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The effect of harvest time of timothy on digestibility in equine with in sacco and in vitro methods

Effekt av høstetid på fordøyelighet av timotei hos hester ved bruk av in sacco og in vitro metoder

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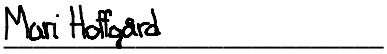
Master of Science in animal nutrition

Preface

This master thesis is written as the final part of a master's degree in Animal nutrition science at the Faculty of Biosciences at Norwegian University of Life Sciences in May of 2022. I wanted to write this thesis to get a wider knowledge in horse nutrition. Target group for this thesis are students, researchers, veterinarians, farmers, horse owners and others with interest of horse nutrition. This thesis gives a brief description of the maturity process in grasses and how it affects chemical composition and digestibility. Additionally, an evaluation was performed on methods to estimate digestibility of forages in horses; in sacco and in vitro methods. The thesis has intention to be a helping tool for decision making of the most optimal harvest time when harvesting grasses for horses, and to discuss the digestibility methods usability.

Several people have supported and helped me through this thesis. First of all, I would like to thank my supervisor Rasmus Bovbjerg Jensen for your support, guidance and eagerly sharing of knowledge. I would also like to thank Elise Fure and Mette Henne, for practical guidance through the digestibility experiments. Further, a huge thanks to Katrine Eikanger, Emma Folkestad and Linn Prime for using your time to carefully review my thesis. Extra thanks to Katrine for sharing your knowledge and always having time to answer my endless questions. Also, I would like to thank "drøvtyggergruppa" for having great feedback comments during our meetings. For help and guidance with the statistical work, I want to thank Hilde Vinje, for being supportive and making the statistical work both educational and not so scary as I intended it to be. Finally, a huge thanks to my partner, friends and co-students for your support. Additionally, it has been an honour to work with the unique horses at NMBU.

Ås, May, 2022.



Mari Hoffgård

Abstract

The objective of the present thesis was to investigate how harvest time influence digestibility in horses, in addition, to compare three different methods evaluating digestibility. Timothy grass was harvested eight times during the summer of 2021 and analyzed for chemical and sugar composition. The techniques used to measure of digestibility were the in sacco (IS) and two in vitro methods: daisy incubation (DI) and gas production (GP).

Forage is the most essential feedstuff in the equine diet. While excessive intake of sugars is associated with metabolic disorders, fiber is essential for the hindgut microbiota. A late harvest time ensures a high fiber content while compromising digestibility and nutritional value.

The reference method of determining digestibility is the in vivo technique. Unfortunately, in vivo techniques are time-consuming and expensive to operate. In the IS method, feedstuff is placed in small bags for incubation in the horse's hindgut. The in vitro methods DI and GP, include incubating a feedstuff with a buffer solution and inoculum (cecal fluid) in different apparatuses. The GP method also records gas production provided by microbes fermenting carbohydrates. The digestibility is eventually calculated based on the amount of feed disappearing during incubation.

Chemical analysis showed a decrease in crude protein (CP) and ash and an increase in neutral detergent fiber on an organic matter basis (NDFom), acid detergent fiber on an organic matter basis (ADFom) and acid detergent lignin on an organic matter basis (ADLom) from early to late harvest, as evidence of progressive maturity in the grass. Further, water-soluble carbohydrates (WSC) content was high, low and thereafter high in early, middle, and late harvest, respectively. Fructan content was high in late harvest time.

All digestibility methods showed a linear decrease in dry matter (DM) digestibility with postponed harvest time, and they were highly correlated. However, the methods showed differences for digestibility estimates, where IS gave the highest values, followed by GP and thereafter DI. Practical recommendations generated through this study are that early harvest is suitable for horses with high protein and energy requirements. Middle harvest time is recommended for horses prone to laminitis, insulin resistance or overweight to avoid high sugar content. For leisure horses, a middle and late harvest time are suitable.

All methods have been evaluated as valuable methods to quantify forage digestibility in horses. Furthermore, IS is the recommended method as it is most similar to the actual biological occasion. However, cannulated horses are rare. Therefore, the GP and DI methods are good alternatives.

Sammendrag

Formålet med denne oppgaven var å undersøke hvordan høstetid påvirker fordøyelighet hos hester, i tillegg til å sammenligne tre ulike metoder for å evaluere fordøyelighet. Timoteigress ble høstet åtte ganger sommeren 2021, analysert for kjemisk- og sukker komposisjon. Teknikkene som ble brukt for å måle fordøyelighet var in sacco (IS) og to in vitro metoder: daisy inkubasjon (DI) og gassproduksjon (GP).

Grovfôr er det mest essensielle fôret i hestens diett. Mens et høyt inntak av sukker er assosiert med metabolske forstyrrelser, er fiber avgjørende for baktarms mikrobiotaen. En sen høstetid sikrer et høyt fiberinnhold samtidig som det går på bekostning av fordøyelighet og næringsverdi.

Referansemetoden for å bestemme fordøyelighet er in vivo-teknikker. Dessverre er in vivo-teknikker både tidkrevende og dyrt. I IS-metoden legges fôr i små poser for inkubering i hestens baktarm. In vitro-metodene DI og GP inkluderer inkubering av fôr med en bufferløsning og inokulum (blindtarmsvæske) i forskjellige apparater. Gassproduksjons-metoden registrerer også mengde gass som akkumuleres fra mikrober som fermenterer karbohydrater. Fordøyeligheten beregnes deretter ut i fra mengde fôr som har forsvunnet under inkubasjonen.

Den kjemiske analysen viste en reduksjon i råprotein (CP) og aske, og en økning i nøytral løselig fiber på organisk stoff basis (NDFom), syreløselig fiber på organisk stoff basis (ADFom) og syreløselig lignin på organisk stoff basis (ADLom) fra tidlig til sen høsting, som bevis på progressiv modenhet i gresset. Videre var innholdet av vannløselige karbohydrater (WSC) høyt, lavt og deretter høyt i henholdsvis tidlig, middels og sen høsting. Fruktaninnholdet var høyt i sen høsting.

Alle fordøyelighetsmetoder viste en lineær nedgang i tørrstoff-fordøyelighet (DMd) med utsatt høstingstid, og de var sterkt korrelerte. Metodene viste imidlertid forskjeller for fordøyelighets-estimater, hvor IS ga høyeste verdier, etterfulgt av GP og deretter DI. Praktiske anbefalinger generert gjennom denne studien er at tidlig høsting er egnet for hester med høyt protein- og energibehov. Middels høstetid anbefales for hester som er utsatt for forfangenhet, insulinresistens eller overvekt for å unngå høyt sukkerinnhold. For hobbyhester passer en middels og sen høstingstid.

Alle metodene har blitt evaluert som nyttige metoder for å kvantifisere fordøyelighet hos hester. Videre er IS den anbefalte metoden da den er nærmest den faktiske biologiske hendelsen. Derimot er kannulerte hester imidlertid sjeldne. Derfor, er GP- og DI-metodene gode alternativer.

Contents

1 Introduction

The horse (*Equus Caballos*) have a digestive tract developed to utilize fermentable, low energy-density, fiber-rich forages (Hoffman, 2009). To meet their nutritional needs, the wild equids would graze for 12-18 hours per day (Krueger et al., 2021). This led to the development of horses' digestive tract requiring adequate amounts of forage, supplied continuously through the day to function properly (minimum 1.5 kg dry matter (DM)/100kg bodyweight (BW) daily) (Harris et al., 2017). Forages has therefore become the most important and basal feedstuff in diets to horses. Challenges of controlling excess feed intake, in addition to limited access to pasture, many horses are today not allowed grazing, and are therefore fed preserved forages, such as hay, haylage or silage (Harris et al., 2017).

Digestion of forages provides energy for the horse as a result of microbes in the hindgut producing volatile fatty acids (VFA). Forages consist of mainly fibrous material which is essential for hindgut health, while excessive intake of non-structural carbohydrates (NSC), or having a restricted forage intake is associated with various intestinal disorders such as colic (Gonçalves et al., 2002), gastric ulcers (Andrews et al., 2005), laminitis (Garner et al., 1975) and hindgut acidosis (Rowe et al., 1994).

Digestibility measures the degree of nutrients that are digested and absorbed, which is strongly related to the nutritional value of the feed. The chemical content of forages varies greatly across grass species, climate conditions and by time of harvest. Characterization of how chemical content and digestibility varies through the different harvest times, aims to optimize and balancing diets to horses with individual requirements.

Traditional in vivo digestibility methods, such as the total collection of faeces are the gold standard method for measuring digestibility. In vivo methods are time-consuming and expensive to operate (Stern et al., 1997). Alternative techniques, as for example the in sacco (IS) or in vitro methods; gas production (GP) and Daisy incubation (DI) are more effective and economical than in vivo methods. In the IS method, bags with feed are incubated in the animal digestive tract, whereas the two other methods are performed in the laboratory. The in vitro techniques are based on incubating the feedstuff in a buffer solution with an inoculum source (caecal fluid or faeces from the horse) with various devices that are simulating the biological occasion of digestion in the animal. These methods estimate digestibility by disappearance of feed after incubation and gas production from a fermentation process.

1.2 Objective and hypothesis

The objective of this thesis was to examine nutritional value in forages (timothy grass) harvested at different maturity stages with near infrared spectroscopy (NIRS) and chemical analysis, and how maturity of grass affects the digestibility in the horse. Three digestibility methods; IS, DI and GP were used to evaluate the dry matter digestibility (DMd) of grasses. A second aim was to compare and evaluate the correlation between the digestibility methods.

The hypothesis are as follows:

1. Plant maturity and harvest time will influence the chemical composition in grasses
2. Plant maturity and harvest time will influence the digestibility negatively
3. There is good correlation between the methods used for measuring digestibility

2 Literature review

2.1 Grasses

Grasses originates from the family Poaceae. A mix of timothy, meadow fescue and tall fescue grasses are typically used when growing forages intended for horses in northern countries (Saastamoinen & Hellämäki, 2012). These grasses are characterized as cool-season grasses. Cool-season grasses are well adapted to grow well in cold climates, and have their optimal growth between 16-24°C.

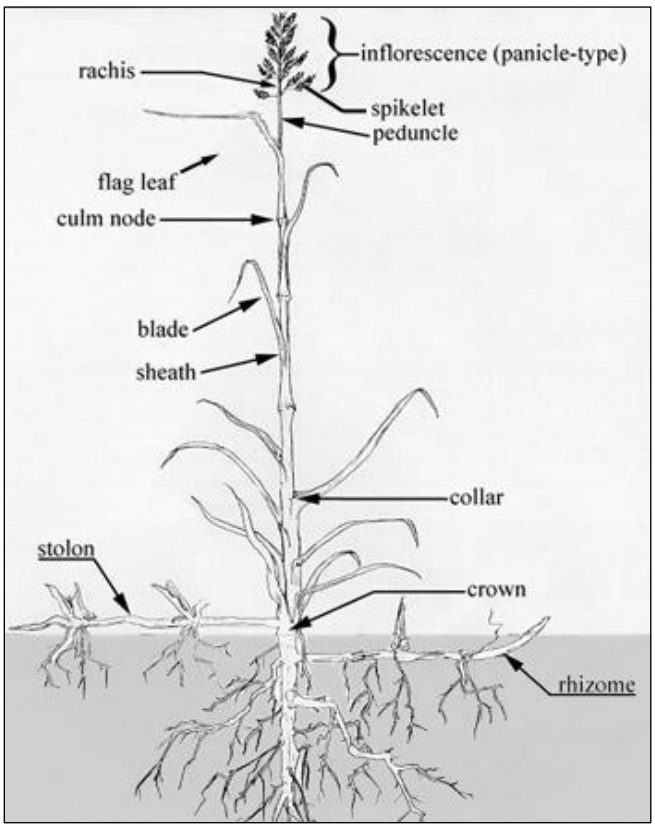


Figure 1. Structures in a grass plant, from Oregon State University (2022)

Timothy (*Phleum pratense*) is a perennial grass and has been used for forage production for horses and ruminants for centuries (Valberg, 1975). Timothy grasses are intolerant of intensive grazing, due to its low crown position (figure 1). Intense grazing will reduce the grasses' food reserves, yield and life length (Lacefield et al., 1980). It is a leafy, bunch-type tall-growing grass that grows in a clumping habit with tall, thin stems, that are smooth and hairless. Timothy grass can reach a height of 30-40 cm at heading state but may grow up to 150 cm at a highly mature stage (Valberg, 1975). Timothy grass does not have rhizomes or stolon as shown in figure 1, but have a

shallow, fibrous root system, and therefore have a bunchy growing habit. Timothy may have between three to six nodes, and short internodes. During maturity, the lower internodes are enlarging, termed as the haplocorm which serves as an important storage organ for carbohydrates (Valberg, 1975). The leaves are flat, smooth and hairless with a pale green to greyish-green colour. The flowering unit (termed inflorescence) on timothy is spikelike, one-flowered, condensed and cylindrical with a green colour, but turn light brown with maturity and grow 6-18cm long (Valberg, 1975). The stem section bearing the flowering unit is termed the peduncle. During the boot stage, the flag leaf is the leaf covering the seed head.

2.1.1 Nutrients in grasses

Forage nutritional value varies greatly among grass species (table 1). Factors like stage of growth (maturity), management practices, harvest date, fertilization and environmental conditions are some effects that modifies nutritional composition in grasses. The most important factor affecting nutritional value is the maturity of grasses (McDonald et al., 2011) which is more described in section 2.1.2.

Table 1. DM, ash, CP and NDF content in some common forage grass types for horses. Value for DM is in %, the others are in % of DM. From Norfor (n.d)

	Timothy grass	Kentucky bluegrass	Orchard grass	Brome grass	Rye grass
DM	19.1	18.5	16.7	15.1	18.6
Ash	8.2	9.5	9.4	9.6	5.7
CP	20.0	29.2	19.1	21.5	11.1
NDF	53.5	54.2	59.7	54.8	61.6

DM= dry matter, CP=crude protein and NDF= neutral detergent fibre.

Carbohydrate description

Grasses are rich in carbohydrates. Carbohydrates are cyclic structures of carbon, hydrogen, and oxygen. Originally, carbohydrates can be classified in terms of their chain length, or degree of polymerization; (1) mono-, (2) di-, (3-10) oligo- or (>10) polysaccharides. Some important mono- and disaccharides (also called sugars) in grasses are glucose, fructose, and sucrose. Monosaccharides that are linked together gives rise to di-, oligo- and polysaccharides. For example, glucose is the main component in the polysaccharides starch and cellulose, being distinctive due to their linkages, which are the most common polysaccharides in horse diets (NRC, 2007). Carbohydrates in plants can be separated into the cell wall and cell components (figure 2). Hoffman et al. (2001) proposed a system for partitioning carbohydrates relevant for equine nutrition into three main fractions: hydrolysable carbohydrates (CHO-H), rapidly fermentable carbohydrates (CHO-F_R) and slowly fermentable

carbohydrates (CHO-F_S) (figure 2). This partitioning reflects the utilization of different carbohydrate fractions in the gastrointestinal tract (GIT) of the equine and how their attendant analytical measures differ. Carbohydrates with α-1,4 linkages are mainly subject to enzymatic hydrolysis (CHO-H) (but may in varying degree be fermented), while ß-1.4 linked molecules are fermented (CHO-F_S and CHO-F_R).

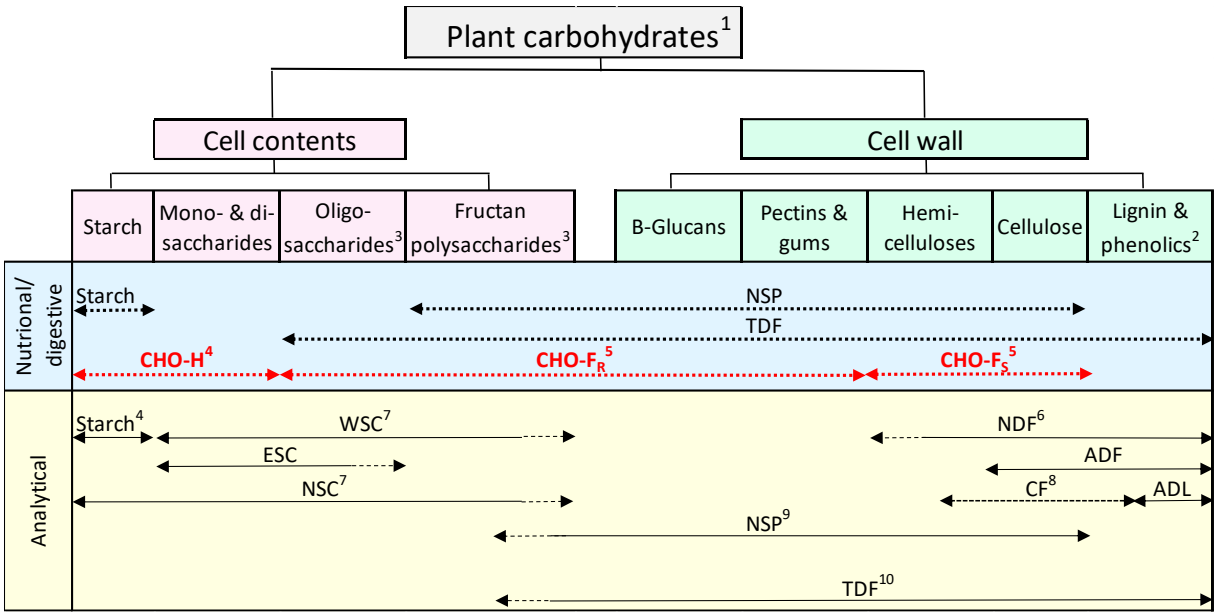


Figure 2. Overview of plant carbohydrates and related compounds relevant to equine nutrition, modified from NRC (2007) and Hoffman (2001). Current and proposed systems for partitioning dietary carbohydrates based on current analytical methods (lower, yellow area) and nutritional or physiological definitions (upper, blue area) relative to equine digestive function. Dashed lines in analytical area indicate that recovery of included compounds may be incomplete.

ADF= acid detergent fiber, ADL= acid detergent lignin, CF= crude fiber, ESC= ethanol-soluble carbohydrates, CHO-H= hydrolysable carbohydrates, CHO-F_S= slowly fermentable carbohydrates, CHO-F_R= rapid fermentable carbohydrates, NDF= neutral detergent fiber, NSC= non-structural carbohydrates, NSP= non-starch polysaccharides, TDF= total dietary fiber and WSC= water-soluble carbohydrates

[1]Major categories of carbohydrates and associated substances are shown. These categories may not include all carbohydrates produced by plants.

[2]Some noncarbohydrate components are included here as they are components of the specific analytical fractions.

[3]This fractions include the fructooligosaccharides, as specific fructans can be categorized as fructooligosaccharides or fructan polysaccharides, depending on the degree of polymerization.

[4]A variable fractions of total starch can be resistant to enzymatic hydrolysis, and thus, some starch may appear in other nutritional fractions.

[5]Fermentability of gums may be variable.

[6]Some hemicellulose may be soluble in neutral detergent and thus recovered in the TDF fraction, rather than the NDF fraction.

[7]Recovery of compounds in the analytical WSC (and then thus the NSC fraction when NSC is approximated as starch + WSC), may depend on the methodology used.

[8]Amount of cell wall constituents included in the CF analysis varies by feed.

[9]From a nutritional perspective, NSP includes all polysaccharides except starch. However, the analytical method for NSP may recover a variable amount of fructan polysaccharides.

[10]From a nutritional perspective, TDF includes all carbohydrates resistant to mammalian digestion. However, the analytical method for TDF does not recover oligosaccharides and may recover a variable amount of fructan polysaccharides.

Carbohydrate components in the plant cell wall

Plant cell wall surrounds the membrane of plant cells and is the fibrous fraction of the plant. Carbohydrates in the cell wall are resistant to digestive enzyme degradation and are therefore

subject to fermentation in the equine hindgut. Hemicellulose and cellulose constitute the CHO-F_S fraction. The CHO-F_R fraction includes both some structures from the cell wall content (pectin, gums and ß-glucans) and the cell content (long-chained fructans and oligosaccharides). The main part of the plant cell wall carbohydrates in grasses are cellulose and hemicelluloses, which, together with lignin, make up the neutral detergent fiber (NDF: lignin, cellulose and hemicellulose) fraction (figure 3). Other fiber fraction is determined as acid detergent fibre (ADF: lignin and cellulose) and acid detergent lignin (ADL: lignin). Cellulose are polymers of ß-1,4 linked glucose, whereas hemicelluloses are polymers of several sugar components such as glucose, arabinose, galactose, xylose and mannose. Lignin, which is not a carbohydrate, but an important part of the cell wall structure, is a polymer built up by different phenylpropanoids. The lignin fraction greatly impacts how digestible the plant cell wall structures are (Pagan, 2009). Pectin contains mainly ß-linked galacturonic acid units but may also contain galactose and arabinose. The ß-glucans are a term of ß-linked polymers of glucose. Gums are viscous galactopolysaccharides. Cool-season grasses, such as timothy, consist of 35-65% cell wall carbohydrates of DM (NRC, 2007). Grasses has low (2-5% of DM) content of pectin (Silva et al., 2016).

