UNIVERSITÀ CATTOLICA DEL SACRO CUORE

PIACENZA

PHYSICAL AND CHEMICAL FEATURES AFFECTING STARCH DIGESTION IN RUMINANTS

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Abbreviations

ATP – Adenosin triphosphate

NADPH – Nicotinamide adenine nucleotide phosphate

RuBO – Ribulose Biphosphate

DSC-Differential scanning calorimetry

VFA – Volatile fatty acids

RDS – Rapid digestible starch

SDS – Slowly digestible starch

RS – Resistant starch

TS – Total Starch

SFS – Steam flake sorghum

DRS – Dry rolled sorghum

SF24 –Steam flake 24

SF28 –Steam flake 28

TMR – Total mixed ration

PS – Particle size

DSA – Degree of starch access

PAF – Processing adjusting factor

DM – Dry matter

AAM - Apparent amylose

TAM – Total amylose

LAM – Lipid complexing capacity of amylose

T&T – Tilley and Terry method

WEC – Whole ear corn

GM – Ground Maize

RSP – Rolled Shrivelled Pea

RTSP – Rolled Toasted Shrivelled Pea

CRTB – Cold Rolled Toasted Barley

CRTW - Cold Rolled Toasted Wheat

NaOHTW – NaOH-Treated Wheat

M1 – Maize waxy

M3 – Maize flint

HMSc – High moisture shelled corn

HMEc1 and 2 – High moisture ear corn 1 and 2

NDF – Neutral Detergent fibre

CP – Crude Protein

TA – Acetate buffer

TB – Bicarbonate buffer

EM – In vitro enzymatic method

CG – corn grains

ADF – Acid detergent fibre

TCA –Trichloroacetic acid

NPN – Non-protein nitrogen

SD –Standard deviation

Min. - Minimum

Max. – Maximum

EE – Ether extract

Alb/Glob – Albumin and globulin in % of crude protein

aiDM – Acetone insoluble dry matter

1 Introduction

Carbohydrates or saccharides are the most abundant class of biomolecules, and make up most organic matter on Earth due to their wide-ranging roles in all forms of life. They serve as energy stores and suppliers, are present as structural elements of cell walls in bacteria and plants, and form part of the structural framework of RNA and DNA (Ribose and Deoxyribose). Naturally-occurring compounds that consist of carbon, hydrogen and oxygen are produced by one of the most complex, vital, and amazing processes in the physical world - photosynthesis. Each year, photosynthesis converts more than 100 billion metric tons of CO_2 and H_20 into cellulose and other plant products (Lehningher, 2005).

Chemically, carbohydrates are polyhydroxyl aldehydes or ketones, or substances that yield such compounds on hydrolysis. Many but not all carbohydrates have the empirical formula $(CH_20)_{n;}$ some contain nitrogen, phosphorus or sulphur.

Carbohydrates fall into one of three classes: monosaccharides, oligosaccharides and polysaccharides. Monosaccharides, or simple sugars, consist of a single polyhydroxyl aldehyde or ketone unit. The most abundant monosaccharide in nature is the six-carbon sugar D-glucose, sometimes referred to as dextrose. Because of the presence of an active aldehyde or ketone grouping, monossaccharides act as reducing substances. An important property of the monossaccharaides is their reaction with phosphoric acid. A number of sugar-phosphates occur naturally in both plants and animals, and have a very important role in cell metabolism (ex: glucose-6-phosphate and glucose-1-phosphate).

Oligosaccharides consist of short chains of monosaccharide units or residues joined by characteristic linkages called glycosidic bonds. The most common are disaccharides (such as maltose, lactose, sucrose and cellobiose), with two monosaccharide units.

The polysaccharides are sugar polymers containing more than 20 or so monosaccharide unit and some have hundreds or thousands of units. Some polysaccharides, such as cellulose, consist in linear chains; others, such as hemicelluloses, pectins, starch and glycogen, are branched. These polysaccharides consist of a recurring unit of D-glucose, but they differ in the type of glycosidic bond and consequently have strikingly different properties and biological roles.

Most carbohydrates found in nature occur as polysaccharides, polymers of medium to high molecular weight. Polysaccharides, also called glycans, differ from each other in the identity of their recurring monosaccharide units, in the length of their chains, in the type of bonds linking the units, and the degree of branching. They can be divided into homopolysaccharides, containing a single type of monomer, and heteropolysaccharides, which contain two or more different kinds. Some homopolysaccharides serve as a storage form for monosaccharides used as fuels: starch and glycogen are homoplysaccharides of this type, as are cellulose and chitin, which are structural elements of plant cell walls and animal exoskeletons. Heteropolysaccharides provide extracellular support for organisms in all kingdoms.

In animal nutrition carbohydrates are the major source of energy for monogastrics and ruminants. In fact in the latter case they are the largest component of the diet, forages or grains contributing 60 to 70% of net energy used for milk production. Most animal feed is of plant origin and carbohydrates are the predominant component.

Carbohydrates, as mentioned above, are composed of structural units which may be hexoses, pentoses or sugars with acid or amino functional groups or even both, joined to each other by glycosidic bonds (Czerkawski, 1986). The carbohydrates fall into two major groups, sugars and non-sugars, according to their chemical composition (McDonald and Greenhalgh, 1975), and have

recently been classified into non-structural and structural carbohydrates in accordance with their function in plants (Czerkawski, 1986; Mertens, 1992).

Non-structural carbohydrates are located mostly in seeds and sometimes in leaves and stems as reserve carbohydrates that are used by plants to reproduce, grow and survive during stress conditions. Non-structural carbohydrates are primarily starches, fructans, beta glucans and simple sugars.

Starch is present in many plants as a reserve carbohydrate found mostly in seeds but also in fruits, tubers and roots, and occurs in nature as granules, whose shape and size vary among plants. Starch is a highly digestible carbohydrate and is therefore widely used in animal feeding, to increase energy yield and animal performance.

Fructans are polysaccharides of relatively low molecular weight and are found in roots, stems, leaves and seeds as reserve material. These polysaccharides are present particularly in the Compositae and Gramineae plant families. They consist of beta-D-fructose units joined by 2,6 or 2,1 linkages. Besides D-fructose, fructan hydrolysis yields small amounts of D-glucose derived from the terminal sucrose unit in the fructan molecule (McDonald and Greenhalgh, 1975).

Beta glucans are polysaccharides consisting of units of D-glucose joined by ß (1-3) and ß (1-4) glycosidic bonds and in the branching point ß (1-6). They occur naturally in cereal grains (mainly oats and barley and to a lesser extent in wheat and rye), in baker's yeast (Saccharomyces cerevisiae), in fungi and algae (Sealey et al., 2008).

Structural carbohydrates are present in plant cell walls and provide the structural support needed for plants to grow upright. The most important structural carbohydrates are cellulose, hemicellulose, and pectic substances (McDonald and Greenhalgh, 1975; Czerkawski, 1986; Mertens, 1992).

Cellulose is the most abundant constituent of plants, and contributes most to the fibrous structure of plant cell walls. It is a homopolysaccharide of high molecular weight, in which the repeating unit is cellobiose, dissacharide of d-glucose units linked to one another by β1-4 glycosidic bonds. There are certain enzymes that are able to degrade cellulose into cellobiose which can then be hydrolysed into its sub-units of glucose. However these enzymes are

only produced in germinating seed, fungi and bacteria; animals are not able to segregate them. Indeed, cellulose is digested to some extent by microbial fermentation in animals, particularly ruminants.

Hemicelluloses are a complex mixture of polysaccharides which together with cellulose are present in the leafy and woody structures of plants and in certain seeds. The hemicelluloses are based on a backbone of xylose (five carbon) residues; arabinose (five carbons) and uronic acid are also part of the complex. On average the proportions of xylose, arabinose and uronic acid are in a ratio of 80:15:5.

Pectic substances are a group of complex heteropolysaccharides characterised by large proportions of polymethylgalacturonic acid. Pectic substances occur as a component of cell walls and intercellular layers of land plants (Mc Donald & GreenHalgh, 1975; Czerkawski, 1986; Mertens1992).

The nutritional quality of starch is much dependent on its processing and state. The glucose released as a source of energy for the body, and the time-line of digestion, are the major physiological properties of starch (Owens, 2009).

Maize is one of the greatest sources of starch among cereals and is widely used in ruminant nutrition. It can be fed as grain, silage and/or high moisture corn (Michaelet-Doreau and Philippeau, 1999). In the European Community, the area devoted to maize crops is equally divided between grain and silage production, grain being produced mainly by southern countries and silage by northern ones (Michaelet-Doreau and Philippeau, 1999). Grain processing implies the reduction of particle size, with or without the application of heat and steam. Grinding and rolling are the most commonly-used processing methods. Water and steam can be added, as in steam-rolled and steam-flaked grains. Steam and heat application partially gelatinises starch, making degradation by ruminal bacteria and microorganisms faster and more complete, and rendering it more accessible to intestinal enzymes. The effect of processing on the site and extent of starch digestion vary with the processing conditions, grain moisture, screen size, fermentation moisture and time, steaming time, etc. In less-processed grains feed value is influenced by grain variety, agronomic conditions etc.

The intrinsic characteristics of the grain, such as vitreousness, protein matrix, amylose/amylopectin ratio, and lipid content, are described as factors affecting the site and extent of starch digestion. Floury and opaque endosperm types are found to be more degradable than vitreous types. Moreover grain degradability is influenced by the proportion of floury and vitreous endosperm within the grain. As an example, the highest vitreousness, associated with the flint genotype, implies a greater proportion of vitreous compared to floury endosperm, and this grain is therefore less degradable compared to normal dent and waxy grain, as demonstrated by Phillipeau et al. (1997).

The presence of the protein matrix involving the starch granules influences starch degradability as well, by limiting enzyme access to the granules.

In cereals, storage proteins are grouped according to their solubility. Albumins are water-soluble, globulins are extracted using salt solutions, prolamins are obtained with an aqueous-alcohol mixture and glutelins are extracted by acid or alkali solutions.

In corn grains prolamin-zeins make up 50-60% of the total protein and are located in the external surface of the starch granules. During maturation zeins tend to extend and encapsulate starch granules, forming a network. Philippeau *et al*. (2000) observed that zeins were negatively correlated to starch degradability, due to the fact that they surround the granules and limit enzyme degrading.

The amylose to amylopectin ratio also influences starch degradability. Waxy varieties (low content or absence of amylose) are more degradable compared to normal and amylose extender varieties. The amylopectin cluster structure allows greater efficacy of enzyme action, compared to a large linear chain of amylose. Moreover, amylose can form double helices or even complex with lipids, which reduces starch digestibility.

In vivo, *in situ* and *in vitro* techniques give the best estimations of starch degradability, being based on a real ruminal environment. However, the fact that they are laborious, time-consuming, expensive and totally animal-dependent is leading researchers to look for new approaches. Improved

enzymatic methods have been presented to estimate feed starch degradability. The enzymatic methods presented so far have proved to be sensitive to the major factors affecting starch degradability, fast, simple and with high repeatability. However, no comparison with *in vivo* results has been made, making it hard to say whether the values obtained represent feedstuff digestibility or not. However, enzymatic methods can give a rapid and rough indication as to whether a starch is fast, medium or slow to degrade.

The aims of this thesis are: 1) to evaluate the starch degradability of several treated or untreated feeds using *in situ* and *in vitro* rumen techniques; 2) to obtain an accurate and repeatable measure of starch degradability by using an enzymatic evaluation; 3) to compare the enzymatic method with the *in situ* and *in vitro* techniques; 4) to verify the factors determining starch enzymatic digestibility of maize samples at different stages of maturity.

2 Starch

Starch is the principal energy reserve in carbon form in plants, and is one of the most abundant carbohydrates in the biosphere. It is a commonplace material of great nutritional and industrial importance. Starch has an important role as an energy source in both human and animal consumption. Moreover, due to its versatility and use in various industrial applications, it is one of the most well-known polymers in terms of chemical and physical characterisation (Martin & Smith, 1995; French, 1972; Tester et al., 2004; Blennow, 2004).

2.1 Occurrence of starch

Native starches are deposited in granules in various types of plant tissues and organs, including pollens, leaves, stems, roots, tubers, bulbs, rhizomes, fruits and seeds (Preiss, 2004; Tester *et al.,* 2004; Martin & Smith, 1995). Starch is synthesized in leaves during the day from photosynthetically-fixed carbon and is mobilised during the night. It is also synthesised transiently in other organs, such as meristems and root cap cells, but its major site of accumulation is in storage organs, including seeds, fruits, tubers and storage roots. In storage organs, starch synthesis occurs during their development and maturation (Martin and Smith, 1995).

Starch polymers are produced within the plastids, self-replicating organelles surrounded by a double membrane envelope and divided into amyloplasts and chloroplasts on the basis of their function. In chloroplasts starch is synthesised and temporarily stored in the case of sugar abundance

during photosynthesis, whereas the starch generated in amyloplasts acts as a permanent storage of carbohydrates.

Chloroplasts are found mostly in the leaves and have a complex internal structure within the double membrane of the chloroplast envelope which forms the border between the chloroplast stroma and cytoplasm (Figure 2.1). In the inner space there is a complicated membrane body embedded in the fine granular matrix, stroma. These membranes appear as flat stacked visicles and are called granal thylakoids; a stack makes up a granum. The unstacked double membranes forming multiple connections between the grana are called stroma thylakoids (Mohr and Schopfer, 1995). Knowledge of chloroplast structure helps in understanding how and where starch is synthesised, and how its precursors arrive at the storage tissues to be resynthesised and accumulated.

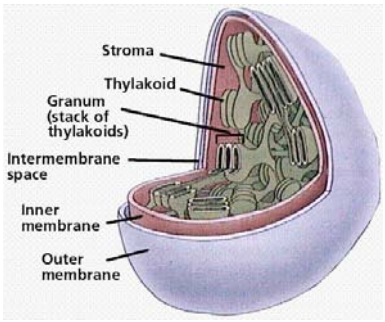


Figure 2.2: Diagram of chloroplast structure.

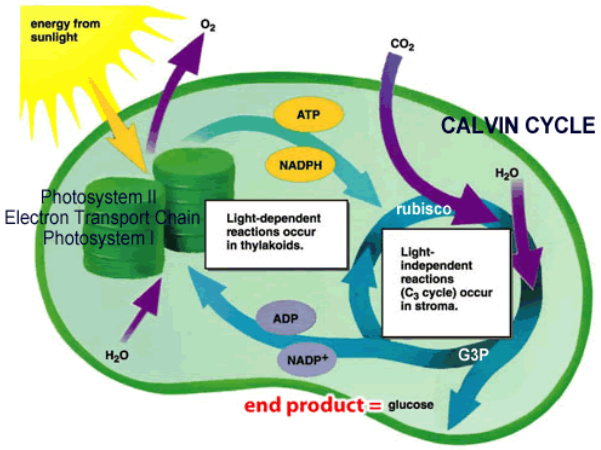


Figure 2.1: Diagram of the photosynthesis process within chloroplasts.

The most complex process in nature, photosynthesis, is based on starch polymer synthesis. Photosynthesis has two distinct stages:

- the light (during daylight) reaction, which converts light energy to ATP and NADPH;
- the dark (during night-time) reaction, which converts CO_2 to carbohydrates using ATP and NADPH.

Both types of reaction occur within chloroplasts (Figure 2.2). The membranes of the lamellae contain photosynthetic pigments and an electron transport system in which the light stage takes place (Figure 2.2). In the light reaction, light strikes the chlorophyll-a in such way as to boost electrons to a higher state of

energy. Each electron is attached to a primary electron acceptor which begins a series of redox reactions, passing the electron through a series of electron carriers. The energy is converted into ATP and NADPH, which are the energy carriers of the dark reaction. In the light reaction water is split, releasing oxygen as a by-product of the reaction.

The dark reaction, also known as the Calvin cycle, occurs in the stroma of the chloroplasts. The Calvin cycle is divided into three phases:

- Phase 1: The carbon from carbon dioxide (CO2) captured from the atmosphere is incorporated into a five-carbon sugar, ribulose biphosphate (RuBP). The reaction is catalysed by the RuBP carboxilase or rubisco. The result form this phase is a six-carbon intermediate, which splits immediately to form two molecules of phosphoglycerate.
- Phase 2: In this phase, ATP and NADPH from the light reactions are used to convert the 3-phosphoglycerate into glyceraldahyde 3-phosphate, the three-carbon precursor of glucose and other sugars.
- The third phase involves using ATP to convert the remaining glyceraldahyde 3-phosphate back to ribulose biphosphate (RuBP), completing the cycle and starting a new one (Mohr and Schopfer, 1995).

The transport of both glucose and fructose in the plants is carried out by sucrose, due to the fact that these two hexoses have no affinity with the phloem, and therefore are not taken up by the sieve tubes to the demanding tissues. Plant tissues such as growing apices, mersitems, young leaves, vegative storage organs, seed and fruits demand a continuous stream of organic molecules, such as glucose, for their development.

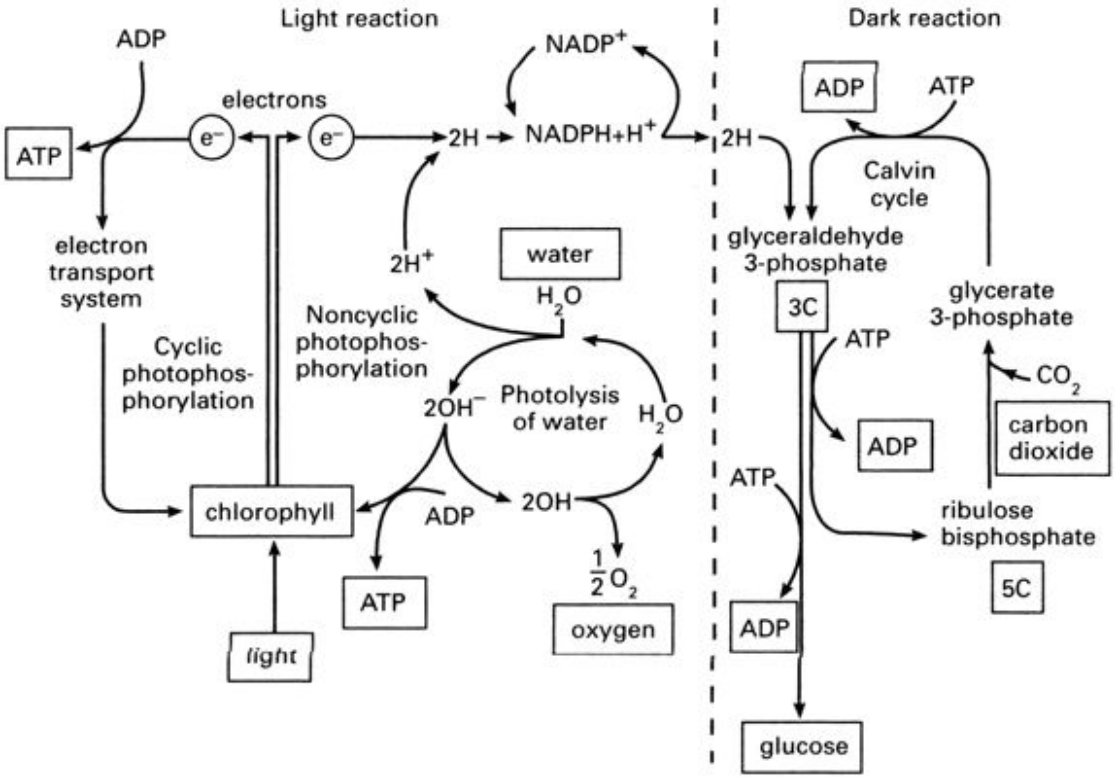


Figure 2.3: Diagram summary of light and dark reactions from the photosynthesis process within chloroplasts. Available at the following URL:
http://content.answers.com/main/content/img/oxford/Oxford_Chemistry/0192801015.photosynthesis.1.jpg

Amyloplasts are found in non-photosynthetic tissue, such as in the cells of the endosperm, and it is in this tissue that starch is synthesised and stored. Starch synthesis in grains starts simultaneously with the second stage of cereal grain development, i.e. filling (Martin and Smith, 1995; Preiss and Sivak, 1996).

The starch precursor is sucrose, which needs to be converted to glucose so it can be taken into the amyloplast, given that the inner envelope is practically impermeable to sucrose. Sucrose is also the main carbohydrate transported in higher plants, and the phloem provides a means for long-distance movement.

The sucrose is loaded into the phloem by two basic mechanisms. The first is the symplasmic pathway, in which metabolites are moved between cells via plasmodesmata. Plasmodesmata are intracellular structures which provide potential symplasmic continuity between adjacent cells; they connect the mesophyll cells and elements of the phloem such as bundle sheath cells, phloem parenchyma cells, companion cells and sieve elements. The second

mechanism is the apoplast pathway. In this mechanism metabolites are first exported to the phloem apoplast from non-phloem cells by an unknown process. The organic N and C compounds are then taken up into a sieve element/companion cell complex of the phloem by an energy-requiring plasma membrane transport step. Both loading mechanisms result in an increase in solutes in the sieve elements, which is important in generating a driving force for phloem translocation (Tegeder and Weber, 2006). Cereal grains have no direct vascular connections with the parent plant and so a short-distance mechanism operates to move sucrose from the vascular tissues to the endosperm (Ermes et al., 2003).

In maize grains, the transport of sucrose from the phloem to the endosperm occurs via sieve elements of the pedicel. Assimilates entering the pedicels of maize are transferred symplastically from the phloem to the palcentochalazal region. The pedicel is overlaid by a compressed cell layer and a group of large cells, the placentochalazal cells. The sucrose moves simplastically through the pedicel to unload in the free space formed by the placentochalazal zone. This zone contains the major storage pool within the pedicel. Sucrose exported from the phloem is converted into hexoses by a very active acid invertase located near the pedicel tissue. The sucrose is then resynthesised in the endosperm after the entry of the hexoses. The invertase enzyme controls the concentration gradient of hexoses between the phloem and pedicel apoplast, and mediates the soluble carbohydrate gradient across the placentochalazal zone and the endosperm, important for the structural stability of this zone and for grain filling (Prioul, 1996).

In wheat grains, assimilate transport occurs within a furrow running along the length of the kernel, with a vascular bundle embedded at the bottom. Assimilates are first transferred symplastically through the chalaza and then into the apoplast via the specialised transfer cells in the nucellus, before reaching the outer layer of the endosperm where transfer cells in the aleurone layer redirect the solutes back to the symplasm and into the endosperm cells (Emes et al., 2003).

In the first step of starch biosynthesis, sucrose is metabolised into fructose and uridine diphosphate glucose (UDP-Glucose). This conversion takes place into the cells cytosol; as mentioned before, only hexoses can be translocated into the amyloplast. Fructose is then converted into fructose-6-phosphate (Fruc-6-P) by a fructokinase, and further into glucose-6-phosphate (Glc-6-P), by the cytosolic phosphate hexose isomerase. The UDP-glucose is metabolised into glucose-1-phosphate (Glc-1-P) by the UDP-glucose phosphorilase and further into Glc-6-P by a phosphoglucomutase (P-glucomutase). The two hexoses are transferred from the cytoplasm into the amyloplasts via translocators present in the amyloplast membranes. Inside the amyloplast the hexoses are metabolised into Glc-1-P by plastidial P-glucose mutase and then converted into ADP-glucose by ADP-glucose phosphorilase to the non-reducing end of the growing α-(1,4) glucans and is involved on amylose and amylopectin synthesis.

ADP glucose has been identified as an exogenous precursor of starch biosynthesis. Most scientific works have indicated ADPGlc as the starch precursor within the plastids. In recent works this idea has been abandoned, as the result of the identification of the isoenzyme ADPGlc phosphorilase (ADPGlcPase) in the cytosol of barley and maize endosperm, which is the enzyme responsible for Glc-1-P phosphorilation into ADPGlc in the plastids. Therefore there is reason to believe that this metabolite could also be metabolised in the cells cytosol. Some scientific works have confirmed the presence of this enzyme in the endosperm cells of cereals such as wheat, maize and barley and that it is responsible for 85 - 95% of the starch synthesis activity (Prioul .J.L., 1996; Preiss and Sivak, 1996; Bulèon et al., 1998; Emes et al, 2003; James et al. 2003; Tester et al.

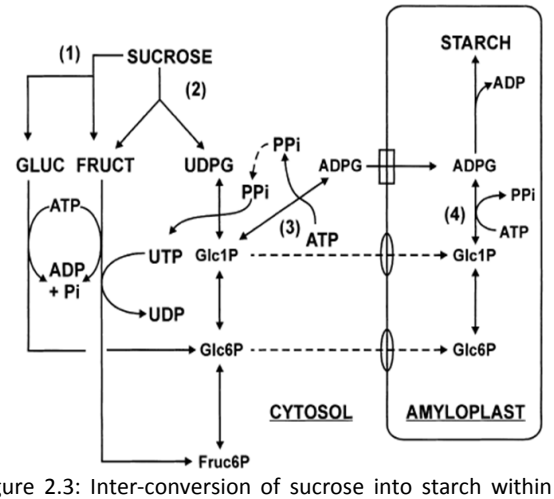


Figure 2.3: Inter-conversion of sucrose into starch within the developing endosperm. Enzymes shown are (1) invertase, (2) sucrose synthase, (3) cytosolic AGPase, and (4) plastidic AGPase. (From: Emes *et al*. 2003, *J Exp. Bot.* Vol. 54, **No**.382, pp. 569-575).

2004). Figure 2.4 illustrates starch biosynthesis in storage tissues as has been described.

2.2 Starch Granule Structure

Most naturally-occurring starch granules, regardless of plant source or tissue, consist of two main types of polysaccharides, namely amylose and amylopectin. Both are polymers of only α-D-glucose, connected by (1-4) (1-6) linkages into shorter or longer chains.

2.2.1 Amylose

Amylose consists entirely of α-(1-4) linked glucose units, though some studies have revealed that it also can be slightly branched in proportions of 99% (1-4) and 1% (1-6) bonds (French, 1973; Buléon, 1998; Tester *et al.*, 2004; Bertof, 2004). Molecular weight measurements indicate that amylose contains hundreds or thousands of glucose units, approximately $1*10^5$ – $1*10^6$ (French, 1973; Tester *el al.*, 2004).

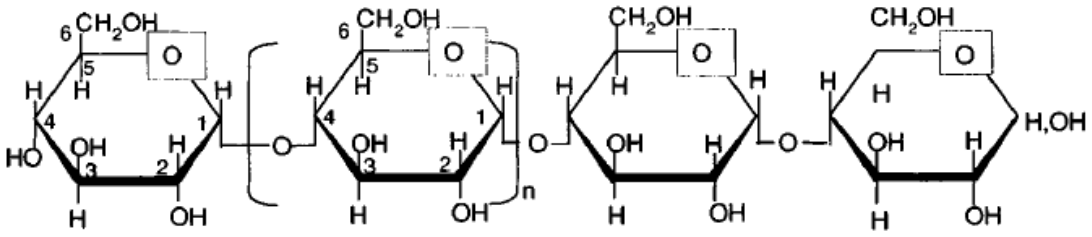


Figure 2.5 Amylose structure, α (1-4)-glucan. Adapted from: Tester *et al*, 2004. Journal of Cereal Science 39:151-165.

Each amylose chain is composed of at least of 1000 glucose units. Simplified models of the structure of amylose are shown in Figure 2.5; though it is typically illustrated as a straight chain structure, amylose is actually often helical. The interior of the helix contains hydrogen atoms and is therefore hydrophobic, allowing amylose to form a type of clathrate complex with fatty acids, fatty acid components of glycerides, some alcohols and iodine. Iodine complexation is an important diagnostic tool for the characterisation of starch; it allows a distinction to be made between amylose and amylopectin, and the

quantification of amylose content in native starches (Rooney and Pflugfelder, 1986; Bulèon *et al*., 1998, Parker and Ring, 2001; Tester *et al*., 2004).

Linear chains of amylose form a dark blue complex with polyiodide ions in aqueous solution at room temperature. This reaction provides a basis for defining amylose, which under standardised conditions binds 20% of its weight of iodine, while under the same conditions amylopectin binds less than 1% (w/w), (Parker and Ring, 2001). The content of amylose in most starches ranges from 20 to 30%; however, there are certain mutant plants carrying a particular gene, commonly known as 'waxy', which have a lower content (<1%), or even a complete lack of amylose, and others, such as amylomaize, which contain around 65% amylose (Buléon 1998; Parker and Ring, 2001).

Based on amylose content, a classification of starch has been established: *waxy*, when the ratio of amylose/amylopectin is lower than 15%; *normal*, when amylose represents 16-35% of all starch, and *high-amylose* or (*amylo-*), when amylose content exceeds 36%; Table 2.1 shows the amylose content of some major plants sources (Buléon et al, 1998).

Table 2.1: Amylose and Amylopectin content, and ratio of amylose/amylopectin of major plant sources

Source	Amylose (AM) content (% of total starch)	Amylopectin(AP) content (% of total starch)	Ratio AM:AP
Barley normal	21-24	79-76	1:4
Wheat normal	25-29	75-71	1:3
Wheat Waxy	1.2-2.0	98.8-98	1:50
Maize normal	25-28	75-72	1:3
Maize waxy	0.5	99.5	1:99
Maize high-amylose	60-73	40-37	2:1
Potato normal	18-21	82-79	1:5
Potato amylose free	1	99	1:99

Adapted from: Buléon *et al*., 1998. International Journal of Biological Macromolecules 23:85-112.

Waxy mutants are commonly available for barley, maize and rice and have been developed more recently for wheat and potato. High-amylose mutants are predominantly from maize (Fench, 1973, Buléon 1998, Tester *et al.,* 2004).

Breeding plants with different ratios of amylose/amylopectin helps further understanding of the role of each molecule within the granule, as well as providing immense commercial value, because these plants provide a 'natural' means of modifying and extending the range of functionality of a specific starch type, as illustrated in Table 2.2 (Oates, 1997).

Table 2.2: Influence of the major starch fractions on the properties of maize starch

Source	Amylose content (%)	Properties
Waxy maize	0-1	Non-gelling, low-setback and clear paste; paste that is resistant to syneresis; elastic and stringy paste.
Maize	27	Firm gel; opaque paste; short paste texture.
Amylomaize	50-70	Granule that is resistant to swelling; opaque paste; high paste temperature.

From: Oates, C.G., 1997 – *Trends in Food Sci. & Tech.* Vol.8

2.2.2 Amylopectin

Amylopectin is one of the largest molecules found in nature and is the major component of starch; it forms up to 80% of starch. Amylopectin consists of a highly-branched polymer, composed of D-glucose units linked together by alpha-1,4 linkages, but with 5-6% of alpha- 1,6 bonds and the branch points, as shown in Figure 2.6 (French D., 1973; Martin & Smith, 1995; Géraldine *et al.,* 2003; Karkalas & Tester, 2004). Amylopectin is a much larger molecule than amylose, its molecular weight ranging between $1*10^7$-$1*10^9$, given that this

polysaccharide can be composed of up to two million glucose units (Tester *et al.*, 2004, French 1973).

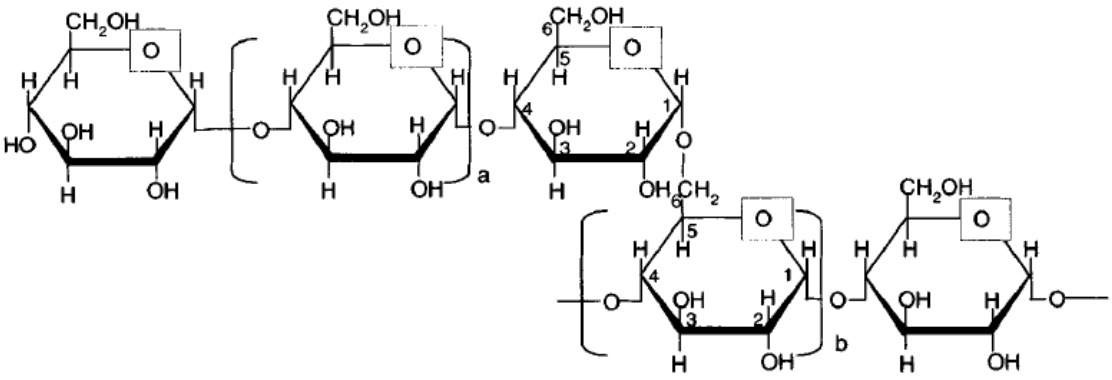


Figure 2.4: Amylopectin structure, α (1-4)-glucan with α (1-6) in the branch points. Adapted from: Tester *et al.*, 2004. Journal of Cereal Science 39:151-165.

Amylopectin is built of three types of chain, A, B and C. The outer, A-chains are glycosidically linked at their potential reducing group through C_6 of a glucose residue to an inner, B-chain. These chains are in turn defined as chains bearing other chains as branches. The macromolecule contains a single C-chain which likewise carries the other chains as branches but contains the sole reducing end group, as shown in Figure 2.7. Amylopectin, like amylose, is able to form double helices but only with the exterior, short A-chains. The outer chains from cereal amylopectin molecules, with chain lengths of 14-20 glucose units, favour the formation of A-type double helices, whereas longer outer chains, such as in tubers, favour B-type double helices, as represented in Figure 2.8 (Tester *et al.*, 2004).

