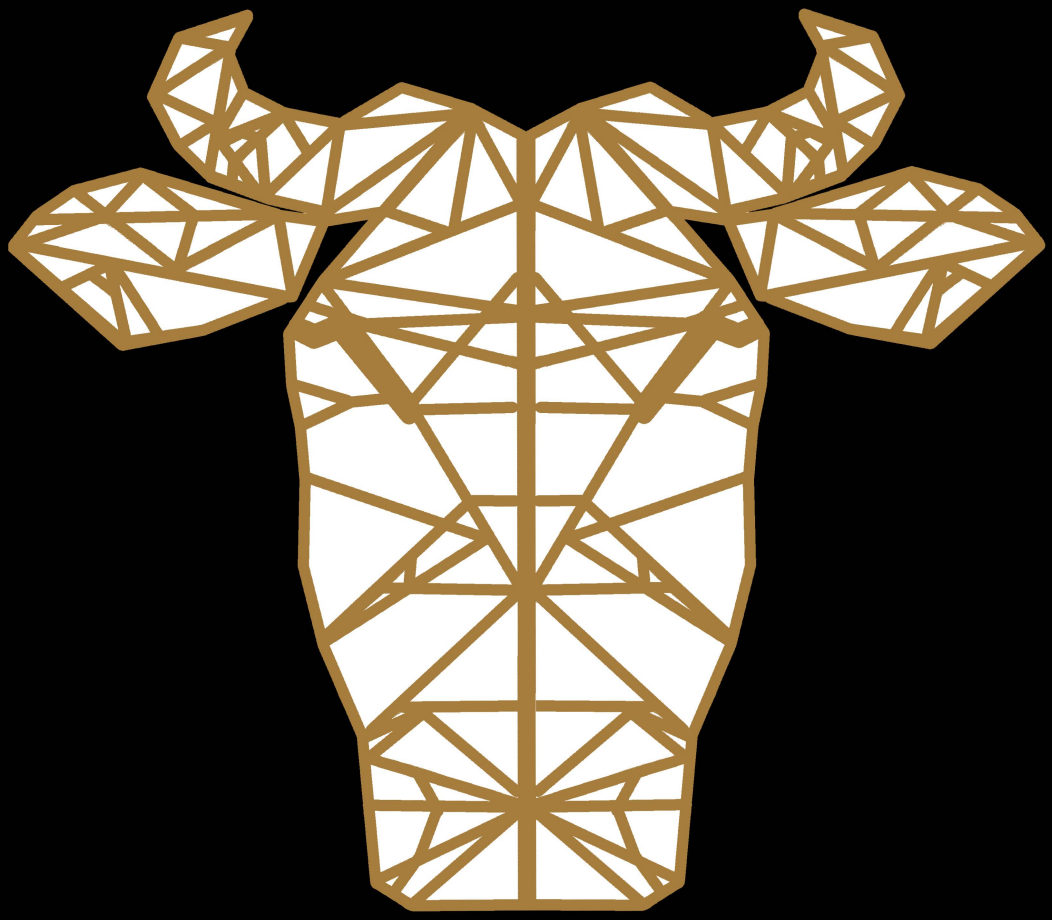
Ruminal degradation characteristics of barley, rye, and triticale grains assayed *in situ* and *in vitro*, and by near-infrared spectroscopy



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The cover was designed in collaboration with Laura Sommerfeld.

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Ruminal degradation characteristics of barley, rye, and triticale grains assayed *in situ* and *in vitro*, and by near-infrared spectroscopy

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Table of Contents

List of Tables

List of Figures

Abbreviations

Abbreviations following the international system of units, the international union of pure and applied chemistry (IUPAC) and the system of symbols for chemical elements are not listed.

a	*In situ* washout fraction
AA	Amino acids
A_λ	Absorbance at a certain wavelength λ
aCP	*In situ* washout fraction of crude protein
aDM	*In situ* washout fraction of dry matter
aST	*In situ* washout fraction of starch
a+b	*In situ* maximal degradable fraction
a+bCP	*In situ* maximal degradable fraction of crude protein
a+bDM	*In situ* maximal degradable fraction of dry matter
a+bST	*In situ* maximal degradable fraction of starch
b_{gas}	Potential gas production in the Hohenheim Gas Test
c	*In situ* degradation rate
cCP	*In situ* degradation rate of crude protein
cDM	*In situ* degradation rate of dry matter
c_{gas}	Gas production rate in the Hohenheim Gas Test
CNCPS	Cornell Net Carbohydrate and Protein System
CP	Crude protein
cST	*In situ* degradation rate of starch
CV	Coefficient of variation
CVB	Centraal Veevoederbureau
DM	Dry matter
DNA	Deoxyribonucleic acid
dOM	Digestibility of the organic matter
ED	*In situ* effective ruminal degradability
ED_5	*In situ* effective ruminal degradability at a ruminal passage rate of 5%/h

ED_8	*In situ* effective ruminal degradability at a ruminal passage rate of 8%/h
ED_5CP	*In situ* effective ruminal degradability of crude protein at a ruminal passage rate of 5%/h
ED_5DM	*In situ* effective ruminal degradability of dry matter at a ruminal passage rate of 5%/h
ED_5ST	*In situ* effective ruminal degradability of starch at a ruminal passage rate of 5%/h
ED_8CP	*In situ* effective ruminal degradability of crude protein at a ruminal passage rate of 8%/h
ED_8DM	*In situ* effective ruminal degradability of dry matter at a ruminal passage rate of 8%/h
ED_8ST	*In situ* effective ruminal degradability of starch at a ruminal passage rate of 8%/h
ED_kCP	*In situ* effective ruminal degradability of crude protein at a ruminal passage rate of k%/h
ED_kDM	*In situ* effective ruminal degradability of dry matter at a ruminal passage rate of k%/h
ED_kST	*In situ* effective ruminal degradability of starch at a ruminal passage rate of k%/h
ε_λ	Wavelength dependent molar absorption
fOM	Fermented organic matter
GfE	Gesellschaft für Ernährungsphysiologie
HGT	Hohenheim Gas Test
HLW	Hectolitre weight
l	Path length of the light through the sample
k	Ruminal passage rate
λ	Wavelength
MCP	Microbial (crude) protein
MLR	Multiple linear regression
ME	Metabolisable Energy
n	Sample number
NIR	Near infrared
NIRS	Near-infrared spectroscopy
NRC	National Research Council
PCR	Principle component regression

PLS	Partial least square
R	Reflectance
r	Pearson correlation coefficient
R^2	Coefficient of determination
RPIQ	Ratio of the standard error of prediction to the inter-quartile distance of validation samples
SCFA	Short chained fatty acids
SE	Standard error
SEC	Standard error of calibration
SECV	Standard error of cross validation
SEP	Standard error of prediction
SNV	Standard normal variate
ST	Starch
uCP	Utilisable crude protein at the duodenum
UDP	Ruminally undegraded crude protein

1 General introduction

In the last decades, milk production of dairy cows has increased continuously. Along with the increased milk production, the energy and nutrient requirements of the animals have risen. However, the potential feed intake has not increased to the same extent, due to physiological and anatomical restrictions. In an attempt to fulfil the high energy requirements, the energy density in rations of dairy cows has been increased. This is mainly realised by including high proportions of easily fermentable carbohydrates in the form of starch (**ST**)-rich feed. To obtain high ST concentrations, rations comprise high proportions of cereal grains. Maize, barley, sorghum, and wheat are the most studied and used cereal grains in dairy nutrition (Eastridge, 2006). Rye and triticale are less researched, but in 2014, together with barley, they comprised a quarter of the total cereal production in the EU. In Germany, rye and triticale are of higher importance than they are in the EU (Food and Agriculture Organization of the United Nations (FAO), 2017).

Barley, rye, and triticale grains have a similar structure. The structure of cereal grains and that of ST in cereal grains are well-characterised and reviewed (Smith, 2001a, Smith, 2001b; Tester *et al.*, 2004b; Pérez and Bertoft, 2010; Koehler and Wieser, 2013; Visakh, 2016). In brief, cereal grains (caryopses) mainly consist of the endosperm and the germ. The endosperm includes the starchy endosperm and the aleurone layer. The germ and the endosperm are surrounded by seed coats and the pericarp. The whole caryopsis is enclosed within the hull (lemma and palea), which remains connected to barley grains, but is removed from triticale and rye grains while threshing (Rodríguez *et al.*, 2015). Quantitatively, the biggest part of cereal grains is the starchy endosperm; hence, the most abundant chemical component of cereal grains is ST (Koehler and Wieser, 2013). Crude protein (**CP**) represents approximately 12% of dry matter (**DM**) in barley, rye, and triticale grains (Deutsche Landwirtschaftsgesellschaft (DLG), 1997; Rodehutscord *et al.,* 2016). Hence, when cereal grains comprise a high proportion of the rations, they can also contribute notably to the total protein intake. The ruminal CP and ST degradation can influence the performance and health of dairy cows.

The possible negative effects of fast and extensive ST fermentation by microbial glycosidases—which leads to an extensive production of short-chained fatty acids (**SCFA**), and hence, a decrease in ruminal pH—are well-described (Zebeli and Metzler-Zebeli, 2012; Krause and Oetzel, 2006; Owens *et al.*, 1998; Kleen and

Cannizzo, 2012; Kleen *et al.*, 2003). Although ruminal acidosis has already been a subject of many studies, possible influencing factors, prevention strategies (Offner *et al.*, 2003), and yet unknown effects on animal performance are still part of recent research (e.g. Abaker *et al.* (2017); Mickdam *et al.* (2016); Sulzberger *et al.* (2016)). An often-discussed strategy to avoid fast and extensive ruminal ST degradation and its negative effects is to shift the ST degradation to the small intestine. It is assumed that ruminally undegraded ST is, in principle, digested in the small intestine. Nozière *et al.* (2010) described values in literature between 10 and 90% for intestinal ST digestibility. Matthé *et al.* (2001) reported variations in intestinal ST digestibility for wheat ST in dairy cows between 53 and 74%, and a decreased digestibility for high amounts of ST reaching the intestine. Hence, ruminally undegraded ST would—to a certain extent—contribute to the energy supply of the animal without the energy loss that occurs during ruminal fermentation (Owens *et al.*, 1986; Deckardt *et al.*, 2013). Dietary CP is partly degraded in the rumen, and microbial (crude) protein (**MCP**) is synthesised, which requires energy. The approach of shifting the ST degradation/digestion to the small intestine can be in conflict with the aim of attaining a high MCP synthesis, because less energy is available for rumen microorganisms if ST is fermented in the small intestine instead of the rumen. In Germany, feed evaluation for ruminants—with a focus on the CP supply—is performed on the basis of the utilisable CP at the duodenum (**uCP**). The uCP consists of ruminal undegraded CP (**UDP**) and MCP reaching the duodenum, corrected for the endogenous protein (Gesellschaft für Ernährungsphysiologie (**GfE**), 2001; Steingass and Südekum, 2013). The amount of MCP depends on the available nitrogen, as well as on the energy available for the synthesis of MCP, which is mainly obtained from carbohydrate fermentation. In the Dutch feed evaluation system, a specific efficiency of the MCP synthesis is assumed for different energy sources, and a relatively high efficiency is assumed for ST. Accordingly, different mathematical models—including those in the Dutch feed evaluation system—also include information about the ruminal ST degradation, in order to model the CP supply of the animal (Offner *et al.*, 2003; Centraal Veevoederbureau (**CVB**), 2011).

Apart from the ST source, the temporal arrangement of CP and ST degradation influences the MCP synthesis. Crude protein and carbohydrate degradation should take place, preferably in a synchronic manner, to achieve high MCP yields (Cole and Todd, 2008). Consequently, if rapidly degradable nitrogen sources are included in the

ration, rapidly degradable carbohydrates such as ST should also be available. The rate at which ST gets degraded is influenced—amongst several other factors—by the diet composition, feed intake per time (and therefore ruminal passage rate), adaption of the microbial community to the feed, and the source of ST (Huntington, 1997). Like ST degradation, ruminal CP degradation is also influenced by the feed, amongst various other factors (Clark *et al.*, 1992). Therefore, a proper description of ruminal degradation kinetics of ST and CP for different feed sources is required for meeting the nutritional requirements of the animal.

Various studies showed that both environment and genetic backgrounds influence the ruminal degradation of cereal grains (Silveira *et al.*, 2007; Ramos *et al.*, 2009; Zhao *et al.*, 2016; Seifried *et al.*, 2016). Barley is one of the feeds for ruminants well-researched (Nikkhah, 2012). Fewer studies have been conducted with triticale and rye, but the influences of environmental and genetic factors on different traits have also been shown for these grain species (Varughese *et al.*, 1996; Hansen *et al.*, 2004). Some studies describe the ruminal degradation of CP and/or ST from barley (Offner *et al.*, 2003; Nikkhah, 2012; Zhao *et al.*, 2015), rye (Lund *et al.*, 2008; Polat *et al.*, 2014), and triticale (Offner *et al.*, 2003; Liu *et al.*, 2012) grains. Only few studies have investigated different, defined samples of different species at the same time; in most studies, the origin or composition of the samples is not defined at all.

A comparison between different studies and the literature survey is complicated by methodological differences between *in situ* studies. If the methodological differences are not considered, ruminal degradation would vary widely within one grain species. For example, Offner *et al.* (2003) reviewed studies that have researched the ruminal ST degradation of different feedstuff and reported results for the effective ruminal degradability (**ED**) of ST in ground barley from three studies, resulting in a mean value of 86% with a standard error (**SE**) of 10.6%, which underlines the high variation in the scarce data that can be found in literature. This variation can be caused by the samples—e.g. the genotype—as well as by differences in methodology. Hence, a systematic investigation of the ruminal degradation of cereal grains is needed, which takes genetical variation into account, but also avoids the variation caused by the use of different methods. To realise this, a defined sample set and standardised methodology to investigate the ruminal degradation behaviour of cereal grains are required.

A commonly used methodology to obtain information about the ruminal degradation of concentrate feeds is the ruminal incubation of feed samples in porous bags—also called the *in situ* technique. The feed sample is incubated in the rumen over defined timespans and the cumulative degradation of the DM or the nutrient of interest is calculated. To describe the degradation, a mathematical function is fitted to the experimental data. If an exponential function is used, the degradation rate (**c**), washout fraction (**a**), and the maximal degradable fraction (**a+b**) for DM (**cDM**, **aDM,** and **a+bDM**) and different nutrients can be estimated (for CP: **cCP**, **aCP,** and **a+bCP**; for ST: **cST**, **aST,** and **a+bST**). Based on these parameters, the effective degradability (**ED_k**: for DM: **ED_kDM;** for CP: **ED_kCP;** for ST: **ED_kST**) is calculated, assuming a ruminal passage rate of **k**.

In situ studies are associated with intensive experimental efforts. Hence, *in vitro* incubations are often used as alternatives to estimate the ruminal degradation of feeds. An often used and standardised technique is the Hohenheim Gas Test (**HGT**), which is based on the gas production that occurs during incubation of the sample with a ruminal fluid-buffer mixture. Analogous to *in situ* studies, the gas production can be described by fitting exponential curves, from which the gas production rate (**c_{gas}**) and the potential gas production (**b_{gas}**) can be derived. Additionally, the metabolisable energy concentration (**ME**) and digestibility of the organic matter (**dOM**) can be estimated from the *in vitro* gas production, in combination with crude nutrient concentrations (Menke and Steingass, 1988).

To obtain ruminal fluid for this *in vitro* technique, ruminally fistulated donor animals are needed. Animal trials are attended by strict legislative regulations and have a negative image among the public. Therefore, alternative methods are essential to evaluate feeds for ruminant nutrition. One way to minimise animal experiments is to predict the degradation and/or digestibility of feeds by *in vitro* incubations, in which the ruminal fluid is substituted by a mixture of enzyme solutions, such as cellulases (Aufrere and Michalet-Doreau, 1988; Cone *et al.*, 1996; Carro *et al.*, 2002; Cheli *et al.*, 2012). These methods are still relatively laborious, and thus, not really applicable for routine feed analysis. Different studies showed, that nutritional values like ruminal degradability (Kitessa *et al.*, 1999; Garnsworthy *et al.*, 2000; Nordheim *et al.*, 2007; Foskolos *et al.*, 2015) and digestibility or energy/nutrient content of feeds (Xiccato *et al.*, 2003; Mentink *et al.*, 2006; Owens *et al.*, 2009; Glencross *et al.*, 2015) can be predicted from the near-infrared (**NIR**) spectra of the feed. Hence, near-infrared spectroscopy (**NIRS**) might be

used to reduce the numbers of animal trials, through the prediction of ruminal degradation characteristics. Near-infrared spectroscopy could also be used to simplify *in situ* studies by substituting chemical analyses. To achieve this, calibrations need to be developed to predict the nutrient concentration in samples from *in situ* studies and the ruminal degradation characteristics of feeds.

2 Overview and aims of the included studies

The present thesis had two major aims. The first aim was to provide comprehensive data on the ruminal CP and ST degradation of barley, rye, and triticale grains to describe inter- and intra-species variation of the feeding value for ruminants. To achieve this aim, ruminal *in situ* and *in vitro* degradation characteristics were obtained by using standardised *in situ* and *in vitro* incubation techniques. In combination with the extensive chemical and physical characterisation of the samples within the GrainUp project, the linkages between chemical and physical parameters and the ruminal degradation of the grain samples were investigated.

The first study included in this thesis covers the ruminal DM and ST degradation *in situ* and the *in vitro* gas production in the HGT (Manuscript 1). The second study addresses the variation of the ruminal CP degradation *in situ* and compares different methods to estimate the uCP. The uCP was predicted by two different approaches, which used the *in situ*-derived UDP, and by a third method based on an extension of the HGT (Manuscript 2).

The determination of ruminal degradation characteristics by the *in situ* and *in vitro* techniques is relatively elaborate, and hence, not applicable for routine feed analyses. Thus, the second aim and the subject of the third manuscript was the development of NIRS calibrations to enable an easier assessment of ruminal degradation of cereal grains (Manuscript 3). In order to achieve this, data from Manuscripts 1 and 2 were used, together with data from other *in situ* experiments, to develop NIRS calibrations. First, calibrations were established to facilitate the determination of the CP and ST concentration of *in situ* residues and cereal grains. The calibrations to estimate ruminal degradation parameters and the ED of cereal grains were also developed.

3 General discussion

One aim of this thesis was the establishment of NIRS calibrations to estimate the CP and ST concentration of cereal grains and their *in situ* residues. Since this step was successful, the data obtained by applying these calibrations were used in the *in situ* studies of this thesis (Manuscripts 1 and 2). Hence, the NIRS predictions of CP and ST concentrations (Manuscript 3) are discussed before the results of *in situ* and *in vitro* experiments (Manuscripts 1 and 2), where they have been implemented.