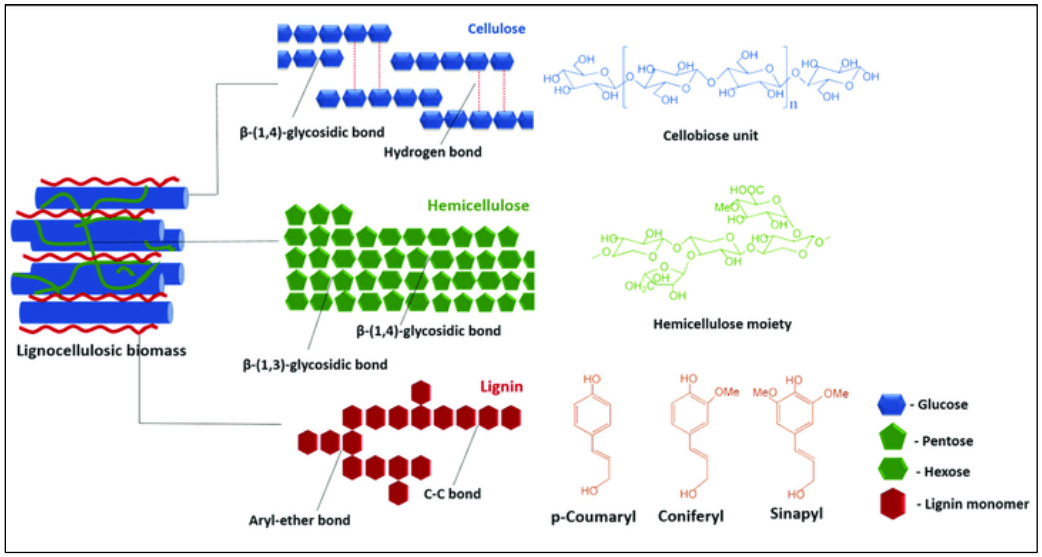


Figure 3. Components of the NDF fraction in the plant cell wall; cellulose, hemicellulose, and lignin. From Baruah et al. (2018).

Components in the plant cell content

The cell content contains all of starch, sugars, organic acid, and other nutrients, such as most of the protein, lipids, and ash. In general, the cell content is highly digestible (Pagan, 2009). Non-structural carbohydrates (NSC) cover the carbohydrates in the plant cell content (sugars and starch). Sugars, which is mainly glucose, fructose, sucrose and fructans can be divided into two subgroups: ethanol soluble carbohydrates (ESC: sugars and short chain fructans) and water-soluble carbohydrates (WSC: sugars and fructans), depending on their solubility and degree of polymerization (NRC, 2007). A low

content of ESC is observed in timothy grass (10% of DM) (Brøkner et al., 2012b). The average value of NSC in timothy, tall fescue and bromegrasses are also low with a range from 5.2-11.8 % of DM (Pelletier et al., 2010). The NSC content is intermediaries in grass metabolism and important for energy transport and -storage (Undersander, 2013). Stress is a factor for accumulation of NSC in grasses, and grasses that accumulate NSC are more viable under drought stress (Watts, 2009). Cool-season grasses like timothy tend to have a high capacity to accumulate NSC, because factors like cold climate and drought are stressors for the plant. Warm-season grasses are entirely inactive during stress, while cold-season grasses needs to accumulate energy for survival during periods of low intensive metabolism, such as wintertime (Watts, 2010). High NSC content in forages is a potential trigger factor for obesity, laminitis, insulin resistance (Treiber et al., 2006) and polysaccharide storage myopathy (PSM) (Firshman et al., 2003) in equines.

Fructans, which are the main storage carbohydrate in cool-season grasses, are polymers of fructose with no or one internal or terminal glucose unit and can have different linkages, where ß-2,1 (termed inulin) and ß-2,6 (termed levans or phlein) (figure 4) are the most common in equine feed stuffs (Hoffman, 2013). Fructans are resistant to mammalian enzyme hydrolysis and will therefore be rapidly fermented by microbes, mainly in the equine hind gut, hence a part of the CHO-F_R fraction. Fructans in grasses improve cold and drought tolerance, and may act as a coolant by stabilization the cell membranes to reduce water leakage (Hincha et al., 2007) and contributes to osmotic adjustment upon freezing (Krasensky & Jonak, 2012). In cold-season grasses, there are no self-limiting mechanism for the production of fructans (Longland & Byrd, 2006).

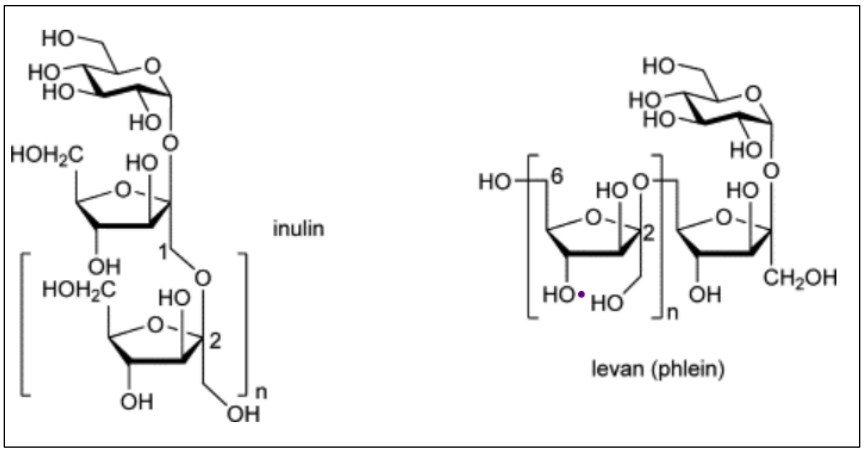


Figure 4. Fructan structures. From Williams and Stick (2009).

Starch, which is the main storage carbohydrate in grains, warm-season grasses and legumes (Hoffman, 2013) are polymers of glucose with two different linkages α-1,4 (termed amylose) and α-1,6 (termed amylopectin) (figure 5). Starch is normally subject to enzymatic hydrolysis in the equine small intestine but can be rapidly fermented in hindgut for two reasons: 1) given in large amounts

(>2g/kg BW) (Julliand et al., 2006) which exceeds the small intestine capacity to digest starch, or 2) the starch is resistant (termed resistant starch) to enzymatic hydrolysis, and this is explained by its chemical or physical structure. Resistant starch is defined as the fraction which escapes digestion in small intestine, and is the starch that is not hydrolysed after 120 min of incubation with an in-vitro method (Sajilata et al., 2006). However, average starch content in grass pasture is low (2.8% of DM) (McDonald et al., 2011).

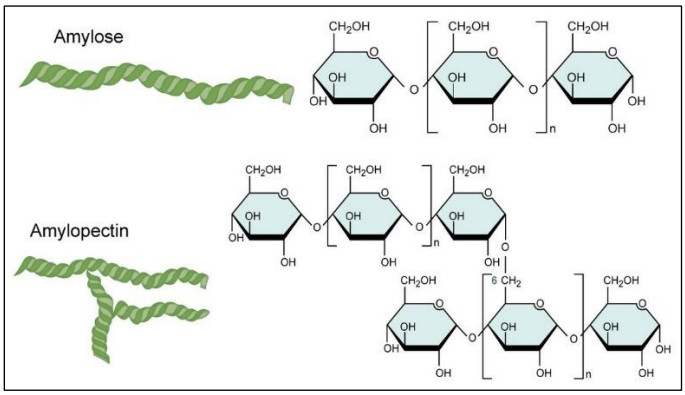


Figure 5. Chemical composition of amylose and amylopectin in starches. From Taylor's University (n.d)

Proteins are molecules consisting of amino acids linked together with peptide bonds. Each amino acid consists of a carbon atom attached to a carboxyl group, an amino group and a sidechain, which make the amino acid unique (figure 6). About 20 different amino acid are identified. Forages have variable content of protein. Crude protein content in timothy grass can range from 16-11% of DM (Bernes et al., 2008) in early to late cut. To improve CP content in forages, red clover is often added. Legumes has ability to fixate N_2, and have a crude protein content decreasing from 21-18% of DM in early to late cut (Vanhatalo et al., 2009).

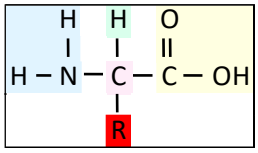


Figure 6. Amino acid structure. The "R" reflects the variable sidechain.

Lipids are built up of glycerol attached to three fatty acids, which in forages often have a carbohydrate chain length of 12-22 carbon atoms (McDonald et al., 2011). Lipids are present in only small amounts (1-3% of DM) in grasses, but have important roles in the protection and metabolism of the plant (Hatfield et al., 2007). Lipids in plants are of two main structures: structural or storage lipids. Storage lipids are mainly triacylglycerols in the seeds. Membrane lipids are mainly galactosylglycerides or phosphoglycerides. Lipids at the surface of the plant are mainly waxes and cutins which are indigestible.

Minerals are inorganic molecules, and the total mineral content is termed ash content. Mineral content in forages is low compared to concentrates, but may vary considerably (table 2). Zhao and Müller (2016) found that forages not fertilized or organic fertilizers used had higher Ca content,

compared to use of a combination of inorganic and organic fertilizer were used. Forages from grass-legume had higher content of Ca, Mg and Cu than grass forage. It is also observed that several minerals were significantly higher in the regrowth (second or third cut) versus the primary growth. However, forage harvested at an early stage had higher content of Ca, P, Mg, K, Na and I (Zhao & Müller, 2016). Ash content in Timothy and Rye grasses are reported to be 5.8 and 9.1% of DM, respectively (Brøkner et al., 2012b).

Table 2. Mineral content in spring grasses from Norway and Sweden, from Zhao and Müller (2016) and in concentrate

	Macrominerals in grass, g/kg DM	**Macrominerals in concentrate*, g/kg**
Ca	5.3 ± 3.4	8.0
P	2.7 ± 0.8	4.0
Mg	1.8 ± 0.8	2.5
K	21.8 ± 7.4	-
Na	0.3 ± 0.6	6.0
	Microminerals in grass, mg/kg DM	**Microminerals in concentrate, mg/kg***
Co	0.09 ± 0.1	0.3
Cu	4.9 ± 1.6	39.0
Fe	194.0 ± 288.9	112.0
Mn	85.0 ± 49.3	70.0
Zn	23.2 ± 9.5	130.0
I	0.25 ± 0.04	0.8
Se	0.03 ± 0.05	0.46

**The concentrate is Champion Komplett (Felleskjøpet, Norway), a concentrate that covers the horses' vitamin and mineral requirements if fed 0.5-0.6kg/100kg bodyweight, from (Felleskjøpet, 2022)*

2.1.2 Effect of harvest time on grass nutrients

As grasses mature, their chemical composition changes (figure 7). During the growth season grasses develop from leafy to stemmy stages which decreases the leaf:stem-ratio. Correspondingly, DM content and -yield and fiber increases, whereas protein, NSC, minerals and lipids content decreases (Hoffman et al., 2001; Saastamoinen et al., 2012). The outcome of this shift in chemical content results in lower nutritional quality and reduced digestibility in late cut grasses (Virkajärvi et al., 2012). As the stem in the grass grows, there is an accumulation of the cell wall material and cell walls get ligninificated, which makes the stem rigid (table 3). When grasses start to grow in the spring, it uses of its own storage reserves and this process may require the total of its energy reservoir. As the grass reaches maturity, the grasses' own production of carbohydrates is in a positive energetic balance, which means carbohydrates (mainly sugars) accumulate in the grass. For example, fructans accumulate when carbohydrate supply exceeds the demand (Housley & Pollock, 1985).

Table 3. Chemical content of fiber (NDF, ADF and ADL) in % DM from northern Italian pasture grass and tall fescue from grass from different harvest times

	Northern Italian pasture grass		Tall fescue	
Harvest time	Early cut	Late cut	Early cut	Late cut
NDF	46.7	57.4	54.3	62.5
ADF	24.6	30.7	29.3	34.4
ADL	2.1	4.2	2.0	3.8
Reference	Superchi et al. (2010)		Särkijärvi et al. (2012)	

NDF= neutral detergent fibre, ADF= acid detergent fibre and ADL= acid detergent lignin

Grass development can be summarized into three main phases: 1) vegetative stage, 2) elongation stage and 3) reproduction phase. In the vegetative stage**,** grasses reproduce vegetatively by forming new shoots from the lowest joint of the straw. Grasses grow only from the crown, which is the point located at the base of the stem. The grass has short, unelongated, non-reproductive tillers that shoots at the crown and produces only leaves. The elongation phase is the stage when nodes start to become visible or touchable and the stem internodes are lengthening. Stem formation contributes to higher DM content and increases the act of photosynthesis. During late boot to early flowering state, the digestibility may decrease about 0.5% units per day (Lacefield et al., 1980), as grass in this stage has reached its maximum yield but has not yet declined in nutritional value (Watts, 2010). The reproduction stage begins when the upper shoot starts flowering, which initiate development of seeds. The leafy stage is similar to vegetative phase, the boot stage is comparable to the elongation phase, and reproductive stage includes heading and blooming (figure 7).

All green plants carry out photosynthesis, which means they converts atmospheric carbon dioxide (CO_2) and water (H_2O) to glucose and other simple sugars. Oxygen (O_2) is the by-product. The photosynthesis must have sunlight to occur, which result in low accumulation of energy in the plant at night. Plant respirations happens mainly at the night, where sugars produced from photosynthesis are utilized for energy or growth, and as substrates for essential components such as hemicellulose and cellulose (Watts, 2004). The grass uses energy gained from the photosynthesis until it meets its energy requirement. Subsequently, energy is stored as storage carbohydrates, mainly starch or fructans. As a function of photosynthesis in plants, diurnal variations in NSC in grasses occurs. Normal trends are concentrations increase during the day when exposure to sunlight and decreasing concentrations overnight. There is reported small increased values for NSC in grass species with delayed cutting time compared to cutting early in the day. For example, NSC in timothy grasses and tall fescue increased from 5.6-7.5 and 9.4-10.1% of DM, respectively, from morning to the afternoon cut (Pelletier et al., 2010).

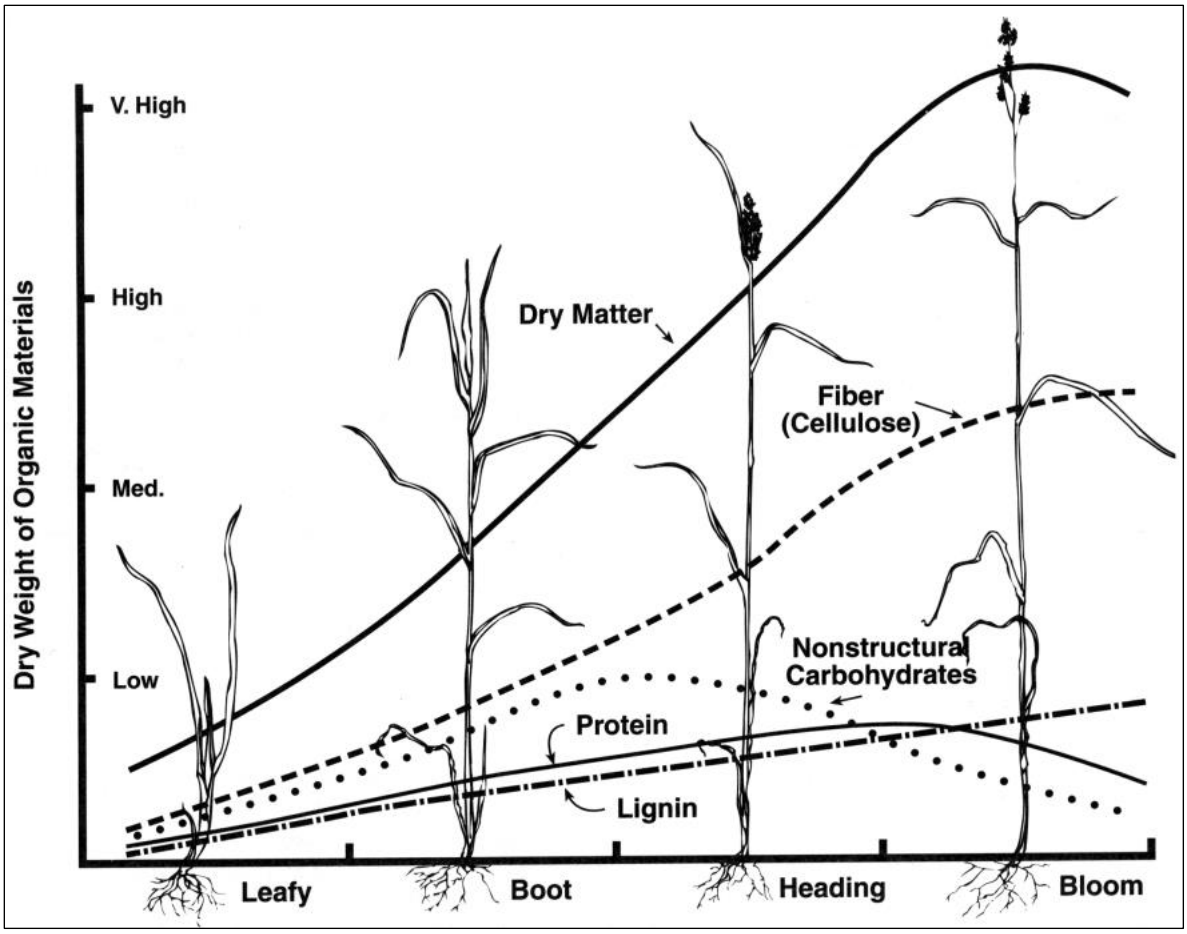


Figure 7. Schematic overview in grasses growth from leafy to stemmy stages, resulting in an increase in DM yield, cell walls materials (fiber and lignin), and decreases in protein and NSC. From White and Wolf (2009).

Fructans may not accumulate in young leaves and tend to increase as the plant matures, where maximum levels are at heading (Undersander, 2013). Lower fructans level are reported when the growth is supported by fertilization, defoliation and irrigation (Undersander, 2013). In Timothy grass, the fructans are mainly of a high degree of polymerization (DP), while in ryegrasses and fescues, the fructans are in the form of both oligomeric and large polymers (Gallagher et al., 2007). Seasonal variations of WSC content has some discrepancy, where high WSC and/or fructan contents are reported as highest for winter, spring/early summer, or autumn (Gallagher et al., 2007). In Orchard and Bermuda grasses, WSC content declined from 21-9% (Kagan et al., 2011a) and 9-5% of DM (Kagan et al., 2011b), respectively, in early to late harvest (table 4). Non-structural carbohydrates content often rises during the spring and declines during the summer, and then increases again in the autumn, as illustrated in figure 8 (Watts, 2010).

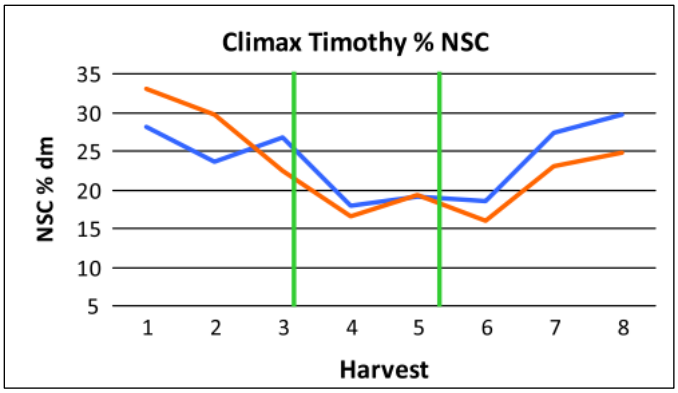


Figure 8. Seasonal variation of NSC in Timothy grass from USA. Green lines represent removal of hay crop and the application of nitrogen fertilizer. The blue line is from 2005, the orange line is from 2005. Harvest (H) 1-3; may, H4; July, H5; August, H6; September, H7; October and H8; November. From Watts (2010).

Cool-season grasses tends to be higher in NSC content than warm season grasses, and this is possibly a result of the plants own survival mechanism in cold and drought climates (Watts, 2010). However, there are many other factors than maturity that alter the NSC content. For example, fertilizing with N reduced the NSC content in a range of 0-118 g/kg DM (Jacobs et al., 1989), and shading of pasture reduced NSC content with 64 g/kg DM (Ciavarella et al., 2000).

Table 4. Contents of the constituents of NSC (% of DM) in Orchard and Bermuda grass from different harvest times.

Grass	**Orchard grasses**		**Bermuda grasses**	
Harvest time	Early	Late	Early	Late
WSC	21.0	9.0	9.0	5.4
ESC	11.2	4.3	6.4	4.7
Fructan (calorimetry)	9.8	4.6	-	-
Fructan (HPLC)	8.0	1.7	-	-
Glucose	2.2	0.8	1.2	0.8
Fructose	2.0	0.9	1.2	0.8
Sucrose	5.7	1.1	2.1	2.0
Starch	-	-	0.5	2.5
Reference	Kagan et al., 2010a		Kagan et al., 2010b	

WSC= water-soluble carbohydrates, ESC= ethanol soluble carbohydrates and HPLC= high-performance liquid chromatography.

Total protein content decreases with maturity (Randby et al., 2010). Crude protein content in timothy is reported to decrease from 13-10% (Yu et al., 2003) and from 17.5-9.3% of DM in early to late cut (Ragnarsson & Lindberg, 2008). The amino acid profile remains stable even though there is a decrease in total protein content. Mature grasses may have lower protein digestibility because the protein is bound to fiber, and are then termed acid-detergent insoluble nitrogen (ADIN) (McDonald et al., 2011).

