human digestion in this type of bred.

It is to be concluded that the majority of phytate was degraded during production of rye bread made with sourdough. Doughs with 30% or 50% added whole grains had a slightly lower degradation rate at 82% and 94%, respectively. Even though substantial amounts of IP<sub>5</sub> and IP<sub>4</sub> still was present in the rye breads with added whole rye grains it must still be recommended to eat whole grains products instead of refined breads, because of the many other factors such as dietary fibre content and rye plant sterols which gives nutritional benefits of whole grain products.



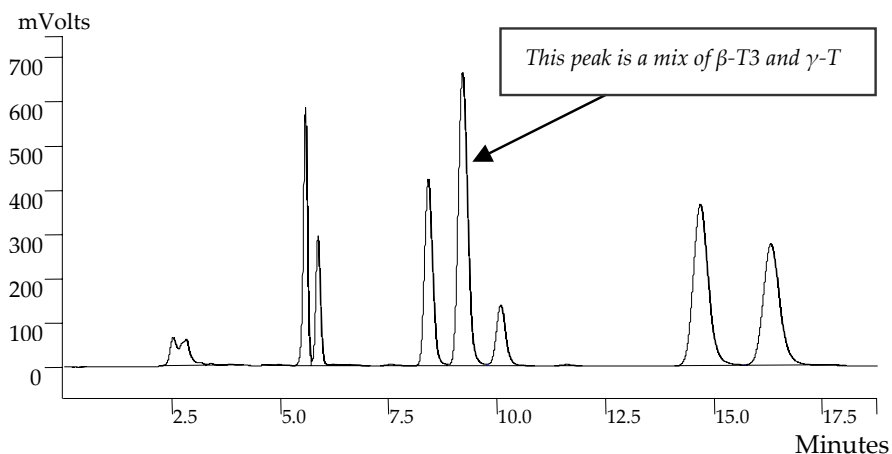


## 6. Vitamin E in Wheat

In this chapter a new optimised method for determination of all eight vitamin E isomers in cereals (PAPER IV) will be described and subsequently applied to evaluate the content, distribution (PAPER V) and degradation of vitamin E during storage (PAPER VI) in wheat and wheat flour products.

### *6.1 An improved method for determination of all vitamin E isomers in cereals*

When examining the literature describing methods for determination of vitamin E, not only in cereals, but in foods in general, large differences exist in which vitamin E isomers that are analysed. Tocotrienols are found in larger amounts than tocopherols in wheat, oat and barley and, as discussed in Chapter 2.6.2, they might have a stronger antioxidant effect than the tocopherols. It is therefore important to be able to analyse both the tocopherols and the tocotrienols. In this respect, the prime difficulty is to chromatographically separate the two isomers,  $\gamma$ -tocopherol and  $\beta$ -tocotrienol, within an acceptable analysis time because of their similar molecular properties. An example from our own laboratory obtained during the optimisation illustrates the problem (Figure 6.1).



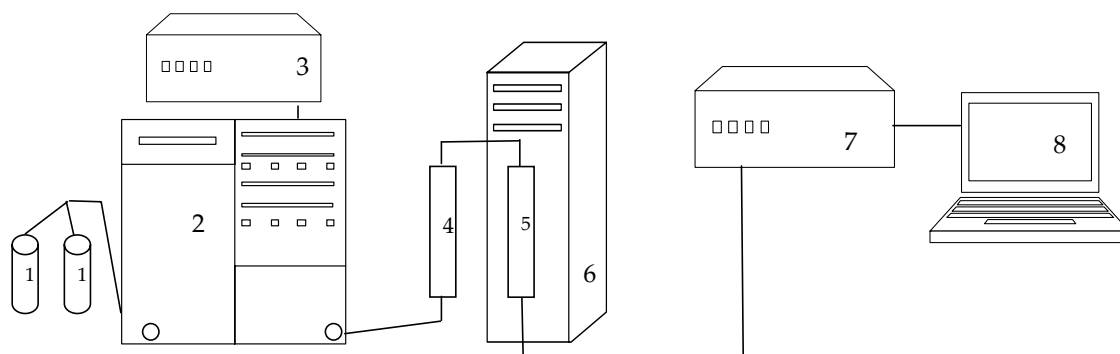
**Figure 6.1:** Example of the difficulties that can arise during NP-HPLC short time but no separation.

The difficulty in separation of the two isomer has led to studies which only report one of the components or report them as a mixture (Chen and Bergman, 2005; Lee et al., 2004; Murkovic et al., 1996; Zielinski et al., 2001). Other researchers decided not to measure the tocotrienols (Bustamante-Rangel et al., 2007; De Greyt et al., 1998; Fukuba and Murota, 1985; Grela, 1996; Panfili et al., 1994; Ramadan and Morsel, 2002; Rodrigo et al., 2002; Shen and Sheppard, 1986) all of which led to lack of important information about health properties of the product.

A significant part of this study was devoted to the development and validation of a high performance liquid chromatography (HPLC) method capable of measuring all eight vitamin E isomers present in cereals and within a short time of analysis (PAPER IV).

### 6.1.1 High Performance Liquid Chromatography (HPLC)

In HPLC, a liquid sample, or a solid sample dissolved in a suitable solvent, is carried through a chromatographic column by a liquid mobile phase. A schematic diagram of a HPLC instrument is shown in Figure 6.2.

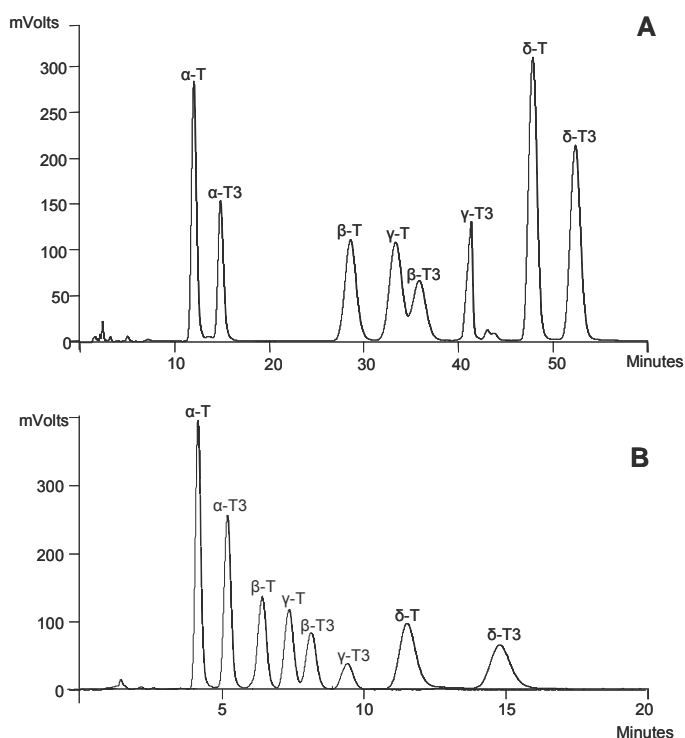


**Figure 6.2:** A schematic diagram of the HPLC used in our laboratory. 1: mobile phase reservoirs, 2: pump system, 3: auto sampler and loop, 4: guard column, 5: stationary phase column, 6: oven, 7: fluorescence detector and 8: computer

HPLC can be operated in two different modes: reverse phase (RP) HPLC and normal phase (NP) HPLC. In NP-HPLC the column, also called the stationary phase, consists of polar particles, while the mobile phase, the solution is nonpolar. The most commonly used polar chromatographic stationary phase used in NP-HPLC was packed with silica particles and differences in the strength of interaction between the stationary phase and the different vitamin E isomers mean that a separation is achieved. The more

polar mobile phase, the more it interacts with the stationary phase and the faster the vitamin E isomers elute from the column. A typical mobile phase used in NP-HPLC is a mixture of nonpolar *n*-hexane with addition of a few percent of polar solvents such as iso-propanol, ethyl acetate or diethyl ether. However, a study has shown that hexane without problems can be substituted by the less harmful heptane (Conkerton et al., 1995), but it is still not commonly used. In RP-HPLC the opposite is valid. This method uses a nonpolar chromatographic stationary phase and polar mobile phases, such as methanol and water.

When optimising the new and improved method different concentrations of hexane:2-propanol were tested as mobile phase, but as Figure 6.3 (A) shows, this solvent did not improve the separation within an acceptable time period. For this reason a mobile phase consisting a mixture of hexane, ethyl acetate and acetic acid with a higher polarity was tested and the results were excellent (Figure 6.3, B).



**Figure 6.3:** Chromatograms of all eight vitamin E isomers measured using (A) the former method (hexane:2-propanol) and (B) the optimised method (hexane:ethyl acetate:lactic acid) (PAPER IV)

Changing HPLC parameters, such as lowering the column temperature and slowing the flow rate to a minimum, also resulted in a better separation. The total time of analysis, when requiring baseline separation of all eight

isomers, was decreased by a factor of 3 (from 60 minutes to 20 minutes). The time of analysis could be further reduced by 8 minutes to a total time of analysis of 12 minutes if the delta isomers are ignored, e.g. already known to be insignificant (PAPER IV).

As detector for the HPLC method a fluorescence detector was chosen because of its selectivity. The fluorescence detector has been reported to be at least ten times more sensitive than UV detection (Thompson and Hatina, 1979). In the method the detector was set to an excitation wavelength of 290 nm and an emission wavelength of 330 nm, which is specific for vitamin E and thereby only peaks of interest will appear in the chromatogram.

Linearity of the analysis was assessed for all eight vitamin E isomers in the concentration range from 1 to 20 µg/mL. The long-term stability of the method is excellent, as retention time of each of the eight peaks varied less than approx. 0.2 seconds over a period of one year. This is to be compared to a retention difference between the two critical isomers  $\beta$ -T3 and  $\gamma$ -T of approx. 0.8 minutes, providing a good margin of reliability for the identification of the two critical isomers in cereal samples.