3.1 Near-infrared spectroscopy for estimating crude protein and starch concentration in samples of *in situ* studies

3.1.1 Principle of near-infrared spectroscopy

Near-infrared spectroscopy is a spectrometric method based on the absorption of electromagnetic radiation from the sample, in the wavelengths between 750 and 2500 nm. If radiation in this wavelength segment is applied to a sample, a certain part of the energy is absorbed by atomic bonds. Due to the energy input, atomic bonds are excited, which leads to vibrational movement. The absorption bands in an NIR spectrum are based on the theory of the harmonic oscillator and its extension to the anharmonic oscillator (Davies, 2005; Burns and Ciurczak, 2008). According to this theory, absorption bands in the NIR area are caused by the transmission from one vibrational energy level of a molecule to a higher level. There are different forms of transmission that lead to different bands. If the energetic level of a bond is excited from the fundamental status to the next higher energy level, a so-called fundamental band is visible in the spectrum. If an already excited bond (e.g. by high temperature) is excited to a higher energy level, hot bands are visible in the spectrum (Bokobza, 1998). The frequency of hot bands is lower than the frequency of fundamental bonds. The excitement of a bond from the fundamental level to the second energy level—leaving the first higher level out—is called the first overtone. If a bond is excited from the fundamental level to the third energy level, it is called the second overtone, and so on. Combination bands are caused if multiple fundamental vibrations are excited at the same time. In NIRS, the absorption bands of interest are caused by overtone and combination bands from C-H, N-H, and O-H bonds. Other possibly excited bonds are C-C and C=O (Davies, 2005; Burns and Ciurczak, 2008).

Generally, there are two types of vibrational motion that can be caused by the excitement of atomic bonds—stretching and bending vibrations. Stretching vibrations can be subdivided into symmetrical or asymmetrical vibrations. Bending vibrations can be plane or out-of-plane. Plane vibrations can be separated into scissoring and rocking. Out-of-plane bending vibrations can again be divided into wagging and twisting. Depending on the energetic level of the bonds and the admitted radiation, the bonds are excited differently. The energy that is not absorbed by the sample is specular-reflected, diffuse-reflected, transmitted, refracted, or scattered (Burns and Ciurczak, 2008). The signal detected in NIRS is the diffuse-reflection, expressed as the logarithm of the reciprocal reflection **(log (1/R)**). Based on a modification of the Beer–Lambert law of reflectance measurements, the concentration of a constituent determines the reflection by: $A_\lambda = -\log_{10}(R) \cong \varepsilon_\lambda \times l \times \text{concentration}$, where $\mathbf{A_\lambda}$ describes the absorbance, dependent on the wavelength (**λ**), **R** the detected reflectance, $\boldsymbol{\varepsilon_\lambda}$ describes wavelength dependent molar absorption, **l** is the path length of the light through the sample, and concentration is the concentration of the constituent (Rinnan *et al.*, 2009). Therefore, the detected signal is influenced by chemical and physical characteristics of the sample, e.g. particle size or the concentration of a certain organic constituent (Bokobza, 1998; Davies, 2005; Burns and Ciurczak, 2008). Most bands in the NIR region are caused by the absorption of energy by bonds of functional groups with H atoms, which leads to stretching and bending vibrations. The large number of overtones detected in NIRS lead to the typical broad, overlapping peaks in the NIR spectra. The overlap of peaks leads to difficulties in estimating the height and width of the bands (Davies, 2005; Burns and Ciurczak, 2008).

This theoretical background leads to steps that need to be considered during sample preparation and the recording of spectral data. As indicated by the occurrence of the so-called hot bonds, sample temperature was found to influence the prediction performance of calibrations (Paul *et al.*, 2002). To avoid errors due to the sample temperature, spectral data in this work were recorded after the samples had adapted to room temperature, at least overnight. One of the most important steps to be standardised during sample preparation is the particle size of the sample. Due to the different stages of degradation resulting from ruminal incubations over various timespans, samples from *in situ* studies show a broad particle size distribution. Hence,

in the present work, samples were pulverised using a vibrating cup mill, to enable chemical analyses and avoid errors due to differences in particle size in NIRS predictions (Manuscript 3). The defined particle size is—at first glance—an advantage of the established calibrations, but it also leads to limitations due to the time-consuming sample preparation and the strong limitation of sample matrices. An adaption to a wider range of particle sizes might be possible by using different mathematical treatments of spectra, as described by Rinnan *et al.* (2009) or Mi *et al.* (2013). Starch in cereal grains is organised in granules that may differ in size (Chapter 3.2.1, Table 1). It can be assumed that—although the samples were finely ground—ST granules were still intact in non-incubated samples. Hence, theoretically, the estimation could be affected by structural variations between native and partly degraded ST granules. This could lead to differences in reflection and/or absorption of the NIR radiation. However, nothing indicating such an influence was reflected in the performance of the established calibrations. The differences between chemically determined and NIRS-estimated CP and ST concentrations were at a consistent level over the whole range of concentrations (Calibration (17) from Tables 3 and 4, respectively, in Manuscript 3; Figure 1). Since the grain samples had a higher concentration in both nutrients compared to incubated samples, the calibrations were presumably uninfluenced by the structure of the ST granules.

As stated above, the principle of NIRS is based on a modification of the Beer–Lambert law. Hence, the sample volume should be big enough to obtain a closed layer of sample material for reflectance measurements. Very little sample material for bag residues was available in the present study. Thus, the spectra were recorded using inlays for small sample volumes. Comparisons between spectra recorded with and without inlays showed no difference (data not shown). Another point to be considered is the influence of the water content of the samples. Tillmann (1996) concluded, that changes in the water content are negligible if calibrations are based on reference values on DM basis. Nonetheless, changes in the water content could lead to modification of atomic bonds, for example through the formation of hydrogen bonds. Changes in the water content might occur during sample storage and preparation, e.g. milling prior to the recording of the spectra. This should be considered when the calibrations of the present thesis are applied to new samples (Williams, 2009; Peiris *et al.*, 2016).

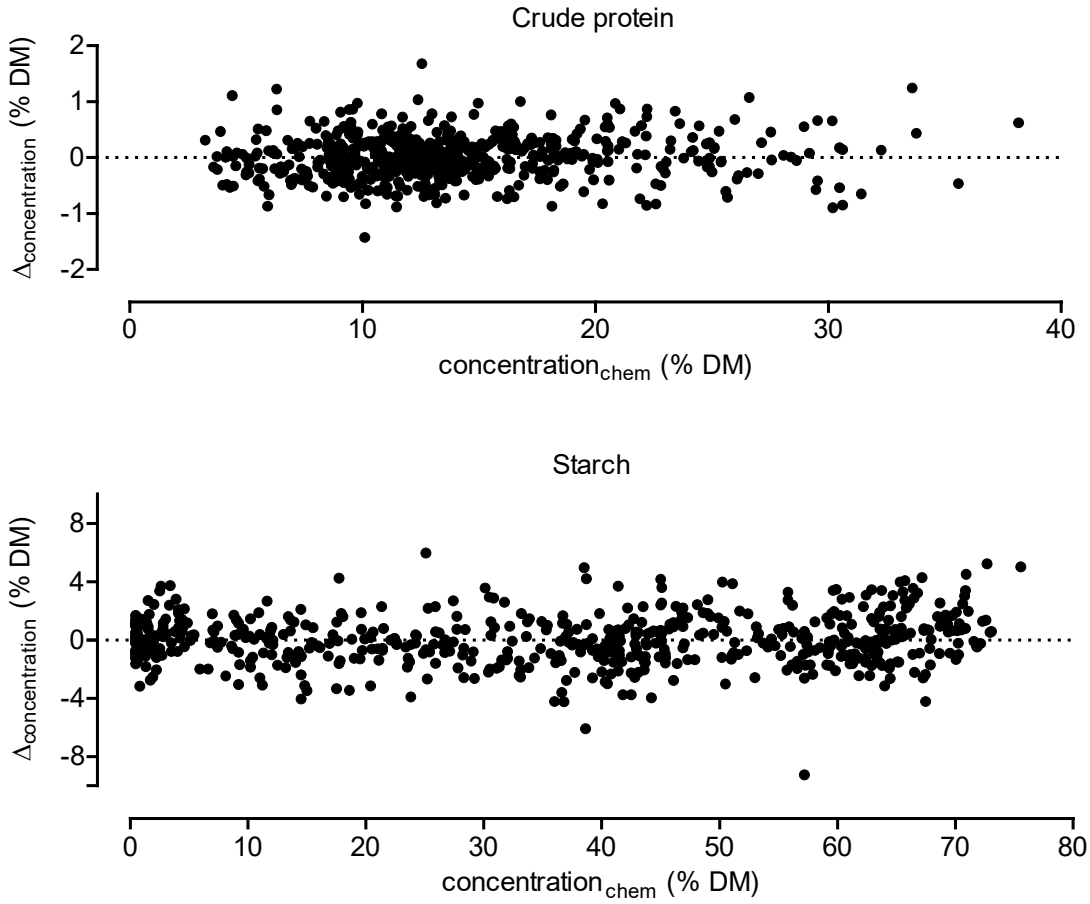

Figure 1: Difference between chemically analysed and NIRS-estimated (Calibration 17 from Tables 3 and 4, respectively, in Manuscript 3) crude protein and starch concentration ($\Delta_{concentration}$), in dependency of the respective chemically analysed concentration ($concentration_{chem}$).

3.1.2 Mathematical pre-treatment of near-infrared spectra and calibration development

Since NIRS utilises the energy absorption and reflection caused by the molecular structure of a sample, a direct interpretation of the peaks of a spectrum, as known from chromatograms for example, is not possible. Thus, different mathematical procedures are needed to interpret the spectral data. Prior to the calculation, a mathematical treatment of spectral data can improve the performance of calibrations. Based on the publications of Rinnan *et al.* (2009) and Tillmann (1996), standard normal variate (**SNV**) and different derivatives of the spectra were used as the mathematical treatment in the present study (Manuscript 3).

The SNV treatment leads to a reduction in the scattering effects of the reflected radiation. The calculation of derivatives includes the usage of points to smooth the spectral data. Depending on the points excluded to smooth the spectrum, a better

performance of calibrations can be achieved due to the depletion of the noise, but if a large number of data points are regressed, important information can also be lost (Agelet and Hurburgh Jr, 2010). Hence, the gap between two points that are used to smooth the spectrum should be as small as possible, but as large as required for an improved performance. The main aim of calculating derivatives is to remove the baseline (first-order derivative) and/or to remove the baseline and linear trends (second-order derivative) (Rinnan *et al.*, 2009). To illustrate the effect of mathematical pre-treatments, Figure 2 shows changes in the spectrum of a ground barley grain (Genotype 1) by using the SNV/detrend pre-treatment (Figure 2 (b)), followed by first- and second-order derivatives (Figure 2 (c) and (d)). As shown, the mathematical pre-treatment of the spectra leads to smaller, more defined peaks and eliminates the positive trend from low to high wavelengths. In the calibrations included in Manuscript 3, in addition to the SNV treatment the mean centre from all spectra within the calibration was subtracted from each spectrum. As stated by different publications—such as those by Agelet and Hurburgh Jr (2010), Rinnan *et al.* (2009), and Delwiche and Reeves (2004)—there is no universal spectral treatment that is suitable for every dataset; the best pre-treatment therefore needs to be found by trial and error for every calibration. Optimal pre-treatment depends on many factors, such as the spectrometer, the estimated constituent, and the sample itself. Thus, in the present thesis, different derivatives of spectra were used to calculate calibrations, and their performances are compared.

In this thesis, the derivations were varied between no derivation, first-order derivation, and second-order derivation. A derivative interval of 8 (the number of wavelength points over which the derivative is computed), with a smoothing of 8, was chosen for all calibrations in Manuscript 3. These settings were applied according to the recommendations of the manufacturer. For a description of other available methods for mathematical pre-processing and their advantages and disadvantages, it is referred to Agelet and Hurburgh Jr (2010), Brereton (2007), and Rinnan *et al.* (2009).

After the pre-treatment of the spectra, a regression of the reference data (CP and ST concentrations) and the processed spectra was computed by the partial least square (**PLS**) method. The best known and most often used methods are multiple linear regression (**MLR**), principle component regression (**PCR**), and PLS (Burns and Ciurczak, 2008; Agelet and Hurburgh Jr, 2010). Relatively new techniques involve artificial neuronal networks or support vector machines (Agelet and Hurburgh Jr, 2010).

Since detailed knowledge of techniques other than PLS is not needed to discuss the results of this work, for further information the reader is referred to Agelet and Hurburgh Jr (2010). Partial least square regression is still the most commonly used method. The advantage of PLS over MLR is the ability to deal with correlated wavelengths in the spectra. The CP and ST concentrations in cereal grains were negatively correlated. Hence, a negative correlation between wavelengths that are typical for the nutrients might have occurred in the CP and ST calibrations (Manuscript 3). Compared to PCR, PLS leads to models with higher precision and is faster than PCR. Possible negative points using PLS are the danger of overfitting a model and thereby including the noise of the spectra used for calibration in the model. This can be prevented by cross-validation and monitoring the residual error sum of squares. These values decline with every factor added to the model, but will rise if the number of factors lead to an overfitting (Agelet and Hurburgh Jr, 2010). This control against overfitting is included in the used software package (UCalibrate, version 3.0.0.23, Unity Scientific, St. Milford, MA, USA) and was additionally controlled by the plot of the residual errors of sum squares against the factors included in the model. Due to its advantages over MLR and PCR, PLS is used in the present study. A more detailed description of the settings and calculations used is included in Manuscript 3.

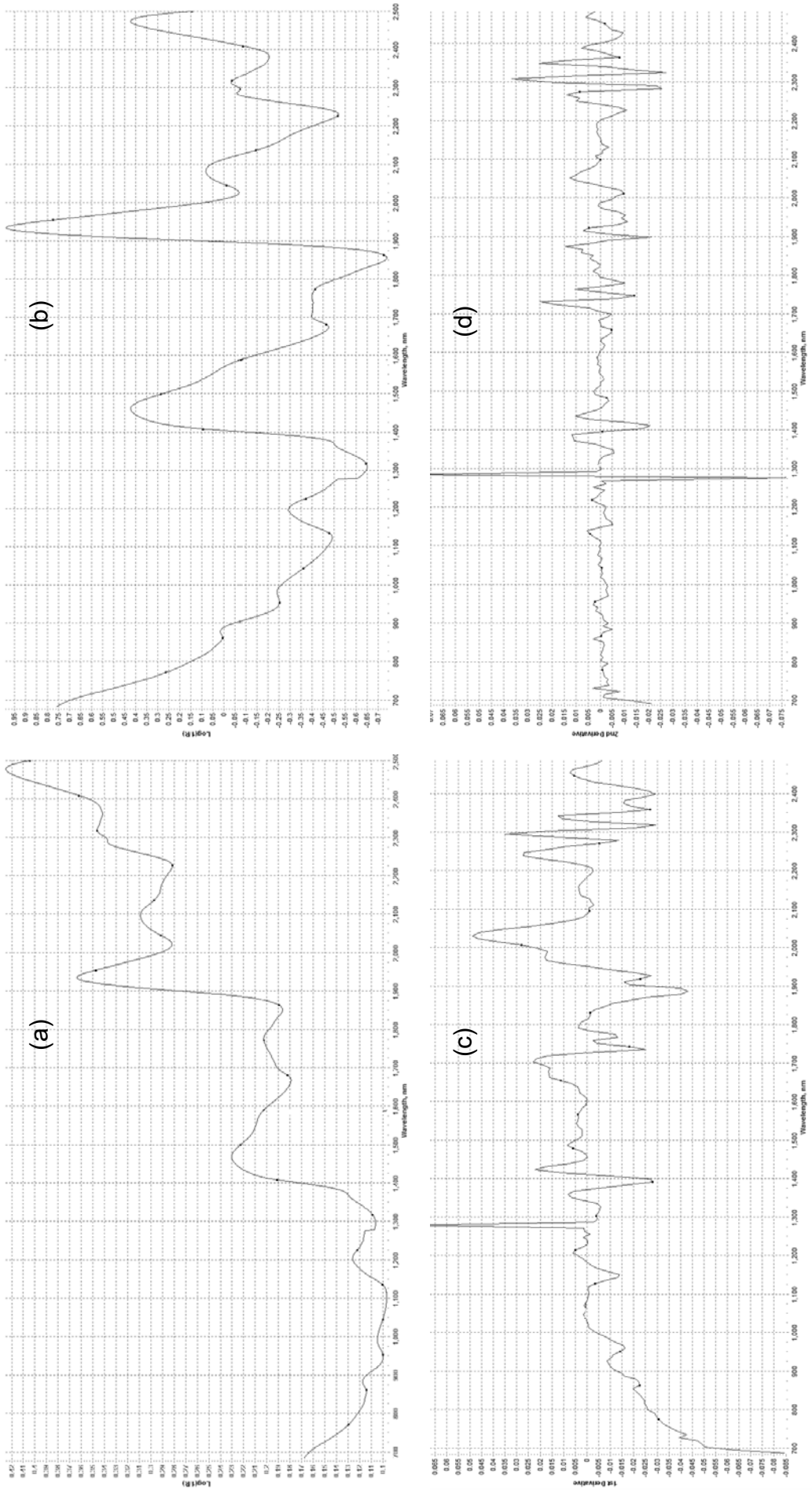

Figure 2: Absorbance (log 1/reflectance) of a barley grain sample before mathematical treatment (a), after correction for scattering effects and variation in particle size by SNV-detrend (b) and after the calculation of the first (c) and second (d) derivative (after SNV-detrend).

3.1.3 Calibrations for estimating crude protein and starch concentration by near-infrared spectroscopy

The CP fraction mostly consists of amino acids (**AA**) (Manuscript 3) and ST of glycosidic-joined glucose molecules. Both CP and ST consist of C-C, C-H, and O-H bonds. These groups/bonds can be detected by NIRS (Burns and Ciurczak, 2008). On a molecular basis (despite structural differences), only the molecules included in the CP analysis exhibit N-H bonds; the number of hydroxyl groups is higher in ST than in CP. As outlined in Chapter 3.1.1 of this thesis, the measured absorption in NIRS depends on the chemical structure. Theoretically, the absorption caused by N-H bonds should be included in the estimation of CP, but not in the ST calibration. As discussed in Manuscript 3, it is possible that the ST concentration might have been estimated indirectly by the CP concentration through the negative correlation between these constituents. Evidence for this assumption is given by the positive coefficient of the wavelengths at 1961 and 1969 nm (14.95 and 17.54, respectively) in the calibration for the CP concentration and negative coefficients of these wavelengths in the ST calibration (−87.76 and −89.71, respectively). This observation was also made at other wavelengths (data not shown). In accordance with this, Bokobza (1998) found that wavelengths near 1500 and 2000 nm were related to the concentration of primary amides. This can be seen as further evidence that the estimated ST concentration is influenced by the absorption of wavelengths connected to the functional groups of compounds included in the CP fraction. An influence of the CP concentration on the ST concentration would not necessarily negatively affect the performance of the calibration, but would underline the tight connection between the CP and ST concentrations in cereal grains.