2.1.3 Forages for horses

Forage is the horses' primary feed and may contribute to 50-100% of the horses diet (Saastamoinen et al., 2012). Almost all types of grass have a valuable contribution as forage (Gupta et al., 2016). The term forage (often also called roughage) includes pasture and all conserved grass species such as hay, haylage, silage or some legumes (mainly lucerne), or crop residue and by-products such as straw and cereal hulls (Harris et al., 2017). Hay has been a traditional forage for horses, but today haylage is also a popular forage used for horses (Müller, 2007). Forage is classified as either hay, haylage, or silage, depending on the DM content (table 5). Hay should always have a DM content above 85% to achieve stable conditions, hygienic quality, avoidance of microorganisms and mould contamination.

Table 5. Dry matter content in different types of forages. From Harris et al. (2017).

Type of forage	Dry matter content (%)
Hay	> 85
Haylage	50-85
Silage	30-50
Grasses	< 30

Climate conditions, restricted area for pasture and grassland production, and challenges during harvesting and storing hay under dry conditions have developed a need for alternative methods for preserving fresh forage such as haylage and silage. Haylage and silage needs to be packed airtight to be storable, which promotes a fermentation process. A successful ensiling process involves anaerobic conditions with organic acids, which decrease pH (if DM is 35%, pH should be a maximum of 4.6 and a maximum 5.0 if DM is 50%) (Weissbach (1968), reported in Müller (2005)). Anaerobic conditions and low pH are critical to minimize content of harmful microorganisms (fungi, yeast or bacteria) and to prevent nutrient loss. Haylage and silage are either wrapped up in bales with plastic layers or stored in silo bunkers.

The fermentation processes

The ensilage process can be separated into four principal phases. The first phase (also termed the aerobic phase) is the phase immediately after harvesting. This phase involves active aerobic microorganisms (proteases and carbohydrases) that degrade and consumes WSC and proteins, which produce acetic- and lactic acid, NH_3, and heat. The main goal in the first phase of ensiling is to stop the respiration and obtain anaerobic conditions in the plant mass, which ideally should be achieved in a few hours after harvesting. Silage additives, such as formic acid or propionic acid, will control or prevent certain types of fermentation, which results in reduced nutrient loss and enhance silage stability (Yitbarek & Tamir, 2014). Formic acid may stimulate lactic acid and propionic acid fermentation and have an antifungal effect (Randby et al., 2010).

In phase two, the retaining O_2 in the plant mass is consumed, and there is establishment of anaerobic microorganisms, such as lactic acid bacteria, which continues fermenting soluble carbohydrates and yield lactic acid. The lactic acid microorganism is highly desired in this phase because it is a strong acid that efficiently contributes to the drop in pH, which is the preferred fermentation pattern. There may be some aerobic microorganisms left, which compete with the anaerobic bacteria for substrate, until true anaerobic conditions are achieved. Adequate WSC content for the lactic acid bacteria will accelerate reduction in pH, and consequently outcompete other microorganisms.

However, successful preservation in haylage with DM above 50% relies on an airtight packaging and not a low pH. Therefore, plant mass with high DM content may require more plastic layers than forages with lower DM content (Harris et al., 2017). In phase three (also termed the stable phase), pH should be below five, which leads to decline in the fermentation process, because the growth of lactic acid bacteria stops. Consequently, the silage will be stable with minimal DM and energy loss, as long as the forage is properly airtight packaged. Phase four is the feed-out phase, where plant mass gets exposed to O_2, henceforth aerobic degradation initiates. Therefore, a quick feed-out is necessary when applying haylage or silage.

2.1.4 Quantitative amounts of forage to horses

For all post-weaned horses, forage should be the foundation of the diet regardless of training intensity. There are two main reasons for minimum recommendations for forage to horses: 1) support of gastro-intestinal function and health and 2) benefit to behaviour (Geor et al., 2013). There are variations on the recommended level of forage horses should be fed daily. Old recommendations from NRC (1989) mention that horses should receive minimum of 1.0 kg DM forage or pasture per 100 kg BW daily, which equals to 1% of BW. Harris et al. (2017) suggests lower limit of daily forage intake should be 1.5 kg DM/100kg BW, with an absolute minimum of 1.25 kg DM/100 kg BW. Additionally claims that previous minimum recommendations at 0.8- 1.0 kg DM/100 kg BW are not adequate in terms of the latest understanding of equine ethological demands and gut health concerns. When horses have free access to pasture, it is estimated they have a voluntary dry matter intake of between 1.5- 3.1 % of BW, where lactating mares have the average highest intake (NRC, 2007).

2.2 Evaluating chemical composition and energy value in horse feeds

2.2.1 Evaluating chemical composition

The three main methods for analysing horse feeds are chemical evaluation (wet chemistry), NIRS and in vitro methods.

Near infrared spectroscopy

Near infrared spectroscopy is a spectroscopic method which relates to a sample reflectance of near infrared light (wavelength 800-2.500 nanometres) to its chemical composition (NRC, 2007). Eurofins use NIRS as the main method for forage analysis (Eurofins, 2022). Organic molecules will absorb differently and reflect near-infrared light, hence the NIRS method got a "fingerprint" of the sample of interest. Each nutrient has a specific peak or slope, and this change of slope with respect to wavelength directly reflects the physical composition of the feed (Harris et al., 2018). Figure 9 illustrates how different nutrients reflects near infrared light. In NIRS, an unknown sample is compared to a database of known results (prediction models), and the method needs to be calibrated against local laboratory analysis and local samples, so the sensor has a starting point base. This method has evolved to predict chemical content, in vivo digestibility and voluntary feed intake of forages (Andueza et al., 2011). The pre- and post-treatment of the sample, together with instrumental variations (manufacturer and methodology) can affect the results obtained and might be a source of errors in the NIRS method (Le Cocq et al., 2022). In addition, methods for the development of calibration models are not standardized. For example, variations in WSC have been reported to differ by up to 20% between NIRS methods but have agreeable correlations with wet chemistry (Harris et al., 2018). Le Cocq et al. (2022) reported similar; poor predictions of WSC with NIRS methods. This may be explained of restricted sample database, low prediction range for this nutrient and the fact that the calibration model is adapted to ruminant forage that differ from forage used for horses (lower DM, NDF and WSC in ruminants' feeds than horse feeds). With that in mind, the advantages of NIRS compared to chemical methods, is that the method are labour-saving, inexpensive and no need for reagents or chemicals.

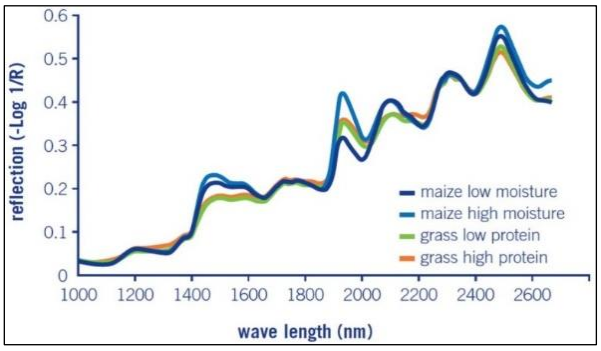


Figure 9. Example of a NIR-spectrum (Eurofins, 2022).

Chemical evaluation

There is a large variety in feedstuffs determination methods and partitioning, since the nutrients are defined by the method applied for its analysis. For example, for evaluating fibre content there are several methods available.

Forages to horses differ greatly in DM content (table 5), symbolizing that DM determination and evaluating on DM basis is essential for correct feed evaluation. Dry matter is defined as the proportion of the sample residual after drying to a constant weight at a defined temperature (Åkerlind et al., 2011). To determine DM content, oven-drying is commonly used, where the principle is to make water evaporate from the sample. Temperature for drying can vary from 45-103°C, depending on what the sample is used for. To not harm the sample, lower temperatures (45°C) are often used for experimental feeds. For chemical evaluation, high (103°C) temperatures are used to make the sample completely dry, in minimum four hours or more (Berg, 2011b). NorFor uses 60°C when determining DM in silage to avoid large losses of VFA during oven drying (Volden, 2011; Åkerlind et al., 2011). Drying temperature of 100°C may underestimate DM content in silages by 2-16% due to VFA and ammonia loss (Dewar & McDonald (1961) in Minson and Lancaster (1963)). Additionally, low pH induce greater loss; therefore, correcting equitations is conducted for final DM determination of silage with pH below five (Porter & Murray, 2001). When DM content is determined, it consists of an organic and a smaller inorganic (ash) fraction. Combustion in oven at 550°C for 5.5 hours is used to quantify the ash content (Berg, 2011c).

Fiber analysis

The fiber fraction can be analyzed by 1) detergent methods described by Van Soest et al. (1991) to NDF, ADF and ADL, 2) sequential extraction with acid and alkali to crude fiber (CF), or 3) extraction method to dietary fibre (DF).

The Van Soest method is frequently applied as it provide a reliable characterization of the cell wall constituents, thus provides the most satisfactory method for fiber analysis (Southgate, 1977). The detergent method of Van Soest quantifies fiber into fiber that is insoluble in neutral detergents (NDF: hemicellulose, cellulose, and lignin) and in acid detergents (ADF: cellulose and lignin). The method may underestimate fiber content in some feedstuffs since soluble fibers may not fully recover in the detergent method. For example, some of the non-starch polysaccharides (NSP), pectin, gums, ß-glucans, mucilage and hemicellulose are soluble in neutral detergent (Knudsen, 2001). To measure NDF, the sample is boiled in a neutral detergent solution (sodium sulphate is often used), where the soluble fraction is termed the neutral detergent solubles (NDS: mainly soluble carbohydrates, proteins, lipids, minerals, and vitamins) (Berg, 2013). To remove starch content, amylase is added to the neutral detergent solution, then the NDF is termed amylase-corrected NDF (aNDF). In addition, the NDF fraction may constitute some ash, and this can be removed by pre-ashing the sample as described for determining the ash content, and then the fraction is termed NDF on organic matter basis (NDFom). ADF is found after extraction with acetyltrimethylammoniumbromide (CTAB) (Berg,

2012). As with NDF analysis, the content of ADF should also be combusted in an oven to remove inorganic materials like ash. The ADL content is the residue after extraction with CTAB and H_2SO_4 for three hours with continuously stirring and thereafter rinsed with water and pre-ashing for correction of inorganic material (Johnsen, 2018). Content of ADL is determined gravimetrically.

An older method is the CF determination from the Weende method. The carbohydrates are split into NFE and CF, where extraction methods determine CF content. This method has several weaknesses when estimating plant cell wall carbohydrates. It only represents a small fraction of the fiber content (average 80% of hemicellulose or pentosans, 50-90% lignin and 50-80% cellulose recovery) (Van Soest & McQueen, 1973). However, CF is still commonly used in forage analysis for horses, in addition to the NDF fraction for determining fiber. The CF method has a complete recovery of pectins (Möller, 2014), while this is not recovered in the NDF fraction.

The method of DF includes all non-starch polysaccharides (NSP) and lignin that are not degraded by endogenous secretions of the human digestive tract (Southgate, 1977). This method quantifies NSP into soluble (S-NSP: gums, ß-glucans and pectin) and insoluble NSP (I-NSP: cellulose and hemicellulose). Analysis of DF are based on one or more of three diverse principles: weighing after removal of non-fiber components, calorimetric carbohydrate determinations and gas-liquid chromatography (GLC) or high-performance liquid chromatography (HPLC) (Asp, 1987).

Protein analysis

Protein content can be measured by the Kjeldahl-N method, which estimates the nitrogen (N) content in the sample, except from nitrogenic oxides and nitrogen atoms in heterocyclic compounds (Berg, 2011a). The sample is dissolved and boiled with concentrated sulfuric acid (H_2SO_4) and lye, which is trapped in a container with a known amount acid. Nitrogen in sample will then be converted to ammonia, and the nitrogen content is then determined by back titrating with sodiumhydroxide. The CP content is then calculated from the analyzed N content with the assumption that protein contains 160g nitrogen/kg, by CP= N*6,25. This method assumes all N content derives from true protein. These assumptions may over- or underestimate the CP content, because feeds have different content of N depending on the amino acid composition, and do include some N that does not derive from protein (McDonald et al., 2011). However, CP is only a measure of N content, and does not provide a value of utilization. Therefore, digestible crude protein (DCP) is included in feed analysis for horses, based on amino acid composition and pre-caecal digestion trials (McDonald et al., 2011).

Sugar analysis

Sugars are measured as either WSC or ESC. The main difference being WSC also contains complex chain of fructans. Fructans of a variety in DP (3-300) (Longland et al., 2012) are water-soluble, hence included in the WSC fraction, whereas only fructans with low DP are included in the ESC fraction (Kagan et al., 2014). Content of WSC and ESC can be measured by calorimetric assays, enzymatic assays combined with calorimetry, titration or chromatography (Kagan et al., 2014). Whereas fructans can be determined directly by HPLC with pulsed amperometric detection (PAD) (Kagan et al., 2014). The HPLC methods can quantify both low- and high- molecular-weight fructans and the other mono- and disaccharides, but the method is expensive to operate, and it requires specialized expertise. The method of colorimetry is based on hot water solubilization of WSC content to remove starch and simple sugars, followed by enzymatic hydrolysis of fructans. The technique can separate the mixture of fructans and their oligomers (Longland et al., 2012). Fructan content determined by colorimetric method is reported to provide lower values than the HPLC method (Longland et al., 2012).

2.2.2 Evaluating energy value

In general, the energy value is based on the digestibility of a feed, and the protein value is based on the amount of amino acids absorbed from the small intestine (Virkajärvi et al., 2012). Energy value in feed for animals should always be in the same unit as energy requirements for animals, these need to reflect each other. In Scandinavia, feed unit for horse (HFU) (Norwegian; Fôrenhet Hest, (FEh)) is used. One HFU corresponds to the net energy (NE) value (2250 kcal) in one kg barley with 87% DM in a horse at maintenance. When measure energy value, several losses during animal metabolism must be accounted for (figure 10). The feeds total energy is termed gross energy (GE) (table 6).

Table 6. Gross energy (KJ/g) in carbohydrates, fats and protein

Nutrient	Gross energy (KJ/g)
Carbohydrates	17.9
Fats	39.8
Protein	23.9

Gross energy reflects the energy in feed released by complete combustion during bomb calorimetry. This is not a practical measure for energy value in feeds but can be described as the feed's energy potential. All type of carbohydrates yields the same amount of gross energy, but they have a different metabolism in the gut, result in higher net energy (NE) in CHO-H than CHO-F (Geor, 2007). Fats are higher in GE per unit weight than both proteins and carbohydrates. Gross energy in horse

feed can be predicted from calculations of the crude nutrients in g/kg DM as GE MJ/kg= 0.0239 crude protein + 0.0398 crude fat + 0.0201 crude fibre + 0.0175 N-free extract, from Anon (1995) in Kienzle and Zeyner (2010).

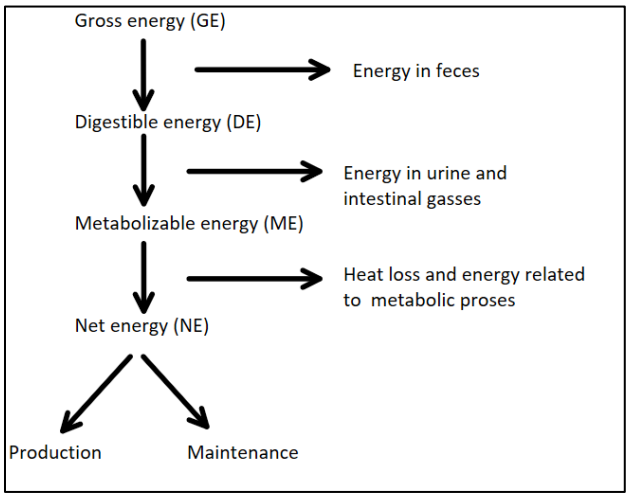


Figure 10. Energy flow diagram

Step two in energy turnover is to subtract the energy released in faeces from GE. This equals the digestible energy (DE). Limitations with the DE system is overestimating energy value in feeds with high gaseous- or fermentative losses like forages, as a result of microbial gas production in the equine hindgut. The DE between feedstuffs may differ by over 50% (Kienzle & Zeyner, 2010).

Step three in energy turnover is to subtract for energy lost through urine and intestinal gases, such as methane (CH4). This equals metabolizable energy (ME). A respiration chamber and analysis of urine are needed to measure ME. Energy losses (% of GE) for urinary energy in maintenance and working horses is found to range between 3.8- 5.2%, and for methane energy its range from 1.9-2.2 (Kienzle & Zeyner, 2010). When extracting for energy loss in heat increment (energy used in digestion processes and absorbing of nutrients) the NE is conducted. Net energy is the feeds most correct energy value that the horse can utilize for energy required for body maintenance, or to additional training, growth or foetus- or milk production. This is complicated to measure because heat production is challenging to measure. It can be done by the TCM or with respirometry methods. Net energy of a feed can be calculated from its ME content where efficiency of ME utilization for maintenance (Km) can be calculated as: NE(MJ/kg)= 0.85EGL + 0.80 ELCFA + 0.70EAA + (0.063 to 0.68)EVFA * ME (MJ/kg), where EGL, ELCFA, EAA and EVFA represents energy absorbed as either glucose (EGL), long-chain fatty acids (ELCFA), amino acids (EAA) and volatile fatty acid (EVFA) (McDonald et al., 2011).

2.3 Digestion of forage in the equine gastro-intestinal tract

The equid digestive system is unique among monogastric animals. Characterized by its ability to directly utilize simple nutrients as well as more complex nutrients with the help of its hindgut microbiota. The entire equine gastro-intestinal tract (figure 11) of an adult horse compasses a total length of over 30 meters and makes up a volume of 150 litres (Ericsson et al., 2016). Enzymatic digestion of CHO-H occurs mainly in the equine foregut, while fermentation of CHO-F_{s+r} predominantly occurs in the hindgut, but may appear in other segments of the GIT where microorganisms has established (Hoffman, 2009).

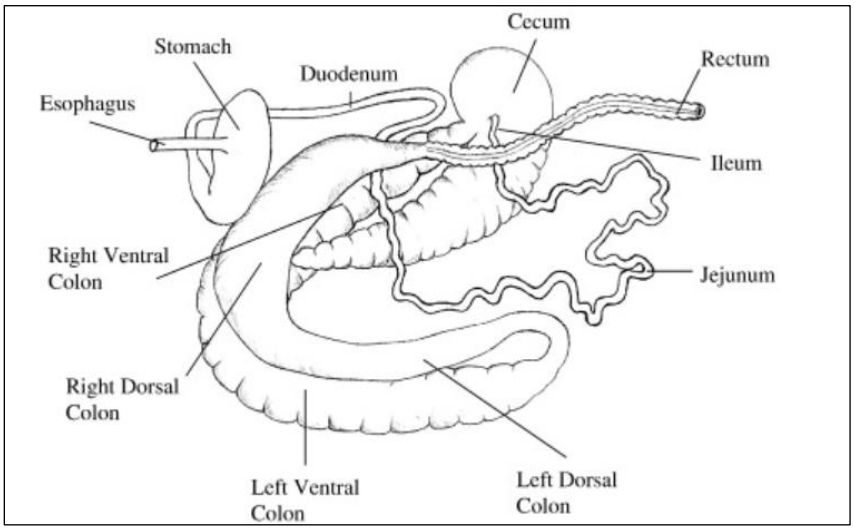


Figure 11. The gastro-intestinal tract of an adult horse, from Van Weyenberg et al. (2006).

2.3.1 Pre-cecal enzymatic digestion

Oral cavity

The digestion of forages starts with oral cavity by mastication to finely grounded particles. Mastication leads to secretion of 35-40 litres saliva daily (Meritt, 2013). More than 99% of the horse saliva is water, whereas the rest of it are minerals, bicarbonate, and trace amounts of the digestive enzyme amylase. The minerals and bicarbonate from saliva act as an important buffer to neutralize the pH in the stomach. The mastication reduces particle size and increase surface area of the feed which facilitates improved digestion further in the GIT. Rate of saliva secretion are depended on DM intake and chewing duration. Feeding with one kg hay or wrap generates 3500 chewing motions and 10-12 litres of saliva (Luthersson, 2013). Comparingly, one kg grain or concentrates generates only 800-1200 chewing motions and three litres of salvia (Luthersson, 2013).

Stomach

The equine stomach constitutes only eight percent of the total gastrointestinal tract (Meritt, 2013)

which means it holds restricted amounts of feed. A small stomach results in that large meals (over 2-2.5 kg feed) may pass nearly undigested further in the gastro-intestinal tract (GIT), and hence reduce digestion, may subsequently lead to digestion disorders (Luthersson, 2013). The equine stomach is divided into two regions: the non-glandular (also termed the squamous region) and the glandular region separated by the area termed margo plicatus. The pH in the stomach vary; in the upper, non-glandular area, pH is between five to seven, and in the glandular region, lower area, the pH is low, between two to three (Husted et al., 2008). In grazing horses, gastric pH is normally above 4.0 throughout the day (Geor & Harris, 2007). In the glandular region, hydrochloric acid (HCl) is the major secretory product (Meritt, 2013). Digesta retains in the stomach for a short time (2-6h) (Frape, 2008; Van Weyenberg et al., 2006), but the stomach is rarely completely empty. The main function of the stomach is to initiate mixing and degradation of feed, destroying bacterial pathogens and function as a temporary storage for digesta. Protein degradation starts in the stomach, where protein is degraded to amino acids of pepsinogen. Degree of protein degradation in the stomach is low in horses fed a hay-based diet with NH_3 concentrations at 0.17-0.23 mmol/l, whereas horses fed starch-based concentrates resulted in a tenfold higher concentration of NH_3 (Meritt, 2013).

