



## **Aroma interactions with starch**

Induction of carbohydrate acting enzymes from *Aspergillus nidulans*

**Jørgensen, Anders Dysted**

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”Aroma interactions with starch”  
&  
“Induction of carbohydrate acting enzymes from *Aspergillus  
nidulans*”

Ph.D. Thesis

2011

**Anders Jørgensen**

*Enzyme and Protein Chemistry, Department of Systems Biology,  
Technical University of Denmark*

&

*Lyckeby Culinar AB*

Supervisors:

Professor Birte Svensson (DTU)

Associate Professor Andreas Blennow (KU-LIFE)

Industrial contacts:

Jan-Olof Lundberg

Susanne Rask

10 µm



Cover image:  
SEM image of maize starch granules with citral (image taken by Susanne Langgård Jensen)



## Preface

The present PhD thesis with the title “Aroma interactions with starch” & “Induction of carbohydrate acting enzymes from *Aspergillus nidulans*” represents the work of my PhD-study carried out under the supervision of Professor Birte Svensson (Enzyme and Protein Chemistry, Institute for Systembiology, Technical University of Denmark) and Associate Professor Andreas Blennow (Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen (KU-LIFE)) at the Enzyme and Protein Chemistry (EPC), Institute of Systems Biology, Technical University of Denmark (DTU). The project was funded by DTU, the PhD school LMC FOOD, and the industrial collaborator Lyckeby Stärkelsen, represented by flavourist Jan-Olof Lundberg and B.Sc. Susanne Rask.

The Ph.D. project has resulted in the following manuscript (see Appendix):

Jørgensen, A.D., Jensen, S.L., Buléon, A., Svensson, B., Blennow, A., 2011. Structural and physical effects of aroma binding to native starch granules. To be submitted to *Starch/Stärke*.

Furthermore during the Ph.D. study a manuscript involving the proteome technologies used in the second part of the Ph.D. study has been published:

Yang, F., Jørgensen, A.D., Li, H., Søndergaard, I., Finnie, C., Svensson, B., Jiang, D., Wollenweber, B., Jacobsen, S., 2011. Implications of high-temperature events and water deficits on protein profiles in wheat (*Triticum aestivum* L. cv. Vinjett) grain. *Proteomics* 11, 1684-1695

Kgs. Lyngby August 11<sup>th</sup> 2011

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Anders Jørgensen

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I would also like to thank the following persons for their help during my PhD-study:

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Alain Buléon for help with planning the DSC and X-ray experiments, his insightful interpretation of data, and interesting discussions.

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The PhD-school LMC FOOD for co-funding the PhD-project.

Anne Knudsen, Birgit Andersen, and Marie Møller, for listening to my problems and giving me support when I needed it most. And last, but definitely not least, all the staff of the EPC, past and present, for providing a wonderful atmosphere to work in. I will miss you guys.

I want to thank my family and friends. Without their continued interest, love, and support, throughout these hard times, this thesis would not exist.

## Summary

Starches are used to enhance aroma perception in low-fat foods. Aroma compounds can bind physically to the starch in grooves on the surface or they can form complexes inside amylose helices. This study has been divided into two parts: one part regarding binding of aromas to starches and their aroma-release, and another part regarding stimulation of a fungal secretome using different carbohydrates.

In the first part, nine aromas and one aroma-mixture were mixed with nine different starches, including genetically modified starches. The objective of this sub-project was to bind aromas to the starches to 15 weight-percent. Aroma binding was tested on both amorphous starches and on native starch granules. A series of aldehydes and alcohols were also tested for binding to the starches.

The aromas with the highest volatility were positively retained by starch, whereas for aromas with a lower volatility the starch had a negative effect on retention. No trends were observed that could relate aroma binding or retention to physical qualities of the starch such as amylose or phosphate content. Still, the physical state of the starch was shown to influence retention of some aromas, possibly by diffusion through the outer layers of the starch granule or binding to grooves on the surface. Chemical reactivity of some of the aromas also influenced the retention. Analysing a series of aldehydes and alcohols revealed an influence of the size of the molecules on retention. Hexanal showed a remarkable drop in retention for all starches, and pentanol showed a favoured retention by native starch granules.

The aroma compounds bound to the starch proved very difficult to release as only a minute fraction of the aroma added could be released from the starch, even under conditions favouring aroma release, as monitored by GC-MS and solvent extractions. Addition of water to the extraction lowered the amount of aroma released, and addition of a starch-degrading enzyme,  $\alpha$ -amylase, did not significantly change the amount extracted.

Studies by differential scanning calorimetry and wide-angle X-ray scattering did not show complete complexation of aromas in the amylose helices, but instead changes were observed that could be evidence of partial complexation. This complexation is not exclusive to the amylose helices, but also appears to include interactions in the amylopectin double helices. In particular, one of the analysed aromas showed a very noticeable reduction in melting temperature, but showed only a minor reduction in melting enthalpy and no evidence of amylose complexation.

Using an enzyme-discovery approach in the second sub-project, the industrially relevant fungus *Aspergillus nidulans* was stimulated with different carbohydrates. Stimulation with starch induced expression of starch-degrading enzymes, while stimulation with the hemicellulose xylan induced expression of xylanases. One particular hypothetical protein was ubiquitously expressed. This protein had no apparent homology with known proteins, but may

be involved in attacking other organisms as a weak homology with other proteins involved in membrane attack was detected. Degradation of secreted proteins was observed in some cultures. Identification of a glycoside hydrolase family 61 using xylan as carbon-source was not successful despite previous evidence for the induction of this enzyme.

## Dansk resumé

Stivelse bliver brugt til at fremhæve smagen af aromastoffer i fedtfattige fødevarer. Aromastoffer kan bindes fysisk til stivelsen i forsænkninger i overfladen eller de kan danne komplekser inde i amylosens helixer. Dette studium er blevet opdelt i to dele: en del som omhandler bindingen af aromastoffer til forskellige stivelser og frigivelsen af aromastofferne fra disse stivelser, samt en anden del, som omhandler sekretion fra en filamentøs svamp efter stimulering med forskellige kulstof-kilder.

I den første del blev ni aromastoffer og en aroma-blanding blandet med ni forskellige stivelser, heriblandt genetisk ændrede stivelser. Formålet med dette del-projekt var at binde 15 vægtprocent af aromastofferne til stivelsen. Aromabindingen blev testet på både amorfe stivelser og native stivelseskorn. Bindingen af en henholdsvis en serie alifatiske aldehyder og en serie af alifatiske alkoholer blev ligeledes undersøgt.

De mest flygtige aromastoffer blev positivt tilbageholdt af stivelsen, mens stivelsen havde en negativ effekt på aromastoffer med lavere flygtighed. Der blev ikke observeret sammenhænge mellem aroma-bindingen eller retentionen, som kunne skyldes stivelsens fysiske egenskaber, i denne sammenhæng dens amylose- eller fosfat-indhold. Dog blev det vist, at stivelsens fysiske tilstand påvirkede retentionen af nogle aromaer, muligvis ved diffusion gennem stivelsens yderste lag eller ved binding til huller i stivelsens overflade. Den kemiske reaktivitet af nogle af aromastofferne påvirkede også retentionen. Et bemærkelsesværdigt fald i retention blev observeret for hexanal med alle de afprøvede stivelser, mens pentanol viste størst retention med native stivelseskorn.

GC-MS og solvent ekstraktioner viste at aromastoffer bundet til stivelsen var svære at frigøre, idet kun en lille brøkdel af de tilsatte aromastoffer blev frigivet fra stivelsen, selv under betingelser som er fordelagtige for aromafrigivelse. Tilsætningen af vand til ekstraktionen sænkede mængden af den frigivne aroma, og tilsætning af et stivelsesnedbrydende enzym,  $\alpha$ -amylase, ændrede ikke på mængden af frigivne aromastoffer.

Studier med differentiell skanning kalorimetri og vidvinkel røntgen spredning viste ikke en komplet kompleksdannelse af aromastoffer i amylosehelixerne, men i stedet blev der observeret ændringer, som kunne være bevis for en delvis kompleksdannelse. Denne kompleksdannelse finder ikke udelukkende sted i amylose helixerne, men involverer også vekselvirkninger med amylopektin dobbelthelixerne. I særdeleshed viste et af de analyserede aromastoffer en betydelig sænkning af smeltepunkts-temperaturen, men viste kun en mindre sænkning af smelteenthalpien og intet bevis for amylose-kompleksdannelse.

Ved brug af en *enzyme discovery* tilgang i den anden del af PhD studiet blev den industrielt relevante filamentøse svamp *Aspergillus nidulans* stimuleret med forskellige kulhydrater. Stimuleringen med stivelse inducerede udtryk af stivelsesnedbrydende enzymer, mens stimulering med hemicellulosen xylan inducerede udtryk af xylanaser. Et bestemt hypotetisk



protein blev udtrykt af alle kulturer. Dette protein havde homologi med kendte proteiner, men kan være involveret i svampens angreb på andre organismer, da det viste en svag homologi med andre proteiner involveret i angreb på celle-membraner. Nedbrydning af udskilte proteiner blev observeret i nogle kulturer. På trods af tidligere induktion af dette enzym i laboratoriet, kunne stimulering med xylan som kulstof-kilde ikke påvise biosyntese af en glycosid-hydrolase fra familie 61.

## List of abbreviations

2DE	2-dimensional gel electrophoresis
A.u.	Arbitrary units
BLAST	Basic Local Alignment Search Tool
CAZy	Carbohydrate-Active EnZymes Database
Da	Dalton (basically identical to g/mol)
DSC	Differential Scanning Calorimetry
EPC	Enzyme and Protein Chemistry
GC-MS	Gas Chromatography Mass Spectrometry
GH	Glycoside Hydrolase family
IEF	Iso-Electric Focusing
INRA	French National Institute for Agricultural Research
KU-LIFE	University of Copenhagen, Faculty of Life Sciences
LD <sub>50</sub>	Lethal dose, 50%
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight
MDa	Mega-Dalton/Million Dalton
MS	Mass Spectrometry
MW	Molecular Weight
pI	Isoelectric Point
ppb	Parts-per-billion
ppm	Parts-per-million
rpm	Revolutions per minute
SDS-PAGE	Sodium-Dodecyl-Sulphate-Polyacrylamide Gel Electrophoresis
SN	Supernatant
WAXS	Wide-Angle X-ray Scattering

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## PART 1: Aroma interactions with starch

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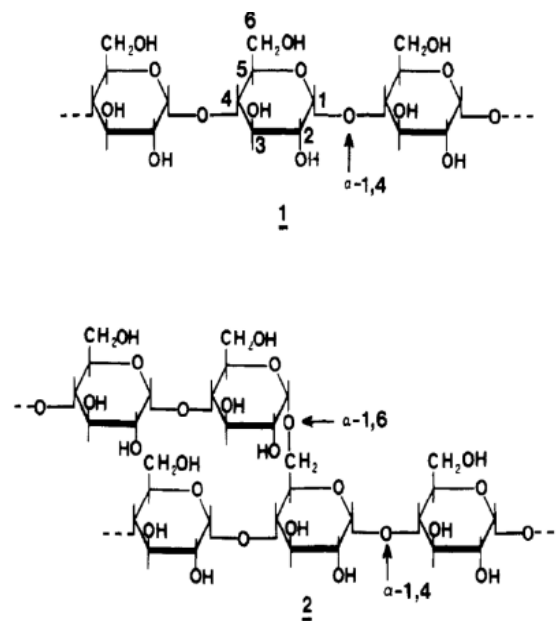


# 1 Introduction

## 1.1 Introduction to starch

Starch is the main energy reserve in plants, trees, algae and some unicellular or colonial eukaryote organisms including dinoflagellates (Ball *et al.*, 2011). Glucose is produced during photosynthesis, but since storage of glucose is limited and unfavorable, the plants convert excess glucose into starch. When the plant needs energy, the starch can be hydrolysed back into glucose.

Starch is composed of two types of molecules: amylose and amylopectin (Figure 1). Amylose is glucose-residues linearly linked by  $\alpha$ -1,4 bonds. Amylopectin is also  $\alpha$ -1,4-linked glucose-residues, but includes one  $\alpha$ -1,6 linkage per 20-25 residues. This creates a very different type of molecule. Whereas amylose forms a helical structure, amylopectin forms a structured, but disorderly bunch of branches. Amylose has a molecular weight of about 1 MDa, whereas amylopectin has a molecular weight of about 100 MDa (Parker and Ring, 2001). The ratio of amylose to amylopectin is important in determining the functionalities of the starch, e.g. gel viscosity, shear strength, solubility and retrogradation (Frederikson *et al.*, 1998). The content of amylopectin is dependent on the botanical origin of the starch, and can vary from around 40% to as much as 100% (waxy maize).



**Figure 1** Amylose (1) and amylopectin (2). Amylose is almost entirely made of  $\alpha$ -1,4-linked glucose residues, while amylopectin contains one  $\alpha$ -1,6-linkage per 20-25 glucose residues.

While starch containing only amylopectin is formed because of the lack of granule-bound starch synthase, starch containing only amylose is more difficult to achieve since amylopectin is the product of several enzymatic reactions of, e.g., branching and debranching enzymes. Knocking out two starch branching enzymes in potato, gives a variant with around 60% amylose, so-called high-amylose potato starch (Jobling *et al.*, 2002). High amylose maize can contain 80% amylose, but a 99% barley amylose has been generated (Carliofi *et al.*,

unpublished). Starches with low amylose content have higher freeze-thaw stability than normal, unmodified starches and are therefore industrially favored (Zheng and Sosulski, 1998).

Apart from amylose and amylopectin, starch also contains 0.2% to 0.4% (w/w) of phosphate, equivalent to approximately one phosphate per 200 glucose residues (Hizukuri *et al.*, 1970). It is the only known naturally occurring modification of starch, and occurs mainly in starches which form a B-polymorph, e.g. potato (see Chapter 1.2) (Blennow and Engelsen, 2010). Starches are phosphorylated by an enzyme called glucan water dikinase. This enzyme phosphorylates the glucose residues at the C-3 and C-6 position (Tabata and Hizukuri, 1971). The phosphorylation takes place in the amylopectin part of the starch suggesting that phosphate influences the crystalline starch (Blennow and Engelsen, 2010). The content of phosphate in the starch is a critical factor in the ability of the starch to form gels and high phosphate contents give high viscosity starch gels (Muhrebeck *et al.*, 1991; Veselovsky, 1940; Viksø-Nielsen *et al.*, 2001).

Increasing the phosphate content in starches is desirable because a high phosphorylation level reduces the requirement for industrial treatment of the starch (Blennow *et al.*, 2002). Interactions between aromas and phosphate are also possible if the phosphate groups are surface exposed, thereby allowing for electrostatic interactions between the negatively charged phosphate and reactive groups in the aromas. Whereas tuber starches, e.g. potato, are characterised by their phosphate content, cereal starches, e.g. maize, are characteristic by having free fatty acid and lysophospholipids in amounts correlated with amylose content (Morrison and Gadan, 1987; South *et al.* 1991).

Amylose is a linear molecule that forms a helical structure held together by hydrogen-bonds. The helix has an overall diameter of about 13 Å, and an internal diameter of approximately 5 Å (Immel and Lichtenthaler, 2000). The hydrophobic core of the amylose helix is known to form complexes with a wide range of molecules e.g. iodine, fatty acids and aroma compounds (Biliaderis *et al.*, 1985).

The botanical origin of the starch determines the chain length of the branches of amylopectin, and if this chain length is sufficiently short, amylopectin can form a double helix (Hizukuri, 1985; Li *et al.*, 2001; Franco *et al.*, 2002). The diameter of the inner hydrophobic core of amylopectin double helix is only about 1 Å, and therefore not relevant for aroma-complexation.

Starch can be isolated from many sources (Table 1), but is mainly isolated from cereals (wheat, rice, barley, maize), tubers (potato) and legumes (pea), though production of starch from other sources is increasing, e.g. from cassava roots. Production worldwide in 2008 was 66 millions tons with maize being the largest source of starch ([www.starch.dk](http://www.starch.dk)). USA is the main producer of maize starch, while potato and wheat starch are mainly produced in Europe. Applications range from use as thickeners, stabilisers and texturisers, but also include uses as

flavour carriers, nutrition, and for mouthfeel. In addition, starch is used in paper milling, textile and renewable energy industries (Ellis *et al.*, 1998).

**Table 1 Sources and variations of starches and their amylose, phosphate content, and their crystallinity. Crystallinity will be explained in Chapter 1.2. Adapted from Glaring *et al.*, 2006.**

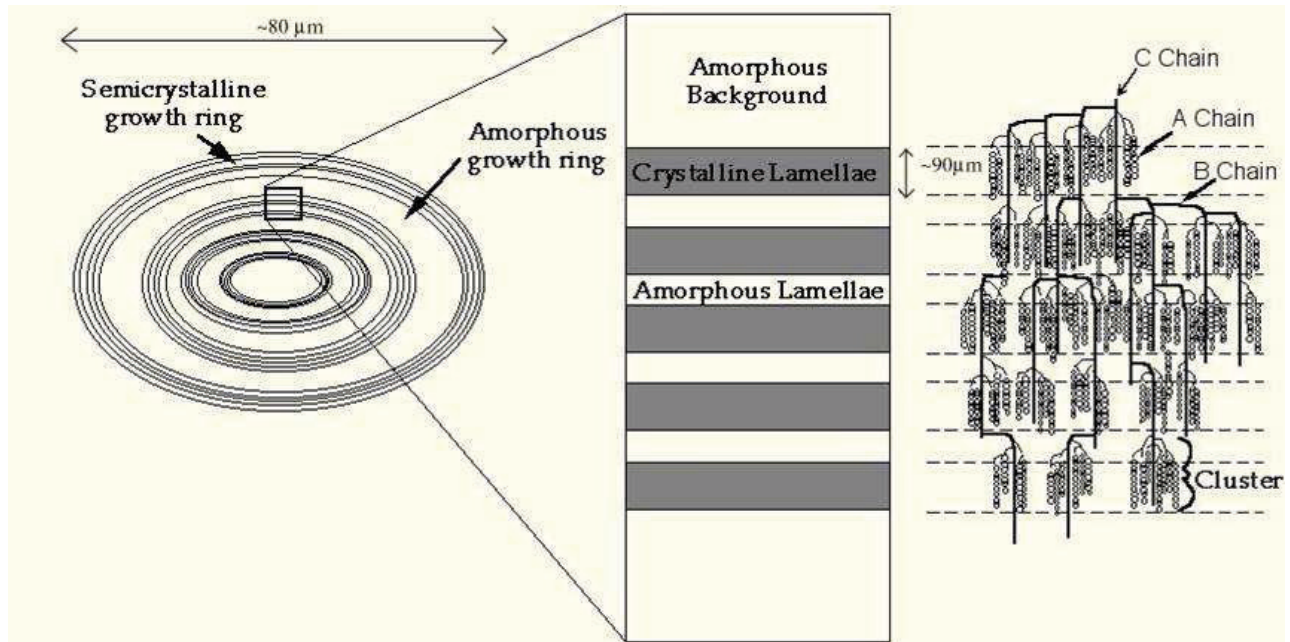
Starch	Amylose content (%)	Phosphate content (%)	Crystallinity/polymorph
Normal potato	27.6	23.2	B
Low phosphate potato	38.5	1.40	B
High amylose and high phosphate potato	41.0	60.0	B
High amylopectin potato	2.1	18.1	B
Normal maize	45.3	0.11	A
High amylose maize	60.3	1.32	B
High amylopectin maize	N.D.	0.09	A
Pea	55.0	0.48	C
Wheat	45.3	0.20	A

A key feature of starch is its ability to gelatinize. Although starch granules (see Chapter 1.2) are insoluble in water, but when starch is heated beyond a specific temperature in the presence of water, the starch granules swell irreversibly. This temperature is called the transition or pasting temperature. It is at this point that the starch forms a viscous gel. Beyond this temperature the helices unwind and the crystalline regions melt, and the starch is now amorphous. If cooled slowly the amylopectin and amylose chains reforms their respective structures (retrograding), but the starch maintains its gel structure, and cannot revert to the granule structure. Retrogradation is important because it affects properties such as texture, flavour and shelf-life of food products. Also retrograded starch is less readily degraded in the intestine, and has a beneficial effect in humans. This is called resistant starch (Garcia-Alonso and Goñi, 2000; Thompson, 2000; Sajilata *et al.* 2006).

### ***1.2 Starch organisation in the granule and crystallinity***

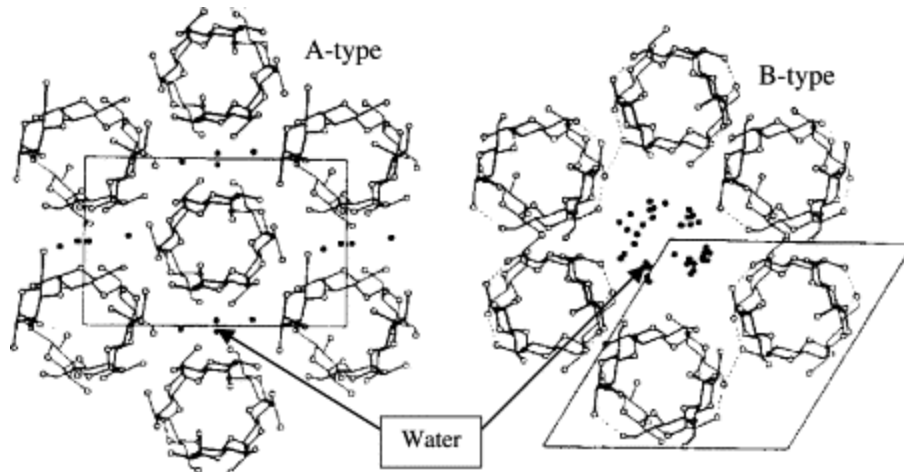
The starch granule is a spherocrystal (fibers radiating from the center of the crystal) of concentric layers or growth rings and it is composed of amorphous regions and semi-crystalline regions (Figure 2). The semi-crystalline regions only contain amylopectin, whereas

amylose is believed to be in both the amorphous and crystalline lamellae (Jacobs and Delcour, 1998). The semi-crystalline region is composed of the short amylopectin double-helices, which can be further organised in crystalline structures (Imberty *et al.*, 1991). In cereal starches the amylose exists either in a lipid-free or lipid-complexed form (Morrison *et al.*, 1993) and the content of amylose and lipids increases towards the periphery of the granule (Morrison and Gadan, 1987).



**Figure 2 Organisation of the starch granule. The starch granule forms growth rings of alternating crystalline and amorphous regions. Adapted from Jenkins and Donald, 1995.**

Channels or pores have been observed in the starch granules from maize using Scanning electro microscopy, but no channels were seen for potato starch (Fannon *et al.*, 1992; Huber and Bemiller, 1997). The digestion of starch granules is related to the accessibility of enzymes in these channels. Therefore maize starch is more readily digested than potato starch (Leach and Schoch, 1961). These channels have a diameter of about 1,000 Å and are important in chemical modifications of the starch, and may therefore also be important by allowing access of guest molecules such as aromas. Three different types of starch crystallinity were identified using X-ray analysis (Zobel, 1988a): A-, B-, and C-type (Table 1). A-type starches are found in cereals (e.g. maize), and consist of closely packed amylopectin double helices. B-type starches mostly come from tubers (e.g. potato), and have a looser structure than A-type starches, and also include structural water (Figure 3). C-type is a mixture of A- and B-type and is mainly found in legumes (e.g. pea) (Imberty *et al.*, 1991).

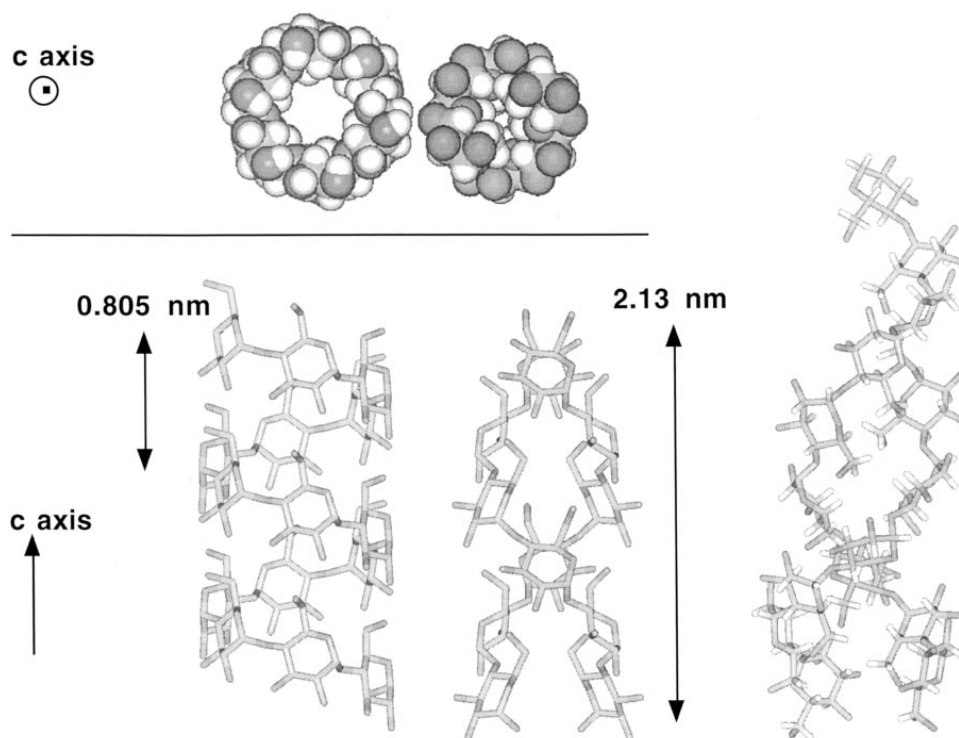


**Figure 3 Crystalline packing of A- and B-type starch including structural water. Note the looser structure of the B-type starch and the water located in the channel formed by the glucose residues. Adapted from Walter, 1998 cited in Nankean and Meenune, 2010.**

### ***1.3 The $V_H$ helix***

A fourth type of crystallinity also exists, though this type only rarely exists in nature. It mainly exists in high amylose cereal starches in a complex with lipids (Buleon *et al.*, 1984). This type of starch is called  $V_H$ . It is found after gelatinisation and is composed of the amylose helix, which forms an inclusion complex with guest molecules, e.g. aroma compounds (Buleon *et al.*, 1990; Rondeau-Mouro *et al.*, 2004; Le Bail *et al.*, 2005; Katzbeck and Kerr, 1950). In the presence only of water, amylose forms a double helical B-type crystallinity, but upon adding a small ligand, e.g. aroma, an A crystallinity is induced. Adding even more ligand induces formation of the  $V_H$  helix (Buleon *et al.*, 1990). It is a left-handed helix with six glucose units per turn and has a pitch height of about 8 Å (Rappenecker and Zugenmaier, 1981; Brisson *et al.*, 1991).

Besides forming complexes with iodine (Rundle and Edwards, 1943; Bluhm and Zugenmaier, 1981), many other ligands have been complexed in starch helices (fatty acids by Godet *et al.*, 1993; pentanol by Helbert and Chanzy, 1994; butanol by Hinkle and Zobel, 1968; isopropanol and acetone by Buleon *et al.*, 1990; decanal and others by Nuessli *et al.*, 1997; Nuessli *et al.*, 2003; Takeo *et al.*, 1973; selected alcohols and acids by Osman-Ismail and Solms, 1972; Le Bail *et al.*, 2005). The  $V_H$  helix mainly forms complexes with linear alcohols or acids. Other complexes can be formed with butanol and isopropanol, where the crystallographic unit cell is larger than for the  $V_H$  helix. X-ray scattering can determine the type of complex.



**Figure 4 (Top) End-view of the helices below. (Bottom) A 6-turn single  $V_H$  helix (left), an amylose double helix (middle), and an amylopectin double helix (right). Note the cavity in the middle of the single helix which has a diameter of approximately 5 Å. From Buleon *et al.*, 1998.**

The complexation described here is usually intra-helical, but interhelical complexation is also possible. Interhelical complexation occurs when the ligand, rather than inserting itself inside the helix, binds to the grooves on the exterior surface of the helix. It is possible to distinguish between these two forms using NMR and X-ray scattering (Rondeau-Mouro *et al.*, 2004). Addition of molecules to the starch helix causes changes in the thermostability, therefore differential scanning calorimetry (DSC) can be used to study the stability of aroma-starch complexes (Biliaderis *et al.*, 1985).

### ***1.4 Starch modification and E-numbering***

Four strategies exist to modify starches for food production: chemical modification, physical alteration, genetic engineering, and enzymatic modification. Native starches have many unwanted properties in food processing, especially retrograding. Therefore starches are chemically modified to many different variants which reflect improvements of these properties. Examples of these modified starches are listed in Table 2 alongside their respective E-numbers. E-numbers are assigned to food additives throughout the European Union.

The chemically modified starches have altered chemical properties and hydrophobicities. All the modifications have an effect on the physical character of starch with regard to pasting/gelatinisation properties, thereby introducing new qualities to the starch that are more desirable industrially than the qualities of the starch from which the new starch was derived.

Especially desirable properties include resistance to heat, freezing, shear, or other factors that give an increase in the shelf-life (Bemiller, 1997). All these modifications use the reactive hydroxyl groups on the glucose moieties to create new functionalities. One of the more common forms of chemically modified starch is acid treated starch (simply called “modified starch”), where an acid, e.g. hydrochloric acid, is used to treat starch granules to break down the starch chains, resulting in decreased viscosity and increased solubility.

**Table 2 List of E-numbered starches in industrial use today. Due to the chemical treatment of the starches, the products need to be labelled.**

<b>E-number</b>	<b>Modification</b>
E1400	Dextrin
E1401	Modified starch (Acid treated starch)
E1402	Alkaline modified starch
E1403	Bleached starch
E1404	Oxidized starch
E1410	Monostarch phosphate
E1412	Distarch phosphate
E1413	Phosphated distarch phosphate
E1414	Acetylated distarch phosphate
E1420	Acetylated starch, mono starch acetate
E1421	Acetylated starch, mono starch acetate
E1422	Acetylated distarch adipate
E1430	Di starch glycerine
E1440	Hydroxy propyl starch
E1441	Hydroxy propyl distarch glycerine
E1442	Hydroxy propyl distarch phosphate
E1450	Starch sodium octenyl succinate
E1451	Acetylated oxidised starch

One of the simplest physical modifications of starch is gelatinisation, where the starch granules undergo irreversible swelling when heated in the presence of water. A pre-gelatinised (pre-cooked) starch will thicken immediately when cold water is added. Because physical modification does not change the chemical structure of the starch, the physically

modified starches do not need to be labelled with E-numbers like the chemically modified starches do. Other examples of physical modification are extrusion and spray drying.

Genetic modifications can involve overexpression of foreign genes or suppression of gene expression using RNAi technology (Safford *et al.*, 1998; Regina *et al.*, 2006). It is also possible to create variants with knock-out mutations of, e.g., starch branching enzymes to give high amylose starches. Some of the modifications have been briefly described in an earlier chapter (Chapter 1.4 and Table 1). However, increasing consumer concerns regarding the safety of using genetically modified organisms has given high importance to finding new ways to improve starch functionalities.

Enzymatic modifications of starch can create many different kinds of modifications depending on the enzymes used. Enzymes catalyse chemical reactions that are highly specific, and the reaction conditions are very mild compared with the chemical modifications discussed above. Recombinant protein technologies can produce enzymes in large enough amounts to be applied on an industrial scale. In addition, mutagenesis can produce enzymes with highly improved specificities and/or reaction velocities, or enzymes with novel reactivities can be designed. Starch hydrolyzing enzymes mainly fall into two different enzyme nomenclature classes: glycosyl transferases (EC 2.4.x.y) and glycosyl hydrolases (EC. 3.2.1.x). The former group transfers, e.g., a short linear chain to form an  $\alpha$ -1,6-linked branch on an amylopectin chain. This transfer can alter the structure and content of amylopectin. The latter group of enzymes hydrolyses the bonds between glucose residues to form smaller products. Hydrolysis can take place at  $\alpha$ -1,4 and  $\alpha$ -1,6 bonds. Hydrolysis can be either exo-acting (from the ends of the molecule) or endo-acting (internal cleavage).  $\alpha$ -amylase (EC 3.2.1.1) is an endo-acting hydrolase that degrades starch to maltodextrins, so the end-product resembles that of the hydrolysis of starch by hydrochloric acid, but takes place at a more neutral pH. Also treating starch granules with  $\alpha$ -amylase leads to a highly porous structure (Madene *et al.*, 2006).

Other starch acting enzymes include dikinases (EC 2.7.9.x) responsible for phosphorylation of starch (Chapter 1.1), phosphorylases (EC 2.4.x.y; dephosphorylation of starch) and lyases (EC 4.1.x.y) which remove e.g. fructose from starch under the formation of a double bond (Yu *et al.*, 1993).

As mentioned, all chemically modified starches have to be labelled with E-numbers according to their treatment, because they are considered additives, and physically modified starch only needs to be labelled as starch. Enzymatically modified starches are not considered additives and therefore do not require E-numbering. Replacing chemical modifications with enzymatical modifications, eliminates the requirement for labelling starch containing food products with E-numbers, thereby creating a product that is closer to being *label-free* and thus more consumer-friendly. Furthermore, starch can act as a replacer of fat, thereby allowing for low- or no-fat products. However, from a consumer perspective, taste is the prime factor, and for the industry consumer-appreciated taste equals higher profit.

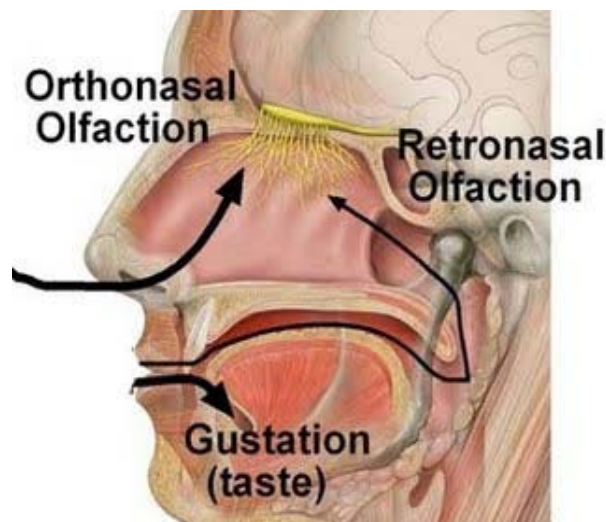


Another industrially relevant group of molecules mimic starch and can bind aromas in an internal cavity. These molecules are called cyclodextrins and are used in pharmaceutical and food industries as carrier of drugs and aromas, respectively (Szejtli, 1997). Specifically these molecules resemble the helix structure of amylose, but instead of being a long chain of molecules, they are closed ring systems of glucose. The most common cyclodextrin is  $\beta$ -cyclodextrin, which is a cyclic heptamer of glucose.

### ***1.5 Aroma perception***

It is critical to distinguish between flavour and taste. Taste is the sensation of the food item in the mouth and can only be sweet, salt, bitter, sour and umami. Flavour is a much more versatile descriptor, and can be one of thousands different sensations (e.g. caramel, corn, grass, apple etc.) as aroma compounds bind to receptors in the olfactory epithelium.

The perception of the flavour starts with chewing, but most of the aroma compounds are carried retro-nasally to the olfactory epithelium as the food items are swallowed, which is followed by an exhalation of air that carries the aroma compounds to the olfactory epithelium (Figure 5). Because only the aroma compounds that are released to the gas phase (headspace) during ingestion and mastication are sensed, it is important that though the aroma compounds need to be retained during storage, they also need to be released immediately upon ingestion. Otherwise if the aroma compounds are either too loosely bound during storage, or too strongly bound during ingestion and mastication, no flavours are sensed. Flavour perception is very individual, especially because the time when the flavour is released is dependent of how long the food item, either liquid or solid, stays in the oral cavity. For example solid food items take 10-20 seconds to chew and swallow, whereas liquids usually take only a few seconds.



**Figure 5 Schematic diagram of the perception of flavour and taste. The primary sense of flavour occurs by retronasal olfaction, where aroma compounds released during mastication are transported to the olfactory bulb.**

Retention of aroma compounds in food items is influenced by many different factors: fat (lipids and triglycerids), protein, carbohydrate, viscosity (if liquid), structure (i.e. porosity), and, in the case of processed foods, the method of preparing the food. Fat is the key player when it comes to aroma delivery and sensation. However, starch has several advantages over fat: higher consumer-friendliness, easy access, low cost, and high diversity (Madene *et al.*, 2006). Also starch can replace the smooth mouth-feel that fat gives. The retention of aromas in starch has long been known (Solms, 1986) and has been characterised in many different experimental setups using the whole range of chemical groups (Jouquand *et al.*, 2006a; Arvisenet *et al.*, 2002a; Arvisenet *et al.*, 2002b; Kollengode and Hanna, 1997; Pozo-Bayon *et al.*, 2008; Nongonierma *et al.*, 2006; Tietz *et al.*, 2008).

The chemistry of the aroma compound and other many factors such as molecular weight (chain length), hydrophobicity, is important for the retention and release (Goubet *et al.*, 1998; Nankean and Meenune, 2010). Furthermore the chemistry of the carrier affects retention, for example the molecular size of starch, its conformation, chemical groups such as phosphates, or its physical state, either amorphous or crystalline (Goubet *et al.*, 1998) are important.

The formation of a complex between aroma compounds and the food carrier, e.g. starch, can be the result of covalent bonding (e.g. the double ketone in diacetyl can react with amino groups in proteins), hydrogen bonding between polar groups, hydrophobic interactions from apolar groups, such as the relatively long carbon-chain of citral, and formation of inclusion complexes. Encapsulation of volatile aroma before processing prevents aroma degradation, e.g. by oxidation (Madene *et al.*, 2006). In starch, two modes of binding can occur: formation of inclusion complexes (intrahelical) or binding of aroma to starch hydroxyl groups (extrahelical). Other encapsulation products include colourants, vitamins, oils, and minerals. The complexation primarily involves the amylose helix, but can, to a lesser extent, also involve the amylopectin double helix.

Although starch is composed of long chains of glucose, glucose itself and other short carbohydrates only possess few aroma retaining qualities (McGorin and LeLand, 1996). Polysaccharides have some aroma retaining qualities (Jouquand *et al.*, 2006b; Jouquand *et al.*, 2008), and thereby reduce the concentration of aroma in the headspace, and thus have an impact on the perception of flavour.

While carbohydrates can be used to enhance certain flavours, they can also be used to mask, or even remove, unwanted aroma, so-called off-flavours. It is especially cyclodextrins that are used for this, though the use is limited (Szejtli, 1997).

## ***1.6 Aroma compounds***

Aroma compounds are generally small, hydrophobic molecules, though some are water-soluble. Their molecular weight does not exceed 400 Da (Landy *et al.*, 1996). They are primarily comprised of carbon, oxygen, sulphur and nitrogen, and include several classes of chemicals, e.g. aldehydes, ketones, alkenes, aromatic rings, acids, sulphides, and esters. The

human nose is finely tuned to perception of aroma compounds, and it can detect most aroma compounds in parts-per-million (ppm), but in some cases also in parts-per-billion (ppb).

In this project the binding of the following aroma compounds to starch will be examined (Table 3):

Acetaldehyde (systematic name: ethanal) is a very low molecular weight compound with a boiling point of only 21 °C. The low boiling point means care must be taken to avoid evaporation during handling. Besides a low boiling point, acetaldehyde has a very high vapour pressure (740 mm Hg = 0.99 Bar). Acetaldehyde smells like fresh fruit and fresh bread. In lower concentration it can also have a smell of yoghurt.

Dimethyl sulphide is another high volatility compound, but easier to work with due to its higher boiling point of 38 °C. In high concentrations this compound smells of cabbage, but in low concentrations it smells of corn. In general, sulphur compounds are considered off-flavours, and masking them may be necessary. However, in beer and cheese products sulphur compounds contribute dramatically to the end-flavour, and removing them will reduce the flavour experience.

Diacetyl (systematic name: 2,3-butanedione) is a diketone. It has a very low odour threshold (limit of detection by the human nose) of only six ppb, equivalent to 6 nL of aroma in 1 L of water. In low concentrations it smells like caramel, but in higher concentrations it has an unpleasant, overpowering smell of butter. For example, diacetyl is used by the popcorn industry to produce butter-flavoured popcorn.

Allyl isothiocyanate (systematic name: 3-isothiocyanato-1-propene) is used naturally by plants as a repellent against animals. It is slightly toxic with a LD<sub>50</sub> of 151 mg/kg (LD<sub>50</sub> is a toxicity index which indicates the amount at which 50 percent of a population is killed). Allyl isothiocyanate is responsible for the characteristic odour of mustard, horseradish and wasabi.

Ethyl butyrate is an ester made from a reaction between butanoic acid and ethanol. It has a very strong (synthetic) fruity pineapple smell.

Citral (systematic name: 3,7-dimethyl-2,6-octadienal) is a mixture of two isoforms: E-citral (geranial) and Z-citral (neral). The mixture has a lemon odour. Geranial has the strongest lemon odour, but neral has a sweeter lemon smell. This shows how the subtleness in a structure can give surprising differences in odour. Even though other compounds are present in higher amounts in lemons (Chamblee *et al.* 1991), citral is responsible of the characteristic lemon odour.

Lemon oil is composed of many different constituents. Besides the above-mentioned citral, 85% of the volatiles of lemon oil are  $\beta$ -pinene,  $\gamma$ -terpinene and limonene (Chamblee *et al.*, 1991), though as stated above citral dominates the lemon flavour.

Cinnamaldehyde (systematic name: 3-phenylprop-2-enal) gives cinnamon its unique odour. It has a structure that is very similar to benzaldehyde, which gives almonds their odour. It can undergo an autoreaction, which replaces the sharp cinnamon smell with a more flat and dull smell.










Octanol is a linear fatty alcohol, and depending on concentration, can smell like waxy citrus or mushroom. It is immiscible with water, and therefore it is often used as a solvent for chemicals.

Butyric acid is a short-chain fatty acid, which is produced when glycerides in butter undergo hydrolysis, giving butter the rancid smell. Due to the carboxylic group butyric acid is quite water soluble.

The aromas listed here (Table 3) represent many different chemical reactivities and have a broad range of odour characteristics from the sweet caramel to rancid butter and from fruity lemon to strong mustard.

The aroma compounds used in the present project represent many classes of chemical compounds. Some have a low molecular weight, while others have a high molecular weight; some are highly volatile, others are not; some are polar, others are apolar.

Table 3 Overview of the aroma compounds used. The unique identification number (CAS), boiling point (Bp) in °C, molecular weight (MW), logarithm of the solubility of aroma in octanol divided by solubility in water, vapour pressure at 25 °C unless otherwise specified, chemical formula, and the odour threshold of detection in parts-per-billion (ppb). N.A.: not available. <sup>1</sup> adapted from <http://www.cschi.cz/odour/files/world/Thresholds%20table.pdf>, <sup>2</sup> adapted from <http://datasheets.scbt.com/sc-252361.pdf>, <sup>3</sup> adapted from <http://toxnet.nlm.nih.gov>.

Compound	CAS	Bp (°C)	MW	Log P (Oct/water)	mm Hg@ 25°C	Formula	Odour threshold /ppb
Acetaldehyde	75-07-0	21	44.05	0.4	740		15 <sup>1</sup>
Dimethyl sulphide	75-18-3	38	64.14	0.92	502		1 <sup>1</sup>
Diacetyl	431-03-8	88	86.10	-1.34	56.8		2.3 <sup>1</sup>
Allyl isothiocyanate	57-06-7	150	99.16	2.11			7 <sup>2</sup>
Ethyl butyrate	105-54-4	119-121	116.18	1.85	12.8 (20°)		1 <sup>1</sup>
Citral	5392-40-5	220-229	152.26	3.45	0.1		30 <sup>1</sup>
Lemon oil	8008-56-8	177	N.A.	N.A.	N.A.	N.A.	N.A.
Cinnamaldehyde	104-55-2	247	132.17	1.90	0.03		50 <sup>3</sup>
Octanol	111-87-5	196	130.26	3.15	0.14 (20°)		110 <sup>1</sup>
Butyric acid	107-92-6	162	88.10	0.8	0.84 (20°)		240 <sup>1</sup>

## ***1.7 Aroma retention studies***

Many studies focus on the aroma retention in hydrated food systems, e.g. yoghurts, but very few studies involve investigations on dry systems (Hau *et al.*, 1998). Also studies tend to focus on model food systems that can be far from the foods experienced by the consumers.

Hau *et al.*, 1996 have studied the retention of a series of aromas in native starch granules using static and semi-dynamic headspace extraction techniques. However, in their experiments the binding of aromas takes place by taking the headspace above a solution of aroma, and then injecting this into a sealed container of starch, before allowing an equilibrium to settle. This procedure only binds very small amounts of aroma to the starch, but, as described in the previous paragraph, the binding only needs to happen in ppm amounts. If more complex mixtures of aroma are needed, this method is less suited, and it might be more feasible to mix one aroma component with starch in very high concentrations, and then mix many individual starch/aroma complexes.

A study of aroma release in a model food system with water using relatively low concentration of starch (7%), uses around 0.1% (mol%) of aromas, and one or two aromas at a time (Arvisenet *et al.*, 2002a). This procedure is excellent for studying the influence of single components such as amylose content of starch, but is less suited for with more complex systems, where the flavour experience is a symphony of many aromas and tastes. However, a strong disadvantage of using a hydrated system is the increase both reactivity and volatility of the aromas, thus giving shorter shelf-life. Thus for products that need a long shelf-life, there is a need to remove water to prevent changes in the flavour experienced by the consumer.

A study of the release of aromas from rehydrated dried vegetables, showed that compared to water, aroma release is slightly higher when a saliva-mimic containing  $\alpha$ -amylase is added compared to a saliva-mimic lacking this enzyme (van Ruth *et al.*, 2000). However, this study also shows a very large difference when comparing the absence or presence of mastication on aroma release. This study suggests that mastication is a major player when it comes to releasing aromas from the food matrix, even though the  $\alpha$ -amylase found in the saliva must also have a role in the aroma release, it seemed less pronounced.

## ***1.8 Techniques applied in this study***

### **1.8.1 Wide-Angle X-ray Scattering (WAXS)**

WAXS on starch uses X-ray radiation from a copper tube to show the diffraction intensity of a starch sample as a function of the diffraction angle. A starch powder is used and rotated perpendicularly to the X-ray radiation. The radiation is scattered according to type of starch, and results in a pattern similar to those shown in Figure 6.

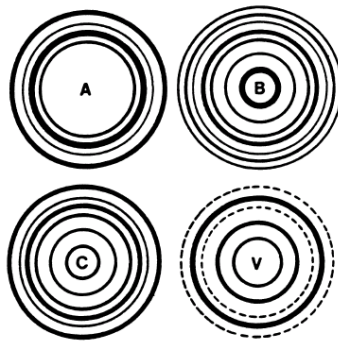


Figure 6 X-ray powder diffraction of starch with either A-, B-, C-, or V-type crystallinity. From Zobel *et al.*, 1988b.

