

**UNDERSTANDING THE CHANGES IN THE BREAD
COMPONENT OF READY-TO-EAT SANDWICHES WHERE
CONSUMERS' OBSERVATION OF "DRY & STALE" LIMITS
SHELF LIFE**

**By
Evgenia Fragkiadaki
BSc, MSc**

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**Division of Food Sciences
School of Biosciences
University of Nottingham
Sutton Bonington Campus
Loughborough
LE12 5RD
United Kingdom**

Abstract

This research constitutes a scientific insight to consumers' feedback indicating that the bread in two commercial ready-to-eat sandwiches becomes drier over storage. These sandwiches were i) ham, mustard and mayonnaise sandwich and ii) ham, farmhouse cheese and pickle sandwich. The techniques used in this project were Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA) and Fourier Transform Infrared spectroscopy (FTIR). The provided sandwiches were packaged and cut in two equal triangles following the shape of the packaging. They were stored at 4°C and each day a different sandwich was used for analysis. During sample preparation, sandwich bread was isolated from the filling and the applied barrier (signature mustard & mayonnaise for the ready-to-eat ham, mustard and mayonnaise sandwich and butter and ploughman's pickle layered on top of the one slice and signature 30% mayonnaise for the other slice of the ready-to-eat ham, farmhouse cheese and pickle sandwich). Samples of bread were taken from three different parts of the sandwich, namely two different samples were isolated from the slices that were in contact with the packaging and one from the slices that were in the middle of packaging. In the case of the ready-to-eat ham, mustard and mayonnaise sandwich, the analysis was taken over 4 days. A considerable increase in total amount of water in all the three samples acquired from the ready-to-eat ham, mustard and mayonnaise sandwich was noticed (oven drying results) and it is probably related to moisture migration from mustard and mayonnaise sauce to sandwich bread over storage. A significant increase of in the FW fraction and a decrease in the UFW fraction (DSC results) was monitored only in the case of middle slices of bread. TGA results detected a higher water release rate in the case of middle slices and in the one slice that was in contact with the packaging, while for the other the results were inconclusive. An upward trend in the amylopectin melting enthalpy in all samples indicated starch retrogradation over storage. Analysis of samples from the ready-to-eat ham, farmhouse cheese and pickle sandwich was taken over 3 days. The slice of bread spread with signature 30% mayonnaise showed an increase in the total amount of water (oven drying results) and an upward trend in water release rate over storage (TGA results). The slice of bread spread with butter exhibited a decrease in the same variable while the TGA results were inconclusive. FW, UFW and amylopectin melting enthalpy were statistically equal over storage. Changes in starch crystallinity could not be detected through FTIR technique for none of the samples. Overall, experiments in ready-to-eat ham, mustard and mayonnaise sandwiches implied that despite the physicochemical changes in water, amylopectin retrogradation is probably the main reason for the drier texture of sandwich bread, while consumers' feedback on ham, farmhouse cheese and pickle sandwich could not be experimentally explained.

Thesis structure

The structure of the thesis is as follows:

Chapter 1 describes the properties of the main ingredients present in bread and their impact on bread ageing. The role of storage temperature as well as the effect of barriers spread onto sandwich bread are also analysed in this chapter. The chapter ends with the objectives of this research. **Chapter 2** presents the preparation of samples, the principles of the instrumental techniques used and the experimental procedures followed. In **Chapter 3**, the results of this project are illustrated and described. This chapter is followed by **Chapter 4** which is the discussion chapter of the thesis. Finally, **Chapter 5** summarises the findings of this project comparing them with the initial objectives of the research and gives suggestions for future work.

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List of Abbreviations and Symbols

A-granules	Starch granules with diameter > 10 µm
ANOVA	Analysis of Variance
AS	Air Side slice
ATR	Attenuated Total Reflectance
B-granules	Starch granules with diameter ≤ 10 µm
BSA	Sandwich Association
DP	Degree of Polymerisation
DRIFT	Diffuse Reflectance Infrared Transform
DSC	Differential Scanning Calorimetry
DTG	Differential thermogravimetric
DTGmax	Maximum rate of weight loss
DVS	Dynamic Vapour Sorption
FTIR	Fourier Transform Infrared spectroscopy
IR	Infrared
MRE	Meal, Ready-to-Eat
MRI	Magnetic Resonance Imaging
MS	Middle Side slices
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
PS	Packing Side slice
SWB	Standard White Bread
TG	Thermogravimetric
TGA	Thermogravimetric Analysis
W/O	Water-in-oil emulsion
w.w.b.	Wet weight base
T _g	Glass transition temperature of the polymer
T _m	Melting temperature of the polymer
P+1	Samples delivered on day 'Production +1'
P+2	Samples delivered on day 'Production +2'
P+3	Samples delivered on day 'Production +3'
P+4	Samples delivered on day 'Production +4'
W ₁	Sample weight before drying
W ₂	Sample weight after drying
To	Onset temperature in DSC procedure
Tp	Peak temperature in DSC procedure

T_e	Endset temperature in DSC procedure
FW	Freezable Water
UFW	Unfreezable Water
ΔH	Enthalpy of specific latent heat of water
mc	Moisture content of samples
ν	Vibrational frequency
k	Force constant of the bond
μ	Reduced mass of the system
ΔE	Energy difference between two successive vibrational levels
h	Planck's constant
θ_c	Critical angle
n_1	Reflective index of the crystal
n_2	Reflective index of the sample
T_1	Spin-lattice relaxation rate
T_2	Spin-spin relaxation rate

1. Introduction

Although ageing of white bread has been studied extensively over the years, bread ageing in more complex systems, such as sandwiches, has not been described thoroughly in the published literature. The complexity of the samples used in this project is due to the utilization of three different flours in every sandwich bread as well as the fact that the bread interacts with a filling. Moreover, storage conditions play a crucial role in bread staling. This experimental project has been conducted at 4°C as relevant to ready-to-eat sandwiches, whereas in many published scientific works have chosen room temperature as storage temperature (Hallberg & Chinachoti, 2002; Baik & Chinachoti, 2000; Czuchajowska & Pomeranz, 1989). In addition, in the present work different techniques, such as Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA) and Fourier Transform Infrared spectroscopy (FTIR) have been used to monitor changes of bread over storage and comparison amongst them has been made in the discussion section of every chapter. Apart from the scientific importance of the study there is an industrial significance as nowadays, according to Sandwich Association (BSA), 6.8 million sandwiches are produced every week in the UK. The shelf-life of a sandwich is approximately 3 to 4 days limited by eating quality of sandwich related to the bread rather than microbial spoilage (Cauvain & Young, 2011). Thus, retardation of bread ageing could lead to shelf-life extension which would have a major economic impact for industries. Furthermore, customers will benefit from a possible reduction in sandwich price and finally a significant resource and energy conservation would be achieved.

Starch, gluten and water are the major components of bread and undergo major modifications during storage of bread. However, non-starch polysaccharides and native lipids contribute to bread ageing. All of these components have been studied widely and their role in bread as well as in bread ageing will be further analysed in this chapter. The amount of starch, proteins, non-starch polysaccharides and lipids in bread is determined by their content in the flour used. Table 1.1 summarises the components of wheat flour, malted barley flour and oat flour, used in sandwich bread production.

Table 1.1. Composition of wheat, wholegrain barley and oat flour expressed on wet weight basis (Goesaert, et al., 2005; Zhu, 2017; Gupta, et al., 2010).

Components	Wheat flour (%)	Malted barley flour (%)	Oat flour (%)
Starch	54-75	51-64	67-73
Non-starch polysaccharides	2-3	4-9	2-8
Proteins	10-12	10-17	9-15
Lipids	2	2-3	3-11

Water	14	10	12
Minerals and vitamins	1	1	1

1.1 Starch in flour

Starch is the most abundant carbohydrate stored in plants and is solid solar energy produced by photosynthesis (Hizukuri, et al., 1997). It can be found in leaves, flowers, fruits, seeds, different types of stems and roots in the form of discrete particles, called granules. The main location of starch synthesis and storage in cereals is the endosperm (Smith, 2001). The following sections focus on the properties of cereal starches and specifically of wheat, malted barley and oat.

1.1.1 Starch structure

Starch consists of two main polysaccharides, amylose and amylopectin. These are both composed of α -D-glucopyranosyl units, or simply called glucose units. However, glucose molecules are linked in a different way resulting in the two different compounds with major distinct functional properties which are listed in Table 1.2.

Table 1.2. Amylose and amylopectin characteristics (table adapted from Thomas, D. J and Atwell, W. A, 1997).

Characteristic	Amylose	Amylopectin
Shape	Essentially linear	Branched
Linkage	α -1,4 (some α -1,6)	α -1,4 & α -1,6
Degree of Polymerisation (DP)	1,500-6,000	300,000-3,000,000
Films	Strong	Weak
Gel formation	Firm	Non-gelling to soft

The total amount of starch as well as the ratio of amylose to amylopectin varies among different cereals and different varieties of the same cereal. Table 1.3 presents the ratios that correspond to starches relevant to this project namely, wheat, malted barley and oat.

Table 1.3. Percentages of total amount of starch, amylose and amylopectin content in wheat, malted barley and oat flours, expressed on dry weight basis. (Maningat, et al., 2009; Autio & Eliasson, 2009; Vasanathan & Hoover, 2009; Fredriksson, et al., 1998; Holtekjølén, et al., 2006; Stevenson, et al., 2007).

Starch Type	Total starch in the cereal (%)	Amylose content (%)	Amylopectin content (%)
Wheat	54-75	25	75
Malted Barley	51-64	30-48	52-70
Oat	67-73	17-33	67-83

Amylose

Amylose is an essentially linear polymer comprised of α -1, 4-linked glucose as depicted in Figure 1.1. It also contains some α -1, 6-glucosidic bonds, thus is slightly branched. Although amylose is often illustrated as a straight chain, in reality it is often helical due to the development of hydrogen bonds in the interior. The hydrophobic nature of the internal part of amylose molecules explains its ability to form complexes with a variety of hydrophobic molecules (e.g free fatty acids, alcohols) or hydrophobic side chains of molecules inside the helices.

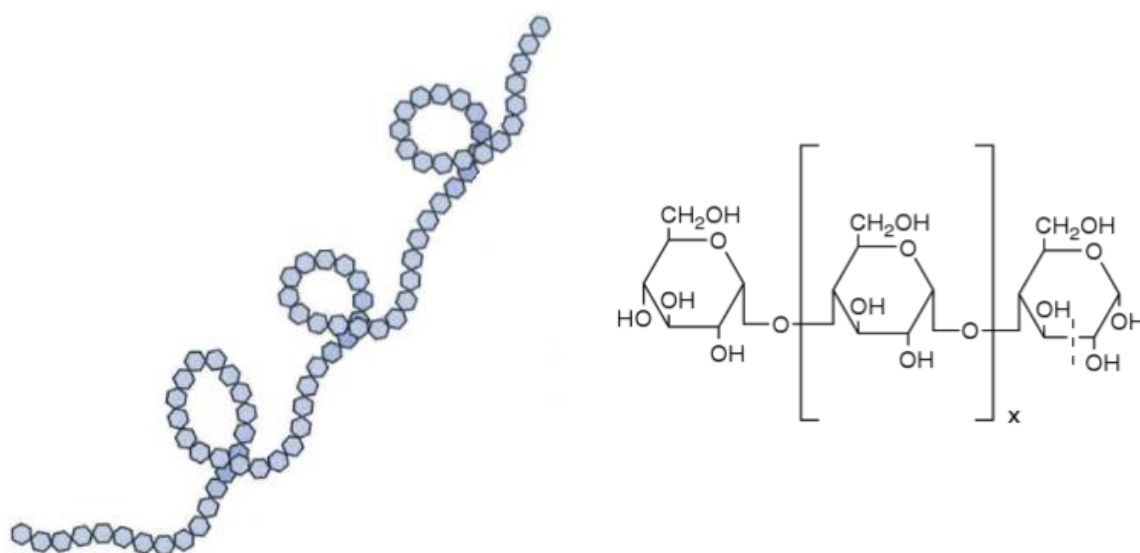


Figure 1.1. Structure of amylose. Macromolecular structure of amylose (on the left) and molecular structure that depicts the repetition of glucose units (on the right) (Perez, et al., 2009).

Amylopectin

Amylopectin is a highly branched polymer that is much larger than amylose. It is composed of α -1, 4-linked glucose segments which are connected by α -1, 6-glucosidic bonds. An average amylopectin molecule contains more than 20,000 branches and approximately 1 million glucose molecules. There are three types of amylopectin chain architecture, A, B and C. According to Peat et al (1952), A-chains are the “outer” unbranched chains, B-chains are the inner branched chains and a C-chain is a single chain per amylopectin molecule that contains the single reducing end (Figure 1.2).

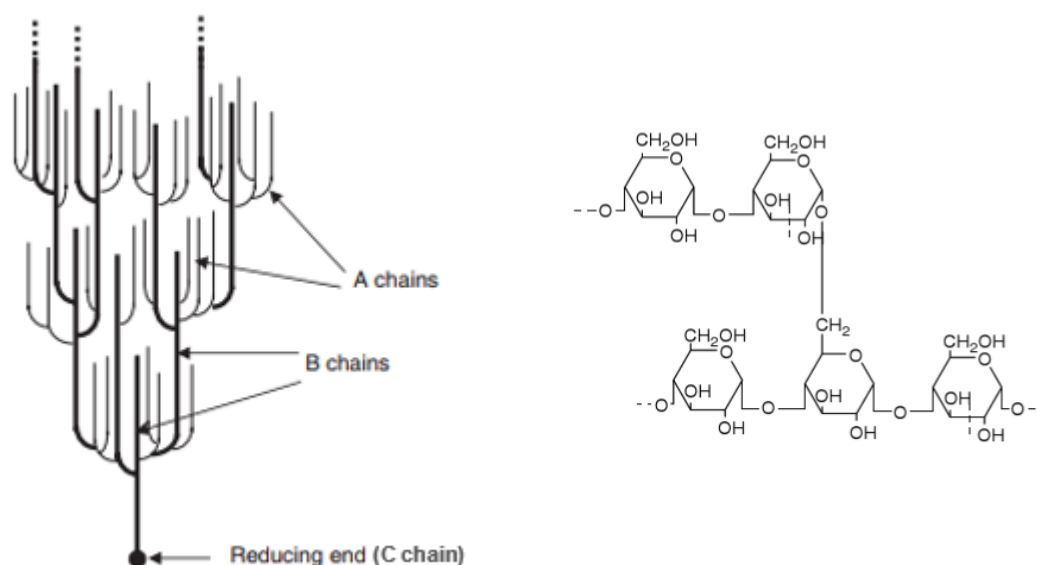


Figure 1.2. Structure of amylopectin. Macroscopic structure of amylopectin with the presence of A-, B- and C- chains (on the left) and molecular illustration of the way that glucose units are linked together (on the right) (Perez, et al., 2009).

Starch granules

Starch in cereals is synthesised and stored in amyloplasts that are mainly located in the endosperm. The way that starch is stored is in discrete partially crystalline particles, called granules. The accumulation pattern of starch granules in each plant tissue, the shape, the size, the structure and the composition is unique to each botanical species (Smith, 2001). However, granules are usually of irregular rounded shape with a size ranging in size from 2-100 μm . By using different types of microscopy, it was revealed that starch granules consist of a series of concentric rings, which are widely called growth rings, and contain a pattern of alternating crystalline and amorphous regions (French, 1972). Their origin is not entirely clear but it was suggested that they derive from diurnal fluctuations. Amylopectin molecules are in a radial arrangement within the granule, with the reducing end of the C-chain towards the centre (Robin, et al., 1974). The A-chains of amylopectin, namely the smaller chains in the molecule, interact strongly with each other resulting in crystalline regions (Robin, et al., 1974). It was also found that in this region neighbouring chains are paired forming short double helices of 6 glucose molecules per turn (French & Murphy, 1977; Robin, et al., 1974). The amorphous regions contain most of the 1,6 branching points. Regarding amylose, it is believed that amylopectin “clusters” are associated with amylose within both crystalline and amorphous regions (Thomas & Atwell, 1999). Figure 1.3 illustrates the above described structure of the granules.

Starch granules are classified according to their size on two categories. Large granules with a diameter of greater than 10 μm are called “A- granules”, while smaller granules with a

diameter of less than 10 μm are called “B-granules” (Jane, et al., 1994). The difference in size is accompanied by difference in their composition and physicochemical properties. A-granules contain less amylose and lipids than B-granules and gelatinise at lower temperature but retrogradation occurs faster than in B-granules (Eliason, 1989). The amount of A- and B-granules differs among the different varieties of the same cereals. Analysis of four wheat starches showed that B-granules are present at a percentage of approximately 30% by weight (BeMiller, et al., 2009), while in barley B-granules constitute 80-90% of the total amount of granules but this percentage corresponds to only 10-15% by weight (Vasanthan & Hoover, 2009). Finally, in oat 60% of the total amount of granules consists of B-granules, while the rest contains clustered granules of 20-150 μm diameter (Autio & Eliasson, 2009; Saccomanno, et al., 2017).

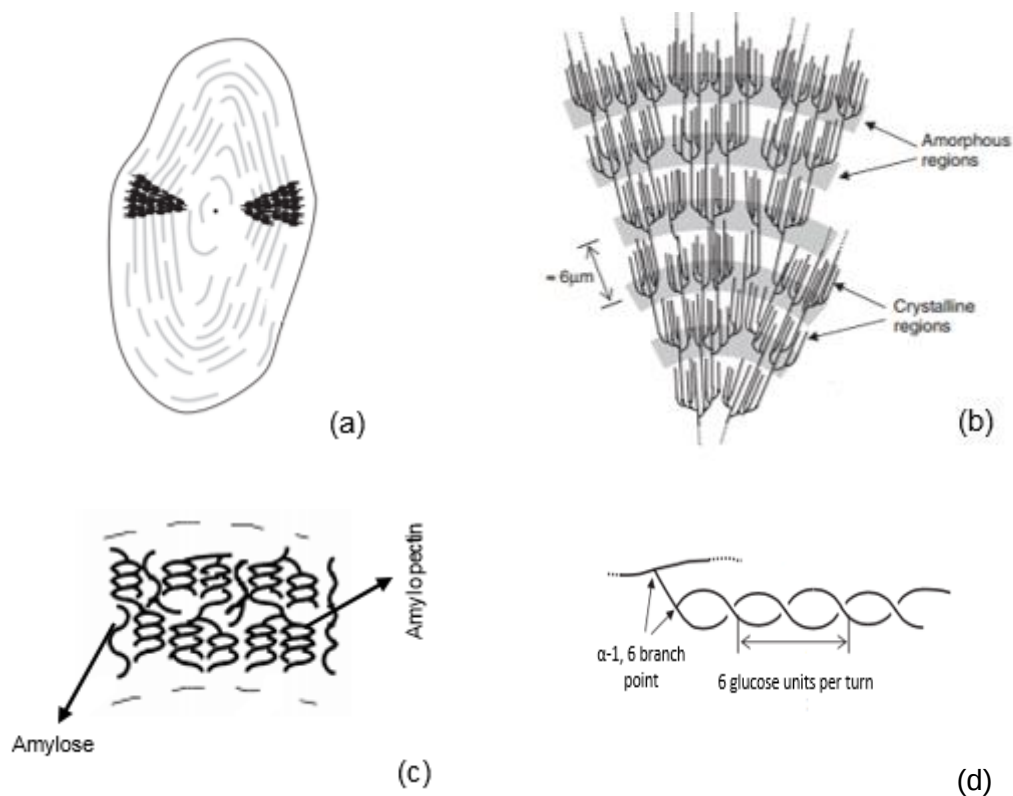


Figure 1.3. Starch granular structure: (a) structure of the whole granule, (b) a closer view of the granular structure, depicting the layout of amylopectin molecules and the formation of amorphous and crystalline regions, (c) amylose and amylopectin molecules inside the granule (d) the double helices that are formed by neighbouring chains in the granules. (Adapted from Thomas & Atwell (1999) and Buleon, et al. (1998)).

Starch crystallinity

The crystalline region of starch granules can contain two different types of crystals depending on the aggregation of amylopectin double helices. Amylopectin A-chains form left-handed double helices containing at least 10 glucose units with either adjacent branched in the same

amylopectin molecule or between neighbouring amylopectin clusters (Robin, et al., 1974). If the helices are made of short chains the “A-type” polymorph results. If long A-chains participate in the helices, they create the “B-type” polymorph (Hizukuri, 1985). A “C-type” polymorph has also been observed in legume starches and consists of intermediate A-chains so is believed to be a combination of the A- and B-type (Bogacheva, et al., 1998). Finally, the last known pattern is the V-type and it is composed of complexed amylose, usually with fatty acids (Zobel, 1988). In cereal starches A- and B-type crystallites differ in the crystallite arrangement and water content. The A-type is a monoclinic unit cell containing 4 molecules of water, while the B-type crystal is arranged into a hexagonal lattice unit cell containing 36 water molecules (Sarko & Wu, 1978). The structure, the unit cell and water molecules of A- and B-type of crystals are illustrated in Figure 1.4. Wheat, oat and barley starches are A-type starches, as are most of the cereal starches (Autio & Eliasson, 2009; Vasanathan & Hoover, 2009). However, certain barley cultivars exhibit a C-type polymorph. During bread making the crystals melt and on cooling the V-type pattern is usually the first that appears, revealing the formation of amylose-lipid complex (see 1.1.3) as well as B-type crystals that are created by the recrystallisation of the amorphously melted starch (Ribotta, et al., 2004).