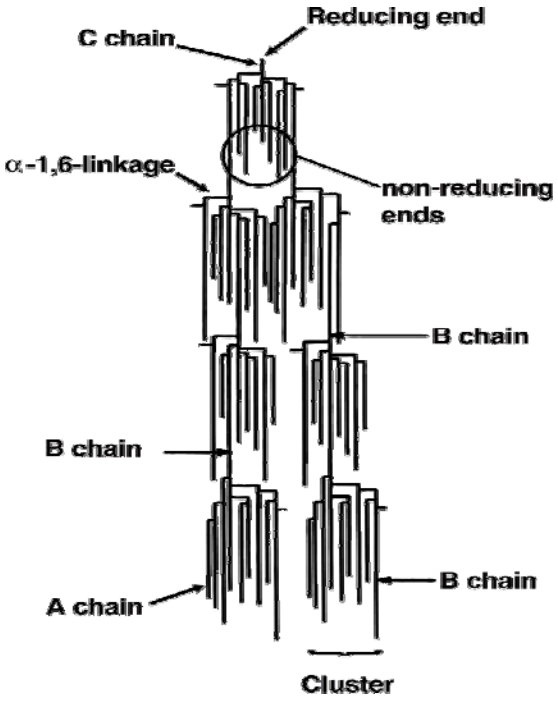


Figure 2.7: Schematic representation of Amylopectin structural organization. Available at URL: http://www.jic.ac.uk/staff/cliff-hedley/graphics/cluster.gif

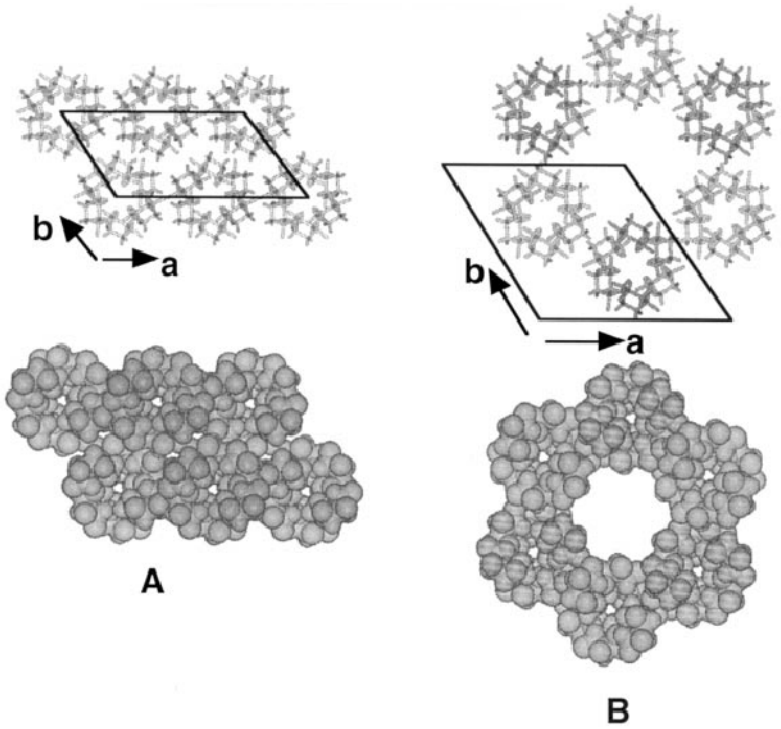


Figure 2.8: Differences in packing densities of the helices in A- and B-type starches. The view is looking down into the double helices. From Buléon *et al.*, 1998 – Int.J. of Biol. Macromolecules 23: 85-112.

2.2.3 Granules

Starch is accumulated in plant tissues as discrete particles or granules. Depending on their origin, granule diameter ranges from 1μm up to 100μm. As mentioned before, starch is composed of two types of polymers, amylose and amylopectin, which are organised in granules as alternating semi-crystalline and amorphous layers, as shown in Figure 2.9. The semi-crystalline layers consist of ordered regions composed of double helices formed by short amylopectin branches, most of which are further ordered into crystalline structures. The amorphous regions of the semi-crystalline layers and the amorphous layers are composed of both amylose and amylopectin α-(1-6) branch regions, as can be observed in Figure 2.9. Starch from storage organs shows internal semi-crystalline growth rings that are differentially sensitive to chemical and enzymatic attack. The formation of these rings may result from periodic differences in the rate of starch synthesis.

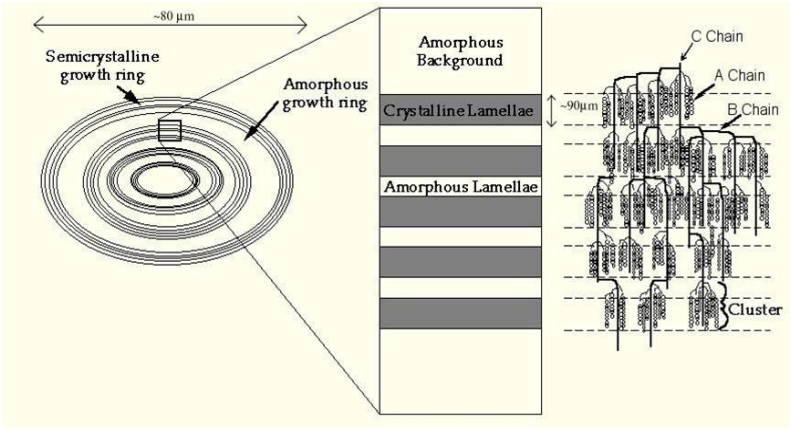


Figure 2.9: Schematic view of the structure of a starch granule, with alternating amorphous and semi-crystalline zones constituting the growth rings. Adapted from: Jenkins and Donald, 1995. International Journal of Biological Macromolecules, 17:315-321.

The crystallinity is determined by wide-angle X-ray diffraction scattering and is referred to as 'A-type' (found in most cereals), 'B-type' (found in most tuber starches) or 'C-type' (a mixture of both forms found mostly in legume starch). This characterisation derives from the way the double helices comprising the crystallites are packed. In the A-type, crystallites are densely packed in an orthogonal pattern, while in B-type they are less densely packed in a hexagonal pattern. Both types of crystallite contain structural water, the amount and mobility of which is greater in B-type crystallites, as shown in Figure 2.7. Starch shape varies according to its botanic origin, from spherical, as in potato starch, to polyhedral, as in maize starch; see Table 2.3.

Table 2.3: Characteristics of native starch granules from common sources

Starch	Size (μm)	Shape	Distribution
Barley	2-5 (B-granules)	Spherical	Bimodal
	15-25 (A-granules)	Lenticular	
Maize (waxy and normal)	2-30	Spherical/Polyhedral	Unimodal
Amylomaize	2-30	Irregular	Unimodal
Millet	4-12	Polyhedral	Unimodal
Oat	3-10 (single)	Polyhedral	Unimodal
	80 (compound)		
Pea	5-10	Rentiform	Unimodal
Potato	5-100	Lenticular	Unimodal
Rice	3-8 (single)	Polyhedral	Unimodal
	150 (compound)		
Rye	5-10 (B-granules)	Spherical	Bimodal
	10-40 (A-granules)		
Sorghum	5-20	Spherical	Unimodal
Tapioca	5-35	Spherical/Lenticular	Unimodal
Triticale	1-30	Spherical	Unimodal
Wheat	2-10 (B-granules)	Spherical	Bimodal
	15-35 (A-granules)	Lenticular	

(Adapted from R.F. Tester *et al.*, 2004). Unimodal: possess single type of starch granule; Bimodal possess two types of granule (Type A and Type B).

According to Buléon *et al.* (1998), the biosynthetic pathway can shape the granule in accordance with physical constraints encountered during cellular starch deposition due to the presence of other subcellulae compounds, such as

the shape assumed by the amyloplast membrane. Some sources contain compound granules, corresponding to the fusing of different granules developing simultaneously within a single amyloplast. Other special forms occur in high-amylose types where non-symmetrical shapes are observed. Some cereals present large (A-granule) and small (B-granule) granules, whose biosynthesis occurs at two different stages of development, as shown in Figure 2.10.

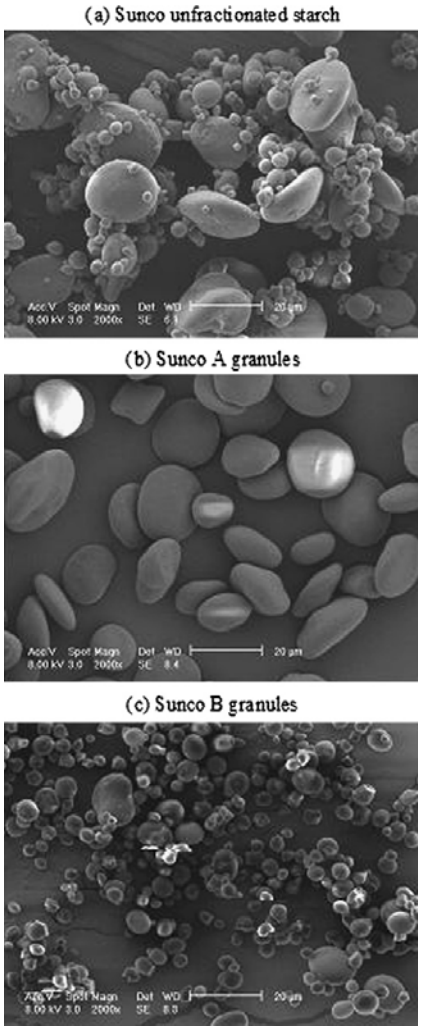


Figure 2.10. Representation of A- and B-type granule from Wheat adapted from: Salman *et al.,* 2009. *Carbohydrates Polymers.*

When observed under a polarised light microscope, starch granules exhibit a characteristic shadow, called the 'Maltese Cross', which is a phenomenon known as birefringence. This expresses a high degree of order, i.e. molecular orientation within the starch granule, which is not to be mistaken with crystallinity (Horseney, 1986; Rooney and Pflugfelder, 1986; Tester *et al.*, 2004).

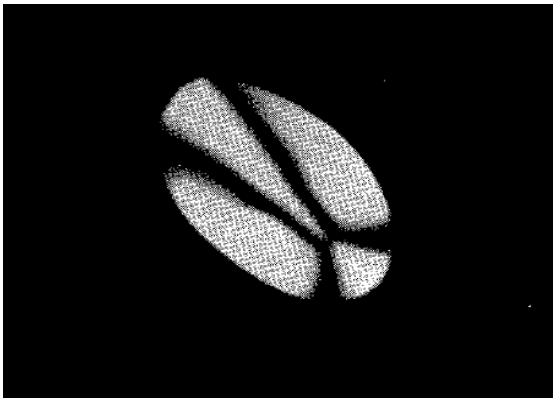


Figure 2.11 : Potato starch granule under the polarised microscope, crossed polariser, showing the 'Maltese Cross' pattern. Adapted from: French, 1973. Journal of Animal Science 37:1048-1061.

The apparent intensity of birefringence depends on the thickness, size, shape, molecular structure, and orientation of the granule. Normally when a starch granule presents birefringence it is considered to be in its native state. Starch granules lose their birefringence when gelatinisation occurs (Horseney, 1986; Rooney and Pflugfelder, 1986).

2.3 Minor components of starch

Minor components such as proteins, lipids, water, and ash (minerals and salts) are found in the starch granules in very small quantities, but have profound effects on the properties of the starch. Moisture content in starches ranges between 10-12% in cereals, and 14-18% in some tubers and roots. Ash content, although variable, is typically less than 0.5% (dry basis) (Buléon *et al.*, 1998; Tester R.F. et al., 2004)

2.3.1 Lipids

Lipids are the most important fraction associated with starch granules. Generally the highest contents are observed in cereal starches (0.8-1.2% and 0.6-0.8% for wheat and normal maize, respectively) (Buléon *et al.*, 1998). Lipids can be found on the surface and inside the granules (Buléon et al. 1998; Tester et al. 2004). Starch granules can also be contaminated with surface lipids, including triglycerides, glycolipids, phospholipids and free fatty acids derived from the amyloplast membrane and non-starch sources (Morrison W.R. 1995, Buléon *et al.* 1998; Tester R.F. *et al.*, 2004). The location of the lipids on the surface is still unknown (Buléon *et al.*, 1998); the presence of internal lipids is a characteristic of cereal starches (Buléon *et al.*, 1998).

Cereal starch is characterised by the presence of integral lipids, lysophospholipids (LPL) and monacyl lipids (free fatty acids (FFA)), and their structure allows them to form a complex with amylose. The amount of LPL may account for up to 1.36% of starch weight. The amount of these lipids is positively correlated with the starch amylose fraction (Morrison WR, 1993, Tester *et al.*, 2004). Starches from different botanical origins have different species of lipids: wheat, barley and rye and other triticale starches contain mainly LPL, whereas other cereal starches such as normal maize starch contain mainly free fatty acids, glycerides and few phospholipids (Buléon *et al.*, 1998; Tester *et al.*, 2004).

Buléon *et al.* (1998) report that in starch from wheat and barley harvested at different stages of development, the amylose and LPL content increase with maturity. Cereal waxy starch contains few lipids, whereas high-amylose starches contain substantially more lipids, as shown in Table 2.4. Root and tuber starches contain very few lipids and no detectable phospholipids (Buléon *et al.*,1998).

Table 2.4: Free fatty acids and lysophospholipids present in purified cereal starches.

Source		Free fatty acid content range	Lysophospholipids content range
Barley			
	Waxy	0.03-0.04	0.12-0.75
	Normal	0.03-0.05	0.47-1.14
	High-amylose	0.05-0.09	0.86-1.36
Maize			
	Waxy	0.01-0.05	0.01-0.03
	Normal	0.30-0.53	0.16-0.35
	High-amylose	0.38-0.67	0.26-0.61
Rice	Normal	0.22-0.50	0.41-0.86
Wheat			
	Normal	0	0.78-1.19
	Waxy	0	0.07-0.17

Values represent % total dry weight; data quoted in Morrison and co-workers. Adapted from: Buléon *et al.*, 1998. International Journal of Biological Macromolecules 23:85-112.

2.3.2 Proteins

Starch granule proteins can be divided into two types on the basis of the possibility of extracting them from the granules. Surface starch granule proteins can be extracted with salt solutions, whereas integral starch granule proteins require more rigorous extraction, for example using the detergent sodium dodecyl sulphate or an alkaline solution (Thomas, D.J. and Atwell, W.A., 1998).

The presence of proteins in starch is highly correlated with the storage proteins that are accumulated in cereal seeds during maturation and starch deposition. Seeds storage proteins have been grouped based on their solubility characteristics. Albumins are water soluble, therefore extracted with water; Globulins are obtained with salt-solutions; Prolamin extraction is carried out with alcohol-water mixtures; finally Glutelins are released with diluted acids or alkali solutions. This distinction is no longer valid, because proteins that are structurally similar but belong to different groups are considered as being in the same group. For example, prolamins lacking interchain disulfide bonds are soluble in an aqueous-alcohol mixture, as expected, but those having disulfide bonds are alcohol-insoluble and are classified, incorrectly, as belonging to the glutelin groups. This inconsistency has been eliminated by redefining glutelins as part of a different class of prolamins.

Prolamins occur only in cereal endosperm and in other grasses (*Poaceae*) where they are the major storage protein, with the exception of rice and oats where glutelin-type prolamins and globulins are predominant. Prolamins from barley, rye and wheat consist of highly polymorphic mixtures of polypeptides with molecular masses of 30 to 90 KDa. They are divided into three groups according to the amino-acid sequence: sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular weight (HMW) (Shewry and Halford, 2001). The S-rich make up 90% of known prolamin proteins.

Prolamins are characterized by high glutamine and proline content. Proline is a highly hydrophobic amino acid capable of complex folding; thus proteins with high proline contents develop tertiary structures that are intensely hydrophobic and are soluble in aqueous alcohol solutions (Momany *et al.*, 2006). They are named according to the cereals where they are found, i.e.

wheat – gliadins; oats – avenins; barley – hordeins; maize – zeins and sorghum – karifin (Mahanna B. 2009).

In maize, prolamin-zeins comprise 50 to 60% of the total protein of whole grain. Prolamins are synthesised in the rough endoplasmic reticulum from the amyloplast. In maize four sub-classes of zeins have been indentified (α, β, γ, δ) (Mu-Foster and Wasserman, 1998; Buchanam *et al.,* 2000). Prolamin-zeins were found associated with the external surface of the starch granules, encapsulating them. Studies have revealed that with the advance of maturity zeins enlarge and distend, forming a kind of network involving endosperm starch granules, often called a protein matrix (Mu-Foster and Wasserman, 1998; Buchanam *et al.,* 2000). Prolamins are generally associated with the corn grain endosperm type. Floury and opaque endosperms have been proven to have a lower prolamin content compared to flint and normal dent corns (Hammaker *et al.,* 1995; Landry *et al.,* 2000). Philippeau *et al*. (2000) suggest that prolamin-zein content in corn grains is linked to grain vitreousness, where vitreous flint corns contain more prolamin-zeins compared with less vitreous counterparts. Starch granules in vitreous endosperm are more extensively incapsulated compared to floury and opaque endosperms (Hoffman and Shaver, 2009). Prolamin-zein content in corn grains is also associated with lower digestibility of starch by ruminants; this issue is further developed in the chapter on Digestion.

Albumins are present in the seeds of both mono- and dicotyledons. The 2S albumins are widely distributed in dicot seeds. They are present in cereals and act as inhibitors of trypsin, α-amylase or both. The 2S albumins are compact globular proteins with conserved cystein residues.

Globulins are the most widespread group of storage proteins, and can be divided into two groups according to their sedimentation coefficient: 7S vicilins, and 11S legumins. The 7S vicilins are trimetric proteins of 150 to 200 KDa, and are located only in the embryo and aleurone layer in cereals. These globulins are stored in protein bodies and appear to function solely as storage proteins (Shewry and Halford, 2001). Mature 11S legumins have molecular masses of 300 to 400 KDa, and are more abundant that 7S vicilins in dicots. Oats, rich in

globulins, contain the 11S storage protein within the endosperm region. Globulins present in the aleurone layer and in the embryo, when compared with those present in starchy endosperms, have a limited impact on the end use properties of the grain (Shewry and Halford, 2001).

2.3.3 Minerals

Starches also contain trace amounts (less than 0.4%) of minerals such as calcium, magnesium, phosphorus, potassium and sodium, a part forming phosphorus of little functional significance. Phosphorus in cereal starch is mainly present in the form of phospholipids. Phosphate monoesters and inorganic phosphates are less common. Root and tuber starches are unique in that they contain phosphate monoesters, with an exceptionally high level in potato (0.089%), (Buléon *et al.*, 1998; Tester R.F., 2004).

2.4 Gelatinisation

Native starch granules are relatively inert and insoluble in cold water. When heated above a critical temperature in abundant water (>90% w/w), starch granules lose their native crystalline structure, a phenomenon termed gelatinisation (Parker and Ring, 2001). Starch granules undergo this process, or irreversible loss of their native structure, when sufficient energy is applied to disrupt intermolecular bonds in the crystalline areas (Rooney and Pflugfelder, 1986; Tester *et al.*, 2004). Gelatinisation starts in the amorphous layers, while within the crystalline regions the penetration of water and heat occurs slowly (Rooney and Pflugfelder, 1986). During gelatinisation the internal structure of the starch granules disintegrates, and some of the polysaccharide (amylose) leaches out of the granules into the surrounding media. Significant swelling takes place during the process (Donald, 2001, Svihus *et al.*, 2005). Gelatinisation commences in water and with temperatures of between 50 and 60ºC, and granule swelling is reversible. However, if the temperature is raised gradually from 60 to 80ºC, most starches undergo irreversible gelatinisation, losing their crystallinity, also termed birefringence (French, 1973, Svihus *et al.*, 2005). The gelatinisation of cereal starches can be caused by physical,

mechanical and thermal, or chemical agents, or by various combinations of these agents, (Rooney and Pflugfelder, 1986). Gelatinisation resulting from physical methods depends on grain moisture content, and normally occurs during the milling and grinding of cereals (Rooney and Pflugfelder, 1986).

Physicochemical treatments such as expanding, steam-flaking and extrusion, involve steam (water and heat) application, in which grains are subjected to high pressure, heat and moisture. These processes hydrate and swell the amorphous and crystalline layers of starch granules, and polysaccharides become more susceptible to enzyme degradation, for example during animal digestion (Svihus *et al.*, 2005). Chemical and physical treatments are normally used to process food/feed cereals to enhance digestibility by enzymes and microorganisms.

The determination of starch gelatinisation can be carried out by enzymatic measurements of starch degradation and by differential scanning calorimetry (DSC). As reported by Svihus *et al.*, (2005), the enzymatic method consists in the measurement of the amount of starch degradation after incubation with amyloglucosidase for a certain time. This method is based on a linear relationship between the extent of gelatinisation and susceptibility to enzymatic degradation.

Instead DSC is a technique that measures the energy absorbed by a substrate and by an inert reference material, as both are subjected to identical temperatures in an environment heated or cooled at a controlled rate. The energy absorbed by the gelatinised sample can be quantified as the area of the curve plotted against the temperature axis. This method can also provide the onset, peak and end temperature for gelatinisation.

2.5 Retrogradation

The term ‘starch retrogradation’ has been used to describe changes in physical behaviour following gelatinisation. Retrogradation is a reassociation of starch molecules separated during gelatinisation, resulting in the liberation of bound water from the paste or gel. The hydrogen bonds between the amylose and the A-chains of amylopectin are reformed, although the retrogradated starch does

not regain is native structure (Rooney and Plfugfelder, 1986). Retrogradation occurs when gelatinised starch is rapidly cooled to room temperature or even lower, favouring crystallization (Parker and Ring, 2001).

The extent of retrogradation depends upon several factors, including the fine structure of amylose and amylopectin, moisture content, temperature, complexing agents (especially lipids) and concentration of starch in the system (Rooney and Pflugfelder, 1986; Svihus *et al.*, 2005). Retrogradation can be beneficial, as in case of edible amylose films for protecting candies and boil-in-the-bag vegetables. However, it is more disadvantageous in foods such as starch-based pie fillings which increase in density and release water when retrograded (Rooney and Plfugfelder, 1986). Starch retrogradation in feed may decrease digestibility, but probably increases the durability of steam-flaked grains. Retrogradation will be further discussed in Chapter 3, with reference to the effect of processing methods on digestibility.

3 Methods for Estimating Starch Digestibility

Starch is a major energy-yielding component of cereal grains, which have an important dietary role in intensive livestock production. Starch digestibility and the site of starch digestion have a great effect on the amount and profile of substrates absorbed from the digestive tract (Huhtanen and Sveinbjornsson, 2006). The efficiency of starch digestion by ruminants is of major importance, in terms of nutrient availability. The rate and site of starch digestion in the total digestive tract of ruminants has been discussed (Offner *et al.*, 2003; Offner and Sauvant 2004; Huhtanen and Sveinbjornsson, 2006).

The rumen is the main site of starch digestion, where 60-95% of the starch intake is fermented into volatile fatty acids (VFA) and gas incorporated into microbial matter, (Ørskov, 1986; Nocek and Tamminga, 1991). The rate and extent of starch digestion in the rumen depends on complex interrelations between several factors, well reviewed by Nocek and Tamminga (1991), Huntington (1997), Offner *et al.* (2003), Offner and Sauvant (2004), Huhtanen and Sveinbjornsson, (2006). The dynamics of nutrient digestion in the reticulo-rumen are one of the major determinants of the utilisation of feedstuffs by

ruminants (Offner *et al.*, 2003). Feed evaluation systems have improved, moving towards dynamic models and the prediction of production in response to nutrient substrate.

Several approaches have been adopted with the aim of finding a dynamic model of starch digestion in an attempt to improve the prediction of starch supplies on the profile of absorbed substrates, taking into account the effects of the site and rate of starch digestion (Huhtanen and Sveinbjornsson, 2006). Prediction method accuracy depends greatly on the precision and accuracy of the methods used to determine the site of digestion and the kinetic parameters of starch digestion. Improvements in this field are limited by the possibility of obtaining reliable *in vivo* reference data, technical difficulties in duodenal starch measurements and alternative laboratory methods. Feed digestibility can be estimated using *in vivo* and *in vitro* techniques. Almost all the digestibility methods used so far require fistulated and/or cannulated animals.

3.1 *In vivo* measurements

In vivo trials require fistulated and/or cannulated animals, where feed digestibility is estimated by rumen absorption patterns using the *in situ* nylon bag technique, the rumen evacuation technique and duodenum flow.

The best perception of feed behaviour along the digestive tract is achieved when data regarding starch digestion from different segments of the digestive tract are also available. Starch digestibility measurements in the total tract can be made by total faecal collection or by using marker techniques (Huhtanen and Sveinbjornsson, 2006). The determination of ruminal starch digestibility is typically carried out by collecting omasal and abomasal samples or by a duodenal cannula or by using markers.

The most commonly-used cannula is the T-piece cannula. However, as Huhtanen and Sveinbjornsson (2006) reported in their review, simple T-piece cannulas have been widely criticised because they result in the separation of fluid and particles, compared to true digesta, leading to unrepresentative samples.

The use of markers was suggested to overcome the problem of representativeness of sampling, though marker inaccuracies can induce erroneous flow estimates. Readers are referred to a work by Owens and Hanson (1992), in which goals, procedures and other aspects related to the use of markers for site and extent evaluation of digestion in ruminants are described. The close T-piece cannula is reported to be more efficient given that it permits a complete diversion of the flow, but not for heterogeneous samples. The unrepresentativeness problem persists, and it is difficult to overcome the problems of diurnal and random variation in the composition of digesta (Harmon and Richards, 1997). Moreover there is no specific marker for each type of feed, once feed particle physical characteristics are different from each other. For example, in starch digestion studies, none of the makers showed specific affinity with starch, so the problem of unrepresentative sampling persists (Huhtanen and Sveinbjornsson, 2006).

3.1.1 Omasal and abomasal cannulas

Omasal and abomasal cannulas have also been used to investigate ruminal digestion in cattle (Harmon and Richards, 1997). Huhtanen *et al*. (1997) develop an alternative system that estimates the nutrient flow entering the omasal canal. Omasal canal sampling, when compared to duodenal and abomasal sampling, has the advantage that surgical intervention is reduced and measurements are less influenced by endogenous secretions (Ahvenjärvi *et al*., 2000). Experimental access to the omasum, although possible, is difficult. Procedures directly cannulating the omasum, although suitable for obtaining samples of omasum digesta, would be unsuitable for obtaining representative sampling of ruminal flow (Harmon and Richards 1997). In order to improve the accuracy of omasal canal measurements, Ahvenjärvi *et al*. (2003) developed a system using double and triple markers, although due to separation of fluid and particles and also the fact that the sampling device might discriminate large particles, the problem of unrepresentative samples persists (Huhtanen and Sveinbjornsson, 2006).

Although used in digestion studies, abomasal cannulas are less popular, because of many physiological aspects of the animal, as well as possible sampling difficulties compared to other methods. Physiological aspects include the low pH within the abomasum, which makes it difficult to choose the most appropriate cannula material. Moreover the motility of the abomasum is prone to prolapse after extended periods (Harmon and Richards, 1997).

Some studies have used measurements of portal-drained visceral (PDV) glucose flux and observed that glucose absorption is low relative to the amount of starch flowing into the small intestine (Reynolds et al., 1997).

3.1.2 Rumen evacuation technique

The rumen evacuation technique has been used to estimate the passage and digestion rate of the neutral detergent fibre (NDF), and it has also been used to estimate the rumen digestion rate of starch, which assumes a first-order kinetic and steady-state conditions (Huhtanen and Sveinbnjornsson 2006). Ruminal digestion is a very dynamic process in which several factors must be taken into account, in order to have reliable data for use in prediction models. Tothi *et al*. (2003) used the *in vivo* rumen evacuation technique to study the effect of expander processing and fractional rates of maize and barley on starch degradation. They observe that the results obtained were markedly lower compared to those obtained with the *in situ* technique for barley. Conversely, maize starch degradation was higher using the rumen evacuation technique compared to *in situ*. They concluded that the *in situ* method overestimates the digestion rate of rapidly-degradable barley starch and underestimates that of slowly-degradable maize starch. The faster barley starch degradation *in situ* compared with the values derived from rumen evacuation may be related to assumptions made for the digestion and passage of the soluble fraction (fraction a). Though it is an expensive, time consuming and laborious analysis, it can provide better and more useful information than other methods. To overcome variable conditions, the enhancement of evacuation frequency considering the better time to do it. This variability is more evident with rapidly-

digestible material, such as starch, given that diurnal variations in the rumen starch pool can be very large (Tothi et al., 2003).

3.1.3 *In situ* Technique

The dynamic interactions within the rumen are difficult to simulate. *In situ* methods permit the study of digestion within the rumen and reduce the need for ruminal simulation (Vanzant et al. 1998). The *in situ* method is a relatively simple and direct technique widely used to estimate ruminant degradation of nutrients or other components of feedstuffs, such as starch, protein, NDF etc, permitting their comparison and a better understanding of ruminal digestion (Stern *et al*., 1997; Huhtanen and Sveinbjörnsson 2006). Moreover, this is a relatively simple, low-cost method compared with methods involving intestinal cannulated animals (Stern *et al*., 1997). *In situ* methods have been commonly used to determine the degradation characteristics of starch and effective ruminal starch degradability (ESD), because they involves the actual rumen environment (Vanzant *et al*., 1998; Offner *et al*., 2003; Huhtanen and Sveinbnjörnsson, 2006).

The principle of the method is based on suspending nylon bags containing different feedstuffs within the rumen and measuring the decrease in nutrient at intervals of time. The advantage of this method compared to laboratory-based alternatives is that it involves the digestive process occurring in the rumen of the animal (Stern *et al*., 1997). However, several aspects that affect *in situ* nutrient estimates need to be considered and controlled to standardise the method as well as possible. Among the aspects that need to be considered are bag pore and sample size, sample processing, diet composition, feeding level and frequency and procedural considerations (Vanzant *et al*., 1998). Readers are referred to an excellent review by Vanzant *et al*. (1998) for a better explanation of the many factors that could influence *in situ* method determinations and suggestions on how to improve and standardise the *in situ* technique.

In situ kinetics of starch degradation can be determined following the model proposed by Ørskov and McDonald (1979) where: $ESD=a + b (1- e^{ct})$; the

formula corrected for the transit time (particles escaping rumen digestion) is ESD=a + b *[c/(c+Kp)] + [1-$e^{-(c+Kp)*t}$]. Parameters a, b, c and Kp represent the immediately degraded fraction, the potentially degradable fraction, the degradation rate of fraction b and constant of rate passage (Huhtanen and Sveinbnjörnsson, 2006).

Comparisons between *in vivo* and *in situ* measurements of starch digestion showed that the *in situ* method tends to underestimate *in vivo* ruminal starch digestibility of slowly degradable starch feedstuffs, whereas the digestibility of rapidly degrading starch tends to be overestimated. Tothi *et al.* (2003) compared RDS estimated by *in situ* and rumen evacuation techniques, with the predicted values of the *in vivo* RSD determinate using the duodenal sampling technique. They conclude that the *in situ* method overestimates that for maize compared with *in vivo* rumen evacuation data.

Nevertheless it is important to consider the variations in RSD influenced by other factors besides the starch source, such as processing methods, grain varieties, feeding levels, diet composition and methodological problems associated with the *in vivo* RSD determinations, as mentioned above.

3.2 *In vitro* measurements

Starch degradation is commonly measured *in vitro* either directly, by measuring starch disappearance after incubation at various time intervals, or indirectly, by measuring gas production (Menke, 1979). Most of the methods involve incubation of the feed samples in rumen fluid, more or less based on the original method proposed by Tilley and Terry (1963) for predicting digestibility. Some attempts have been made to estimate starch degradation rates *in vitro* by incubation with enzyme preparations (Karkalas, 1985; Cone and Volt, 1990; Xiong *et al.*, 1990).

3.2.1 Tilley and Terry Method

This method involves estimating feedstuff degradability by measuring the disappearance of the sample, which is incubated in buffered rumen fluid over a certain number of incubation periods. Important factors regarding incubation

with rumen fluid are: ratio of feed to rumen fluid, ratio of rumen fluid to *in vitro* media, composition of the *in vitro* media, type of mill used for grinding, grinder screen size and diet of the donor animal.

Richards *et al*. (1995) observed that the best results of feedstuff degradability were obtained when ruminal fluid was diluted in proportion of 1:3 and 1:4, however to the dilution 1:3 is the most commonly-used. Cone *et al*. (1989) estimate starch degradability following the Tilley and Terry method, and observed that the ruminal liquid from donor cows fed with concentrate rations produced better results on starch degradability compared to hay-fed donor cows. They also found that starch disappeared faster when the milling screen size was reduced from 1mm to 0.1 mm.

Table 3.1: The percentage of starch (mean± SEM) of different feedstuffs degraded after 6 hours' incubation in rumen fluid taken from hay-fed cow (HFC), or from two different concentrate-fed cows (CFC). The concentrates consisted mainly of tapioca and steam-flake maize (A) or maize and milocorn (B).

Samples	HFC	CFC.A	CFC.B
Lupin	0.0 ±0.0	13.9 ± 4.8	18.5 ± 0.1
Pea	3.1 ± 0.2	33.3 ± 2.8	36.1 ± 0.1
Maize	3.5 ± 0.5	21.6 ± 1.6	21.6 ± 1.7
Milocorn	5.5 ± 0.5	23.6 ± 1.5	25.1 ± 1.8
Potato	6.6 ± 0.1	25.1 ± 1.0	36.5 ± 2.7
Millet	6.8 ± 1.7	25.7 ± 3.2	31.0 ± 0.4
Tick-Bean	8.2 ± 2.6	29.0 ± 3.9	36.0 ± 0.2
Wheat	9.9 ± 1.1	42.3 ± 2.7	41.2 ± 2.5
Barley	10.3 ± 3.2	33.8 ±3.7	41.0 ± 1.0
Oat	15.9 ± 1.3	51.0 ± 3.2	55.6 ± 1.9
Tapioca	23.3 ± 0.9	47.0 ± 2.5	52.8 ± 2.4
Paselli	55.9 ± 2.4	60.4 ± 2.3	58.9 ± 1.8

Adapted from Cone et al., 1989. Journal of Food Agriculture, pp. 173-183.