The resulting diffraction seen in Figure 6 can be deconvoluted to a diagram showing the intensity of the scattered radiation versus the diffraction angle ( $2\theta$ ). If amylose/aroma inclusion complexes are present in the starch, diffraction intensities will appear at  $2\theta = 6.7^\circ$ ,  $12.5^\circ$ , and  $17.8^\circ$  (Arvisenet *et al.*, 2002a) (Figure 7). These peaks indicate the presence of the  $V_H$  helix.

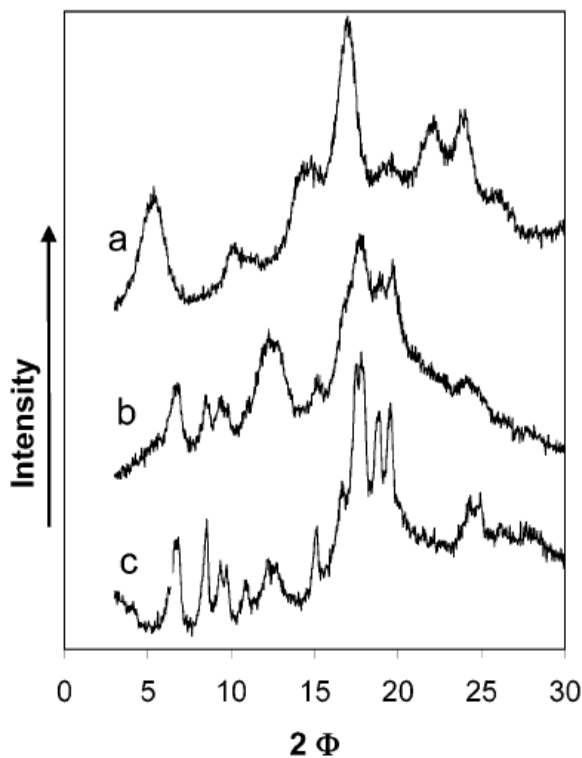


Figure 7 X-ray diffractograms for amylose crystallized with isoamyl acetate (a), linalool (b), and ethyl hexanoate (c). Isoamyl acetate does not form a complex and therefore does not diffract at  $6.7^\circ$ ,  $12.5^\circ$ , and  $17.8^\circ$ . From Arvisenet *et al.*, 2002a.

### **1.8.2 Differential Scanning Calorimetry (DSC)**

As mentioned previously, starch undergoes a gelatinisation when heated in the presence of water. This process includes complete dissolution of the starch granule mainly driven by uncoiling and dissociation of double helical amylopectin chains. Since the gelatinization process is endothermic, it can be studied by DSC. The endotherms can be interpreted as the amount of double helices present in the starch granule and the melting temperature to the perfection of the ordering of these helices in the crystalline lattice (Cooke and Gidley, 1992; Tester and Debon, 2000). Therefore starch of different botanical origin show differing gelatinisation temperatures and melting enthalpies (Silverio *et al.*, 2000; Srichuwong *et al.*, 2005). The amylopectin chain length also influences melting temperature (Jane *et al.*, 1999).

The gelatinization process can be described by both the melting temperature ( $T_m$ ) and the melting enthalpy. Guest molecules that affect the amylose or amylopectin helix structures, thereby also the crystallinity, the crystalline lattice, or chain mobility, will influence either the melting temperature and/or enthalpy. The presence of a mixture of aromas in a starch suspension has been shown to change the thermal profile (Blaczak *et al.*, 2007). Apart from the endotherm seen for the gelatinisation, another endotherm can be observed at around 110 °C – 120 °C. This latter endotherm is caused by the melting of the amylose-complexes (Biliaderis *et al.*, 1985).

### **1.8.3 Gas Chromatography-Mass Spectrometry (GC-MS)**

The GC-MS instrument is composed of two units: First the GC-part where the volatiles, such as aromas, are separated on a column, followed by the second part, the mass spectrometer. Here the volatiles are fragmented and ionized and then detected according to their mass-to-charge ratio. GC-MS is a very versatile technique that can be used for studying aroma release, quantification of specific aromas and identification of unwanted flavours in food products (Arvisenet *et al.*, 2002a).

#### ***1.8.3.1 Multiple headspace extraction (MHE)***

MHE (Kolb, 1982) is a variation of dynamic headspace, where a carrier gas is constantly passed over the sample to remove the volatiles. The volatiles will be captured on traps composed of 2,6-diphenylene-oxide polymer with a surface area of 35 m<sup>2</sup>/g. During the course of a MHE experiment, the headspace above the sample is extracted repeatedly thereby gradually depleting the sample of volatiles. This is due to equilibrium constants between the volatiles in gas phase and volatiles bound in matrix.

The depletion of volatiles from the sample follows an exponential decay. And by plotting the intensity of an extraction as a function of the extraction number, the total integrated intensity of this plot is total amount of aroma that can be extracted from the sample. In other words, the sum of all theoretical extractions continued to infinity gives the total intensity of aroma in the sample (for a more comprehensive explanation of MHE and the mathematics behind, see Kolb and Ettre, 1991). The total amount of aroma can be calculated by the following formula:



$$\sum_{i=1}^{i=\infty} A_{total} = \frac{A_1}{1 - e^q}$$

, where  $i$  is the extraction number (i.e. the second extraction is extraction number two),  $A_{total}$  is the total amount of aroma,  $A_1$  is the first extraction,  $e$  is the natural logarithm, and  $q$  is the slope of the linear regression in a plot of the logarithm of the peak intensity of the aroma measured by GC-MS as a function of the extraction number. The total area under this linear regression curve is the total amount of aroma that can be released.

By comparing the total amount of aroma that can be released with the intensity of a known amount of aroma (standard), preferably the same aroma that is analysed if the identity of the aroma is known, it is possible to convert the total intensity to an amount, i.e. weight or volume. If a food product of many different aroma is analysed a non-naturally occurring standard internal standard is used instead.

### **1.9 Project aim**

The aim of this PhD-project is to examine aroma retention in natural starches, meaning non-chemically modified starches, thereby taking a step towards introducing *label-free* food products on the market. Rather than using chemicals to modify the starches, the starches used in this study are from natural sources, where genetic engineering has been used to create variants with differing amylose, amylopectin or phosphate content. As described above, because these starches are not modified after isolation, no E-numbers are required to label the final product.

In the following section the terms binding and retention is used in the broadest possible way, meaning adsorption, absorption, and physical and chemical binding to the starch. Binding includes diffusion through the matrix, surface adherence, or diffusion through the outer layers of the starch granule.

In the following the term “percent binding” refers to the weight of aroma or other chemical compound per total weight of aroma plus starch:

$$\% \text{ binding} = \frac{\text{weight (aroma)}}{\text{weight (aroma)} + \text{weight (starch)}}$$

For example adding 100 mg aroma to 200 mg starch gives 33% (=100 mg/(100 mg +200 mg)).

The overall aim of the project is to incorporate aroma into starches to a concentration of 15%.

The term “percent aroma retained of added aroma” refers to the percentage of the amount of left at the end of the experiment relative to the amount of aroma added at the beginning of the experiment:

$$\% \textit{ retention} = \frac{\textit{ weight of aroma left}}{\textit{ weight of aroma added}}$$

This thesis uses the terms binding and retention to refer to the respective formulas above. Thus the term binding will only be used when starch is used in the calculations, whereas retention will only be used when comparing the amount of aroma in the experiment without taking into account the amount of starch used.

This study differs from previous studies by using very large concentrations of aroma. Using these high concentrations of aroma is an advantage in the production processes if the final food product requires use of minimal amounts of starch without compromising flavour and taste. Previous studies usually use aroma concentration in the sub-percent range and in hydrated model systems. These experiments focus on binding to dried starches, both in their native granule state and their amorphous state.

## 2 Materials and methods

### 2.1 Materials

Normal potato starch was purchased from Sigma Aldrich. All maize starches were provided by Cerestar-AKV I/S (Denmark), and pea starch was obtained from KMC, Brande (Denmark). The high amylopectin potato was obtained from Lyckeby Stärkelse AB (Sweden). High amylose phosphate was obtained from Danisco (Denmark). Wheat starch was provided by Semper AB (Sweden).

Aroma compounds were purchased from Sigma Aldrich. The purity of the compounds was 93% or higher.

### 2.2 Preparation of starches

Water was added to starch (10 g) to a final volume of 200 mL to make a 5% slurry, which was then heated to the boiling point. The slurry was then autoclaved at 121 °C for 15 min to get amorphous starches. This was first placed on water with ice, and subsequently frozen at -80 °C to preserve the amorphous structure. The frozen starch slurry was finally freeze-dried to dryness (excluding structural water). The native starch granules were frozen and freeze-dried.

Starch (200 mg  $\pm$ 2 mg) was added to 14 mL tubes (three tubes per aroma per starch (Table 1) and three tubes without aroma per starch). The tubes including the lid were weighed before and after adding starch, as well as after aroma addition and aroma evaporation, to enable calculation of the exact amount of starch present and the aroma added, respectively, as well as the amount of aroma released and the amount of aroma retained.

Each aroma (100  $\mu$ L) was added 200 mg starch to give approximately 33 weight-percent, except acetaldehyde where 200  $\mu$ L was added due to difficulties with handling this compound. Aroma-starch mixtures were put on an ultra-sonication bath on ice for one hour. Thereafter, aroma was allowed to evaporate from the starch mixture by passive diffusion by leaving the open 14 mL tubes in a fume hood for six days.

After the experiments, the tubes including the lid were weighed. Water absorption was calculated by subtracting the weight of the tubes with starch without aroma after six days from their starting weight. This was then subtracted from the tubes with the same type of starch.

### 2.3 Multiple Headspace Extraction

For the experiments with water acting as saliva substitute (van Ruth *et al.*, 2000), amorphous pea starch was used, while amorphous maize starch was used for the dry experiments.

The starches with aroma were transferred into a purge flask (3-4 aromas per flask; there is no overlap between retention times of the aromas on the GC column; lemon oil was analysed

alone). A magnet and 10 mL of de-ionized water was added and the flasks were closed by inserting a purge head and sealed using a clamp. Flasks were put in a thermostated water bath at 37 °C with magnetic stirring (200 rpm). A Tenax TA (Scientific Instrument Services) column was attached to the purge head and nitrogen flow through the purge flask was set to approximately 80 mL N<sub>2</sub>/min. Samples were adsorbed for 15 min onto the Tenax TA columns at 30 min intervals for 90 min, then at one hour intervals for two hours and finally at two hour intervals for a total of six and a half hours. Thus one 15 min interval defines one extraction, and an extraction after one hour is extraction number five (60 min of purging + 15 min adsorption time). For the dry experiments no water was added.

The retained aroma compounds on the Tenax TA columns were thermally desorbed by an automated thermal desorber (ATD 400, Perkin Elmer) by heating the columns to 250 °C. Desorption time from the column to the cold trap was 15 min. The cold trap was then heated rapidly to from 5 °C to 300 °C to release the aroma compounds into the GC. Compounds were separated and identified on a Hewlett-Packard G1800A GC-MS with a J&W scientific DB-Wax column using helium as a carrier gas (1 mL/min). No split was used, unless otherwise stated. Column temperature was kept at 45 °C for 10 min before heating by 6 °C/min to 240 °C and holding this temperature for 10 min. The GC-MS used an electron ionization detector and mass range (mass/charge ratio) was set from 15 to 300. MS data were analyzed by Agilent ChemStation. Spectra were auto-integrated and all relevant peaks were checked for integration errors and corrected manually if necessary.

The experiments performed in the presence of water were conducted in duplicate (triplicate was preferred, but starch sample shortage prevented this) and the dry experiments were conducted in triplicate.

Standards were made by diluting aromas 1,000 times (10 µL in 10 mL), unless otherwise stated, in heptane. Aromas were grouped in the same way as for the actual analysis (i.e. 3-4 aromas per analysis and lemon oil was analysed alone). 2 µL of the dilution were injected directly by syringe onto the Tenax traps. Analysis of standard was identical to the analysis of samples, except for a delay of 2.5 min, to protect the GC-MS from overloading on the heptane peak. No droplets of aroma were observed in the heptane solution (similar to oil:water emulsions), which could otherwise have corrupted the results (personal communication with laboratory technician Mehdi Farastani).

Enzymatic digestion was done in a 50 mM potassium phosphate buffer pH 6.8 on amorphous high amylose maize starch mixed with aroma. Pancreatic  $\alpha$ -amylase was added to the solution.

For the ether/pentane extraction a 1:1 mixture of ether/pentane was made. 60 mL was added to a blue cap bottle with one starch including all aromas (i.e. nine times 200 mg starch) except lemon oil. The extraction proceeded for 60 min before the ether/pentane solution was evaporated by air flow to a volume of about 2 mL (during the evaporation the solution is cooled by the evaporating ether/pentane so no aromas should be lost in this process). The 2

mL extraction medium was transferred to tubes and sealed before being analysed by GC-MS equipped with an autosampler.

## ***2.4 Differential Scanning Calorimetry***

### **2.4.1 Initial experiments (KU-LIFE)**

Starches-aroma mixtures were prepared as described above (Chapter 2.2). The following aromas were analysed at KU-LIFE: acetaldehyde, dimethyl sulphide, diacetyl, allyl isothiocyanate, ethyl butyrate, citral, and butyric acid. At Institut National de la Recherche Agronomique (INRA) only citral, octanol, and butyric acid were analysed. Samples were analysed using Perkin Elmer Diamond DSC operated from 30 °C to 100 °C at a scanning rate of 10 °C per minute. The starches were analysed in slurries of 3 mg native starch and 12 µL 10 mM NaCl in duplicates. Perkin Elmer Pyris 7.0 software was used to determine the peak temperature ( $T_p$ ) and the enthalpy change ( $\Delta H$ ). Starch/octanol mixtures were not analysed at KU-LIFE due to technical issues. These experiments were performed by PhD-student Susanne Langgård Jensen.

### **2.4.2 Further experiments (INRA)**

Starch-aroma mixtures with citral, octanol, and butyric acid were prepared as described. The samples were analysed in a TA instrument DSC Q100 with indium used to calibrate the instrument. 10 mg of sample was mixed with 40 µL MilliQ water in stainless steel pans. The reference pan was 50 µL of MilliQ water. The temperature range was 10 °C to 120 °C with a scan rate of 3.0 °C/min. These experiments were performed by laboratory technician Marion de Carvalho.

## ***2.5 Wide-Angle X-ray Scattering***

Diffraction diagrams were monitored by recording X-ray diffraction diagrams every 10 min on a Bruker D8 Discover diffractometer. Cu  $K\alpha_1$  radiation with a wave-length of 1.5405 Å was produced in a sealed tube at 40 kV and 40 mA and parallelized using a Göbel mirror parallel optics system and collimated to produce a 500 µm beam diameter. Hydrated samples (100 mg) were sealed between two aluminium foils to prevent any significant change in the water content during the measurement. The diffracted beam was collected with a two-dimensional GADDS detector and recording time was 600 sec. The distance from the sample to detector was 100 mm. The resulting diffraction diagrams were normalised to the same total integrated area between 3° and 30°. These experiments were performed by laboratory technician Bruno Pontoire.

## 3 Results

### *3.1 Experimental considerations regarding work with aromas*

Of the ten aromas and aroma-mixtures, four aromas are very volatile (see Table 3; acetaldehyde, dimethyl sulphide, diacetyl, and ethyl butyrate with boiling points of 21, 38, 88, and 120 °C, respectively). Especially acetaldehyde is very volatile with a boiling point of only 21 °C and therefore is very difficult to handle at room temperature and a low retention must be expected already before beginning the experiments. Also dimethyl sulphide, diacetyl and ethyl butyrate can be expected to show relatively low retentions, due to their low boiling points and high volatilities. As mentioned in the Introduction, lemon oil is composed of many different components, including aldehydes, ester, alcohols, and terpenes, the latter of which are derived from 2-methyl-1,3-butadiene, so-called isoterpene (Chamblee *et al.*, 1991). The physical and chemical differences of the components, e.g. volatility, will undoubtedly give a different composition of the lemon oil at the end of the experiments than the composition found in the starting mixture. The primary factor for this difference is the differences in volatility, but it can also be caused by other factors, e.g. chemical reactivity.

Autoreactivity is a process whereby a chemical compound reacts with itself to form a new product. Cinnamaldehyde is such a compound, where the aldehyde-group is responsible for the reactivity. It has a sharp cinnamon smell, whereas the autoreaction product has a duller and flatter cinnamon smell. The identity can be shown by GC-MS.

In the following the term “the most volatile aromas” covers the aromas acetaldehyde, dimethyl sulphide, diacetyl, and ethyl butyrate, whereas the term “the least volatile aromas” covers citral, cinnamaldehyde, octanol, and to a lesser extent also butyric acid.

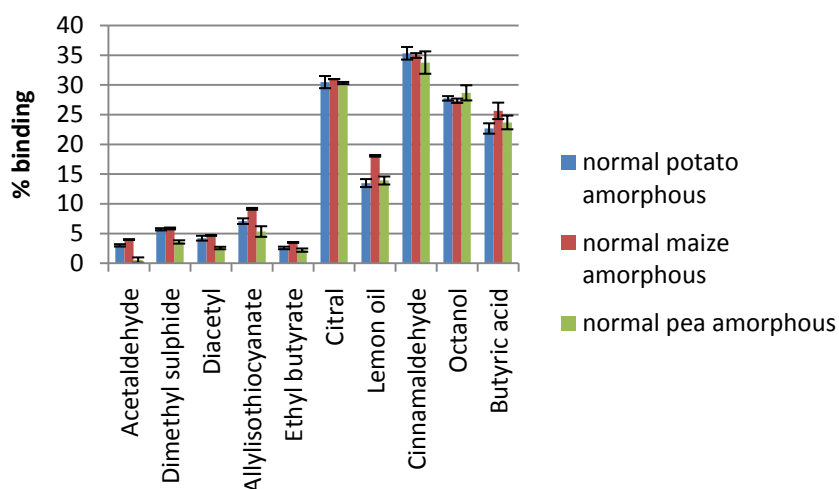
### *3.2 Aroma-binding studies*

#### **3.2.1 Amorphous starches**

Throughout the experiments the most volatile compounds do not surprisingly show the lowest binding, whereas the less volatile compounds show a larger binding, indicating that vapour pressure and volatility is a main factor in the binding. For the most volatile compounds amorphous maize starch (see Figure 8) appears to be slightly better at binding the aromas, whereas pea starch shows the lowest binding. However, for the less volatile compounds, there is no substantial difference, though maize starch is better at binding butyric acid than potato starch.

During the course of the experiments, the tubes containing starch with allyl isothiocyanate began to develop a browned appearance, similar to burning food on a stove. Allyl isothiocyanate contains two double-bonds and a nucleophilic nitrogen and sulphur, so it is possible that it reacts with the starch to form a modified starch. The Maillard reaction (Martins *et al.*, 2000) is a process whereby food items are browned, e.g. frying a steak or baking bread, caused by reaction between an amino-group and a sugar. The existence of this process makes a reaction between the allyl isothiocyanate and starch very likely.

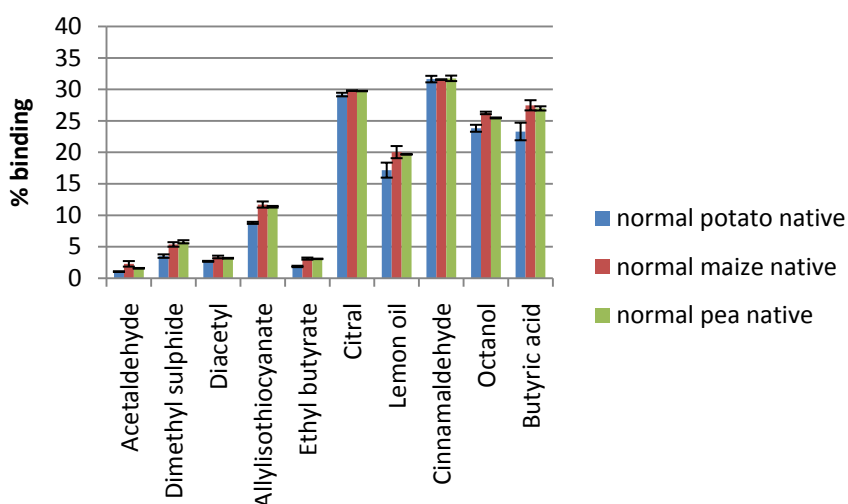
The water absorption for the starches used as negative control in this experiment was between an equivalent of about five to ten microlitres of water.



**Figure 8** Binding of aromas to amorphous potato (blue), maize (red), and pea (green) starch. Binding is defined as weight of aroma per total weight of starch including aroma. The error bars are the standard error of the mean of triplicates.

### 3.2.2 Native starches

For the native starches (Figure 9), potato starch shows a lower binding than both maize and pea, both for the most volatile compounds and for octanol and butyric acid. However, for citral and cinnamaldehyde the binding is similar for all three starches. Allyl isothiocyanate, lemon oil and butyric acid show a larger binding for the native starches than the amorphous starches, whereas cinnamaldehyde and octanol show reduced binding. The most volatile aromas only show comparatively minor differences relative to the amorphous starches.



**Figure 9** Binding of aromas to native potato (blue), maize (red), and pea (green) starch. Binding is defined as weight of aroma per total weight of starch including aroma. The error bars are the standard error of the mean of triplicates.

### 3.3 Aroma-retention studies

By comparing the retention of aromas observed with a control where no starch is added, a direct comparison of the effect of the addition of starch is possible. The retention calculations only take into account the amount of aroma left after the experiment, whereas the aroma binding results above also take the weight of starch into account. Comparisons can be made either on the amount of aroma in weight left in the samples, and also on the amount of aroma left relative to the amount added.

The results from the two forms of calculations give different representations of the same data, but show similar results, therefore only the representation regarding percentage of aroma left compared to the starting amount will be discussed.

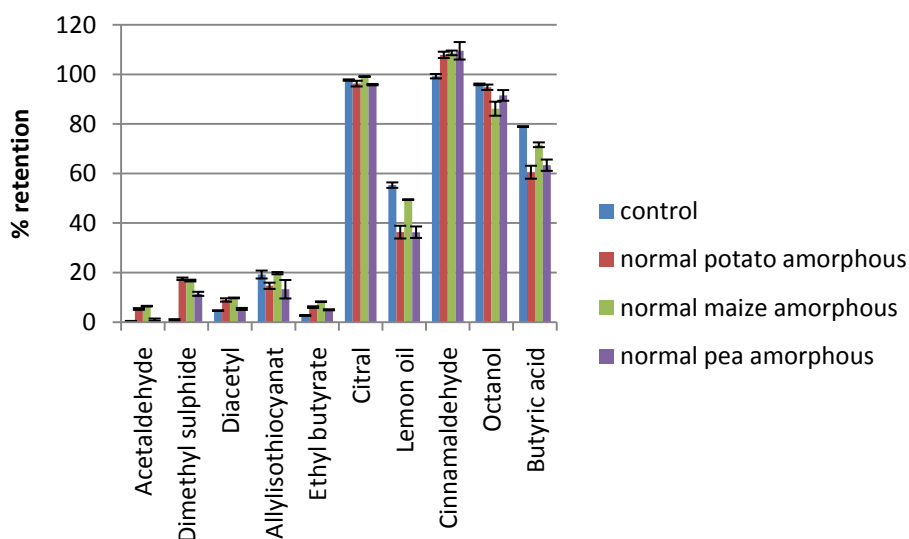
**Table 4 Comparison of the amounts of aroma left in the tubes after six days calculated in percent of the amount added. For the starch samples the amount of aroma is per 200 mg starch. For the control, volumes of aroma identical to volumes used for the starch samples were added to an empty tube. Water adsorption during the experiment to starch samples without aroma, has been subtracted from the starch samples with aroma. The percentage is calculated as one hundred times the amount left after the experiment divided by the amount added before the experiment. The standard deviations are the standard error of the mean of triplicates.**

Percent retained of added aroma	Amorphous starch				Native starch		
	Control	Potato	Maize	Pea	Potato	Maize	Pea
Acetaldehyde	0.4±0.0	5.3±0.3	6.5±0.1	1.0±0.5	1.4±0.2	3.3±0.5	2.2±0.1
Dimethyl sulphide	0.8±0.4	17.5±0.5	16.8±0.4	11.4±0.8	11.3±0.3	16.2±0.6	16.4±0.3
Diacetyl	4.7±0.1	9.0±0.7	9.8±0.1	5.3±0.4	5.7±0.1	7.5±0.5	6.5±0.1
Allyl isothiocyanate	19.2±1.6	14.7±1.3	19.8±0.4	13.3±3.7	18.7±0.4	26.2±1.0	25.1±0.6
Ethyl butyrate	2.7±0.2	6.1±0.3	8.3±0.1	5.0±0.2	4.3±0.3	7.5±0.1	7.1±0.3
Citral	97.7±0.3	96.3±1.1	99.1±0.1	95.9±0.3	91.6±0.3	94.0±0.5	92.2±0.7
Lemon oil	55.3±1.1	36.3±2.6	49.5±0.0	36.3±2.3	48.3±3.3	57.9±2.0	57.2±0.7
Cinnamaldehyde	99.3±0.9	107.9±1.3	108.7±0.9	109.5±3.5	95.8±0.6	97.5±0.3	95.6±0.4
Octanol	96.0±0.3	94.8±1.1	86.1±2.8	91.5±2.1	80.0±2.5	88.1±1.4	84.1±0.4
Butyric acid	78.9±0.2	60.5±2.6	71.6±1.0	63.3±2.3	63.2±5.1	77.5±1.9	75.1±1.1

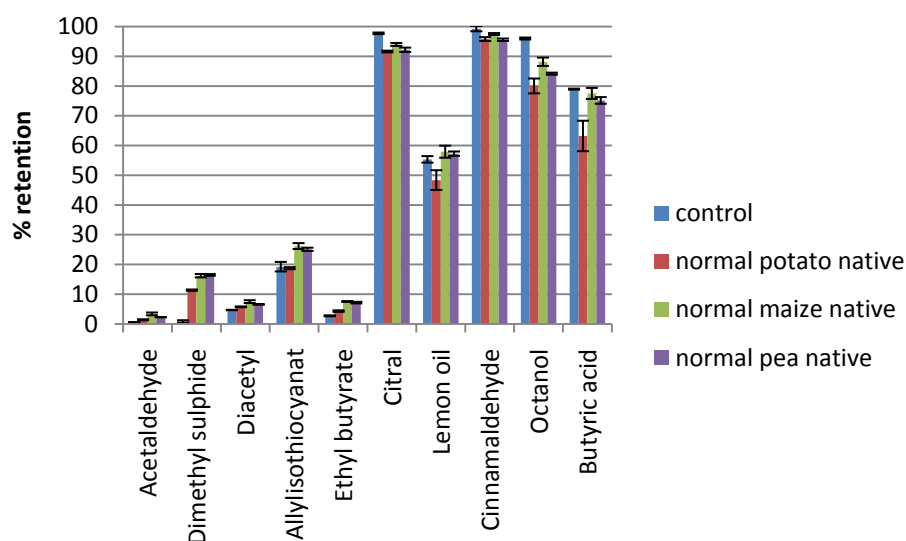


The amount of aroma left in the tubes after six days of evaporation for the most volatile compounds, i.e. acetaldehyde, dimethyl sulphide, diacetyl, and ethyl butyrate, is larger when starch is present compared to the sample without starch (Table 4). The amorphous pea starch stands out with comparatively lower amounts of the most volatile compounds than the rest, but this may be due to problems with water content in the sample, i.e. the starch control without aroma shows negative water absorption evidenced by a decrease in weight during the experiment of three milligrams, whereas the other starches, including native starches, show an increase in weight of five to seven milligram.

The most noticeable difference is for dimethyl sulphide, which shows 15 to 24-fold better retention compared to the control without starch (Table 4, Figure 10 and Figure 11). Acetaldehyde also shows a large increase in retention (from 2.5 to 16-fold increase), but primarily for amorphous potato and maize starch. Diacetyl and ethyl butyrate show a minor, but for most starches still a substantial, increase in the amount of aroma retained compared to the control.



**Figure 10 Retention of aroma by amorphous potato (red), maize (green), pea (purple) starches relative to a control (blue) without starch added. Please note the difference in Y-axis scale between this and the following figure. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The error bars are the standard error of the mean of triplicates.**



**Figure 11 Retention of aroma by native potato (red), maize (green), pea (purple) starches relative to a control (blue) without starch added. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The error bars are the standard error of the mean of triplicates.**

Allyl isothiocyanate appears to be slightly better retained by native starches, especially maize and pea starch, than by amorphous starches (Table 4), although there is a relatively large uncertainty on this type of results, when only three points (one for each starch) are considered and the values are slightly scattered, even though all of these data points are an average of triplicates.

Generally for the rest of the less volatile compounds (citral, lemon, oil, cinnamaldehyde, octanol, and butyric acid), a reduction of retention relative to the control is observed, with the exception of cinnamaldehyde with amorphous starches (Figure 10). Especially lemon oil and butyric acid show a marked reduction in retention compared to the control, noticeably this effect is largest for the amorphous starches.

As mentioned above, cinnamaldehyde behaves differently for the amorphous starches with an increase in weight during the experiment beyond the starting weight of aroma. After the six days in fume cupboard, the starch/cinnamaldehyde mixture appeared to have crystallized slightly to form a semi-solid gelatinous crystal. As the retention is around 110% for all amorphous starches, water must be preferentially absorbed in the amorphous starches relative to the native starches. This is possibly an effect of larger area of cinnamaldehyde-gel/starch in the amorphous starches to which water can absorb.

### **3.4 Starch-screening**

#### **3.4.1 Amorphous starches**

The starches with different contents of amylose and/or phosphate listed in Table 1 in the Introduction were used to examine the influence of these parameters on aroma-retention. It should, however, be pointed out that these experiments were actually performed after the aroma-binding and -retention experiments described above (Chapter 3.2 and 3.3), therefore a

repetition may provide a different result. A very high water absorbance was observed for the amorphous starches, which is reflected in the results which are very erratic (Table 5), i.e. the retention of ethyl butyrate is minus 16% for wheat starch in one end of the scale, and 130% retention for citral for normal potato starch in the opposite end of the scale. This water absorbance is between an equivalent of about 10  $\mu\text{L}$  of water to about 30  $\mu\text{L}$  of water in the samples, except for normal maize starch and normal potato starch which have a water absorption of -0.4  $\mu\text{L}$  and 60  $\mu\text{L}$ , respectively. Despite the large variations in the data, there are some apparent tendencies.

The most volatile aromas (acetaldehyde, dimethyl sulphide, diacetyl, and ethyl butyrate) still show generally low binding, though dimethyl sulphide has a relatively high retention for potato starch. Interestingly, maize starch shows a retention of about 21% for ethyl butyrate, and also shows the highest retention for the later experiments (Aroma-retention studies, Chapter 3.3), though only 8% in those studies.

When comparing the three maize starches (Table 5), it is apparent that the content of amylose (and hence also amylopectin by inverse relationship, i.e. the content of amylose plus the content of amylopectin is 100%) has little noticeable effect on retention, as both the high amylose and the high amylopectin waxy maize have generally similar retentions for the aromas. The higher retention for the normal maize is probably caused by a water absorption of close to zero, presumably flawed as the other starches have higher water absorptions. Also comparing normal maize and wheat starch shows a large variation in the retention, even though these starches only differ by a small difference in phosphate content (Table 1).

**Table 5 Percent retention of aroma in amorphous starches. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The standard deviations are the standard error of the mean of triplicates.**

<b>Percent aroma retained of added</b>	<b>Normal potato</b>	<b>High amylose + high phosphate potato</b>	<b>High amylopectin potato</b>	<b>Low phosphate potato</b>	<b>Normal maize</b>	<b>High amylose maize</b>	<b>Waxy maize</b>	<b>Normal pea</b>	<b>Normal wheat</b>
<b>Acetaldehyde</b>	0.9±0.0	-3.8±2.0	1.3±2.4	-10.9±1.8	-0.3±0.2	1.9±0.6	3.1±0.9	3.1±0.6	4.4±0.6
<b>Dimethyl sulphide</b>	12.3±0.0	-3.7±2.8	5.9±2.0	-8.6±2.1	1.5±0.3	3.1±1.1	4.5±1.8	6.9±1.8	3.9±0.3
<b>Diacetyl</b>	4.1±0.0	-2.7±1.5	-0.5±0.4	-1.8±1.4	13.7±0.8	1.8±0.2	1.4±0.3	4.2±0.1	-5.8±7.1
<b>Allylisothiocyanate</b>	20.7±0.0	21.4±8.8	40.3±7.0	51.2±3.2	47.2±4.5	54.3±3.0	57.1±2.2	49.0±4.9	26.1±11.7
<b>Ethyl butyrate</b>	7.3±0.1	2.7±3.7	7.8±5.1	-0.9±3.7	21.5±2.3	10.4±1.9	-1.7±4.7	9.4±1.1	-16.2±12.6
<b>Citral</b>	76.1±0.0	116.0±3.1	117.8±2.2	119.8±3.8	129.8±13.6	122.4±0.8	112.9±1.0	126.1±1.9	108.2±15.9
<b>Lemon oil</b>	42.1±0.0	56.0±2.5	68.8±6.0	70.5±3.9	81.0±3.8	68.6±2.5	66.6±4.4	62.7±7.2	66.5±7.1
<b>Cinnamaldehyde</b>	81.6±0.1	103.3±1.8	112.7±1.6	102.4±0.6	119.4±3.6	113.6±1.7	108.0±1.3	110.1±0.4	108.7±6.6
<b>Octanol</b>	50.7±0.1	89.0±3.3	99.9±2.5	98.1±1.5	113.6±1.4	99.3±2.9	97.5±1.3	99.6±0.9	89.2±25.1
<b>Butyric acid</b>	64.8±0.0	75.6±2.0	67.0±11.9	78.2±1.6	103.3±2.8	85.2±1.1	85.2±1.1	85.7±0.4	85.6±4.2

For the potato starches, the high amylose and high phosphate starch, and the low phosphate starch show comparable retentions, except for allyl isothiocyanate and lemon oil, indicating that for the other aromas, the phosphate content is of little significance, because these two starches have similar amylose content (Table 1). It is, however, not clear if the amylose content has any effect on retention for the amorphous potato starches. However, given the large variations within these data, interpretations must be taken with caution.

### **3.4.2 Native starches**

The native starches all show a water absorption of between an equivalent of 40 to 60  $\mu\text{L}$  of water, which is substantially higher than the water absorption for the amorphous starches of between 10 to 30  $\mu\text{L}$  of water.

It appears that high amylose maize is slightly better at retaining the most volatile aromas (acetaldehyde, dimethyl sulphide, diacetyl, and ethyl butyrate) than both the normal maize and the high amylopectin waxy maize, indicating an effect of amylose content on aroma retention (Table 6). Though for the rest of the aromas there is no observable effect on retention for the maize starches. Instead normal maize shows a marginally better retention than the other two maize starches.

However, despite the possible effect of amylose seen for the maize starches, no such effect is observed for the potato starches. The high amylose and high phosphate starch and the high amylopectin starch show roughly similar retentions, except for dimethyl sulphide and allyl isothiocyanate. Nor does it appear to be an effect of phosphate as the normal potato starch and the high amylopectin starch have similar phosphate contents, but show retentions equivalent to the high amylose and high phosphate starch with much higher phosphate content (Table 1). However, as for the amorphous starches, the variation is too large to make conclusive arguments towards favorable retention as a function of either amylose or phosphate content.

**Table 6 Percent retention of aroma in native starches. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The standard deviations are the standard error of the mean of triplicates.**

<b>Percent aroma retained of added</b>	<b>Normal potato</b>	<b>High amylose + high phosphate potato</b>	<b>High amylopectin potato</b>	<b>Low phosphate potato</b>	<b>Normal maize</b>	<b>High amylose maize</b>	<b>Waxy maize</b>	<b>Normal pea</b>	<b>Normal wheat</b>
<b>Acetaldehyde</b>	10.3±0.9	18.8±12.0	6.8±0.4	-4.9±1.2	-18.1±7.3	-3.8±1.3	-18.6±6.4	-5.6±1.6	24.6±8.8
<b>Dimethyl sulphide</b>	35.6±6.6	20.5±2.5	20.2±2.4	15.8±3.2	-5.8±1.0	1.8±2.0	-4.2±2.7	8.3±1.7	24.4±0.4
<b>Diacetyl</b>	13.8±4.4	11.2±0.5	13.2±0.6	7.8±0.4	-5.6±0.4	1.6±1.0	-5.3±3.0	2.4±0.5	8.0±0.2
<b>Allylisothiocyanate</b>	24.5±5.3	33.1±1.6	49.3±1.8	29.2±1.3	41.7±1.7	38.0±1.4	38.8±3.0	36.0±0.6	43.5±6.1
<b>Ethyl butyrate</b>	23.6±1.6	15.4±1.3	16.0±1.2	6.5±1.1	-0.6±1.3	6.0±3.8	-0.8±2.5	4.4±2.9	12.8±0.7
<b>Citral</b>	140.2±1.5	88.9±0.9	91.9±0.3	83.7±0.8	96.0±2.1	81.6±0.4	74.5±0.8	103.9±0.8	118.7±3.0
<b>Lemon oil</b>	56.1±3.9	62.4±3.1	66.3±3.0	55.5±4.5	52.7±5.8	56.8±1.9	58.6±5.1	63.2±2.0	67.1±1.8
<b>Cinnamaldehyde</b>	121.9±0.7	92.0±1.3	95.0±0.5	85.0±0.9	100.2±2.0	101.8±3.0	96.3±3.3	91.7±1.2	108.3±2.2
<b>Octanol</b>	116.6±0.4	84.9±2.1	86.5±0.5	74.2±1.1	90.6±4.5	81.9±3.3	71.5±3.1	76.1±3.0	94.0±2.1
<b>Butyric acid</b>	78.4±0.5	83.6±0.6	85.2±0.7	69.8±1.1	80.7±2.2	75.7±1.3	76.2±4.0	76.5±2.2	82.7±3.1

### 3.5 Saturation of starch with aroma

During the aroma-experiments, an effect of the density of aroma was noticed, i.e. a starch with a low density absorbed the aroma much more readily than a starch with a higher density. So rather than adding a limited, but still very high, amount of aroma to the starch, adding an extreme amount of aroma to the starches will reveal the effects of saturating the starch with aroma. For example, 200 mg of a native starch has a volume of approximately 0.5 mL, whereas an amorphous starch has a volume of approximately 4 mL. This undoubtedly reflects a larger surface area to which the aroma can be absorbed. However, the question remains if the retention is improved, keeping in mind that the results above showed that relative to a control the retention dropped for the least volatile aromas, but increased for the most volatile aromas.

**Table 7 Retention of aromas in amorphous aroma-saturated starches. 500  $\mu$ L aroma was added to 50 mg starch. Due to limitations in aroma supply, experiments were only performed in duplicate.**

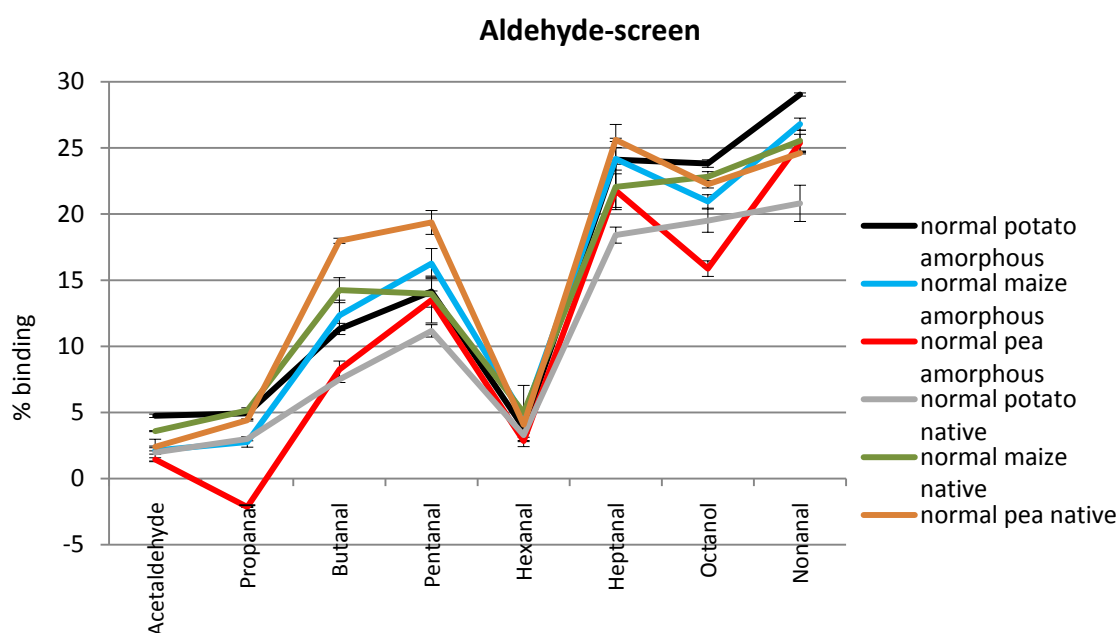
	Control	Amylose	Potato	Maize
Acetaldehyde	0.3 $\pm$ 0.0	0.7 $\pm$ 0.2	0.7 $\pm$ 0.0	0.9 $\pm$ 0.0
Dimethyl sulphide	1.9 $\pm$ 0.1	6.5 $\pm$ 0.5	2.2 $\pm$ 0.0	7.3 $\pm$ 0.0
Diacetyl	5.1 $\pm$ 0.0	4.9 $\pm$ 0.1	5.4 $\pm$ 0.1	5.2 $\pm$ 0.0
Allyl isothiocyanate	78.2 $\pm$ 0.8	72.1 $\pm$ 1.1	78.5 $\pm$ 0.0	69.2 $\pm$ 2.2
Ethyl butyrate	12.3 $\pm$ 0.1	5.3 $\pm$ 0.1	6.4 $\pm$ 0.1	6.6 $\pm$ 0.6
Citral	100.3 $\pm$ 0.3	100.2 $\pm$ 0.8	99.9 $\pm$ 0.0	100.4 $\pm$ 0.4
Lemon oil	87.4 $\pm$ 0.1	84.9 $\pm$ 0.1	86.0 $\pm$ 0.4	80.3 $\pm$ 2.9
Cinnamaldehyde	100.8 $\pm$ 0.0	87.5 $\pm$ 13.6	106.5 $\pm$ 0.2	101.3 $\pm$ 0.1
Octanol	99.2 $\pm$ 0.3	97.8 $\pm$ 0.1	125.5 $\pm$ 26.4	97.4 $\pm$ 0.1
Butyric acid	95.3 $\pm$ 0.1	93.7 $\pm$ 0.0	94.7 $\pm$ 0.5	82.5 $\pm$ 9.4

There are no clear differences or trends apparent for these data (Table 7), and all starches appear to retain all aromas equally well almost as well as compared to the control, though there are small differences for dimethyl sulphide and ethyl butyrate. The retention of dimethyl sulphide to amylose and maize starch is about five percent higher than for the control and potato starch. For ethyl butyrate the control shows the largest retention, whereas the starches show all show a retention of about half the retention of the control.

### 3.6 Aldehyde-screening

#### 3.6.1 Aldehyde-binding

Because the aroma-binding results are not easy to explain, a screening of aldehydes from ethanal (acetaldehyde) to nonanal was performed with the purpose of examining the effect of molecular size with only one chemical group. Methanal (formaldehyde) was omitted, because the highest purity obtainable from the supplier was only 37% which is insufficient for this type of analysis. The experiments were performed both on potato, maize and pea starches that were either in their amorphous and on native state.



**Figure 12** The percent binding of the aldehydes acetaldehyde (ethanal) through nonanal to potato, maize, and pea starch in both amorphous and native states. Note the sharp drop in the binding that occurs at hexanal. Binding is defined as weight of aroma per total weight of starch including aroma. The error bars are the standard error of the mean of triplicates.

##### 3.6.1.1 Amorphous starches

An interesting trend is seen for the amorphous starches: disregarding hexanal, two sets of data appear – one for the aldehydes with an even number of carbons (acetaldehyde, butanal, octanal) and one for the aldehydes containing an odd number of carbons (propanal, pentanal, heptanal, nonanal; see Table 8 and Figure 27). The effect is most noticeable when comparing the amorphous starches with aldehydes with an odd number of carbons and amorphous starches with aldehydes with an even number of carbons. The effect is not present for the native starches, except for maize starch, as the curves for the native starches with aldehydes with an odd number of carbons almost intersects the curves for the native starches with aldehydes with an even number of carbons. However, hexanal stands out noticeably from this trend, as the binding drops to between three and five percent, compared to about 15% for pentanal and 24% for heptanal.



**Table 8 Percent binding of a series of aldehydes, from acetaldehyde with two carbons to nonanal with nine carbons, to amorphous potato, maize and pea starches. Binding is defined as weight of aroma per total weight of starch including aroma. The standard deviations are the standard error of the mean of triplicates.**

	Acetaldehyde	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal
<b>Potato</b>	4.7±0.1	4.9±0.4	11.3±0.4	14.1±1.2	3.8±0.1	24.1±2.7	23.8±0.3	29.0±0.1
<b>Maize</b>	2.1±0.8	2.8±0.4	12.3±1.2	16.3±1.1	4.7±2.3	24.2±0.9	21.0±0.5	26.8±0.5
<b>Pea</b>	1.5±0.1	-2.2±0.0	8.2±0.6	13.5±1.7	2.8±0.0	21.8±1.3	15.9±0.6	25.4±0.7

### 3.6.1.2 Native starches

The binding of aldehydes to the starches increases as the aldehyde increases in length, even though the binding seems to decrease a few percent for pea starch with octanal and nonanal (Table 9). However, like the amorphous starches, hexanal shows a remarkably lower binding than the rest of the aldehydes.

**Table 9 Percent binding of a series of aldehydes, from acetaldehyde with two carbons to nonanal with nine carbons, to native potato, maize and pea starch granules. Binding is defined as weight of aroma per total weight of starch including aroma. The standard deviations are the standard error of the mean of triplicates.**

	Acetaldehyde	Propanal	Butanal	Pentanal	Hexanal	Heptanal	Octanal	Nonanal
<b>Potato</b>	2.0±0.1	3.0±0.1	7.5±0.2	11.2±0.3	3.3±0.2	18.4±0.6	19.5±0.9	20.8±1.4
<b>Maize</b>	3.6±0.0	5.1±0.0	14.3±0.9	14.0±0.2	5.0±0.2	22.1±1.7	22.8±0.4	25.6±0.8
<b>Pea</b>	2.4±0.1	4.4±0.1	18.0±0.2	19.4±0.9	4.1±0.2	25.6±0.1	22.3±0.3	24.6±0.1

### 3.6.2 Aldehyde-retention

When comparing the percentage of aldehyde retained relative to the amount added (Table 10), it becomes apparent that a trend similar to the aroma binding appears, i.e. the most volatile compounds (acetaldehyde to hexanal) are better retained by the starches relative to the control, whereas the less volatile (heptanal to nonanal) are, generally speaking, less retained by starches relative to the control.

