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Estimation of the protein value of pig feeds from fibre-bound crude protein fractions

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„Du bist tapferer als du glaubst,
stärker als es scheint und
klüger als du denkst.“

Winnie Puuh

Für meine Eltern

Summary

Estimation of the protein value of pig feeds from fibre-bound crude protein fractions

Crude Protein (CP) and Amino acids (AA) are essential nutrients for diverse processes in the pig's body therefore an adequate CP and AA supply for pigs is important to sustain performance and animal health. The utilisation of CP and AA depends on their digestibility and absorption. Therefore, methods to determine reliably the CP and AA supply to pigs are critical for precise feed evaluation. The evaluation of CP and AA supply has so far been based on *in vivo* determination of standardised precaecal digestible (pcd) CP (pcdCP) and AA (pcdAA) and *in vitro* estimates of pcdCP and pcdAA applying time-consuming and complex chemical-enzymatic methods. The objective of this study was to develop and establish a rapid laboratory method for estimating pcdCP and pcdAA based on the determination of CP (N · 6.25) insoluble in neutral-detergent (ND) or acid-detergent (AD) (NDICP, ADICP) and AA insoluble in ND or AD (NDIAA, ADIAA).

A unique, large sample pool of 82 feed ingredients (cereal grains, differently heat-treated legume grains) was available on which *in vivo* pcdCP were determined in cannulated pigs. The CP was determined for cereal grains on the ND residue and for all protein feeds on the AD residue, because N compounds such as Maillard products or N bound to tannin or in phytate complexes are retained in the AD insoluble fraction. Crude protein was determined in feed ingredients and in their ND or AD residues. The concentrations of ND- and AD soluble CP (NDSCP, ADSCP) were calculated by difference of NDICP or ADICP to CP in feed. For the estimation of the concentrations of *in vivo* pcdCP for the entire dataset, a linear relationship was established between the concentrations of NDSCP or ADSCP and the *in vivo* pcdCP: y = 0.8640 (standard error [SE] 0.019) x - 13.37 (SE 7.479), where y represents the *in vivo* pcdCP (g/kg dry matter [DM]) and x represents the NDSCP (cereal grains) or ADSCP (protein feeds) value (g/kg DM). The coefficient of determination (R^2) of this equation was 0.962. A validation with literature values showed a good fit of the equation to an independent data set (n = 20; R^2 = 0.955).

For the estimation of pcdAA the same procedure which was previously applied to estimate pcdCP was used. Of the same sample pool 74 feed ingredients were available. Amino acids in feed ingredients and in ND or AD residues of feed ingredients were determined by an HPLC method. The concentrations (g/kg DM) of NDSAA, ADSAA were calculated by difference to total AA in feed. For the estimation of the concentrations of *in vivo* pcdAA for total AA and the entire dataset (n = 74), a linear relationship was established between the concentrations of NDSAA or ADSAA and the *in vivo* pcdAA: y = 0.823 (SE 0.018) x + 10.52 (SE 4.420), where y represents the *in vivo* pcdAA (g/kg DM) and x represents the NDSAA (cereal grains) or ADSAA (protein feeds) value (g/kg DM). The R^2 of this equation was 0.968. For the 17 individual AA the R^2 ranged from 0.895 to 0.984. This study shows that based on chemical analysis alone, namely determination of NDICP/ADICP and NDIAA/ADIAA, from which NDSCP/ADSCP and NDSAA/ADSAA are calculated, *in vivo* pcdCP and pcdAA values can be estimated with a standardised and rapid laboratory method.

Kurzfassung

Schätzung des Proteinwerts von Futtermitteln für Schweine aus fasergebundenen Rohproteinfraktionen

Rohprotein (XP) und Aminosäuren (AS) sind essentielle Bestandteile für diverse Prozesse im Körper des Schweins, somit ist eine adäquate XP- und AS-Versorgung wichtig, um die Leistung und Tiergesundheit sicherzustellen. Die Nutzbarkeit von XP und AS ist abhängig von der Verdaulichkeit und Aufnahme. Daher sind Methoden, die zuverlässig die XP- und AS-Versorgung des Schweins bestimmen unerlässlich für die präzise Futterbewertung. Die Bewertung der XP- und AS-Versorgung basiert bisher auf *in vivo* Bestimmungen des standardisiert praecaecal verdaulichen (pcv) XP (pcvXP) und AS (pcvAS) und auf *in vitro* Schätzungen von pcvXP und pcvAS durch zeitintensive und aufwändige Labormethoden. Ziel der Studie war es eine schnelle Labormethode zur Schätzung des pcvXP und pcvAS auf der Basis von XP unlöslich in Neutral-Detergenz oder Säure-Detergenz (NDUXP, ADUXP) und AS unlöslich in Neutral-Detergenz oder Säure-Detergenz (NDUAS, ADUAS) zu entwickeln und etablieren.

Es stand ein einzigartig großer Probenpool von 82 Einzelfuttermitteln (Getreide, verschieden hitzebehandelte Leguminose Saaten zur Verfügung an denen bereits *in vivo* die pcvXP an dünndarm-fistulierten Schweinen bestimmt wurde. Für Getreide wurde das XP bei Neutral-Detergenzien (ND) Rückständen und für Proteinkomponenten bei Säure-Detergenzien (AD) Rückständen bestimmt, da N-Verbindungen wie Maillardprodukte oder N-Bindungen an Tannin oder in Phytat-Komplexen in der säurelöslichen Fraktion miterfasst werden. Rohprotein (N · 6.25) wurde in den Komponenten und in ihren ND und AD Rückständen bestimmt. Die Konzentration von ND- und AD löslichem XP (NDLXP, ADLXP) wurden durch Differenzbildung von XP im Futter und NDUXP bzw. ADUXP berechnet. Für die Schätzung der Konzentration von *in vivo* pcvXP für den gesamten Datensatz wurde eine lineare Beziehung erstellt zwischen der Konzentration von NDLXP oder ADLXP und dem *in vivo* pcvXP: y = 0,8640 (Standard Fehler [SE] 0,019) x – 13,37 (SE 7,479), wobei y die *in vivo* pcvXP (g/kg Trockenmasse [TM]) und x Gehalte an NDLXP- (Getreide) oder ADLXP (Proteinkomponenten) (g/kg TM) repräsentiert. Das Bestimmtheitsmaß (R^2) dieser Gleichung lag bei 0,962. Eine Validierung mit Literaturwerten zeigte, dass die Gleichung eine gute Übereinstimmung mit einem unabhängigen Datensatz aufwies (n = 20; R^2 = 0,955).

Für die Schätzung von pcvAS wurde die gleiche Vorgehensweise, die für pcvXP beschrieben wurde, verwendet. Vom gleichen Datensatz standen hierfür 74 Futterkomponenten zur Verfügung. Aminosäuren in Futterkomponenten und in deren ND und AD Rückständen wurden durch eine HPLC Methode bestimmt. Die Konzentration (g/kg TM) von NDLAS und ADLAS wurde aus der Differenz zu den Gesamtaminosäuren im Futter berechnet. Für die Schätzung der Konzentrationen von *in vivo* pcvAS für die Gesamtaminosäuren und den Gesamten Datensatz (n = 74) wurde eine lineare Beziehung zwischen den Konzentrationen von NDLAS oder ADLAS und den *in vivo* pcdAS festgestellt. y = 0,823 (SE 0,018) x + 10,52 (SE 4.420), wobei y die *in vivo* pcvAS (g/kg TM) und x die Gehalte an NDLAS (Getreide) oder ADLAS (Proteinkomponenten) (g/kg TM) darstellt. Das R^2 dieser Gleichung lag bei 0.968. Für die 17 einzelnen AS befand sich das R^2 zwischen 0,895 bis 0,984. Diese Studie zeigt, dass alleine die chemische Analyse ausreicht, nämlich die Bestimmung von NDUXP/ADUXP und NDUAS/ADUAS, woraus NDLXP/ADLXP und NDLAS/ADLAS berechnet werden, um *in vivo* pcvXP und pcvAS mit einer standardisierten und schnellen Labormethode zu schätzen.

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List of abbreviations

AA	Amino acids
AD	Acid detergent
ADF	Acid detergent fibre expressed inclusive of residual ash
ADFom	Acid detergent fibre expressed exclusive residual ash
ADIAA	Acid-detergent-insoluble amino acids
ADICP	Acid-detergent-insoluble crude protein
ADL	Acid detergent lignin determined by solublisation of cellulose with sulphuric acid
ADSAA	Acid-detergent soluble amino acids
ADSCP	Acid-detergent soluble crude protein
AFZ	Association Francaise de Zootechnie
Ala	Alanine
aNDFom	Neutral detergent fibre assayed with heat stable amylase and expressed exclusive residual ash
ANF	Anti-nutritional factors
apcD	Apparent precaecal digestibility
Asn	Asparagin
Asp	Aspartic acid
Arg	Arginine
B	Barley
BGT	Barley genotype
BLE	Federal Office for Agriculture and Food; Bundesanstalt für Landwirtschaft und Ernährung
BMEL	Federal Ministry of Food and Agriculture; Bundesministeriums für Ernährung und Landwandwirtschaft
°C	Temperature in Celsius

CCellulose	Cellulose calculated
CHemicellulose	Hemicellulose calculated
CP	Crude protein
Cr_2O_3	Chromic oxide
CVB	Centraal Veevoeder Bureau
Cys	Cysteine
DM	Dry matter
e.g.	Exempli gratia
FFSB	Full fat soybean
FM	Fish meal
g	Gram
GfE	Society of Nutrition Physiology
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GSL	Glucosinolates
GT	Genotype
HCL	Hydrocloric acid
His	Histidine
HPLC	High performance liquid chromatography
IArabinoxylan	Insoluble Arabinoxylan
IBGlucan	Insoluble ß-Glucan
i.e.	Id est
Ile	Isoleucine
INSP	Insoluble non starch polysaccharides
kg	Kilogram
Leu	Leucine

Lys	Lysine
mm	Millimetre
Met	Methionine
N	Nitrogen
n.d.	Not determined/detectable
ND	Neutral detergent
NDF	Neutral detergent fibre
NDIAA	Neutral detergent insoluble amino acids
NDICP	Neutral detergent insoluble crude protein
NDSAA	Neutral detergent soluble amino acids
NDSCP	Neutral detergent soluble crude protein
NH_3	Ammonia
NIRS	Near Infrared Spectroscopy
NO_3^-	Nitrate
N_2O	Nitrous oxide
NRC	National Research Council
NSP	Non starch polysaccharides
PCA	Principal component analysis
pcd	Precaecal digestible
pcdAA	Precaecal digestible amino acids
pcdCP	Precaecal digestible crude protein
pcDCP	Precaecal digestibility of crude protein
pcDAA	Precaecal digestibility of amino acids
PeaP	Pea protein
Phe	Phenylalanine
Pro	Proline
Pyl	Pyrrolysine

R	Rye
R^2	Coefficient of determination
RGT	Rye genotype
rpm	Rounds per minute
RSC	Rapeseed cake
RSM	Rapeseed meal
RMSE	Root-mean-square error
SArabinoxylan	Soluble Arabinoxylan
SBGlucan	Soluble ß-Glucan
SBM	Soybean meal
SBC	Soybean cake
SCFA	Short chain fatty acids
SE	Standard error
Sec	Selenocysteine
Ser	Serine
SNSP	Soluble non-starch polysaccharides
spcD	Standardised precaecal digestibility
SPC	Soy protein concentrate
SPI	Soy protein isolate
T	Triticale
TArabinoxylan	Total Arabinoxylan
TBGlucan	Total ß-Glucan
TGT	Triticale genotype
Thr	Threonine
TI	Trypsin inhibitor
TIA	Trypsin inhibitror activity
TiO_2	Titanium dioxide

TNSP	Total non-starch polysaccharides
tpcD	True precaecal digestibility
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
W	Wheat
WB	Wheat bran
WF	Wheat feed
WG	Wheat gluten
WGT	Wheat genotype
WM	Wheat middlings
VDLUFA	Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten e. V.; Association of German Agricultural Analytic and Research Institutes

Chapter 1. General introduction

General introduction

Pig production has come under increasing criticism from environmentalists and the public due to high nitrogen (N) emissions, especially ammonia (NH_3), nitrate (NO_3^-) and nitrous oxide (N_2O). Also, legal requirements, such as the federal immission control act (Technical Instruction on Air Quality Control) (TA Luft, 2021) and the fertiliser ordiance (DüV, 2017) set guidelines in Germany. Environmentally harmful and odorus emissions are emitted both during manure and slurry handling (Lampe et al., 2006; Jha and Berrocoso et al. 2016) and directly from urine (Powell et al., 2011). Released N compounds contribute to the greenhouse effect and to input of N into soil and water. The resulting NH_3 leads to eutrophication of waters, which ultimately disrupts the ecosystem and threatens biodiversity. Therefore, a goal of pig production must be to reduce N inputs into the production cycle and to increase N utilisation, thus reducing N excretion via urine. This is a great opportunity to reduce N emissions from agriculture and to practice sustainable, environmentally and climate friendly agriculture.

Adequate ration planning is an opportunity to sustainably improve N use efficiency. The aim of compound feed and ration planning is to meet animal requirements. Optimising the crude protein (CP) and amino acid (AA) supply to ensure performance and animal health is an ongoing task in animal nutrition. Therefore, in order to feed the animal optimally the composition of the ration must be adapted to the requirement depending on age, size, sex and performance. Knowledge of the feed ingredients is essential for this. Therefore, in addition to routine chemical analysis, the determination of AA patterns is also used to evaluate feed ingredients. Depending on the type, pre-treatment and composition, feeds have different nutrient and in particular AA digestibilities, which have to be taken into account when planning a ration. Until now, this cannot be estimated from chemical analysis alone.

Reducing CP in the rations of weaned piglets is essential to reduce microbial protein degradation and fermentation in the hindgut (Nollet et al., 1999; Htoo et al., 2007; Wellock et al., 2008). Fermentation of undigested and endogenous N compounds can also produce toxic metabolites (Htoo et al., 2007; Moughan et al., 2014), which can lead to diarrhoea in weaned piglets (Heo et al., 2009; Marchetti et al., 2023). Reducing the level of CP in the ration and supplementing with limiting AA can reduce the production of toxic metabolites by microbes in the large intestine (Htoo et al., 2007), thereby reducing the incidence of diarrhoea. In addition, AA supplementation can reduce urinary N and total N excretion (Zervas and Zijlstra, 2002) without adversely affecting performance or carcass quality (Jha et al., 2013; Morales et al., 2015).

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Chapter 2. Scope of the thesis

Scope of the thesis

In recent years there have been major advances in the assessment of crude protein (CP) quality in feed. For example, precaecal digestible crude protein (pcdCP) and precaecal digestible amino acids (pcdAA) are key variables for protein evaluation in pigs in Germany (GfE, 2008). This protein evaluation system is similar to evaluation systems in the Netherlands (CVB, 1998), the United States (NRC, 1998) or France (AFZ, 2000). It is clear that the number of cannulated animals required for the determination of pcdCP and pcdAA is very large and that a validated laboratory analytical method is of great interest and can therefore be widely used.

In this work, a fast and simple laboratory chemical method for the estimation of precaecal digestible CP and AA from horse diets will be tested for its potential application in pigs. The following hypotheses have been formulated.

Hypothesis:

- The laboratory method is suitable for the estimation of standardised precaecal digestible crude protein and amino acids
- The laboratory method enables a more targeted use of feeds for a sustainable and optimal protein supply taking into account feed-specific properties
- The laboratory analytical method for estimating pcdCP and pcdAA in horse feeds is also applicable to pig feeds and for poultry feeds, however, the estimation equation has to be derived from *in vivo* data for the respective species.
- The laboratory method is expected to be easier to implement and faster than known *in vivo* and *in vitro* methods to estimate *in vivo* pcdCP and pcdAA

The workflow of this thesis is pictured and described in Figure 1.

Chapter 3 introduces the state of knowledge and gives an overview of protein and amino acid digestion in pigs and the *in vivo* and *in vitro* methods to determine protein and amino acid digestibility.

Chapter 4 presents the performance of the laboratory method to determine the NDSCP and ADSCP, the resulting regressions between the *in vivo* pcdCP data given in reference and the analytical data and the subsequent validation of the regression.

Chapter 5 presents the performance of the laboratory method for the analysis of NDSAA and ADSAA, the resulting regressions between *in vivo* pcdAA data given in reference and the analytical data.

Chapter 6 includes the final considerations about the feed ingredients and the used sample pool, the methods like the selection criteria, analytical procedure, data evaluation and NIRS.

The final chapter, Chapter 7, gives the conclusion and outlook.

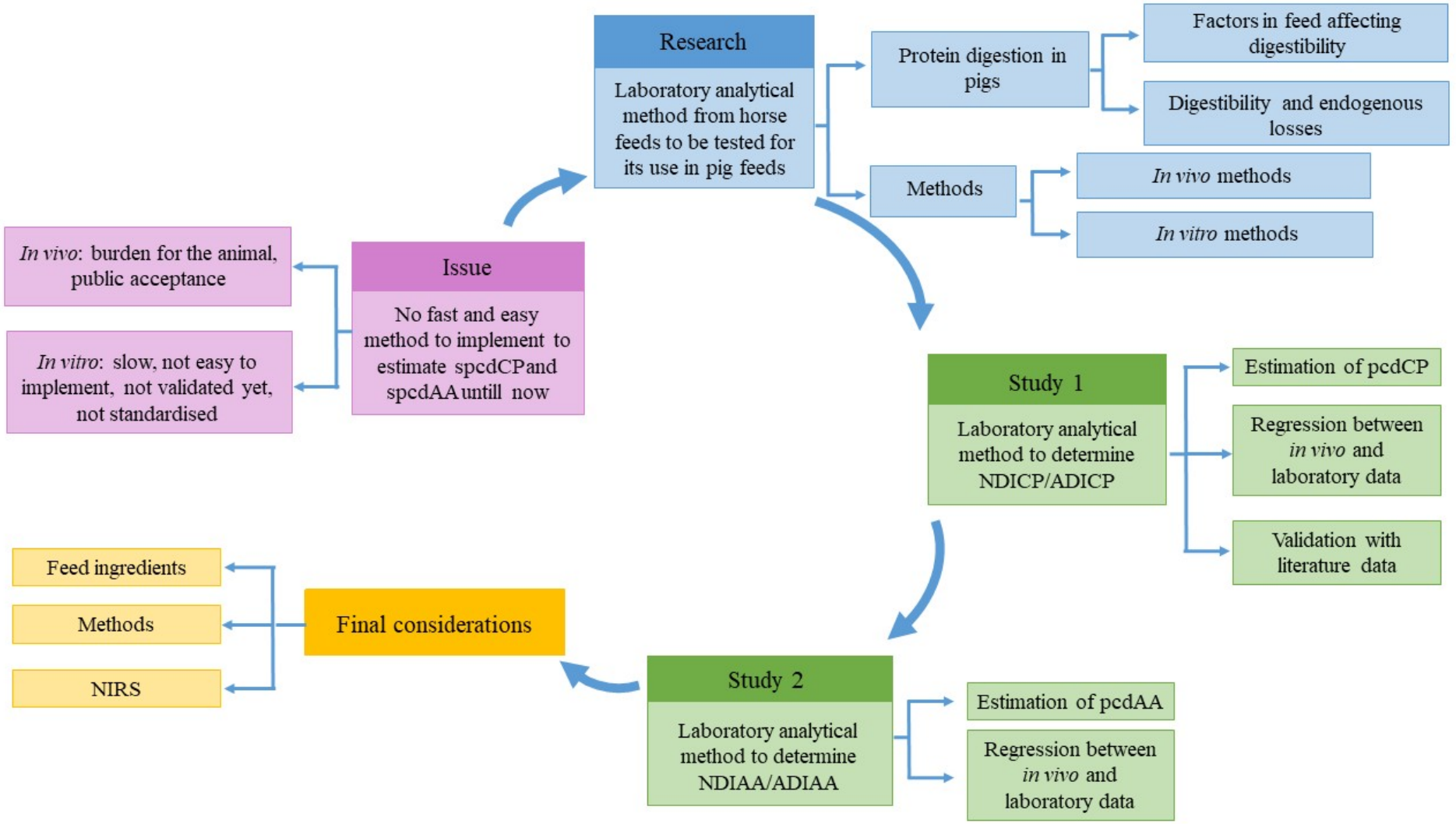

Figure 1: Workflow of this thesis.

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Chapter 3. State of knowledge

State of knowledge

3.1 Overview of protein and amino acid digestion in pigs

3.1.1 Proteins and amino acids

Proteins are essential components for all living organisms. They are built of a set of only 22 AA, which are connected with peptide bonds. Amino acids consist of an amino group, carboxyl group and a side chain. The structure of the protein is determined by the interaction between the side chains of the AA and other AA. The 22 AA can be divided into essential, semi essential and non-essential AA (Table 1). There are nine essential AA, which have to be supplied with the feed and ten non-essential AA, which can be synthesized by the pig itself. Three semi-essential AA can be synthesized from essential AA but not in the sufficient quantity as needed.

Table 1: Amino acids divided into essential, semi-essential and non-essential for pigs (Nørgaard, 2012).

Essential	Semi-essential	Non-essential
Histidine (His)	Arginine (Arg)	Alanine (Ala)
Isoleucine (Ile)	Cysteine (Cys)	Asparagine (Asn)
Leucine (Leu)	Tyrosine (Tyr)	Aspartic acid (Asp)
Lysine (Lys)		Glutamic acid (Glu)
Methionine (Met)		Glutamine (Gln)
Phenylalanine (Phe)		Glycine (Gly)
Threonine (Thr)		Proline (Pro)
Tryptophan (Trp)		Serine (Ser)
Valine (Val)		Selenocysteine (Sec)*
		Pyrrolysine (Pyl)*

*(until now selenocysteine (Johansson et al. 2005) and pyrrolysine (Zhang et al. 2005) are not taken into account for AA demand and supply).

Both for conservation and performance, AA are needed in the pig's organism. Depending on the stage of growth of the pigs, AA must be provided in the ration in an appropriate quantity and ratio to each other. The protein supply is therefore not only determined by the crude protein (CP) in the diet but requires rather consideration of the AA content (Mößeler-Witte et al., 2024). The correct supply of dietary AA is of great importance, as the undersupply of only one of the essential AA and first limiting AA results in growth decline (Nørgaard, 2012). A first limiting AA is labelled as the essential AA which is present in the diet in the lowest concentration

(Mößeler-Witte et al., 2024). In pig diets, the first limiting AA is mostly lysine. However, depending on the AA pattern of the feed ingredients the ranking can change and methionine or cysteine can become the first limiting AA. This can be the case for diets with components with high lysine levels like for example in diets with high graine legumes proportion (Witten et al., 2024) or high protein plasma diets for piglets (Wu et al., 2018).

The AA pattern determines the quality of the feed component and diets for the protein supply. The required AA pattern can be obtained through the combination of different feed components (complementary effect) and/or the supplementation of crystalline AA (Mößeler-Witte et al., 2024). For example, crystalline AA such as lysine, methionine, threonine, tryptophan and valine are used as feed additives in pig diets to compensate for the low AA content in feedstuffs such as cereal grains and to reduce the CP concentrations in the ration (Nørgaard, 2012). For reasons of economy (high prices for protein feeds) and environment (reduction of N input via excreta) the fundamental objective is to cover the AA requirements with the lowest possible CP content in the diet (Mößeler-Witte et al., 2024).

3.1.2 Protein and amino acid digestibility in pigs

Physiologically, digestion in pigs is divided into a precaecal digestion by endogenous enzymes and a post-ileal digestion by microbial digestion and fermentation (Figure 1). Digestion begins in the mouth and continues in the stomach and small intestine through mechanical and enzymatic mechanisms. The microbial activity mainly takes place in the large intestine (Figure 1). Most food components, such as starches and sugars, are digested and absorbed before the end of the ileum. In order to be absorbed in the small intestine, protein must be broken down with the help of enzymes. In the stomach, protein is denatured from hydrochloric acid and broken down from pepsin to peptides and AA. In the small intestine endo- and exopeptidases hydrolyse the proteins to oligopeptides and peptidases of the brush border membrane and split them into di- and tripeptides as well as AA, which are the resorbable end products (Scharrer and Wolffram, 2005). Smith and James (1976) described that the absorption of AA in the large intestine was possible for new born piglets but this ability vanishes rapidly shortly after birth and is very limited in grown pigs (Sepúlveda and Smith, 1979; Just et al., 1981). Components that are not digested before the ileum, such as complex carbohydrates or more precisely cell wall carbohydrates (hemicellulose, cellulose and pectins), as well as undigested proteins and endogenous proteins, are degraded and eventually fermented in the large intestine with the help of microbiota (Wiesemüller and Leibetseder, 1993).

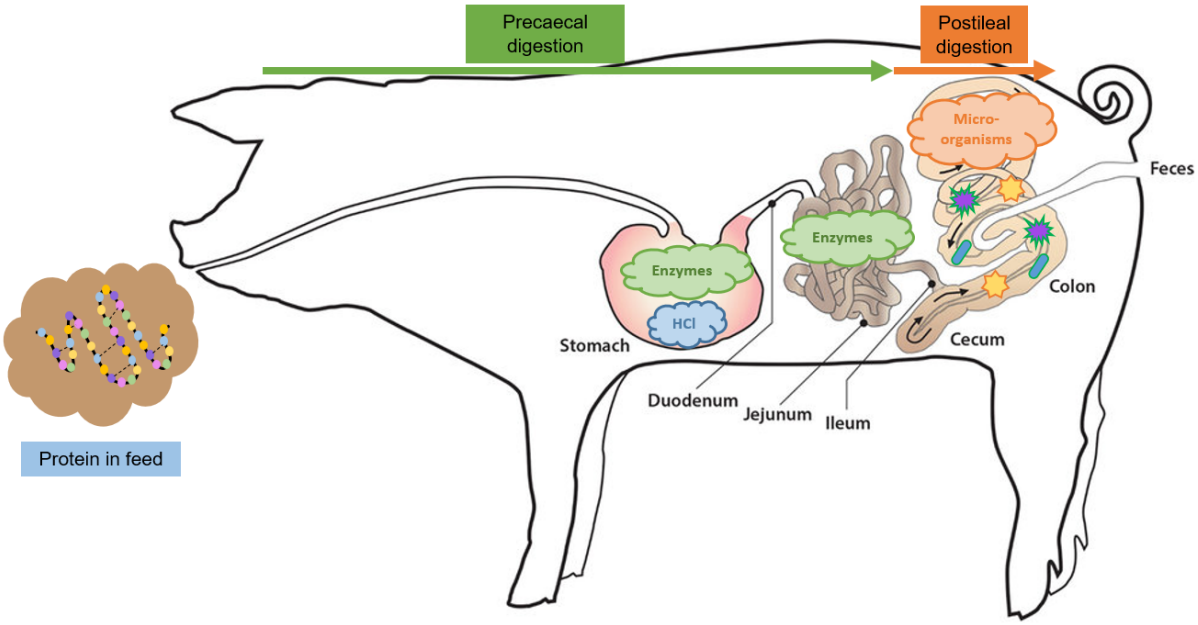

Figure 1: The gastro-intestinal tract of the pig (modified from Holman et al., 2017).

The end products of microbial fermentation are short-chain fatty acids (mainly acetate, propionate and butyrate), which can be absorbed and utilised in the intermediate metabolism in the pig. Further methane, carbon dioxide and the potential toxic metabolic product ammonia (NH_3) (Figure 2) are released, which can have a negative influence on performance and gut health (Williams et al., 2001). According to Visek (1984) and Nousiainen (1991), NH_3 can negatively influence the development of the intestinal mucosa and reduce the height of the villi. On the other hand, NH_3 can also be absorbed and transferred to the liver where it is detoxified to urea and either excreted in urine. After secretion into the large intestine, it is an important N-source for *de novo* synthesise of microbial AA respectively, protein which is excreted via faeces.

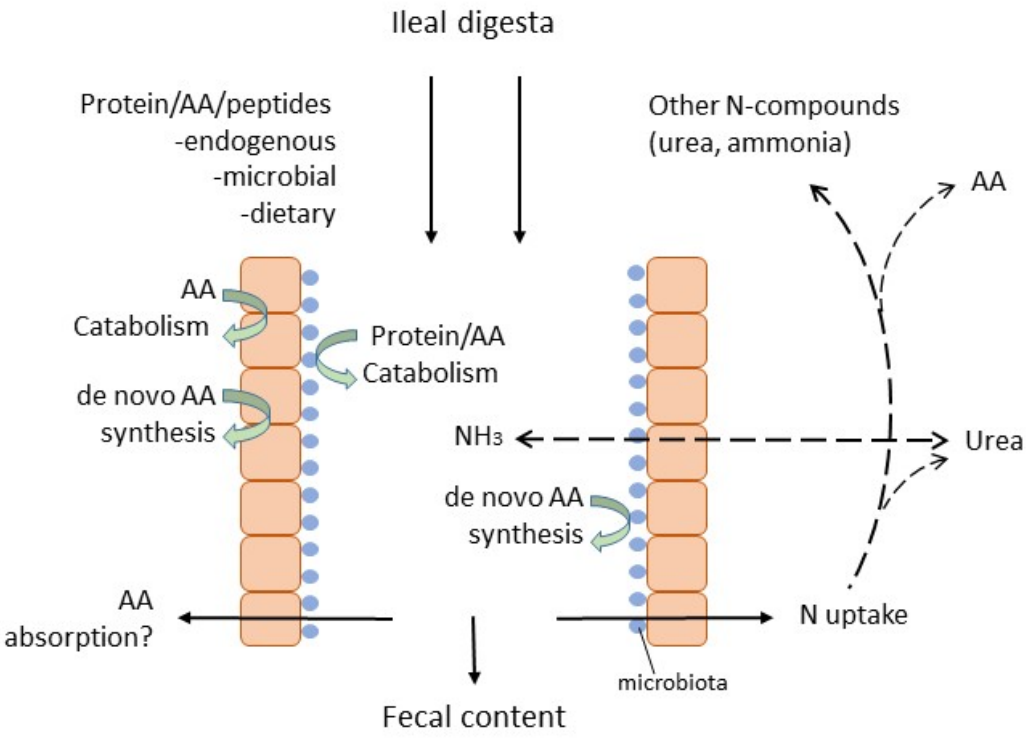

Figure 2: Peptide and AA metabolism in the large intestine (modified from van der Wielen et al., 2017).

3.1.3 Digestibility and endogenous losses

The protein supply is not only depending on the AA pattern of a feed component/diet but also on the digestibility of the AA (Williams, 1995). As described in the previous section, only AA which are absorbed before the terminal ileum are available for the protein supply of the pig.

A distinction must be made between AA that are digestible and those that are available. Apparent precaecal digestibility of AA is defined as the proportion of AA intake that does not appear in the digesta at the terminal ileum. This means that no correction is made for endogenous losses (described in the following). In contrast, availability is defined as the proportion of dietary AA that is associated with compounds involved in digestion, absorption and utilisation for tissue growth (ARC, 1981). According to Wiseman et al. (1991) and Batterham (1992), there may be an overestimation of precaecal (synonymously termed ‘ileal’) AA digestibility compared to availability if, for example, the ration contains AA that have been partially damaged by heat treatment and are not available for intermediate AA metabolism.

Material (structural carbohydrates like cellulose and hemicellulose) that has not been digested precaecally or endogenous protein (described in the following) can be fermented partially in the large intestine. End products of this fermentation are short chain fatty acids, which can be

absorbed and used in the pig's metabolism (can contribute to cover 15-30% of the basal energy demand (Breves and Diener, 2005) and ammonia, which is partly used for the new synthesis of microbial AA. However, these microbial AA are not utilised by the host animal, which means they are not available to the animal itself as a source of AA for maintain or tissue accretion. They are excreted as such via faeces or in case of ammonia after the absorption detoxified in the liver to urea and excreted via urine (Jha and Berrocoso, 2016). Finally, this means that only a very small proportion of the AA that is excreted in the faeces is derived from the AA that is recovered from the distal ileum. Therefore, precaecal digestible AA (pcdAA) give a more precise estimate of the AA available to the animal compared to AA that have disappeared from the total length of the digestive tract (Sauer and Ozimek, 1986; Williams, 1995; Parsons, 1996).

The precaecal digestibility of CP and AA (pcD) can be expressed as apparent (apcD), true (tpcD) or standardised (spcD) precaecal digestibility. The term used depends on which part of the precaecal outflow respectively, which AA losses are included in the calculation (Stein et al., 2007b). Sauer and Ozimek (1986) calculated the values for the pcdAA by subtracting the amount of AA in the outflow of the precaecal digesta from the amount that was ingested by the animal. The precaecal digesta contains both unabsorbed exogenous AA from dietary sources as well as precaecal endogenous AA losses (AA from endogenous sources). Jansman et al. (2002) described that endogenous N compounds arise from salivary, gastric, pancreatic, bile and small intestinal secretions as well as mucoproteins and exfoliated cells. As a result of microbial activity in the small intestine, the digesta at the terminal ileum also contains microbial CP derived from dietary protein and endogenous protein (Blok et al., 2017). Strictly speaking, this microbial CP is not endogenous. However, as it does not come from the diet, it is usually taken into account when determining endogenous protein (Moughan et al., 2005; Miner-Williams et al., 2009). The precaecal endogenous losses can be divided into basal endogenous losses and specific endogenous losses (Jansman, et al., 2002; Stein et al., 2007a; McDonald et al., 2011).

Basal endogenous losses are inevitable losses that are closely related to the metabolic function of the animal and are independent of ration composition but strongly influenced by dry matter (DM) intake (Boisen and Fernández, 1995; Jansman et al., 2002; Moter and Stein, 2004; McDonald et al., 2011). Therefore, basal endogenous losses are expressed in relation to DM intake. Stein et al. (2007a) stated, that they also could be affected by the physiological state of the animal or the experimental conditions. Basal endogenous losses come from various sources such as saliva, pancreatic and biliary secretions, sloughed epithelial cells and mucus (Souffrant, 1991). The value of basal endogenous CP or AA losses is constant at different levels of CP or AA in the ration with a constant DM intake (Figure 3). Stein et al. (2007a) described, that the

source of the dietary protein and the level of inclusion determines the amount of unabsorbed exogenous dietary protein in the precaecal digesta. However, there exist no techniques for the direct measurement of all undigested dietary AA in the ileal digesta. Nevertheless, Boisen and Fernández (1995) reported that approaches to measure the digestibility *in vitro* can also provide estimations of undigested dietary protein in the precaecal digesta. Basal endogenous losses can be estimated in digestibility trials with standard N-free diets or highly digestible purified diets and the peptide alimentation or regression technique (Jansman et al., 2002; Moughan, 2003; Stein et al., 2007b). Whereby standard N-free diets are preferred over the other methods and used most frequently (Stein et al., 2007b).

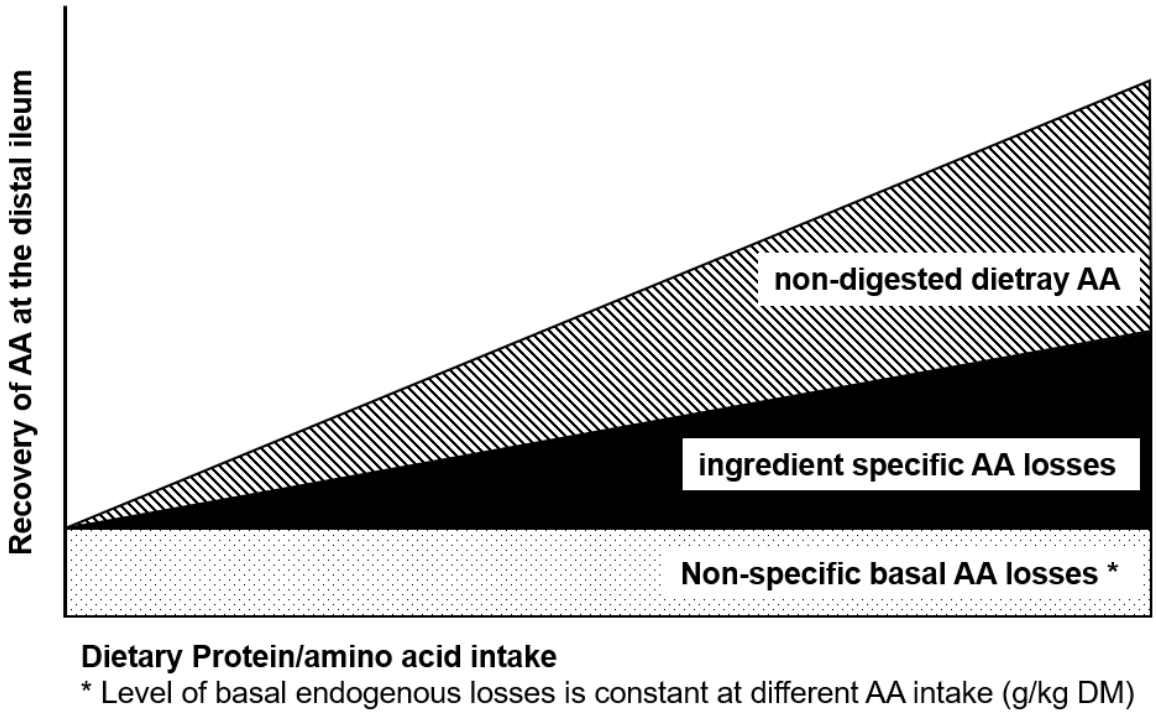

Figure 3: Precaecal amino acid losses affected by dietary protein and amino acid intake at constant DM intake (modified from Lemme et al., 2004).