Table 3.2: The percentage degradation of starch of different feedstuffs with different particle size after 6h incubation in rumen fluid from concentrate-fed cow.

Sample	Particle size (mm)					
	0 – 0.1	0.1 – 0.25	0.25 – 0.5	0.5 – 0.8	0.8 – 1.0	> 1.0
Maize	-	28.0	21.1	14.4	10.5	7.7
Wheat	47.8	42.2	34.5	21.6	14.4	17.5
Oat	54.8	45.8	46.4	40.2	39.4	38.1
Tapioca	49.2	42.6	49.2	48.8	44.1	-
Potato	26.0	19.6	18.2	15.5	12.7	8.3

(Standard errors of the mean less than ±4.0, have been omitted for clarity). Adapted from Cone *et al.*, 1989. Journal of Food Agriculture, pp. 173-183.

The determination of starch residues after incubation in rumen fluid requires complete hydrolysis of the starch to glucose and determination of the α-linked glucose polymers. The *in vitro* estimated degradability is on average about one third lower than the *in situ* values and also lower than would be expected *in vivo*. However, the *in situ* method in general overestimates starch degradation by assuming that starch is degraded when it disappears from the nylon bags, but *in vitro* gravimetric methods only consider starch as degraded when it is completely degraded to glucose.

3.2.2 *In vitro* gas production method

The gas production technique implies the use of ruminal liquid. This method measures the gas produced during feedstuff fermentation at several time intervals, instead of measuring sample disappearance. Anaerobic carbohydrate fermentation by ruminal microbes produces VFA, CO_2, CH4 and some traces of H_2, (Stern et al., 1997).The gas production technique was proposed by Menke (1979), and since then has been adapted to more sophisticated technology in an attempt to measure the kinetics of gas production accurately (Cone *et al.*, 1996). Automated gas production systems have been used extensively to describe digestion kinetics of intact feeds or feed fractions (Huhtanen and Sveinbjörnsson, 2006). This system has some advantages relative to other *in vitro* methods, such as:

-the possibility of making frequent measurements, by using electronic pressure sensors and data logging equipment, allowing the collection of a sufficient number of observations for accurate parameter estimation;

-the possibility of estimating digestion rates of different fractions by fractionating the feed before incubation;

-compared to the *in situ* technique, the estimation of digestion kinetics is based on end-product formation instead of material disappearance from the bags, which appears to be confusing (Huhtanen and Sveinbjörnsson, 2006).

Recent studies have used this technique to evaluate the site and extent of starch degradation from different sources. Hindle et al. (2005) observed that wheat ferments faster compared to maize and potato. Although all samples were almost completely degraded, potato starch presented the highest degradation, due to the fact that it was pure starch (99.7%), whereas the others were ground raw materials containing 59-69% starch. In a further work, Lanzas *et al*. (2007) studied the digestion kinetics of cereals grains, observing that barley and wheat cereal grains were faster degraded faster compared to corn and sorghum. These results are consistent with the findings of van Hindle *et al*. (2005) for wheat and maize (Figures 3.1 and 3.2).

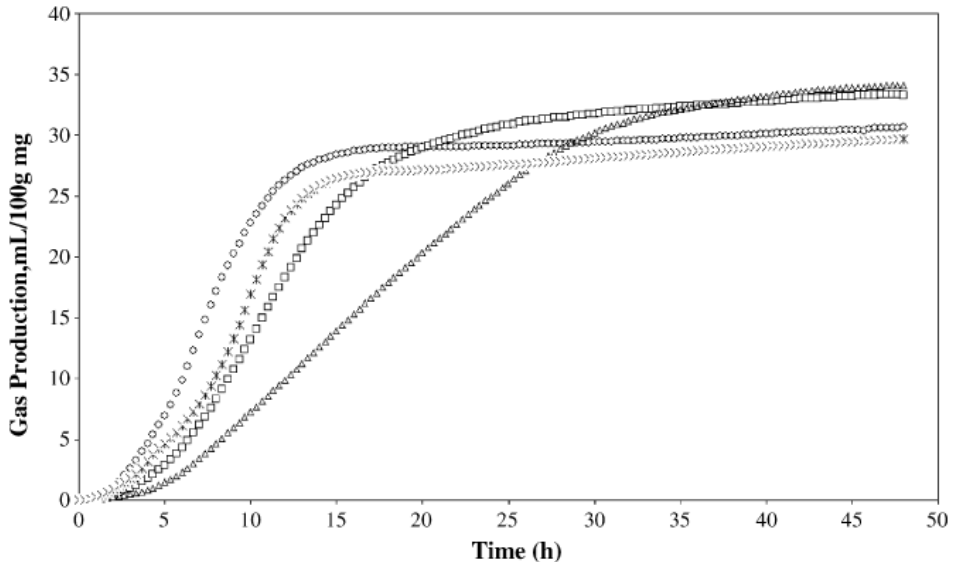


Figure 3.1:Average cumulative gas production of: barley (*) (*n* = 20), corn (□) (*n* = 99), sorghum (△) (*n* = 23), and wheat (○) grain (*n* = 53). Adapted from Lanzas *et al*., 2007. *Animal Feed Science and Technology 136:256-280*.

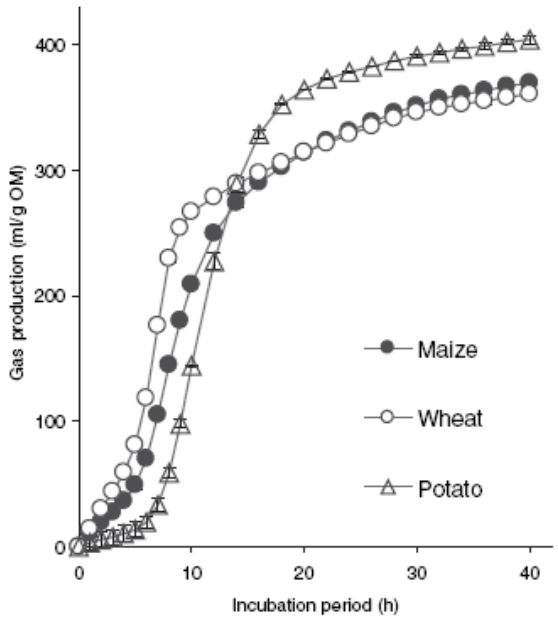


Figure 3.1: Gas production profiles of maize, wheat and potato starch samples. Adapted from van Hindle et al., 2005. *Journal of Animal Physiology and Animal Nutrition 89:158-165.*

The gas production technique allowed authors to rank cereal digestion according to their fractional rates, where wheat and barley produced more gas per hour compared to corn and sorghum. Chai *et al*. (2004), in their study of the possible estimation of starch fermentation in rumen fluid, found that starchy feed samples were almost completely degradable after 24 hours of incubation. They also found that starch digestion in maize silage samples followed the same pattern, varying from 82.4% to 97.7%, which means that inside the same material matrix, the digestion pattern is variable. They also found a good correlation between starch degradation and gas production, $0.96<r^2<0.99$, demonstrating a linear relationship between them as well. This indicates that the fermentation of starch and other non-soluble components occurs simultaneously and not sequentially as happens during *in situ* and *in vivo* trials, (Chai *et al*., 2004; Hindle *et al*., 2005; and Lanzas *et al*., 2007).

Gas production can be used as a fast screen of individual samples to obtain an impression of starch fermentation kinetics, and also to observe the effect of several factors, such as processing, genotype, harvest date, etc, though this method is not considered sufficiently accurate to be used for feed evaluation and ration composition purposes.

3.2.3 Enzymatic techniques

Predicting the digestibility of feedstuff is the common and main interest of livestock producers and feed companies. Methodologies to study the site and extent of feedstuff degradation are dependent on cannulated animals, and are laborious, time consuming, expensive and difficult to standardise. Enzymatic methods could represent the solution to overcome the problems mentioned above. In fact several researchers have been trying to develop an enzymatic technique for the prediction of the degradation of feedstuff nutrients such as proteins, starch, etc.

The first works with enzymes were developed to determine starch content. Karkalas (1985) improved on a sequential hydrolysis using bacterial α-amylase and fungal amyloglucosidase. The author claims that this is an accurate method, reproducible, fast and inexpensive. Moreover it is a method that overcomes problems of starch determination, such as resistant starch and amylose-lipid complexes formed during the processing of food/feed. However, it was not developed to estimate starch degradability.

Cone and Volt (1990) approached the possibility of the application of enzymes for starch degradability predictions. They compared the results of starch degradation by α-amylase, pancreatin and amyloglucosidase with *in vitro* ruminal starch degradation results. Enzymatic degradation produced similar results to rumen fluid degradation, ranking feedstuffs in the same order; starches classified as degrading faster, at a mid rate and more slowly correspond between the two methods, although the results with rumen fluid were highest. Moreover, enzyme behaviour with respect to processed and unprocessed feeds was similar to that observed with rumen fluid, in which processed feed proved to be more degradable compared to raw materials (see Table 3.3). Among the enzymes tested, pancreatin gave better correlations with rumen fluid results independently of the type of diet of the donor cow, compared to α-amylase (r=0.77 and 0.65 respectively).

In a further work Cone (1991) studied the effect of cell-free enzyme extract from rumen fluid on feedstuff starch degradation compared to starch rumen fluid degradation, and observed a correlation of 0.96.

Table 3.3: The percentage of starch in different feedstuffs (500mg): Degraded after 4h of incubation with 100mg α-amylase, 100mg pancreatin or 100µl amylglucosidase at 39ºC in 50ml of buffer solution; degraded after 6h of incubation at 39ºC in 50ml of diluted rumen fluid solution. (Rumen taken from two different cows, a hay-fed cow (HFC) and a concentrate-fed cow (CFC), with ph kept constant at 6.5 during incubation)

Samples	α-amylase	Pancreatin	Amyloglucosidase	HFC	CFC
Potato	6.3 (0.6)	8.1 (0.4)	0.1 (0.4)	10.5 (2.6)	23.0 (0.8)
Tick-bean	7.9 (1.3)	14.9 (1.5)	1.2 (0.5)	4.9 (2.2)	19.7 (1.1)
Millet	8.6 (0.9)	18.4 (1.9)	0.7 (0.2)	5.5 (4.2)	14.0 (4.6)
Milocorn	10.7 (0.6)	23.3 (1.8)	1.1 (0.1)	4.6 (2.3)	12.5 (1.1)
Pea	18.4 (3.6)	26.7 (3.0)	0.8 (0.8)	1.4 (1.0)	24.6 (1.7)
Maize	18.1 (0.4)	33.1 (5.1)	3.0 (0.4)	1.6 (1.6)	17.9 (2.9)
Flake meal	27.6 (1.6)	43.9(0.6)	4.5 (0.4)	16.2 (1.5)	30.6 (3.1)
Gluten feed	43.8 (3.1)	57.5 (1.8)	8.9 (0.7)	11.7 (1.5)	31.6 (1.7)
Steam flaked	61.8 (1.7)	80.0 (3.0)	25.4 (1.7)	7.9 (1.8)	26.1 (3.4)
Flake	62.6 (4.4)	73.4 (1.6)	38.7 (3.2)	19.5 (2.3)	42.1 (1.3)
Popped	64.0 (4.6)	72.1 (0.9)	58.9 (5.5)	51.2 (0.2)	69.8 (0.5)
Wheat	17.4 (0.7)	10.1 (0.4)	1.9 (0.2)	14.0 (4.6)	41.7 (2.4)
Middlings	60.6 (2.8)	56.6 (2.1)	9.3 (0.8)	14.2 (6.6)	34.8 (3.7)
Feed meal	57.6 (2.4)	61.6 (3.8)	9.0 (0.8)	18.1 (2.1)	40.6 (2.4)
Popped	62.3 (1.1)	75.5 (2.4)	87.3 (3.2)	66.5 (0.5)	72.6 (0.4)
Barley	20.9 (0.4)	29.0 (2.3)	2.1 (0.3)	6.3 (1.1)	27.6 (1.9)
Oat	26.2 (2.3)	54.3 (2.1)	2.1 (0.4)	14.6 (0.5)	48.2 (4.0)
Tapioca	59.3 (1.9)	74.9 (3.1)	36.5 (3.4)	28.4 (1.9)	57.5 (2.3)
Rice	10.1 (0.5)	22.6 (1.6)	1.6 (0.2)	6.0 (1.1)	13.4 (1.2)
Feed meal	37.8 (1.8)	47.7 (2.3)	11.1 (1.4)	29.1 (1.6)	33.8 (2.4)
Popped	67.3 (3.3)	79.9 (0.6)	90.9 (4.2)	59.6 (4.1)	67.6 (2.4)

Standard deviations are given in brackets. Adapted from Cone and Volt (1990). *Journal of Animal Physiology and Animal Nutrition* 63:142-148.

In all the works by Cone and co-workers the conclusions are that enzymes provide valid information on how starch would be degraded in the rumen, and that pancreatin and amyloglucosidase would be the best options, although the results are not accurate enough to lead to the adoption of the enzymatic method to predict rumen starch degradability: the authors concluded

that the enzymatic method could only give a rough indication of whether starch degrades slowly, at a mid rate or fast (Cone *et al.,* 1989; Cone and Volt, 1990, Cone, 1991). Regarding the cell-free extract from rumen fluid, the valid results do not overcome the fact that this method is still dependent on fistulated animals, but they did suggested that complete starch degradation requires several amylolytic enzymes, and that therefore more than a single enzyme is needed to improve the accuracy of the enzymatic method (Cone 1991).

Xiong *et al.,* (1990) improved a modified method to measure processing effects and starch availability in steam-flaked sorghum grain. Sample starch availability was tested by incubating with amyloglucosidase. The authors used this enzyme due to the fact that the end product of the enzymatic reaction is only glucose, and a starch equivalent can only be calculated from glucose. The glucose released was measured at different incubation times (0 to 48h), and the samples tested were uncooked, fully cooked, steam-flaked and a mixture of cooked and uncooked sorghum grains. Results were expressed by a component of the equation to determine the glucose released, rate constant K. Fully cooked samples were found to be degraded faster by amyloglucosidase compared to uncooked and steam-flaked sorghum grains, 1.781/h, 0.119/h and 0.368/h respectively ($r^2 \simeq 0.98$). The mixture, (50:50 cooked:uncooked), showed two different starch components, one with a fast rate (2.624/h) and the other with a slow constant rate (0.066/h) ($r^2=0.99$). The authors recommended this method because it was simple, fast and sensitive to the different types of starch that can be found in feedstuffs.

Stern *et al.* (1997) in their review report several works using the enzyme method to determine protein degradability and fewer works on carbohydrate enzymatic degradation. The advantage of using enzymatic methods is that they are completely independent from the animals. The fact that the enzymatic technique is animal-independent should result in less variation, making it relatively simple to standardise; however the biological validity of the results may be compromised by incomplete enzymatic activity compared with the ruminal environment (Stern *et al.,* 1997). When enzymatic techniques are used to predict microbial feedstuff fermentation it is extremely important that enzyme

concentration is sufficient, otherwise this is a limiting factor leading to an accumulation of end-products during incubation that can lead to a progressive inhibition of enzyme activity.

Recent studies on feed degradation are testing new approaches applying enzymes as an alternative to *in vivo*, *in situ* and *in vitro* ruminal techniques, not only for the reasons enumerated above but also because people have became more sensitive and concerned about the wellbeing of animals used in scientific research. Blasel *et al.* (2006) tested whether the degree of starch access (DSA) technique could provide accurate results to be used as an index of evaluation of relative starch digestion potentials, in this case on corn grain and silage. According to the authors this method is very simple, requiring minimum equipment, and is sensitive to factors affecting starch degradability, such as particle size, vitreousness and dry matter content, while also being a quantifiable method with acceptable reproducibility. However, the lack of *in vivo* data to establish the relationship with DSA values, and the fact that it cannot distinguish aspects of starch digestion such as the presence of other oligosaccharides which could result in the overestimation of the DSA results, are weaknesses of this method that may be overcome by integrating it with controlled research studies evaluating *in vivo* starch digestion (Blasel *et al.,* 2006).

Enzymatic methods have also been developed in research studies for the estimation of starch digestion in monogastrics, and in studies aiming to understand the molecular structure of starch, e.g. in order to measure its amylose and amylopectin content, determine resistant starch, as well as to measure carbohydrates in feed/food grain, etc. The majority of the methods have in common the enzymes used, α-amylase, amyloglucosidase and pancreatin, which are responsible for fractioning and degrading starch into its single unit, glucose, as was already discussed above in the chapter on digestion (Englyst *et al.,* 1992; Séne *et al.,* 1996; Wilfart *et al.,* 2003; Stevenebø *et al.,* 2005; McCleary *et al.,* 2006; Anker-Nilssen *et al.,* 2006). However, more studies are needed in order to achieve the goal of being able to predict feedstuff degradability and reducing or even eliminating the need for animals in the tests.

4 Digestion

Carbohydrates are as important to the ruminant animal as they are to non-ruminants, since they provide the glucose necessary for the adequate functioning of cells (Cerrila and Martinez, 2003). Carbohydrates are the major source of energy for animals and comprise 60 to 70% of the total diet fed to dairy cows (Mertens, 1992). Plants tissues contain about 75% carbohydrates, and form part of animals' diet (Czerkawasky, 1986; Cerrila and Martinez, 2003). Carbohydrates found in plants are primarily polysaccharides: cellulose, hemicelluloses, pectins, fructans, and starches, with minor amounts of other components (Cerrilla and Martinez, 2003). Carbohydrates are broadly classified as structural and non-structural. Structural carbohydrates comprise cellulose and hemicelluloses and lignins, and are found in the cell walls. Non-structural carbohydrates consist of starches, β-glucans, pectins and simple sugars, and are present inside the plant cells (Czerkawsky, 1986; Mertens, 1992; Jung, 1997).

Structural carbohydrates are the major compounds in forages, and are not digestible by mammals, unless like ruminants, they possess enzymes able to break down β- (1,4) linked polysaccharides, present in this kind of diet (Jung, 1997). Ruminants are specialized herbivores which exploit a symbiotic relationship with microorganisms that inhabit the rumen, and so are able to hydrolyze and use large proportions of structural carbohydrates (Czerkawsky,

1986). Grains are fed to livestock primarily to supply energy, and the major energy source in cereal grains is starch (Owens, 2005). Starch represents rapidly-digestible energy and is widely used in livestock diets in order to improve performance; as an example, dairy cow milk production improves with increased starch intake (Firkins, 2001).

Carbohydrate digestion consists in the hydrolysis of α-(1,4), α-(1,6), ß-(1,3) and ß-(1,6) linkages, depending on the carbohydrate that is to be digested. Briefly, carbohydrate digestion by monogastrics occurs principally in the intestine through enzymatic action, whereas in ruminants, carbohydrates are first fermented in the rumen by bacteria and protozoa, and then digested in the intestine (Call *et al.,* 1975). In the rumen, carbohydrates are fermented into volatile fatty acids (VFA, propionic, acetic and butyric acid etc), which provide more than 70% of the energy supply for maintenance and synthesis of milk and tissues. After leaving the rumen, unfermented carbohydrates such as starch are digested by enzymic hydrolysis during passage through the animals' intestine. Enzymes break down the glycosidic bonds of the polysaccharides, so they can be used for different functions in the animal's body system; for example cellulases and hemicellulases are the most important enzymes for hydrolyzing the β-1:4 linkages of structural carbohydrates; amylases are used for reducing starch into smaller molecules like dextrin, maltose and glucose (Czerkawsky, 1986).

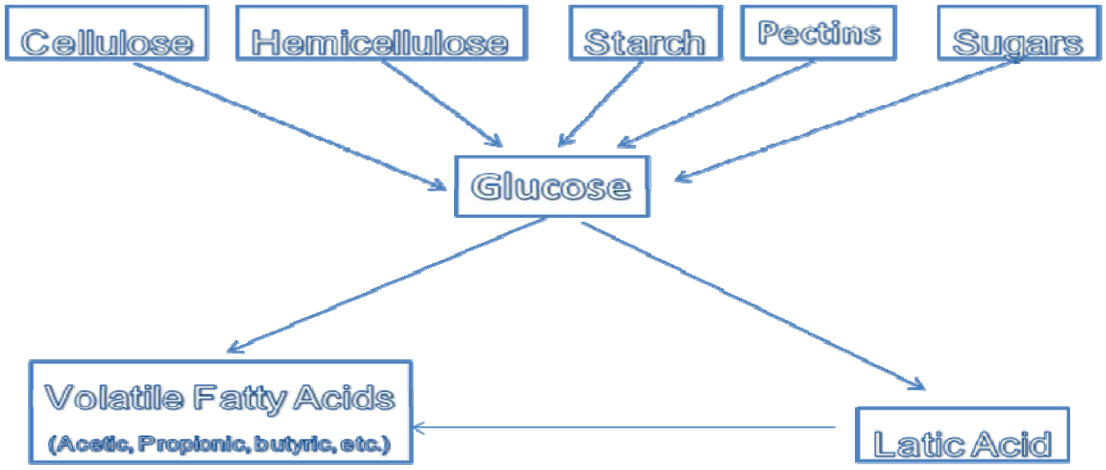


Figure 3.1 Ruminal plant carbohydrate hydrolysis into main units and the sub-products for microorganism use.

4.1 Starch digestion

Cereals are widely used as feed for ruminants, especially in intensive production systems with high-yielding animals. Cereal grain provides an appreciable amount of starch for both ruminal and intestinal digestion, depending on starch resistance to microbial rumen digestion (Cerrilla and Martinez, 2003). Cereal grains such as wheat, maize, sorghum, oats and barley contain between 57 to 77% of dry matter as starch. Data provided by Huntington (1997) shows that wheat has the highest content, followed by maize and sorghum, then by barley and oats.

Starch degradation into glucose requires the action of several enzymes produced by the salivary glands, rumen microorganisms, the pancreas and small intestine mucosa (Cerrilla and Martinez, 2003, Tester *et al.*, 2003). Degradation starts in the mouth apparatus, where amylase from saliva hydrolyses accessible starch, though because of the short time spent in the mouth, saliva only causes small changes (Cerrilla and Martinez, 2003). Major starch digestion occurs in the rumen and the small intestine. Inside the rumen starch is fermented by live microorganisms and bacteria, where ruminal bacterial are mainly responsible for the bulk of fermentation (Huntington, 1997; Cerrila and Martinez, 2003). The action of α-amylase from the microorganisms reduces starch into its main compounds, amylose and amylopectin. Amylose and amylopectin are degraded into maltose, maltotriose and some glucose, but because amylopectin is a branched molecule with α-(1,6) glycosidic bonds, it also releases some α-limiti dextrins. The α-amylase enzymes are not able to hydrolyze α-(1,6) glycosidic bonds and therefore specialized enzymes, such as pullulanase, maltase, α-limit dextrinase, are needed to break down these bonds (Cerrila and Martinez, 2003). The final products of starch fermentation in the rumen are glucose and volatile fatty acids (VFA). Starch that escapes rumen fermentation is then partially or totally digested in the small intestine. Enzymatic digestion of starch in the small intestine of ruminants proceeds much as it does in other species (Huntington 1997). Digestion and absorption in the small intestine occurs in three distinct phases. Digestion begins in the lumen of the

duodenum due to the action of α-amylase (EC3.2.1.1) secreted by the pancreas, which breaks down α-(1-4) bonds and thus produces glucose, oligosaccharides of two or three units, and dextrins, as described above in the rumen.

Glucose can be absorbed by the small intestine but maltose, maltoriose and dextrins cannot. Other enzymes, oligosaccharidases associated with the 'brush border', complete the hydrolysis of these materials, allowing them to be absorbed. These enzymes are not released in the lumen, but are bound to the membrane of the microvilli (Huntington, 1997, 2006, Cerrilla and Martinez, 2003). Brush border enzymes include: sucrase (converts sucrose into glucose and fructose); trehalase (converts trehalose into two molecules of glucose); maltase (converts maltose, generated form α-amylase activity, into glucose); isomaltase, also known as α-dextrinase, (hydrolyses α-(1,6) linkages from isomaltase and α-dextrins), which continues the digestion process, converting available starch into glucose (Tester *et al.,* 2003). Unlike non-ruminants, ruminants do not have measurable sucrase activity and therefore depend on maltase and isomaltase activity to produce glucose units for absorption (Huntinton 1997). The third and final phase of intestinal starch digestion and absorption is the transport of glucose out of the intestinal lumen into the portal circulation (Huntington, 2006).

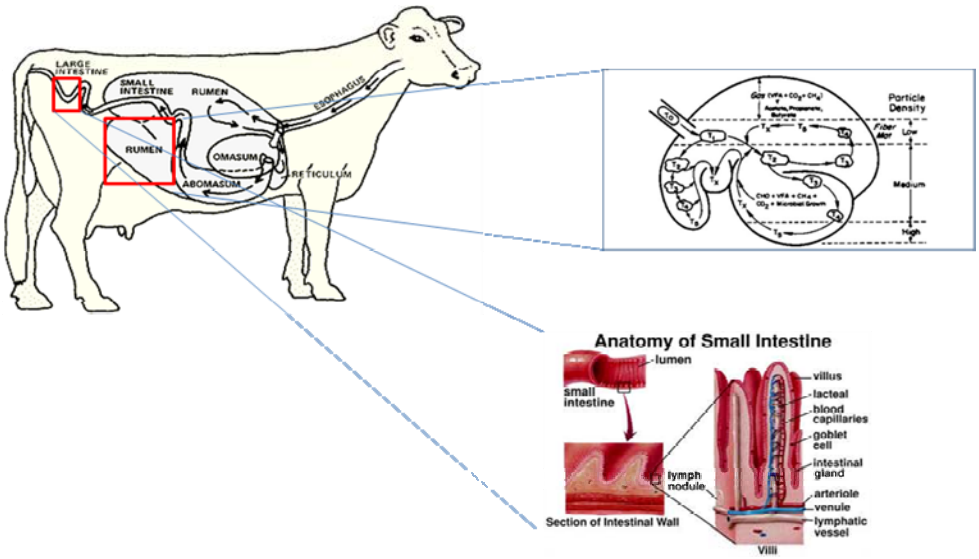


Figure 3.2: Ruminant digestive system, in detail the rumen section, and brush-border details.

4.2 Enzymatic breakdown of starch polymers

In general enzymes erode the entire granule surface or sections of it by a process named exocorrosion, or digest channels form selected points on the surface towards the centre of the granule, by endorcorrosion, (Oats, 1997).

Most of the starches are initially hydrolyzed on the surface, like B-type potato starch; however, starches such as wheat, barley and rye have specific points where hydrolysis initiates owing to endocorrosion (Oats, 1997). After the initial hydrolysis there are some regions of the granules which are more digestible than others. The susceptible areas correspond to a less organised granule structure in the amorphous rings, whereas the crystalline layers offer more resistance to enzyme hydrolysis. The variation in the pattern of enzyme attack is evident between starch types and within the same population of granules, and is influenced by the type of amylase. The amylolytic enzyme for a specific starch is also dependent, to some extent, on the source of the enzymes. Early works suggested that pancreatic α-amylase is more effective as regards native starch compared to bacterial and fungal amylases (Kimura and Robyt, 1995). Enzyme activity is also determined by the starch features. Tester *et al*. (2006) report that porcine pancreatic α-amylase activity on native starches is a function of the surface area of the granule than the concentration of the substrate. Ring *et al.* (1988) quantified the rate and extent of starch hydrolysis from different sources and observed that cereal starch degradation decreases with the increase in granule size. Potato granule size ranges between 5 and 100μm, whereas maize, pea and wheat are on average 20-30μm; corresponding differences on the surface are ≈20:1; see Table 4.1, for details. Assuming that the surface is a major site of enzyme activity, potato granule starch would be hydrolyzed slowly compared to starch granules from wheat, maize and pea (Ring *et al.,* 1988). However digestion trials have demonstrated the contrary. Clary *et al*. (1968) observed that potato starch was degraded faster and completely compared to wheat, corn and rice starches.

The surface area of starch granules depends on their shape, which varies with botanical origin. To measure the starch surface it would be

necessary to assume that they are of specific shapes such as a sphere, or a polyhedral e.g. a cube, for which it is possible to calculate areas and volumes, as described in Table 4.1. It is also important to remember that enzymes hydrolyse starch granules differently, as mentioned above.

Table 4.1: Characteristics of native starch granules from common sources

Starch	Diameter (μm)	Surface area (μm^2)[a]	Volume (μm^3)[b]	SA:V Ratio[c]
Barley	2-5 (B-granules)	12.6-78.5	4.2-65.4	1.2-3.0:1
	15-25 (A-granules)	707-1964	1767-8181	0.2-0.4:1
Maize (waxy and normal)	2-30	12.6-2827	4,2-14137	0,2-0,3:1
Amylomaize	2-30	12.6-2827	4,2-14137	0,2-0,3:1
Millet	4-12	50.3-452	33,5-905	0,5-1,5:1
Oat	3-10 (single)	28.3-314	14,1-524	0,6-1,2:1
	80 (compound)	20106	268083	0,08:1
Pea	5-10	78,5-314	65,4-524	0,6-1,2:1
Potato	5-100	78,5-31416	65,4-523599	0,06-1,2:1
Rice	3-8 (sinlge)	28,3-201	14,1-268	0.8-2.0:1
	150 (compound)	70686	1767146	0.04:1
Rye	5-10 (B-granules)	78,5-314	65,4-524	0,6-1,2:1
	10-40 (A-granules)	314-5027	524-33510	0,15-0,6:1
Sorghum	5-20	78,5-1257	65,4-4189	0,3-1,2:1
Tapioca	5-35	78,5-3849	65,4-22449	0,17-1,2:1
Triticale	1-30	3,1-2827	0,5-14137	0,2-6,2:1
Wheat	2-10 (B-granules)	12,6-314	4,2-524	0.6-3.0:1
	15-35 (A-granules)	707-3849	1767-22449	0,17-0,4:1

[a] from $4\pi r^2$, where r is the granule radius; [b] from $4/3\pi r^3$. [C] Surface area-volume ratio. Adapted from Tester *et al.,* 2006. *Journal of Animal Feed Science and Technology.*

Another important aspect is the diversity in the source of enzymes, which brings a genetic variation into the structure and subsequently into the activity (Tester *el al.,* 2006). Alpha-amylases are the main enzymes involved in the hydrolysis of α-(1-4) starch bonds (amylose, amylopectin) and various maltodextrins (Colonna *et al*., 1992). Alpha-amylase is an endoglucosidase, which means it is capable of hydrolysing internal α(1-4) glycosidic bonds and does not require free ends of amylose chains for activity (Harmon, 2009). All enzymes are able to bypass the α-(1,6) branching points but not to cleave them.

The nature and distribution of the hydrolysis products (maltose, glucose and dextrins of high molecular weight) depend on the source of the amylases (Colonna *et al.,* 1992). The α-amylases have a molecular weight of between 50000 and 60000. The optimum pH and temperature for their activity depend on their origin; Table 4.2 presents the main biochemical characteristics of some α-amylases from different sources (Colonna *et al.,* 1992).

Table 4.2: Main biochemical characteristics of α-amylases

α-Amylases from:	Mol.wt	pH optimum	T(ºC) optimum
Animals			
Human Saliva	62000	6.9	50
Human pancreas	56000	6.9	50
Pig pancreas	50000	6.9	35
Vegetables			
Barley malt	45000	5.5	51-60
Wheat malt	41500	5.5	60-66
Rice malt	44000	6.0	45-60
Microorganisms			
Bacillus subtilis	47300	5.3	<55
Bacillus amyloliquefaciens	48900	5.9	<80
Bacillus lichenformis	59000	6.0	90
Bacillus stearothermophilus	62650	7-9	65-73
Bacillus acidocaldarius	66000	3.5	70
Aspergillus niger	61000	5-6	35
Aspergillus oryzae	52600	5.5-6.9	40

Table adapted from Colonna *et al.,* 1992. European Journal of Clinical Nutrition 46(Suppl.2): S17-S22.

The activity of α-amylases from different sources (ovine, bovine, porcine and fungal) were analysed in different substrates. Porcine and fungal α-amylases proved to be more efficient in hydrolysing starch molecules compared with ovine and bovine α-amylases (see Figure 4.3) (Clary *et al.,* 1968).