Hexanal, which also acts as a low-volatility compound, shows a reduced retention relative to both pentanal and heptanal. There is no substantial difference in retention between amorphous and native starches. To remove doubts of the identity of the contents of the hexanal bottle, nuclear magnetic resonance was performed and confirmed the identity.

The amorphous pea starch shows a negative retention for propanal, but a higher than average water absorption observed for the negative control used for subtracting water content, may have skewed the results.

There is not substantial difference between the retention of aldehydes with amorphous or native starches up to pentanal, but for heptanal and up the amorphous starches show a generally better retention.

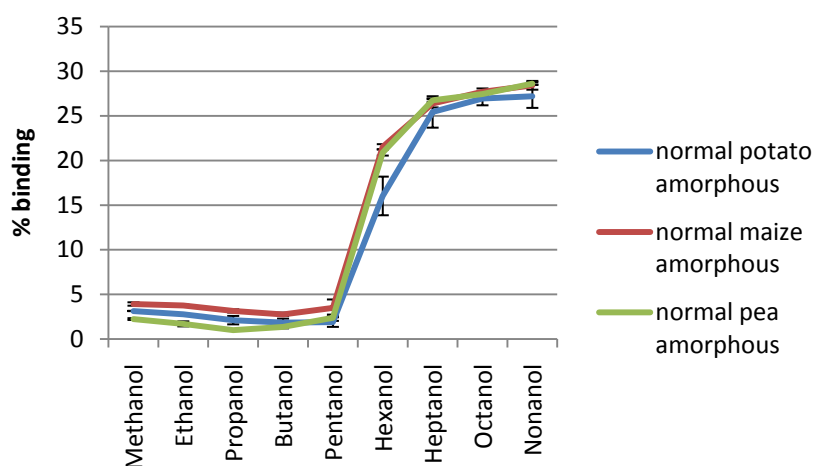
**Table 10 Percentage retention of aldehydes added to either amorphous or native starch. Hexanal shows a different behaviour than the aldehydes of similar length, e.g. pentanal and heptanal. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The standard deviations are the standard error of the mean of triplicates.**

Percent retained of added aroma	Amorphous starch				Native starch		
	Control	Potato	Maize	Pea	Potato	Maize	Pea
<b>Acetaldehyde</b>	0.5±0.2	8.7±0.6	3.8±1.2	2.4±0.1	4.0±0.2	6.1±0.3	4.6±0.2
<b>Propanal</b>	4.5±0.3	13.8±1.1	7.7±0.7	-6.4±0.3	7.6±0.3	15.3±0.2	12.6±0.2
<b>Butanal</b>	16.5±0.8	30.0±0.8	35.1±3.0	29.0±6.4	18.7±0.6	40.5±2.9	51.2±0.7
<b>Pentanal</b>	28.6±0.9	38.1±3.2	46.6±2.9	39.7±4.4	29.2±1.2	36.6±0.7	58.3±4.8
<b>Hexanal</b>	5.7±0.1	9.2±0.3	12.8±5.2	7.4±0.2	8.0±0.5	12.4±0.3	9.9±0.2
<b>Heptanal</b>	68.4±0.5	74.0±1.2	75.4±2.9	66.9±3.0	52.9±0.9	60.9±1.7	79.1±1.6
<b>Octanal</b>	75.7±0.4	70.6±0.7	61.8±0.4	45.3±2.5	55.2±1.1	66.6±1.3	62.3±1.1
<b>Nonanal</b>	88.0±0.8	97.0±0.6	86.7±1.8	81.9±0.8	61.4±3.5	77.0±1.8	73.8±0.7

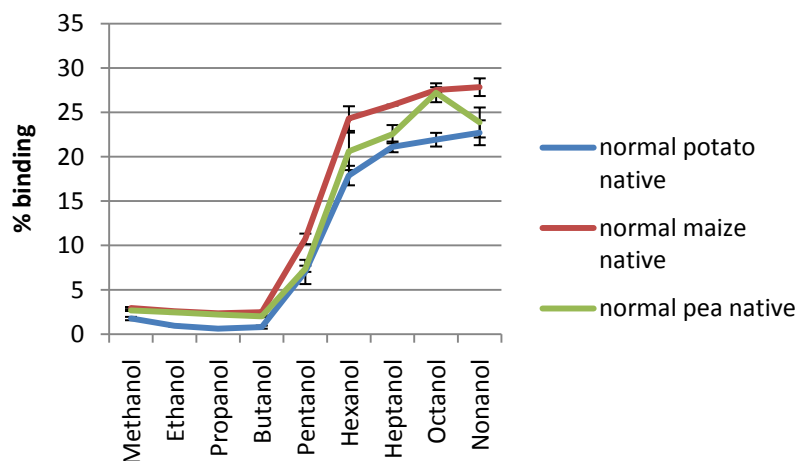
### **3.7 Alcohol-screening**

#### **3.7.1 Alcohol-binding**

In a similar way to the aldehyde-screen, an alcohol-screening was performed using alcohols from methanol to nonanol. Like the aldehyde screen, these experiments were performed both on potato, maize and pea starches in an amorphous or native state.



**Figure 13** Percent binding of alcohols to amorphous potato (blue), maize (red), and pea (green) starches. Binding is defined as weight of aroma per total weight of starch including aroma. The error bars are the standard error of the mean of triplicates.



**Figure 14** Percent binding of alcohols to native potato (blue), maize (red), and pea (green) starches. Binding is defined as weight of aroma per total weight of starch including aroma. The error bars are the standard error of the mean of triplicates.

### 3.7.1.1 Amorphous starches

There is a small decrease in the binding taking place for methanol up to butanol (Figure 13 and Table 11). However, the apparent binding is very low, so there is a relatively high level of uncertainty due to the low values, despite similarly low standard errors. At pentanol the binding is roughly similar to that of butanol. From hexanol to nonanol the binding increases gradually.

None of the starches stands out noticeably from the others, except for potato starch with hexanol, which shows a slightly lower binding than the others.

**Table 11 Percent binding of alcohols to amorphous starches. Binding is defined as weight of aroma per total weight of starch including aroma. The standard deviations are the standard error of the mean of triplicates. Note the shift in binding occurring between pentanol and heptanol.**

	Methano	Ethano	Propano	Butano	Pentano	Hexano	Heptano	Octanol	Nonano
<b>Potat</b>	3.1±0.0	2.8±0.1	2.1±0.5	1.8±0.4	1.9±0.5	16.0±2.	25.4±1.8	26.9±0.	27.2±1.3
<b>Maize</b>	3.9±0.2	3.8±0.1	3.1±0.2	2.8±0.2	3.5±0.9	21.5±0.	26.4±0.5	27.6±0.	28.4±0.5
<b>Pea</b>	2.2±0.1	1.7±0.3	1.0±0.1	1.4±0.2	2.4±0.3	20.9±0.	26.7±0.2	27.2±0.	28.6±0.2

### 3.7.1.2 Native starches

As seen for the amorphous starches it seems that a very small decrease of the apparent binding of the shortest alcohols is taking place (Figure 14 and Table 12). Even though this decrease is very small, it is present for the three starches tested, both amorphous and native.

At pentanol the binding starts to increase sharply from 2.5% and below for butanol to about 18% and above for hexanol. The binding of pentanol to native starches is very different to the amorphous starches (Table 11 and Table 12, respectively), as the native starches show seven to ten percent binding, but the amorphous starches only show two to four percent binding.

Native maize and pea starch shows a higher binding than potato starch, especially for pentanol and above.

**Table 12 Percent binding of alcohols to native starches. Binding is defined as weight of aroma per total weight of starch including aroma. The standard deviations are the standard error of the mean of triplicates. Note the shift in binding that occurs between butanol and hexanol.**

	Methano	Ethano	Propano	Butano	Pentano	Hexano	Heptano	Octanol	Nonano
<b>Potat</b>	1.8±0.2	0.9±0.1	0.6±0.1	0.8±0.2	7.0±1.4	17.9±1.	21.1±0.6	21.9±0.	22.7±1.4
<b>Maize</b>	2.9±0.1	2.6±0.0	2.3±0.0	2.5±0.1	10.7±0.6	24.3±1.	25.8±0.1	27.5±0.	27.8±1.0
<b>Pea</b>	2.7±0.0	2.4±0.2	2.2±0.0	2.0±0.1	7.4±0.3	20.6±2.	22.5±1.0	27.2±1.	23.8±1.7

### 3.7.2 Alcohol-retention

The behaviour of alcohols from methanol to nonanol is similar to that of both the aromas and the aldehydes (Table 4, Table 10, and Table 13). Relative to the control, both types of starches show an increase in retention for methanol through butanol. Likewise there is a drop in retention for the less volatile compounds. The amorphous starches appear better at retaining the longer alcohols (heptanol through nonanal) than the native starches, whereas there is little difference for the shorter alcohols.

However, as mentioned in Chapter 3.7.1.2 there is an interesting effect occurring at pentanol (Table 13 and Figure 41, Appendix 1 (Chapter 14.1)) where the amorphous starches still show

a relatively weak retention, whereas the native starches have a substantially better retention, although both types of starches show retentions well below that of the control.

**Table 13 Percentage retention of alcohols in potato, maize and pea starch in either an amorphous or native state. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The standard deviation are the standard error of the mean of triplicates.**

Percent retained of added aroma	Amorphous starches			Native starches			
	Control	Potato	Maize	Pea	Potato	Maize	Pea
<b>Methanol</b>	-0.2±0.0	8.8±0.3	10.7±0.4	6.0±0.2	5.0±0.6	8.2±0.3	7.3±0.2
<b>Ethanol</b>	-0.1±0.1	7.6±0.4	10.0±0.3	4.4±0.6	2.4±0.2	6.8±0.2	6.1±0.4
<b>Propanol</b>	-0.3±0.0	5.5±1.1	8.0±0.4	2.5±0.2	1.6±0.3	5.7±0.1	5.4±0.1
<b>Butanol</b>	0.0±0.1	4.7±0.8	6.9±0.4	3.4±0.3	1.9±0.4	5.9±0.3	4.8±0.3
<b>Pentanol</b>	46.1±1.5	4.9±1.3	8.8±2.1	5.9±0.7	18.1±2.8	29.3±1.1	18.3±0.8
<b>Hexanol</b>	80.1±0.3	47.3±4.3	65.9±0.2	63.9±1.3	50.7±1.9	69.0±3.3	57.5±6.6
<b>Heptanol</b>	92.0±0.8	86.4±3.0	87.0±0.1	88.9±0.5	64.5±1.4	83.3±0.8	71.9±3.6
<b>Octanol</b>	95.7±0.5	94.6±1.2	95.8±0.7	95.0±0.5	69.8±1.9	91.1±0.7	89.9±6.9
<b>Nonanol</b>	97.1±0.5	97.1±2.0	96.8±0.4	98.0±0.2	71.8±4.8	88.8±4.2	77.7±7.9

### ***3.8 Multiple headspace extraction of aromas from starch***

#### **3.8.1 Extraction with and without water**

The purpose of performing the multiple headspace experiments was to confirm the aroma binding experiments, as the amount of aroma bound to the starches is not necessarily the same as the amount of aroma that can be released. However, a correlation might exist between the amounts of aroma observed to be bound in the aroma binding experiments and the amounts of aroma released during the headspace experiments.

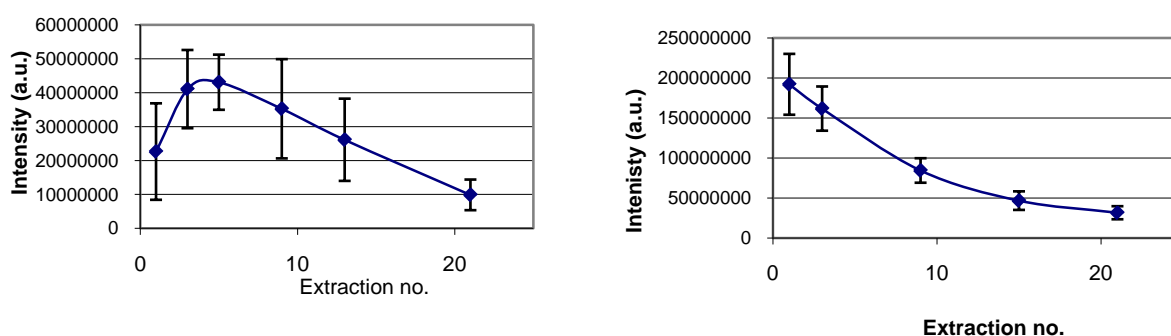
At first all ten aromas listed in Table 3 were tested, but because octanol repeatedly overloaded the GC-MS, octanol was omitted from further analysis. Also acetaldehyde was not positively identified during the data analysis following the extraction, i.e. the identification score was three with 70 being the threshold for a confident identification. This is partly due to its molecular weight of 44 g/mol, which is identical to atmospheric carbon dioxide, and partly due its very low boiling point, which gives very low adsorption of the columns used to trap the aromas. Also the low amount of acetaldehyde bound in the starches may either be too low

for detection or may have evaporated during the time from the binding experiments were completed until the headspace experiments were started.

Another highly volatile compound, dimethyl sulphide, was detected for up to 90 min after the extraction was started, but since heptane was used as solvent for the standards, dimethyl sulphide could not be quantified, as the GC-MS peak of dimethyl sulphide in the standard was hidden by the heptane peak.

The experiments were initially performed with and without water (see Materials and Methods, Chapter 2.3) present during the extraction phase. It was expected that the experiments performed with water present should show a higher extraction yield, because water was expected to disturb the starch matrix, thereby releasing more aroma into the headspace. In the following the extractions performed in the absence of water will be referred to as dry, and the extractions performed in the presence of water will be referred to as wet.

The results seen from the wet extractions show a much larger experimental uncertainty than the data from the dry extractions (Figure 15). However, this is not unexpected as the reproducibility of the experiments is lowered when water is added to the system, due to added complexity and risk of inhomogeneous distribution of water in the sample (personal communication with Mikael Agerlin Petersen).



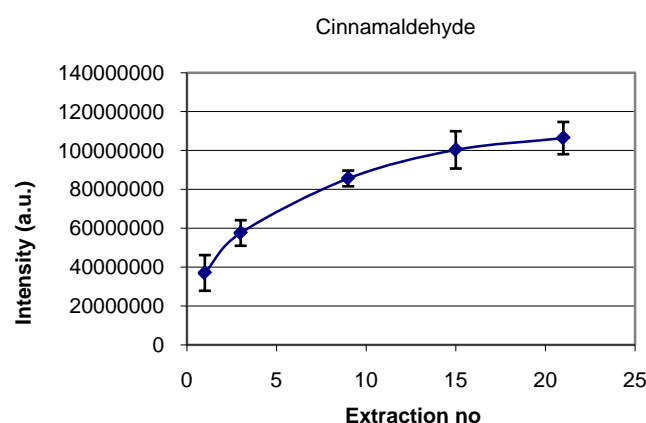
**Figure 15** Raw data of the wet (left) and dry (right) experiment of diacetyl with the intensity of the extraction in arbitrary units (a.u.) plotted against the extraction number. Note the difference in the development of the curve and the difference in the size of the error bars.

The results of the dry and wet extractions are listed in Table 14. For most aromas the dry extraction gives a two-to-four fold higher extraction yield than the wet extraction. The large experimental uncertainty of the wet experiments does not change the fact that there is a substantial difference between the two types of extraction.

Cinnamaldehyde shows a different behaviour during the MHE than the other aromas (Figure 16). As the extraction progresses, for the dry and non-enzymatic (described later in Chapter 3.8.3) samples, the intensity of cinnamaldehyde continues to increase; therefore no amount can be calculated, as the calculations require an exponentially decreasing intensity. For the wet and enzymatic (described in Chapter 3.8.3) samples a similar effect is observed, but a small drop in intensity is observed after approximately two hours of extraction. Thus, because of this behaviour the results for cinnamaldehyde must be taken with care.

**Table 14** Comparison of the amount of aroma in nL obtained on a dry sample set and a wet sample set (only water added). Estimated amount of aroma calculated using the standard curve using the highest dilution-factors of aroma. N.D. not determined. The conversion from nL of aroma to  $\mu\text{g}$  is done under the assumption that the density of the all the aromas is identical to water.

	Dry (nL aroma = $\mu\text{g}$ )	Wet (nL aroma = $\mu\text{g}$ )
<b>Diacetyl</b>	126.7	53.36
<b>Allyl isothiocyanate</b>	41.40	24.78
<b>Ethyl butyrate</b>	17.67	4.621
<b>E-citral</b>	115.1	31.69
<b>Z-citral</b>	129.1	36.11
<b>Cinnamaldehyde</b>	N.D.	107.3
<b>Butyric acid</b>	2493	81.96

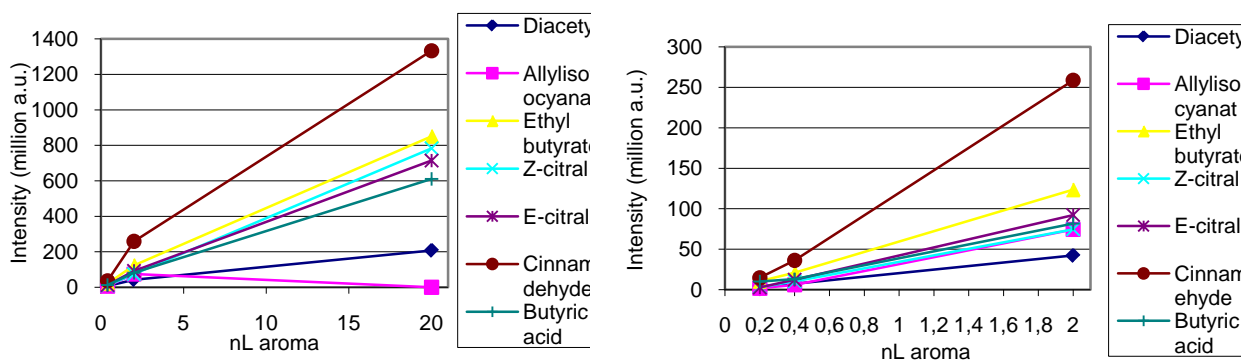


**Figure 16** Raw data of the extraction of cinnamaldehyde in a dry environment. Intensity in arbitrary units (a.u.) is plotted as a function of the extraction number. For cinnamaldehyde 100,000,000 intensity units is roughly equal to one nano-litre of pure aroma.

### 3.8.2 Making a standard curve

The amounts observed by multiple headspace extraction are a factor of 1,000 below the amounts seen in the aroma-binding and retention experiments (Chapter 3.2 and 3.3) when the calculations are based on a single 1,000-fold dilution of aroma as standard. In other words, the amounts seen by multiple headspace extraction are in the  $\mu\text{g}$ -range, whereas the initial binding experiments are in the mg-range. To further clarify this difference, rather than just using single aroma dilution for quantifying results, a series of dilutions was made.

The normal dilution used for quantification of aroma is 1,000-fold. The dilutions used for making the standard-curve were 100-fold, 1,000-fold, 5,000-fold, and 10,000-fold. A kink is observed in the standard-curve when going from the lowest dilution-factor to the higher dilution-factors (Figure 17).



**Figure 17** Graphical representation of the whole standard-curve (left) and a curve created using the three most dilute concentrations of aroma (right). The 100-fold dilution is omitted from the rightmost curve, because it masks the subtleness of the curve at the highest dilutions.

Because the analysed samples show intensities similar to the intensities observed for the highest dilutions, a standard-curve was made using only the most dilute concentrations. The choice of basing calculations on a 100-fold dilution, a 5,000-fold dilution, the whole standard-curve, or just a part of the standard-curve made from the most dilute dilutions of aromas, has a substantial impact on the calculation of amount of aroma (data not shown).

### 3.8.3 $\alpha$ -amylase treatment

During mastication, the food item is subjected to physical and chemical forces. The grinding of the food between the teeth disrupts the physical structure, and the saliva contains enzymes, e.g.  $\alpha$ -amylase, which degrades the starch into oligo-saccharides. This disruption of the structural integrity of the food increases the speed of the aroma release.

To test if adding  $\alpha$ -amylase would increase the amount of aroma released, a single experiment was performed (enzymatic). For comparison purposes a parallel experiment was done without both water and enzyme (non-enzymatic), where identical samples were analysed simultaneously. The results are listed in Table 15. The results from the above-mentioned dry and wet experiments (Chapter 3.8.1) are listed alongside these results.

**Table 15** Comparison of the four experiments performed on aroma release. Analysis of the dry and non-enzymatic experiment were both performed without the presence of water, while wet and enzymatic were performed in the presence of water and buffer plus enzyme, respectively.

(nL aroma= $\mu$ g)	Dry	Wet	Non-enzymatic	Enzymatic
<b>Diacetyl</b>	126.7	53.36	92.4	42.8
<b>Allyl</b>	41.40	24.78	2.60	22.1
<b>Ethyl butyrate</b>	17.67	4.621	N.D.	2.65
<b>E-citral</b>	115.1	31.69	26.0	31.4
<b>Z-citral</b>	129.1	36.11	24.9	27.4
<b>Cinnamaldehyde</b>	N.D.	107.3	N.D.	940
<b>Butyric acid</b>	2493	81.96	271	69.4



The experimental conditions used for the wet and the enzymatic experiments are identical except for the presence 50 mM of a potassium phosphate buffer and enzyme. The amount of both diacetyl and butyric acid released is lower for the enzyme-treated sample than the non-treated sample. For the other aromas (allyl isothiocyanate, ethyl butyrate, citral, cinnamaldehyde), there is an increase in the amount released for the enzyme-treated sample, except for ethyl butyrate and cinnamaldehyde where the amount released from the non-treated sample can not be calculated.

Also the experimental setup in the dry and non-enzymatic is identical. However, there is a difference in the amounts extracted, which is not readily explainable.

### 3.8.4 Ether/pentane extraction

To extract as much aroma as possible from the starches, an ether/pentane extraction was made (Chapter 2.3), but the aroma extracted from this extraction medium actually shows lower values of aroma than the MHE results. Most of the aromas tested could not be detected, and those that could be detected only show an extraction of hundreds of nanograms, with the exception of diacetyl with potato starch, which show an extraction of micrograms (Table 16).

**Table 16 Amounts of aroma extracted from the ether/pentane extraction. Only diacetyl, citral, and butyric acid are found in the extraction. The other aromas and the internal standard could not be detected. The amount of aroma extracted was calculated by comparing it to a known amount of aroma dissolved in heptane.**

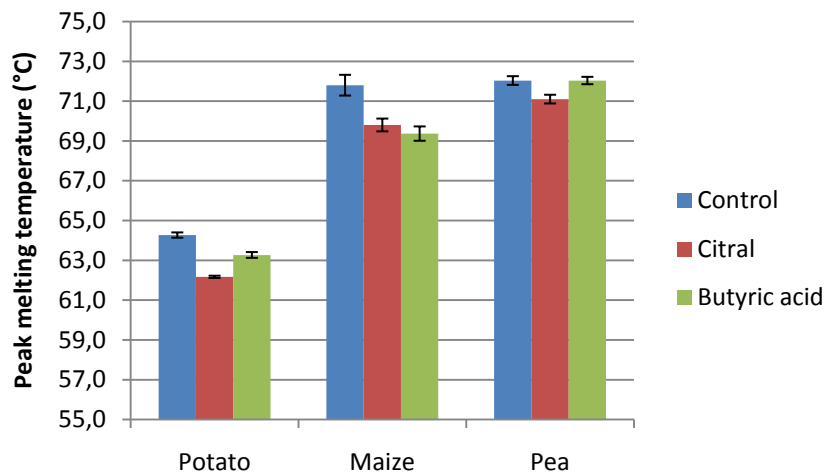
Amount of aroma recovered ( $\mu\text{L}$ )	Potato	Maize	Pea
<b>Diacetyl</b>	16.3	0.2	0.1
<b>E-citral</b>	0.4	0.009	0.009
<b>Z-citral</b>	0.4	0.1	0.1
<b>Butyric acid</b>	0.4	0.1	0.1

## 3.9 DSC on aroma-starch complexes

### 3.9.1 Initial experiments (KU-LIFE)

Experiments with DSC was initially performed at KU-LIFE by PhD-student Susanne Langgård Jensen on native potato, maize, and pea starch mixed with either acetaldehyde, dimethyl sulphide, diacetyl, allyl isothiocyanate, ethyl butyrate, citral, or butyric acid.

The addition of citral affects the melting temperature of all three starches, though less pronounced for pea starch (Figure 18). The effect of butyric acid is less pronounced for potato starch, but for maize starch butyric acid shows a larger reduction in melting temperature than citral. The reduction in melting temperature for pea starch with butyric acid is not significant.

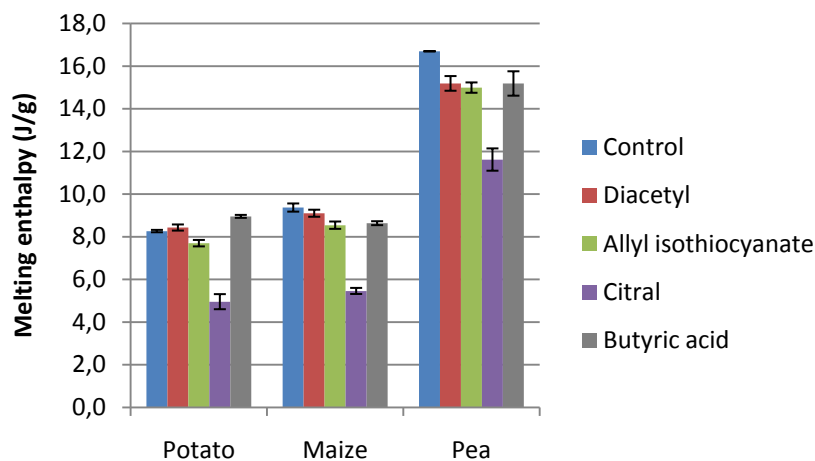


**Figure 18** Graphical representation of the peak melting temperature of potato, maize, and pea starch granules with either no aroma (control), citral, or butyric acid added. The error bars are the standard error of the mean of triplicates.

No effect on melting enthalpy or temperature was observed on starches with acetaldehyde, dimethyl sulphide, diacetyl, or ethyl butyrate (data not shown), though pea starch does show a reduction in melting enthalpy of 1.5 J/g for diacetyl (Figure 19).

Citral has a large influence on the melting enthalpy of all three starches (3.3 J/g, 3.9 J/g, and 5.1 J/g for potato, maize, and pea, respectively). Relative to the control diacetyl shows a reduction in melting enthalpy for pea starch, though not for the other two starches. Allyl isothiocyanate shows a reduction for all three starches (0.6 J/g, 0.9 J/g, and 1.7 J/g for potato, maize and pea starch, respectively). Butyric acid increases the melting temperature of potato starch (+0.7 J/g), but a reduction for maize and pea starch (0.8 J/g and 1.5 J/g, respectively).

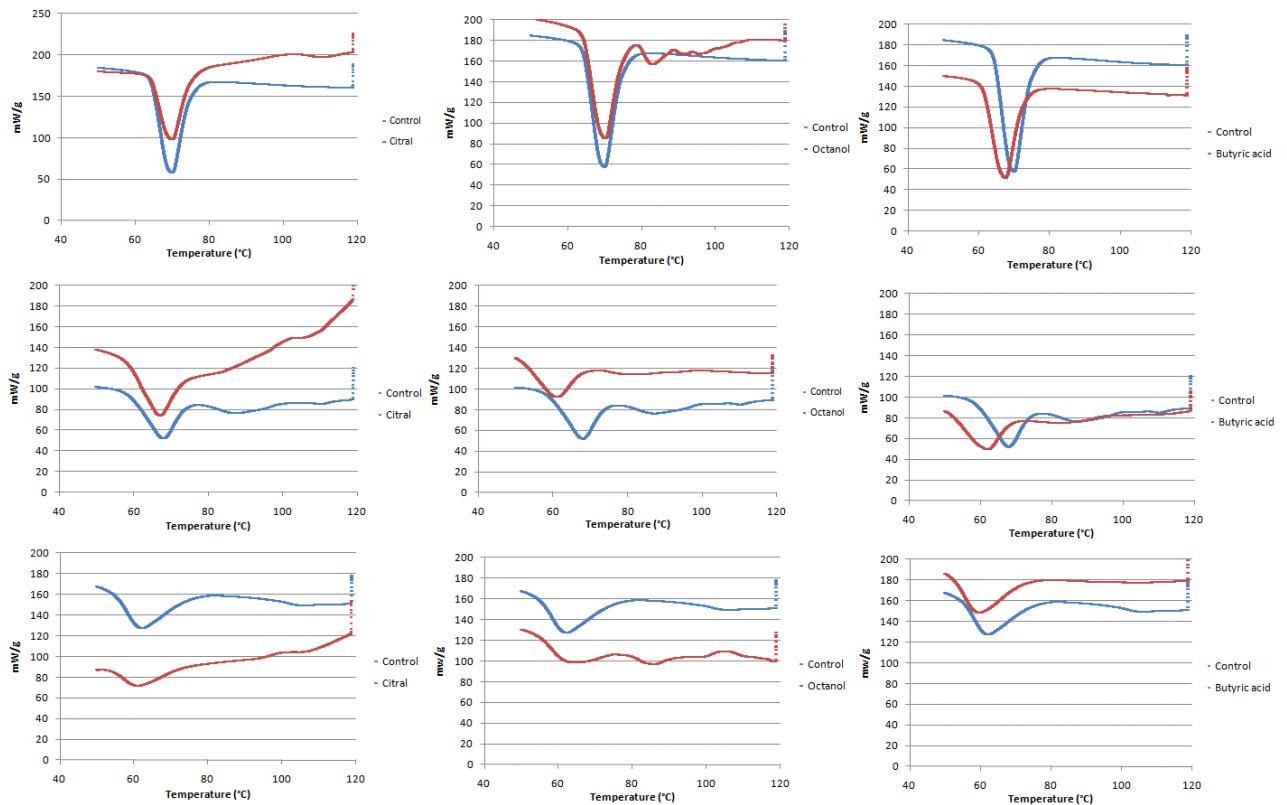
Amorphous starches with aroma were also tested, but no endotherms were detected (data not shown).



**Figure 19** Graphical representation of the melting enthalpy (Joules per gram starch) of potato, maize, and pea starch with either no starch (control), diacetyl, allyl isothiocyanate, citral, or butyric acid. The error bars are the standard error of the mean of triplicates.

### 3.9.2 Further experiments (INRA)

DSC on starch with citral shows a small reduction in the melting temperature, and a broad peak occurring at after the gelatinisation peak (around 70 °C) at about 100 °C (Figure 20). It is unclear why there is a continuous increase in heat flow for maize starch with citral, though pea starch also shows this increase. For octanol there is a small endotherm (reduction in heat flow) for all three starches occurring at around 90 °C. This may indicate unfolding of starch/octanol-complexes. There is a large drop in the gelatinisation temperature for maize starch with octanol. There is a large reduction in melting temperature for maize starch with butyric acid, and smaller reductions for potato and pea starch. A small endotherm is also observed for maize at around 90 °C indicating the unfolding of a complex.

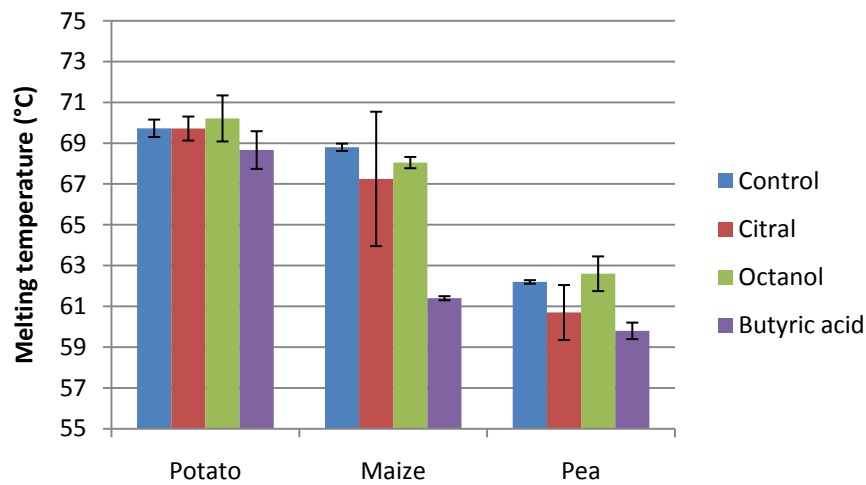


**Figure 20** DSC data performed on native starch granules with the heat flow (milliwatts per gram starch) as a function of temperature (°C). Analysis was done on potato (top), maize (middle), and pea starch (bottom) with aromas citral (left column), octanol (middle column), and butyric acid (right column). The DSC endotherms of starch/aroma mixtures are compared to a control starch (blue line in all DSC endotherms) without aroma. Only a single endotherm is depicted per mixture because there is a large variation in the absolute scale of the heat flow of the duplicates.

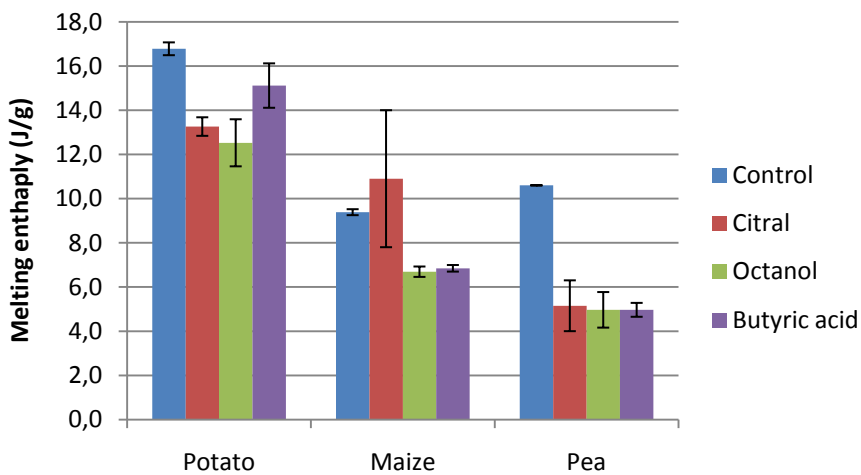
A large variation between the two maize starch samples mixed with citral is seen in both the peak melting temperature (Figure 21) and the melting enthalpy (Figure 22), represented by the large error bars.

In these results, citral does not appear to affect melting temperature of any of the starches (Figure 21), which was otherwise observed in the experiments performed at KU-LIFE.

However, a noticeable effect is observed in the melting enthalpy (Figure 22), especially for maize and pea starch. Octanol shows a marginal reduction in melting temperature for maize starch (-0.6 °C), but no reduction for potato or pea starch (+0.5 °C and +0.4 °C and thus within the standard error), though all starches show reductions in melting enthalpy of respectively 4.3 J/g, 3.3 J/g, and 5.6 J/g for potato, maize, and pea (Figure 21 and Figure 22, respectively). Butyric acid shows a very large reduction in melting temperature (Figure 21) for maize starch (7.3 °C), and small reduction for pea starch (2.4 °C), and a reduction within the standard error for potato starch (0.9 °C). Also the maize and pea starches with butyric acid have substantial reductions in melting enthalpy (2.6 J/g and 5.6 J/g, respectively; Figure 22), whereas potato starch only shows a marginal reduction (1.7 J/g).



**Figure 21** The starch gelatinisation peak temperature of native potato, maize, or pea starch granules mixed with either no aroma (control), citral, octanol, or butyric acid.



**Figure 22** The starch gelatinisation melting enthalpy of potato, maize, and pea starch granules, with either no aroma (control), citral, octanol, or butyric acid.

### 3.10 Wide-Angle X-ray Scattering

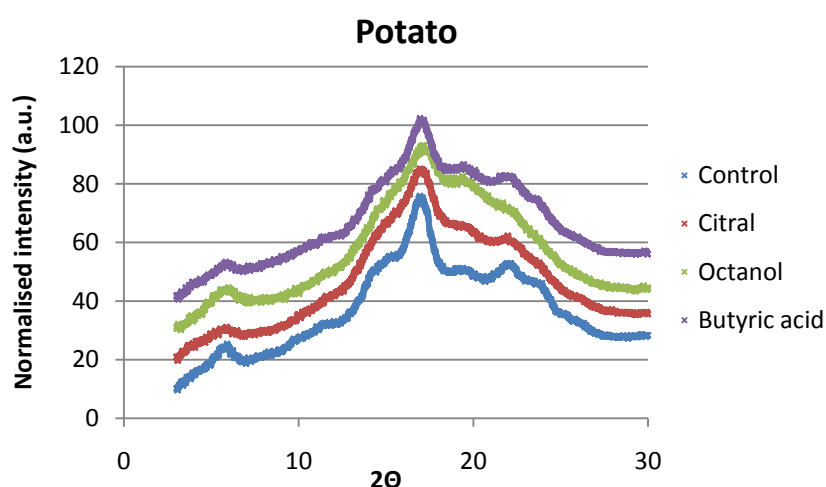
In connection with the DSC experiments performed above, X-ray analysis was performed (Wide-Angle X-ray Scattering; WAXS) on the same samples as the DSC experiments.

Several peaks are characteristic of the formation of complexes between starch and guest molecules, e.g. aromas (Arvisenet *et al.*, 2002a; Jouquand *et al.*, 2006a). In particular, peaks around 7°, 13°, and 18° are relevant for studying complex formation.

For potato starch no distinct changes are observed relative to the control. However, there are noticeable changes in the intensity of some of the peaks observed for the control. In particular, a small peak at around 18° changes in the presence of aromas, most dramatically for octanol (Figure 23). Citral shows a flattening of peaks throughout the spectrum, indicating that there are interactions that are not necessarily of the amylose-complex type. Octanol shows the same flattening, but the most prominent feature is the shoulder following the largest peak. This shoulder starts around 18° and ends at around 24°, thereby indicating that the interactions taking place between the starch and octanol affect the granule structure dramatically. The effect of butyric acid is less dramatic, though the peaks are substantially flatter than for the control, especially at around 22°.

Relative to the potato and pea spectra, the maize spectrum has more rounded peaks (Figure 23), possibly indicating heterogeneity of the possible interactions with residual lipids in the starch (Morrison and Gadan, 1987). There is hardly any effect of citral on maize starch, except a broad peak at around 5°. Octanol shows a similar shoulder as observed for potato starch at between 18° and 24°. As seen for citral there is a peak appearing at around 5°. Butyric acid does not seem to have an effect on maize starch.

The effect of citral on pea starch is limited, except for a marginal flattening of a peak at around 10°, and a shoulder at around 20° (Figure 23). Octanol still shows a very large shoulder as already seen for the potato and maize starches, but the shoulder is largest for pea starch, again indicating changes in the granule structure and integrity occurring when octanol is added to starch. As for maize starch, butyric acid does not appear to affect the spectrum, though there is a small shoulder at 20°.



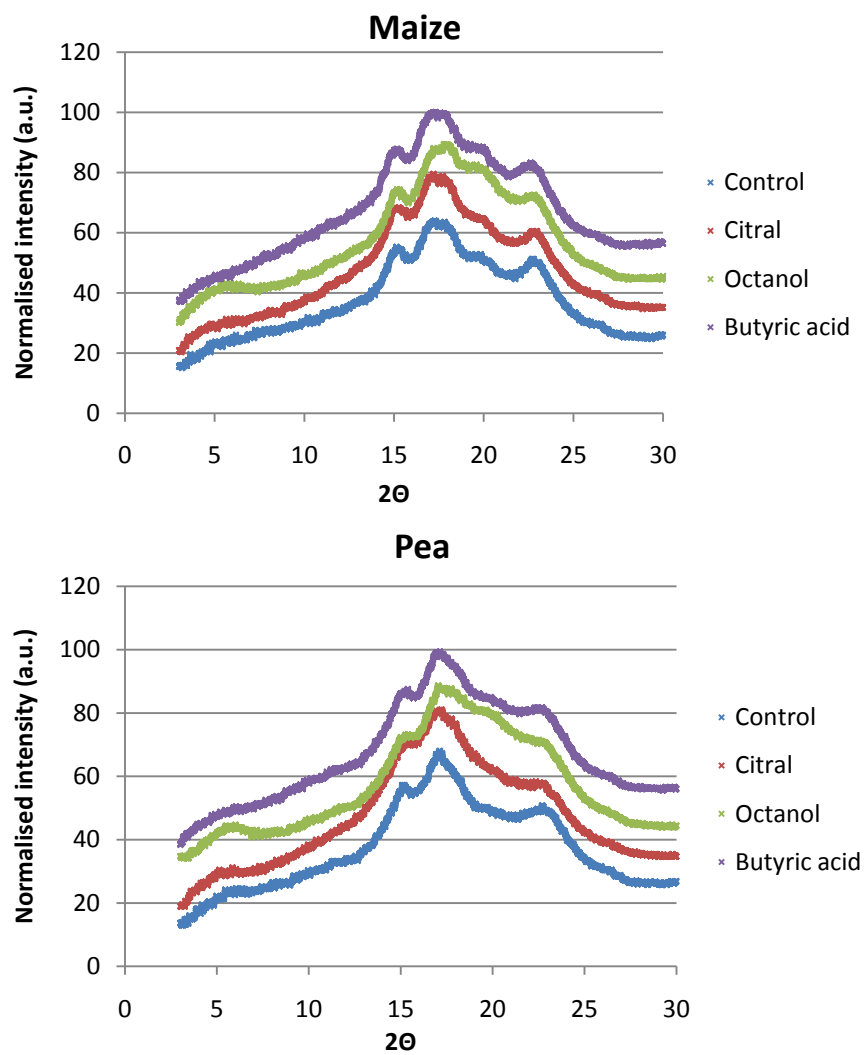


Figure 23 WAXS of potato (top), maize (middle), and pea (bottom) starch with either no aroma (control, blue), citral (red), octanol (green), or butyric acid (purple). The normalised intensity (in arbitrary units, a.u.) is plotted as a function of the diffraction angle  $2\theta$  (degrees). The curves have been shifted along the y-axis by zero, 10, 20, or 30 a.u. for control, citral, octanol, and butyric acid, respectively.

## 4 Discussion

### 4.1 Aroma binding studies

The low binding of the most volatile aromas (acetaldehyde, dimethyl sulphide, diacetyl, ethyl butyrate) is not surprising given their high vapour pressure. Likewise it is not surprising that compounds with a gradually lower vapour pressure show a gradual increase in binding, as the rate of evaporation decreases.

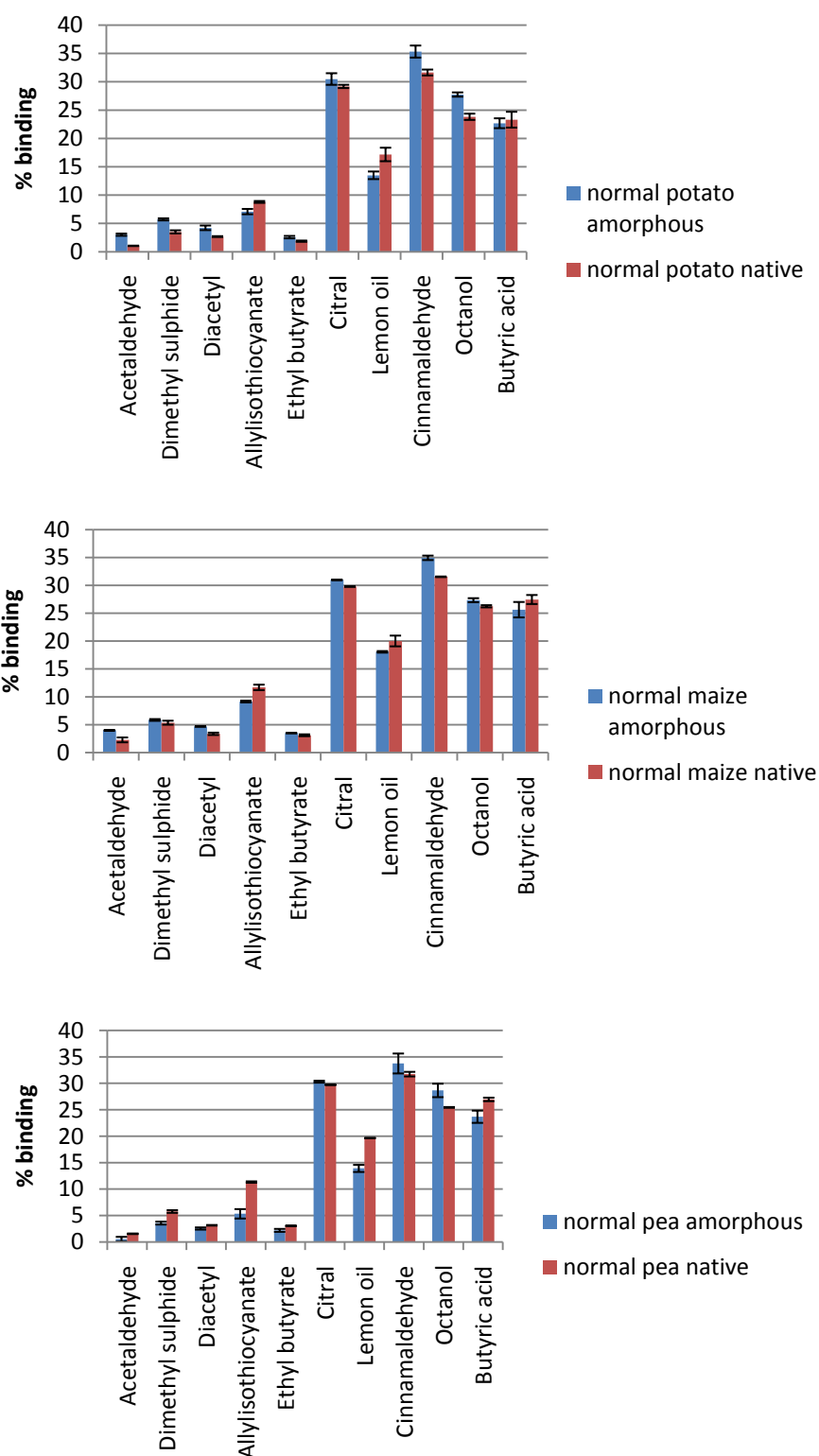
For the amorphous starches, maize starch showed a slightly better binding of the most volatile aromas than both potato and pea starch. This may be caused by either a larger surface area to adhere to or a higher content of residual lipids in the starch (Buléon, 1998; Forss, 1969).

Conversely, pea starch showed the lowest binding of the most volatile compounds of the three amorphous starches (Figure 8), though this may be the effect of a high water adsorption in the samples without aroma used for subtracting water relative to the samples with aroma. Thus the presence of aroma can hinder or slow down the water adsorption, thereby yielding a result where the weight of aroma is subtracted, giving lower results. However, it is not clear why this should only be true for pea starch, so other factors such as the crystallinity (C-type for pea starch; Imberty, 1991), or the amylose-content of the three starches influence the results and thereby cause the lower binding.