Specific endogenous N losses represent the losses above the basal endogenous losses (Figure 3). They are caused by the ingestion of feed with specific composition, are variable and depend on factors in the ration such as CP content, fibre type and anti-nutritional factors (ANF) (Cowieson and Ravindran, 2007; Stein et al., 2007b). In the case of highly digestible proteins, the specific endogenous losses are minimal. However, when feeding a ration rich in fibre and ANF, specific endogenous losses can contribute more than 50% to the total endogenous losses (Souffrant, 1991; Moughan, 2003; Stein et al., 2007a). With an estimation of the total endogenous losses (basal + specific) and subtracting the basal endogenous losses from them, it is possible to

calculate the specific endogenous losses (Stein et al., 2007b). Methods to estimate the total endogenous losses like the homoarginine technique (Rutherford and Moughan, 1990) and the isotope tracer dilution technique (de Lange et al., 1990) have limitations (Leterme et al., 1998) and are labour intensive, costly and dependent on special equipment (Stein et al. 2007b). Therefore, total endogenous losses are not determined in the routine evaluation of feed ingredients, which is why no routine procedures exist for the direct measurement of specific endogenous N losses (Stein et al., 2007a; Stein et al., 2007b). As there are sometimes inaccuracies in the termination of apparent, true and standardised precaecal digestibility, in the following definition for terminology in literature used in this report will be given:

The apcD represents the net disappearance of CP or AA from the precaecal gastro intestinal tract (Stein et al., 2007a) and is calculated by subtracting the total precaecal CP or AA outflow (not digested and endogenous losses) from the CP or AA intake:

$$\text{apcD (\%)} = \frac{\text{CP or AA intake - (precaecal CP or AA outflow)}}{\text{CP or AA intake}} \text{ x } 100 \qquad [1]$$

The term “apparent” is used to describe undigested dietary CP and AA and into the gastrointestinal tract secreted endogenous CP and AA which are not absorbed precaecally, therefore they contribute to the total precaecal CP and AA outflow (Stein et al., 2007b).

The tpcD represents the portion of CP or AA that disappear from the precaecal gastro-intestinal tract and which does not include any endogenous losses (Stein et al., 2007a) and is calculated according to the equation:

$$\text{tpcD (\%)} = \frac{\text{CP or AA intake - (precaecal CP or AA outflow - total endogenous CP or AA losses)}}{\text{CP or AA intake}} \text{ x } 100 \qquad [2]$$

In previous works of de Lange et al (1990) and Souffrant (1991) the tpcD has also been described as real ileal digestibility. However, in order to maintain the consistency of the term between species and nutrients, the term true is preferred (Stein et al., 2007b).

The spcD is calculated by subtracting only basal endogenous losses form the precaecal outflow of CP and AA (Jansman et al., 2002; Stein et al., 2007a):

$$\text{spcD (\%)} = \frac{\text{CP or AA intake - (precaecal CP or AA outflow - basal endogenous CP or AA losses)}}{\text{CP or AA intake}} \text{ x } 100 \qquad [3]$$

The values for spcD are between the values for the apparent and true precaecal digestibility, because only the values for the basal endogenous losses are subtracted from the total precaecal CP and AA outflow. Therefore, the values for spcD are not depending on the CP and AA in the feed (Stein et al., 2007b). Unfortunately, there were also inaccuracies in the use of this term, as

e.g., NRC (1998) used the term tpcD to describe the spcD, which led to confusion about the correct use and interpretation of precaecal digestibility values (Stein et al., 2007b). The disadvantages and limitations of apcD and tpcD are on the one hand the lack of additivity of pcD of individual components to mixtures (Jansman et al., 2002; Stein et al., 2005) and on the other hand the difficulty in measuring the total endogenous losses, respectively, there is a method to measure basal endogenous losses but no routine method to measure specific endogenous losses. In contrast, the spcD has advantages: N-secretions induced by specific feed components are included in the calculation as only basal endogenous losses are subtracted from the outflow, which means the spcD is independent of basal endogenous losses (Stein et al., 2005). Mosenthin et al. (2000) and Stein (2005) already pointed out that the values of spcD of feed ingredients are more additive in mixtures than values which are based on apcD. Due to the previously described reasons, the GfE (2008) decided to evaluate the precaecal digestibility on the basis of standardised precaecal digestibility, and agreed on the term pcdCP and pcdAA.

3.1.4 Feed factors affecting digestibility

As mentioned above, the digestibility of CP and AA depends on the feed and the type of protein. Protein digestion and absorption can be impaired by ANF from secondary plant substances and limits nutritional value (Clarke and Wiseman, 2000). These compounds are often found in grain legumes and rapeseed, which are part of chemical plant defence mechanisms and include protease inhibitors, such as trypsin inhibitors (TI), or tannins as well as phytate, the latter forming complexes with proteins and glucosinolates. Non-ruminants are more vulnerable for ANF induced declines in performance than ruminants, because they lack the ruminal microbes which enables the degradation of ANF (Ohm and Südekum, 2024). There are several procedures, categorized in chemical-biological (e.g., soaking, germination, fermentation) and physical (e.g., dehulling, heat treatment), depending on the feed and animal species, to reduce these ANF (Ohm and Südekum, 2024). Pal et al. (2016, 2017) and Sangronis and Machado (2007) described the induced germination of disinfected seeds as a method to reduce the content of TI and tannins in lentils and beans. Fermentation, which is based on naturally occurring microorganisms, was also described as a method to reduce TI and tannins in grain legumes (Coda et al., 2015; James et al., 2020; Sakandar et al. 2021). Soaking (various soaking media, time and temperature) is used in grain legumes as single method or in combination with heat or pressure to reduce the tannin content in the feed component (Ohm and Südekum, 2024; Khandelwal et al., 2010). In combination with the previous soaking in water Pal et al. (2017) could confirm a reduction of TI and tannins in dehulled lentils. Pal et al. (2016) could prove

similar results for dehulled beans. Another procedure to reduce ANF in feed components is the treatment with heat during toasting, roasting and extruding. For example, soybeans were treated with heat to reduce their TI activity (Messerschmidt et al., 2012). But the thermal treatment during the processing of feed can have potentially negative impact on CP availability caused by formation of Maillard products. In the following, the antinutritional factors and the procedures to reduce them will be described in more detail.

Trypsin inhibitors

Trypsin inhibitors form enzyme inhibitor complexes with the pancreatic enzymes trypsin and chymotrypsin, which lead to a reduction in protein digestion (Grala et al., 1998) leading to a negative impact on growth performance, which can vary between species (Batterham et al., 1993). Jezierny et al. (2010) analysed trypsin inhibitor activity (TIA) in white and coloured faba beans. The white flowered faba beans showed higher TIA values compared to the coloured flowered faba beans. The TIA is the most important ANF in soybeans (Kuenz et al., 2022). To avoid a reduction of performance by TI in pig diets including soybean products, TIA must be deactivated considerably. For pig feed, Batterham et al. (1993) recommended a reduction to 4.7 mg TI/g for grain legumes (chickpeas and pigeon peas). Hansen et al. (1987) also observed no significant differences in digestibility due to TI in a range of 1.6 to 5.3 mg TI/g.

Tannins

Tannins can be roughly divided into two groups. The hydrolysable tannins which are recovered in the neutral-detergent (ND) residue during detergent fibre analysis and the condensed tannins are recovered in acid-detergent (AD) residue (van Soest, 1994). Condensed tannins are known to form complexes with proteins and enzymes and thus have a negative influence on the digestibility of CP (Jansman, 1993). Therefore, the specific endogenous excretions are higher here and thus pcDCP is lower.

Glucosinolates

The use of rapeseed products in pig feed is often limited by degradation products of glucosinolates (GSL). They are formed by the enzyme myrosinase and have a negative influence on the liver, thyroid gland and kidney function of the pig (Bell, 1993). According to McCurdy (1992), the majority of myrosinases are inactivated during the steps conditioning, mechanical oil extraction, toasting and solvent extraction of oil. The hydrothermal treatment during the dissolving and toasting processes greatly reduced the GSL content in rapeseed meal (RSM) compared to rapeseed cake (RSC). Eklund et al. (2015) and Kaewtapee et al. (2017b) also observed a decrease in GSL content with increasing time of heat treatment.

Maillard products

Strong thermal treatment of the feed to reduce TIA and GSL content can cause a condensation of the free amino groups of the AA with reducing sugars (e.g., glucose, fructose) depending on heating time and moisture during heat treatment. In this reaction, the so-called Maillard products are formed, which can also lead to a negative impact on the intestinal availability of CP and AA, respectively, to the animal (Messerschmidt et al., 2012). Excessive heat treatment stimulates the formation of Maillard products and due to the strong heat exposure of oilseed products, an increased amount of ND insoluble N can be detected (Eklund et al., 2015; Pastuszewska et al., 2003; Kaewtapee et al., 2017a).

3.2 Methods to determine protein and amino acid digestibility

The literature frequently uses the pcD to describe the quality of feed, however this unit is an exclusively calculated quantity form the digestible CP and AA. Therefor the precaecal digestible (pcd) CP and AA is a more accurate unit, as it describes which proportion was absorbed at the ileum.

The primary objective of planning rations is to ensure that animals are supplied with the required protein, more precisely AA, to maintain performance and health, which is an ongoing challenge for animal nutrition. Pigs can be categorized anatomically as large intestine fermenters. Therefore, AA are primarily absorbed in the small intestine up to the terminal ileum. Therefore, precaecal digestible AA, which are available to the animal, is of particular importance in this context. Protein evaluation for pig feeds in Germany is based on pcdCP (GfE, 2008). For pcdCP apparent digestible CP is corrected for basal endogenous CP losses, but specific endogenous CP losses, which depend on feed properties, such as fibres, tannins and protease inhibitors, are not considered (Mosenthin and Rademacher, 2003; Adeola et al., 2016). So far, there are several approaches to estimate pcdCP and pcdAA both *in vivo* and *in vitro*.

3.2.1 *In vivo* approaches

The methods used to determine pcdCP and pcdAA *in vivo* are often time-consuming and invasive. But they are the so called “gold standard” as they are performed directly on the animal and can therefore reflect the digestion processes most accurately (Zaefarian et al., 2021; Santos-Sánchez et al., 2024). The digesta collection at the terminal ileum is the basis for the determination of *in vivo* digestibility (GfE, 2002; GfE, 2005; Mosenthin et al., 2007), which often involves marker methods using indigestible markers such as chromic oxide (Cr_2O_3) or titanium dioxide (TiO_2), as well as direct total chyme sampling methods (Mroz et al., 1996). In

the future the use of these markers may face restrictions due to concerns about health hazards for humans (Sedman et al., 2006; Bampidis et al., 2021). A cannulation at the terminal ileum is often used to determine pcdCP and pcdAA *in vivo*. Methods to analyse small intestinal chyme sampled by cannulas are the most accurate. The cannula is inserted during a surgery into the terminal ileum trough which ileal digesta can be collected and subsequently analysed (Mroz, 1996; Mosenthin et al., 2007). The GfE (2005) stated that in principle there are three different cannulation techniques with different modifications: simple cannulation techniques (Cunningham et al., 1963; Furuya and Kaji, 1989), re-entrant cannulation techniques (Cunningham et al., 1962; van Leeuwen et al., 1988a) and ileo-rectal anastomosis techniques (Fuller and Livingston, 1982; Roth et al., 1999). In Zhang et al. (2013) these invasive *in vivo* methods and the respective surgical procedures can be found described in more detail. The different cannula methods at the terminal ileum are depending on the structure and CP content of the feed (Yin et al., 1991; Mroz et al., 1996; Zhang et al., 2004; GfE, 2005; Mosenthin et al., 2007; Metzler-Zebeli et al., 2020). But, as different cannula methods lead to different results, the results are only comparable when using the same cannula method. The simple T-cannula (Figure 4) is the most common method to collect digesta (GfE, 2005). However, there are concerns about receiving representative samples and the potential shortage of markers for this method (GfE, 2005; Yin et al., 2000).

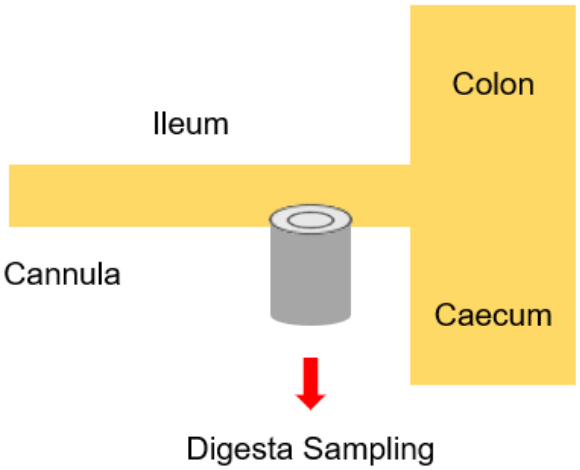

Figure 4: Simple t-cannula (modified from Moughan and Miner-Williams, 2013).

Modifications to improve the representative sampling of the digesta are the post-valve t - caecum cannula (van Leeuwen et al., 1988b) (Figure 5) and the steered ileo-caecal valve (Mroz et al., 1991) (Figure 6).

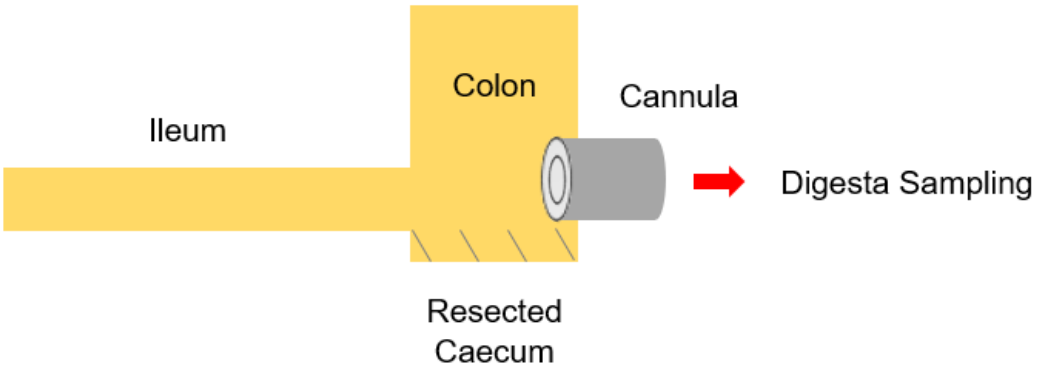

Figure 5: Post-valve t-caecum cannula (modified from Moughan and Miner-Williams, 2013).

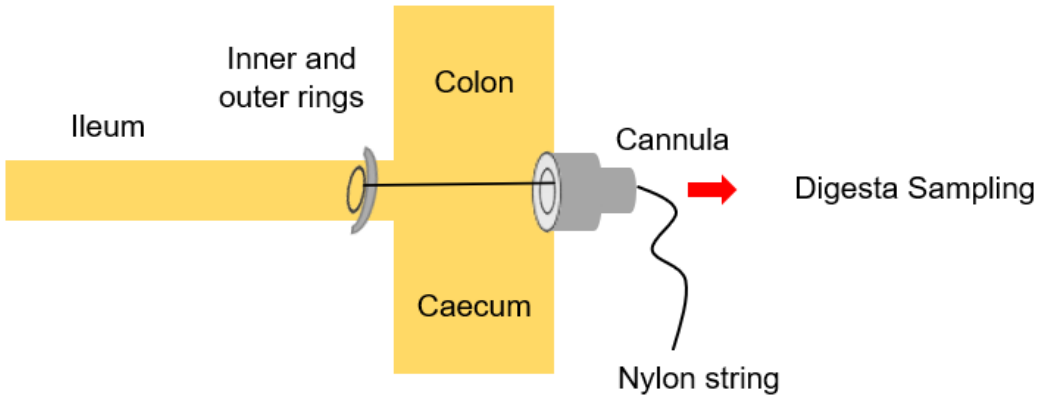

Figure 6: Steered ileo-caecal valve (modified from Moughan and Miner-Williams, 2013).

With re-entrant cannulas like the ileo-ileal re-entrant cannulas or ileo-caecal re-entrant cannula (Cunningham et al., 1962) (Figure 7 and Figure 8) the possible problems described above can be avoided (GfE, 2005). But, also these two methods have problems like blockage of the cannulas with digesta for example trough increasing particle size or crude fibre content (GfE, 2005), which is resulting in labour intensive inspection and cleaning of the cannula (van Leeuwen et al., 1987; Köhler, 1992).

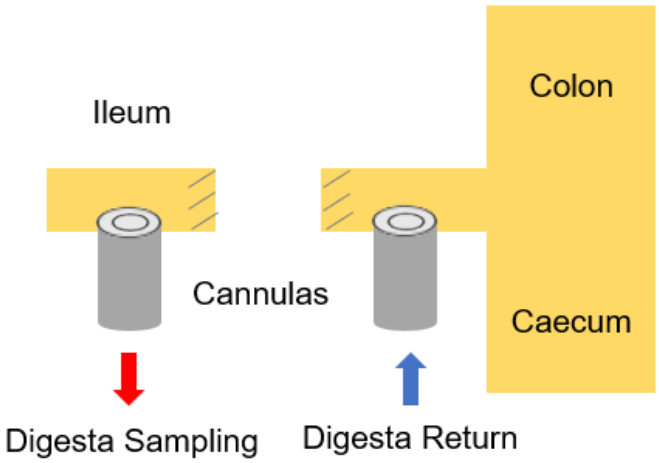

Figure 7: Ileo-ileal re-entrant cannula (modified from Moughan and Miner-Williams, 2013).

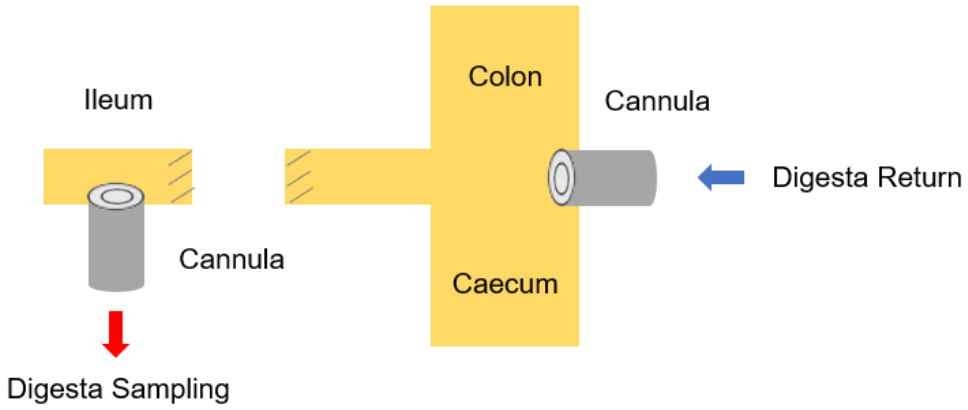

Figure 8: Ileo-caecal cannula (modified from Moughan and Miner-Williams, 2013).

The ileo-rectal anastomosis technique (Figure 9) described in Fuller and Livingston (1982) represents a good alternative with many advantages compared to the previous described methods (GfE, 2005): The collection is easy to handle and quantitative because the ileal digesta is excreted via the rectum. Pigs equipped with this kind of procedure are less time consuming and labour-intensive in the canula care than pigs with re-entrant cannulas, pigs equipped with this method can also be used for a longer time period compared to other cannula methods and diets with a high fibre content can be tested without problems. However, important points like the daily cleaning of the anus area to prevent skin irritations from ileal digesta have to be taken into account (GfE, 2005). Due to the missing of the caecum and colon and their functions the water intake of the pigs is two to three times higher compared to normal pigs (GfE, 2005). To

compensate the losses it is important, that the diets for those pigs contain several electrolytes (GfE, 2002).

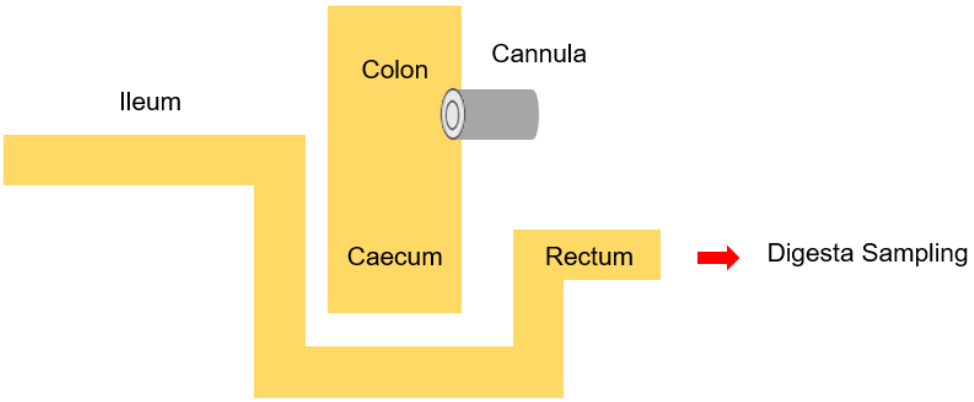

Figure 9: Ileo-rectal anastomosis (modified from Köhler, 1992).

The collection of the chyme after the animals have been killed is disadvantageous, as there is a possibility that the small intestinal chyme may be mixed with the contents of other intestinal segments (GfE, 2002). Both, generally after death and also caused by electrical current, there could be a shedding of mucosal cells which enter in the gastro intestinal tract and could affect the N and AA content of the digesta (Fell, 1961; Sauer and de Lange, 1993; Albin et al., 1999). Furthermore, only one sample can be collected per animal, which is why more animals are used to achieve reliable results (Zhang et al., 2013).

There have also been attempts to estimate CP digestibility in pigs using rats as model animals, but when fed high-fibre diets, digestibility in rats is lower than in pigs (Eggum et al., 1982; Balle et al., 2000; Pastuszewska et al., 2003; Jørgensen and Lindberg, 2006). Although some works described similar ileal AA digestibility coefficients for example in barley meal, fishmeal and meat and bone meal (Moughan et al., 1984), the rat does not seem to be suitable for all feed components (Donkoh et al., 1994). There were found differences between pigs and rats in legumes for example peas (Moughan et al., 1984) and beans and peas (Huisman et al., 1991). Rutherford and Moughan (2003) concluded that the rat has potential to be a good model animal for pigs for a rapid screening of the ileal amino acid digestibility (specific range of feed components), but is not suitable when a high degree of accuracy is required. Another important point is, that there were also different methods for rats to determine digestibility like after slaughter (Donkoh et al., 1994; Rutherford and Moughan, 2003) or with an antibiotic-treatment (nebacitin) to reduce the intestinal flora (Wisker and Bach Knudsen, 2003), which can have an influence on the collected digesta and further on the results.

In vivo approaches on animals play an essential role for the precise determination of pcdCP and pcdAA in pigs. However, with regard to animal welfare they should be kept to the indispensable minimum. An important guideline in this respect forms the 3-R-concept: Replace, Reduce, Refine after Russell and Burch (1992). The consistent realisation of the 3-R-concept creates the basis for the authorisation of animal experiments. More precisely, the three terms mean that animal experiments should be replaced with alternative methods, if possible. The number of experimental animals should be reduced to the indispensable minimum and a constant refinement of the research methods should reduce stress and suffering for the animal.

In the previous passages it was described that *in vivo* experiments require a large amount of feed, experimental animals or model animals and a considerable amount of equipment and labour. In addition, public acceptance of animal testing is low, regardless of the actual burden on the test animals. As a result, the authorisation and realisation of invasive animal experiments in Germany and other European countries is becoming increasingly difficult, since they are very complex and the 3-R-concept has to be implemented consistently (Santos-Sánches et al., 2024). Therefore, rapid *in vitro* methods are needed as an alternative to simulate digestion processes in laboratory environment and estimate pcdCP and pcdAA (Moughan et al., 2014; Santos-Sánchez et al., 2024).

3.2.2 *In vitro* approaches

In vitro digestion procedures are considered as acceptable alternatives to animal experiments because they are less labour-intensive and time-consuming (Boisen and Fernández, 1995; Regmi et al., 2009; Kim et al., 2021; Palowski et al., 2021). *In vitro* methods simulate – at least partly – digestive processes in the pig's gastro intestinal tract by incubation with enzymes in a laboratory setting. Boisen and Eggum (1991) gave an overview of some *in vitro* methods for estimating the nutrient digestibility of diets in pigs. They described and compared dialysis cell methods, pH-drop and pH-stat methods, colourimetric methods, filtration methods and multi-enzyme methods. They conclude that for general and routine feed evaluation the multi-enzyme method, which was later optimized by two (Boisen and Fernández, 1995) or three (Boisen and Fernández, 1997) incubation steps followed by separation of the undigested material by filtration, is the most suitable method (Figure 10). *In vitro* techniques should be able to provide a general estimation of the nutrient digestibility of diets. According to Boisen and Eggum (1991), the multi-enzyme method makes this possible for a wide range of feeds. But variations can easily occur, because the effect of enzymes is highly dependent on external influences, e.g., ambient temperature. Therefore, multi-enzyme methods may require complex handling,

especially when a large number of samples has to be analysed. Although this procedure has been used increasingly since 1995, there is still a lack of validation studies to support its suitability for estimating the pcdCP and pcdAA (Pujol and Torrallardona, 2007; Eklund et al., 2013; Rosenfelder-Kuon et al., 2020). Eklund et al. (2015) pointed out the lack of research on *in vitro* pcdCP and pcdAA estimation in thermally processed feed. Which can also be attributed to the large variations of the *in vitro* results between laboratories and between experiments (Boisen and Fernández, 1995; Pujol and Torrallardona, 2007). Further, the different digestion conditions like the selection of enzymes, pH value and digestion time, which were used in distinct ways by several authors require optimisation and standardisation (Santos-Sánchez et al., 2024). Also, the different sample preparation (grinding, pre-treatment) needs to be standardised. This also makes it difficult to compare the results with each other over the past 40 years and makes clear why a harmonisation of *in vitro* methods is so essential (Moughan et al., 2014).

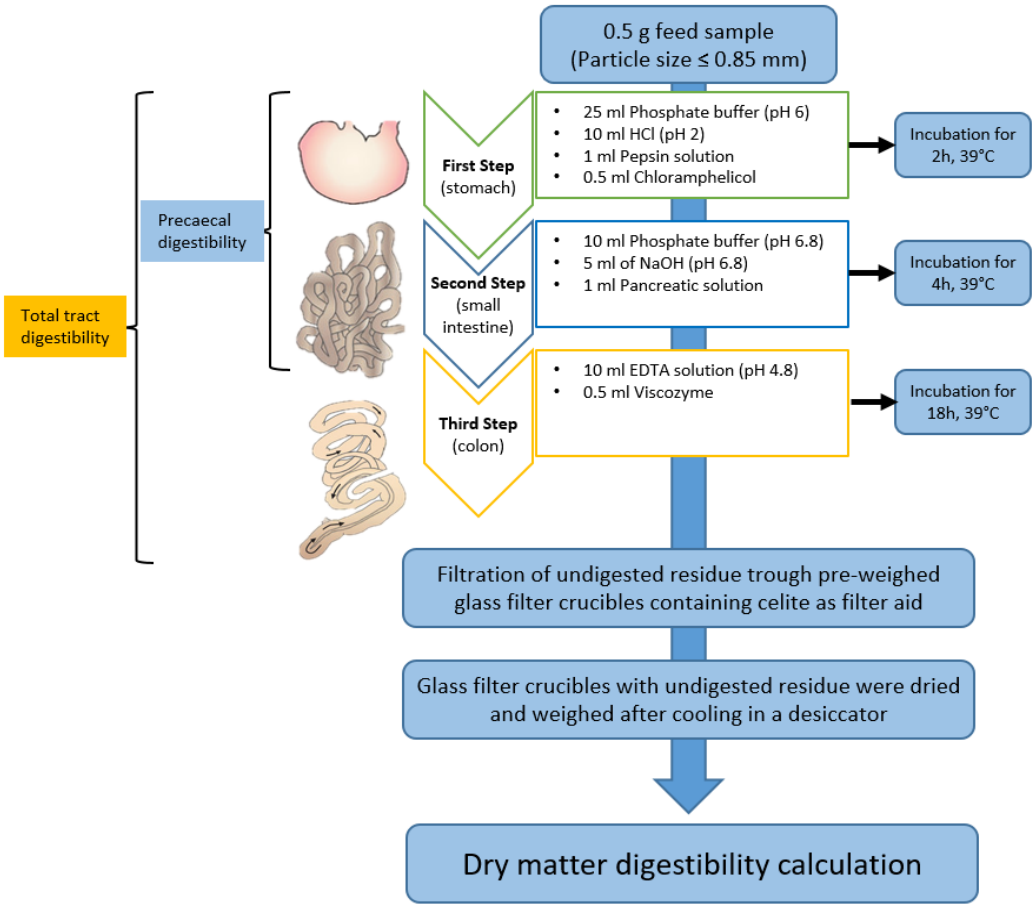

Figure 10: Schematic representation of the *in vitro* two- and three-step multi-enzyme method of Boisen and Fernández (1995, 1997) (modified from Recharla et al., 2019; images of the stomach and intestines from Holman et al., 2017).

The pcdCP and pcdAA can only be determined with the two-step method (incubation with porcine pepsin and pancreatin) and mimics the pcD (Boisen and Fernández, 1995), whereas the three-step procedure (with an additional incubation step using a multi-enzyme mixture) mimics the total tract digestibility (Boisen and Fernández, 1997) (Figure 10). In the meantime, the two-step procedure has been applied in a number of studies to estimate pcdCP e.g., Pujol and Torrallardona (2007), Eklund et al. (2013), Salazar-Villanea et al. (2016), Bachmann et al. (2021), Kim et al. (2021), Kim et al. (2022) and Song et al. (2024) and to estimate pcdAA e.g., Boisen and Fernández (1995), Pujol and Torrallardona (2007), Jezierny et al. (2010), Eklund et al. (2013), Eklund et al. (2015) and Rosenfelder-Kuon et al. (2020). Unfortunately, in the above studies, the procedural specifications of the multi-enzyme method varied considerably among laboratories such that data of these studies could not be merged into one single dataset for further evaluation. Generally, the aim is a standardisation of these *in vitro* methods for animals and humans, but so far over the past 40 years this has only been implemented in the human nutrition by the INFOGEST digestion protocol (Minekus et al., 2014; Brodkorb et al., 2019). However, the INFOGEST protocol is rather complex in its application and not practicable for the routine use in animal nutrition.

Near Infrared Spectroscopy

Near Infrared Spectroscopy (NIRS) can be mentioned as another method for estimating CP or AA digestibility (Harrison et al., 1991; van Leeuwen et al., 1991; Pujol et al., 2007; Rahman et al., 2015). With this method, the molecules in a feed component are brought to vibrate with electromagnetic light in the near infrared range. The radiation which is absorbed respectively, reflected gives information about the composition of the molecules (Zaefarian et al, 2021, Eder et al., 2024). This method provides results much faster than chemical methods because it is a physical method, which needs a minimum of sample preparation and reagents (Zaefarian et al., 2021), therefore it is used frequently (Owens et al. 2009).

However, the NIRS method is not independent and requires an ongoing calibration based on a very large number of *in vivo* reference data (Pujol et al., 2007; Patience et al., 2009; Rahman et al., 2015, Zaefarian et al., 2021) for, e.g., different feeds, genotypes and treatments to allow robust and accurate estimates (Rahman et al., 2015). This reference data must come form *in vivo* digestion studies, which is why the NIRS method is dependent on animal studies (Jha and Tiwari, 2016). Also reference data of chemical analyses and *in vitro* studies are required for accurate calibration (Zaefarian et al., 2021). An advantage is, that one calibration can be transferred and used to further NIRS instruments in the same field (Fernandez-Ahumada et al.,

2008). The quality of the calibration improves constantly with a wider spectrum and larger amount of data (van Kempen and Simminis, 1997). Therefore, the quality of the NIRS determination is dependent on the reproducibility and precision of the data which were used for the calibration (Owens et al., 2009; Jha and Tiwari, 2016). Also, a certain expertise is required to calibrate and validate the results (Jha and Tiwari, 2016).

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Chapter 4. A simple laboratory method for estimating the standardised precaecal digestible crude protein in pig feeds

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4.1 Abstract

Adequate protein supply for pigs to sustain performance and animal health can be determined *in vivo* from standardised precaecal digestible (pcd) crude protein (pcdCP). Until now, only time-consuming methods are available to estimate pcdCP from laboratory measurements without *in vivo* experiments. Therefore, the objective was to develop and establish a rapid laboratory method for estimating pcdCP based on the determination of neutral-detergent-insoluble or acid-detergent-insoluble crude protein (NDICP, ADICP). The CP was determined for cereal grains on the neutral detergent residue and for all protein feeds on the acid detergent residue, because N compounds such as Maillard products or N bound to tannin or in phytate complexes are retained in the acid detergent-insoluble fraction. A unique, large sample pool of 82 feed ingredients (cereal grains, differently heat-treated legume grains) was available on which *in vivo* pcdCP were determined in cannulated pigs. Crude protein (N · 6.25) was determined in feed ingredients and in their ND or AD residues. The concentrations of ND- and AD-soluble CP (NDSCP, ADSCP) were calculated by difference. For the estimation of the concentrations of *in vivo* pcdCP for the entire dataset, a linear relationship was established between the concentrations of NDSCP or ADSCP and the *in vivo* pcdCP: y = 0.8640 (standard error [SE] 0.019) x - 13.37 (SE 7.479), where y represents the *in vivo* pcdCP (g/kg dry matter) and x represents the NDSCP (cereal grains) or ADSCP (protein feeds) value (g/kg dry matter). The coefficient of determination (R^2) of this equation was 0.962. A validation with literature values showed a good fit of the equation to an independent data set (n = 20; R^2 = 0.955). This study shows that based on chemical analysis alone, namely determination of NDICP and ADICP, from which NDSCP and ADSCP are calculated, *in vivo* pcdCP values can be estimated with a standardised and rapid laboratory method.

Keywords: Chemical analysis, Digestibility, Feed evaluation, *Sus scrofa domesticus*

4.2 Introduction

The primary objective of ration planning is to ensure that animals are provided with the energy and nutrients they need to maintain performance and health, which is an ongoing challenge for animal nutrition. In pigs, protein and amino acids are primarily digested and absorbed in the small intestine, i.e., precaecally. Therefore, precaecal (synonymously termed "ileal") digestible crude protein (pcdCP), more precisely its constituent amino acids, is the key variable of protein evaluation of pig feeds (GfE, 2008). For the determination of *in vivo* standardised pcdCP values, apparent digestible CP is corrected for basal endogenous CP losses, but specific losses, which depend on feed properties, such as fibre, lectin, tannins and protease inhibitors, are not considered (GfE, 2005; Adeola et al., 2016).

Several approaches exist to determine pcdCP with *in vivo* and *in vitro* methods. *In vivo* determination of pc digestibility of CP (pcDCP) in pigs often involves marker methods using indigestible markers such as chromic oxide (Cr_2O_3) or titanium dioxide (TiO_2), as well as direct total digesta collection methods (Mroz et al., 1996). Some of these methods are based on sample collection after the animal has been euthanized, which has been shown to be inaccurate because the digesta from the small intestine can mix with contents of other intestinal sections (GfE, 2005). Additional, invasive approaches utilizing different cannula methods (depending on the structure and CP content of the feedstuff) at the terminal ileum (Mroz et al., 1996; GfE, 2005; Mosenthin et al., 2007; Metzler-Zebeli et al., 2020) have been used. *In vivo* experiments require large quantities of feed, equipment and labour and rely on animal experiments. *In vitro* digestion tests are often considered as alternatives to animal experiments, as these are less costly and time-consuming (Boisen and Fernández, 1995; Kim et al., 2022). These tests intend to simulate the digestion processes in the pig's gastro-intestinal tract using enzymes in a laboratory setting. In this process, the choice of enzymes and incubation conditions depends on the objective. Boisen and Eggum (1991) compared different enzymatic methods (dialysis cell, pH-drop and pH-stat, colourimetric and filtration) and concluded that multi-enzyme methods are able to predict nutrient digestibility of all relevant feedstuffs and mixtures. In the meantime, a two-step multi-enzyme method according to Boisen and Fernández (1995) has been used in a number of studies, e.g., Pujol and Torrallardona (2007), Eklund et al. (2013), Kim et al. (2022) and Song et al. (2024). The method requires complex handling, is time-consuming and, despite its widespread application in particular on cereal grains, has not yet been established with a strictly standardised protocol (Rosenfelder-Kuon et al. (2020).

This study aimed to develop and evaluate a simple laboratory method to estimate the pcdCP in pig feed without the need to use animals or animal-derived substrates like digestive juice or enzymes, digesta or faeces. The method assumes that neutral-detergent-insoluble CP (NDICP) or acid-detergent-insoluble CP (ADICP) are largely indigestible in the small intestine (Rezvani et al., 2012; Zeyner et al., 2015) and therefore ND-soluble CP (NDSCP) or AD-soluble CP (ADSCP) in the feed, represent the (potentially) digestible CP in the small intestine.

4.3 Materials and methods

4.3.1 Material

4.3.1.1 Samples

A large sample pool was accessible of 82 feed ingredients on which standardised pc digestibility of CP had been determined *in vivo* in pigs at the University of Hohenheim (Table 1). The pcdCP values were then calculated as CP · pc digestibility of CP, these values are also shown in Table 1. The sample pool consisted of cereal (wheat, triticale, rye, barley) and legume grains (faba beans, peas and lupins), each represented by several varieties, and of untreated and heat-treated rapeseed commodities, namely rapeseed meal (RSM) and rapeseed cake (often synonymously denoted “expeller", soybean products and miscellaneous samples (Table 1).

Table 1: The number, sample group, reference, pre-treatment, crude protein (CP; [g/kg DM]), fractional standardised precaecal digestibility of crude protein (pcDCP; [g/g CP]) and calculated standardised precaecal digestible crude protein concentration (pcdCP; [g/kg DM]) *in vivo* of the 82 assayed feedstuffs.