When horses ingest forage, it provides a fiber-mat on top of the gastric content in the squamous region, hence make a neutral pH for microbes to establish. However, only small, unimportant amounts of cellulolytic bacteria is observed in the stomach, and the plant cell wall degradation in the stomach may be nonessential (Meritt, 2013).

Small intestine

The small intestine is divided into three parts: duodenum, jejunum and ileum which in total compromises 21-25m length and have a short (approximately 3h) passage time (Van Weyenberg et al., 2006). In the duodenum, jejunum and ileum the pH is 6.32, 7.10 and 7.47, respectively(Mackie & Wilkins, 1988). The small intestine provides mainly enzymatic degradation and is the primary site of digestion and absorption for most nutrients, except fiber. The most studied digestive enzymes in the small intestine of the horse are amylase and trypsinogen for degradation of starch and protein, respectively, is found to be low compared to other animals (Meritt, 2013). As a result of equines having reduced capacity of absorbing glucose, low amylase activity and rapid passage in the small intestine, large meals with CHO-H may pass undigested to the hindgut. In general, pre-caecal DM digestion of hay is low, and found to be 32% when fed hay cubes (Silva et al., 2009), 40% when fed timothy hay first cut (Thorringer et al., 2022), and more precisely, the amylase corrected NDF (aNDF) degradation of hay is 20% (Thorringer et al., 2022). Degradation of proteins continues in the small intestine by the action of peptidases into amino acids and small peptides. Free amino acids are absorbed in the small intestine, while undigested proteins pass further into the hindgut.

α-amylase, α-glucosidases (sucrase, glucoamylase, maltase) and ß-galactosidases (lactase) are the main groups of enzymes that breaks down CHO-H in the small intestine. Amylase cleaves α-1,4 linkages, and amylopectinase cleaves α-1,6 linkages in starches to disaccharides and oligosaccharides. Further will other enzymes like sucrase and maltase secreted from intestinal mucosa cleave the last linkages to free glucose units, which are further absorbed to the bloodstream. Activity of amylase is both variable (increases when horses are fed grain diets) and low compared to other species (Meritt, 2013). Pre-cecal digestibility of NSC is found to be high (88-89%) (Varloud et al., 2004).

The degradation of fructans in the equine GIT is not fully known. The ß-2,6 bonds in fructans cannot be degraded by enzymes, but apparently of intestinal microbes, both in small intestine and in hindgut, in different rates. Ince et al. (2014) found that grass-fructan extract was partially degraded into oligomeric fructans by in vitro method with equine gastric or small intestinal digesta. Strauch et al. (2017) did also observe pre-cecal fructose digestbility by in vitro method, where the highest loss of fructans occurred at a lower pH and mentions that the plant fructose hydrolase enzyme can be contributing to the degradation of fructans.

2.3.2 Fermentation in the hindgut

The hindgut constitutes about 75% of the total gastrointestinal tract and can be separated into cecum, the left and right ventral colon, the left and right dorsal colon, small colon and rectum. The cecum is a large blind sac located at the distal end of ileum and have a capacity to hold average 33litres whereas the great colon averages 80 litres (Meritt, 2013). Digestion in hind gut relies almost entirely on the microbial activity. Hind gut transit time of hay (timothy, first cut) is reported to be 32.3 hours (Thorringer et al., 2022). The pH in the cecum and colon is 6.70 and 6.67 (Mackie & Wilkins, 1988), respectively, but may decrease to 6.0 when fed 3-4g/kg BW barley or corn (Willard et al., 1977).

About 75-85% of plant cell wall material are digested in the hindgut, making the hindgut as primary site for degradation of forages. Fermentation results in the end products VFA (also termed short chain fatty acids (SCFA)). Fluctuations in pH and VFA concentration are substrate dependent (table 7). The main VFA end products from fermenting of forage are acetate, propionate, and butyrate (figure 12). Lactate, isobutyrate, valerate and isovalerate are also VFA produced, but to a lesser extent. Volatile fatty acids together with water and minerals are absorbed across the hind gut wall.

The microbes produce enzymes to hydrolyse plant cell wall material. Degradation of forage in hind gut can be separated in two steps; 1) Bacteria and fungi attach to plant cell walls and releases

enzymes to start hydrolysis of the complex polysaccharides to simple sugars (glucose, cellobiose and xylose) which led to formation of pyruvate and 2) fermentation of the yielded simple sugar from the hydrolysis to VFA. Fermentation also produces gases such as CO_2, H_2 and CH_4. There are several microorganisms in the hindgut that are essential for degradation of cell wall carbohydrates: fungi, cilica protozoa and bacteria. Around 72 protozoa species are found from the genera of *Buetschlia, Cecyloposthium, Blepharocorys* and *Paraisotricha.* The bacteria active in digestion of feed can be separated into fibrolytic microorganism which degrade plant cell wall, amylolytic bacteria that degrade starch, and proteolytic microorganisms that degrade protein. The major fibrolytic microorganism are bacterial species of the *Ruminococcus* and *Fibrobacter* genera and the fungal species of *Piromyces* genus (Julliand & Grimm, 2017).

Miyaji et al. (2008a) found that VFA concentration did increase rapidly from cecum to the right and ventral colon, then constant until right dorsal colon and decreased against the small colon. Digestibility of DM, OM and NDF from forage did follow the same pattern (Miyaji et al., 2008a).

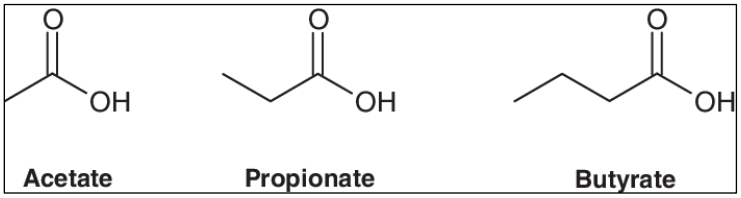


Figure 12. Chemical structure of the three most important volatile fatty acids (VFA), Acetate, Propionate and Butyrate from equine hindgut fermentation of forages. From Darzi et al. (2011).

Starch and WSC will normally be degraded in the small intestine but may also be subject to fermentation in the hindgut. This may induce lowered pH and altered microbiota in the hindgut. However, this is normally not a problem in a forage only diet The main amylolytic bacteria that degrade starch and WSC in hindgut belongs to the *Streptococcus* and *Lactobacillus* genera (Julliand & Grimm, 2017). The main products from fermentation of starch or WSC is lactate. Comparingly, degradation of plant cell wall materials will yield only small, or zero amounts lactate (table 7). Lactate is a substrate for the lactate-utilizing bacteria, that produces mainly propionate (Julliand & Grimm, 2017). Hindgut fermentation of fructans seems to be readily fermented. Ryegrass with high content of fructans provided more rapidly fermentation with high concentrations of lactate, compared to ryegrass with low content of fructans (Ince et al., 2014).

Table 7. Effect of diet on pH, VFA production and on microbial growth in cecum and ventral colon of the horse 7 hours after a meal, from Frape (2008)

		VFA (mmol/L)				
Diet	**pH**	**Acetate**	**Propionate**	**Butyrate**	**Lactate**	**Total bacteria per mL*10-7**
Hay	6.90	43.0	10.0	3.0	1.0	500
Concentrate and minimal hay	6.25	54.0	15.0	5.0	21.0	800
Fasted	7.15	10.0	1.0	0.5	0.1	5

VFA= volatile fatty acids

Proteins that reach the hindgut, are little or not utilized by the host animal. The microbial growth is dependent on available N sources such as dietary protein or urea. Proteases generated from the microbiota cleaves proteins into smaller peptides and free amino acids, where microbial protein can either be used for microbial protein synthesis or degraded further to carbon skeleton and ammonia.

2.4 Methods to estimate digestibility

Digestibility can be measured with different techniques and can be separated into three main methods: in vivo-, in sacco- and in vitro methods. Independent of the method used, it is essential to compare them with in vivo results for critical evaluation and validation of the accuracy of the method (Goldman et al., 1987; McDonald et al., 2011). This chapter will describe these techniques and how these are used in experiments with horses.

2.4.1 In vivo Methods

In vivo methods refer to methods performed on a living animal. This can be approached with the total collection method (TCM), marker method, or with the mobile nylon bag technique (MBT). The gold standard is the TCM. The first and most important loss of nutrients are those excreted in faeces, and therefore the TCM is established as the reference method to estimate digestibility. In TCM, feeds are analysed and given to the animal in known amounts and faecal output is analysed and measured. This method requires at least seven days of introduction of feeds (Cichorska et al., 2014) before the period of collection which may consist of four to six days. In the period of collection, metabolism stalls can be used, or horses get equipped with a harness for total collection of excreta (Brøkner et al., 2012a; Goachet et al., 2009; Goldman et al., 1987). The harness is normally emptied three to four times daily (Goldman et al., 1987; Ragnarsson & Lindberg, 2010). The general formula for calculation of apparent digestibility is shown in equitation 1 (McDonald et al., 2011).

$$Apparent\ digestibility = \frac{nutrient\ consumed - \ nutrient\ in\ feces}{nutrient\ consumed} \quad (1)$$

Partial total collection methods have evolved since this total collection method as mentioned earlier are time consuming, leads to a need for alternative methods. Partial collection methods involves taking a few periodic spot samples of faeces, either from the ground or by rectal sampling, based on the marker method (Goachet et al., 2009). The marker method relies on a known concentration of marker that is either naturally present in the diet or administrated to the animal as an external marker. To measure digestibility this way, the marker concentrations and chemical content in feed and faeces must be known. The change in ratio of each nutrient with reference to the marker in feed and faeces are used to estimate the digestibility. Unfortunately, in vivo experiments are time consuming, laborious with low repeatability (Goldman et al., 1987) and ability to precisely maintain experimental conditions (Getachew et al., 1998) compared to in vitro methods. In addition, they are often impractical, especially with sport horses (Cichorska et al., 2014). It may also interrupt animals' normal routines and behaviour (Sales & Janssens, 2003).

2.4.2 In vitro methods

In vitro means "in glass" and these methods refers to trials done outside the living animal, trying to simulate the in vivo occasion. The Daisy incubation (DI) and gas production (GP) are examples of in vitro methods. The methods are in general based on incubating feed in inoculum from the animal with a buffer solution. The buffer solution should ensure the correct pH and access to ammonia to ensure proper environment for the microbes. These methods are widely used to study digestibility of feed stuffs in ruminants, and to a lesser extent in horses.

Daisy Incubation

The principal of the DI method is to incubate feed samples with inoculum and buffer in a metal cabinet with rotating digestion jars in a defined temperature (39°C) (figure 13).

Figure 13. The ANKOM Daisy incubator with four digestion jars. From (ANKOM, n.d -b)

The inoculum can be of microbial source such as rumen- or cecum fluid, faeces, or enzymes. Small amounts (0.25-0.50g) (Lattimer et al., 2007) of feed are placed into filter bags and sealed. Before incubation, the digestion jars are merged with CO_2 to simulate the anaerobic environment from the digestive tract. The digestibility coefficient is calculated based on the weight of bags, where the fraction that has disappeared is assumed to be digestible DM. Incubation time can vary, and it should reflect transit time in the gut of the horse. Earing et al. (2010) used 30, 48 and 72 hours of incubation. An example of digestibility results from Daisy incubation with different feeds for horses are shown in figure 14. The DI method has earlier provided valid estimates of in vivo digestibility in horses (Earing et al., 2010; Lattimer et al., 2007). However, studies also shows that DI results are slightly lower than in vivo digestibility (Earing et al., 2010; Tassone et al., 2020b). Equine faeces have been frequently used as a source of inoculum instead of cecum fluid (Earing et al., 2010) as this is an inexpensive and easily available alternative to the equine caecal fluid. In addition, there are few cannulated horses available.

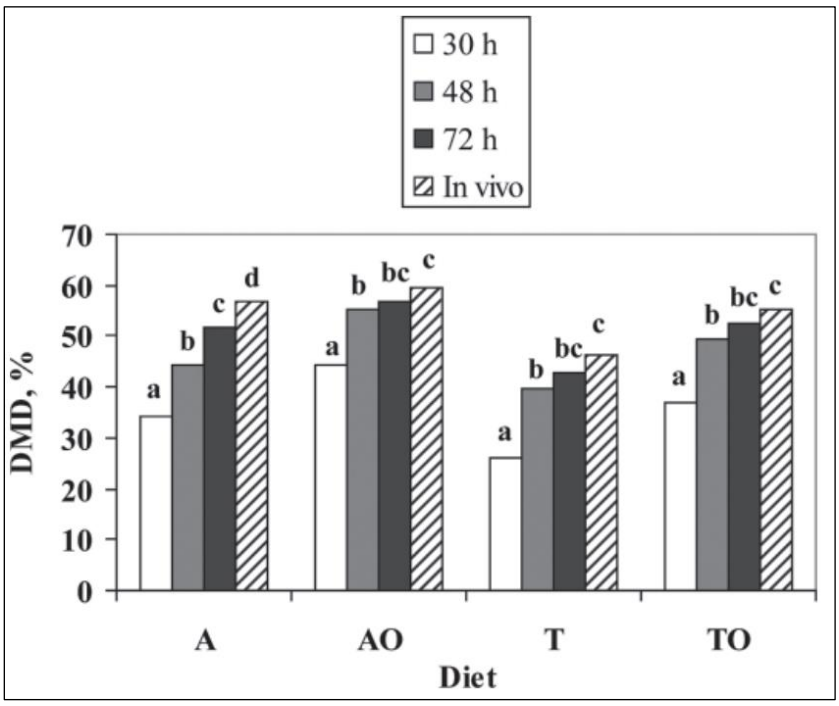


Figure 14. In vitro and in vivo estimates of DM digestibility (DMd %) for A: alfaalfa, AO: alfaalfa+oat, T: timothy, TO: timothy+oats. Estimates lacking common letters differ (P <0.05). From Earing et al. (2010)

The main sources of variation in the DI method are inoculum source, sample size, sample preparation and bag type (Tassone et al., 2020a). Additionally, there is a lack of standardised procedures for collection, storage and transportation of inoculum or faeces, and for the washing procedure of bags after digestion (Tassone et al., 2020a). An important advantage with the DI method is the possibility of having multiple samples in one container, making it an effective method to test several feed

samples at similar time. It is easier (less preparation), cost-saving and more labour saving than both IS and GP.

Gas Production

The GP method involves a system that measure gas production, which is proportional to DM degradability. Gas production is the result of fermentation of carbohydrates to acetate, propionate, and butyrate, which generates CO_2 and CH_4. However, gas is produced mainly when substrates are fermented to acetate and butyrate (Getachew et al., 1998). Feedstuff is incubated with inoculum and buffer solutions. There is reported good linear correlation between loss of NDF and GP in forages (Schofield & Pell, 1995). Figure 15 is an example of how a graph looks after use of the gas production technique, where different types of inoculums are used. For monogastric animals as the horse, faeces is a representative inoculum (Bauer et al., 2004). This may be not the case for ruminants, because the microbial population in faeces differs from the rumen fluid since faeces has been influenced by both gastric and cecum population. Source of inoculum is the largest cause of variations in gas production (Rymer et al., 2005), but other factors that also contribute to variation is donor animal effects, sampling time and source, as well as the sample preparation (Mould et al., 2005). Important aspects for a successful in vitro gas production is to achieve anaerobiosis, correct temperature and pH and a proper buffer solution (Getachew et al., 1998).

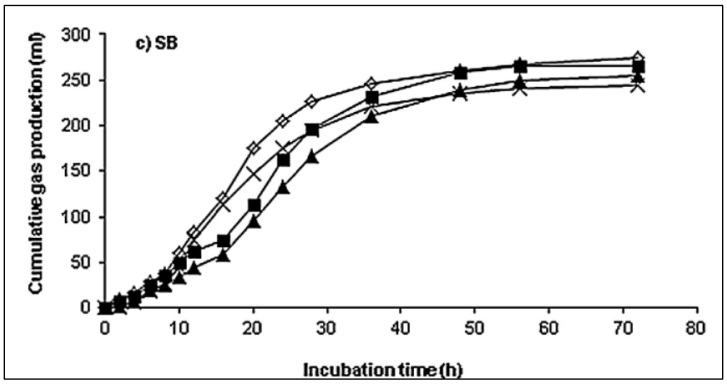


Figure 15. Example on a graph from gas production technique, cumulative values (ml/ g DM) for unmolassed sugar beet pulp (SB) incubated with inoculum from cecum (-Δ-), ventral colon (-▪-), dorsal colon (-x-) or feces (-◊-). From (Murray et al., 2014).

To describe and interpret the accumulative gas production, several mathematical models to describe and interpret the cumulative gas production profiles has been described (France et al., 2000). From the model of Groot et al., (1996); $G=A/1+(B^c/t^c)$, GP curves can be explained by three parameters, A, B and C. The A value represents total gas accumulation (higher gas production corresponds to higher digestibility, or specific high degree of fermentation), the B value explains timepoint for half of gas production, the C value is a constant that illustrates how the curve is switching, i.e., it's sharpness and t is time of incubation. These parameters will vary with inoculum source and substrate. Typically,

in early fermentation there is little or no fermentation before a steady increase where the CHO-F_R components are fermented. A slower gas production appears when microbes are fermenting the CHO-F_s components, until the profile approaches an upper asymptote (France et al., 2000; Groot et al., 1996) (figure 15).

2.4.3 In Sacco method

In sacco means "in bag" and are a part of in situ or the nylon bag techniques. The principle is to fill bags with feed and incubate them in the animal's digestive tract for a period of time to create a degradation profile. The method can be used as the fixed IS method, where bags are recovered the same place they were inserted, or as the mobile nylon bags technique, where the bags are inserted by a nasogastric tube into the stomach and collected either in the cecum (if cannulated animal) or faeces (Rosenfeld & Austbø, 2009). Cannulated animals are needed when performing the fixed IS method. Nylon bag techniques has been used to investigate digestive processes in the rumen of ruminants for years (Hyslop et al., 1999) and are the main routine method in NorFor to measure both potential degradable fraction and degradation rate of CP, NDF and starch (Åkerlind et al., 2011). In sacco methods are not widely used in horse nutrition, compared to ruminants, but effort is done to adapt these techniques for horse nutrition (Hyslop, 2006). The method has earlier showed high correlation with in vivo results in horses (Miraglia et al., 1988).

Typically, feed samples of three to five g DM are placed in permeable polyester, nylon or dacron bags with a pore size from 5-50 um. The recommended pore size is 30-50um (Nozière & Michalet-Doreau, 2000). The weave structure and pore size of the bag is of importance. The weave structure influences how the bag is affected by physical pressure under incubation, and the pore size affects how microorganism in the rumen or cecum can exchange through the bag. The pore size should be large enough to make microbes flow through easily, but also small enough to avoid loss of feed particles that are undegraded from the bag. Feeds used for the IS bags should be milled to achieve a homogenous sample and to compensate for the lack of mastication of the feed.

In sacco is used to determine the feed of interest in three parts, a soluble fraction (S), a potentially degradable fraction (Pd) and the rate of disappearance of the Pd fraction (Kd) (Åkerlind et al., 2011), with the model from Ørskov and McDonald (1979): $D= S+Pd\ (1-e^{-kd*t})$, where D is the degradation through incubation at time t. Example on a degradation curve from IS are presented in figure 16. The upper level in the graph is the fraction not digested, i.e., still left in the in bag after incubation. To estimate the actual value of indigestible NDF (iNDF), a long (288 hours) incubation time is needed (Jančík et al.). The mid-level in the graph illustrates the fraction that is degraded over time (Pd),

according to first-order kinetics. The lower level illustrates the fraction that is degraded instantly, i.e., the soluble fraction (S), which is determined by bags washed in the washing machine. Variation to consider are factors due to sample and bag, rumen or cecum environment or exchange through the IS bag and the rumen or cecum.