For the native starches, potato starch shows a reduced binding of aromas relative to the other starches. There is no clear explanation for this. Again, this may be due to surface area. Noticeably, potato starch has the lowest content of amylose (28% for potato, 45% for maize, and 55% for pea; Table 1), which might strongly influence the results, though the effect is absent for the amorphous starches, and maize and pea starch show roughly the same binding, despite a higher content of amylose in pea starch.

For the aromas allyl isothiocyanate, lemon oil, and butyric acid, the native starches are better at binding than the amorphous starches (Figure 24), with the exception of butyric acid for potato starch. However, the starches mixed with allyl isothiocyanate, gradually gain a browned appearance over the course of the experiments, so any interpretation of the results of this compound must be taken with precautions. The larger binding of lemon oil and butyric acid may be caused by diffusion of these compounds into the starch granule, thereby preventing the aromas from evaporating. Butyric acid is relatively small and hydrophilic, and lemon oil contains many different chemicals of many sizes and hydrophobicities, whereas citral, cinnamaldehyde, and octanol are large and very hydrophobic, thus preventing them from diffusing through the layers of the starch granule.

The difference in binding for octanol where the amorphous starch has a higher binding is probably due to a larger surface area of the amorphous starch relative to the native. A larger surface area will give a larger area from which octanol can evaporate. The higher binding for cinnamaldehyde with amorphous starches (Figure 10, Figure 11, and Figure 24) will be discussed in the next section.



**Figure 24 Comparison of the binding of aromas to the three starches (top: potato; middle: maize; bottom: pea) in either an amorphous or native state. Binding is defined as weight of aroma per total weight of starch including aroma. The error bars are the standard error of the mean of triplicates.**

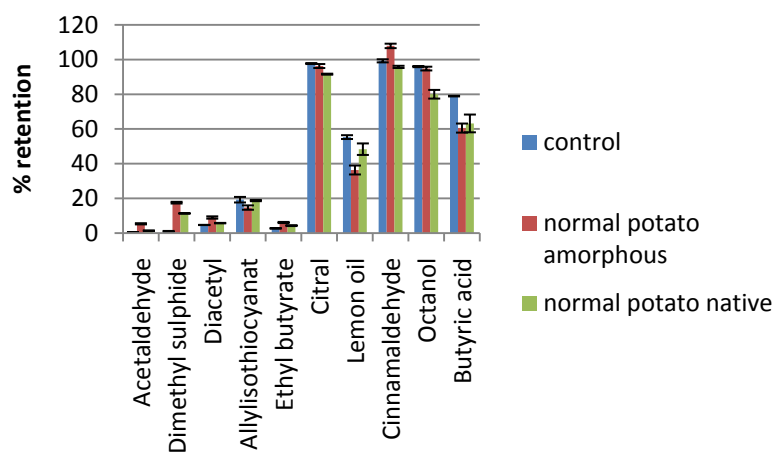


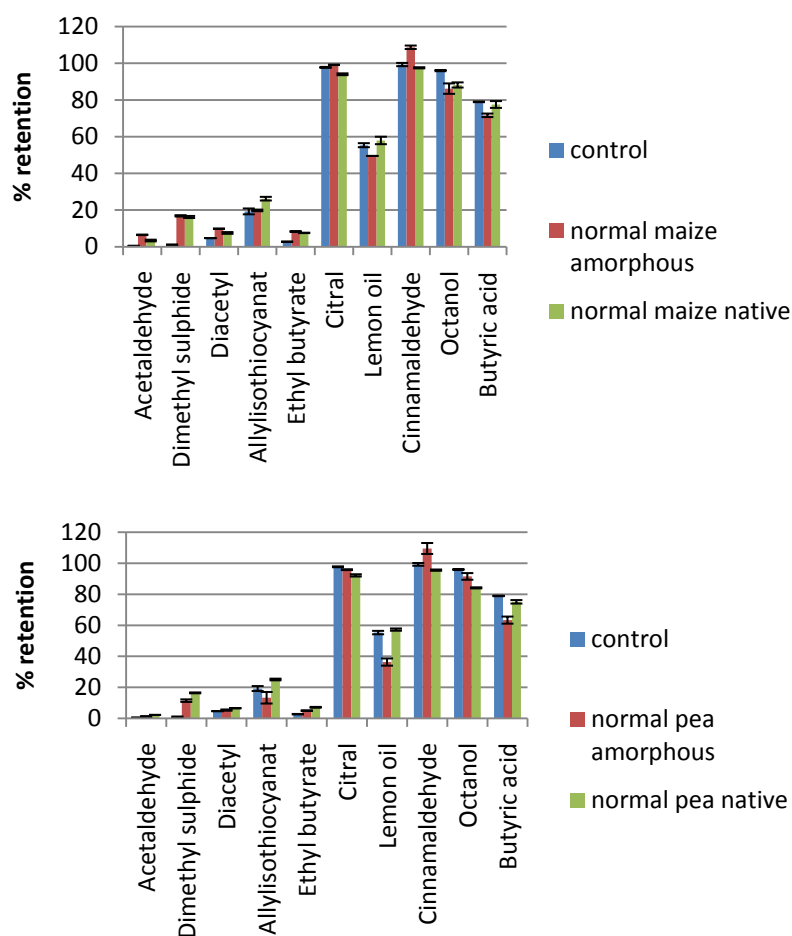
## 4.2 Aroma retention studies

The increase in retention seen for the most volatile compounds is most likely caused by adherence to the surface of the starch. The amorphous starches have a much larger surface area and therefore more starch for the aromas to adhere to. The native starches have grooves and dimples in their surface and a large inner surface (Fannon *et al.*, 1992; Conde-Petit, 2006), which are ideal for the adherence and/or binding of aromas to the surface. Starch granule surface chemistry, i.e. phosphates or lipids, is also likely to be an important part in the aroma binding.

Of the four most volatile aroma compounds, acetaldehyde and dimethyl sulphide have a better retention than diacetyl and ethyl butyrate relative to the control (Table 4). Both of these two aromas are very small molecules, and it is possible that apart from having easier access to grooves on the starch granule (Fannon *et al.*, 1992), they might also diffuse through the outer layers of the native starch granules more easily than other molecules, e.g. citral, which has a larger molecular size.

It has previously been shown that polarity has an influence on retention (Rosenberg *et al.*, 1990). However, in this study for this theory to be true, the retention of ethyl butyrate and cinnamaldehyde, and dimethyl sulphide and butyric acid, should have pair wise equal retentions, when comparing the hydrophobicity index “Log P” (Table 3), which is related to polarity. These results do not show this trend, which demonstrates that aroma retention is a complex interplay of many factors acting together, and no single chemical or physical property determines the retention.





**Figure 25** Comparison of the retention of aromas in the presence of amorphous and native potato (top), maize (middle), and pea (bottom) starch relative to a control. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The error bars are the standard error of the mean of triplicates.

Maize starch shows a slightly larger retention than the other starches for most aromas (Figure 10 and Figure 11). This may be an effect of residual lipids present in the maize starch, as these lipids are generally found in cereal starches (Buléon, 1998). Aromas are generally hydrophobic and thus may have favourable interactions with these lipids.

Allyl isothiocyanate appears to be slightly better retained by the native starches than the amorphous starches (Figure 25). However, as mentioned, this compound gives the starches a browned appearance, possibly due to a Maillard reaction, so the difference between the two types of starches may very well be a result of this reaction, i.e. if the reaction proceeds more rapidly for the native starches, then these starches will show a larger relative retention.

The higher than 100 percent retention of cinnamaldehyde in the amorphous starches is a direct consequence of the formation of a semi-crystalline gel. This gel is probably very effective at adsorbing water, thereby increasing the weight and giving a falsely high binding. The gel is most likely the product of the auto-reaction described in the Introduction (Chapter 1.6). The auto-reaction is a condensation reaction that releases water so the weight increase is not a

result of this. The effect is only seen for amorphous starches, and may be a result of the increased surface area for this type of starch.

For lemon oil and butyric acid, a reduction in retention is observed when amorphous starch is present relative to the control (Figure 10). The binding of these aromas to the surface of the starch is probably not very effective, and therefore it gives the aroma a larger surface area to evaporate from compared to the control, where the liquid aroma sets on the bottom of the tube with a small surface area exposed. This also explains why the amorphous starches, with a very large surface area, show lower retention than the native starches. Butyric acid and most of the compounds in lemon oil are bulky compared to acetaldehyde and dimethyl sulphide, which might prevent them from diffusing through the outer layers of the granule, thereby also increasing their exposed surface area compared to the control.

There is a slight tendency for the amorphous starches to be better at retaining the most volatile aromas (acetaldehyde, dimethyl sulphide, diacetyl, and ethyl butyrate), as well as citral and octanol, than the native starches, whereas the native starches are better at retaining lemon oil and butyric acid than the amorphous starches (Figure 25). Cinnamaldehyde, is better retained by amorphous starches than native starches, but due to the strong absorption of water of the crystalline gel formed by cinnamaldehyde, this compound is an outlier from the remaining aromas. It has previously been shown that aromas can form supramolecular structures with gelatinised starches (Golovnya *et al.*, 2001; Misharina *et al.*, 2004), though for these experiments there is no clear effect. It is possible that the most volatile aromas form these supramolecular structures, whereas the more bulky aromas are retained by other mechanisms, e.g. diffusion into pores (Fannon *et al.*, 1992).

### **4.3 Starch screening**

The starch screening data (Table 5 and Table 6) show very large variations between samples, and include negative retentions and retentions above 100%. Water absorption is very high compared to other experiments performed later with an equivalent of 60  $\mu\text{L}$  of water being absorbed by some starches compared to 10  $\mu\text{L}$  for later experiments. The humidity of the starch is influential in the absorption or desorption of aromas (Boutboul, 2000). However, maize starch shows close to no water being absorbed. Overall these variations give these data a large degree of uncertainty. This makes it difficult to conclude if the retention of aroma is dependent on either amylose or phosphate content in both amorphous and native starch.

The most volatile aromas (acetaldehyde, dimethyl sulphide, diacetyl, and ethyl butyrate) still show relatively low retention, though for normal potato these data show a large retention of dimethyl sulphide, however, later experiments show much lower retention. Conversely, the less volatile aromas, especially citral, cinnamaldehyde, and octanol, show large retentions, in many cases above 100%.

It is not possible to determine if the starch state, i.e. amorphous or native, influences retention. During the course of these and later experiments, much experience has been gained regarding work with starches and aroma in this type of experiment, so repeating these experiments will undoubtedly give different, less variable, and thus more reliable results.

#### ***4.4 Saturation of starch with aroma***

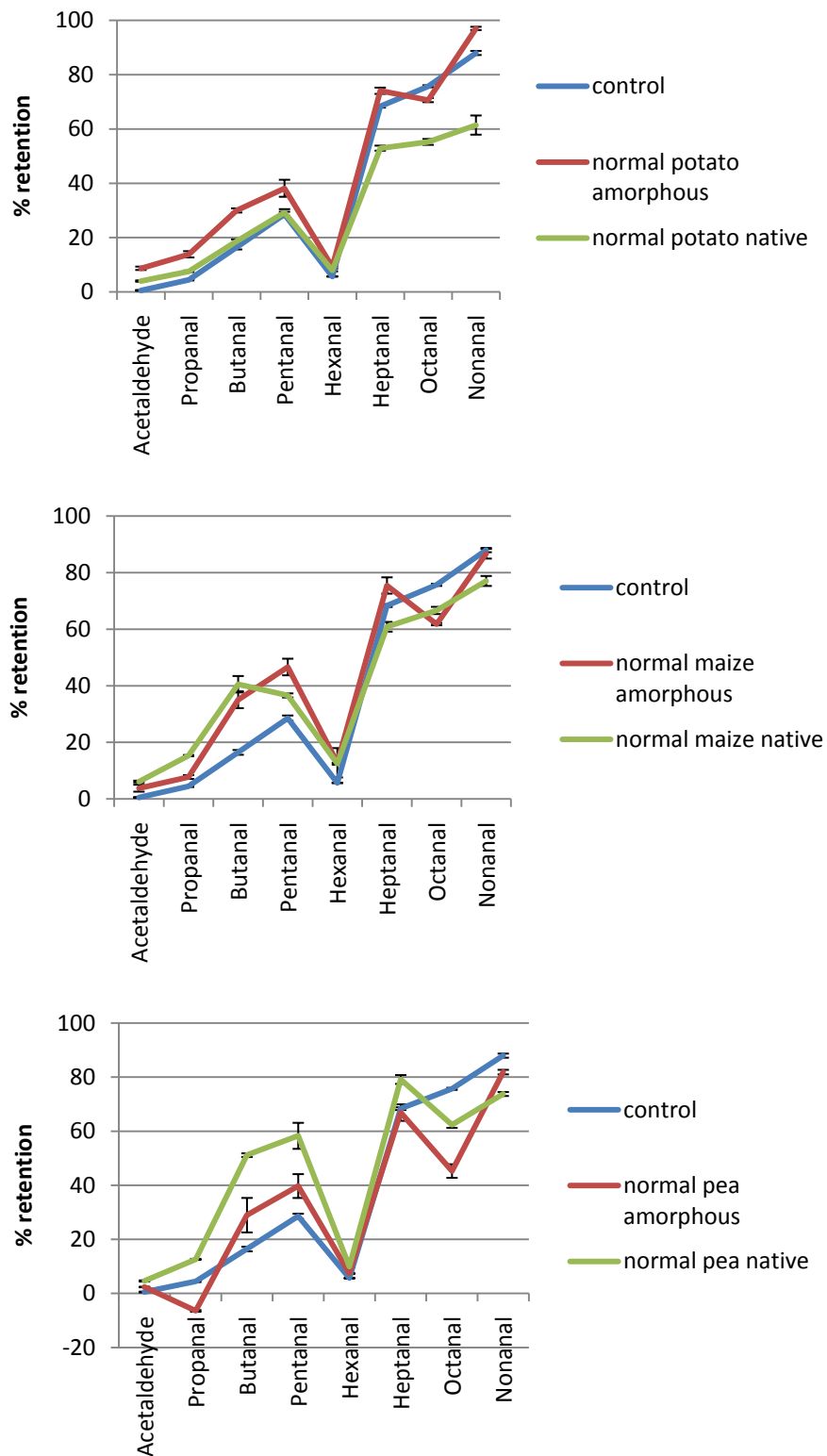
There are no conclusive results, though the data seem to indicate that the botanical origin of the starch is generally only of little importance in this type of experiment. The difference seen for dimethyl sulphide is interesting, but may equally well be an outlier since none of the other of the most volatile aromas shows a similar pattern. Ethyl butyrate shows a larger retention in the absence of starch relative to the presence of starch. This may also be an outlier, but since amylose and the two starches retain equally well, it is possible that with high concentrations of aroma used relative to starch, an increase in evaporation is observed from the increased surface area created by the amorphous starch. However, it is unclear why none of the other aromas show the same tendency.

#### ***4.5 Aldehyde-screening***

The most interesting trend seen for the aldehydes is the reduction in binding (Figure 12) and retention (Figure 26) seen for hexanal. This reduction stands out as it is lower than the retention for the aldehydes closest to hexanal, i.e. pentanal and heptanal. It is possible that hexanal forms a ring-like structure similar to that of pentanol (Pietrzycki *et al.*, 1991) that can affect the physical properties relative to the linear hexanal molecule, i.e. vapour pressure and boiling point.

A previous study of the retention of aldehydes hexanal through decanal (Terenina *et al.*, 2003), show no drop in the retention of hexanal. It should be noted that the experimental setup is different in those experiments and this may influence the results. By using a frozen 3% starch gel, they show a correlation between chain length and the binding of aldehyde, and a correlation between starting concentration and desorbed concentration. Another study (Maier and Bauer, 1972) reports on the partial oxidation of hexanal (and propanal), though such effects are likely to occur for all aldehydes.

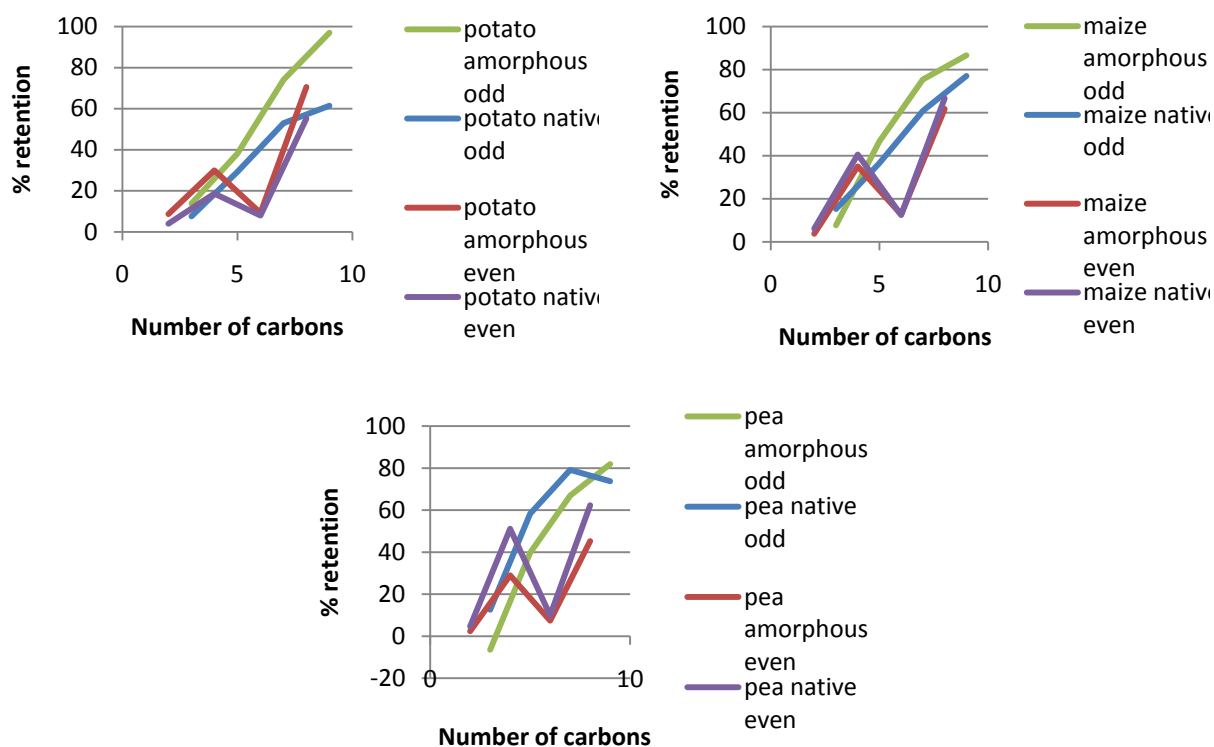
Like the most volatile compounds in the aroma-binding studies, the most volatile aldehydes also show an increased retention relative to the control (Figure 26). It is likely that, just like the aromas, this phenomenon relates to surface binding of both amorphous and native starches. Like the aroma-binding studies, the less volatile compounds show indications of reduced retention relative to control, but for the aldehydes this trend is less clear.



**Figure 26 Retention of aldehydes by amorphous and native potato (top), maize (middle), and pea (bottom) starches. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The error bars are the standard error of the mean of triplicates.**

The data presented for the aldehyde-screen are not adequate to determine if amorphous starches are better at retaining aldehydes than native starches because no general trends can be seen for all three starches. However, for heptanal, octanal, and nonanal, the amorphous starches show a better retention than the native starches for potato and maize starch, but not for pea starch (Figure 26). Likewise acetaldehyde, propanal, and butanal appear to be better retained by the native starches than amorphous starch for maize and pea starch, but not for potato starch.

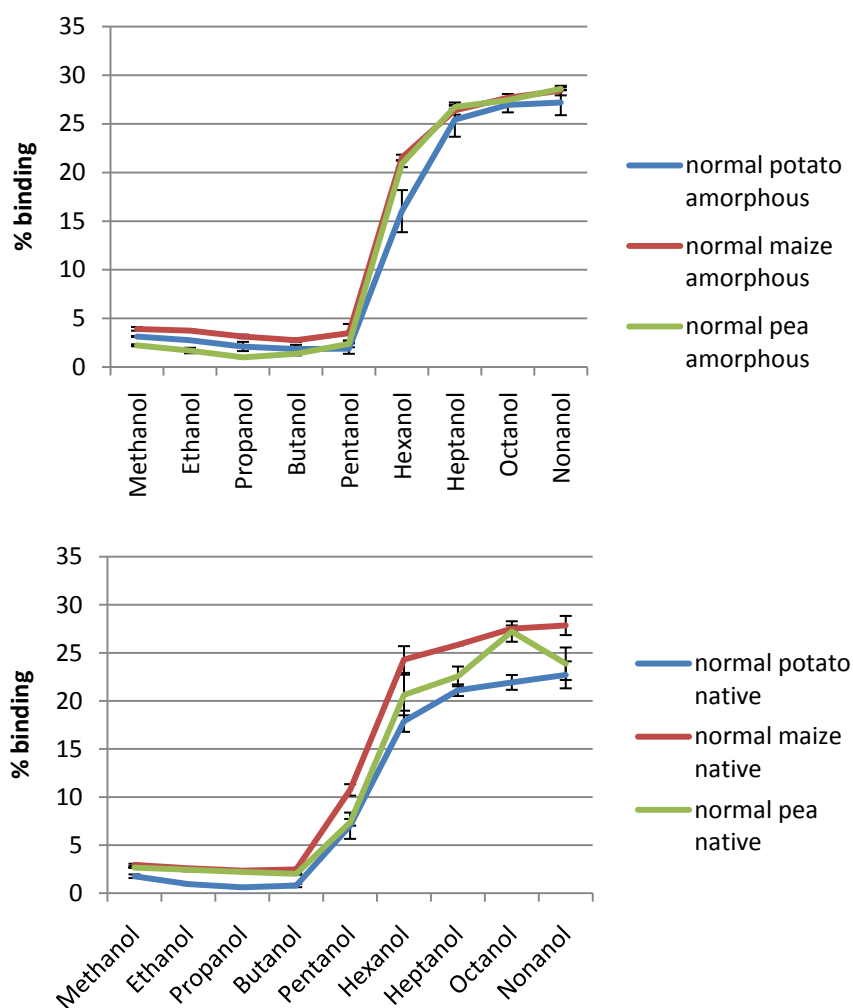
There is no apparent explanation for the double set of binding curves seen for the amorphous starches, i.e. one for aldehydes with an even number of carbons and one for aldehydes with an odd number of carbons (Figure 27). The effect is seen both for percentage binding and percentage retention. It is not clear why the binding and retention is slightly better for aldehydes containing an odd number of carbons, than aldehydes with an even number of carbons, and it is also not clear why this trend is not observed for the native starches. It is possible that these aldehydes have a molecular length that corresponds to the pitch in either an amylose or amylopectin helix, thereby making it possible for the aldehyde to insert itself extra-helically in the grooves of a helix. Or perhaps phosphates groups or lipids are exposed on the surface as the starch becomes amorphous, thereby providing attachment sites for every 2.5 Å, equivalent to the increase in molecular size when two methylene groups are added to the aldehyde. Structural data are needed to validate this possibility.



**Figure 27** Percentage retention of potato (top left), maize (top right), and pea (bottom) starch as a function of the number of carbons in the aldehyde with an odd number (3, 5, 7, 9) or with an even number (2, 4, 6, 8). The graphs are divided into four lines: amorphous or native starch, and odd or even number of carbons in the aldehyde. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment.

#### 4.6 Alcohol-screening

The apparent decrease in retention seen when going from methanol to butanol is evident for both native and amorphous starches (Figure 28). Despite the low retention percentages that give the data a higher level of uncertainty than the other alcohols, the trend is generally present for all starches and must therefore be considered substantial. Surface binding is probably the cause for this effect, i.e. methanol has easier access to less accessible surfaces, whereas longer alcohols have gradually less access to surface sites, which gives a higher evaporation rate, and thus lower retention.



**Figure 28** Percentage binding of alcohols to amorphous (top) and native (bottom) potato, maize and pea starches. Note the difference in binding between the amorphous and native starches occurring at pentanol. Binding is defined as weight of aroma per total weight of starch including aroma. The error bars are the standard error of the mean of triplicates.

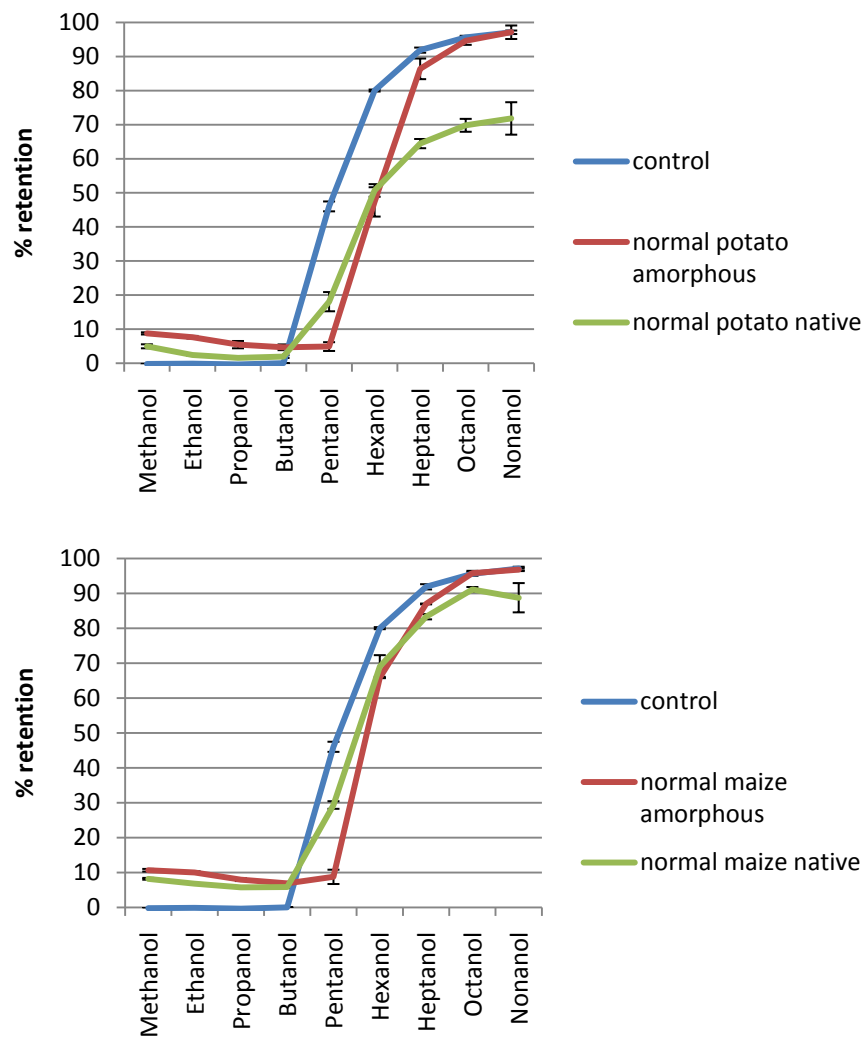
Pentanol is markedly better retained by the native starches than the amorphous starches. However, in both cases the control showed higher percentage retention. The drop in retention is much larger than the drop seen for the less volatile alcohols i.e. hexanol and longer. It is likely that pentanol has a physical quality that improves its retention for the native starches relative to the amorphous starches. Polaczek *et al.*, 1999 demonstrate that addition of pentanol to potato starch granules results in expulsion of the amorphous content from the granules as the pentanol penetrates the granule. Simultaneously they speculate that complexation of propanol occurs in the amylopectin helices of the granule, whereas methanol is too short to induce any helix formation.

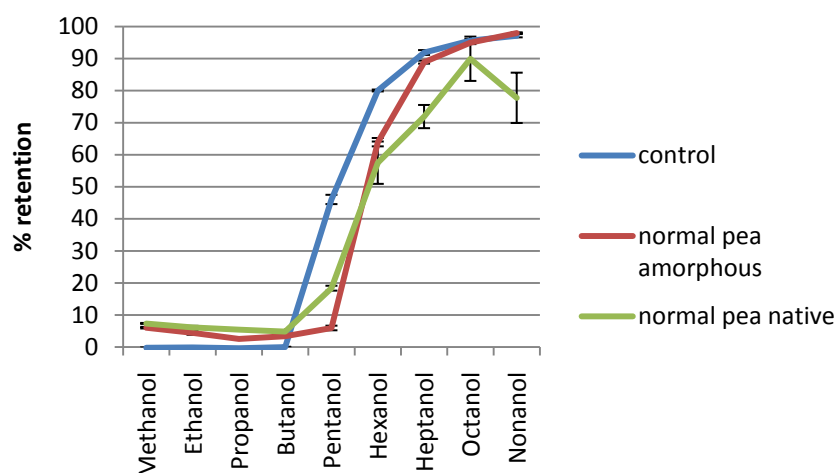
Perhaps the molecular length and diameter is just right for pentanol to insert itself in or on the surface of the crystalline amylopectin helices, thus expelling the amorphous (amylose)



content from the granules. Because the amylopectin helices in the amorphous starches have been re-organised, they can no longer absorb the pentanol, and therefore show a lower retention than the native starches. However, it is intriguing that the control shows a larger retention than either type of starch. This may be caused by the cyclic conformations of pentanol (Pietrzycik *et al.*, 1991), which might be more abundant in the presence of starch or actually induced by the starch. Though butanol is reported also to have a cyclic conformation, no difference is seen here for the native and amorphous starches.

Relative to the control, the starches show an improved retention for the most volatile alcohols (methanol to butanol), whereas the less volatile alcohols (hexanol to nonanol) show reduced retention (Figure 29). This is just like both the aromas and the aldehydes, so surface binding to either the amorphous or native starches is probably still the cause for this effect.





**Figure 29** Percent retention of alcohols to amorphous and native potato (top), maize (middle), and pea (bottom) starches. The error bars are the standard error of the mean of triplicates.

The amorphous starches appear to retain the alcohols better than the native starches for all alcohols, though it is clearest for the longer alcohols. This may yet again be attributed to a larger surface area available. It should, however, still be remembered that compared to the control, the longer alcohols still show reduced retention for both types of starches, also most likely caused by the surface area, as more surface area means more evaporation. Misharina *et al.* also showed a preferential retention on amorphous starches using maize starches on hexanol through nonanol, and simultaneously observe that a high amylose maize shows a binding similar to that of normal maize starch, thus suggesting that amylose is not involved in complexation of the aromas (Misharina *et al.*, 2006). The results in the present study do not indicate amylose as a key player in complexation as the retention in native starches are similar, and in the amorphous starches have an order of potato>pea>maize (Figure 28; amylose content of 25%, 55%, and 45%, respectively).

The fact that the amorphous starches appear to be slightly better at retaining alcohols, makes the higher retention of pentanol by native starches relative to amorphous starches very interesting. The expulsion of the amorphous content from the granule seen by Polazcek *et al.* may provide more binding sites for the pentanol, for example by disrupting the layer structure of the starch granule.

## ***4.7 Multiple headspace extraction of aromas from starch***

### **4.7.1 GC-MS linearity**

A linearity in the response on the GC-MS was expected, but instead a kink was observed in the standard-curve when going from dilute aroma concentrations to a more concentrated aroma concentration. A lower than expected response is observed, which is probably caused by a slight overloading of the GC-MS. Because of this overloading, care must be taken when calculating the amount of aroma released. A 10,000-fold dilution may overcome the

overloading problem, but instead present problems with accuracy in pipetting the correct volume. In these experiments the 10,000 fold dilution was made by making two consecutive 100-fold dilutions of 10  $\mu$ L aroma in 1 mL heptane. Any error in pipetting accuracy will be amplified and give very unaccurate results. However, in these experiments I believe that using 5,000-fold and 10,000-fold dilutions gives the most correct amounts of aroma extracted.

#### **4.7.2 Comparing experiments with and without water added**

The dry and non-enzymatic experiments were both performed in the absence of water. Likewise the “wet” and the “enzymatic” were performed in the presence of water. However, even though both the dry and non-enzymatic should be comparable, there is a difference in the amounts calculated, whereas the two experiments performed in water show more similar results (Table 15).

The aromas diacetyl and butyric acid both noticeably show lower amount for the wet experiments than for the dry experiments (Table 15). This is opposite to expected as the presence of water should help to soften the starch structure and release more aromas into the headspace. Instead it appears that the aromas are retained by the water. Because both of these aromas are water-soluble, it is very likely that they are dissolved in the water. Aroma dissolved in the water phase cannot be detected by the GC-MS. It has previously been observed that more polar compounds tend to have a low volatility in water, while less polar compounds have a high volatility, and vice versa for hydrophobic solvents (Forrs, 1969). Furthermore, the total amount of aroma in the starch can influence the degree to which aroma is released into the gas phase, as it is likely only a critical amount of aroma can be dissolved in water. Therefore if this critical amount is reached, any excess aroma will readily be released into the gas phase (a “salting out”-effect), where it can be detected by the GC-MS.

From a consumer point of view, the lower release of aroma in the presence of water is not desirable as this reduces the flavour experience. However, mastication in the mouth is not truly hydrated system, but rather “semi-wet”, and both the physical shearing and enzymatic attack on the food matrix will doubtlessly increase the amount of aroma released compared to a fully hydrated system like the one used here, though it is not clear whether or not this amount will also exceed that of a dry.

#### **4.7.3 Influence of enzyme treatment on aroma release**

The amounts of both diacetyl and butyric acid are lower for the  $\alpha$ -amylase treated sample and the sample analysed in the presence of water, compared to the two samples analysed with neither enzyme nor water added (Table 15). This is probably also an effect of diacetyl and butyric acid being dissolved in the water, compared to being released when no water is present.

For the non-enzymatically treated sample only a single data point was measured during the extraction for ethyl butyrate and for cinnamaldehyde the intensity continues to increase during the experiment. Because ethyl butyrate is absent in all but the first extraction of the non-enzyme treated sample, and given that more ethyl butyrate is released in the absence of water, it is very likely that all the ethyl butyrate is released in the first extraction, whereas in the

presence of enzyme and water the release is slower, thus allowing for more data points to be collected.

The amount of cinnamaldehyde released might be higher for the non-treated sample, but due to the continuing increase in intensity during the experiment, it is not possible to calculate this amount.

There is a small increase for citral for the enzymatically treated sample compared to the non-enzymatically treated sample, but this is not large enough to be considered substantial, even though the hydrophobic nature of citral might release more of the aroma into the headspace rather than dissolving in the water (Forss, 1969).

There is a ten-fold difference for allyl isothiocyanate (Table 15) which might be explained if all aroma is trapped inside the starch matrix, and then released when a starch-degrading agent is added. It is also possible that the browning reaction seen for allyl isothiocyanate somehow can release the aroma when water is added. It should, however, be noted that the amount of allyl isothiocyanate released in the first dry experiment is twice that for both the wet and the enzyme-treated, so there might be some uncertainty regarding the sample used for the non-treated control.

However, because no measurements were done to estimate the extent of the enzymatic degradation of the starch, the efficiency of the  $\alpha$ -amylase is unclear. It is therefore not possible to determine whether or not the enzymatic treatment actually had an effect or if other factors are involved in producing the observed differences.

#### **4.7.4 The scale of aroma release**

All the MHE experiments show that aromas can be released in microgram-amounts, even though the aroma retention studies showed milligram-amounts retained, so there is still a difference of a factor of one thousand between the two types of measurements. However, it is necessary to remember that the binding and retention studies only measures the amount of aroma adsorbed or in any other way remaining in the starch matrix, whereas multiple headspace extraction measures the amount of aroma that can be released again. In other words, MHE actually shows the amount of aroma that can be released during mastication, even though this is a very small part of the available aroma. If assuming a release from a MHE-experiment of 100 microgram from 200 mg starch, then this equals 0.05% using the formula for calculating binding described in the introduction. Assuming for simplicity that the volume in the mouth cavity is 100 mL, then 100 micrograms (if the density of the aroma is identical to water) is equal to one part-per-million. This is very similar to the limit of detection for most aromas in the human nose, meaning that the amount of aroma released from the starch in the MHE experiments is similar to the amount of aroma experience during ingestion of food items.

#### **4.7.5 Ether/pentane extraction**

Because aromas are generally hydrophobic, the ether/pentane extraction medium should be more than adequate to extract all aromas. However, this is not the case and only diacetyl (very hydrophilic), citral (very hydrophobic), and butyric acid (neither exclusively hydrophilic nor -phobic) are detected in the extraction medium, and then only in lower amounts than for the

other extractions (using either nitrogen gas as carrier or water plus nitrogen gas). The low extraction yield of the above aromas, and complete absence of the others aromas, suggests that aromas are not released during the extraction, but instead is strongly bound to the starch, and therefore not released to the extraction medium.

#### 4.8 Differential scanning calorimetry

The presence of citral in all three starches gives a reduction in melting temperature in the analysis performed at KU-LIFE, but not those performed at INRA (Table 17). This is quite interesting as both sets of samples show a reduced melting enthalpy for all starches, except the maize starch analysed at INRA where there is a large difference between the two samples in the melting enthalpy (6.2 J/g difference). Remember, that the endotherms can be interpreted as the amount of double helices present in the starch granule and the melting temperature as the perfection of the ordering of these helices in the crystalline lattice (Cooke and Gidley, 1992; Tester and Debon, 2000). Therefore, based on the analysis performed at KU-LIFE, it appears that citral either unfolds some of the amylopectin double helices in the starch before the starch reaches its gelatinisation temperature, or that citral aides in the unfolding during heating. Since the citral affects the crystalline amylopectin parts of the granule, a local unfolding in parts of the crystalline layers makes these parts amorphous, which will be reflected by a lowering of the melting enthalpy.

**Table 17** Difference in peak melting temperature (°C) of native potato, maize, and or starch granules mixed with citral, octanol, or butyric relative to a starch control without aroma added. Data are an average of triplicates. N.A. Not analysed.

$\Delta T$ (°C)	KU-LIFE			INRA		
	Potato	Maize	Pea	Potato	Maize	Pea
<b>Citral</b>	-2.1	-2.0	-0.9	0.0	-0.8	-1.5
<b>Octanol</b>	N.A.	N.A.	N.A.	+0.5	-1.6	+0.4
<b>Butyric acid</b>	-1.0	-2.4	0.0	-1.1	-7.4	-2.4

**Table 18** Difference in peak melting enthalpy (J/g starch) of native potato, maize, and or starch granules mixed with citral, octanol, or butyric relative to a starch control without aroma added. Data are an average of duplicates. N.A. Not analysed.

$\Delta \Delta H$ (J/g)	KU-LIFE			INRA		
	Potato	Maize	Pea	Potato	Maize	Pea
<b>Citral</b>	-3.3	-3.9	-5.1	-3.5	+1.5	-5.5
<b>Octanol</b>	N.A.	N.A.	N.A.	-4.3	-2.7	-5.6
<b>Butyric acid</b>	+0.7	-0.8	-1.5	-1.7	-2.5	-5.6

The DSC endotherms for octanol (Figure 20) show endothermic peaks after the gelatinisation peak. These peaks likely correspond to the formation or reorganisation of amylose/octanol-complexes (Itthisoponkul *et al.*, 2007). A large reduction in melting enthalpy is seen for all three starches, though there does not appear to be a connection between amylose/amylopectin content and the scale of the reduction. The reduction is probably caused by interactions by unfolding of amylopectin double helices, and is possibly also related to the penetration of octanol into the granule leading to irreversible change of the granule structure (Polaczek *et al.*, 1999).

Butyric acid shows a very large drop in melting temperature for maize starch (-2.4 and -7.4 °C; Table 17). This large difference may be the results of lipids found in cereal starches (Morrison and Gadan, 1987), which the non-polar end of butyric acid can interact with. Alternatively the second endotherm observed (Figure 20) is the unfolding of the amylose/lipid complexes. However, pea starch shows the largest reduction in melting enthalpy of the three starches, suggesting that lipid do not account for the whole explanation. Pea starch has the lowest amount of amylopectin of the three starches, so unfolding a similar amount of the amylopectin double helices will give a relatively percentage unfolding relative to a starch with a higher amylopectin content. This makes the pea starch more sensitive for factors that affect the crystalline, amylopectin containing layers of the granule, which is reflected by the largest reduction in melting enthalpy of the three starches for citral, octanol, and butyric acid. The differences seen between the samples analysed at KU-LIFE and those analysed at INRA may be caused by differences in equipment, handling, and experimental parameters.

#### **4.9 Wide-Angle X-ray Scattering**

Octanol shows a dramatic effect on all three starches, whereas citral and butyric acid show minor effects (Figure 23). If a pure amylose/aroma-complex was formed very sharp and distinct peaks would have appeared, similar to those observed by Arvisenet and co-workers (Arvisenet *et al.*, 2002a). In addition, citral and butyric acid do influence the spectrum, but to a lesser degree than octanol, despite the large reduction in gelatinisation temperature seen by DSC on maize starch with butyric acid (Table 17). The small changes in peak height and width observed in these experiments are small relative to those reported by other studies (Nuessli *et al.*, 2003; Jouquand *et al.*, 2006a; Itthisoponkul *et al.*, 2007). This suggests that instead of having pure complexes, a mixture of a least two species in the sample: one starch-species with complexation and one starch-species without complexation. In this context, when working with native starch granules, complexation can be the classical insertion into the amylose helix, the interaction and unfolding of amylopectin helices seen in the DSC experiments, or diffusion into the layers of the granules.

The largest influence on the spectrum is octanol with pea starch, whereas octanol with maize starch only results in minor differences, and octanol with potato starch showing an intermediate of the two other starches. This makes it very likely that, given their relative amylose content of 28%, 45%, and 55% (potato, maize, and pea, respectively), octanol does not interact with the amylose content in a systematic way, but this only excludes more interactions with more amylose, and not interactions with amylose altogether. The fact that

both citral and butyric acid only showed minor effects on the spectra suggests that these compounds are more likely to interact with the crystalline amylopectin layers of the granule, whereas octanol is more likely to interact with the amorphous amylose layers.

Combining these results with those achieved by DSC (Chapter 3.9 and 4.8), it is likely that the addition of these aromas interfere with the granule structure, resulting in unfolding of the amylopectin helices and possible minor complexation with the aromas. Overall these effects are local and do not affect the whole of the starch granule, thus some parts or layers of the granule remain unaffected by the large concentrations of aroma. An expulsion of some parts of the granule as observed by Polaczek and co-workers (Polaczek *et al.*, 1999) with pentanol is also a possibility, which does not exclude the previous possibilities.

## 5 Conclusion

The degree of binding of all types of compounds to the starches is roughly determined by the vapour pressure. The most volatile compounds show a relatively low binding, whereas compounds with a higher vapour pressure show a larger binding. However, other factors influence the degree of binding: lipid content of the starch, access to grooves on starch, surface area, amylose and amylopectin content. Therefore the binding is not determined by a single factor, but rather a complex interplay of many factors.

Whether it is aromas, aldehydes, or alcohols, the retention of the most volatile compounds increases in the presence of starch compared to a control, regardless of the type of starch. The increase in retention relative to the control is in some cases very substantial, but in terms of binding these cases do not exceed ten percent. The increase in retention is probably caused by interactions between the aromas and the surface of the starch, either chemically via interactions with amylose helices, phosphates or lipids, or physically by adhering or attaching in grooves on the surface of the starch (Fannon *et al.*, 1992).

The less volatile compounds, though they show a relatively large binding, have a lower retention than the control. The positive effect seen for the most volatile compounds retained by starch, becomes a minus for the less volatile compounds, i.e. the increase in surface area of the starch relative to the control gives a larger area from which the aroma can evaporate. Likewise, the molecular size of the less volatile compounds, may give a larger steric hindrance in the starch, thus preventing the molecules from reaching pockets that are available for the smaller compounds. It is also possible that the shorter compounds can diffuse through the outer layers of the starch granule or the amorphous starch matrix.

Some compounds act very differently than the rest: Allyl isothiocyanate “burns” the starch possibly through a Maillard reaction (Martins *et al.*, 2000), cinnamaldehyde forms a gel-like crystal that absorbs water, hexanal shows a big drop in retention, and pentanol is better retained by native than amorphous starches. The behaviour of the two former compounds means that the results seen for these compounds are questionable, whereas hexanal and pentanol show no signs of reacting with the starch or in another way become modified and the results are therefore credible.

No literature can presently be found to explain the drop in the retention of hexanal, whereas the drastically better retention of pentanol by native starches is undoubtedly caused by the expulsion of the amorphous content of the starch granule observed by Polaczek and co-workers (Polaczek *et al.*, 1999). Pentanol has been observed to form ring-like structures (Pietrzycki *et al.*, 1992), and it is likely that hexanal also forms this type of structure, though more evidence is needed to support this.

The multiple headspace extraction experiments were designed to examine how much aroma could be released once the aroma was mixed with the starch. However, the multiple headspace extractions show that despite having aroma present in milligrams, only micrograms can be released, even in the presence of either starch degrading agents or strong extractors. This suggests that the interaction of aroma with starch is very strong.



DSC revealed interactions between citral, octanol, and butyric acid with the native starch granules, presumably by unfolding the amylopectin double helices. The most pronounced difference was in the melting enthalpy which is affected by the amount of double helices in the granule. In other words, adding these compounds “melts” the amylopectin helices so the crystalline lattice is more imperfect. These compounds may also irreversibly disrupt the starch granule in a manner similar to that seen for pentanol (Polaczek *et al.*, 1999).

WAXS shows large effects when octanol is added to the starch granule, but only minor effects are observed when citral or butyric acid is added. For all aromas and starches, it is clear that no complete complexation takes place, but rather a localised interaction either with the amylose or amylopectin that does not affect the whole granule. Instead it is possible that the aromas affect different parts of the granule, so octanol may interact more with amylose (amorphous) layers, whereas citral and butyric acid affect the amylopectin (crystalline) layers. The 15% binding objective was not accomplished, though further work may improve the binding seen for the most volatile aromas acetaldehyde, dimethyl sulphide, diacetyl, and ethyl butyrate. Some interesting effects have been observed, and homing in on these parameters to find the variables that lead to the specific bindings for each aroma will provide a good platform for further work.

## **6 Future work and perspectives**

These results are useful for the flavour industry because they show an unexpected behaviour of the low-volatility compounds which have a lower retention in the presence of starch than a control. Oppositely the very volatile compounds are better retained in the presence of starch. Taken together these findings can be used when designing foods to compensate for aroma loss during processing. However, the project aim of reaching a 15% binding was not obtained for the most volatile aromas. Performing experiments to illuminate the causes of the specific binding, e.g. granule structure, size, amylose content etc., is an obvious continuation of this work.

The multiple headspace extractions performed should be extended in a way, where the starch integrity, e.g. by reducing sugar assay, can be examined in the presence of a starch degrading enzyme while the aroma release is monitored by headspace experiments.

Scanning electron microscopy of the starch might reveal if citral, octanol and butyric acid influences granule structure in a way that the DSC and X-ray data suggest. Knowledge of this can also help to understand why the least volatile compound show an increase in evaporation when starch is added.

All these results combined reveal an intricate interplay of factors that determine the aroma-retention in the presence of starch. Once the aroma has bound, it can be difficult to release it again, and this may easily complicate aroma-release during ingestion of food items.