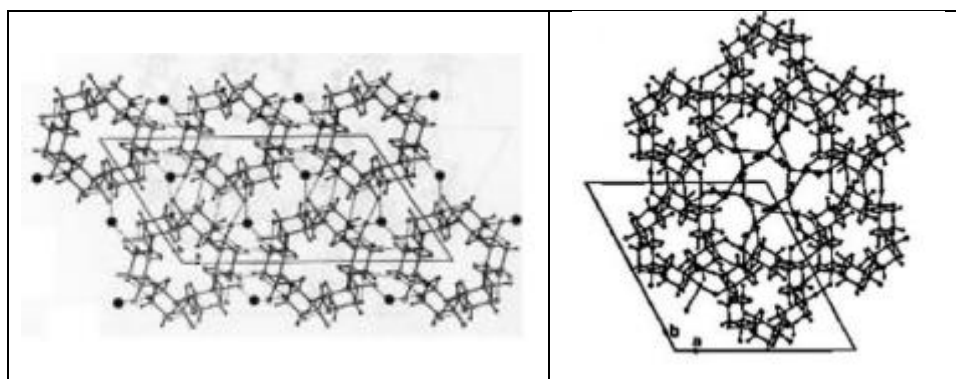


Figure 1.4. Type of crystals in starch. A-type crystals (on the left) and B-type crystals (on the right). The unit cell is presented in both pictures, while water molecules are pointed with black closed circles. Projection of the structure onto the (a, b) plane (adapted from Pérez & Bertof, 2010).

1.1.2 Starch gelatinisation

Starch gelatinisation is the deformation of starch granules within an aqueous environment over heating (Zanoni, et al., 1991). The result of gelatinisation is the conversion of starch from a semi-crystalline form, which is relatively indigestible, to an amorphous, digestible form (Tester & Debon, 2000). This change takes place in many food processes, such as baking and affects the final quality of the product. Starch gelatinisation involves two principal phenomena: loss of crystallinity and irreversible swelling. During this process, swelling starts in the least organised regions within the granule, the amorphous regions (French, 1984). As a result of swelling, the amorphous region exerts pressure onto the crystals and the layered organisation is lost, a fact

that causes loss of birefringence in the granules (French, 1984). In addition, heating leads to the disruption of the hydrogen bonds in the double helical regions and eventually amylopectin crystals melt (Liu, et al., 1991). Afterwards, the disassociated side chains of amylopectin become hydrated and swell. It has been shown that A-type crystals melt and dissolve at approximately 20°C higher temperature than B-type crystals (Kokini, et al., 1994). Gelatinisation is not an instant process. Monitoring of the gelatinisation process by thermal methods, such as DSC, showed that the onset temperature of gelatinisation of wheat starch is approximately 45°C, peak temperature is typically around 60°C, while conclusion temperature is generally around 75°C (Tester & Debon, 2000). Furthermore, in the case of barley these temperatures are about 52-61°C, 58-65°C and 62-74°C respectively (Vasanthan & Hoover, 2009). Oat starch gelatinises at higher temperatures, namely the process starts at 60°C and ends at 70°C, while the peak of gelatinisation was noticed at 64°C (Autio & Eliasson, 2009). Reaching conclusion temperature, although amylopectin chains are disassociated and granules are swollen, they maintain their integrity. Further heating and swelling of the disrupted parts result in solubilisation of small linear amylose molecules and eventually diffusion of these out of the granule (Leach, et al., 1959). The temperature at which amylose solubilises differs among cereals and is also different from the gelatinisation temperature (Banks & Greenwood, 1975). The increase in the volume fraction of starch granules and amylose leaching lead to an increase in viscosity (Leach, et al., 1959). Ultimately, swollen granules occupy the available volume, forming a viscoelastic material, whose properties depend on deformability of the granules and amylopectin content in them (Parker & Ring, 2001). Finally, at a critical point of swelling and heating granules cannot resist mechanical and thermal tension and they rupture. Hence, a large amount of molecules is released and a paste is eventually formed. The remaining starch molecules and the outer layers of the disintegrated granules are called “ghost remnants” and also exhibit elastic properties (Atkin, et al., 1998). On cooling, some starches form a gel, such as in the case of bread baking. During this process, starch-starch hydrogen bonds are formed leading to the creation of a gel network and increased viscosity of the system, called starch gelation (Gidley, 1989). The difference between a paste and a gel is that a paste shows fluidity, while a starch gel has a defined shape without fluidity (Tabilo-Munizaga & Barbosa-Canovas, 2005).

Despite the above description, in the case of more complex products that apart from starch contains other compounds, starch gelatinisation might not lead to the complete rupture of starch granules due to water deficiency. For instance, in the case of bread there is insufficient free water to fully gelatinise the starch. Thus, starch granules firstly absorb any free water available in the dough and then start to use the water from protein membranes but the swelling is still limited and although birefringence under polarised light is lost, some granules can be recognised on microscope inspection (Cauvain, 2015; Schiraldi & Fessas, 2001).

1.1.3 Starch retrogradation

The term “retrogradation” is used to describe changes that occur during storage of gelatinised starch at lower temperatures than the melting temperature of the starch crystals. It is a process of reassociation and recrystallisation of amylose and amylopectin chains during ageing and it was firstly described in 1852 (Boussingault, 1852). After starch gelatinisation and cooling, the amorphous starch is not in a state of equilibrium so it starts to retrograde. This process has a negative impact on the quality of food products containing starch as it results in a product with drier texture and reduced aroma (Cauvain, 2015). In the case of bread, starch retrogradation leads to bread staling, which is defined as the time-dependent loss in quality of flavour and texture of the bread.

X-ray diffraction experiments have shown that the starch crystals formed during retrogradation are B-type crystals (Ribotta, et al., 2004; Aguirre, et al., 2011). The mechanism of starch recrystallisation, according to Goodfellow and Wilson (1990), consists of two stages, a fast stage and a slow stage and involves phase separation, water syneresis, and increase in viscosity and gel formation which are related to molecular recrystallisation. The term phase separation is referred to amylose and amylopectin indicating the formation of amylose-rich and amylopectin-rich phases created mainly due to their different extent of branching (Kalichevsky & Ring, 1987). On the other hand, water syneresis is a term related to the phenomenon of the breaking up of gels occurring over storage or when disrupted. The result of this process is the transition from a homogeneous mass to distinct solid lumps surrounded by a thin liquid layer (Graham, 1876).

Although retrogradation is a natural process, the presence of lipids, the storage temperature and the water present in the final product affect it significantly thus, their impact will be further analysed below in this section. (Impact of water in bread will be presented in section 1.5).

Amylose retrogradation

Amylose retrogradation is relatively rapid, sometimes it is completed within about 20min after the end of baking (Ianson, et al., 1988). The fast stage of amylose retrogradation involves transformation of the molecules from a random coil configuration to a double helical structure creating a gel network, while during the slow stage, amylose helices aggregate to form crystals. According to Gidley and Bulpin (1989), mainly the long double helices trigger the creation of an extensive cross-linked network, which finally leads to gelation. With regards to bread, the slow stage of amylose retrogradation is particularly important as it contributes to the loaf structure and initial hardness of the bread (Cauvain, 2015). The significance of amylose retrogradation in crumb formation was shown in an experiment where only waxy starch, namely amylose-free starch, was used in the bread making process resulting in the

collapse of bread loaves on cooling (Hoseney, et al., 1983). It is considered that the final structure of amylose molecules remains stable during further storage of bread because the amorphous regions are linked to the aggregated regions in a stable way (Cauvain, 2015). This was in agreement with DSC results showing that amylose crystals demand high temperature to melt, approximately 120°C (Miles, et al., 1985a).

Amylopectin retrogradation

Amylopectin retrogradation can last from days to weeks probably due to the highly branched structure of the molecule (Goodfellow & Wilson, 1990). Specifically, during the fast stage the side chains of amylopectin form intramolecular helices with no significant effect in viscosity of the product, while in the slow stage, the helical side chains form rigid sections within the molecule resulting in an increase in gel strength and viscosity (Goodfellow & Wilson, 1990). It is believed that the double helices that are formed initially in the molecule are associated to the same backbone of the polymer via $\alpha(1\rightarrow6)$ glycosidic bond. Thus, even after gelatinisation they are in close proximity allowing to form double helices easily (Goodfellow & Wilson, 1990; Klucinec & Thompson, 1999). Eliasson *et al.* (1987) showed that the length of the linkage formed by helices belonging to a $\alpha(1\rightarrow6)$ branch point was comparable to the van der Waals distance formed between the double helices. Furthermore it was proven that the stereochemistry of the $\alpha(1\rightarrow6)$ branch point in amylopectin and the two chains connected by it favours the formation of a stable double helix, as in this way the minimum distortion is achieved (Imberty & Perez, 1989). It is worth mentioning that amylose helices are comprised of 40-70 glucose units (Liu, et al., 1991; Gidley, et al., 1995), whereas double helices of amylopectin are shorter due to the restrictions imposed by the branching structure and the existence of short length of branches (Miles, et al., 1985). The structure of retrograded amylopectin has been modelled in two different ways. The first is called “fringed micelle” and was developed by Levine and Slade (1988). According to this, every amylopectin chain contains both helical and random coil regions with water acting as a plasticizer playing a crucial role in the formation of the final structure of starch, where crystals are surrounded by amorphous starch regions. The second model is named “chain-folded polymer model” and was developed for synthetic polymers (Lauritzen & Hoffman, 1973), but it has been applied to starch (Marsh & Blanshard, 1988; Farhat, et al., 2000b). The crystalline regions of starch are made up of adjacent double helices and the branching regions create folds (Farhat, et al., 2000b). Miles *et al.* (1985a) showed that amylopectin crystallinity was reversible in temperature lower than 100°C. Miles *et al.* (1985a) suggested and Ring *et al.* (1987) experimentally confirmed that the relatively low melting temperature of amylopectin crystals at approximately 50-60°C was due to the weak interaction between smaller and longer

amylopectin chains as the presence of branching points hindered a more stable attachment of the chains.

Regarding bread, it is worth mentioning that since the amount of amylopectin in wheat, barley and oat starch is greater than that of amylose and as amylopectin retrogradation lasts longer than that of amylose, amylopectin is considered to play a more crucial role in bread staling.

Amylose and amylopectin co-crystallisation

In 1990 Gudmundsson and Eliasson observed a disruption in the amylopectin melting peak, appearing in the DSC thermogram when amylose was present at a level of less than 50% (w/w). On the other hand, there was not a significant impact on the results when the amylose content was larger than 50%. Furthermore, van Soest et al. (1994) detected that potato starch was retrograding faster than waxy maize, suggesting that the presence of amylose in potato starch formed an ordered matrix and acted as nucleus for amylopectin retrogradation. Later, Klucinec & Thompson (1999) proposed two possible ways for amylose-amylopectin co-crystallisation. The first suggestion was that some double helices consist of one chain of amylose and one chain of amylopectin. However from a kinetic point of view this is unlikely, since it is more likely that two amylopectin chains that are connected to the same $\alpha(1\rightarrow6)$ branch point create a chain. The second proposal was that amylose and amylopectin double helices are joined creating a crystal unit cell. The way that these helices are connected depends on the proximity of the double helices as well as their flexibility. Finally, the ratio of amylose to amylopectin and the length of amylopectin chains affect the interactions between amylose and amylopectin (Klucinec & Thompson, 1999).

Amylose-lipid and amylopectin-lipid complex formation

Apart from amylose and amylopectin, flours contain a small amount, typically between 2-3%, of mainly free fatty acids and lysophospholipids (Buleon, et al., 1998a). However, oat flour contains approximately 3-11% lipid, which is a very high lipid content (Zhu, 2017). In the case of an amylose-lipid complex, the hydrophobic tail of a lipid enters into the internal helical space, while the hydrophilic head remains external as illustrated in Figure 1.5 (Carlson, et al., 1979). According to molecular modeling, the reason why the hydrophilic head is not entering the helical cavity but remains near the entrance of it is the steric and electrostatic repulsions (Godet, et al., 1993). As a result, in longer amylose chains, where two fatty acids can complex with the same molecule, the hydrophilic part can cause deformation of the chain. Every amylose-lipid complex usually consists of three turns of the amylose helix where each turn is made of six glucose units but in the presence of bigger lipid molecule, glucose units are seven or eight (French & Murphy, 1977). Although the amylose-lipid complex has been found in native starches, so it exists even before gelatinisation, it is not in a crystalline packing as the

V-type diffraction pattern has not been observed (French, 1984; Morrison, et al., 1993a; Morrison, et al., 1993b). The crystalline form of this complex is formed upon heating of the sample. Specifically, Le Bail et al. (1999) suggested that during heating the already existing amylose-lipid complexes aggregate to create crystals. The amylopectin-lipid complex that is also depicted in Figure 1.5 was observed many years after the amylose-lipid complex. According to Gudmundsson & Eliasson (1990) both complexes have the same arrangement with the difference that the linear structure of amylose can more efficiently form complexes with lipids thus, it will be the first molecule to create a complex.

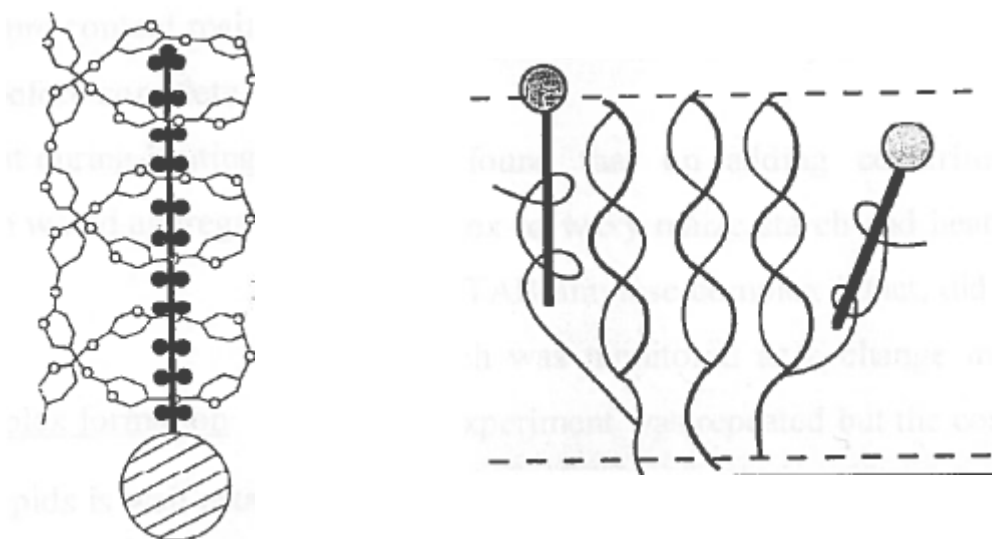


Figure 1.5. Model of amylopectin-lipid complex, on the left (Eliasson & Larsson, 1993) and schematic diagram of amylose-monoglyceride complex, on the right (Carlson, et al., 1979).

The role of starch-lipid complexes in retrogradation

The presence of lipids and the formation of the starch-lipid complexes generally retards starch retrogradation (Copeland, et al., 2009). Specifically, the shorter the fatty acid chain, the more efficient is the decrease of the retrogradation rate (Germani, et al., 1983). A starch-lipid complex hinders the cross-linking between helices of neighbouring starch molecules resulting in reduced crystallinity. This is why oat starch with its high level of lipids retrogrades to a lesser extent (Autio & Eliasson, 2009). An investigation on the impact of monoglyceride addition on wheat bread staling showed that although monoglycerides interacted preferably with amylose, when all amylose was complexed, an increase in the interaction with amylopectin occurred, leading to less firm crumb during bread ageing (Krog, et al., 1989). The importance of amylopectin-lipid complex was also illustrated in an experiment using cetyltrimethylammonium bromide (CTAB)-amylose complex to waxy maize starch (Gudmundsson, 1992). CTAB is an emulsifier consisting of a hydrophobic backbone and a cationic end. Hence, its structure imitates the structure of lipids. When the system was heated up to a temperature where the starch was gelatinised but the (CTAB)-amylose complex was still intact, retrogradation did not

decrease significantly. On the other hand, when the starch was gelatinised and the complex was melted, probably allowing a direct interaction between CTAB and amylopectin, the retrogradation rate decreased (Gudmundsson, 1992). Becker et al. (2001) provided another explanation for the anti-firming effect that the addition of an amylose-emulsifier complex can have in starch systems. It was indicated that the addition of amylose-lipid complex to starch granules limited water penetration in the granules and granule swelling, when sample was heated without shear. Hence, due to reduced amylose leaching from the granules, the retrogradation process was restricted. Finally, Conde-Petit et al. (1998) proposed when starch sample containing emulsifier was heated at low shear conditions, only the formation of an amylose-emulsifier complex was observed. However, this complex accelerated the gelation process and led to the creation of a gel characterised by weak cohesion. This fact hindered the re-association of amylopectin branches resulting in the retardation of amylopectin retrogradation.

Storage temperature and starch retrogradation

Storage temperature of a product containing starch is significant in bread staling and it has been proved that the rate of bread staling is negatively correlated to storage temperature (Colwell, et al., 1969). In other words, the lower the temperature, the higher the staling rate. According to the polymer crystal growth theory, which was adapted for starch retrogradation by Slade & Levine (1991), storage temperature affects significantly starch recrystallisation. Polymer crystal growth theory states that the crystallisation of a polymer consists of three phases: nucleation, propagation and maturation (Szczesniak, 1998). The importance of storage temperature lies on the impact that it has on the nucleation and propagation phases in a polymer. It has been found that nucleation takes place at temperature close to the glass transition temperature (T_g) of the polymer, while propagation happens at a temperature slightly lower than the melting temperature (T_m) of the polymer (Slade & Levine, 1987). In the case of starch T_g is approximately -5°C for 50% of water, while T_m of amylopectin crystals is around 60°C (Marsh & Blanshard, 1988; Miles, et al., 1985). According to Marsh & Blanshard (1988), starch retrogradation rate mainly depends on nucleation and to a lesser extent on propagation. As a result, the highest retrogradation rate of starch was found to be at 5°C (Slade & Levine, 1987). More recently, Aguirre et al. (2011) conducted an experiment where different storage temperatures (25 , 4 and -18°C) were applied to breads prepared from the same dough and baked them simultaneously. This study concluded that storage at 4°C resulted in the highest and fastest retrogradation level. Storage at 25°C was used as reference. The lowest retrogradation rate and level was noticed at -18°C , which is a temperature remarkably lower than the T_g of starch. It is worth mentioning that B-type crystals were clearly detected even

after one day of storage when bread was stored at 4°C whereas bread kept at -18°C exhibited an almost constant crystallisation rate after the third day of storage.

1.2 Non-starch polysaccharides in flour

Non-starch polysaccharides are the polysaccharides which cannot be hydrolysed by mammalian α -amylase and together with lignin they are regularly referred to as dietary fibres (Englyst, et al., 1983). Arabinoxylans are the non-starch polysaccharides of wheat, while in malted barley and oat flour, apart from arabinoxylans, β -glucans also exist (Goesaert, et al., 2005; Zhu, 2017; Gupta, et al., 2010). β -glucans are linear D-glucose chains linked via $\beta(1,3)$ - and $\beta(1,4)$ -glycosidic linkages, while arabinoxylans consist of linear $\beta(1,4)$ - D -xylopyranosyl chains (Koehler & Wieser, 2013). Arabinoxylans and β -glucans belong to the hydrocolloid class of “hemicellulose”. Although β -glucans are soluble in water, arabinoxylans are further classified into water-extractable and water-unextractable. During the last decade a lot of studies have focused on the role of hemicelluloses in bread staling. It has been reported that the addition of β -glucans in the bread retards crumb hardening and results in higher specific volume of the loaf of bread after three days of storage (Fadda, et al., 2014; Symons & Brennan, 2004; Skendi, et al., 2010; Biliaderis, et al., 1995). In the case of arabinoxylans, previous results are contradictory. Some (Kulp, 1968; Denli & Ercan, 2001) suggest that water-unextractable arabinoxylans are responsible for an increased initial volume and softness in bread, whereas another (Jeleca & Hlynka, 1972) proposed that water-extractable arabinoxylans also play an active role in this process. Sharma & Gujral (2014) showed that the bigger the amount of dietary fibres in the bread, the lower the staling rate. This was revealed by making chapatti from combining barley and wheat flour or only wheat flour enriched with β -glucan and then storing the breads at 4°C for 24h. It was found that the former combination retarded chapatti staling due to the additional soluble and insoluble dietary fibres in barley flour. The mechanism that non-starch polysaccharides act in bread indicated that they have the capacity of to retain a large amount of water due to their asymmetrical rod or worm-like configuration in aqueous solutions and release it gradually after baking, as well as the possible contribution to amylopectin recrystallisation retardation (Biliaderis, et al., 1995; Skendi, et al., 2010; Jeleca & Hlynka, 1972; Cleemput, et al., 1997). A further suggestion from Skendi et al. (2010) was that the addition of β -glucans to bread increased the total area of gas cells explaining the greater specific volume of the loaves, while some interactions between arabinoxylans and gluten are considered responsible for the increased volume of the bread (Jeleca & Hlynka, 1972; Cleemput, et al., 1997). With regard to starch-arabinoxylans interactions, although older studies proposed that the water-extractable arabinoxylans reduced amylose and amylopectin retrogradation, while water-unextractable arabinoxylans had an impact only on amylopectin retrogradation, more recent experiments showed that

arabinoxylans do not affect starch recrystallisation (Fessas & Schiraldi, 1998). Finally, apart from scientific works supporting the positive impact of the addition of non-starch polysaccharides to bread, there were some studies underlining that the presence of arabinoxylans and β -glucans in bread enhanced the qualitative characteristics of it but did not have an impact on the staling rate (Jacobs, et al., 2008). Hager et al. (2011) also detected an increase in hardness of bread over storage when β -glucans were added to the dough, proposing that the increased water-binding capacity of the non-starch polysaccharide probably affected in a negative way the formation of the gluten network.