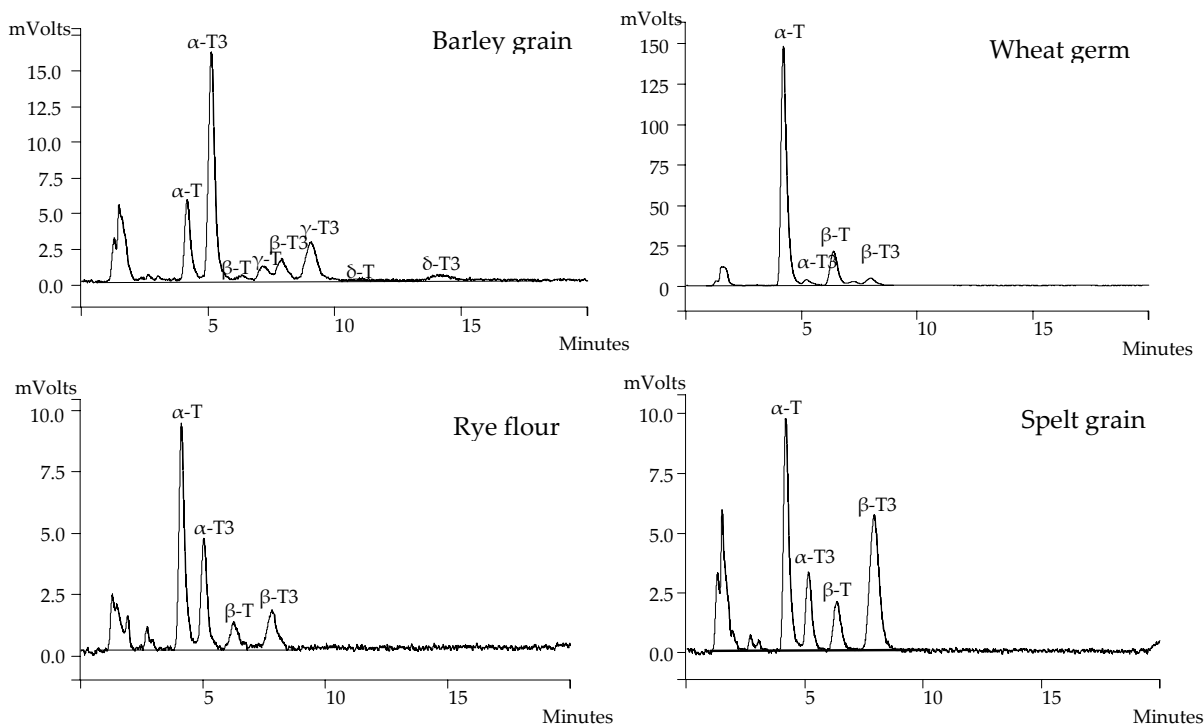
### **6.1.2 Extraction of vitamin E**

The extraction method used in this study is a slightly modified procedure adapted from (Murkovic et al., 2004). The time of extraction on the ultrasound bath was reduced from 30 to 10 minutes and the use of a drying agent was obsolete due to the use of dried hexane (PAPER IV). The extraction phase can cause substantial loss of vitamin E due to its sensitivity to light and oxygen. Since vitamin E is fat-soluble, a saponification step has in former studies been used to secure quantitative extraction of vitamin E from the grains (Franke et al., 2007; Panfili et al., 2003). In this study it was found that, for wheat and rye, nothing was gained by this step, possibly because of the increased exposure to oxygen during a saponification step and due to the low content of lipids (2-3%) in the measured cereals. The extraction method was evaluated by recovery experiments of standard compounds and was found to be in the range of 96-100%.

### **6.1.3 Identification of vitamin E in cereal products**

After careful optimisation, the extraction and NP-HPLC method was validated by application to a set of various cereal samples for identification and quantification of vitamin E isomers. Whole barley and spelt grains, rye

flour and wheat germ were analysed (see Figure 6.4) to illustrate the potential of the optimised method.



**Figure 6.4:** Chromatograms of (A) whole barley grains, (B) wheat germ, (C) rye flour and (D) whole spelt grains. All samples were measured using NP-HPLC (PAPER IV)

In all the investigated cereals the optimised NP-HPLC method resulted in good baseline separation of all eight analytes, which was a major improvement on previous methods, in particular on the very reliable separation of the  $\beta$ -T3 and  $\gamma$ -T peak. The identification was made by the retention time, which throughout this study was very reproducible.

It is observed in Figure 6.4 that the content and composition of vitamin E vary strongly from cereal to cereal. The measured wheat, spelt and rye samples only contain  $\alpha$  and  $\beta$  forms of vitamin E, whereas the barley samples contain all eight vitamin E forms, in agreement with earlier studies (Eitenmiller and Lee, 2004a; Michalska et al., 2007b; Wang et al., 1993; Panfili et al., 2003). The most predominant vitamin E isomers in barley grains are  $\alpha$ -T3 and  $\gamma$ -T3, which were found in much higher concentrations than in any of the other measured samples. In contrast,  $\beta$ -T and  $\beta$ -T3 are found at much lower levels compared to wheat and spelt. Wheat germ has a very high content of  $\alpha$ -T and  $\beta$ -T and is an excellent source of vitamin E.

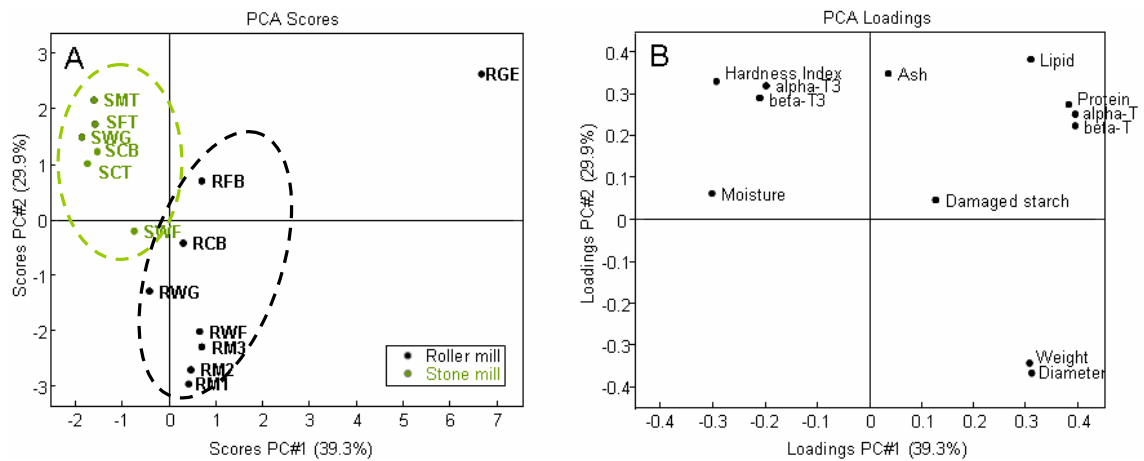
After having optimised the NP-HPLC method and demonstrating it to be a very useful method for measuring all vitamin E isomers in cereals, different wheat flour samples from two different mills were analysed for content and distribution of vitamin E isomers.

## ***6.2 The fate of vitamin E during wheat milling***

The aim of investigating the flour from two different mills was to determine whether the content and distribution of vitamin E depend on the milling method (PAPER V). The two different milling methods differ, as described in Chapter 3, among other things, in the way that stone milling used at Aurion A/S was not able to separate the germ fraction from the flour fraction.

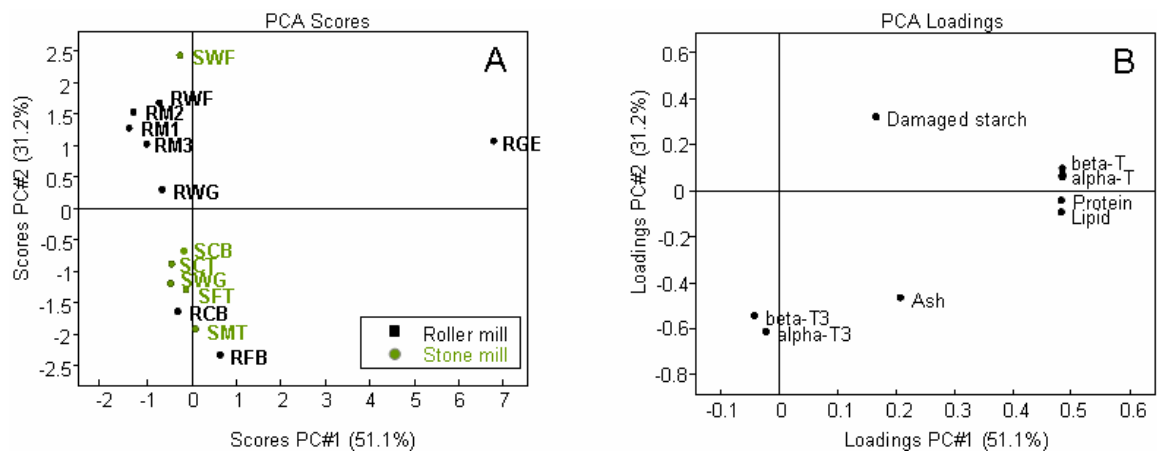
### **6.2.1 Content of vitamin E in wheat flour as a function of milling method**

Expressed as total  $\alpha$ -tocopherol equivalents ( $\alpha$ -TE), the investigated stone-milled WF had a total content of 14.3 $\alpha$ -TE, which was higher than the total vitamin E content of roller-milled WF of 12.2 $\alpha$ -TE. However, it has to be considered that the content of vitamin E was higher in the wheat grain samples used for stone milling compared to the wheat grain samples used for roller milling. Thus, if only the total content of vitamin E is evaluated without taking the biological activity into consideration, the level of vitamin E in WF was higher for roller-milled WF (37.8 $\mu$ g/g d.m.) than stone-milled WF (31.4 $\mu$ g/g d.m.). This is presumably due to the fact that the germ will still be present in the stone-milled WF and therefore a higher content of tocopherols is present. To elucidate whether or not there was a difference in milling methods in the content of vitamin E a PCA was performed on all the parameters measured (vitamin E isomers, lipid, protein, ash, hardness, moisture, damaged starch, grain weight and grains diameter). As seen in the PCA score plot in Figure 6.5 (A), the samples from the two milling methods are almost separated, except for the stone-milled WF which is positioned near the roller-milled samples.



**Figure 6.5:** PCA score (A) and loading (B) plots of all measured quality parameters (damaged starch, ash, lipid, protein, vitamin E) and grain hardness, moisture, diameter and weight in the different milled fractions of wheat (PAPER V)

Inspection of the loading plot in Figure 6.5 (B) reveals that the grain hardness, grain weight and grain diameter are the primary variables spanning both PC1 and PC2. In other words, the wheat grains used for stone milling were harder, but smaller than the wheat grains used for roller milling. When these three variables were removed from the model, the PCA picture became quite different. This is displayed in the score plot in Figure 6.6 (A) which shows no clear grouping of samples from the two mills. When looking at the loading plot in Figure 6.6 (B) it is observed that differences in the vitamin E isomers account for 51% of the described variation along PC1, while 31% of the variation along PC2 primarily describe the milling parameters (damaged starch and ash content).