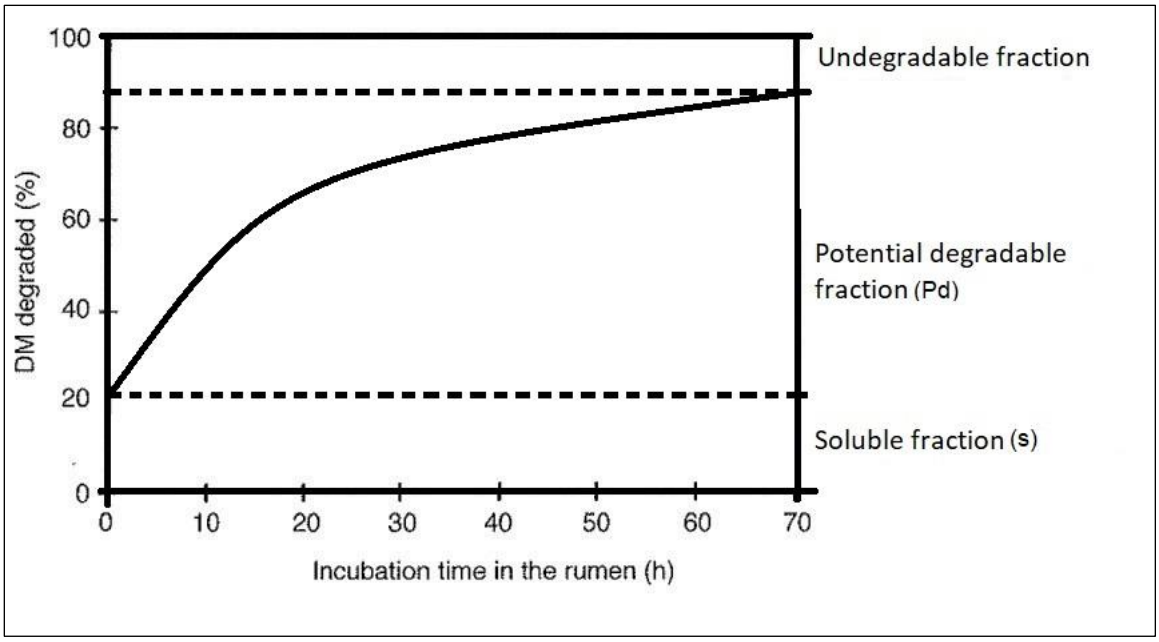


Figure 16. In sacco degradation of forage DM in the rumen, adjustment to first-order kinetics. Modified from Nozière and Michalet-Doreau (2000).

3 Materials and methods

3.1 Experimental design

The experimental design is presented in figure 17. Harvesting of grasses were conducted summer (may-july) 2021 at Vollebekk, Ås from three fields at eight harvesttimes, results in total 24 grass samples. Chemical and NIRS evaluation were performed on all samples, but only samples from field number "26" were used for digestibility trials; ANKOM Daisy^{II} incubation, ANKOM RF Gas production system and in sacco. Digestibility experiments were conducted at the metabolism unit at the Norwegian University of Life Sciences (NMBU) in December (2021) and January (2022).

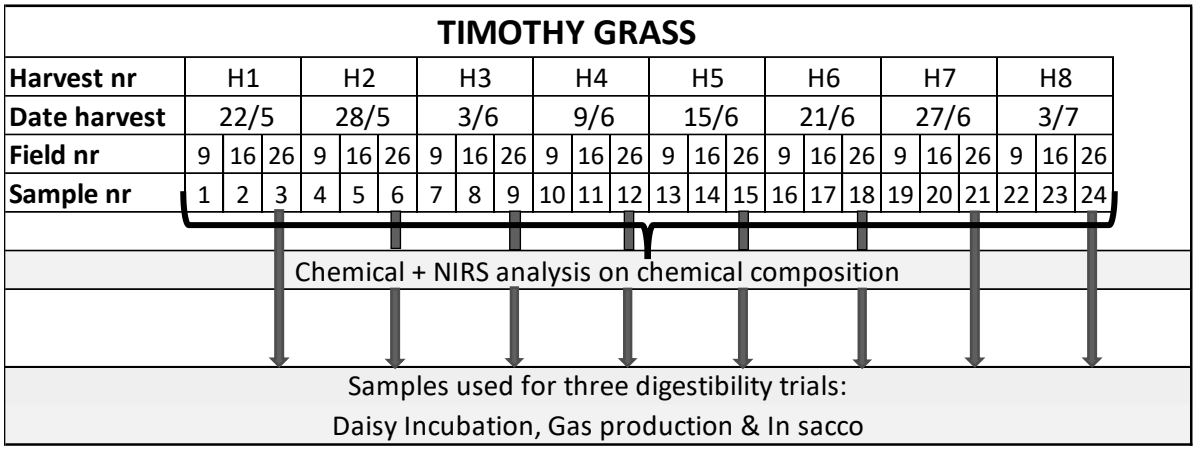


Figure 17. Illustration of the experimental design. Timothy grass was harvested every sixth day during 22/5-3/7 the summer 2021, from three fields yielding three replicates from eight harvest times, resulting in total 24 grass samples. Chemical compositions were analyzed by chemical evaluation and NIRS analysis on all samples. The cuts from field nr 26 from each harvest time was used for the digestibility experiments: Daisy^{II} incubation, gas production and in sacco.

3.2 Animals

Three cecum-cannulated 16-28 years old Norwegian coldblooded trotters were used as experimental animals. Average bodyweight was 558 ± 27 kg. The horses were fed three meals per day at 6.20, 14.00 and 19.00. Two of the horses were fed 7.5 kg hay and one 8 kg hay in addition to 100g of pelleted vitamins and minerals (Champion Multitilskudd pellets, Felleskjøpet, Lillestrøm, Norway). Horses had ad libitum access to salt lick stones and water from automatic drinking throes. The horses were stabled at individual 3x3 m stalls with wood shavings as bedding material and were outside in a group paddock for 9-11 hours through the day, divided into two visits. Meals were offered inside in the stall, as a normal routine at the stall. During procedure with IS bags and extracting cecum inoculum for DI and GP, horses were restrained inside the stall, with one person holding the horse and the other doing the experiments. The horses' normal routines and stabling were minimally interrupted during the study. Horses was fitted with a permanent cannula (length 15cm) at the base of the cecum, close to the ileocecal junction. All housing, management and experimental procedures

followed the laws and regulations for experimental animals in Norway (Norwegian Government, 2015).

3.3 Harvesting of grasses

An overview of the experimental field is shown in figure 18. Grasses was harvested from three different fields (field number 9, 16 and 26) at Norwegian University of Life Sciences, Ås, Norway every sixth day during 22.may to 3. July summer 2021. The plots were fertilized with 30 and 100 kg N/ha in the autumn 2020 and spring 2021, respectively. Grasses were cut in the late afternoon (17.00 pm) manually with scissors. Plant biomass harvested from each cut and field was then oven-dried at 45°C for 48 hours for DM determination. Further, feeds were milled and stored in sealed plastic bags until experiments and analysis.

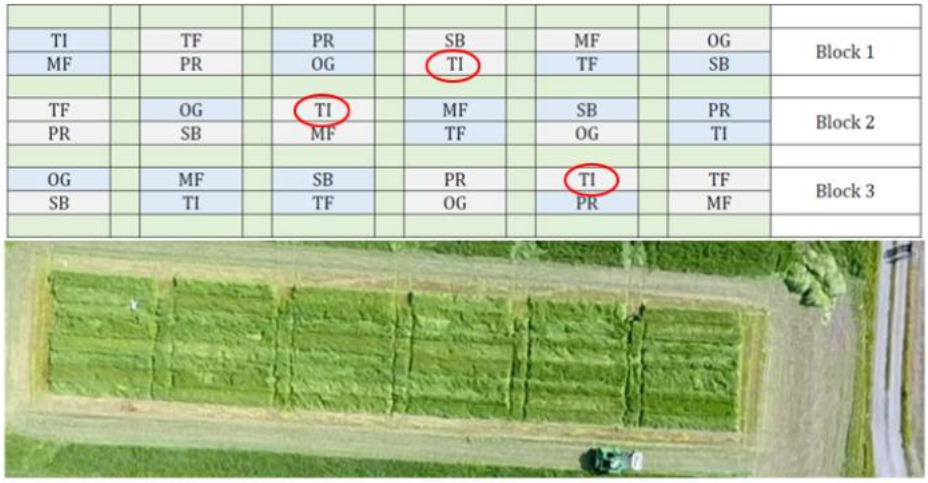


Figure 18. A schematic illustration and photo of the experimental field June 2019 before the early 1st cut.

3.4 Experimental feed

The experimental feed consisted of 100% timothy grass. An internal control feed (silage) was included in all digestibility studies. Chemical and sugar composition are presented in table 9 and 10 and in figure 23.

3.5 Chemical analysis and preparation

Feeds got milled to pass a 1 mm screen, and dried in a heat cabinet at 45°C for 48 hours before analysis and digestibility experiments. Analysis was performed in duplicates. Chemical analysis was conducted at NMBU, Labtek, Ås, Norway for DM, CP, NDFom, ADFom, ADLom and ash. Dry matter was determined by drying at 103°C to constant weight, as described in Berg (2011b). Ash was determined by combustion at 550C for 16 hours (Berg, 2011c). Crude protein were analysed by the Kjeldahl-N method as described in (Berg, 2011a) and calculated as N*6.26. The NDF, ADF and ADL

were analysed by the Van Soest detergent methods as described in Berg (2013), Berg (2012) and (Johnsen, 2018). Water-soluble carbohydrates (glucose, sucrose, fructose, fructans and total WSC) were analysed at SLU, Uppsala, Sweden, with a colorimetric method as described in Larsson and Bengtsson (1983). Near infrared spectrometry analysis were conducted at Eurofins Agro Testing Sweden AB (Kristiansand) on DM, CP, ESC, fructans, NDF, CF, fat, ash, in vitro digestibility of organic matter (IVOS), digestibility and Cl. Metabolizable energy (MJ/kg DM) and DCP (g/kg DM) were calculated.

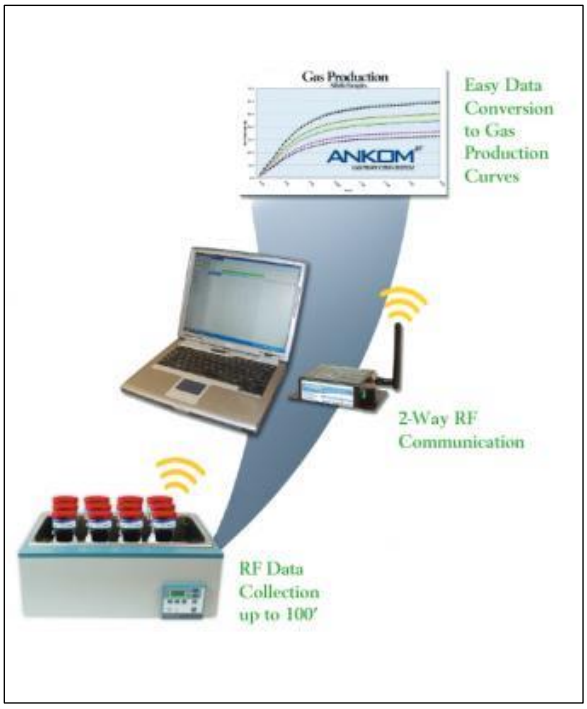


Figure 19. The gas production system. From ANKOM (n.d - a)

3.6 Gas Production method

This technique was conducted using the ANKOM RF Gas Production System (Version 9.8.3, ANKOM technology, Macedon, NY, USA) (figure 19). This system consists of bottles (250 ml) with head units containing pressure sensor modules. Before filling bottles with feed, buffer and inoculum, the bottles were flushed as a standard procedure before incubation to ensure the system is working. One gram feed sample with three replicates from each harvest time were weighed and put into bottles covered with parafilm. Three replicates of one gram control feed and three blank bottles were also included. The blank bottles contained only buffer solution and inoculum, needed for correcting for the gas produced by inoculum. In total 30 bottles were used. To make sure the feed had the right temperature before adding inoculum and buffer, they were kept in a heat cabinet at 39°C before the experiment started. Five solutions were made for the GP experiment according to Goering and Van Soest (1970). Figure 20 illustrates the quantity of chemicals used for the solutions. All solutions were stirred and mixed properly by use of a magnet in the solution placed on a magnetic stirrer. Solutions were made two days prior incubation, but solution number five were made the same day as incubation. Eventually, solution number one to four were mixed into a five litres bottle, placed into a 39°C water bath for two hours. In addition, the solution got flushed with CO_2 for two hours, to remove the oxygen. This step is to achieve an anaerobic environment, for simulating the environment of the hindgut. The mineral mixes are also for the growth of microbes. Solution number 5 (reducing solution) were added at the end. After 20 minutes the colour changed from pink to blank, which indicate the content is anaerobic. The final buffer solutions gave a total volume 2700 ml.

(1) Macromineral solution	g or ml
$Na_2HPO_412H_2O$	9.707
KH_2PO_4	4.19
$MGSO_47H2O$	0.41
Distilled water	675.0

(2) Buffer solution	g or ml
NH_4HCO_3	2.700
$NAHCO_3$	23.625
Distilled water	675.0

(3) Micromineral solution	g or ml
$CaCl_22H_2O$	6.600
$MnCl_24H_2O$	5.000
$CoCl_26H_2O$	0.500
$FeCl_36H_2O$	4.000
Distilled water	50.0

(4) Resazurin solution	g or ml
Resazurin	0.025
Distilled water	25.0

(5) Reducing solution	g or ml
Cysteine hydrochloride	1.145
1N N NaOH	7.329
Na_2S3H_2O	0.630
Distilled water	183.0

Figure 20. Composition of the Goering & Van Soest (1970) buffer solutions used for the ANKOM RF Gas Production experiment.

The inoculum was taken from the three cecum cannulated horses immediately prior to the trial, approximately five hours after morning feeding. Approximately 0.5 litres caecal fluid were extracted from each horse, where in total 1400mL caecal fluid was used for the trial. Inoculum was collected via the canula, using a thin plastic hose connected to a pump to extract out the inoculum into a pre-warmed thermos, to create correct temperature for the microbes. Caecal fluid got filtered through a nylon cloth (SEFAR NITEX, Sefar AG, Heiden, Switzerland) with pore size 200µm before it was poured in a two litres bottle and placed in the water tube at 39°C. To easily get the correct doses from buffer solution and caecal fluid into the bottles, they were equipped with a dispenser. Immediately before filling the bottles with inoculum and buffer solution, the batteries were connected into each individual head unit. Eventually, 66 ml of buffer solution and 33 ml of cecum inoculum were placed in all bottles, flushed with CO_2 in proximately 10-15 seconds before the pressure sensor head went on. Three people helped under this sequence, to make sure it was done as fast as possible and to avoid drop in temperature. When all bottles were filled up, they were placed in a heat cabinet at 39°C on a slowly moving-gyro rocker (Cole-Palmer Ltd, Staffordshire, UK), to stimulate caecal mixing. The system was set to record and update the computer every 10 minutes, and to release pressure when over 0.75 psi. The pressure sensor modules measured the gas production during incubation as pressure (psi), which was recorded on a computer automatically when the system is running.

Immediately after 48 hours incubation, bottles were taken out of the incubator and pH was measured. Thereafter, bottle content was put into nylon bags with pore size of 12µm closed with rubber bands and soaked in cold water to stop the fermentation process. Distilled water was used to rinse the bottle content. Eventually, bags were washed in the washing machine at a cold wool

program without centrifugation. After washing, bags were placed into a heat cabinet at 45°C to dry for 48 hours. After, bags were weighed directly, as well as after 24 hours equilibration on the bench.

3.7 In Sacco method

Figure 21. In sacco bags.

One gram of feed with three replicates for each harvest time and incubation time were inserted to nylon bags with size 6x15cm and pore size of 36µm (figure 21). In total there was made 120 bags for incubation in horses. Additional one gram of feed with 4 replicates of each forage, in total 32 "zero-bags" were made for determining particle loss at zero hours. All bags were marked with numbers. For every incubation period, four bags at the time got attached to a 78 cm long string, by using rubber bands. For inserting of bags, a thin plastic tube was used. The end of the string (approximately 10cm) where hanging outside of the canula plug for recovery of the bags after incubation. Bags got incubated in the cecum for 0, 2, 4, 8, 16 and 48 hours. The horses' cannula was fitted with a rubber plug, connected to a 20 long tube. Immediately after incubating, bags were rinsed in cold tap water and washed in washing machine at cold wool program without centrifugation, to stop fermentation. After washing, the bags got placed in a heat cabinet at 39°C to dry at 48 hours. After, bags were treated in same way as explained for the GP method. Finally, bags with the similar feed and incubation time got pooled into a new set of jars.

Figure 22. The Ankom F57 filter bags with feed

3.8 Daisy^{II} Incubator

This experiment was conducted using a ANKOM Daisy^{II} Incubator (ANKOM Technology, Macedon, New York, USA). The F57 filter bag (ANKOM Technology, Macedon, New York, USA) used had an 25µm pore size and was 50mm long, 50mm wide at the open top and 30mm wide at the bottom (figure 22). The bags were pre-rinsed in acetone for five minutes and then air-dried before they were filled with feed. For each test feed there was made eight replicates and eight controls with one gram feed, in addition to four blank bags. Two of each feed sample were put into each digestion jar. The bags were closed by heat sealing. In each digestion jar there was placed 19 filter bags (16 with experimental feed, 2 controls and 1 blank). The inoculum was collected and filtered using the same procedure described for the GP method.

Inoculum was purged with CO2 for 30 seconds. Digestion jars were placed in the preheated incubator for 20 min before each jar was filled with 1600ml buffer solution and 400ml cecum inoculum, and then purged with CO2 for 30 seconds. Digestion jars was in the incubation chamber for four periods: 8, 16, 24 and 48 hours. The quantity of chemicals used in the buffer solutions is shown in table 8a and b. Buffer solutions were made the same day as the incubation started. After mixing the buffer solutions, they were stirred and placed in a water bath of 39°C. Final pH was measured to 6.8. After each jar finished incubation, the filter bags were placed in a net, closed with rubber bands and thereafter washed, weighed, and dried in same procedure as the GP method.

Table 8a and b. Chemical composition for the buffer solution A and B for the Daisy method.

Buffer solution A	**g/L**
KH_2PO_4	55
$MgSO_4*7H_2O$	2.75
NaCl	2.75
$CaCl_2*2H_2O$	0.55
Urea	2.75
Distilled water	5.5 l

Buffer solution B	**g/L**
Na_2CO_3	16.5
Na_2S*3H_2O	0.65
Distilled water	1.1 l

3.9 Calculations and statistical analysis

Dry matter degradability for the IS (ISDMd) method was calculated by

$$In\ Sacco\ DM\ degradability\ (\%) = 100 - \frac{feed\ residue\ after\ incubation}{feed\ before\ incubation * DM} * 100 \quad (2)$$

Degradability values were used for DM degradation curves, that were fitted to the model according the Ørskov and McDonalds (1979) model, by using the solver function in Excel.

$$D = S + Pd * (1 - e^{-kd*t}) \quad (3)$$

Where D= degradation through incubation time t, S=soluble fraction of feed stuff and the curve interception, Pd= potential/slowly degradable fraction, Kd= degradation rate of Pd and t=incubation time. S, Pd and Kd are constants fitted by an iterative least square's procedure.

Gas production is expressed as cumulative pressure in psi, and to standardize the gas production, the gas is converted from psi into moles using the ideal gas law:

$$n = p\left(\frac{V}{RT}\right) \quad (4)$$

P= cumulative pressure in kilopascal (kPa), V=headspace volume in the bottle in litres (l), N= gas produced in moles, R= ideal gas constant (8,314472 l*kPa*K^{-1} *mol^{-1}) and T= temperature in kelvin (K).

To convert moles into mL, Avogadro's law was used:

$$Gas\ produced\ in\ mL = n * 22{,}4 * 1000 \quad (5)$$

mL gas produced was corrected for DM and expressed as mL gas per g DM. Gas production curves were fitted to the model by Groot et al. (1996) by use of NLIN procedure in SAS, to obtain values for A, B, and C.

$$G = A/(1 + \left(\frac{B^C}{t^C}\right)) \quad (6)$$

Where G= amount gas produced per g DM at time t after incubation, A= asymptotic gas production in mL per g DM, B= time after incubation at which half of the asymptotic amount of gas has been formed, C= constant determining the sharpness of the switching characteristics of the profile and t= incubation time in hours.

Dry matter degradability for the GP (GPDMd) method was calculated by

$$Gas\ Production\ DM\ degradability\ (\%) = \frac{I - O}{I} * 100 \quad (7)$$

Where I= amount of feed added to the bottle before incubation and O= amount of feed residue after incubation.

Dry matter degradability for DI (DIDMd) method was calculated by

$$Daisy\ Incubation\ DM\ degradability\ (\%) = 100 - (\frac{\left(W3 - (W1 * C1)\right) * 100}{W2 * DM}) \quad (8)$$

Where w1= Bag weight, W2= Feed + bag weight before incubation, W3= Bag + feed weight after incubation and drying and C1= Blank bag correction (dried oven weight/original bag weight).

Statistical analysis

Gas production parameters (A, B and C) and curves fitted to the model by Groot et al. (1996) were performed by use of NLIN procedure in SAS (version 9.4, SAS Institute Inc., Cary, North Carolina, USA), to obtain values for A, B, and C. To test if there were differences for digestibility and chemical

content between harvest times, a one-way ANOVA analysis was performed in RStudio (version 1.2.5033, R Studio Inc.). The model comprised the effect of harvest time (H1-H8). Differences between harvest time were tested for significance using the Tukey's tests, where effects were considered significant if $p<0.05$. The three digestibility methods were tested for correlation with the spearman correlation coefficient.

4 Results

4.1 Chemical composition

The chemical composition in the experimental feeds by chemical evaluation are presented in table 9, where harvest time did have an effect on chemical composition ($p<0.001$). Dry matter content increased from 17.4-39.6% in H1-H8. The NDFom content increased with later harvest time vary from 48.4-65.5% of DM, where the increase is largest in H1-H4, thereafter the NDFom content was stable. The ADFom content follows same pattern, increased in H1-H4, and no large differences in the late harvests. The lignin (ADLom) content is low (vary from 1.2-4.2% of DM) in H1-H8, however, the content is increasing by harvest time, and have almost a five-fold doubling in content. For ash and CP there was a linear decrease from H1-H8. The CP content did decrease (18.2-5.6% of DM) over two thirds from H8-H1. Ash content decreases from 7.5-4.8% of DM for H1-H8.