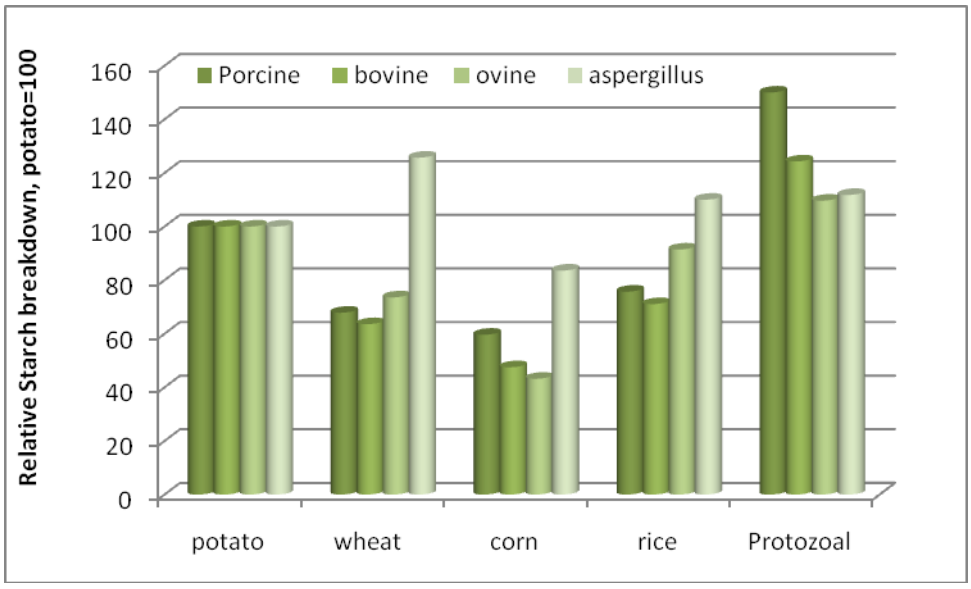


Figure 4.3: Hydrolysis of different starch sources by porcine, bovine, ovine and *Aspergillus* α-amylases, measured using the iodine binding method. Data adapted from Clary et al., (1968).

However, ruminant α-amylases proved to be more efficient compared with their non-ruminant counterparts when the production of maltose and α-limit dextrins was determined (based on starch-iodine molecular complex), (Figure 4.4) (Clary *et al.,* 1968; Harmon, 1992).

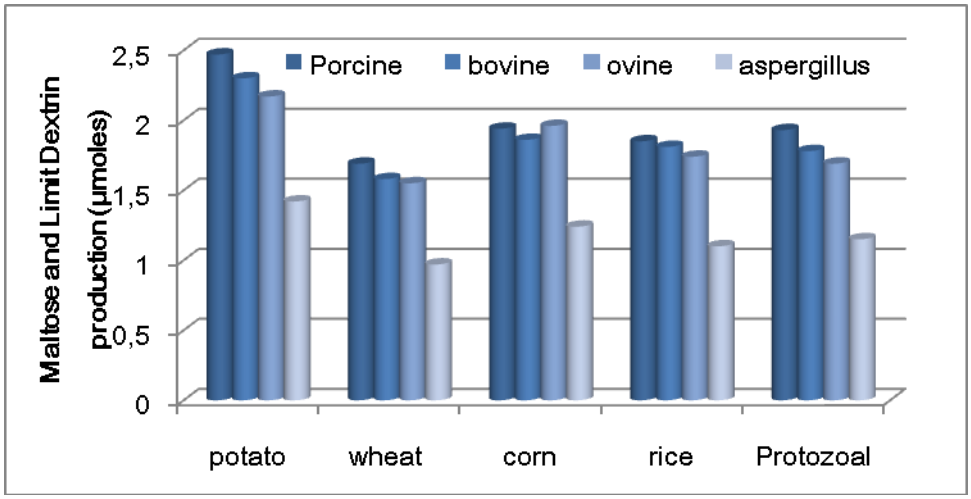


Figure 4.4: Maltose and limit dextrin production from different starches by porcine, bovine, ovine and *Aspergillus* amylases.

Starch digestion has been broadly studied for humans and livestock. The structural and functional features of starch make it a complex molecule, which is hydrolysed and absorbed more slowly than simple sugars or even disaccharides. From the human nutritional point of view, starch in food is

classified into: Rapidly Digestible Starch (RDS), Slowly Digestible Starch (SDS) and Resistant Starch (RS) (Englyst *et al.,* 1992). In the earliest studies it was assumed that food starch was completely hydrolysed and absorbed within the small intestine; however it could be slow to degrade due to the fact it consists in large and complex polymers. The classification of starch in food was first approached after observing enzyme resistance when improving a technique to determine non-starch polysaccharides. Englyst *et al.* (1992) report a resistance to hydrolysis with pancreatic amylase and pullulanase in some types of processed food, and also that starch resisted digestion in the human small intestine. Therefore they developed a classification of starch fractions based on measuring the glucose released by controlled enzymic hydrolysis. Total Starch (TS) is determined as glucose released by enzymatic hydrolysis following gelatinisation in boiling water and a treatment with potassium hydroxide to disperse retrograded amylose. TS is corrected for free glucose but includes maltose and maltodextrins. RDS and SDS are measured after incubation with pancreatic amylase and amyloglucosidase at 37ºC for 20 min and a further 100min, respectively. RS is starch not hydrolysed after 120 min of incubation. Resistant starch is further classified according to different characteristics with reference to resistance to digestion, as described in Table 3.1. This starch classification system is also followed in starchy feed digestion studies, normally carried out on cooked, not native starch.

Knowing how the process occurs inside the animals' digestive tract and indentifying the factors influencing digestion, helps improve feeding programs and subsequently enhances animals' performance.

4.3 Factors Affecting Starch Digestibility

The ruminal digestion process is a dynamic sequence and synergy of events that influences fermentation, the end product and delivery of carbohydrate sources consumed by the animal (Nocek and Tamminga 1991). Starch digestibility depends more on grain processing and particle size than on native chemical/structural characteristics.

Table 4.3: *In vitro* nutritional classification of starch

Type of Starch	Example of occurrence	Digestion in the small intestine.
Rapidly Digestible Starch (RDS)	Freshly cooked starchy food	Rapid
Slowly Digestible Starch (SDS)	Most ram cereals	Slowly but complexly
Resistant Starch (RS)		Resistant
Type I: Physically inaccessible. Starch granules embedded in plant tissue, e.g. protein matrix form the endosperm.	Partially milled grains and seeds.	
Type II: native starch granules such as potato and banana are more resistant compared to other starches	Raw potato and banana.	
Type III: Retrograded starch. Starch molecules that recrystallised through hydrogen bonding.	Cooked potato bread and corn flakes.	
Type IV: new chemical bonds are formed (other than α-(1-4) or α-(1-6).		

Adapted from: Englyst *et al.,* (1992), E. J. Clin. Nut. 46: S33-S45, and Tester *et al.,* (2004), W. Poultry Sci. J. 60:186-195.

4.3.1 Grain Processing

The effect of cereal processing on starch digestion has been widely studied. Processing methods consist in physical and/or chemical treatments applied to cereals, which can affect starch availability by altering its properties or by interacting with other components in the feed.

Physical treatments consist of breaking, cracking, grinding, rolling or pelleting dried grains. Physicochemical treatments consist of the application of heat, pressure and water, which affect starch structure by hydrating and swelling the amorphous and crystalline layers of the starch granule, leaving it more accessible to microorganisms and enzymatic action (Nocek and Tamminga, 1991). It has been observed that the degree of processing is an important factor that influences the degree of cereal grain digestibility in the rumen and post-rumenl (Cerrilla and Martínez, 2003).

Early *in vivo* and *in vitro* studies reported that steam-flaking and pressuring cooked grain improved dry matter and starch ruminal and total tract digestibility in sorghum and corn grains (Hale 1973; Owens *et al.,* 1986; Theurer 1986), whereas the major starch digestion occurs in the rumen (Hale, 1973). As an example, sorghum grain *in vitro* digestibility increased from 63% to 71-86%, for untreated, medium and flat steam-flaked respectively (Hale 1973), and corn grain digestibility in the rumen varied between 58 - 82.8% for whole and steam-flaked processing (Owens *et al.,*1986). Theurer (1986) reported the digestibility values of sorghum and corn grain from several studies, steam-flaked processing revealing the greatest rumen starch digestibility percentage in both grain sources when compared to other treatments. Table 3.2 shows starch digestibility (adapted from Theurer, 1986), in support of the statements above. Treatments such as cracked and whole grain in corn, and ground and dry rolled sorghum grains resulted in the lowest values of ruminal and total tract starch digestibility when compared to the other processing methods, as shown in Table 4.4 (Theurer, 1986).

The effect of processing methods is more evident in the rumen; however it also influences the intestinal digestibility of grains. Less processed grains have low digestibility in the rumen, for example whole grains 59%, increasing the starch flow to the intestine and subsequently increasing the availability of starch for further digestion in the small intestine (17%), as is shown in Figure 4.5 (Owens *et al.,* 1986).

Table 4.4: Starch digestibility of corn and sorghum processed grains.

Grains	Processing methods	% Starch digestibility in rumen	% Starch digestibility in total tract.
Corn	Dry rolled	76	95
	Ground	85	95
	Whole	64	89
	Cracked	61	94
	Steam flaked	86	99
Sorghum	Ground	64	94
	Dry rolled	68	91
	Steam rolled	90	96
	Steam flaked	82	99

Adapted from: Theurer C.B., 1986. J. Ani. Sci. 63:1649-1662. The data reported are the means of the values from several references reported by Theurer.

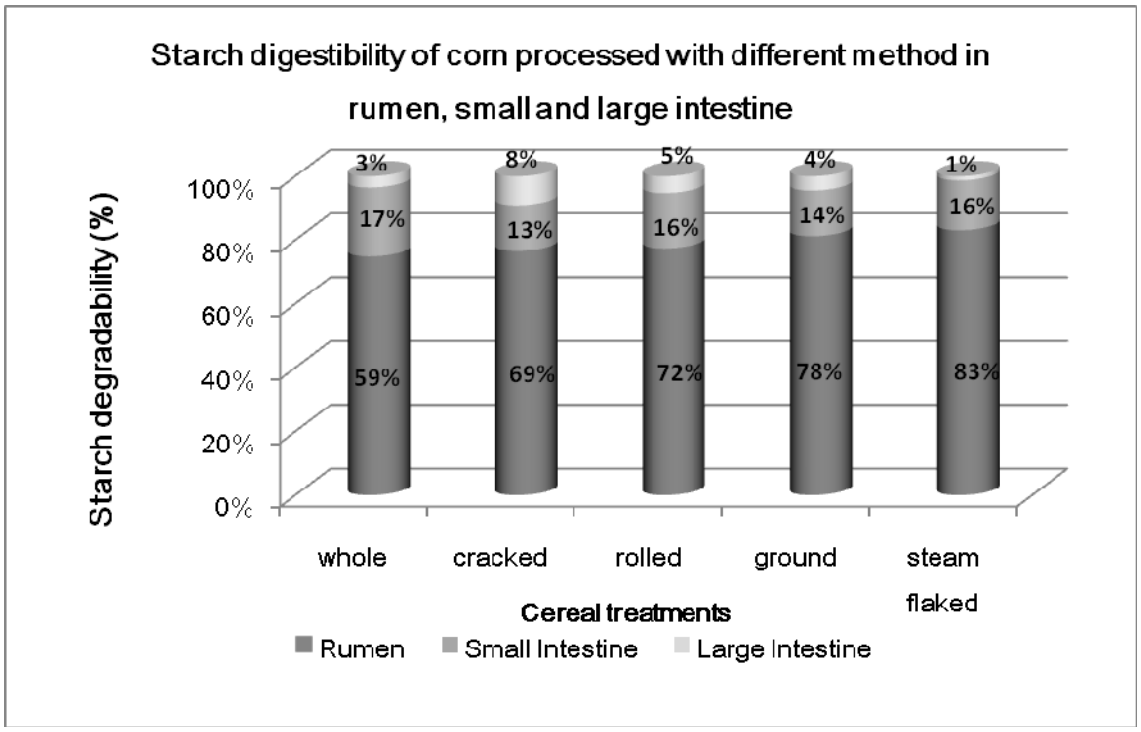


Figure 4.5 starch digestibility of different processed corn grains in the rumen, small and large intestine. Modified from Owens et al., 1986.

When physical treatments are combined with temperature, water content and pressure changes, and with the reduction of particle dimensions, they not only fractionate starch granules but also alter the internal features of starch.

Table 4.5 summarise the results of both *in vivo* and *in vitro* digestibility studies considering processing treatments, reported by Nocek and Tamminga (1991) in a bibliographic review, supporting what was mentioned above.

Studies conducted during the 60s, 70s and 80s regarding the effect of processing on feed digestion compared the digestibility of processed feeds by the type of processing method, disregarding the implications of each treatment for cereal structure. In recent studies (Cramer *et al.*, 2003; Svihus *et al.*, 2005; Sind *et al.*, 2005; Sun *et al.*, 2006; Zimonja and Svihus, 2009) the processing method has also been considered, together with the changes in cereal starch granules caused by the specific conditions of the treatment.

Table 4.5: rumen degradable starch (% total starch) values for various feedstuffs determined by *in vitro*, *in situ* or *in vivo* methods.

Feedstuff	*in situ* and *in vitro* (mean value)	*in vivo* (mean value)
Barley		
Ground	89.9	87.9
Rolled		87.2
Corn		
Whole		62.6
Cracked		65.0
Ensiled-shelled	72.0	86.0
Ground	58.4	76.4
Rolled wet		68.3
Rolled	51.0	76.8
Steam flaked	87.0	85.6
Silage	69.6	82.0
Corn gluten feed	80.6	
Corn gluten meal	86.5	
Oats	94.3	84.0
Sorghum		
Grain Ground	54.3	67.3
Ensiled		86.2
Rolled		64.0
Steam flaked		82.6
Rye		90.2
Wheat		
Bran	88.2	89.3
Grain	90.5	
Flour	88.3	
Middlings	88.8	

Adapted from Nocek and Tamminga, 1991, *Journal of Dairy Science*.

Treatments such as breaking, grinding, cracking, dry pelleting and rolling involve a reduction of grain size, while those requiring the applications of heat and water, such as steam-flaking, expanding and extrusion, cause modification to the basic structure of the starch granule, by hydrating and swelling the

amorphous and crystalline layers (Nocek and Tamminga, 1991). Gelatinisation is the most common change resulting from processing treatments that require the application of heat and water. Gelatinisation, defined above in the chapter on starch, modifies starch structure by altering its organisation and denaturating proteins which are normally associated, thus leaving the starch more vulnerable to microbial and enzymic hydrolysis (Rooney and Pflugfelder, 1986; Nocek and Tamminga, 1991). The combination of water and high temperature determines the extent of gelatinisation which will then increase the susceptibility of starch to degradation in the digestive tract, although a linear relationship between the extent of gelatinisation due to processing and digestibility has not been found (Svihus *et al.,* 2005).

Steam flaking is one of the processing methods which combines heat and water, widely used in feedlot operations to improve the efficiency of feed utilisation (Theurer *et al.,* 1999). Many studies provide information about the effect of steam flaking on starch digestion along the digestive tract as well as its effect on milk production. Theurer *et al.* (1999a) compare the starch digestibility of steam flake sorghum (SFS) versus dry rolled sorghum (DRS). They observed that SFS had a higher ruminal and total tract starch digestibility, 82% and 98,9% respectively, than DRS, 67% and 96.5% (P=0.01), although in the small and large intestine this tendency was not observed. Indeed, DRS was more digestible: 28% and 1.2% for the small and large intestines respectively, compared to 16% and 0.5% found for SFS. They also found that the sorghum and corn flake density linearly increased starch digestibility in the rumen (P=0.03, and P=0.02, respectively) and total tract (P=0.03, and P=0.09, respectively) and linearly decreased it in the small intestine (P=0.04, P=0.09, respectively).

Sindt *et al.* (2005) found that enhancing the percentage of moisture (0.6 and 12%) in steam processing tended to linearly increase the percentage of available starch (51, 51.5 and 52.5% respectively, for SF28; 58.2, 59.4, and 60.9% respectively for SF24) and linearly decrease particle size in both densities SF28 and SF24 (5.2, 5.1 and 4.9 μm; 5.0, 4.5 and 4.2 μm, respectively). Also, decreasing flake density increased (P<0.001) starch

availability, although it is observed that steers consumed less DM of diets containing steam flakes of low density (9.38, 10.06 and 8,75 kg/d) compared to those with high density (9.16, 9.38 and 9.92 kg/d), even with an increase in moisture levels.

Moreover, steam flaking compared to steam rolling or dry-rolling of grains improves milk production and milk protein production (Theurer *et al.,* 1999b). The authors reported the results of comparisons in four trials where steam flaking corn increased total starch digestion by 9%, milk yield by 6%, milk protein content by 0.07% and protein yield by 8%. However, the authors maintain that additional studies are needed, using animals fed with steam-flaked corn in TMR containing corn silage and alfalfa haylage or silage. These results are due to greater proportions of starch fermented within the rumen and an increase in small fractions of starch in the small intestine and subsequently of total starch, as shown in Table 4.6 (Theurer *et al.,* 1999b).

Table 4.6: Steam-flaking of corn and sorghum grains: increases in ruminal, post-ruminal and total starch digestibility of lactating dairy cows

Grain	Processing Method	Rumen % Starch Digestibility	Post-Ruminal % Starch Digestibility (%of entry)	Total % Starch Digestibility
Corn	Dry Rolled	35	61	77.5
	Steam-Flaked	52	93	96.6
Sorghum	Dry Rolled	54	74	88.7
	Steam-Flaked	76	90	97.9

Modified from: Theurer *et al.,* 1999b, *Journal of dairy Science* 82: 1950 – 1959.

On the other hand, Svihus *et al.* (2005) in their literature review report that in steam conditioning and pelleting small amounts of starch are gelatinised. Due to a low extent of gelatinisation, this does not have a marked effect on starch digestibility or on the physical quality of feed.

Briggs *et al.* (1999) observed that pellet quality decreases with an increase in the starch content of maize in pellet diets. However, the inverse

effect is observed by Zimonja and Svihus (2009), who found that pellet quality was slightly improved in ($p<0.05$) starch-containing (oat and wheat) diets due to the addition of gelatinised starch. The durability of the pellet was reduced in starch-containing diets compared to non-starch diets ($p<0.05$) (Zimonja and Svihus, 2009). No differences in starch digestibility were found in a wheat diet before and after pelleting. Pelleting increased the apparent metabolisable energy of the diets from 11.6 to 11.8 MJ/kg, but this is not reflected in starch digestibility (Svihus *et al.,* 2004).

In expander processing, feeds are subjected to temperatures of around 80-100ºC under pressure (30-40 bar) with application of steam for a short time (Goelema *et al.,* 1999; Cramer *et al.,* 2003), increasing starch gelatinisation. Cramer *et al.* (2003) observe that the percentage of starch gelatinisation varies from 9.82% to 35.37% for standard and expanded diets, respectively. However, this processing method is reported to have little or no effect on starch digestibility (Svihus *et al.,* 2005). In fact, low efficiency (g Gain/g Feed) is observed in broiler performance, 0.569 to 0.575 from standard and expanded diets, respectively (Cramer *et al.,* 2003).

The extrusion process is a more severe treatment which results in more complete gelatinisation and disintegration of starch of granules (Svihus *et al.,* 2005). This process involves applying large quantities of water and high temperatures (between 100-130ºC) under pressure (80-100 bar) to feed, and apparently it increases starch digestibility. Extrusion, when compared with other processing procedures, results in a higher degree of gelatinisation; Holm and Björck (1988) observed that steam-flaked and dry-autoclaved samples were less susceptible to enzymatic hydrolysis compared to extrusion-cooked samples, due to a low degree of gelatinisation. However, the results were enhanced when high processing conditions were applied, subsequently increasing starch availability to enzymic hydrolysis, as is shown in Figure 4.6. Sun *et al.* (2006) found that extrusion cooking had effects on the different fractions of starch: it increased the proportion of rapidly digested starch (RDS) and reduced the proportion of slowly digested starch (SDS) and resistant starch (RS), as shown in Table 4.7. Digestion rates and the amount of raw cereals

reveal major differences compared to the digestion profiles of extruder products, which are almost identical (see Figure 4.7).

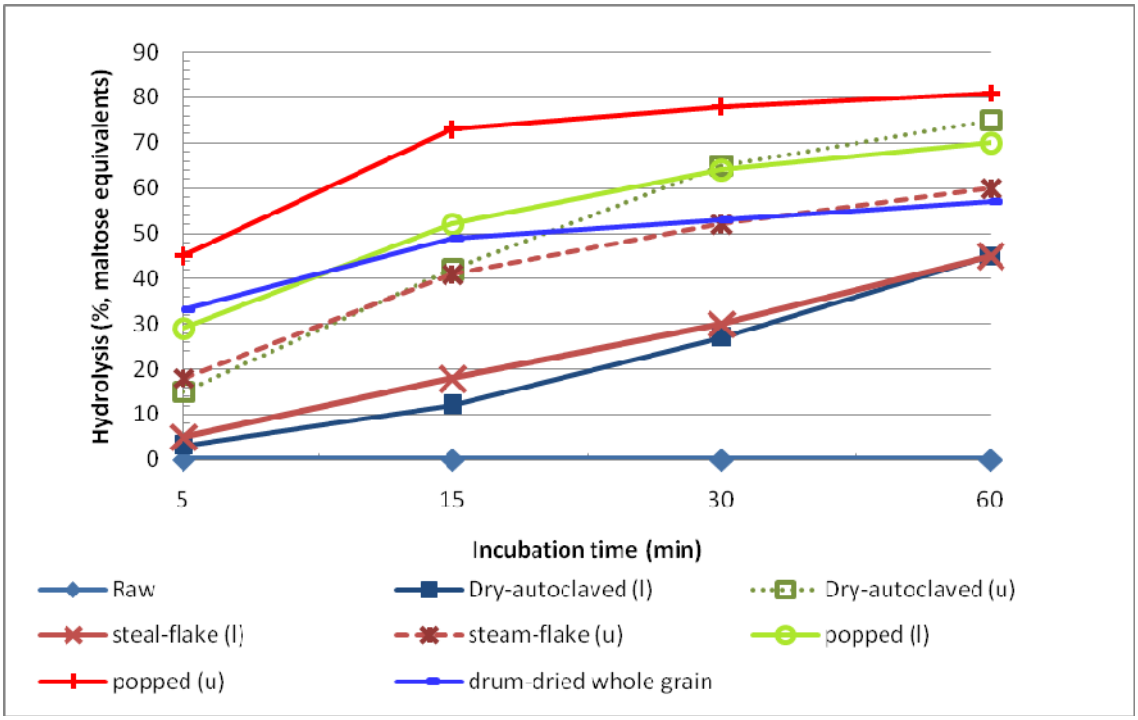


Figure 4.6: starch hydrolysis in milled wheat samples with porcine pancreatic α-amylase (200units/g starch). (l) and (u) correspond to the lower and upper limits of processing conditions. Modified from Holm and Björck, 1988, *Journal of Cereal Science* 8: 261-268.

Table 4.7: contents of starch fractions (g/Kg DM) and their relative composition in native and extruded barley, peas and the mixture of potato starch and wheat bran (PSWB)

Ingredient	Processing	TS	RDS	SDS	RS	RS_1	RS_2	RS_3
Barley	Raw	635	98	456	81	61	20	4
	Extruded	617	608	0	13	1	6	3
Peas	Raw	458	69	171	219	153	57	6
	Extruded	491	453	17	22	5	8	11
PSWB	Raw	818	106	332	380	14	354	3
	Extruded	837	810	9	19	1	6	12

Adapted from Sun T. *et al.*, (2006). *Animal Feed Science and Technology* 131: 66 – 85. (TS, total starch; RDS, rapidly digested starch; SDS, slowly digested starch; RS, resistant starch; RS_1, physically inaccessible starch; RS_2, resistant starch granule; RS_3, retrograded starch. RDS, SDS and RS are expressed based on TS. RS_1, RS_2 and RS_3 are expressed as a fraction of RS.

Zimonja and Svihus (2009), in agreement with previous works, reported that extrusion improves starch digestibility ($P<0.05$) in their study on oat diets; the effect was more evident at duodenal level in broilers.

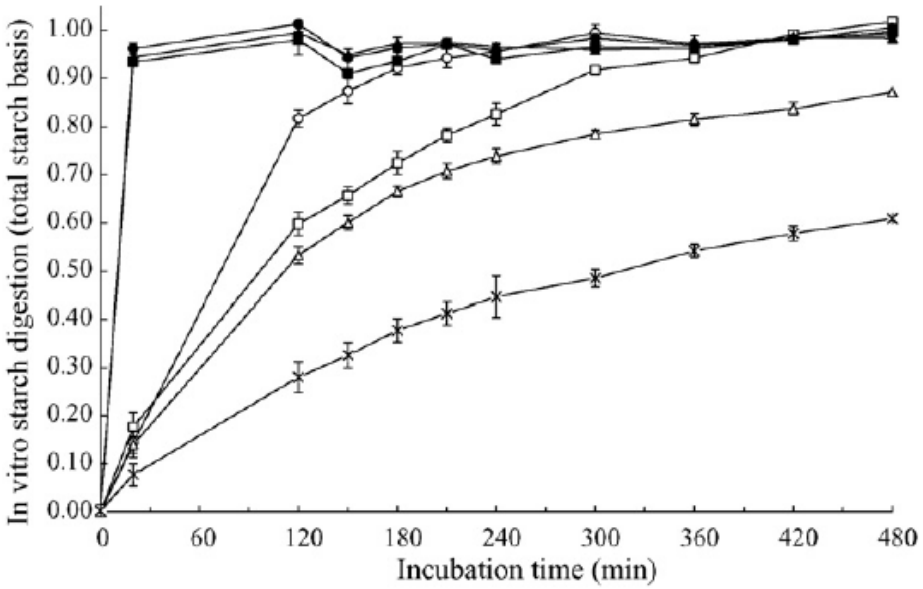


Figure 4.7: Time course of *in vitro* starch digestion (proportion of total starch) of barley (○, raw; ●, extruded), Pea (□, raw; ■ extruded) and PSWB (△, raw; ▲,extruded). Lintner potato starch as reference. Copied from: Sun T. *et al.,* (2006). *Animal Feed Science and* Technology 131: 66 – 85.

Retrogradation, like gelatinisation, is a factor that needs to be considered after processing (Svihus *et al.,* 2005). Retrogradation consists in a reorganisation of gelatinised starch into organised aggregates by molecular chain association, in which amylose and amylopectin form new hydrogen bonds (French, 1973; Rooney and PFlugfelder, 1986; Svihus *et al.,* 2005). Retrogradation is promoted by cooling starch to room temperature or by freezing materials. However, retrogradation may be reversed by heating, which will break up all or most of the refractory aggregates (French, 1973). The extent of retrogradation depends on several factors, including the fine structure of amylose and amylopectin, moisture content, temperature, complexing agents (especially lipids), and the concentration of starch (Rooney and Pflugfelder, 1986). When gelatinised starch is cooled to room temperature, amylose precipitation occurs rapidly (French, 1973; Park and Ring, 2001). Amylose forms strong aggregates; however, because this is a fast process it is not conducive to the formation of a crystalline material. Fast cooling favours the

formation of an amorphous matrix, similar to an elastic gel (French, 1973; Rooney and Pflugfelder, 1986; Park and Ring, 2001). There is also slow crystallisation over a few days after the fast cooling. The crystallites do not have a marked effect on the material properties (Park and Ring, 2001).

The linear structure of amylose, forms reasonably though films and fibres, similar to those of regenerate cellulose (French 1973). Retrograded amylose is stable up to 120ºC; above this temperature the solution initiates materials dissolution, the process is more difficult to reverse than for amylopectin (French, 1973; Park and Ring, 2001). In contrast, amylopectin retrogradation is a very slow process; it can take days or weeks (Eerlingen *et al.,* 1994; Park and Ring, 2001). Amylopectin retrogradation involves the crystallisation of outer short chains; the longer the chains and the more abundant this fraction, the greater the tendency to retrograde and crystallise (Park and Ring, 2001).

The amylopectin branch structure forms weak brittle films, and is associated with the development of a stiffness of material, typically at higher water contents (French 1973; Park and Ring, 2001). At the end of amylopectin retrogradation the extent of crystallinity is comparable to that found in native starch granules, i.e. around 30% (Park and Ring, 2001). Due to the fact that retrograded short chains of amylopectin exhibit low stability of the crystallites formed compared to those formed by amylose (Eerlingen *et al.,* 1994), crystallisation and the associated firming can be eliminated by reheating the retrograded material to 60ºC (Parker and Ring, 2001).

Eerlingen *et al.* (1994) studied the effect of retrograded starch of waxy to enzyme susceptibility, and observed that increasing retrogradation (higher melting temperatures, melting enthalpies, and higher crystalline levels), reduced starch susceptibility to pancreatic alpha-amylase and amyloglucosidase enzymes at 37ºC. The authors tested the effect of storage time and temperature in the retrogradation process and the effect on enzymic hydrolysis. They foundnd that freshly-gelatinised starch was almost fully digested in less than one hour of incubation with pancreatic α-amylase at 37ºC. However, the samples stored at 6ºC for 24 hours, and subsequently stored for 48 hours and

29 days at 40ºC, proved to be more resistant to enzymic hydrolysis. Samples stored for 48 hours were degraded faster (>3hours) that the ones stored for 29 days, which needed more than 6 hours of incubation to reach the plateau. The gelatinised samples, stored for 48 hours at room temperature, were completely hydrolysed after 2 hours of incubation (Figure 4.8). Eerlingen *et al.* (1994) also found that the effect of retrograded amylopectin on starch enzyme resistance depends on the *in vitro* definition of resistance starch is taken in account. For instance, if the authors follow the definition established by Englyst *et al.* (1992), they have to consider the influence of retrograded amylopectin on enzymatic digestion as reported above. Instead, if they consider resistant starch determined by hydrolysis with heat-stable α-amylase at 100ºC, no resistant starch is found; retrograded amylopectin loses its molecular order with high temperatures, and therefore would be easily digested.

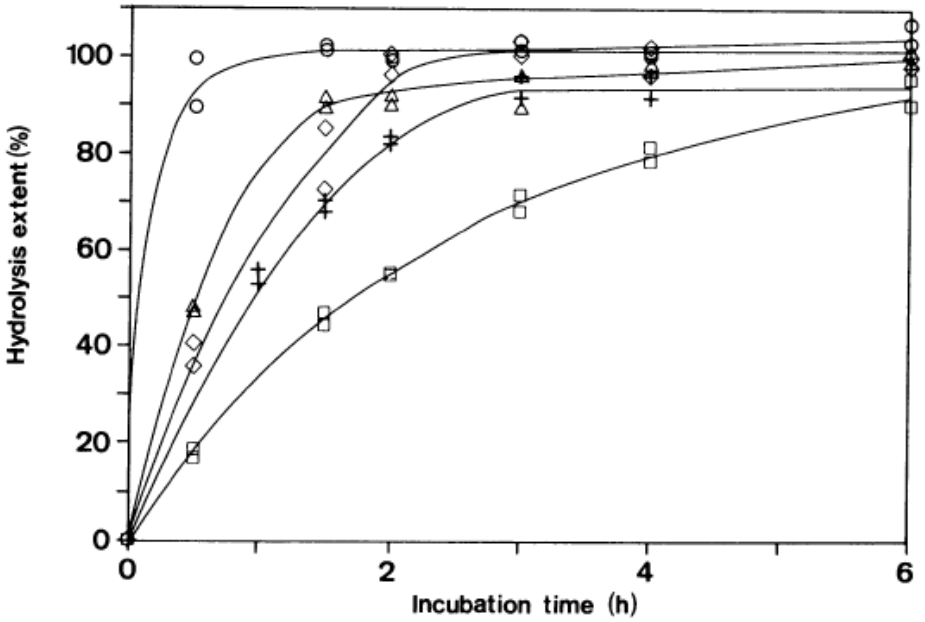


Figure 4.8: Enzymic hydrolysis at 37ºC by pancreatic α-amylase and amyloglucosidase of waxy maize starch samples: native waxy maize starch (△), freshly gelatinised waxy maize starch (o), gelatinised waxy maize starch stored for 48 h at room temperature (◇), gelatinised waxy maize starch stored for 24 h at 6ºC and for 48H at 40ºC (+), gelatinised waxy maize starch stored for 24h at 6ºC and for 29 days at 40ºC (□). Copied from Eerlingen *et al.,* 1994, *Cereal Chemistry* 71 (4):351-355.

Further work is required to evaluate the effect of retrogradation on rice starch enzyme hydrolysis; it has been demonstrated that the longer the holding

time after steaming, the greater the extent of retrogradation and the lower the degree of enzymatic digestibility. Samples with short-chain amylopectin exhibit a slow decrease in enzyme digestibility (Okuda *et al.*, 2006). Retrogradation may occur in feed but under very specific circumstances, for example immediately after extrusion and before the feed has been dried and cooled, but current feed processing methods will not usually favour retrogardation (Svihus *et al.,* 2005). More studies are needed to better understand the influence of retrograded starch on starch degradability.

Other effects of processing have been cited as increasing and decreasing starch digestibility; for example the possible denaturation of alpha-amylase inhibitors may increase digestibility. On the other hand, processing may increase amylose-lipid complexes, reducing digestibility. In fact severe processing conditions have been reported as increasing amylose-lipid complexes and reducing starch digestibility. However, there is no data available in support of the effect mentioned above (Svihus *et al.,* 2005).

4.3.2 Particle size

Particle size (PS) is one of the factors affecting starch digestibility in the rumen and total tract. Particle size reduction increases surface area and makes particles more fragile and accessible for digestion. Particle size trials have demonstrated that small particles are highly degradable compared to larger particles. These conclusions were reached by McAllister *et al.,* (1993), Bird *et al.,* (1999), Rémond *et al.,* (2004), and Blasel *et al.*, (2006).