**Figure 6.6:** PCA score (A) and loading (B) plots based on the relative numbers of ash, protein, lipid, damaged starch and vitamin E contents (PAPER V)

Stone milling is normally regarded as more gentle to the grains and soft wheat can be milled with essentially no starch damaged (Hoseney, 1994a). The level of damaged starch in this study was 4.8% for both milling methods and thus at an appropriate level for baking, which is between 4.5% and 8% (Dexter and Wood, 1996). The fact that the level of damaged starch was the same for both milling methods is ascribed to the fact that the grains used in this part of the study from roller milling were softer than the grains used for stone milling.

As described in Chapter 4, the major difference between the two milling techniques is the capability of the roller mill to separate the germ and bran fractions from the endosperm. The ash content might therefore be used as an indicator of the presence of bran in the flour. The wheat flour (WF) from the stone milling had a higher content of ash (1.2%) compared to the level expected for WF and indeed higher than the ash content from the roller-milled WF (0.6%). This difference in ash is ascribed to the removal of the bran and germ from the flour fraction in roller-milled flour, which was not possible using a stone mill.

In conclusion, it was not possible to differentiate the two milling methods by inspection of single parameters such as damaged starch, ash content, lipid, protein or vitamin E content. In contrast, the study underlined that the effectiveness of milling to a great extent depends on grain parameters such as hardness (Greffeuille et al., 2005; Hrusková et al., 2006; Edwards et al., 2007).

### **6.2.2 Distribution of vitamin E in the roller mill streams**

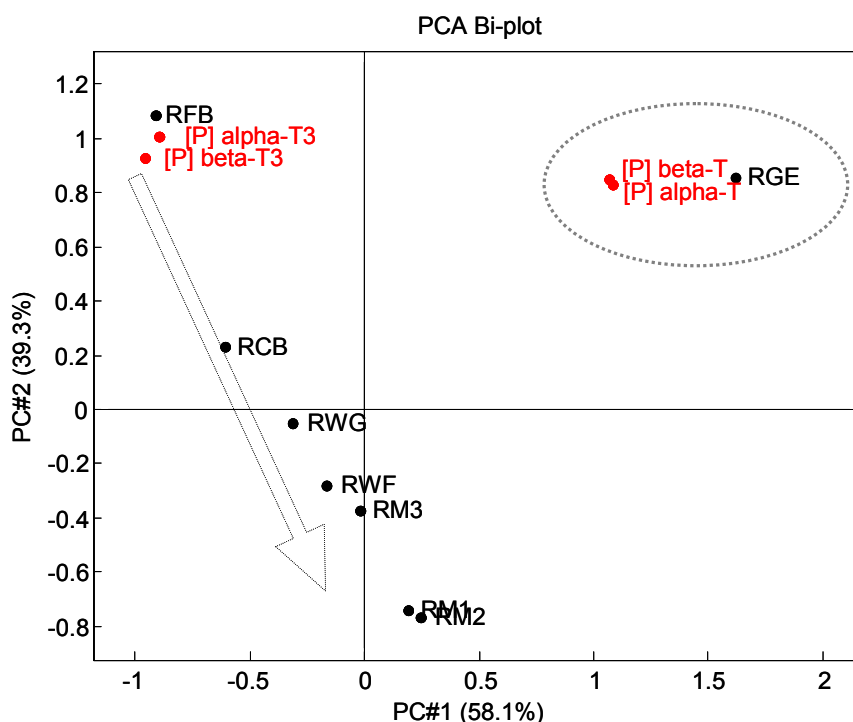
Knowledge about the distribution of tocopherols and tocotrienols in the wheat milling fractions is of prime importance, if different millstreams are to be used to fortify cereal products with vitamin E. Especially the knowledge about differentiation of tocopherol and tocotrienol is important due to the possible health benefit of tocotrienols.

Vitamin E in wheat grain and wheat milling fractions generally consists of the four vitamin E isomers  $\alpha$ -T and  $\beta$ -T and  $\alpha$ -T3 and  $\beta$ -T3 (Barnes, 1983). It has been reported that wheat contains  $\gamma$ -T, but no  $\beta$ -T3, but this was erroneously concluded from experiments with poor or no chromatographic separation of the  $\beta$ -T3 and  $\gamma$ -T peaks as mentioned previously (Bustamante-Rangel et al., 2007; Chen and Bergman, 2005; Lee et al., 2004).



In order to investigate the distribution of vitamin E in different wheat fractions the roller-milled fractions were chosen, because this milling method is capable of providing more well-defined fractions than the stone-milled wheat fractions.

The vitamin E content of the roller-milled fractions which gave the “cleanest” fractions were analysed by PCA. From the PCA bi-plot, Figure 6.7, the distribution of vitamin E isomers in the different fractions was clearly manifested.



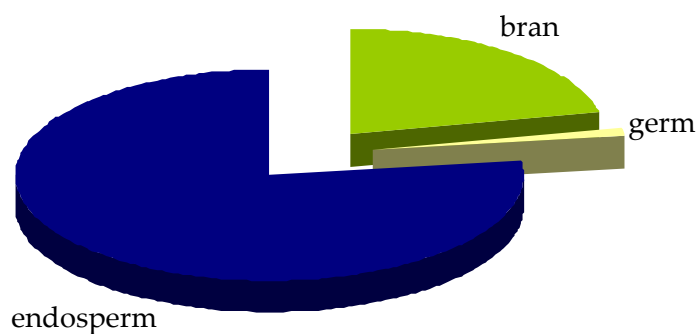
**Figure 6.7:** PCA bi-plot for roller-milled wheat fractions (PAPER V)

Figure 6.7 shows, that the  $\alpha$ -T and  $\beta$ -T contents covary almost perfectly and that the germ fraction (RGE) is unique by containing large amounts of tocopherols. Also the content of  $\alpha$ -T3 and  $\beta$ -T3 covaries strongly, a variation which is able to discriminate the other fractions in a trend from the fine bran (RFB) with high content of tocotrienols to the middlings (RM1, RM2 and RM3) with low content of tocotrienols.

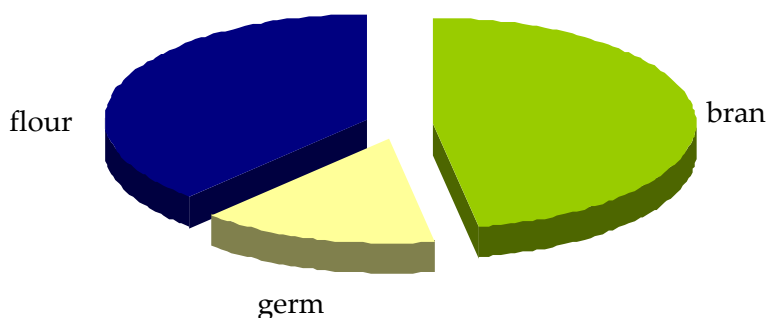
It can be concluded that there is an exceptionally high concentration of tocopherols in the germ while the tocotrienols are more equally distributed throughout the grain with a slightly higher content in the bran. The relative low tocopherol content in roller-milled wheat flour is thus the results of a

highly refined and sophisticated milling process where the high-value germ fraction has been removed from the principal flour product used for baking.

To illustrate how important the germ fraction is with respect to  $\alpha$ -T content, Figure 6.8 shows the mass distribution of the three parts: bran, germ and endosperm and Figure 6.9 show the distribution of  $\alpha$ -T in the three parts.



**Figure 6.8:** Mass distribution of the three roller-milled parts in wheat; endosperm, bran and germ.



**Figure 6.9:**  $\alpha$ -T distribution in germ, bran and flour fractions

While the germ only represents 1% of the grain mass it contains 20% of the total  $\alpha$ -T content in the flour (Liyana-Pathirana and Shahidi, 2007; Ko et al., 2003). It has been reported that the bran and feed (middlings and tailings) fractions of wheat have the highest antioxidant capacity followed by shorts and flour fractions, but that the germ fraction had the absolute highest concentration of  $\alpha$ -T. These results imply that grinding grains into wheat flour leads to a decrease in vitamin E content (Eitenmiller and Lee, 2004a; Zielinski et al., 2007; Bryngelsson et al., 2002). The study of rye flour fractions has revealed the same tendency, meaning that the content of

tocopherols and tocotrienols depends on the extraction rates of the rye flour (Michalska et al., 2007a; Nyström et al., 2007).

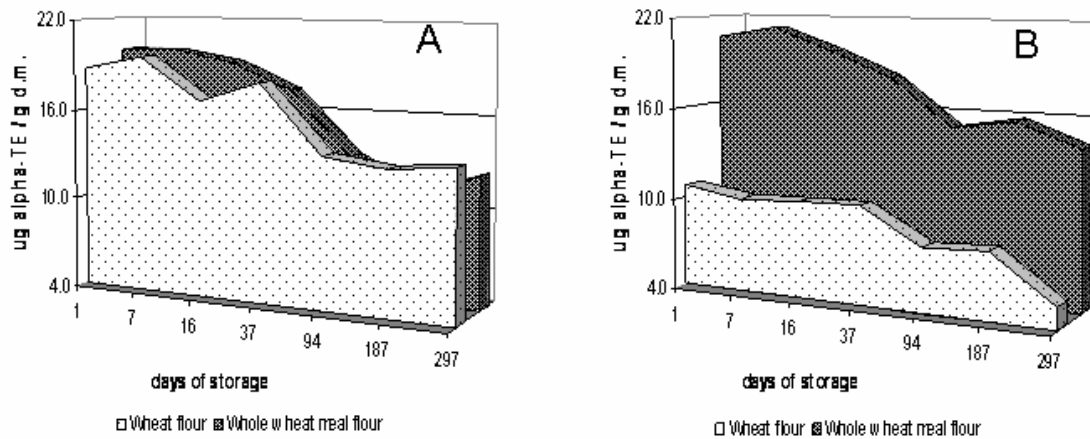
This study showed that tocopherols are located primarily in the germ fraction of wheat flour. In contrast, the tocotrienols were almost equally distributed throughout the wheat grain, but with a higher amount in the bran fractions. Due to the recipe used at the mill it was not possible to distinguish between roller-milled and stone-milled flour solely on the basis of vitamin E content and isomer distribution. However, the study clearly demonstrated that the possibility of removing the germ fraction in the sophisticated commercial mills will lead to significantly lower vitamin E content in the flour fraction. Enriched or fortified tocopherol products thus need to have large fractions of germ or fine bran fractions, whereas fortified tocotrienol products are less obvious to produce.