Table 9. Chemical composition by chemical evaluation. Values are presented as mean ± SD. Numbers are average from the three cuts from each field within each harvest time. Dry matter are in % and all other nutrients are in % of DM.

	DM	Ash	NDFom	ADFom	ADLom	CP*
H1	17.4 ± 0.4^{g}	7.5 ± 0.4^{a}	48.4 ± 0.4^{e}	23.6 ± 0.4^{e}	1.2 ± 0.0^{e}	18.1 ± 0.8^{a}
H2	18.8 ± 0.1^{f}	7.3 ± 0.3^{a}	52.3 ± 0.6^{d}	27.0 ± 0.7^{d}	1.4 ± 0.1^{e}	14.7 ± 0.5^{b}
H3	20.9 ± 0.1^{e}	6.8 ± 0.2^{ab}	60.0 ± 0.3^{c}	32.3 ± 0.3^{c}	2.0 ± 0.2^{d}	11.0 ± 0.3^{c}
H4	23.1 ± 0.3^{c}	6.3 ± 0.1^{bc}	64.7 ± 0.9^{ab}	36.3 ± 0.8^{ab}	2.7 ± 0.1^{c}	9.4 ± 0.1^{d}
H5	27.6 ± 0.5^{d}	5.8 ± 0.3^{cd}	64.1 ± 0.5^{ab}	36.4 ± 0.4^{ab}	3.6 ± 0.1^{b}	8.2 ± 0.5^{d}
H6	24.4 ± 1.0^{c}	5.3 ± 0.2^{de}	65.5 ± 0.2^{a}	37.5 ± 0.1^{a}	4.0 ± 0.1^{ab}	7.1 ± 0.1^{ed}
H7	32.2 ± 0.1^{b}	5.1 ± 0.3^{de}	63.5 ± 0.5^{b}	35.9 ± 0.3^{b}	3.8 ± 0.1^{b}	6.4 ± 0.3^{ef}
H8	39.6 ± 1.1^{a}	4.8 ± 0.4^{e}	63.6 ± 1.1^{b}	36.1 ± 1.0^{ab}	4.2 ± 0.3^{a}	5.6 ± 0.6^{f}
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a, b, c, d, e, f, g}$ Values within columns differ if superscripts are different ($p<0.05$)
*DM= Dry matter, NDFom= Neutral detergent fiber on organic matter basis (ash-corrected), ADFom= Acid detergent fiber on organic matter basis (ash-corrected), ADLom= Acid detergent lignin on organic matter basis (ash-corrected) and CP= Crude protein. *CP is calculated as N*6.25*

A complete table from the NIRS analysis are shown in Appendix 1. Results from the analysis by the NIRS method are presented in table 10, where harvest time did have an effect on all parameters ($p<0.001$). The same pattern of increase or decline in nutrients can be seen as in table 9. The digestibility parameters (IVOS) declined from 77.3-55.6% in H1-H8. Ash content also followed a declining pattern of 7.9-4.1% of DM but was highest in H2. Crude protein declined from 14.9-4.5% of DM in H1-H8. The NDF content increased from 38.4-56.8% of DM in H1-H8, however, H4-H8 were non-different. The ESC and fructan content declined from H1-H4 and thereafter increased in H4-H8, where the largest increase was observed for the fructan content. The ESC content was high (14.7%) in H1, declined to 7.8% in H4, followed by an increase to 10.2% of DM in H8, however, H5-H8 were non-different.

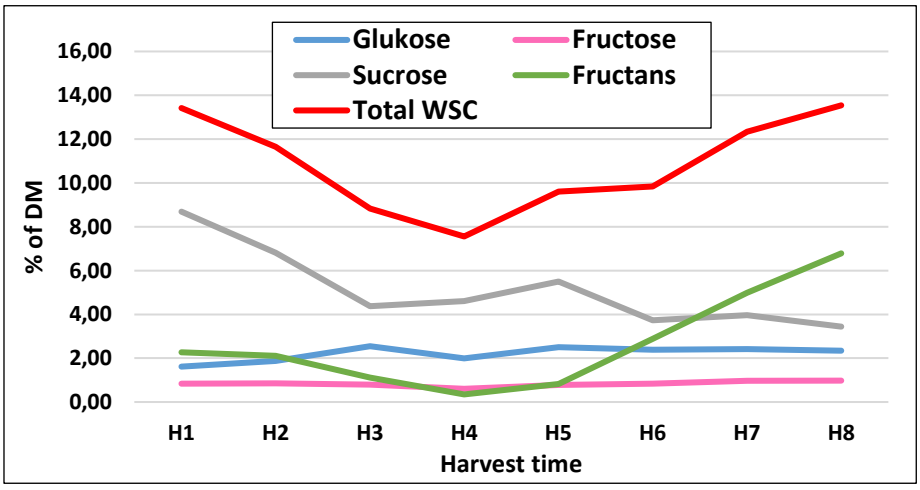
Table 10. Forage analysis by NIRS evaluation of the experimental feeds. Values are presented as mean ± SD. Numbers are average from the three cuts from each field within each harvest time. Nutrients are in % of DM and digestibility and IVOS are in %.

	Ash	NDF	CP	ESC	Fructans	IVOS
H1	7.6 ± 0.2^{a}	38.4 ± 0.6^{d}	15.0 ± 0.2^{a}	14.7 ± 0.8^{a}	3.5 ± 0.4^{c}	77.3 ± 0.6^{a}
H2	7.9 ± 0.4^{a}	42.2 ± 0.3^{c}	12.1 ± 0.4^{b}	12.8 ± 0.5^{b}	2.5 ± 0.2^{ce}	73.4 ± 0.6^{b}
H3	6.7 ± 0.1^{ab}	48.1 ± 1.0^{b}	9.3 ± 0.2^{c}	9.5 ± 0.4^{cd}	1.5 ± 0.2^{e}	67.3 ± 0.3^{c}
H4	6.2 ± 0.2^{bc}	54.3 ± 0.7^{a}	8.8 ± 0.2^{cd}	7.8 ± 0.7^{d}	1.3 ± 0.1^{e}	63.6 ± 1.9^{d}
H5	5.3 ± 0.4^{cd}	55.8 ± 0.8^{a}	7.8 ± 0.3^{de}	9.4 ± 0.4^{cd}	2.2 ± 0.2^{de}	61.8 ± 0.5^{de}
H6	4.9 ± 0.4^{de}	56.6 ± 1.3^{a}	6.8 ± 0.2^{e}	9.0 ± 0.3^{cd}	2.8 ± 0.1^{cd}	58.9 ± 1.0^{ef}
H7	5.0 ± 0.4^{de}	55.8 ± 1.5^{a}	5.7 ± 0.5^{f}	10.1 ± 0.5^{cd}	5.2 ± 0.8^{b}	57.8 ± 0.8^{fg}
H8	4.1 ± 0.3^{e}	56.9 ± 0.6^{a}	4.6 ± 0.3^{g}	10.2 ± 0.6^{cd}	6.6 ± 0.2^{a}	55.6 ± 0.8^{g}
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a, b, c, d, e, f}$ Values within columns differ if superscripts are different (p<0,05).
NDF= neutral detergent fibre, CP= crude protein, ESC= ethanol soluble carbohydrates and IVOS= In Vitro organic matter digestibility

Sugar contents are presented in figure 23. Comparing to table 10, the total WSC content shows similar pattern, a U-shape. Total WSC is high (13.4%) in H1, declined to its lowest content of 7.5% in H4, followed by an increase to its highest content at 13.5% of DM in H8. Fructan content was low (2.3-0.3%) in H1-H4 and increased to the highest content at 6.8% of DM in H8, being the major component of the total WSC content. Sucrose was high in H1 (8.7%) and decreases to 3.4% of DM in H8. The glucose and fructose content were low and had minor variation between harvest times vary from 1.6-2.5% and 0.6-1.0% of DM, respectively.

Figure 23. Illustration of sugar content in the experimental feeds. Numbers are average from the three cuts from three field within each harvest time. All nutrients are in % of DM.



WSC= water soluble carbohydrates

The DM yield for the different harvest times is presented in table 11, did vary from 261.1-1030.6g DM m^2 and was increasing by delayed harvest time.

Table 11. Dry matter yield (g DM m²) from the different harvest times. Numbers are presented as mean ± standard deviation from the three cuts from three fields within eight harvest dates.

	DM yield
H1	261.1 ± 20.9
H2	363.0 ± 15.6
H3	550.7 ± 23.9
H4	671.1 ± 63.5
H5	791.7 ± 43.3
H6	1030.6 ± 24.8
H7	931.9 ± 26.5
H8	1014.7 ± 59.5

The temperature and precipitation for the days before, and during the eight harvest times are presented in figure 24. Mean daily temperature were overall increasing during the period and varied from 8.5-21.9°C, with the lowest temperature (3.5°C) on 28th of May around harvest time one, and the highest temperature (29.2°C) on 3rd of July, around harvest time eight.

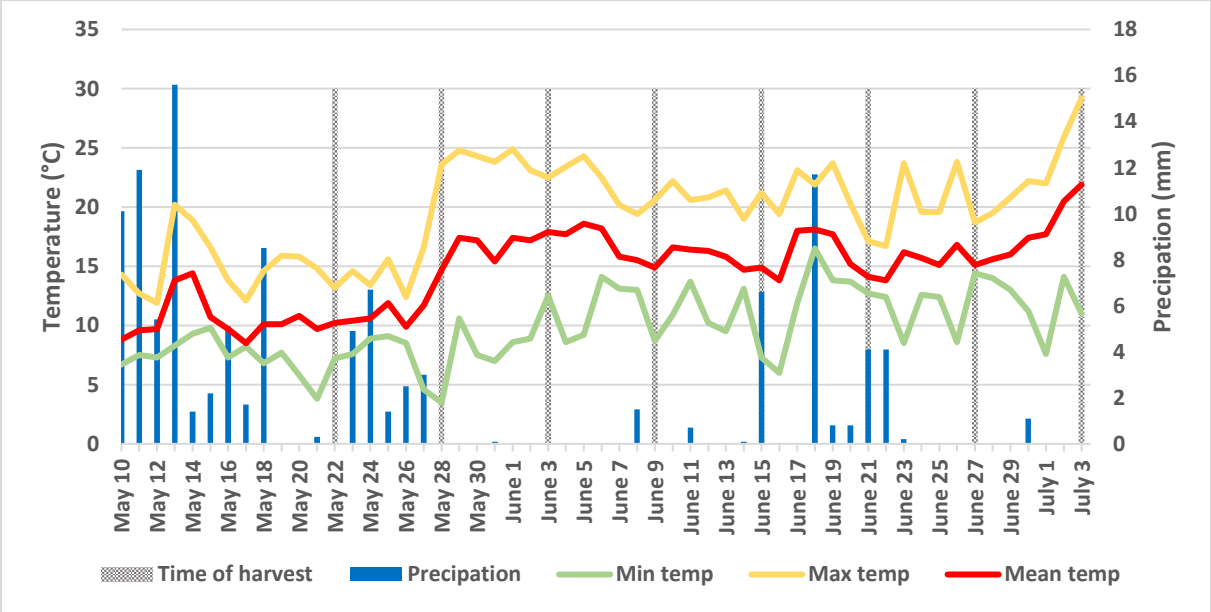


Figure 24. Maximum, minimum, and mean temperature and precipitation measured in Ås, Norway in 12 days before and throughout the period of harvest times. Modified from Yr.no (2021a, b, c)

4.2 In Sacco

All incubations of IS bags were completed successfully. The parameters from the IS experiment presented in table 12 are based on the degradation curves shown in figure 25. Harvest time had an effect on all parameters ($p<0.001$). Overall, the parameters in table 4.2.1 illustrates a declining linear pattern of all parameters, specifically for the earliest harvests (H1-H4). The S fraction was highest in H1 (42.7%), decreased to 36.1% in H3 and was non-different in H4-H8. The Pd fraction followed a clear declining pattern, vary from 49.5-23.3% in H1-H8. The Kd fraction was highest in H1 and H2

(15.6%/h and 12.9%/h respectively) and was non-different for H3-H8. In sacco DM degradability were highest in H1 and declined with delayed harvest time with 89.5-52.8% in H1-H8, with all being statically different from each other, except of H7, which was similar to H6 and H8.

Table 12. Parameters from the In Sacco experiment, from Ørskov & McDonald (1979). Numbers are presented as mean ± SD. S, Pd and ISDMd are in % and Kd are in %/h.

	S	Pd	Kd	IsDMd
H1	42.8 ± 0.3^{a}	49.5 ± 1.2^{a}	15.6 ± 0.018^{a}	89.5 ± 0.2^{a}
H2	40.8 ± 0.3^{b}	48.8 ± 0.6^{a}	12.9 ± 0.010^{ab}	86.3 ± 0.6^{b}
H3	36.1 ± 0.1^{c}	45.6 ± 1.3^{ab}	11.2 ± 0.007^{b}	77.6 ± 1.2^{c}
H4	31.8 ± 0.3^{d}	42.0 ± 1.5^{b}	9.5 ± 0.011^{b}	68.7 ± 1.3^{d}
H5	31.5 ± 0.5^{d}	35.2 ± 0.2^{c}	9.3 ± 0.005^{b}	62.9 ± 0.7^{e}
H6	31.0 ± 0.2^{d}	29.1 ± 1.8^{d}	10.6 ± 0.016^{b}	57.8 ± 0.6^{f}
H7	31.8 ± 0.5^{d}	25.8 ± 2.0^{de}	10.5 ± 0013^{b}	55.4 ± 0.5^{fg}
H8	31.8 ± 0.3^{d}	23.3 ± 0.7^{e}	9.7 ± 0.004^{b}	52.9 ± 0.1^{g}
P-value	<0.001	<0.001	<0.001	<0.001

$^{a, b, c, d, e, f, g}$ *Values within columns differ if superscripts are different (p<0.05).*
S= soluble fraction, Pd=potential degradable fraction, Kd= degradation rate of Pd, and ISDMd=In sacco dry matter degradability at 24hours of incubation, calculated after equitation 2.

The degradation curves for the IS method are presented in figure 25. The digestibility is linearly decreasing with postponed harvest time. For H1-H3, their starting point (S-fraction) was separated while the others have clustered in the starting point, illustrating a relative similar S-fraction as also can be seen in table 12.

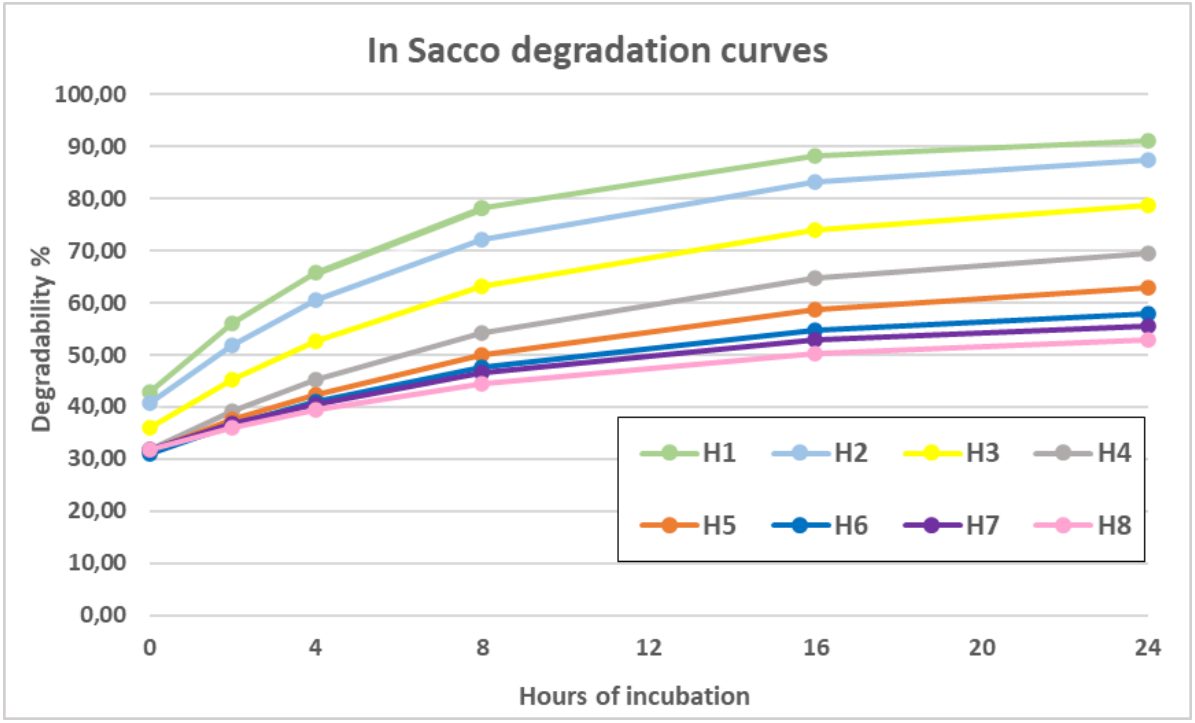


Figure 25. In sacco degradation curves, fitted from Ørskov & McDonald (1979).

4.3 Gas Production

Some results from GP were eliminated because battery voltage was too low to register all cumulative gas produced, which reduced replicates from three to two for H1, H2, H4 and H5. One mistake was detected for the one replicate of H8 under statical analysis, which also was removed from the data. The blank samples from GP were not used for correction, since they were decreasing, indicating no fermentation or gas production. The control samples behaved as normal, hence not included in the results.

The fitted gas production curves from the Groots model are presented in figure 26, where variable GP curves are seen as the effect of different harvest times. The cuts from H1-H3 clusters, as they all have a sharper curve and increased faster than the other cuts and may tend to flattening out when reaching 48 hours of incubation. The other cuts, H5-H8, clusters as they have a slower increase in gas production and seem to continue increasing when reaching 48 hours of incubation.

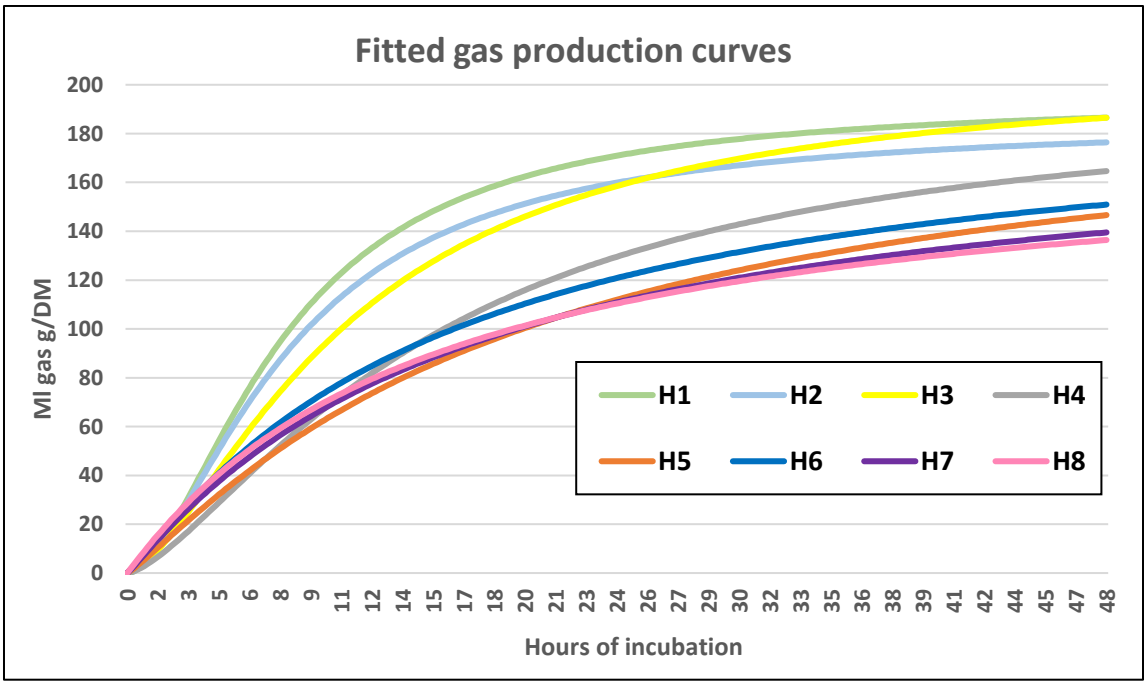


Figure 26. Fitted gas curves from the Groot model (Groot et al., 1996) from the gas production of the eight harvest times.

The corresponding parameters (A, B, C, time of maximal degradation rate (tRM)), pH and GPDMd from the GP experiment are presented in table 13. The asymptotic gas production (parameter A) ranged from 176.5-205.6 ml/g, with a trend of decreasing with delayed harvest time, where the lowest value was seen in the latest cut H8. However, the A parameter was non-significant. The half-time of gas production (parameter B) did increase from H1-H5, as it was fastest in H1-H3 with values from 7.7-10.9 hours, and the lowest value was observed for H5 (19 hours). From H6-H8 the B varies

from 14.6-16 hours. The constant for the switching characteristics for the curves (C) decreased with delayed harvest time. The pH varies from 6.37-6.48. The lowest pH (6.37) was observed in H2, and the highest pH (6.48) was observed in H7 and H8, which indicates a trend of increase in pH by delayed harvest time. The digestibility from the GP experiment decreased from 76.2-56.2% in H1-H8. The time of maximal degradation rate did vary from 0.4-8.7 hours, with the highest and lowest tRM in the early harvest and late harvests, respectively.