Number	Sample group	Sample	Reference	Pre-treatment	CP* (g/kg DM)	pcDCP	pcdCP *in vivo* **
1	Wheat	Skalmeje	[1]	Incubation	130	0.84	109
2		Tommi			140	0.84	118
3		St. Tobak			129	0.84	108
4		Event			137	0.87	119
5		Mulan			132	0.83	110
6		Tabasco			124	0.83	103
7		Adler			151	0.85	128
8		KWS Erasmus			129	0.85	110

9	Triticale	Grenado	[2]	Incubation	118	0.81	96
10		Tarzan			128	0.85	109
11		HYT Prime			131	0.83	109
12		Massimo			126	0.85	107
13		Cultivo			134	0.84	113
14		SW Talentro			134	0.83	111
15		Cando			119	0.83	99
16		Agostino			121	0.83	100
17	Rye	Conduct	[2]	Incubation	116	0.74	86
18		Visello			113	0.73	82
19		Helltop			120	0.73	88
20		Bellami			115	0.70	81
21		Palazzo			111	0.72	80
22		Dukato			116	0.73	85
23		Guttino			108	0.72	78
24		Dankowski Diament			127	0.73	93
25	Barley	Yool	[3]	Incubation	113	0.71	80
26		Ack 2927			117	0.74	87
27		Lomerit			117	0.73	85
28		Campanille			119	0.69	82
29		Canberra			126	0.72	91
30		Antisette			127	0.74	94
31		Metaxa			128	0.74	95
32		Fridericus			129	0.73	94
33	Pea	Santana	[4]	Incubation	252	0.81	204
34		Jutta			260	0.81	211
35		Phönix			247	0.79	195
36		Harnas			255	0.79	201
37		Rocket			254	0.78	198
38		Hardy			224	0.76	170
39	Lupin	Probor	[4]	Incubation	377	0.90	339
40		Boregine			359	0.86	309
41		Boruta			339	0.84	285

42		Idefix			383	0.84	322
43	Faba bean	Aurelia	[4]	Incubation	314	0.81	254
44		Divine			300	0.81	243
45		Gloria			337	0.80	270
46		Limbo			319	0.73	233
47		Fuego			292	0.71	207
48		Espresso			285	0.70	200
49	Full-fat soybean/ soybean product	FFSB K0	[5]	Fat extraction	392	0.46	180
50		FFSB K1			394	0.52	186
51		FFSB K2			394	0.73	205
52		FFSB K3			394	0.80	288
53		FFSB Z1			398	0.82	315
54		FFSB Z2			402	0.83	326
55		FFSB Z3			397	0.84	334
56		FFSB Z4			402	0.82	333
57		FFSB	[6]	Fat extraction	351	0.53	330
58		FFSB (roasted)			384	0.72	388
59		SBC			467	0.85	276
60		SBM (Austria)		/	473	0.82	397
61		SBM (GMO-free)		/	542	0.79	428
62		SBM (standard)		/	481	0.81	390
63	Rapeseed meal and cake	RSM48	[7]	/	381	0.66	251
64		RSM64		/	375	0.65	244
65		RSM76		/	381	0.62	236
66		RSM93		/	381	0.60	229
67		LOW-GLS RSM		/	381	0.62	236
68		RSC	[6]	Fat extraction	361	0.73	269
69		RSM		/	402	0.67	264

70	Various samples	Soybean (extruded)	[8]	Fat extraction	433	0.73	316
71		SBM (high protein)	[9]	/	555	0.80	444
72		SPC (A coarse)		/	713	0.86	613
73		SPC (A fine)		/	693	0.86	596
74		SPC (B coarse)		/	723	0.87	629
75		SPC (B fine)		/	785	0.85	667
76		SPI (hydrolysed)		/	916	0.86	788
77		PeaP		Fat extraction	878	0.87	764
78		WG1		/	894	0.90	805
79		WG2 (hydrolysed)		/	866	0.88	762
80		WG3 (hydrolysed)		/	848	0.87	738
81		FM1		Fat extraction	779	0.87	678
82		FM2 (extracted)		/	851	0.79	672

FFSB, Full-fat soybean; FM, Fish meal; GLS, Glucosinolates; PeaP, Pea protein; RSC, Rapeseed cake; RSM, Rapeseed meal; SBC, Soybean cake; SBM, Soybean meal; SPC, Soy protein concentrate; SPI, Soy protein isolate; WG, Wheat gluten, / no pre-treatment. *Incubation: carried out according to McQueen and Nicholson (1979). *Fat extraction: carried out according to Regulation (EG) 152/2009 Annex III, C* 1.1. **The precaecal digestible crude protein concentration was calculated from CP and pcDCP given in reference.References: [1] Rosenfelder et al. (2015), [2] Strang et al. (2017), [3] Spindler et al. (2016), [4] Jezierny et al. (2010), [5] Kaewtapee et al. (2017), [6] Kaewtapee et al. (2018), [7] Eklund et al. (2015), [8] Urbaityte et al. (2009a), [9] Urbaityte et al. (2009b).

* Incorrectly stated in the publication the correct letter for the method is H 1.1; without hydrochloric acid.

4.3.1.2 Characterisation of samples

A condensed overview of anti-nutritional compounds compiled from the references given in Table 1 is presented in the Appendix (Tables A1 - A4). Data presented in these Tables were needed as a basis for the selection criteria described below (4.3.2.1). Table A1 provides data on anti-nutritional compounds in the three legume grain species. Table A2 summarizes analytical data on trypsin inhibitor activity (TIA) of soybeans and soybean products. Table A3 presents the glucosinolate concentrations in rapeseed commodities, i.e., RSM and rapeseed cake. Table A4 depicts the results of TIA determination in the miscellaneous feed ingredients.

4.3.2 Methods

4.3.2.1 Selection criteria for assigning samples to the neutral-detergent- or acid-detergent-insoluble crude protein procedures

Based on data provided in the Appendix Tables, feed samples containing specific N compounds, e.g., Maillard products or/and tannin-protein complexes, which are recovered as part of the acid-detergent-insoluble CP (ADICP) but not in the neutral-detergent-insoluble CP (NDICP; see Table 2) fraction, were assigned to the determination of ADICP, whereas on all other samples NDICP was analysed. Consequently, NDICP was analysed on all cereal grain samples and ADICP on all other samples, which primarily can be referred to as protein feeds. The a priori assignment of samples to either ADICP or NDICP determination implies that less analytical steps are needed to generate results.

Table 2: The selection criteria Maillard-CP, condensed tannin-CP, isoelectric point protein and phytate-CP complexes for neutral-detergent-insoluble (NDICP) or acid-detergent-insoluble crude protein (ADICP).

	Literature	NDICP	ADICP
Maillard-CP	van Soest and Mason (1991) Licitra et al. (1996) Classen et al. (2004)	/	X
Condensed tannin-CP	van Soest (1994)	/	X
Isoelectric point protein	Csonka et al. (1926) Csonka and Jones (1927) Morales et al. (2013)	X	X
Phytate-CP complex	Morales et al. (2013)	X	X
		Cereal grains	**Protein feeds**

ADICP, Acid-detergent-insoluble crude protein; CP, Crude protein; NDICP, Neutral-detergent-insoluble crude protein; /, Not included; X, Included

4.3.2.2 General analyses

The samples were milled through a 1-mm screen using an ultra-centrifugal mill at 18.000 rpm (ZM 200, Retsch, Haan, Germany). Dry matter (DM) was determined by oven-drying at 103 °C (Regulation (EG) 152/2009 Annex III, C*). The determination of NDICP or ADICP was generally carried out according to Licitra et al. (1996), following van Soest et al. (1991) for specifications of ND residue separation and VDLUFA (2023) for the AD residue (both procedures used Fibretherm (FT12; FibreBags ADF, 30 µm pore size; C. Gerhardt,

Königswinter, Germany). The N determination on the residues was done according to the Kjeldahl method (Regulation (EG) 152, 2009 Annex III, C) using Vapodest (VAP 50sc, C. Gerhardt). Crude protein was expressed as N · 6.25.

* Incorrectly stated in the publication the correct letter for the method is A.

4.3.2.3 Sample pre-treatment for removal of starch or fat

In starch-containing samples (cereal and legume grains; samples 1-48 in Table 1), treatment with hot ND may result in the formation of starchy gels, leading to clogging of bag pores, which impedes the detergent flow into the bags and may negatively affect dissolving of non-fibre materials inclusive CP (McQueen and Nicholson, 1979). Starchy samples were, therefore, pre-treated overnight with α-amylase (Termamyl 2X, Novozymes, Novo Industrials, Bagsværd, Denmark) in a buffer (pH 7) at 40°C in a shaking (80 rpm) water bath (SW22, JULABO, Seelbach, Germany) according to McQueen and Nicholson (1979). Then, the pre-treated samples were used to isolate the ND residue.

High fat content can have a negative impact on the preparation of the AD residue due to limited capacity of the detergent to solve fat (Mertens, 2002). Therefore, the pre-treatment according to Regulation (EG) 152/2009 Annex III, H (1.1; without hydrochloric acid pre-treatment) was applied to samples with fat content > 80 g/kg DM (full-fat soybeans, soybean cake, rapeseed cake, fish meal, pea protein; samples 49-59, 68, 70, 77, 81). The samples, weighed in bags, were extracted in a Soxtherm device (Sox 404, C. Gerhardt) for 1.5 h. The pre-treated bags were afterwards used to isolate the AD residue.

4.3.3 Calculations and statistical analysis

The concentrations of NDSCP or ADSCP were calculated as follows:

$$\text{NDSCP (g/kg DM)} = \text{CP (g/kg DM)} - \text{NDICP (g/kg DM)} \quad [1]$$

and

$$\text{ADSCP (g/kg DM)} = \text{CP (g/kg DM)} - \text{ADICP (g/kg DM)} \quad [2]$$

4.3.3.1 Regression analysis

The statistical data evaluation was carried out with the programme R 2.2 (R Foundation for Statistical Computing, Vienna, Austria). First, a raw data analysis was performed and means and standard deviations were calculated. Then a linear model was applied for regression analysis:

$$y = a\,x + b \qquad [3]$$

where y = *in vivo* pcdCP (g/kg DM) and x = NDSCP or ADSCP (g/kg DM) as categorised above (4.3.2.1).

This model was applied to the entire dataset (n = 82) and separately to cereal grains at large (n =38) and in different combinations of cereal grain species. For this largest subgroup in the dataset, an additional ANOVA, followed by a Tukey-Test to separate means for wheat, rye, barley and triticale, was carried out to further evaluate the data.

4.3.3.2 Validation

The relationship between *in vivo* pcdCP and the concentrations of NDSCP or ADSCP (equation [3]), was validated using data (n =20) published by Jondreville et al. (2000), Leterme et al. (2000) and Jondreville et al. (2001). Table 3 presents information on types of cereal grains and co-products of grain processing that were studied. The *in vivo* pcdCP, CP and NDICP values were given in the publications, and NDSCP values were calculated using equation [1]. The pcdCP values estimated according to selected equations derived from the dataset used for establishing relationships shown below ([4] and [7].) and the *in vivo* pcdCP values were then used to compare and validate the relationship.

Table 3: The data for the validation. The data of CP [g/kg DM], NDICP [g/kg DM] and NDSCP [g/kg DM] for the references of wheat middling, wheat feed, wheat bran (Jondreville et al., 2000), cereals (Jondreville et al., 2001) and barley (Leterme et al., 2007) for the validation.

Reference	Sample	CP (g/kg DM)	NDICP (g/kg DM)	NDSCP (g/kg DM)
Jondreville et al. (2000)				
Wheat middlings	WM1	172	6	166
	WM2	143	7	136
	WM3	158	9	149

Wheat feed	WF1	185	11	174
	WF2	191	11	180
	WF3	179	13	166
	WF4	204	16	188
Wheat bran	WB1	219	36	183
	WB2	161	35	126
	WB3	208	40	168
Jondreville et al. (2001)				
Cereals	Wheat	141	14	127
	Triticale	119	15	104
	Rye	96	7	89
	Barley	131	15	116
	Maize	104	15	89
	Sorghum	112	12	100
Leterme et al. (2000)				
Barley	Taiga naked	131	13	118
	Volga spring	116	9	107
	Krimhild winter	93	11	82
	Crete winter	121	12	109

CP, Crude protein; DM, Dry matter; NDICP, Neutral-detergent-insoluble crude protein; NDSCP, Neutral-detergent-soluble crude protein; WM, Wheat middlings; WF, Wheat feed; WB, Wheat bran

4.3.3.3 Principal component analysis

A principal component analysis (PCA) [Prcomp] was performed with the following fibre fractions as factors to increase the interpretability of the results (data from Rodehutscord et al., 2016): Crude fibre, dietary fibre (calculated as the sum of non-starch polysaccharides [NSP] and Klason lignin), arabinoxylan (soluble, insoluble, total), cellulose, β-glucan (soluble, insoluble, total), Klason lignin, NDF assayed with a heat-stable amylase and expressed excluding residual ash (aNDFom), ADF expressed excluding residual ash (ADFom), acid-detergent lignin (ADL), NSP (soluble, insoluble, total). Calculated values for hemicellulose (from non-sequential analyses of aNDFom and ADFom) and cellulose (from ADFom and ADL) were also included in the PCA.

4.4 Results

4.4.1 All feed ingredients

The entire dataset (n = 82) was used to display the relationship between *in vivo* pcdCP values and corresponding NDSCP values for cereal grains and ADSCP values for protein feeds (Fig. 1), resulting in the following linear equation to estimate pcdCP from NDICP or ADICP:

y = 0.864 (standard error [SE] 0.019) x – 13.38 (SE 7.479) [4]

R^2 = 0.962

Root-mean-square error (RMSE) = 38.61

y = *in vivo* pcdCP (g/kg DM) and x = NDSCP or ADSCP (g/kg DM)

The cereal grains were in the lower range and the protein feeds in the upper range of values (Fig. 1), with four particularly striking values. Three of them were thermally processed full-fat soybeans which, based on their TIA values (> 6.4 g/kg DM; Table A2), appear insufficiently processed. One out of three wheat gluten data was also noticeable. This sample was a hydrolysed wheat gluten for which no specific information on processing conditions was available (Table A4). Since ADICP was very low, i.e., very little AD residue remained for analysis after preparation and consequently the ADSCP value is high. After removal of the four conspicuous samples, the coefficient of determination further increased slightly from R^2 = 0.962 to R^2 = 0.989 with concomitant, small changes in slope and moderate changes in intercept of the linear equation (y = 0.849 [SE 0,010] x – 6.86 [SE 3.972]; RMSE = 20.36).

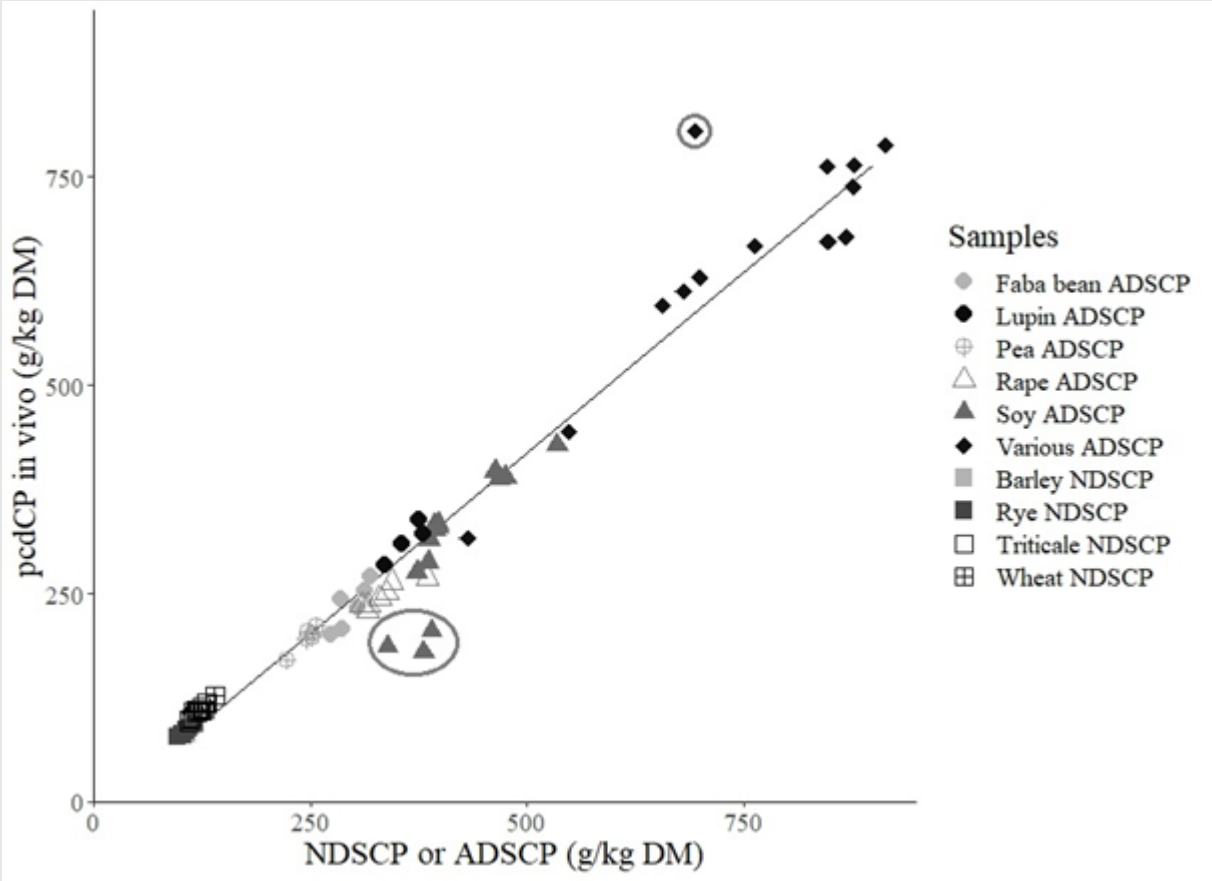

Figure 1: Relationship between NDSCP and ADSCP data from the laboratory method (X) and *in vivo* pcdCP (Y) for cereal grains and protein feedstuffs for all 82 samples. With the regression equation y = 0.864 (SE 0.019) x – 13.38 (SE 7.479). The encircled symbols represent four particularly striking values. See text for further information (4.4.1).

4.4.2 Validation

The validation of equation [4] (generated from the entire dataset, n = 82) with independent data produced a high coefficient of determination (R^2 = 0.955) and an RMSE value of 6.246. The relationship between the *in vivo* pcdCP and the estimated pcdCP is illustrated in Fig. 2. The relatively high RMSE value may be due to, at least in part, the fact that the NDICP analyses were performed in different laboratories. The validation using equation [7] for all cereal grains (y = 0.972 (SE 0.050) x – 10.55 (SE 6.359) showed similar values as the use of equation [4] (entire dataset) for both the R^2 (0.955) and the RMSE (6.671) for the relationship between the *in vivo* pcdCP and the estimated pcdCP. Despite the apparent slight systematic underestimation and the lower range of in vivo pcdCP values in the validation dataset, this further underpins the applicability of the relationships established from strictly standardised *in vivo* measurements and laboratory methods.

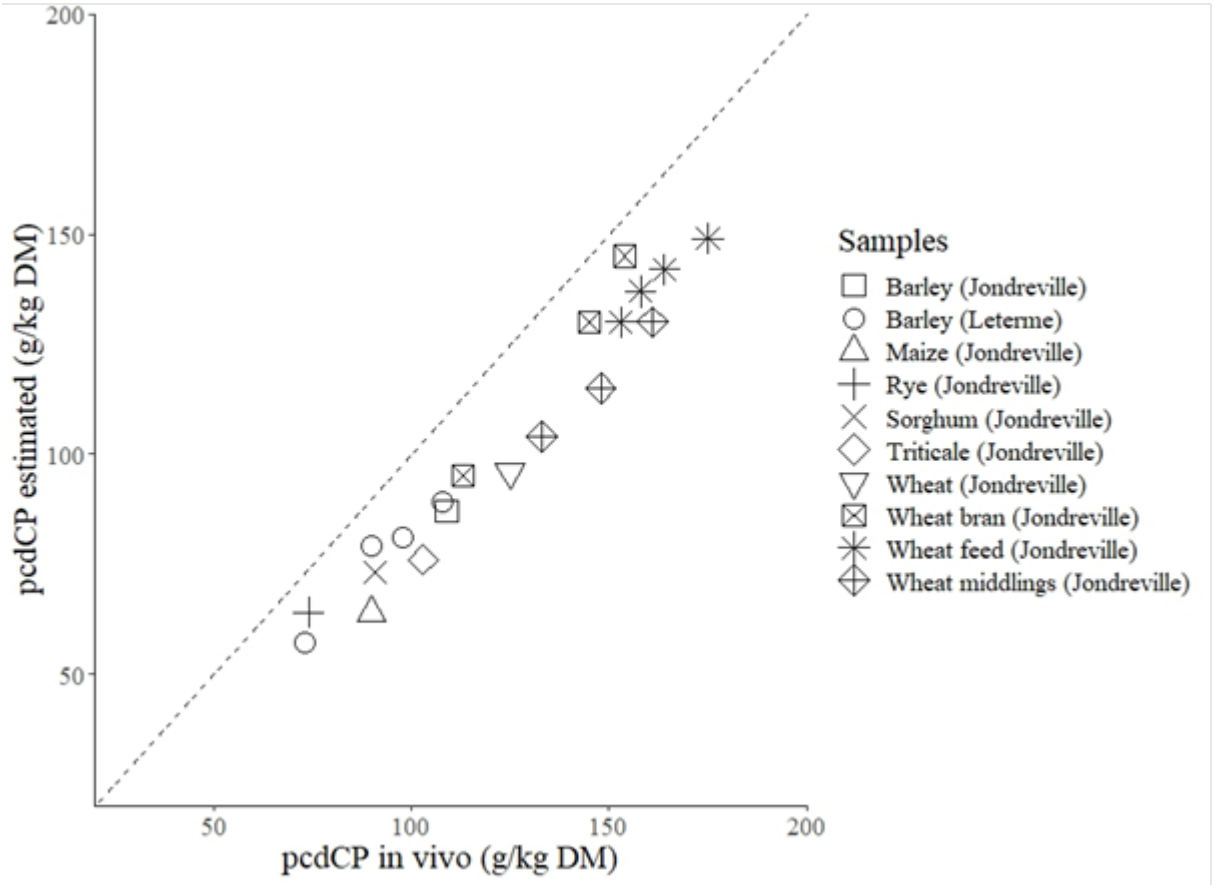

Figure 2: Comparison of *in vivo* pcdCP from data of Jondreville et al. (2000), Leterme et al. (2000) and Jondreville et al. (2001) (X), and estimated *in vivo* pcdCP (Y), derived from the regression equation for all cereal grain samples [7]: y = 0.917 (SE 0.054) x – 13.26 (SE 6.043). The angle bisector is shown as the dashed line.

4.4.3 Cereal grains

First, the *in vivo* pcdCP values (Table 1) were plotted against NDSCP for all cereal grains. The plot revealed a visual clustering of wheat/triticale and rye/barley. The ANOVA showed differences between grain types ($P < 0.001$) and the post hoc Tukey-Test revealed that the regression equations of rye and barley were similar ($P = 0.3762$) and could therefore be clustered together. Regressions of wheat and triticale were significantly different ($P = 0.0084$). However, because the visual appraisal of the diagram suggests a grouping of wheat and triticale, it can be assumed that the observed significant difference was either an artifact or due to the small sample size. This view is corroborated by the PCA which shows that, based on the large range of fibre fractions, wheat and triticale can be grouped well (Fig. 3). Ultimately, based on visual appraisal, usefulness and practicality, we decided to cluster wheat with triticale and barley with rye (Fig. 4).

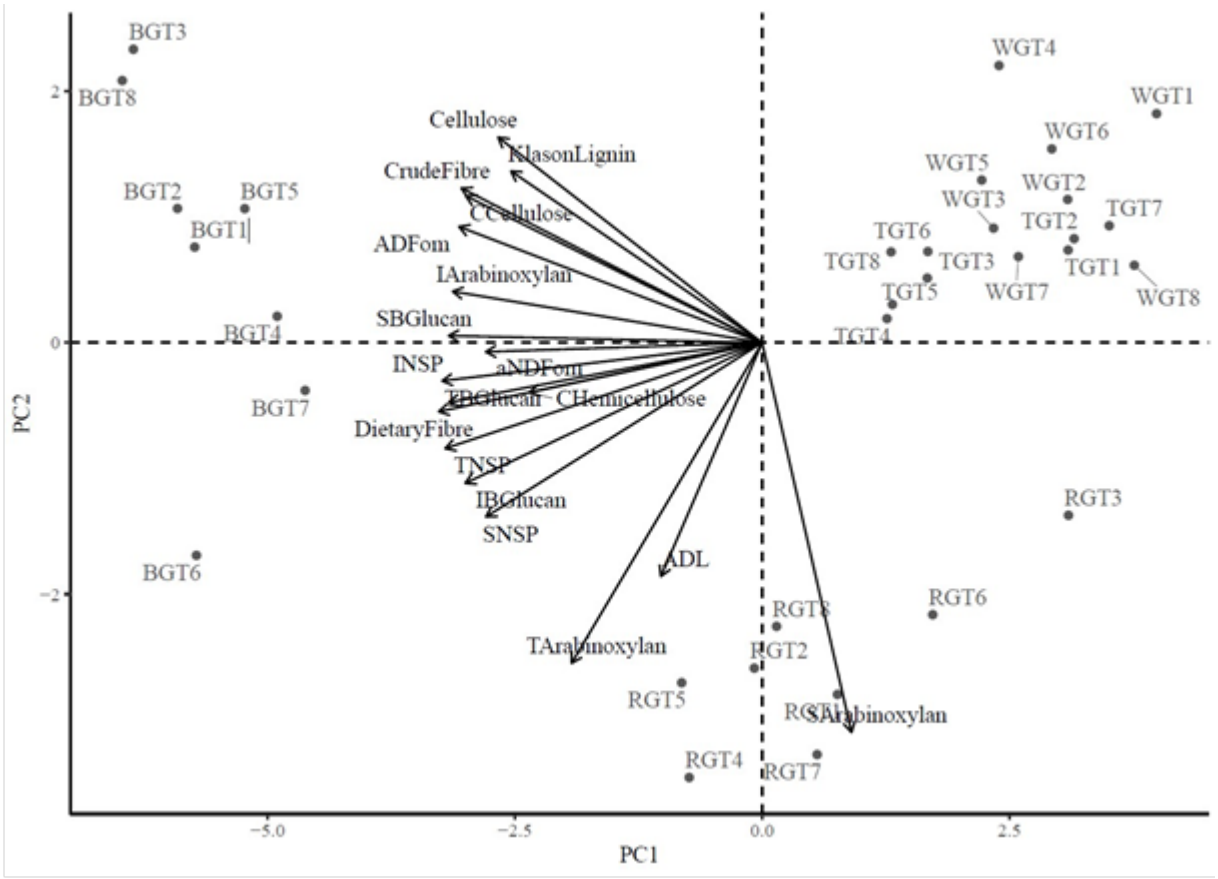

Figure 3: Principal component analysis of fibre components in the eight genotypes each of wheat, triticale, barley and rye, using data from Rodehutscord et al. (2016).

List of abbreviations: ADFom, Acid-detergent fibre expressed exclusive of residual ash; ADL, Acid-detergent lignin; aNDFom, Neutral-detergent fibre amylase treated expressed exclusive of residual ash; BGT, Barley genotype; Cellulose, Cellulose analysed; CCellulose, Cellulose calculated (ADFom – ADL); CHemicellulose, Hemicellulose calculated (aNDFom – ADFom); Dietary Fibre, calculated as the sum of non-starch polysaccharides and Klason lignin; IArabinoxylan, Insoluble Arabinoxylan; IBGlucan, Insoluble ß-Glucan; INSP, Insoluble non-starch polysaccharides; NSP, Non-starch polysaccharides; RGT, Rye genotype; SArabinoxylan, Soluble arabinoxylan; SBGlucan, Soluble ß-Glucan; SNSP, Soluble non-starch polysaccharides; TArabinoxylan, Total arabinoxylan; TBGlucan, Total ß-Glucan; TGT, Triticale genotype, TNSP, Total non-starch polysaccharides; WGT, Wheat genotype.

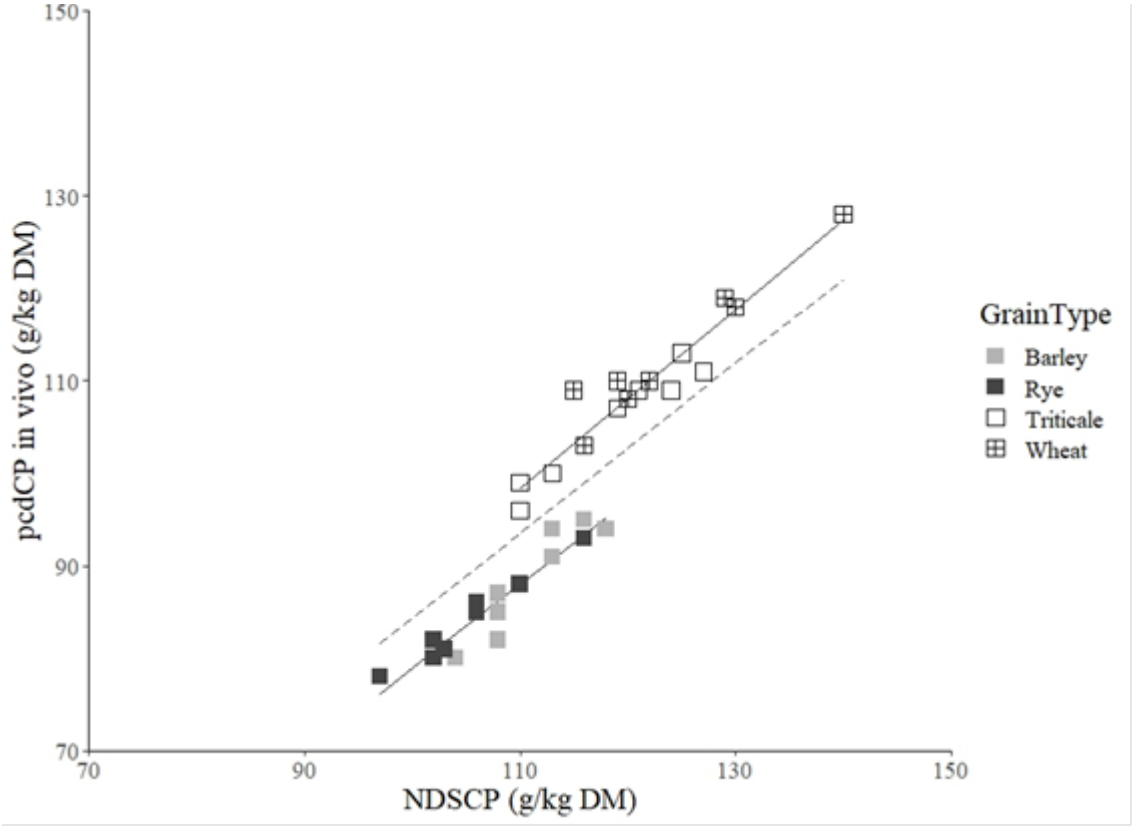

Figure 4: Relationship between NDSCP data from the laboratory method (X) and *in vivo* pcdCP (Y) for cereal grains for wheat, triticale, barley and rye with grouping according to wheat/triticale and barley/rye. The dashed line shows the regression line over all cereal grain samples. See text for detail.

The resulting linear relationships to estimate pcdCP from NDICP were as follows:

Wheat and triticale:

$y = 0.963$ (SE 0.078) $x - 7.405$ (SE 9.435) [5]

$R^2 = 0.916$

RMSE = 2.231

y = *in vivo* pcdCP (g/kg DM) and x = NDSCP (g/kg DM)

Rye and barley:

$y = 0.908$ (SE 0.083) $x - 11.83$ (SE 8.938) [6]

$R^2 = 0.896$

RMSE = 1.767

y = *in vivo* pcdCP (g/kg DM) and x = NDSCP (g/kg DM)

All cereal grains:

$y = 0.917$ (SE 0.054) $x - 13.26$ (SE 6.043) [7]

$R^2 = 0.983$

RMSE = 1.739

y = *in vivo* pcdCP (g/kg DM) and x = NDSCP (g/kg DM)

4.5 Discussion

4.5.1 General considerations

The approach to estimate pcdCP from chemical fractions alone, which formed the backbone of this study. refers to a method for estimating pcdCP in horse feed (GfE, 2014; Zeyner et al., 2015) with the understanding that both pigs and horses digest fibre and cell wall carbohydrates solely in the large intestine with the help of microbial enzymes (Fernández et al., 1986; Zeyner et al., 2015). Only the fibre-associated-CP fractions (e.g., cell wall protein, Maillard products), are assumed to be almost completely indigestible precaecally (Schulze et al., 1994). Vice versa, non-fibre-associated CP is highly digestible in the small intestine and supplies the majority, if not all, of amino acids to cover requirements of the animal.

Depending on the type of feedstuff, fibre-associated CP was estimated from either NDICP or ADICP. Importantly, all samples were obtained from the same facility, ensuring consistency in data generation and analytical characterisation and minimizing variation in pcdCP determination. This study benefitted from the opportunity to conduct specific chemical analyses, namely NDICP and ADICP, on feed ingredients on which not only the *in vivo* pcdCP had been determined using standardised and consistent methods but also a large range of chemical analyses had been conducted by the same laboratory. The large range of analytical data allowed a grouping of ingredients, which was instrumental in assigning samples to either a NDICP of ADICP determination. The grouping thus avoided analytical challenges related to potential filtration problems with protein feeds and implies that less analytical steps are needed to generate results.

4.5.2 Analytical procedure

The samples had been stored at -20°C for a long time period commencing in 2006. A possibility therefore exists that duration of storage had an influence on chemical compounds. However, we did not detect any visible changes during inspection of samples and the aNDFom or ADFom values which were re-analysed were not different from values reported in the published studies.

4.5.2.1 Grinding

The majority of the samples were ground trough a 1 mm screen. Some of the 82 samples had been ground before using a 1 mm screen or no information on grinding was provided, therefore samples may have been ground finer. With a finer grinding, it can be assumed that the finest particles are washed out of the bags during processing and, therefore, the NSDCP or ADSCP values may be overestimated (Mertens, 1992). Another point is that NDSCP or ADSCP can be also overestimated the finer the sample material is milled, as there is a larger surface area and thus more surface for the solvents to attack (Mertens, 1992). The particle size distribution in the sample material depends on several factors, such as the nature of the sample material, e.g. with or without husk, the mill type and the pore size of sieves (Mertens, 2002), and also the grinding speed (rpm). Therefore, depending on several factors, samples can disappear from the assay bags which may also happen with other suitable equipment for fibre isolation.

4.5.2.2 Sample pre-treatment

The pre-treatment of starch-rich samples depends on the equipment used for fibre isolation. Kehraus and Südekum (2015) recommended an additonal pre-treatment with amylase for samples containing starch (McQueen and Nicholson, 1979) in systems using bags (F57 Fiber Filter Bag, ANKOM Technology, Macedon, NY, USA or Fibretherm FT12 FibreBags ADF, 30 µm pore size; C. Gerhardt, Königswinter, Germany) due to unrealistically high analysed aNDFom contents which sometimes have been observed when these systems were applied. This may be caused by starch gelatinisation inside the bags and choking of pores from the inside. As a result, detergent and amylase are unable to penetrate the bag surface and starch and other non-fibrous components are incompletely dissolved. If analyses are performed with the ANKOM system, closed bags are used and the detergent does not have the possibility to act on the sample also from top to bottom as is the case with the open bags of the Fibretherm system. The beaker method using filter paper does not require any specific pre-treatment either, as the detergents can completely rinse the sample material during boiling. Therefore, it seems that additional pre-

incubation incubation is not absolutely necessary when using open fibre bags, but was carried out to be on the safe side. However, it is not excluded that some additional N-containing compounds will be dissolved with incubation longer than 10 h leading to overestimated NDSCP values.

4.5.3 Validation

The validation with literature data (Jondreville et al., 2000; Leterme et al., 2000; Jondreville et al., 2001) shows a generally satisfying result. This view is corroborated by the observation that the validation against the entire dataset (n = 82) yielded a similar outcome as the validation against cereal grains only. The large RMSE may be related to the diverse sample pool, e.g., wheat middlings, wheat feed and wheat bran, and cereal grains such as wheat, triticale, barley, rye, maize and sorghum. These samples were also differently ground than samples of our study, namely in a hammer mill using a 2 mm screen, and included also genetically diverse barley samples (naked, spring, winter and winter with high content of ß-glucan). A comparison of validation results with other studies and feeding stuffs with a higher CP concentration is needed but was impossible because no studies exist which addressed the suitability of *in vitro* methods for the prediction of pcdCP in feed ingredients for pigs, which confirmed a previous conclusion by Jezierny et al. (2010).

4.5.4 Clustering of cereal grain samples

For estimation of pcdCP a clustering of the cereal grain species was carried out into a wheat/triticale and a barley/rye cluster. Clustering by cereal grain species may thus allow to estimate more accurately the pcdCP values for different cereal grains resulting in an improved ration planning and, eventually, precision feeding of pigs. Clustering of cereal grains was facilitated and underpinned by the PCA which helped recognise patterns and subsequent grouping. Although it is likely that, also based on practical considerations, the general, non-specific equation for all feeds may be used primarily to estimate pcdCP of pig feeds, results that specific equations, inclusive clustering of cereal grain species has potential for increased estimation accuracy should more *in vivo* data become available.

4.5.5 Comparison with other methods

In vivo, the pcdCP is determined using time-consuming and invasive methods. The determination of pcdCP often uses indigestible markers such as Cr_2O_3 or TiO_2, as well as direct total digesta sampling methods (Mroz et al., 1996). However, due to concerns about health

hazards for humans, the future use of Cr_2O_3 (Sedman et al., 2006) and TiO_2 (Bampidis et al., 2021) also in experiments on animals may face restrictions. Furthermore, *in vivo* methods which are based on sample collection after the animal has been killed, have proved to be inaccurate because the digesta from the small intestine can mix with contents of other intestinal sections (GfE, 2005). Fistulated pigs are often used to determine the pcdCP *in vivo*. During surgery a cannula is inserted into the terminal ileum trough which ileal digesta can be collected and subsequently analysed (Mroz et al., 1996; Mosenthin et al., 2007). There have also been *in vivo* attempts in which rats were used as model animals to estimate protein digestibility for pigs (Jørgensen and Lindberg, 2006). However, already Eggum et al. (1982) described that the use of the rat as a model animal is limited because digestibility is lower than that in pigs when fed high-fibre diets.

In vitro multi-enzyme methods are considered to be acceptable alternatives to *in vivo* experiments because they are less labour-intensive and time-consuming (Boisen and Eggum, 1991; Boisen and Fernández, 1995). These procedures use different enzymes and incubation conditions, depending on the objective, to simulate digestion processes in the pig's gastro-intestinal tract in a laboratory setting (Boisen and Fernández, 1995). However, because the effect of enzymes is highly dependent on external influences, e.g., ambient temperature, variations can easily occur. Therefore, multi-enzyme methods may require complex handling, especially when a large number of samples has to be analysed. Provided that these methods are strictly standardised, multi-enzyme methods may be able to predict nutrient digestibility of all relevant feedstuffs and mixtures (Boisen and Eggum, 1991).