### ***6.3 The degradation of vitamin E during storage of wheat flours***

The degradation of vitamin E during storage of wheat flours (WWF and WF) from both stone mills and roller mills were studied by direct measurements of the actual content of the vitamin E isomers in the samples, but also indirectly by measuring the degree of lipid oxidation expressed by the lipoxygenase activity and by the formation of hexanal.

#### **6.3.1 Loss of vitamin E during storage of wheat flour**

The content of vitamin E in both stone-milled and roller-milled flours (WWF and WF) was determined various times during a storage period of approx. 10 months. This was done to determine if the loss of vitamin E during storage is dependent on the milling method. As seen in Figure 6.10 (A), the loss of vitamin E in WWF and WF milled at stone mills were greatest after approx. three months from which point the vitamin E content remained constant. The total loss for WWF was 36% and 24% for WF (PAPER VI). Figure 6.10 (B) shows that there was a difference in the vitamin E content dependent on flour type in the case of roller-milled flours. The WF had almost half the initial content of vitamin E compared to WWF.



**Figure 6.10:** Total loss of vitamin E expressed as  $\mu\text{g } \alpha\text{-TE/g d.m.}$  (PAPER VI)  
 A: stone-milled samples and B: roller-milled samples

The loss of vitamin E in the roller-milled flour followed the same pattern as for stone-milled flour. The total loss of vitamin E in WWF was only 31%, compared to the loss of 50% for roller-milled WF. These observations are in agreement with experiments performed by Wennermark and Jagerstad (1992) who observed a loss of 40% for WWF and 38% for WF after 12 months of storage (Wennermark and Jagerstad, 1992), whereas Piironen et al. (1988) reported even greater losses (80% of  $\alpha$ -isomers) in WF after one year of storage at room temperature. Normally, nine months is registered as the shelf life date for flour in Denmark, but in accordance with the results obtained in this study it should be recommended that the consumers use their flour within a period of three months in order to maintain a good starting content of vitamin E before baking. The results also show that the loss of vitamin E occurs continuously over the first three months for the flour types containing more bran and germ.

### 6.3.2 Lipid oxidation and activity of lipoxygenase (LOX)

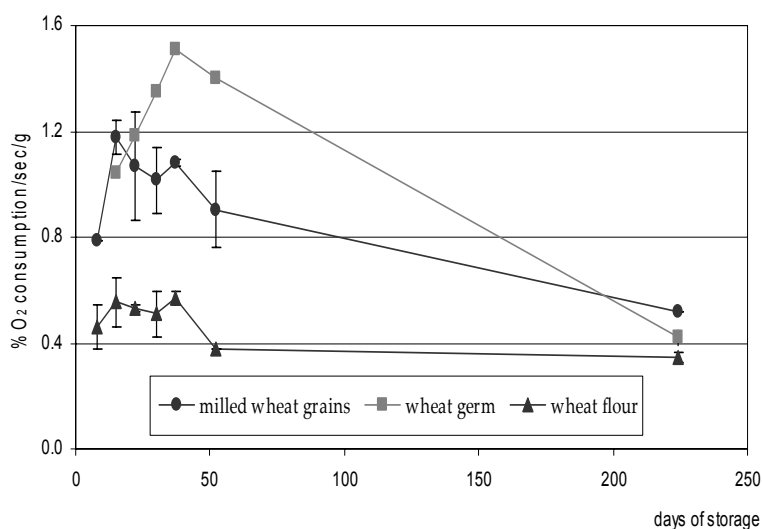
In order to investigate the relation between degradation of vitamin E and lipid oxidation during storage the activity of lipoxygenase were also measured in the storage experiment. In this study the oxidation was monitored by measuring the lipoxygenase activity and the oxidative stability of the flour was simultaneously monitored by measuring formation of hexanal.

Autoxidation occurs when unsaturated fatty acids (UFA), such as like linoleic acid and linolenic acid are exposed to oxygen (Belitz et al., 2004) and secondary oxidation products like ketones and aldehydes are formed. This is an autocatalytic chain reaction that proceeds through a free-radical chain reaction mechanism involving three stages: initiation, propagation and termination. In the initiation reaction, a carbon-centred lipid radical is produced by the abstraction from an UFA moiety of lipid. The initiation process is very slow and can be catalysed by heat, light, trace of metal ions and certain enzymes. In the propagation step, the alkyl radical reacts with molecular oxygen at a very high rate, yielding a peroxy radical. The peroxy radical, a chain-carrying radical, is able to attack another polyunsaturated lipid molecule. Although the initial peroxy radical is converted to a hydroperoxide, this process produces a new alkyl radical, which is rapidly converted into another peroxy radical. Thus, the chain reaction does not stop until the chain-carrying peroxy radical meets and combines with another radical to form non-radical products (termination reaction) (Angelo, 1996; Frankel, 2005; Belitz et al., 2004; Chung, 1991). Alpha-tocopherol is known to be a chain-breaking antioxidant which inhibits the propagation step (Niki et al., 1991; Yamauchi, 2007).

Phytic acid and vitamin E have some links, and similarities have been reported in literature. They both act as antioxidants and in a study by Soares and co-workers (2004) it was found that dietary  $\alpha$ -T inhibited the lipid oxidation in meat at the initiation stage, whereas phytic acid acted on at the propagation stage and that a synergetic action occurred between the antioxidants (Soares et al., 2004).

Enzymatic oxidation of UFA also occurs by lipoxygenase (LOX). LOX is located in many plants and is an enzyme which catalyses the oxidation of some UFA. Since activity of LOX is specific to UFA with a 1-cis, 4-cis-pentadien system, linoleic and linolenic acid will oxidise easily (Belitz et al., 2004). Experiments with wheat flour have shown that activity of lipoxygenase was relatively high in WWF due to the fact that both the bran and the germ were rich in lipoxygenase. Wheat germ and wheat bran have 17 and 4 times as much lipoxygenase activity as the endosperm, respectively (Rani et al., 2001).

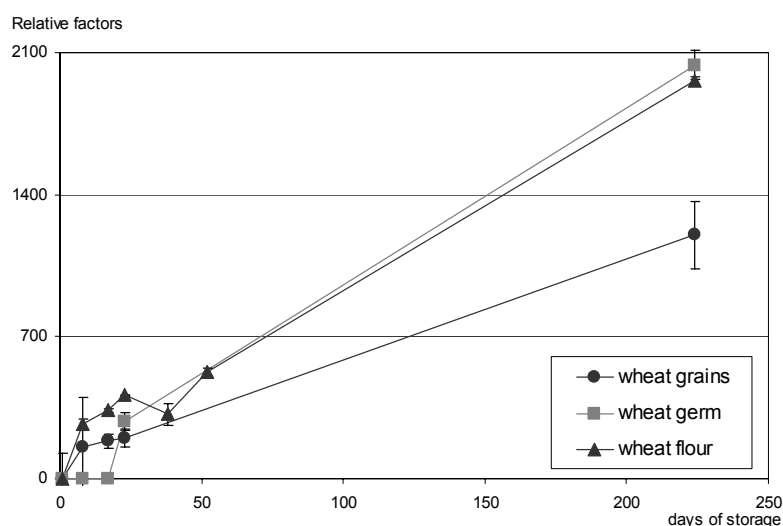
The activity of LOX in milled wheat grains, WGM and WF from the roller mill was examined during the storage period of approx. 10 months (PAPER VI). The results revealed (Figure 6.11) that the activity of LOX was lowest in WF and that the activity after 224 days was not significantly different from day 1. This is due to the fact that almost no bran and no germ were present in the WF fraction and thus not much enzyme was present. The milled wheat grains showed twice the amount of activity than WF, which was expected, because the grains were freshly milled before analysis and still contained both germ and bran. Previous studies have shown that activities of LOX in bran fractions were much higher than in flour and that the activity of LOX was highest in germ fractions (Galliard, 1986; Gökman et al., 2007).



**Figure 6.11:** Activity of lipoxygenase in milled wheat grains, wheat germ and wheat flour during 297 days of storage (PAPER VI)

In this study the activity of LOX in WG increased to the highest level of activity after 37 days from which point the activity decreased rapidly. As lipase is located in the bran and LOX is primarily located in the germ (Galliard, 1983) it was expected that the activity of LOX would be highest in the germ fraction. The results from monitoring LOX activity as a function of storage time showed a high activity of LOX in the germ and in the milled wheat grains and a lower activity in the wheat flour, meaning that significant lipid oxidation occur during storage.

Lipid oxidation is often quantified in terms of volatile secondary lipid oxidation products, and hexanal is a characteristic oxidation product that is present in high amounts and therefore often been used to indicate lipid oxidation (Fritsch and Gale, 1977; Frankel, 2005). Hexanal is a good indicator of the degree of lipid oxidation in cereals and has along with other volatiles, like 1-octen-3-one, pentanal and 2(E)-nonanal, been applied to various cereal products to follow the quality deterioration (Jensen and Risbo, 2007; Ullrich and Grosch, 1987). In this study pentanal, hexanal, heptanal, nonanal, 1-octen-3-one and decanal were detected, but since hexanal previously had shown to be the best indicator of oxidation of especially linoleic acid and linolenic acid (data not shown), only hexanal is shown in Figure 6.12.



**Figure 6.12:** Formation of hexanal during storage of milled wheat grains, wheat flour and wheat germ (PAPER VI)

The result presented in Figure 6.12 shows that the level of hexanal increase throughout the whole storage period. The formation of hexanal was apparently not correlated to the LOX activity, since the formation of hexanal was increasing through the whole storage period. It is also observed that WG exhibited a lag period of 17 days before the formation of hexanal became measurable, which means that vitamin E in this intermediate storage period functions as an effective antioxidant that is able to suppress the lipid oxidation.

It is to be concluded from this study that the loss of vitamin E was not dependent on the milling method used, but related to the flour products, since WWF from both stone milling and roller milled displayed the same loss of vitamin E. The content of vitamin E was halved during storage of roller-milled WF, but for the three wheat flours with a certain amount of bran and germ the content decreased 30% during the first 94 days, and then the content remained constant. Consumers should therefore recommended to use their WWF or stone-milled WF within the first 3 months after milling in order to retain as much vitamin E functionality and consequently prevent as much lipid oxidation as possible in the bread.