PART 2: Induction of carbohydrate acting enzymes from  
*Aspergillus nidulans*

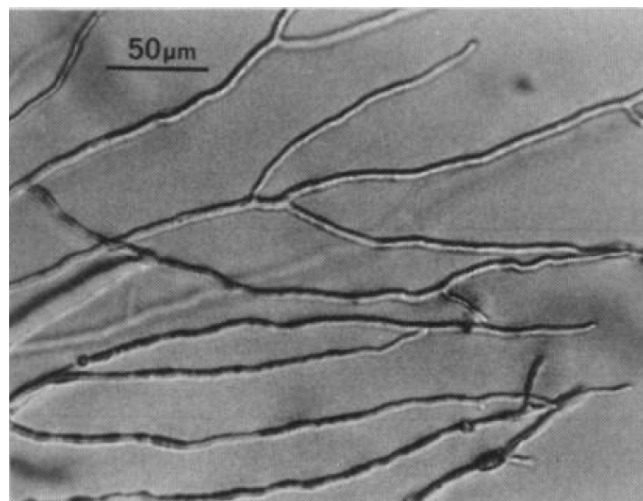
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## 7 Introduction

### 7.1 Fungi

Fungi are a broad class of organisms that have both beneficial and detrimental effects for humans. Fungi are used to produce antibiotics like penicillin (Timberlake and Marshall, 1989), but at the same time they are known pathogens both in humans (Cutler *et al.*, 2007) and in plants (Maor and Shirasu, 2005). Fungi are used in production of food items such as bread and in production of enzymes and organic acids (MacCabe *et al.*, 2002; Kim *et al.*, 2007), but have also been implicated in diseases (Meyer *et al.*, 2004) and crop devastation. The industrial utilisation of fungi is responsible for an annual billion USD market (Kim *et al.*, 2007), thus giving the fungi a substantial commercial relevance. Furthermore advances are being made for the heterologous protein expression by filamentous fungi (Nevalainen *et al.*, 2005), taking advantage of a vast amount of proteins secreted from fungi (Gibbs *et al.*, 2000).

Some fungi such as *Aspergillus nidulans* are filamentous fungi which form multinucleated, tubular filaments (hyphae) that grow by apical extensions (Figure 30). These hyphae are the fungus' main weapon for quickly colonising large areas, because the hyphae secrete chemicals and enzymes that favour fungal growth. As nutrients at the centre of the fungal colony become depleted, the hyphae extend to search for more nutrients.

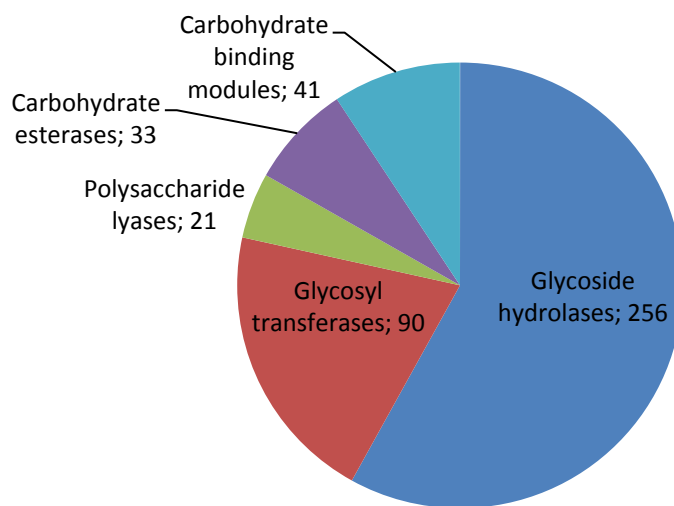


**Figure 30** Hyphae of *A. nidulans* at 100 times magnification. Adapted from Timberlake and Marshall, 1989.

There are three ways in which a fungus can interact with an organism: Saprotrophic, pathogenic, and symbiotic. A saprobe feeds on non-living organisms, pathogens cause disease in the host organism, and symbionts depend on a host organism for survival and may even benefit the host. These different fungal lifestyles require different gene and protein

expressions for survival. For example the concentration of carbohydrate has been shown to have adverse effects on protein expression when the concentration of carbohydrate exceeded 1 mM, but was unaltered at all carbohydrate concentrations in a mutant where a protein suspected of being involved in carbohydrate transport was knocked out (de Vries *et al.*, 1999). This suggests a tight regulation of all proteins involved in pathways including carbohydrate metabolism.

*A. nidulans* is a model organism and as such has been used for studying many aspects of the genetics of fungi, and since it is fully sequenced *A. nidulans* is also excellently suited for proteomics studies (Galagan *et al.*, 2005). Searching in the CAZy database (a database of carbohydrate active enzymes; [www.cazy.org](http://www.cazy.org); Cantarel *et al.*, 2009), *A. nidulans* reveals 256 encoded glycoside-hydrolase (GH) enzyme family members (Figure 31) in 56 families. The most dominant of these families are GH2, 3, 5, 13, 16, 18, 31, and 43 whose 115 members include galactosidases, glucosidases, xylanases, amylases, chitinases, and xylosidases. The optimal degradation of polysaccharides to their minor components usually requires more than one enzyme (de Vries *et al.*, 2000).



**Figure 31** Number of carbohydrate active proteins in *A. nidulans*. Glycoside hydrolases hydrolyse the glycosidic bond between two carbohydrate residues. Glycosyl transferases transfer parts of a carbohydrate from an activated donor to a specific acceptor. Lyases create an unsaturated residue and a new reducing end in the cleaved polysaccharide. Esterases deacylates substituted carbohydrates. Carbohydrate binding modules are separate protein domains within carbohydrate active enzymes that bind to oligosaccharides and polysaccharides.

## 7.2 Gene regulation

When used as production host for enzymes etc., the fungus is some times grown in on a solid medium and some times in a liquid medium. However, it is worth remembering that fungi grow naturally on solid “substrates” such as plants or animals. It has been shown that secretion of a heterologous protein was 500-fold higher when grown on solid medium rather

than on liquid medium (Tsuchiya *et al.*, 1994). Further, by using DNA technology genes expressed specifically in solid or liquid media have been identified, and those identified as being up-regulated in liquid medium includes enzymes involved in the glycolytic pathway (Akeo *et al.*, 2002; Maeda *et al.*, 2004). The up-regulation of these enzymes is most likely caused by a higher water activity, thus removing the catabolites of the enzyme degradation by diffusion before they can suppress gene expression.

### **7.3 Proteomics**

Proteomics is the analysis of all the proteins expressed by a cell. Proteomics is very well suited for studying differences in protein expression under varying environmental conditions (Aebersold and Mann, 2003). If the fully sequenced genome is available, it is possible to give a positive identification using only peptide mass fingerprinting by cleaving unique proteins separated by 2-dimensional gel electrophoresis (2DE) with trypsin. If no genome data exist in databases, tandem mass spectrometry (see Chapter 7.5.2.2) can sequence the peptides accurately to allow identification.

Proteomes can be divided into several sub-proteomes categories, including the secretome which is all proteins transported to the outside of the cell and the transport-proteins involved therein (Kim *et al.*, 2007). The secretome is very important in understanding the mechanisms that allow fungi to invade and colonise other organisms, because the fungus secretes the enzymes to break down the plant cell wall of the invaded organism. Thereafter the uptake of partially degraded cell wall polysaccharides and final break down to its minor components takes place inside the fungus.

There is usually a difference between the expression levels identified by DNA techniques and those observed by proteomics because factors such as splicing and protein modifications affect the protein expression. Oda and co-workers (Oda *et al.*, 2006) have studied the secretome of *A. oryzae* grown on solid and in liquid media. In the liquid medium they find enzymes such as glucoamylase,  $\alpha$ -amylase, and  $\beta$ -glucosidase. Also they find that many of the secreted protein are glycosylated, thereby making them resistant to trypsin cleavage, but performing an in-gel deglycosylation made it possible to identify them. The secretion of enzymes on solid medium was shown to be four to six-fold higher than in liquid state (mg protein secreted per g mycelium), due to the fact that on a solid medium the fungus needs to secrete more enzymes to receive nutrients. However, they do not mention the actual amounts of protein secreted in either medium, which might be more even as the volume of the liquid culture is larger and the requirement for nutrients is the same.

### **7.4 Carbohydrate induction of secreted proteins**

Studies using various carbohydrate sources showed induced expression of specific enzymes capable of degrading these carbohydrates so the fungi can take full advantage of the nutrients available in a changing environment (Keller and Hohn, 1997). Medina and co-workers (Medina *et al.*, 2004) have grown *Aspergillus flavus* on rutin (a glycoside), glucose, and

potato dextrose. In total they identify 51 proteins, of which ten are rutin-specific, five are glucose-specific, and one is potato dextrose specific. The unique proteins identified when the fungus is stimulated with glucose include glucosidases, the single unique protein identified when the fungus is stimulated with potato dextrose is a ribonuclease, whereas  $\alpha$ -amylase and glucoamylase are identified in all three cultures. Chitinase is only identified in the rutin culture. One of the components of the cell walls of fungi is chitin, and chitinase is therefore important when the fungi invade another organism and in the re-organisation of the cell wall during growth (Horiuchi, 2009).

Han *et al.*, 2010 have grown *Aspergillus terreus* on sucrose, glucose, and starch (undefined soluble starch) and performed a secretome study. In this study,  $\beta$ -glucosidase is expressed with all three carbohydrates, arabinosidase with sucrose and starch, and xylanase and chitinase only when sucrose is added to the medium. They also tested the effect of higher concentrations of sucrose and glucose on protein expression and found a slightly lower protein concentration in the media, similar to results observed by De Vries (De Vries *et al.*, 1999). Simultaneously, they also find a larger cell mass and hence a two-fold lower protein secretion per weight, though they find no differences in the expression pattern despite a higher concentration of carbohydrate.

Amylase production has been shown to be dependent not only on the carbohydrate source, but also carbohydrate concentration with a 2% glucose stimulation resulting in lower  $\alpha$ -amylase activity than a control without glucose and 0.25% giving the highest activity (Nahas and Waldemarin, 2002). This again points to an effective repression of gene expression in the presence of catabolite. The same study identified lactose as the best stimulator of  $\alpha$ -amylase activity followed by maltose, xylose, and starch (from an undefined source) in that order.  $\alpha$ -amylase activity in cultures stimulated with starch was also shown to peak after two days and then drop to a baseline level.

No secretome studies on *A. nidulans* have yet been reported.

## ***7.5 Methods used in this study***

### **7.5.1 2-dimensional gel electrophoresis (2-DE)**

2DE is used to separate many proteins from e.g. a supernatant or an extract, by a combination of separation according to mass and isoelectric point (pI; O'Farrell, 1975).

#### ***7.5.1.1 First dimension electrophoresis (Iso-Electric Focusing; IEF)***

All proteins have electrically charged amino acids, which gives the whole protein a charge. At a specific pI the overall net charge is zero, and if an electrical field is applied in the presence of ampholytes physically attached to the gel, the proteins will migrate to their respective pI's (Rabilloud *et al.*, 2009).

### **7.5.1.2 Second dimension electrophoresis**

Second dimension electrophoresis separates proteins according to their mass by coating the proteins in a negative charge using sodium dodecyl sulphate and applying another electric field perpendicularly to the first field. The proteins then migrate towards the anode. The proteins migrate in an acrylamide matrix which helps to separate larger proteins from smaller proteins, so the smaller proteins migrate faster than the larger proteins (Shapiro *et al.*, 1967; Rabilloud *et al.*, 2009). The protein spots can then be excised and digested with trypsin in preparation for mass spectrometry.

## **7.5.2 Matrix-Assisted Laser Desorption/Ionisation-Time of Flight (MALDI-TOF) Mass Spectrometry**

MALDI-TOF begins with the ionisation of trypsin-cleaved proteins by MALDI using a high-intensity laser, followed by analysis of the mass of the peptide in a vacuum where the mass of the peptide is correlated with the time it takes for the peptide to travel to the detector. This method only requires down to femtomoles ( $10^{-15}$  moles) of protein for analysis.

### **7.5.2.1 MALDI**

MALDI using organic acids as matrix was shown by Karas and Hillenkamp (Karas and Hillenkamp, 1988) to be suitable for proteins in 1988. As the laser fires onto a target, the sample protein (with matrix) is desorbed and ionised. This creates a charge state where the proteins gain a proton (one mass and one charge), and is brought into gas phase. The ionised sample is then transferred through a vacuum before hitting a detector plate (TOF-part of the instrument; Aebersold and Mann, 2003).

The requirement of a matrix complicates matters as the matrix solution is not always homogenous, and the presence of salts can make the matrix unsuitable for MALDI.

### **7.5.2.2 TOF**

The TOF-instrument is basically a tube with vacuum followed by a detector that measure the time it takes for the peptide ionised by a laser to travel to the detector (single MS mode). These peptides can be fragmented further into smaller peptide by tandem mass spectrometry (MS/MS). This is achieved by first ionising the peptides as normal, and then only allowing the peptide of interest through for fragmentation by gating the peptide through at a specific time (ion trap), before being fragmented by laser (Aebersold and Mann, 2003). These fragments also travel according to their mass-to-charge ratio and because fragmentation is not complete, sequencing is possible by comparing the loss in mass from one fragment to the next. The accuracy of the instrument in single MS mode is less than 80 ppm, and in tandem MS mode it is less than one Da.

## **7.6 Project aim**

This project was originally intended to be support for the aroma-studies by identifying proteins and enzymes secreted from *Aspergillus nidulans* when stimulated with carbohydrates of various sources and with various linkages. The enzymes thus induced could then be used



for modifying the starches used in the aroma-binding part of this PhD-project for enhancing the aroma-binding. Therefore the differences in the starches tested may result in differences in induction of carbohydrate-active enzymes. The enzymes secreted are probably related to carbohydrate metabolism and are therefore carbohydrate-active enzymes. Also different concentrations of carbohydrate may change the expression level of the enzymes so this is also relevant for modifying starches, because enzymes normally found only in small, undetectable amounts may be up-regulated.

In the present study a wide variety of carbohydrates will be used, differing in size from monosaccharides to starch, and in their bond types. The supernatant from *A. nidulans* cultures stimulated with these carbohydrates will be analysed by 2DE and MALDI-TOF MS.

## 8 Materials and methods

### 8.1 Cell culture

*Aspergillus nidulans* FGSC A4 was inoculated on minimal media (12 mM potassium phosphate buffer pH 6.8 with 70 mM NaNO<sub>3</sub>, 7 mM KCl, and 2 mM MgSO<sub>4</sub> including 1 mL Hutner's trace elements (76 mM ZnSO<sub>4</sub>, 178 mM boric acid, 25 mM MnCl<sub>2</sub>, 18 mM FeSO<sub>4</sub>, 7 mM CoCl<sub>2</sub>, 1 mM (NH<sub>4</sub>)Mo<sub>7</sub>O<sub>24</sub>, 14 mM Na<sub>2</sub>EDTA per litre of culture) with 10 g carbohydrate per L for solid media and 3.6 g carbohydrate per L for liquid media, unless otherwise specified. In addition, solid media contains 12.5 g/L agar. Mycelia grew for 5 days at 30 °C before being washed from the plates with 2-3 mL 0.1% Tween-20. 200 mL liquid cultures were inoculated with 1.5 OD-units (=1.5 mL of a suspension with OD 1) per culture and grown for five days at 30 °C with shaking (150 rpm). Supernatants (SN) were harvested and frozen immediately. The carbon-sources used were glucose; potato, maize and pea starch (contains  $\alpha$ -1,4 and  $\alpha$ -1,6 linkages); oat spelt xylan ( $\beta$ -1,4); tamarind xyloglucan ( $\beta$ -1,4 and  $\alpha$ -1,6); wheat arabinoxylan ( $\alpha$ -1,2 and  $\alpha$ -1,3); sugar beet arabinan ( $\alpha$ -1,5); larchwood arabinogalactan ( $\alpha$ -1,3); guarin galactomannan ( $\alpha$ -1,6); and konjac glucomannan ( $\beta$ -1,4). Glucose was purchased from Sigma Aldrich, the origin of the starches is described in Chapter 2.1, other carbohydrates were purchased from Carl Roth GmbH (Germany).

### 8.2 Preparation of extracellular proteins

Protein concentrations were determined using Bradford reagent (Bradford, 1976) by the following procedure: 50  $\mu$ L SN per culture was pipetted into a microtiter-plate in duplicates. 200  $\mu$ L of Bradford reagent (0.01% Coomassie Brilliant Blue G-250, 5% ethanol, 8.5% phosphoric acid) was added and mixed with the SN by repeated pipetting of the solution. A standard-curve was made using a 2 mg/mL BSA stock diluted to 0.01, 0.02, 0.04, 0.06, 0.08, and 0.10 mg/mL. Protein concentration was measured by reading the absorbance at 595 nm in a microtiter plate reader (Microtek Powerwave XS).

50  $\mu$ g of protein was precipitated by chloroform/methanol precipitation (Wessel and Flugge, 1984): to one volume of sample, two volumes of methanol, half a volume of chloroform, and one-and-a-half volume of water is added and vortexed. After centrifugation at 20000 x g, the upper phase is removed carefully, and two volumes of methanol were added, followed by vortexing. Following centrifugation, the supernatant is removed and the precipitate is washed twice with two volumes of methanol to remove salt. The precipitate is then air-dried before proceeding.

### 8.3 Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Pure culture supernatant is mixed with 4x LDS sample buffer (Invitrogen) and dithiothreitol (final concentration 40 mM). Samples are heated to 80 °C for 10 minutes, before being loaded onto the gel. Gels were run 35 minutes at 200 V followed by fixing in 30 % ethanol with 2% phosphoric acid and staining in colloidal Coomassie staining solution (Candiano *et al.*, 2004; 0.12% Coomassie blue G250, 10% ammonium sulphate, 10% phosphoric acid, 20% ethanol).

## 8.4 2-dimensional gel electrophoresis

The precipitate from the chloroform/methanol precipitation was redissolved in 200  $\mu\text{L}$  rehydration solution (8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, 0.5% (v/v) IPG-buffer pH 3-10 (GE Healthcare), 0.3% dithiothreitol, trace amounts of Orange G). First dimension was carried out on an IPGphor (Amersham Pharmacia) over night using 11 cm IPGstrips pI 3-10 (GE Healthcare).

Second dimension was prepared by first reducing and alkylating proteins in the IPGstrips with dithiothreitol and iodoacetamide, respectively, in 5 mL of an equilibration solution (6 M urea, 30% (v/v) glycerol, 50 mM Tris pH 8.8, 0.01% (w/v) bromophenol blue, 2% sodium dodecyl sulphate). IPGstrips were cut 0.5 cm from the cathodic end and 2.5 cm from the anodic end and placed in NuPAGE ZOOM gels (Invitrogen). Electrophoresis and staining was done as described for SDS-PAGE. Mark12 (Invitrogen) was used as protein standard.

## 8.5 Mass spectrometry

Gel pieces were excised and digested by trypsin essentially as described (Shevchenko *et al.*, 1996): the excised gel pieces were washed in 40% ethanol in Eppendorf<sup>®</sup> tubes, followed by shrinking by 100% acetonitrile; then trypsin solution (12.5 ng/ $\mu\text{L}$ ; Promega) in 25 mM  $\text{NH}_4\text{CO}_3$  was added to the tubes and then incubated on ice for 45 min, before 5  $\mu\text{L}$  25 mM  $\text{NH}_4\text{CO}_3$  was added. The tubes were left over-night at 37 °C, and then the supernatant was transferred to new tubes.

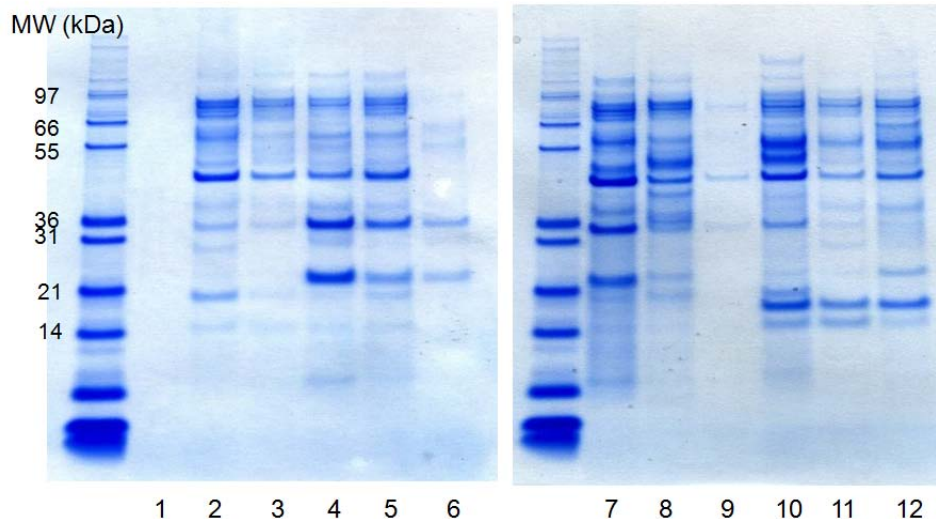
Tryptic peptides were prepared for MS as described (Zhang *et al.*, 2007): on a 600  $\mu\text{L}$  AnchorChip target (Bruker-Daltonics) one  $\mu\text{L}$  was added to a given spot on the target and letting it dry. Then one  $\mu\text{L}$  0.5  $\mu\text{g}/\mu\text{L}$   $\alpha$ -cyano-4-hydroxycinnamic acid in 90% acetonitrile, 0.1% trifluoro-acetic acid was added and left to dry. The spots on the target are then washed by adding two  $\mu\text{L}$  0.5% trifluoro-acetic acid and removing it after about 30 sec.

Tryptic peptides were analysed in Ultraflex II Matrix Assisted Laser Desorption/Ionisation-Time of Flight-Time-of-Flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) in positive reflector mode for peptide mass mapping or peptide fragment ion mapping. Calibration was performed externally (pre-MS) using  $\beta$ -lactoglobulin digested with trypsin, and internally (post-MS) using trypsin autolysis peaks 842.509, 1045.564 and 2211.104  $m/z$ . Instrument control and analysis were done on FlexControl and FlexAnalysis respectively. Database searches were performed using an in-house Mascot server (<http://www.matrixscience.com>) in the National Centre for Biotechnology Information database. The parameters used for searching were: global modifications: carbamidomethyl; variable modifications: methionine oxidation; missed cleavages, 1; peptide tolerance, 80 ppm and MS/MS tolerance  $\pm 1$  Da. Hits were considered significant if both MS and MS/MS hits, or two MS/MS hits, identify the same protein, and the score was above the significance threshold set by the FlexAnalysis software.

## 9 Results

### 9.1 Preliminary studies on protein secretion

Prior to the proteomics studies, preliminary trial experiments were performed on various oligo- and polysaccharides as carbon-source, including potato starch. These experiments were performed by post doctoral fellow Hiroyuki Nakai and then PhD-student Kenji Maeda, both at EPC.



**Figure 32** SDS-PAGE of culture supernatant of *A. nidulans* stimulated with either no carbohydrate (lane 1) or various carbohydrates. Lane 1: no carbon source; lane 2: glucose; lane 3: potato starch; lane 4: oat spelt xylan; lane 5: birchwood xylan; lane 6: tamarind xyloglucan; lane 7: wheat arabinoxylan; lane 8: sugar beet arabinan; lane 9: larchwood arabinogalactan; lane 10: guarin galactomannan; lane 11: carbin galactomannan; lane 12: konjac glucomannan. An equivalent of 18  $\mu$ L of undiluted culture supernatant was loaded in each lane.

When no carbon-source was added, no secreted protein was detected (Figure 32). The other eleven supernatants show eight different types of expression patterns. Both oat spelt xylan and birchwood xylan, as well as tamarind xyloglucan show the same expression pattern though the bands from the stimulation with xyloglucan are weaker. The difference seen between glucose and potato starch is a band at about 28 kDa. There is almost no difference between galactomannan and glucomannan, except for a band at about 66 kDa.

Activity assays confirmed between a 5-fold and a 25-fold increase in activity of arabinofuranosidase when stimulated with oat spelt xylan, wheat arabinoxylan and sugar beet arabinan. Galactosidase activity was stimulated 10-fold and 45-fold with sugar beet arabinan and guarin galactomannan, respectively. Xylosidase activity was stimulated around 15-fold in supernatants from oat spelt and birchwood xylan, as well as wheat arabinoxylan (data not shown; Nakai, unpublished results).

Trial 2DE-experiments on cultures stimulated with oat spelt xylan, sugar beet arabinan, and guarin galactomannan showed a different expression pattern relative to a culture stimulated with glucose (data not shown).

The following protein identifications were made (Table 19):

**Table 19 Identification of proteins in the preliminary experiments**

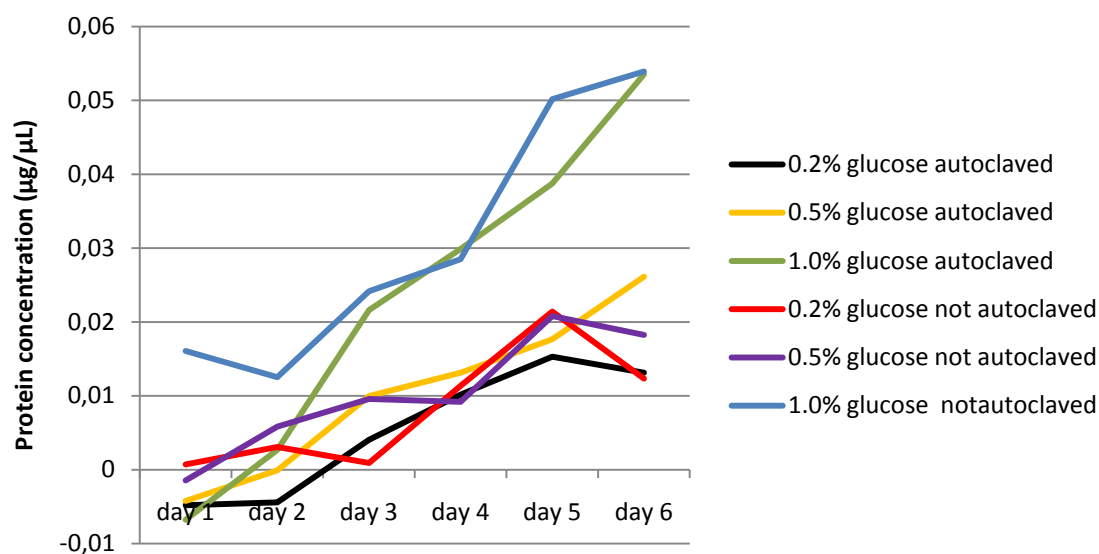
Glucose	Oat spelt xylan	Sugar beet arabinan	Guarin galactomannan
$\beta$ -galactosidase (GH2)	endo-1,4- $\beta$ -xy lanase (GH10 and GH11)	$\alpha$ -galactosidase (GH27)	endo-1,4- $\beta$ -mannanase (GH5)
$\beta$ -glucosidase (GH3)	endo-glucanase (GH61)	endo-1,5- $\alpha$ -arabinanase (GH43), $\alpha$ -L-arabinofuranosidase (GH54) $\alpha$ -L-rhamnosidase (GH78) $\alpha$ -1,2-mannosidase (GH92)	

These results indicated that many different enzymes can be induced in the presence of different carbohydrates, and also suggest that different enzymes may be induced by different starches.

## ***9.2 Protein secretion as a function of time***

During one experiment, five identical glucose cultures were started and protein concentration was measured as the cultures were progressively harvested one per day. Cultures were stimulated with 0.2%, 0.5%, or 1% glucose, either autoclaved with the media or sterilized and then added to autoclaved media. These concentrations were used to give a broad representation of concentrations of glucose which might be reflected in the amount of specifically expressed proteins.

There is a continuous increase in the extracellular protein concentration throughout the experiment (Figure 33). However, both the 0.2% and 0.5% glucose cultures show a minor drop in protein concentration at day six, possibly due to experimental uncertainty. There is a large difference in the extracellular protein concentration of the cultures stimulated with 1% glucose and the two lower concentrations. However, there is also a difference between the 1% cultures, where the culture on glucose autoclaved with the media shows a larger increase in secreted protein concentration from day one to day six than the culture where glucose was not autoclaved. No apparent differences in the protein pattern are observed on the SDS-PAGE gel, though 1% glucose supernatants show clearer bands as a result of the higher protein concentration (data not shown).

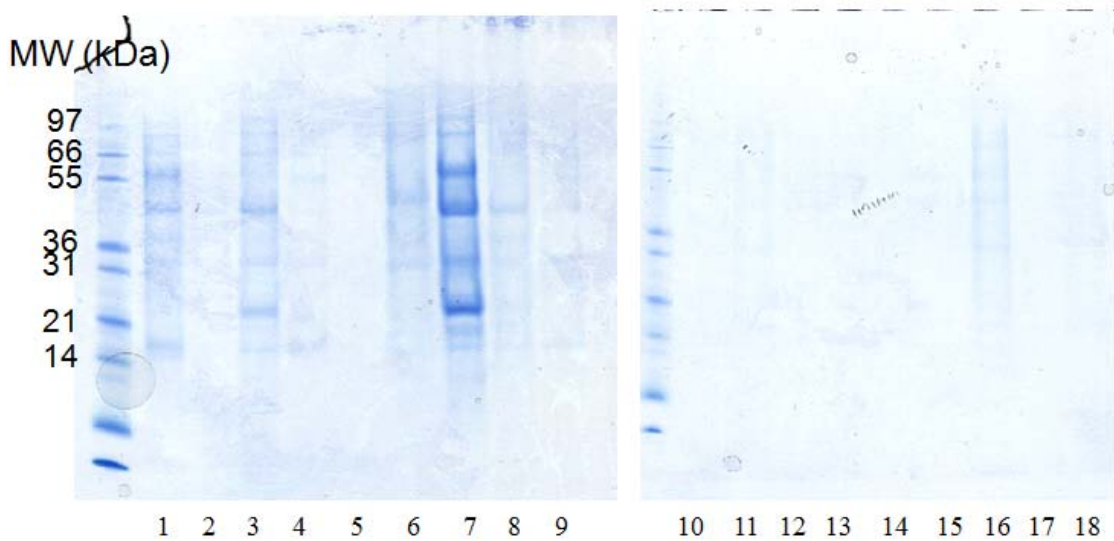


**Figure 33** Extracellular protein concentration as a function of time. *A. nidulans* was stimulated with glucose in three concentrations (0.2, 0.5 or 1.0%). The glucose was either autoclaved with the media or sterilized with 70% ethanol and dried, and then added to the autoclaved media (not autoclaved). Protein concentration was determined by the Bradford method (Bradford, 1976).

### 9.3 Carbohydrate induction of secreted proteins

During the growth of the different liquid fungi cultures, a big difference in the morphology of the cultures was observed. *A. nidulans* forms small spherical aggregates. In the culture stimulated with glucose, large aggregates are seen, but for the hard-degradable pullulan small, barely discernable threads are observed (data not shown). Cultures grown on starches have a morphology and cell density similar to glucose, only with smaller aggregates.

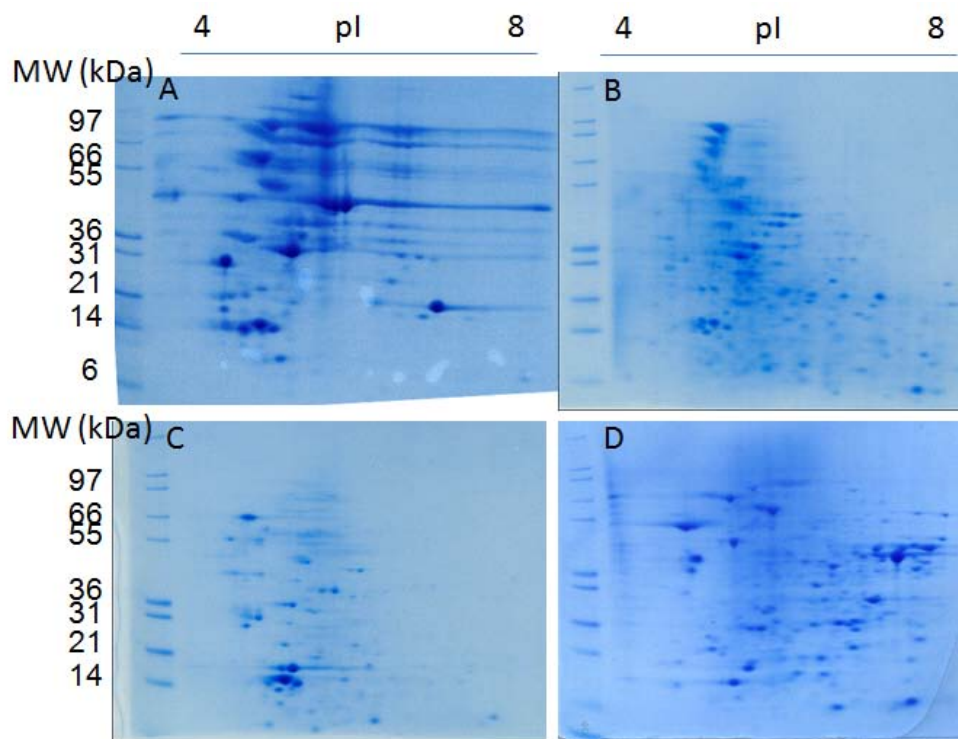
SDS-PAGE of these culture supernatants also shows that some carbohydrates induce secretion of more protein (Figure 34), however, supernatants from cultures stimulated with starch show very low protein concentrations. Protein concentration determinations show concentrations of around 0.02 µg/µL for all cultures, except wheat arabinoxylan, which shows a protein concentration around 0.4 µg/µL (data not shown). Of the supernatants with visible protein expression shown in Figure 34, konjac glucomannan, oat spelt xylan, sugar beet arabinan, and wheat arabinoxylan show a similar pattern to those observed in Figure 32.



**Figure 34** SDS-PAGE of the supernatants of cultures stimulated with: 1: konjac glucmannan; 2: larch wood arabinogalactan; 3: oat spelt xylan; 4: guarin galactomannan; 5: tamarind xyloglucan; 6: sugar beet arabinan; 7: wheat arabinoxylan; 8: soy bean rhamnogalacturonan; 9: high amylose maize starch; 10: high amylopectin maize starch; 11: high amylopectin maize starch; 12: normal maize starch; 13: high amylopectin potato starch; 14: high amylose and high phosphate potato starch; 15: low phosphate potato starch; 16: normal pea starch; 17: normal wheat starch; 18: glucose. An equivalent of 15  $\mu$ L culture supernatant was loaded in each lane.

Experiments performed later using different concentrations of carbohydrate do not show the same low protein concentrations for the starch samples (data not shown), though stimulation with potato starch does not give a visible protein expression as seen by 2DE (data not shown).

Comparisons of the 2DE spot pattern (Figure 35) of four of the carbohydrates reveal many differences. Of the many cultures grown on different carbohydrates only a few were analysed by 2DE before extensive sample degradation was observed. This degradation was presumably caused by active uninhibited secreted proteases. Also a shift in focus in the PhD-project towards the project regarding aroma-binding to starch, meant that all of the carbohydrate stimulated cultures analysed by SDS-PAGE, were not analysed by 2DE.



**Figure 35** Stimulations with glucose (A), wheat arabinoxylan (B), konjac glucomannan (C), and wheat starch (D).

Despite many well-resolved and distinct peaks in the MS results, only around one-third of the excised spots gave significant hits in database searches. The possible explanations for this low success-rate are discussed in Chapter 10.1. The spots on the 2D-gels that were excised and identified are shown in Appendix 2 (Chapter 14.1).

**Table 20** Identification of protein from cultures stimulated with different carbohydrates. The theoretical MW and pI were calculated using the “Compute MW/pI” tool on the ExPASy Bioinformatics website. The observed MW and pI were assessed by manually. Homology searches were done using Basic Local Alignment Search Tool on the National Centre for Bio-Informatics website. The score is calculated based on an algorithm which is based on the database size and number of specific identifications of peptides from a given protein. In this study a score above 70 is a unique identification.

	Identification	MW (obs.; kDa)	MW (theo.; kDa)	pI (obs.)	pI (theo.)	Score	Homology (BLAST)
<b><u>Glucose</u></b>	CATB_EMENI	80	79	4.2	4.92	284	Catalase
	CATB_EMENI	80	79	5.4	4.92	116	Catalase



	AN7657	48	49	5.0	4.67	158	Glucanosyltransferase (GH2)
	AN4871	44	44	5.4	5.33	163	GH18 chitinase
	AN4871	44	44	5.4	5.33	245	GH18 chitinase
<b><u>Wheat</u></b>	AN4871	34	44	5.0	5.33	163	GH18 chitinase
<b><u>arabino-</u></b>							
<b><u>xylan</u></b>							
	AN4871	44	44	5.4	5.33	245	GH18 chitinase
	AN3983	15	26	5.0	6.74	90	Hypothetical protein
	AN3983	15	26	5.0	6.74	81	Hypothetical protein
	AN3983	15	26	5.0	6.74	71	Hypothetical protein
	AN3983	15	26	5.0	6.74	71	Hypothetical protein
	AN3983	15	26	5.0	6.74	71	Hypothetical protein
	AN7349	34	48	5.0	4.59	75	GH71 $\alpha$ -glucanase
	AN9177	23	43	5.0	5.75	70	NADH/flavin oxidoreductase
	AN7908	38	36	4.4	4.27	134	GH43 arabinofuranosidase
	AN8218	44	42	5.4	5.05	97	Thioredoxin reductase
	AN1152	14	19	5.8	5.51	90	Hypothetical protein
	gi 67515919	15	19	6.4	6.44	103	Superoxide-dismutase
	HEX1_EMENI	8	25	6.8	6.70	152	Woronin body
	HEX1_EMENI	9	25	6.8	6.70	159	Woronin body
	HEX1_EMENI	14	25	7.0	6.70	80	Woronin body
	HEX1_EMENI	18	25	7.0	6.70	107	Woronin body
<b><u>Konjac</u></b>	AN2710	18	21	5.0	4.85	76	Hypothetical protein
<b><u>gluco-</u></b>							
<b><u>mannan</u></b>							

	AN3983	16	26	5.0	6.74	91	Hypothetical protein
	AN3983	16	26	5.0	6.74	82	Hypothetical protein
	AN3983	17	26	5.0	6.74	70	Hypothetical protein
	gi 67522723	34	34	5.2	4.93	100	GH10 xylanase
	AN4871	44	44	5.4	5.33	95	GH18 chitinase
	AN4871	44	44	5.4	5.33	76	GH18 chitinase
	gi 67515919	22	19	7.0	6.44	89	Superoxide-dismutase
<b><u>Wheat starch</u></b>	AN6103	40	36	4.6	4.73	110	Starch binding protein related to glucoamylase (GH15)
	AN3402	10	69	4.6	4.78	147	GH13 $\alpha$ -amylase
<b><u>Pea starch</u></b>	gi 67540710	40	42	6.0	6.24	86	Formate dehydrogenase
	AN7895	38	33	6.0	5.13	144	Oxidoreductase
	AN2366	40	26	4.6	4.22	93	Serine protease
	AN3983	22	26	5.0	6.74	98	Hypothetical
<b><u>Barley beta-glucan</u></b>	XYNC_EMENI	35	34	5.2	4.93	123	GH10 xylanase
	AN3983	16	26	5.0	6.74	106	Hypothetical protein

Enzymes specific to a certain type of carbohydrate are identified, e.g. wheat arabinoxylan induces expression of arabinofuranosidase and wheat starch induces expression of glucoamylase. Induction with konjac glucomannan induces expression of the hypothetical protein AN2710, which has no known function, but is a protein is exclusively induced in cultures stimulated with glucomannan, suggesting an involvement in degradation of this

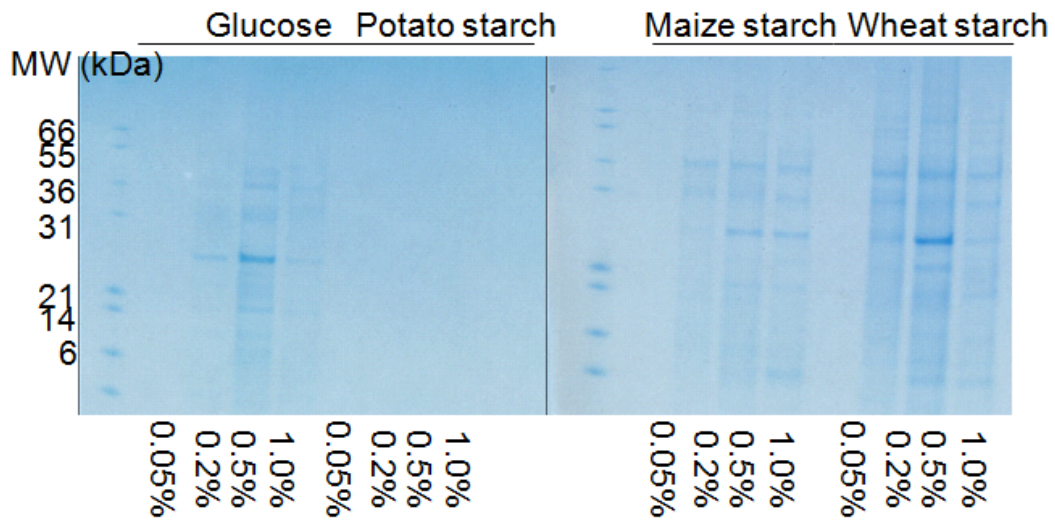
carbohydrate. Many of the proteins are expressed ubiquitously and are therefore found in more than one supernatant, e.g. chitinase. However, most of the supernatants show a protein pattern where the majority of proteins are found at lower observed molecular weights than could theoretically be expected. This could be the result of degradation by extracellular proteases.

#### ***9.4 Induction of protein at different concentration of carbohydrates***

As shown by the studies by Nahas and Waldemarin, and Kubrak and co-workers (Nahas and Waldemarin, 2002; Kubrak *et al.*, 2010), the concentration of the carbohydrate added can change the expression level of amylases.

For the present study glucose and three starches (potato, maize, and wheat) are chosen. Glucose has previously been shown to induce  $\alpha$ -amylase (Nahas and Waldemarin, 2002), which are needed to degrade starch to glucose. Therefore it is possible that the presence of glucose probably induces expression and secretion of amylases to degrade starch. These three starches represent two types of starch crystallinities (see Chapter 1.2), and may therefore also induce different expressions of certain enzymes such as amylases.

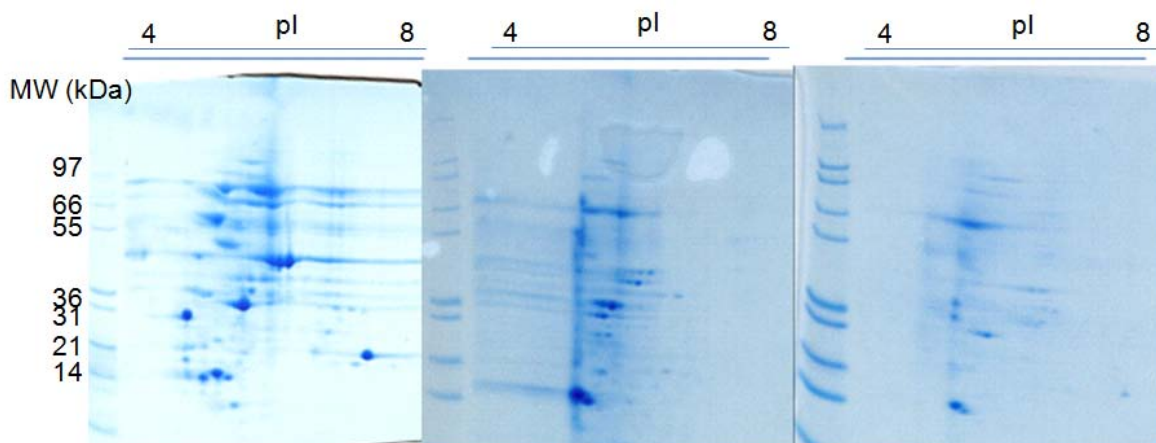
The cultures were stimulated with four different concentrations of carbohydrate, but for all types of carbohydrates the cultures stimulated with 0.05% showed no growth and no extracellular protein was detected (data not shown). For glucose little, if any, difference between the cultures stimulated different concentrations can be seen on a SDS-PAGE gel (Figure 36). For potato starch no protein expression is observed at all concentrations (Figure 36), and protein analysis by the Bradford method (Bradford, 1976) also shows lower amounts of protein observed for the other samples (data not shown). Maize and wheat starch show little variation, both when comparing the effect of the different concentrations and between these two starches (Figure 36). However, it is very likely that 2DE will reveal more differences as one band on a SDS-PAGE can easily contain more than one protein to be resolved in 2DE due to differences in pI.



**Figure 36** SDS-PAGE on supernatants from cultures stimulated at four different concentrations of carbohydrate (glucose, potato starch, maize starch, or wheat starch).

#### 9.4.1 Comparisons of the 1% cultures

By directly comparing the cultures that were stimulated with 1% carbohydrate (excluding potato starch due to very low protein concentrations) it is possible to identify differences originating solely from the nature of the carbohydrate. However, the presence of salts in the sample which cause streaking, complicate interpretations (Figure 37). Despite this streaking, it is possible to observe many similarities, but also some differences for all three carbohydrates. In particular, a group of proteins at around 14 kDa and around pI 5 is roughly visible on all three gels. All of these proteins were identified as AN3983, which gives no significant homology hits but may be involved in membrane attack complexes (see Chapter 10.3).



**Figure 37** Comparison of the supernatant of 1% cultures stimulated with glucose (left), maize starch (middle), and wheat starch (right). 50  $\mu$ g was loaded on each gel as measured by Bradford assay.

### 9.4.2 Influence of carbohydrate concentration on protein secretion

The protein concentration in the supernatants does not appear to be concentration-dependent as the 0.2%, 0.5%, and the 1% cultures show almost the same concentration (data not shown). There is, however, a difference between the types of carbohydrate used, as glucose and maize starch show protein concentrations of about 0.04  $\mu\text{g}/\mu\text{L}$ , whereas wheat starch shows a protein concentration around 0.06  $\mu\text{g}/\mu\text{L}$ .