Although the increasing use of this procedure since 1995, there is still a lack of validation studies to support its suitability for estimating the pcdCP (Pujol and Torrallardona, 2007; Eklund et al., 2013; Rosenfelder-Kuon et al., 2020). For example, Eklund et al. (2015) pointed out the lack of research on *in vitro* pcdCP estimation in thermally processed feed. This can also be attributed to the large variations of the *in vitro* results between laboratories and between experiments (Boisen and Fernández, 1995; Pujol and Torrallardona, 2007). In addition, the changing nature of the enzymes and incubation conditions in the different experiments makes it difficult to standardise the procedure and compare the results with each other. In comparison to *in vivo* and *in vitro* methods the chemical laboratory method has advantages. In contrast to all *in vivo* approaches, this method is rapid and non-invasive. Compared to multi-enzyme methods, it is easy to perform, even with large numbers of samples, and provides plausible values even for feeds, which were heat-treated or with high concentrations of antinutritive substances.

In addition, the amount of samples/data for evaluation is greater with the laboratory method (n=82) than with other methods using the multi-enzyme method of Boisen and Fernández (1995) (n = 17) in Pujol and Torrallardona (2007) (n = 7), Jezierny et al. (2010) (n = 17), Eklund et al. (2013), (n = 16), and Rosenfelder-Kuon et al. (2020) (n = 32), Kim et al., 2022 (n = 5). Unfortunately, in the above studies, the procedural specifications of the multi-enzyme method varied considerably among laboratories such that data of these studies could not be merged into one single dataset for further evaluation.

As the determination of NDICP and ADICP is a routine analytical step in a CP fractionation procedure (Licitra et al., 1996) of ruminant feeds and is already standardised and established in laboratories, the laboratory method described herein is easy to apply. Interlaboratory comparisons (VDLUFA, 2023) showed a very good between-laboratory comparability of NDICP and ADICP values (standard deviation of the comparison: 9.2 g/kg DM for NDICP and 2.4 g/kg DM for ADICP) for mean values of 30.8 g/kg DM (NDICP) and 10.0 g/kg DM (ADICP).

4.6 Conclusions

In vivo determined standardised pcdCP of cereal grains and protein feeds was predicted with a high accuracy by a chemical procedure. The procedure encompassed the determination of NDICP on cereal grains and ADICP on other feedstuffs, mainly protein feeds. The soluble fractions were used to predict *in vivo* determined standardised pcdCP by linear regression. This procedure can be used in any laboratory equipped for standard feedstuff analysis and does not involve multiple analytical steps. The method is also applicable to predict standardised precaecal digestibility of AA which will be described in a follow-up paper. The analytcical procedure is the same as for CP, because the residues obtained with the neutral or acid detergent can also undergo AA analysis. A major limitation of this study is that the validation was only applicable on samples of cereal grains and grain co-products which generally had low concentration of CP. Future studies, therefor, should include higher protein ingredients as well. An extension of the database of *in vivo* pcdCP values may then yield predictions for specific types of feeds which would aid in further improving the accuracy of the prediction of pcdCP without compromising robustness.

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CRediT authorship contribution statement

Valérie Schumacher: Writing – original draft, Investigation, Formal analysis, Data curation. **Markus Rodehutscord:** Writing – review & editing. **Karl-Heinz Südekum:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Saskia Kehraus:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors have no conflict of interest.

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APPENDIX

Table A1: The analysed contents of the anti-nutritional factors trypsin inhibitor activity [mg/g crude protein], trypsin inhibitor activity [mg/g DM], condensed tannins [g/kg DM] and processing (given in reference) in the assayed peas, lupins and faba beans (Jezierny et al., 2010).

Number		Trypsin inhibitor activity (mg/g crude protein)	Trypsin inhibitor activity* (mg/g DM)	Condensed tannins (g/kg DM)	Processing
	Peas				
33	Santana	2.4	0.61	n.d.	X
34	Jutta	1.8	0.47	n.d.	X
35	Phönix	5.0	1.23	n.d.	X
36	Harnas	< 0.2	0.05	n.d.	X
37	Rocket	3.9	0.99	n.d.	X
38	Hardy	4.5	1.01	n.d.	X
	Lupins				
39	Probor	2.9	1.09	n.d.	X
40	Boregine	< 0.2	< 0.07	n.d.	X
41	Boruta	< 0.2	< 0.07	n.d.	X
42	Idefix	< 0.2	< 0.07	n.d.	X
	Faba beans				
43	Aurelia	3.9	1.23	n.d.	X
44	Divine	1.4	0.42	2.1	X
45	Gloria	3.3	1.11	n.d.	X
46	Limbo	< 0.2	< 0.06	7.0	X
47	Fuego	< 0.2	< 0.06	7.4	X
48	Espresso	< 0.2	< 0.06	4.2	X

CP, Crude protein; DM, Dry matter; n.d., Not determined; TIA, Trypsin inhibitor activity; X, No processing.

*Calculated considering CP content (g/kg DM) in references (CP*TIA (mg/g CP)/1000).

Table A2: The analysed content of the anti-nutritional factor trypsin inhibitor activity [mg/g DM] and processing (given in reference) in the assayed soybeans and soybean products (Kaewtapee et al., 2017, Kaewtapee et al., 2018).

Number	Soybean/soybean product	Trypsin inhibitor activity (mg/g DM)	Processing
			Wet heating:
49	FFSB K0	29.5	raw
50	FFSB K1	7.8	80°C, 1 min
51	FFSB K2	6.4	100°C, 6 min
52	FFSB K3	5.7	100°C, 16 min
			Autoclaving 110°C
53	FFSB Z1	2.6	15 min
54	FFSB Z2	1.6	30 min
55	FFSB Z3	1.1	45 min
56	FFSB Z4	0.9	60 min
57	FFSB	24.5	70°C until DM 880 g/kg
58	FFSB (roasted)	2.6	110-115°C, gas-fire chamber
59	SBC	3.8	Expelling of oil, 102°C 10 min, extruded 125-145°C, 1-5 s
60	SBM (Austria)	3.0	Conditioning 50-70°C, 10-20 min; flaking 50-70°C; oil removing 55-65°C 40-55 min; dissolving 100-110°C, 5-10 min; drying 60-70°C, cooling 20-30°C, 15-20 min
61	SBM (GMO-free)	2.8	No information
62	SBM (standard)	1.8	No information

DM, Dry matter; FFSB, Full-fat soybean; SBC, Soybean cake; SBM, Soybean meal.

Table A3: The analysed content of the anti-nutritional factor glucosinolate [µmol/g DM] and processing (given in reference) in the assayed different heat-processed rapeseed meals and cake (Eklund et al., 2015, Kaewtapee et al., 2018).

Number	Rapeseed product	Glucosinolate (µmol/g DM)	Processing
			Flaking, cooking, pressing, dissolving:
63	RSM48	15	48 min >100°C
64	RSM64	12	64 min >100°C
65	RSM76	8	76 min >100°C
66	RSM93	6	93 min >100°C
67	LOW-GLS RSM	4	39 min >100°C
68	RSC	27.3	Pre-heating 40°C; oil removing
69	RSM	8.8	Flaking, cooking 110°C; oil removing; dissolving 75°C; cooling 30°C

DM, Dry matter; n.d., Not determined; RSC, Rapeseed cake; RSM, Rapeseed meal.
Processing for 63 to 67 according to Mosenthin et al. (2012).

Table A4: Trypsin inhibitor activity [mg/g DM] and processing information (given in reference) in miscellaneous straight feedingstuffs (Urbaityte et al., 2009a, 2009b).

Number	Various samples	Trypsin inhibitor activity (mg/g DM)	Processing
70	Soybean (extruded)	7.36	Extruded
71	SBM (high protein)	2.22	
72	SPC (A coarse)	0.93	
73	SPC (A fine)	1.73	
74	SPC (B coarse)	1.59	
75	SPC (B fine)	0.86	
76	SPI (hydrolysed)	1.92	
77	PeaP	0.00	
78	WG1	n.d.	Hydrolysed
79	WG2 (hydrolysed)	n.d.	
80	WG3 (hydrolysed)	n.d.	
81	FM1	n.d.	Cooking 90°C, 1h; air-dried 400°C, 10-15 s
82	FM2 (extracted)	n.d.	Cooking 90°C, 1h; vacuum-dried 70-100°C, 1h

DM, Dry matter; FM, Fish meal; n.d., Not determined; PeaP, Pea protein; SBM, Soybean meal; SPC, Soy protein concentrate; SPI, Soy protein isolate; WG, Wheat gluten.

Chapter 5. A rapid laboratory method for estimating the standardised precaecal digestible amino acids in pig feeds

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5.1 Abstract

Amino acids (AA) are essential nutrients for diverse processes in the pig's body. The utilisation of AA depends on their digestibility and absorption. Therefore, methods to determine reliably the AA supply to pigs to sustain performance and animal health are critical for precise feed evaluation. The evaluation of AA supply has so far been based on *in vivo* determination of standardised precaecal digestible (pcd) AA (pcdAA) and *in vitro* estimates of pcdAA applying time-consuming and complex laboratory methods. The objective of this study was to develop and establish a rapid laboratory method for estimating pcdAA based on the determination of AA insoluble in neutral-detergent (ND) or acid-detergent (AD) (NDIAA, ADIAA). The laboratory method used the same procedure which was previously applied to estimate standardised pcd crude protein (pcdCP). The hypothesis was that the method was similarly suitable to estimate pcdAA. A sample pool of 74 feed ingredients (cereal grains, differently heat-treated legume grains) was available on which *in vivo* pcdAA were determined in cannulated pigs. Amino acids in feed ingredients and in ND or AD residues of feed ingredients were determined by an HPLC method. The concentrations (g/kg dry matter) of ND- and AD-soluble AA (NDSAA, ADSAA) were calculated by difference to total AA in feed. For the estimation of the concentrations of *in vivo* pcdAA for total AA and the entire dataset (n = 74), a linear relationship was established between the concentrations of NDSAA or ADSAA and the *in vivo* pcdAA: y = 0.823 (standard error [SE] 0.018) x + 10.52 (SE 4.420), where y represents the *in vivo* pcdAA (g/kg dry matter) and x represents the NDSAA (cereal grains) or ADSAA (protein feeds) value (g/kg dry matter). The coefficient of determination (R^2) of this equation was 0.968 and ranged from 0.895 to 0.984 for the 17 individual AA. This study shows that *in vivo* pcdAA values can be estimated following the same standardised and rapid laboratory procedure previously established for pcdCP, based on chemical analyses, namely determination of NDIAA and ADIAA, from which NDSAA and ADSAA values are calculated.

Keywords: Chemical analysis, Digestibility, Feed evaluation, Ileal, *Sus scrofa domesticus*

5.2 Introduction

An ongoing challenge for animal nutrition is to compose and feed rations which provide the animals with an adequate quantity of amino acids (AA) to sustain performance and health. In pigs, protein digestion and AA absorption primarily occur in the small intestine, i.e. precaecally. Therefore, standardised precaecal (synonymously termed "ileal") digestible amino acids (pcdAA) are the key variable of AA evaluation of pig feeds (GfE, 2008). For the determination of *in vivo* pcdAA values, apparent digestible AA are corrected for basal endogenous AA losses, but specific losses, which depend on feed properties, such as content of fibre, lectin, tannins or protease inhibitor activity, are not considered (GfE, 2005; Adeola et al., 2016). *In vivo* methods are the most precise approaches, often called "gold standard", for the evaluation of pcdAA, however they have some disadvantages. Animal trials can be difficult to perform, are time and cost intensive, have ethical limitations and are limited in their ability to cover the required high number of tests for new or differently treated feed ingredients (Brodkorb et al., 2019; Santos-Sánchez et al., 2024). Therefore, *in vitro* methods have been developed over the last decades to simulate digestion processes in a laboratory environment (Moughan et al. 2014). Santos-Sánchez et al. (2024) described and compared the scope and limitations of several *in vitro* methods and modifications to estimate the crude protein (CP) and AA digestibility in animals and humans. Only the study of Jezierny et al. (2010), which was based on a two-step incubation method with pepsin and pancreatin following the method of Boisen and Fernández (1995), was mentioned as being able to estimate pcdAA in pig feeds. Not cited by Santos-Sánchez et al. (2024), Eklund et al. (2015) and Rosenfelder-Kuon et al. (2020) were also using the two-step enzymatic method of Boisen and Fernández (1995) to estimate pcdAA. However, these studies used small sample pools which consisted of either cereal grains or protein feeds.

Recently, Schumacher et al. (2025) have demonstrated that pcdCP can be reliably estimated from the analysis of neutral-detergent insoluble (NDI) CP. Therefore, the aim of this study was to extend this method and estimate pcdAA based on the same sample pool used to estimate pcdCP which included different cereal grains, thermally treated protein feeds and other samples (Schumacher et al., 2025). The hypothesis was that the method developed and established to estimate pcdCP was similarly suitable to estimate pcdAA. The assumption was that insoluble AA determined as NDIAA or ADIAA are largely indigestible in the small intestine (Rezvani et al., 2012; Zeyner et al., 2015) and, therefore, soluble AA determined as ND-soluble (NDSAA) or AD-soluble (ADSAA) in the feed, represent the (potentially) digestible AA in the small intestine.

5.3 Materials and Methods

5.3.1 Material

5.3.1.1 Samples

A large sample pool was accessible of 82 feed ingredients on which pcdAA had been determined *in vivo* in pigs at the University of Hohenheim, Stuttgart, Germany. The sample pool consisted of cereal (wheat, triticale, rye, barley) and legume (faba beans, peas, lupins) grains, each represented by several varieties, untreated and heat-processed rapeseed meal and cake, soybean products and miscellaneous samples (Table 1). Based on the results presented by Schumacher et al. (2025), the following feeds listed in Table 1 were excluded from the AA analyses: 3 full-fat soybeans (no. 49, 50, 57) with high trypsin inhibitor activity, resulting in low *in vivo* concentrations of pcdCP and pcdAA. Additionally, soy protein isolate (hydrolysed), pea protein, wheat gluten 1, wheat gluten 3 (hydrolysed) and fish meal 1 (no. 76, 77, 78, 80, 81) were also excluded due to the high *in vivo* digestibility and therefore expected very low amounts of AD residues for AA analyses which would reduce the accuracy of the AD determination and introduce variability not related to intrinsic feed characteristics. Ultimately, 74 feed ingredients were used for the analyses in this study. The pcdAA values for total and individual AA were then calculated by multiplying the fractional standardised precaecal digestibility of AA (pcDAA) by AA content. These values are shown in the Appendix, Tables A1 - A7.

Table 1: The number, sample group, reference and pre-treatment of 82 feed ingredients of which 74 could be assayed in this study (see Section 5.3.1.1 for exclusion reasons).

Number	Sample group	Sample	Reference	Pre-treatment*
1	Wheat	Skalmeje	[1]	Incubation
2		Tommi		
3		St. Tobak		
4		Event		
5		Mulan		
6		Tabasco		
7		Adler		
8		KWS Erasmus		
9	Triticale	Grenado	[2]	Incubation
10		Tarzan		
11		HYT Prime		
12		Massimo		
13		Cultivo		
14		SW Talentro		
15		Cando		
16		Agostino		
17	Rye	Conduct	[2]	Incubation
18		Visello		
19		Helltop		
20		Bellami		
21		Palazzo		
22		Dukato		
23		Guttino		
24		Dankowski Diament		
25	Barley	Yool	[3]	Incubation
26		Ack 2927		
27		Lomerit		
28		Campanille		
29		Canberra		
30		Antisette		
31		Metaxa		
32		Fridericus		
33	Pea	Santana	[4]	Incubation
34		Jutta		
35		Phönix		
36		Harnas		
37		Rocket		
38		Hardy		
39	Lupin	Probor	[4]	Incubation
40		Boregine		
41		Boruta		
42		Idefix		
43	Faba bean	Aurelia	[4]	Incubation
44		Divine		
45		Gloria		
46		Limbo		
47		Fuego		
48		Espresso		

49	Full-fat soybean/ soybean product	FFSB K0	[5]	Fat extraction
50		FFSB K1		
51		FFSB K2		
52		FFSB K3		
53		FFSB Z1		
54		FFSB Z2		
55		FFSB Z3		
56		FFSB Z4		
57		FFSB	[6]	Fat extraction
58		FFSB (roasted)		
59		SBC		
60		SBM (Austria)		/
61		SBM (GMO-free)		/
62		SBM (standard)		/
63	Rapeseed meal and cake	RSM48	[7]	/
64		RSM64		/
65		RSM76		/
66		RSM93		/
67		LOW-GLS RSM		/
68		RSC	[6]	Fat extraction
69		RSM		/
70	Various samples	Soybean (extruded)	[8]	Fat extraction
71		SBM (high protein)	[9]	/
72		SPC (A coarse)		/
73		SPC (A fine)		/
74		SPC (B coarse)		/
75		SPC (B fine)		/
76		SPI (hydrolysed)		/
77		PeaP		Fat extraction
78		WG1		/
79		WG2 (hydrolysed)		/
80		WG3 (hydrolysed)		/
81		FM1		Fat extraction
82		FM2 (extracted)		/

FFSB, Full-fat soybean; FM, Fish meal; GLS, Glucosinolates; PeaP, Pea protein; RSC, Rapeseed cake; RSM, Rapeseed meal; SBC, Soybean cake; SBM, Soybean meal; SPC, Soy protein concentrate; SPI, Soy protein isolate; WG, Wheat gluten; / no pre-treatment;
*Incubation: carried out according to McQueen and Nicholson (1979).
*Fat extraction: carried out according to Regulation (EG) 152/2009 Annex III, H 1.1.
References: [1] Rosenfelder et al. (2015), [2] Strang et al. (2017), [3] Spindler et al. (2016), [4] Jezierny et al. (2010), [5] Kaewtapee et al. (2017a), [6] Kaewtapee et al. (2017b), [7] Eklund et al. (2015), [8] Urbaityte et al. (2009a), [9] Urbaityte et al. (2009b).

5.3.1.2 Characterisation and selection for assigning samples to the neutral- or acid-detergent insoluble amino acid procedure

A condensed overview of anti-nutritional compounds compiled from the references given in Table 1 is presented in Schumacher et al. (2025; Appendix Tables A1 - A4) which were used as a basis for the selection criteria of NDIAA or ADIAA (Table 2): Feed ingredients containing specific N compounds, e.g., Maillard products or/and tannin-protein complexes, which are

captured in the AD residue but not in the ND residue (Table 2), were analysed for ADIAA, whereas on all other samples NDIAA were analysed. Hence, NDIAA was analysed on all cereal grain samples and ADIAA on all other samples, which primarily can be referred to as protein feeds.

5.3.2 Methods

5.3.2.1 General analyses

The samples were milled through a 1-mm screen using an ultra-centrifugal mill at 18,000 rpm (ZM 200, Retsch GmbH & Co. KG, Haan, Germany). Dry matter (DM) was determined by oven-drying at 103°C (Regulation (EG) 152/2009 Annex III, A). The ND or AD residues for NDIAA or ADIAA determination were isolated as specified in VDLUFA (2023) using Fibretherm (FT12; FibreBags ADF, 30 µm pore size; C. Gerhardt, Königswinter, Germany). The residues were dried at 103°C, thereafter scraped out of the bags, ground with a coffee grinder (MX 32, Braun AG, Frankfurt, Germany) and collected in glass containers until a quantity of at least 2.3 g of the dried residue for AA analyses was reached in each case. Depending on feed ingredient, the boiling cycles had to be repeated 2 to 4 times to obtain this quantity. Afterwards, AA were analysed on the residue by HPLC according to Regulation (EG) 152/2009 (Annex III, F, G) by LUFA Nord-West, Oldenburg, Germany. Amino acids were separated on a PEEK column (for tryptophan, a Phenomenex Synergi Hydro RP column was used) with the following mobile phase: 6 ml acetic acid + 1,800 ml H_20 + 100 ml trichloro-2-methyl-2-propanol, adjusted to pH 5 with ethanolamine and brought to 2 l volume with distilled water. Different buffers were used as eluents. After post-column ninhydrin derivatization, AA were detected by fluorescence at 280 nm excitation wave length and 356 nm emission wave length.

Table 2: The selection criteria Maillard products, condensed tannin-CP, isoelectric point protein and phytate-CP-complexes for neutral-detergent insoluble (NDIAA) or acid-detergent insoluble amino acids (ADIAA).

	Literature	NDIAA	ADIAA
Maillard products	van Soest and Mason (1991) Licitra et al. (1996) Classen et al. (2004)	/	X
Condensed tannin-CP	van Soest (1994)	/	X
Isoelectric point protein	Csonka et al. (1926) Csonka and Jones (1927) Morales et al. (2013)	X	X
Phytate-CP-complex	Morales et al. (2013)	X	X
		Cereal grains	**Protein feeds**

CP, Crude protein; ADIAA, Acid-detergent insoluble amino acid; NDIAA, Neutral-detergent insoluble amino acid; /, not included; X, included

5.3.2.2 Sample pre-treatment for removal of starch or fat

As listed in Table 1 and described in detail by Schumacher et al. (2025), starchy samples were pre-treated overnight with α-amylase (Termamyl 2X, Novozymes, Novo Industrials, Bagsværd, Denmark) in a buffer (pH 7) at 40°C in a shaking (80 rpm) water bath (SW22, JULABO, Seelbach, Germany) according to McQueen and Nicholson (1979). Samples with fat content >80 g/kg DM (full-fat soybeans, soybean cake, rapeseed cake, samples no. 52-56, 58-59, 68, 70) were pre-treated to extract fat according to Regulation (EG) 152/2009 Annex III, H (1.1; without hydrochloric acid pre-treatment). The samples, weighed in bags, were extracted in a Soxtherm device (Sox 404, C. Gerhardt) with petrolether (40-60°C) for 1.5 h. The bags with pre-treated samples were afterwards used to isolate the ND and AD residue.

5.3.3 Calculations and statistical analysis

The concentrations of NDSAA or ADSAA of feeds were calculated as follows:

NDSAA (g/kg DM) = AA (g/kg DM) - NDIAA (g/kg DM) [1]

and

ADSAA (g/kg DM) = AA (g/kg DM) - ADIAA (g/kg DM) [2]

5.3.3.1 Regression analysis

The statistical data evaluation was carried out with the programme R 2.2 (R Foundation for Statistical Computing, Vienna, Austria). First, a raw data analysis was performed and means and standard deviations were calculated. Then, a linear model was applied for the regression analysis:

$$y = a\,x + b \qquad [3]$$

where y = *in vivo* pcdAA (g/kg DM) and x = NDSAA or ADSAA (g/kg DM) as categorised above (5.3.1.2).

This model was applied to the entire dataset (*n* = 74) and separately to cereal grains at large (*n* = 38) and in different combinations of cereal grain species and separately to protein sources (*n* = 36). For the large subgroup of cereal grains in the dataset, an additional ANOVA, followed by a Tukey-Test to separate means for wheat, rye, barley and triticale, was carried out to further evaluate the data in accordance with the procedure reported for pcdCP by Schumacher et al. (2025). The relationship between residues and fitted values of data of all 74 samples for total, indispensible, dispensable and individual AA were plotted.

5.4 Results

5.4.1 All feed ingredients

Total amino acids

The entire dataset (*n* = 74) was used to display for total AA the relationship between *in vivo* pcdAA values and corresponding NDSAA values for cereal grains and ADSAA values for protein feeds (Fig.1), resulting in the following linear equation to estimate pcdAA from NDIAA and ADIAA, respectively:

y = 0.823 (standard error [SE] 0.018, confidence interval [CI] 0.788; 0.858) x

+ 10.52 (SE 4.420, CI 1.709; 19.33) [4]

$R^2 = 0.968$

Root mean square error (RMSE) = 17.13

y = *in vivo* pcdAA (g/kg DM) and x = NDSAA for cereal grains and ADSAA (g/kg DM) for protein feeds

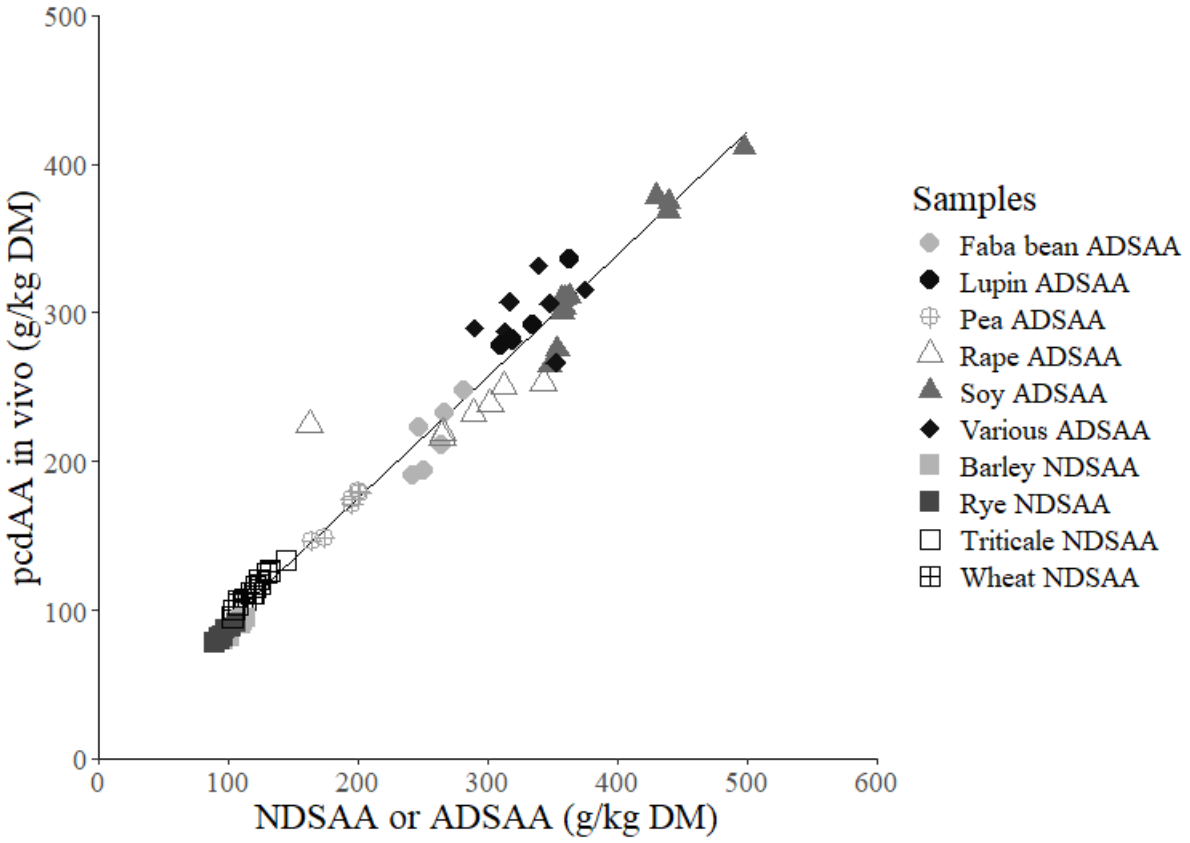

Figure 1: Linear relationship between NDSAA and ADSAA data from the laboratory method (x) and *in vivo* pcdAA (y) for cereal grains and protein feed ingredients for all 74 samples and total AA (n = 17): y = 0.823 (SE 0.018, CI 0.788; 0.858) x+ 10.52 (SE 4.420, CI 1.709; 19.33).

Cereal grains were in the lower range and most of the protein feeds in the middle and upper range of values, with one particularly striking rapeseed meal value (RSM76).

The entire dataset (n = 74) was also used to evaluate the relationship between *in vivo* pcdAA values and corresponding NDSAA values for cereal grains and ADSAA values for protein feeds of indispensable AA (Fig. 2A) and dispensable AA (Fig. 2B), resulting in the following linear equation to estimate pcdAA from NDIAA and ADIAA, respectively:

Sum of indispensable AA:

y = 0.853 (SE 0.017, CI 0.819; 0.887) x + 1.754 (SE 2.021, CI -2.274; 5.783) [5]

R^2 = 0.972

RMSE = 8.610

y = *in vivo* pcdAA (g/kg DM) and x = NDSAA or ADSAA (g/kg DM)

Sum of dispensable AA:

$$y = 0.797 \text{ (SE } 0.019, \text{ CI } 0.759; 0.834) \; x + 8.905 \text{ (SE } 2.481, \text{ CI } 3.958; 13.85) \qquad [6]$$

$R^2 = 0.962$

RMSE = 8.884

y = *in vivo* pcdAA (g/kg DM) and x = NDSAA or ADSAA (g/kg DM)

For the group of indispensable AA, cereal grains were in the lower range and protein feeds in the upper range of the values (Fig. 2A). The value for rapeseed meal (RMS76) was again visually striking. Concerning the dispensable AA, cereal grains and peas were in the lower range of the values (Fig. 2B). The protein samples were in the middle and upper range of the values. For dispensable AA a striking value was also noticeable for the sample RSM76.

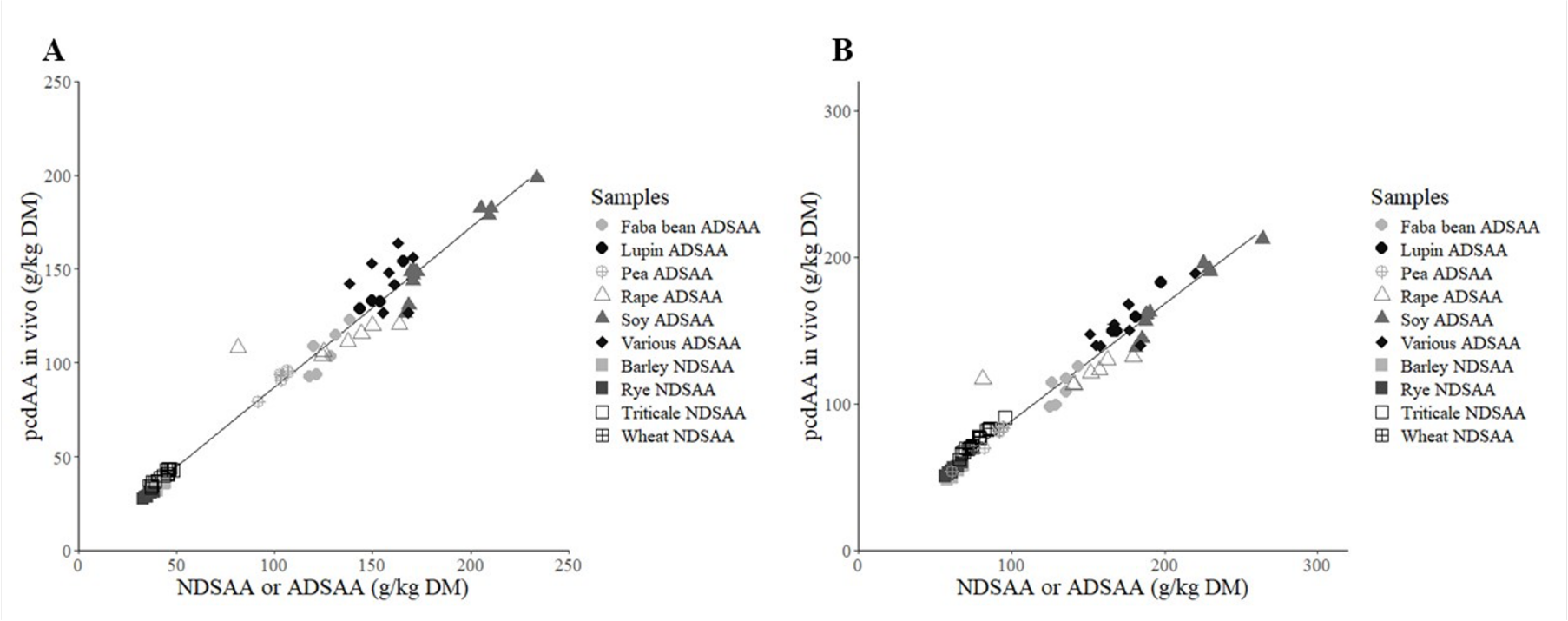

Figure 2: (A) Linear relationship between NDSAA and ADSAA data from the laboratory method (x) and *in vivo* pcdAA (y) for cereal grains and protein feed ingredients for all 74 samples and indispensable AA (n =10): y = 0.853 (SE 0.017, CI 0.819; 0.887) x + 1.754 (SE 2.021, CI -2.274; 5.783). **(B)** Linear relationship between NDSAA and ADSAA data from the laboratory method (x) and *in vivo* pcdAA (y) for cereal grains and protein feed ingredients for all 74 samples and dispensable AA (n = 7): y = 0.797 (SE 0.019, CI 0.759; 0.834) x + 8.905 (SE 2.481, CI 3.958; 13.85).

5.4.1.2 Individual amino acids

The entire dataset (*n* = 74) was also used to display, for each individual AA, the relationship between *in vivo* pcdAA values and corresponding NDSAA values for cereal grains and ADSAA values for protein feeds (Fig. 3 and Fig. 4), resulting in linear equations to estimate pcdAA from NDIAA or ADIAA, exemplified hereafter for Lys and Met:

Lys:

y = 0.863 (SE 0.019, CI 0.825;0.902) x - 0.272 (SE 0.311, CI -0.893; 0.348) [7]

R^2 = 0.965

RMSE = 1.505

y = *in vivo* pcdLys (g/kg DM) and x = NDSLys or ADSLys (g/kg DM)

Met:

y = 0.936 (SE 0.019, CI 0.897; 0.975) x - 0.141 (SE 0.083, CI -0.306; 0.024) [8]

R^2 = 0.970

RMSE = 0.394

y = *in vivo* pcdMet (g/kg DM) and x = NDSMet or ADSMet (g/kg DM)

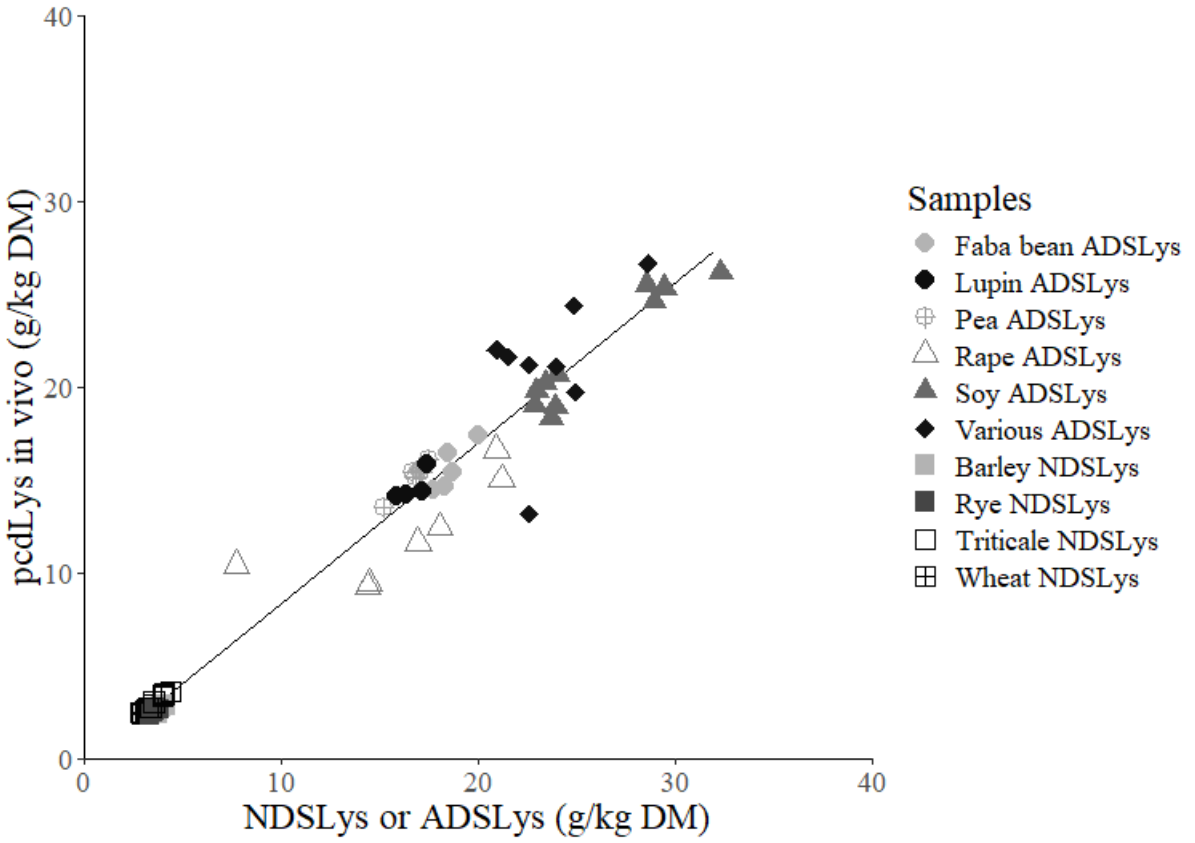

Figure 3: Linear relationship between NDSLys and ADSLys data from the laboratory method (x) and *in vivo* pcdLys (y) for cereal grains and protein feed ingredients for all 74 samples: y = 0.863 (SE 0.019, CI 0.825;0.902) x - 0.272 (SE 0.311, CI -0.893; 0.348).