## 7. Conclusions and Perspectives

This project has contributed with knowledge about the influence of processing on the nutritional quality of rye and wheat flour products.

The degradation of phytic acid during rye bread making was studied and it was shown that phytase had good conditions for degrading phytate during rye bread making with the use of sourdough. The sourdough process led to a sufficient degradation of phytate to make the minerals bioavailable in the whole grain product. The degradation of phytic acid by phytase was also studied in a model system and it was shown that the degradation of phytic acid was a stepwise process, which could be followed in detail by NMR. It was concluded that the combination of NMR and chemometrics is a most powerful tool to measure the degradation pattern of phytate during processing.

Secondly, the distribution, content and degradation of vitamin E in wheat during milling and storage were studied for stone-milled and roller-milled flour. The vitamin E isomers were measured with the use of the optimised NP-HPLC method, which was capable of detecting all eight isomers. It was concluded that it was not possible to differentiate flour from the two milling methods neither in terms of content of vitamin E nor in terms of degradation of vitamin E during storage. For roller-milled wheat flour the content of vitamin E was reduced by 50% during storage, whereas wheat flours with bran and germ present, the vitamin E content was reduced by 30% the first 94 days, from which point the vitamin E content remained constant. These results gives the small bakeries and millers, who use stone mills, an opportunity to promote their products, and may in time support the labelling of their products as being health promoting? Finally, it can also be concluded that the tocopherols were exceptional high concentrated in the germ fraction whereas that the tocotrienols are more equally distributed

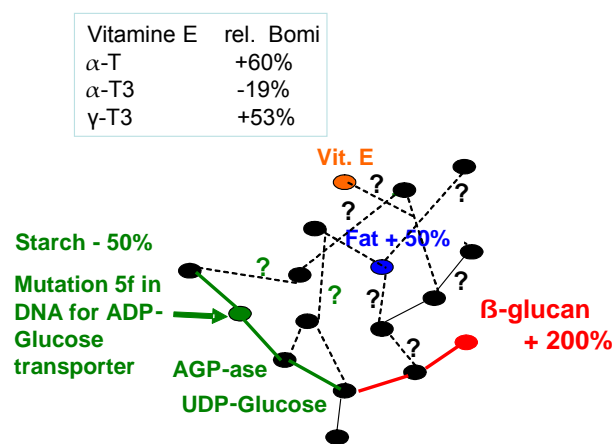
throughout the grain with a decrease in content when moving away from the bran.

The research area covered by this study gives many perspectives. As mentioned in the introduction the European “Healthgrain” project deals with many aspects of making our cereal food healthier. One of the aspects is to evaluate the processing tools in order to optimise our cereal foods. This study has pointed out some simple but very useful tools for optimising our products. The use of sourdough in rye bread making has been demonstrated to give the natural occurring enzymes optimal conditions and by that, the mineral bioavailability will increase. With regard to vitamin E this study has shown, that by mixing the flour fractions from industrial roller mills the vitamin E content in the flour can be altered drastically for example by adding more of the germ fraction. The problem about adding more germ fraction to the flour is the increased lipid oxidation and hence rancidness, but if the consumers are using their flour products fresh, this will not be a problem. For the industry there can be some large problems according to the changed baking performance of the flours, but these are technological problems that can be solved.

Another aspect of mineral-phytic acid complexes, besides the mineral bio-unavailability, are that most of the phosphate is bound in phytic acid and since the mineral-phytate complex is indigestible for non-ruminant animals including humans the phytic acid excretes in the manure. The phosphate thus contributes to a significant phosphate load on the environment, leading to increased ecological problems. It is therefore of great interest to find ways to reduce this problem. One possible way to solve this problem is to breed for new rye varieties with decreased content of phytic acid for fodder and food. It is also possible to develop low phytic acid rye mutants using genetic engineering or *nutrigenomics* approaches but attempts to solve the “problem” in this manner may lead to pleiotropic side effects that can have a great impact on the outcome of the breeding (Jespersen and Munck, 2008).

The study of pleiotropic effects of cereal mutant breeding is in fact an extremely interesting area that has not yet received the attention it deserves. As an example on pleiotropic effects, we have studied how the vitamin E content of the different isomers changes in different barley mutants, in the example here a low starch barley mutant. In this mutant the pleiotropic side effects of the changed starch synthesis is, as seen in the simplified Figure 7.1,

a very high  $\beta$ -glucan content and a high fat content, but in this context most interestingly, also a strongly increased content of  $\alpha$ -T and  $\gamma$ -T3 and a decrease in  $\alpha$ -T3 content relative to the mother barley variety. Breeding can therefore be very useful and maybe have health beneficial side effects that are unknown. Spectroscopy and chemometrics has shown to be important tools to get an overview of the whole physical-chemical composition of the seed, and relate it to genetics (Munck, 2008). While NIR spectroscopy provide a most reproducible fingerprint of the whole plant phenome and thus is useful for mutant identification and screening, NMR spectroscopy such as used in this study is a most powerful method to screen the detailed plant metabolome for bioactive substances.



**Figure 7.1:** A modified graphic over the primary and secondary effects of endosperm synthesis (Jespersen and Munck, 2008)

There are also great future perspectives in using NMR spectroscopy combined with chemometrics for cereal quality control. This is an area which is of growing interest, because the food industry wants to produce as healthy and nutritionally adequate products as possible and not least be able to prove it.

In conclusion the results of this study suggest that it should be recommended that consumers eat whole grain products rather than refined products due to the benefits of vitamin E, other phenolic compounds and phytic acid. Together with the dietary fibres these substances may in part be responsible for the reduced risk of cancer and coronary heart disease associated with intake of high-fibre diets containing whole grains. Because vitamin E and phytic acid are most associated with the fibre in the outer layers of the grain, the intake of whole vs. refined grain is emphasized for optimum health benefits.

## References

- American Association of Cereal Chemists (1999) Definition of whole grain; [aaccnet.org/definitions/wholegrain.asp](http://aaccnet.org/definitions/wholegrain.asp).
- Angelo, A.J. (1996) Lipid oxidation on foods. *Critical Reviews in Food Science and Nutrition* 36(3), pp. 175-224
- Anjum, F.M., Butt, M.S., Ahmad, N., and Ahmad, I. (2002) Phytate and mineral content in different milling fractions of some Pakistani spring wheats. *International Journal of Food Sciences and Technology* 37, pp. 13-17
- Bach Knudsen, K.E., Johansen, H.N., and Glitsø, V. (1997) Rye Dietary Fiber and Fermentation in the Colon. *Cereal Foods World* 42(8), pp. 690-693
- Ball, G.F.M. (2004) Vitamin E. In *VITAMINS: Their Role in the Human Body*, Ed G.F.M. Ball, Blackwell Publishing Ltd, London, pp. 234-252
- Barnes, P.J. (1983) Non-saponifiable Lipids in Cereals. In *Lipids in Cereal Technology*, Ed P.J. Barnes, Academic Press Inc., London, pp. 33-53
- Bartnik, M., Jakubczyk, T., and Wrzeciono, A. (1987) Phytase activity in fermentative microflora of dough. *Acta Alimentaria Polonica* 13(1), pp. 67-73
- Bass, E.J. (1988) Wheat flour milling. In *Wheat: Chemistry and Technology*, Ed Y. Pomeranz, AACC, Inc., St. Paul, USA, pp. 1-68
- Bechmann, I.E., Pedersen, H.T., Nørgaard, L., and Engelsen, S.B. (1999) Comparative chemometric analysis of transverse low-field  $^1\text{H}$  NMR relaxation data. In *Advances in Magnetic Resonance in Food Science*, Eds. P.S. Belton, B.P. Hills, and G.A. Webb, The Royal Society of Chemistry, Cambridge, UK, pp. 217-225

- Belitz,H.-D., Grosch,W., and Schieberle,P. (2004) Lipids. In *Food Chemistry*, Eds. H.-D.Belitz, W.Grosch, and P.Schieberle, Springer-Verlag, Berlin, pp. 157-244
- Bender,D.A. (2003) The Vitamins. In *Nutritional Biochemistry of the Vitamins*, Ed D.A.Bender, Cambridge University Press, London, pp. 3-29
- Brugger,R., Simões Nunes,C., Hug,D., Vogel,K., Guggenbuhl,P., Mascarello,F., Augem,S., Wyss,M., van Loon,A.P.G.M., and Pasamontes,L. (2004) Characteristics of fungal phytases from *Aspergillus fumigatus* and *Sartorya fumigata*. *Applied Microbiology and Biotechnology* 63, pp. 383-389
- Brune,M., Rossander-Hultén,L., Hallberg,L., Glerup,A., and Sandberg,A.-S. (1992) Iron Absorption from Bread in Humans: Inhibiting Effects of Cereal Fiber, Phytate and Inositol Phosphates with Different Numbers of Phosphate Groups. *Journal of Nutrition* 122, pp. 442-449
- Bryngelsson,S., Dimberg,L.H., and Kamal-Eldin,A. (2002) Effects of commercial processing on levels of antioxidants in oats (*Avena sativa* L.). *Journal of Agricultural and Food Chemistry* 50(7), pp. 1890-1896
- Bustamante-Rangel,M., Delgado-Zamarreño,M.M., Sánchez-Pérez,A., and Carabias-Martínez,R. (2007) Determination of tocopherols and tocotrienols in cereals by pressurized liquid extraction-liquid chromatography-mass spectrometry. *Analytica Chimica Acta* 587, pp. 216-221
- Camire,A.L. and Clydesdale,F.M. (1982) Analysis of Phytic Acid in Foods by HPLC. *Journal of Food Science* 47, pp. 575-578
- Carlsson,N.-G., Bergman,E.-L., Skoglund,E., Hasselblad,K., and Sandberg,A.-S. (2001) Rapid Analysis of Inositol Phosphates. *Journal of Agricultural and Food Chemistry* 49, pp. 1695-1701
- Casey,A. and Walsh,G. (2004) Identification and characterization of a phytase of potential commercial interest. *Journal of Biotechnology* 110, pp. 313-322

Centeno,C., Viveros,A., Brenes,A., Canales,R., Lozano,A., and de la Cuadra,C. (2001) Effect of Several Germination Conditions on Total P, Phytate P, Phytase, and Acid Phosphatase Activities and Inositol Phosphate Esters in Rye and Barley. *Journal of Agricultural and Food Chemistry* 49, pp. 3208-3215