NIRS calibrations were shown to be suitable for CP and ST prediction of samples when used for *in situ* studies without affecting the results, e.g. the ED (Manuscript 3). The a fraction and a+b fraction—either based on chemical or NIRS analysis of the samples and incubation residue of barley, rye, and triticale—are shown in Figure 3. For comparing the used analytical methods, degradation kinetics were fitted for each of the three animals used for *in situ* incubations (Manuscripts 1, 2, and 3) and the mean is plotted in Figure 3. With the exception of the aST of Triticale Sample 1, all differences between the estimated degradation parameters were within the SE of the estimate, and did not differ ($P > 0.1$, Wilcoxon Two-Sample Test using the NPAR1WAY procedure of the SAS software version 9.3 of the SAS system for Windows; SAS

Institute Inc., Cary, NC, USA). The aST of Triticale Sample 1 differed numerically between the chemically determined and the NIRS-determined concentrations by 9 percentage points. The difference of the aST for Triticale Sample 1 is high compared to other samples and is outside the SE of the estimate, but the difference can be explained by the SE of the laboratory method. To evaluate the difference between the estimates based on chemical analysis and NIRS prediction, the possible variation that might be caused by the error of the laboratory method should be considered (Tillmann, 1996). The a fraction represents the percentage loss from the incubation bags, which occurs without ruminal incubation during a washing step (0 h sample). If the chemically analysed concentrations for the grain and 0 h sample would be elevated and NIRS estimations reduced by the laboratory error (2.1% DM), the estimates for the aST fraction would no longer differ between chemically analysed and NIRS-analysed samples. This shows that the laboratory error and the variation between incubated bags within one animal and the variation between animals overlay the variation caused by using NIRS for sample analysis. Hence, NIRS is not seen as an error source in the *in situ* studies of this thesis and will not be discussed separately as a methodological error for *in situ* studies (Chapter 3.2). It is concluded that the use of NIRS for the prediction of CP and ST concentrations of *in situ* residues and grains is possible without influencing *in situ* degradation parameters or ED.

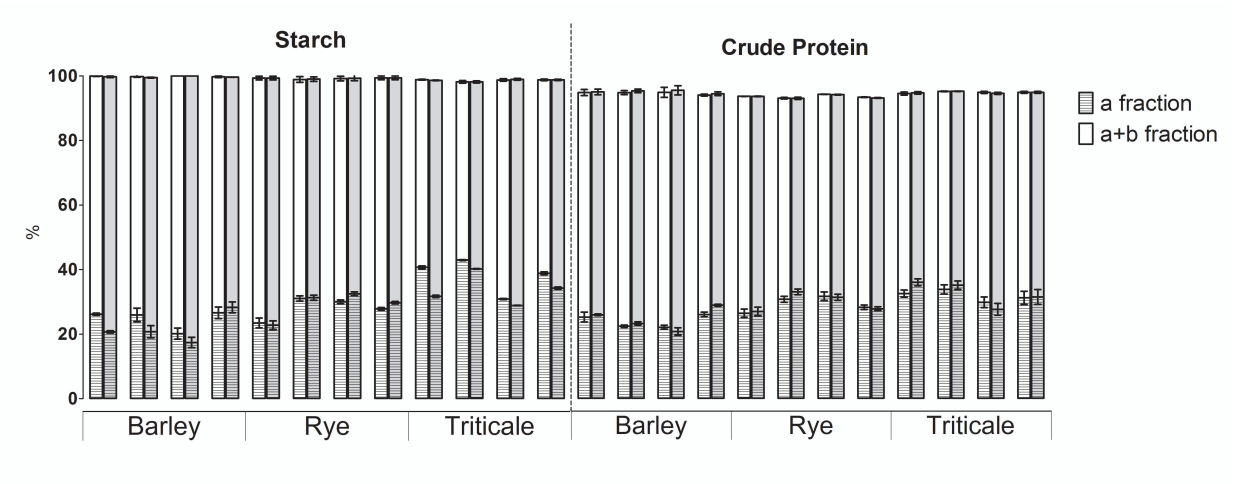

Figure 3: Washout fraction (a fraction) and maximal degradable fraction (a+b fraction) of starch and crude protein from *in situ* studies using chemical methods (white bars) or near-infrared spectroscopy (grey bars) for sample analysis (means and variation of n=3 animals; four different genotypes per grain species).

3.2 Factors influencing ruminal crude protein and starch degradation *in situ* and *in vitro*

Morphological properties of ST granules—as well as enzymatically in general and specifically ruminal ST degradation of cereal grains—have been discussed in many publications (Kotarski *et al.*, 1992; Jane *et al.*, 1994; Tester *et al.*, 2004a; Tester *et al.*, 2004b; Svihus *et al.*, 2005; Jane, 2006; Pérez *et al.*, 2009; Jane, 2009; Giuberti *et al.*, 2014; Moharrery *et al.*, 2014) and recently in the work of Seifried (2016). The work of Seifried (2016) includes an extensive discussion of *in vivo*, *in situ,* and *in vitro* techniques to study ruminal degradation. Hence, in order to prevent repetitions of the work of Seifried (2016) in terms of the methodological considerations of different techniques to study ruminal degradation and a detailed description of interactions between the protein matrix and ST granules in the endosperm of cereal grains, the reader is referred to Seifried (2016).

The saliva of ruminants is known to lack amylase. Thus, the dietary ST degradation is initiated in the reticulorumen (hereinafter called rumen) by microbial debranching enzymes, endo- and exoamylases, and glucosidases. They degrade ST to dextrins, maltose, and glucose (Mackie *et al.*, 2013). After the hydrolysis of ST to glucose molecules, glucose is further degraded to SCFAs acetate, butyrate, and propionate, and fermentation gases (CO_2 and—through further steps—to methane) (Mackie *et al.*, 2013). For a detailed description of ruminal glycolysis and the end products of the fermentation, the reader is referred to the diverse reviews available, e.g. Owens *et al.* (1998) Huntington (1997), or Ørskov (1986). Whilst amylolytic bacteria enzymatically degrade ST, entodiniomorphid protozoa can take up whole ST granules, transform them into a storage polymer, and gradually degrade the carbohydrate. With this step, entodiniomorphid protozoa are able to slow down ruminal ST degradation (Nozière *et al.*, 2010; Mackie *et al.*, 2013). The microbial breakdown of proteins to peptides and AA occurs mostly through cysteine and serine proteases. Ruminal microorganisms can utilise AA, peptides, and ammonia. Amino acids that are not utilised by the ruminal microorganisms can be further degraded to ammonia, fatty acids, and CO_2. Proteases are expressed by the most common ruminal microorganisms, such as *Prevotella ruminicola* or *Butyrivibrio fibrisolvens*. As already mentioned for ST granules, protozoa are also able to engulf particle-bound proteins or other microorganisms for N utilisation (Mackie *et al.*, 2013; Gresner *et al.*, 2014).

The ruminal CP and ST degradation of cereal grains are closely connected. This is because of the protein matrix surrounding ST granules in cereal grains and its ability to limit ST degradation (McAllister *et al.*, 1993; Seifried, 2016). Hence, the ruminal degradation of CP and ST are discussed together. There are a multitude of possible factors that might influence the rate and extent of ruminal ST degradation. One possible explanation for the differences between and within grain species could be differences in the structure of the ST granules.

3.2.1 Characteristics of starch granules

Differences in the density of ST granules were found to influence the accessibility of enzymes and thus the degradation of the granules, which might also apply to ruminal degradation (Tester *et al.*, 2006; Seifried, 2016). Starch is formed from the macromolecules amylose and amylopectin, which contribute approximately 22–28% and 72–78% of the ST respectively, but with high variation in grains. Amylose and amylopectin are α-glucans that consist of α- (1→4)-linked glucose molecules, with a few additional α-(1→6) bonds in amylose and more abundant α-(1→6) branches (approximately 5%) in amylopectin (Pérez *et al.*, 2009; Visakh, 2016). Starch in cereal grains is allocated in the plastids of plant cells as ST granules. Multiple amylopectin molecules lead to the specific shape of the ST granules. The α- (1→4)-linked exterior amylopectin chains form double helices that make up the so-called crystalline part of the ST granule. The crystalline parts of the granule alternate with amorphous regions, where the α-(1→6) branches are located within the amylopectin molecule. The alternating amorphous and crystalline regions of the granule (with different intermediate organisation structures) form the lamellae-like rings of the ST granule. Whether amylose also forms double helices and is located in the crystalline region or in the amorphous region of the granule is not clear (Smith, 2001a; Smith, 2001b; Tester *et al.*, 2004b). For further information about the organisation of ST granules, the reader is referred to Tester *et al.* (2004b), Nakamura (2015), and Pérez *et al.* (2009). The share of crystalline regions can be used to describe starches and is an often-reported characteristic referred to as crystallinity. Buléon *et al.* (1998) pointed out that data on crystallinity in literature varies strongly due to the used methods and ST hydration. It was shown that with an increasing amylopectin:amylose ratio and crystallinity, the enzymatic degradability of ST granules decreases. This could be due to the higher density of ST granules with high proportion of amylopectin, or to differences in the lipids and proteins connected with the respective carbohydrate molecule. Different studies

gave evidence that a higher amylopectin:amylose ratio or associated lipids and proteins on the surface of ST granules decrease ST degradation in non-ruminants and ruminants (Svihus *et al.*, 2005; Foley *et al.*, 2006; Giuberti *et al.*, 2014). On the other hand, Stevnebø *et al.* (2009) did not find an effect of the amylose content of barley ST on the *in vitro* ST degradation in rumen fluid. The discrepancies might be due to differences in the used incubation technique and the animal species. Since some of the studies showed effects of the amylopectin content on ruminal ST degradation, a corresponding influence might have occurred in the present thesis. Based on the analyses performed for the samples of this thesis, it was not possible to conclude whether the crystallinity differed between the samples. Thus, it could not be determined whether the crystallinity and/or the amylopectin:amylose ratio influenced the ruminal *in situ* ST degradation and *in vitro* gas production, but these factors should be considered in future studies.

Other characteristics of ST granules in cereal grains that were reported to differ between grain species are the shape of ST granules (e.g. spherical or disc shaped) and the distribution of ST granule sizes (bimodal vs unimodal) (Jane, 2009). As seen in Table 1, the ST granule size distribution described in literature varies strongly. Thus, no tendency can be derived towards a typical granule size. Variation in ST granule size distribution can be caused by differences in ST isolation, differences between genotypes, and differences induced by varying growing conditions (Tester, 1997; Lindeboom *et al.*, 2004; Gomand *et al.*, 2011; Makowska *et al.*, 2014).

Table 1: Form and diameter of starch granules from different grain species, as described in literature.

	Form	Big (µm)	Small (µm)
Barley	Symmetrical disc[1]	12–32[2]	2–3[2]
	Lenticular (A-type), spherical (B-type)[3]	15–25[3]	2–5[3]
		10–15[4]	2–4[4]
Rye	Symmetrical sphere[1]	22–36[2]	2–3[2]
	Lenticular/disc shaped (A-type), spherical (B-type)[3, 5]	10–40[3]	5–10[3]
		10–35[5]	2[5]
		30–31[6]	
Triticale	Symmetrical disc[1]	22–36[2]	5[2]
	Spherical[3]		1–30[3,#]
	Disc shaped (A-type), spherical (B-type)[5]	10–35[5]	2[5]

[1]Jane *et al.* (1994); [2]Lindeboom *et al.* (2004),[3] Tester *et al.* (2004b), [4] Tester (1997), [5] Ao and Jane (2007), [6] Gomand *et al.* (2011), [#] unimodal distributed

The ST granule size distribution was not measured in the sample set of the present study, but may have influenced enzymatical ST degradation, and hence, the results of the *in situ* and *in vitro* experiments (Dhital *et al.*, 2017). Based on the strong variation of granule sizes in literature and the described factors that might influence the granule size, a variation in the present sample set cannot be certainly assumed, but also cannot be ruled out.

Theoretically, the pores of the bags used in the present thesis (pore size of 50 µm), had (under the assumption of a quadratic pore shape) a diagonal of approximately 70.7 µm. Hence, ST granules might have been washed out from the bags after being released from the surrounding protein matrix. A fast release of ST granules from the protein matrix has been shown for barley and wheat (McAllister *et al.*, 1990), and therefore is also conceivable for triticale and rye. Similarly, Seifried *et al.* (2015) showed that undegraded ST of barley can be washed out from the bags. There are different reports regarding whether the granule size influences the enzymatical degradation of ST granules (Dhital *et al.*, 2017) or not (Stevnebø *et al.*, 2009). Hence, the size of the ST granules might also have influenced the degradation of the retained granules. An influence of the ST granule size distribution on the ruminal degradation of the ST granules might be given, but cannot be proved. The influence of other factors—such as the engulfment of ST granules by protozoa or the possible ruminal outflow of undegraded particles via the liquid phase (Manuscript 1)—should also be kept in mind when the role of the ST granule size on ruminal degradation is evaluated. Moreover, the enzymatic degradation of ST granules might be influenced by the surface of the granules (Giuberti *et al.*, 2014) as well as by the molecular structure of the ST (Svihus *et al.*, 2005). Amylases degrade ST granules of wheat from the inside to the outside by entering pores, which is why the occurrence of pores on the surface also probably influences the degradation rate of ST (Svihus *et al.*, 2005). Analogous to wheat, surface pores were found on the ST granules of rye and barley (Fannon *et al.*, 1992). Although the results of McAllister *et al.* (2006) and Stevnebø *et al.* (2009) suggest that the differences in ST granule structure and the amylose proportion of ST are not the main reason for the differences in ruminal ST degradation, variations in *in situ* ST degradation due to morphological differences of ST granules, and especially the concomitant losses of undegraded ST granules from the bags, cannot be ruled out. All in all, the influence of ST granule structure and size do not seem to be the main

reasons for variations within and between grain species in the present study, but might contribute to differences in losses of undegraded ST granules from the bags.

3.2.2 Protein matrix and grain hardness

Proteins in the endosperm of cereal grains can be divided into endosperm storage proteins and ST-granule-associated proteins. Endosperm storage proteins make up the major part of proteins in the endosperm and are located on the surface of the ST granules. Starch-granule-associated proteins occur on the surface of as well as inside the ST granules (Baldwin, 2001; Giuberti et al., 2014). Apart from endosperm storage proteins and ST, there are also minor components in the endosperm. These are associated with amylose and amylopectin molecules, contribute up to 1–3% of isolated ST, and are—in declining parts—lipids, proteins, phosphorus, and other mineral compounds (Berry et al., 1971; Robyt, 2008). The proteins of the endosperm form the protein matrix, which surrounds the ST granules and can thus influence ruminal ST degradation by preventing ST degradation by amylases (McAllister et al., 1993). The protein matrix is the most commonly mentioned characteristic that is assumed to influence the ruminal ST degradation of cereal grains. Based on their solubility in different solvents, proteins in cereal grains can be divided into albumins, globulins, prolamins, and glutelins (Osborne, 1907; Nikokyris and Kandylis, 1997). Glutelins and prolamins (endosperm storage proteins) show a lower ruminal degradability compared to the other protein fractions (Van Barneveld, 1999). For barley and rye, prolamins and glutelins are named differently—prolamins are called hordeins in barley and secalins in rye and triticale. Prolamins and glutelins are the main proteins of the endosperm, whereas albumins and globulins are located in the aleurone layer, bran and germ (Koehler and Wieser, 2013). As stated in Manuscript 2, protein fractions differ in terms of their AA patterns. Prolamins and glutelins are high in Pro and Glu, but low in Lys compared to albumins and globulins. Accordingly, the significantly negative correlation of the Lys concentration (g/16 g N) to the CP concentration could indicate a shift in the protein fractions towards the endosperm storage proteins—prolamins and glutelins—at the expense of albumins and globulins (Manuscript 2). No correlations between the ruminal in situ degradation characteristics of DM, CP, and ST, and AA concentrations that could indicate a change in protein fractions, were found within grain species. Based on the data of the present thesis, there is no apparent influence of different protein fractions on ruminal degradation. Hence, it can be assumed that variation in ruminal degradation within grain species was not due to differences in the protein

matrix. On the other hand, an influence of the protein matrix on the variation between grain species might be possible.

Starch granule damage

Svihus *et al.* (2005) stated, that the strength of bonds between ST granules and the protein matrix can lead to a different share of broken ST granules in ground cereal grains, which are more easily degraded as compared to intact ST granules. According to the studies reviewed by Svihus *et al.* (2005), differences in ST granule damage by grinding are the reason for a stronger increase of ruminal ST degradation of maize, compared to soft grains (Svihus *et al.*, 2005). Hence, more intact ST granules should have been found in ground rye samples, which are generally known for their rather soft endosperm texture (Bhave and Morris, 2008). According to the aforementioned theory, this would have been reflected in a slower and/or lower ruminal degradation of rye grains. In contrast to this hypothesis, rye showed a remarkably fast degradation in the present thesis (Manuscripts 1 and 2). Differences in ST granule damage therefore do not seem to be the reason for differences between grain species; however, the variation within one grain species might still be attributed to a different share of damaged ST granules. This might be due to differences in the regulation of grain hardness among the grain species.

In wheat, grain hardness is well-researched and determined by the absence or presence of puroindolins (Morris, 2002), which are proteins located on the surface of ST granules. Their absence leads to the hard endosperm texture of durum wheat. Orthologues of puroindolines were found in barley (hordoindolins), rye (secaloindolins), and triticale (secaloindolins) (Nadolska-Orczyk *et al.*, 2009). A connection between grain hardness and the presence of hordoindolin in barley grains could not be clearly proved. Other substances—e.g. β-glucans as main component of endosperm cell wall in barley—are also discussed as reasons for differences in grain hardness (Baik and Ullrich, 2008; Bhave and Morris, 2008). Accordingly, Nair *et al.* (2011a) described thicker cell walls in hard barley grains than soft barley grains. There is very little information about the influence of secaloindolins on the grain hardness of rye, possibly due to the low variation in the grain texture of rye (Bhave and Morris, 2008). In triticale, the influence of secaloindolins on grain hardness is also not completely understood. Studies in which the expression of secaloindolins was suppressed indicate that they do not influence the grain hardness in triticale (Bhave and Morris, 2008; Gasparis *et*

al., 2013). For a more detailed description of the role of puroindolins in grain hardness of wheat and other cereals, the reader is referred to Seifried (2016), Bhave and Morris (2008), and Morris (2002).