Table 13. In vitro gas production parameters (A, B, C and tRM), pH measurements and degradability from the gas production technique for the eight harvesttimes, presented as means ± standard deviation (SD).

	Nb	A	B	C	pH	GPDMd	tRM
H1	n=2	194.0 ± 6.1	7.7 ± 0.2^{c}	1.8 ± 0.1^{a}	6.42 ± 0.01^{abc}	76.2 ± 2.7^{ab}	6.6 ± 0.2^{a}
H2	n=2	185.0 ± 9.3	8.0 ± 0.3^{c}	1.7 ± 0.1^{ab}	6.37 ± 0.01^{c}	82.4 ± 1.2^{a}	6.4 ± 0.1^{a}
H3	n=3	205.6 ± 4.3	10.9 ± 0.2^{bc}	1.5 ± 0.0^{bc}	6.39 ± 0.01^{bc}	74.1 ± 6.1^{ac}	7.2 ± 0.1^{a}
H4	n=2	195.1 ± 2.5	15.0 ± 1.0^{ab}	1.5 ± 0.1^{c}	6.42 ± 0.03^{abc}	69.1 ± 0.5^{bc}	8.7 ± 0.7^{a}
H5	n=2	197.5 ± 0.5	19.0 ± 0.7^{a}	1.1 ± 0.0^{d}	6.43 ± 0.06^{ac}	61.9 ± 2.0^{cd}	3.4 ± 0.6^{b}
H6	n=3	193.5 ± 7.4	15.1 ± 2.2^{ab}	1.1 ± 0.0^{d}	6.47 ± 0.02^{ab}	53.4 ± 2.8^{d}	1.7 ± 0.7^{bc}
H7	n=3	182.6 ± 11.0	16.0 ± 0.9^{a}	1.1 ± 0.0^{d}	6.48 ± 0.01^{a}	57.7 ± 0.5^{d}	1.3 ± 0.6^{bc}
H8	n=2	176.5 ± 3.8	14.6 ± 1.2^{ab}	1.0 ± 0.0^{d}	6.48 ± 0.01^{a}	56.5 ± 0.9^{d}	0.4 ± 0.1^{c}
P-value		NS	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a, b, c, d}$ *Values within columns differ if superscripts are different (p <0.05).*
Nb= number of replicates, A= asymptotic gas production (mL gas /g DM), B= time of where half of A is produced (h), C= constant for switching characteristics of the curve, GPDMd= gas production dry matter degradability (%), tRM= time of maximal degradation rate (h) and NS= non-significant.

Gas production at 24 and 48 hours is presented in table 14. Gas production was affected by harvest time (p<0.001). For 24 and 48 hours, the GP did decrease from 171.0-106.7 and 186.6-129.7 ml gas g/DM for H1-H8, respectively.

Table 14. Gas production (ml gas g/DM) at 24 and 48 hours for the eight harvest times, presented as means ± standard deviation (SD).

	24h	48h
H1	171.0 ± 2.8^{a}	186.6 ± 4.7^{a}
H2	159.9 ± 10.9^{a}	176.4 ± 10.3^{a}
H3	158.5 ± 3.6^{a}	186.5 ± 4.0^{a}
H4	129.6 ± 4.1^{a}	164.6 ± 2.7^{ab}
H5	112.0 ± 2.8^{b}	146.6 ± 3.1^{bc}
H6	120.9 ± 5.1^{b}	150.9 ± 4.0^{bc}
H7	110.9 ± 4.9^{b}	139.5 ± 6.3^{c}
H8	106.7 ± 0.9^{b}	129.7 ± 0.5^{c}
P-value	<0.001	<0.001

$^{a, b, c}$ *Values within columns differ if superscripts are different (p <0.05).*

4.4. Daisy incubation

Incubation with the Daisy method was successful, but there was one blank value for the 8 hours which had an unexplainable error in weight, so the average of the other three blanks were used for the bags incubated for 8 hours.

Dry matter digestibility values from the Daisy Incubation (DIDMd) method are presented in figure 27. Overall, DIDMd decreased with delayed harvest time and increased with incubation time. An effect of harvest time ($p < 0.001$) on DIDMd was observed for all incubation times. H1 had the highest digestibility, followed by H2 in all incubation times. However, there is only significant difference between H1 and H2 for the 8 hours incubation time. For the 48-hour incubation, DIDMd was highest for H1 and H2 with 57.3 and 53.5%, respectively, followed by H3 with 46.2%, and for H4-H8 the digestibility decreased from 39.3-35.9% without significant difference. Incubation of 48 hours within harvest times was significant different from all other incubation times (see appendix D), but less significant differences were seen between the 8, 16 and 24 incubation hours. For example, significant differences between 8 and 16 hours of incubation were only observed for H2. Additionally, H1, H5, and H7 was nondifferent for incubation times 8, 16, and 24 hours, while the others did have significant differences between incubation times (appendix D).

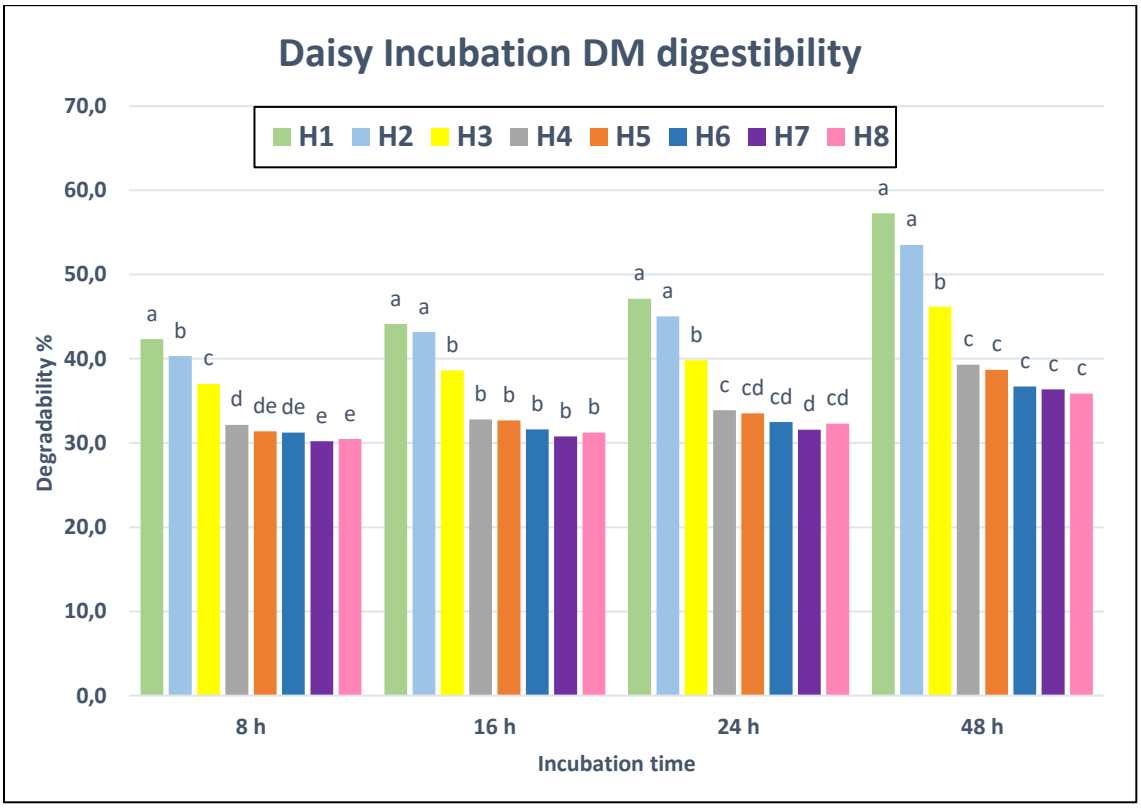


Figure 27. Dry matter digestibility (%) from the Daisy Incubation experiment (DIDMd), illustrates the variance within all incubation times (8, 16, 24, and 48 hours) for all harvest times.
[a, b, c, d, e] Values within incubation time differ is superscripts are different ($p < 0.05$).

4.5 Comparison of methods

The correlation matrix for the three digestibility experiments is presented in table 15. A high positive correlation was found for all methods. The DI and IS method had the highest correlation coefficient (0.96).

	DI	GP	IS
DI	1.00	0.85	0.96
GP	0.85	1.00	0.85
IS	0.96	0.85	1.00
P-value	<0.001	<0.001	<0.001

Table 15. Correlation matrix for the three digestibility methods. DI= Daisy incubation, GP= gas production and IS=In Sacco

Correlations plots for the three methods are presented in figure 28. The plot comparing GP and IS illustrates a straight line, whereas the plots comparing DI against GP and IS illustrate a more curved line.

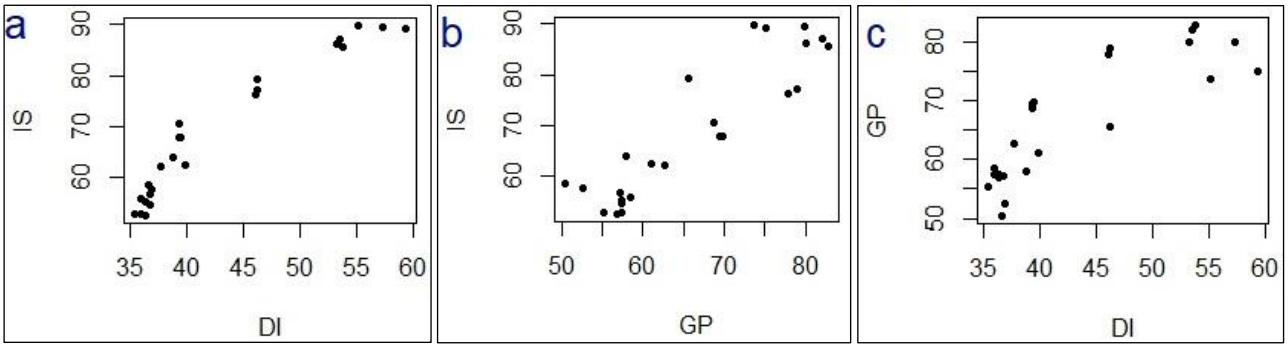


Figure 28 a, b and c. Correlation plots for the Daisy Incubation (DI), Gas Production (GP) and In Sacco (IS) method.

A comparison plot for the endpoints in digestibility from the DI, GP and IS method are presented in figure 29. Overall, DMd for all methods decreased with later harvest time, but the GP method did have some discrepancy in the linearity. The IS and DI method provided overall highest and lowest digestibility estimates, respectively.

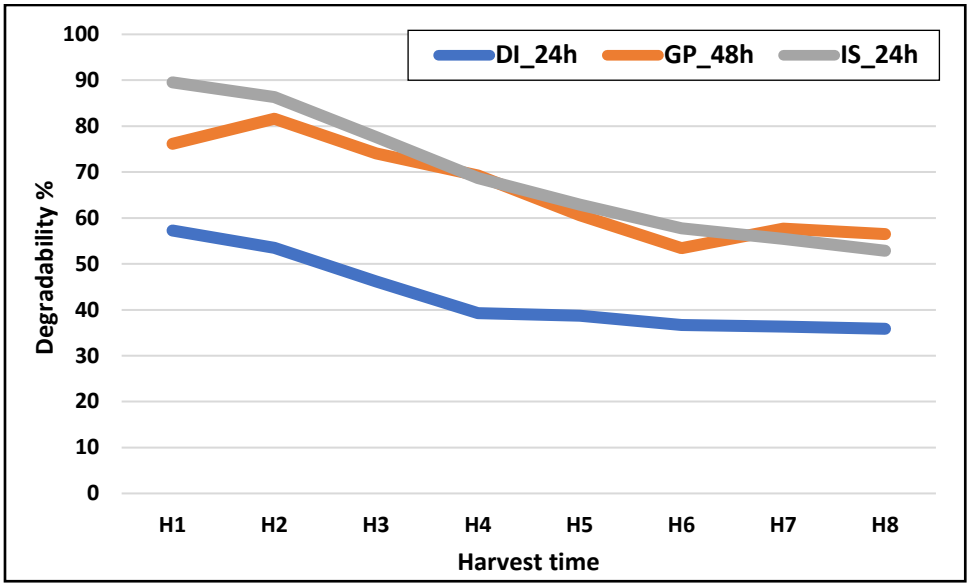


Figure 29. The measured endpoints digestibility from Daisy incubation, gas production and in sacco methods.
DI_24h= dry matter digestibility from the daisy incubation method at 24 hours incubation for all harvest times, GP_48h= dry matter digestibility from the gas production method at 48 hours of incubation for all harvest times and IS_24h= dry matter digestibility from the in sacco method at 24 hours of incubation for all harvest times.

5 Discussion

5.1 Chemical composition in grasses

One of the aims of the present study was to evaluate the nutritional value of timothy grass from different harvest times. In the present study, significant effect of harvest time on chemical content is seen, as expected (table 9). The increase in NDFom, ADFom, ADLom and a corresponding decrease in CP and ash with later harvest are in accordance with normal plant maturity as the leaf:stem ratio decreases and a evidence of a progressive maturity in grasses, and in agreement with others (Maeta et al., 1993; Ragnarsson & Lindberg, 2008; Särkijärvi et al., 2012). Dry matter yield increased with postponed harvest time (table 11), and this negative relationship between nutritional value and DM yield is well acknowledged (Bélanger et al., 2001). Both the DM yield and nutritional value are critical in the selection of the most optimum harvest time as an early harvest provide high nutritional value but low DM yield.

Alterations in the WSC content are of multifactorial causes. Factors like cool temperatures, short day length, intense sunlight, drought and limited nutrients may cause an increase in WSC content (Chatterton et al., 1988). The WSC content during maturity from the present study agree with the findings in the studies of Watts (2010). Chatterton et al. (1988) reported peak sucrose concentrations simultaneously as fructan content were low, as seen in the present study. The high WSC content in early harvested grasses can be explained by sunny weather which supports photosynthesis and accumulations of WSC (figure 23). The low WSC content in middle harvest may be explain of optimum growth conditions, allowing sugars to be metabolized into structural components, i.e., used for growth. Appropriate precipitation in the period before the first harvest may also have facilitated to growth in the early harvests (figure 24). Rapid growth can be an important explanation because this induce respiration in the grasses to increase faster than the photosynthesis, which reduces the WSC content (Undersander, 2013). The high WSC and fructan content in late harvest can be explained by sugars accumulating because the grass is more mature, which leads to reduced growth in grasses, hence resulting in low consumption, but high production of WSC in the grass. Additionally, sunny weather fuels the photosynthesis even though growth is restrained, causes WSC accumulate. The increase in fructan content in late harvests can also be supported by the theory of fructans being an essential constituent for freezing tolerance in the grass, as it prepares for lower temperatures for autumn. As long as there's still green leaf material and sufficient sunny weather, production of sugars will remain (Watts, 2009).

These variations observed in the grass growing season are crucial and should be taken into consideration when grasses are harvested for horses, but also for horse owners when choosing forage for horses, as horses' energy requirements differ with workload, growth and physiological conditions. Early harvests had high nutritional value, recommended as forage for horses with high protein and energy requirements, which includes lactating mares, growing youngsters, horses in intense training or horses with a low body condition score. For healthy leisure horses, a middle and late harvest time is appropriate. For horses that is prone to laminitis or insulin resistance, the middle harvest time would be the safest choice to ensure a low sugar and fructan intake. Although the daily fructan intake (based on a high DM intake of forage (3kg DM/100kg BW) corresponding horse on pasture) from all harvest times would be far below the level of 7.5 g/kg BW, which did induce laminitis in horses (Van Eps & Pollitt, 2006).

The analysis from chemistry and NIRS was evaluated visually. Overall, the content of nutrients was similar and followed a parallel pattern for nutrients and digestibility in both methods. However, some differences were observed for NDF and CP content. The NIRS method predicted these to be lower than the chemical method, where the NDF content had the largest differences between the two methods, indicating that NDF content is challenging to measure and needs further investigations for better prediction with the NIRS method. For the ESC content, the NIRS method predicted slightly higher values. For the fructan and ash content there no visual differences.

5.2 In Sacco

The digestibility coefficients from IS are undoubtedly affected by harvest time, as the ISDMd decreased with delayed harvest. To the authors knowledge, the digestibility (89.5%) for H1 with the IS method in the present study is higher than found for other studies investigating digestibility of forages in horses. High (above 80%) DMd values for other forages are seen with the legume peanut (*Arachis pintoi cv. Amarillo)* with 82.8% digestibility with corresponding S, Pd and Kd values at 29.57, 53.05 % and 10.36%/h respectively (Silva et al., 2010). The S-fraction in that study is comparingly low to those generated in this thesis (ranging 31.0-42.8%), while the Pd fraction is comparingly high to those of this study (ranging 23.3-49.5), where the Kd fraction is more similar from those of this thesis as they had a mean of 11.16%/h. However, the peanut is a legume plant that do differ from grasses as legumes tend to have lower fiber content, higher protein content and lower cell wall digestibility than grasses (Ball et al., 2001), and therefore may not be ideal to compare with. However, the ISDMd of early harvest can extend almost up to ATTD of starch in grains (oats, barley and maize) which are nearly completely digested (91-99%) (Rosenfeld & Austbø, 2009). It is also comparable to hindgut

disappearance of sugar beet pulp (SBP) with values varying from 81-90% for a variable transit time (Thorringer et al., 2022).

Thorringer et al. (2022) did also measure hindgut disappearance of Timothy from first cut with the MBT technique, where the DMd, S, Pd and Kd was 62.3, 28.6, 44.0% and 7.3%/h, respectively (for a transit time of 20-29 hours as comparative for this study). Digestibility of 62.3% is very similar to that of H5 (62.9%), but 27% units lower than of the first cut (H1; 89.5%) of the present study. The S fraction are again comparingly low, as also observed when comparing to the Silva et al., (2010) study. The Pd fraction is comparative for H3 and H4 from the present study. The Kd fraction is lower than those reported in the present study as they ranged from 9.3-15.6 %/h.

Another perspective to discuss is the IS methods capacity to predict ATTD, as the method only includes incubation in the hindgut. Estimates for digestibility are often based on in vitro or IS methods as a predictor for the ATTD. These concerns consider the lack of pre-caecal influence. Efforts to compensate for the absence of mastication are properly grinding of feed samples. However, the enzyme influence from the equine saliva is minimal as mentioned in section 2.3.1, and therefore may not be of importance for the estimates of ATTD when performing the mentioned methods. As mentioned in section 2.3.3, pre-caecal digestibility of forage is low. After all, it does not seem like there is any great difference in digestibility of forage from the ATTD and hindgut digestibility, as described in Thorringer et al. (2022), where the MBT method was a successful method to predict ATTD when using bags found between 20 and 39 hours after administration. Additionally, those methods aid specific information of feed stuff evaluation (such as rate, extent and site of disappearance of feed stuffs) than TCM. Therefore, the IS method is useful for predicting ATTD of forage for horses.

Comparing in vitro and IS methods with in vivo is crucial for validation of the results. Since there was no in vivo experiment with the feed stuffs in the present study, results from the literature on similar feed stuffs are the best alternative in this situation. In vivo digestibility from the literature varies. Apparent total tract digestibility of DM is in the literature reported to be 55.9% for hay (Thorringer & Jensen, 2021), 62.5% for timothy hay of first cut (Thorringer et al., 2022), 52.8% for mature timothy hay (Jensen et al., 2014) and 71.6% for timothy hay ensilage of first cut (Ragnarsson & Lindberg, 2008). These results are consistent with the present study, although none of them are comparable for H1. However, H1 from this study was from a very early harvest date, which does not seem to be common practice when harvesting of grasses to horses.

There are some challenges to the IS method. It is assumed that feed leaving the bag is digested, absorbed, and utilized by the animal. This may not be true, as feed that disappear from the bags in

cecum can be utilized by the microbes and not utilized by the host by absorption and provide the animal host energy, or it may pass undigested to faeces, may resulting in overestimated results in the IS method (Hyslop, 2006). The feed that has disappeared from the bag may also be undigested particles. A high loss of undigested particle could potentially have overestimated the IS results in this study. Also, a problem with attachment of microbes for low quality forage at an early stage of fermentation can result in higher weight of bags (Getachew et al., 1998). The S fraction is also a topic for discussion because this is assumed to be rapid degraded, utilized and absorbed by the bloodstream, but this is may not true, as same reason for the assuming of feed leaving the bag are digested. The S-fraction is determined by the washing loss, i.e., the nutrients that disappears after a round in the washing machine which is supposed to simulate the very rapid digested nutrients. The washing loss consists of mainly ash, CP and WSC (Thorringer & Jensen, 2021), as is in correspondence with this study, with lower S fraction in grasses with lower content of ash, CP and WSC. Additionally, disadvantage with the IS method is it requires cannulated animals, only a few feed samples can be incubated at any one time, the need of at least three cannulated animals to justify for variations caused by animals and the necessity of a high numbers of samples (Getachew et al., 1998).