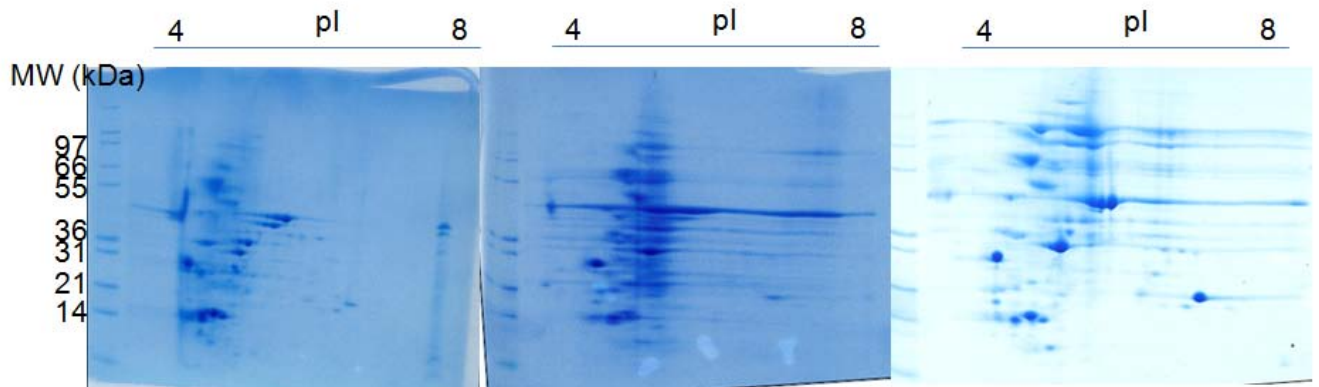
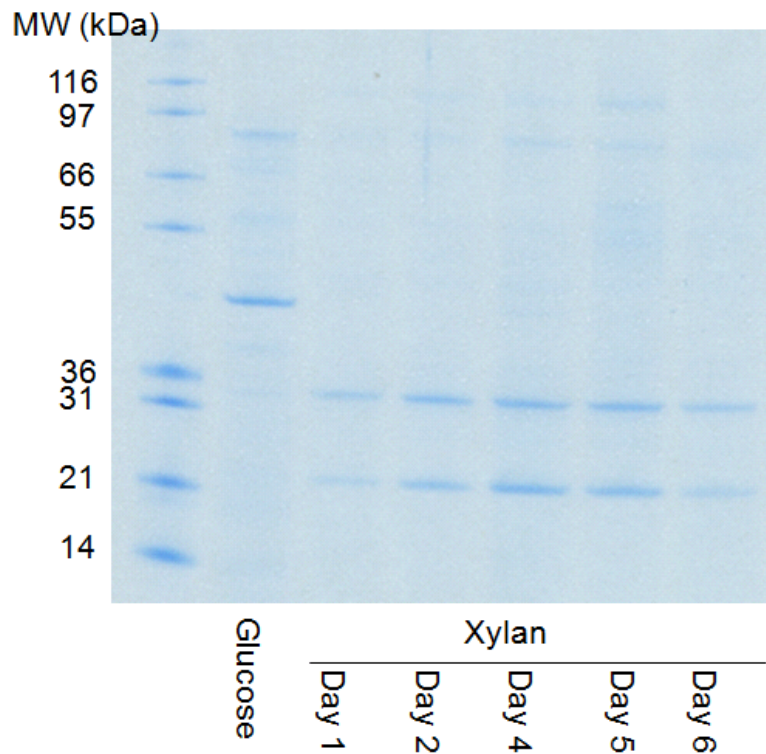


Figure 38 Comparison of supernatant from cultures stimulated with 0.2% glucose (left), 0.5% glucose (middle) and 1.0% glucose (right).

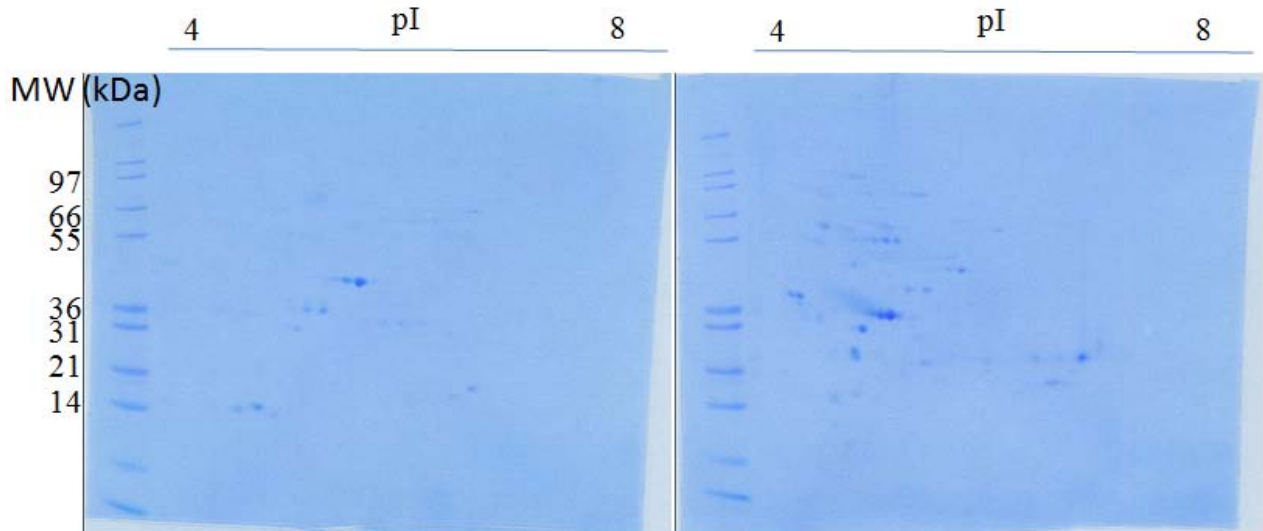
### 9.5 Attempts of induction of GH61 protein

As mentioned in the preliminary studies (Chapter 9.1; Table 19), stimulation with oat spelt xylan induced expression of a GH family 61 protein. The function of this protein is unknown, but it is speculated to be a  $\beta$ -glucanase (Bauer *et al.*, 2006). Alternatively, it is speculated to play a role in binding cellulose for degradation by other enzymes (Koseki *et al.*, 2008).

Monitoring the protein expression during the stimulation with xylan only revealed minor differences in the proteome. However, these minor differences may be very important in the degradation of xylan. The bands on the SDS-PAGE gel appear most intense at day 4 and slightly decreasing thereafter, possibly due to proteolytic activity. Relative to glucose at least two protein bands at 21 kDa and 32 kDa, respectively, are induced in the presence of xylan.



**Figure 39** Small changes in protein expression are observed during the growth of *A. nidulans* stimulated with 0.36% oat spelt xylan as seen by SDS-PAGE. An equivalent of 15  $\mu$ L of culture supernatant is loaded in each lane.



**Figure 40** Supernatants from cultures stimulated with 0.36% glucose (left) and 0.36% oat spelt xylan (right). 50  $\mu$ g protein was loaded on each gel. In total 64 spots were excised from the two gels.

All visible spots were excised from the two gels in Figure 40, and of these 64 spots excised, 19 could be identified (Appendix 2 and Table 21). Chitinase and a superoxide-dismutase are

found in both cultures, whereas two types of xylanases and an arabinofuranosidase are only found in the xylan stimulated culture. Stimulation with glucose induces expression of arabinase and  $\beta$ -glucanase. Glucanase has previously been shown to be ubiquitously expressed in other *Aspergilli* in the presence of many different carbohydrates (Medina *et al.*, 2004; Tsang *et al.*, 2009).

**Table 21** Identifications from 2DE gels from supernatants from cultures stimulated with glucose or oat spelt xylan. All visible proteins were excised from the gels and analysed. Of 64 spots excised and analysed by MS and MS/MS, 19 gave positive identifications. The observed molecular weights and pI's were assessed by eye using the gel images in Figure 40. Obs.: observed; Theo.: theoretical. Homology searches were performed using protein BLAST at the National Center for Bio-Informatics (NCBI). In this study a score above 42 is a unique identification.

	Identification	MW (obs.; kDa)	MW (theo.; kDa)	pI (obs.)	pI (theo.)	Score	Homology (BLAST)
<b><u>Glucose</u></b>	AN7950	36	47	4.6	4.47	135	GH17 endo- $\beta$ -glucanase
	AN8007	36	35	5.4	5.21	76	GH43 arabinase
	AN8007	36	35	5.6	5.21	67	GH43 arabinase
	AN8007	14	35	5.0	5.21	65	GH43 arabinase
	AN3983	14	26	4.8	6.74	44	Hypothetical protein
	AN4871	44	44	5.8	5.33	116	GH18 chitinase
	AN4871	44	44	5.8	5.33	126	GH18 chitinase
	AN4871	44	44	5.8	5.33	126	GH18 chitinase
	AN7962	18	38	6.6	4.97	67	Peptidase
	gi 67515919	17	19	6.4	6.44	63	Superoxide-dismutase
<b><u>Xylan</u></b>	XYNC_EMENI	34	34	5.0	4.93	119	GH10 xylanase
	XYNC_EMENI	34	34	4.8	4.93	119	GH10 xylanase
	XYNC_EMENI	35	34	4.8	4.93	119	GH10 xylanase
	AN9380	23	26	4.6	4.60	46	Polysaccharide deacetylase (carbohydrate esterase family 4)

AN0779	47	109	4.6	4.48	67	GH55 exo- $\beta$ - glucanase
AN4871	44	44	5.8	5.33	63	GH18 chitinase
gi 67526311	22	24	6.6	5.87	223	GH11 xylanase
AN7908	38	36	4.0	4.27	68	GH62 arabinofuranosidase
AN7908	38	36	4.2	4.27	74	GH62 arabinofuranosidase
gi 67515919	18	19	6.4	6.44	61	Superoxide-dismutase

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Some of the proteins show a lower molecular and a shift in pI compared to their theoretical values, indicating that degradation may have occurred. On the basis of the present data, it cannot be clarified if this degradation is happening before or after secretion.



## 10 Discussion

### ***10.1 General remarks on the results and reproducibility of in the present study***

There have been many technical issues during this study that were not all solved fully satisfactorily. This includes problems with accuracy in determining the protein concentrations, problems with salt causing incomplete isoelectric focusing, and problems with sample degradation. Combined these problems resulted in further problems with reproducing some of the results. Solutions to these problems have been extensive desalting, adding protease inhibitors, and lowering pH after harvesting the cultures. The solutions to these problems have not completely solved all technical issues, but they have introduced a larger degree of reproducibility to the experiments. The problems have becoming progressively less during the study, and the reliability of the results has increased as a result thereof. The proteins identified as being induced by specific carbohydrates are still induced, even if they are proteolytically degraded later. Streaking may mask some of the proteins induced, but especially in the induction of the GH61 protein, the problem with streaking on the gels is sufficiently resolved to see clear differences in protein pattern.

Many of the MS-spectra in this study showed many clear and well separated peaks, but no identification was possible, either in single MS mode or tandem MS mode. The success-rate for identification was around 33%. The peptides may either be glycosylated as seen previously for another *Aspergillus* (Oda *et al.*, 2006), partial proteolysis caused by secreted proteases has occurred, or the protein spots analysed may be a mixture of two or more proteins. The two latter possibilities will still result in positive identifications by tandem MS, whereas glycosylations on the protein will prevent identification, possibly by preventing degradation by trypsin. Enzymatic deglycosylation of proteins before MS analysis (Oda *et al.*, 2006) may result in more identifications, but deglycosylation was not attempted in the present study.

### ***10.2 Protein concentration in the cultures as a function of time***

The sharp increase in protein concentrations for the two 1% glucose cultures (glucose added before and after autoclaving, respectively), show that there is plenty carbon-source for continued growth, whereas the lower increase for the cultures stimulated with 0.2% and 0.5% glucose, indicate that these cultures have limited access to the carbon-source, and continued growth after day six is perhaps not possible. The difference in the increase of the protein concentration between the two 1% glucose cultures is probably caused by the autoclaving process which helps to dissolve carbohydrates. This makes the carbohydrate more readily accessible for degradation, thus giving an advantage to the culture stimulated with autoclaved glucose.

Nahas and Waldemarin (2002) show an increase in mycelium weight as a function of glucose concentration, but simultaneously show a decrease in  $\alpha$ -amylase activity. In a similar study in *Bacillus sp. BKL20*, the  $\alpha$ -amylase activity decreased when the starch concentration in the

medium was increased, despite increasing cell mass and protein concentration (Kubrak *et al.*, 2010). Although no activity measurements were performed in the present study, it is very likely that there is a continued production of enzymes other than  $\alpha$ -amylase in the cultures stimulated with 1% glucose. At the same time, it is also likely that in cultures stimulated with lower concentrations of glucose, the secretion of enzymes at day six is stopped or slowed as the carbohydrate-source is close to depletion.

### ***10.3 Carbohydrate screening***

The differences in morphology of the cultures stimulated with different carbohydrate is a clear indicator that *A. nidulans* is very well suited to grow on substrates such as glucose and starch. These carbohydrates resulted in large spherical aggregates, whereas other carbohydrates such as pullulan did not stimulate any visible growth. This relates to the saprotrophic lifestyle of fungi, where starch is often found in plants, and glucose is the main product in the degradation of starch.

The Woronin body identified for the culture stimulated with arabinoxylan (Table 20) is a protein secreted during cell disruption, where it function as a plug to stop the cell bleeding (Tenney *et al.*, 2000). This could indicate that at least some of the fungal cells in the culture have undergone auto-lysis. This may also explain the presence of cytosolic proteins thioredoxin b and NADH oxidoreductase in the extracellular medium. Further, the high representations of low molecular weight proteins in 2DE, suggest these are fragments formed by proteases. The most noteworthy example of degradation is  $\alpha$ -amylase which has theoretical MW of 69 kDa, but is identified on the 2D-gel as a protein with a MW of 10 kDa.

Chitinase is found in the cultures stimulated with glucose, oat spelt xylan, wheat arabinoxylan, and konjac glucomannan, suggesting that this enzyme is expressed ubiquitously. Fungal cell walls contain chitin and expression of chitinase can re-organise cell structures during formation of new branches in filamentous fungi. Chitinases are also involved in the pathogenesis of a fungus (Punja and Utkhede, 2003). However, Medina and co-workers only find chitinase induced in an *A. flavus* culture stimulated with a glycoside (Medina *et al.*, 2004), suggesting that the chitinase expression may be regulated by stimulation with certain carbohydrates. Chitinases have also been shown to be involved in auto-lysis (Yamazaki *et al.*, 2007).

The induction of glucoamylase by wheat starch has previously been identified in liquid cultures (Medina *et al.*, 2004), but at least one of the glucoamylase gene is only expressed in solid state cultures in *A. oryzae* (Ishida *et al.*, 2000). This is probably related to the saprotrophic lifestyle where more enzyme is needed to maximize the utilisation of solid nutrients.

The hypothetical protein AN2710 was only observed in cultures stimulated with konjac glucomannan. Homology searches do not show any similarities with other known proteins, and no conserved domains are found. Therefore it is possible that this protein can have

domains specific for konjac glucomannan, which have not previously been identified and characterised.

The identification of a superoxide dismutase and catalase is evidence of the presence of reactive oxygen species, such as hydrogen peroxide. Carbon starvation has been shown to induce expression of proteins like superoxide dismutases and catalases (Emri *et al.*, 2004). The protein AN3983 is found in almost all of the cultures tested. Homology searches on this protein only significantly identify this protein as hypothetical, but non-significant hits include membrane attack complexes and perforins. In the present study this protein is found as a group of three to four spots on the 2D-gel. This could indicate that this protein may be involved cell lysis of the invaded tissue. This may be done by forming protein complexes with a central cavity that insert themselves into the membrane and allowing the contents of the cells to flow out through the protein complex cavity. The nutrients and salts can then be easily absorbed by the fungus. Because this type of protein is vital for the saprotropic behaviour of the fungi, no special carbohydrate requirements must be fulfilled before AN3983 is expressed.

Neither of the two hypothetical proteins, AN2710 or AN3983, or proteins believed to have a similar function, have been observed in previous studies.

Technical problems such as streaking complicate the interpretation, however, if experiments had run technically perfect, it is very likely that both  $\alpha$ -amylase and glucoamylase would have shown the highest level of secretion in the cultures with the lowest concentration of starch, and the lowest level with the highest concentrations of starch. This is probably again related to maximizing the use of nutrients, and if there is plenty carbon-source available the fungus has no incentive to secrete more enzyme.

#### ***10.4 Induction of the GH61 protein***

No GH61 protein was found in the present study, whereas both glycosidase hydrolase family 10 and family 11 xylanases were identified. These two xylanases were also identified in the preliminary studies leading to the present study, thus confirming these previous results (Chapter 9.1). However, in this study there were repeated problems with the iso-electric focusing, especially in the low pI range, which is where the GH61 protein was found in the preliminary study by Nakai and Maeda. It is possible that these focusing-problems have masked the possible presence of GH61 or any other proteins found in the low pI range.

The presence of arabinofuranosidase in the xylan stimulated culture may well be a result of expression of a gene similar to that found in *A. niger*, where the XlnR gene regulates expression of xylanase, xylosidase, and arabinofuranosidase (van Peij *et al.*, 1998). A similar mechanism of regulation has also been found in *A. oryzae* (Marui *et al.*, 2002).

The functionality of the GH61 protein is unknown, but in the preliminary studies leading up to the present study, GH61 (AN1602) was induced on xylan, suggesting its involvement in the degradation of this hemicellulose. It may be a glucanase (Bauer *et al.*, 2006). The gene encodes a GH61 consisting of a catalytic domain and a carbohydrate binding domain

connected by a linker. The reported lower activity of GH61 compared to other  $\beta$ -glucanases (Karlsson *et al.*, 2001), and the presence of a carbohydrate binding domain, suggest that this protein may also function to aid other enzymes in the degradation of, e.g., xylan by making it more readily digestible. An enzyme with structural similarity to GH61 has been shown to cleave glycosidic bonds in crystalline chitin, suggesting that GH61 may have an similar function (Vaaje-Kolstad *et al.*, 2010).

## 11 Conclusions

Since some of the problems seen early on in the present study were solved at a relatively late stage, it is likely that repeating some of the earliest experiments may provide different results than those observed in the present study. Degradation of proteins is revealed by many low molecular weight proteins on the gels and slowing or stopping the degradation, i.e. by adding protease inhibitors, will provide a clearer expression pattern and more intense protein spots. Glycosylations on the protein may have prevented identification of some of the protein analysed by mass spectrometry.

Differences were found in the cultures stimulated with types of carbohydrates as shown by SDS-PAGE. However, due to the problems with accurate protein concentration determination, these differences were hard to identify by 2DE. Stimulation with wheat starch induced expression of  $\alpha$ -amylases and glucoamylases, which is not surprising as these enzymes degrade starch to glucose. These enzymes could not be identified in the other starch induced cultures, but this is probably due to degradation issues. Likewise stimulation with arabinoxylan induced expression of arabinofuranosidase, and stimulation with glucomannan induced expression of hypothetical protein AN2710, which was only observed in cultures stimulated with glucomannan.

Growing cultures in different concentrations of glucose and starches did not clearly induce expression of enzymes not seen in the other concentrations, though as above difficulties with protein concentration determination complicates interpretation of these data.

Stimulation with oat spelt xylan induced xylanases and arabinofuranosidases, presumably through activation of the same gene (van Peij *et al.*, 1998), whereas the target of this sub-study, a glycoside hydrolase family 61 protein of unknown function, was not detected. Problems with focusing in the low pI range may have masked this protein, which was originally observed with a pI of around 4.

The objective of the present study was to identify enzymes from *A. nidulans* with relevance for the modification of starch. No such enzymes were found, though their presence may have overlooked by low amounts of enzyme secreted or partial degradation. Alternatively the carbohydrate may be partially degraded outside the cell by other enzymes, followed by uptake and final degradation to monosaccharides inside the cell.

## 12 Future work and perspectives

The industrial use of many species of *Aspergilli* is well-documented and is a billion dollar industry. However, despite some proteomics studies on other *Aspergilli*, such as *A. oryzae* and *A. flavus* (Oda *et al.*, 2006; Medina *et al.*, 2004) a true elucidation and understanding of the vast and complex machinery of enzymes used by fungi for carbohydrate degradation is far from completion. The present study attempted to disclose a central portion of this machinery, but more work is needed for that to be fulfilled.

Continued work will probably benefit from deglycosylating the analysed protein before performing mass spectrometry analysis (Oda *et al.*, 2006). Other issues that will definitely improve the success-rate are lowering pH when cultures are harvested, extensive filtering of the culture supernatant, and addition of protease inhibitors. These steps were introduced in the present study at a relatively late stage.

The results achieved in the present study are not directly applicable for further studies. However, if the GH61 protein had been found in the oat spelt xylan stimulated culture, this knowledge could be applicable in the processing of biomass for production of bio-fuels.

## 13 References

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## **14 Appendix**

List of appendices:

### **Supplementary figures and tables**

Appendix 1: Retention of alcohols by starches

Appendix 2: Spots on 2D-gels excised and identified

### **List of presentations and publications**

Appendix 3: List of presentations and publications

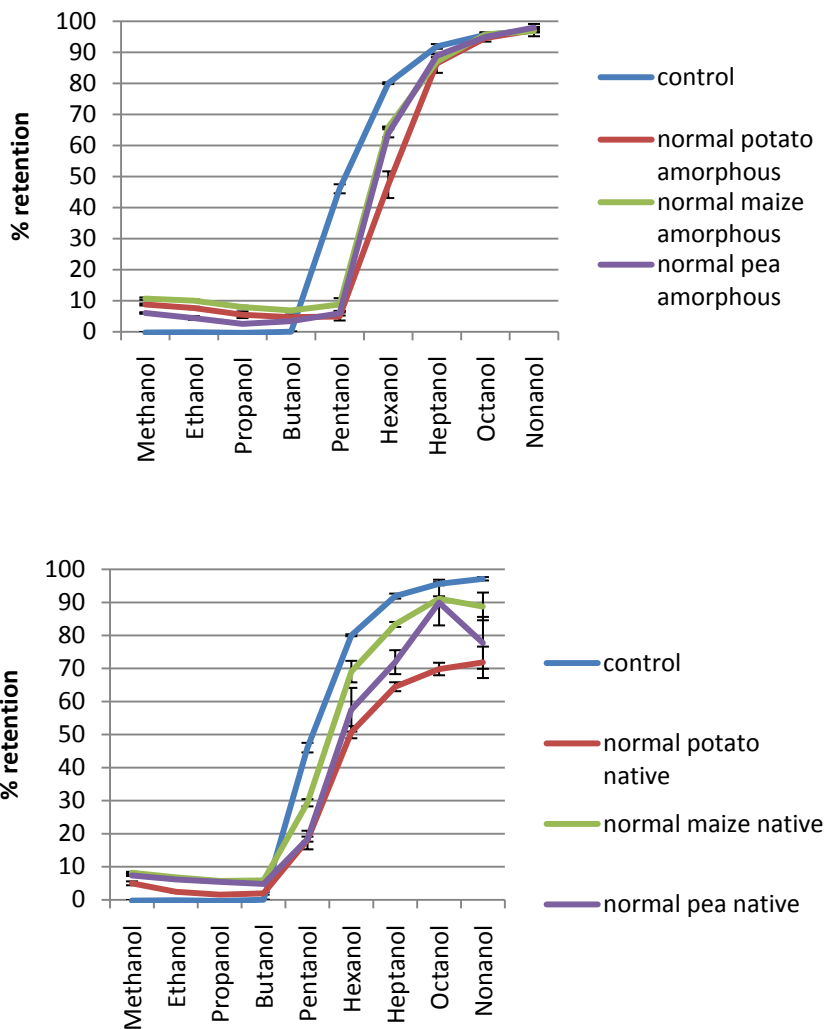
Appendix 4: Manuscript I

Appendix 5: Manuscript II

Appendix 6: Enclosed poster

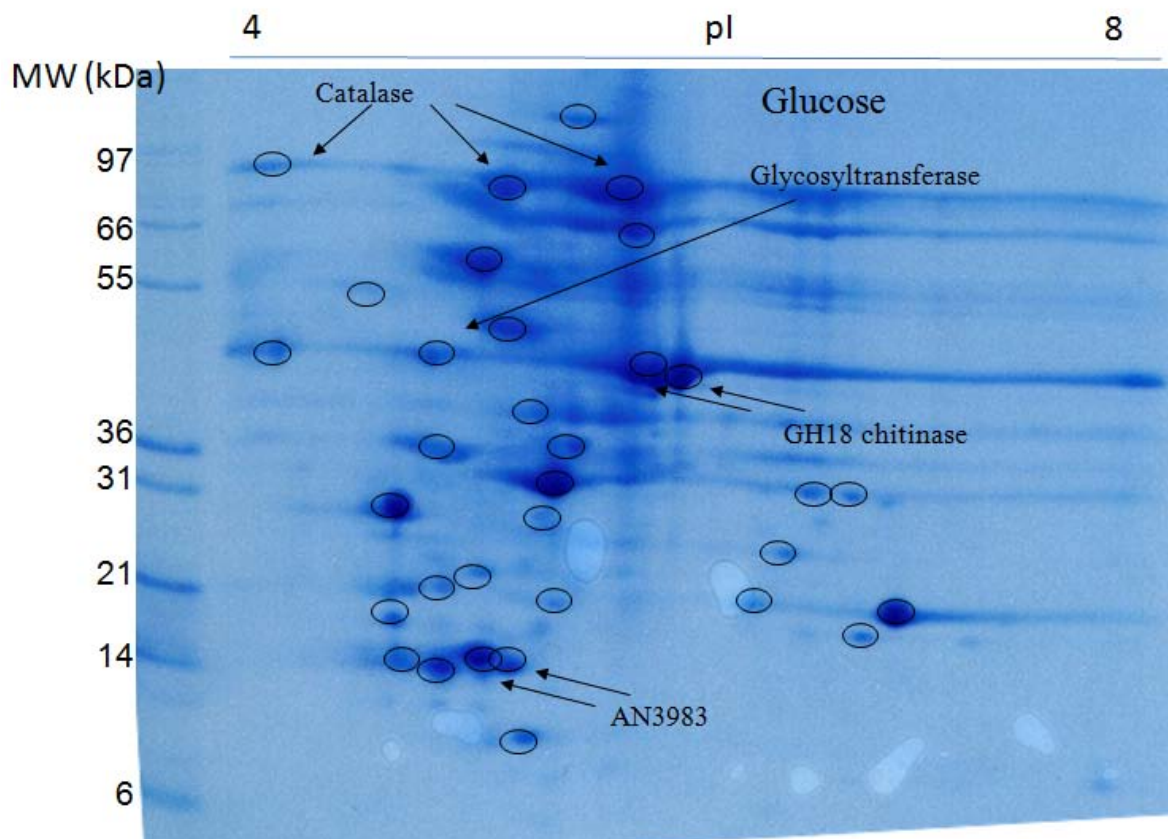
### 14.1 Supplementary figures

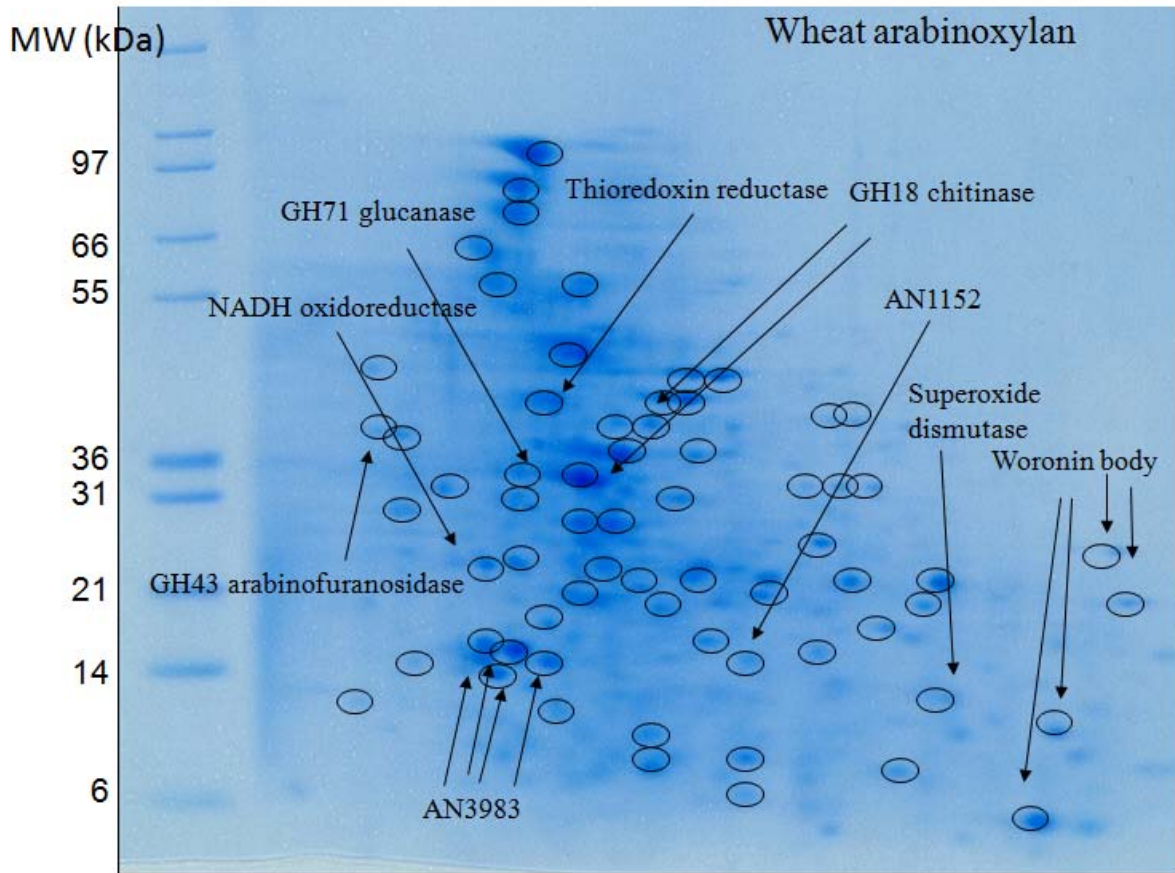
**Appendix 1:** Retention of alcohols by starches. A graphical representation of the data presented in Table 13.



**Figure 41** Graphical representation of the data presented in Table 13. The amorphous starches (top) show a better retention for hexanol through nonanol than the native starches (bottom). Retention is defined as the amount of weight of aroma left at the end of the experiment divided by the amount of aroma added at the starch of the experiment. The error bars are the standard error of the mean of triplicates.

**Appendix 2:** The following eight figures show which protein spots were excised from the gels of supernatants of *A. nidulans* stimulated with different carbohydrates (Chapters 9.3 and 9.5) and the resulting identifications. 50  $\mu$ g is loaded onto each gel. The carbohydrate used from stimulation is listed in the top right corner of each gel. The gels appear in the order they are listed in Table 20 and Table 21.

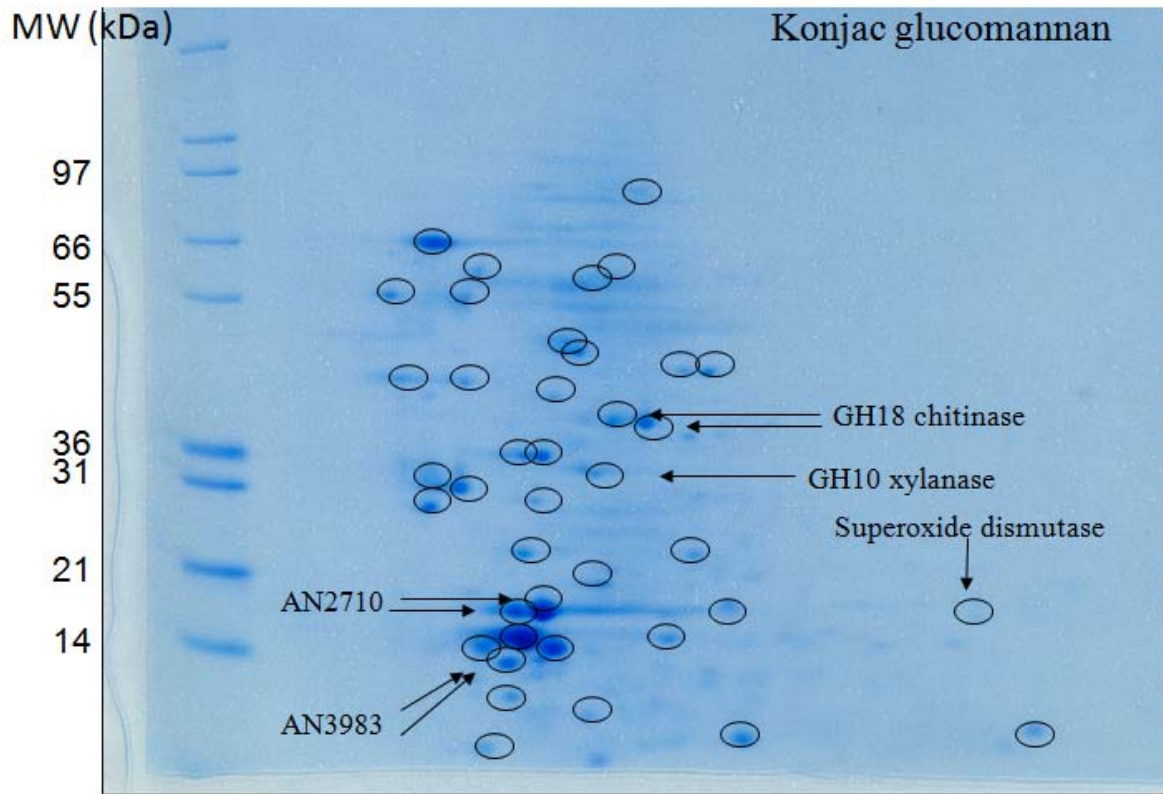




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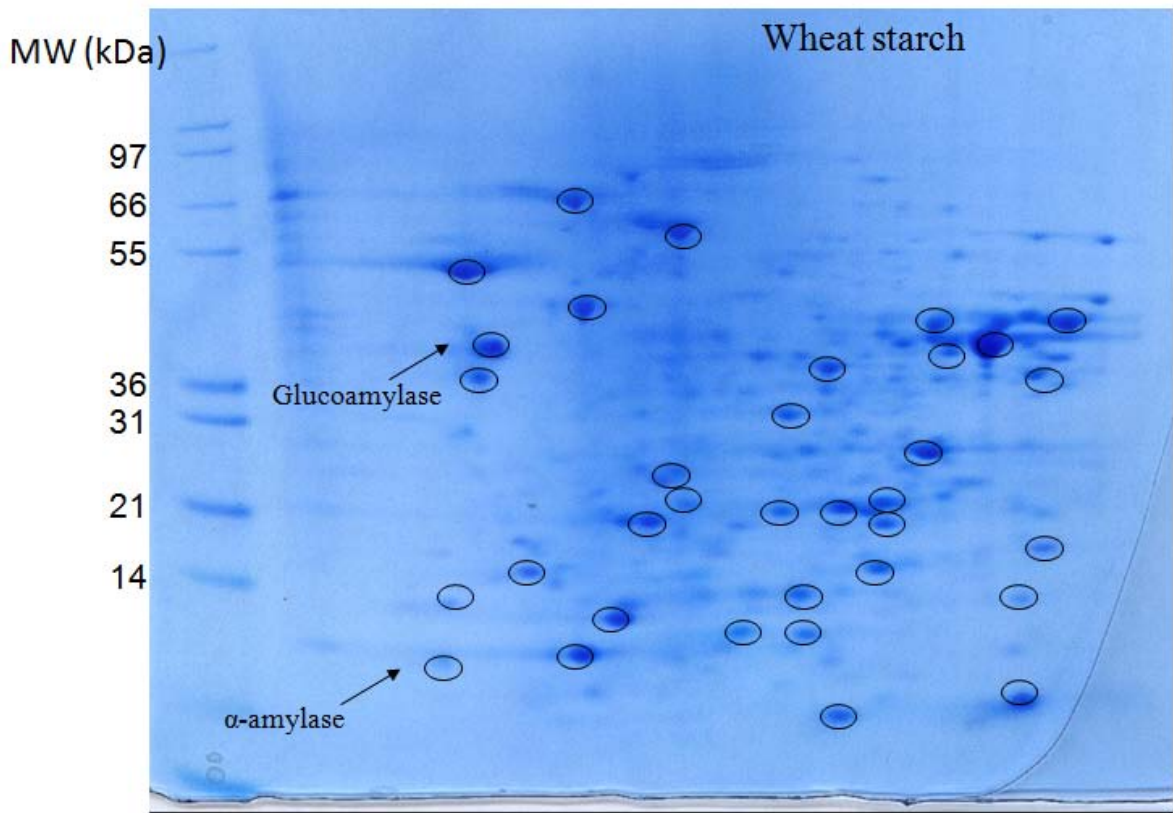
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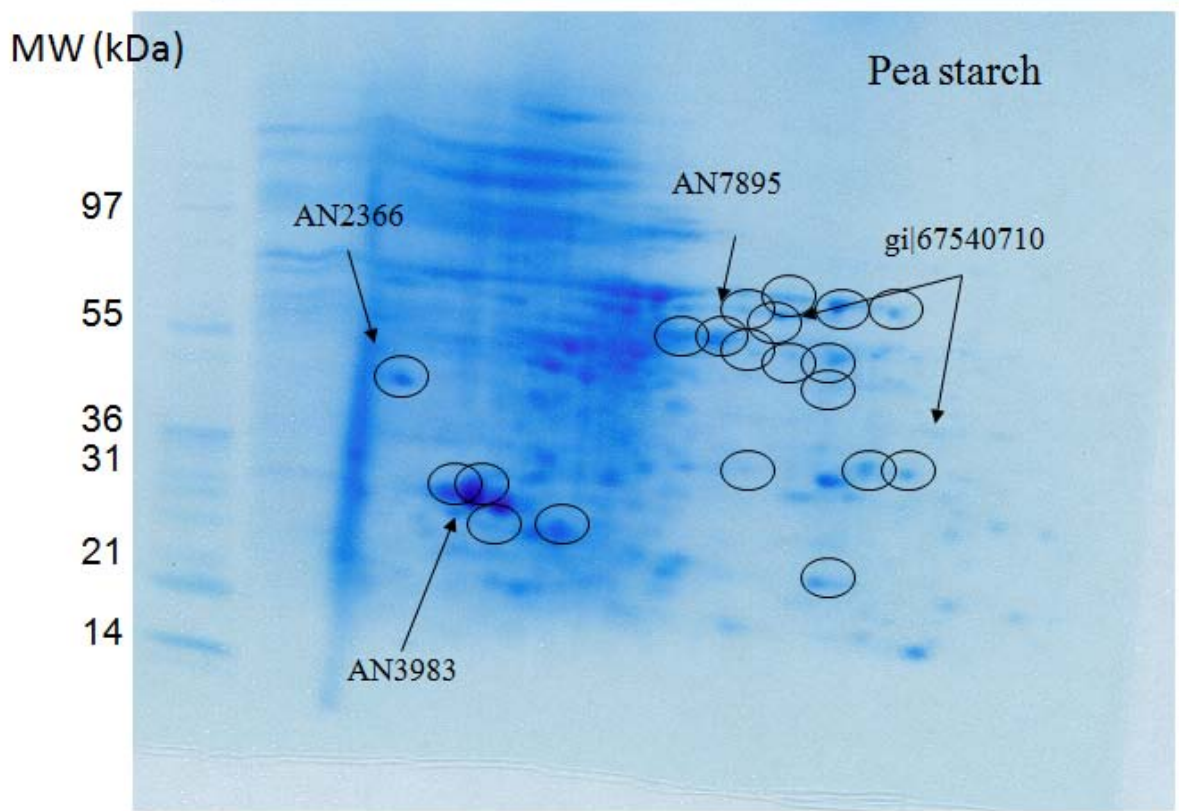
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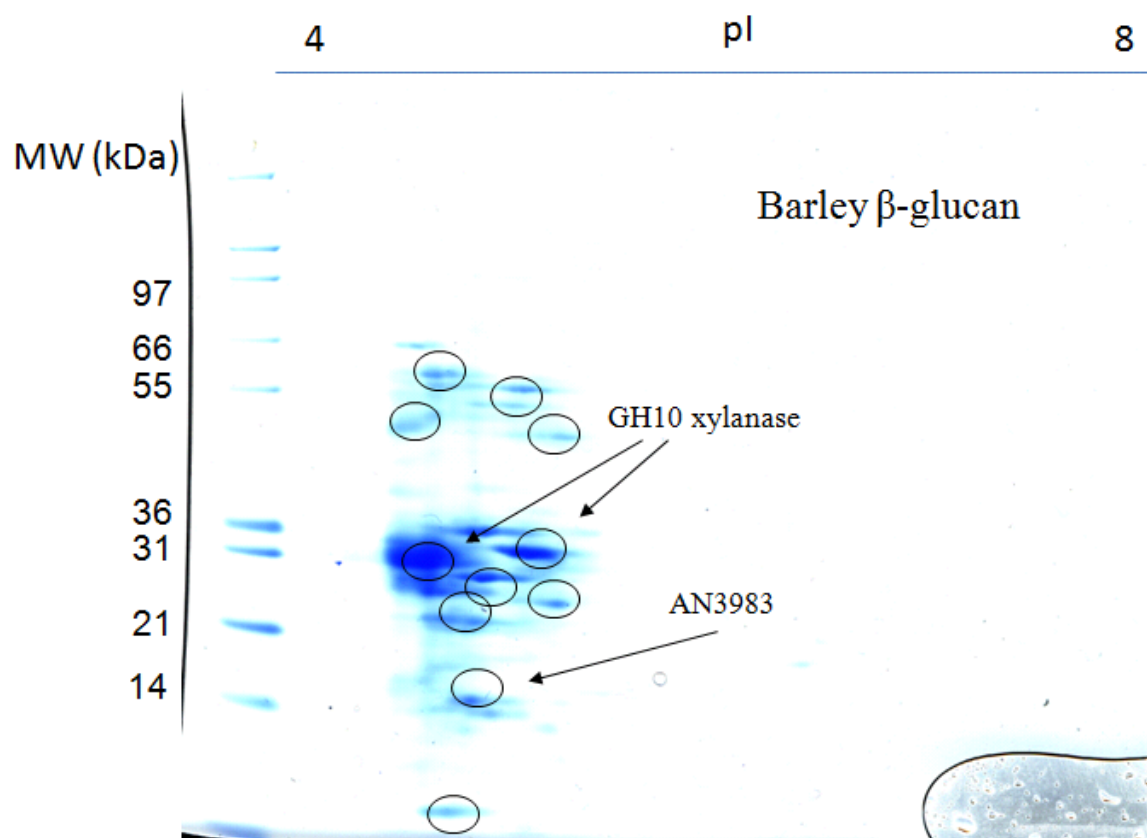
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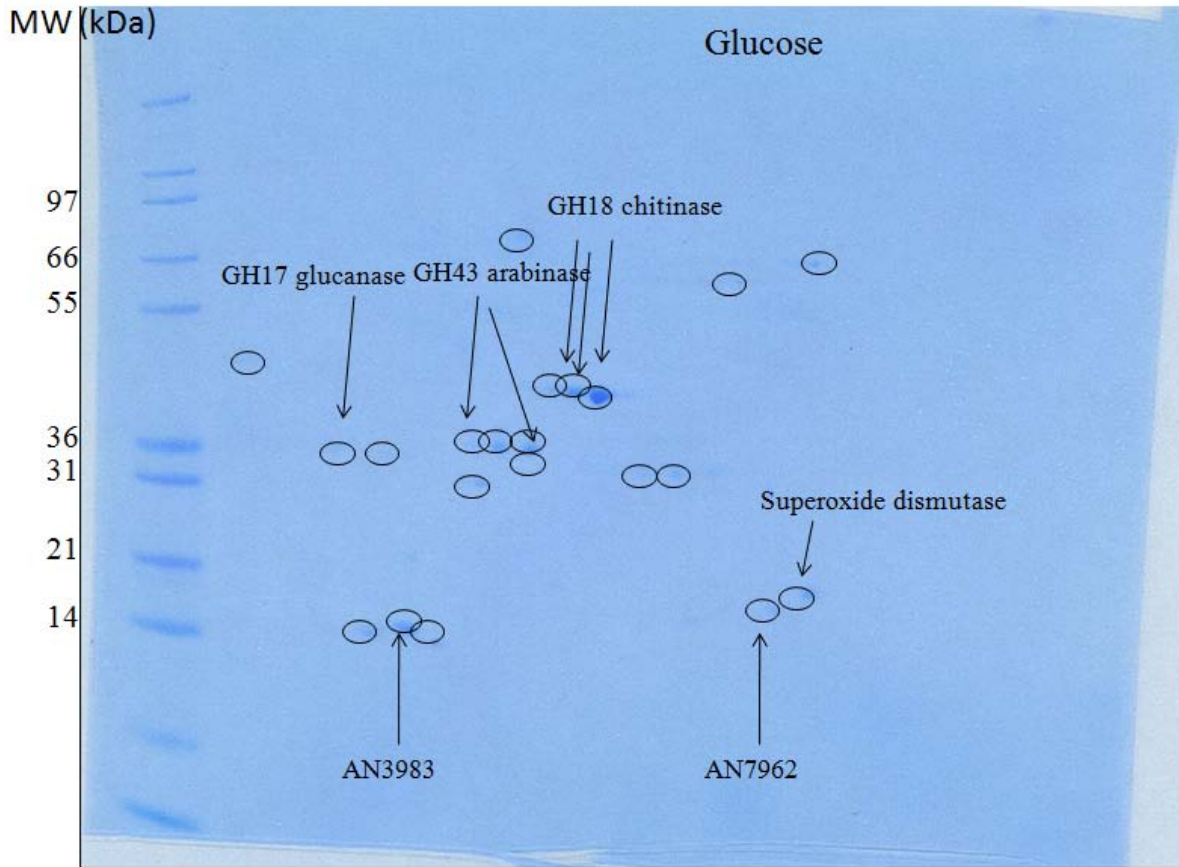








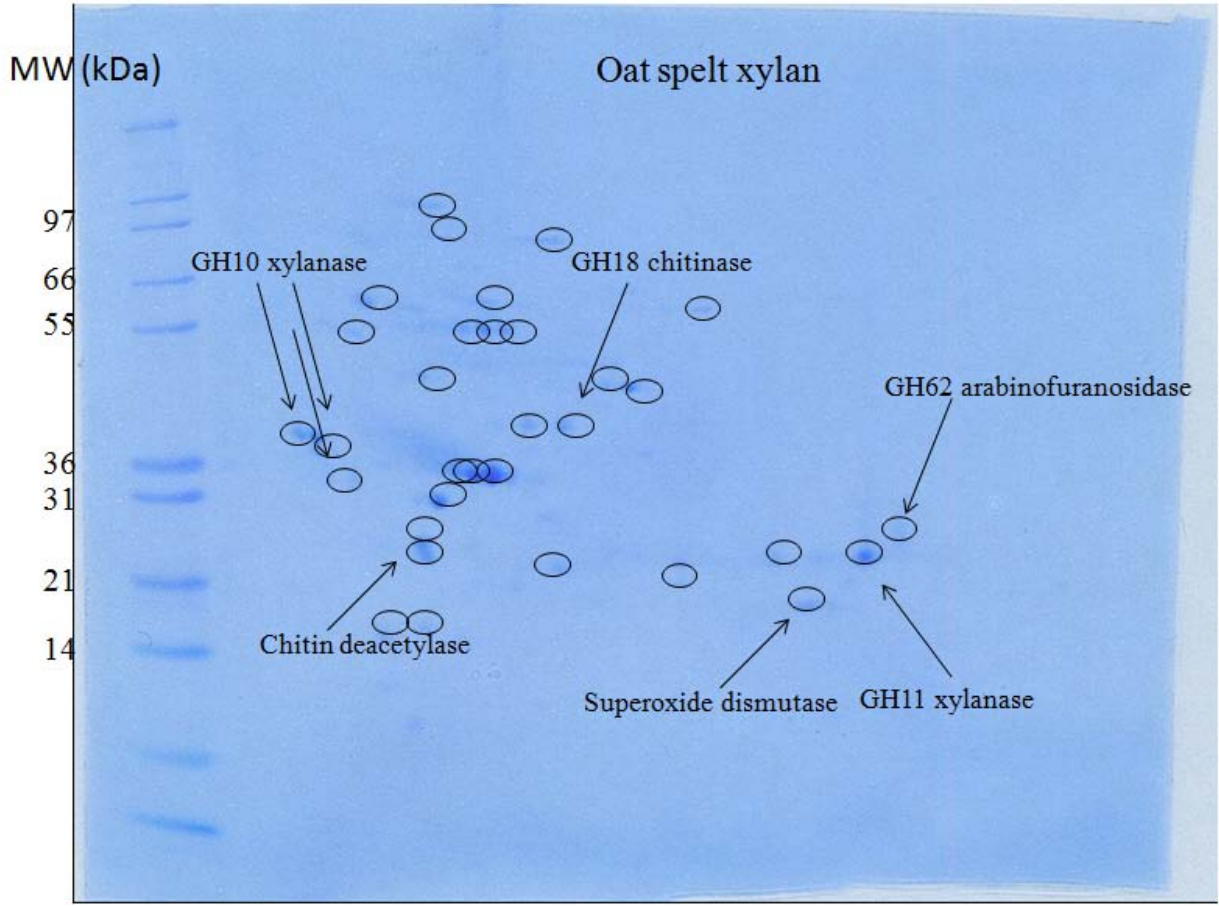




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## ***14.2 List of presentations and publications***

### **Appendix 3**

#### **14.2.1 Oral presentations**

7 Nov 2008 Protein Science Meeting, Tisvildeleje, Denmark. “Use of two-dimensional electrophoresis on *Aspergillus nidulans* supernatant for discovery of novel carbohydrate modifying enzymes and binding modules”.