Although the determination of grain hardness in triticale grains is not clear, analogous to wheat, ground triticale and barley grains with a hard endosperm showed a higher share of damaged ST granules than soft grains of the respective grain species (Ramírez *et al.*, 2003; Nair *et al.*, 2011b). In barley grains, this is due to a tighter connection between the ST granules and the protein matrix in hard grains (Nair *et al.*, 2011a).

Different ratios of damaged ST granules would be reflected in the cST but not in the cCP. Accordingly, the coefficient of variation (**CV**) in cCP is lower than that in cST for rye (CV = 8% and 17%, respectively) and triticale (CV = 10% and 23%, respectively). For barley, cCP and cST varied to the same extent (CV = 14%, each). Typically, barley shows relatively little variation in endosperm structure, while triticale shows high variation (Bhave and Morris, 2008). This corresponds with the slightly stronger variation of c_{gas} that was observed for rye and triticale compared to barley. A higher variation in triticale and rye grains than in barley grains was also reflected in the higher variation in the falling number (barley: CV = 13%, rye: CV = 33%, triticale: CV = 55%) (Rodehutscord *et al.*, 2016).

Particle size distribution

Another hypothesis regarding the different degradation characteristics *in situ* and *in vitro* is based on the variations in grain hardness. The high cCP and cST *in situ* and c_{gas} of rye could be due to the reduced particle size of rye samples, as a soft grain, compared to harder grains (Manuscript 2). Accordingly, a lower particle size distribution for soft grains compared to hard grains has been demonstrated for barley (Nair *et al.*, 2011a; Nair *et al.*, 2011b). Other researchers report a higher share of small particles in the ground samples of hard barley grains compared to soft barley grains (Ding *et al.*, 2015) or did not find a significant influence of the grain hardness on particle size distribution (Ramsey *et al.*, 2001). This might be due to differences in the grinding step (Ding *et al.* (2015): ground to 6 mm; Nair *et al.* (2011b): ground to 0.5 mm). A transfer of the findings regarding the particle size distribution from other studies to the present thesis is—due to differences in the mill type and sieve size—hardly possible. Nonetheless, findings which show that samples with a higher share of small particles

exhibit higher *in situ* degradation underline the influence of the particle size on ruminal in situ degradation (Ding *et al.*, 2015). This theory could also explain the higher c value *in situ* and c_{gas} *in vitro* of rye, compared to barley and triticale. The gas production in the HGT is an indicator of the ruminal fermentation of feeds. The gas production of grains can be attributed mainly to the degradation of ST (Menke and Steingass, 1988). Hence, a slow ruminal ST and DM degradation, should be reflected in a slow *in vitro* gas production. Accordingly, c_{gas} was higher for rye grains than for barley and triticale grains (Manuscript 1). This is in accordance with ranking of the grain species regarding their cST, cCP, and cDM *in situ*. The high c_{gas} for rye matches the theory of a faster fermentation due to a higher surface:volume ratio caused by a reduced particle size (Giuberti *et al.*, 2014). A reduced particle size would also lead to higher primary and (assuming the same or a higher c for small particles) secondary particle losses *in situ* (Seifried, 2016). Hence, a high variation in particle size distribution between the samples would cause high variation in particle losses. The higher variability of *in situ* degradation characteristics compared to variations of *in vitro* gas production can be seen as an indication of the involvement of particle losses in the variability of *in situ* results. Contrary to this theory, the a fraction—and hence probably also primary particle losses—were not higher for rye than for the other grains. It cannot be determined whether, to what extent, and by what mechanisms the particle size distribution of the samples may have influenced the results of *in situ* and *in vitro* experiments in the present study.

Kernel density

A physical measurement for the strength of the endosperm compression is the kernel density, which takes the vitreousness and hardness of the grains into account. Vitreous kernels appear glassy and translucent and are denser and more compressed than non-vitreous, opaque kernels (Dowell, 2000; Topin *et al.*, 2008). Growing conditions mainly influence the vitreousness, whereas genetical information mostly influences the hardness and—to a small part—the vitreousness of cereal grains. Due to the similar growing conditions within one species, the biggest differences in kernel density are probably due to variation of grain hardness (Seifried, 2016). Accordingly, various studies confirm a positive correlation between grain hardness and kernel density of barley grains (Walker and Panozzo, 2016). The kernel density of the samples in the present thesis was measured using a pycnometer, as described by Correa (2002). The mean kernel density of the grain species is shown in Figure 4. The data for all samples

is listed in Annex 1. As seen in the boxplots, the mean kernel density decreased from barley (1.38 g/cm^3) over rye (1.33 g/cm^3) to triticale (1.30 g/cm^3), but overlapped between the species.

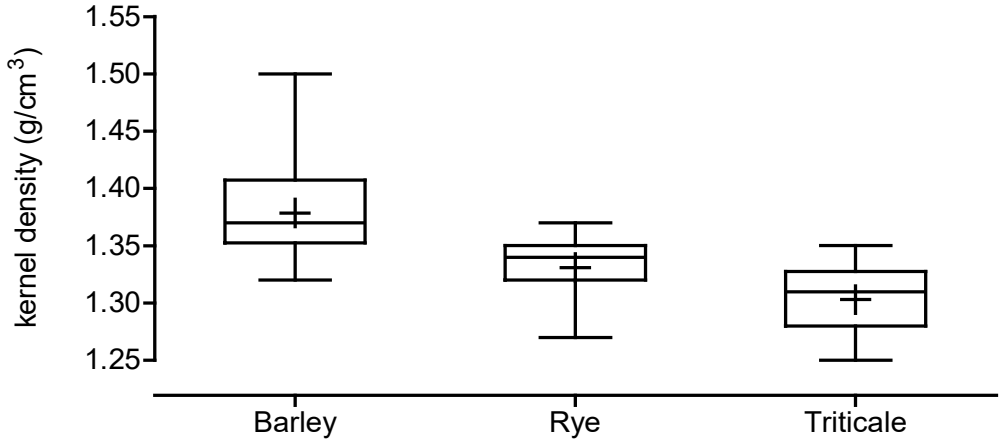

Figure 4: Mean (crosses) and variation of the density of barley, rye, and triticale grains (n = 20 genotypes per grain species; box = median, upper and lower quartile, whiskers = minimal, and maximal value for each species)

Correlations between the kernel density and results of the *in vitro* and *in situ* incubations are shown in Table 2. Similar to other sample characteristics (Manuscripts 1 and 2), the kernel density was significantly correlated to all measurements of *in situ* degradation and *in vitro* gas production, if samples from all cereal species were considered. Within grain species, the cST, EDST, and c_{gas} were significantly negatively correlated to the kernel density for barley and rye, but not for triticale. Correlations to all other ruminal degradation characteristics showed no consistent pattern over the grain species. Assuming that the kernel density mainly reflects the grain hardness, this could imply a slower ruminal degradation in hard barley and rye grains compared to softer grains from the respective species. This could be explained by a reduced enzymatic accessibility by the protein matrix or the aforementioned changes in particle size distribution. No significant correlations between the density and the concentration of Pro, Glu or Lys (g/16 g N), which would indicate a change in protein fractions were found. This reflects the results of correlation analysis between ruminal *in situ* degradation and the AA pattern (Manuscripts 1 and 2) and is in accordance with the hypothesis that fractions other than proteins might play a role in the determination of grain hardness, such as β-glucans in barley (Baik and Ullrich, 2008; Nair *et al.*, 2011b; Walker and Panozzo, 2016).

It is plausible to assume that all of the aforementioned theories regarding differences in endosperm structure may have led to differences in ST granule damage, particle size distribution, washed-out ST granules, or alterations in enzymatic accessibility, and hence, differences in ruminal degradation characteristics. Based on the discussed points, it seems possible that differences in grain hardness may have influenced the ruminal degradation in different ways; variation in particle size distribution, enzymatic accessibility, and loss of ST granules could explain the variation between and within grain species. Different levels of ST granule damage and variation in the protein matrix might have led to variations in the ruminal degradation within grain species, but this does not seem to be the reason for differences between the grain species. The degree at which one or different ways may have affected the results of this thesis is not determinable and it is assumed that interactions between different mechanisms led to the observed variation of ruminal CP and ST degradation.

Table 2: Significant Pearson´s correlation coefficients between the density of barley, rye and triticale grains (n = 20, each) and their ruminal *in situ* degradation and *in vitro* gas production kinetics.

		All	Barley	Rye	Triticale
DM	a[1]	−0.53***			
	b[1]	0.52***			
	a+b[1]	−0.39**	0.50*		
	c[1]	−0.48***			
	ED_8[2]	−0.57***			
CP	a[1]	−0.36**			
	b[1]	0.38**		0.53*	
	a+b[1]	0.28*		0.46*	
	c[1]	−0.33**			
	ED_8[2]	−0.47***			
ST	a[1]	−0.52***			0.50*
	b[1]	0.54***			−0.50*
	a+b[1]	0.58***	0.62**		
	c[1]	−0.53***	−0.53*	−0.64**	
	ED_8[2]	−0.64***	−0.46*	−0.52*	
Gp24[3]		−0.50***			
c_{gas}[4]		−0.32*	−0.49*	−0.59**	
b_{gas}[4]		−0.39**		0.47*	

***p<0.001; **p<0.01; *p<0.05

[1] from Equation 1 in Manuscript 1, with a = washout fraction (%); b = potentially degradable fraction (%); c = degradation rate (%/h); [2] from Equation 2 in Manuscript 1, ED_k = effective degradability at a ruminal passage rate of k = 8%/h, respectively; [3] Gp24 = Gas production after 24 h of incubation (ml/200 mg DM); [4] from Equation 3 in Manuscript 1, b_{gas} = potential gas production (ml/200 mg DM), c_{gas} = gas production rate (%/h)

Although the results of *in situ* and *in vitro* degradation and gas production lead to the same ranking of grain species, the cDM, cCP, and cST were at different levels compared to the c_{gas} (manuscripts 1 and 2). Seifried *et al.* (2016) used the same techniques that were used in this thesis and reported degradation rates for maize grains to be at the same level as the corresponding gas production rates. In accordance with the results in Manuscript 1 and 2, the differences between degradation and gas production rates were observed for wheat grains (Seifried *et al.*, 2017). Since the c_{gas} and the *in situ* c are similar for roughages and maize, the observed differences for soft grains might—at least partly—be due to an overestimation of c by particle losses during the *in situ* incubation of cereal grains (Seifried, 2016). In short, the reason gas production rates differ from *in situ* degradation rates cannot be certainly distinguished. Hence, it is concluded that the values obtained in the HGT and *in situ* studies should both be used to rank feedstuff regarding their feeding value and should not be seen as a reflection of the *in vivo* commodities.

3.2.3 Other possible influences on ruminal crude protein and starch degradation *in situ* and *in vitro*

Alkylresorcinols

As stated in Manuscript 1, the alkylresorcinol content of rye grains may have been involved in the remarkably high degradation rates of some rye genotypes. Alkylresorcinols are secondary plant components belonging to the phenolic lipids, which were shown to affect the metabolism of microorganisms and animals (Oishi *et al.*, 2015; Luís *et al.*, 2016). They are located between the pericarp and the testa (outer layer of the seed coats) of the grain. They act as a ‘barrier’ against microorganisms and occur in higher concentrations in rye than in other cereals (Bartlomiej *et al.*, 2012; Landberg *et al.*, 2014). Apart from the possible alteration of enzyme activities—as noted in Manuscript 1—alkylresorcinols may have influenced the *in situ* and *in vitro* degradation through changes in the microbial activity. The concentration of alkylresorcinols in rye grains may have led to a shift in the microbiota inside the bags during *in situ* incubations and the HGT towards ST-degrading, gram-negative bacteria through a reduced activity of protozoa (Seifried, 2016). Since entodiniomorphic protozoa can slow down ST degradation, a higher ST degradation rate might have occurred (Seifried, 2016). Since alkylresorcinols form an antimicrobial layer around the grain, rye grains may exhibit less protection mechanisms inside the endosperm, e.g.

in the form of a protecting protein matrix. Hence, when the outer layers are destroyed, for instance by grinding, the endosperm can be degraded relatively easily. This could be another reason for the high CP and ST degradation rates of ground rye grains. Whether alkylresorcinols play a role in rumen CP and ST degradation of cereal grains *in situ* and *in vitro*, and if these potential influences also exist under *in vivo* conditions, cannot be inferred by the results of this thesis. The same rye genotypes that were used in the present thesis were studied regarding the AA digestibility for laying hens and alkylresorcinols were also discussed as a possible reason for the variation found between the genotypes (Zuber *et al.* 2016). Especially in light of the fact that alkylresorcinols may also influence the feeding value of rye for other animal species, the influence of alkylresorcinols from rye on the ruminal degradation and intestinal digestion of nutrients should be a subject of future research.

Hectolitre Weight

In contrast to triticale and rye, the barley samples were hulled. The hulls in the barley samples led to a relatively low hectolitre weight (**HLW**, also called test weight, Figure 5) compared to triticale and rye (Andersson *et al.*, 1999; Rodehutscord *et al.*, 2016). Reports on the possible effects of the HLW of barley on the feeding value and/or performance of ruminants are not consistent. Grimson *et al.* (1987) and Mathison *et al.* (1991) investigated whether the HLW shows a connection with the performance of steers. Hunt (1996) concluded from these studies that the HLW appears to be connected to the animal performance for barley samples ranging within relatively low HLW, but not for samples with high HLW. Despite the inconsistent data, HLW is used by the food and feed industry to classify barley and high HLW is used as an indicator for good malting and feed quality (Fox *et al.*, 2009). As shown in Figure 5, the ED_8ST and the ED_8CP (Manuscripts 1 and 2) increased with a higher HLW of the grains, but regression analysis showed low coefficients of determination (**R^2**, R^2 = 0.49 and 0.46 for ST and CP, respectively) and SE of the estimates = 3.0 and 2.8, respectively. If the correlations between HLW and the ED were calculated for triticale together with rye grains, no significant correlation was found. If the data of Seifried (2016) was included in the correlation analysis (20 genotypes of wheat and maize, each), a slightly significant positive correlation (r = 0.201; P = 0.045) was found, only between ED_8ST and HLW. If the correlation was calculated only for soft grains (barley, rye, triticale, and wheat), ED_8ST and HLW were also found to be significantly positive correlated (r = 0.449; P < 0.001), but regression analysis revealed a very low coefficient of

determination (R^2 = 0.202) and a relatively high SE (SE = 3.403). Contrary to correlations that include different grain species, the HLW was negatively correlated to the ED_8ST within rye grains (r = −0.45, P = 0.032). Negative correlations between the HLW and the EDCP and EDST were also found for wheat and maize (Seifried, 2016). No significant correlations were found within barley and triticale grains. These results indicate that the HLW is connected to the ED, but is not an appropriate tool to reliably estimate ruminal *in situ* CP and ST degradation within the used dataset. This is in accordance with findings of Seifried (2016). The data of Fox *et al.* (2009) were used to calculate correlations between the HLW and *in situ* DM of barley grains. Similar to the results in this thesis, no significant correlation was found (P = 0.30, n = 35 barley grain samples).

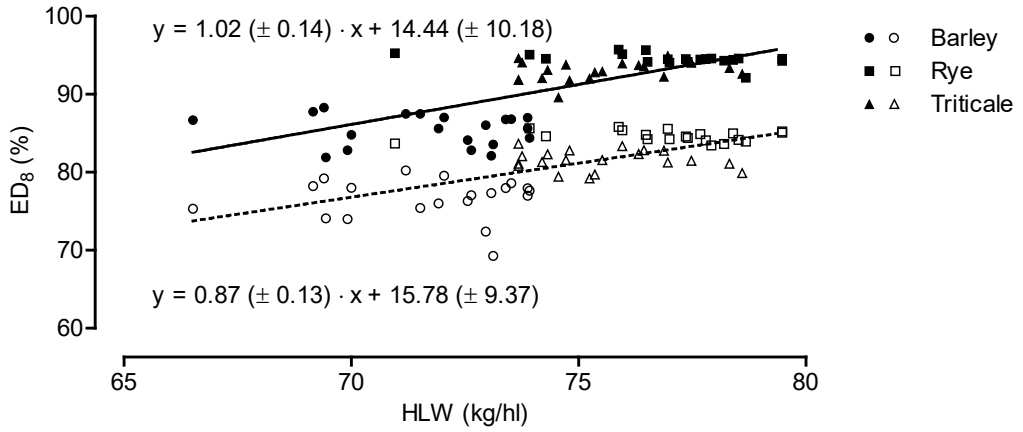

Figure 5: Effective ruminal *in situ* degradability at a ruminal passage rate of 8%/h (ED_8) of starch (filled symbols) and crude protein (open symbols) from barley, rye and triticale grains as function of the hectolitre weight (HLW) of the grains (n = 3 for ED_8; regression parameters ± standard error).