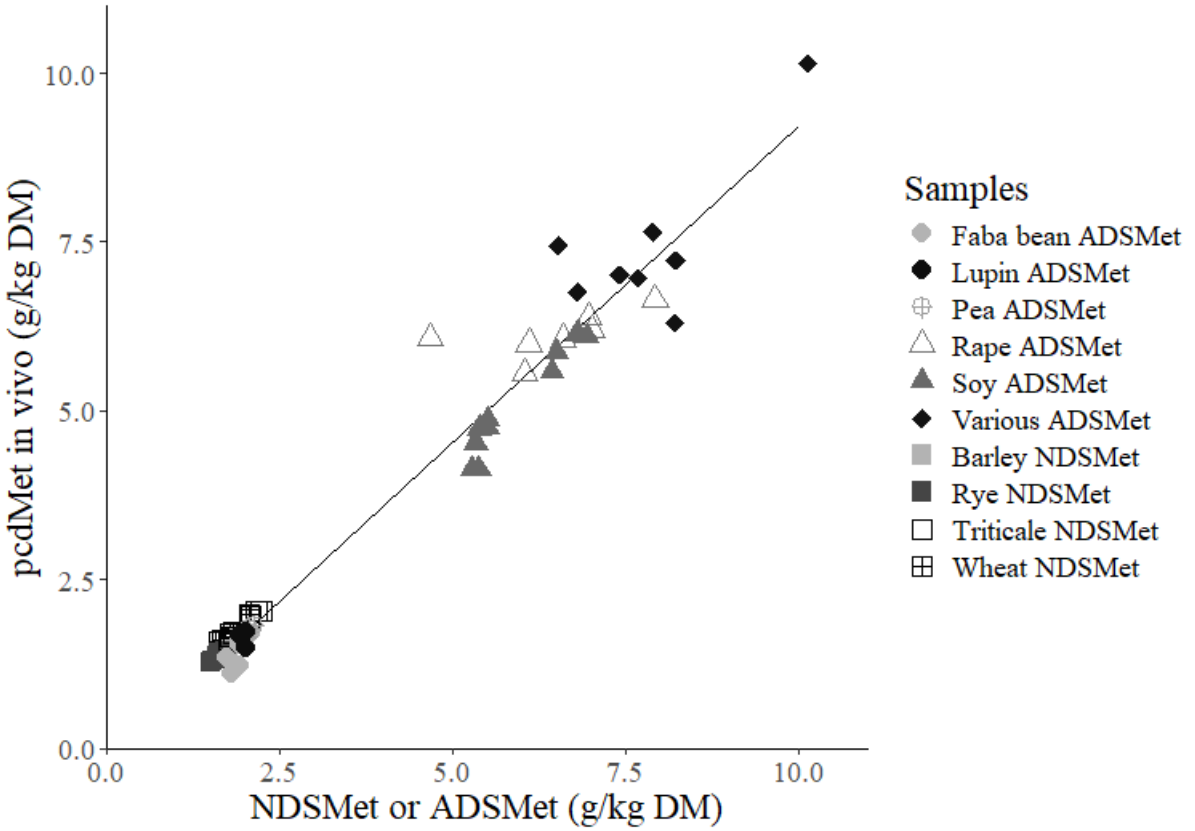

Figure 4: Linear relationship between NDSMet and ADSMet data from the laboratory method (x) and *in vivo* pcdMet (y) for cereal grains and protein feed ingredients for all 74 samples: y = 0.936 (SE 0.019, CI 0.897; 0.975) x - 0.141 (SE 0.083, CI -0.306; 0.024). The encircled symbols represent four particularly striking values.

The regression equations and R^2 values for indispensable and dispensable AA are summarised in Table 3. The coefficients of determination were very high for almost all individual AA, both for indispensable and dispensable AA, with values between 0.895 for Pro and 0.984 for Arg.

Table 3: Linear relationship between NDSAA and ADSAA data from the laboratory method (x) and *in vivo* AA (pcdAA) for all samples (cereal grains and protein feeds, *n* = 74): Regression equation (y = a x + b), standard error (SE), confidence interval (CI), coefficient of determination (R^2) and root mean square error (RMSE).

Amino acids	Samples		
	y =	R^2	RMSE
Indispensable AA			
Arginine	0.914 (SE 0.014, CI 0.886; 0.941) x + 0.287 (SE 0.282, CI -2.753; 0.850)	0.984	1.363
Histidine	0.845 (SE 0.018, CI 0.810; 0.880) x + 0.180 (SE 0.125, CI -0.069; 0.429)	0.970	0.496
Isoleucine	0.873 (SE 0.017, CI 0.840; 0.907) x + 0.011 (SE 0.191, CI -0.365; 0.396)	0.974	0.841
Leucine	0.854 (SE 0.016, CI 0.822; 0.885) x + 0.320 (SE 0.317, CI -0.311; 0.952)	0.976	1.317
Lysine	0.863 (SE 0.019, CI 0.825; 0.902) x – 0.272 (SE 0.311, CI -0.839; 0.348)	0.965	1.505
Methionine	0.936 (SE 0.019, CI 0.897; 0.975) x – 0.141 (SE 0.083, CI -0.306; 0.024)	0.970	0.394
Phenylalanine	0.818 (SE 0.024, CI 0.770; 0.866) x + 0.657 (SE 0.305, CI 0.049; 1.265)	0.941	1.227
Threonine	0.785 (SE 0.021, CI 0.742; 0.827) x + 0.214 (SE 0.227, CI -0.238; 0.667)	0.949	0.985
Tryptophan	0.784 (SE 0.025, CI 0.735; 0.834) x + 0.038 (SE 0.083, CI -0.128; 0.204)	0.932	0.364
Valine	0.817 (SE 0.021, CI 0.775; 0.860) x + 0.438 (SE 0.272, CI -0.103; 0.980)	0.953	1.132

Amino acids	Samples		
	y =	R^2	RMSE
Dispensable AA			
Alanine	0.811 (SE 0.023, CI 0.766; 0.857) x + 0.190 (SE 0.247, CI -0.302; 0.683)	0.946	1.016
Aspartic acid	0.822 (SE 0.016, CI 0.790; 0.855) x + 0.354 (SE 0.420, CI -0.481; 1.190)	0.973	2.155
Cysteine	0.721 (SE 0.028, CI 0.665; 0.776) x + 0.299 (SE 0.122, CI 0.056; 0.541)	0.904	0.417
Glutamic acid	0.818 (SE 0.018, CI 0.782; 0.853) x + 4.135 (SE 0.947, CI 2.248; 6.022)	0.967	3.160
Glycine	0.739 (SE 0.019, CI 0.700; 0.777) x + 0.491 (SE 0.218, CI 0.056; 0.925)	0.953	0.879
Proline	0.840 (SE 0.034, CI 0.772; 0.907) x + 1.187 (SE 0.595, CI 0.000; 2.374)	0.895	1.756
Serine	0.813 (SE 0.019, CI 0.775; 0.851) x + 0.533 (SE 0.248, CI 0.039; 1.028)	0.961	1.057

y = estimated pcdAA (g/kg DM) and x = NDSAA or ADSAA (g/kg DM).

For pcdLys, cereal grains were in the lower range and protein feeds in the middle and upper range of the values (Fig. 3). In contrast, values for pcdMet were in the upper range of values for rapeseed and rape products, soy and various samples and in the lower range for cereal grains and legume grains such as peas, faba beans and lupins (Fig. 4). The values for RSM76 and soy protein concentrate B fine (SPCBf) deviated remarkably from the regression line. A closer look at the lower range scatter plot with a different scaling showed that the values of the legumes are almost completely among those of the cereals (Fig. 5). There were noticeably high ADSMet values for three colour-flowering faba beans which had higher tannin contents than the white-flowering faba beans.

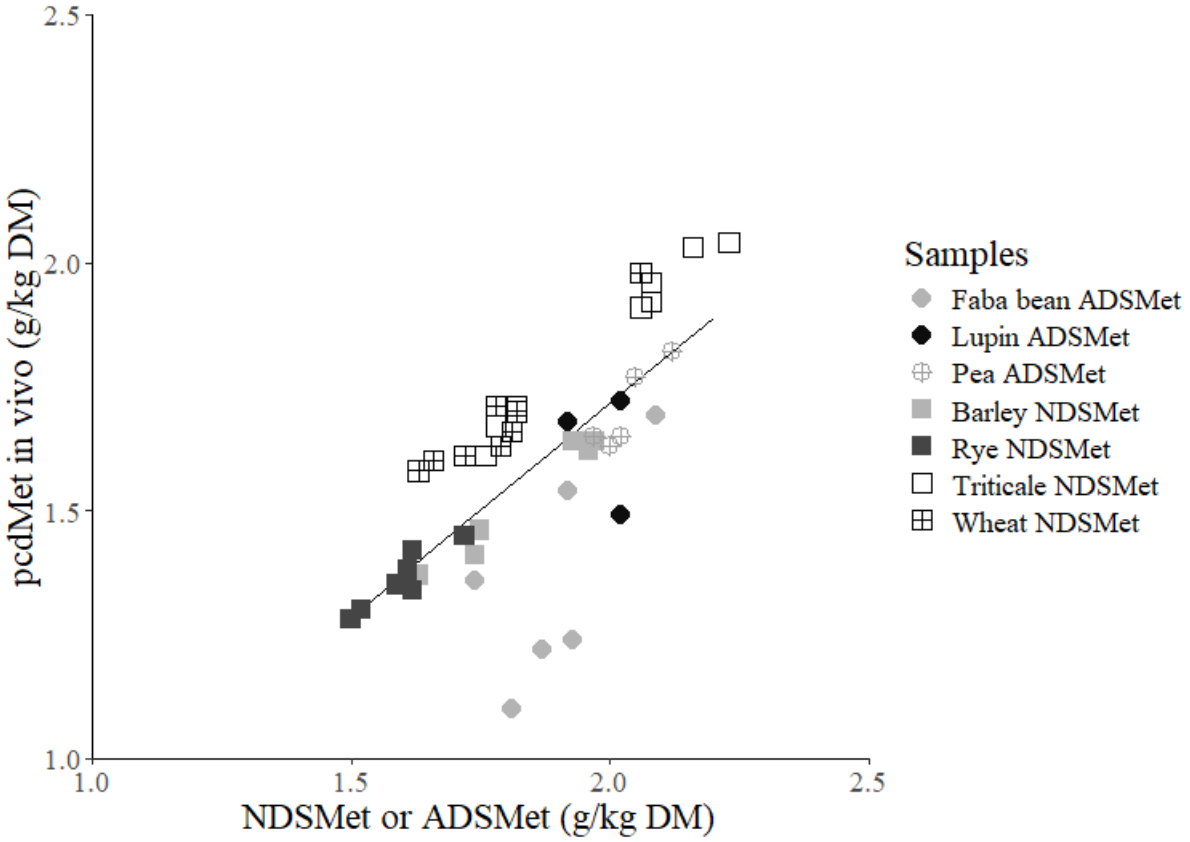

Figure 5: Linear relationship between NDSAA and ADSAA data from the laboratory method (x) and *in vivo* pcdAA (y) for cereal grains and the legume grains faba bean, pea and lupin zoomed in from Fig. 4.

The illustration of the linear relationship between *in vivo* pcdAA values and corresponding NDSAA values for cereal grains and ADSAA values for protein feeds for all other AA can be found in the Appendix (Fig. A1- Fig. A15). For Met, Thr, Trp, His and Cys the values for RSM76 and soy protein concentrate B fine (SPCBf) were visually striking (Fig. 4, A1, A2, A6,

A11). In addition, the sample RSM76 was visibly divergent for Val, Arg, Phe, Ala, Asp, Glu, Gly and Ser (Fig. A3, A5, A8, A9, A10, A12, A13, A15). In general, all linear regression equations had high coefficients of determination.

5.4.2 Cereal grains

5.4.2.1 Total amino acids

In accordance with *in vivo* pcdCP values in Schumacher et al. (2025), the *in vivo* pcdAA values of feed ingredients were grouped in cereal grains and protein feeds, just as forming clusters of wheat with triticale and barley with rye. The entire cereal grain dataset (n = 32) was used to display the relationship of the total AA between *in vivo* pcdAA values and corresponding NDSAA values for the two groups (Fig. 6), resulting in the following linear equation to estimate pcdAA from NDIAA:

Wheat and triticale:

y = 0.904 (SE 0.045, CI 0.807; 1.001) x + 5.841 (SE 5.389, CI -5.717; 17.40) [9]

R^2 = 0.966

RMSE = 1.992

y = *in vivo* pcdAA (g/kg DM) and x = NDSAA (g/kg DM)

Barley and rye:

y = 0.696 (SE 0.069, CI 0.547; 0.844) x + 15.60 (SE 6.978, CI 0.631; 30.56) [10]

R^2 = 0.879

RMSE = 1.813

y = *in vivo* pcdAA (g/kg DM) and x = NDSAA (g/kg DM)

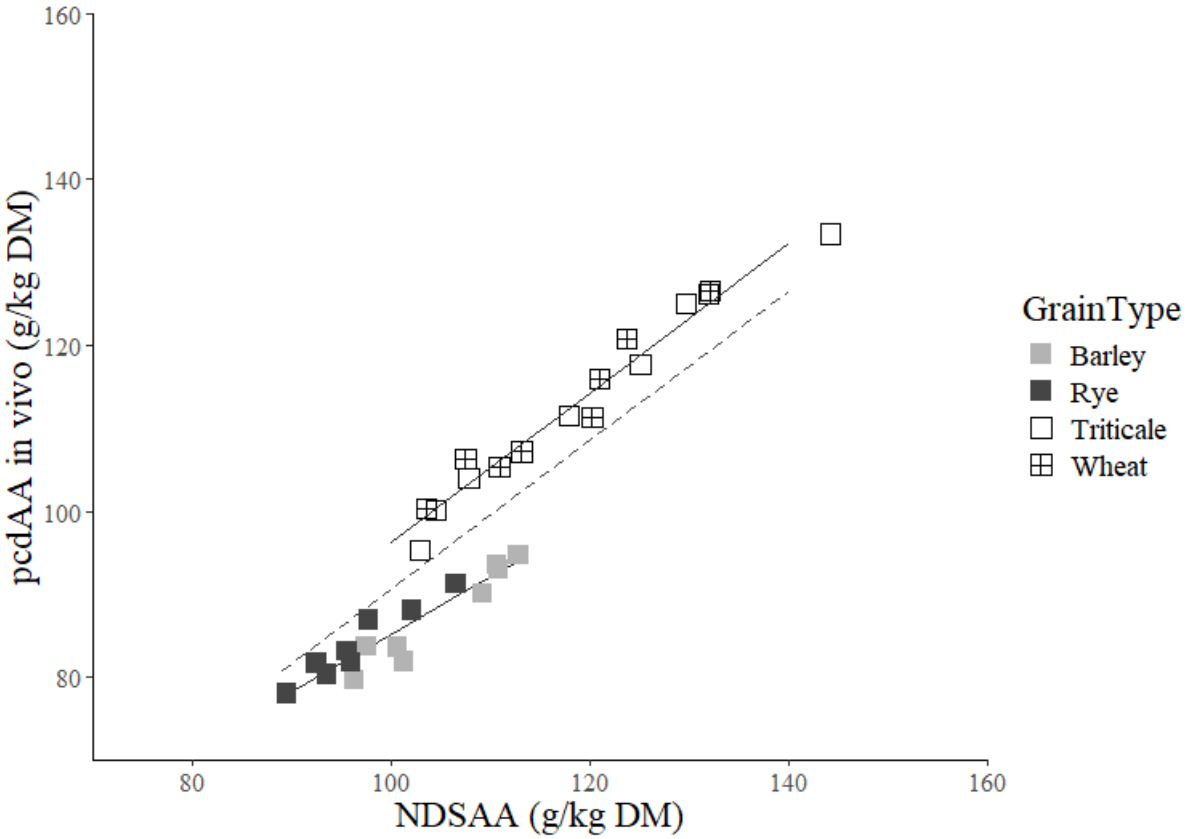

Figure 6: Linear relationship between NDSAA data from the laboratory method (x) and *in vivo* pcdAA (y) for cereal grains for wheat, triticale, barley and rye with clustering of wheat with triticale and barley with rye. The dashed line shows the regression line for all cereal grain samples without clustering.

The relationship between residues and fitted values of data of all 74 samples for all 17 AA, indispensable and dispensable AA, Lys, Met and for total AA for alle cereal grains are shown in Fig. 7.

The cereal grain dataset was also used to display the relationship between *in vivo* pcdAA values of indispensable and dispensable AA and the corresponding NDSAA values in the two cereal grain groups (Appendix Fig. A16 and A17), resulting in the linear regression equations to estimate pcdAA from NDSAA shown in Table A8. The R^2 values for cereal grains in general were very high, with 0.989 for the total AA, 0.980 for the indispensable AA and 0.993 for the dispensable AA. Comparing R^2 values of groups, the values for the total AA were higher for the wheat and triticale cluster (0.966 > 0.879). The values for indispensable AA were higher for the barley and rye cluster (0.969 > 0.933) and the values for dispensable AA were higher in the wheat and triticale cluster (0.979 > 0.775).

The relationship between residues and fitted values of data of all 74 samples for individual AA are shown in Fig. A18, Fig. A19, Fig. A20.

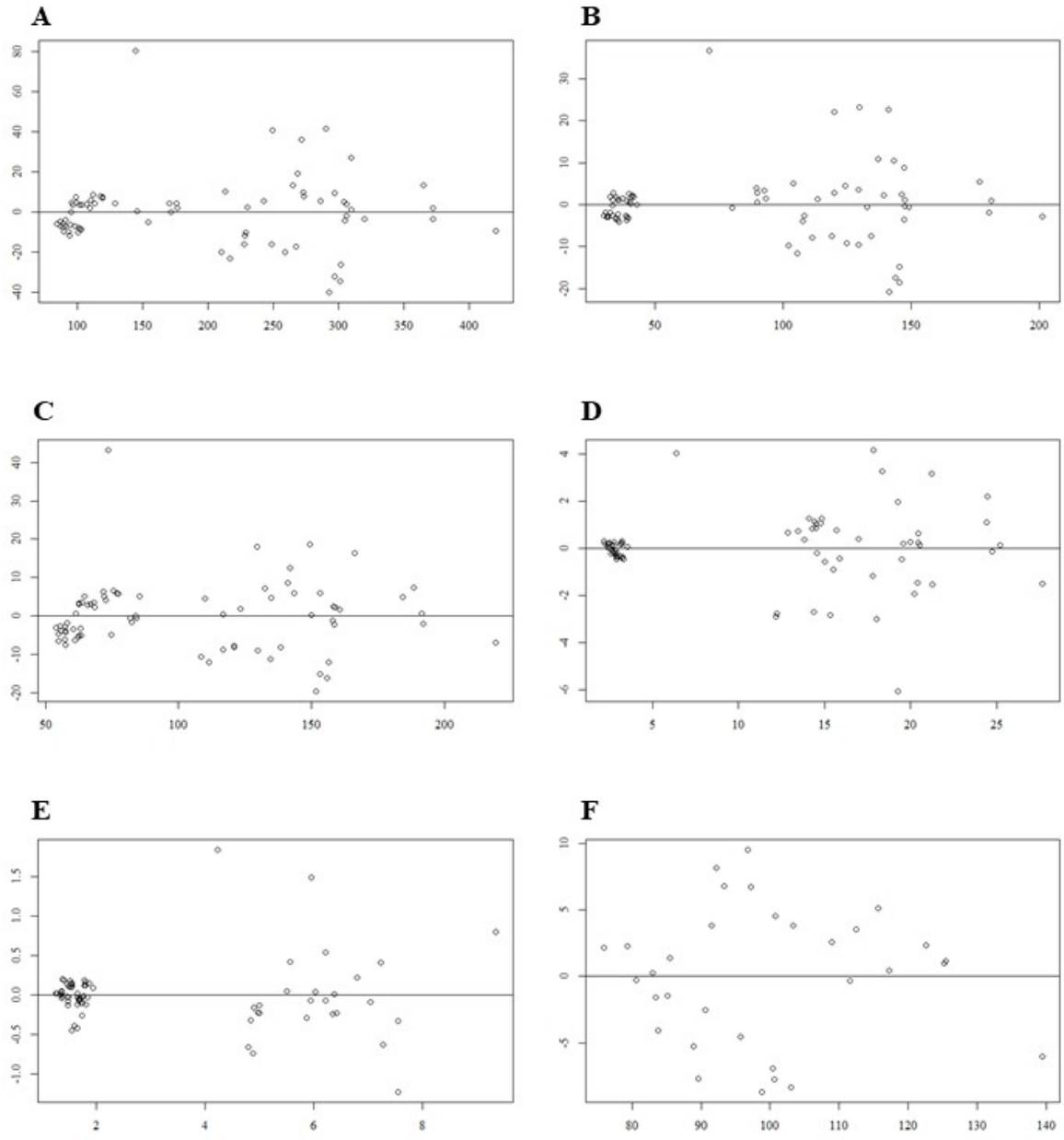

Figure 7: Relationship between residues (y; g/kg DM) and fitted values (x; g/kg DM) of data of all 74 samples for all 17 amino acids (A), indispensable amino acids (B), dispensable amino acids (C), lysine (D), methionine (E) and for total amino acids for all cereal grains (F).

5.4.2.2 Individual amino acids

The regression equations and R^2 values for the individual AA for the two cereal grain clusters are summarised in the Appendix Tables A9 and A10. The R^2 values of the wheat and triticale cluster were high for indispensable and dispensable AA, with values ranging between 0.817 for Gly and 0.999 for Trp. Similarly, the barley and rye cluster had high R^2 values for almost all AA, with values ranging between 0.730 for serine and 0.988 for leucine. Remarkably lower values were observed for the dispensable AA Gly (0.687) and Pro (0.402).

5.5 Discussion

5.5.1 General considerations

The main objective of this study was to investigate if the estimation of pcdAA could be based on the same rapid laboratory procedure developed and established for the estimation of pcdCP (Schumacher et al., 2025). Tedeschi et al. (2001) reported that NDSAA and NDIAA of various forages (different genotypes of grass and different legumes, among others soybeans and lucerne) had similar AA profiles. However, this was not observed for the untreated and differently heat-treated feed ingredients used in this research analysing NDIAA or ADIAA, which Tedeschi et al. (2001) already reported for AD residues. The discrepancy in the AA profiles of the AD-soluble and AD-insoluble fractions are attributable to complexes of proteins with anti-nutritional components like tannins and phytate, which are recovered in the AD-insoluble fraction and reflected in the ADIAA pattern (Table 2).

This study benefitted from a data on 74 feed ingredients, which were obtained from the same facilities, ensuring consistency in data generation and analytical characterisation and minimising assay effects on variation in pcdAA determination. Moreover, the specific chemical analyses for NDIAA and ADIAA were performed on the identical feed ingredients on which the *in vivo* pcdAA and general chemical characteristics had been determined using standardised and consistent methods.

5.5.2 Comparison with other methods

In vivo methods are the so called “gold standard”, for the evaluation of AA digestibility of feed ingredients, however they pose several challenges and demanding features. Animal intervention trials can be difficult to perform as they require expensive and lengthy experiments, have ethical limitations and should adhere to policies on experimental animals which follow the principle

of the 3R concept, namely replacement, reduction and refinement (Santos-Sánchez et al., 2024). Further, presumably *in vivo* experiments have insufficient capacity to cover the required high number of tests for new, differently processed and treated feed ingredients (Brodkorb et al., 2019; Santos-Sánchez et al., 2024). The pressure from consumers to reduce the number of experimental animals, due to environmental and animal welfare perspectives, is growing currently (Santos-Sánchez et al., 2024). Because of the aforementioned reasons, *in vitro* methods have been developed and used over the last decades to simulate protein digestion in the gastro-intestinal tract of several species (Santos-Sánchez et al., 2024). These methods show several advantages compared with *in vivo* methods. *In vitro* methods are rapid and more cost-effective, the variable effects of the animals are removed, they can simulate processes of different gastrointestinal segments and ethical limitations and policies on experimental animals are no longer barriers (Moughan et al., 2014; Zaefarian et al., 2021). Also factors like livestock farming and management, environment, genotype and diseases do not affect the *in vitro* evaluation (Zaefarian et al., 2021). Santos-Sánchez et al. (2024) described and compared the scope and limitation of different *in vitro* methods and their modifications to estimate CP and AA digestibility in animals and humans; the majority of these approaches was based on the use of different digestive enzymes. Most studies determined apparent pcD, three studies reported standardised pcD in pig feed (Meunier et al., 2008; Jezierny et al., 2010; Salazar-Villanea et al., 2016). Jansman et al. (2002) described the lack of additivity of the apparent pcD for feed ingredients in mixed diets, because the apparent digestibility is influenced by the AA content in the feed (Furuya and Kaji, 1989; Jansman et al. 2002). In contrast, the standardised pcD is independent of the concentration of AA in the feed, because it is corrected for basal endogenous losses (Moter and Stein, 2004). Therefore, compared to the apparent pcD, the standardised pcD enables a more precise estimation of digestible AA in mixed feeds (Jansman et al., 2002).

Only in the study of Jezierny et al. (2010), which was based on the Boisen and Fernández (1995) two-step incubation method with pepsin and pancreatin, pcDAA in pig feed was estimated. Salazar-Villanea et al. (2016) also estimated standardised precaecal digestibility of CP and AA but did not show any results for AA. Another valuable reference is the INFOGEST digestion protocol, developed by a consortium for application to human nutrition (Minekus et al., 2014; Brodkorb et al., 2019), which represented a standardisation of the diverse *in vitro* methods to simulate, in a laboratory setting, digestion in the upper gastro-intestinal tract (oral, gastric, duodenal) (Minekus et al., 2014). Santos-Sánchez et al. (2024) quoted three adaptions of the method (number of feed ingredients in parentheses): Ariëns et al., 2021 (n = 8); Sousa et al., 2022 (n = 7); Martineau-Côté et al., 2024 (n = 5), which allowed to determine *in vitro* pcdAA.

The *in vitro* methods showed good comparability with the *in vivo* pcD of CP and AA in pigs (Santos-Sánchez et al., 2024). However, Santos-Sánchez et al. (2024) again pointed out explicitly that different digestive conditions like the selection of enzymes, pH value and digestion time, which were used in distinct ways by various authors, require optimisation and standardisation. This point also makes it difficult to compare results over the past 40 years and reinforces the statement that a harmonisation of *in vitro* methods is essential (Moughan et al. 2014).

The different *in vitro* methods characterized by Santos-Sánchez et al. (2024) were only applied to small sample numbers. In an extended literature search, more studies were identified in which also the two-step enzymatic method of Boisen and Fernández (1995) was employed to estimate pcDAA in pig feed (number of feed ingredients in parentheses), namely Boisen and Fernández (1995; n = 9), Pujol and Torrallardona (2007; n = 7), Jezierny et al., (2010; n = 17), Eklund et al., (2013; n = 16), Eklund et al. (2015; n = 6) and Rosenfelder-Kuon et al. (2020; n = 32). Although the total number of feeds investigated in the studies mentioned above (n = 87) outperformed the number of feeds in this study (n = 74), the major drawback appears that the specific procedures and analytical methods varied between laboratories (analytical steps and specifications of enzymes, incubation conditions such as duration and temperature) which hinders or even precludes to merge data into one large dataset for joint evaluation. Taken together, the portrayed situation further strengthens the need for a standardisation and harmonisation of *in vitro* methods both for application to animals and humans. This has – over the past 40 years – only been achieved for human nutrition studies by the INFOGEST digestion protocol (Minekus et al., 2014; Brodkorb et al., 2019). Unfortunately, the INFOGEST protocol is rather complex in structure and application and not practicable for routine use in (farm) animal nutrition studies. Therefore, there is still a need for laboratory methods with a tolerable degree of complexity and sufficient ease of handling. *In vitro* procedures with a satisfying degree of standardisation and reproducibility across laboratories have advantages compared with chemical methods because they basically allow to adjust the conditions (e.g., pH, temperature, duration, type of enzymes and enzyme activity) both to different animal species and different segments with the gastro-intestinal tract of a given species.

The determination of NDSAA and ADSAA is based on procedures for isolating ND and AD residues which have been developed, refined and standardised over decades mainly by Van Soest and co-workers (see, e.g., Van Soest et al., 1991). These procedures have been applied to analytical schemes for CP fractionation (Licitra et al., 1996) and integrated into agricultural chemical analysis handbooks such as VDLUFA (2023). Analytical quality assurance is

routinely performed, e.g., in ring tests following international standards (VDLUFA, 2023). An advantage is that AD and ND residues can be determined by instrumental analysis. This facilitates processing of large sample sets which currently appears unfeasible for *in vitro* incubations. The AA determination on ND and AD residues follows the approved routine method for feed AA analysis (Regulation (EG) 152/2009 Annex III, F, G) and does not require new or modified procedures. Therefore, consistent with the procedures characterised by Schumacher et al. (2025) for NDICP and ADICP, the determination of NDIAA or ADIAA appears robust and reliable.

In general, data in the present study showed a close relationship between NDSAA and ADSAA data from the laboratory method and *in vivo* pcdAA, which is reflected in the close alignment of the vast majority of data to the regression line for total AA, indispensible and dispensible AA and also individual AA. For only two feed ingredients, notable exceptions from the general observation occurred, one thermally treated rapeseed meal and one soy protein concentrate. The rapeseed meal (RSM76) had a residence time of 76 minutes in the desolventizer/toaster with unsaturated steam (Eklund et al., 2015), and all AA except Lys, Ile, Leu and Pro showed a remarkable distance from the regression line. This is surprising because other RSM samples treated similarly with desolventizer/toaster residence times from 48 to 93 minutes were not notably distant from the regression line, therefore it can be assumed that the particular values of RSM76 were not due to a too short or too long thermal treatment, which in the latter case would have increased the risk of heat damage with the formation of Maillard products. Hence, no explanation for the divergent values of this specific sample can be given.

The other exception from the close alignment of data to the regression line was soy protein concentrate B fine (SPCBf), which – for the indispensible AA Thr and Trp – had *in vivo* pcd values above the regression line, i.e., ADSThr and ADSTrp values were low. The first assumption was that this could be explained with fine grinding, as the same sample treated similarly, but with coarser grinding, soy protein concentrate B coarse (SPCBc) did not show the same distinct feature. For ND and AD residues, Mertens (1992) mentioned that in analyses, finer particles can be washed out of the bag during processing. Also, there is a larger surface for the solvent to attack in finer milled sample material. This, however, would have resulted in overestimated ADSAA values, in contrast to the observed low ADSAA values. Again, it remains unclear why the values of this specific soy protein concentrate were in contrast to expectations, and moreover, no rationale is obvious why only two AA were affected.

The results of this study suggest that the estimation of pcdAA based on procedures for isolating ND and AD residues can be carried out reliably using equations developed for the entire dataset which consisted mainly of ceral grains and protein feeds. These equations are recommended currently for routine analysis of feed ingredients. Specific equations for particular groups of feed ingredients may improve the quality of pcdAA estimation which was exemplarily shown for cereal grains. To avoid bias, however, it appears advisable to use the general equations until specific estimates for other particular groups of feed ingredients have been developed. This was not possible in this study due to limited sample size.

5.6 Conclusion

In vivo determined standardised pcdAA of cereal grains and protein feeds was predicted with high accuracy by chemical procedures. The procedure encompassed the determination of NDIAA on cereal grains and ADIAA on other feedstuffs, mainly protein feeds. This procedure can be used in any laboratory equipped for standard feedstuff analysis, including AA. The hypothesis could thus be maintained that the method developed and established to estimate pcdCP (Schumacher et al., 2025) is similarly suitable to estimate *in vivo* determined pcdAA of cereal grains and protein feeds. However, independent validation of the established regressions was impossible because no independent in vivo data set could be identified. An extension of the database of *in vivo* pcdAA values may yield predictions for specific types of feeds which would aid in further improving the accuracy of the prediction of pcdAA without compromising robustness. The applicability of the method to poultry feeds applying the same rapid laboratory method reported in this study is also conceivable and currently thoroughly examined.

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CRediT authorship contribution statement

Valérie Schumacher: Investigation, Formal analysis, Data curation, Writing – original draft. **Markus Rodehutscord:** Writing – review & editing. **Karl-Heinz Südekum:** Funding

acquisition, Project administration, Conceptualization, Supervision, Writing – review & editing. **Saskia Kehraus:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors have no conflict of interest.

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APPENDIX

Figures

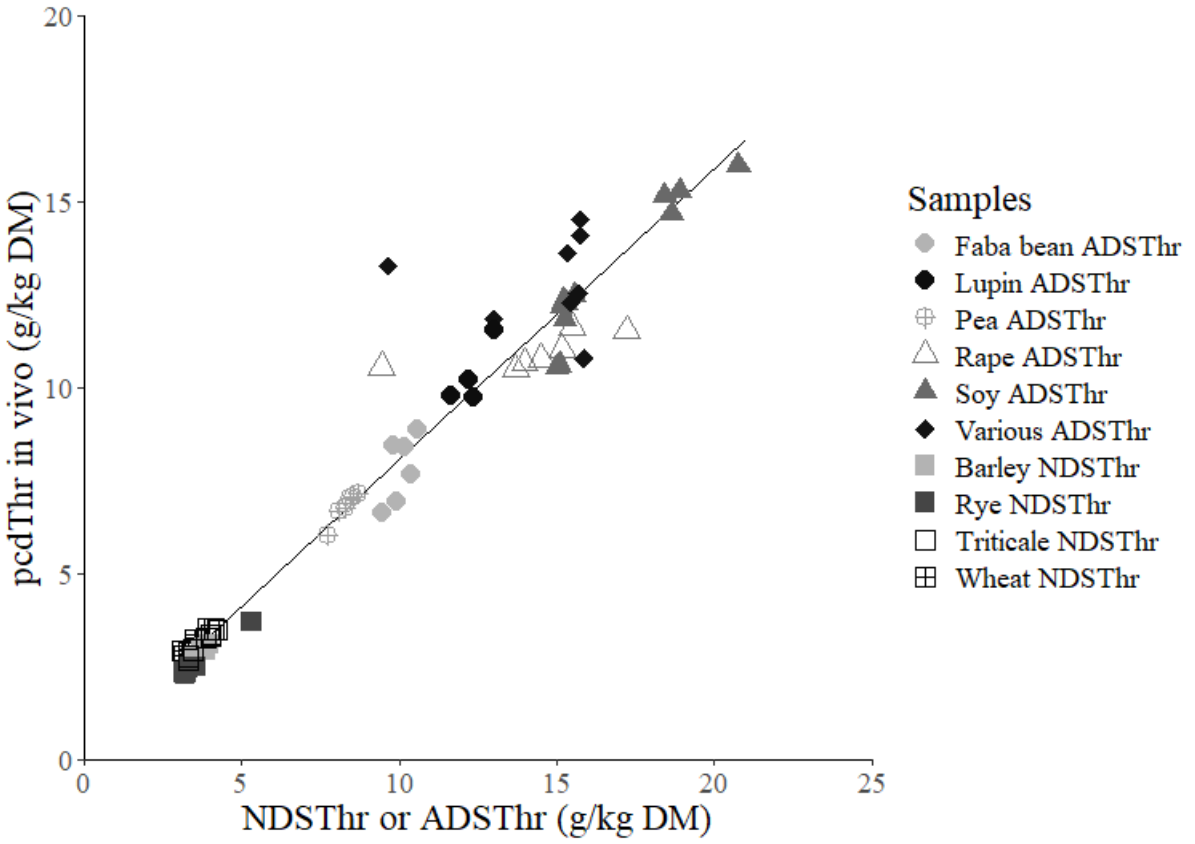

Figure A1: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for threonine (Thr). With the regression equation y = 0.785 (SE 0.021, CI 0.742; 0.827) x + 0.214 (SE 0.227, CI -0.238; 0.667), R^2 = 0.949, RMSE = 0.985.

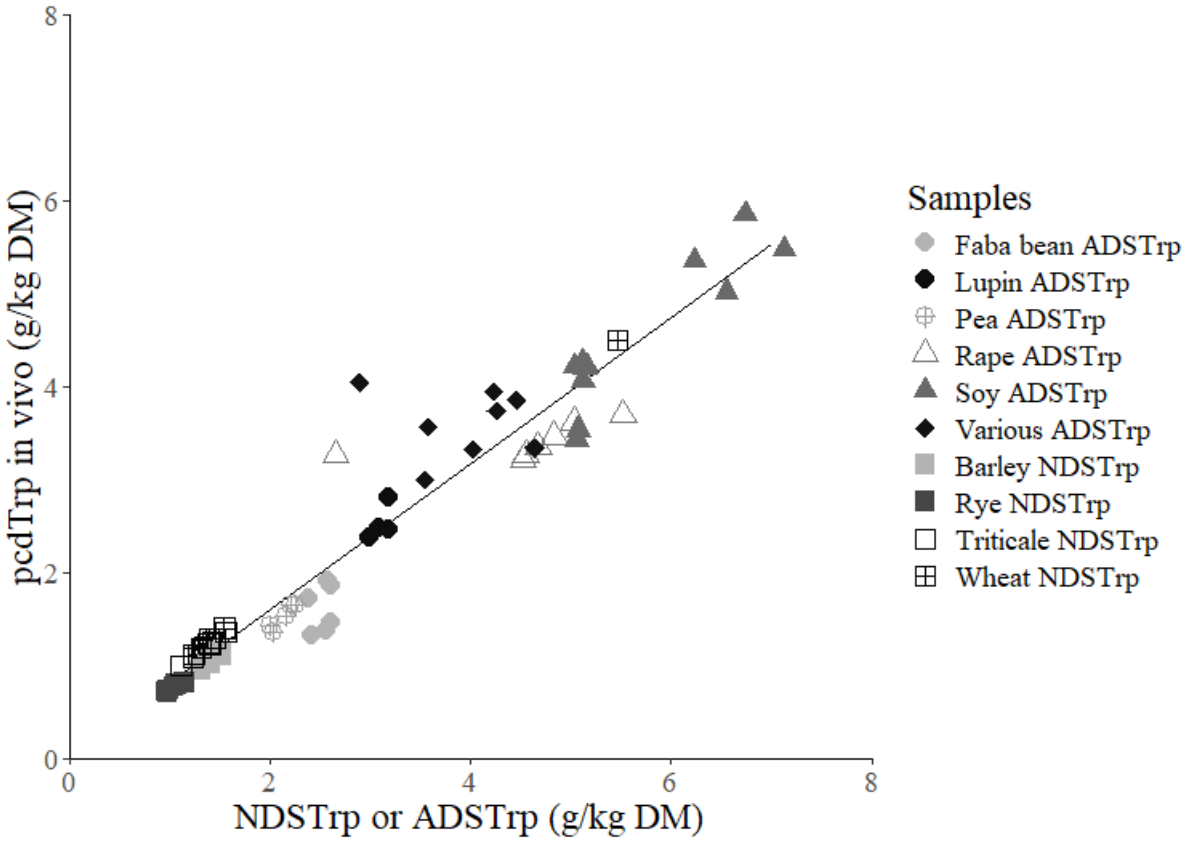

Figure A2: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in* vivo pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for tryptophan (Trp). With the regression equation y = 0.784 (SE 0.025, CI 0.735; 0.834) x + 0.038 (SE 0.083, CI -0.128; 0.204), R^2 = 0.932, RMSE = 0.364.