Dry matter digestibility does not quantify which nutrients that are digested. The results could be improved by measuring NDF in the residue to provide NDF digestibility, as this is a more precise measure for the true digestibility of the cell walls components in forages.

5.3 Gas Production

The large variation between asymptotic gas production (A parameter) within the replicates for harvest times may explain why the A parameter from GP was not significantly different, as illustrated in figure A in Appendix 3. However, for the GP for 24 and 48 hours there were significant differences. This confirms that there were diversities in GP between harvest time, as reflected by chemical composition in grasses, because GP was decreasing with harvest time.

Earlier studies have reported A values of 234 ml gas g/DM for grass hay at 72 hours (caecal inoculum) (Murray et al., 2014), 292 ml gas g/DM for oat straw at 48 hours (CP 27% and NDF 67% of DM) (faecal inoculum) (Kholif et al., 2016), 253 ml gas g/dm for mature grass hay for 96 hours (CP 12% and NDF 54% of DM) (faecal inoculum) (Gandarillas et al., 2021) and approximately 110 ml gas g/DM for grass haylage (CP 10% and NDF 65% of DM) (faecal inoculum) at approximately 48 hours (Lowman et al., 1999). These values except the last one, are all higher compared to the GP generated in the present study. Explanations for this can be different chemical content and incubation times.

Half-time of gas production (B parameter) is smallest in the three first cuts, which may be explained by the chemical content, i.e., lower NDF, ADF, ADL content and higher WSC and CP, which generate

more soluble nutrients for microbial activity and hence rapid fermentation in the beginning of the fermentation process. Grasses from late harvest do probably have lower digestibility of NDF, therefor longer incubation is needed to reach B. A high C-value will be more sigmoidal with an increasing slope. This corresponds with the results in this thesis because the earliest harvest did have a more S-shaped and faster increasing slope as their C-values were significantly higher than the later cuts (H3-H8). Extensive fermentation and formations of VFA can explain changes in pH, where higher pH corresponds to a restrictive fermentation. Lower pH and higher VFA concentration as seen in faeces from horses feed early cut forage, as reflected by a higher potential of fermentability in early cut forages (Jensen et al., 2010). In the case of feeds from this study, the early harvests should be contributing to a higher concentration of VFA's and hence lower pH. This pH measurements do not show a strict clear pattern of low pH in the early harvest and high pH in the late harvests, but there may be a hint of it as H2 had the lowest pH and H7-H8 had the highest pH.

5.4 Daisy Incubation method

The digestibility values for the DI method were low, compared to GP and IS. Also, there was low variation between DMd from 8, 16 and 24 hours of incubation with minor increases, whereas samples incubated for 48 hours are significantly different from the aforementioned (figure 27 and appendix 3). This suggest that minimum 48 hours of incubation is needed when comparing DI to in vivo values.

Lattimer et al. (2007) reported that DI was lower than in vivo estimates. Lower estimates for DI can be related to large (1g) feed sample, high sample size to bag surface area and short (8, 16, 24 and 48hours) incubation time. The ratio of sample-size to bag surface has an effect on the accuracy of the degradability predictions, as a lower ratio seems to give higher digestibility values (Adesogan, 2005). Higher digestibility is observed with a bag sample of 0.25g compared to 0.5g (Coblentz et al., 2019; Lattimer et al., 2007). ANKOM (2021) recommends a bag sample of 0.25g, but also 0.5g is acceptable for 48 hour of incubation. Smaller sample size could, therefore, might improve the digestibility estimates for the DI method. A bag sample of 1g as used in this study is also used in separate master theses (Brustad, 2020; Fure, 2019). Additionally, a measure of pH after incubation could provide information about the degradation process were successful or not. Also, it could be an idea to include an element that stimulate the foregut digestion before performing DI, for example some elements from the Tilley and Terry method (Tilley & Terry, 1963).

Dry matter digestibility (animal: donkeys) of timothy grass by the DI method is in a single study reported to be approximately 38 and 58% for 30 and 72 hours of incubation, respectively (Tassone et al., 2019). Comparingly, DI digestibility for grasses in this thesis was slightly lower, ranging between

32-47% and 36-57% for 24 and 48 hours, respectively. Another study reported DI digestibility of Orchard grass and fescue grasses to be just below 40% and approximately 24-30% for 48 hours of incubation time (O'Donnell et al., 2021). This is in accordance with the results from DI digestibility in this thesis.

A potential disadvantage with the DI method is the concern of the free movement of particles through bags and the fact that the samples can influence each other when incubated in the same digestion jar (Tassone et al., 2020a). This effect would probably be minimal when incubating similar feeds, but as for this thesis, the early harvest with a high content of soluble nutrients released into caecal fluid in the digestion jar, may have improved the degradation of the later harvest as they were incubated in the same digestion jar. Also, free movement of particles through the bags might disturb the digestibility estimate, as there may be a potential loss of undigested particles. The pore size should be large enough, so protozoa and bacterial populations have access, but not too large to risk the loss of undigested particles.

The ambition of in vitro studies is to represent the in vivo process. Incubation time should reflect passage time in the animal of interest. To obtain results similar to in vivo, a longer incubation time than the natural passage time may be applied. Mean retention time in the equine varies, but the literature suggest 23 (Miyaji et al., 2008b) and 26 hours when fed hay (Rosenfeld et al., 2006), but higher values are also reported (59.6 hours) when fed alfalfa hay (Cuddefordl et al., 1995). Earing et al., (2010) suggests that an incubation time of 72 hours should be used when studying the digestibility of feeds of greater fiber content and that 72 hours of incubation was the most similar to the in vivo results. However, a significant difference was not seen from 48-72 hours of incubation time for timothy hay in the experiment of Earing et al. (2010), which may indicate that 48 hours is an appropriate incubation time.

Diet of donor animal may affect the results, because the GIT microbiota can be affected by for example the NDF:starch ratio (Julliand et al., 2001; Kern et al., 1973). Lower pH and deprived cellulolytic bacteria were seen when level of barley was increased in the diet (Julliand et al., 2001), which may reduce the digestibility of forages. This might not be relevant in this study, as horses were fed hay only in addition to a multi-supplement with vitamins and minerals. On the contrary, diets with grains in addition to hay are also observed to improve DIDMd compared to forage only diet (Godwin et al., 2021). This is also seen in ruminants (Holden, 1999). Accordingly, the low DIDMd in this thesis could have been affected of the diet as they did not receive any concentrates.

5.5 Comparison of methods

All three digestibility experiments showed a high positive correlation, indicating that they all are useful to predict the linear decreasing digestibility caused by postponed harvest time. However, the smaller correlation for GP and the two other methods might be explained of GP did not follow the very strict pattern of linearity in decreasing digestibility by delayed harvest time, as seen in IS and DI. For example, GP predicted higher DMd for H2 than H1 and H7 to be higher than H6. However, these differences were not significantly different, therefore might be irrelevant (table 13). Also, correlations do not consider the differences in digestibility estimates, only how they whether increases or decreases at the same time. For example, results from DI were lower compared to IS, yet they had a high correlation. For example, IS predicted digestibility for H1 and H8 to be 89 and 53%, respectively, where DI predicted this to be 57 and 36%, respectively (figure 29). Underestimated results of DI compared to IS are also observed in the study of Trujillo et al. (2010).

Many factors make the methods investigated different and therefore provides varying estimates for digestibility within methods. The most important factor may be the absence of true biological stimulation when feeds are incubated outside the living animal as in DI and GP. However, there was not any large differences in DMd for IS and GP (figure 29). There were some high standard deviations (SD) in the GP parameters (table 13), whereas these were overall low in the IS parameters and DIDMd (table 12 and Appendix 4), which may indicate lower repeatability in GP method. Even though the DI method did not reach as high digestibility values as IS, it proves to be a useful method for comparing degradation potential within feedstuffs, considering their high correlation.

To summarize, the IS technique may be the most correct method to estimate digestibility of the methods compared in this thesis. On the other hand, cannulated horses are scarce, and therefore the DI and GP are methods more applicable, often used with a faecal inoculum instead of caecal inoculum due to the limited availability of cannulated horses. Advantages with the DI and GP are that they are less laborious, easier to study multiple different feeds simultaneously in one run of experiment, making DI and GP more effective than IS method. Furthermore, all methods need further research.

6 Conclusion

Plant maturity and harvest time did significantly influence the chemical composition in grasses. Fiber and lignin were increasing, and CP and ash were decreasing with delayed harvest time. This consequently caused a significant reduced digestibility and nutritional value by postponed harvest time. Total WSC were high in early harvest, low in in middle harvest, and thereafter high, with fructans as the main component in the late harvest time. Digestibility coefficients were significantly reduced by delayed harvest time independent of method used. All methods used for digestibility measurements were highly correlated where IS and DI were most correlated. However, the DI method was lower compared with IS, GP and in vivo results from literature. The IS technique is the recommended method if cannulated horses are available. Further, DI and GP are good alternative methods as they are cheaper, more effective, less time consuming than the IS method and do not require cannulated horses. Finally, there is a need for further research and standardisation in IS and in vitro methods.

7 Perspectives

Improving the equine health from a nutritional point of view is a key driving force to further investigate research on effect of harvesting times on nutritional value of forages, in addition to different techniques to quantify digestibility. There is a need for standardization of the methods to obtain as true as possible digestibility predications. Methodical studies should be conducted to investigate the factors that induce variation in the methods examined. To fully evaluate the potential of the methods used, it should be compared with in vivo experiments, such as total collection method or MBT. The method with highest correlation with in vivo, would be the most recommended method. As concluded from this thesis, the IS method is the recommended method, but it is highly desirable that the DI and GP methods proves to be good alternative methods for measuring digestibility, as they reduce the need of research animals, labour intensity and economy required. The DI method should be investigated with aim to improve the method, to be similar to in vivo results in horses, as it has a high potential to be a rapid and easy method to determine digestibility.

Other issues that have raised during the present thesis is the concern of the low NDF content predicted by NIRS versus chemical method. Also, fructan content in grasses at late harvest times should be further investigated, because many horses are often fed a late harvested forage. It is also a need for classification of sugar and fructan content, whether it is quantified as either high or low, in terms of safe intakes without negatively altering the metabolic responses in hindgut, as it is recommendations for starch intake.

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Appendix 1

Table 1. Sugar composition in the experimental feeds. Numbers are presented as mean ± SD from the three cuts from three fields within eight harvest dates. All nutrients are in % of DM.

	Glucose	Fructose	Sucrose	Fructans	Total WSC
H1	1.6 ± 0.0^{de}	0.8 ± 0.0^{ab}	8.7 ± 0.2^{a}	2.3 ± 0.2^{cd}	13.4 ± 0.3^{a}
H2	1.9 ± 0.1^{ce}	0.9 ± 0.1^{ab}	6.8 ± 0.4^{b}	2.1 ± 0.1^{cd}	11.6 ± 0.7^{b}
H3	2.5 ± 0.1^{a}	0.8 ± 0.1^{ac}	4.4 ± 0.2^{de}	1.1 ± 0.2^{de}	8.8 ± 0.3^{cd}
H4	2.0 ± 0.1^{bcd}	0.6 ± 0.1^{c}	4.6 ± 0.1^{d}	0.3 ± 0.3^{ef}	7.6 ± 0.5^{d}
H5	2.5 ± 0.1^{ad}	0.8 ± 0.1^{bc}	5.5 ± 0.4^{c}	0.8 ± 0.5^{df}	9.6 ± 0.9^{c}
H6	2.4 ± 0.3^{ab}	0.8 ± 0.1^{ab}	3.7 ± 0.2^{ef}	2.9 ± 0.3^{c}	9.8 ± 0.5^{c}
H7	2.4 ± 0.3^{ab}	1.0 ± 0.0^{ab}	4.0 ± 0.1^{df}	5.0 ± 1.0^{b}	12.3 ± 0.7^{ab}
H8	2.3 ± 0.2^{ac}	1.0 ± 0.0^{a}	3.4 ± 0.1^{f}	6.8 ± 0.9^{a}	13.5 ± 0.7^{a}
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a, b, c, d, e}$ *Values within columns differ if superscripts are different (p<.05).*
WSC= water soluble carbohydrates

Table 2. Forage analysis by NIRS evaluation of the experimental feeds. Values are presented as mean ± SD. Numbers are average from the three cuts from each field within each harvest time. Nutrients are in % of DM, except of DCP (g/kg DM), and digestibility and IVOS are in %.

	Ash	NDF	CF	CP	DCP	ESC	Fructans	Digestibility	IVOS	Fat	Cl
H1	7.6 ± 0.2	38.4 ± 0.6	18.3 ± 0.1	15.0 ± 0.2	111.6 ± 1.9	14.7 ± 0.8	3.5 ± 0,4	77.9 ± 0.6	77.3 ± 0.6	2.8 ± 0.1	0.9 ± 0.1
H2	7.9 ± 0.4	42.2 ± 0.3	21.0 ± 0.5	12.1 ± 0.4	85.0 ± 3.5	12.8 ± 0.5	2.5 ± 0,2	74.2 ± 0.5	73.4 ± 0.6	2.4 ± 0.2	0.8 ± 0.0
H3	6.7 ± 0.1	48.1 ± 1.0	24.8 ± 0.8	9.3 ± 0.2	59.3 ± 1.3	9.5 ± 0.4	1.5 ± 0,2	68.3 ± 0.3	67.3 ± 0.3	2.1 ± 0.0	0.7 ± 0.0
H4	6.2 ± 0.2	54.3 ± 0.7	27.8 ± 0.6	8.8 ± 0.2	53.7 ± 2.3	7.8 ± 0.7	1.3 ± 0.1	64.7 ± 1.8	63.6 ± 1.9	2.1 ± 0.1	0.7 ± 0.0
H5	5.3 ± 0.4	55.8 ± 0.8	28.8 ± 0.2	7.8 ± 0.3	43.9 ± 3.0	9.4 ± 0.4	2.2 ± 0.2	63.0 ± 0.5	61.8 ± 0.5	1.8 ± 0.1	0.7 ± 0.0
H6	4.9 ± 0.4	56.6 ± 1.3	29.2 ± 0.5	6.8 ± 0.2	34.1 ± 1.7	9.0 ± 0.3	2.8 ± 0.1	60.3 ± 0.9	58.9 ± 1.0	1.7 ± 0.2	0.6 ± 0.0
H7	5.0 ± 0.4	55.8 ± 1.5	28.6 ± 0.6	5.7 ± 0.5	24.2 ± 4.7	10.1 ± 0.5	5.2 ± 0.8	59.2 ± 0.8	57.8 ± 0.8	1.7 ± 0.0	0.7 ± 0.1
H8	4.1 ± 0.3	56.9 ± 0.6	29.4 ± 0.6	4.6 ± 0.3	13.6 ± 2.9	10.2 ± 0.6	6.6 ± 0.2	57.2 ± 0.8	55.6 ± 0.8	1.4 ± 0.0	0.7 ± 0.1

NDF= neutral detergent fibre, CF= crude fiber, CP= crude protein, DCP= digestible crude protein, ESC= ethanol soluble carbohydrates and IVOS= In Vitro organic matter digestibility.

Appendix 2

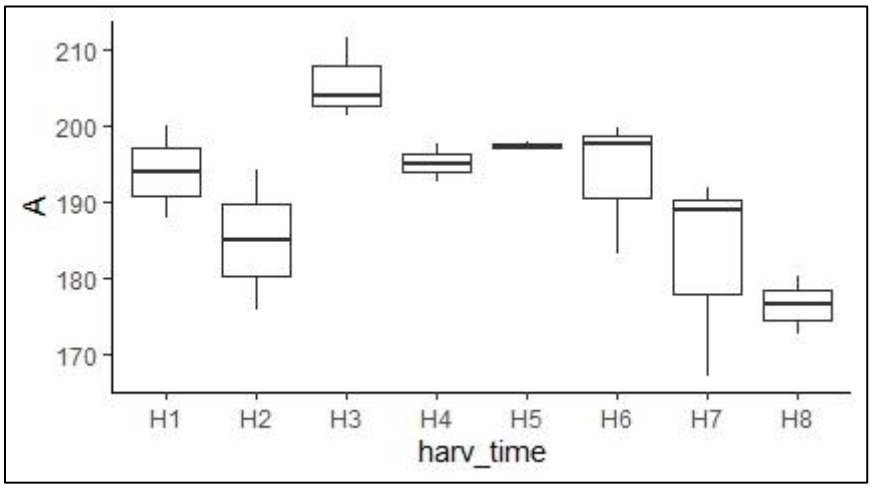

Figure 1. GGplot illustrating the mean and variance for parametre A for all harvest times. A= Gas production (ml gas g/DM)

Appendix 3

Table 3. Dry matter degradability (%) from the Daisy Incubation experiment (DIDMd) for 8, 16, 24 and 48 hours of incubation, presented as means ± standard deviation (SD).

	8 h	16 h	24 h	48 h
H1	42.3 ± 0.1^{a}	44.1 ± 0.2^{a}	47.1 ± 0.6^{a}	57.3 ± 2.1^{a}
H2	40.3 ± 0.2^{b}	43.2 ± 0.2^{a}	45.0 ± 0.6^{a}	53.5 ± 0.3^{a}
H3	37.0 ± 0.6^{c}	38.6 ± 0.0^{b}	39.8 ± 0.6^{b}	46.2 ± 0.1^{b}
H4	32.2 ± 0.1^{d}	32.8 ± 0.2^{b}	33.9 ± 0.3^{c}	39.3 ± 0.1^{c}
H5	31.4 ± 0.2^{de}	32.7 ± 0.0^{b}	33.5 ± 0.0^{cd}	38.7 ± 1.1^{c}
H6	31.2 ± 0.0^{de}	31.6 ± 0.1^{b}	32.5 ± 0.0^{cd}	36.7 ± 0.1^{c}
H7	30.2 ± 0.1^{e}	30.8 ± 0.3^{b}	31.6 ± 0.1^{d}	36.4 ± 0.4^{c}
H8	30.5 ± 0.2^{e}	31.2 ± 0.1^{b}	32.3 ± 0.1^{cd}	35.9 ± 0.5^{c}
P-value	<0.001	<0.001	<0.001	<0.001

$^{a, b, c, d, e}$ *Values within a column lacking common superscript differ with p <0.05.*

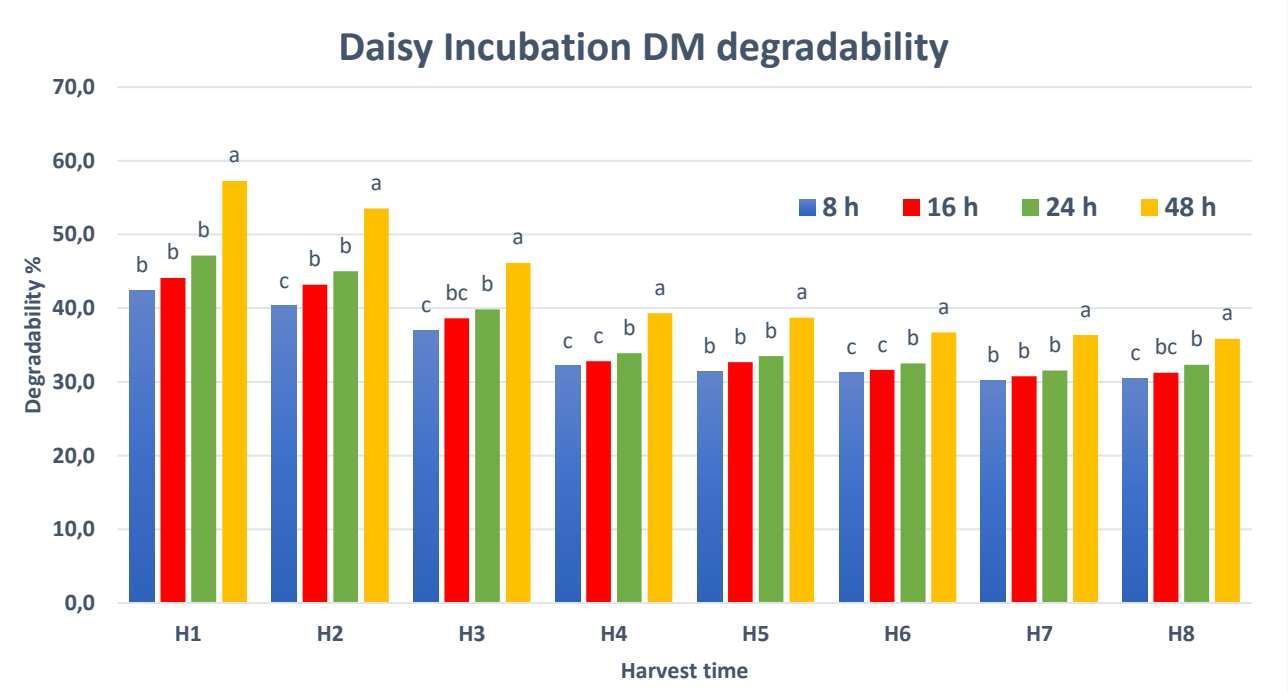

Figure 2. Dry matter degradability (%) from the Daisy Incubation method (DIDMd), illustrating the variance within all harvest times, for the different incubation times (8, 16, 24 and 48 hours).

$^{a, b, c}$ *Values within harvest time lacking common superscript differ with p <0.05.*

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