Non-starch polysaccharides

Further physical characteristics that were recorded include thousand-kernel weight, falling number, yield point, consistency index, extract viscosity, and shear rate (Rodehutscord *et al.*, 2016). Additional constituents analysed in the samples of the present thesis and not already discussed in the manuscripts include minerals (Ca, Fe, Mg, Mn, K, Na, P, Zn, As, Cd, Cu, and Pb), inositol phosphates, and—for a subset of samples (Barley Samples 1–8, all rye and triticale samples)—carbohydrate fractions (as published by Rodehutscord *et al.* (2016): glucose, fructose, sucrose, total sugars,

fructans; cellulose; concentration of total and soluble: β-glucans, arabinoxylans, total-non-starch polysaccharides; Klason Lignin). The correlations to results of *in situ* and *in vitro* incubations were calculated for all these physical characteristics and chemical constituents. Since the data for carbohydrate fractions of barely grains were limited, no separate correlations within barley grains were calculated for these characteristics. As for most other grain characteristics, correlations to *in situ* degradation and *in vitro* gas production were only significant if calculated for all samples, but no consistency in correlations was found within species (data not shown). For rye grains, the arabinoxylan concentration was significantly negatively correlated to the ED_5CP and ED_8CP ($r = -0.62$ and -0.45, $p = 0.004$ and 0.044, respectively) as well as the ED_5DM ($r = -0.54$, $p = 0.013$). In Figure 6, the ED_5CP is plotted in dependency of the arabinoxylan content of the rye grains. Although there is a negative correlation between the arabinoxylan content and the ED_5CP, the graph shows that the connection is weak and the low variation in the ED_5CP (3 percentage points) is not explainable by the arabinoxylan content. The ED_8CP and ED_5DM showed similar patterns. Since arabinoxylans are known to increase the viscosity of the digesta in pigs (Lærke *et al.*, 2015) and broilers (Annison, 1993), an influence on the rheological properties of the sample inside the bags—and hence on the *in situ* degradation—cannot be ruled out. But since ruminal fungi (Akin and Borneman, 1990), protozoa (Béra-Maillet *et al.*, 2005), and bacteria (Hespell *et al.*, 1987; Flint *et al.*, 1991; Mackie *et al.*, 2013) are known to synthesise xylanases, an influence on the ruminal degradation of cereal grains seems unlikely. Accordingly, there was no correlation between the arabinoxylan concentration and the ST degradation that would indicate a limitation of the ruminal degradation of rye grains. Hence, the correlations between the arabinoxylan concentration and the ED_5CP, ED_8CP and ED_5DM are not seen as causal relationships within the present dataset. Compared to the data from literature describing arabinoxylan contents for rye grains of 65–122 g/kg DM (Vinkx and Delcour, 1996), the variation in the present sample set was relatively small (74–96 g/kg DM). Hence, to certainly rule out an influence of arabinoxylans on *in situ* degradation, samples with a wider range in arabinoxylan concentration should be investigated regarding their influence on ruminal *in situ* degradation.

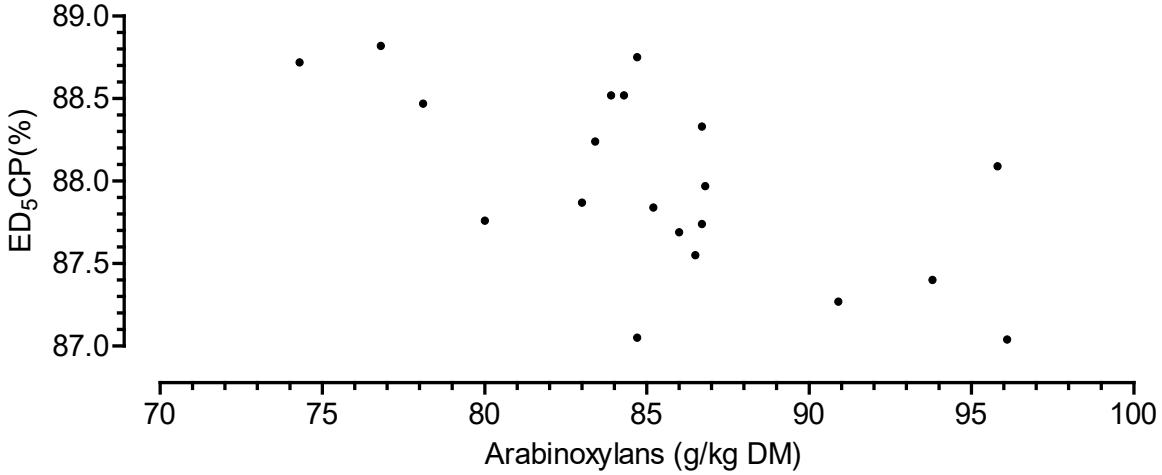

Figure 6: Effective ruminal *in situ* degradability (ruminal passage rate = 5%/h) of CP (ED_5CP) in dependency of the arabinoxylan concentration of rye grains (n = 20).

3.3 Utilisable crude protein at the duodenum

During ruminal fermentation, dietary CP is (partly) degraded while MCP is synthesised. Hence, the amount and composition of CP changes during ruminal fermentation. The digestion of MCP and UDP to AA and peptides and their absorption by the animal take place in the small intestine. Consequently, the requirement of the animal and the supply of the CP (and AA) are evaluated at the intestinal level. Although the nomenclature and exact definitions differ among different systems, the evaluation of feeds and the definition of animal requirements based on the post-ruminal CP or AA supply is consistent for many protein evaluation systems (e.g. National Research Council (**NRC**) (2001), GfE (2001), Lanzas *et al.* (2007), CVB (2012) and Tedeschi *et al.* (2015)).

According to GfE (2001), the CP supply of ruminants is evaluated on the basis of the uCP. For an extensive review of the German uCP system, the methods used therein, and the derivation of the requirements, the reader is referred to Steingass and Südekum (2013) and GfE (2001). In brief, the CP that reaches the duodenum consists of MCP, UDP, and endogenously secreted CP. Utilisable CP consequently includes the MCP and UDP corrected for endogenous losses (Lebzien and Voigt, 1999; Steingass and Südekum, 2013). In routine feed evaluation, the UDP and the uCP can be predicted through a modification of the HGT or by utilising the data from *in situ* studies. These estimation methods were established using the data of extensive *in vivo* studies as reference (Steingass and Südekum, 2013).

The *in vivo* measurements of the uCP and the portion of MCP and UDP can be conducted with ruminal and duodenal or abomasal-fistulated animals. In these *in vivo* studies, the flow of uCP can be determined by a total collection of the digesta or using markers. For marker-based studies, different markers are available (e.g. tris-(1,10-phenanthroline)-ruthenium(II) chloride (^{103}Ru-phen) for the solid and ^{15}CrEDTA for the liquid phase) (Faichney, 1992; Firkins *et al.*, 1998; Faichney, 2005). The amount and portion of microbial CP can be calculated by the additional use of internal markers like DNA (Deoxyribonucleic acid) or external markers like ^{15}N (Broderick and Merchen, 1992). Although these methods are based on *in vivo* measurements, all of them are connected to certain errors—such as the unequal distribution of the markers (Faichney, 2005)—and are indirect measurements of actual *in vivo* commodities. Since abomasal and duodenal fistulation often leads to complications and underlies strict

legal regimentations, alternative methods are mostly used. A method that allows *in vivo* estimations of the uCP without duodenal or omasal fistulation is the digesta collection from the omasum via a rumen cannula. However, the errors mentioned for the sample techniques utilising omasal- or duodenal-fistulated animals (usage of markers and sampling) might also occur when this technique is used (Steingass and Südekum, 2013; Huhtanen *et al.*, 1997). Another alternative, which is independent of the fistulation of animals, is the estimation of the *in vivo* MCP synthesis from the urinary excretion of diaminopimelic acid, purines, and purine degradation products (allantoin and uric acid). However, reports about the quality of the estimations differ (Broderick and Merchen, 1992; Fujihara and Shem, 2011; Stentoft *et al.*, 2015).

3.3.1 Estimation of the utilisable crude protein at the duodenum *in situ* and *in vitro*

In the present thesis, two different estimation approaches—based on the *in situ* UDP and *in vitro* method—are compared. The used methods based on *in situ* UDP differ in the estimation of the MCP synthesis: the estimation based on GfE (2001) uses the *in vitro*-estimated ME of the feed to estimate the MCP synthesis, in the second method, the MCP synthesis is estimated from the *in situ*-fermented organic matter (**fOM**) (Lebzien and Voigt, 1999). The comparison showed significant differences between the estimation methods (Manuscript 2). In Manuscript 2, it was concluded that the use of a ME estimate that does not change with the ruminal passage rate probably does not reflect ruminal conditions correctly. However, Schwab *et al.* (2005) compared different protein evaluation systems and confirmed the good performance of the German system. The equation they used differed from those used in the present thesis, but the ME of the feed was utilised in both equations to estimate the MCP.

A more variable estimation of MCP was performed in the present thesis by utilising the fOM. The amount of MCP formed per kg of fOM varies widely in literature. The NRC (2001) summarised the literature and reported values of 75–338 g MCP per kg fOM. Other reviews described values of 63–313 g MCP per kg fOM (Stern and Hoover, 1979; Lebzien and Voigt, 1999). Tamminga *et al.* (1994) reported, for the Dutch protein evaluation system, a value of 150 g MCP/kg fOM, while the French system assumes a MCP synthesis of 154 g/kg fOM. Although other researchers reported MCP synthesis at a comparable level to the one used in Manuscript 2—e.g. 187 g MCP per kg fOM as reviewed by Jarrige and Alderman (1987)—the overall strong variation of MCP

synthesis in literature has led the NRC (2001) to the conclusion that the fOM seems unsuitable to estimate the MCP synthesis and other factors need to be considered. Accordingly, Oldham (1993) concluded that there is variation in MCP synthesis per fOM, which is hardly predictable. Boguhn *et al.* (2006) reported a high variation in the efficiency of ruminal MCP synthesis in an *in vitro* approach, depending on the composition of the total mixed ration. The group around Lebzien and other researchers stated, that the estimation from MCP separately from UDP might lead to higher errors than the direct estimation of uCP by more complex mathematical models (Clark *et al.*, 1992; Lebzien *et al.*, 1996; Lebzien and Voigt, 1999; Flachowsky and Lebzien, 2006). This error source might apply to both the *in situ*-based methods used in this thesis and hence would be an advantage of the *in vitro* method over the *in situ*-based methods. A more detailed list of the advantages and disadvantages of different methods to predict or determine the uCP is included in the work of Flachowsky and Lebzien (2006).

Another point that needs to be considered is the applicability of estimation methods to different samples. Basically, there are two different approaches to modelling ruminal protein degradation and MCP synthesis—mechanistic and empirical models (Dijkstra and Bannink, 1999). Extensive reviews over different models to estimate the MCP and/or the uCP have been published, amongst others, by Dijkstra and France (1996), Dijkstra *et al.* (1998), and Dijkstra and Bannink (1999). In short, empirical models are established by regression using experimental data, which leads to a restriction on their applicability to the conditions (e.g. samples and animals) during data collection. Since all models of the present thesis can be assigned to empirical models, this limitation needs to be considered for all of them. Mechanistic models are more complex, but also applicable to a broader range of data (Dijkstra and Bannink, 1999). Dijkstra *et al.* (1998) concluded, that mechanistic models lead to a better estimation compared to models based on experimental data and noted that the type of substrate as well as the ruminal microbiota might influence the MCP synthesis, and should therefore be considered in models. Firkins *et al.* (1998) concluded, that—based on the currently available data—empirical models should be used rather than mechanistical models, although mechanistical models are promising for certain questions. A detailed insight of the different models used in different countries is given by Tedeschi *et al.* (2015). More recently, models have been published by Petruzzi *et al.* (2002) and Van Duinkerken *et al.* (2011). The high number of factors included in these models are typical for

mechanistic models, and might represent a limitation in the application of these models to routine feed evaluation.

Various publications described factors that influence the ruminal MCP synthesis, including for example the feeding level/feed intake (and therefore the ruminal passage rate), the synchrony of ruminal protein and carbohydrate fermentation, the adequate availability of micronutrients (e.g. sulphur), and the formulation and quality of the ration compounds (Stern and Hoover, 1979; Dewhurst *et al.*, 2000; Steingass and Südekum, 2013). If the various factors that might influence ruminal MCP synthesis and the variations in literature regarding MCP synthesis per fOM are considered, it appears questionable whether it is appropriate to estimate the contribution to MCP synthesis of a single feedstuff based on its fOM or ME. A consideration of interactions between different components of a ration seems more correct, but would lead to highly complex calculation models (e.g. the NorFor Model). The methods used in the present thesis enable the fast and easy evaluation of feeds for ruminants. Considering more influencing factors might lead to a more accurate prediction of uCP, but the advantages of an easy interpretable and applicable value would no longer be there. Hence, based on the current knowledge and the need for further improvement of mechanistical models, the use of empirical models seems reasonable.

A possible future opportunity to easily estimate the uCP at the duodenum might be the NIRS. Near-infrared spectroscopy techniques have already been used to estimate different feeding values, such as the protein fractions of the Dutch feed evaluation system, protein that escaped ruminal degradation and fOM, as described by De Boever *et al.* (2003), or the EDCP of herbs (Waters and Givens, 1992). Near-infrared spectroscopy is similar to empirical prediction models, which is why it is only suitable for a defined range of values and defined sample material. Hence, the prediction of input parameters for mechanistic models by NIRS would be an alternative to sustain the broad applicability of mechanistic models and minimise the analytical effort required. However, the accuracy of the estimation methods for uCP used in this thesis needs to be evaluated by future studies.

3.3.2 Intestinal digestibility and amino acid pattern of the utilisable crude protein at the duodenum

Like non-ruminants, ruminants are unable to synthesise essential AA and are therefore dependent on an adequate supply of these AA at the small intestine from the MCP or

UDP. The uCP estimation methods used in the present thesis do not consider the intestinal digestibility or the AA pattern of the uCP. Feed evaluation on the basis of praecaecal-digestible AAs is applied for pigs and chicken (Stein *et al.*, 2007; Ravindran *et al.*, 2017). Since the AA requirement of dairy cows is not yet clearly defined, feed evaluation on the basis of AAs is difficult (NRC, 2001; Flachowsky and Lebzien, 2006). Based on the metabolisable protein (postruminally digested true protein and the intestinally absorbed component AA (NRC, 2001)) values for the optimal amount of the two first limiting AAs Met (2.4-2.5% of metabolisable protein), and Lys (7.2% of metabolisable protein) for dairy cows that were fed maize-based diets were published (NRC, 2001; Schwab, 2012). The optimal ratio of Lys:Met in the metabolisable protein was reported to be 3:1 for dairy cattle (NRC, 2001; Schwab, 2012), but further research is needed to provide stable requirement values for dairy cows on the basis of the intestinally available AAs (NRC, 2001). A detailed discussion of AA metabolism—including the consequences on the AA requirement for dairy cows—can be found in Arriola Apelo *et al.* (2014).

Like the uCP, the AAs at the duodenum consist of a microbial synthesised part and a feed-derived, ruminally undegraded part. Although the AA pattern of ruminal bacteria might differ (Clark *et al.*, 1992; Hildebrand *et al.*, 2011), the AA pattern of the MCP at the duodenum is reported to vary within a relatively small range (Steingass and Südekum, 2013). More recent findings showed differences in the ruminal microbiota composition through various influencing factors (Lengowski *et al.*, 2016; Paz *et al.*, 2016), but the variation of the microbial community does not seem to be reflected in the AA pattern of the MCP; hence, mean values for the AA concentration in the MCP seem applicable (Steingass and Südekum, 2013). As already highlighted, the amount of MCP synthesised varies in dependency of different factors, and hence seems to play a more important role than changes in the relatively constant composition of MCP. Methodologically, the intestinal digestibility of UDP and AAs can be determined *in vivo, in situ,* or *in vitro*. Since *in vivo* methods are not feasible in standard feed evaluation, the *in situ* mobile bag technique or *in vitro* techniques are mostly used (Calsamiglia *et al.*, 2010). A deeper insight and comparison between different available methods is given by Calsamiglia *et al.* (2010). Steingass and Südekum (2013) concluded, that the benefits of a deeper knowledge of the AA pattern and digestibility do not seem to be sufficient to justify extensive *in vivo* studies.

Unlike the AA pattern of the MCP, the AA pattern of the UDP varies strongly. Differences between the AA pattern of the feed and the UDP complicate the evaluation of the UDP fraction in terms of the AA composition (Steingass and Südekum, 2013). Contrary to the German protein evaluation system, which assumes a constant intestinal digestibility of the UDP of 85% for all feedstuffs (GfE, 2001), Prestløkken and Rise (2003) showed, that the intestinal digestibility of AAs differs in the UDP from barley. In the present study, UDP concentrations of cereal grains (13–38 g/kg DM including values for ruminal passage rates of 5 and 8%/h, Manuscript 2) and the contribution of UDP to the estimated uCP were low (Manuscript 2). Hence, the contribution of undegraded feed AAs from cereal grains to the total AAs at the duodenum is presumably at a similarly low level, and the impact on the AA supply to the animal seems rather small. For a detailed overview of the UDP and its digestibility, the reader is referred to Steingass and Südekum (2013) and Arriola Apelo *et al.* (2014). As a consequence of the low UDP/ruminally undegraded AAs from cereal grains, the major part of uCP and AAs at the duodenum are of microbial origin. Hence, a proper evaluation of microbial CP and AA synthesis and their intestinal digestibility is needed and should be part of the future research.

However, the data regarding the uCP and ruminal degradation characteristics gained in the present thesis can be used within the German protein system and might also be adapted to future systems that are based on the AA availability for the animal, for example by the application of factors for the share of AAs on the uCP or the intestinal digestibility of AAs (Steingass and Südekum, 2013).

3.4 Estimation of ruminal in situ degradation characteristics by near-infrared spectroscopy

To minimise the number of animal trials and provide a quick method for the characterisation of different grain batches in ration formulation, different methods to predict the ruminal degradation parameters are available. Most approaches are based on the prediction of degradation parameters from the chemical or physical characteristics of the grains (Lanzas *et al.*, 2007; Seifried *et al.*, 2016; Seifried *et al.*, 2017). Another approach is to estimate the feeding value using NIRS. In Manuscript 3, it was shown that it is possible to estimate the EDCP and EDST of cereal grains from their NIR spectra. For a better interpretation of the performance statistics, DIN EN ISO 12099:2016-02 (DIN-Normenausschuss Lebensmittel und landwirtschaftliche Produkte (NAL), 2016) supposed to plot estimated against reference values. Accordingly, the NIRS-estimated ED values are plotted against the corresponding experimentally obtained ED in Figure 7. The graphs illustrate the good performance of the calibrations, but also visualise the need for an expansion of the dataset with values between 60 and 80% of ED_8CP and ED_8ST. The calibrations cover ED values of approximately 50–85% for CP and up to 95% for ST, with considerable differences in the ED between the grain species. To enable the application of the calibrations under practical conditions, more variable samples and more feeds should be included.

As discussed in Chapter 3.1, the absorption measured in NIRS is based on the chemical and physical structure of the sample. The chemical constituents of a sample can be estimated by the excitement of chemical bonds and, therefore, the specific absorption of radiation. Under the assumption that the ED is influenced by the physical and chemical properties of a grain (Philippeau, 1999; Seifried, 2016) the prediction of ED by NIRS might follow the same path as the prediction of ED by chemical analysis. Estimations of ruminal degradation by NIRS can therefore be seen as indirect estimation via chemical constituents and would hence be comparable to ‘classical’ regression methods of prediction using analytical characteristics. In the calibrations developed in the present thesis, the samples were finely ground before spectra recording. An influence of the physical properties of the whole grain therefore seems unlikely. The development of a calibration using intact or coarsely ground cereal grains might also take the structure of the endosperm and thereby its physical properties into

account. Dowell (2000), for example, demonstrated that it is possible to predict the vitreousness of whole durum wheat kernels by NIRS. Since different studies showed a connection between the physical properties of cereal grains and their ruminal degradation, this might improve the calibration performance. On the other hand, wider variation in physical properties may also lead to a less favourable performance (Bokobza, 1998; Davies, 2005) and the utilisation of whole grains for NIRS estimations increases the difficulty of generating a homogeneous, representative subsample. Further studies are needed to evaluate how the use of non-processed grains affects the performance of the established NIRS calibrations.