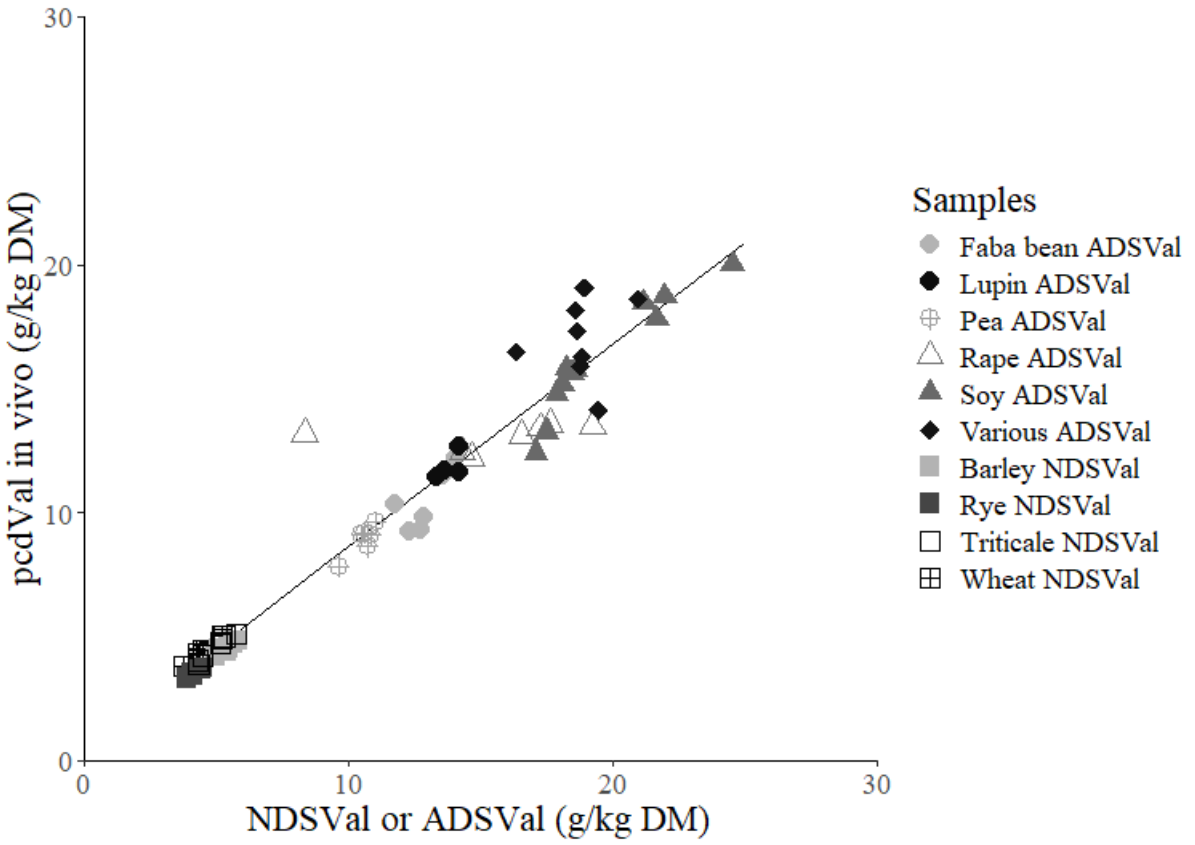

Figure A3: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for valine (Val). With the regression equation y = 0.817 (SE 0.021, CI 0.775; 0.860) x + 0.438 (SE 0.272, CI -0.103; 0.980), R^2 = 0.953, RMSE = 1.132.

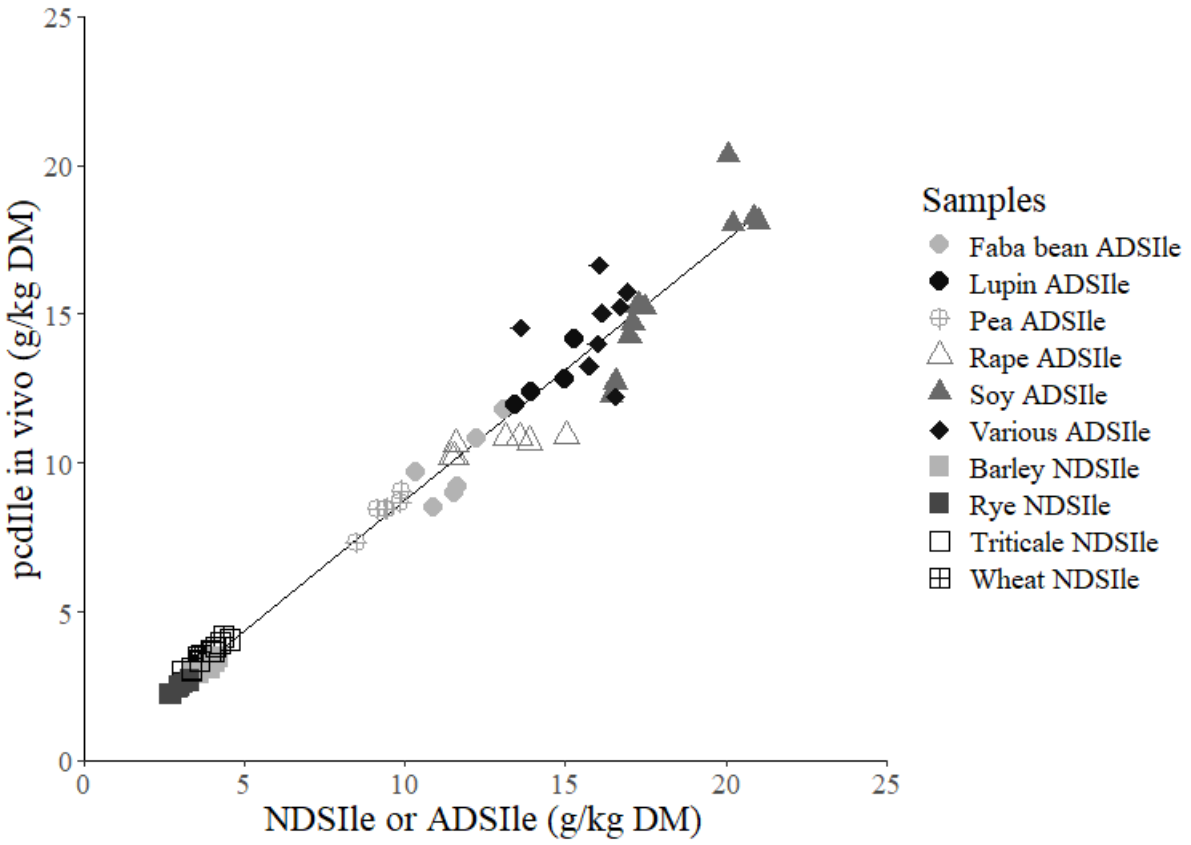

Figure A4: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for isoleucine (Ile). With the regression equation y = 0.873 (SE 0.017, CI 0.840; 0.907) x + 0.011 (SE 0.191, CI -0.365; 0.396), R^2 = 0.974, RMSE = 0.841.

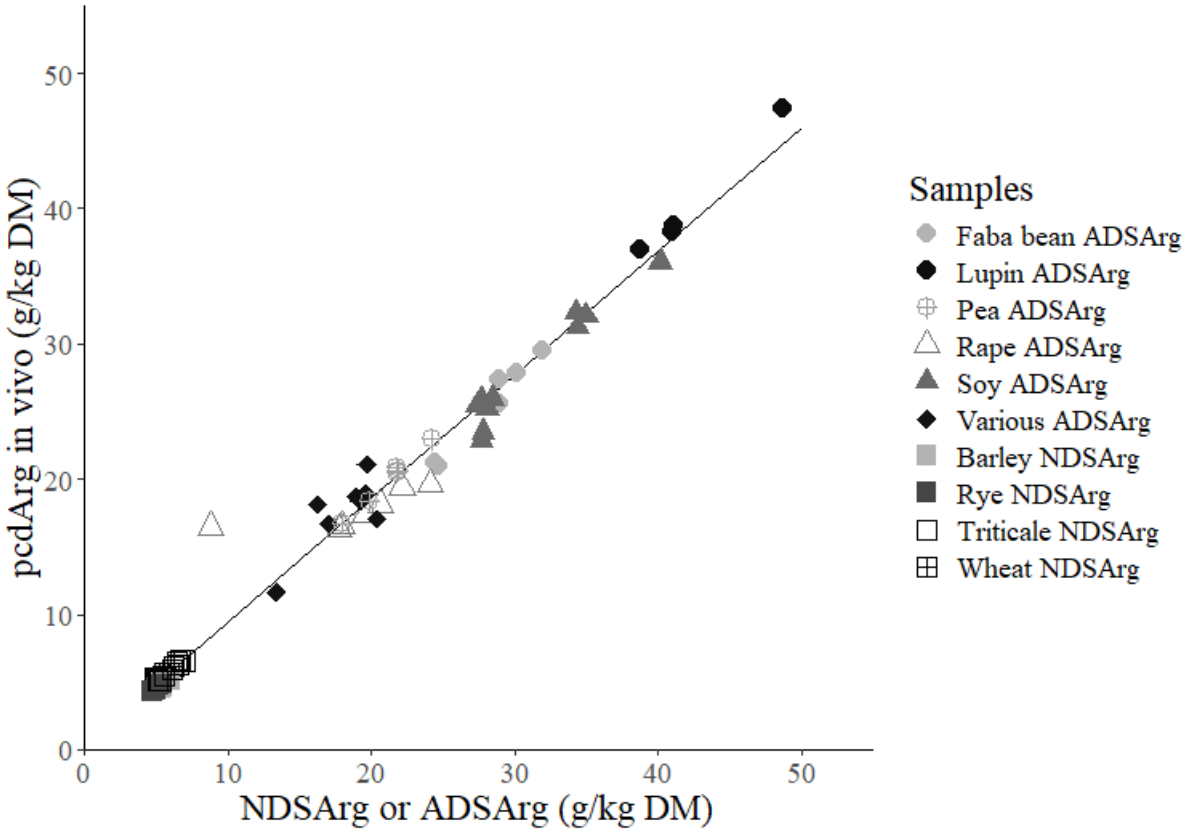

Figure A5: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in* vivo pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for arginine (Arg). With the regression equation y = 0.914 (SE 0.014, CI 0.886; 0.941) x + 0.287 (SE 0.282, CI -2.753; 0.850), R^2 = 0.984, RMSE = 1.363.

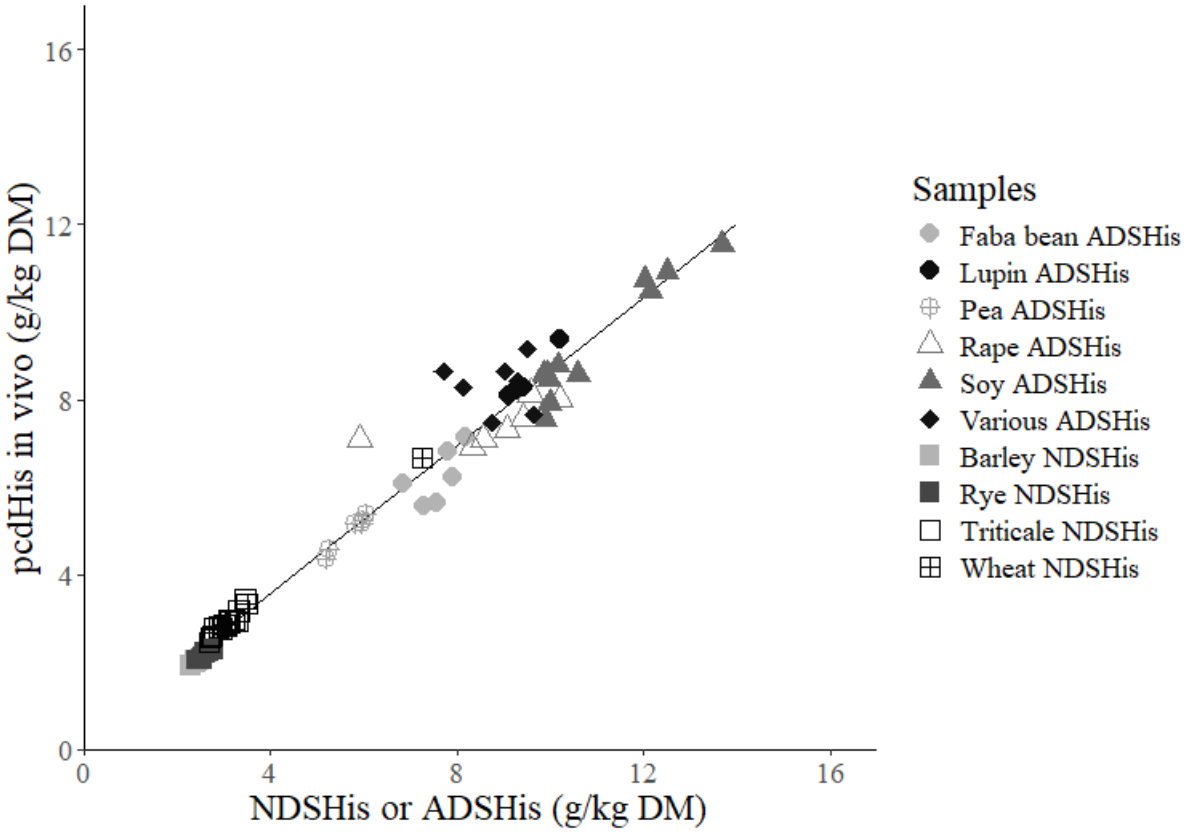

Figure A6: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for histidine (His). With the regression equation y = 0.845 (SE 0.018, CI 0.810; 0.880) x + 0.180 (SE 0.125, CI -0.069; 0.429), R^2 = 0.970, RMSE = 0.496.

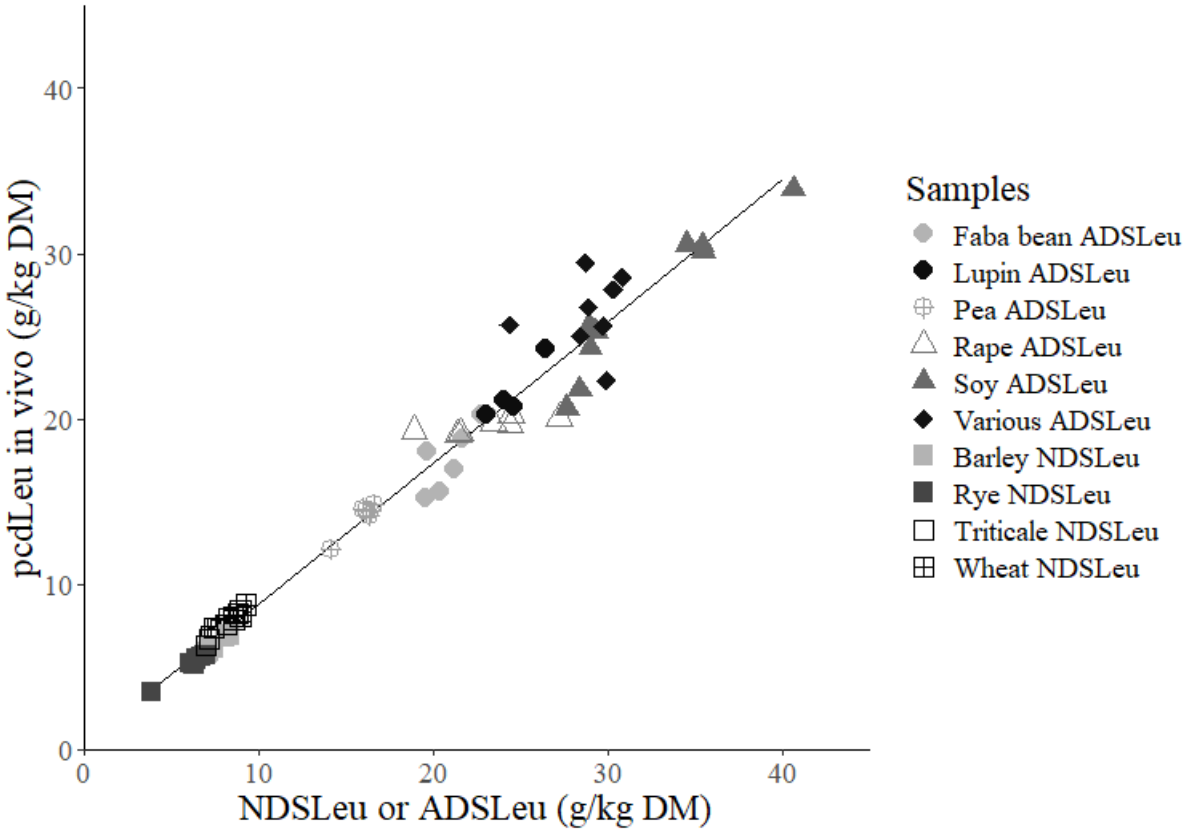

Figure A7: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for leucine (Leu). With the regression equation y = 0.854 (SE 0.016, CI 0.822; 0.885) x + 0.320 (SE 0.317, CI -0.311; 0.952), R^2 = 0.976, RMSE = 1.317.

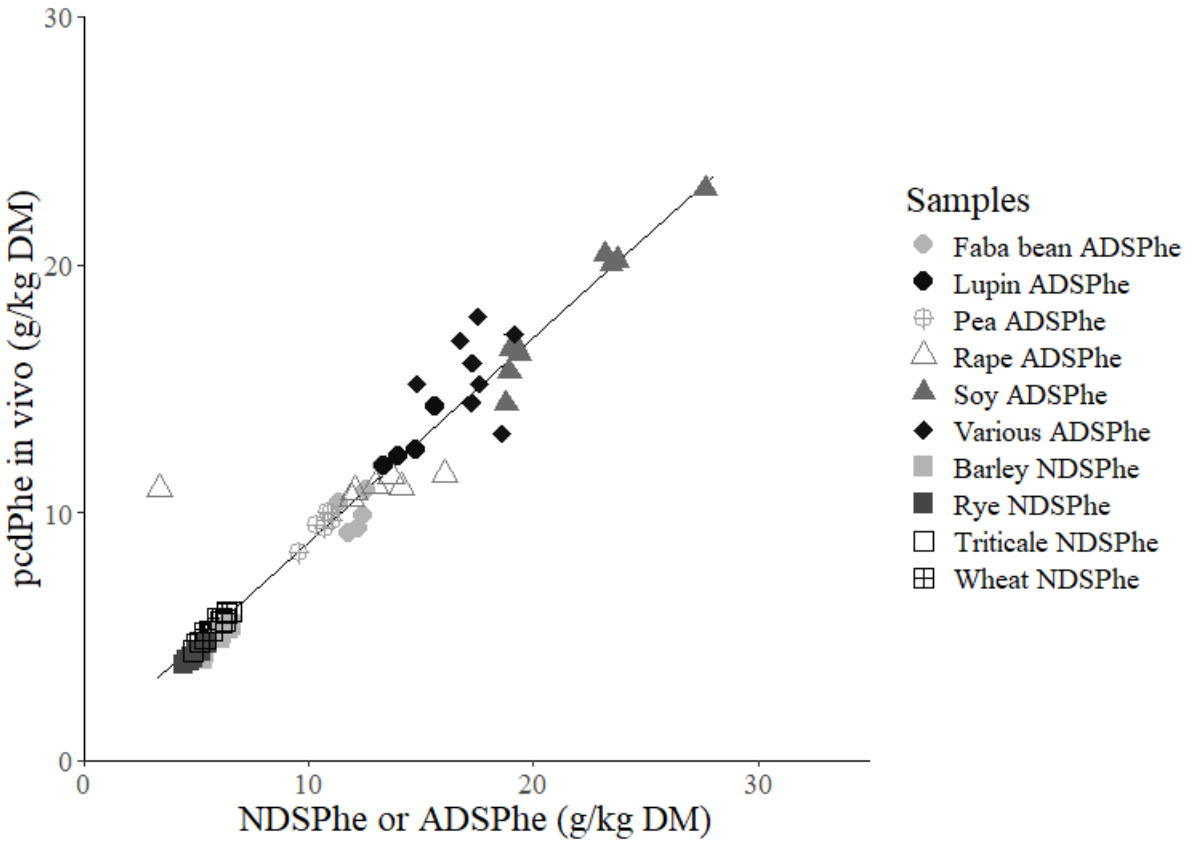

Figure A8: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for phenylalanine (Phe). With the regression equation y = 0.818 (SE 0.024, CI 0.770; 0.866) x + 0.657 (SE 0.305, CI 0.049; 1.265), R^2 = 0.941, RMSE = 1.227.

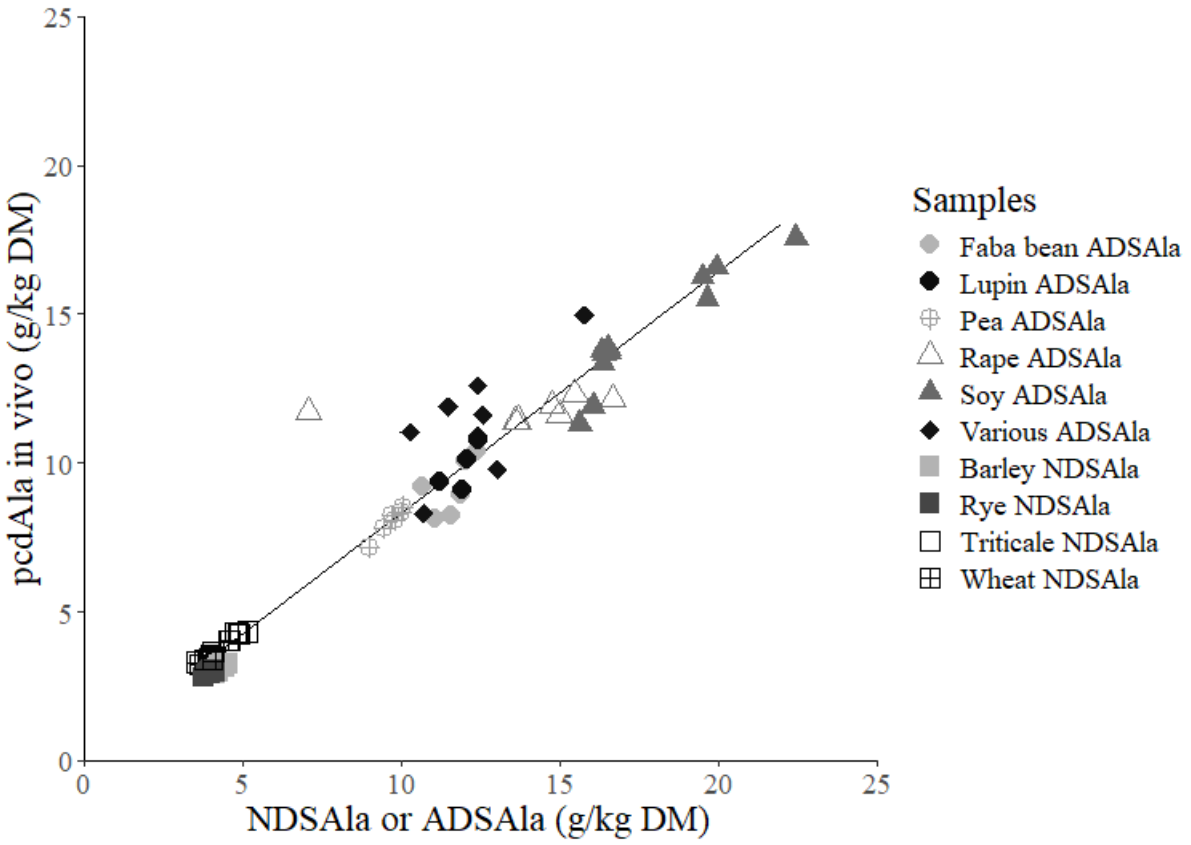

Figure A9: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for alanine (Ala). With the regression equation y = 0.811 (SE 0.023, CI 0.766; 0.857) x + 0.190 (SE 0.247, CI -0.302; 0.683), R^2 = 0.946, RMSE = 1.016.

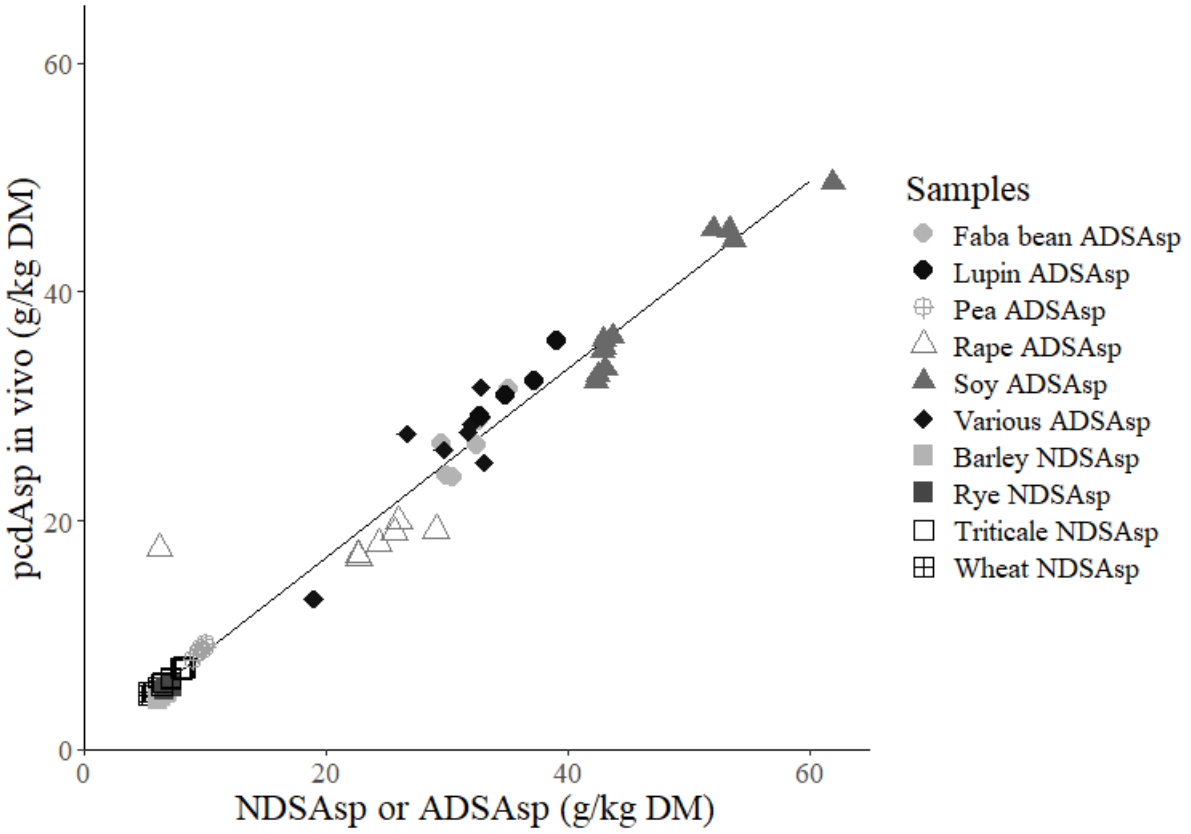

Figure A10: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for aspartic acid (Asp). With the regression equation y = 0.822 (SE 0.016, CI 0.790; 0.855) x + 0.354 (SE 0.420, CI -0.481; 1.190), R^2 = 0.973, RMSE = 2.155.

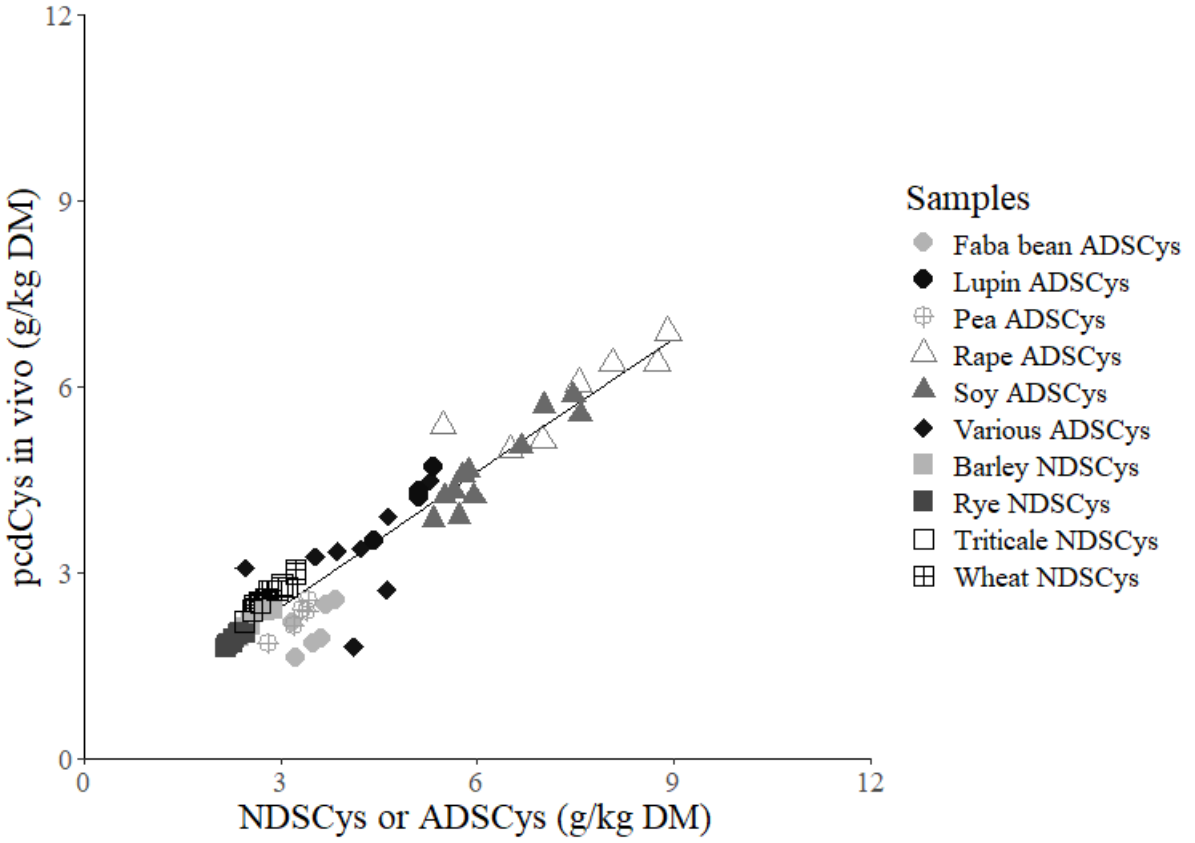

Figure A11: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for cysteine (Cys). With the regression equation y = 0.721 (SE 0.028, CI 0.665; 0.776) x + 0.299 (SE 0.122, CI 0.056; 0.541), R^2 = 0.904, RMSE = 0.417.

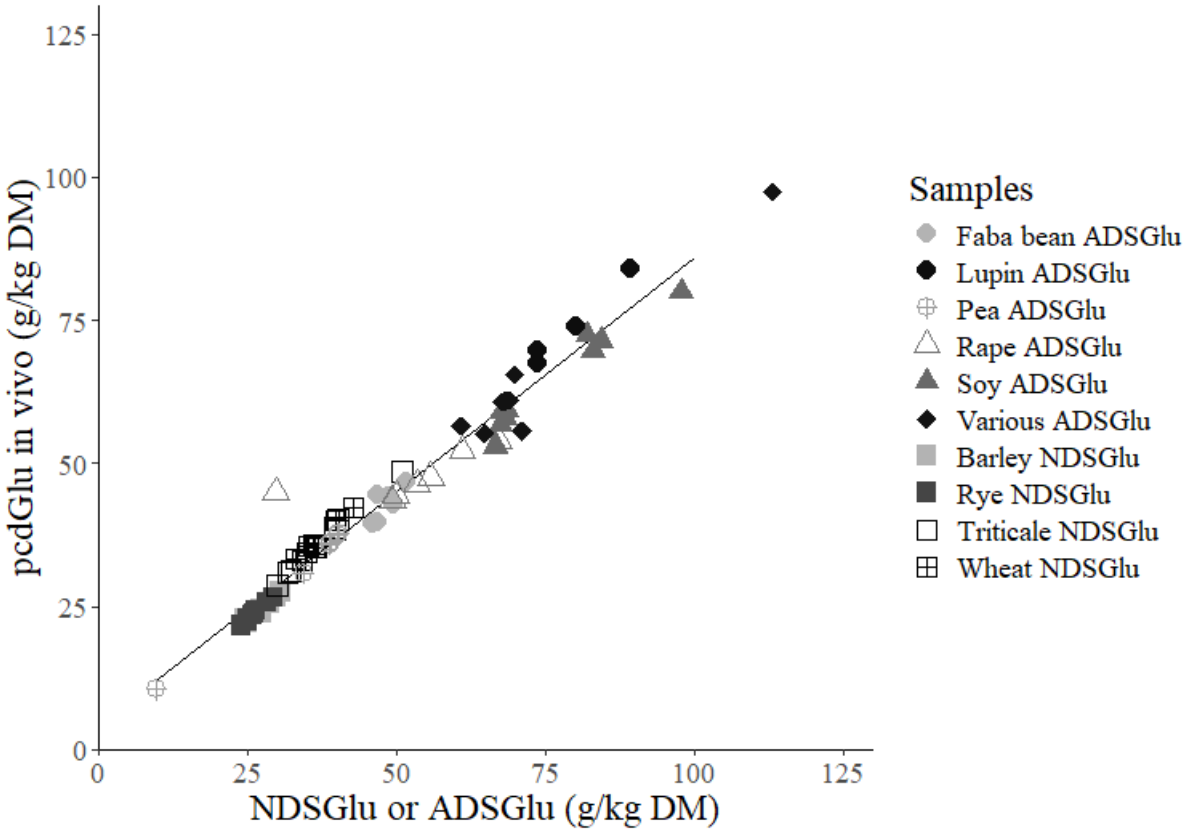

Figure A12: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for glutamic acid (Glu). With the regression equation y = 0.818 (SE 0.018, CI 0.782; 0.853) x + 4.135 (SE 0.947, CI 2.248; 6.022), R^2 = 0.967, RMSE = 3.160.

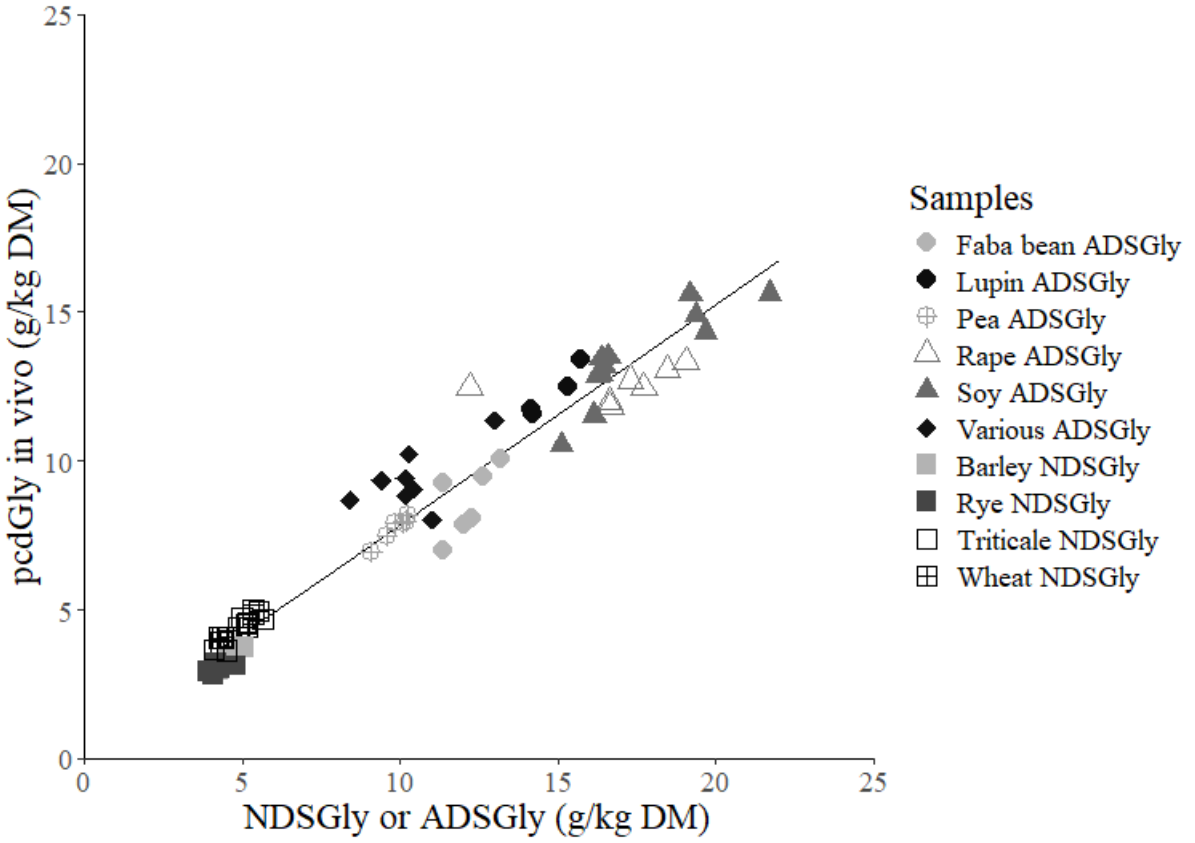

Figure A13: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for glycine (Gly). With the regression equation y = 0.739 (SE 0.019, CI 0.700; 0.777) x + 0.491 (SE 0.218, CI 0.056; 0.925), R^2 = 0.953, RMSE = 0.879.