1.3 Proteins in flour - Gluten

Cereal flours contain hundreds of different proteins which are classified into four fractions, called Osborne fractions. Albumins that are soluble in water, globulins that are soluble in salt solution, prolamins that are soluble in aqueous alcohol and glutelins that are only soluble in alcohol when reducing agents are present (Schalk, et al., 2017). Albumins and globulins comprise approximately 20-25% of the total cereal flour proteins, while the prolamins and glutelins comprise the rest (Schalk, et al., 2017). In wheat, prolamins and glutelins are called gliadins and glutenins, respectively, while in barley they are named hordeins and in oats avenins (Koehler & Wieser, 2013). Table 1.4 illustrates the exact amount of prolamins and glutelins in wheat, barley and oats flour.

In wheat flour, prolamins and glutelins play a crucial role as they participate in the formation of gluten. "As gluten can be defined the rubbery mass that remains when wheat dough is washed to remove starch granules and water-soluble constituents" (Wieser, 2007). The low amount of prolamins and glutelins in barley and oat flour result in the lack of gluten network formation (Gujral, et al., 2003; Salehifar & Shahedi, 2007) and only wheat flour forms this crucial protein structure. Although baking products cannot be exclusively made from barley or oat flours, small amounts of them are often mixed with wheat flour to improve the nutritional quality. The high amount of β -glucans in oat and barley flour is beneficial for crumb softness and loaf specific volume (section 1.2) and for chronic disease conditions. Oat flour is also rich in lysine which is limited in other cereals enhancing the nutritive value of the final product (Salehifar & Shahedi, 2007; Gill, et al., 2002).

Table 1.4. Grams of prolamins and glutelins in 100g of wheat, barley and oats flour. Adapted from (Schalk, et al., 2017).

g/100g of flour	Wheat	Barley	Oat
Prolamins	5.94 $\pm$ 0.07	3.13 $\pm$ 0.06	1.29 $\pm$ 0.03
Glutelins	2.98 $\pm$ 0.04	1.10 $\pm$ 0.02	1.01 $\pm$ 0.05

In terms of dry solid mass, wheat gluten consists of 75-85% of proteins and 5-10% of lipids, while starch and non-starch polysaccharides are also present (Wieser, 2007). Gluten network formation starts when proteins become hydrated. Mixing breaks glutenin molecules to smaller units (Graveland, et al., 1985). The point of scission is thought to be at the disulphide bonds, which in turn are reformed between neighbouring molecules that are oriented along the direction of the stretching action (Redman, et al., 1966; Wieser, 2007). While mixing continues, proteins interact two-dimensionally than just along the individual strand axes leading to the creation of a continuous film. The elasticity and cohesiveness of it is determined by the amount of glutenin in the flour (Toufeili, et al., 1999). Hydrated gliadins contribute to the viscosity of the dough (Toufeili, et al., 1999). The protein also incorporates lipids from the flour and added emulsifiers. Polar and most of the non-polar lipids become tightly associated with gluten proteins (Chung, et al., 1978). The final cohesivity, viscosity, elasticity and up to a certain extend the water absorption capacity of a dough are determined by the gluten. Hence, gluten is vital in bread making process and the quality of the final product.

After baking, the gluten network and the amorphous amylose have formed the continuous phase of bread whose formation and hydration determines to a great extent the texture and the perceived characteristics of bread. During bread storage, it is often suggested that gluten retards bread staling (Kim & D'Appolonia, 1977). It has been indicated that an increase in gluten content of bread results in a lower staling rate due to the dilution of the starch in the product (Kim & D'Appolonia, 1977). However, it is believed that gluten and starch develop hydrogen bonds (Erlander & Erlander, 1969). The significance of these interactions is contradictory. Martin et al. (1991) suggested that gluten-gelatinised starch interactions increased bread firming due to restricted crumb flexibility. On the contrary Every et al. (1998), who created bread with different gluten content, showed that bread firming rate was not affected by gluten content demonstrating that gluten-gelatinised starch interactions do not play a significant role in bread staling. This is in agreement with Ottenhof & Farhat (2004) who did not observe any change in amylopectin recrystallisation rate over storage nor in crumb firming. It was proposed that, as the amount of starch in bread is remarkably higher than the protein content, starch-starch interactions are considerably more than gluten-starch interactions (Every, et al., 1998). Finally, Eliasson (1983a) revealed that the higher the level of gluten in bread, the lower the amylopectin retrogradation over storage and hence, the softer the bread. On the other hand, Curti et al. (2014) detected that higher amounts of gluten resulted in softer bread, over seven days of storage, while also larger amount of recrystallised amylopectin was noticed. These observations were accompanied by a higher mobility of water molecules and stronger interactions between water and gluten. Hence, it was concluded that the presence of gluten increased water mobility, a fact that favoured starch retrogradation but also retained

bread softness. The starch-water and gluten-water interactions will be further analysed in section 1.5.

1.4 Native lipids in flour

The role of native lipids in bread staling was discussed in section 1.1.3. However, it is worth mentioning that it has been shown that lipids have a negative impact on bread volume. This phenomenon has been connected to protein-lipid interaction (Rogers, et al., 1988).

1.5 Water in bread

Water is essential in bread making and bread staling. It plays a key role in the formation of a homogenous dough, which in turn is significant for all chemical reactions and interactions among the ingredients during bread processing. Furthermore, flour hydration allows the creation of a gluten network that provides viscoelastic properties to the dough.

Water migration and/or water redistribution are certainly crucial in bread ageing. Specifically, there are three processes that have to deal with moisture: drying out, moisture migration from crust to crumb and moisture redistribution among the bread components. Because in the present work sandwiches were packaged and the sandwich bread did not have crispy crust, it is considered that only the latter process can significantly affect bread ageing and is further described. According to Zeleznak & Hoseney (1986) the optimum level of total moisture in bread so that to encourage starch recrysallisation is 35-40% and as stated by Schiraldi & Fessas (2001) due to the remarkable high values of water activity (a_w) in the bread, water is rather mobile and can act as a plasticizer. Due to this property bread components are more flexible hence, when water is removed, crumb becomes more firm. However, in order to study the contribution of water in bread staling, water activity and moisture content measurements are not enough. Thus, the exact mechanism of water transfer among bread components has been investigated, in numerous studies, by utilising Nuclear Magnetic Resonance (NMR), X-ray diffraction and thermal methods. The majority of them suggest that water migrates from gluten to starch. Specifically, since 1927 there has been the belief that gluten is the component that hardens over storage (Alsberg & Griffing, 1927) and this proposal was firstly confirmed in 1947 when an increase in the moisture-sorption capacity of starch was detected (Bachrach & Briggs, 1947). Since then it has been revealed that starch changes to a more crystalline structure over storage, adapting B-type crystalline conformation that incorporates the highest number of water molecules among all the types of crystals in starch (entraps 36 molecules of water) (Leung, 1981; Leung, et al., 1983). Thus, water is becoming less available over time, as molecules that are enclosed in the crystalline lattice do not have the ability to act as plasticizers. This results in drier texture of bread (Chen, et al., 1997a; Wynne-Jones & Blanshard, 1986; Slade & Levine, 1991).

1.6 Literature review on methods to study physicochemical changes on bread over storage

As bread staling has been studied extensively there are different techniques used to monitor physicochemical changes over storage. As it has already been stated, amylopectin retrogradation and water redistribution play a crucial role in bread ageing. One of the methods used to monitor changes in water incorporated in a bread over storage is Thermogravimetric analysis (TGA). TGA experiments can determine the total amount of water in bread sample but can also monitor transitions in water mobility when heated over 100°C (Schiraldi, et al., 1996; Fessas & Schiraldi, 1998). It has been stated that water molecules which interact strongly with constituents of a product are released at higher temperature than more mobile water molecules. Thus, different fractions of water can be detected and changes in their percentages can be monitored over storage (Schiraldi, et al., 1996).

Differential Scanning Calorimetry (DSC) has been utilised in several scientific works on bread staling (Czuchajowska & Pomeranz, 1989; Baik & Chinachoti, 2000; Hallberg & Chinachoti, 2002; Ribotta & Le Bail, 2007) due to its ability to monitor every phase transition occurring in a sample. When a sample is cooled below 0°C and then heated above ice melting temperature, an endothermic peak appears in the thermogram that is related to the ice melting enthalpy. A DSC thermogram can provide the amount of Freezable Water (FW) which is the water that is integrated in the matrix but with low binding energy and when cooled forms detectable crystals (FW) (Clubbs, et al., 2008). This type of water is available for chemical reactions and microbial growth and as a result is the type of water that can be perceived when a product is consumed. Hence, detecting changes on its population is important for monitoring changes in bread texture. In complex systems, a proportion of water tends to develop very strong interactions with constituents present in the system and is not able to crystallise during cooling (Simatos & Faure, 1975). This type of water is called Unfreezable Water (UFW). By determining the amount of FW, the amount of UFW can be calculated if the total amount of water in the sample is known. Another important peak in a DSC thermogram in the case of bread is the endothermic peak between 50-60°C. This peak corresponds to melting of amylopectin crystals (Miles, et al., 1985; Goodfellow & Wilson, 1990; Rogers, et al., 1988). An increase in the enthalpy of the peak indicates an increase in the amount of amylopectin crystals in the sample. Although this peak has been used widely in the literature to monitor bread staling over storage (Hallberg & Chinachoti, 2002; Ribotta & Le Bail, 2007; Baik & Chinachoti, 2000), it is worth mentioning that Appelqvist, et al. (1993) supported that is not necessarily associated to amylopectin. In detail, it was suggested it is a general probe of water-carbohydrate interactions appearing between 50-80°C and that experimental work

detected the endotherm in granular starches, amylopetin, amylose, dextran, pullulan, xanthan gum, pectin, alginate, agar, guar gum.

A third technique for detecting changes in bread over storage is Fourier Transform Infrared spectroscopy (FTIR) which is suitable to identify changes between the amount of amorphous and crystalline starch. Specifically, the bands at 1000 cm^{-1} , 1022 cm^{-1} and 1047 cm^{-1} have been used widely to monitor starch retrogradation (Htoon, et al., 2009; van Soest, et al., 1994; Flores-Morales, et al., 2012; Van Soest & Vliegenthart, 1997; Wilson, et al., 1987; Warren, et al., 2016; Capron, et al., 2007; Waigh, et al., 1998). The region of the spectra between 1200 and 1000 cm^{-1} is assigned to C-O, C-C and C-H stretching modes and C-O-H bending (Capron, et al., 2007; Warren, et al., 2016). According to Warren, et al. (2016), who investigated starch ordered structure by using FTIR, DSC, NMR and XRD methods, the peak at 1000 cm^{-1} is more sensitive to changes in starch structure than the 1047 cm^{-1} peak. Furthermore, a strong correlation between the $1022:1000\text{ cm}^{-1}$ ratio and the DSC results for both dry and hydrated starches was observed and the bands at 1000 cm^{-1} and 1022 cm^{-1} were linked to short range interactions. Warren, et al. (2013) noticed a distinct but not linear shift of the peak at 1000 cm^{-1} to 1022 cm^{-1} during gelatinisation of native starch. Hence, they suggested that the peak at 1000 cm^{-1} is related to the ordered structure of starch, while the 1022 cm^{-1} corresponds to the amorphous starch structure. Capron, et al. (2007) analysed the FTIR spectra of amorphous, crystalline and semi-crystalline starches and observed a broad peak at 1000 cm^{-1} on dry starch spectra which narrowed when starches were hydrated. On the other hand, in the former condition, the spectra of the same samples did not show any peak at 1047 cm^{-1} , while in the latter only a shoulder appeared. As a result, the band at 1000 cm^{-1} was related to the development of hydrogen bonds among starch helices when starch is exposed to water. This indication agreed to van Soest, et al. (1995) and Kacurakova & Mathlouthi (1996) who suggested that the peak 1000 cm^{-1} is sensitive to plasticising effect of water and monitors hydrogen bonding of hydroxyl groups. On the other hand, the absorbance at 1047 cm^{-1} is composed of two overlapping bands at 1040 and 1053 cm^{-1} . The former, corresponds to the formation of starch double helices over the fast stage of retrogradation and the absorbance of the latter increases over longer storage due to aggregation and crystallisation (Goodfellow & Wilson, 1990). Nevertheless, the band at 1047 cm^{-1} was found essentially independent of changes in the degree of structure in the starch, while the bands at 1000 cm^{-1} and 1022 cm^{-1} managed to monitor changes in starch structure more accurately according to Capron, et al. (2007), Warren, et al. (2013) and to Warren, et al. (2016).

1.7 Barriers on sandwich

One of the main problems of multiple component products such as sandwiches, is the moisture migration taking place from a “wet” compound to the relatively dry bread. Hence, to maintain

the moisture levels in sandwiches, physical barriers are introduced to sandwiches. These barriers are either butter or water in oil emulsions and are applied in the surface of the bread in contact with the filling. Some of the main characteristics that a barrier should have are low moisture permeability preventing moisture redistribution within the product and the ability to adhere well in the food surface. The hydrophobic properties of these ingredients are responsible for their water impervious features. A more recent approach regarding barriers indicated that barriers must also be imperceptible. In detail, a thin layer of lipophilic compounds should be added on the product surface. This compound should also be sufficiently strong but also soft and flexible to form a homogeneous thin surface above the product that will not crack upon handling procedures (Kraft Foods Holdings, Inc., 2004; Cain, et al., 2002). These thin, not apparent by consumers, products are used in numerous food snacks such as ice cream sandwiches, cookies and cakes (Kraft Foods Holdings, Inc., 2004).

An interesting patent published by Kraft Foods Holdings (2004) describes a system of bagel with creamy cheese that contains a controlled moisture supply layer and an edible moisture barrier to protect the product against staling and/or sogginess. In detail, the controlled moisture supply layer was a water-in-oil emulsion (W/O) composed of approximately 60% of water and 40% fat or fat and oil. The fat was either animal fat or vegetable fat. The emulsion contained in the aqueous phase a low-hydrophile-lipophile balance emulsifier (less than 8) and an edible hydrophilic colloid as stabiliser. The moisture supply layer was spread on the internal side of the bagel so that to constantly supply the bagel surface with water but without encouraging sogginess. The ideal thickness for the layer was stated to be between 500 and 1000 microns. The edible moisture barrier was added between the moisture supply layer and the cream cheese so that to prevent any moisture migration between the moisture supply layer to the cream cheese. This barrier was comprised of lipid or oil, with a low melting point, combined with a high melting lipid and/or fat crystal growth retarding agent such as diglycerol or triglycerol monoleate emulsifier. There was no addition of any hydrophilic substance in the lipid-based barrier since the presence of these substances reduces the ability of the barrier to prevent moisture migration.

1.8 Research objectives

This thesis constitutes an investigation into the physicochemical processes that take place in sandwich bread, isolated from two ready-to-eat sandwiches, over storage. The main aim was to understand consumers' feedback for this sandwich that the bread becomes drier over storage. The results were then to be analysed in view to advising on the necessary characteristics of a novel barrier that would extend the shelf-life of sandwiches. This was to be accomplished by utilising different techniques to monitor physicochemical changes on sandwich bread during ageing.

Consumers' feedback regarding the ready-to-eat ham, mustard and mayonnaise sandwich and the ready-to-eat ham, farmhouse cheese and pickle sandwich mostly referred to textural deterioration of bread. The two sandwiches are made from different sandwich bread. The first is made from oatmeal sandwich bread, while the second from malted wholegrain sandwich bread. However, both of the breads contain wheat, barley and oat flour. Each internal side of the sandwich bread is spread with a barrier that prevents contact between the sandwich bread and the filling. In the case of the ready-to-eat ham, mustard and mayonnaise sandwich the barrier used is signature mustard and mayonnaise sauce, whereas in the ready-to-eat ham, farmhouse cheese and pickle sandwich, butter and then ploughman's pickle is spread onto the one slice and signature 30% mayonnaise is added in the other. The filling of both sandwiches consists of a slice of cooked meat, while the ready-to-eat ham, farmhouse cheese and pickle sandwich also contains cheese.

As stated in the beginning of the chapter, the main compounds affecting the texture of bread are starch, gluten and water. Most of the experiments reported in the literature regarding the impact of gluten on the bread are focusing on baking breads with different gluten content and then monitoring changes on starch retrogradation, moisture distribution and moisture mobility on the product. Hence, the investigation of physicochemical changes in gluten network on a ready-to-eat sandwich is challenging. On the other hand, there are numerous scientific works on starch recrystallisation, particularly amylopectin recrystallisation, in bread and the role of water in this process. Amylopectin retrogradation has been investigated in the literature because it lasts from days to weeks contributing significantly to bread staling. As a result, the aim of this project were:

- to investigate amylopectin retrogradation over storage
- to detect physicochemical changes in water over storage.
- to obtain reliable and reproducible results that would provide insights into the mechanism of sandwich bread staling in these sandwiches.
- if possible to advice on characteristics of new barrier that will retard bread staling.

2. Materials and Methods

2.1 Materials

Ready-to-eat ham, mustard mayonnaise and ham, farmhouse cheese and pickle sandwiches were provided by Marks and Spencer plc (UK). The composition of the sandwiches is presented in Table 2.1. All samples were delivered on day “Production +1” (P+1), which corresponded to the first day that they would have been available in the market place.

Table 2.1. Sandwich manufacturing.

Ham, mustard and mayonnaise	Ham, farmhouse cheese and pickle
Supersoft Oatmeal Bread (11mm)	Malted wholegrain bread (13mm)
10±2g Mustard and mayonnaise mixture	2±1g of butter
60±6g Smoked salt square gammon	20±3g of signature ploughman’s pickle
10±2g Mustard and mayonnaise mixture	42±3g of 9 Month Farmhouse Mature Cheddar Cheese
Supersoft Oatmeal Bread (11mm)	48±6g of Mid Tier Smoked Ham
	8±1g of signature 30% mayonnaise
	Malted wholegrain bread (13mm)

2.2 Methods

2.2.1 Sample preparation

Both sandwiches were stored within their packaging at 4°C after their production. The humidity inside the walk-in fridge was approximately 85%. Figure 2.1 illustrates the sandwiches that were used in this project as well as the different slices of bread that were analysed separately according to their place in the packaging. Samples were analysed from the day after their production (P+1) until a day after their expiry day (P+4 in the case of the ham, mustard & mayonnaise sandwich and P+3 in the case of the ham, farmhouse cheese and pickle sandwich). A different sandwich (from each type) was used every day for the analyses. Each slice of sandwich bread was isolated from the filling and processed separately from the others. The inner side of every bread slice, which was in contact with the barrier (signature mustard & mayonnaise or butter or signature 30% mayonnaise), was separated from the outer side by using a food slicer set to 4mm (Lakeland Easy-Store Food Slicer, Lakeland, UK). Only the crumb of the outer side of the bread was kept for analysis. Specifically, the crust was removed and discarded and the remaining crumb was grinded in a coffee grinder (De’Longhi KG49 Coffee Grinder, De’Longhi, S.p.A, Italy) with three grinding options: coarse, medium and fine. Grinding stopped when fine setting reached. The so produced breadcrumbs were sealed in a

plastic bag and stored again at 4°C, for up to 1 hour, until the analysis. The aforementioned process was conducted as fast as possible to minimise changes in sample temperature. Before every analysis, small pieces of cereal grains contained within the sandwich bread were removed so that to not interfere in the measurements. The sample preparation and the methods used during this project are presented in a flow chart see Figure 2.2.