Chen,M.-H. and Bergman,C.J. (2005) A rapid procedure for analysing rice bran tocopherol, tocotrienol and  $\gamma$ -oryzanol contents. *Journal of Composition and Analysis* 18, pp. 139-151

Chen,Q.C. (2004) Determination of Phytic Acid and Inositol Pentakisphosphates in Foods by High-Performance Ion Chromatography. *Journal of Agricultural and Food Chemistry* 52(15), pp. 4604-4613

Cho,J.S., Lee,C.W., Kang,S.H., Lee,J.C., Bok,J.D., Moon,Y.S., Lee,H.G., Kim,S.C., and Choi,Y.J. (2003) Purification and Characterization of a Phytase from *Pseudomonas syringae* MOK1. *Current Microbiology* 47, pp. 290-294

Chung,O.K. (1991) Cereal lipids. In *Handbook of cereal science and technology*, Eds. K.J.Lorenz and K.Kulp, Marcel Dekker, Inc., New York, pp. 497-554

Conkerton,E.J., Wan,P.J., and Richard,O.A. (1995) Hexane and Heptane as Extraction Solvents for Cottonseed: A Laboratory-Scale Study. *Journal of the American Oil Chemists' Society* 72(8), pp. 963-965

Courtin,C.M. and Delcour,J.A. (2002) Arabinoxylans and Endoxylanases in Wheat Flour Bread-making. *Journal of Cereal Science* 35, pp. 225-243

De Angelis,M., Gallo,G., Corbo,M.R., McSweeney,P.L.H., Faccia,M., Giovine,M., and Gobbetti,M. (2003) Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus enfranchises* CD. *International Journal of Food Microbiology* 2723, pp. 1-12

De Greyt,W.F., Petrauskaite,V., Kellens,M.J., and Huyghebaert,A.D. (1998) Analysis of tocopherols by gas-liquid and high-performance liquid chromatography: a comparative study. *Fett/Lipid* 11, pp. 503-507

Dexter,J.E. and Wood,P.J. (1996) Recent applications of debranning of wheat before milling. *Trends in Food Science & Technology* 7, pp. 35-41

- Edwards,M.A., Osborne,B.G., and Henry,R.J. (2007) Investigation of the effect of conditioning on the fracture of hard and soft wheat grain by single-kernel characterization system: A comparison with roller milling. *Journal of Cereal Science* 46, pp. 64-74
- Eeckhout,W. and De Paepe,M. (1994) Total Phosphorus, phytate-phosphorus and phytase activity in plant feedstuffs. *Animal Feed Science and Technology* 47, pp. 19-29
- Eitenmiller,R. and Lee,J. (2004a) Food Composition - Vitamin E. In *Vitamin E: Food Chemistry, Composition, and Analysis*, Eds. R.Eitenmiller and J.Lee, Marcel Dekker, Inc., New York, pp. 425-505
- Eitenmiller,R. and Lee,J. (2004b) Nutrition and Health Implications of Vitamin E. In *Vitamin E: Food Chemistry, Composition, and Analysis*, Eds. R.Eitenmiller and J.Lee, Marcel 2, Inc., New York, pp. 39-88
- Eitenmiller,R. and Lee,J. (2004c) Vitamin E: Chemistry and Biochemistry. In *Vitamin E: Food Chemistry, Composition, and Analysis*, Eds. R.Eitenmiller and J.Lee, Marcel Dekker, Inc., New York, pp. 1-38
- Eliasson,A. and Larsson,K. (1993). Cereals in Breadmaking - a molecular collidal approach. Marcel Dekker, Inc.,
- Engelen,A.J., van der Heeft,F.C., Randsdorp,P.H.G., and Smit,E.L.C. (1994) Simple and Rapid Determination of Phytase Activity. *Journal of AOAC International* 77(3), pp. 760-764
- Fødevaredirektoratet (1999) Danskernes kostvaner 1995; foedevarestyrelsen.dk/fdir/publications/2000006/Rapport1.asp.
- Fødevaredirektoratet (2002) Danskernes kostvaner 2000-2001; foedevarestyrelsen.dk/FDir/Publications/2002010/Rapport.pdf.
- Franke,A.A., Murphy,S.P., Lacey,R., and Custer,L.J. (2007) Tocopherol and Tocotrienol Levels of Foods Consumed in Hawaii. *Journal of Agricultural and Food Chemistry* 55, pp. 769-778
- Frankel,E.N. (2005). Lipid Oxidation., Ed. The Oily Press Lipid Technology,

- Fretzdorff, B. and Brümmer, J.-M. (1992) Reduction of Phytic Acid During Breadmaking of Whole-Meal Breads. *Cereal Chemistry* 69(3), pp. 266-270
- Fretzdorff, B. and Weipert, D. (1986) Phytinsäure in Getreide und Getreideerzeugnissen. *Zeitschrift fuer Lebensmittel-Untersuchung und -Forschung* 182, pp. 287-293
- Fritsch, C.W. and Gale, J.A. (1977) Hexanal as a Measure of Rancidity in Low Fat Foods. *Journal of American Oil Chemists Society* 54, pp. 225-228
- Frølich, W., Drakenberg, T., and Asp, N.-G. (1986) Enzymic Degradation of Phytate (*myo*-inositol Hexaphosphate) in Whole Grain Flour Suspension and Dough. A Comparison between <sup>31</sup>P NMR Spectroscopy and a Ferric Ion Method. *Journal of Cereal Science* 4, pp. 325-334
- Frølich, W., Wahlgren, M., and Drakenberg, T. (1988) Studies on Phytase Activity in Oats and Wheat using <sup>31</sup>P-NMR spectroscopy. *Journal of Cereal Science* 8, pp. 47-53
- Fukuba, H. and Murota, T. (1985) Determination of Tocopherols in Foodstuffs, Especially Nuts and Spices, by High-Performance Liquid Chromatography. *Journal of Micronutrient Analysis* 1, pp. 93-105
- Galliard, T. (1983) Enzymatic Degradation of Cereal lipids. In *Lipids in Cereal Technology*, Ed P.J.Barnes, Academic Press, New York, pp. 111-147
- Galliard, T. (1986) Hydrolytic and Oxidative Degradation of Lipids During Storage of Wholemeal Flour: Effects of Bran and Germ Components. *Journal of Cereal Science* 4, pp. 179-192
- García-Estapa, R.M., Guerra-Hernández, E., and García-Villanova, B. (1999) Phytic acid content in milled cereal products and breads. *Food Research International* 32, pp. 217-221
- Gökman, V., Serpen, A., Atli, A., and Köksel, H. (2007) A Practical Spectrophotometric Approach for the Determination of Lipoxxygenase Activity of Durum Wheat. *Cereal Chemistry* 84(3), pp. 290-293
- Graf, E. and Dintzis, F.R. (1982) High-Performance Liquid Chromatographic Method for the Determination of Phytate. *Analytical Biochemistry* 119, pp. 413-417



- Graf,E. and Eaton,J.W. (1990) Antioxidant functions of phytic acid. *Free Radical Biology and Medicine* 8, pp. 61-69
- Greffeuille,V., Abecassis,J., Bar L'Helgouac'h,C., and Lullien-Pellerin,V. (2005) Differences in the Aleurone Layer Fate Between Hard and Soft Common Wheats at Grain Milling. *Cereal Chemistry* 82(2), pp. 138-143
- Greiner,R. and Egli,I. (2003) Determination of the activity of acidic phytate-degrading enzymes in cereal seeds. *Journal of Agricultural and Food Chemistry* 51(4), pp. 847-850
- Greiner,R., Jany,K.-D., and Almingier,M.L. (2000) Identification and Properties of myo-Inositol Hexakisphosphate Phosphohydrolases (Phytases) from Barley (*Hordeum vulgare*). *Journal of Cereal Science* 31, pp. 127-139
- Greiner,R., Konietzny,U., and Jany,K.-D. (1998) Purification and properties of a phytase from rye. *Journal of Food Biochemistry* 22, pp. 143-161
- Grela,E.R. (1996) Nutrient Composition and Content of Antinutritional Factors in Spelt (*Triticum spelta* L) Cultivars. *Journal of the Science of Food and Agriculture* 71, pp. 399-404
- Håkansson,B., Jägerstad,M., and Öste,R. (1987) Determination of Vitamin E in Wheat Products by HPLC. *Journal of Micronutrient Analysis* 3, pp. 307-318
- Hansen,H.B., Andreasen,M.F., Nielsen,M.M., Larsen,L.M., Knudsen,K.E.B., Meyer,A.S., Christensen,L.P., and Hansen,Å. (2002) Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread-making. *European Food Research and Technology* 214, pp. 33-42
- Hansen,H.B., Møller,B., Andersen,S.B., Jørgensen,J.R., and Hansen,Å. (2004) Grain Characteristics, Chemical Composition, and Functional Properties of Rye (*Secale cereale* L.) As Influenced by Genotype and Harvest Year. *Journal of Agricultural and Food Chemistry* 52(8), pp. 2282-2291
- Hansen,H.B., Rasmussen,C.V., Knudsen,K.E.B., and Hansen,Å. (2003) Effects of genotype and harvest year on the content and composition of dietary fibre in rye (*Secale cereale* L.) grain. *Journal of the Science of Food and Agriculture* 81, pp. 1-10