16 Mar 2010 3<sup>rd</sup> Protein Engineering Workshop, DTU, Denmark. “Targeted enzyme modification of starch for encapsulation of flavours”.

9 Mar 2011 Protein Engineering Workshop at EPC, DTU, Denmark. “Targeted enzyme modification of starch for encapsulation of flavours”.

11 Nov 2011 Planned final presentation for the industrial collaborator Lyckeby, Lyckeby Culinar AB, Fjälkinge, Sweden.

Five presentations for the industrial collaborator Lyckeby,; four at DTU, Denmark, one at Fjälkinge, Sweden.

Nine presentations at EPC group meetings, EPC, DTU, Denmark.

#### **14.2.2 Poster presentations**

16 Mar 2010 3<sup>rd</sup> protein engineering workshop, DTU, Denmark (see enclosed poster in Chapter 14.2.3.1.3).

17-20 May 2010 Summer Course in Glycosciences 2010, Wageningen, The Netherlands.

22-23 Nov 2010 LMC FOOD PhD congress, KU-LIFE, Denmark.

#### **14.2.3 Manuscripts and publications**

Jørgensen, A.D., Jensen, S.L., Buléon, A., Svensson, B., Blennow, A., 2011. Structural and physical effects of aroma binding to native starch granules. To be submitted to *Starch/Stärke*. See Chapter 14.2.3.1.1

Yang, F., Jørgensen, A.D., Li, H., Søndergaard, I., Finnie, C., Svensson, B., Jiang, D., Wollenweber, B., Jacobsen, S., 2011. Implications of high-temperature events and water deficits on protein profiles in wheat (*Triticum aestivum* L. cv. Vinjett) grain. *Proteomics* 11, 1684-1695. See Chapter 14.2.3.1.2

### ***14.2.3.1 Enclosed manuscripts, publications, and posters***

#### **Appendix 4**

##### ***14.2.3.1.1 Manuscript I***

Jørgensen, A.D., Jensen, S.L., Buléon, A., Svensson, B., Blennow, A., 2011. Structural and physical effects of aroma binding to native starch granules. To be submitted to *Starch/Stärke*.

## **Structural and physical effects of aroma binding to native starch granules**

*Anders D. Jørgensen<sup>1</sup>, Susanne L. Jensen<sup>2</sup>, Alain Buléon<sup>3</sup>, Birte Svensson<sup>1</sup>, Andreas Blennow<sup>2</sup>*

<sup>1</sup> Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

<sup>2</sup> Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, 1870 Frederiksberg, Denmark

<sup>3</sup> INRA, UR1268 Biopolymères Interactions Assemblages, Nantes F-44316, France

## Abstract

The interaction and physical/structural effects on dry native starch granules of eight selected model aroma compounds, acetaldehyde, dimethyl sulphide, diacetyl, allyl isothiocyanate, ethyl butyrate, citral, octanol and butyric acid were studied. The starch types used from maize, potato and pea represent typical structural and chemical starch characteristics. Retention of aroma at high concentrations varied from a few percent to one hundred percent for the different aromas. Furthermore the starch type influenced the retention behaviour. As deduced from differential scanning calorimetry (DSC), powder X-ray diffraction (XRD) and scanning electron microscopy (SEM) analyses, citral, butyric acid and octanol exerted specific effects on the starch granules manifested in local melting of crystalline layers and partial disruption of the granular meso structure. The most prominent effect was obtained with citral that generated surface wrinkles on B- and C-type polymorphic granules and clustering of A-type polymorphic granules, decreased melting temperature and suppressed crystallinity.

## 1. Introduction

Aroma compounds are generally small, hydrophobic molecules of molecular weight below 400 Da (Landy *et al.*, 1996) comprising aldehydes, ketones, alkenes, aromatic rings, acids, sulphides, and esters. When included in food systems or aroma formulations, retention of aroma compounds is mainly influenced by the matrix constituents like fat, protein and carbohydrate as well as by the physical and structural properties, e.g. viscosity and porosity. Lipids are the most prominent aroma retention component, but in many low fat foods starch is being used to replace some of the properties of fat such as aroma-retention/interaction (Madene *et al.*, 2006). Thus retention of aromas in starch has been reported (Solms, 1986) and demonstrated for a variety of systems including native starches (Hau *et al.*, 1996), starch suspensions (Jouquand *et al.*, 2006), extruded starch (Kollengode and Hanna, 1997), and complex formation with amylose (Pozo-Bayon *et al.*, 2008). In a study on starch with low water contents, the water content has been suggested to have an effect on volatile binding (Hau *et al.*, 1998). However, systematic analysis of pure starch systems with high aroma concentrations and very low water contents as it is found in aroma formulations has not been performed and gets increasingly relevant as low-fat food products continues to gain importance and consumer attention. In this study we use starches with 10% to 30% water content, and aroma-concentration of around 33% of the total weight.

To gain knowledge on specific interaction between starch and aromas well-defined starch structures must be tested using single, pure aroma compounds. Molecularly well defined starch systems are not readily available and foreign compounds can induce structural alterations in the starch to change interaction. At high concentrations, specific foreign compounds can act as chaotropic agents. An example is DMSO (Englyst and Cummings, 1984) which interacts with macromolecules including starch and other polysaccharides. These



compounds disrupt the semi-crystalline starch granules leading to gelatinization. At high concentration of chaotropic aroma compounds used for e.g. concentrated aroma formulations these aromas can induce amorphisation and re-arrangements to create new sites for aroma interaction.

It is anticipated that aroma compounds form complexes with specific parts of the starch granules including the crystalline and amorphous regions. For native starch granules, surface area and porosity can be expected to be important for initial interaction. An effect of surface area on enzymatic starch granule degradation has been demonstrated suggesting that the interaction of aroma compounds with starch may also be affected by the surface area (Tahir *et al.*, 2010). Channels or pores have been observed in the starch granules from maize, barley and wheat by using SEM, and confocal laser light microscopy but no channels are seen in potato starch granules (Glaring *et al.*, 2007; Fannon *et al.*, 1992; Huber and Bemiller, 1997). These channels have a diameter of about 100 nm and play a central role in chemical modifications of starches (Fannon *et al.*, 1992), and they probably are also important in allowing access of guest molecules such as aromas. The structural units at these surfaces can include amorphous amylose or amylopectin, crystalline or semi-crystalline amylopectin including double helical or single helical segments.

Hence, the molecular composition of starch is also considered to be important for its capacity to complex with and host guest molecules. Amylose is an inherent component of native starch and hence it can be expected that the binding capacity of a guest molecule is correlated with the amylose content of the starch. The amylose:amylopectin ratio, which has a rather narrow range in normal seed and tuber starches, is dependent on the botanical origin of the starch (Frederikson *et al.*, 1998). Variations in this ratio are expected to have significant impact on aroma binding (Arvisenet *et al.*, 2002) as reflected by the ability of amylose to form the so

called  $V_H$  helices identified by XRD. The  $V_H$  helix is a left-handed single helix with six glucose units per turn, a pitch height of about 8 Å and an internal diameter of 10 Å (Rappenecker and Zugenmaier, 1981; Brisson *et al.*, 1991). The  $V_H$  helix is important conformational element of amylose forming the classical inclusion complexes with iodine (Rundle and Edwards, 1943; Bluhm and Zugenmaier, 1981). The  $V_H$  helix is also excellent for accommodating many other ligands, such as fatty acids (Godet *et al.*, 1993), pentanol (Helbert and Chanzy, 1994), butanol (Hinkle and Zobel, 1968), selected alcohols and acids (Osman-Ismail and Solms, 1972), decanal and more (Nuessli *et al.*, 1997) can interact with this starch conformation. As deduced by NMR foreign molecules can be accommodated either inside the amylose helix as described above or on the helix surface, in particular between two amylose helices (Rondeau-Mouro *et al.*, 2004). For less specific interactions and at high concentrations of chaotropic agents, DSC can be useful for monitoring and quantifying complexation and induced molecular disorder. Water acts as a plastisiser of the amorphous regions of starch, so increasing the water content lowers the melting/transition temperature (Biliaderis, 1985). Thus addition of polar aroma compounds or compounds with polar groups, *i.e.* butyric acid, can influence the thermal behaviour by increasing amorphous chain mobility, thereby lowering the melting temperature.

## **2. Materials and Methods**

### **2.1 Materials**

Maize starches were provided by Cerestar-AKV I/S (Denmark), pea starch was obtained from KMC, Brande Denmark. Potato starch was purchased from Sigma-Aldrich. Aroma compounds were of 93% purity or higher (Sigma-Aldrich).

### **2.2 Aroma complexation**

Native starch granules were frozen at -80 °C, followed by freeze-drying.

Starch (200 mg  $\pm$  2 mg) was added to 15 mL polypropylene tubes with screw-cap (Greiner Bio-One GmbH) (three tubes per aroma per starch and three tubes without aroma per starch). The tubes including the cap were weighed before and after adding starch, as well as after aroma addition and aroma evaporation. This allowed calculation of the exact amount of starch used and aroma added, as well as the amount of aroma released retained, respectively. 100  $\mu$ L aroma (Sigma Aldrich; purity 93% or higher) was added to 200 mg starch to give approximately 33 weight-percent aroma, except for acetaldehyde where 200  $\mu$ L was added. The aroma-starch mixtures were placed on an ultra-sonication bath on ice for one hour. Thereafter, the aroma was allowed to evaporate from the mixture by passive diffusion by leaving the open tubes in a fume hood for six days. At the end of this period, the tubes were closed with the cap and weighed. Water absorption was calculated by subtracting the weight of the tubes with each starch type without aroma after six days from their starting weight and then subtracted from the tubes with the same type of starch. Binding is calculated as the difference in weight of a starch/aroma sample relative to the sample with starch, divided by the weight of the starch.

### **2.3 Differential scanning calorimetry (DSC)**

The starches were analysed in slurries of 3 mg native starch and 12  $\mu$ L 10 mM NaCl in duplicates using a Perkin Elmer Diamond DSC operated from 30 to 100°C at a scanning rate of 10°C/min. Perkin Elmer Pyris 7.0 software was used to determine the parameters onset temperature ( $T_O$ ), peak temperature ( $T_P$ ), conclusion temperature ( $T_C$ ) and enthalpy change ( $\Delta H$ ).  $\Delta T$  is defined as  $T_O - T_P$ .

### **2.4 Powder X-ray diffraction (XRD)**

Powder X-ray diffraction was performed as described (Tawil *et al.*, 2011). Starch water content was equilibrated to 90% air moisture for 10 days using saturated barium chloride and 20 mg of hydrated samples were analysed to generate XRD diagrams recorded on a Bruker (Wissembourg, France) D8 Discover diffractometer. Relative crystallinity was determined after normalization of the diffractograms between 3 and 30° (2 $\theta$ ). B-type re-crystallized amyloses were used as crystalline standards and dry extruded potato starch was used as the amorphous standard as described (Tawil *et al.*, 2011).

### **2.5 Scanning electron microscopy (SEM)**

Native and aroma complexed starch granules were analysed directly or after removal of citral by acetone washing. The dried starch granule preparations were gently distributed in a thin layer over a carbon sticker on an aluminium stub, coated with gold/palladium for two min at 2.1 mV and microscopy was performed using an XL30 ESEM FEG SEM (FEL Company, Boston) instrument operating at 10 kV and a working distance of approximately 10 mm.

### **2.6 Brightfield (BF) and polarized light (PL) microscopy and iodine complexation**

Citral treated granules were analysed with BF microscopy before and after removal of citral remnants with acetone. Samples were stained with a solution of 0.026% I<sub>2</sub>, 0.26% KI in 100 mM HCl, inspected under BF and PL and images recorded using Leica DC 300F camera and IM50 software. Scaling was accomplished using a stage micrometer.

## **3. Results and Discussion**

### **3.1 Retention of aroma to starch granules**

The aromas acetaldehyde, dimethyl sulphide, diacetyl, allyl isothiocyanate and ethyl butyrate showed an increase in aroma retention (Figure 1). This demonstrates an interaction with the

starch granules at the surface or internally of the starch granules. Conversely, the aromas citral, octanol and butyric acid showed a reduction in retention relative to a control. Dimethyl sulphide had the largest improvement in retention, whereas octanol and butyric acid had the largest reduction in retention with potato starch showing the largest reduction.

The increased evaporation found for citral, octanol, and butyric acid found for all three starch types is possibly an effect of starch-induced exposure of surface area from which aroma can evaporate. Further, these compounds are bulkier than e.g. acetaldehyde and dimethyl sulphide, and may therefore diffuse less readily through the granule matrix. Lower retention of all aromas was found for potato starch as compared to maize and pea starch. This effect can be due to absence of channels known to be present in the other starches (Fannon *et al.*, 1992; Huber and Bemiller, 1997; Glaring *et al.*, 2007) or more smooth surface structure for these granules. No difference between the starches in retention was noted for gelatinized, amorphous starch showing that the granule architecture is important for interaction.

### **3.2 Differential Scanning Calorimetry (DSC)**

Interaction between foreign molecules with starch granules can result in disruption or creation of crystalline material in the granules. The thermic transition of the starch granule upon heating in e.g. water can be interpreted to disclose the number of crystalline double helices present in the starch granule and the melting temperature can be related to the perfection of these helices in the crystalline lamellae (Tester and Debon, 2000). Strong interaction with aroma compounds can affect crystallinity and crystallite perfection and hence, such changes can be monitored by DSC. The three different granular starches tested (Table 1) had distinctly different thermal properties (Table 3). Thus incubation with aroma compounds induced different effects on thermal behaviour depending on the starch type and

the structure of the aroma compound. The most pronounced effect of aroma complexation on melting enthalpy was observed for citral, which suppressed the melting enthalpy of all three starch types. The maximum suppression of melting enthalpy caused by citral is observed on maize starch, which showed a melting enthalpy of 42% below the control (Figure 2B). Also the melting enthalpy of potato and pea starch showed significant suppression induced by citral (40 and 30% respectively). Amylopectin constitutes the major crystalline part of the native starch granules and since the data shows a strong influence on the crystalline parts of the starch granule, citral interacts strongly with the amylopectin fraction of the starch. Further, allyl isothiocyanate and butyric acid seemed to slightly suppress the melting enthalpy of maize and pea starches, but not potato starch (Figure 2D). Octanol showed a reduction of 53% melting enthalpy for pea starch, and 29% and 25% enthalpy reduction for maize and potato, respectively. The other aroma compounds did not significantly influence the melting enthalpy of the starches demonstrating insignificant effects on starch granule integrity for these compounds.

The effect of aroma complexation on peak temperature was generally moderate. The effect of citral, octanol and butyric acid was most pronounced, but these three starch types were affected differently by these compounds (Figure 2A). The effect of citral resulted in a distinct suppression of melting peak temperature of all three starch types; however pea starch seemed to be less affected than maize and potato starch. Likewise, a complexation with butyric acid suppressed the peak temperature for maize and potato starches, but not for pea starch (Figure 2C). Maize starch was most sensitive to this aroma compound showing suppression of peak temperature of 2.4 °C, whereas peak temperature of potato starch showed 1.0 °C suppression (Figure 2C). Minor changes were observed for octanol which suppressed the melting temperature of maize starch by 0.8 °C, and increased the melting temperature of potato and

pea starch by 0.5 and 0.4 °C, respectively. The differential effect of starch type was demonstrated by aroma complexation with allyl isothiocyanate that gave a slight suppression of potato starch peak temperature while an opposite effect was observed of pea starch and no effect was seen on maize starch. The observed variation between starch types cannot generally be explained by amylose content, crystalline polymorph type, or phosphate content. However, the peak temperature of pea starch seems to be less affected by both citral and butyric acid than the other starches, which could be an effect of its lower amylopectin content. The width of the endothermic peaks indicates heterogeneity in the granular crystallite structure (Tester and Debon, 2000). Peak widths were different for the three different starch types (Table 3). However, it did not change upon aroma complexation despite altered enthalpy supporting that heterogeneity of remaining lamellar crystallites in the starch granules was unaffected. Hence, aroma complexation supposedly affects only specific parts of the granule leading to complete local amorphisation. Alternatively, the aroma complexation could occur in amorphous parts of the granule and/or with amylose.

For all three starches the presence of citral gave additional broad peaks following the gelatinization endotherm, thus strongly suggesting some form of interaction, possibly with amylose. Also octanol generated a minor melting endotherms at approximately 90 °C as well as a decrease in melting enthalpy, corresponding to formation and/or improvement of complexes formed during heating of the sample (data not shown).

For butyric acid there is no indication of complexation with potato and pea starches. However, for maize starch the presence of a second endotherm can either be caused by complexation or simple complexes between amylose and residual lipids in the starch (data not shown).

No endotherm peaks were detected for aromas mixed with pre-gelatinised, amorphous starches (data not shown). This indicates that possible complexes formed with amorphous starch (as indicated by melting endotherms at higher temperatures of native starch granules mentioned above) are not well defined in a crystalline sense and possibly are amorphous in its nature. This also substantiates the conserved peak width observed for the granular starch.

### **3.3 Powder X-ray diffraction (XRD)**

In order to further deduce the origin of the DSC endothermic transitions in a crystalline sense, XRD analysis was carried out. XRD data is not as readily quantified as DSC. For maize starch only the diffraction of starch with octanol differs from the control showing a slightly smoother diffractogram. The differences are most evident at about  $6^\circ$  and  $20^\circ$  (Figure 3A). Butyric acid also shows a minor difference at around  $20^\circ$ , although this difference was not substantial. For the citral and butyric acid complexed with potato starch the peak at around  $6^\circ$  seems to be slightly diminished (Figure 3B), whereas for all the samples the clear peak in the control at around  $16^\circ$  is broadened backwards. Also the features in the diffraction for the control around both  $10^\circ$ - $14^\circ$  and  $18^\circ$ - $25^\circ$  were flattened for all three samples.

For pea starch there was a major amorphising effect induced by octanol indicated at  $20^\circ$  (Figure 3C). Also citral showed a substantial difference at this angle though less pronounced. For both compounds the peak at  $15^\circ$  was also affected. Butyric acid induced a minor difference at  $20^\circ$ .

In summary, XRD data does not support the presence of specific aroma complex specimens, although octanol does show some form of interaction, especially with pea starch granules, but not with potato and maize granules, possibly due to a higher amylose content of pea starch.



Citral and butyric acid seem to have only a small influence on diffraction, thus not indicating the full formation of a complex, although minor specific and non-specific complexation cannot be excluded.

### **3.4 Scanning electron microscopy (SEM)**

The micro scale effects of aroma treatment of potato, maize and pea starch granules were assessed visually using SEM and compared to controls. The control potato starch granules (Figure 4A) appeared as rather large (up to 100  $\mu\text{m}$ ) spherical or elongated granules with smooth surfaces. Treated with citral the potato starch granules showed rippled surfaces at specific sites (Figure 4B). Further, parts of the surface appeared slightly swollen indicating partial gelatinisation. Material was identified deposited between the granules, at granule surfaces with frequent irregular and eroded parts. The observed partial granular gelatinization corroborates with the decreased melting enthalpy as monitored by DSC found for the citral treated starch granules. Potato starch treated with butyric acid and octanol, respectively, had no detectable microscopic influence on the granule surface (data not shown).

The control maize starch granules (Figure 5A) had a size of 2-15  $\mu\text{m}$ , were slightly angular and had rough surfaces. After treatment with citral, maize starch granules showed smoother surface as if covered by material, possibly citral, in mixture with gelatinized starch (Figure 5B). They also appeared clumped together in clusters. For this starch type specific sites of the surface appeared wrinkled and swollen. However, this effect was less pronounced than for potato starch. The surface of maize starch granules treated with octanol and butyric acid, respectively, (Figure 5C and Figure 5D) appeared smoother and more even than the maize starch control but showed no tendency for aggregation induced by the aroma compounds.

Native pea starch granules (Figure 6A) were in the range of 5-40  $\mu\text{m}$ , were smooth and the granules were rounded and multi-lobed. The citral treated pea starch granules (Figure 6B) appeared to have a disrupted surface, slightly swollen and wrinkled at specific sites, as for the potato starch granules. The granule irregularities observed for the control sample were less pronounced following treatment with citral. Just as for the maize granules this effect is possibly due to deposited a layer of citral complexed with gelatinized starch covering dents and cavities. No structural effects were observed for pea starch granules treated with octanol or butyric acid (data not shown).

Citral, octanol, and butyric acid show large effects on starch in DSC, and minor complexation was observed by X-ray diffraction. This combined with the SEM data, might indicate citral may interact with surface sites on the starch granules, whereas octanol and butyric acid may be involved in interactions under the surface.

### **3.5 Brightfield microscopy and iodine complexation**

In order to identify the nature of deposited material, samples were stained with iodine and investigated with brightfield light microscopy. Pea starch is shown as an example in Figure 7A. Here it was observed that there is matter present between the granules, which do not stain with iodine. It was suspected that this could be citral and this could also be what we observe on the SEM images between the granules (Figs. 4B, 5B, and 6B). To confirm that the granules were partly gelatinized and/or had leaked amylose as postulated above, the starch samples were washed with acetone to remove citral (Figure 7B). Here it is clear that the transparent matter has been removed, and blue-stained starch is now clearly present between granules and at the granule surface. Further it can be seen (Figure 4B) that some of the granules were

cracked, exposing internal parts of the granules. It is likely that the unstained matter observed in the unwashed sample is a mixture of starch and citral and that the starch does not stain with iodine due to the high hydrophobicity of citral in complex with starch. This effect can also explain the weaker iodine staining of the citral-containing starch granules (Figure 7). Inspection with polarised light showed no main change in birefringence of the granular structures indicating that glucan chain directions within the granules were not affected by the citral treatment (data not shown). SEM analysis of citral-treated pea starch granules washed with acetone showed cracked and surface-disrupted structures confirming the interpretation of the brightfield data (data not shown).

The light microscopic data substantiate the DSC, XRD and SEM data to support a partial destruction and solubilisation of the starch granules induced by specific aroma compounds.

#### **4. Conclusions**

Aroma interaction were analysed with starch in their native granular form. Both retention and evaporation of aroma induced by starch granules were found dependent on starch and aroma type. As judged by SEM interaction was primarily at specific surface sites on the granule, possibly followed by a certain diffusion through the outer layers of the granule leading to partial amorphisation of the starch granule. DSC indicated that minor complexation took place in the crystalline layers of the starch granule resulting in partial destruction of the granular matrix for some starches resulting in amylose leakage.

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## Figure captions

**Figure 1.** Relative retention of aromas in native starches. The retention is defined as the weight difference in milligrams relative to a control without aroma, divided by the weight (in grams) of the starch

**Figure 2.** DSC Make in the figures A, B, C, and D.

Average melting enthalpy of aroma treated starch relative to control of same starch type. ■ pea starch, ■ potato starch and ■ maize starch. Error bars show combined standard deviations.

Average deviation of peak temperature between aroma treated starch and control starch of same type. ■ pea starch, ■ potato starch and ■ maize starch. Error bars show combined standard deviations.

**Figure 3. XRD** X-ray diffraction of starch mixed with no aroma (■), citral (■), octanol (■), or butyric acid (■)

**Figure 4.** SEM images of potato starch granules (A) and potato starch granules treated with citral (B).

**Figure 5.** SEM images of maize starch granules (A), and maize starch granules treated with citral (B), octanol (C), and butyric acid (D), respectively.



**Figure 6.** SEM images of untreated pea starch granules (A) and pea starch granules treated with citral (B).





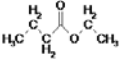
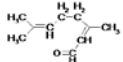
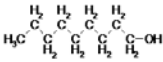

**Figure 7.** Partly destructed pea starch granules as result of citral treatment viewed by light microscopy. A) Citral treated starch granules. Arrow indicates unstained matter between the granules. B) Citral treated pea starch granules after acetone washing. Unaffected granules (UG), leaked starch (LS), and cracked granules (CG)

## Tables

**Table 1.** Chemical and crystalline properties of the starch types used (adapted from Glaring *et al.*, 2006)

Starch	Amylose content (%)	Phosphate content (nmol/mg starch)	Crystalline polymorph
Normal potato	27.6	23.2	B
Normal maize	45.3	0.11	A
Pea	55.0	0.48	C

**Table 2.** Chemical and physical properties of the aroma compounds

Compound	Bp (°C)	MW	Log P (Oct/water)	Formula
Acetaldehyde	21	44.05	0.4	
Dimethyl sulphide	38	64.14	0.92	
Diacetyl	88	86.10	-1.34	
Allyl isothiocyanate	150	99.16	2.11	
Ethyl butyrate	119-121	116.18	1.85	
Citral	220-229	152.26	3.45	
Octanol	196	130.26	3.15	
Butyric acid	162	88.10	0.8	

**Table 3.** Average melting enthalpies and peak temperatures and  $\Delta T$  of the endotherms for the three native granular starch types.

	$\Delta H$ (J/g)	Peak Temperature ( $^{\circ}\text{C}$ )	$\Delta T$ ( $^{\circ}\text{C}$ )
Pea	$16.69 \pm 0.02$	$72.0 \pm 0.3$	$8.9 \pm 0.2$
Potato	$8.26 \pm 0.10$	$64.3 \pm 0.2$	$17.5 \pm 0.4$
Maize	$9.37 \pm 0.33$	$71.8 \pm 0.1$	$12.8 \pm 0.3$

## Figures

Figure 1

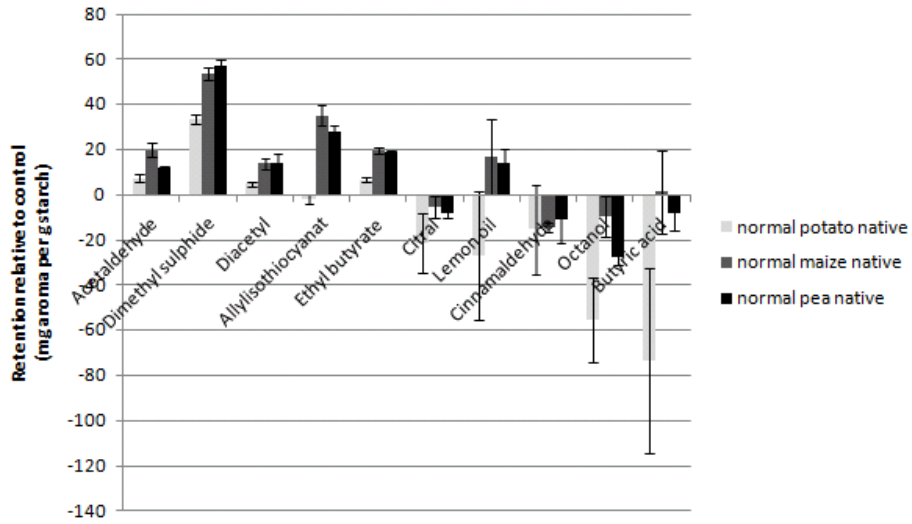


Figure 2

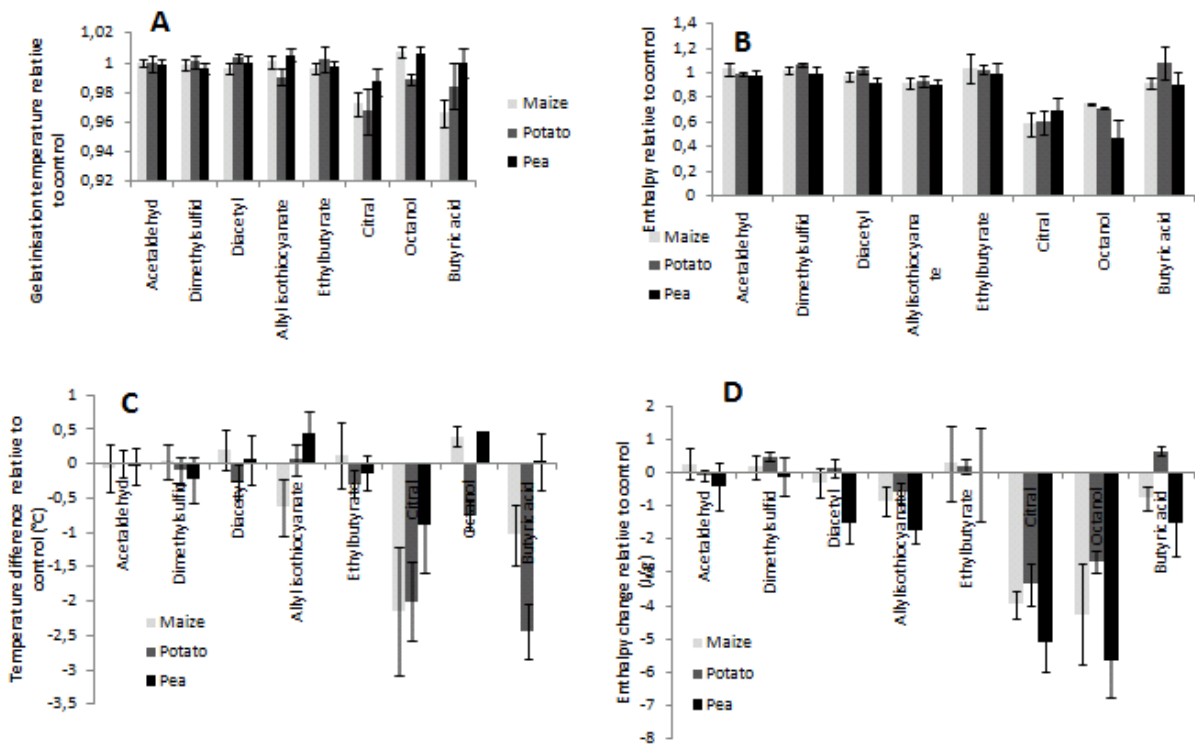


Figure 3

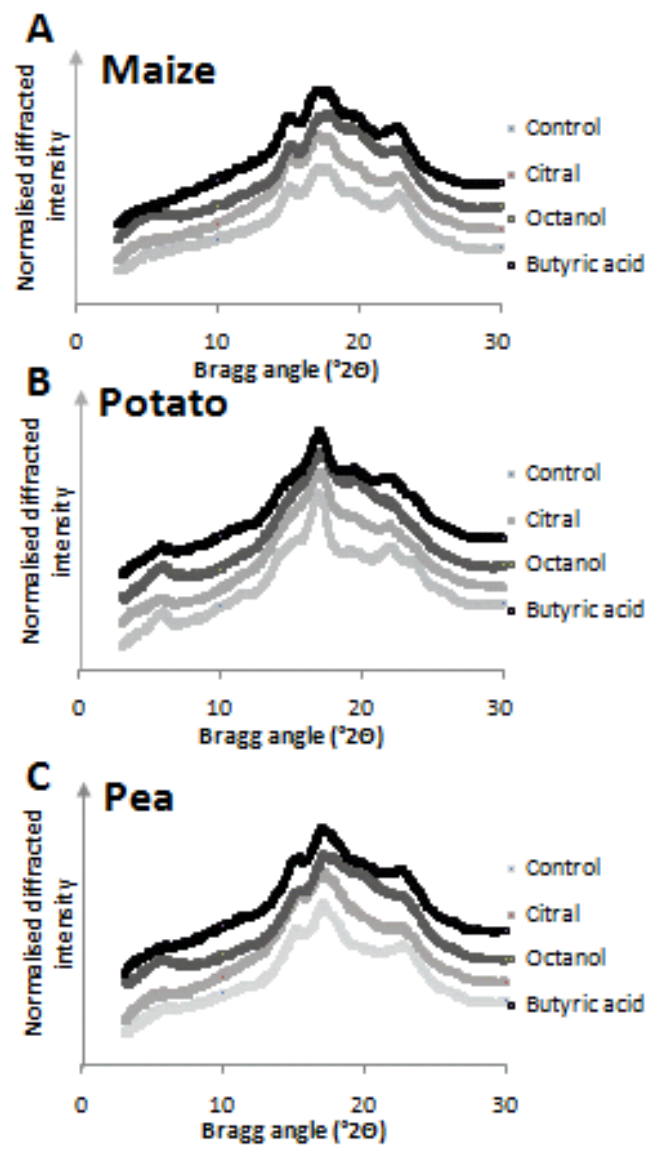


Figure 4

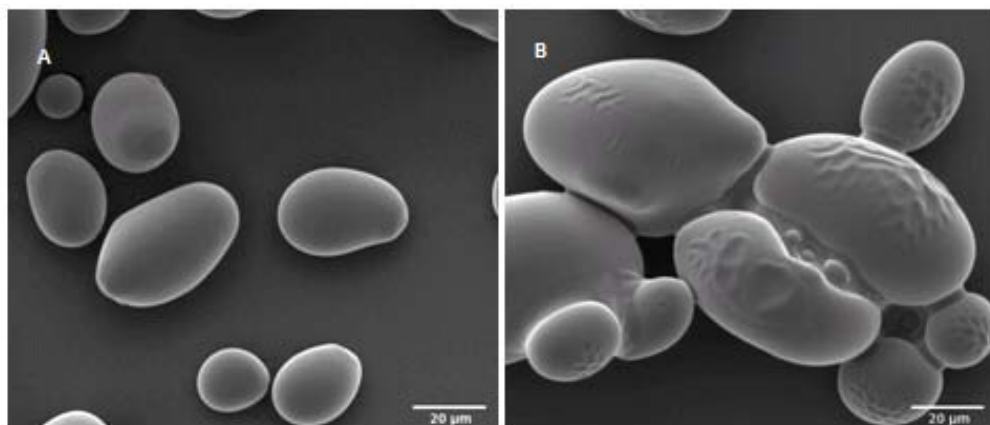


Figure 5

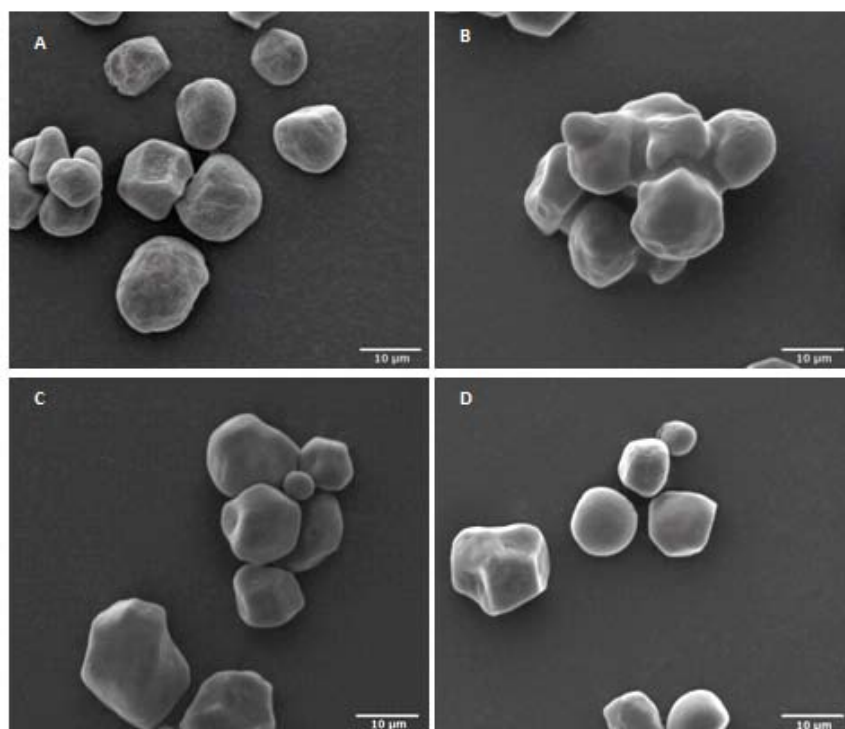


Figure 6

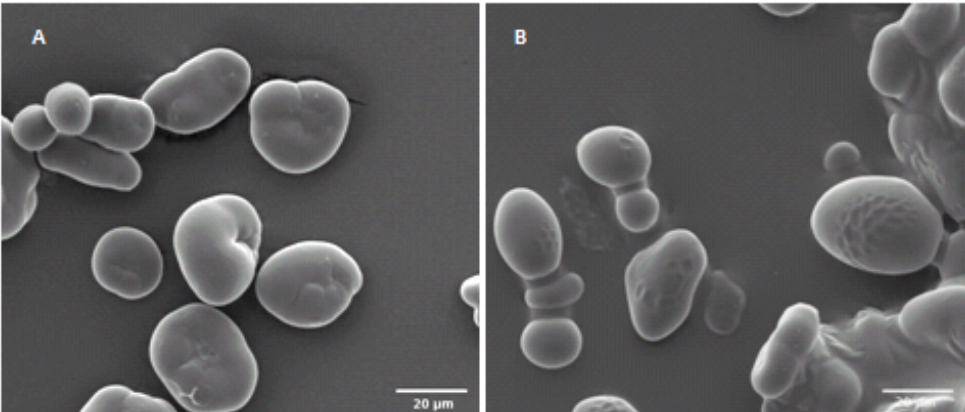
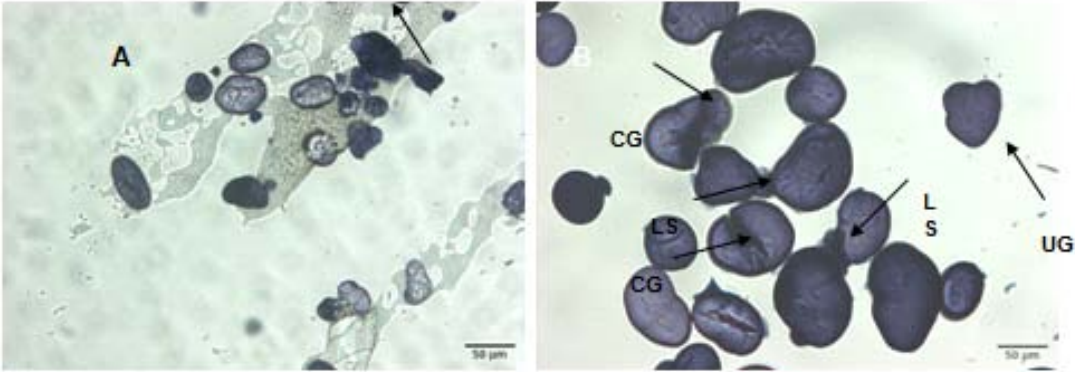


Figure 7





## **Appendix 5**

### ***14.2.3.1.2 Manuscript II***

Yang, F., Jørgensen, A.D., Li, H., Søndergaard, I., Finnie, C., Svensson, B., Jiang, D., Wollenweber, B., Jacobsen, S., 2011. Implications of high-temperature events and water deficits on protein profiles in wheat (*Triticum aestivum* L. cv. Vinjett) grain. *Proteomics* 11, 1684-1695

## RESEARCH ARTICLE

# Implications of high-temperature events and water deficits on protein profiles in wheat (*Triticum aestivum* L. cv. Vinjett) grain

Fen Yang<sup>1</sup>, Anders D. Jørgensen<sup>1</sup>, Huawei Li<sup>1,2,3</sup>, Ib Søndergaard<sup>4</sup>, Christine Finnie<sup>1</sup>, Birte Svensson<sup>1</sup>, Dong Jiang<sup>3</sup>, Bernd Wollenweber<sup>2</sup> and Susanne Jacobsen<sup>1</sup>

<sup>1</sup> Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark

<sup>2</sup> Department of Genetics and Biotechnology, Faculty of Agricultural Sciences, Aarhus University, Slagelse, Denmark

<sup>3</sup> College of Agriculture, Nanjing Agricultural University, Nanjing, Jiangsu Province, P. R. China

<sup>4</sup> Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark

Increased climatic variability is resulting in an increase of both the frequency and the magnitude of extreme climate events. Therefore, cereals may be exposed to more than one stress event in the growing season, which may ultimately affect crop yield and quality. Here, effects are reported of interaction of water deficits and/or a high-temperature event (32°C) during vegetative growth (terminal spikelet) with either of these stress events applied during generative growth (anthesis) in wheat. Influence of combinations of stress on protein fractions (albumins, globulins, gliadins and glutenins) in grains and stress-induced changes on the albumin and gliadin proteomes were investigated by 2-DE and MS. The synthesis of individual protein fractions was shown to be affected by both the type and time of the applied stresses. Identified drought or high-temperature-responsive proteins included proteins involved in primary metabolism, storage and stress response such as late embryogenesis abundant proteins, peroxiredoxins and  $\alpha$ -amylase/trypsin inhibitors. Several proteins, e.g. heat shock protein and 14-3-3 protein changed in abundance only under multiple high temperatures.

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Environmental stress / Grain protein fractions / Plant proteomics / Wheat

## 1 Introduction

Climate change is likely to become an even more acute problem than previously anticipated [1]. Accelerated climatic variability is resulting in an increase of both the frequency

and the magnitude of extreme events [2, 3]. More frequent extreme climatic episodes result in cereals being exposed to several stress events during the growing season, ultimately affecting cereal production [4–6]. The most sensitive growth stages in cereals are stem elongation, booting and anthesis [7]. Extreme heat episodes after anthesis increase development of the crops, shorten the grain filling period [8], resulting in reduction of kernel weight and of grain quality parameters (starch and protein) [9]. Moderately high temperatures after cold winter periods increase cereal yields [10]. An extreme heat event at the double-ridge stage did not affect the response of wheat to heat stress at anthesis [2]. Drought has also become a limiting factor for cereal

**Correspondence:** Dr. Susanne Jacobsen, Enzyme and Protein Chemistry, Department of Systems Biology, Building 224, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

**E-mail:** sj@bio.dtu.dk

**Fax:** +45-4588-6307

**Abbreviation:** dpa, days post anthesis

production worldwide, resulting in significant grain yield losses [4, 11, 12] and increased heavy rainfalls lead to waterlogging and nutrient leaching, resulting in reductions of cereal growth and quality [5, 13].

Wheat grain quality receives increasing attention because of its economic and nutritional importance [14]. Protein fractions including albumins, globulins, gliadins and glutenins are the most important components of wheat grains for end-use quality [15]. In order to maintain grain yield and quality under increased climatic variability it is important to understand the effect of both single environmental constraints and the combination of extreme climate events on grain protein composition. It has been shown that increases in temperature induce synthesis of gliadins at the expense of glutenins in wheat [16]. Furthermore, high temperature applied from anthesis to maturity decreased the contents of the albumin and globulin fractions, while drought applied after anthesis decreased the albumin and globulin fraction, but had no significant effect on gliadins and glutenins [17]. Several studies have been performed to investigate the response of wheat grain proteins to either long-term drought during vegetative growth or long-term high temperature applied during grain filling, showing up-regulation of pathogenesis-related proteins, allergens, protease inhibitors, heat shock proteins and proteins involved in oxidative stress and starch biosynthesis [14, 18, 19]. However, little is known about the effect of the combination of short-term (days) water deficits and high temperature during grain development on wheat grain proteins. Although grain protein content and composition – the key parameters of grain quality – are primarily genetically controlled, environmental factors can influence the synthesis of protein throughout grain growth [20]. The interactions of multiple episodes as well as multiple forms of stress so far have not been investigated extensively.

The aim of the present study was to investigate implications of the interactions of short-term drought and high-temperature events applied at two different growth stages of wheat (terminal spikelet and anthesis). Changes in grain proteins were monitored by 2-DE MALDI-MS-based proteomics. This approach has been shown to be very useful in characterizing changes in protein profiles of plants under stress episodes [19]. The results are discussed in relation to the general use of proteomics to identify traits relevant for stress tolerant cereals and identification of wheat cultivars with drought and high-temperature tolerance.

## 2 Materials and methods

### 2.1 Plant growth

A pot experiment was conducted at the “semifield” station of Research Center Flakkebjerg, Department of Genetics and Biotechnology, Faculty of Agricultural Sciences, Aarhus University, Denmark in the growing season of 2007. Pots

with both depth and diameter of 25 cm were filled with 4.2 kg of a 1:2:1 v/v/v mixture of peat substrate, loamy soil and sand. A dose of 5.25 g  $K_2SO_4$ , 3.5 g  $(NH_4)_2SO_4$ , 4.67 g  $NH_4NO_3$ , 1.9 g  $CaSO_4$ , 1.9 g  $MgSO_4$ , 0.4 g  $MnSO_4$ , 0.4 g  $CuSO_4$  and 11.67 g  $CaCO_3$  per pot was mixed in the soil. Spring wheat (*Triticum aestivum* L. cv. Vinjett) was sown (15 seeds per pot) and thinned to five seedlings per pot at the three-leaf stage. The main shoot of each plant was labeled. From the third-leaf stage, three to five seedlings were taken for observation of spike initiation by dissection at an interval of about 2–3 days, until the terminal spikelet stage was identified.

An automatic irrigation system was used, and the total (not for individual pots) applied water was recorded. Water deficits and high-temperature stresses were implemented at terminal spikelet (the first stage, denoted A) and anthesis (the second stage, denoted B) as specified below. At the first stage, three treatments were implemented in three growth chambers, namely, temperate conditions as control (cA), water deficits (wdA) and high temperature (htA). At the second stage, plants from each of the above treatments were further exposed to three treatments in the three chambers, as control (cB), water deficit (wdB) and high temperature (htB). In total, nine combinations of stress types were implemented: cA-cB, cA-wdB, cA-htB, wdA-cB, wdA-htB, htA-cB, htA-wdB, htA-htB and wdA-wdB. The experiment was a completely random block design with three biological replicates.