Observations made *in vitro* with ruminal liquid and enzymatic methods have led to the same conclusions. Tests involving incubation with rumen microorganisms show that barley fermentation has a particular effect on small particles, which reveal a high percentage of ruminal digestibility, as is also found for corn (Figure 4.9) (McAllister *et al.,* 1993).

Small particle size has the greatest influence in rumen digestion; for example, small barley particles increase the production of total VFA, e.g. a 0.5mm particle size produces around 79 mmol, whereas larger particles (4mm) produce 41 mmol, indicating that small particles ferment completely in the

rumen; instead sorghum and oats present low rates of fermentation in all particle sizes, between 30-20 mmol for sorghum and 50-40 mmol for oats total acid production (Bird *et al.,* 1999).

Rémond *et al.* (2004) conducted a trial with cannulated cows to observe the effect of corn particle size on the site and extent of starch digestion. Organic matter, starch and protein digestibility increase with the decrease in particle size (from 3.7 to 0.7 mm screen). In the specific case of starch digestibility, the influence of (small) particle size was more evident in the rumen ($P<0.001$) compared to the small and large intestine, and enhances total starch digestibility as reported in Table 4.8.

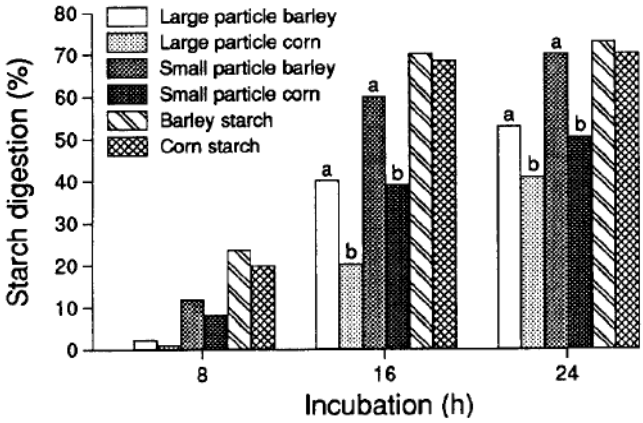


Figure 4.9: Effect of particle size and isolation of starch granules on the digestion of corn and barley starch by mixed ruminal microorganisms. (Small particle size 0.25 to 0.89 mm; large particle size 2 to 3 mm, SEM=±3.7). Bars with different letters within grain type at the same incubation time ($P<0.05$). McAlister *et al.,*1993.

Table 4.8: Apparent starch digestibility in lactating dairy cows fed diets differing in corn processing (experiment1; n=6)

Apparent Digestibility	Mean Particle Size (mm)			P
(%intake)	0.7	1.8	3.7	
Rumen	58.6	49.8	35.5	0.001
Small intestine	28.9	31.5	30.6	0.803
Large intestine	4.0	4.7	3.4	0.841
Total Tract	91.4	86.0	69.5	0.001

Modified from: Rémond et al., 2004. Journal of Dairy Science 87:1389-1399.

Blasel *et al.* (2006) studied the effect of particle size using an enzymatic method. The degree of starch access (DSA), based on enzymatic hydrolysis, determines the potential degradable starch of cereal grains. The authors observed that, as found for the rumen, an increase in particle size lowers the degree of starch access. In fact, particle size greatly influences DSA ($P<0.0001$), with results ranging from 1068 to 15 g/kg for corn grains, for a range of particle sizes from 370 to 4000μm, respectively (see Table 4.9). They observed that for each 100nm increase in particle size, the DSA is reduced by 2.68%.

Table 4.9: Mean particle size effect on DSA

	Mean Particle Size (nm)					
	370	500	640	1100	3140	>4000
DSA %	106.8	103.3	99.6	87.3	32.6	1.5

Modified from Blasel *et al.,* 2006. *Animal Feed Science and Technology* 1-2: 96-107.

This method emerged from the need for an indication of relative starch digestion potentials for cereals considering aspects such as particle size that affect digestibility. So far, such an attempt has been made by NRC (2001), proposing an empirical processing adjustment factor (PAF) which takes into consideration the sources of variation influencing ruminal and total tract digestion. This PAF value is used to adjust non-fibre carbohydrate digestion coefficients for high-starch feeds. The processing adjusting factor was determined based on *in vivo* starch digestibility data obtained from studies performed in the last 10 years (before 2001). The factor permits the correction of the energy value of some feeds. It is a reference correction value that establishes a ranking of feeds processed in different ways, and which corresponds to a major or minor effect on final feed energy value; however it cannot be determined or estimated (NRC, 2001). To overcome the use of a reference value, Blasel *et al.* (2006) attempt to find a simple, quantifiable method involving minimum laboratory equipment and sensitive to factors affecting starch digestion, even though as with 'PAF' their method is subjective and gives an empirical result. A limitation of this method is the lack of data available to establish a relationship between measured values with DSA and *in*

vivo starch digestion. The results obtained in the study of the factors affecting starch digestion (particularly particle size) coincided with those observed in the published *in vivo* and *in vitro* studies mentioned above.

A recent work by Allen *et al.* (2009) evaluated the effect of particle size on rumen kinetics and rumen and total starch degradability, and observed that fine particles increase the rate of starch digestion (19.5 vs. 9.51%/h, $P>0.01$), as well as the apparent ruminal digestibility (47.2 vs. 31.3 %/h, $P=0.03$) compared to medium particle size. The total tract starch digestibility (22%/h) was not affected by differences in particle size, due to greater post-ruminal starch digestion for medium-sized particles compared to fine ones (57.2vs. 41.9%/h).

Dorshort *et al.* 2009 confirmed the effect of particle size in corn silages, where it was negatively related to the starch hydrolysis potential (r=-0.64). Greater particle dimensions reduced the enzymatic hydrolysis, though thiswas not only influenced by particle size, but also by starch and grain structure.

4.3.3 Type of endosperm

Cereal grains are broadly classified according the type of endosperm. The endosperm distinction is based on visual analysis of the texture of the grain. Most grain endosperm is classified into floury or vitreous. In yellow dent corn grain, as well as in sorghum kernels, 25 to 80% of the starch is present in the horny (hard or vitreous) endosperm where starch granules are densely packed within a protein matrix. The remaining starch, as well as most starch present in other grains (barley, oats, and wheat), is deposited as floury starch (Owens, 2005). Figure 4.10 shows a maize kernel and the localisation of each endosperm fraction, horny and floury, respectively. Corn grains have also been described as: flint, translucent, horny, glassy, dense, opaque, not vitreous, soft textures, moshy and porous. The reason for so many endosperm designations is that there is no specific laboratory method for vitreousness quantification, and also there are no chemical properties that can be analysed. Therefore the characterisation of the endosperm is based on the ratio of vitreous horny/floury

endosperm and determined as vitreousness (Dombrink-Kurtzman and Bietz, 1993).

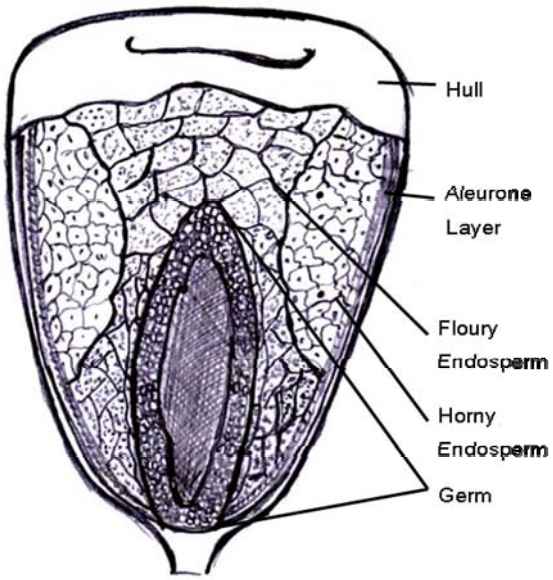


Figure 4.20: Diagram of maize dernel internal structure.

The ratio of Horny/Floury, or vitreousness, can be estimated through physical dissecation of kernels, by measuring the density of the grain or by grinding with a sterwert mill (Owens, 2005).

The studies that have evaluated the effect of vitreousness on starch degradation (Philippeau C. and Michalet-Doreau B., 1997, Philippeau C. *et al.,* 1998, 1999, 2000; Correa *et al.,* 2002) followed the procedure described by Drombrink-Kurtzman and Bietz (1993) to measure the horny/floury endosperm ratio. The method consists in soaking kernels in water for five minutes and then removing the pericarp and germ. The kernel is dried overnight, and the following day the floury proportion is drilled out with help of a 'Dremel Moto Tool'. The horny proportion is ground, approximately 3 minutes for each kernel, then both fractions, horny and floury, are passed through a 250µm mesh screen. The hard and soft fractions are weighed, in order to determine the percentage of hard endosperm. In this analysis between 10 and 100 grains are used (Drombrink-Kurtzman and Bietz, 1993).

Some studies have been carried out in an attempt to observe the effect of the type of endosperm on starch degradability. Most of the studies used dent and flint genotypes, and observed that ruminal starch degradability is greater for dent than flint, (Philippeau C. and Michaleti-Doreau B., 1997; Philippeau C. *et al.,* 1998, 1999, 2000; Correa *et al.,* 2002). In fact Philippeau C. and Michaleti-Doreau B. (1997), Philippeau *et al.* (2000) and Correa *et al.* (2002) found that on average flint genotypes had around 72% of vitreousness, and dent genotyes 49%. Correa *et al*. (2002) observed that vitreousness varied among the dent hybrids. Greater ruminal starch availability was observed for low vitreousness dent hybrids (Correa *et al.,* 2002, Figure 4.11), which on the other hand seemed to be dependent on of maturity stage of grains (Philippeau *et al.,* 1997) Table 4.10. Correa *et al.* (2002) observed that dent cultivar vitreousness showed greater correlation with the days from planting to mature stage.

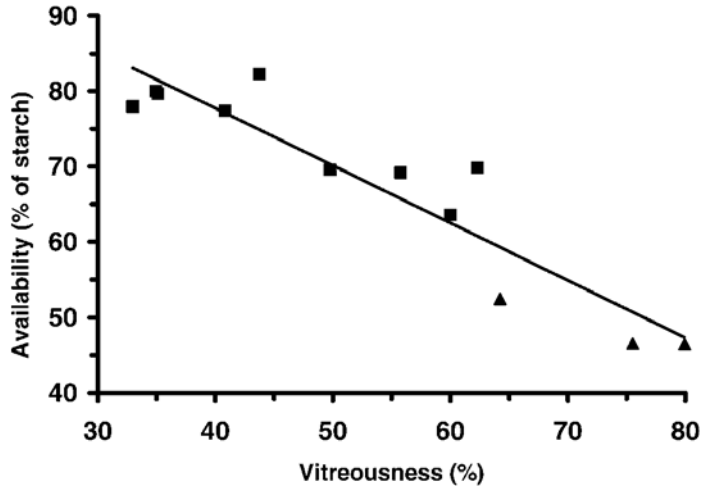


Figure 4.11: Relationship between corn kernel vitreousness and ruminal *in situ* starch availability measured in three U.S. dent (_) and three Brazilian flint (▲) hybrids harvested at the mature stage of maturity and two U.S. dent (_) hybrids harvested at half milk line, black layer, and mature stages of maturity. From Correra *et al.,* 2002, *J. Dairy Sci*.

Table 4.10: influence of the genotype and stage maturity on chemical composition (%DM) and physical parameters of grain

Genotype	Days after silking	DM content		Chemical composition		Physical Parameters
		Whole Plant	Grain	CP	Starch	Viteousness
Dent	22	29.0	38.7	12.5	61.0	26.5
	37	36.5	56.0	10.1	63.1	28.0
	59	39.8	66.0	11.1	68.5	45.4
	78	50.7	75.7	10.0	68.6	48.1
Flint	22	29.7	36.9	13.3	58.6	38.3
	37	30.2	53.9	11.4	62.1	53.3
	59	35.9	63.3	10.4	67.9	61.5
	65	39.3	70.5	11.6	67.3	66.2
	78	40.1	75.1	11.3	67.2	72.3

From: Philippeau and Michalet-Doreau, 1997. J*. An.Feed Sci. Tech.* 68:25-35.

Blasel *et al.* (2006) confirmed previous findings, measuring the effect of vitreousness on starch degradability by applying an enzymatic method to DSA (degree of starch access). They observed that the greater the percentage of vitreous endosperm, the lower the starch degradability. The effect of vitreousness on DSA was determined using the following equation: DSA= 1.385(v) + (-0.0267(v^2) + 646.5, r^2=.59. Results are shown in Table 4.11.

In more recent works, Ngonyamo-Majee *et al.* (2008), Alen *et al.* (2009) and Lopes *et al.* (2009) confirm results from previous findings, where the type of endosperm has a great influence on DM and starch digestibility. Ngonyamo-Majee *et al.* (2008) observed a strong negative correlation of vitreousness with degradability in *in situ* measurements, in particular for more mature, black layer samples (-0.728, -0.770 and -0.603) versus milk layer (-0.569, -0.541 and -0.338) for 0h of disappearance, rumen dry matter and total dry matter degradability. Vitreousness was highly correlated with corn degradability especially in more kernels.

Alen *et al.* (2009), using the *in situ* technique, studied the effect of type of endosperm in rumen kinetics as well as ruminal and total tract starch digestibility. The authors observed that the rate of starch digestibility and

apparent ruminal starch digestibility increased with floury endosperm; conversely the rate of passage decreased compared to vitreous endosperm. The total tract starch digestibility also increased with floury endosperm although post-ruminal digestion increased with vitreous endosperm, as shown in Table 4.12.

Table 4.11: Effect of % of vitreous endosperm on DSA determination.

	Vitreousness (% of the endosperm)					
	0	20	40	60	80	100
DSA (% of Starch)	64.6	66.2	65.8	63.2	58.2	51.7

Modified from Blasel *et al.*, 2006. Animal Feed Science and Technology 1-2: 96-107

In contrast, Lopes *et al.* (2009) studied the effect of the type of corn endosperm on lactating dairy cows. In agreement with previous findings, the authors confirm that floury and opaque corn endosperm is further degraded compared to vitreous ones. The results obtained with DSA, ruminal *in vitro* and rumen *in situ* techniques confirm the statement above and are consistent with each other; DSA and *in vitro* ruminal methods give results 32 and 42% higher for floury and opaque corn than for vitreous, and 24 and 32% greater for floury and opaque than for vitreous corn endosperm, after 8h of rumen *in situ* incubation. The authors found no effect of type of endosperm on milk yield.

Table 4.12: Effect of type of endosperm in dry rolled corn on starch digestibility in ruminants.

	Type of Endosperm		
	Floury	Vitreous	P
Rate of digestion (%/h)	19.9	9.9	<.01
Apparent ruminal Digestion	53.7	24.6	<.001
Passage rate (%/h)	16.1	25.7	<.001
Post-ruminal digestion (%/h)	38.4	60.7	<.01
Total tract starch Digestion	92.2	85.1	<.0001

Modified from Alen *et al.,* 2009. *Journal of Animal Science* 86 (E-suppl.2/J).

4.3.4 Other factors affecting starch degradability

Cereal grains contain relatively little protein compared to legume seeds, on average about 10-14%. Storage proteins account for about 50% of the total protein in mature cereal grains and have an important impact on their nutritional quality for human and livestock consumption and on their functional properties in food processing (Shewry and Halford, 2002). Early studies refer to the protein matrix as a possible factor affecting starch degradability. The protein matrix binds starch granules together, reducing the susceptibility of native and processed starch to microorganism and/or enzyme action (Rooney and Pflugfelder, 1986; Owens *et al.*, 1986). Research has been carried out in attempts to evaluate the effect of the protein matrix on starch digestibility. McAllister *et al.* (1993) observed that maize and barley samples pre-incubated with protease had higher percentages of starch in *in vitro* digestion using mixed ruminal microorganisms compared to untreated samples, as shown in Figure 4.12.

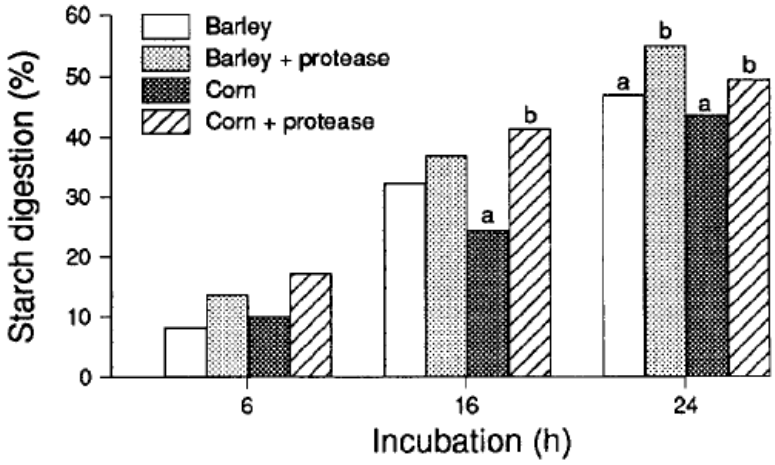


Figure 4.12: Effect of pre-incubation with protease on the digestion of barley and corn by mixed microorganisms. Bars with different letters within the grain type at the same incubation time differ (P<0.05). From: McAllister *et al.,* 1993. J. Anim. Sci.

As stated above, seed proteins are divided into four fractions according to their solubility in: water (albumins), salt solutions (globulins), ethanol solution (prolamins) and dilute alkali or acid (glutelins) (Romagnolo *et al.,* 1994). *In vitro* studies prior to and after incubation in the rumen of corn and corn meal

demonstrated that prolamins were resistant to ruminal degradation (Romagnolo *et al.,* 1994). Philippeau *et al.* (1998) found no relation between the amount of protein and starch degradability. However, they found that the degradable starch and protein fractions of starch were correlated. In fact the salt-soluble protein fraction was found to be positively related to rapidly degradable starches as well as true glutelins. A further study on the effect of the protein distribution of the maize endosperm on ruminal starch degradability confirmed a positive correlation between glutelins and starch degradability and a negative correlation with (α, β, γ) zeins (prolamins of maize), consistently with the findings of Romagnolo *et al*. (1994), Figure 4.13 (Philippeau *et al.,* 2000).

Recently Dorshort *et al.* (2009) have confirmed the negative relation of zein contents (r=-0.51) and the positive relation of albumin-globulin protein (R=0.35) with starch hydrolysis potential in corn silages, in agreement with Philippeau *et al.* (2000), suggesting that starch hydrolysis potential in corn silage decreases with advancing maturity, because of increasing encapsulation of starch by zein proteins.

Hoffman and Larson (2008) observed that the content of prolamin-zeins in high moisture corn, expressed on starch basis, was 2.5 times lower compared to the prolamin-zein content in normal dent corn. Some explanations were advanced to justify such differences. The first was the maturity stage at harvest time: high-moisture corn is harvested earlier compared to dry corn. However, Hoffman and Shaver (2009) reported a work where maximum prolamin content in corn grain harvested near to black-layer formation was observed; at this phase moisture content is similar to the ensiling moisture content of high-moisture corns. The second justification for lower prolamin-zein content in high-moisture corn, more plausible according to Hoffman and Shaver (2009), was that fermentation acids and proteolysis degrade the prolamin-zein content. Besides aqueous alcohol solutions, fermentation acids are also recognised as prolamin solvents. Therefore prolamins are likely to be dissolved by the acids formed during the ensiling process of high-moisture corn, subsequently reducing zein content. Moreover, bacterial proteolysis is an

intrinsic mechanism in corn-grain fermentation, inducing corn protein degradation (Hoffman and Shaver, 2009).

Philippeau and Michalet-Doreau (1998) observed increased ruminal starch degradation with ensiling grains, suggesting starch availability as a consequence of partial degradation involving protein zeins. This hypothesis is supported by further work where starch degradability in kernel grains increases from 70.2% to 92.3 before and after ensiling respectively (Jurjans and Monteils, 2005).

One of the structural features affecting starch degradability is the amylose/amylopectin ratio. In fact several studies have shown that amylose content is negatively correlated with starch digestion (Cone and Wolters 1990, Li *et al.,* 2004, Anker-Nilssen *et al.,* 2006). *In vitro* enzymatic studies using three cultivars of barley (waxy, normal and high-amylose) have shown that waxy cultivars are more digestible compared to normal and high amylose cultivars (Björck *et al.,*1990, Li *et al.,* 2004, Anker-Nilssen *et al.,* 2006).

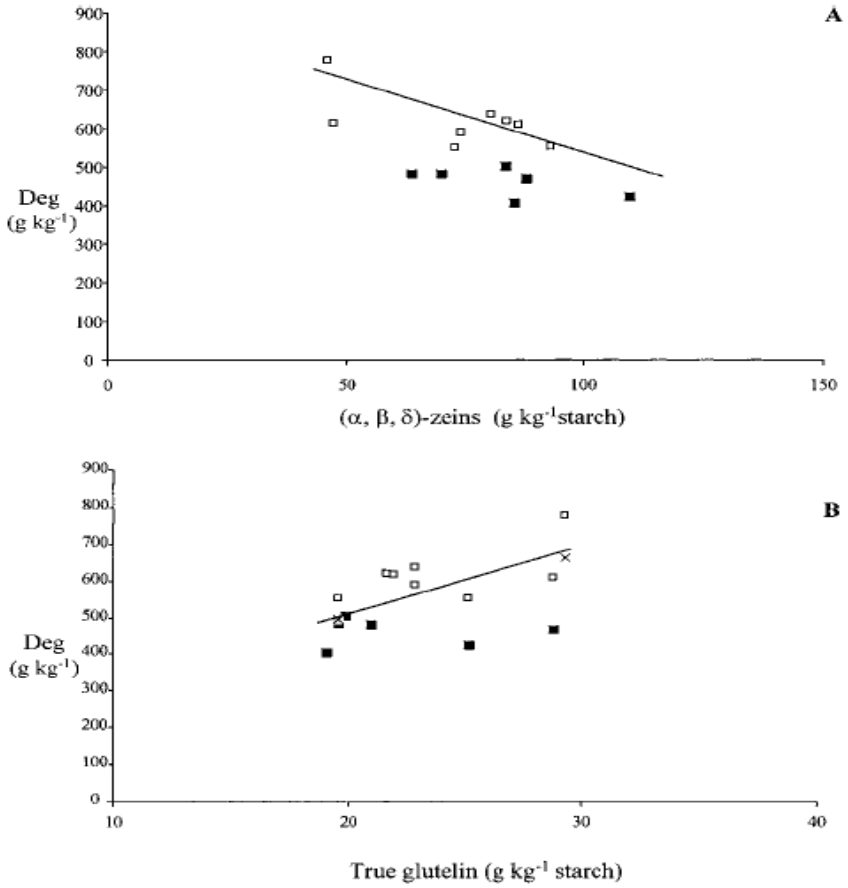


Figure 4.13: Relationships between ruminal starch degradability (Deg) and (α β γ)-zeins (A) and true glutelins (B) for dent (□) and flint (■) maize. Philippeau. *et al.,* 2000*. J. Sic. Food Agric 80*:404-408.

Waxy cultivars are degraded faster and to a greater extent compared to normal and high-amylose cultivars, and these differences are more evident with an increase in the incubation time (Anker-Nilssen *et al.,* 2006), as illustrated in Figure 4.14. Waxy genotype ultra-structure appears to be more accessible to enzymic hydrolysis (Li *et al.,* 2004).

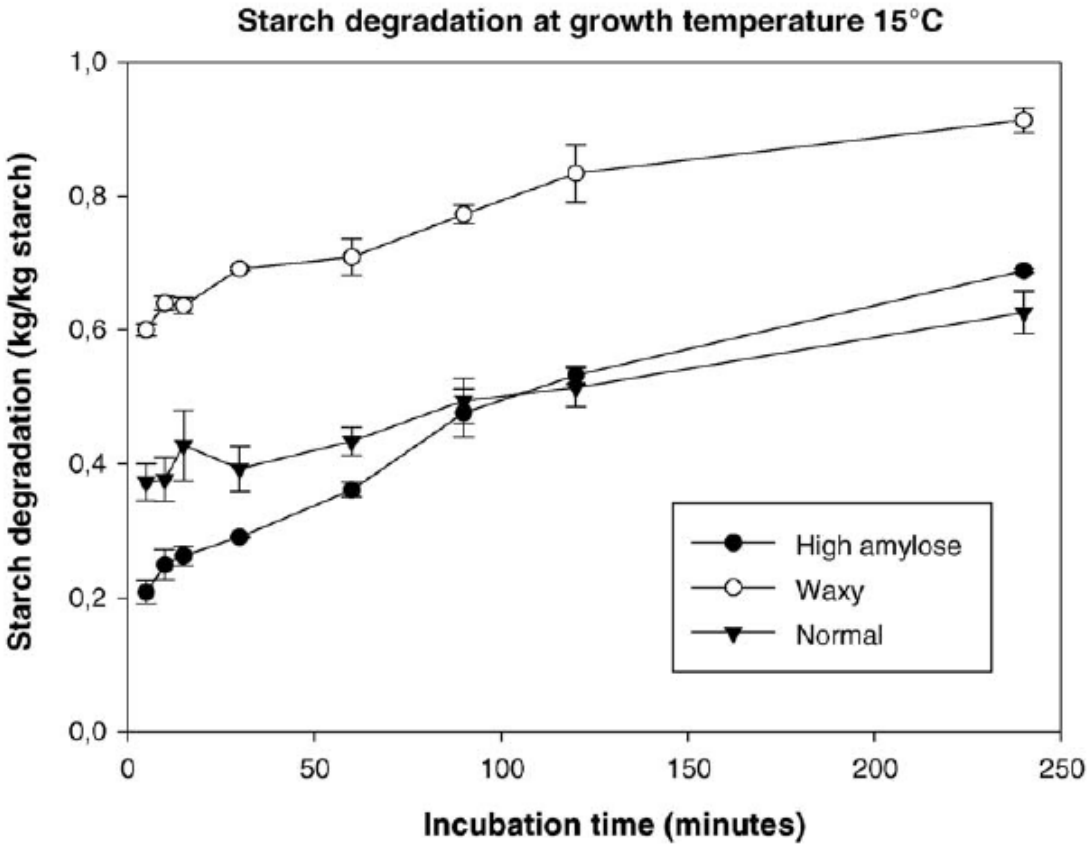


Figure 4.14: The cumulative increase in starch degradation with increasing incubation time at 15ºC for the three amylose genotypes (waxy, normal and high amylose). Adapted from Anker-Nilssen *et al.,* 2006. *Animal Feed Science and Technology* 130:3-22.

A recent *in vitro* study of ruminal starch digestion, using different genotypes of barley (low, normal and high amylose) has shown that low amylose genotypes have higher effective starch digestion rates (0.148/h) compared to normal amylose (0.115/h) and high-amylose (0.102/h) cultivars. Once more, lower amylose content genotypes exhibit greater and faster starch digestibility (Stevenebø *et al.,* 2009).

As mentioned before, severe processing treatment has a remarkable effect on starch structure. Htoon *et al.* (2009) observed that high-amylose starches undergo a significant reduction in resistant starch when subjected to extrusion cooking, enhancing the accessibility of enzymes to starch. However, after such treatment there is a reorganisation of starch polymers, with the establishment of new hydrogen bonds and the formation of double helices, resulting in a different resistance to enzyme hydrolysis, with subsequently low

processed starch digestibility. Amylose affects starch degradability not only because of its presence in starch granules but also due to the native lipid-complexed amylose, or formations of lipid complexes under special conditions. The lipid content in cereal starches is generally proportional to their amylose content. In barley, wheat and rye starches, the starch lipids are almost exclusively lysophospholipids (LPL), which have been shown to exist as amylose-lipid inclusion complexes in native starch granules rather than being formed during starch gelatinisation (Morrison *et al.,* 1993a, b). The effect of lipids in gelatinised starch hydrolysis is lower than that of the lipids present in granular starch. In fact, Shamekh *et al.* (1998) observed that the peak of hydrolysis achieved for gelatinised starch was 80%, compared to almost 20% for native starches.

Anker-Nilssen *et al.* (2006) characterised three barley genotypes by measuring the amylose content divided into three important fractions. The measurements were performed following a colorimetric method, where was the following were determined: apparent amylose (AAM-measured on flour with all lipids present) and total amylose (TAM-measured on defatted flour). The lipid complexing capacity of amylose (LAM) was determined by the difference between the AAM and TAM, as shown in Table 4.13.

Table 4.13: The average values of some important characteristics for amylose genotype waxy, normal and high amylose

Amylose Genotype	Total Starch (g/kg flour)	TAM (g/kg flour)	AAM (g/kg flour)	LAM (g/kg flour)
Waxy	630	7	4	3
Normal	673	278	237	41
High Amylose	607	461	387	74

From: All values are given on dry matter basis. Anker-Nilssen *et al.,* 2006. *Animal Feed Science and Technology* 130:3-22.

The high amylose genotype, besides having the lowest starch degradability in all incubation times compared with waxy and normal varieties, also presents the highest content of lipid complexing amylose. Consistently with

the above, the high digestibility of high-amylose barley could be a consequence of amylose-lipids complex, although more studies are needed in this field to be certain of such conclusions. Dry matter content and conservation methods were shown to influence starch degradability.

Oba and Allen (2003) studied the effect of corn grain conservation on ruminal digestion kinetics using two dietary starch concentrations. They observed that corn grain with a higher content of starch and moisture was degraded more in the rumen compared to low starch corn, independently of the moisture content. They also showed that independent of the starch content, dry corn is less degradable in the rumen compared with high-moisture corn, and subsequently the passage rate and intestinal digestion increase, as described in Table 4.14.

Table 4.14: Effects of corn grain conservation methods on starch digestibility for two dietary starch concentrations

	High starch (31%)		Low starch (21%)	
	High Moisture	Dry	High Moisture	Dry
Intake (Kg)	6.2	7.0	3.9	4.1
Ruminal Digestion (%)	71.1	46.9	58.5	45.9
Passage to duodenum (Kg/day)	2.2	4.2	1.9	2.4
Digestion Duodenum (%)	86.2	89.6	83.8	86.9
Total tract Digestion (%)	95.8	94.2	93.3	93.0

Modified from Oba and Allen, 2003. *Journal of Dairy Science* 86:184-194.

Dorshort *et al.* (2009), studying the effect of factors on starch hydrolysis potential, observed that it was negatively correlated to plant dry matter (r=-0.53), suggesting that with an increase in dry matter starch hydrolysis decreases. This confirms the findings of Blasel *et al.* (2006), who showed that for each 1% of increased DM the starch hydrolysis potential decreases by 2%. Schwab *et al.* (2003) observed that starch digestibility predictions was are highly dependent on DM content, and at 35% DM they predicted that the apparent total starch digestibility for unprocessed and processed corn silage

was 0.86 and 0.91 respectively, thus suggesting that starch in dried kernels is less available for digestion, therefore influencing the lactation energy value of corn silage. Table 4.15 shows the variation in silage net lactation energy with DM content and the effect of processing.

Table 4.15: Effect of maturity stage and processing to estimate net energy value of lactation using empirical and multi-component summative equations (Rohweder *et al.,* 1978) of corn silages, varying the whole-plant dry matter concentration.

Dry Matter (%)	Rohweder (1978)	Processed	Unprocessed
30	1.52	1.63	1.63
35	1.52	1.61	1.56
40	1.52	1.56	1.47
45	1.52	1.52	1.41

Modified from Schwab *et al.*, 2003. Animal Feed Science and Technology 109:1-18.

5 Trials

Preface

Livestock production is an important component of food industry and Nutrition is the key factor to improve animal performance, health and welfare (NRC 2001). Fulfilling animal nutritional requirements is a crucial aspect in the management of a lactating dairy cow's husbandry, diets formulation takes in account not only the nutrients requirement of animals but and also the values of feed digestibility. The feed digestibility values used by NRC 2001 and Cornell models for diets formulation were obtained from in vivo studies, also the in Situ and in Vitro Tilley and Terry method have been used for feeds digestibility determination; however as reviewed in chapter 3, this techniques are time consuming, expensive and require the use of animals. The application of in vitro enzymatic techniques has been studied in order to obtain a new approach, simple, easy and less expensive, for feeds digestibility determination.

Starch represents the major energy components of cereals grains (Huntington 1997) and the most important source of energy for lactating dairy cows (Zebelli et al., 2010). Starch digestibility varies among type of cereals (Herrera and Saldana et al., 1990; Lanzas et al., 2007) and with the type of treatment that cereals grains were submitted.

In the last decade, cereals intrinsic physical and/or chemical characteristics (genotype, vitreousness, particle size, amylose/amylopectin ratio, amylose-lipids complexes) led researchers to study their potential effect on the rate of starch degradation by enhancing or reducing starch availability to bacteria and microorganisms in the rumen (Phillipeau and Michalet-Doreau, 1997; Correa *et al.* 2002; Callison *et al.,* 2001; Oba and Allen, 2003; Rémond *et al*, 2004; Li *et al.,* 2004; Svihus *et al.,* 2005; Blasel *et al.,* 2006). Based on these findings, a correct evaluation of starch rumen degradability and intestinal degradability is essential in ruminants ration formulation.

In this thesis experimental part, the aim was to study factors affecting corn starch degradability by applying an enzymatic technique; however preliminary studies were needed. The first trial consisted in the study of feeds starch ruminal digestibility by using in Situ technique and in Vitro Tilley & Terry

method. In the second trial was studied and improved an enzymatic method to evaluate feeds starch degradability. Foreseeing a potential correlation between the enzymatic method and the ruminal starch degradation, the same set of samples used in the first trial was used for enzymatic starch degradability determination. The results obtained were studied statistically and compared with the results of starch degradability from the first trial. The good correlations obtained between the in vitro Tilley & Terry and the enzymatic technique led us to proceed in our research and evaluate corn starch degradability. A set of samples composed by: corn grains, high moisture and high moisture ear corn was used to evaluate the intrinsic factors affecting starch degradability.