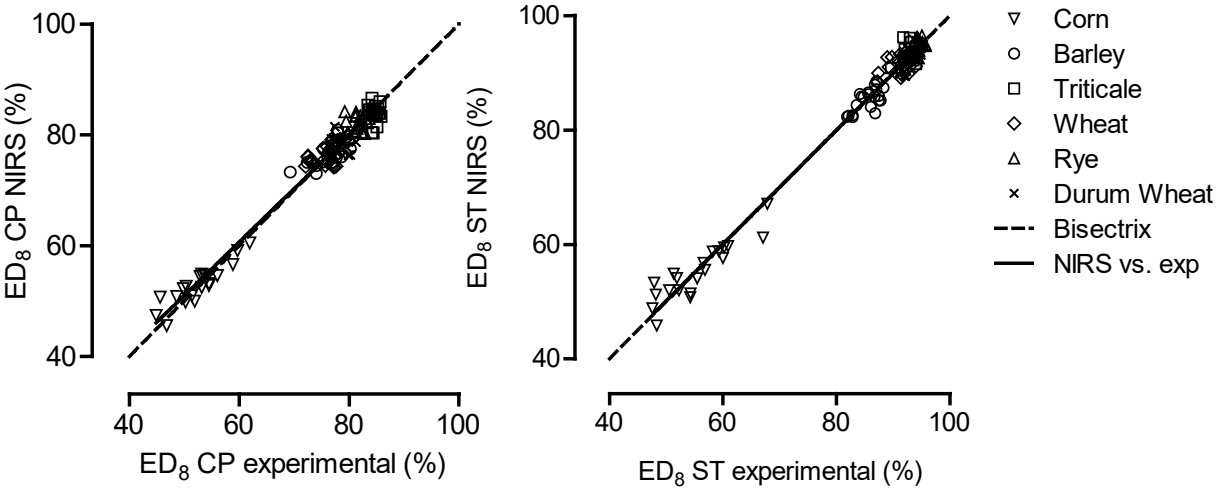

Figure 7: Experimentally determined vs. near-infrared spectroscopically estimated effective ruminal crude protein and starch degradability at a ruminal passage rate of 8%/h (ED_8CP and ED_8ST, respectively) of cereal grains.

The similarity between the prediction of the ED by NIRS or through chemical and physical analysis is reflected in the performance of the calibrations. Recently published equations for the prediction of the ruminal degradation of wheat grains from diverse chemical constituents and *in situ* DM degradation had an adjusted R^2 of 0.99 and 0.80 and a RMSE of 0.25 and 0.94 for ED_5ST and ED_5CP respectively (Seifried *et al.*, 2017). If only chemical constituents—including AAs—, were used, the equations yielded an adjusted R^2 of 0.70 and 0.72, and a RMSE of 1.10 and 1.34 for ED_8ST and ED_8CP, respectively (Seifried *et al.*, 2017). Equations for maize that only included chemical and physical parameters showed an adjusted R^2 of 0.76 and 0.91 and a RMSE of 2.53 and 1.26 for the corresponding ED. An inclusion of parameters of *in vitro* gas production and *in situ* DM degradation led to high adjusted R^2 of 0.99 for ED_8ST and ED_8CP and

a decrease in the RMSE to 0.46 and 0.39, respectively (Seifried *et al.*, 2016). Offner *et al.* (2003) published equations to predict the ED_6ST only from the *in situ* a-fraction of different cereal grains. This led to a less favourable performance (R^2=0.61) than the results of Seifried *et al.* (2016) and Seifried *et al.* (2017) and the NIRS calibrations established in the present thesis.

Effective degradability estimation equations utilising the chemical and physical grain characteristics were also calculated on the basis of all the grains studied within the GrainUp project regarding their ruminal degradation. To obtain the equations for ED_8CP with a comparable performance like NIRS calibrations data for *in vitro* gas production after eight hours, aNDFom concentration, ADFom concentration, polarimetrical determined ST concentration, and *in vitro* gas production after 24 h of incubation were necessary (adjusted R^2 = 0.90, RSME = 3.89). The characteristics used to establish an equation for estimating ED_8ST with the most desirable performance were: *in vitro* gas production after eight hours, aNDFom concentration, *in vitro* gas production after 24 hours, ADFom concentration, and crude fat concentration (adjusted R^2 = 0.94, RMSE = 3.71). In both equations, most of the variance of ED_8 was explained by the *in vitro* gas production after eight hours (partial R^2 = 0.87 for ST and 0.78 for CP) (Seifried, personal communication), which underlines the connection between *in vitro* and *in situ* results. The NIRS calibrations for EDCP and EDST established within this thesis (Manuscript 3) showed a performance that is comparable to that of the equations from Seifried *et al.* (2016) and Seifried *et al.* (2017), if all available data was used for multiple regression.

In addition to the calibrations included in Manuscript 3, DM *in situ* degradation parameters, ED_5DM and ED_8DM, were estimated using NIRS. The used datasets are shown in Table 3. As for the other calibrations, the samples were split into two groups—one group to calculate the calibrations and a second group to act as an independent validation sample set to describe the performance of the estimation. According to the calibrations included in Manuscript 3, values in the calibration and validation datasets had the same range. The division in calibration and validation datasets was carried out as described for the other calibrations (Manuscript 3).

Table 3: Number (n), mean, range, and coefficient of variation (CV (%)) of *in situ* dry matter (DM) degradation parameters from six different cereal grain species used for calibration and validation (data from Krieg *et al.* (2015), Seifried *et al.* (2016), Seifried *et al.* (2017), Krieg *et al.* (2017a) and Krieg *et al.* (2017b)).

	Calibration					Validation				
	n	Mean	Min	Max	CV	n	Mean	Min	Max	CV
DM										
a (%) [1]	85	27.5	16.3	39.9	19.9	30	27.3	16.6	39.4	21.1
b (%) [1]	85	66.2	52.6	83.7	10.6	30	65.6	54.1	83.5	10.9
c (%/h) [1]	85	43.0	4.8	102.8	56.6	30	42.9	4.8	98.0	56.9
ED_5 (%) [2]	85	81.6	57.6	90.0	10.8	30	82.0	57.7	89.5	10.6
ED_8 (%) [2]	85	77.3	48.0	88.3	14.4	30	77.6	48.2	88.2	14.4

[1] from Equation (2) in Manuscript 3, with a = washout fraction; b = potentially degradable fraction; c = degradation rate; [2] from Equation (3) in Manuscript 3, ED_k = effective degradability at a ruminal passage rate of k = 5 or 8%/h, respectively

The performance of calibrations that were considered most suitable (based on the evaluation criteria used in Manuscript 3) are shown in Table 4. As already seen for calibrations of the ruminal *in situ* degradation of CP and ST, ED_5DM and ED_8DM was estimated with a high R^2 and relatively low error, but the degradation parameters showed higher error measurements and lower R^2. An estimation of degradation parameters would enable the prediction of the ED for different ruminal passage rates without the need for specific calibrations for every ruminal passage rate. Although cDM was estimated with a satisfying accuracy, there was a negative estimate for a maize sample with a low experimentally obtained cDM (5.6%/h). The difference between the NIRS-estimated and experimentally obtained value was not noticeably high and the spectral data showed no remarkable deviation from other spectra. Hence, errors from spectra recording could be excluded as the reason for the negative estimate. To overcome the problem of negative estimations for cDM, cCP, and cST (Manuscript 3), an expansion of the calibrations might be useful.

The ED_5DM and ED_8DM calibrations showed a slightly higher SE of prediction (**SEP**) and lower R^2 in the validation step than the corresponding calibrations for CP and ST. A possible explanation for the better performance of ED calibrations for CP and ST might be the correlations between the experimentally determined EDCP and EDST and the respective concentration of the nutrient. No correlations were seen between the EDDM and the ST or CP concentrations. This would indicate a mutually indirect contribution of CP or ST concentrations for the estimation of the ED. Hence, it appears possible that the spectral data used for estimating the concentration of the nutrients are also involved in the estimation of the corresponding ED.

Table 4: Performance of calibrations for *in situ* dry matter degradation kinetics of cereal grains. Selection by the R^2 of validation, SEP (standard error of prediction), RPIQ (Ratio SEP to the inter-quartile distance of validation samples) and the bias of the validation. Cross-validation groups: 6.

	Settings		Calibration				Cross-Validation		Validation					
	Wavelength (nm)	D,G,S	Factors	Samples Available/ used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept	RPIQ
a (%) [1]	730-2450	0,8,8	11	85/85	2.80	0.74	3.63	0.53	3.11	0.72	−0.04	1.00	−0.08	2.6
b (%) [1]	730-2450	2,8,8	6	85/85	2.92	0.83	3.74	0.70	3.00	0.83	−0.82	1.01	-1.64	2.8
c (%/h) [1]	680-2500	1,8,8	14	85/85	4.22	0.97	8.65	0.87	8.09	0.89	0.15	1.00	0.30	3.8
ED_5 (%) [2]	1250-2450	1,8,8	13	85/85	1.21	0.98	2.29	0.93	2.58	0.91	0.00	1.00	0.00	3.1
ED_8 (%) [2]	680-2500	0,8,8	15	85/85	1.77	0.97	3.10	0.91	2.88	0.93	−0.02	1.00	−0.05	3.3

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

[1] from Equation (2) in Manuscript 3, with a = washout fraction; b = potentially degradable fraction; c = degradation rate

[2] from Equation (3) in Manuscript 3, ED_k = effective degradability at a ruminal passage rate of k = 5 or 8 %/h, respectively

Even though the performance of NIRS calibrations of the present thesis were at a comparable level to ‘classical’ estimation methods, the estimation by NIRS has various advantages over the prediction based on chemical and physical traits. The biggest advantage for routine use is the time-saving aspect achieved by the use of NIRS instead of chemical analysis. The low running costs of NIRS equipment and measurement also favour NIRS over classical approaches. To provide a calibration that is useable in routine quality control of cereal grains—e.g. for feed industry or farmers—calibrations need to be suitable, preferably for whole cereal grains. A re-development of the calibrations using whole or cracked cereal grains should be considered in the development of further calibrations. Recently, it has been shown that the chemical and physical treatment of cereal grains influences their chemical structure (Rahman *et al.*, 2016). To achieve calibrations that are applicable under practical conditions, an expansion with processed cereals is therefore necessary, even if the ruminal degradation characteristics of the samples are in the range of the calibration.

As indicated in Manuscript 3, the number of samples used to establish calibrations for degradation parameters and the ED are on the lower boundaries of needed samples to establish a calibration (Sapienza *et al.*, 2008); hence, it should be expanded by more samples (DIN-Normenausschuss Lebensmittel und landwirtschaftliche Produkte (NAL), 2016). Despite the error associated with estimation methods, a general improvement of the description of feeds—e.g. in feeding tables—can be achieved by the increased number of samples that can be classified using NIRS.

Genetical markers for ruminal *in situ* degradation parameters of DM, CP and ST have been found for barley (Gous *et al.*, 2012). This is a promising approach to adapt ruminal degradation characteristics to plant breeding, but QTL analyses are relatively expensive. Nonetheless, it demonstrates that the ruminal degradation of cereal grains can be influenced by plant breeding. In plant breeding experiments, often only small sample amounts, which are not sufficient for feeding trials or *in situ* studies, are available; hence, rapid, non-destructive methods for potential breeding traits are needed. Thus, the prediction of ruminal degradation parameters by NIRS might help to establish the feeding values as traits in cereal breeding. The possibility of using NIRS-estimated nutritional values in plant breeding should also be kept in mind for other feeds and animal species.

3.5 Conclusions and outlook

In the present thesis, *in situ* degradation parameters of DM, CP, and ST of genotypes from barley, triticale, and rye genotypes have been found to differ. This variation is not reflected in the ED. Hence, using mean values for the ED for each grain species for ration planning seem reasonable. The *in vitro* gas production showed the same ranking for the grain species as *in situ* studies, which underlines the suitability of the HGT to assess and compare the feeding value of cereal grains for ruminants. Since the variation in ruminal degradation may have been partly caused by particle losses from *in situ* bags, further standardisation of the *in situ* technique is recommended. Methods for the estimation of MCP synthesis *in vitro* and from *in situ* differed, thus, the used methods should be validated and—if necessary—modified.

It has been demonstrated that NIRS is a suitable method to predict the CP and ST concentrations of cereal grains and their incubation residues from *in situ* studies, without influencing the resulting ED. Further, NIRS can be used to predict the ED_8CP, ED_8ST, and ED_8DM of cereal grains. This opens new possibilities for the precise calculation of rations for ruminants.

Although new insight has been gained in this thesis in regard to the variation of ruminal ST and CP degradation of a multitude of genotypes from barley, rye, and triticale genotypes, and a basis for the estimation of ruminal CP and ST degradation by NIRS has been laid, further research is needed.

The standardisation of the *in situ* incubation technique needs to be further developed. Seifried *et al.* (2015) investigated the influence of particle losses during *in situ* incubations of cereal grains and reported a pore size of 50 µm as the most suitable for avoiding the bloating of the bags and keeping particle losses as small as possible. Nonetheless, particle losses occur during *in situ* incubations and mathematical correction for them is still an issue when *in situ* studies are performed (Manuscripts 1 and 2). A possible entry point to meet the challenge of correcting for particle losses in *in situ* studies could be a deduction of the pore sizes that should be used for incubation, in dependency of the particle size distribution of the sample. In combination with a defined sieve size for sample preparation, this could unify the particle losses, enable an appropriate mathematical correction for particle losses, and thus contribute to a further standardisation of the technique. As mentioned in Chapter 3.2.2, the particle size of ground cereal grains can be influenced by the grain hardness. Hence, the

particle size distribution after grinding should be considered in future *in situ* studies and seen as a possible influence on the results.

At present, the correction for washout losses is carried out under the assumption that the degradation of particles outside the nylon bag follows the same kinetics as that of particles inside the bag. Since the particle size, and thereby the surface:volume ratio, differs strongly between washed-out particles and particles inside the bag (theoretically < 70.7 µm for washed-out particles and 70.7 µm-2 mm for particles inside the bag), different degradation kinetics or ruminal outflow of undegraded particles can be assumed (Manuscript 1). Studies on the degradation behaviour of particles of different sizes under ruminal conditions are needed to overcome this error and equations for the correction of particle losses should be developed. This could be investigated by using *in vitro* systems—e.g. the RUSITEC—as already done by Seifried *et al.* (2015) and a subsequent derivation of formulas that estimate the degradation kinetics for washed-out particles from the kinetics of the particles inside the bag. It must be considered that the microbial community might change in *in vitro* systems (Lengowski *et al.*, 2016), and hence not reflect the actual ruminal microbiota. Nonetheless, the use of *in vitro* techniques seems adequate for investigating the degradation kinetics of small particles (Huhtanen and Sveinbjörnsson, 2006).

Following the standardisation of the *in situ* technique, further studies should be conducted to evaluate the influence of different cultivation areas and their interaction with different genotypes of cereal grains. Besides the enhanced knowledge of variation in ruminal degradation, the results of these studies would also contribute to an expanded database as basis for the improvement of estimation methods. The broader variability in grain characteristics might further help to obtain a deeper understanding of factors that influence the ruminal degradation of cereal grains. One of the reasons the equations to estimate the ruminal degradation from chemical composition using the present dataset showed relatively poor performance is possibly the low variation within grain species. This might be solved by the use of a more variable reference dataset. Corresponding to this, the NIRS calibrations for the ED_8ST, ED_8CP, and ED_8DM need to be expanded by more variable samples. To enable the routine use of NIRS calibrations, the sample preparation should be minimised. This can be accomplished by a mathematical correction for differences in particle sizes or at best by establishing calibrations for whole cereal grains (Manuscript 3). In further steps, NIRS calibrations for compound feeds or rations should be attempted.

Based on the high variation of the share of MCP on the uCP and MCP that is formed per kg fOM in literature, a standardisation of the method for measuring the MCP synthesis seems recommendable (Chapter 3.3). In this context, factors that influence the MCP production, such as feed intake and ration composition or interrelations with the ruminal microbiome, should be considered. Ideally, the *in vivo* measurements should be conducted using a defined sample material under different feeding regimes. Samples from the same batch should be used afterwards for *in situ* and *in vitro* studies to derive estimation equations or expand existing estimation methods. Due to the accompanying workload and the legal regulations for animal welfare, this is hardly realisable. Further, problems during digesta sampling due to the separation of liquid and solid phases of the digesta have been reported when T-cannulas are used (Huhtanen and Sveinbjörnsson, 2006). Hence, alternative techniques to withdraw samples of the ruminal outflow should be further investigated, such as the omasal sampling technique of Huhtanen *et al.* (1997).

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4 Included manuscripts

4.1 Manuscript 1

In situ and *in vitro* ruminal starch degradation of grains from different rye, triticale and barley genotypes

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Abstract

In recent years, advances in plant breeding potentially led to modified nutritional values of cereal grains. The present study was conducted in order to obtain a broad overview of ruminal digestion kinetics of rye, triticale and barley grains, and to highlight differences between the grain species. In total, 20 genotypes of each grain species were investigated using *in situ* and *in vitro* methods. Samples were ground (2 mm), weighed into polyester bags, and incubated 1 to 48 h in three ruminally cannulated lactating dairy cows. The *in vitro* gas production of ground samples (1 mm) was measured according to the 'Hohenheim Gas Test', and cumulative gas production was recorded over different time spans for up to 72 h. There were significant differences ($P<0.05$) between the grain species for most parameters used to describe the *in situ* degradation of starch (ST) and dry matter (DM). The *in situ* degradation rate (c) and effective degradability (assuming a ruminal passage rate of 8%/h; ED8) of ST differed significantly between all grains and was highest for rye (rye: 116.5%/h and 96.2%; triticale: 85.1%/h and 95.0%; barley: 36.2%/h and 90.0% for c and ED8, respectively). With respect to DM degradation, the ranking of the species was similar, and predicted c values exhibited the highest variation within species. The *in vitro* gas production rate was significantly higher ($P<0.05$) for rye than for triticale and barley (rye: 12.5%/h; triticale: 11.5%/h; barley: 11.1%/h). A positive relationship between the potential gas production *in vitro* and the maximal degradable DM fraction *in situ* was found using all samples ($r=0.84$; $P<0.001$) as well as rye ($P=0.002$) and barley ($P<0.001$) alone, but not for triticale. Variation in ruminal *in situ* degradation parameters within the grain species was not reflected in the ED estimates. Therefore, the usage of mean values for the ED of DM and ST for each species appears reasonable. Estimated metabolisable energy concentrations (ME, MJ/kg DM) and the estimated digestibility of organic matter (dOM, %) were significantly lower ($P<0.05$) for barley than for rye and triticale. Rye and triticale dOM and ME values were not significantly different ($P=0.386$ and 0.485).