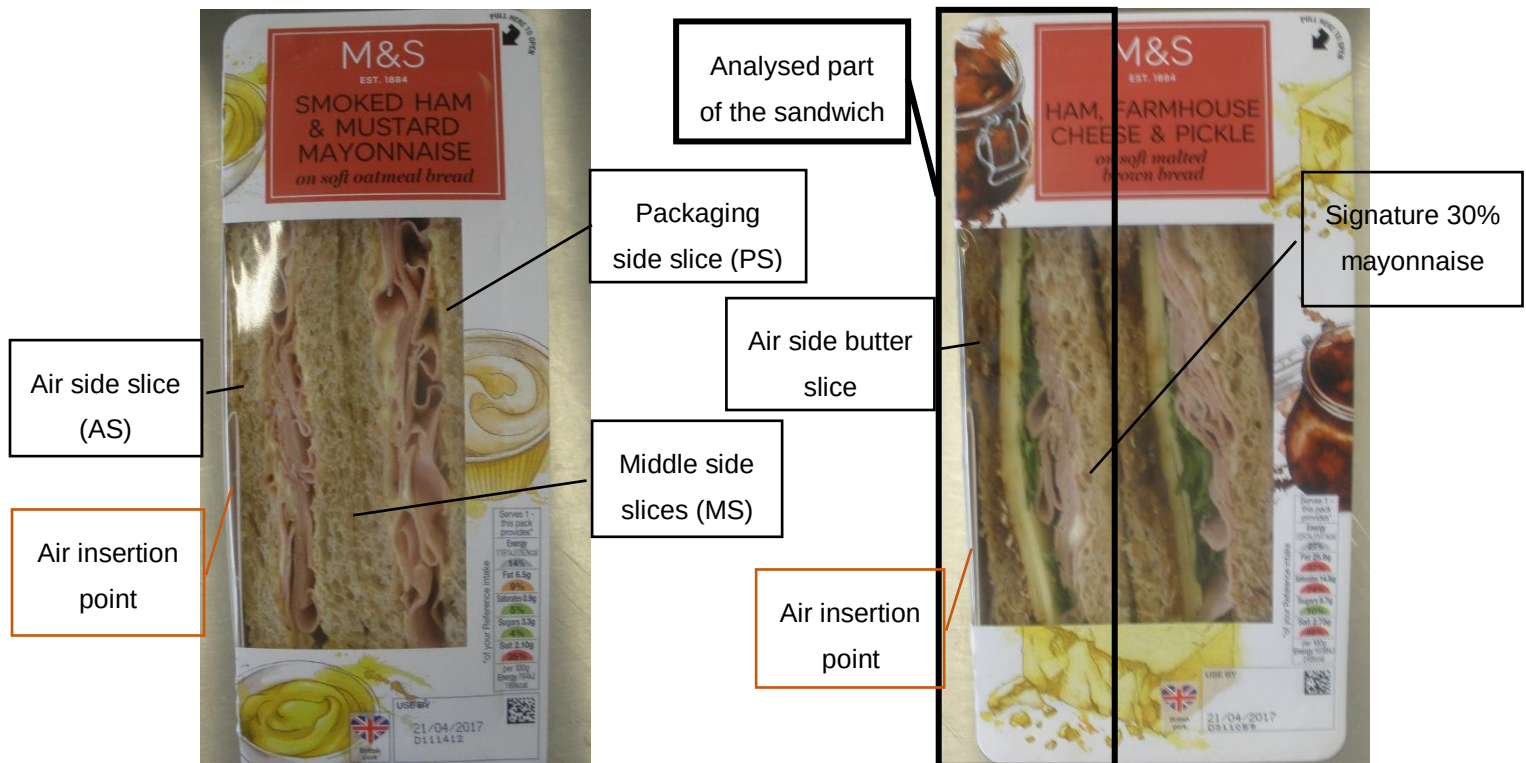


Figure 2.1. Picture of the sandwiches used in this project and names of the different samples utilised for the analyses. The ready-to-eat ham, mustard and mayonnaise sandwich is depicted on the left, while the ready-to-eat ham, farmhouse cheese and pickle sandwich is illustrated on the right. The part of the latter sandwich that was analysed during this project is specified on the sandwich.

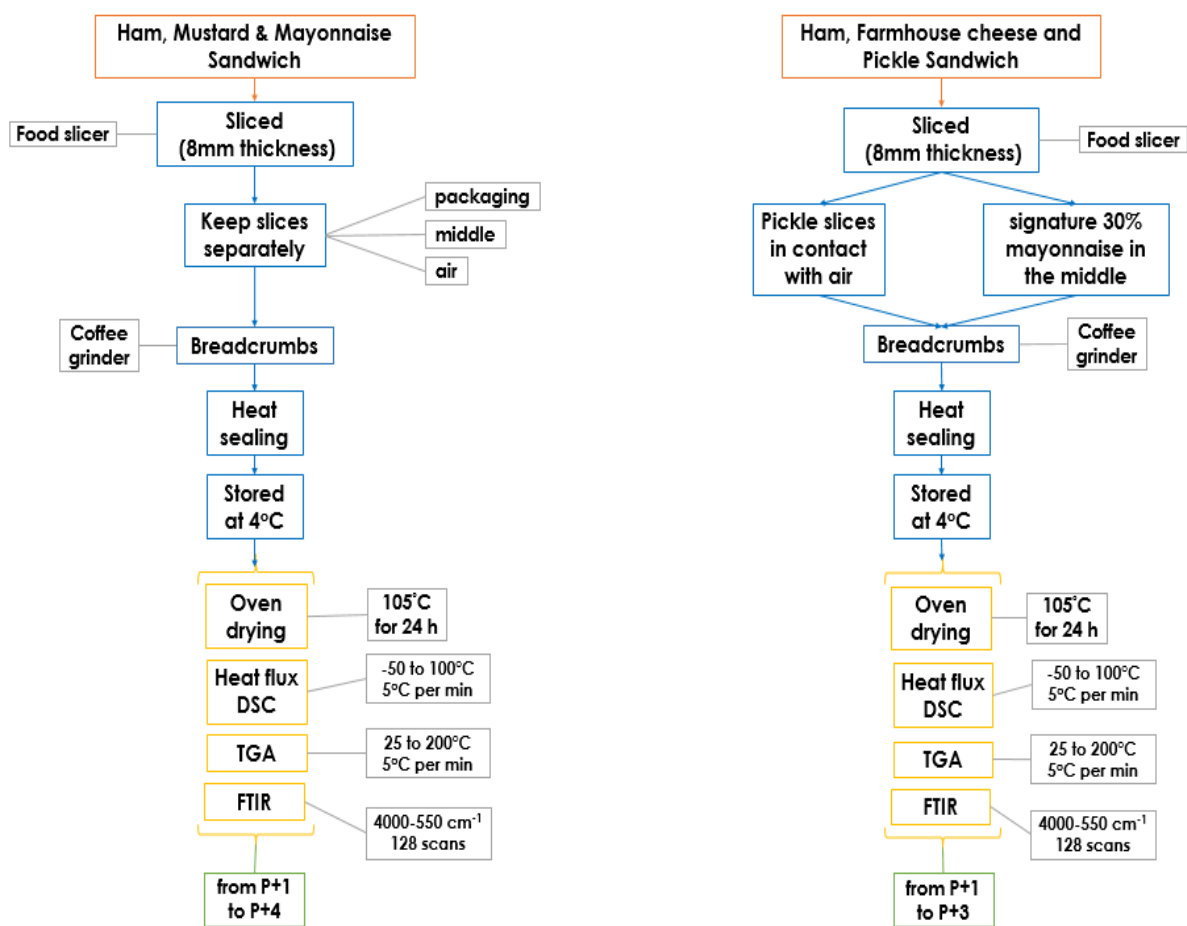


Figure 2.2. Flow chart depicting sample preparation and the methods used during this project.

2.2.2 Oven Drying Method

Oven drying method is a widely used technique for the determination of the total amount of moisture that is contained within a sample. It is considered as a simple and reliable technique thus, it has been used in most of the scientific works.

Experimental procedure

3.000±0.07g of sample (W_1) were added into a pre-weighted aluminium pan and then placed into an oven and held at 105°C for 24 hours. Samples were then removed and weighed again (W_2) and the % moisture was calculated with the following equation (Eq. 1):

$$\text{Moisture Content (g/100g)} = \frac{W_1 - W_2}{W_1} \times 100 \quad (1)$$

Every experiment was repeated six times and the results are presented as a mean average.

2.2.3 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) is a technique measuring changes in the mass of a sample as a function of sample temperature or time. Mass loss occurs for many different reasons such as degradation and/or decomposition, evaporation of bulk liquids or liquids absorbed by solids, oxidation of metals in air or oxygen, while mass gain can be detected in adsorption gases and/or in reactions of solids with reactive gasses (Vyazovkin, 2012). Since TGA provides limited qualitative information, as it requires the knowledge of the components of a substance or the mechanism of sample decomposition, it is often combined with other spectroscopic techniques or with DSC (Vyazovkin, 2012).

TGA apparatus

In TGA experiments samples are either solids or low volatility liquids and their size is usually within the range of 1-100mg. A sample is loaded into a crucible which is placed on a thermobalance. Crucibles for TGA are made of thermally and chemically resistant materials of good thermal conductivity such as aluminium, platinum, quartz (Vyazovkin, 2012). A thermobalance is a combination of a sensitive analytical balance with a temperature-controlled furnace that can be heated from ambient temperature to 1000-1600°C (Vyazovkin, 2012). In a TGA experiment, the temperature can be changed linearly with time so that the heating rate is constant (dynamic measurement) but a sample may also be subjected to a non-linear temperature program. Finally, it can also be held at a constant temperature (isothermal measurement) or temperature can be changed as a combination of the three options (Bottom, 2008). In the present work, a dynamic measurement was performed.

The heating rate varies but most of the times is between 1-20°C/min. A decrease in heating rate usually results in higher resolution curves but increases the time of the experiment

significantly (Vyazovkin, 2012). The TGA instruments, furnace can be in either horizontal or vertical arrangement with respect to the balance (Vyazovkin, 2012). In this study, the furnace was in horizontal arrangement with the balance (Figure 2.3). TGA experiments are conducted in controlled gaseous atmosphere so that to assure constant pressure that will not affect the reaction kinetics (Vyazovkin, 2012). The gas can be either inert such as nitrogen, which was utilised in the present work, argon and helium or reactive such as oxygen for oxidation of metals and hydrogen or reduction of metal oxides (Vyazovkin, 2012).

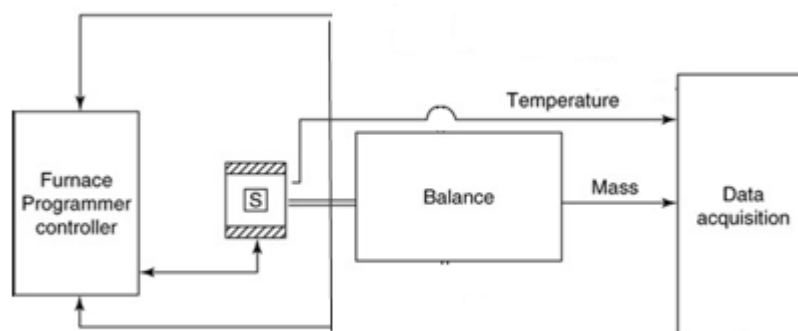


Figure 2.3. Schematic representation of TGA apparatus, where the balance is in horizontal arrangement with the sample.

TG and DTG curves

Changes in the mass of the sample are depicted as thermogravimetric (TG) and differential thermogravimetric (DTG) curves. As an example, Figure 2.4 illustrates TG and DTG curves obtained from sandwich bread analysis. A TG curve is a plot of the mass against temperature or time while a DTG curve is a graph of the first derivative of TG curve as a function to temperature or time. The latter illustrates the rate at which the mass changes and is used complementary in data interpretation (Bottom, 2008). Sample response to heating ends when the rate of weight loss becomes 0 mg/°C. The DTG_{max} value represents the maximum rate of weight loss.

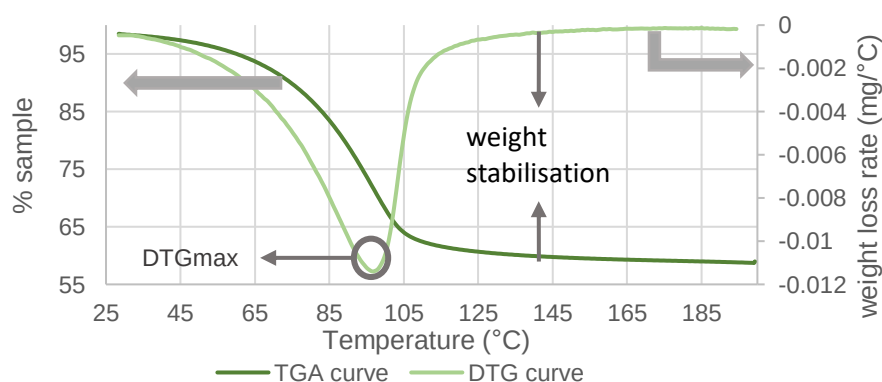


Figure 2.4. TG and DTG curves obtained from the analysis of sandwich bread.

Experimental procedure

10±1 mg of breadcrumbs was placed in a pre-weighted aluminium pan of 100 µl (Mettler Toledo, UK) and an aluminium lid (Mettler Toledo, UK) was added. The crucible was sealed and loaded to a TGA/DSC 3+ (Mettler Toledo, UK). Samples were heated from 25°C to 200°C at 5°C/min. An empty aluminium crucible was used as a reference. Plots of the sample weight loss (TG) and its first derivative (DTG) as a function of temperature were utilised for analysis. The total weight loss and the number of the peaks were determined by utilising STAR^e Evaluation Software (Mettler Toledo, UK). Every experiment was performed six times and the results are presented as a mean average.

2.2.4 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a widely used thermo-analytical technique that measures the energy changes that occur as a sample is heated, cooled or held isothermally (Gabbott, 2008). The energy changes represent phase transitions, order-disorder transitions and/or chemical reactions in the sample (Wang, et al., 2015). A DSC apparatus enables the user to measure these changes quantitatively. One of its applications is for studying the crystallisation, melting, glass-rubber transition and physical ageing phenomena in samples containing carbohydrates.

DSC apparatus

There are two different modes of DSC, heat-flux DSC and power-compensation DSC. In Heat-flux DSC the pan containing the sample and the reference pan are enclosed in a single furnace and are connected by a low resistance heat flow path (a metal disk) (Figure 2.5). Both pans are hermitically sealed so that there is no loss of mass and they are heated or cooled simultaneously so that the heat flow of the sample can be compared to that of the reference pan (Vodovotz, et al., 2001). As heat is the type of energy provided to the system, the resulting thermogram illustrates the change of enthalpy as a function of time or temperature. There is a large variety of construction materials for the pans used in DSC. Some of them are aluminium, stainless steel, graphite, cooper, silver, gold and platinum (Schick, et al., 2012). In power-compensation DSC, the temperature of the sample and the reference are controlled independently using separate, identical furnaces. The temperature measuring system controls the power of the two heaters so that the sample temperature and the reference temperature in the two furnaces to be equal (Schick, et al., 2012). In this project, heat-flux DSC was utilised. Sample pan and reference pan were made from aluminium.

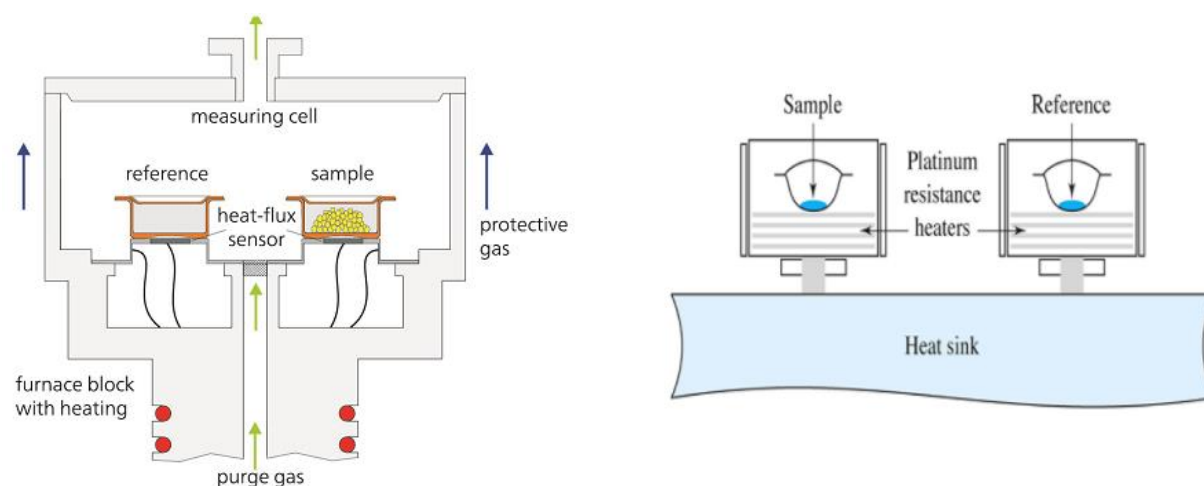


Figure 2.5. Schematic representation of the heat-flux DSC on the left (adapted from (Anon., 2017)) and power-compensation DSC on the right (adapted from (Kodre, et al., 2014)).

DSC thermogram

A thermogram is the visual presentation of the energy changes that take place when a sample is heated, cooled or held isothermally in a sealed environment. Most of the times the graph illustrates changes in the enthalpy as a function of sample temperature. For instance, Figure 2.6 depicts the major peaks appearing for oatmeal sandwich bread. These are the ice melting peak at approximately 0°C and an endothermic peak at approximately 50°C corresponding to amylopectin melting crystals. Integration of these peaks leads to the determination of the enthalpy needed for these transitions.

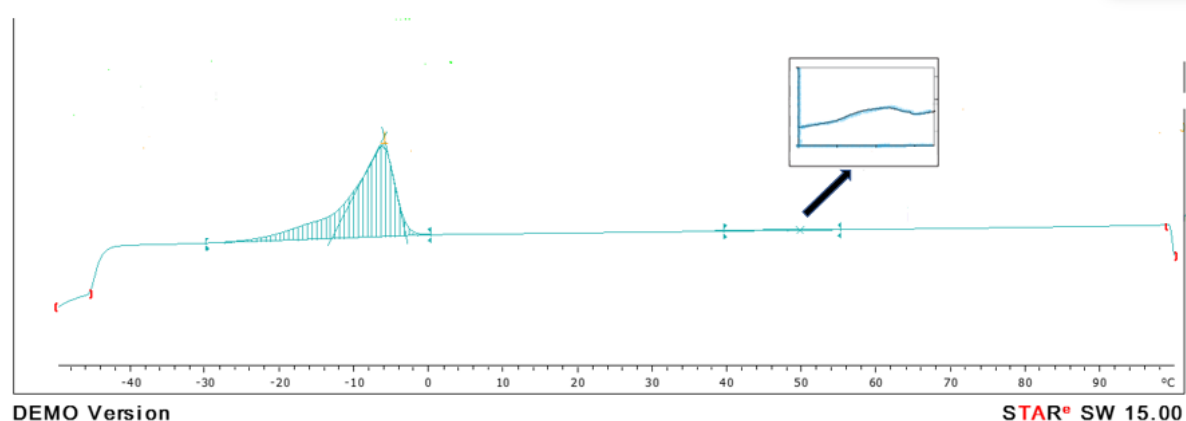


Figure 2.6. DSC thermogram of oatmeal bread illustrating changes of the enthalpy as a function of sample temperature.

Experimental procedure

12±3 mg of breadcrumbs was placed in aluminium pans and pressed. Afterwards, the pans were sealed hermetically and analysed with DSC823^e (a heat-flux DSC apparatus) containing a TS0801RO sample robot (Mettler Toledo, UK). The DSC was calibrated by using Indium

and cyclohexane. An empty pan was used as a reference and a pan containing pure water was used to determine the latent heat of fusion of water. Samples were fast cooled from 25°C to -50°C and held at that temperature for 5min and then heated at 5°C/min from -50°C to 100°C as it was proposed by Ribotta and Le Bail (2007). The onset (To), peak (Tp) and Endset (Te) temperatures, as well as the transition enthalpy (J/g) of ice melting and the enthalpy of the peak that corresponds to amylopectin retrogradation were calculated by utilising the STARE Evaluation Software (Mettler Toledo, UK). The enthalpies were normalised per unit weight of sample. FW expressed as percentage weight of total water basis in bread, was calculated with Equation 2. The difference to 100% was characterised as the %UFW on total water basis (Eq. 3). FW and UFW were also expressed in grams per gram of total amount of sample see Equation 4. Data from oven drying method were used in Equations 2 and 3. All the experiments were performed six times and the results presented as a mean average.

$$\%FW \text{ in total water basis} = \frac{\Delta H_{\text{of endothermic peak corresponding to water}}}{\Delta H_{\text{of specific latent heat of fusion of water}} \times mc} \times 100 \quad (2)$$

$$\%UFW \text{ in the sample} = 100\% - \%FW \quad (3)$$

where ΔH of specific latent heat of fusion of water equals 309.70 J/g, mc is the moisture content of the sample ($g_{\text{water}}/g_{\text{bread}}$) determined by oven drying.

$$FW \text{ or } UFW (g/g) = \frac{mc \times \%FW \text{ or } \%UFW}{100} \quad (4)$$

2.2.5 Fourier Transform Infrared spectroscopy

Infrared (IR) spectroscopy is a technique based on the vibrations of particular functional groups within a substance. Specifically, it measures transitions between molecular vibrational energy levels as a result of the absorption of IR radiation. The raw data occurring from the exposure of the sample to the IR radiation need to be converted by a Fourier transform, a mathematical process, to form the actual spectrum. Hence, this type of infrared spectroscopy is called Fourier Transformed Infrared spectroscopy (FTIR). A major advantage of IR spectrometry in general is that it can be used to analyse a variety of different samples including liquids, solutions, powders, fibres and gases (Gaffney, et al., 2012).

Principle of IR spectroscopy

Infrared radiation is electromagnetic radiation in the wavelength region of approximately 0.78-1000 μm or 14,000-10 cm^{-1} in terms of wavenumber. Due to its length, the IR spectrum is divided into three regions: near ($>4000 \text{ cm}^{-1}$), mid ($650\text{-}4000 \text{ cm}^{-1}$) and far ($<650 \text{ cm}^{-1}$) (Gaffney, et al., 2012). This study was conducted in the mid-infrared region.