5.1 Trials carried out for the present work

The first experiment was carried out *at Foulum Research Centre (Aahrus University, Aarhus, Denmark*) to evaluate the starch degradability of different feeds using the in situ nylon bags and in vitro Tilley & Terry methods.

The second experiment was carried out *at Raggio di Sole Industrial mill (Fiorenzuola, Italy*) to improve an enzymatic technique to estimate starch degradability of cereal grains and the effect of buffer solution media and enzyme mixture.

The third experiment was carried out in both *Foulum Research Centre and ISAN* (*Piacenza, Italy)* to compare values of starch degradability produced by the enzymatic technique and in vitro rumen methods.

The forth experiment was carried out at *ISAN,* creating a data set of maize samples collected at different maturity, to evaluate the main factors affecting enzymatic starch degradability.

5.2 Trial 1- Evaluation of starch degradability by using in situ and in vitro methods

The *in situ* nylon bags and *in vitro* Tilley & Terry trials were organized and carried out at Foulum Research Centre facilities (Foulum, Denmark).

5.3.1 Experimental feeds

The tested feeds were provided by the Department of Animal Health and Bioscience, Faculty of Agricultural Sciences – AarhusUniversity (Denmark) and consisted of dried whole-ear corn (WEC), ground maize (GM), rolled shrivelled pea (RSP), rolled toasted shrivelled pea (RTSP), cold rolled toasted barley (CRTB), cold rolled toasted wheat (CRTW), NaOH-treated wheat, waxy maize (M1), flint maize (M3), high moisture shelled corn (HMS) and two high moisture ear corn (HME1 and HME2). These samples were selected to represent feeds having different starch content, processing treatments (chemical and physical treatments) and conservation. Among maize samples, different genotypes (waxy, flint and yellow corn) were used in current trial.

5.3.2 Animals

The research protocols and animal care were performed in accordance with the EC council directive guidelines regarding the use of animals for experimental and other scientific purposes (EEC, 1986). Three non-lactating Holstein cows fitted with a permanent rumen fistula, housed in a tie stalls with rubber mats were used in this trial. Cows were fed with a maintenance diet and fed twice a day (8:00 am and 16:00 pm) and had free access to water.

5.3.3 In Situ Starch Degradation

Principle of method: Feed were incubated in the rumen with nylon bags, which have pores that are small enough to retain feed particles and big enough to allow rumen microorganism enter the bags, providing similar conditions within the bags as found in the surrounding rumen content.

This procedure was divided in three parts:

1- Samples preparation,

2- Samples incubation in the rumen,

3- Determination of digested starch.

1) Samples preparation

After air-drying at 65ºC, the samples were milled through a 1.5-mm screen and then weighted (1.0 g for 0h, 2h, 4h, 8h, 16h and 1.5 g for 24h, 48h, 72h incubation time) into polyester bags with pore size of 36 µm (dried at 60ºC and weighted before weighting out). The samples were replicated three times for each incubation time. The replicates were incubated in rumen of fistulated dry cows, one for each cow.

2) Rumen incubation

Bags were incubated just before morning and afternoon feeding time, and before placing bags inside the rumen, they were soaked in warm water (35-37°C) for 5 min. At the end of each incubation time, bags were taken out from the rumen, washed with cold water in a washing machine for 10-15 min and frozen until all incubations were ended.

At the end of the trial, samples were defrosted and washed to eliminate any residue from rumen remaining in the outer part of the nylon bags.

Afterwards samples were dried at 60ºC until constant weight for estimation of dry matter degradability. Then analyzed to determine the residual starch content.

3) Undegraded starch determination:

The three replicates residues of each sample were mixed together and ground with a mortar and pestle.

Then, residual starch was measured by enzymatic hydrolysis. Residues were incubated in acetate buffer 2M (164 g of sodium acetate dissolved in 500 ml of distilled water and 75 ml of acetic acid 100%. pH was adjusted to 5.0 using NaOH 12.5M, afterwards solution was diluted to 1L with distilled water), and 200 µl of heat stable α-amylase (Ankom), in a 100 ºC glycerol bath for 90 minutes, then samples were cool down on ice till 60ºC, and the

amyloglucosidase solution 1087 U/ml, (1 ml of amyloglucosidase, Megazyme International catalogue nr. E-AMDGDF, 140.000: 3260 u/ml into 2 ml of acetate buffer 0.1M: 8.641 g of sodium acetate dissolved in 500 ml of distilled water and 2.088 ml of acetic acid, adjusting pH to 5.0 with NaOH) was added. Samples were incubated for another two hours in water bath at 60ºC and agitated after the first hour. Enzymatic reaction was stopped by placing the samples in glycerol bath at 100ºC for 10 minutes and cool down on ice for 15-20 minutes.

Samples were centrifuged and the supernant was analysed for glucose content, with Hitachi 912 instrument.

The digested starch was calculated as follow:

Digested Starch (% of Total Starch) = 100 – (Indigested Starch / Incubated Starch) x 100.

5.2.4 In Vitro Tilley & Terry Starch Degradation:

Principle of method: Samples were subjected to in vitro incubation at different times with a diluted and buffered rumen liquor to simulate rumen conditions.

This procedure was divided in three parts:

1- Samples preparation,

2- Samples incubation in the diluted rumen inoculum,

3- Determination of digested starch.

1) Samples preparation

Samples were ground with 1-mm screen. Then, 360 mg were weighted into test tube. For each sample and incubation time (0, 1, 2, 4, 8, 24, 48 and 72 hours), two replicates were weighted. Two blank (rumen without samples) were used at each time.

2) Incubation with rumen inoculum

Rumen liquor was collected from three fistulated cows in the early morning of the day trial, before morning feeding time and mixed. Rumen contents were taken by hand and the liquid was pressed out and filtered through a layer of gauze.

The buffer solution (29.1g $Na_2HPO_4.2H_2O$, 49g $NaHCO_3$, 2.35g NaCl, 2.85g KCl, 0.64g $MgCl_2.6H_2O$ and 0.26g $CaCl_2.2H_2O$, mixed into 5 litres of distilled water, solution was saturated with CO_2 to pH 6.8-6.9) was prepared the day before the beginning of the trial.

Buffer-rumen solution was mixed in proportion of 2:1 (buffer:rumen) respectively.

To each sample 40 ml of buffer-rumen solution were added and than placed into a 39ºC water bath. Samples were shaken every hour for the first eight hours of incubation.

At the end of incubation time, microbial activity was stopped by placing samples into a 100ºC glycerol bath for 10 minutes, and then cooled for 15-20 minutes on ice. Samples were treated enzymatically to determine residual starch (Tilley &Terry, 1963, Sveijorsson *et al.*, 2007).

3) Determination of residual starch

Each sample was added with 10 ml of acetate buffer 2M, as described above and 200 µl of heat stable α-amylase (Ankom). After agitation with vortex, samples were placed for 90 minutes into a 100ºC glycerol bath then conserved at 4°C. At the end of the experiment, the samples were centrifuge (3000 g) at 4ºC for 10 minutes, 5 ml of supernatant was transferred to a test tube and 50 µl of the amyloglucosidase solution (see point 3 from in situ trial) was added. Samples were incubated in a 60ºC water bath for 2 hours and agitated after 1h.

Enzyme action was stopped after 2 hours by placing the test tubes in 100ºC glycerol bath for 10 minutes and cool down on ice for 15-20 minutes.

After centrifugation (3000g at 4ºC) glucose content was determined on the Hitachi 912 instrument. Glucose measured was converted into indigestible starch by the following equation:

Indigestible starch= mg of glucose*0.9

Digested Starch (% of Total Starch) = 100 – (Indigested Starch / Incubated Starch) x 100.

5.2.5 Results and Discussion

The chemical characteristics of the experimental feeds are presented in table 2. The NDF content of WEC and HMC samples were high due to the presence of the cob, with values over 28.0% on DM. The starch content ranged from 29.92 (Pea) to over 70% (corn) and resulted higher in treated or native cereals (maize, barley and wheat) than legume (pea) samples. On the contrary, a higher CP content was measured for TRS and RSPS (25.52 and 25.49, respectively) compared to cereals. Among corn genotypes, the starch content was similar and equal to 69.60, 71.82, and 71.22 % of DM, respectively for waxy, flint and yellow corns. In the present trial, three corn samples harvested before black layer stage of maturity were tested. At harvest the HMS had lower moisture content of 32.5%; compared to the HME1 and HME2, being 47.31 and 45.82% moisture content, respectively (Table 5.1). Moreover, the HMS had a higher starch content than HME1 and HME2 due to the dilution effect of the cob in HME (72.51 vs. 49.60 and 44.041 % of DM; respectively). The WEC contained 55% of starch and was characterized by having the complete Ear (grain + cob) dried and ground. For this reason the starch quality and type can be considered similar to the starch of commercial yellow corn grains.

Table 5.1: Chemical characterization of samples, on a dry matter basis.

Samples	Composition, % of Dry Matter				Moisture
	Ash	Crude Protein	Starch	NDF	
WEC	2.10	7.8	55.87	29.03	
GM	1.82	9.6	71.22	9.94	
RTSP	3.79	25.52	31.31	14.52	
RSP	3.87	25.49	29.92	14.31	
CRTB	2.09	11.4	56.21	16.74	
CRTW	1.99	11.9	59.77	10.08	
NaOH-T W	5.14	9.8	68.30	7.79	
Waxy	1.78	9.66	69.60	10.02	
Flint	2.12	9.73	71.82	9.57	
HMS	1.83	7.19	72.51	8.91	30.28
HME 1	2.13	8.49	49.60	28.73	45.46
HME 2	2.15	8.30	44.04	33.05	43.56

5.3.5.1 In Situ Starch Degradation

In the present thesis, different starchy feeds were evaluated for DM and starch degradability by in situ method, performed in agreement with standardized protocol used at Foulum Research Centre. The results are shown in figure 5.1 and 5.3. The lowest ruminal DM degradability were measured for the two HME1 and HME2. Also the WEC and HMS samples had DM degradability lower than other cereals (Wheat, barley and dried maize), figure 5.1, contrarily to what was observed for starch degradability, figure 5.3. As described above, these samples were characterized by a high NDF content.

The DM digestion kinetic tended to increase linearly, reaching the maximum value only after 72h of incubation. For WEC, the DM disappearance is limited by the undigested cob residues in the bag and could not be related to the starch degradation pattern. The in situ DM degradability curves of treated cereals (cold rolled toasted barley, cold rolled toasted wheat and NaOH-treated wheat) reached the peak earlier than other samples, just after 8h of incubation.

Intermediate digestion kinetics was observed for the three types of corn (waxy, flint and yellow) and the two treated pea samples. However, the cumulative DM degraded after rumen incubation resulted higher for treated pea (RSP and RTSP) samples than corn samples at each time. Among maize genotypes, DM degradability decreases from the waxy to the yellow and to the flint samples. The DM degradabilities values were interpolated with the exponential model $Y= a + b (1- e^{-ct})$ proposed by Ørskov and McDonald (1979) and by comparing the fractional degradation rate ($c\ h^{-1}$) with the total degraded DM; a rank can be done as described in table 5.2.

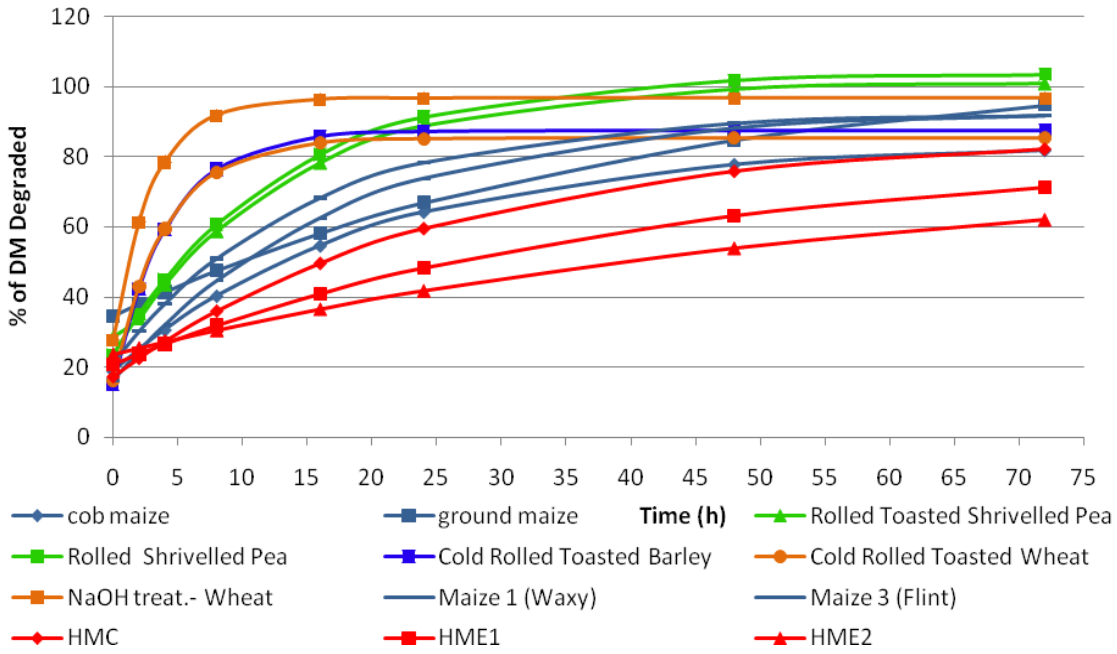


Figure 5.1: Percentage of degraded dry matter determined by in situ technique

In situ methods have been extensively used in many studies conducted to evaluate the digestion rates and rumen starch digestibility. Different results were reported by Tothi *et al* (2003), suggesting that in situ method tend to overestimate ruminal degradability of rapidly fermentable starch and underestimate degradability of slowly fermentable starch when compared with the in vivo method consisting on rumen evacuations. These could be explained by data of van Zwieten *et al*. (2008) reporting a reduction of the disappearance rate of starch in nylon bags in presence of protozoa. Also Hindle *et al*. (2005) reported a reduction of corn starch degradability when evaluated by using in situ and in vivo techniques (53% and 75%; respectively).

In figure 5.2, the more representative starch degradability kinetics of experimental feeds was described. Similar to results of DM degradability, the three treated starchy feeds (cold rolled toasted barley, cold rolled toasted wheat and NaOH-treated wheat) were faster digested than others, being more than 90% of total starch degraded after 8h of incubation.

Similar starch degradation kinetics were measured for high moisture corns, WEC and native maize (waxy, flint and yellow), even if the cumulative starch degraded from 2h to 8h resulted higher in WEC, HMS and HME 1.

Different starch degradation kinetic were observed the for two treated pea samples. After 8h of incubation the RSP resulted more degradable than RTSP: being the digested starch 80% compared to only 20% for RSTP.

All starch was completely degraded after 72h of rumen incubation.

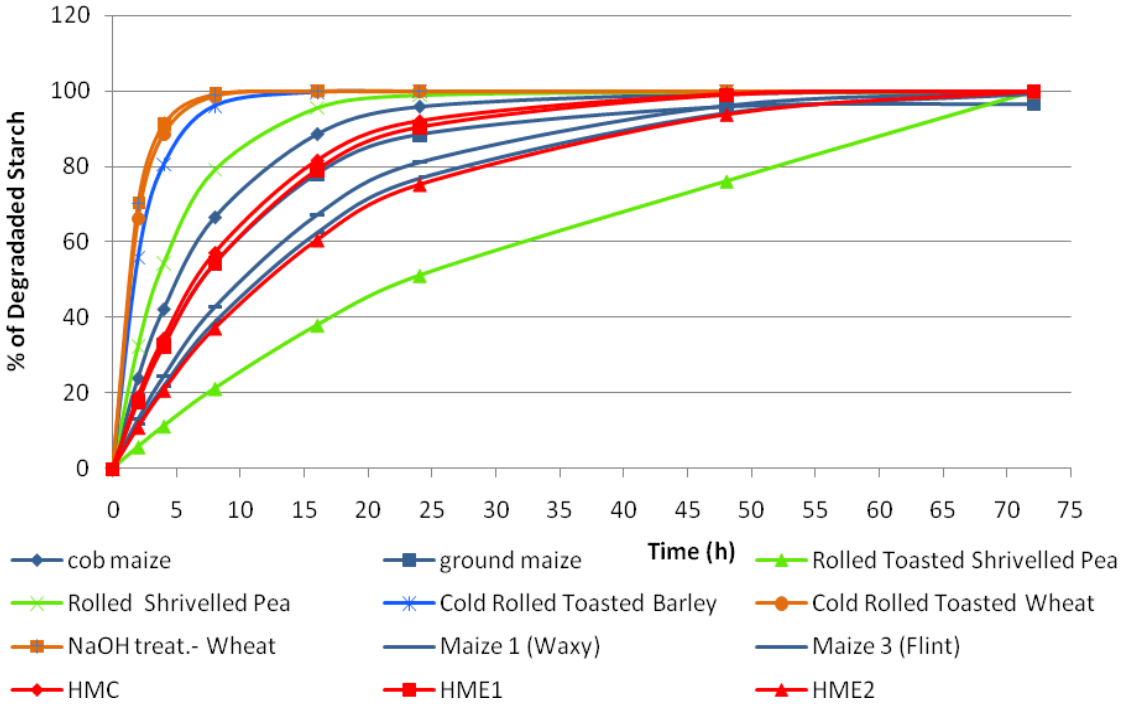


Figure 5.2: Percentage of starch degraded by in situ technique

Table 5.2: Samples ranking base on starch fractional degradation rate c (h^{-1}).

Feed	RSTP<	P36<	M1<	M3<	P32<	GM<	P26<	CM<	RSP<	CRTB<	CRTW<	NaOH TW
C (h^{-1})	0.03	0.06	0.06	0.07	0.09	0.103	0.106	0.14	0.19	0.41	0.54	0.61

The rank of rate of starch degradability among feeds showed how physical-chemical processing of cereal starches affected in situ and in vitro digestibility compared to untreated raw materials. This is in agreement with data reported by other authors. In particular, subjecting grains to moisture, pressure and heat makes the starch granules more accessible to digestion in rumen compartment (Huntington, 1997). About 100% of starch of CTRW and CTRB

was degraded after 16h of incubation. Similar results were measured for the chemical treated wheat (NaOHTW); however, since this treatment acts over the whole grain by disruption of the shell, and in this work samples were fine ground, this result is due to the wheat natural digestibility and not a consequence of the grain treatment. The fractional rate values calculated in present work for treated samples (0.41, 0.54, and 0.61 for CRTB, CRTW, and NaOHTW; respectively) resulted in agreement with results reported by other authors (Tothi *et al.*, 2003).

Besides grain processing, also the endosperm texture were showed to influence the in situ starch degradability. Philippaeu *et al* (1998) reported a 22% effective starch degradability of dent corn, higher than flint corn. Our results were in agreement with these results, having the dent corn (CM) a starch fractional degradation rate 2-fold higher than flint corn (0.14 vs. 0.07 h^{-1}).

Despite few published data (Larsen *et al.*, 2009), the starch from legumes are found to have lower availability in situ than starch from cereals (Offner *et al*, 2003). This was confirmed by in situ test, being the starch of RSP less degraded in rumen compartment than other samples (see picture 5.2).

Some authors suggest that in situ techniques could not be precise to evaluate starch degradation rates, because of the proportion of water soluble materials that can leave unfermented from the nylon bags (Stern *et al.*, 1997). In our experimental condition the amount of the materials escaping from nylon bags (a fraction) range from 17% to 38%. Our samples were ground through a 1,5 mm screen. Similar results were reported by (Tothi *et al*, 2003).

Despite the high amount of feed released from the nylon bag, the method seemed to be adequate to rank the starch degradability of feeds.

5.3.5.2 In vitro Tilley & Terry Starch Degradation

To evaluate the cereal starch degradability of experimental feeds, an in vitro Tilley and Terry (1963) method was used. This method it is largely used by labs to predict the digestibility of feeds both forages and concentrates.

The results of the Tilley & Terry starch degradability are shown in figure 5.3. The starch evaluation by using the T&T method showed differences among

feeds, in agreement with different work carried out by (Grant and Mertens1992b; Tománková and Homolka, 2004). Differences observed were less evident than with the in situ method. The samples did not show a pronounced lag phase, and the major differences in starch degradability among samples were measured at 8h of the in vitro incubation. All the samples were completely degraded after 48h of incubation, earlier than what was observed for in situ trial.

In agreement with digestion kinetics observed using in situ method, the CRTB and CRTW samples had the highest percentage of starch degradability at 8h (60-70 %). Native maize was less degraded, with starch degradation ranging from 30% to 55%. Also using this method, higher starch degradation was measured for RSP than RTSP.

Tomáková and Homolka (2004) studied the in vitro ruminal starch degradability comparing untreated with NaOH treated cereals. They observed that NaOH treated cereals were slightly less degradable than the untreated grains. The results obtained in our work with the NaOHTW were in agreement with the results reported by the authors; at 8h and 24h of incubation 49% and of 94.9% starch was degraded in our trial similar to the 6h and 24h of incubation reported by the authors 46% and 96.4%.

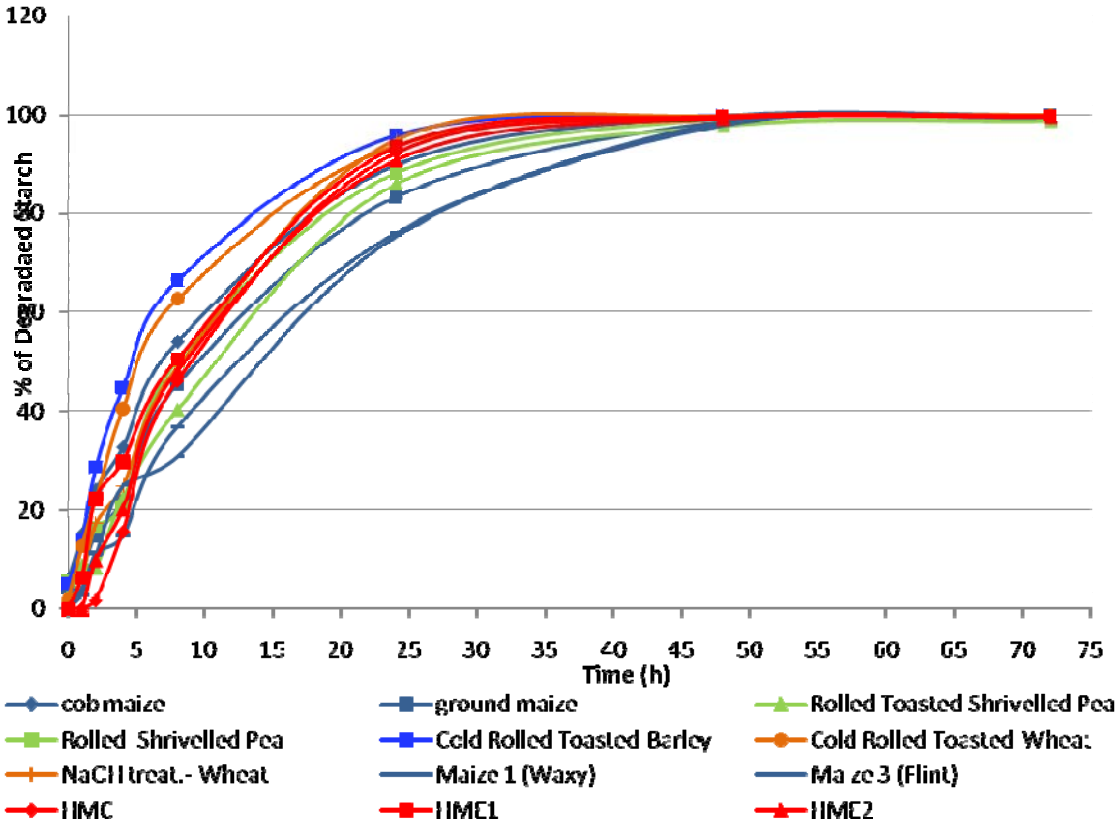


Figure 5.3: Starch degradability determine by using Tilley and Terry Method.

5.3.5.3 Comparison of in situ starch degradability values with the in vitro Tilley and Terry results.

The Pearson correlation coefficients were estimated using starch degradability data obtained with both in situ and in vitro T&T method.

The highest relationships could be measured comparing starch degradation at 4h and 8h of incubation (r=0.68 and r=0.77, respectively 4h of in vitro T&T vs. 4h of in situ technique and 8h in vitro T&T vs. 8h in Situ technique; $P < 0.01$). However, as showed in pictures 5.4 and 5.5, a high dispersion of the data could be observed when comparing starch degradation values measured at 4h by the two methods.

A lower correlation coefficient was calculated at 2h (r=0.59; respectively $P<0.05$).

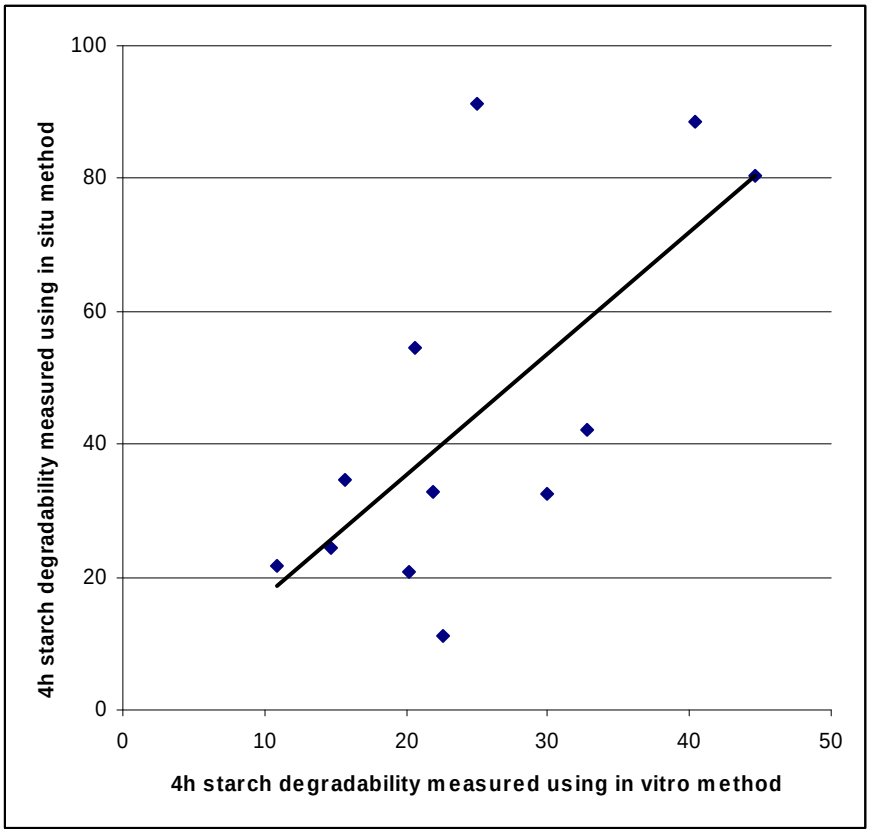


Figure 5.4: Relationship between starch degradability measured at 4h incubation by using in situ and in vitro techniques.

On average, the in vitro Tilley and Terry method gave a high starch degradability response. The in situ procedure allowed us to better evidenciate the differences of starch degradability values among feed samples, in particular for 4 to 24h of incubation, having a direct effect on the degradation curves parameters (a, b and c h^{-1}). With this method we were unable to determine the lag phase, due to the fine particle loss from the nylon bags in the early stages of incubation. The particle loss could be due to the type of starch (starch granules, structure and size, solubility) and physical form of the samples (grinding). Also the presence of cob and peels in some samples as (HME) could interfere on the starch degradability, altering the ratio of starch incubated to the bag surface area among samples. (Vanzant *et al.*1998).

In the in vitro Tilley and Terry method, the tubes were agitated every hour till 8 hours of incubation, and the samples well dispersed within the test tube,

giving an optimal sample surface to enzyme contact without loss of starch. This showed a lag phase for some feeds before starting the exponential digestion.

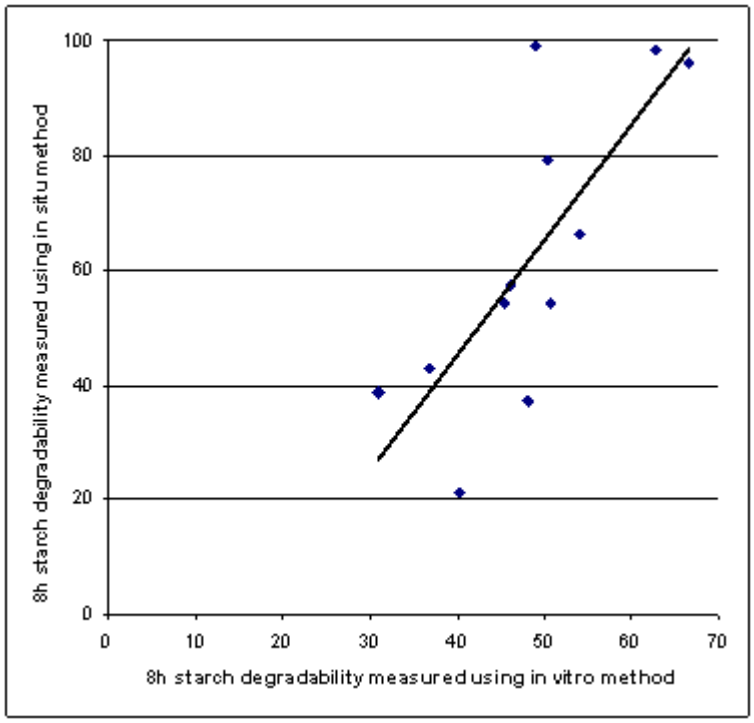


Figure 5.5: Relationship between starch degradability measured at 8h incubation by using in situ and in vitro techniques.

One of the disadvantages of the in situ method pointed out by several authors (Sveinbjörnsson and Huhtanen, 2006; Sveinbjörnsson *et al*., 2007) is the overestimation of digestibility of starchy feeds. Also in the present work the in situ results of digestibility were higher respecting to in vitro T&T results. At 8 h of incubation with in situ technique starch degradability ranged from 20 to 100%, instead with in vitro method the results varied from 20 to 60%.

5.3 Trial 2: Improvement of an Enzymatic Method: Preliminary tests

Two preliminary tests were performed to choose which would be the most performing buffer and enzymes combination to obtain reliable results on feeds starch degradability, able to well separate and rank native starches among cereals.

5.3.1 In Vitro Enzymatic Digestion:

The principle: Samples were subjected to an in vitro enzymatic digestion, by incubation in a buffer solution containing a cocktail of amylolytic enzymes.

1) Sample preparation:

Wheat (59.7% of starch content) was ground with 1mm screen and weighted and transferred into a 100 ml glass batch, where starch enzymatic digestion took place.

2) Enzymatic procedure:

Bicarbonate solution was prepared by weighting 8g of $NaHCO_3$ and diluted into 1L of distilled water, and pH was corrected to pH 6.5-6.6 with H_2PO_4. To avoid fermentation during enzymatic digestion antibiotics were added: 100 mg chloramphenicol (Sigma Aldrich EC200-287-4), 50 mg amoxicillin (Sigma Aldrich A8523), 600 mg of sodium metabisulphite (Carlo Erba Cod. 481287).

To the bicarbonate solution 50 mg of Pancreatine (porcine pancreas Sigma Aldrich Co St. Louis) and 50 mg amyloglucosidase (from *Aspergillus niger Sigma* Aldrich, 70U/mg) were added. 40 ml of bicarbonate solution,

containing both enzymes were added to each sample and incubation was carried at 39ºC with light agitation. After 8 hours 50µl of α-amylase (heat stable, Sigma Aldrich, A3306) was added until 24 hours.

At 4, 8 and 24h sampling times, 1 ml of solution was collected and diluted into 3 ml of TCA 5% (5 g trichloroacetic acid dissolved in 100 ml of distilled water), in order to stop enzymatic reaction (Salman *et al* ., 2009).

Solution was then analysed for glucose content, by a colorimetric reaction with GODPOD (Glucose SL, colorimetric enzymatic method, GIESSE DIAGNOSTICS, reference 4058, Rome), measured with spectrophotometer. Absorbance was read at 510 nm and converted to the amount of glucose released by comparison with a standard curve. The extent of hydrolysis was calculated as the amount of glucose released from the total amount of glucose equivalents in the starch sample.

5.3.2 Test 1: Buffer solution effect

Feed sample was incubated with two buffers: bicarbonate solution (described above) and acetate buffer (164 g of sodium acetate dissolved in 925 ml of distilled water and 75 ml of acetic acid 100%. pH was adjusted)

Both buffers contained pancreatin and amyloglucosidase. After the incubation time starch degradability was determined by measuring the amount of glucose released during the incubation time.

Starch digested = (glucose produced (mg/g) *0.9)

% of Digested starch = (ST digested/ST incubated*100)

5.3.3 Test 2: Enzyme mixtures

This trial consisted an enzymatic digestion at two incubations times (4 and 24hours) with bicarbonate solution containing two enzymes mixtures pancreatin and amyloglucosidase or lichenase and betaglucosidase).

Starch degradability determination was done as described for test 1.