- Hatzack,F., Hübel,F., Zhang,W., Hansen,P.E., and Rasmussen,S.K. (2001) Inositol phosphates from barley low-phytate grain mutants analysed by metal-dye detection HPLC and NMR. *Biochemical Journal* 354, pp. 473-480
- Heinonen,J.K. and Lahti,R.J. (1981) A New and Convenient Colorimetric Determination of Inorganic Orthophosphate and Its Application to the Assay of Inorganic Pyrophosphatase. *Analytical Biochemistry* 113, pp. 313-317
- Hemery,Y., Rouau,X., Lullien-Pellerin,V., Barron,C., and Abecassis,J. (2007) Dry processes to develop wheat fractions and products with enhanced nutritional quality. *Journal of Cereal Science* 46, pp. 327-347
- Hoseney,R.C. (1994a) Dry milling. In *Principles of cereal science and technology*, Ed R.C.Hoseney, AACC, Inc., St. Paul, USA, pp. 125-146
- Hoseney,R.C. (1994b) Minor constituents. In *Principles of cereal science and technology*, Ed R.C.Hoseney, St. Paul, USA, pp. 81-102
- Hoseney,R.C. (1994c) Structure of Cereals. In *Principles of cereal science and technology*, Ed R.C.Hoseney, AACC, Inc., St. Paul, USA, pp. 1-28
- Hotelling,H. (1933) Analysis of a complex of statistical variables into principal components. *Journal of Educational Psychology* 24, pp. 417-441
- Hrusková,M., Švec,I., and Jirsa,O. (2006) Correlation between milling and baking parameters of wheat varieties. *Journal of Food Engineering* 77, pp. 439-444
- Jacob,H.E. (1997). Six thousand years of bread - Its holy and unholy history. The Lyons Press
- Jensen,P.N. and Risbo,J. (2007) Oxidative stability of snack and cereal products in relation to moisture sorption. *Food Chemistry* 103, pp. 717-724
- Jespersen,B.M. and Munck,L. (2008) From prediction of individual analytes to identification of genes and functional factors for food in cereal science and technology. In *IR Spectroscopy in Foods*, Ed D.-V.Sun, Elsevier
- Kamal-Eldin,A. and Appelqvist,L.-Å. (1996) The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* 31(7), pp. 671-701

- Katz,S.H. and Weaver,W.W. (2003). Encyclopedia of food and culture. Charles Scribner's Sons
- Kemme,P.A., Lommen,A., Jongbloed,A.W., Mroz,Z., and Beynen,A.C. (1999) Quantification of Inositol Phosphates Using  $^{31}\text{P}$  Nuclear Magnetic Resonance Spectroscopy in Animal Nutrition. *Journal of Agricultural and Food Chemistry* 47, pp. 5116-5121
- Kent,N.L. and Evers,A.D. (1994) Botanical aspects of cereals. In *Technology of cereals*, Eds. N.L.Kent and A.D.Evers, Pergamon, pp. 29-52
- Kline,K., Yu,W., and Sanders,B.G. (2004) Vitamin E and Breast Cancer. *Journal of Nutrition*
- Ko,S.-N., Kim,C.-J., Kim,H., Kim,C.-T., Chung,S.-H., Tae,B.-S., and Kim,I.-H. (2003) Tocol Levels in Milling Fractions of Some Cereal Grains and Soybean. *Journal of the American Oil Chemists' Society* 80(6), pp. 585-589
- Lásztity,R. and Lásztity,L. (1990) Phytic Acid in Cereal Technology. In *Advances in Cereal Science and Technology*, Ed Y.Pomeranz, American Association of Cereal Chemists, St. Paul, Minnesota, USA, pp. 309-372
- Lee,Y.-C., Oh,S.-W., Chang,J., and Kim,I.-H. (2004) Chemical composition and oxidative stability of safflower oil prepared from safflower seed roasted with different temperatures. *Food Chemistry* 84, pp. 1-6
- Leenhardt,F., Levrat-Verny,M.-A., Chanliaud,E., and Rémésy,C. (2005) Moderate Decrease of pH by Sourdough Fermentation Is Sufficient To Reduce Phytate Content of Whole Wheat Flour through Endogenous Phytase Activity. *Journal of Agricultural and Food Chemistry* 53, pp. 98-102
- Liyana-Pathirana,C.M. and Shahidi,F. (2007) Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chemistry* 101, pp. 1151-1157
- Lolas,G.M. and Markakis,P. (1977) The phytase of navy beans (*Phaseolus vulgaris*). *Journal of Food Science* 42(4), pp. 1094-1106
- Lorentz,K. (2000) Rye. In *Handbook of Cereal Science and Technology*, Eds. K.Kulp and J.G.Ponte, Marcel Dekker, Inc., New York, pp. 223-256

Michalska,A., Ceglinska,A., Amarowicz,R., Piskula,M.K., Szawara-Nowak,D., and Zielinski,H. (2007a) Antioxidant Contents and Antioxidative Properties of Traditional Rye Breads. *Journal of Agricultural and Food Chemistry* 55, pp. 734-740

Michalska,A., Ceglinska,A., and Zielinski,H. (2007b) Bioactive compounds in rye flours with different extractions rates. *European Food Research and Technology* 225, pp. 545-551

Micklander,E., Thygesen,L.G., Pedersen,H.T., van den Berg,F., Bro,R., and Engelsen,S.B. (2003) Multivariate analysis of time domain NMR signals in relation to food quality. In *Magnetic Resonance in Food Science: Latest Developments* , Eds. G.A.Webb, P.S.Belton, and D.N.Rutledge, The Royal Society of Chemistry, pp. 239-254

Munck,L. (2008) Breeding for Quality Traits in Cereals - a Revised Outlook on Old and New Tools for Integrated Breeding. In *Cereals*, Ed M.J.Carena, Springer Publisher, New York

Munck,L., Norgaard,L., Engelsen,S.B., Bro,R., and Andersson,C.A. (1998) Chemometrics in food science - a demonstration of the feasibility of a highly exploratory, inductive evaluation strategy of fundamental scientific significance. *Chemometrics and Intelligent Laboratory Systems* 44(1-2), pp. 31-60

Murkovic,M., Hillebrand,A., and Winkler,J. (1996) Variability of vitamin E content in pumpkin seeds (*Cucurbita pepo* L.). *Zeitschrift fuer Lebensmittel-Untersuchung und -Forschung* 202, pp. 275-278

Murkovic,M., Piironen,V., Lampi,A.M., Kraushofer,T., and Sontag,G. (2004) Changes in chemical composition of pumpkin seeds during the roasting process for production of pumpkin seed oil (Part 1: non-volatile compounds). *Food Chemistry* 84(3), pp. 359-365

Nakamura,Y., Fukuhara,H., and Sano,K. (2000) Secreted Phytase Activities of Yeasts. *Bioscience, Biotechnology, and Biochemistry* 64(4), pp. 841-844

Nakano,T., Joh,T., Narita,K., and Hayakawa,T. (2000) The Pathway of Dephosphorylation of *myo*-Inositol Hexakisphosphate by Phytases from Wheat Bran of *Triticum aestivum* L. cv. Nourin nr. 61. *Bioscience, Biotechnology, and Biochemistry* 64(5), pp. 995-1003

- Nawar,W.W. (1996) Lipids. In *Food Chemistry*, Ed O.R.Fennema, Marcel Dekker, Inc., New York, pp. 225-320
- Nayini,N.R. and Markakis,P. (1983) Effect of Fermentation Time on the Inositol Phosphates of Bread. *Journal of Food Science* 48, pp. 262-263
- Nayini,N.R. and Markakis,P. (1984) The Phytase of Yeast. *Lebensmittel Wissenschaft und Technologie* 17, pp. 24-26
- Nesaretnem,K., Guthrie,N., Chambers,A.F., and Carroll,K.K. (1995) Effect of Tocotrienols on the Growth of a Human Breast Cancer Cell Line in Culture. *Lipids* 30(12), pp. 1139-1143
- Nesaretnem,K., Yew,W.W., and Wahid,M.B. (2007) Tocotrienols and cancer: Beyond antioxidant activity. *European Journal of Lipid Science and Technology* 109(4), pp. 445-452
- Niki,E., Yamamoto,Y., Komuro,E., and Sato,K. (1991) Membrane damage due to lipid oxidation. *American Journal of Clinical Nutrition* 53, pp. 201S-205S
- Nørgaard,L., Hahn,M.T., Knudsen,L.B., Farhat,I.A., and Engelsen,S.B. (2005) Multivariate near-infrared and Raman spectroscopic quantifications of the crystallinity of lactose in whey permeate powder. *International Dairy Journal* 15, pp. 1261-1270
- Nyström,L., Paasonen,A., Lampi,A.-M., and Piironen,V. (2007) Total plant sterols, steryl ferulates and steryl glycosides in milling fractions of wheat and rye. *Journal of Cereal Science* 45, pp. 106-115
- Pallauf,J. and Rimbach,G. (1997) Nutritional significance of phytic acid and phytase. *Archives of Animal Nutrition* 50, pp. 301-319
- Pandey,A., Szakacs,G., Soccol,C.R., Rodriguez-Leon,J.A., and Soccol,V.T. (2001) Production, purification and properties of microbial phytases. *Bioresource Technology* 77(203), pp. 214
- Panfili,G., Fratianni,A., and Irano,M. (2003) Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. *Journal of Agricultural and Food Chemistry* 51(14), pp. 3940-3944

- Panfili,G., Manzi,P., and Pizzoferrato,L. (1994) High-Performance Liquid Chromatographic Method for the Simultaneous Determination of Tocopherols, Carotenes, and Retinol and its Geometric Isomers in Italian Cheeses. *Analyst* 119, pp. 1161-1165
- Peers,F.G. (1957) The Phytase of Wheat. *Biochemical Journal* 53, pp. 102-110
- Persson,H., Nyman,M., Liljeberg,H., and Önning,G. (1991) Binding of Mineral Elements by Dietary Fibre Components in Cereals - *In vitro* (III). *Food Chemistry* 40, pp. 169-183
- Peterson,D.M. and Qureshi,A.A. (1993) Genotype and Environment Effects on Tocols of Barley and Oats. *Cereal Chemistry* 70(2), pp. 157-162
- Phillippy,B.Q. (1989) Identification by Two-Dimensional NMR of *myo*-Inositol Tris- and Tetrakis(phosphates) Formed from Phytic Acid by Wheat Phytase. *Journal of Agricultural and Food Chemistry* 37, pp. 1261-1265
- Phillippy,B.Q. and Graf,E. (1997) Antioxidant functions of inositol 1,2,3-trisphosphate and inositol 1,2,3,6-tetrakisphosphate. *Free Radical Biology and Medicine* 22(6), pp. 939-946
- Piironen,V., Syvaöja,E.L., Varo,P., Salminen,K., and Koivistoinen,P. (1986) Tocopherols and Tocotrienols in Cereal Products from Finland. *Cereal Chemistry* 63(2), pp. 78-81
- Posner,E.S. (2005) The Grinding Process. In *Wheat Flour Milling*, Eds. E.S.Posner and A.N.Hibbs, AACC, Inc., St. Paul, USA, pp. 185-221
- Ramadan,M.F. and Morsel,J.-T. (2002) Direct isocratic normal-phase HPLC assay of fat-soluble vitamins and beta-carotene in oilseeds. *European Food Research and Technology* 214, pp. 521-527
- Rani,K.U., Rao,U.J.S.P., Leelavathi,K., and Rao,P.H. (2001) Distribution of enzymes in wheat flour mill streams. *Journal of Cereal Science* 34(3), pp. 233-242
- Ravindran,V., Selle,P.H., and Bryden,W.L. (1999) Effects of Phytase Supplementation, Individually and in Combination with Glycanase, on the Nutritive Value of Wheat and Barley. *Poultry Science* 78, pp. 1588-1595