Each molecule that is composed of N atoms has $3N$ independent degrees of freedom of motion. However, by subtracting the three modes of translation and the three modes of rotation that exist in the three-dimensional Cartesian Coordinate system, the final vibrational independent degrees of freedom are $3N-6$ for non-linear molecules and $3N-5$ for linear molecules. Although, the vibrational motions of the atoms are restricted due to the existence of the bonds and the bond angles which keep the atoms joined within the molecules, molecules vibrate constantly. There are two predominant types of normal vibrational modes, stretching and bending modes. The former includes a change in the interatomic distance along the axis of the bond and consequently is sub classified by the symmetry of the vibration as either symmetrical or asymmetrical. The latter involves a change in the bond angles between the atoms. Stretching vibrations always occur at higher frequencies than bending vibrations as there is higher demand of energy to stretch a bond than to bend it (Larkin, 2011). The vibrational frequency (ν) of a bond can be calculated by using Hooke's law (Eq. 6):

$$\nu = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \quad (6)$$

where k is the force constant of the bond; μ is the reduced mass of the system ($\mu = \frac{m_1 m_2}{m_1 + m_2}$, m_1, m_2 are the masses of the two atoms participating in the bond).

The basic principle behind infrared spectroscopy is that when a molecule is exposed to infrared radiation, it will absorb the energy of the radiation that is equal to the energy of a vibrational transition of the molecule (Eq. 7).

$$\Delta E = h\nu \quad (7)$$

where ΔE is the energy difference between two successive vibrational levels, $h=6.6 \times 10^{-34}$ Js (Planck's constant) and ν is the vibrational frequency (Hz or s^{-1}).

A single vibrational energy transition is accompanied by a number of rotational energy states which together give the IR absorption bands their overall band shape. As it was illustrated in Equation 6, the wavelength of the absorption depends on the relative masses of the atoms, the geometry and force constants of the bonds (Larkin, 2011). The absorption intensity of a certain frequency depends on the effectiveness of energy transfer from infrared radiation to the molecule, which in turn depends on the change in dipole moment of the bond during vibration (Gaffney, et al., 2012). Infrared photons generate an oscillating electric field which generates oscillation in the molecule due to oppositely directed forces on the positive and negative charges in the molecular dipole. As a result, molecules without dipole moment are IR inactive (Larkin, 2011).

FTIR Apparatus-Sampling techniques

There are different sampling techniques depending on the physical state and form of the sample. Briefly, liquids or very thin films can be analysed in transmission mode where a sample is placed on a cell with transparent to mid-infrared radiation windows (Larkin, 2011). Powders can be analysed by Diffuse Reflectance Infrared Fourier Transform (DRIFT) where there are mirrors placed in an arrangement that allows the infrared radiation only to be diffused, providing structural information for the sample (Larkin, 2011). Furthermore, infrared microscopy which combines a microscope with an FTIR spectrometer, gives the opportunity to analyse microscopically both reflection and transmission modes. Finally, one of the most popular sampling techniques is Attenuated Total Reflectance (ATR) that can analyse both solid and liquid samples that are thick or strongly absorbing and will be explained in the next paragraph as it was the one utilised in this study. ATR involves a crystal with high refractive index and remarkable IR transmitting properties. The refractive index of the crystal must be higher than the sample being measured. Thus, there are several different materials that can be utilised, such as diamond, germanium, zinc selenide and silicon (PerkinElmer, 2004). This technique is based on the spectral information that can be collected from reflection phenomena (Larkin, 2011). The infrared beam passes through the diamond prism and total internal reflection can be achieved only if the angle of incidence is greater than the critical angle, see Figure 2.7. The critical angle is a function of the real parts of the refractive indices of both the sample and the crystal (Eq. 8) (Larkin, 2011). Diamond, as used in the current study has a higher refractive index than organics resulting in internal reflection at moderate angles of incidence.

$$\theta_c = \sin^{-1} \frac{n_2}{n_1} \quad (8)$$

where n_1 is the refractive index of the crystal and n_2 is the refractive index of the sample.

At frequency within an absorption band the reflection will be attenuated, otherwise all light is reflected. A good contact between the sample and the crystal is required in ATR and thus an anvil containing a rubber O-ring is utilised. Anvil not only assures the excellent sample-crystal contact but also prevents, up to a certain extent, water loss from the sample.

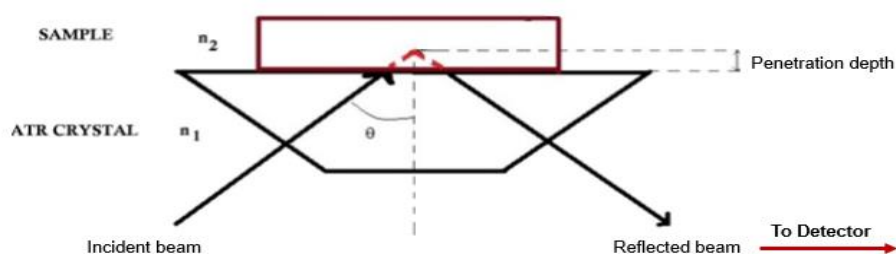


Figure 2.7. Schematic representation of the layout between the sample and the ATR crystal. The incident beam, the critical angle and the reflected beam are also illustrated. Adapted from (Atitar, et al., 2015).

FTIR spectrum

The plot containing the absorption bands of a molecule exposed to IR radiation is called IR spectrum and it can be used for both qualitative and quantitative analysis. The plot presents the absorbance of the radiation as a function of wavenumber (cm^{-1}). The peaks illustrated in Figure 2.8 are the peaks related to amorphous (1022cm^{-1}) and ordered starch structure (1000cm^{-1} and 1047cm^{-1}) (Htoon, et al., 2009; van Soest, et al., 1994; Flores-Morales, et al., 2012; Van Soest & Vliegenthart, 1997; Wilson, et al., 1987; Warren, et al., 2016; Capron, et al., 2007; Waigh, et al., 1998).

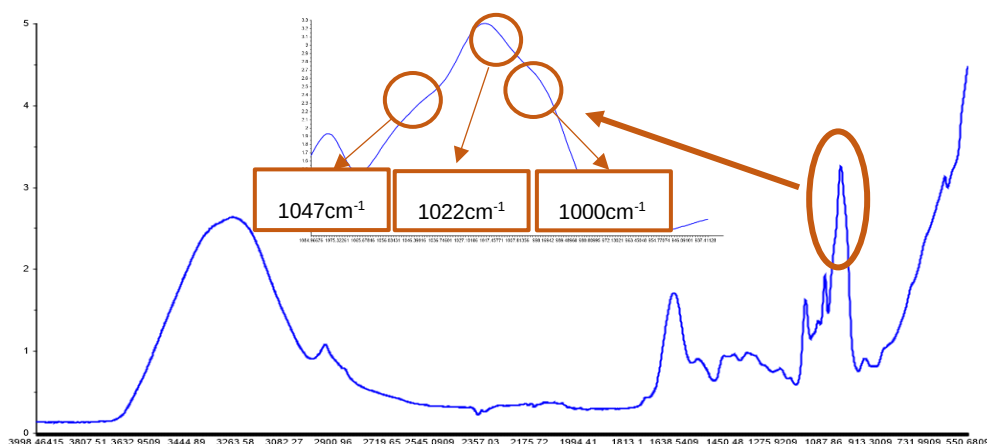


Figure 2.8. FTIR spectrum obtained from oatmeal sandwich bread.

Experimental Procedure

Mid-infrared spectra were collected by using a Bruker IFS48 infrared spectrophotometer (Bruker, UK) equipped with a DTGS detector and a heated single reflectance ATR cell with a diamond crystal (Graseby-Specac Ltd, UK). A sealed sapphire anvil with a rubber O-ring was used to minimise moisture loss from the sample during measurement. For each spectrum 128 scans were acquired at a resolution of 4cm^{-1} and co-added. The ATR cell, apart from the

sample compartment which was sealed, was being purged with dry air so that to minimise water vapour contribution to the spectra. Sample spectra was obtained by using the standard direct mode where the background was recorded with no sample in the ATR cell. The experiment was repeated at least six times for each sample. Data analysis was carried out using OPUS 3.0 software (Bruker, UK). Mean normalisation of the spectra and Principal Component Analysis (PCA) to identify outliers was conducted by The Unscrambler® X software, Version 10.4 (CAMO Software AS, Norway) and the results were presented as a mean average.

2.2.6 Statistical Analysis

The statistical analysis of the results was performed by using single factor ANOVA at Microsoft Excel 2016. $P\text{-value} \geq 0.05$ indicated statistically equal results, while $P\text{-value} < 0.05$ underlined significantly different results. In the case of significantly different results, a Tukey Honest Significant Differences test took place at Microsoft Excel 2016.

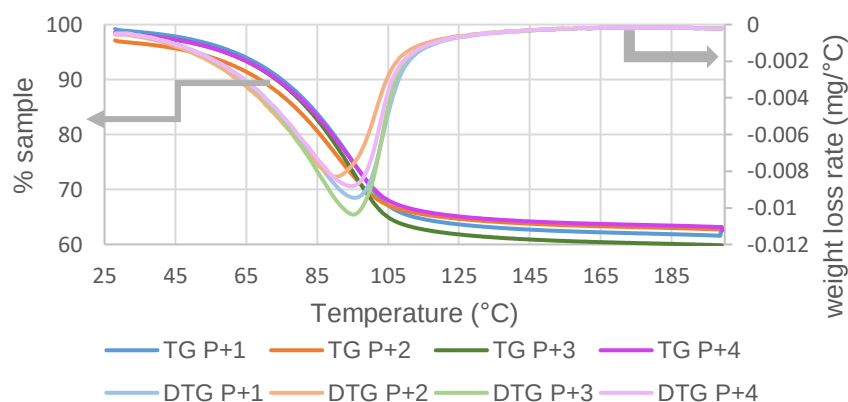
3. Results

3.1 Ready-to-eat ham, mustard and mayonnaise sandwich

Ready-to-eat ham, mustard and mayonnaise sandwiches, made of oatmeal bread, were analysed for four days (P+4) after their production (P). Their shelf-life was three days (P+3). Sandwich bread was divided into three sections as it was shown in Figure 2.1. Each section was analysed separately.

3.1.1 Water evaporation process

Figure 3.1 depicts the graphs obtained from the TGA experiments conducted in ready-to-eat ham, mustard and mayonnaise sandwich. Presence of only one sigmoidal drop was noticed, which occurred over a broad temperature range and was then stabilised at approximately 140°C. This drop can be assigned to water loss from the sample. The TG curve from the Packaging Side (PS) slice showed that the total amount of water remained relatively stable from P+1 to P+4, as the %sample value in the end of the experiment was very similar among the TG curves (the exact values are shown in Table 3.1). In the case of the Middle Side (MS) and the Air Side (AS) slices there was a clear upward trend in the amount of water during bread ageing (see Table 3.2 and Table 3.3). The DTG traces for PS slice did not reveal a clear pattern regarding changes in water evaporation rate. The highest water releasing rate was noticed on P+3, the second highest was indicated on P+1, while the lowest was detected on P+2. In the case of MS and AS water loss speed followed an upward trend from P+1 to P+3, while on P+4 the speed decreased. Finally, the DTG_{max} value shifted to higher temperature over storage for all the samples.



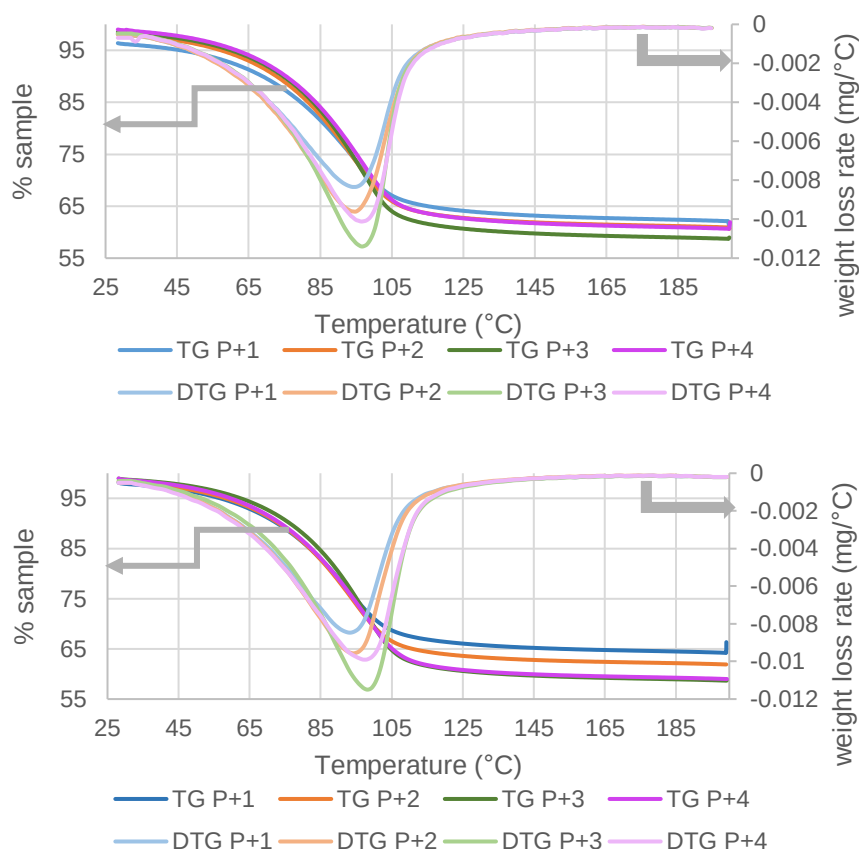


Figure 3.1. Graphs corresponding to TG and DTG traces of all the experiments with the ready-to-eat ham, mustard and mayonnaise sandwich. The top curves refer to the Packaging Side (PS) slice of the sandwich. The middle graph depicts the weight loss process in the Middle Side (MS) slices while in the bottom diagram, the results from the analysis of Air Side (AS) slice are presented. The curves are normalised with the initial weight of the sample and correspond to the averages of the TG and DTG curves from each day, as every experiment was conducted six times.

3.1.2 Water content and types of water

Table 3.1 illustrates oven drying, TGA and DSC results regarding the water content and types of water from the packaging side slice (PS). It can be observed that the total amount of moisture increased over storage according to the oven drying method, a fact that is not in agreement with the TGA results that did not reveal a clear change during bread ageing. Statistical analysis between oven drying and TGA data for each day, indicated that the figures differ significantly. Due to the higher standard deviation of the TGA results, data from the oven drying method were used to calculate FW and UFW water. Furthermore, statistical analysis of ice melting enthalpies characterised the data as equal. However, due to the high standard deviation in enthalpies, the reliability of the result is questionable. Considering the %FW expressed in total water basis and in total amount of sample, a decrease was noticed over storage that led to an increase in UFW (Figure 3.2). Finally, a slight freezing point depression was detected in ice melting temperatures.

Table 3.1. Results related to water obtained from the PS of ready-to-eat ham, mustard and mayonnaise sandwiches, collected by oven drying method, TGA & DSC experiments. Each measurement repeated six times. P-values in the last row, were calculated by using One-way Anova in Excel. P-value $\geq$ 0.05 indicated statistically equal results, while P-value $<$ 0.05 underlined significantly different results. FW and UFW values were calculated by using the oven drying data. Different lower-case letters represent statistical differences between days after production according to Tukey test.

DSC results ready-to-eat ham, mustard and mayonnaise sandwich-packaging side (PS)										
	oven	TGA	DSC	DSC ice melting temperature						
Days After Production	moisture (g/100g) (w.w.b)	moisture (g/100g) (w.w.b)	Enthalpy of ice melting (J/g)	%FW total water basis	%UFW total water basis	FW g/g of sample	UFW g/g of sample	Onset To (°C)	Peak Tp (°C)	Enset Te (°C)
P+1	41.27 $\pm$ 0.83	36.38 $\pm$ 3.01	48.93 $\pm$ 6.42	38.23 $\pm$ 4.52	61.77 $\pm$ 4.52	0.16 $\pm$ 0.02	0.25 $\pm$ 0.02	-11.59 $\pm$ 0.69	-4.48 $\pm$ 0.31	-0.97 $\pm$ 0.17
P+2	40.71 $\pm$ 1.05 ^a	35.04 $\pm$ 3.57	46.08 $\pm$ 10.40	36.96 $\pm$ 7.98	63.04 $\pm$ 7.98	0.15 $\pm$ 0.03	0.25 $\pm$ 0.03	-12.34 $\pm$ 1.36	-5.10 $\pm$ 0.77	-1.30 $\pm$ 0.68
P+3	41.77 $\pm$ 1.18	37.29 $\pm$ 1.92	45.56 $\pm$ 7.30	35.14 $\pm$ 4.95	64.86 $\pm$ 4.95	0.15 $\pm$ 0.02	0.27 $\pm$ 0.02	-11.94 $\pm$ 1.00	-4.92 $\pm$ 0.68	-1.55 $\pm$ 0.57
P+4	42.89 $\pm$ 1.18 ^b	36.14 $\pm$ 3.26	47.90 $\pm$ 11.51	36.08 $\pm$ 8.82	63.92 $\pm$	0.15 $\pm$ 0.04	0.27 $\pm$ 0.04	-12.34 $\pm$ 1.67	-5.07 $\pm$ 0.77	-1.55 $\pm$ 0.52
P-value	$<$ 0.05	0.44	0.29	0.38	0.38	0.29	0.29	0.17	0.10	0.18

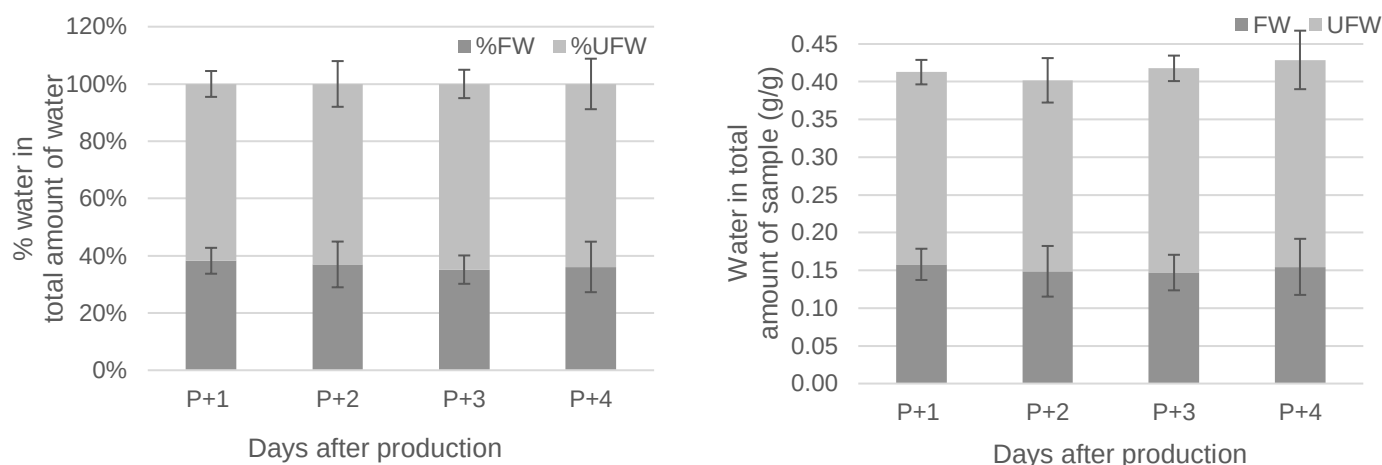


Figure 3.2. Column chart representing the fractions of %FW and %UFW water in total amount of water (on the left) and FW, UFW in total amount of sample (on the right) as it was obtained from PS slice of ready-to-eat ham, mustard and mayonnaise sandwiches. Each column represents a different day of storage. Each measurement repeated six times. Error bars correspond to the standard deviation values for these data.

According to the results presented in Table 3.2, in the case of the middle side slice (MS) the total amount of moisture increased significantly over storage, as it was revealed from the oven drying results. TGA data did not differ significantly over days, possibly due to the high variance of the measurements. It is worth mentioning that statistical analysis between oven drying and TGA results pointed out that the data are not equal at none of the days of the experiment. Due to the higher standard deviation of the TGA results, data from the oven drying method were used to calculate FW and UFW water. In addition, a remarkable increase in ice melting enthalpies was noticed. However, standard deviation was high. The increase in %moisture and ice melting enthalpies resulted in a significant increase in FW in the sample and consequently in a decrease in UFW (Figure 3.3). Finally, a slight freezing point elevation was detected with the passage of the days.

Table 3.2. Results related to water obtained from the MS of ready-to-eat ham, mustard and mayonnaise sandwiches, collected by oven drying method, TGA & DSC experiments. Each measurement repeated six times. P-values in the last row, were calculated by using One-way Anova in Excel. P-value $\geq$ 0.05 indicated statistically equal results, while P-value $<$ 0.05 underlined significantly different results. FW and UFW values were calculated by using the oven drying data. Different lower-case letters represent statistical differences between days after production according to Tukey test.