5.3.4 Results and Discussion

The need of new approaches to estimate starch degradability, avoiding animals used, being less expensive and fast, is leading research to enzymes application on starch degradability estimation.

In the 90s some authors performed enzymatic digestion trials (Cone and Volt, 1990; Xiong *et al.*, 1990; Cone, 1991), underestimating on average digestibility data obtained with the in vitro ruminal techniques: Cone and Volt (1990) tested feedstuffs starch digestibility using several enzymes and ruminal liquid. The authors observed that, despite the low percentage of starch degradation obtained with enzymes hydrolysis, it ranked feedstuff starch degradability in the same way as the results obtained using the T&T method.

In the first trial was compared the effect of two different buffers on starch degradability at two incubations times. The bicarbonate buffer gave higher degradability results.

Acetatec buffer was used in many published trials which tested enzymatic starch hydrolysis (Mohan and Malleshi, 2006; Blasel *et al.*, 2006; Htoon *et al.*, 2009; Salman *et al.*, 2009). In figure 5.6 is described the effect of bicarbonate respecting to acetate buffer.

The differences observed between bicarbonate and acetate buffer were 33% at 4 hours of incubation and 20% higher at 24h of incubation, as showed in picture 6. Based on the results obtained, the bicarbonate solution was chosen to continue the enzymatic trials.

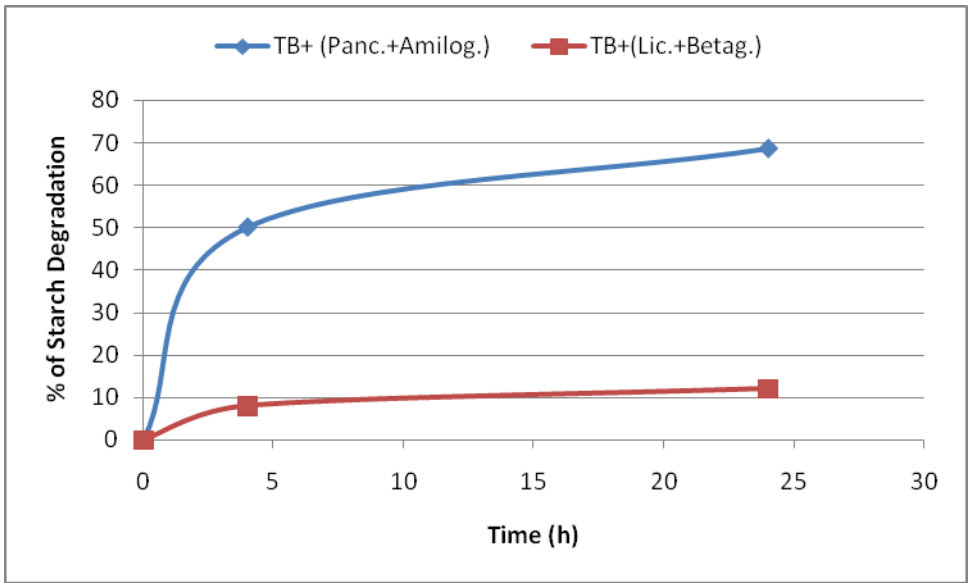


Figure 5.6: Wheat starch enzymatic degradability, using Acetate (TA) and Bicarbonate (TB) buffer.

Bicarbonate is one the most important and prevalent buffers inside the rumen, coming from the saliva avoiding the possibility of lowering ruminal pH and acidosis (Kokn and Dunlap, 1998; Bilk *et al.*, 2005). Bicarbonate in the rumen buffering system represents about the 6-8%. Therefore using the bicarbonate buffer could approach the experiment to rumen conditions. Moreover bicarbonate has been used in enzymatic trials even if is applied for measurement of specific characteristics of starch or measurement of starch digestion by monogastrics (Stevnebo *et al.*, 2006; Kim *et al.* 2008).

The second assay was conducted to choose which would be the enzyme mixture giving the most reliable starch degradability results.

From the enzyme mixtures tested, pancreatin/amyloglucosidase mixture digested 40 and 60% more, at 4 and 24 hours respectively, when compared to the mixture of lichenase /betaglucosidase, figure 5.7.

The combination of Pancreatin and Amyloglucosidase gave the best results. These enzymes were chosen due to their action on starch molecules; pancreatin α-amylase hydrolyses the α-1;4 (Czerkawski, 1986) bonds within the starch molecules and amyloglucosidase acts within both α-1;4 and α-1;6 glycosidic bonds(Pazur and Kleppe, 1962); Cone and Volt (1990) reported that

even if the starch degradability values were lower the best results were obtained with pancreatin. On the other hand and contrary to observations of these authors, Xiong *et al.*, (1990a) showed that the amyloglucosidase was sensitive to all aspects affecting the starch degradability therefore could be used for starch digestion estimates. Moreover, Xiong *et al.* (1990b) demonstrate a high positive correlation between enzymatic digestibility of starch and gas production by Mencke (1979).

Cone (1991) studied the enzymatic digestion in several samples, including a wheat sample. Enzymatic trials were done by the author using a single enzyme (i.e. α-amylase or pancreatin). Starch degradabilities obtained at 4h of incubation were of 18.9 and 7%, for α-amylase and pancreatin respectively. Both results were lower than that obtained with the combined effect of pancreatin/amyloglucosidase used in the present thesis, where 50% of incubated starch was degraded.

However the starch degradability of the wheat sample was 50% when measured with the enzymatic method and close to the value estimated by Cone *et al.*, (1989), using the Tilley and Terry technique (41.2%).

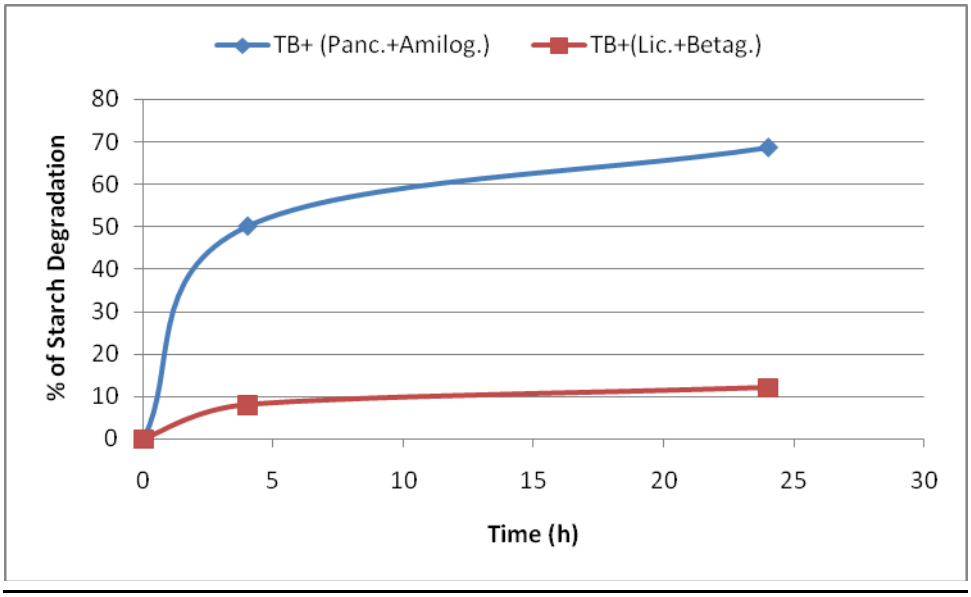


Figure 5.7: Wheat starch enzymatic degradability using Bicarbonate buffer and two different enzymes cocktails (pancreatin + amyloglucosidase) and (lichenase + betaglucosidase).

Recent study used a sequential application of two enzymes to digest starch, but a partial gelatinization of starch is foreseen, which enhances the access of enzymes for starch digestion, (Blasel *et al.*, 2006).

Starch enzymatic digestion has been target of many researchers (Li *et al.*, 2004; Anker-Nilssen *et al.*, 2006; Stevnebø *et al*, 2006; Mohan and Malleshi 2006; Kim *et al.*, 2008; Wilfart *et al.*, 2008; Htoon *et al.*, 2009; Salman *et al.* 2009) unfortunately only few was addressed in aspects of starch digestion of the ruminants (Xiong *et al.*, 1990 and Blasel *et al.*, 2006). Some studies suggested the use of Pullulanase for starch digestion studies (Englyst *et al.*, 1992; Mohan and Malleshi, 2006); however the results obtained using this enzyme (data not shown) were lower than the ones obtained by the combined effect of the pancreatin and amyloglucosidase. Moreover the price*unit of this enzyme is so high, that the goal of finding a less expensive method for a routine starch digestion estimates would never be achieved.

Based on the preliminary test results, it was defined that further enzymatic determinations would be carried on using a mixture of pancreatin and amyloglucosidase and the bicarbonate solution. The procedure was tested on three different cereals (maize, wheat and barley) and a maize flake to evaluate the competence of the method for a good separation of the cereals. The digestion trial ranked the four samples with the following decreasing order: corn flake, barley, wheat and corn with the degradabilities values of 78> 62> 44> 22% respectively after 4 hours of incubation (figure 5.8).

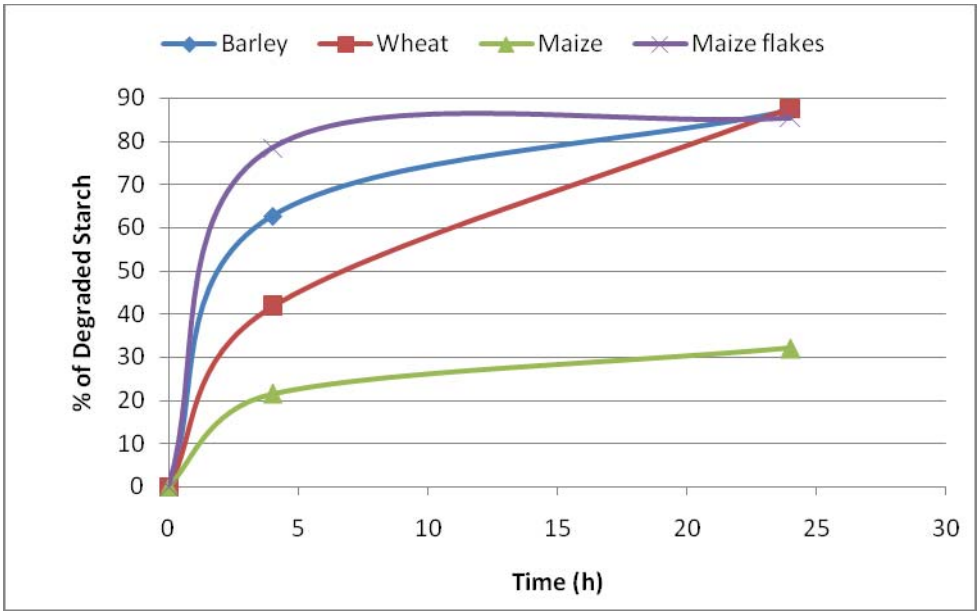


Figure 5.8: Cereal starch degradability using bicarbonate buffer containing pancreatin and amyloglucosidase.

The results showed that the enzymatic method was able to rank cereals based on their starch degradability, as mentioned above and in agreement with Lanzas *et al.*, (2006) ranking different cereals using the gas production technique. Corn flakes, barley and wheat reached a digestibility of 85 % after 24 hours of incubation, at the same time native maize starch showed only a 31% digestibility, highlighting the effect of the starch granule matrix characteristics and the interference of the vitreousness on the enzyme hydrolysis process.

5.4 Trial 3: Comparison of Enzymatic Method with In vitro Rumen Inoculum

5.5.1 Comparison of starch degradability values obtained using enzymatic an in vitro “Tilley & Terry” methods

Few works (Cone and Volt 1990; Cone 1991) have compared the results of enzymatic estimates with in vitro starch digestion. In the present thesis were compared the results of starch degradability from T&T method with the estimates obtained with the enzymatic method.

The starch degradability of the same 12 samples tested in the first trial was estimated with the in vitro enzymatic method described in section “Trial 2”. The chemical composition was showed in table 5.1 (“Trial 1”).

The differences between measurements from each method were calculated as an indicator of bias, whereas the standard error of differences was used as random fluctuations about these means (Bland & Altman 1986). The results are showed in table 5.3.

Table 5.3: Comparison between enzymatic method and Tilley & Terry technique to evaluate the on starch degradation feeds.

Methods Comparison	Mean difference	Standard deviation of difference	95% limit of Agreement	
			Lower limit	Upper limit
4h ENZ vs. 4h T&T	-3.80	8.12	-19.72	12.12
8h ENZ vs. 8h T&T	-1.75	15.33	-31.31	28.30

Comparing starch degradability values at 4h in the two tested methods, the mean of differences was -3.8 with a standard deviation of differences equal

to 8.12. The negative value of the mean of difference was a consequence of a slightly overestimation of enzymatic method with respect to in vitro method.

Calculating the 95% limits of agreement between methods (mean ± standard deviation of differences) for the 4h incubation time, it was possible to calculate the lower (-19.72) and the upper (+12.12) limit values. This suggest that if a sample had a starch degradability of 20% when measured with T&T method, the enzymatic degradability would be >0.28 and <32.12, in agreement with data reported by Cone (1991), an in vitro rumen degradability of 20% corresponded to dry corn grains. On the other if higher in vitro starch degradation was measured (i.e 46%, typical for a dry wheat grain sample as reported by Cone (1991), the enzymatic starch degradability would be >26.28 and <58.12%. the two ranges of the enzymatic measurements estimated by applying 95% limit of agreements showed that both methods (T&T and EM) yielded practically similar results, and were able to differentiate the starch degradability among the different samples analyzed. The correspondence between both methods as it was confirmed by the high relationship between starch degradability values obtained using the two methods (r^2=0.80) as showed by the regression represented in figure 5.9.

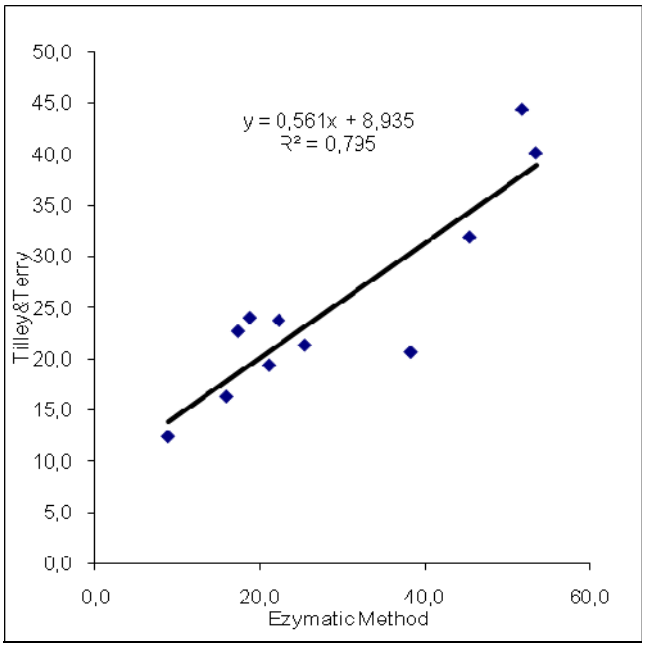


Figure 5.9: Comparison between 4h of incubation with enzymatic method and with Tilley & Terry Technique. Y=0.561x+8.935; R^2=0.795.

The comparison between data obtained at 8h time incubation T&T and 8h enzymatic starch degradability estimates also showed a good relationship. However the comparison of estimates (mean of differences and standard error) was slightly higher than those obtained comparing both methods at 4h. Moreover, the limits of agreement were low for the lower limit and high for the upper limit, which means that the range of starch degradation estimates with EM become larger, loosing accuracy on the predictions (figure 5.10).

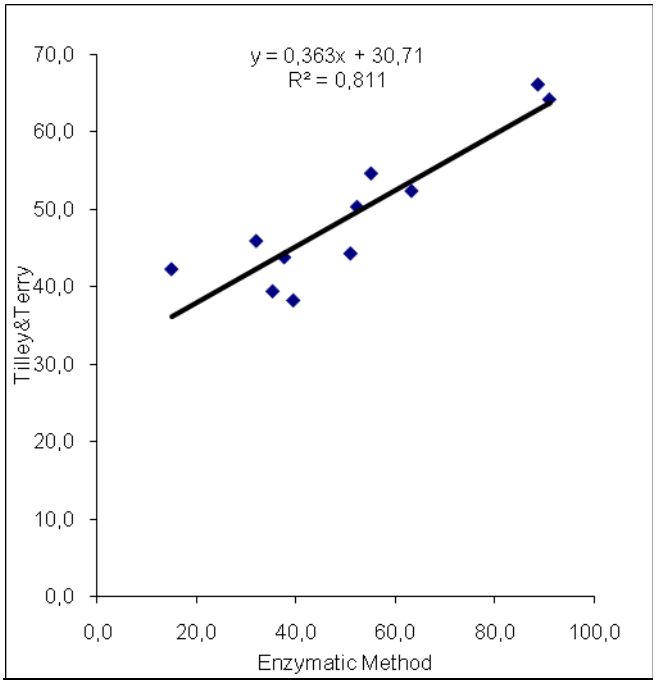


Figure 5.10: Comparison between 8h of incubation with Enzymatic Method and with Tilley & Terry Technique. Y=0.363x+30.71 R^2= 0.811

Similar results were reported by Cone (1991) when compared the results from two enzymatic solutions, one using α-amylase and the other pancreatin, with in vitro values using ruminal fluid, (r=0.77 and r=0.65, respectively). Moreover the author observed a good correlation between the cell-free enzyme extraction and the values from the T&T method (r=0.96), concluding that complete degradation of starch requires the presence of several enzymes, that were present in the cell-free enzyme extraction. Bird *et al.*, (1999) compared the enzymatic starch degradability with the in vitro starch fermentation and observed a positive correlation r=0.67. The enzymatic method tested by the

authors is similar to the method used in the present thesis. Differences on time of digestion and the buffer solution could be the reason for the higher correlations obtained with our method (r=0.80 and r=0.81, for 4h EM *vs.* 4hT&T and 8h EM *vs.* 8hT&T, respectively). Moreover these results suggest that our methodology could be a good approach of the rumen conditions; also supports the idea that an enzymatic evaluation of starch degradability could be useful tool to rank starch degradability among feedstuff.

5.5 Trial 4: Factors affecting Maize Starch Enzymatic Degradations:

The aim of this experiment was to evaluate the main factors affecting the enzymatic starch degradability in native and ensiled maize starch samples.

5.6.1 Experimental feeds

Forty two corn samples (growing season 2007) were collected from different farms (n=32) in the Po Valley (Northern Italy) during December 2007. Thirty-two of these were ensiled as high moisture ground corn (HMC, n=17) or as high moisture ground-ear corn (HME, n=15), while 12 were dried corn grains (CG). Only one of the HMC was in a tower-silo, all the other HMC and HMEC were ensiled in bunker silos.

The moisture content of high moisture corns ranged from 27.9% to 60.2%, showing an earlier time of harvesting (from 10 to 15 days) compared to dry maize grains, usually harvested at the black layer stage of maturity and with 20 to 24 %of moisture. Considering a harvest time for ensiling at late August, at least 90 days of in silo conservation can be estimated for the high moisture samples.

5.6.2 Chemical Analysis:

Samples were dried in a ventilated oven at 65ºC until they reached a constant weight, and then ground in a laboratory mill (Thomas-Wiley, Arthur H. Thomas Co., Philadelphia, PA) equipped with a 1 mm sieve and stored for analysis. Samples (CG, HMC and HMEC, respectively) were assayed in duplicate according to AOAC (1990) for dry matter (procedure 930.15), crude protein (procedure 975.06), and acid detergent fibre (ADF) exclusive of residual ash (procedure 973.18). Neutral detergent fibre (NDF) was assayed with heat stable amylase with correction for residual ash according to Mertens, 2002,

without sodium sulphite and using Ankom device (Ankom 220, USA) for extraction and filtering.

Soluble protein was quantified following the method described in Licitra *et al.* (1996), which gives soluble protein as the difference between the total crude protein content and the insoluble fraction.

The zein content was determined on defatted samples in agreement with Larson and Hoffman (2008) method. Acetone-insoluble DM (aiDM) was retained and used in zein content calculation. A 200 mg of DM sample was solubilised using 20 ml of 55.0% aqueous isopropyl solution with 0.6% 2-mercaptoethanol (ME) on a magnetic stir plate for 4 h. After mixing, the solution was centrifuged at 4,500 x *g* for 20 min and 0.5 ml of the supernatant was added to a spectrophotometer tube containing 5.5 ml of 0.15M TCA solution and mixed. The turbidity was allowed to equilibrate for 45 min. Zein were quantified by using a standard absorbance curve developed from purified zein (Acros Organics; 179311000, Thermo Fisher Scientific, Waltman, MA). The absorbance was read at 440 nm on a double beam spectrophotometer (PerkinElmer, San Jose, CA, USA).

Albumine-globuline protein fraction was determined by the improved method of Landry *et al.,* (2000). Samples were primarily treated with 10% TCA in order to isolate non-protein nitrogen (NPN). Afterwards samples were extracted with a 0.5M NaCl solution followed by two extractions with distilled water. The salt extract was mixed with the first distilled water extract, and then analysed for total nitrogen content by Kjeldahl method (A.O.A.C. 960.52) and converted to CP (percent N x 6.25).

Starch content was determined both by using the AOAC polarimetric method (2000) and a modified enzymatic method proposed by Basel *et al.* (2006). Briefly, 0.5g of dried ground samples were weighted into a 100ml flask, 15ml of distilled water was added and swirled vigorously ensuring sample dispersion. Twenty millitres of phosphate buffer (14g NaH2PO4.H20/986ml H2O, pH 6.5) was added, placed on a heating unit at 90ºC for 24 minutes in light agitation. Then samples were removed from the heat, 10ml of distilled

water were added. When temperature reached 80ºC, 0.3 ml of alpha-amylase (Sigma A-3306), was added and agitated. At a temperature of 50ºC, 10 ml of a 4.5 acetate buffer and 50 µl of amyloglucosidase (Sigma 10115) solution containing 60U/ml were added. The sample flask was stirred for one hour and then 1ml of sample solution was transferred to 3ml of TCA 5%. Samples were read at 510nm, after incubation for 30 minutes with GODPOD (Glucose SL, Giesse Diagnostics REF.4058).

HMC and HMEC samples were analyzed for pH and volatile fatty acid (VFA) contents according to Muck and Dickerson (1988). Briefly, a 20-g sample was placed in a blender jar, diluted with distilled water swirled for 60 minutes. The diluted sample was filtered through four layers of cheesecloth, and pH was measured with a pH meter immediately. Volatile fatty acids were determined by an high-performance liquid chromatography (Muck and Dickerson, 1988) using Pivalic acid as internal standard.

In vitro enzymatic starch degradability was determined by using the improved method tested in the second trial.

Table 5.4: Chemical characterization of maize samples [10 corn grains (CG); 16 high moisture corns (HMC); 16 high moisture ear corns (HMEC)]

	CG			HMC			HMEC		
	mean ± SD	Min	max	mean ± SD	min	Max	mean ± SD	min	max
Dry matter (DM)	90.05 ± 0.97	88.38	91.58	66.01 ± 5.04	54.77	72.05	51.87 ± 4.44	39.85	58.28
Crude Protein (% DM)	9.72 ± 0.64	8.27	10.31	8.92 ± 0.85	7.34	10.40	8.36 ± 1.28	6.05	11.22
Soluble Protein (% PG)	25.86 ± 7.51	15.28	34.42	59.03 ± 19.23	17.82	87.74	65.26 ± 11.62	35.87	80.51
Albumin + Globuline (% PG)	20.15 ± 5.04	11.87	28.48	62.38 ± 16.43	38.27	93.98	67.50 ± 12.84	49.22	95.09
Zein (% PG)	38.14 ± 8.96	16.94	48.30	15.36 ± 5.20	7.33	25.71	21.81 ± 9.07	7.72	38.8
NDF (% DM)	12.50 ± 2.07	9.56	16.53	10.23 ± 2.51	5.72	15.63	25.95 ± 4.78	18.98	35.75
ADF (% DM)	3.72 ± 0.32	3.35	4.27	2.51 ± 0.63	1.33	3.42	11.71 ± 3.96	5.43	18.88
Crude Lipid (% DM)	4.26 ± 0.37	3.82	4.88	5.68 ± 0.32	5.30	6.06	6.36 ± 0.92	5.19	7.79
Polarimetric Starch (% DM)	65.79 ± 5.05	54.77	72.05	65.80 ± 3.05	59.55	69.79	50.66 ± 5.31	42.48	57.64
Enzymatic Starch (% DM)	59.10 ± 2.82	54.54	62.05	64.80 ± 4.65	54.68	73.59	50.10 ± 7.23	33.60	64.10

5.6.3 Results and Discussion

The chemical composition of CG, HMc and HMEc was reported in table 5.4. The DM content of CGs was 90.05%, while the high moisture corns (HMc and HMEc) were characterized by lower DM values (66.01% and 51.87%, respectively). These two products are normally harvested at the same moment, but with two different harvesting processes. In the HMEC the presence of cob determine the lower DM content, being the cob higher in moisture content than the kernels. Moreover, the HMEC had a different chemical composition with respect to HMC due to cob presence. In any case, should be taken in consideration that in the Po Valley differences in moisture content within the HMC categories could be due to differences in physiological stage of maturity of kernels at the harvested time. The HMC's are harvested between 8 to 10 days after corn silage, and 15 days before harvest time of corn grain.

The CP content of CG was higher (9.72% DM) compared to lower values of HMC (8.92%) and HMEC (8.36%). However, a major variability characterized the CP of HMEC, with values ranging from the lowest value (i.e. 6.05% DM) to the highest (11.22% DM) of dataset. The chemical characteristics of the CP of the three typologies of feeds were quite different. In particular, the soluble fraction of CP was 25.66, 59.03 and 65.26% in CG, HMC and HMES; respectively both related to the moisture content and ensiling pH. Also the different protein matrix fractions showed differences among feeds: the albumine/globuline content in CG was lower than in HMC and HMEC (20.15 *vs.* 62.38 and 67.50% of CP, respectively). Differences were obtained for zein content (%CP), having CG a higher value than the two high moisture corns (38.14 *vs.* 15.36 and 21.81% of CP, respectively). The zeins are storage proteins and normally increase in the endosperm during grain ripening and maturation while the albumins are less variable. For this reason zein and albumins/globulins ratio depends on the stage of the maturity of the plant.

The NDF and ADF content of samples was similar for CG and HMC, being the HMC the moist ground kernels without residues of cob and ears, whereas

HMEC had a higher fibre content due to cob presence, (NDF and ADF content 25.95% and 11.71%, respectively).

The starch content of corn samples was evaluated with two methods. No difference in starch quantification should be attributed to used methods (polarimetric vs. enzymatic methods), in particular in HMC (65.80 *vs.* 64.80% DM, respectively) and HMEC (50.66 *vs.* 50.10% DM, respectively) samples. A discrepancy between methods was observed for starch determination in dried corn grains. The dilution effect due to the cob presence in the HMEC was evident on the starch content, being about 30-35% lower that starch in CG and HMC.

The ether extract was higher in HMEC (6.36% DM) with respect to 4.26 and 5.68% DM, for CG and HMC.

Table 5.5: pH, lactic acid, Total VFA, mean values standard deviation, maximum and minimum values.

	HMC			HMEC		
	mean ± SD	min	max	mean ± SD	Min	max
pH	4.05 ± 0.169	3.62	4.35	3.86 ± 0.16	3.63	4.19
Lactic acid (% of DM)	1.46 ± 0.78	0.33	2.71	2.17 ± 0.75	0.63	3.48
Total VFA[1] (% of DM)	2.31 ± 0.92	1.23	4.02	3.46 ± 1.39	1.01	5.41

[1] Total VFA = acetic + propionic +isobutiric +butiric + isovaleric + valeric +Latic.

The ensiled samples of dataset were characterized for fermentation parameters, in particular pH, lactic acid and total VFA content (% of DM). The pH values were similar and around 4.00 in both HMC and HMEC. Instead, a higher total VFA and lactic acid content was observed in HMEC (3.46%) comparedto HMC (2.31%), probably due to higher water content of HMEC (48.13% vs. 33.99%, respectively). Consequently, the total VFA content resulted about 1.5 fold higher in HMEC than HMC samples.

Thornton *et al*. (1977) measured a relationship between these fermentation parameters and moisture content of ensiled corn grain. As shown in Figure 5.11 and 5.12, a positive correlation was found by the author pH and DM content (%), whereas a negative trend was reported between DM content (%) and production of lactic acid (% DM) in ensiled products.

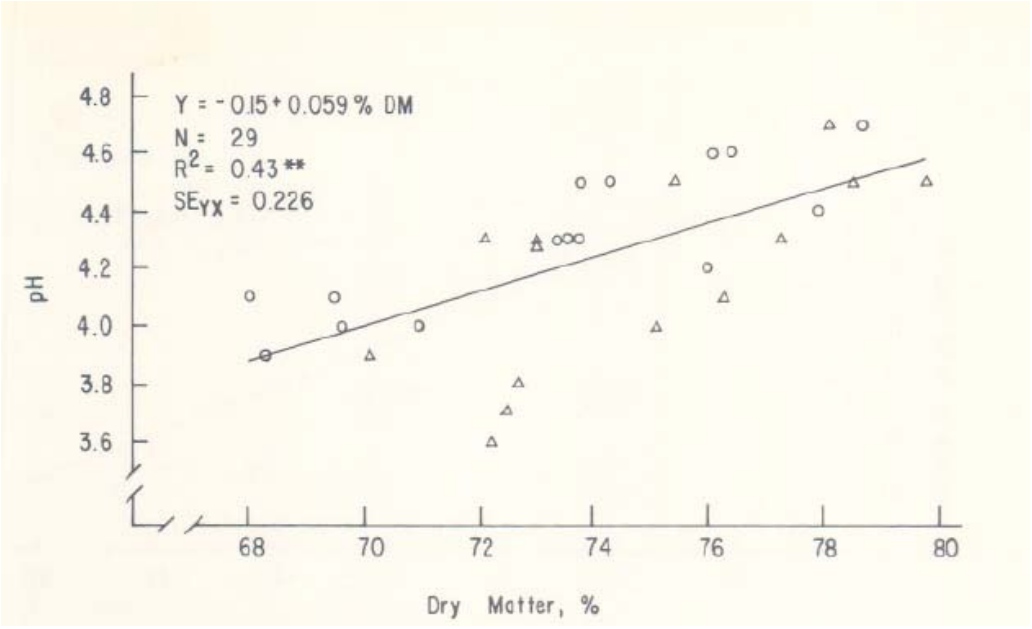


Figure 5.11: Relationship between pH and dry matter content. Adapted from Thornton *et al.*, (1977). Animal Research Report Oklahoma Agricultural Experimental Station.

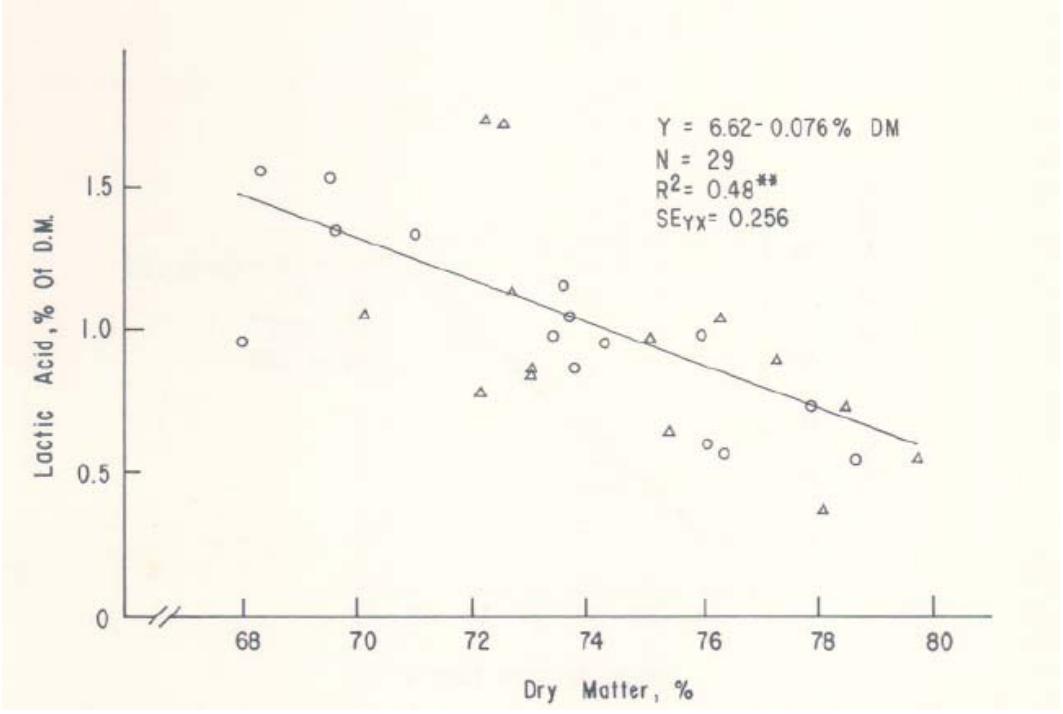


Figure 5.12: Relationship between Dry Matter and Lactic acid. Adapted from Thornton *et al.*, (1977). Animal Research Report Oklahoma Agricultural Experimental Station.

The DM content of samples analyzed by Thornton *et al.* (1977) ranged from 68% to 80%. In the dataset presented in present thesis, a wider range of DM content was measured. In any case, plotting the moisture content of samples in agreement with Thornton *et al.* (1977), similar interactions between DM content and pH or lactic acid contents could be observed (figure 5.13).