In the high-temperature treatment at both stages the temperature in the growth chambers was set to increase from 24°C at night (8 h) to a maximum of 32°C for 2 h during the day (16 h). The temperature in the control and water-deficit treatments was set to increase from 12 to 20°C at the first stage and from 16 to 24°C at the second stage. The heat treatment lasted for 10 days. Water supply was withheld in the pots in the semifield for 7 days (at the three-leaf stage) before the first stage and for 6 days (just after the spike emerged) before the second stage. Thereafter, the pots were moved into the growth chambers in order to apply the different treatments.

### 2.2 Protein extraction and quantification

Grain fresh weight and raw protein% (derived from total N \* 5.7) were measured and shown in Supporting Information Figs. s1 and s2. Kernels from five individual ears of the main tillers in five individual pots were harvested at 19, 26, 32 and 52 days (at maturity) post anthesis (dpa). For each treatment, biological triplicates of wheat kernels were freeze dried and ground to a fine powder in liquid nitrogen. Based on the solubility in a series of solvents, the classified grain protein fractions of albumins, globulins, gliadins and glutenins [15] were extracted as described in [21] with minor modifications. Briefly, albumins were extracted from 100 mg flour that was mixed continuously with 1 mL of buffer containing 25 mM sodium phosphate (pH 7.5) for 60 min at 4°C. The flour was re-extracted with 1 mL of a solution

containing 0.1 M NaCl and 20 mM DTT by incubation in an ultrasonic bath (2210 BRANSON) for 60 min at 4°C to obtain the globulins. The gliadins were extracted from the resulting pellet using 1 mL of a solution containing 50% v/v 1-propanol, 1% v/v acetic acid, 2% w/v DTT by incubating the samples in the ultrasonic bath for 60 min at 4°C. Then, the glutenins were extracted from the resulting pellet with 1 mL of 1 mM HCl by mixing for 60 min followed by ultrasonication for 1 min at 4°C. After each extraction, the suspension was collected by centrifugation at  $20\,800 \times g$  for 10 min. The residue was washed and re-extracted with the same buffer twice before the extraction of the following fraction. Protein concentration of albumins, globulins and gliadins from supernatants was determined using the Amido black method [22] with bovine serum albumin as standard. As this method is not ideal for determination of the concentration of acid-soluble proteins, the concentration of glutenins was determined using the Bradford assay [23]. Statistical analysis of differences in contents of protein fractions between stress and control treatments was performed by Student's *t*-test ( $p < 0.05$ ).

### 2.3 2-DE

2-DE was run according to [24] with minor modifications. Albumins (150 µg depending on the sample in a volume of 200–300 µL) were precipitated by four volumes of acetone at  $-20^{\circ}\text{C}$  overnight. The albumin pellets or gliadins (100 µg corresponding to a volume of 25–30 µL depending on the sample) were added to 350 µL of rehydration buffer (8 M urea (GE Healthcare), 2% w/v CHAPS (Sigma), 0.5% v/v IPG buffer, pH 3–10 (GE Healthcare), 0.3% w/v DTT (Sigma) and a trace of orange G). The solution was thoroughly vortexed and centrifuged ( $20\,800 \times g$  for 10 min). The supernatant was applied to an IPG strip, pH 3–10 (18 cm; GE Healthcare) and IEF was run (Ettan IPGphor; GE Healthcare) as previously described [25]. After IEF, the strips were equilibrated first with a solution containing 6 M urea (GE Healthcare), 30% v/v glycerol (GE Healthcare), 50 mM Tris (Sigma), pH 8.8, 0.01% w/v Bromophenol Blue (Sigma), 2% w/v SDS (BDH/Merck) with 1% w/v DTT (Sigma) for 15 min and then with a similar solution without DTT, but containing 2.5% w/v iodoacetamide (Sigma) for 15 min. The strips were placed on top of 12.5% acrylamide gels (3% C/0.375% bisacrylamide) and the second dimension was run (Ettan DALTsix electrophoresis unit; GE Healthcare) according to the manufacturer's protocol. The gels were stained in Blue Silver stain [26]. One gel was run for each biological replicate.

### 2.4 Image analysis

The gels were scanned using a ScanMaker 9800XL, Microtek at 300 dpi resolution in both color and grayscale (16 bits).

All grayscale gel images were imported into the Progenesis SameSpots software (Nonlinear Dynamics, Newcastle upon Tyne, UK). One gel image from the cA-cB control treatment was chosen as a reference image and all gel images were warped and matched to the reference image. Three gel images representing three biological replicates for each treatment were grouped to obtain the average volume of each spot. Images from each stress treatment were compared to images from cA-cB. A threshold of ANOVA,  $p < 0.05$ , and at least 1.5-fold change in average spot volume between a stress treatment and the corresponding control treatment was used to select the protein spots for further MS analysis.

### 2.5 Enzymatic digestion and MS

In-gel digestion was performed as previously described [25], albumins and gliadins being digested by trypsin (Promega) and chymotrypsin (sequencing grade, Roche) [27], respectively. The peptide mixtures were prepared for MS analysis on a washed MALDI 600 µL AnchorChip target plate (Bruker-Daltonics, Bremen, Germany) as previously described [25]. A tryptic digest of  $\beta$ -lactoglobulin was used for external calibration. Tryptic and chymotryptic peptides were analyzed in Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker-Daltonics) in positive reflector mode for peptide mass mapping or peptide fragment ion mapping. Internal calibration was carried out using trypsin autolysis products ( $m/z$  842.51 and 2211.10). An in-house MASCOT server (<http://www.matrixscience.com>) was used for database searches in the NCBI nr (National Center for Biotechnology Information) and the TaGI wheat gene index Release 11.0 (<http://compbio.dfci.harvard.edu/tgi>). The following parameters were used for searching: allowed global modification, carbamidomethyl cysteine; variable modification, oxidation of methionine; missed cleavages, 1; peptide tolerance, 80 ppm and MS/MS tolerance  $\pm 0.5$  Da. The positive identification had to meet the following criteria: a significant MASCOT score and at least four matched peptides in MS analysis or two matched peptides in MS/MS analysis.

## 3 Results and discussion

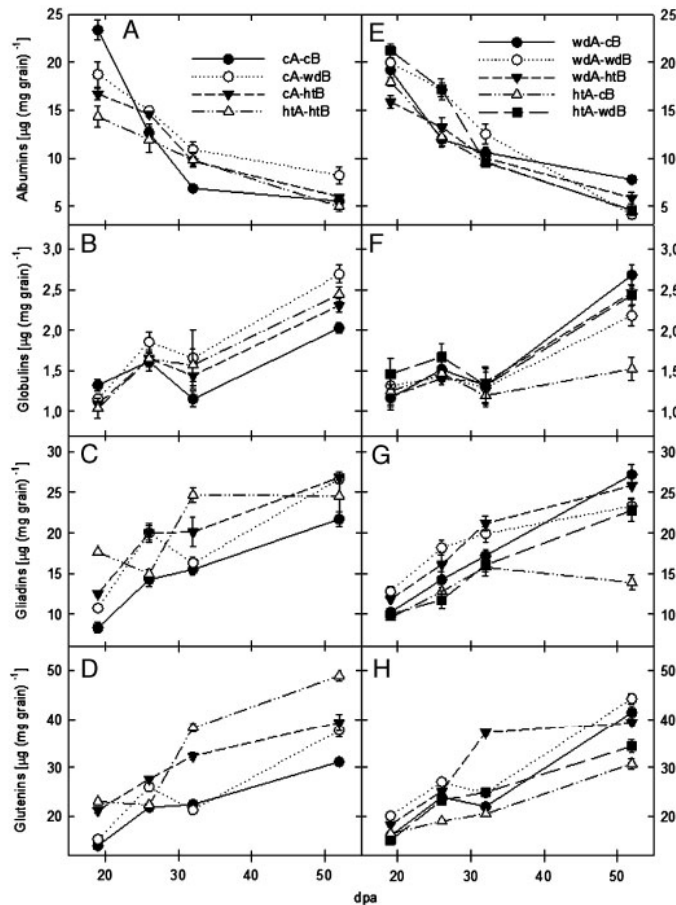
Overall, no significant differences in final grain weights have been found (Supporting Information data, Fig. s1). When heat was applied in the vegetative stages (htA), the grain weight at 12 dpa was significantly higher than in the other treatments (Supporting Information Fig. s1 C), probably indicating enhanced growth during these treatments. No significant differences in the total protein content of these grains have been found, although the protein content was more variable when heat was applied during the vegetative stage (Supporting Information data, Fig. s2).

**3.1 The effect of stress on accumulation of protein fractions**

The albumin content in all treatments decreased significantly (56–80%) during grain filling (Fig. 1A and E). At 19 dpa, all stress treatments applied had decreased the albumin fractions significantly, where the lowest contents were found in the htA-htB treatment. By contrast, at 32 dpa the albumin content in all of the stress treatments were higher than for the control and at maturity, only the albumin contents in cA-wdB and wdA-cB treatments were significantly higher than in the control. High-temperature stress resulted in higher albumin contents at maturity when applied at anthesis than at terminal spikelet. By contrast, no significant difference in albumin contents was observed when drought was applied at both growth stages.

The relatively low globulin content increased differently (22–135%) under treatments during grain filling. Compared to the earlier growth stages, greater differences in globulin contents between stress and control were observed at maturity (Fig. 1B and F). The stress treatments except htA-cB (Fig. 1F) significantly increased the contents of globulins at maturity. A larger increase in globulins at maturity was observed when drought was applied at anthesis or terminal spikelet than high temperature (Fig. 1B and F).

Gliadins showed with the exception of the htA-cB treatment increased (39–166%) contents during grain filling, but stress treatment did not significantly affect the rate of accumulation of gliadins (Fig. 1C and G). At maturity only htA-cB treatment decreased gliadin contents, similar to globulins. The highest contents of gliadins were observed at 19 dpa in htA-htB and at maturity in cA-wdB, cA-htB and in wdA-cB.



**Figure 1.** The accumulation of albumins (A+E), globulins (B+F), gliadins (C+G) and glutenins (D+H) in wheat grain under high-temperature and/or water-deficit stress treatments applied at terminal spikelet and/or anthesis. The treatments are denoted as outlined in Section 2. The cA-cB, cA-wdB, cA-htB and htA-htB treatments selected for proteomic analysis are shown in the left column, other treatments are shown in the right column.

The glutenins accumulated during grain filling increased (85–159%) in response to different stress treatments (Fig. 1D and H). The htA-htB treatment resulted at maturity in higher contents of glutenins as well as gliadins, which is contrary to findings by others that in wheat high temperature can induce synthesis of gliadins at the expense of glutenins [16]. As with the globulins, stress caused greater differences in glutenin contents at maturity than at 19 dpa. In contrast to the albumin and gliadin fractions, multiple high-temperature events (htA-htB, Fig. 1D) or water deficits (wdA-wdB, Fig. 1H) applied at both terminal spikelet and anthesis resulted in significantly higher contents of glutenins at maturity than in the single stress treatments.

At maturity the highest contents of albumins, globulins and gliadins were obtained under the single drought stress applied at either stage whereas glutenins increased under the htA-htB treatment, indicating differences in the response of grain protein fractions to drought and heat stress. High temperature was capable of causing substantial changes in the accumulation level of gluten proteins during grain filling, in agreement with previous findings [9]. In addition, higher contents of albumins, globulins, gliadins and glutenins were observed when high temperature was applied at anthesis than terminal spikelet. This is in contrast to the finding of no significant differences in protein fractions under drought stress applied at either stage, which indicates that grain protein contents were greatly affected by both the type and the exposure time of the stress applied. During the period from terminal spikelet to anthesis, high-temperature stress is reported to have a damaging effect on the viability of pollen, resulting in failure of fertilization [20]. As a consequence, the poor development of fertilized ovaries into caryopses may affect protein synthesis during grain filling. Water deficit was found to inhibit photosynthesis at both stages, which can influence the formation of generative organs and consequently grain protein synthesis [20, 28]. It has previously been shown that both grain yield and grain number were influenced more by stress at anthesis than at heading [9]. Furthermore, it was observed that the combination of drought and heat stress applied at two stages did not have a greater effect on the albumin, globulin and gliadin contents during grain filling, compared with either of the stresses applied individually. It has been reported that different stresses may require antagonistic responses and that a combination of drought and heat stress may alter plant metabolism, resulting in a new pattern of defense response compared to single stress [20, 29]. Thus, the combination of different stresses might not cause more changes to grain protein content than a single stress event.

### 3.2 Drought or high-temperature-responsive albumins

Albumins and gliadins from mature grains (52 dpa) in the cA-wdB, cA-htB, htA-htB and cA-cB (control) treatments

were chosen for detailed 2-D-based proteome analysis, because these stress scenarios resulted in significant increases of albumins, globulins, gliadins and glutenins at maturity.

The representative 2-D protein patterns of albumins and gliadins for all the treatments (Figs. 2 and 3) allowed detection of approximately 250 and 100 spots, respectively. ANOVA was applied to evaluate spots differing significantly in abundance between stress treatments and control and to confirm the reproducibility of the data. Based on the selection criteria (at least 1.5-fold change, see Section 2.4), in the cA-wdB, cA-htB and htA-htB treatments, 16/5, 10/11 and 31/8 spots exhibited significant changes (between 1.5- and 2.4-fold) in intensity in the albumin/gliadin fractions due to stress, respectively. All differentially displayed spots were identified by MS except spot a3 of decreased intensity in cA-wdB (Fig. 2) and spots e14, e18 and e26 in htA-htB (Fig. 2 and Table 1). The difference of pI and MW between experimental and theoretical data may have resulted from experimental error, PTM or protein turnover occurring in the cell. According to the function, the proteins identified from different stress treatments were mainly involved in stress response, carbohydrate metabolism and storage in addition to protein synthesis.

#### 3.2.1 Stress-related albumins

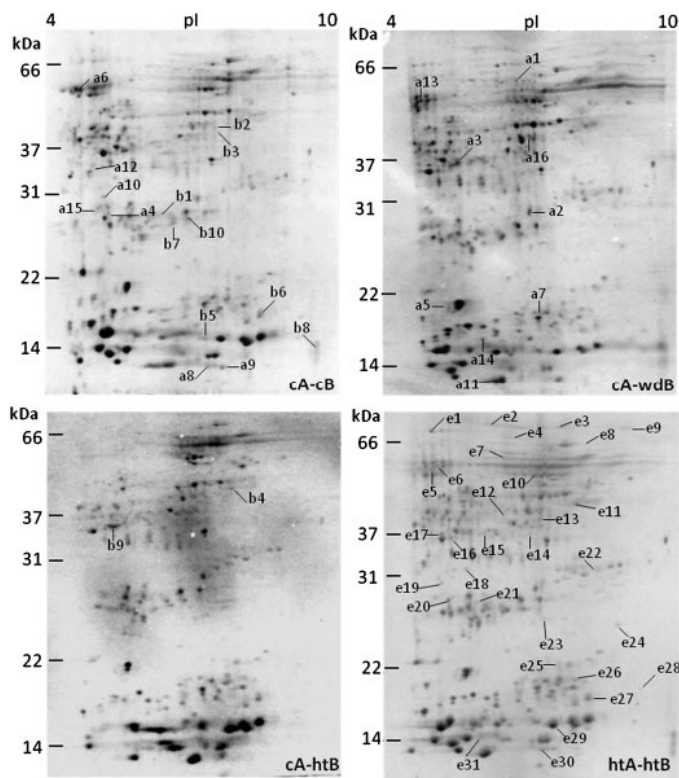
The first major group of the responsive albumins had functions related to stress and defense. A late embryogenesis-abundant protein that is associated with desiccation stress was up-regulated in response to drought stress (1.8-fold, spot a9, Fig. 2), possibly due to its role in protection of proteins and cell membranes from disruption or damage in the near-dry state [30]. A number of proteins related to oxidative stress showed a different expression behavior in response to temperature or drought stress. For instance, 1-Cys peroxiredoxin was down-regulated under drought treatment (2.4-fold, spot a4, Fig. 2), whereas up-regulation of 1-Cys peroxiredoxin (1.9-fold, spot b1, Fig. 2) and a 27 kDa hypothetical protein belonging to the thiol reductase superfamily (1.5-fold, spot b7, Fig. 2) and down-regulation of a hypothetical protein belonging to the thioredoxin superfamily (1.5-fold, spot b5, Fig. 2) were observed in plants under single high-temperature stress but not with multiple high-temperature stress. During exposure to abiotic stress, ROS (reactive oxygen species) are often produced in plants and can cause oxidative damage to proteins, DNA and lipids in the cells [19, 31]. The quantitative alteration of the above ROS-scavenging enzymes suggests their different roles in oxidative stress. Similar observations were made in wheat leaves after a 7-day exposure to drought [31]. Lipoxigenase, which is considered to be partly responsible for the formation of lipid peroxidation products, as well as ROS were down-regulated with high temperature probably as a consequence of the inhibition of ROS production [32].

Additionally, lipoxygenase is known to be involved in jasmonic acid biosynthesis, which often accumulates in plants in response to biotic and abiotic stress [33].

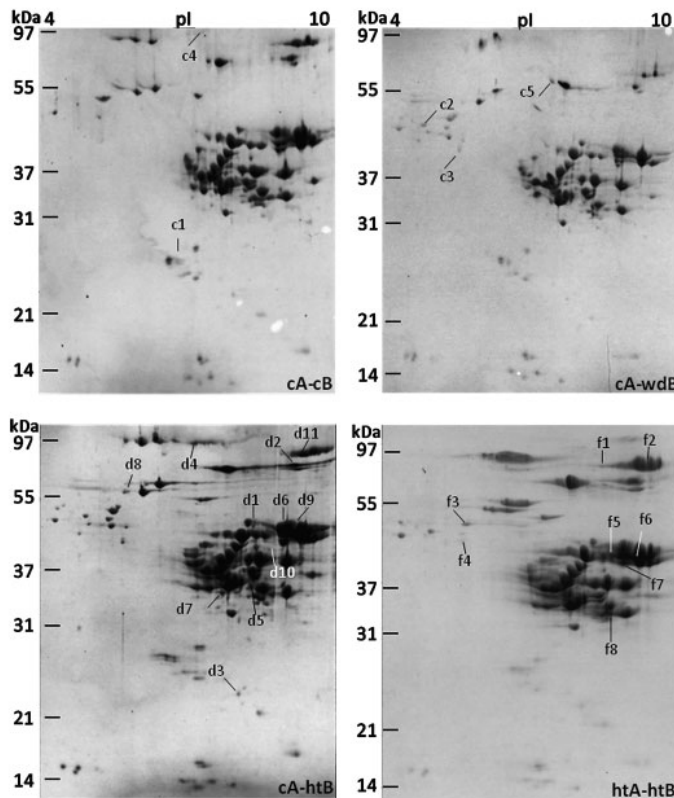
Several spots changing in abundance were identified as defense-related proteins, including  $\alpha$ -amylase inhibitors,  $\alpha$ -amylase/trypsin inhibitors CM1, CM3, CM17, serpin and cystatin. Similar results were also observed in seeds of durum wheat subjected to heat for 5 days after anthesis [34]. The  $\alpha$ -amylase/trypsin protease inhibitors have been shown to guard against digestive enzymes of insects and fungi and are often accumulated in response to biotic stress [19, 34]. Interestingly,  $\alpha$ -amylase inhibitor in one spot (1.5-fold, spot a8, Fig. 2) was up-regulated and down-regulated in another spot (1.5-fold, spot a11, Fig. 2) under drought. This may be due to PTMs of the protein such as dephosphorylation resulting in  $pI$  shift from acid to base. It is well known that the phosphorylation of proteins can change in response to stress [31]. Furthermore, the up-regulation of three  $\alpha$ -amylase inhibitors and the down-regulation of  $\alpha$ -amylase inhibitor CM1 and cystatin under htA-htB may reflect their different roles in response to high-temperature treatments.

### 3.2.2 Albumins involved in primary metabolism

The second group of identified albumins is involved in primary metabolism. In response to drought, 3-phosphoglycerate kinase involved in the glycolysis was up-regulated (1.6-fold, spot a16, Fig. 2) and glucose/ribitol dehydrogenase, which is related to glucose degradation and has a role in desiccation and salinity tolerance in barley seeds [35, 36] (2.1-fold, spot a12, Fig. 2), was down-regulated, suggesting changes in carbohydrate metabolism. In addition, enzymes such as triosephosphate isomerase (1.5-fold, spot b10, Fig. 2) and succinyl-CoA ligase (1.9-fold, spot b2, Fig. 2) participating in glycolysis and in citric acid cycle, respectively, were down-regulated by a single high-temperature event, whereas fructose-6-phosphate-2-kinase (2.0-fold, spot e8, Fig. 2) enolase 2 (1.9-fold, spot e10, Fig. 2) and  $\beta$ -amylase (1.6-fold, spot e7, Fig. 2) increased and aldose reductase decreased (1.9-fold, spot e16, Fig. 2) in abundance with multiple high-temperature events. It has been reported by others that several glycolytic enzymes including triosephosphate isomerase were increased in the grain in response to high temperature initiated at 10 dpa [19]. This is



**Figure 2.** Albumin profiles of mature wheat grain. The representative 2-D gels ( $pI$  range 4–10) are shown for control (cA-cB) and samples under water-deficit (cA-wdB), single high-temperature (cA-htB) and multiple high-temperature (htA-htB) treatments. Molecular size markers are indicated. Protein spots changing in intensity in response to stress are numbered.



**Figure 3.** Gliadin profiles of mature wheat grain. The representative 2-D gels (pI range 4–10) are shown for control (cA-cB) and samples under water-deficit (cA-wdB), single high-temperature (cA-htB) and multiple high-temperature (htA-htB) treatments. Molecular size markers are indicated. Protein spots changing in intensity in response to stress are numbered.

in contrast to the present results and can be explained by the duration of stress exposure or be cultivar-specific. Furthermore, changes in the abundance of 40S ribosomal protein S8 and glutamine synthetase in the single high-temperature treatment and of ribosomal protein S2, methionine synthase and glutamate-cysteine ligase in the multiple high-temperature treatment may reflect alterations in protein and amino acid biosynthesis.

### 3.2.3 Albumins with other functions

The identified calmodulin TaCaM2-2 decreased in response to drought (1.7-fold, spot a5, Fig. 2). Many extracellular signals such as biotic and abiotic stress can elicit changes in cellular  $\text{Ca}^{2+}$  concentration in plants [37]. Calmodulin is a  $\text{Ca}^{2+}$ -binding protein involved in calcium signaling under stress conditions [37]. Several proteins changed in abundance only under multiple high temperatures including up-regulated cinnamoyl-CoA reductase, translationally controlled tumor protein, cell division control protein and heat shock cognate 70 (1.6-fold, spot e28; 1.5-fold, spot e24; 1.9-fold, spot

e3; 1.9-fold, spot e19, respectively), and the down-regulated 14-3-3 protein (1.9-fold, spot e22, Fig. 2). Cinnamoyl-CoA reductase is involved in lignin biosynthesis. Lignin is an important factor in plant defense response because pathogens are not able to degrade this mechanical barrier [38]. It has recently been reported that the expression of genes encoding translationally controlled tumor protein, which is related to diverse cellular processes such as apoptosis, microtubule organization and ion homeostasis in eukaryotes, was enhanced by stress including high temperature and salt in cabbage [39]. Up-regulation of cell division control protein suggests enhanced cell division in response to high-temperature stress. Heat shock proteins, known as molecular chaperones assisting in the correct folding of polypeptides, have been shown to have a protective role during abiotic and biotic stress [40]. 14-3-3 proteins function as major regulators of primary metabolism and cellular signal transduction in plants. They were shown to regulate target proteins that are involved in response to stress [41]. Several 14-3-3 genes were differentially regulated in rice in response to biotic and abiotic stress [41]. Transcripts encoding proteins belonging to the 14-3-3 family accumulated in barley after biotic stress [34].



**Table 1.** Identification of wheat grain proteins in response to high temperature (ht) and water deficit (wd)

Spot no. <sup>a1</sup>	Spot relative intensity <sup>b2</sup>	MS score <sup>c1</sup>	Number of matched peptides	Sequence coverage (%)	Theor. MW (kDa)/pI	Exp. MW (kD)/pI	Accession number <sup>d1</sup>	Organism <sup>d2</sup>	Protein and conserved domain	MS/MS (sequence of matched peptides) <sup>d3</sup>
<b>cA-wdB treatment</b>										
a8	1.5	78	7	34	16.6/7.45	13.0/7.40	TC264942	<i>Triticum aestivum</i>	Monomeric $\alpha$ -amylase inhibitor	
a9	1.8	86	7	66	10.0/5.50	12.8/7.67	gil1169516	<i>T. aestivum</i>	Late embryogenesis abundant protein H2	
a16	1.6	82	8	31	31.3/5.05	45.7/7.77	gil28172913	<i>Hordeum vulgare</i>	Cytosolic 3-phosphoglycerate kinase	
a1	1.9	72	6	14	72.6/5.63	62.5/7.63	gil218683839	<i>Isomeris arborea</i>	Phytochrome B	
a2	1.6	102	12	42	26.4/5.58	30.1/7.70	TC235043	<i>Oryza sativa</i>	Embryo-specific protein	
a7	1.6	75	7	35	66.3/7.78	17.0/7.70	TC234137	<i>T. aestivum</i>	Embryo globulin	
a4	-2.4	89	8	44	24.0/6.31	28.6/5.78	TC265183	<i>T. aestivum</i>	1-Cys peroxiredoxin	
a11	-1.5	75	5	30	13.6/5.37	12.9/6.53	gil134034615	<i>T. aestivum</i>	Monomeric $\alpha$ -amylase inhibitor	
a14	-1.7	89	7	57	18.9/7.44	15.3/6.75	gil123957	<i>T. aestivum</i>	$\alpha$ -Amylase/trypsin inhibitor CM3	
a12	-2.1	77	6	20	31.9/6.54	37.1/5.53	gil7431022	<i>H. vulgare</i>	Glucose and ribitol dehydrogenase	
a10	-2.1	133	10	52	25.5/6.66	31.3/5.70	gil16903082	<i>T. aestivum</i>	Small Ras-related GTP-binding protein	
a5	-1.7	76	5	22	16.9/4.10	19.3/5.90	gil1754999	<i>T. aestivum</i>	Calmodulin TaCaM2-2	
a6	-2.1	137	16	28	49.9/6.16	54.8/5.27	TC246874	<i>Zea mays</i>	Globulin-like protein	
a13	-1.6	93	15	36	49.9/6.16	56.1/5.98	TC246759	<i>Z. mays</i>	Globulin-like protein	
a15	-1.5	108	10	22	49.9/6.16	29.4/5.60	TC246874	<i>Z. mays</i>	Globulin-like protein	
c3	1.5	72	5	40	34.6/8.71	43.6/5.39	BQ243369	<i>T. aestivum</i>	Low molecular weight glutenin	
c2	-1.5	72	5	38	32.8/8.50	47.5/5.04	BQ242949	<i>T. aestivum</i>	$\alpha$ -Gliadin	
c4	-1.5	76	4	37	34.6/8.71	68.6/7.37	BQ243369	<i>T. aestivum</i>	Low molecular weight glutenin	
c5	-1.5				32.3/7.16	61.3/7.20	TC250312	<i>T. aestivum</i>	$\gamma$ -Gliadin	RWDIHVAHGW, ATNPRDYAGKW
c1	-1.5				32.3/7.16	26.3/6.50	BJ233925	<i>T. aestivum</i>	$\gamma$ -Gliadin	TRGMTPTMTQVCIVARGY, TRGMTPTMTQVCIVARGY- SCMGSTVVEMVE
<b>cA-htB treatment</b>										
b1	1.9	72	7	31	24.1/6.31	28.4/6.60	gil1710077	<i>H. vulgare</i>	1-Cys peroxiredoxin PER1	
b7	1.5	102	8	31	23.9/6.06	27.1/6.85	TC250400	<i>T. aestivum</i>	27K protein, pfam03227, $\gamma$ -IFN inducible lysosomal thiol reductase	
b4	1.5	76	9	22	43.5/5.11	46.1/8.03	gil224589268	<i>T. aestivum</i>	Serpin 2	
b6	1.8	76	5	35	16.5/5.07	17.2/8.30	gil21711	<i>T. aestivum</i>	$\alpha$ -Amylase/trypsin inhibitor CM17	
b3	-1.8	75	8	17	50.5/5.68	45.3/7.57	gil1495812	<i>Solanum tuberosum</i>	Lipoxygenase	
b5	-1.5	73	6	51	14.8/5.68	14.9/7.37	gil242032659	<i>Sorghum bicolor</i>	Hypothetical protein, cd02947, thioredoxin family	
b2	-1.9	72	7	22	45.6/6.08	46.1/7.57	gil226500228	<i>Z. mays</i>	Succinyl-CoA ligase $\beta$ -chain	
b10	-1.5	109	11	39	26.8/5.38	29.0/7.07	TC246911	<i>T. aestivum</i>	Triosephosphate isomerase	
b9	-1.5	81	9	18	39.4/5.38	40.5/5.93	gil1419094	<i>Nicotiana tabacum</i>	Glutamine synthetase	
b8	-1.8	72	5	20	24.8/10.41	14.1/9.24	TC264449	<i>O. sativa</i>	40S ribosomal protein S8	
d1	1.5				32.8/8.50	47.5/8.25	BQ244309	<i>T. aestivum</i>	$\alpha$ -Gliadin	PIEPGIFPTAFATISIRPGL, GFPCASTIAPVGRNRPD- RVPDPCNVQCL
d6	1.5	77	4	33	32.8/8.50	46.2/8.83	BQ242040	<i>T. aestivum</i>	$\alpha$ -Gliadin	
d7	1.7	79	5	31	34.6/6.62	34.4/7.61	BQ167777	<i>T. aestivum</i>	$\alpha/\beta$ -Gliadin A-V	

Table 1. Continued

Spot no. <sup>a)</sup>	Spot- relative intensity <sup>b)</sup>	MS score <sup>c)</sup>	Number of matched peptides	Sequence coverage (%)	Theor. MW (kDa)/pI	Exp. MW (kD)/pI	Accession number <sup>d)</sup>	Organism <sup>e)</sup>	Protein and conserved domain	MS/MS (sequence of matched peptides) <sup>f)</sup>
d9	1.6	72	4	23	32.8/8.50	45.6/9.01	BE424082	<i>T. aestivum</i>	$\alpha$ -Gliadin	
d10	1.7	74	4	25	34.6/6.62	42.5/8.48	BQ245636	<i>T. aestivum</i>	$\alpha/\beta$ -Gliadin A-V	
d11	1.6	72	4	23	32.8/8.50	71.9/9.18	BE424082	<i>T. aestivum</i>	$\alpha$ -Gliadin	
d2	1.8				32.3/7.16	61.3/9.01	TC250312	<i>T. aestivum</i>	$\gamma$ -Gliadin	RWDIHVAHW, ATNPRDYAGKW
d3	1.6				34.6/8.71	22.2/7.84	BQ243369	<i>T. aestivum</i>	Low molecular weight glutenin	KKIGSP, GVHSLGGGTPPTGGAM- CFPTTAVGGGNW
d4	1.9				34.6/8.71	67.6/7.08	BQ243369	<i>T. aestivum</i>	Low molecular weight glutenin	KKIGSP, GVHSLGGGTPPTGGAM- CFPTTAVGGGNW
d5	1.9				34.6/8.71	35.8/8.07	BQ243369	<i>T. aestivum</i>	Low molecular weight glutenin	WAVVQNP, GVHSLGGGTPPTGGAM- CFPTTAVGGGNW
d8	-2.0	75	5	17	34.6/6.62	54.0/5.77	TC249891	<i>T. aestivum</i>	$\alpha/\beta$ -Gliadin	
<b>htA-htB treatment</b>										
e27	1.6	76	6	35	16.5/5.07	17.1/8.13	gil21711	<i>T. aestivum</i>	$\alpha$ -Amylase inhibitor CM17	
e30	1.6	115	7	65	13.7/6.18	14.5/7.38	gil134034577	<i>T. aestivum</i>	Monomeric $\alpha$ -amylase inhibitor	
e29	1.5	101	8	72	13.7/5.23	14.0/7.58	gil56480630	<i>T. aestivum</i>	0.19 dimeric $\alpha$ -amylase inhibitor	
e8	2.0	74	6	14	84.8/6.05	70.3/8.17	TC249217	<i>O. sativa</i>	Fructose 6- phosphate-2-kinase	
e10	1.9	72	9	31	48.4/5.70	50.9/7.34	gil162460735	<i>Z. mays</i>	Enolase 2	
e7	1.6	94	11	19	59.6/5.66	60.5/6.89	TC249933	<i>H. vulgare</i>	$\beta$ -Amylase	
e11	2.0	86	10	28	40.9/9.35	42.0/8.24	gil81176532	<i>T. aestivum</i>	Ribosomal protein S2	
e28	1.6	77	6	46	18.7/8.62	18.3/8.92	gil15822545	<i>T. aestivum</i>	Cinnamoyl-CoA reductase	
e24	1.5	79	5	33	18.9/4.55	28.7/9.61	gil75246527	<i>T. aestivum</i>	Translationally- controlled tumor protein	
e3	1.9	81	10	13	89.7/5.12	88.7/7.73	TC233240	<i>O. sativa</i>	Cell division control protein 48 homolog E	
e19	1.9	96	11	37	71.6/5.30	32.8/5.86	TC264180	<i>O. sativa</i>	Heat shock cognate 70kDa protein	
e23	1.5	72	4	20	18.7/6.04	29.6/7.49	TC237069	<i>T. aestivum</i>	NADH dehydrogenase subunit J	
e13	1.6	72	7	22	41.2/6.60	38.5/7.40	gil242046234	<i>S. bicolor</i>	Hypothetical protein, pfam03214, reversibly glycosylated polypeptide	
e12	2.0	87	6	23	37.4/5.85	38.7/6.85	gil218197438	<i>O. sativa</i>	Hypothetical protein	
e25	2.2	72	5	51	15.2/11.62	21.9/7.69	CA739630	<i>T. aestivum</i>	Predicted protein	
e9	1.7	78	11	12	134.3/9.05	87.5/8.75	gil224099935	<i>Populus trichocarpa</i>	Predicted protein	
e20	-2.2	112	7	35	23.3/6.06	31.4/5.97	gil30793446	<i>T. aestivum</i>	27K protein, pfam03227, $\gamma$ -IFN inducible lysosomal thiol reductase	
e31	-1.9	82	5	55	13.7/6.73	13.7/6.45	gil253783731	<i>T. aestivum</i>	$\alpha$ -Amylase inhibitor CM1	
e21	-2.0	117	8	34	26.6/6.37	31.1/6.40	gil90959771	<i>T. aestivum</i>	Multidomain cystatin	
e16	-1.9	113	12	34	35.8/6.51	36.1/6.00	TC264995	<i>H. vulgare</i>	Aldose reductase	
e4	-1.5	72	5	11	84.8/5.68	77.2/7.03	gil50897038	<i>H. vulgare</i>	Methionine synthase	
e5	-1.6	94	15	32	59.5/6.27	54.1/5.71	gil3913791	<i>Solanum lycopersicum</i>	Glutamate - cysteine ligase, chloroplastic	
e22	-1.9	95	8	40	29.4/4.83	33.8/8.13	gil40781605	<i>T. aestivum</i>	14-3-3 Protein	
e1	-1.6	72	8	16	71.2/6.25	82.7/5.71	TC252572	<i>O. sativa</i>	Ste20-related protein	
e17	-1.9	75	9	20	66.7/7.78	36.6/5.86	gil215398470	<i>T. aestivum</i>	Globulin 3	
e15	-1.6	92	8	19	66.6/7.78	36.6/6.56	gil215398470	<i>T. aestivum</i>	Globulin 3	
e6	-1.9	75	9	15	49.9/6.16	55.3/5.81	TC246703	<i>Z. mays</i>	Globulin-like protein	
e2	-1.7	73	6	15	57.8/8.19	91.3/6.81	gil225450579	<i>Vitis vinifera</i>	Hypothetical protein, cl03252, PPR repeat	
f1	1.6	75	4	23	32.8/8.50	71.9/8.46	BE424082	<i>T. aestivum</i>	$\alpha$ -Gliadin	

Table 1. Continued

Spot no. <sup>a)</sup>	Spot- relative intensity <sup>b)</sup>	MS score <sup>c)</sup>	Number of matched peptides	Sequence coverage (%)	Theor. MW (kDa)/pI	Exp. MW (kDa)/pI	Accession number <sup>d)</sup>	Organism <sup>e)</sup>	Protein and conserved domain	MS/MS (sequence of matched peptides) <sup>f)</sup>
f2	1.8	81	4	23	32.8/8.50	71.9/9.05	BE424082	<i>T. aestivum</i>	$\alpha$ -Gliadin	VPPECSIIIRAPF, VPPECSIIIRAPFASIVAGI- GGQ
f5	1.5				32.3/7.16	39.2/8.20	TC250312	<i>T. aestivum</i>	$\gamma$ -Gliadin	
f6	1.5	73	4	33	32.8/8.50	39.2/8.67	BQ242040	<i>T. aestivum</i>	$\alpha$ -Gliadin	HNSNHVHSNNHHYRS- NNHHF, HCNKHYRTTNNNPN- NNHHF
f7	1.9				35.0/8.60	38.4/8.52	BQ241281	<i>T. aestivum</i>	Low molecular weight glutenin subunit group 4 type II	
f8	1.6	72	4	8	32.3/7.16	32.3/8.30	TC249991	<i>T. aestivum</i>	$\gamma$ -Gliadin	SSRSKAKPSPMLSML, SVLTEKKITL
f3	-2.0				34.6/6.62	47.9/5.44	BQ244076	<i>T. aestivum</i>	$\alpha/\beta$ -Gliadin	
f4	-1.6				44.6/6.12	44.0/5.33	CA726637	<i>T. aestivum</i>	$\omega$ -Gliadin	IAMARRTNMMK, IAMARRTNMMKVL

a) Spot numbers a, b and e refer to Fig. 2 (albumin fractions), spot numbers c, d and f refer to Fig. 3 (gliadin fractions).

b) +: spots increasing in intensity under stress treatments; -: spots decreasing in intensity under stress treatments.

c) Significant MS score is above 71 for NCBI and TaGI.

d) gi: accession number in NCBI; TC, BQ, BE, BJ: accession number in the TaGI wheat gene index Release 11.0.

e) When the identification was based on EST sequence, the organism with the most homologous sequence is given. The theoretical pI and MW are calculated from the homologous sequence of that organism.

f) Significant MS/MS score is above 26 for TaGI.

Hurkman et al. [19] and Laino et al. [34] have observed the decreased abundance of the spots belonging to the 14-3-3 family in wheat after heat stress. The down-regulation of 14-3-3 protein in the present study may indicate that 14-3-3-interactor negatively regulates factors that mediate stress response.

### 3.3 Drought or high-temperature-responsive storage proteins

Expression of several storage proteins were modulated under stress as well. Some storage proteins including  $\alpha$ -gliadin,  $\gamma$ -gliadin, low molecular weight glutenin and globulins decreased and one globulin and one low molecular weight glutenin increased in abundance in response to water deficits. Several  $\alpha$ -gliadins,  $\gamma$ -gliadins and low molecular weight glutenins increased and  $\alpha/\beta$ -gliadin,  $\omega$ -gliadin and globulins decreased in abundance in response to high temperature. Our results indicate that stress could increase the abundance of some gliadins at the expense of other gliadins that have highly variable sequences [27]. However, the reason why different components of gliadins and globulins vary in response to abiotic stress is still unknown.

### 3.4 Comparison of plant responses under three different stress events

No common spot from the albumin and gliadin fractions was found to respond to both drought and high-temperature stress. Furthermore, 1-Cys peroxidoxin was regulated in

opposite ways at single drought and high-temperature events. The results indicate different responses of wheat grain protein to drought and to high-temperature events. Drought can cause stomatal closure, decrease of photosynthetic activity, increased oxidative stress, alteration of cell wall elasticity and generation of toxic metabolites causing plant death [31]. Here, proteins involved in stress/defense, signaling pathways, redox regulation and energy metabolism were found. High-temperature stress often results in increases of stomatal conductance, respiration, leaf transpiration and oxidative stress [42], shortens the duration of grain filling in cereals and enhances gluten protein accumulation and starch synthesis [19]. Plants respond to heat by signaling via abscisic acid, ethylene and salicylic acid, scavenging of ROS via production of antioxidants, and transcriptional activation of stress-related proteins [43]. Here, a number of proteins including heat shock proteins, carbohydrate metabolism and storage proteins were found to be responsive to high temperature.

Albumin proteins involved in primary metabolism did not change in abundance between the cA-htB and htA-htB treatments. However, a 27 kDa hypothetical protein belonging to thiol reductase superfamily was regulated in opposite ways between these two treatments. Several proteins with other functions than involvement in stress and primary metabolism were found to be regulated only in response to two high-temperature events.

## 4 Concluding remarks

The combination of drought and heat stress applied at two stages of grain filling did not have a significant effect on

albumin contents, compared with either of the stresses applied individually. Globulins and gliadins showed with exception hA-cB increased protein fraction contents from 19 dpa. The glutenins accumulated during grain filling in response to different treatments. Some stress treatments can significantly modify contents of individual proteins in grain. Changes of grain protein contents strongly depended on the type of stress applied. It was possible to identify several proteins responsive to drought and high-temperature episodes. Few common proteins were observed responding to single and multiple high-temperature events. The identified proteins play key roles in anti-desiccation, antioxidation, defense, carbohydrate metabolism and storage. These protein markers identified in this study might be relevant for plant breeding. Thus, the present findings indicating effects of more than one stress event and type on the grain protein composition will contribute to the identification of cultivars with increased tolerance to increasing climate variability.

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*The authors have declared no conflict of interest.*

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## Appendix 6

### 14.2.3.1.3 Posters

# Targeted enzyme modification of starch for encapsulation of flavours



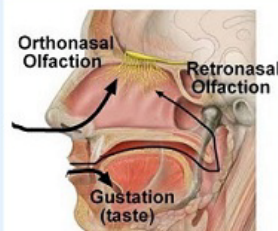
Anders D. Jørgensen<sup>1</sup>, Jan-Olof Lundberg<sup>2</sup>, Susanne Rask<sup>3</sup>, Andreas Blenow<sup>3</sup> and Birte Sørensen<sup>1</sup>  
<sup>1</sup> Department of Systems Biology, Technical University of Denmark, DK-2800 Lyngby, Denmark; <sup>2</sup> Culinar AB, Fjälkinge, Sweden; <sup>3</sup> Faculty of Life Sciences, University of Copenhagen, DK-2100 Copenhagen, Denmark

## Introduction

Taste experiences can be classified as sweet, salty, bitter, sour or umami (savoury).

On the other hand flavour sensations can be counted in the tens of thousands. Some of the aromas that stimulate the flavour receptors, do so in concentrations as low as parts-per billion.

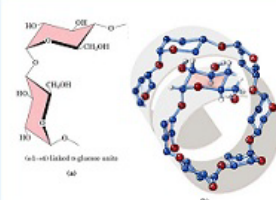
Manufactured foods mostly use starch-derivatives (i.e. acetylated) as flavour-carriers, thickeners or stabilizers. However, modified starches are not favoured by the consumer, so using natural starches is desirable.



**Figure 1** Taste is perceived by taste buds on the tongue, while the olfactory bulb registers all flavours. Most of the flavours are released during mastication leading to retronasal olfaction.

## Starch

Starch is composed to two different molecules, amylose and amylopectin. Amylose forms helices with a diameter of 10 Å where guest molecules, i.e. aromas, can be accommodated. Amylopectin is unsuited for complexation.

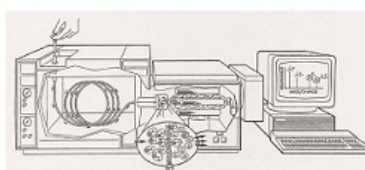


Enzymatic treatment of starch with debranching enzymes creates starches with a higher content of amylose. These modified starches might be better for accommodating aromas.

**Figure 2** The helical structure of amylose has an internal diameter of 10 Å. This structure can be used for accommodation of compounds like aromas, but complexes with fatty acids and alcohols are also common.

## Headspace analysis

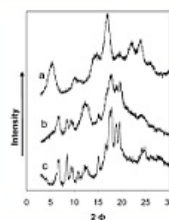
The perception of aromas is determined by the amount of aroma in the gas phase (headspace). Therefore it is critical to know how much aroma is trapped in the starch and how much aroma that can be released again. One way is by gas-chromatography mass spectrometry (GC-MS), which can identify and quantify aroma compounds.



**Figure 3** A schematic of a GC-MS. Sample is injected and passed through a column, where aromas are separated on a hydrophobic column (the GC part). Thereafter the aromas are injected into the mass analyzer, where the ions are fragmented to reveal a unique fingerprint for any given compound.

## X-ray analysis

The interactions between starch and aroma can be analysed by wide-angle X-ray scattering to reveal the starch type. Changes in starch morphology can be observed when aroma is added. X-ray analysis also allows for investigation of the helix structure of amylose.



**Figure 4** X-ray diffraction of starch in the presence of three different aromas. The peaks in trace of the starch with aroma a are symptomatic of B-type amylose, thus indicating no binding of the aroma. On the other hand the traces from the other aromas b and c indicate that complexation is occurring between the starch and the aroma (peak at  $2\theta = 7^\circ, 13^\circ$  and  $17^\circ$ ). From Arvisenet et al, 2002.

## Perspectives

The aim of this study is to create or enhance flavour delivery systems for the industrial production of food products using either natural starches or enzymatically treated starches.



## Litterature

Arvisenet, G.; Le Bail, P.; Voilley, A. and Cayot, N (2002) *J Agr Food Chem* **50** 7088-7093

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This work is a collaboration between Culinar AB, KU-LIFE and DTU. Culinar AB, LMC FOOD and DTU contribute 1/3 each.

#### ***14.2.3.2 Planned publications***

The following publication is planned:

*Anders D. Jørgensen<sup>1</sup>, Mikael Agerlin Petersen<sup>2</sup>, Andreas Blennow<sup>3</sup>, Birte Svensson<sup>1</sup>*

<sup>1</sup> Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

<sup>2</sup> Department of Food Science, Faculty of Life Sciences, University of Copenhagen, 1870 Frederiksberg, Denmark

<sup>3</sup> Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, 1870 Frederiksberg, Denmark

#### **“Factors influencing retention of aromas, aldehydes and alcohols by starch”**

Many factors have been shown to influence aroma retention by starch, and in this study we examine the retention of selected aromas representing a broad range of chemicals, as well as a series of aldehydes and alcohols. The retention is dependent on the volatility of the compounds where highly volatile compounds are positively retained by starch, whereas compounds with lower volatility showed a negative retention. Retention is dependent on the nature of the starch for some compounds, where native starch granules had a higher retention of the compounds tested than pre-gelatinised, freeze-dried starch. Grooves on the surface of the native starch granules may be critical in the retention of the compounds tested.