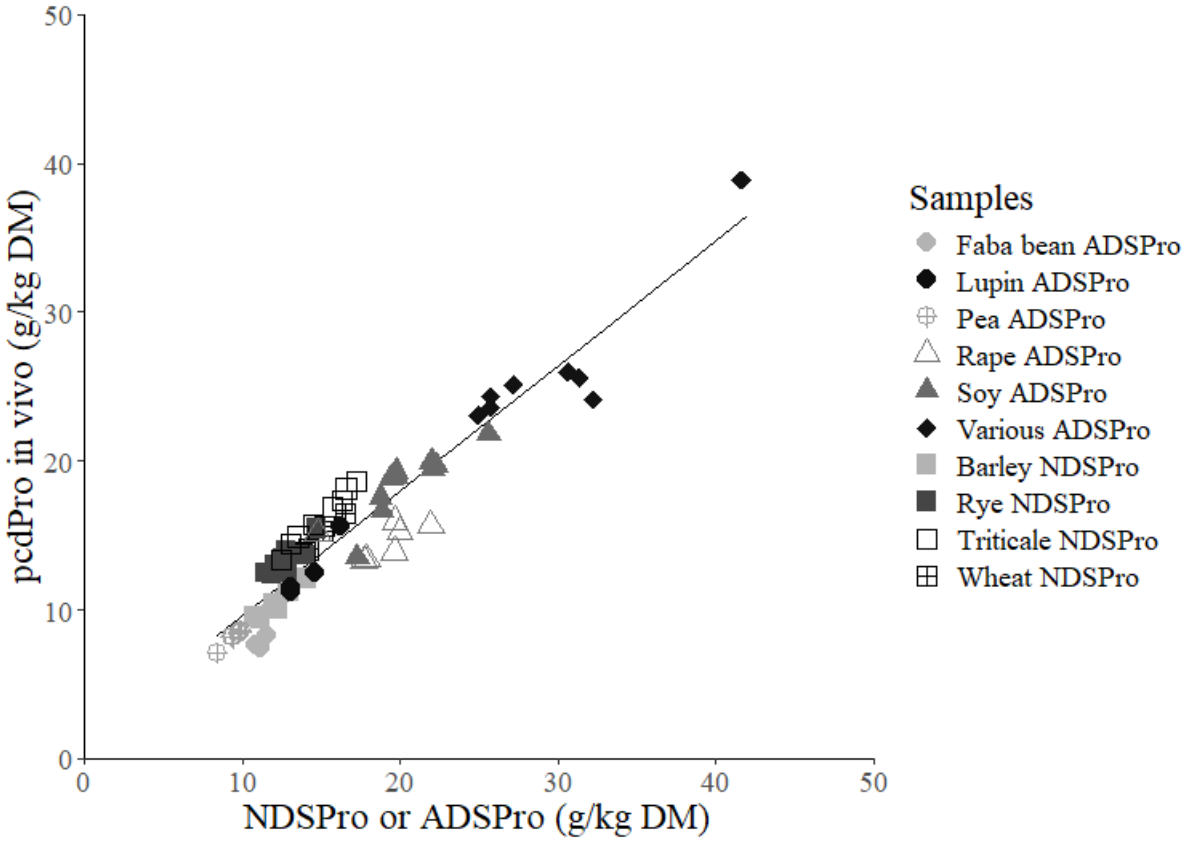

Figure A14: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for proline (Pro). With the regression equation y = 0.840 (SE 0.034, CI 0.772; 0.907) x + 1.187 (SE 0.595, CI 0.000; 2.374), R^2 = 0.895, RMSE = 1.756.

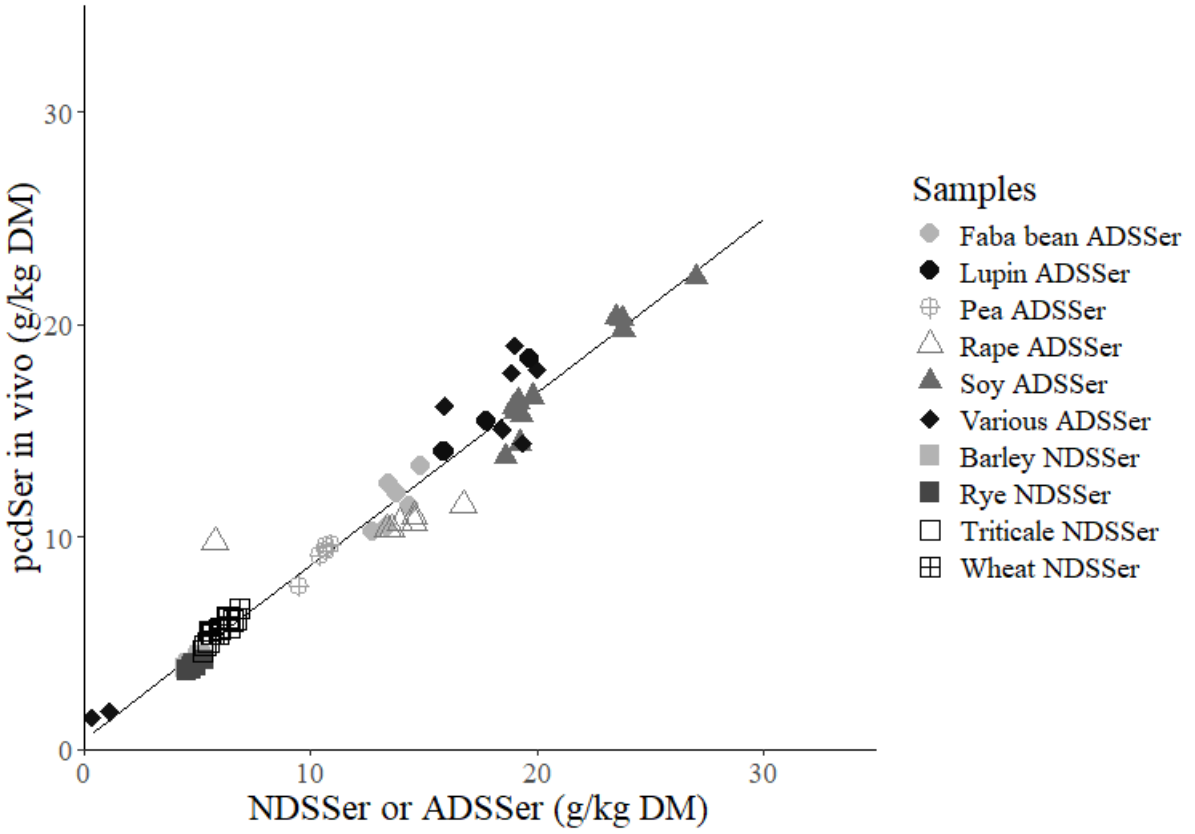

Figure A15: Linear regression of NDSAA and ADSAA data (X) from the laboratory method and *in vivo* pcdAA (Y) for cereal grains and protein feed ingredients from the reference of all 74 samples for serine (Ser). With the regression equation y = 0.813 (SE 0.019, CI 0.775; 0.851) x + 0.533 (SE 0.248, CI 0.039; 1.028), R^2 = 0.961, RMSE = 1.057.

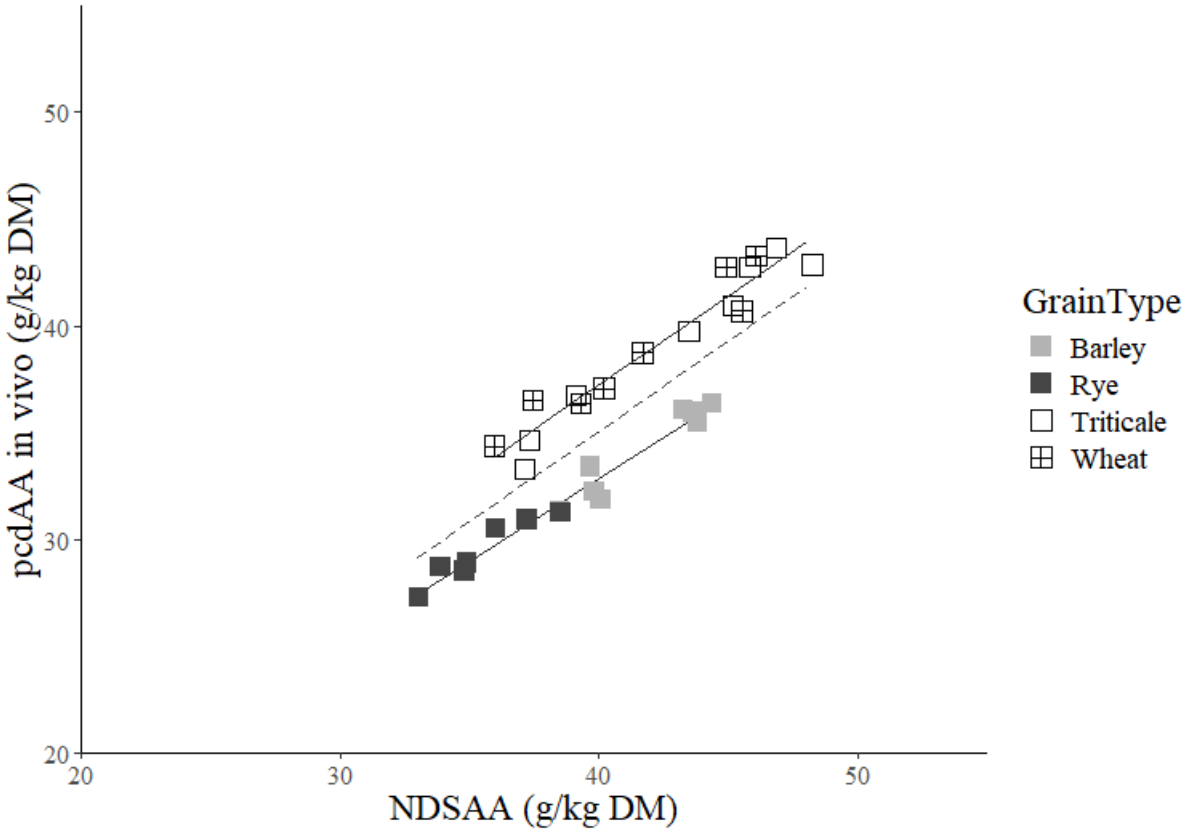

Figure A16: Relationship between NDSAA data from the laboratory method (X) and *in vivo* pcdAA (Y) for cereal grains for wheat, triticale, barley and rye with grouping according to wheat/triticale and barley/rye for 10 indispensable AA. The dashed line shows the regression line over all cereal grain samples. See text and table S8 for details.

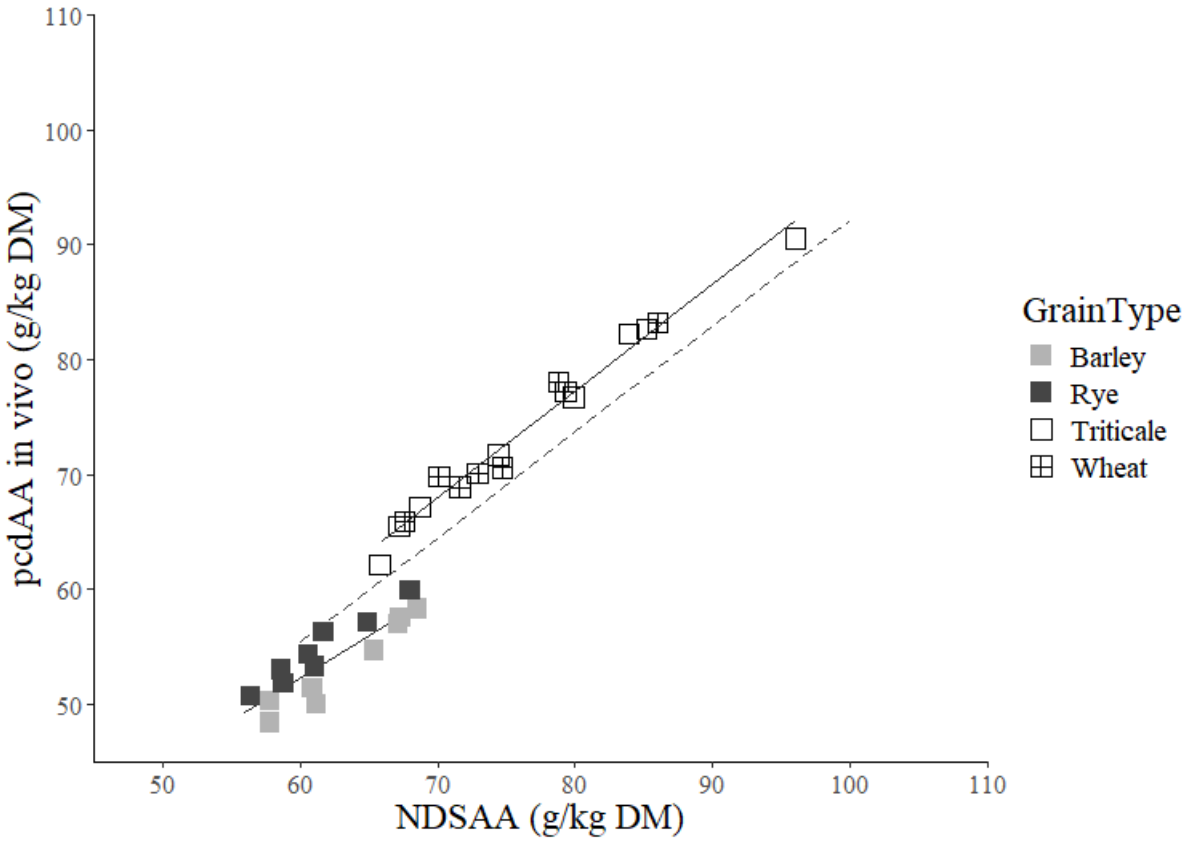

Figure A17: Relationship between NDSAA data from the laboratory method (X) and *in vivo* pcdAA (Y) for cereal grains for wheat, triticale, barley and rye with grouping according to wheat/triticale and barley/rye for 7 dispensable AA. The dashed line shows the regression line over all cereal grain samples. See text and table S8 for details.

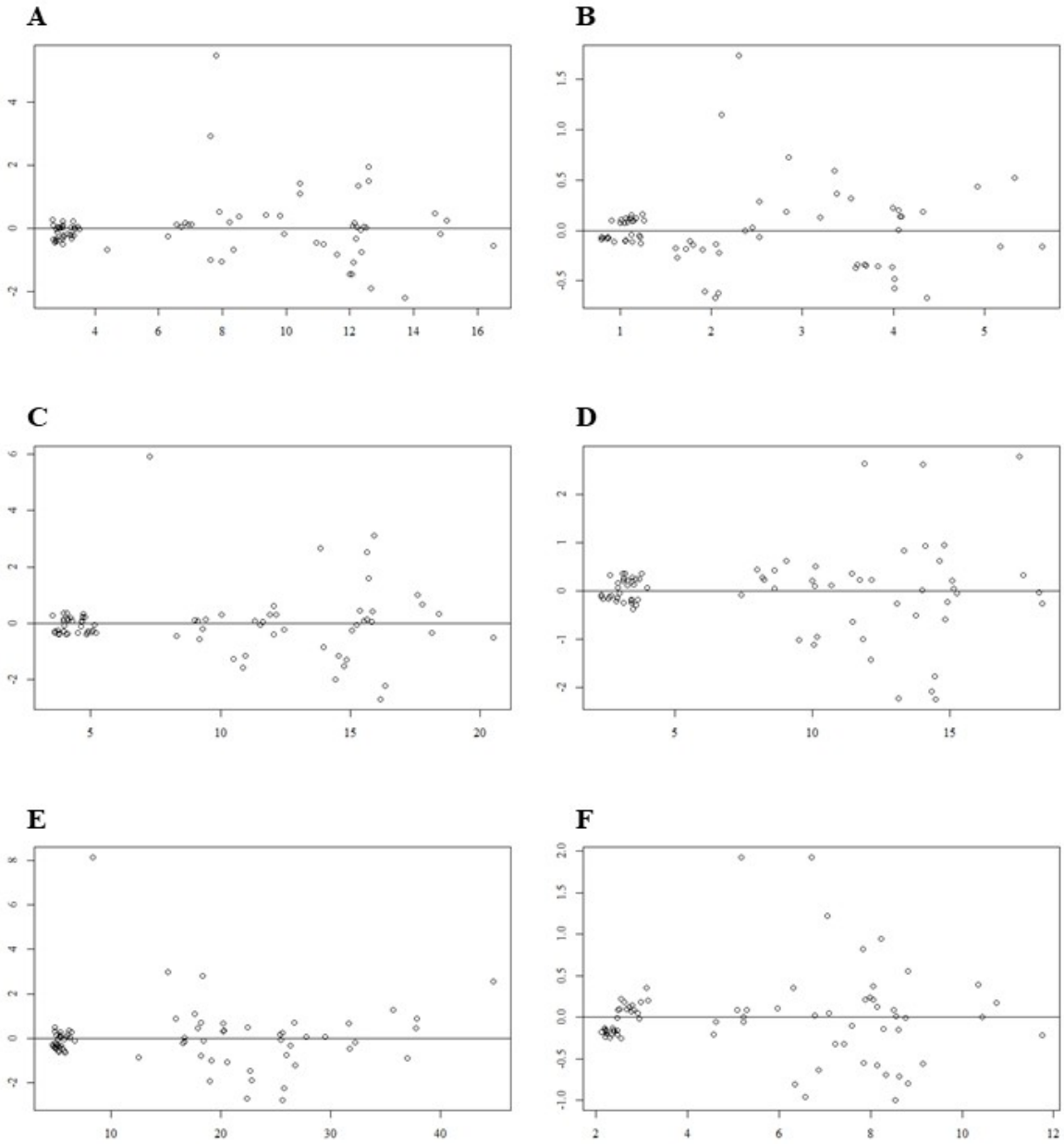

Figure A18: Relationship between residues (y; g/kg DM) and fitted values (x; g/kg DM) of data of all 74 samples for the amino acids threonine (A), tryptophan (B), valine (C), isoleucine (D), arginine (E) and histidine (F).

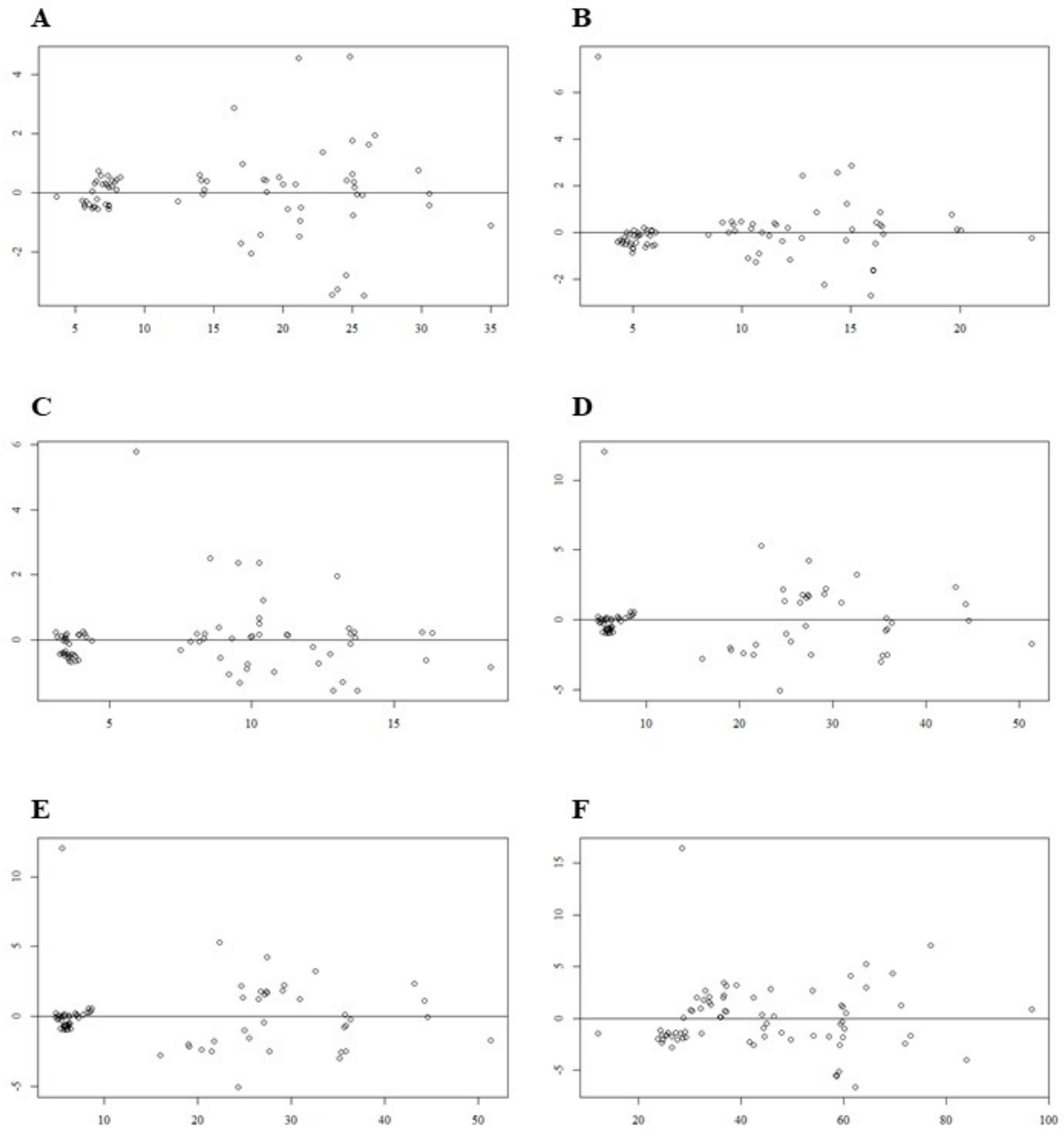

Figure A19: Relationship between residues (y; g/kg DM) and fitted values (x; g/kg DM) of data of all 74 samples for the amino acids leucine (A), phenylalanine (B), alanine (C), aspartic acid (D), cysteine (E) and glutamic acid (F).

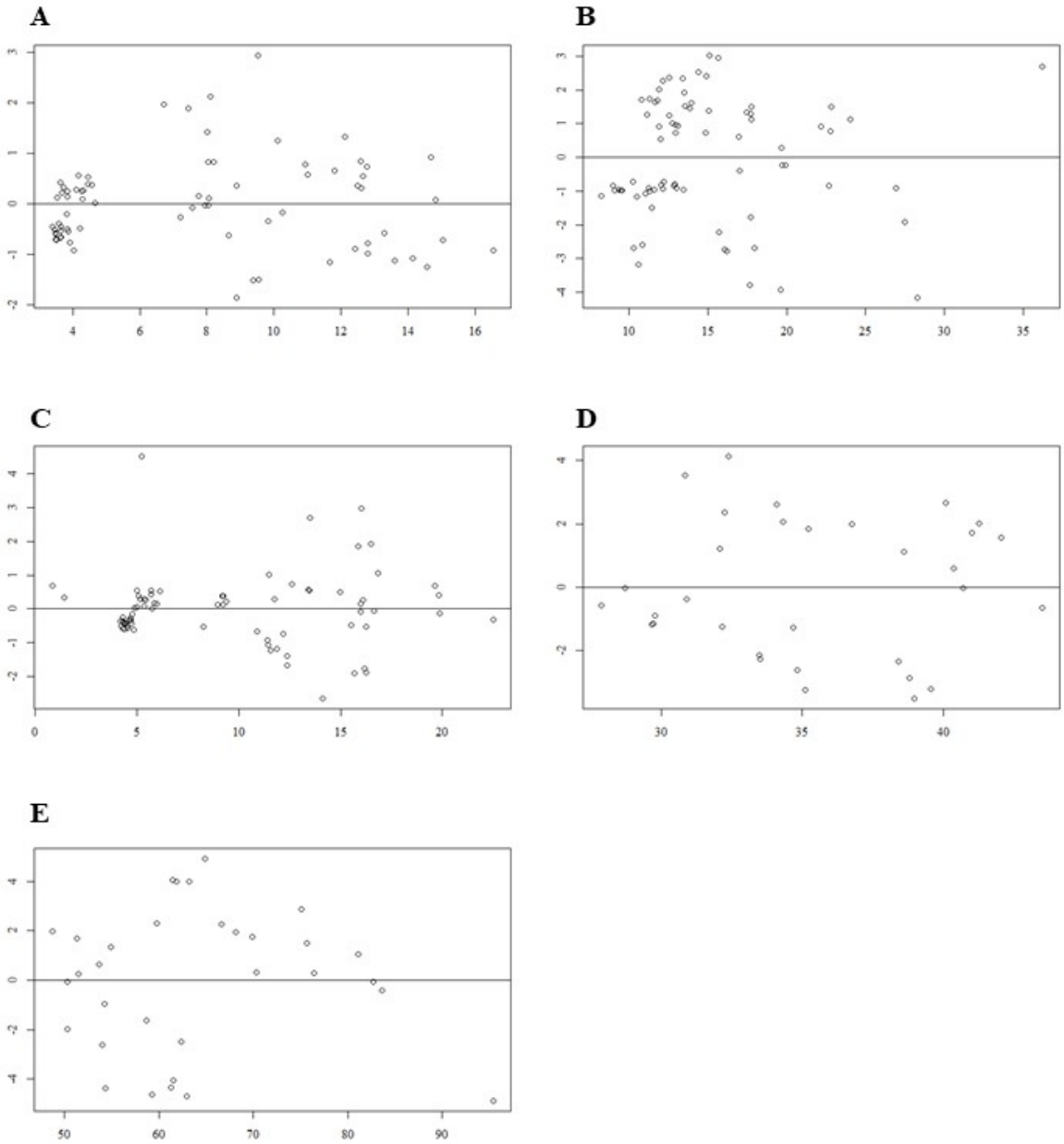

Figure A20: Relationship between residues (y; g/kg DM) and fitted values (x; g/kg DM) of data of all 74 samples for the amino acids glycine (A), proline (B), serin (C) and all cereal grains for indispensable amino acids (D) and dispensable amino acids (E).

Tables

Table A1: The number, sample group, sample, amino acid content (AA; [g/kg DM]), fractional standardised precaecal digestibility of AA (pcDAA; [g/g AA]) and calculated standardised precaecal digestible AA concentration (pcdAA; [g/kg DM]) *in vivo* of the 74 assayed feedstuffs for the indispensable AAs arginine, histidine and isoleucine.

			Arginine			Histidine			Isoleucine		
Number	Sample group	Sample	AA (g/kg DM)	pcDArg	pcdArg *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDHis	pcdHis *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDIso	pcdIso *in vivo* * (g/kg DM)
1	Wheat	Skalmeje	6.27	0.85	5.33	3.21	0.86	2.76	4.05	0.86	3.48
2		Tommi	6.37	0.84	5.35	3.42	0.86	2.94	4.33	0.86	3.72
3		St. Tobak	6.29	0.86	5.41	3.28	0.87	2.85	4.08	0.86	3.51
4		Event	6.40	0.88	5.63	7.57	0.88	6.66	4.03	0.88	3.55
5		Mulan	6.65	0.85	5.65	3.38	0.86	2.91	4.04	0.84	3.39
6		Tabasco	6.01	0.86	5.17	3.25	0.86	2.80	3.46	0.86	2.98
7		Adler	7.18	0.86	6.17	3.83	0.87	3.33	4.74	0.88	4.17
8		KWS Erasmus	6.32	0.85	5.37	3.22	0.86	2.77	3.91	0.87	3.40
9	Triticale	Grenado	6.03	0.84	5.07	2.95	0.83	2.45	3.71	0.81	3.01
10		Tarzan	7.13	0.88	6.27	3.44	0.86	2.96	4.31	0.85	3.66
11		HYT Prime	7.72	0.85	6.56	3.54	0.83	2.94	4.89	0.83	4.06
12		Massimo	6.93	0.86	5.96	3.34	0.85	2.84	4.29	0.85	3.65
13		Cultivo	7.72	0.86	6.64	3.83	0.90	3.45	4.72	0.84	3.96
14		SW Talentro	7.57	0.86	6.51	3.73	0.85	3.17	4.61	0.83	3.83
15		Cando	6.04	0.85	5.13	3.01	0.85	2.56	3.74	0.83	3.10
16		Agostino	6.61	0.83	5.49	3.13	0.83	2.60	4.04	0.83	3.35

17	Rye	Conduct	5.95	0.79	4.70	2.89	0.77	2.23	3.52	0.74	2.60
18		Visello	5.96	0.78	4.65	2.94	0.75	2.21	3.11	0.72	2.24
19		Helltop	6.11	0.78	4.77	2.93	0.76	2.23	3.72	0.74	2.75
20		Bellami	5.93	0.75	4.45	3.04	0.74	2.25	3.18	0.70	2.23
21		Palazzo	5.84	0.76	4.44	2.83	0.73	2.07	3.39	0.72	2.44
22		Dukato	5.74	0.76	4.36	2.88	0.74	2.13	3.42	0.73	2.50
23		Guttino	5.68	0.76	4.32	2.78	0.74	2.06	3.11	0.71	2.21
24		Dankowski Diament	6.34	0.76	4.82	3.13	0.73	2.28	3.74	0.71	2.66
25	Barley	Yool	5.70	0.79	4.50	2.50	0.77	1.93	3.90	0.74	2.89
26		Ack 2927	6.10	0.80	4.88	2.60	0.79	2.05	3.80	0.78	2.96
27		Lomerit	5.80	0.78	4.52	2.60	0.78	2.03	4.20	0.77	3.23
28		Campanille	6.00	0.77	4.62	2.60	0.76	1.98	4.30	0.72	3.10
29		Canberra	6.50	0.79	5.14	2.60	0.78	2.03	4.20	0.77	3.23
30		Antisette	6.20	0.82	5.08	2.60	0.79	2.05	4.50	0.77	3.47
31		Metaxa	6.30	0.81	5.10	2.60	0.79	2.05	4.20	0.76	3.19
32		Fridericus	6.50	0.79	5.14	2.90	0.79	2.29	4.40	0.75	3.30
33	Pea	Santana	22.8	0.90	20.5	6.40	0.84	5.38	10.8	0.84	9.07
34		Jutta	25.2	0.91	22.9	6.30	0.82	5.17	10.2	0.83	8.47
35		Phönix	20.8	0.88	18.3	6.30	0.83	5.23	10.7	0.81	8.67
36		Harnas	23.2	0.90	20.9	6.30	0.82	5.17	10.3	0.82	8.45
37		Rocket	23.2	0.89	20.7	5.70	0.80	4.56	10.6	0.80	8.48
38		Hardy	19.0	0.88	16.7	5.60	0.78	4.37	9.40	0.78	7.33
39	Lupin	Probor	48.8	0.97	47.3	10.3	0.91	9.37	15.4	0.92	14.2
40		Boregine	38.9	0.95	37.0	9.20	0.88	8.10	13.6	0.88	12.0
41		Boruta	41.2	0.94	38.7	9.40	0.88	8.27	14.1	0.88	12.4
42		Idefix	41.1	0.93	38.2	9.50	0.87	8.27	15.1	0.85	12.8
43	Faba	Aurelia	30.6	0.91	27.9	8.00	0.85	6.80	12.7	0.85	10.8
44	bean	Divine	30.8	0.89	27.4	7.50	0.81	6.08	11.8	0.82	9.68
45		Gloria	32.8	0.90	29.5	8.50	0.84	7.14	13.9	0.85	11.8
46		Limbo	29.7	0.86	25.5	8.20	0.76	6.23	12.3	0.75	9.23
47		Fuego	24.9	0.84	20.9	7.70	0.73	5.62	11.8	0.76	8.97
48		Espresso	25.2	0.84	21.2	7.60	0.73	5.55	11.5	0.74	8.51

51	Full-fat soybean/ soybean product	FFSB K2	28.6	0.82	23.5	10.3	0.77	7.92	17.4	0.73	12.7
52		FFSB K3	28.6	0.88	25.2	10.2	0.83	8.47	17.6	0.81	14.3
53		FFSB Z1	28.8	0.90	25.9	10.3	0.85	8.76	17.5	0.84	14.7
54		FFSB Z2	28.1	0.92	25.9	10.1	0.85	8.58	17.9	0.85	15.2
55		FFSB Z3	27.7	0.92	25.5	10.0	0.86	8.60	17.6	0.87	15.3
56		FFSB Z4	27.9	0.91	25.4	10.2	0.84	8.58	17.7	0.86	15.2
58		FFSB (roasted)	28.5	0.80	22.8	10.2	0.74	7.55	17.3	0.71	12.3
59		SBC	34.7	0.93	32.3	12.2	0.88	10.7	20.7	0.87	18.0
60		SBM (Austria)	34.7	0.90	31.2	12.7	0.86	10.9	21.2	0.86	18.2
61		SBM (GMO-free)	40.5	0.89	36.1	13.9	0.83	11.5	24.5	0.83	20.3
62		SBM (standard)	35.2	0.91	32.0	12.3	0.85	10.5	21.3	0.85	18.1
63	Rapeseed meal and cake	RSM48	22.1	0.82	18.1	10.1	0.75	7.58	15.3	0.71	10.9
64		RSM64	21.5	0.81	17.4	10.0	0.73	7.30	15.3	0.71	10.9
65		RSM76	20.6	0.80	16.5	10.0	0.71	7.10	15.2	0.70	10.6
66		RSM93	20.6	0.79	16.3	10.0	0.69	6.90	15.0	0.68	10.2
67		LOW-GLS RSM	20.9	0.79	16.5	10.0	0.71	7.10	14.6	0.70	10.2
68		RSC	22.6	0.86	19.4	9.80	0.83	8.13	14.5	0.74	10.7
69		RSM	24.5	0.80	19.6	10.4	0.77	8.01	15.6	0.70	10.9
70	Various samples	Soybean (extruded)	20.8	0.82	17.1	9.80	0.78	7.64	17.0	0.72	12.2
71		SBM (high protein)	20.6	0.89	18.3	9.80	0.86	8.43	17.3	0.81	14.0
72		SPC (A coarse)	19.7	0.95	18.7	9.50	0.91	8.65	17.1	0.88	15.1
73		SPC (A fine)	19.5	0.93	18.1	9.30	0.89	8.28	16.7	0.87	14.5
74		SPC (B coarse)	22.0	0.96	21.1	10.3	0.89	9.17	18.5	0.90	16.7
75		SPC (B fine)	20.1	0.94	18.9	9.70	0.89	8.63	17.5	0.90	15.8
79		WG2 (hydrolysed)	13.4	0.87	11.7	8.80	0.85	7.48	15.8	0.84	13.3
82		FM2 (extracted)	18.8	0.89	16.7	9.90	0.83	8.22	18.6	0.82	15.3

FFSB, Full-fat soybean; FM, Fish meal; GLS, Glucosinolates; PeaP, Pea protein; RSC, Rapeseed cake; RSM, Rapeseed meal; SBC, Soybean cake; SBM, Soybean meal; SPC, Soy protein concentrate; SPI, Soy protein isolate; WG, Wheat gluten, / no pre-treatment; The samples 49, 50, 57, 76, 77, 78, 80 and 81 were not used for the determination of pcdAA.

*The precaecal digestible AA was calculated from AA content and pcDAA reported in the particular reference.

Table A2: The number, amino acid content (AA; [g/kg DM]), fractional standardised precaecal digestibility of AA (pcDAA; [g/g AA]) and calculated standardised precaecal digestible AA concentration (pcdAA; [g/kg DM]) *in vivo* of the 74 assayed feedstuffs for the indispensable amino acids leucine, lysine and methionine.

	Leucine			Lysine			Methionine		
Number	AA (g/kg DM)	pcDLeu	pcdLeu *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDLys	pcdLys *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDMet	pcdMet *in vivo* * (g/kg DM)
1	8.60	0.86	7.40	3.50	0.71	2.49	1.86	0.85	1.58
2	9.35	0.86	8.04	3.60	0.69	2.48	2.02	0.84	1.70
3	8.72	0.86	7.50	3.60	0.69	2.48	1.95	0.85	1.66
4	9.01	0.88	7.93	3.55	0.73	2.59	1.96	0.87	1.71
5	8.90	0.85	7.57	3.76	0.69	2.59	1.92	0.85	1.63
6	8.07	0.85	6.86	3.43	0.71	2.44	1.86	0.86	1.60
7	10.1	0.87	8.76	4.01	0.73	2.93	2.25	0.88	1.98
8	8.44	0.86	7.26	3.59	0.74	2.66	1.87	0.86	1.61
9	7.59	0.83	6.30	3.86	0.72	2.78	1.92	0.84	1.61
10	9.12	0.86	7.84	4.46	0.77	3.43	2.21	0.87	1.92
11	9.57	0.84	8.04	4.87	0.74	3.60	2.40	0.85	2.04
12	8.89	0.85	7.56	4.46	0.75	3.35	2.22	0.86	1.91
13	9.86	0.85	8.38	4.70	0.73	3.43	2.39	0.85	2.03
14	9.80	0.84	8.23	4.74	0.75	3.56	2.31	0.85	1.96
15	7.89	0.85	6.71	3.77	0.73	2.75	1.96	0.85	1.67
16	8.31	0.89	7.40	4.20	0.73	3.07	2.04	0.84	1.71
17	7.22	0.76	5.49	4.23	0.65	2.75	1.82	0.78	1.42
18	6.96	0.75	5.22	4.11	0.64	2.63	1.71	0.75	1.28
19	7.48	0.75	5.61	4.25	0.64	2.72	1.81	0.76	1.38
20	7.12	0.73	5.20	4.13	0.61	2.52	1.73	0.75	1.30

21	7.05	0.73	5.15	4.07	0.62	2.52	1.81	0.74	1.34
22	7.09	0.74	5.25	4.16	0.63	2.62	1.80	0.75	1.35
23	4.74	0.73	3.46	3.94	0.60	2.36	1.71	0.75	1.28
24	7.80	0.73	5.69	4.43	0.60	2.66	1.93	0.75	1.45
25	7.70	0.76	5.85	4.00	0.64	2.56	1.80	0.76	1.37
26	8.00	0.79	6.32	4.50	0.65	2.93	1.80	0.79	1.42
27	7.70	0.77	5.93	4.00	0.61	2.44	1.90	0.77	1.46
28	8.10	0.75	6.08	4.10	0.62	2.54	1.90	0.74	1.41
29	8.90	0.77	6.85	4.50	0.63	2.84	2.10	0.78	1.64
30	8.70	0.78	6.79	4.10	0.66	2.71	2.10	0.78	1.64
31	8.90	0.78	6.94	4.30	0.65	2.80	2.10	0.78	1.64
32	8.90	0.78	6.94	4.40	0.64	2.82	2.10	0.77	1.62
33	18.1	0.82	14.8	18.5	0.87	16.1	2.30	0.79	1.82
34	17.8	0.81	14.4	17.8	0.85	15.1	2.30	0.79	1.82
35	17.7	0.80	14.2	18.1	0.85	15.4	2.20	0.75	1.65
36	18.0	0.81	14.6	18.1	0.85	15.4	2.30	0.77	1.77
37	18.1	0.80	14.5	18.5	0.84	15.5	2.20	0.75	1.65
38	15.7	0.77	12.1	16.3	0.83	13.5	2.20	0.74	1.63
39	26.6	0.91	24.2	17.6	0.90	15.8	2.00	0.84	1.68
40	23.3	0.87	20.3	16.1	0.88	14.2	2.10	0.82	1.72
41	24.3	0.87	21.1	16.5	0.86	14.2	2.10	0.82	1.72
42	24.7	0.84	20.8	17.3	0.83	14.4	2.10	0.71	1.49
43	22.4	0.84	18.8	18.9	0.87	16.4	2.00	0.77	1.54
44	22.0	0.82	18.0	18.5	0.84	15.5	2.00	0.68	1.36
45	24.1	0.84	20.2	20.7	0.84	17.4	2.20	0.77	1.69
46	22.3	0.76	17.0	19.3	0.80	15.4	2.00	0.61	1.22
47	20.8	0.75	15.6	18.5	0.79	14.6	2.00	0.62	1.24
48	20.6	0.74	15.2	18.3	0.79	14.5	1.90	0.58	1.10
51	29.8	0.73	21.8	24.6	0.77	18.9	5.52	0.75	4.14
52	30.0	0.81	24.3	24.6	0.84	20.7	5.52	0.82	4.53
53	30.1	0.84	25.3	24.3	0.85	20.7	5.61	0.85	4.77
54	29.8	0.85	25.3	23.8	0.85	20.2	5.59	0.85	4.75

55	29.4	0.87	25.6	23.3	0.85	19.8	5.60	0.87	4.87
56	29.6	0.86	25.5	23.2	0.82	19.0	5.51	0.86	4.74
58	29.1	0.71	20.7	24.4	0.75	18.3	5.60	0.74	4.14
59	35.1	0.87	30.5	29.0	0.88	25.5	6.60	0.89	5.87
60	35.9	0.85	30.5	29.8	0.85	25.3	6.90	0.89	6.14
61	41.3	0.82	33.9	32.7	0.80	26.2	7.02	0.87	6.11
62	35.9	0.84	30.1	29.3	0.84	24.6	6.50	0.86	5.59
63	27.0	0.75	20.3	19.5	0.64	12.5	7.60	0.84	6.38
64	26.7	0.74	19.8	18.8	0.62	11.7	7.40	0.82	6.07
65	26.8	0.72	19.3	17.7	0.59	10.4	7.60	0.80	6.08
66	26.8	0.71	19.0	17.2	0.54	9.29	7.04	0.79	5.56
67	26.6	0.72	19.2	17.2	0.55	9.46	7.40	0.81	5.99
68	25.2	0.78	19.7	21.6	0.77	16.6	7.20	0.86	6.19
69	27.9	0.72	20.1	21.8	0.69	15.0	8.10	0.82	6.64
70	30.6	0.73	22.3	25.3	0.78	25.3	8.30	0.76	6.31
71	30.5	0.82	25.0	25.1	0.84	25.1	8.00	0.87	6.96
72	30.4	0.88	26.8	23.3	0.91	23.3	7.80	0.90	7.02
73	29.5	0.87	25.7	24.3	0.89	24.3	7.60	0.89	6.76
74	32.7	0.90	29.4	26.8	0.91	26.8	8.40	0.91	7.64
75	30.9	0.90	27.8	25.3	0.87	25.3	8.10	0.92	7.45
79	29.8	0.86	25.6	22.7	0.58	22.7	8.30	0.87	7.22
82	33.6	0.85	28.6	31.0	0.86	31.0	11.4	0.89	10.2

*The precaecal digestible AA was calculated by AA content and pcDAA given in reference

Table A3: The number, amino acid content (AA; [g/kg DM]), fractional standardised precaecal digestibility of AA (pcDAA; [g/g AA]) and calculated standardised precaecal digestible AA concentration (pcdAA; [g/kg DM]) *in vivo* of the 74 assayed feedstuffs for the indispensable amino acids phenylalanine, threonine and tryptophan.