DSC results ready-to-eat ham, mustard and mayonnaise sandwich -middle side (MS)										
	oven	TGA	DSC	DSC ice melting temperature						
Days After Production	moisture (g/100g) (w.w.b)	moisture (g/100g) (w.w.b)	Enthalpy of ice melting (J/g)	%FW total water basis	%UFW total water basis	FW g/g of sample	UFW g/g of sample	Onset To (°C)	Peak Tp (°C)	Enset Te (°C)
P+1	41.08 $\pm$ 0.27 ^a	34.03 $\pm$ 4.49	41.37 $\pm$ 5.94 ^a	32.45 $\pm$ 4.62 ^a	67.55 $\pm$ 4.62 ^a	0.13 $\pm$ 0.02 ^a	0.28 $\pm$ 0.02 ^a	-12.21 $\pm$ 0.95	-4.92 $\pm$ 0.70	-1.11 $\pm$ 0.58
P+2	42.73 $\pm$ 0.51 ^b	35.50 $\pm$ 4.13	46.19 $\pm$ 14.27	34.78 $\pm$ 10.42	65.22 $\pm$ 10.42	0.15 $\pm$ 0.05	0.28 $\pm$ 0.04	-12.04 $\pm$ 1.68	-4.95 $\pm$ 1.13	-1.69 $\pm$ 0.83
P+3	41.81 $\pm$ 0.41 ^c	38.97 $\pm$ 1.92	53.15 $\pm$ 4.17	41.05 $\pm$ 3.23	58.95 $\pm$ 3.23	0.17 $\pm$ 0.01	0.25 $\pm$ 0.01	-11.08 $\pm$ 0.50	-4.62 $\pm$ 0.40	-1.62 $\pm$ 0.45
P+4	43.69 $\pm$ 0.21 ^d	39.26 $\pm$ 1.57	55.27 $\pm$ 3.27 ^b	40.90 $\pm$ 2.17 ^b	59.10 $\pm$ 2.17 ^b	0.18 $\pm$ 0.01 ^b	0.26 $\pm$ 0.01 ^b	-11.21 $\pm$ 0.25	-4.30 $\pm$ 0.10	-0.94 $\pm$ 0.35
P-value	<0.05	0.09	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.18	0.23

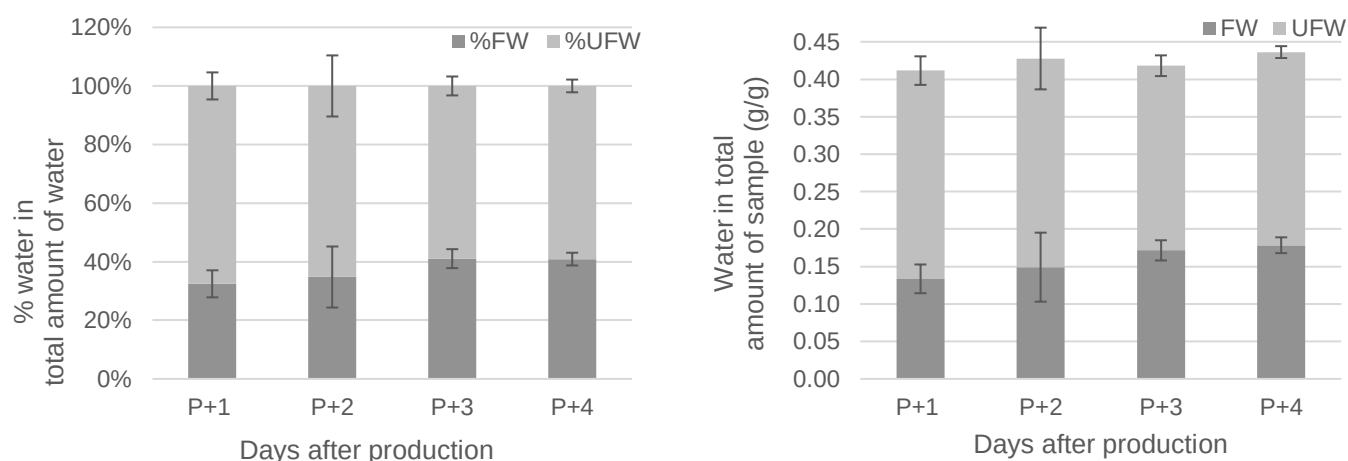


Figure 3.3. Column chart representing the fractions of %FW and %UFW water in total amount of water (on the left) and FW, UFW in total amount of sample (on the right) as it was obtained from MS slice of ready-to-eat ham, mustard and mayonnaise sandwiches. Each column represents a different day of storage. Each measurement repeated six times. Error bars correspond to the standard deviation values for these data.

Regarding the air side slice (AS) of the sandwich, see Table 3.3, an increase in the total amount of moisture over storage was detected from both oven drying and TGA methods. However, only in the case of oven drying method the difference was significant. Due to the higher standard deviation of the TGA results, data from the oven drying method were used to calculate FW and UFW water. The ice melting enthalpy increased during bread ageing, even though not significantly, and resulted in a rise of the total amount of FW in the sample (Figure 3.4). On the other hand, UFW exhibited a downward trend over storage (Figure 3.4), while ice melting temperatures increased, not significantly, over storage.

Table 3.3. Results related to water obtained from the AS slice of ready-to-eat ham, mustard and mayonnaise sandwiches, collected by oven drying method, TGA & DSC experiments. Each measurement repeated six times. P-values in the last row, were calculated by using One-way Anova in Excel. P-value $\geq$ 0.05 indicated statistically equal results, while P-value $<$ 0.05 underlined significantly different results. FW and UFW values were calculated by using the oven drying data. Different lower-case letters represent statistical differences between days after production according to Tukey test.

DSC results ready-to-eat ham, mustard and mayonnaise sandwich-air side (AS)										
	oven	TGA	DSC	DSC ice melting temperature						
Days After Production	moisture (g/100g) (w.w.b)	moisture (g/100g) (w.w.b)	Enthalpy of ice melting (J/g)	%FW total water basis	%UFW total water basis	FW g/g of sample	UFW g/g of sample	Onset To (°C)	Peak Tp (°C)	Enset Te (°C)
P+1	40.72 $\pm$ 0.34 ^a	36.27 $\pm$ 4.32	38.86 $\pm$ 5.81	30.67 $\pm$ 4.70	69.33 $\pm$ 4.70	0.12 $\pm$ 0.02	0.28 $\pm$ 0.02	-12.85 $\pm$ 0.74	-5.38 $\pm$ 0.49	-1.71 $\pm$ 0.30
P+2	41.94 $\pm$ 0.37 ^b	37.79 $\pm$ 3.06	46.33 $\pm$ 11.75	38.75 $\pm$ 7.52	61.25 $\pm$ 7.52	0.16 $\pm$ 0.03	0.26 $\pm$ 0.03	-11.87 $\pm$ 1.08	-4.65 $\pm$ 0.74	-1.05 $\pm$ 0.60
P+3	41.60 $\pm$ 0.22 ^b	39.48 $\pm$ 1.61	54.14 $\pm$ 9.52	42.01 $\pm$ 7.26	57.99 $\pm$ 7.26	0.17 $\pm$ 0.03	0.24 $\pm$ 0.03	-11.24 $\pm$ 1.06	-4.76 $\pm$ 0.46	-1.70 $\pm$ 0.33
P+4	42.88 $\pm$ 0.52 ^c	38.52 $\pm$ 2.43	51.92 $\pm$ 13.66	39.14 $\pm$ 10.48	60.86 $\pm$ 10.48	0.17 $\pm$ 0.04	0.26 $\pm$ 0.05	-11.64 $\pm$ 1.50	-4.89 $\pm$ 1.08	-1.18 $\pm$ 1.03
P-value	<0.05	0.39	0.10	0.13	0.13	0.10	0.10	0.13	0.52	0.69

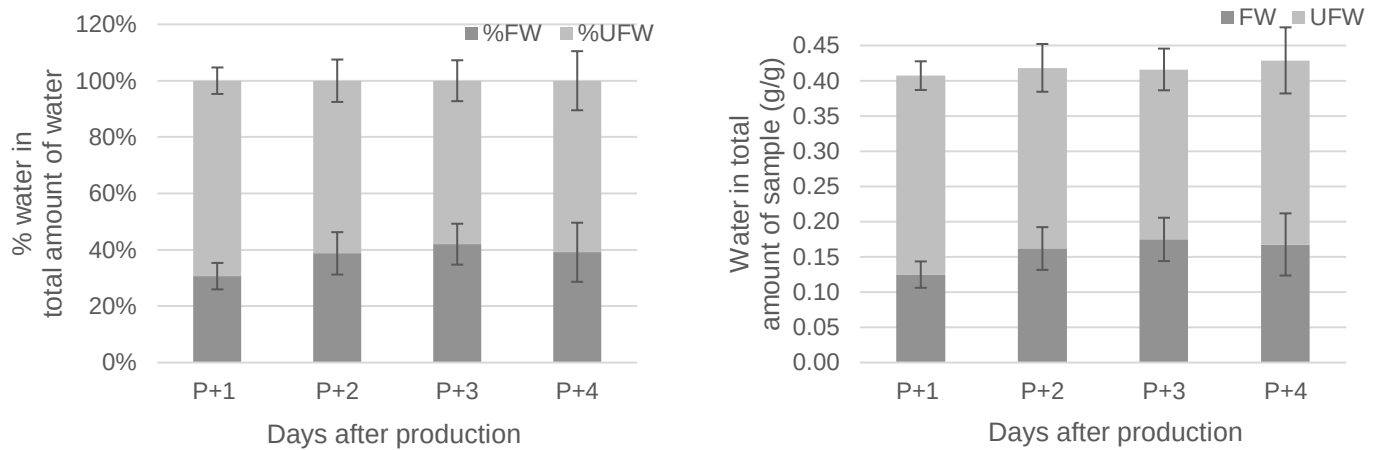


Figure 3.4. Column chart representing the fractions of %FW and %UFW water in total amount of water (on the left) and FW, UFW in total amount of sample (on the right) as it was obtained from AS slice of ready-to-eat ham, mustard and mayonnaise sandwiches. Each column represents a different day of storage. Each measurement repeated six times. Error bars correspond to the standard deviation values for these data.

3.1.3 Amylopectin crystals

Figure 3.5 illustrates changes in amylopectin melting enthalpy over storage in all samples used for the ready-to-eat ham, mustard and mayonnaise sandwich analysis. Despite high values of standard deviation among the measurements, a significant upward trend was noticed in amylopectin melting enthalpy for all three different samples ($P\text{-value}_{PS} < 0.05$, $P\text{-value}_{MS} < 0.05$, $P\text{-value}_{AS} < 0.05$). In the case of AS slice each day differed significantly from the other apart from P+3 and P+4. In PS slice only P+1 and P+4 differed statistically, while in MS slices P+1 enthalpy was unequal to P+4 and P+2 differed from P+2 and P+3. The monitored increase in the enthalpies indicates an increase in the amount of amylopectin crystals in the samples over storage. The amount of amylopectin crystals in the AS slice was the highest throughout the experiment.

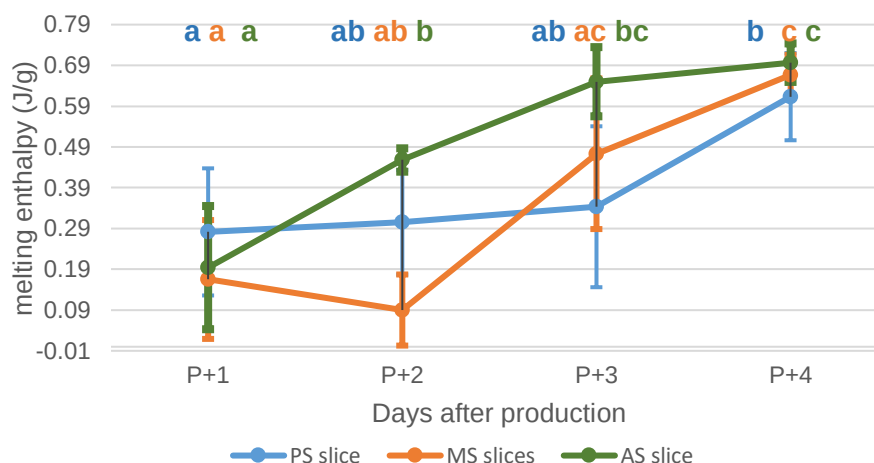


Figure 3.5. Amylopectin melting enthalpy as it was determined by the endothermic peak appeared between 50-60°C in DSC experiments conducted in ready-to-eat ham, mustard and mayonnaise sandwich. The values are expressed on the basis of the total amount of sample. Blue solid line presents the analysis of the PS slice of the sandwich. The orange solid line depicts the melting enthalpy detected in MS slices, while the green solid line corresponds to the AS slice of the sandwich. Each experiment was performed six times. The error bars correspond to the standard deviation among the measurements. Different lower-case letters represent statistical differences between days after production according to Tukey test.

3.1.4 Changes in the population of amorphous and crystalline starch

The spectra in Figure 3.6 represent the average spectra acquired from the analysis of PS slice sampled from ready-to-eat ham, mustard and mayonnaise sandwich. The analysis of the spectra illustrated will be focused on changes in the intensities of the bands at 1047cm⁻¹, 1022cm⁻¹ and 1000cm⁻¹. As it is shown the results are inconclusive as the average spectra from P+1 and P+3 are almost identical. This is also the case for P+2 and P+4.

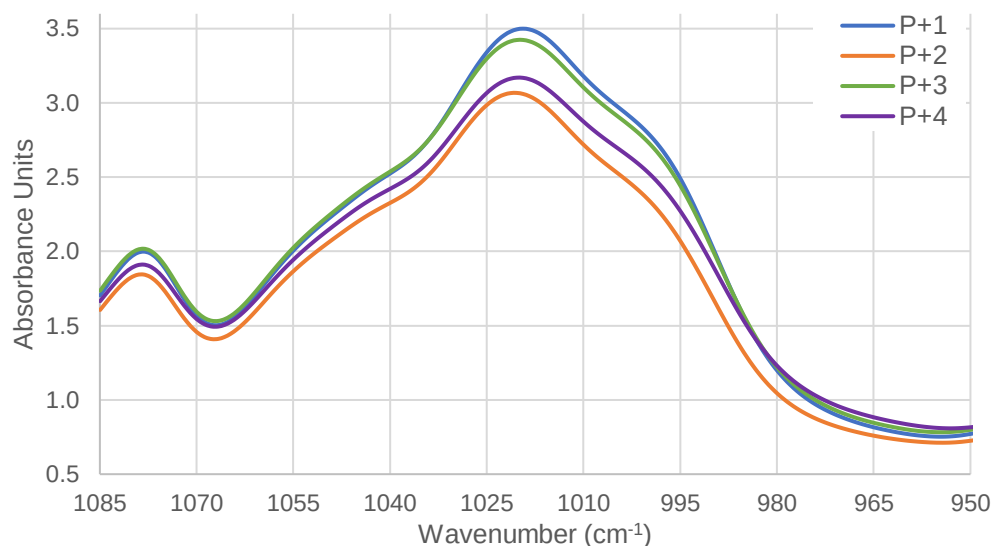


Figure 3.6. Average FTIR spectra between 950-1100 cm^{-1} acquired from the analysis of the PS slice of bread sampled from ready-to-eat ham, mustard and mayonnaise sandwich. The samples presented in this plot are mean normalised and belong to the 90% of the explained variances according to Principal Component Analysis (PCA).

Figure 3.7 depicts a part of the average spectra obtained from the analysis of MS slices acquired from ready-to-eat ham, mustard and mayonnaise sandwich. From P+1 to P+3 samples behaved similarly, whereas on P+4 the absorbance of the sample in the presented region was lower resulting in reduced intensity of the bands at 1047 cm^{-1} , 1022 cm^{-1} and 1000 cm^{-1} .

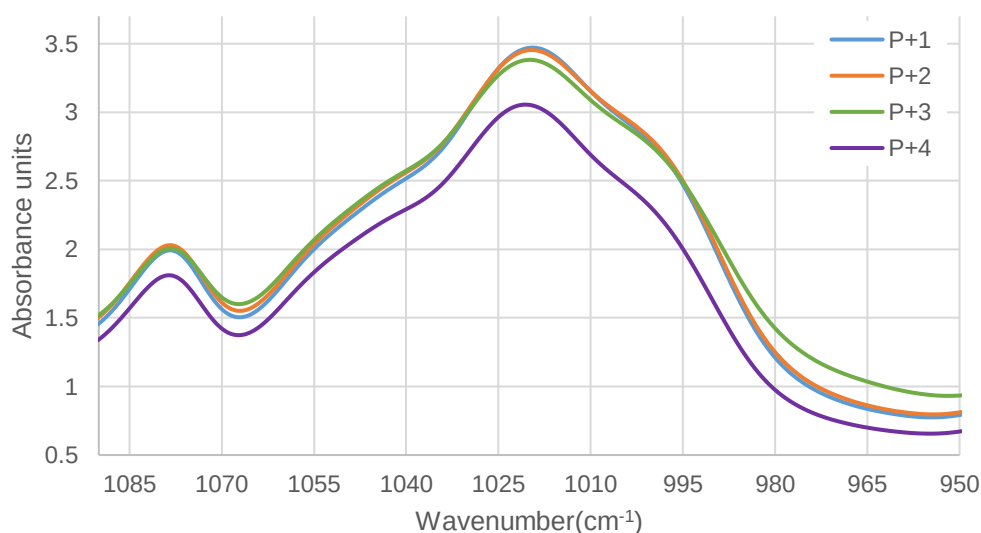


Figure 3.7. Average FTIR spectra between 950-1100 cm^{-1} acquired from the analysis of the MS slices of bread sampled from ready-to-eat ham, mustard and mayonnaise sandwich. The samples presented in this plot are mean normalised and belong to the 90% of the explained variances according to Principal Component Analysis (PCA).

Figure 3.8 illustrates a part the spectra obtained from the analysis of AS slice collected from ready-to-eat ham, mustard and mayonnaise sandwich. On average, the intensities of all the peaks (at 1047cm^{-1} , 1022cm^{-1} and 1000cm^{-1}) decreased over storage.

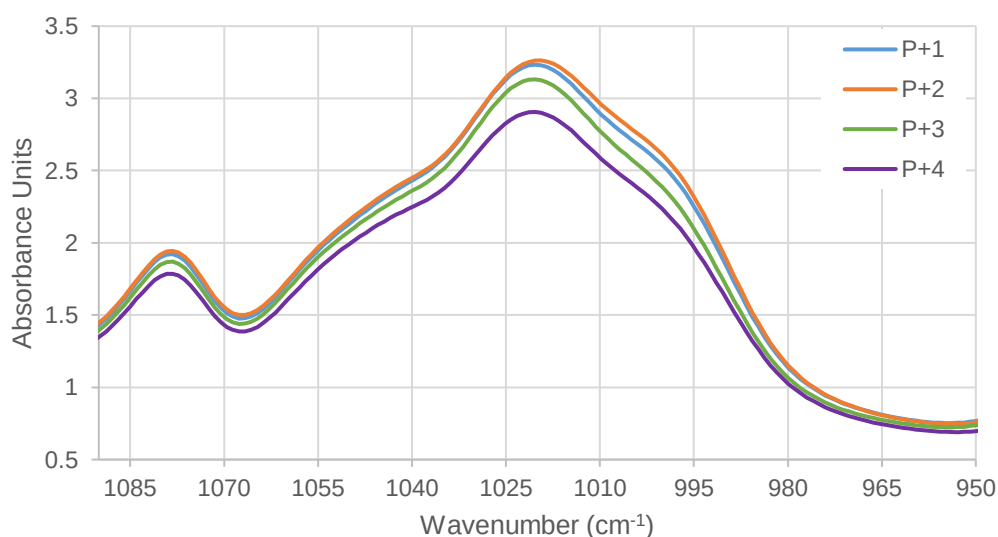


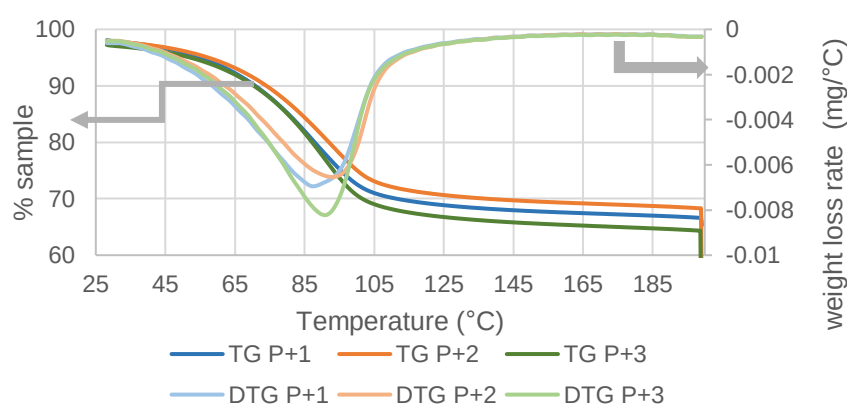
Figure 3.8. Average FTIR spectra between $950\text{-}1100\text{cm}^{-1}$ acquired from the analysis of the AS slice of bread sampled from ready-to-eat ham, mustard and mayonnaise sandwich. The samples presented in this plot are mean normalised and belong to the 90% of the explained variances according to Principal Component Analysis (PCA).

3.2 Ready-to-eat ham, farmhouse cheese and pickle sandwich

Ready-to-eat ham, farmhouse cheese and pickle sandwiches, made of wholegrain malted bread, were analysed for three days (P+3) after their production (P). The shelf-life of the sandwich was two days (P+2). The part of the sandwich that was used for the analysis was illustrated in Figure 2.1.