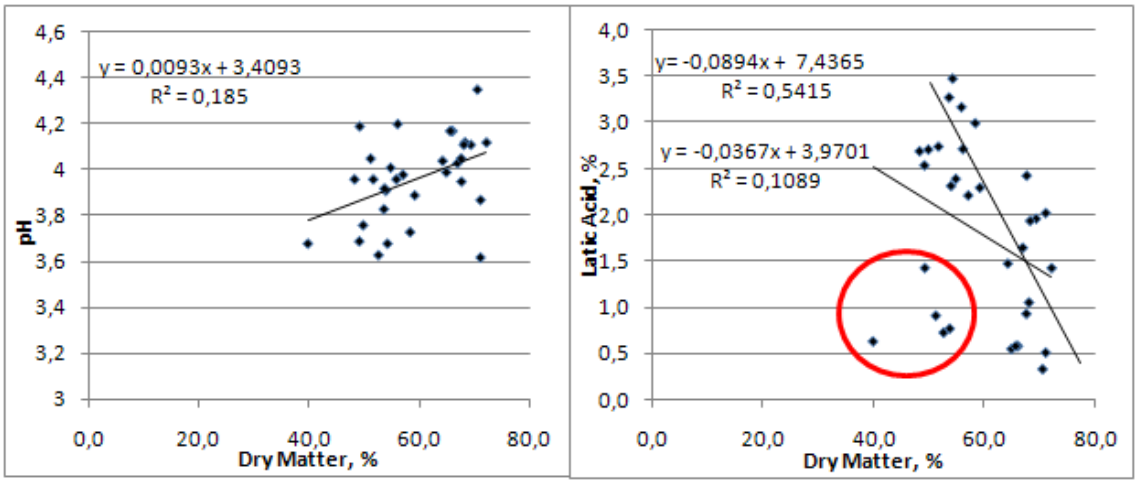


Figure 5.13: Correlations between dry matter content and pH value and Lactic acid content.

5.6.3.1 Evaluation of enzymatic starch degradability

Figure 5.15 summarise the enzymatic starch degradability dynamics of CG, HMC and HMEC samples. Two steam flaked corns were also tested for enzymatic starch availability. The steam-flaking is one of the most important processes to improve grain utilization by ruminants (Xiong *et al.*, 1990). The heat and pressure cause a gelatinization of starch granules and lower the time needed to enzymes to break down starch molecules (Rooney et Pflugfelder, 1986; Sniffen *et al.*, 2009).

In graph A and B the average starch degradability dynamics for each category was shown. The obtained rank was: steam flaked corn > HMEC > HMC > CG. In particular, more than 70% of starch of steam flaked samples were degraded after 8h about 97% after 24h of incubation. Osman *et al.* (1970) reported that steam flaking can increase enzymatic starch degradability approximately threefold with respect to raw cereal.In 1990, Xiong *et al.*, measuring starch availability by using amyloglucosidase incubation on milo flakes, showed a rapid release of glucose in steam flaked samples. Also Cone (1991) measured a similar percentage of degraded starch after 4h incubation with α-amylase or pancreatin (77.1% and 84,.7%, respectively) enzymes. To our knowledge, no published works have tested the starch degradability by using an enzyme cocktail as proposed in enzymatic method used in present thesis.

The degradation of starch in CGs resulted 2-3 times lower than steam flaked corns (table 6). Similar results were reported by Cone (1991). Due to similar moisture content and particle size between samples categories, the

different starch degradability measured after enzymatic incubation supported the idea that native starch was more difficult to degrade than processing starches. Despite a higher degradability at 4h incubation of CGs with respect to HMCs (21.1% and 16.5% respectively), the starch of ensiled products could be considered more easily degraded by enzymes than dry corns. Moreover, of great interest is the coefficient of variation (CV, %) of starch degradability in ensiled products: at 8h and 24h incubation times the CV was 25.0% and 20,9% for HMC and 14.3% and 30,4% for HMEC, respectively. Lower data variability could be observed on CG samples. In particular, this appeared evident in figure 5.18 and 5.19 were the moisture content of samples was plotted in function of enzymatic starch degradability at 8h and 24h, respectively. The CGs showed values ranging from 21.1% and 38.8% at 8h and 39.6% and 60.8% at 24h incubation times.

Among the ensiled products, they were usual considered by animal nutritionist to have similar starch degradability. Moreover, in NRC (2001) the PAF used for the ensiled product does not differ among high moisture corn typologies and resulted equal to 1.04, being 1.0 for ground corn grains. Also the difference in PAF value between HMC and ground corn grains is smaller, respecting to the difference observed in this trial between CG and HMC with enzymatic starch degradability (table 5.6). This suggests that PAF could be recalculated considering the feedstuff starch degradability. The data showed in figure 5.13 demonstrated a quite different situation: the range of variability of high moisture corns was from 19.9 and 51.6 after 8h incubation and 42.6 to 100.0 after 24 h of incubation.

As reported in trial 3, a good relationship was found between rumen in vitro data and enzymatic evaluation at 8h of incubation:

$Y = 0.363 * X + 30.71$

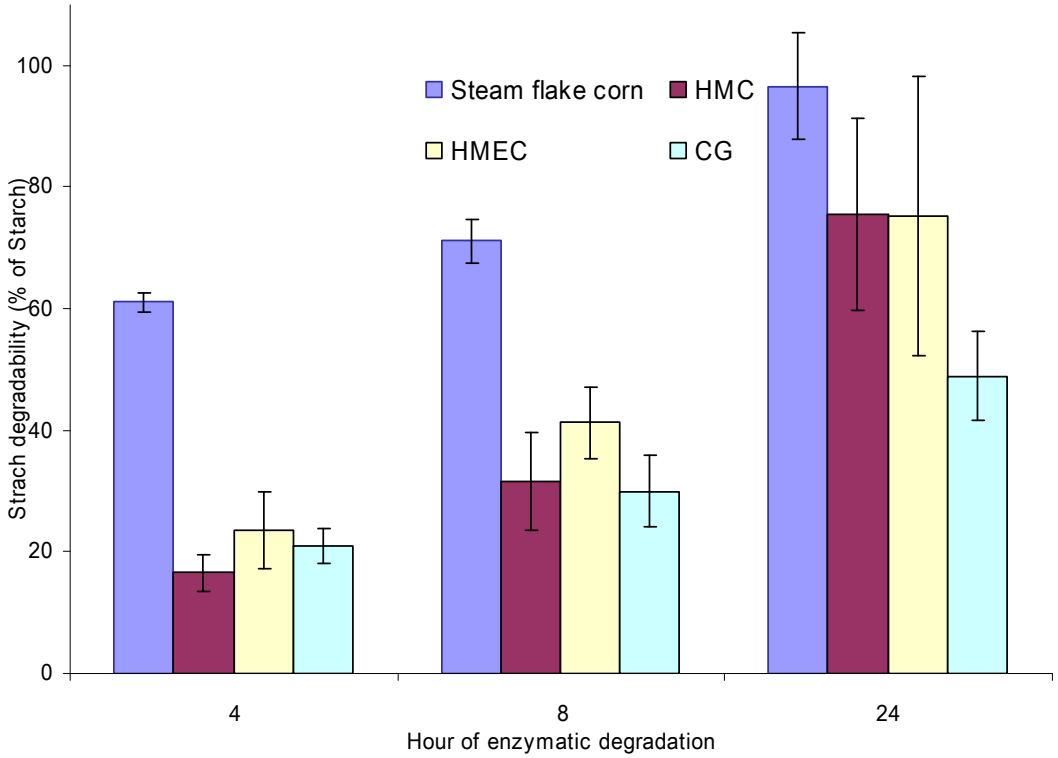
$R^2 = 0.795$

SEM = 7.400

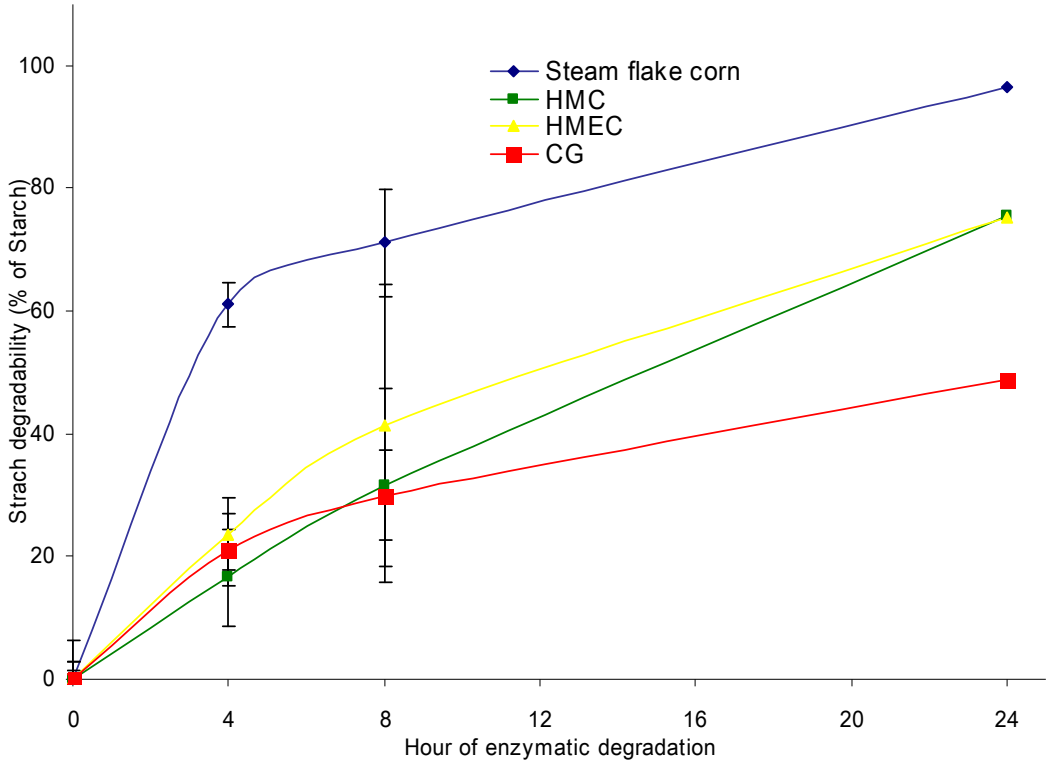
Where Y and X are the starch degradability value obtained with T&T method and Enzymatic method, respectively.

Therefore, using the minimum and maximum values reported above for high moisture corns, the in vitro T&T starch degradability could be estimated, obtaining values of 37.9% and 49.4% for the 8h-starch degradability.

Using these data to estimate the amount of ruminal-degraded starch for a dairy cow fed daily 6 kg of starch (24Kg DM and 25% of starch) a minimum value of 2275g and a maximum value of 2946g could be obtained. A difference of 670g of degradable starch, will traduce in the difference of digestibility when using two HMC having different starch degradation rate.



Graph A



Graph B

Figure 5.14: Graph And B- represent the starch degradability of Steam flaked corn, HMC, HMEC and CG at 4, 8 and 24h of incubation, with the standard deviation.

Table 5.6: Mean value, standard deviation, minimum and maximum values determined for starch degradation of Steam flaked corn (n=2), CG, HMC and HMEC at 4, 8 and 24 hours of enzymatic incubation.

Starch deg.		Steam flake	CG	HMC	HMEC
4h incubation	mean	61.1	21.1	16.5	23.6
	SD[1]	1.5	2.9	2.9	6.3
	min	60.0	17.8	12.1	12.3
	max	62.1	27.0	23.8	34.8
8h incubation	mean	71.1	29.9	31.5	41.3
	SD[1]	3.7	5.9	7.9	5.9
	min	68.6	21.1	19.9	31.9
	max	73.7	38.8	48.8	51.6
24h incubation	mean	96.6	48.9	75.5	75.3
	SD[1]	8.7	7.3	15.8	22.9
	Min	90.4	39.6	42.9	42.6
	Max	102.8	60.8	100	100

The fractional rate of starch degradation (c-value, h^{-1}) for the samples of data set were calculated by the interpolation (Ørsokv model) of the starch degradability values at 0h, 4h, 8h, 24h and 144h incubation times. The 4h, 8h and 24h starch degradability values were measured by using the enzymatic method, whereas the 0h and 144h were considered equal to 0 and 95% of starch degradability, respectively.

The differences observed in the fractional rate of starch degradation were in agreement with rank described above: the steam-flaked corns had the highest c-value (0.210 h^{-1}), resulting about 6.5-fold higher than c-value reported by native corns (CG, 0.032 h^{-1}). The high moisture corns had a fractional rate lower than treated corns, but higher than CG-value (0.031 h^{-1}), being 0.053 h^{-1} and 0.075 h^{-1} for HMC and HMEC, respectively.

Table 5.7: Mean values, standard deviation, minimum and maximum values of the kinetics parameters enzymatically estimated for steam flaked corn, CG, HMC and HMEC.

Kinetics parameters		Steam flake	CG	HMC	HMEC
c (h^{-1})	mean	0.210	0.031	0.054	0.072
	SD[1]	0.006	0.009	0.007	0.013
	min	0.17	0.02	0.04	0.06
	max	0.25	0.046	0.069	0.097

The differences in starch degradability between dry and wet corn samples observed by using enzymatic method were supported by in vivo experiments. Oba and Allen (2003), studying the effect of corn grain conservation method on digestion kinetics, reported that the rumen digestibility of starch was greater for high moisture corn than for corn grains either in high-starch (71.1% vs. 46.9%, respectively) or in low-starch (58.5% vs. 45.9, respectively) diets. However, due to a higher amount of starch digested in the intestine of animal fed corn starch, the total tract starch degradability resulted similar for two corn products. Also Szasz *et al*. (2007) reported that DM and starch degradation measured in situ with nylon bags techniques increased linearly with the moisture of the kernels. In this experiment, the tested corn hybrids were harvested at 28.1%, 31.2% and 35.7% moisture, respectively.

5.6.4 Correlations

The different composition of CG, HMC and HMEC samples was confirmed by the strong relationship (r = -0.92) found between starch and NDF contents. When starch content changed from 50.66 to 65.80 % of DM (average content of starch in HMEC and HMC-CG; respectively), the NDF decreased from 25.95% of DM for HMEC to 10.23 and 12.50% of DM for HMC and CG, respectively.

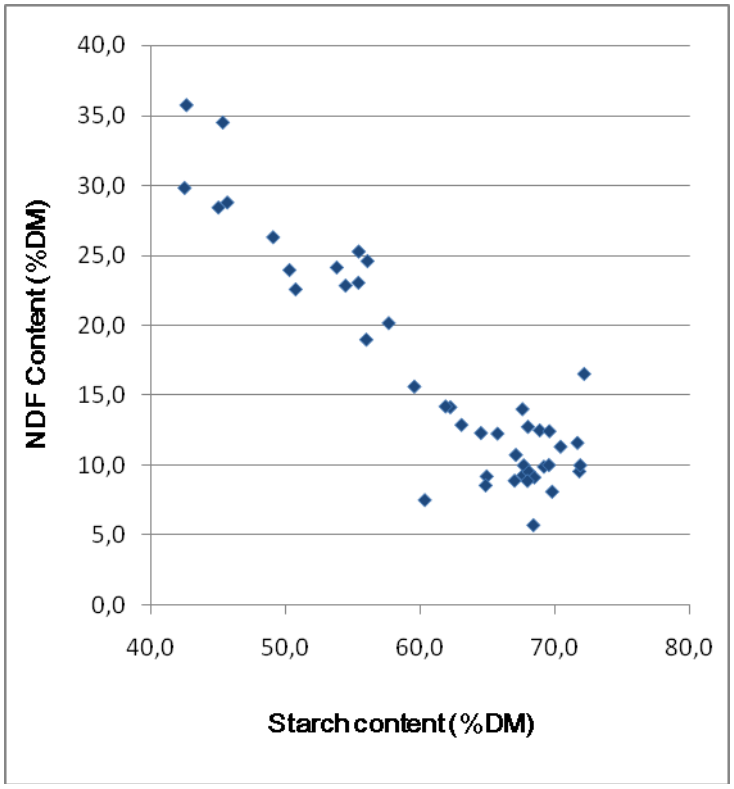


Figure 5.15: Relationship between NDF and starch content.

As already stated, the cobs component could justify the relationship between fibre fractions and moisture content (r = 0.63 and r = 0.60 for NDF and ADF, respectively; P<0.001) and in the same way the starch content (r = -0.77, figure 5.15). The parameters described above could be considered as intrinsic factors characterizing the samples used in the data set and useful to distinguish HMEC from HMC and CG. In table 5.8 was reported the effect of the moisture content of samples on some chemical composition of tested feeds.

Table 5.8: Correlations between moisture content and chemical composition parameters for total feeds, pH and total VFA were determined for (HMc and HMEc).

	Starch (%DM)	CP % DM	Sol. CP % CP	Alb/Glob %CP	Zein % CP	NDF % DM	ADF % DM	EE % DM	pH	Tot VFA % DM
Moisture content	-0.77	-0.37	0.61	0.71	-0.77	0.63	0.60	-0.79	0.43	0.45
P-value	***	*	***	***	***	***	***	***	**	**

*P<0.05; ** P<0.01; ***P<0.001

The moisture content was poorly associated to crude protein (r= -0.37; P<0.05), but highly related to its soluble fraction (r = 0.61; P<0.001). Also the

protein-matrix fractions were highly correlated to the moisture content in corn ($P<0.001$): the albumin to globulin ratio tend to increase ($r = 0.71$), whereas the zein decreased ($r = -0.77$) with the moisture content, as showed in figures 5.16 and 5.17. The change in soluble protein content could be due to maturity of the kernels and, therefore, with the zein deposition and vitreounsness of starch grains (Mahanna, 2009; Hoffman and Shaver, 2009).

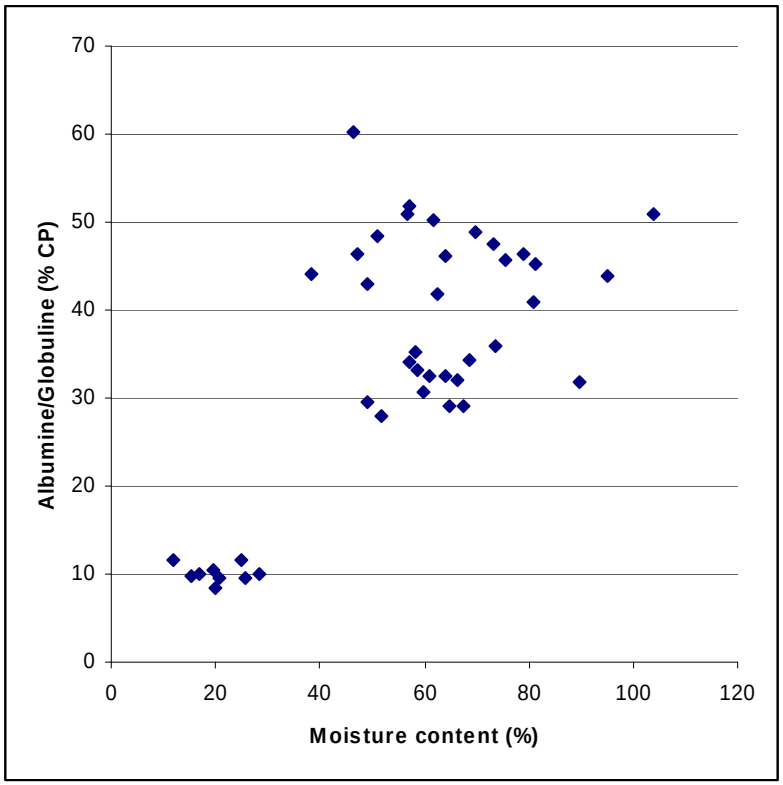


Figure 5.16: Relationship between Albumin/globuline moisture content.

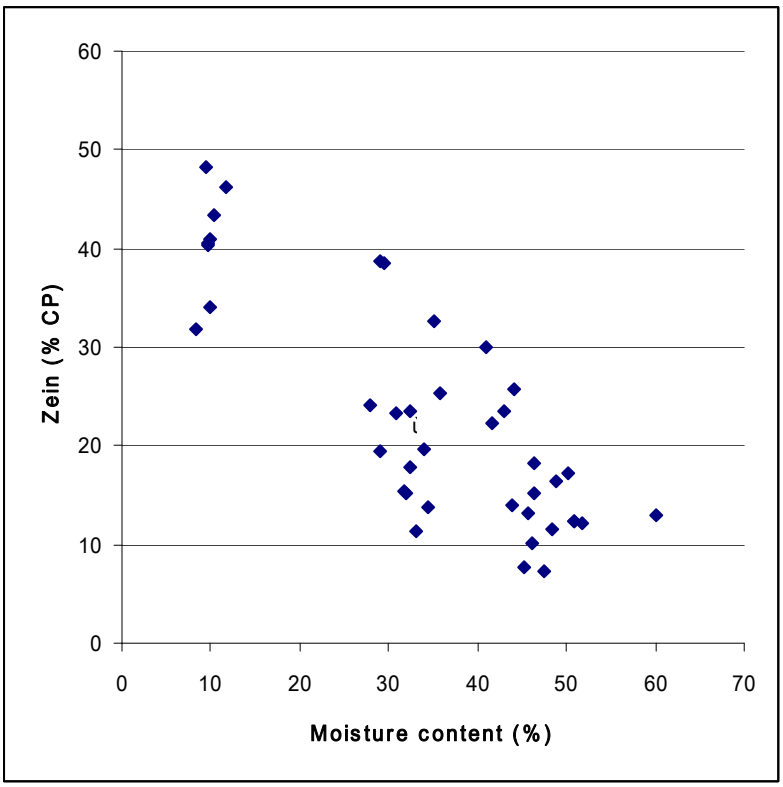


Figure 5.17: Relationship between zein content and moisture content

Among high moisture corns (n=32), the pH and the total VFA were influenced by moisture level (r = 0.43 and r = 0.45; respectively).

Some authors (Hoffman and Shaver, 2009) considered plausible a decrease in zein content due to ensiling processes: the acid fermentation and the proteolysis activity of microorganisms in the silage could degrade the protein, in particular zein. In our experimental condition, the pH was similar between HMC and HMEC (3.86 and 4.05, respectively) and did not influence the zein content of the two products (r=-0.77, P<0.001) (table 8). On the contrary, a slight negative correlation was observed between zein and total VFA (r=-0.39, P<0.05).

Table 5.9: Correlation between enzymatic starch degradability at 4, 8 and 24 hours incubation and rate of starch degradation (c h^{-1}) with chemical characteristics of corn.

	pH	Water (Moisture)	Starch	CP	soluble CP	Albumine Globuline	Zein	NDF	ADF
Starch deg. At									
4h	-0.39*		-0.60***	-0.39*		-0.39**		0.60***	0.62***
8h	-0.55***	0.57***	-0.65***	-0.27*			-0.45**	0.60***	0.68***
24h		0.50***	-0.65***	-0.27*	0.37**	0.43***	-0.55***	0.46***	0.41**
c	-0.41*	0.82***	-0.86***		0.30*	0.43*	-0.63***	0.72***	0.76***

(*)- P<0.05, (**) – P<0.01; (***) – P<0.001

The starch degradability, performed with the enzymatic method described in section 2, was related to chemical characteristics of the feeds.

The moisture content result the most important factor related to starch degradability (Sniffen *et al.*, 2009). In particular, a positive relationship between moisture content and 8h and 24h starch degradation (r=0.57 and r=0.50, respectively; P<0.001) were observed. This relationship showed that, the HMC and HMEC samples were more degradable than CG. This was confirmed by the relationship between fractional degradation rate (c h^{-1}) and the moisture content of corn samples (r= 0.82, P<0.001): the moisture levels of CG, HMC and HMEC were 9.95%, 33.99%, and 48.13%, respectively; whereas, the fractional degradation rate increased from 0.032 to 0.054 and 0.072 $h^{-1}$for CG, HMC and HMEC, respectively. Moisture content and, therefore, maturity of kernels at harvest can affect the vitreousness of grain (Szasz *et al.*, 2009). Philippeau and Michalet-Doreau (1997) showed the vitreousness differing between immature and mature grains (32% vs. 60.2%). Similar observations were reported by Correa el al. (2002): vitreousness of kernels increased with the advancing maturity of dent corns and in situ starch disappearance decreased. However, the effect of moisture content on vitreousness is not still completely clear (Szasz *et al.*, 2009).

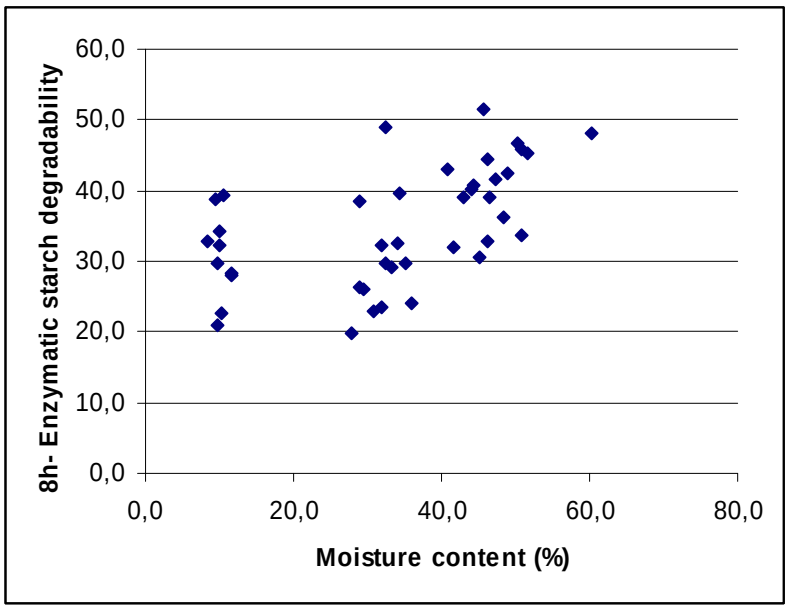


Figure 5.18: Relationship between moisture content and 8h-starch degradabiltiy.

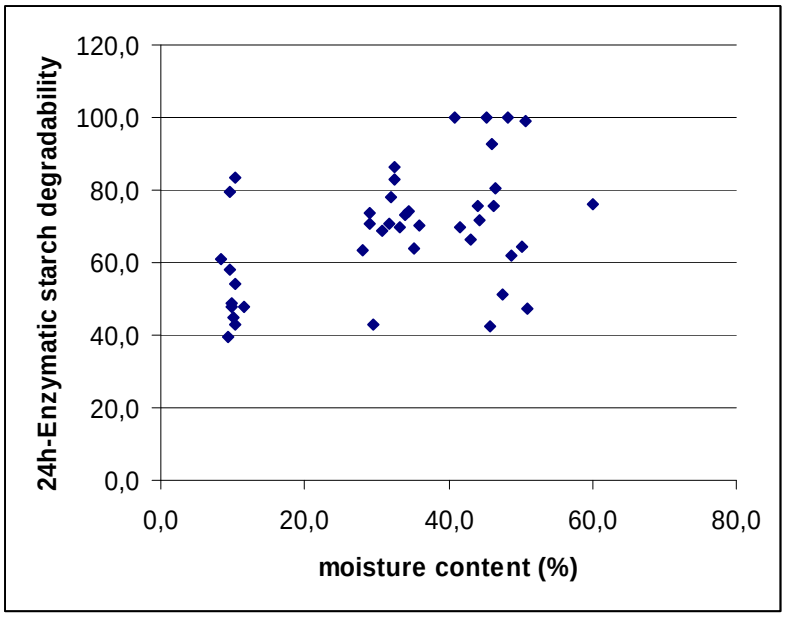


Figure 5.19: Relationship between moisture content and 24h-starch degradability.

Starch, CP and protein fraction could be considered as intrinsic characteristics associated to the DM content of grain, therefore their effect on the starch enzymatic degradation will always be dependent of stage of maturation of the kernel. A very low relationship ($P<0.05$) between crude protein and enzymatic starch degradation was observed at 4, 8 and 24h ($r = -0.37$, $r = -0.27$ and $r = -0.27$ for 4 and both 8, 24 hours respectively). Other authors suggested that protein matrix could be involved in starch degradability (McAllister *et al.*, 1993; Philippeau *et al.*, 1998; Philippeau *et al.*, 2000; Anker-Nilssen *et al.*, 2006).

Recently, Hoffman and Shaver (2009) and Mahanna (2009) discussed about the negative effect that prolamin zein fraction could have on starch degradability. Also in this trial, the protein fractions appeared to be more correlated to the enzymatic starch degradability than crude protein itself. In fact, the soluble proteins (% CP) tended to influence ($P<0.001$) the 24h starch enzymatic degradability ($r=0.37$). A positive relationship was observed also with the fractional degradation rate ($r= 0.30$).

Concerning the endosperm protein matrix, the albumins/globulin content of samples was positively related to amount of starch degraded at 4 and 24h of incubation ($r=0.39$ and $r=0.43$, respectively, $P<0.01$). These proteins are known to be water soluble protein, therefore had similar relationship with starch degradability of those reported for soluble protein fraction. On the contrary, the zein fraction negatively influenced the 8h and 24h enzymatic starch degradation ($r = -0.45$ and $r = -0.55$, respectively; $P<0.01$). Moreover, the zein affected the estimated fractional degradation rate ($r = -0.63$; $P<0.001$). These results supported the idea that interaction with zein-protein could reduce the availability of starch to enzyme hydrolysis (Rooney and Pflegfeder, 1986) and to rumen microorganism (Philippeau *et al*., 2000). Philippeau *et al*. (2000), determining starch degradability by an in situ technique, reported negative relationship between rumen starch degradability and zein content of dry corn samples.

Some authors (Shamekh *et al.,* 1998; Svihus *et al.,* 2005; Anker-Nilssen *et al.,* 2006) suggested that also the lipid matrix could be affecting the starch degradability due to the potential of amylose-lipid complex formation. In agreement with these observations, the EE negatively influenced the starch degradability (data not reported in table). These results seem to confirm previous observations reported by other authors on factors affecting starch degradability of cereals and their ensiled products. In particular, Thornton *et al*., (1977); Nocek and Tamminga, (1991); McAllister *et al.,* (1993), Romagnolo *et al.,* (1994); Schwab *et al.,* (2003); Svihus *et al.,* (2005); Blasel *et al*., (2006), Shaver and Hoffman, 2009, Sniffen 2009, Dorshort *et al.,* (2009).

6 Conclusion

The results obtained in the present work confirm the interference of some protein fractions, as prolamins, in the chemical and physical characteristics of starch. In particular, prolamins starch matrix limit the access to the digestive enzymes and at the same time increase the starch vitreousness. Also particle hydration is impaired; since prolamins are hydrophobic proteins that protect the starch granule they limit the water absorption phase. Based on our results and on that of other autors (Philippeau and Michalet-Doreau, 1997; Philippeau et al. 1998, 1999, 2000; Correa et al. 2002; Hoffman et al. 2009), prolamins are correlated with the grains dry matter and subsequently to the maturity stage of maize grain. The work also highlights the correlation of these proteins with starch enzyme degradability in both dry corn and high moisture ear corn when harvested at early stages of development. Observations showed a positive relation between the level of kernel moisture and an increase in starch degradability. This information is of particular interest and of great applicative use as it allows ranking starch availability/ruminal fermentation which is not accounted for in the NRC_2001, CPM Dairy and Cornell models.

These models as well as the Milk_2006 model, consider only some aspects:

- the processing adjusting factor (PAF) considers the effect of particle size while the kernel processing factor (KPF) and the degree of starch access (DSA) consider only the physical shape of starch.

None of these models accounts for the intrinsic characteristics of starch. According to the most recent literature, PAF compares the effect of different feed typologies in relation to their *in vivo* experimental performances.
PAF as a ranking factor accounts for physical starch characteristics differentiating for example corn flakes or mature corn silage (1.04) from early corn silage (0.98).

The variations in starch degradability obtained in our trials ranged from 20 to 50% in ensiled high moisture corn after 8 hours of incubation, showing that the same commercial product can in fact supply quite different energy amounts to rumen microorganisms. Likewise, the great differences observed for *in vitro* starch degradation, influence the quantity of starch in the intestine. These two aspects must be considered in the models since they strongly interfere with microbial synthesis and therefore with the metabolic amino acid balance of the diet, with the VFA production and the ratio (propionic: acetic+ butyric acid) and with the glucose absorbed in the intestine. These metabolic aspects are of major importance in determining the correct energetic value of the diet and subsequently the amino-acids supply to the mammary gland for milk protein synthesis.

Considering a diet with 26% of starch content, proper for high producing dairy cows, and a daily consumption around 24kg of DM, about 6200g of starch will be ingested. When switching from a low degradable starch source like dried grains with a starch degradability of 20-25%, to a high degradable high moisture corn (55-65% in vitro enzymatic starch degradability) an increase of about 2500g of ruminal degradable starch will result from this change.

In conclusion, indirect ranking methods like DSA or the KPF do not represent accurately what really occurs within the animal but are useful to minimize natural model deviations. The simple *in vitro* enzymatic approach proposed in this work, could be useful to separate the intrinsic starch characteristics from the physical aspects determined by the methods mentioned above. Since starch characteristics were correlated to prolamin presence, which can be determined with rapid colorimetric methods, the combination of both enzymatic and colorimetric methods could be used as a routine method in commercial laboratories leading to a faster qualitative determination of starch degradability.

7 Bibliography

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