4.2 Manuscript 2

In situ and *in vitro* evaluation of crude protein degradation and utilisable crude protein content of barley, rye, and triticale grains for ruminants

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Abstract

Despite their low crude protein (CP) content, grains can contribute considerably to the CP intake of dairy cows. This study was conducted to describe and compare ruminal CP degradation of a broad range of barley, rye and triticale genotypes *in situ* and *in vitro* and different methods to estimate the utilisable CP at the duodenum (uCP). Twenty samples each of rye, barley and triticale were utilised. Exponential regression analyses were used to estimate in situ degradation parameters. Further, the effective degradability (ED), ruminal undegraded CP (UDP) and uCP for ruminal passage rates of 5% and 8% per h were estimated. The uCP was estimated *in vitro* and based on two different approaches using *in situ* UDP data and estimates of microbial synthesised protein (based on fermented organic matter [fOM] or equations of the Gesellschaft für Ernährungsphysiologie). The degradation rate declined from rye (43% per h) to triticale (27% per h) to barley (20% per h), and it exhibited remarkable variation between the genotypes of a single species. The maximal degradable CP fraction also differed between the species, but was overall very high (94%–99%). The lowest washout fraction (26%) and the highest variation in ED (77%–86% and 69%– 80% for a passage rate of 5% and 8% per h, respectively) were found in barley. The *in situ* uCP content (estimated using fOM) was lower for barley than for rye and triticale at ruminal passage rates of 5% and 8% per h (barley: 157 g/kg DM at both passage rates; rye and triticale: 168 (at 5% per h) and 169 (at 8% per h) g/kg DM). *In vitro* estimations of uCP did not differ between the grain species. The uCP estimated according to GfE was higher for triticale than for barley and rye, which did not differ. The low variation within a single grain species and the weak correlations between ruminal CP degradation and grain properties suggested that differentiation of ED and uCP between the genotypes of a single grain species is not necessary.

4.3 Manuscript 3

Prediction of crude protein and starch in residues of ruminal *in situ* incubations and degradation characteristics using near-infrared spectroscopy

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Abstract

Ruminal *in situ* incubations are widely used to assess the nutritional value of feeds for ruminants. In this method, feed samples are ruminally incubated in indigestible bags over a predefined timespan and the disappearance of nutrients from the bags is recorded. To describe the degradation of specific nutrients, information on the concentration of feed samples and undegraded feed after ruminal incubation ('bag residues') is needed. For cereal and pea grains, CP and starch (ST) analyses are of interest. The numerous analyses of residues following ruminal incubation contribute greatly to the substantial investments in labour and money, and faster methods would be beneficial. Therefore, calibrations were developed to estimate CP and ST concentrations in grains and bag residues following *in situ* incubations by using their near-infrared spectra recorded from 680 to 2500 nm. The samples comprised rye, triticale, barley, wheat, and maize grains (20 genotypes each), and 15 durum wheat and 13 pea grains. In addition, residues after ruminal incubation were included (at least from four samples per species for various incubation times). To establish CP and ST calibrations, 620 and 610 samples (grains and bag residues after incubation, respectively) were chemically analysed for their CP and ST concentration. Calibrations using wavelengths from 1250 to 2450 nm and the first derivative of the spectra produced the best results ($R^2_{Validation}$=0.99 for CP and ST; standard error of prediction=0.47 and 2.10% DM for CP and ST, respectively). Hence, CP and ST concentration in grains and their bag residues could be predicted with high precision by NIRS for use in *in situ* studies. No differences were found between the effective ruminal degradation calculated from NIRS estimations and those calculated from chemical analyses (P>0.70). Calibrations were also calculated to predict ruminal degradation kinetics of cereal grains from the spectra of ground grains. Estimation of the effective ruminal degradation of CP and ST from the near-infrared spectra of cereal grains showed promising results (R^2>0.90).

5 Summary

The milk yield of dairy cows and related energy and protein requirements have steadily increased in the last few decades. Since feed intake has not increased to the same extent as nutritional requirements, the concentration of nutrients in mixed rations had to be increased. An increase in energy concentration is often achieved by the inclusion of high levels of cereal grains. In the EU—apart from wheat—barley, rye, and triticale are widely cultivated cereal grains. Starch (ST), followed by crude protein (CP), is the main constituent of cereal grains. The rate and extent of ruminal CP and ST degradation can influence the performance and health of dairy cows, but data that can enable the comparison of ruminal degradation within and between barley, rye, and triticale grains are scarce. Commonly used techniques to explore ruminal degradation of feed are *in situ* and *in vitro* incubations. Both techniques require ruminal-fistulated animals, but alternative methods are being demanded by the community, in order to reduce the number of animal trials. An approach with the potential to estimate the nutritional value of various feeds is near-infrared spectroscopy (NIRS).

The present thesis has two major parts. In the first part, ruminal degradation parameters and the effective degradability (ED) of DM, CP, and ST from barley, rye, and triticale grains were investigated using standardised *in situ* and *in vitro* incubation techniques. A total of 20 genotypes per grain species were used. In the second part, NIRS calibrations were developed with the aim of estimating the CP and ST concentrations of cereal grains and their incubation residues. Subsequently, data from *in situ* experiments were used to establish the calibrations for estimating the ruminal *in situ* degradation of cereal grains from their spectral data.

In situ degradation studies have been conducted by ruminal incubation of ground samples (8 g, 2 mm sieve size) in porous bags (10 x 20 cm; pore size: 50 µm), utilising three lactating cows. The bags were ruminally incubated for 1, 2, 4, 8, 16, 24, and 48 hours. The incubation residues were analysed for their CP and ST concentrations, and ruminal degradation parameters and ED (ruminal passage rate = 8%/h) were calculated. For *in vitro* incubations, the samples were ground (1 mm sieve size) and incubated in a rumen fluid-buffer mixture (‘Hohenheim Gas Test’). The gas production after 2, 4, 6, 8, 12, 24, and 48 hours was recorded for estimating gas production kinetics. *In vitro* gas production—in combination with crude nutrient concentrations—

was used to estimate the metabolisable energy concentration (ME) and digestibility of organic matter (dOM).

The degradation rates differed between and within the grain species for DM, CP, and ST. The variation within grain species was not reflected in the ED of CP and ST, due to the relatively fast and almost complete degradation of the grains. The ED of CP was 77% (69–80%) for barley, 85% (83–86%) for rye, and 82% (79–84%) for triticale. The corresponding ED of ST was 86% (82–88%), 95% (92–96%), and 94% (90–95%). Accordingly, the estimated ME (barley: 13.5 MJ/kg DM, rye: 13.9 MJ/kg DM, triticale: 13.5 MJ/kg DM) showed only relatively minor variation within one grain species. The dOM was overall at a high level (barley: 91.3%, rye: 95.3%, triticale: 95.8%). The relatively small variation within one grain species could not be explained by the chemical and physical characteristics of the samples. Hence, it was concluded that it is feasible to use mean values for every species in feed formulation and ration planning.

In the second part of this thesis, it was shown that it is possible to replace chemical CP and ST analyses of samples from *in situ* studies by NIRS without affecting the calculated ruminal degradation characteristics. NIRS could be used to estimate the ED of CP and ST from cereal grains. The sample set to establish the calibrations included barley, durum, maize, rye, triticale, and wheat grains. Calibrations for the CP and ST concentration were extended to pea samples. The spectra were detected from 680 to 2500 nm and mathematically pre-treated (SND/detrend). For every characteristic the samples were split into two groups. One group was used to establish the calibrations by using PLS regression and the other for the validation of the calibration. The wavelength segments used for calibration (680–2500 nm, 730–2450 nm, 1250–2450 nm) and the mathematical pre-treatment (no derivative, first or second order derivative) were varied. The calibrations with the best validation performance for CP and ST concentration were obtained by using the wavelength segment of 1250 to 2450 nm and the first derivative of the spectra (CP: R^2 = 0.99; SEP = 0.46% DM; bias = 0.03% DM; slope = 1.00; Intercept = 0.07. ST: R^2 = 0.99; SEP = 2.10% DM; bias = 0.08% DM; slope = 1.00; Intercept = 0.17). The results of *in situ* studies did not differ, irrespective of whether chemical or NIRS analysis was used. Like the CP and ST concentration, the ED was estimated with a high accuracy (ED_8 CP: R^2 = 0.95; SEP = 2.43%; bias = −0.49%; slope = 1.01; Intercept = −0.99. ED_8 ST: R^2 = 0.97;

SEP = 2.45%; bias = 0.13%; slope = 1.00; Intercept = 0.62). However, calibrations need to be extended before they can be recommended for routine use.

The present thesis demonstrates that the ED of CP and ST of barley, rye, and triticale grains differ between the species, but variation within one grain species is relatively small and not related to the chemical and physical characteristics of the grain. Hence, under the prevailing cultivation conditions, the mean values for each grain species in feed evaluation are deemed adequate. It is demonstrated that NIRS has the potential to facilitate the evaluation of the nutritive value of cereal grains for ruminants. However, the database needs to be expanded to get calibrations that are suitable for routine use.

6 Zusammenfassung

Die seit Jahrzehnten steigende Leistung von Milchkühen geht mit einem erhöhten Energie- und Proteinbedarf einher. Da die Futteraufnahme nicht im selben Umfang zugenommen hat, wird eine Bedarfsdeckung häufig durch eine erhöhte Nährstoff- und Energiedichte der Ration angestrebt. Eine gesteigerte Energiedichte kann durch den Einsatz leicht fermentierbarer Kohlenhydrate in Form von Getreidestärke (ST) erreicht werden. Eine zu schnelle und umfangreiche ruminale Kohlenhydratfermentation kann allerdings zu einer verminderten Leistung führen und die Tiergesundheit negativ beeinflussen. Bei einem hohen Anteil von Getreide in Milchviehrationen stammt außerdem ein nicht unerheblicher Teil des in einer Ration enthaltenen XP aus Getreide. Um eine bedarfsgerechte Fütterung von Milchkühen sicherzustellen ist es daher notwendig, den ruminalen XP- und ST-Abbau von Getreidekörnern beurteilen zu können. Der ruminale Abbau von Getreide kann sowohl zwischen als auch innerhalb von Getreidearten variieren. Verfügbare Untersuchungen sind jedoch meist auf Proben einer Getreideart oder wenige Proben verschiedener Getreidearten begrenzt und ein Vergleich zwischen verschiedenen Studien ist aufgrund von Unterschieden in der Methodik oft nicht möglich.

Das erste Ziel dieser Dissertation war es daher, die Variation im ruminalen XP- und ST-Abbau von je 20 Genotypen Gerste, Roggen und Triticale unter Verwendung von standardisierten *in situ* und *in vitro* Methoden zu untersuchen und zu vergleichen. Ein weiteres Ziel war es, die Beurteilung des ruminalen *in situ* Abbaus von Getreidekörnern durch die Schätzung der XP- und ST-Konzentration von gemahlenen Getreide- und Erbsenkörnern sowie deren Inkubationsrückständen aus *in situ*-Studien mittels Nahinfrarotspektroskopie (NIRS), zu vereinfachen. Außerdem wurden Kalibrationen zur Schätzung des ruminalen Abbaus von Getreidekörnern erstellt.

In den ersten beiden Studien wurden die Proben hinsichtlich der Parameter des ruminalen *in situ* XP- und ST-Abbaus sowie dem daraus geschätzten effektiven ruminalen Abbau (Passagerate = 8%/h, ED) charakterisiert. Hierfür wurden 8 g des vermahlenen (2 mm Siebweite) Getreides in Beutel (10 x 20 cm, Porengröße = 50 µm) eingewogen und in drei pansenfistulierten, laktierenden Milchkühen über 1, 2, 4, 8, 16, 24 und 48 h inkubiert. Zur Ermittlung der *in vitro* Gasbildungskinetik wurden die gemahlenen (1 mm Siebweite) Proben in einem Pansen-Puffer-Gemisch inkubiert und die Gasbildung nach 2, 4, 6, 8, 12, 24, 48 und 72 Stunden erfasst. Die Gasbildung

wurde zusammen mit Rohnährstoffkonzentrationen verwendet, um die Umsetzbare Energie (ME, MJ/kg TM) und die Verdaulichkeit der Organischen Masse (dOM, %) zu schätzen.

Die *in situ* Abbauparameter für XP und ST variierten sowohl zwischen als auch innerhalb der Getreidearten. Die Variation innerhalb einer Getreideart war aufgrund des raschen und annähernd vollständigen Abbaus nicht im selben Umfang im ED widergespiegelt. Der ED des XP lag bei 77% (69-80%) für Gerste, 85% (83-86%) für Roggen und bei 82% (79-84%) für Triticale. Die entsprechenden Werte für den ED der ST waren 86% (82-88%), 95% (92-96%) und 94% (90-95%). Die in vitro Gasbildung sowie die ME und dOM zeigten eine relativ geringe Variation innerhalb und zwischen den Getreidearten. Für Gerstenkörner wurde eine ME-Konzentration von im Mittel 13,5 MJ/kg TM, für Roggen 13,9 MJ/kg TM und für Triticale 13,5 MJ/ kg TM geschätzt. Die geschätzte dOM lag bei 91,3% für Gerste, 95,3% für Roggen und 95,8% für Triticale. Die Variation im ED und in den *in vitro* Kennzahlen innerhalb einer Getreideart konnte nicht durch chemische oder physikalische Eigenschaften der Proben erklärt werden. Daher scheint unter den gegebenen Anbaubedingungen ein Mittelwert für den ED je Getreideart zur Rationsgestaltung angemessen.

In der dritten Studie dieser Arbeit wurden NIRS Kalibrationen erstellt um die XP und ST Konzentration in Getreidekörnern und Erbsen sowie deren Rückständen nach einer ruminalen Inkubation zu schätzen. Hierfür wurden Gerste-, Hartweizen-, Mais-, Roggen-, Triticale- und Weizen- sowie Erbsenkörner verwendet. Bei der Kalibrationsentwicklung wurde der verwendete Wellenlängenbereich (680-2500, 730-2450 und 1250-2450 nm) sowie die mathematische Behandlung der Spektren (keine, erste oder zweite Ableitung) variiert. Für die XP- und ST-Konzentration zeigten Kalibrationen im Wellenlängenbereich 1250-2450 nm unter Verwendung der ersten Ableitung der Spektren die beste Schätzgüte (Kennzahlen Validierung: XP: R^2 = 0,99; SEP = 0,46% TM; bias = 0,03% TM. ST: R^2 = 0,99; SEP = 2,10% TM; bias = 0,08% TM). Die Verwendung von NIRS anstelle von chemischen Methoden zur XP- und ST-Analyse von Inkubationsrückständen und Körnerproben beeinflusste die Ergebnisse der *in situ* Studien nicht. Des Weiteren wurden Kalibrationen zur Schätzung der ruminalen *in situ* Abbauparameter von Gerste, Hartweizen, Mais, Roggen, Triticale und Weizen mittels NIRS erstellt. Der ED konnte mit hoher Schätzgüte aus den Spektren der vermahlenen Getreidekörner geschätzt werden (Kennzahlen Validierung: ED_8 XP:

R^2 = 0,95; SEP = 2,43%; bias = -0,49%; ED_8 ST: R^2 = 0,97; SEP = 2,45%; bias = 0,13%).

Die Ergebnisse zeigen, dass es hinsichtlich des ED von XP und ST Unterschiede zwischen den untersuchten Getreidearten gibt, die Variation innerhalb der Getreidearten aber relativ gering und nicht durch die durchgeführten chemischen oder physikalischen Analysen zu erklären ist. Es konnte außerdem gezeigt werden, dass sich NIRS zur Schätzung des ED von Getreidekörnern eignet. Die Kalibrationen könnten nach einer Erweiterung um weitere Proben zu einer besseren Beurteilung von einzelnen Getreidechargen in der Praxis und somit einer präziseren Rationsplanung beitragen.

Annex

Annex 1: Kernel density[1] of twenty barley, rye and triticale grains, each.