3.2.1 Water evaporation process

Figure 3.9 presents the results collected from the TGA experiments with regards to the ready-to-eat ham, farmhouse cheese and pickle sandwich. Presence of only one sigmoidal drop was noticed, which occurred over a broad temperature range and was then stabilised at approximately 140°C. This drop can be connected to water loss from the sample. DTG curves highlighted that the water loss occurred over a broad temperature range. In the case of the air side butter (and pickle layered on top) slice of sandwich bread TG curves showed that the highest amount of water was detected on P+3 day but the lowest on P+2 (the exact values are shown on Table 3.4). The DTG traces did not reveal a clear pattern regarding changes in water evaporation rate over storage, as the fastest water release rate was noticed on P+3 and the slowest on P+2. The highest value for DTG_{max} was revealed on P+2. Regarding the middle slice of the ready-to-eat ham, farmhouse cheese and pickle sandwich spread with signature 30% mayonnaise, an upward trend in total amount of water was detected over storage (see also Table 3.5). The DTG curves indicate that the highest water loss rate was noticed on P+3, while the curves for P+1 and P+2 were very similar. The peaks of the DTG curve were noticed almost at the same temperature at all days, however at P+2 the maximum was at a slightly higher temperature compared to other days.



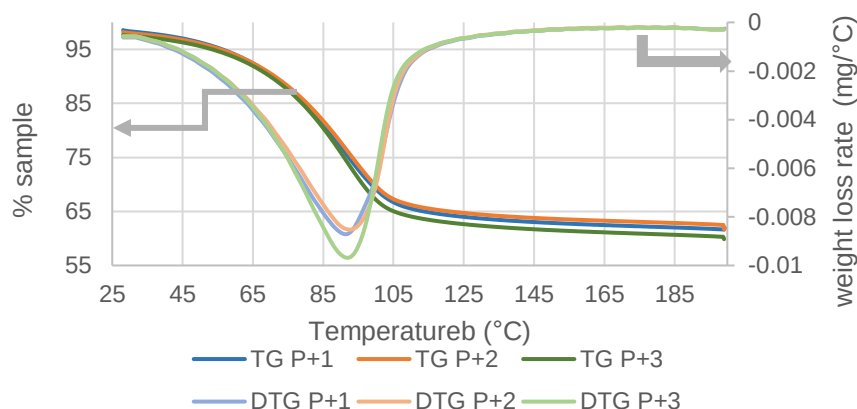


Figure 3.9. TG and DTG traces from the experiments conducted in ready-to-eat ham, farmhouse cheese and pickle sandwiches. The top figure is referred to TGA analysis in the slice of bread that was in contact with butter (and pickle layered on top) and it was in the side of packaging that had access to air. The bottom curves correspond to the analysis of the slice of bread that was in the middle of the packaging and was spread with signature 30% mayonnaise. The curves are normalised with regards to the initial weight of the sample. Each curve presents the average of the TG and DTG curves from each day, as every experiment was conducted six times. Additionally, the curves were normalised.

3.2.2 Water content and types of water

The results acquired from oven drying, TGA, and DSC slice of bread spread with butter and pickle layered on top are presented in Table 3.4. According to the oven drying results, the total amount of water followed a significant downward trend, whereas TGA measurements showed a not statistically significant increase on the same variable. As the TGA results showed high standard deviation, the oven drying results were considered more reliable and utilised to calculate FW and UFW fractions. Ice melting enthalpies underwent a decline that led to a slight decrease in FW and a small increase in UFW (Figure 3.10). It is worth mentioning that none of the above-mentioned changes was statistically different. Finally, a noticeable freezing point depression was detected over storage.

Table 3.4. Results related to moisture content and types of water in the slice of bread that was in contact butter (and pickle layered on top) and had access to air in the packaging), collected from ready-to-eat ham, farmhouse cheese and pickle sandwich. The data obtained from oven drying method, TGA & DSC experiments. Each measurement repeated six times. P-values in the last row, were measured by using One-way Anova in Excel. P-value $\geq$ 0.05 indicated statistically equal results, while P-value $<$ 0.05 underlined significantly different results. FW and UFW values were calculated by using the oven drying data. Different lower-case letters represent statistical differences between days after production according to Tukey test.

Ready-to-eat ham, farmhouse cheese and pickle sandwich-butter side Air										
Days After Production	oven	TGA	DSC	DSC ice melting temperature						
	moisture (g/100g) (w.w.b)	moisture (g/100g) (w.w.b)	Enthalpy of ice melting (J/g)	%FW total water basis	%UFW total water basis	FW g/g of sample	UFW g/g of sample	Onset To (°C)	Peak Tp (°C)	Enset Te (°C)
P+1	39.40 $\pm$ 0.09 ^a	32.15 $\pm$ 5.83	37.85 $\pm$ 11.49	31.01 $\pm$ 9.40	68.99 $\pm$ 9.40	0.12 $\pm$ 0.04	0.24 $\pm$ 0.04	-14.00 $\pm$ 1.87	-6.36 $\pm$ 2.80	-2.93 $\pm$ 1.06
P+2	36.54 $\pm$ 0.63 ^b	31.67 $\pm$ 7.38	30.88 $\pm$ 7.47	27.32 $\pm$ 6.78	72.68 $\pm$ 6.78	0.10 $\pm$ 0.02	0.27 $\pm$ 0.03	-15.83 $\pm$ 0.89	-5.04 $\pm$ 2.94	-2.76 $\pm$ 0.70
P+3	36.80 $\pm$ 0.37 ^b	34.88 $\pm$ 3.10	31.60 $\pm$ 2.08	27.74 $\pm$ 1.95	72.26 $\pm$ 1.95	0.10 $\pm$ 0.01	0.27 $\pm$ 0.01	-16.04 $\pm$ 0.56	-7.83 $\pm$ 0.39	-4.96 $\pm$ 2.29
P-value	$<$ 0.05	0.59	0.30	0.61	0.61	0.30	0.30	0.05	0.17	0.05

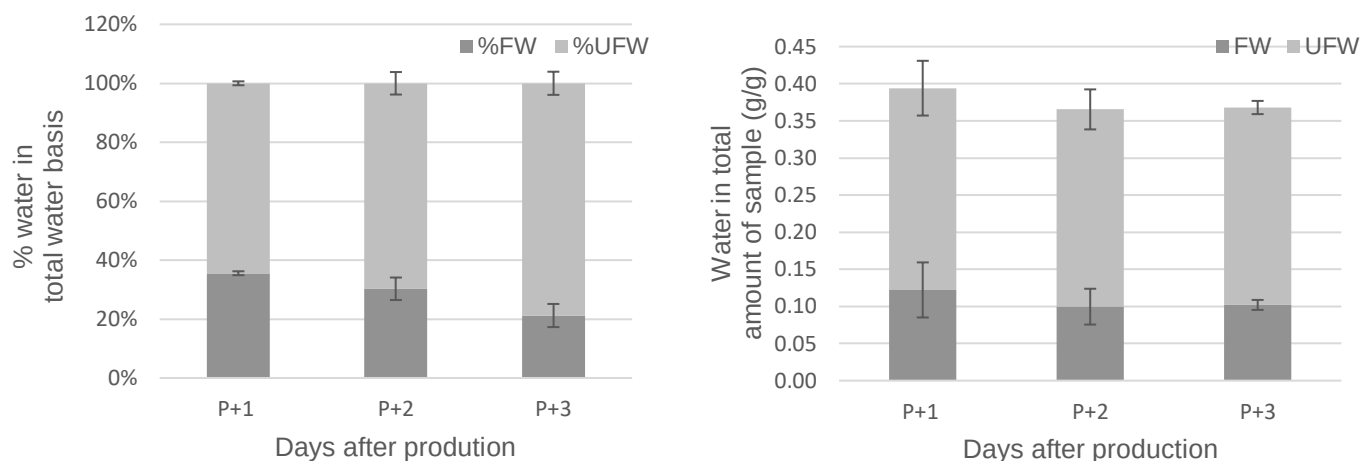


Figure 3.10. Freezable and Unfreezable water, expressed in total amount of water (on the left) and in total amount of sample (on the right) in the slice of bread that was in contact with butter (and pickle layered on top) and its position in the packaging was in the side that had access to air. The sample was obtained from ready-to-eat ham, farmhouse cheese and pickle sandwich. The values were calculated from the DSC and oven drying experiments. The values in the graphs represent the averages of each day. Error bars correspond to the standard deviation.

Table 3.5 shows the results for the slice of bread from the ready-to-eat ham, farmhouse cheese and pickle sandwich that was spread with signature 30% mayonnaise. It can be observed that the results for changes in the total amount of water are inconclusive. According to oven drying method, the total amount of moisture noticed a peak in day P+2 but in days P+1 and P+3 the values were closely aligned. On the other hand, TGA data detected an overall statistically insignificant increase over storage. Ice melting enthalpy increased with the passage of the time revealing an upward trend in FW and a downward trend in UFW (Figure 3.11). However, these trends are statistically equal. Finally, there was a slight freezing point elevation in the water of the sample.

Table 3.5. Results related to moisture content and types of water in the slice of bread that was in contact with signature 30% mayonnaise (and specifically was lay in the middle of the packaging), collected from ready-to-eat ham, farmhouse cheese and pickle sandwich. The data obtained from oven drying method, TGA & DSC experiments. Each measurement repeated six times. P-values in the last row, were measured by using One-way Anova in Excel. P-value $\geq$ 0.05 indicated statistically equal results, while P-value $<$ 0.05 underlined significantly different results. FW and UFW values were calculated by using the oven drying data. Different lower-case letters represent statistical differences between days after production according to Tukey test.

Ready-to-eat ham, farmhouse cheese and pickle sandwich-signature 30% mayonnaise middle side										
Days After Production	oven	TGA	DSC	DSC ice melting temperature						
	moisture (g/100g) (w.w.b)	moisture (g/100g) (w.w.b)	Enthalpy of ice melting (J/g)	%FW total water basis	%UFW total water basis	FW g/g of sample	UFW g/g of sample	Onset To (°C)	Peak Tp (°C)	Enset Te (°C)
P+1	40.19 $\pm$ 0.39 ^a	35.43 $\pm$ 2.71	32.81 $\pm$ 8.43	26.35 $\pm$ 6.52	73.65 $\pm$ 6.52	0.11 $\pm$ 0.03	0.30 $\pm$ 0.03	-15.07 $\pm$ 0.92	-7.29 $\pm$ 1.14	-2.88 $\pm$ 0.99
P+2	41.33 $\pm$ 0.81 ^b	34.95 $\pm$ 3.90	43.58 $\pm$ 9.90	34.03 $\pm$ 7.58	65.97 $\pm$ 7.58	0.14 $\pm$ 0.03	0.27 $\pm$ 0.03	-13.84 $\pm$ 1.49	-5.94 $\pm$ 0.89	-2.05 $\pm$ 0.73
P+3	40.18 $\pm$ 0.93 ^a	36.99 $\pm$ 2.50	43.99 $\pm$ 7.67	35.34 $\pm$ 6.09	64.66 $\pm$ 6.09	0.14 $\pm$ 0.02	0.26 $\pm$ 0.02	-13.62 $\pm$ 0.90	-6.33 $\pm$ 0.55	-3.05 $\pm$ 0.26
P-value	<0.05	0.41	0.07	0.07	0.07	0.07	0.07	0.09	0.05	0.07

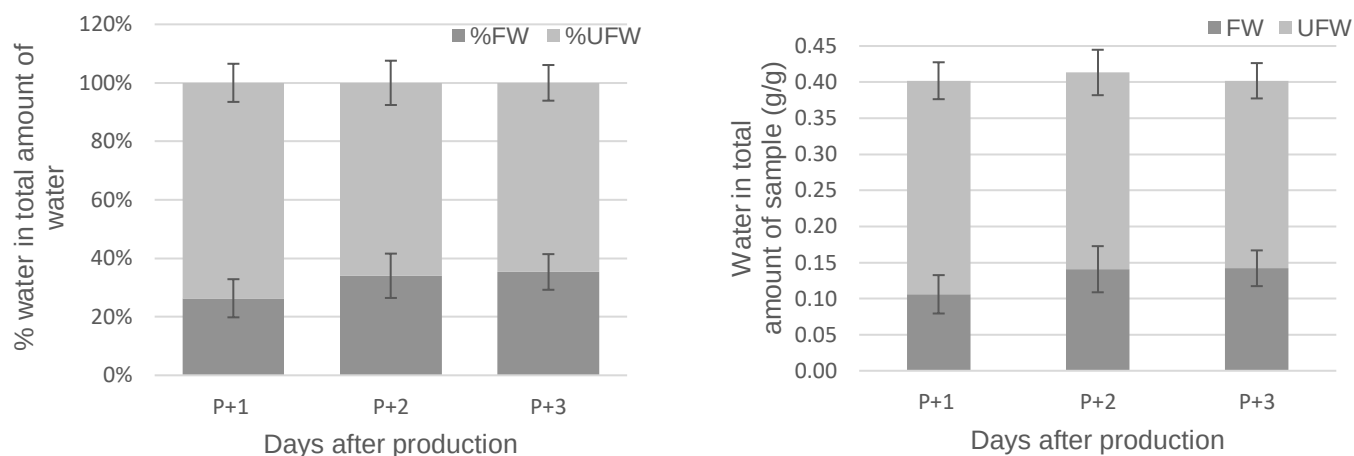


Figure 3.11. Freezable and Unfreezable water, expressed in total amount of water (on the left) and in total amount of sample (on the right) in the slice of bread that was in contact with signature 30% mayonnaise and lay in the middle of the packaging. The sample was collected from ready-to-eat ham, farmhouse cheese and pickle sandwich. The values were calculated from the DSC and oven drying experiments. The values in the graphs represent the averages of each day. Error bars correspond to the standard deviation.

3.2.3 Amylopectin crystals

Figure 3.12 illustrates changes of amylopectin melting enthalpies over storage in the slices of sandwich bread acquired from ready-to-eat ham, farmhouse cheese and pickle sandwich. A gradual but not significant increase in amylopectin melting enthalpy values was noticed in both the slice of bread with butter (P-value=0.43) and with signature 30% mayonnaise (P-value=0.24). This fact indicates the presence of more amylopectin crystals in the samples over storage. The amount of amylopectin crystals in the slice of bread with butter (and pickle layered on top) was higher than in the slice with signature 30% mayonnaise. It is also worth mentioning that the enthalpy values for this sandwich bread are lower than those observed for the ready-to-eat ham, mustard and mayonnaise sandwich. However, the high values of standard deviation among the measurements cannot be ignored.

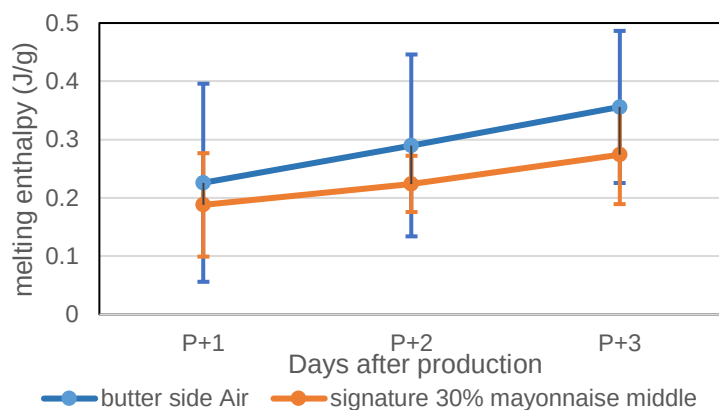


Figure 3.12. Amylopectin melting enthalpy as it was determined by the endothermic peak appeared between 50-60°C in DSC experiments conducted in ready-to-eat ham, farmhouse cheese and pickle sandwich. The values are expressed on the basis of the total amount of sample. The blue solid line presents the analysis of the bread spread with butter (and pickle layered on top) its position in the packaging was in the side that had access to air. The orange solid line corresponds to the bread in contact with signature 30% mayonnaise lay in the middle of the packaging. Each experiment was performed six times. The error bars correspond to the standard deviation among the measurements.

3.2.4 Changes in the population of amorphous and crystalline starch

Figure 3.13 depicts spectra of the analysis conducted on sandwich bread spread with butter sampled from the ready-to-eat ham, farmhouse cheese and pickle sandwich. Spectra from P+2 and P+3 are similar in the case of the band at 1022cm⁻¹, whereas the intensity of the band on P+1 was lower. In the case of the band at 1047cm⁻¹, the absorbance of this peak was similar over storage, while the highest absorbance of the peak at 1000cm⁻¹ was noticed on P+2 and the lower on P+1.

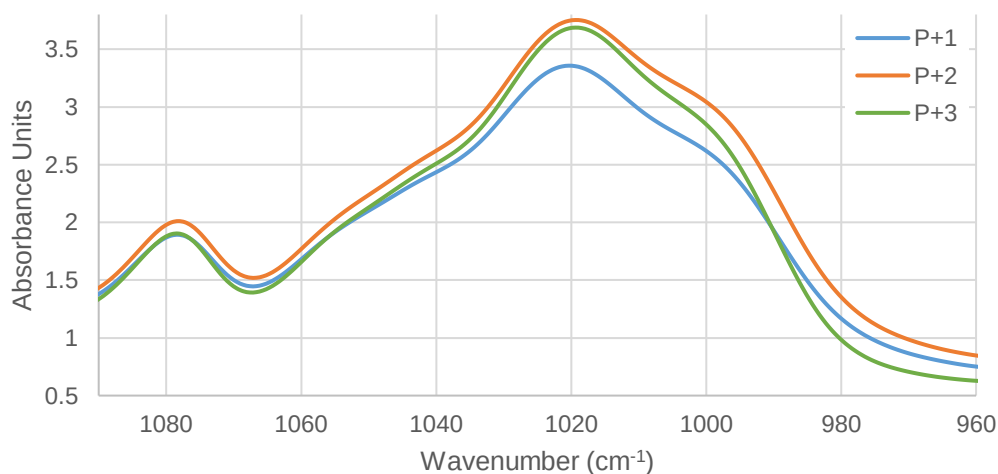


Figure 3.13. Average FTIR spectra between 950-1100cm⁻¹ of the slice of sandwich bread spread with butter obtained from the packaging side that had access to air of ready-to-eat ham, farmhouse cheese and pickle sandwich. The samples presented in this plot are mean normalised and belong to the 90% of the explained variances according to Principal Component Analysis (PCA).

Figure 3.14 illustrates the average spectra corresponding to the slice of bread spread with signature 30% mayonnaise sampled from the ready-to-eat ham, farmhouse cheese and pickle sandwich. The results show that the spectra of P+1 and P+3 are closely aligned, whereas on P+2 the absorbance of the bands was strikingly higher.

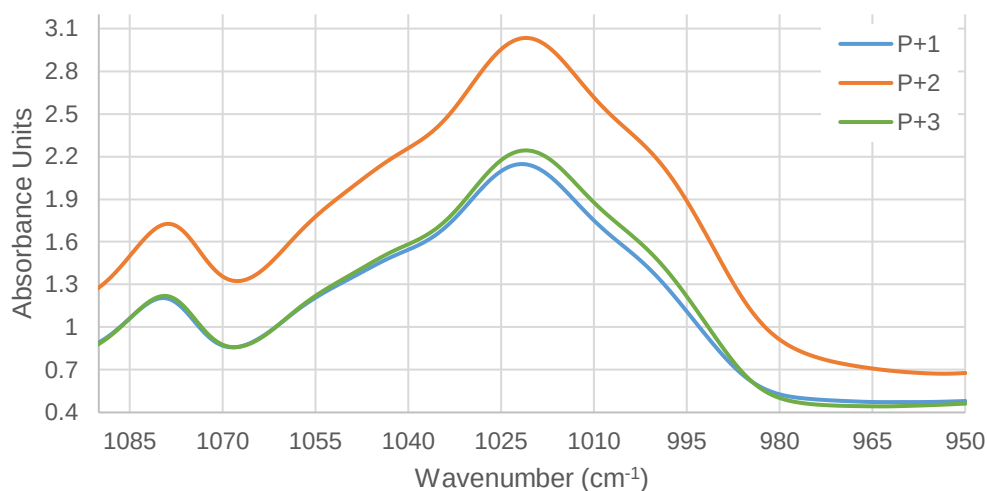


Figure 3.14. Average FTIR spectra between 950-1100cm⁻¹ acquired from the analysis of the signature 30% mayonnaise slice lay in the middle of the packaging of ready-to-eat ham, farmhouse cheese and pickle sandwich. The samples presented in this plot are mean normalised and belong to the 90% of the explained variances according to Principal Component Analysis (PCA).

4. Discussion

4.1.1 Comparison between oven drying method and TGA

Both the oven drying method and TGA are based on weight loss over heating. In the case of the oven drying method, the sample was exposed to constant temperature (105°C) while in the TGA experiments the temperature was gradually increased and weight loss was monitored constantly. According to Schiraldi et. al. (1996) who conducted a research on bread staling by using TGA the water loss process lasts from the beginning of the heating up to approximately 140°C. Hence, heating the sample up to 200°C, which was the maximum temperature of the present work, was adequate to study the total water loss procedure. This was confirmed from the existence of only one peak in the DTG curve and from the fact that weight loss rate reached 0mg/°C at approximately 140°C. Although both methods are reliable as they have been utilised widely for the determination of the total amount of water in bread (Baik & Chinachoti, 2000; Hallberg & Chinachoti, 2002; Lodi & Vodovotz, 2008; Ribotta & Le Bail, 2007; Clubbs, et al., 2008; Schiraldi, et al., 1996; Fessas & Schiraldi, 2001), the discrepancy between moisture values determined from these two methods was noticeable. In all experiments, the moisture values obtained by TGA experiments were lower than these acquired from the oven drying experiment. This might be due to the considerably smaller amount of sample required for TGA analysis (10-15 mg), which increases the risk of drying out while the sample is prepared and loaded into the crucibles. This fact could also explain the higher standard deviations of the TGA results. As a result, as well as the wide use of oven drying method in the published work regarding bread staling, the oven drying data were chosen for the calculation of FW and UFW in the sample.