- Reale,A., Mannina,L., Tremonte,P., Sobolev,A.P., Succi,M., Sorrentino,E., and Coppola,R. (2004) Phytate Degradation by Lactic Acid Bacteria and Yeasts during the Wholemeal Dough Fermentation: a <sup>31</sup>P NMR Study. *Journal of Agricultural and Food Chemistry* 52, pp. 6300-6305
- Reddy,N.R. (2002) Occurrence, Distribution, Content, and Dietary Intake of Phytate. In *Food Phytates*, Eds. N.R.Reddy and S.K.Sathe, CRC Press, Boca Raton, pp. 25-51
- Rodrigo,N., Alegria,A., Barbera,R., and Farre,R. (2002) High-performance liquid chromatographic determinaton of tocopherols in infant formulas. *Journal of Chromatography A* 947, pp. 97-102
- Salovaara,H. and Autio,K. (2001) Rye and Triticale. In *Cereals and Cereal Products*, Eds. D.A.V.Dendy and G.J.Dobraszczyk, Aspen Publishers, Inc., Maryland, USA
- Sandberg,A.-S. and Ahderinne,R. (1986) HPLC Method for Determination of Inositol Tri-, Tetra-, Penta-, and Hexaphosphates in Foods and Intestinal Contents. *Journal of Food Science* 51(3), pp. 547-550
- Sandberg,A.-S., Brune,M., Carlsson,N.-G., Hallberg,L., Skoglund,E., and Rossander-Hultén,L. (1999) Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *American Journal of Clinical Nutrition* 70, pp. 240-246
- Sandberg,A.-S., Carlsson,N.-G., and Svanberg,U. (1989) Effects of Inositol Tri-, Tetra-, Penta-, and Hexaphosphates on *In vitro* Estimation of Iron Availability. *Journal of Food Science* 54(1), pp. 159-161
- Sandberg,A.-S., Rossander-Hultén,L., and Türk,M. (1996) Dietary *Aspergillus niger* Phytase Increases Iron Absorption in Humans. *Journal of Nutrition* 126, pp. 476-480
- Sen,C.K., Khanna,S., Rink,C., and Roy,S. (2007) Tocotrienols: The emerging face of natural vitamin E. *Vitamins and Hormones* 76, pp. 203-261
- Sen,C.K., Khanna,S., and Roy,S. (2006) Tocotrienols: Vitamin E beyond tocopherols. *Life Sciences* 78, pp. 2088-2098

- Shamsuddin,A.M. (1995) Inositol Phosphates Have Novel Anticancer Function. *Journal of Nutrition* 125, pp. 725S-732S
- Shamsuddin,A.M. (2002) Anti-cancer function of phytic acid. *International Journal of Food Sciences and Technology* 37, pp. 769-782
- Shen,C.-S.J. and Sheppard,A.J. (1986) A Rapid High-Performance Liquid Chromatographic Method for Separating Tocopherols. *Journal of Micronutrient Analysis* 2, pp. 43-53
- Sheppard,A.J., Pennington,J.A.T., and Weihrauch,J.L. (1992) Analysis and Distribution of Vitamin E in Vegetable Oils and Foods. In *Vitamin E in Health and Disease*, Eds. L.Packer and J.Fuchs, Marcel Dekker, Inc., New York, pp. 9-31
- Shewry,P.R. and Bechtel,D.B. (2001) Morphology and chemistry of the rye grain. In *Rye: Production, Chemistry, and Technology*, Ed W.Bushuk, AACC, Inc., St. Paul, USA, pp. 69-128
- Siener,R., Heynck,H., and Hesse,A. (2001) Calcium-Binding Capacities of Different Brans under Simulated Gastrointestinal pH Conditions. In Vitro Study with <sup>45</sup>Ca. *Journal of Agricultural and Food Chemistry* 49, pp. 4397-4401
- Skoglund,E., Carlsson,N.-G., and Sandberg,A.-S. (1997) Analysis of Inositol Mono- and Diphosphate Isomers Using High-Performance Ion Chromatography and Pulsed Amperometric Detection. *Journal of Agricultural and Food Chemistry* 45, pp. 4668-4673
- Slavin,J., Jacobs,D., and Marquart,L. (1997) Whole-Grain Consumption and Chronic Disease: Protective Mechanisms. *Nutrition and Cancer* 27(1), pp. 14-21
- Slots,T., Skibsted,L.H., and Nielsen,J.H. (2007) The difference in transfer of *all-rac-α*-tocopherol stereo-isomers to milk from cows and the effect on its oxidative stability. *International Dairy Journal* 17, pp. 737-745
- Soares,A.L., Olivo,R., Shimokomaki,M., and Ida,E.I. (2004) Synergism between Dietary Vitamin E and Exogenous Phytic Acid in Prevention of Warmed-Over Flavour Development in Chicken Breast Meat, *Pectoralis major* M. *Brazilian Archives of Biology and Technology* 47(1), pp. 57-62



- Stauffer,C.E. (1989) Ester Hydrolases. In *Enzyme assays for food scientists*, Ed C.E.Stauffer, AVI BOOK, New York, USA, pp. 187-201
- Sylvester,P.W. (2007) Vitamin E and apoptosis. *Vitamins and Hormones* 76, pp. 329-356
- Tangkongchitr,U., Seib,P.A., and Hosney,R.C. (1982) Phytic Acid. III. Two Barriers to the Loss of Phytate During Breadmaking. *Cereal Chemistry* 59(3), pp. 216-221
- Tauler,R. (1995) Multivariate curve resolution applied to second order data. *Chemometrics and Intelligent Laboratory Systems* 30(1), pp. 133-146
- Thompson,J.N. and Hatina,G. (1979) Determination of Tocopherols and Tocotrienols in Foods and Tissues by High-Performance Liquid-Chromatography. *Journal of Liquid Chromatography* 2(3), pp. 327-344
- Türk, M. (1999) Cereal- and Microbial Phytases. Phytate Degradation, Mineral Binding and Absorption. Chalmers University of Technology
- Türk,M., Carlsson,N.-G., and Sandberg,A.-S. (1996) Reduction in the Level of Phytate During Wholemeal Bread Making; Effect of Yeast and Wheat Phytases. *Journal of Cereal Science* 23, pp. 257-264
- Türk,M., Sandberg,A.-S., Carlsson,N.-G., and Andlid,T. (2000) Inositol Hexaphosphate Hydrolysis by Baker's Yeast. Capacity, Kinetics, and Degradation Products. *Journal of Agricultural and Food Chemistry* 48, pp. 100-104
- Turnbull,K.-M. and Rahman,S. (2002) Endosperm Texture in Wheat. *Journal of Cereal Science* 36, pp. 327-337
- Ullrich,F. and Grosch,W. (1987) Identification of the most intense volatile flavour compounds formed during autoxidation of linoleic acid. *Zeitung für Lebensmittel Untersuchung und Forschung* 184, pp. 277-282
- Valsemøllen A/S (1999). Brød i titusinde år. Erhvervshistorisk Bureau
- Verwimp,T., Vandeputte,G.E., Marrant,K., and Delcour,J.A. (2004) Isolation and characterisation of rye starch. *Journal of Cereal Science* 39, pp. 85-90

- Vieira,E.C. and Nogueira,A.R.A. (2004) Orthophosphate, Phytate, and Total Phosphorus Determination in Cereals by Flow Injection Analysis. *Journal of Agricultural and Food Chemistry* 52(7), pp. 1800-1803
- Viereck,N., Dyrby,M., and Engelsen,S.B. (2005a) Monitoring Thermal Processes by NMR Technology. In *Emerging Technologies for Food Processing*, Ed D.-V.Sun, Elsevier Science / Academic Press, London, pp. 553-575
- Viereck,N., Seefeldt,H.F., Nielsen,M.M., and Engelsen,S.B. (2005b) HR MAS NMR-spektroskopi: En hel ny verden for fødevareforskningen. *Dansk Kemi* 86(11), pp. 1-8
- Vinkx,C.J.A. and Delcour,J.A. (1996) Rye (*Secale cereale* L.) Arabinoxylans: A critical Review. *Journal of Cereal Science* 24, pp. 1-14
- Vucenik,I. and Shamsuddin,A.M. (2003) Cancer Inhibition by Inositol Hexaphosphate (IP6) and Inositol: From Laboratory to Clinic. *Journal of Nutrition* 133, pp. 3778S-3784S
- Wagner,K.-H., Wotruba,F., and Elmadfa,I. (2001) Antioxidative potential of tocotrienols and tocopherols in coconut fat at different oxidation temperatures. *European Journal of Lipid Science and Technology* 103, pp. 746-751
- Wang,L., Xue,Q., Newman,R.K., and Newman,C.W. (1993) Enrichment of Tocopherols, Tocotrienols, and Oil in Barley Fractions by Milling and Pearling. *Cereal Chemistry* 70(5), pp. 499-501
- Weaver,C.M. and Kannan,S. (2002) Phytate and Mineral Bioavailability. In *Food Phytates*, Eds. N.R.Reddy and S.K.Sathe, CRC Press, pp. 211-223
- Wennermark,B. and Jagerstad,M. (1992) Breadmaking and Storage of Various Wheat Fractions Affect Vitamin E. *Journal of Food Science* 57(5), pp. 1205-1209
- Winning,H., Larsen,F.H., Bro,R., and Engelsen,S.B. (2007) Quantitative analysis of NMR spectra with chemometrics. *Journal of Magnetic Resonance* 190, pp. 153-159
- www.healthgrain.org (2007) HealthGrain

Yamauchi,R. (2007) Addition products of  $\alpha$ -tocopherol with lipid-derived free radicals. *Vitamins and Hormones* 76, pp. 309-329

Zielinski,H., Ceglinska,A., and Michalska,A. (2007) Antioxidant contents and properties as quality indices of rye cultivars. *Food Chemistry* 104, pp. 980-988

Zielinski,H., Kozłowska,H., and Lewczuk,B. (2001) Bioactive compounds in the cereal grains before and after hydrothermal processing. *Innovative Food Science & Emerging Technologies* 2, pp. 159-169