Genotype	Kernel density (g/cm^3)		
	Barley	Rye	Triticale
1	1.41	1.35	1.31
2	1.38	1.33	1.33
3	1.41	1.36	1.28
4	1.39	1.34	1.28
5	1.37	1.36	1.25
6	1.37	1.37	1.27
7	1.40	1.34	1.33
8	1.50	1.35	1.32
9	1.41	1.32	1.29
10	1.36	1.32	1.33
11	1.36	1.27	1.31
12	1.33	1.36	1.31
13	1.35	1.30	1.31
14	1.34	1.34	1.30
15	1.35	1.34	1.31
16	1.39	1.32	1.28
17	1.36	1.34	1.35
18	1.41	1.32	1.35
19	1.36	1.27	1.27
20	1.32	1.32	1.28

[1] determined using a pycnometer, according to Correa (2002)

Annex 2: Calibrations to predict *in situ* ruminal degradation parameters by near-infrared spectroscopy – washout fraction

Settings		Calibration				Cross-Validation		Validation				
Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
DM												
680-2500	0,8,8	7	85/85	3.25	0.65	3.92	0.40	3.08	0.71	-0.14	1.01	-0.28
680-2500	1,8,8	8	85/85	2.79	0.74	3.57	0.52	2.90	0.75	-0.21	1.01	-0.42
680-2500	2,8,8	7	85/85	2.69	0.76	3.64	0.54	3.06	0.71	0.18	0.99	0.36
730-2450	0,8,8	11	85/85	2.80	0.74	3.63	0.53	3.11	0.72	-0.04	1.00	-0.08
730-2450	1,8,8	8	85/85	2.84	0.73	3.51	0.52	2.99	0.74	-0.29	1.01	-0.59
730-2450	2,8,8	7	85/85	2.70	0.76	3.61	0.51	3.23	0.69	-0.50	1.02	-1.00
1250-2450	0,8,8	5	85/85	3.38	0.62	3.95	0.37	3.29	0.67	-0.27	1.01	-0.54
1250-2450	1,8,8	6	85/85	3.07	0.68	3.67	0.52	3.10	0.71	-0.21	1.01	-0.37
1250-2450	2,8,8	6	85/85	2.95	0.71	3.61	0.51	3.00	0.72	-0.08	1.00	-0.16
CP												
680-2500	0,8,8	6	85/85	3.17	0.80	3.98	0.68	3.81	0.72	0.22	0.99	0.45
680-2500	1,8,8	11	85/85	2.10	0.91	3.34	0.77	3.67	0.75	0.71	0.97	1.40
680-2500	2,8,8	11	85/85	1.38	0.96	3.01	0.81	4.23	0.68	1.16	0.95	2.27
730-2450	0,8,8	4	85/85	3.66	0.73	4.03	0.61	4.34	0.63	0.08	1.00	0.16
730-2450	1,8,8	10	85/85	2.41	0.88	3.56	0.72	3.59	0.75	0.29	0.99	0.58
730-2450	2,8,8	7	85/85	2.39	0.89	3.48	0.72	3.94	0.70	0.10	1.00	0.19
1250-2450	0,8,8	5	85/85	3.25	0.79	3.84	0.69	3.73	0.73	-0.03	1.00	-0.05
1250-2450	1,8,8	6	85/85	2.91	0.83	3.55	0.73	3.54	0.76	0.05	1.00	0.10
1250-2450	2,8,8	6	85/85	2.48	0.88	3.25	0.77	3.91	0.70	0.02	1.00	0.04

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 2: Continued

Settings			Calibration				Cross-Validation		Validation				
	Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
ST													
	680-2500	0,8,8	8	85/85	4.27	0.73	4.93	0.58	6.03	0.50	0.18	0.99	0.35
	680-2500	1,8,8	7	85/85	5.05	0.62	5.49	0.37	5.43	0.57	-0.21	1.01	-0.41
	680-2500	2,8,8	4	85/84	4.30	0.72	5.07	0.54	5.00	0.64	0.12	1.00	0.24
	730-2450	0,8,8	8	85/85	4.19	0.74	4.83	0.62	6.21	0.46	0.12	1.00	0.23
	730-2450	1,8,8	7	85/85	4.07	0.75	4.93	0.59	5.46	0.57	-0.28	1.01	-0.56
	730-2450	2,8,8	8	85/85	3.10	0.86	4.69	0.64	5.31	0.59	-0.33	1.01	-0.65
	1250-2450	0,8,8	2	85/85	5.39	0.56	5.47	0.29	6.75	0.34	-0.77	1.03	-1.57
	1250-2450	1,8,8	3	85/85	4.76	0.66	5.22	0.43	5.37	0.58	-0.32	1.01	-0.64
	1250-2450	2,8,8	4	85/85	4.43	0.70	5.11	0.53	5.02	0.63	-0.04	1.00	-0.08

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 3: Calibrations to predict *in situ* ruminal degradation parameters by near-infrared spectroscopy – potential degradable fraction

Settings		Calibration				Cross-Validation		Validation				
Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
DM												
680-2500	0,8,8	7	85/85	3.33	0.77	3.93	0.64	3.19	0.80	-0.23	1.00	-0.46
680-2500	1,8,8	5	85/84	3.22	0.78	3.71	0.62	3.15	0.82	-0.89	1.01	-1.78
680-2500	2,8,8	5	85/85	3.14	0.80	3.72	0.68	3.10	0.82	-0.72	1.01	-1.45
730-2450	0,8,8	4	85/85	4.00	0.67	4.40	0.53	3.46	0.77	-0.63	1.01	-1.27
730-2450	1,8,8	7	85/85	3.15	0.80	3.80	0.69	2.96	0.83	-0.53	1.01	-1.07
730-2450	2,8,8	6	85/85	2.92	0.83	3.74	0.70	3.00	0.83	-0.82	1.01	-1.64
1250-2450	0,8,8	5	85/85	3.47	0.75	3.85	0.64	3.46	0.76	-0.29	1.00	-0.57
1250-2450	1,8,8	4	85/83	3.09	0.80	3.38	0.65	2.98	0.83	-0.46	1.01	-0.92
1250-2450	2,8,8	5	85/85	3.19	0.79	3.64	0.71	2.93	0.83	-0.53	1.01	-1.06
CP												
680-2500	0,8,8	6	85/85	3.51	0.82	3.87	0.73	3.68	0.82	0.16	1.00	0.32
680-2500	1,8,8	8	85/85	2.78	0.89	3.63	0.78	3.69	0.83	-0.44	1.01	-0.88
680-2500	2,8,8	8	85/85	2.24	0.93	3.09	0.85	3.66	0.83	-0.82	1.01	-1.65
730-2450	0,8,8	7	85/85	3.27	0.85	3.74	0.78	3.67	0.83	-0.03	1.00	-0.06
730-2450	1,8,8	8	85/85	2.80	0.89	3.38	0.80	3.97	0.81	-0.36	1.00	-0.50
730-2450	2,8,8	9	85/85	2.08	0.94	3.02	0.86	3.96	0.79	-0.36	1.00	-0.73
1250-2450	0,8,8	7	85/85	3.44	0.83	3.86	0.76	3.59	0.83	-0.38	1.01	-0.77
1250-2450	1,8,8	11	85/85	2.26	0.93	3.12	0.85	3.89	0.81	-1.00	1.01	-2.02
1250-2450	2,8,8	11	85/85	2.45	0.91	3.08	0.85	3.88	0.80	-0.52	1.01	-1.04

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 3: Continued

Settings			Calibration				Cross-Validation		Validation				
	Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
ST													
	680-2500	0,8,8	8	85/85	4.27	0.76	4.77	0.67	6.09	0.50	0.04	1.00	0.07
	680-2500	1,8,8	7	85/85	4.06	0.78	4.81	0.66	5.66	0.55	0.09	1.00	0.17
	680-2500	2,8,8	4	85/84	4.35	0.75	4.98	0.60	4.99	0.63	0.21	1.00	0.43
	730-2450	0,8,8	8	85/85	4.41	0.74	4.83	0.67	6.08	0.49	-0.09	1.00	-0.18
	730-2450	1,8,8	7	85/85	4.05	0.78	4.73	0.66	5.78	0.53	0.18	1.00	0.36
	730-2450	2,8,8	7	85/85	3.50	0.84	4.70	0.69	5.66	0.55	0.23	1.00	0.46
	1250-2450	0,8,8	6	85/85	4.56	0.73	4.93	0.65	5.62	0.54	-0.18	1.00	-0.35
	1250-2450	1,8,8	4	85/85	4.64	0.72	4.92	0.64	5.40	0.57	-0.49	1.01	-0.99
	1250-2450	2,8,8	4	85/84	4.34	0.75	4.78	0.61	5.07	0.62	-0.03	1.00	-0.05

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 4: Calibrations to predict *in situ* ruminal degradation parameters by near-infrared spectroscopy – degradation rate

Settings		Calibration				Cross-Validation		Validation				
Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
DM												
680-2500	0,8,8	5	85/83	9.14	0.85	10.24	0.78	9.36	0.86	0.52	0.99	1.02
680-2500	1,8,8	14	85/85	4.22	0.97	8.65	0.87	8.09	0.89	0.15	1.00	0.30
680-2500	2,8,8	11	85/85	3.56	0.98	8.10	0.88	10.53	0.82	0.02	1.00	0.04
730-2450	0,8,8	5	85/83	8.85	0.86	9.91	0.79	9.73	0.85	0.28	0.99	0.56
730-2450	1,8,8	5	85/83	8.42	0.87	9.63	0.82	10.10	0.83	1.19	0.97	2.34
730-2450	2,8,8	9	85/85	4.65	0.96	7.96	0.89	9.18	0.86	0.64	0.99	1.27
1250-2450	0,8,8	14	85/84	5.80	0.94	8.14	0.87	9.16	0.86	1.94	0.95	3.79
1250-2450	1,8,8	12	85/85	5.45	0.95	8.28	0.88	8.49	0.88	1.22	0.97	2.40
1250-2450	2,8,8	9	85/85	5.57	0.95	8.93	0.85	10.06	0.84	2.05	0.95	4.00
CP												
680-2500	0,8,8	13	85/83	2.29	0.96	3.44	0.91	4.43	0.86	0.75	0.97	1.48
680-2500	1,8,8	9	85/83	2.53	0.95	3.53	0.90	4.19	0.87	0.61	0.97	1.20
680-2500	2,8,8	9	85/85	2.30	0.96	3.92	0.87	3.66	0.90	-0.25	1.01	-0.50
730-2450	0,8,8	5	85/84	3.65	0.90	4.19	0.85	4.93	0.82	1.01	0.96	1.98
730-2450	1,8,8	7	85/85	3.22	0.92	4.25	0.85	4.18	0.87	-0.06	1.00	-0.13
730-2450	2,8,8	8	85/85	2.53	0.95	3.78	0.89	3.50	0.91	-0.20	1.01	-0.40
1250-2450	0,8,8	4	85/84	5.59	0.77	6.15	0.64	8.77	0.59	1.36	0.94	2.64
1250-2450	1,8,8	9	85/84	2.93	0.94	3.72	0.89	5.02	0.86	1.08	0.95	2.10
1250-2450	2,8,8	8	85/84	2.63	0.95	3.48	0.89	4.39	0.87	0.44	0.98	0.87

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 4: Continued

Settings			Calibration				Cross-Validation		Validation				
	Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
ST													
	680-2500	0,8,8	5	85/82	16.60	0.80	18.62	0.71	17.46	0.81	2.88	0.96	5.63
	680-2500	1,8,8	4	85/83	17.50	0.78	19.64	0.70	18.28	0.79	1.72	0.97	3.40
	680-2500	2,8,8	9	85/83	9.27	0.94	16.18	0.80	17.23	0.81	1.80	0.97	3.55
	730-2450	0,8,8	5	85/82	16.13	0.81	18.37	0.71	17.40	0.81	1.84	0.97	3.63
	730-2450	1,8,8	12	85/85	11.20	0.92	17.28	0.80	14.70	0.86	1.65	0.98	3.27
	730-2450	2,8,8	9	85/83	8.67	0.95	14.86	0.83	15.70	0.85	2.69	0.96	5.28
	1250-2450	0,8,8	14	85/83	10.65	0.92	15.01	0.82	17.40	0.82	4.24	0.94	8.20
	1250-2450	1,8,8	12	85/83	8.96	0.94	14.07	0.84	17.73	0.82	5.05	0.92	9.71
	1250-2450	2,8,8	9	85/83	9.85	0.93	15.71	0.79	17.83	0.81	4.61	0.93	8.89

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 5: Calibrations to predict *in situ* ruminal degradation parameters by near-infrared spectroscopy – Effective Degradability (ruminal passage rate = 5%/h)

Settings		Calibration				Cross-Validation		Validation				
Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
DM												
680-2500	0,8,8	15	85/85	1.39	0.98	2.43	0.91	2.96	0.88	0.37	1.00	0.74
680-2500	1,8,8	13	85/85	1.40	0.98	2.62	0.91	2.83	0.89	-0.48	1.01	-0.95
680-2500	2,8,8	7	85/85	2.03	0.95	2.95	0.87	3.37	0.85	-0.76	1.01	-1.53
730-2450	0,8,8	15	85/85	1.39	0.98	2.12	0.94	2.57	0.91	0.18	1.00	0.35
730-2450	1,8,8	12	85/85	1.34	0.98	2.35	0.91	2.70	0.90	-0.44	1.01	-0.88
730-2450	2,8,8	8	85/85	1.66	0.96	2.51	0.90	2.89	0.89	-0.51	1.01	-1.02
1250-2450	0,8,8	13	85/85	1.46	0.97	2.01	0.93	2.56	0.91	0.09	1.00	0.17
1250-2450	1,8,8	13	85/85	1.21	0.98	2.29	0.93	2.58	0.91	0.00	1.00	0.00
1250-2450	2,8,8	9	85/85	1.56	0.97	2.43	0.92	2.84	0.89	-0.45	1.01	-0.89
CP												
680-2500	0,8,8	15	85/85	2.23	0.96	3.35	0.88	3.47	0.91	-0.12	1.00	-0.23
680-2500	1,8,8	6	85/85	4.76	0.82	5.55	0.73	6.03	0.72	0.12	1.00	0.24
680-2500	2,8,8	8	85/85	3.24	0.91	4.49	0.81	4.34	0.86	0.10	1.00	0.19
730-2450	0,8,8	15	85/85	2.16	0.96	3.29	0.90	3.01	0.93	-0.17	1.00	-0.34
730-2450	1,8,8	15	85/85	1.37	0.98	2.78	0.93	2.95	0.93	0.23	1.00	0.46
730-2450	2,8,8	11	85/85	1.72	0.98	3.32	0.90	3.55	0.90	0.20	1.00	0.40
1250-2450	0,8,8	13	85/85	2.52	0.95	3.47	0.89	3.30	0.92	-0.59	1.01	-1.18
1250-2450	1,8,8	15	85/85	1.33	0.99	2.69	0.93	2.98	0.93	-0.12	1.00	-0.24
1250-2450	2,8,8	11	85/85	1.98	0.97	3.29	0.90	3.89	0.89	0.47	0.99	0.94

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 5: Continued

Settings			Calibration				Cross-Validation		Validation				
	Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
ST													
	680-2500	0,8,8	15	85/85	1.66	0.98	2.53	0.95	3.06	0.94	0.90	0.99	1.79
	680-2500	1,8,8	12	85/85	1.58	0.98	2.68	0.93	2.86	0.94	0.33	1.00	0.65
	680-2500	2,8,8	6	85/85	2.56	0.95	3.57	0.87	3.15	0.93	0.41	1.00	0.82
	730-2450	0,8,8	15	85/85	1.61	0.98	2.33	0.96	2.83	0.96	0.98	0.99	1.95
	730-2450	1,8,8	13	85/85	1.23	0.99	2.28	0.96	2.25	0.96	0.13	1.00	0.26
	730-2450	2,8,8	9	85/85	1.55	0.98	2.58	0.94	2.34	0.96	0.02	1.00	0.04
	1250-2450	0,8,8	13	85/85	1.57	0.98	2.21	0.96	2.69	0.96	0.91	0.99	1.82
	1250-2450	1,8,8	13	85/85	1.12	0.99	2.02	0.97	2.64	0.95	0.17	1.00	0.33
	1250-2450	2,8,8	11	85/85	1.20	0.99	2.50	0.95	2.59	0.95	-0.01	1.00	-0.01

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 6: Calibrations to predict *in situ* ruminal degradation parameters by near-infrared spectroscopy – Effective Degradability (ruminal passage rate = 8%/h)

Settings		Calibration				Cross-Validation		Validation				
Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
DM												
680-2500	0,8,8	15	85/85	1.77	0.97	3.10	0.91	2.88	0.93	-0.02	1.00	-0.04
680-2500	1,8,8	15	85/85	1.36	0.99	3.18	0.92	2.72	0.94	0.21	1.00	0.41
680-2500	2,8,8	9	85/85	2.13	0.96	3.39	0.90	3.95	0.88	-0.51	1.01	-1.02
730-2450	0,8,8	15	85/85	1.81	0.97	2.87	0.93	2.69	0.94	-0.23	1.00	-0.46
730-2450	1,8,8	12	85/85	1.61	0.98	2.84	0.93	3.06	0.92	-0.25	1.00	-0.50
730-2450	2,8,8	8	85/85	2.17	0.96	3.04	0.92	3.53	0.90	-0.24	1.00	-0.48
1250-2450	0,8,8	12	85/85	1.87	0.97	2.78	0.93	2.85	0.94	-0.19	1.00	-0.38
1250-2450	1,8,8	10	85/85	1.94	0.97	2.97	0.92	3.56	0.90	0.30	1.00	0.60
1250-2450	2,8,8	11	85/85	1.45	0.98	2.80	0.93	3.18	0.92	-0.33	1.00	-0.66
CP												
680-2500	0,8,8	12	85/85	1.92	0.97	3.14	0.91	3.12	0.92	-0.54	1.01	-1.07
680-2500	1,8,8	11	85/85	2.09	0.96	3.19	0.90	3.26	0.91	-0.35	1.00	-0.70
680-2500	2,8,8	7	85/85	2.43	0.95	3.43	0.88	3.19	0.92	-1.03	1.01	-2.07
730-2450	0,8,8	14	85/85	1.99	0.97	3.13	0.90	3.31	0.91	-0.60	1.01	-1.21
730-2450	1,8,8	11	85/85	1.84	0.97	2.93	0.92	2.79	0.93	-0.46	1.01	-0.92
730-2450	2,8,8	7	85/85	2.42	0.95	3.20	0.89	3.22	0.92	-0.75	1.01	-1.52
1250-2450	0,8,8	13	85/85	2.05	0.97	2.95	0.91	2.66	0.94	-0.65	1.01	-1.30
1250-2450	1,8,8	12	85/85	1.83	0.97	3.07	0.91	2.43	0.95	-0.49	1.01	-0.99
1250-2450	2,8,8	8	85/85	2.22	0.96	3.10	0.91	2.86	0.94	-0.82	1.01	-1.66

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

Annex 6: Continued

Settings			Calibration				Cross-Validation		Validation				
	Wavelength (nm)	D,G,S	Factors	Samples Available /used	SEC (% DM)	R^2	SECV (% DM)	R^2	SEP (% DM)	R^2	Bias (% DM)	Slope	Intercept
ST													
	680-2500	0,8,8	15	85/85	2.02	0.98	3.09	0.95	3.25	0.95	0.73	0.99	1.45
	680-2500	1,8,8	12	85/85	2.20	0.98	3.34	0.93	3.13	0.96	0.36	1.00	0.72
	680-2500	2,8,8	9	85/85	2.35	0.97	3.77	0.93	4.05	0.92	-0.75	1.01	-1.51
	730-2450	0,8,8	15	85/85	1.94	0.98	2.93	0.95	2.90	0.96	0.71	0.99	1.41
	730-2450	1,8,8	15	85/85	1.30	0.99	2.75	0.96	2.95	0.96	0.04	1.00	0.08
	730-2450	2,8,8	9	85/85	1.98	0.98	3.06	0.95	3.16	0.95	-0.32	1.00	-0.65
	1250-2450	0,8,8	13	85/85	1.96	0.98	2.84	0.95	3.07	0.96	0.37	1.00	0.73
	1250-2450	1,8,8	13	85/85	1.51	0.99	2.73	0.96	2.45	0.97	0.13	1.00	0.26
	1250-2450	2,8,8	11	85/85	1.61	0.99	2.96	0.95	3.11	0.96	-0.59	1.01	-1.19

D,G,S = Derivation, Gap, Smooth; R^2 = squared correlation coefficient; SEC = Standard Error of Calibration; SECV = Standard Error of Cross-Validation; SEP = Standard Error of Prediction

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