4.1.2 TG and DTG thermograms

The asymmetric nature of the DTG curves can imply the presence of water molecules that were sufficiently difficult to evaporate from the sample. This fact is probably connected to the existence of water molecules that differ in their degree of interaction with the ingredients in the bread. Furthermore, reduced diffusivity of water through a firmed outer layer of bread that has been formed by the so far evaporated water from the outer surface of the crumb can also contribute to the asymmetric shape of DTG curve (Fessas & Schiraldi, 2001). The range of temperature that water loss took place agreed with the findings of Schiraldi et. al. (1996). However, in that research heating of 5h-old white bread at 2°C/min revealed two types of water in the sample. The peaks were identified by deconvolution of the DTG curve, the shape of which was containing a “shoulder”. These peaks were connected to the DSC findings regarding FW and UFW and it was stated that the first peak of water, which appeared at lower temperature, was representing water that acted as a plasticizer and as a result, had the ability

to form weak links, e.g. hydrogen bonds, with macromolecules (Schiraldi, et al., 1996). These molecules must remain mobile so that to be placed and displaced easily in the polymer (Schiraldi, et al., 1996). On the other hand, the second type of water was released at higher temperature, a clue indicating that it was more difficult to evaporate. This type of water was suggested to participate in amylopectin crystallisation (Schiraldi, et al., 1996). In the present work, the heating rate of the sample was 5°C/min and resulted in a DTG curve without a “shoulder” so that deconvolution was not possible to be applied. Heating rate is a significant factor as it affects the drying kinetics of the samples. Saldo *et al.* (2002) concluded that as diffusing elements cannot react immediately to the driving force that is exerted, higher heating rates result in higher temperatures of the DTG peak. In addition, the different types of water cannot be detected accurately, as when the more bound water starts to be released, the less bound water has not been fully evaporated, resulting in the appearance of one peak regular DTG peak. In this way, the selection of a lower heating rate might have led to a differentiation between different types of water in sandwich bread. Another possible reason for the appearance of a DTG curve containing only one peak can be sample mass. Fessas and Schiraldi (2001) stated that the onset slope of DTG trace related to sample mass. It was suggested that for the same heating rate, a different amount of sample results in changes in DTG peak temperature and in the shape of the curve. The amounts of sample used for that experiment were 30, 50 and 70 mg and it was concluded that the smaller the mass of the sample, the greater the water loss rate as diffusion can take place more easily. In the present work, the selected amount of sample was 15 mg. Hence, a greater amount of sample might have led to a different shape of the DTG curve, where deconvolution could have been applied.

Regarding the importance of the results acquired from the TGA experiment, the increase in the total amount of water, noted in all experiments conducted on the bread isolated from ready-to-eat ham, mustard and mayonnaise sandwiches, could be explained by the interaction of the bread with the signature mustard & mayonnaise sauce. Specifically, moisture migrates from the components with higher amount of water to these with lower until equilibrium is reached (Labuza & Hyman, 1998). According to Kraft Foods Holdings, Inc. (2004) during the interaction between an emulsion and a product, the presence of water in the emulsion results in moisture delivery to the product, assuming that the latter has a lower water activity. This moisture migration is controlled without resulting in soggy bread. As the water activity of a regular mayonnaise is approximately 0.93 and of a light one is around 0.95 (Chirife, et al., 1989; Hwang & Tamplin, 2005), whereas water activity of sandwich bread is approximately 0.90 (Barrett, et al., 2005), the above mentioned hypothesis can be supported.

The higher water release rate which was noticed for MS and AS samples (Figure 3.1) over storage, is possibly related to the increase in total amount of water, as the rate of evaporation

depends on the total mass of water (dm_w/dt) (Fessas & Schiraldi, 2001). On the other hand, the DTG curves obtained from the PS slice experiments are inconclusive (Figure 3.1).

Considering, the ready-to-eat ham, farmhouse cheese and pickle sandwich, the increase in the total amount of water in the air side slice of bread spread with butter (and pickle layered on top) was not in agreement with the oven drying results indicating decrease in water population (Table 3.4). Due to the contradictory results and the high standard deviation of the TGA data the result cannot be discussed. The slower releasing rate that was detected from P+1 to P+2 as well as the shift of the DTG_{max} to higher temperature during bread staling suggest a more difficult water evaporation (Schiraldi, et al., 1996). This can be linked with the DSC results showing a slight increase in the amount of UFW in the sample, implying that water formed stronger links within the sample over storage. However, the increase in water release rate from P+2 to P+3 cannot be explained by DSC data.

On the other hand, the rise in total amount of water (Table 3.5), reflected by both TGA and oven drying, detected in the slices spread with signature 30% mayonnaise could be linked with the aforementioned impact that the emulsion might have on the product. Furthermore, the relatively stable temperature of the DTG maximum indicated not significant changes in interactions of water with other ingredients in bread during bread ageing. Nevertheless, the value of the DTG_{max} for P+3 in this slice of bread was higher than in the case of bread spread with butter (and pickle layered on top) probably due to the higher amount of water in the former slice than in the latter. This fact indicated the impact of different barriers on the physicochemical changes of bread.

4.1.3 Changes in total amount of water, Freezable and Unfreezable water

The total amount of moisture changed significantly over sandwich storage in every sample. In the case of the air side slice of bread spread with butter collected from the ready-to-eat ham, farmhouse cheese and pickle sandwich the total amount of moisture decreased, while in all the other experiments the total amount of water increased. Changes in the fractions of FW and UFW were considered statistically significant only for the MS slice bread isolated from the ready-to-eat ham, mustard and mayonnaise sandwich (Table 3.2). In detail, FW increased, while UFW decreased. Hallberg & Chinachoti (2002) investigated the staling of standard white bread (SWB) and Meal, Ready-to-Eat (MRE) bread over 50 months and detected a reduction in total amount of moisture in SWB and loss in FW. An increase in the moisture content was noticed for MRE, probably due to the better packaging material used and the addition of moisturisers so that to maintain the crumb soft. However, the storage conditions (at 25°C and breads were placed in sealed bags enriched with oxygen) and the duration of the experiment differed from the present work. Ribotta and Le Bail (2007) studied the staling process of white bread stored in plastic pouches at 4°C. In that study, the loss of moisture in the crumb and the

decrease in FW fraction from 62.3% on total water basis on 1-day to 52% on 4-day were mainly related to moisture migration from crumb to crust. An increase in the enthalpy of the amylopectin melting crystals was also detected. Consequently, it was suggested that part of the water is also incorporated into starch crystals. Moisture migration in the near-crust area of white bread, but not in the centre of the crumb was also detected by Czuchajowska & Pomeranz (1989).

Baik & Chinachoti (2000) observed that the level of moisture was maintained in bread stored without crust, while the amount of FW slightly declined until day 3 and then stabilised. In bread stored with crust a significant moisture migration from crumb to crust was observed and a gradual decrease in FW until day 7 culminating in a dramatical downward trend was noticed (Baik & Chinachoti, 2000). In bread stored with crust, crumb firming occurred over extended storage was mainly due to migration of FW to crust, driven by a moisture gradient between crumb and crust. Amylopectin retrogradation, did not show a direct correlation with firming of that bread. In bread stored without crust, the remarkable firming monitored over storage was related to starch retrogradation, despite the relatively stable level of moisture.

As the crust of the sandwich bread analysed here was not crispy, the aforementioned moisture gradient that leads to moisture migration in standard bread is not expected to be a significant cause of the consumers' reported drier texture. Thus, results from bread stored without crust are probably more relevant to this study. Indeed, the only case where the total amount of moisture decreased was when bread was in contact with butter in the ready-to-eat ham, farmhouse cheese and pickle sandwich. In all other cases there was a slight increase of moisture content. This was probably due to the moisture delivery taking place when an emulsion containing water is in contact with a product with lower water activity (Kraft Foods Holdings, Inc., 2004). In this project two emulsions were used: a 30% mayonnaise (in ready-to-eat ham, farmhouse cheese pickle sandwich) and a mustard & mayonnaise sauce (in ready-to-eat ham, mustard and mayonnaise sandwich).

As the total amount of water in the sample increased, it was expected that FW will also follow an upward trend and UFW will show a downward trend. This was the trend for MS slices (Table 3.2) from the ready-to-eat ham, mustard and mayonnaise. In all the other experiments changes were not statistically significant. It is worth mentioning that, in general, sandwich samples are characterised by great heterogeneity concerning the spread of the barriers over the sandwich bread. This lack of homogeneity could lead to moisture migration from sandwich bread to other materials in the sandwich with lower water activity. In this experiment, this could be the case for ready-to-eat ham, farmhouse cheese and pickle sandwich where an improperly spread butter could allow moisture to migrate from the sandwich bread to the ploughman's pickle.

4.1.4 Amylopectin retrogradation

Amylopectin retrogradation is related to loss in water, decrease of the FW fraction and increase in the UFW (Baik & Chinachoti, 2000; Ribotta & Le Bail, 2007; Hallberg & Chinachoti, 2002; Ottenhof & Farhat, 2004). However, in bread stored without crust, amylopectin retrogradation was the main cause of bread firming despite the relatively stable percentage of water and FW in the sample, over storage (Baik & Chinachoti, 2000). This fact agrees with the results of this project. An upward trend in the amylopectin crystals melting enthalpy was noticed on all the experiments conducted in both sandwiches regardless the changes in the total amount of water and FW (Figure 3.5 and Figure 3.12). However, only in case of ready-to-eat ham, mustard and mayonnaise sandwich changes were statistically significant. Amylopectin melting enthalpy in bread stored without crust was calculated on total amount of starch and was detected to be 1J/g in the first day of storage (Baik & Chinachoti, 2000). Then the melting enthalpy increased until day-7 when it reached the value of 3J/g and afterwards it showed a stable performance (Baik & Chinachoti, 2000). According to Baik & Chinachoti (2000) the absence of crust in bread results in lower amylopectin retrogradation levels, indicating that the absence of crust retards starch recrystallisation. In this project, amylopectin melting enthalpies were expressed on total amount of sample basis and as a result it is not possible to perform a direct comparison between the above-mentioned research and this project. However, the different basis that the results were expressed can probably explain the fact that the highest value on this project (0.6-0.7J/g noticed on P+4 for all the three samples) is lower than this monitored on bread stored without crust. Another factor differentiating these two studies is the storage conditions. Baik & Chinachoti (2000) stored breads at 25°C, whereas in this project samples were stored at 4°C. It is known that storage of bread at 4°C encourages amylopectin retrogradation (section 1.1.3). Ribotta & Le Bail (2007) investigated staling of white bread stored at 4°C and monitored a clear upward trend in the amount of amylopectin crystals over storage. The enthalpy of the endothermic peak increased to 7J/g within the first 24h, while on day-4 the detected enthalpy was approximately 9 J/g. Although these values seem to be higher than in the case of Baik & Chinachoti (2000) and the present work, the basis on which these data were calculated was not stated hence, the results cannot be compared.

Further evidence of starch retrogradation was aimed to be given through FTIR experiments. Flores-Morales et al. (2012) conducted FTIR experiments attempting to monitor starch retrogradation in maize tortillas. A decrease in the absorbance of the band at 1022cm⁻¹ and a simultaneous increase in the intensity of the band at 1047cm⁻¹ over storage. As the band at 1022cm⁻¹ is related to amorphous starch, while the band at 1047cm⁻¹ corresponds the formation of starch double helices in the sample (Goodfellow & Wilson, 1990) it was suggested

by Flores-Morales et al. (2012) that changes in the intensities of these bands indicated starch retrogradation. However, the FTIR experiments performed in this research did not show this pattern. In the case of PS slice (Figure 3.6) sampled from the ready-to-eat ham, mustard and mayonnaise slice, the results were inconclusive, while in MS (Figure 3.7) and AS (Figure 3.8) slices the decrease in the intensity of the 1022cm^{-1} band was accompanied by a decrease in the intensity of the 1047cm^{-1} over storage. In the air side slice of bread in contact with butter isolated from the ready-to-eat ham, farmhouse cheese and pickle sandwich (Figure 3.13), an increase in the peak related to the amorphous starch was noticed, while the peak corresponding to the crystalline starch remained relatively stable. Finally, in the slice of bread spread with signature 30% mayonnaise the highest absorbance of both peaks among the spectra was noticed in P+2 but spectra from P+1 and P+3 were closely aligned (Figure 3.14). Capron, et al. (2007), Warren, et al. (2013) and to Warren, et al. (2016) observed that the band at 1047cm^{-1} was not sensitive on changes in the structure of starch, whereas the peak at 1000cm^{-1} monitored accurately order transitions upon hydration. As a result they suggested that the band at 1000cm^{-1} could be more useful to investigate changes in the ordered structure of starch. In this way, the band at 1000cm^{-1} also studied in the present work. However, the absorbance of this band changed in a similar way to the absorbance of the 1022cm^{-1} band, namely both increased and decreased in the same way over storage in all the samples. Hence, it was not possible to observe changes in starch structure over storage through FTIR experiments. This is probably related to the duration of the experiments and the complexity of the samples. Flores-Morales et al. (2012) performed a 10-days analysis of the samples, while Capron, et al. (2007), Warren, et al. (2013) and to Warren, et al. (2016), studied structural changes in starches containing different percentage of water and with different level of crystallinity, which are simpler samples than ready products.

Nevertheless, the exact mechanism of firming is not fully understood yet. For instance, Baik & Chinachoti (2000) underlined that bread firming in some cases might not be related to starch retrogradation as bread firming (DMA measurements) and amylopectin melting enthalpy did not increase in the same way. This suggestion agreed with Martin, et al. (1991) and Rogers, et al. (1988). On the other hand, numerous studies have related bread staling to amylopectin retrogradation. Aguirre et al. (2011) by conducting X-ray diffraction experiments detected that over bread storage at 4°C mainly B-type crystals appeared in the sample. As it was mentioned in section 1.1.1, this type of crystal traps 36 water molecules hence, it is believed that during bread ageing water migrates from the amorphous bread matrix to the starch crystals (Ribotta & Le Bail, 2007). Ribotta & Le Bail (2007) suggested that approximately 62% of the FW water loss was due to water incorporation in the crystal lattice (Ribotta & Le Bail, 2007). NMR studies have revealed a reduction in water mobility within the passage of the time. This fact was related to the theory of water encapsulation in the B-type crystals (Leung, et al., 1983; Wynne-

Jones & Blanshard , 1986; Slade & Levine , 1991). However, Kim-Shin et. al. (1991) proposed that the decrease in water mobility is due to redistribution of water between the amorphous starch regions and the gluten matrix.

In this study, water mobility was not investigated apart from water evaporation rate on TGA experiments. Only a trial scanning of the sandwiches was undertaken by Magnetic Resonance Imaging (MRI) showing that this technique can possibly provide interesting insights regarding water mobility in the future. MRI is a non-invasive technique that provides digital images of the product and determines changes in water signal intensity. It is possible to illustrate moisture distribution in the bread and spatial distribution of spin-lattice (T_1) relaxation rate and spin-spin relaxation rate (T_2) through mapping techniques. Relaxation times are important as they are connected to water mobility. Specifically, short T_1 and T_2 relaxation times indicate less mobility of water molecules (Ruan, et al., 1996). Gulati, et al. (2015) monitored the T_2 relaxation times in a sandwich contained bread and chicken. By processing the data of T_2 relaxation times, T_2 mapping images were created that revealed moisture migration from chicken to bread.

All in all, samples from the ready-to-eat ham, mustard and mayonnaise sandwich behaved in a relatively similar way regarding changes in the total amount of water, while FW and UFW changed significantly over storage only in the case of MS slice. However, differences on the amount of amylopectin crystals were more pronounced among these samples as the AS slice exhibited higher level of starch retrogradation over storage. The reason for this observation cannot be explained. Nevertheless, the observed increase in the amount of starch crystals in all the samples can probably be the main reason for the reported drier texture of bread. The ready-to-eat ham, farmhouse cheese and pickle sandwich was a interesting example of the impact that a barrier can exert on the physicochemical properties of bread. The presence of butter on the one slice of bread led to moisture loss, whereas the presence of signature 30% mayonnaise on the other slice of bread resulted in an increase in total amount of water. Furthermore, the water release rate was lower in the bread spread with butter than in the one spread with signature 30% mayonnaise and the amount of amylopectin crystals increased more in bread spread with butter. However, as changes in the values of amylopectin melting enthalpy over storage in the slices of the ready-to-eat ham, farmhouse cheese and pickle sandwich were not significant over storage, consumers' feedback on drier texture could not be explained from these experiments.

4.1.5 Correlation between freezable and unfreezable water with free and bound definitions of water

Although in numerous published experiments related to DSC analysis of bread over storage freezable and unfreezable water were connected to bulk and bound water respectively

(Ribotta & Le Bail, 2007; Ottenhof & Farhat, 2004; Hallberg & Chinachoti, 2002; Lodi & Vodovotz, 2008), according to Belton (1997) this relation is not necessarily correct. Belton (1997) supported that water can interact with a polysaccharide in two ways; water can either be placed in the polysaccharide helices cavities, as an internal part of the structure, or it can be trapped in the area between two helices. In the former case, water is able to be exchanged very slowly, while in the latter water molecules can be altered more rapidly. Only trapped in the biopolymer secondary structure water has been found to deviate from bulk water behaviour, based on measurements of the diffusion coefficient and proton relaxation time. However, these water molecules represent only a small fraction of the total amount of water present in the solution. In an attempt to explain how the rest of the water is held in gels so that drainage does not occur, it was proposed that gels have to be considered as porous solids with flexible walls. Water is held inside gel cavities by capillary rise phenomenon and this capture of water results in water activity reduction.

Although Belton (1997) supported that bound water consists a small amount of the total water, the presence of unfreezable water in polysaccharide gels is undeniable and had also to be explained. Belton's (1997) arguments were based on well-established principles. Specifically, even at -30°C liquid water can co-exist with ice, if the relative vapour pressure of water is less than 0.8. This parameter can be achieved in many biopolymer systems. In addition, it was stated that "when both water and ice have the same vapour pressures, there is no mechanism for the net transfer of matter from one phase to another and hence the freezing process stops and a liquid phase continues to co-exist with it."

4.1.6 Changes in ice melting temperature

Ribotta and Le Bail (2007) as well as Hallberg and Chinachoti (2002) detected a decrease in the onset and peak temperature of the ice melting endotherm during storage. This freezing point depression was explained with the loss in FW. In detail, the decline in FW resulted in an increment in solute concentration in water phase, which in turn caused the observed shift of the ice-melting endotherm to lower temperatures (Ribotta & Le Bail, 2007). This argument was also mentioned in Vodovotz, et al. (1996) in an attempt to present the complexity of bread and the impact that a small amount of sugar could have on the thermomechanical properties of the product. In the current work, ice-melting temperatures followed the same pattern as FW. Specifically, when total amount of FW did not change significantly, the ice melting temperatures also remained statistically equal, whereas in the case of MS slices of ham, mustard and mayonnaise sandwich that the FW increased over storage, freezing point temperature also increased. Thus, it was indicated that changes in the ice-melting temperature were monitored due to changes in the concentration of the solutes in water phase, as a consequence of the changes in the amount of FW.

5. Conclusions

Overall, it appears that amylopectin retrogradation is the main reason for the drier texture in the bread of ready-to-eat ham, mustard and mayonnaise sandwiches. Physicochemical changes of water are not clearly related to amylopectin retrogradation, but future MRI and/or NMR experiments could give insights on that aspect. Amylopectin retrogradation also took place in ready-to-eat ham, farmhouse cheese and pickle sandwich but not in a statistically significant level, hence the consumers' feedback is not understood yet. A greater amount of amylopectin crystals and a decrease in the total amount of moisture in the case of the slice of bread spread with butter (and pickle layered on the top) isolated from ready-to-eat ham, farmhouse cheese and pickle sandwich was observed, compared to the respective amylopectin crystals and the increase of total amount of moisture in the case of the slice of bread spread with 30% mayonnaise. This fact could possibly imply that barriers containing small amounts of water can be beneficial in retarding bread staling. However, the exact mechanism of interaction between the bread and the barrier is not yet fully understood. Dynamic Vapour Sorption (DVS) analysis of sandwich bread without the removal of the barrier could possibly provide information regarding the interactions between the two components. All the three slices of bread spread with mustard and mayonnaise sauce collected from ready-to-eat ham, mustard and mayonnaise sandwich, showed that the position of the slices of bread within the packaging did not have a significant impact on the moisture content of bread, even in the case of AS slice. Nevertheless, the higher amylopectin retrogradation level on that slice cannot be explained. The difference on amylopectin melting enthalpy between breads of the two sandwiches was probably observed due to the different nature of the breads and/or the different composition of the barriers and are indicative of the complexity of this research.

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