	Phenylalanine			Threonine			Tryptophan		
Number	AA (g/kg DM)	pcDPhe	pcdPhe *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDThr	pcdThr *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDTrp	pcdTrp *in vivo* * (g/kg DM)
1	5.97	0.86	5.13	3.66	0.80	2.93	1.38	0.81	1.12
2	6.62	0.87	5.76	3.94	0.78	3.07	1.56	0.80	1.25
3	6.02	0.87	5.24	3.73	0.78	2.91	1.50	0.83	1.25
4	6.43	0.89	5.72	3.91	0.82	3.21	1.51	0.85	1.28
5	5.96	0.85	5.07	3.83	0.78	2.99	5.56	0.81	4.50
6	5.49	0.86	4.72	3.60	0.78	2.81	1.49	0.81	1.21
7	6.86	0.87	5.97	4.36	0.81	3.53	1.69	0.83	1.40
8	6.01	0.86	5.17	3.62	0.79	2.86	1.44	0.81	1.17
9	5.32	0.83	4.42	3.68	0.73	2.69	1.35	0.80	1.08
10	6.50	0.86	5.59	4.33	0.77	3.33	1.50	0.82	1.23
11	6.73	0.84	5.65	4.59	0.76	3.49	1.68	0.81	1.36
12	6.14	0.85	5.22	4.25	0.77	3.27	1.50	0.83	1.25
13	7.16	0.84	6.01	4.68	0.75	3.51	1.62	0.80	1.30
14	6.97	0.85	5.92	4.55	0.74	3.37	1.48	0.80	1.18
15	5.60	0.85	4.76	3.70	0.75	2.78	1.38	0.81	1.12
16	5.94	0.83	4.93	3.93	0.75	2.95	1.27	0.79	1.00
17	5.49	0.80	4.39	3.76	0.66	2.48	1.19	0.67	0.80
18	5.20	0.78	4.06	3.67	0.64	2.35	1.13	0.65	0.73
19	5.74	0.77	4.42	3.83	0.65	2.49	1.21	0.66	0.80
20	5.42	0.76	4.12	3.69	0.62	2.29	1.12	0.63	0.71

21	5.23	0.77	4.03	3.72	0.64	2.38	1.14	0.65	0.74
22	5.34	0.78	4.17	3.72	0.63	2.34	1.19	0.65	0.77
23	4.99	0.78	3.89	5.80	0.64	3.71	1.12	0.65	0.73
24	6.03	0.78	4.70	4.05	0.62	2.51	1.30	0.63	0.82
25	5.70	0.75	4.28	3.90	0.71	2.77	1.50	0.68	1.02
26	5.60	0.77	4.31	4.10	0.72	2.95	1.50	0.72	1.08
27	5.70	0.76	4.33	3.90	0.71	2.77	1.40	0.69	0.97
28	5.70	0.72	4.10	3.90	0.68	2.65	1.40	0.68	0.95
29	6.40	0.77	4.93	4.20	0.70	2.94	1.60	0.69	1.10
30	6.80	0.78	5.30	4.20	0.72	3.02	1.60	0.72	1.15
31	6.90	0.79	5.45	4.20	0.72	3.02	1.60	0.72	1.15
32	6.50	0.79	5.14	4.30	0.72	3.10	1.60	0.72	1.15
33	12.0	0.83	9.96	9.10	0.77	7.01	2.40	0.69	1.66
34	11.6	0.81	9.40	9.30	0.77	7.16	2.40	0.69	1.66
35	12.0	0.81	9.72	8.90	0.76	6.76	2.30	0.67	1.54
36	11.6	0.82	9.51	9.40	0.75	7.05	2.40	0.69	1.66
37	12.2	0.82	10.0	8.90	0.75	6.68	2.20	0.65	1.43
38	10.6	0.79	8.37	8.40	0.72	6.05	2.20	0.62	1.36
39	15.7	0.91	14.3	13.1	0.88	11.5	3.20	0.88	2.82
40	13.5	0.88	11.9	11.8	0.83	9.79	3.10	0.80	2.48
41	14.1	0.87	12.3	12.3	0.83	10.2	3.00	0.79	2.37
42	14.9	0.84	12.5	12.5	0.78	9.75	3.20	0.77	2.46
43	13.0	0.84	10.9	10.5	0.80	8.40	2.70	0.69	1.86
44	12.7	0.82	10.4	10.8	0.78	8.42	2.60	0.66	1.72
45	14.0	0.85	11.9	11.1	0.80	8.88	2.70	0.71	1.92
46	13.0	0.76	9.88	10.8	0.71	7.67	2.70	0.54	1.46
47	12.5	0.75	9.38	10.2	0.68	6.94	2.60	0.53	1.38
48	12.4	0.74	9.18	9.9	0.67	6.63	2.50	0.53	1.33
51	19.7	0.73	14.4	15.6	0.68	10.6	5.20	0.68	3.54
52	19.6	0.80	15.7	15.6	0.76	11.9	5.20	0.78	4.06
53	19.8	0.83	16.4	15.8	0.79	12.5	5.21	0.81	4.22
54	19.6	0.85	16.7	15.4	0.79	12.2	5.19	0.82	4.26

55	19.3	0.86	16.6	15.4	0.80	12.3	5.08	0.83	4.22
56	19.6	0.85	16.7	15.5	0.79	12.3	5.19	0.81	4.20
58	19.7	0.73	14.4	15.5	0.68	10.5	5.20	0.66	3.43
59	23.7	0.86	20.4	18.7	0.81	15.2	6.30	0.85	5.36
60	23.8	0.84	20.0	19.1	0.80	15.3	6.80	0.86	5.85
61	28.1	0.82	23.0	21.0	0.76	16.0	7.20	0.76	5.47
62	24.0	0.84	20.2	18.8	0.78	14.7	6.60	0.76	5.02
63	15.3	0.75	11.5	17.6	0.66	11.6	5.10	0.68	3.47
64	15.0	0.74	11.1	17.1	0.63	10.8	5.00	0.67	3.35
65	15.2	0.72	10.9	17.3	0.61	10.6	5.10	0.64	3.26
66	15.1	0.70	10.6	17.5	0.60	10.5	5.10	0.63	3.21
67	15.1	0.72	10.9	17.5	0.61	10.7	5.10	0.64	3.26
68	14.7	0.75	11.0	16.0	0.69	11.0	5.10	0.71	3.62
69	16.5	0.70	11.6	18.0	0.64	11.5	5.60	0.66	3.70
70	19.1	0.69	13.2	16.1	0.67	10.8	4.70	0.71	3.34
71	19.0	0.80	15.2	16.5	0.76	12.5	4.70	0.82	3.85
72	18.2	0.88	16.0	16.4	0.83	13.6	4.40	0.85	3.74
73	18.1	0.84	15.2	15.2	0.78	11.9	4.30	0.83	3.57
74	20.1	0.89	17.9	17.3	0.84	14.5	4.70	0.84	3.95
75	18.8	0.90	16.9	16.0	0.83	13.3	4.70	0.86	4.04
79	19.3	0.89	17.2	16.6	0.74	12.3	3.90	0.77	3.00
82	19.0	0.76	14.4	17.4	0.81	14.1	4.50	0.74	3.33

*The precaecal digestible AA was calculated by AA content and pcDAA given in reference

Table A4: The number, amino acid content (AA; [g/kg DM]), fractional standardised precaecal digestibility of AA (pcDAA; [g/g AA]) and calculated standardised precaecal digestible AA concentration (pcdAA; [g/kg DM]) *in vivo* of the 74 assayed feedstuffs for the indispensable amino acid valine.

	Valine		
Number	AA (g/kg DM)	pcDVal	pcdVal *in vivo* * (g/kg DM)
1	5.06	0.85	4.30
2	5.26	0.84	4.42
3	5.02	0.85	4.27
4	5.07	0.88	4.46
5	5.22	0.84	4.38
6	4.47	0.85	3.80
7	5.86	0.86	5.04
8	4.84	0.85	4.11
9	4.87	0.80	3.90
10	5.61	0.84	4.71
11	6.31	0.81	5.11
12	5.71	0.83	4.74
13	5.99	0.82	4.91
14	6.10	0.82	5.00
15	4.93	0.82	4.04
16	5.22	0.81	4.23
17	4.95	0.74	3.66
18	4.64	0.72	3.34
19	5.15	0.73	3.76
20	4.75	0.74	3.52

21	4.74	0.72	3.41
22	4.80	0.71	3.41
23	4.61	0.71	3.27
24	5.17	0.71	3.67
25	5.50	0.76	4.18
26	5.70	0.79	4.50
27	5.90	0.77	4.54
28	5.90	0.75	4.43
29	6.10	0.78	4.76
30	6.20	0.78	4.84
31	5.90	0.78	4.60
32	6.30	0.77	4.85
33	12.0	0.80	9.60
34	11.1	0.78	8.63
35	11.7	0.78	9.13
36	11.7	0.78	9.13
37	11.8	0.78	9.20
38	10.6	0.74	7.84
39	14.4	0.88	12.7
40	13.6	0.84	11.4
41	13.9	0.84	11.7
42	14.4	0.81	11.7
43	14.0	0.82	11.5
44	13.1	0.79	10.4
45	14.9	0.82	12.2
46	13.6	0.72	9.79
47	13.1	0.71	9.30
48	13.0	0.71	9.23
51	18.4	0.72	13.2
52	18.5	0.80	14.8
53	18.5	0.82	15.2
54	19.0	0.83	15.8

55	18.6	0.85	15.8
56	18.8	0.83	15.6
58	18.0	0.69	12.4
59	21.7	0.85	18.4
60	22.3	0.84	18.7
61	25.0	0.80	20.0
62	22.0	0.81	17.8
63	19.7	0.68	13.4
64	19.6	0.67	13.1
65	19.7	0.67	13.2
66	19.1	0.64	12.2
67	18.6	0.67	12.5
68	18.6	0.73	13.6
69	20.1	0.67	13.5
70	19.9	0.71	14.1
71	20.1	0.81	16.3
72	19.9	0.87	17.3
73	19.4	0.85	16.5
74	21.4	0.89	19.0
75	20.2	0.90	18.2
79	18.9	0.84	15.9
82	23.0	0.81	18.6

*The precaecal digestible AA was calculated by AA content and pcDAA given in reference

Table A5: The number, amino acid content (AA; [g/kg DM]), fractional standardised precaecal digestibility of AA (pcDAA; [g/g AA]) and calculated standardised precaecal digestible AA concentration (pcdAA; [g/kg DM]) *in vivo* of the 74 assayed feedstuffs for the dispensable amino acids alanine, aspartic acid and cysteine.

	Alanine			Aspartic acid			Cysteine		
Number	AA (g/kg DM)	pcDAla	pcdAla *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDAsp	pcdAsp *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDCys	pcdCys *in vivo* * (g/kg DM)
1	4.41	0.75	3.31	6.48	0.77	4.99	2.81	0.88	2.47
2	4.64	0.75	3.48	6.66	0.75	5.00	3.07	0.88	2.70
3	4.47	0.75	3.35	6.34	0.75	4.76	2.93	0.88	2.58
4	4.57	0.78	3.56	6.43	0.79	5.08	3.00	0.90	2.70
5	4.63	0.74	3.43	6.79	0.75	5.09	2.94	0.88	2.59
6	4.33	0.75	3.25	6.28	0.75	4.71	2.87	0.88	2.53
7	5.18	0.78	4.04	7.38	0.78	5.76	3.42	0.89	3.04
8	4.46	0.77	3.43	6.81	0.79	5.38	2.75	0.88	2.42
9	4.59	0.74	3.40	7.88	0.78	6.15	2.60	0.85	2.21
10	5.37	0.79	4.24	8.68	0.81	7.03	3.17	0.87	2.76
11	5.70	0.76	4.33	9.09	0.78	7.09	3.25	0.85	2.76
12	5.19	0.78	4.05	7.94	0.79	6.27	3.11	0.87	2.71
13	5.66	0.76	4.30	9.23	0.78	7.20	3.47	0.86	2.98
14	5.61	0.77	4.32	9.04	0.79	7.14	3.26	0.86	2.80
15	4.47	0.76	3.40	7.29	0.78	5.69	2.88	0.87	2.51
16	4.84	0.76	3.68	7.58	0.78	5.91	2.81	0.85	2.39
17	4.67	0.66	3.08	8.10	0.71	5.75	2.53	0.79	2.00
18	4.60	0.63	2.90	7.86	0.69	5.42	2.38	0.77	1.83
19	4.76	0.64	3.05	7.97	0.69	5.50	2.57	0.79	2.03
20	4.69	0.62	2.91	8.02	0.68	5.45	2.34	0.76	1.78

21	4.60	0.64	2.94	7.86	0.69	5.42	2.45	0.79	1.94
22	4.68	0.64	3.00	8.13	0.70	5.69	2.47	0.76	1.88
23	4.52	0.62	2.80	7.62	0.68	5.18	2.38	0.78	1.86
24	4.86	0.61	2.96	8.29	0.66	5.47	2.66	0.76	2.02
25	4.70	0.66	3.10	7.10	0.69	4.90	2.50	0.79	1.98
26	4.90	0.67	3.28	7.20	0.69	4.97	2.70	0.80	2.16
27	4.70	0.63	2.96	6.70	0.66	4.42	2.50	0.80	2.00
28	4.70	0.62	2.91	7.10	0.66	4.69	2.40	0.79	1.90
29	4.90	0.64	3.14	7.40	0.67	4.96	3.00	0.80	2.40
30	4.80	0.67	3.22	7.10	0.70	4.97	2.60	0.81	2.11
31	4.90	0.67	3.28	7.20	0.69	4.97	2.90	0.82	2.38
32	5.00	0.65	3.25	7.50	0.68	5.10	2.70	0.81	2.19
33	10.8	0.79	8.53	10.8	0.85	9.18	3.60	0.71	2.56
34	10.7	0.78	8.35	10.7	0.84	8.99	3.60	0.71	2.56
35	10.5	0.77	8.09	10.5	0.83	8.72	3.50	0.69	2.42
36	10.7	0.77	8.24	10.7	0.83	8.88	3.60	0.66	2.38
37	10.4	0.75	7.80	10.4	0.81	8.42	3.00	0.62	1.86
38	9.80	0.73	7.15	9.80	0.80	7.84	3.40	0.63	2.14
39	12.5	0.86	10.8	39.3	0.91	35.8	5.40	0.87	4.70
40	11.4	0.82	9.35	33.1	0.88	29.1	5.20	0.83	4.32
41	12.2	0.83	10.1	35.1	0.88	30.9	5.20	0.81	4.21
42	12.0	0.76	9.12	37.4	0.86	32.2	4.50	0.78	3.51
43	12.4	0.81	10.0	33.4	0.86	28.7	3.80	0.65	2.47
44	11.8	0.78	9.20	32.3	0.83	26.8	3.50	0.63	2.21
45	13.0	0.80	10.4	36.6	0.86	31.5	4.00	0.64	2.56
46	12.4	0.72	8.93	33.6	0.79	26.5	3.80	0.51	1.94
47	11.8	0.70	8.26	31.0	0.77	23.9	3.60	0.52	1.87
48	11.6	0.70	8.12	31.1	0.77	24.0	3.40	0.48	1.63
51	16.8	0.71	11.9	44.2	0.74	32.7	6.30	0.67	4.22
52	16.9	0.79	13.4	44.2	0.81	35.8	6.19	0.75	4.64
53	16.9	0.81	13.7	44.5	0.81	36.0	6.01	0.76	4.57
54	16.9	0.82	13.8	43.9	0.80	35.1	5.91	0.73	4.31

55	16.6	0.83	13.8	43.5	0.80	34.8	5.72	0.74	4.23
56	16.7	0.82	13.7	43.8	0.76	33.3	5.59	0.69	3.86
58	16.4	0.69	11.3	44.0	0.73	32.1	6.10	0.64	3.90
59	19.8	0.82	16.2	52.9	0.86	45.5	7.20	0.79	5.69
60	20.2	0.82	16.6	54.0	0.84	45.4	7.70	0.76	5.85
61	22.8	0.77	17.6	62.7	0.79	49.5	7.70	0.72	5.54
62	19.9	0.78	15.5	54.3	0.82	44.5	6.80	0.74	5.03
63	17.1	0.72	12.3	28.3	0.67	19.0	9.10	0.70	6.37
64	16.8	0.71	11.9	27.7	0.65	18.0	8.90	0.68	6.05
65	17.0	0.69	11.7	27.9	0.63	17.6	8.80	0.61	5.37
66	17.0	0.67	11.4	27.6	0.61	16.8	8.70	0.59	5.13
67	16.8	0.68	11.4	27.4	0.62	17.0	8.30	0.6	4.98
68	15.5	0.75	11.6	27.0	0.74	20.0	9.30	0.74	6.88
69	17.1	0.71	12.1	30.0	0.64	19.2	9.10	0.70	6.37
70	13.4	0.73	9.78	33.9	0.74	25.1	4.70	0.58	2.73
71	13.5	0.81	10.9	34.2	0.81	27.7	4.70	0.72	3.38
72	13.5	0.86	11.6	34.2	0.85	29.1	4.40	0.76	3.34
73	13.0	0.85	11.1	32.5	0.85	27.6	4.40	0.74	3.26
74	14.5	0.87	12.6	37.2	0.85	31.6	5.20	0.75	3.90
75	13.5	0.88	11.9	34.3	0.83	28.5	4.80	0.64	3.07
79	10.8	0.77	8.31	19.1	0.63	13.2	5.40	0.83	4.48
82	17.6	0.85	15.0	32.7	0.80	26.2	4.50	0.40	1.80

*The precaecal digestible AA was calculated by AA content and pcDAA given in reference

Table A6: The number, amino acid content (AA; [g/kg DM]), fractional standardised precaecal digestibility of AA (pcDAA; [g/g AA]) and calculated standardised precaecal digestible AA concentration (pcdAA; [g/kg DM]) *in vivo* of the 74 assayed feedstuffs for the dispensable amino acids glutamic acid, glycine and proline.

	Glutamic acid			Glycine			Proline		
Number	AA (g/kg DM)	pcDGlu	pcdGlu *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDGly	pcdGly *in vivo* * (g/kg DM)	AA (g/kg DM)	pcDPro	pcdPro *in vivo* * (g/kg DM)
1	37.5	0.95	35.6	5.14	0.79	4.06	14.3	0.97	13.8
2	42.3	0.95	40.2	5.57	0.79	4.40	15.8	0.97	15.3
3	37.6	0.95	35.8	5.11	0.80	4.09	14.5	0.96	13.9
4	41.8	0.96	40.1	5.65	0.84	4.75	15.9	0.98	15.6
5	37.6	0.94	35.3	5.72	0.77	4.40	14.7	0.96	14.1
6	35.0	0.95	33.2	5.08	0.77	3.91	13.4	0.96	12.9
7	44.5	0.95	42.3	6.21	0.80	4.97	17.1	0.96	16.4
8	36.3	0.95	34.5	5.12	0.78	3.99	14.2	0.97	13.8
9	31.2	0.92	28.7	5.11	0.71	3.63	12.9	1.03	13.3
10	37.7	0.94	35.4	5.65	0.81	4.58	16.1	1.05	16.9
11	52.3	0.93	48.6	6.25	0.75	4.69	16.8	1.03	17.3
12	35.5	0.93	33.0	5.73	0.79	4.53	15.0	1.05	15.8
13	41.4	0.93	38.5	6.22	0.78	4.85	17.9	1.04	18.6
14	41.7	0.93	38.8	6.24	0.80	4.99	17.3	1.05	18.2
15	33.2	0.93	30.9	4.80	0.76	3.65	13.6	1.06	14.5
16	33.9	0.92	31.2	5.22	0.78	4.07	14.2	1.05	14.9
17	28.0	0.87	24.3	4.93	0.65	3.20	13.4	1.04	14.0
18	26.6	0.86	22.9	4.83	0.61	2.95	12.7	1.03	13.1
19	29.6	0.87	25.8	5.03	0.60	3.02	14.6	0.94	13.7
20	27.7	0.86	23.8	4.91	0.57	2.80	13.5	0.93	12.5

21	26.6	0.85	22.6	4.81	0.58	2.79	12.4	1.00	12.4
22	27.3	0.86	23.5	4.80	0.61	2.93	13.2	1.02	13.5
23	25.5	0.85	21.7	4.73	0.62	2.93	12.0	1.04	12.5
24	31.0	0.86	26.7	5.28	0.59	3.12	15.3	1.01	15.5
25	26.3	0.84	22.1	4.80	0.65	3.12	11.7	0.80	9.36
26	26.1	0.88	23.0	5.10	0.65	3.32	11.5	0.83	9.55
27	28.4	0.87	24.7	4.70	0.63	2.96	12.5	0.83	10.4
28	28.6	0.83	23.7	4.60	0.63	2.90	12.8	0.78	9.98
29	29.6	0.86	25.5	5.10	0.62	3.16	13.4	0.84	11.3
30	31.9	0.86	27.4	4.80	0.67	3.22	14.6	0.83	12.1
31	30.9	0.86	26.6	5.00	0.67	3.35	14.4	0.84	12.1
32	31.8	0.87	27.7	5.50	0.68	3.74	14.5	0.83	12.0
33	42.2	0.89	37.6	10.9	0.75	8.18	10.4	0.81	8.42
34	42.4	0.89	37.7	10.7	0.74	7.92	10.5	0.81	8.51
35	40.8	0.88	35.9	10.8	0.74	7.99	10.6	0.81	8.59
36	42.0	0.86	36.1	10.7	0.74	7.92	10.2	0.80	8.16
37	12.5	0.84	10.5	10.4	0.72	7.49	10.3	0.79	8.14
38	36.6	0.84	30.7	9.80	0.71	6.96	9.10	0.78	7.10
39	89.4	0.94	84.0	15.8	0.85	13.4	16.4	0.95	15.6
40	74.1	0.94	69.7	14.3	0.82	11.7	13.2	0.87	11.5
41	74.1	0.91	67.4	14.3	0.81	11.6	13.2	0.85	11.2
42	80.4	0.92	74.0	15.4	0.81	12.5	14.7	0.85	12.5
43	49.8	0.89	44.3	13.0	0.73	9.49	12.4	0.83	10.3
44	50.5	0.88	44.4	12.5	0.74	9.25	13.0	0.77	10.0
45	53.6	0.87	46.6	13.8	0.73	10.1	13.0	0.82	10.7
46	50.9	0.84	42.8	12.8	0.63	8.06	12.0	0.69	8.28
47	47.3	0.84	39.7	12.3	0.64	7.87	11.4	0.65	7.41
48	47.4	0.83	39.3	11.9	0.59	7.02	11.4	0.67	7.64
51	68.9	0.77	53.1	16.7	0.69	11.5	19.6	0.85	16.6
52	69.1	0.82	56.7	16.8	0.77	12.9	19.3	0.91	17.6
53	69.6	0.85	59.2	16.7	0.79	13.2	20.2	0.94	19.0
54	69.0	0.86	59.3	16.9	0.80	13.5	19.8	0.95	18.8

55	68.5	0.86	58.9	16.6	0.81	13.4	20.1	0.96	19.3
56	69.0	0.84	57.9	16.5	0.78	12.9	20.1	0.94	18.9
58	68.8	0.77	53.0	15.7	0.67	10.5	18.0	0.75	13.5
59	83.3	0.87	72.5	19.5	0.80	15.6	22.4	0.89	19.9
60	83.8	0.83	69.6	19.6	0.76	14.9	22.6	0.87	19.7
61	98.8	0.81	80.0	22.0	0.71	15.6	26.0	0.84	21.8
62	85.0	0.84	71.4	19.9	0.72	14.3	22.4	0.87	19.5
63	60.3	0.79	47.6	20.1	0.65	13.1	22.4	0.62	13.9
64	59.5	0.78	46.4	19.8	0.63	12.5	23.5	0.65	15.3
65	59.9	0.75	44.9	20.1	0.62	12.5	24.0	0.63	15.1
66	59.6	0.73	43.5	20.0	0.60	12.0	22.8	0.59	13.5
67	59.3	0.75	44.5	19.7	0.60	11.8	22.2	0.60	13.3
68	68.3	0.79	54.0	17.9	0.71	12.7	21.0	0.76	16.0
69	62.3	0.84	52.3	19.6	0.68	13.3	23.1	0.68	15.7
70	72.2	0.77	55.6	11.3	0.71	8.02	32.6	0.74	24.1
71	71.7	0.85	60.9	11.3	0.80	9.04	31.7	0.82	26.0
72	71.8	0.85	61.0	11.1	0.85	9.44	27.1	0.87	23.6
73	69.0	0.82	56.6	10.6	0.82	8.69	28.3	0.86	24.3
74	76.1	0.86	65.4	11.9	0.86	10.2	28.9	0.87	25.1
75	71.6	0.85	60.9	11.0	0.85	9.35	27.5	0.84	23.1
79	113	0.86	97.5	10.3	0.86	8.86	41.8	0.93	38.9
82	69.1	0.80	55.3	14.2	0.80	11.4	32.4	0.79	25.6

*The precaecal digestible AA was calculated by AA content and pcDAA given in reference

Table A7: The number, amino acid content (AA; [g/kg DM]), fractional standardised precaecal digestibility of AA (pcDAA; [g/g AA]) and calculated standardised precaecal digestible AA concentration (pcdAA; [g/kg DM]) *in vivo* of the 74 assayed feedstuffs for the dispensable amino acid serine.

	Serine		
Number	AA (g/kg DM)	pcDSer	pcdSer *in vivo* * (g/kg DM)
1	6.18	0.90	5.56
2	6.89	0.89	6.13
3	6.35	0.89	5.65
4	6.84	0.91	6.22
5	6.40	0.89	5.70
6	6.13	0.89	5.46
7	7.39	0.90	6.65
8	6.11	0.89	5.44
9	5.64	0.82	4.62
10	6.79	0.85	5.77
11	6.84	0.84	5.75
12	6.38	0.85	5.42
13	7.32	0.84	6.15
14	7.21	0.84	6.06
15	5.85	0.84	4.91
16	6.08	0.83	5.05
17	5.36	0.75	4.02
18	5.31	0.74	3.93
19	5.45	0.74	4.03
20	5.48	0.72	3.95

21	5.20	0.72	3.74
22	5.29	0.72	3.81
23	5.11	0.73	3.73
24	5.89	0.72	4.24
25	4.90	0.78	3.82
26	5.10	0.80	4.08
27	5.00	0.79	3.95
28	5.10	0.77	3.93
29	5.50	0.78	4.29
30	5.50	0.80	4.40
31	5.40	0.80	4.32
32	5.50	0.79	4.35
33	11.4	0.84	9.58
34	11.6	0.83	9.63
35	11.1	0.82	9.10
36	11.7	0.82	9.59
37	11.7	0.80	9.36
38	10.3	0.75	7.73
39	19.8	0.93	18.4
40	16.1	0.87	14.0
41	16.1	0.87	14.0
42	18.0	0.86	15.5
43	14.2	0.85	12.1
44	14.7	0.85	12.5
45	15.5	0.86	13.3
46	14.9	0.77	11.5
47	13.6	0.77	10.5
48	13.3	0.77	10.2
51	20.0	0.72	14.4
52	19.9	0.79	15.7
53	20.2	0.82	16.6
54	19.4	0.83	16.1

55	19.5	0.84	16.4
56	19.4	0.82	15.9
58	19.4	0.71	13.8
59	23.9	0.85	20.3
60	24.1	0.84	20.2
61	27.4	0.81	22.2
62	24.1	0.82	19.8
63	16.4	0.67	11.0
64	16.2	0.66	10.7
65	16.0	0.61	9.76
66	16.7	0.62	10.4
67	16.7	0.62	10.4
68	17.4	0.66	11.5
69	15.3	0.70	10.7
70	19.7	0.73	14.4
71	1.90	0.80	1.52
72	2.00	0.89	1.78
73	18.8	0.86	16.2
74	21.1	0.90	19.0
75	19.7	0.90	17.7
79	20.1	0.89	17.9
82	19.8	0.76	15.0

*The precaecal digestible AA was calculated by AA content and pcDAA given in reference

Table A8: The regression equation (y = a x + b), coefficient of determination (R^2) and root mean square error (RMSE) for pcdAA for the cereal grains (n = 32) and group wheat and triticale and barley and rye (each N = 8).

	Samples		
Amino acids (g/kg DM)	Y =	R^2	RMSE
	Cereal grains wheat, triticale, barley, rye		
Total AA	0.896 (SE 0.035) x – 6.466 (SE 3.699)	0.989	1.648
Indispensable AA	0.844 (SE 0.043) x – 1.062 (SE 1.793)	0.980	0.678
Dispensable AA	0.918 (SE 0.030) x – 4.612 (SE 1.964)	0.993	0.992
	Group wheat and triticale		
Total AA	0.904 (SE 0.045) x + 5.841 (SE 5.389)	0.966	1.992
Indispensable AA	0.835 (SE 0.060) x + 3.854 (SE 2.525)	0.933	0.879
Dispensable AA	0.932 (SE 0.037) x + 2.725 (SE 2.806)	0.979	1.108
	Group barley and rye		
Total AA	0.696 (SE 0.070) x + 15.60 (SE 6.978)	0.879	1.813
Indispensable AA	0.775 (SE 0.037) x + 1.831 (SE 1.438)	0.969	0.511
Dispensable AA	0.745 (SE 0.107) x + 7.610 (SE 6.690)	0.775	1.568

Table A9: The regression equation (y = a x + b), coefficient of determination (R^2) and root mean square error (RMSE) for pcdAA for the group wheat and triticale (each N = 8).

Amino acids (g/kg DM)	Samples y =	R^2	RMSE
Indispensable AA			
Arginine	0.882 (SE 0.057) x + 0.580 (SE 0.330)	0.964	0.128
Histidine	0.904 (SE 0.021) x + 0.125 (SE 0.074)	0.992	0.083
Isoleucine	0.849 (SE 0.065) x + 0.333 (SE 0.247)	0.925	0.093
Leucine	0.860 (SE 0.069) x + 0.624 (SE 0.563)	0.917	0.179
Lysine	0.871 (SE 0.600) x - 0.130 (SE 0.212)	0.938	0.103
Methionine	0.886 (SE 0.046) x + 0.095 (SE 0.088)	0.963	0.032
Phenylalanine	0.901 (SE 0.055) x + 0.150 (SE 0.316)	0.951	0.105
Threonine	0.727 (SE 0.077) x + 0.481 (SE 0.278)	0.865	0.100
Tryptophan	0.803 (SE 0.006) x + 0.121 (SE 0.011)	0.999	0.022
Valine	0.754 (SE 0.065) x + 0.901 (SE 0.309)	0.906	0.123
Dispensable AA			
Alanine	0.826 (SE 0.060) x + 0.215 (SE 0.257)	0.931	0.104
Aspartic acid	0.860 (SE 0.040) x + 0.109 (SE 0.270)	0.970	0.150
Cysteine	0.895 (SE 0.057) x + 0.097 (SE 0.161)	0.947	0.048
Glutamic acid	0.959 (SE 0.035) x + 0.825 (SE 1.318)	0.981	0.652
Glycine	0.816 (SE 0.103) x + 0.374 (SE 0.506)	0.817	0.612
Proline	1.109 (SE 0.111) x - 0.944 (SE 1.634)	0.877	0.599
Serine	0.931 (SE 0.108) x + 0.053 (SE 0.651)	0.842	0.201

y = estimated pcdAA (g/kg DM) and x = NDSAA (g/kg DM)

Table A10: The regression equation (y = a x + b), coefficient of determination (R^2) and root mean square error (RMSE) for pcdAA for the group barley and rye (each N = 8).

Amino acids (g/kg DM)	Samples y =	R^2	RMSE
Indispensable AA			
Arginine	0.695 (SE 0.071) x + 1.019 (SE 0.376)	0.874	0.097
Histidine	0.777 (SE 0.069) x + 0.167 (SE 0.174)	0.900	0.035
Isoleucine	0.773 (SE 0.033) x + 0.179 (SE 0.114)	0.975	0.064
Leucine	0.791 (SE 0.029) x + 0.342 (SE 0.160)	0.988	0.094
Lysine	0.532 (SE 0.097) x + 0.678 (SE 0.361)	0.681	0.084
Methionine	0.755 (SE 0.043) x + 0.151 (SE 0.073)	0.975	0.025
Phenylalanine	0.728 (SE 0.057) x + 0.606 (SE 0.055)	0.922	0.131
Threonine	0.696 (SE 0.061) x + 0.202 (SE 0.226)	0.902	0.115
Tryptophan	0.766 (SE 0.029) x - 0.022 (SE 0.036)	0.980	0.022
Valine	0.827 (SE 0.026) x + 0.078 (SE 0.127)	0.986	0.067
Dispensable AA			
Alanine	0.545 (SE 0.096) x + 0.790 (SE 0.397)	0.699	0.080
Aspartic acid	0.977 (SE 0.107) x - 1.427 (SE 0.721)	0.857	0.135
Cysteine	0.896 (SE 0.042) x - 0.129 (SE 0.101)	0.970	0.030
Glutamic acid	0.836 (SE 0.050) x + 1.877 (SE 1.368)	0.952	0.406
Glycine	0.640 (SE 0.115) x + 0.329 (SE 0.499)	0.687	0.131
Proline	0.921 (SE 0.301) x - 0.399 (SE 3.838)	0.402	1.264
Serine	0.783 (SE 0.127) x + 0.239 (SE 0.617)	0.730	0.111

y = estimated pcdAA (g/kg DM) and x = NDSAA (g/kg DM)

Chapter 6. Final considerations

Final considerations

An ongoing challenge for animal nutrition is to reduce nitrogen (N) inputs into the production cycle and to optimise the crude protein (CP) and amino acids (AA) supply to the animals. Reduced N inputs without a negative impact on performance lower N emissions and support environmentally friendly agriculture. Adequate ration planning to ensure that animals are provided with the energy and nutrients they need to sustain performance and health plays a key role in this process. During ration planning it has to be taken into account that feeds have different nutrient digestibilities depending on the type, processing and composition. Precaecal digestible CP and AA are the key variables for protein and amino acid evaluation of pig feeds in Germany (GfE, 2008). Several *in vivo* and *in vitro* methods exist to estimate precaecal digestible crude protein (pcdCP) and precaecal digestible amino acids (pcdAA). This study benefitted from the opportunity to conduct specific chemical analyses, namely neutral-detergent-insoluble CP and neutral-detergent-insoluble AA (NDICP/NDIAA) and acid-detergent-insoluble CP and acid -detergent-insoluble AA (ADICP/ADIAA), on feed ingredients on which not only the *in vivo* pcdCP and pcdAA had been determined using standardised and consistent methods but also a large range of chemical analyses had been conducted. The following final considerations intend to summarize and complement the discussion of the previous chapters.

6.1 Feed ingredients

6.1.1 Sample pool

Although the number of samples in the data set was large, it also had an uneven distribution and gaps. The sample pool consisted of cereal grains (wheat, triticale, barley and rye) and legume grains (faba beans, peas and lupins), which were each represented by several varieties, and of oil seeds and co-products of oil-seed processing (rape seed and soy bean products), and untreated or heat-treated miscellaneous samples (fish meal, wheat gluten, pea protein). However, co-products from food processing and cereal grain processing are not represented in the data set. Dairy co-products and insect-based feed ingredients as animal-derived feeds should be investigated in the future using a different analytical approach, because only plant fibre constituents are recovered in detergent fibre fractions which are essential, integral components of the laboratory method reported in this thesis.

Furthermore, Figure 1 shows that data points were clustered more around low and high values for both *in vivo* and laboratory values, whereas gaps are obvious between low and medium as well as between medium and high values.

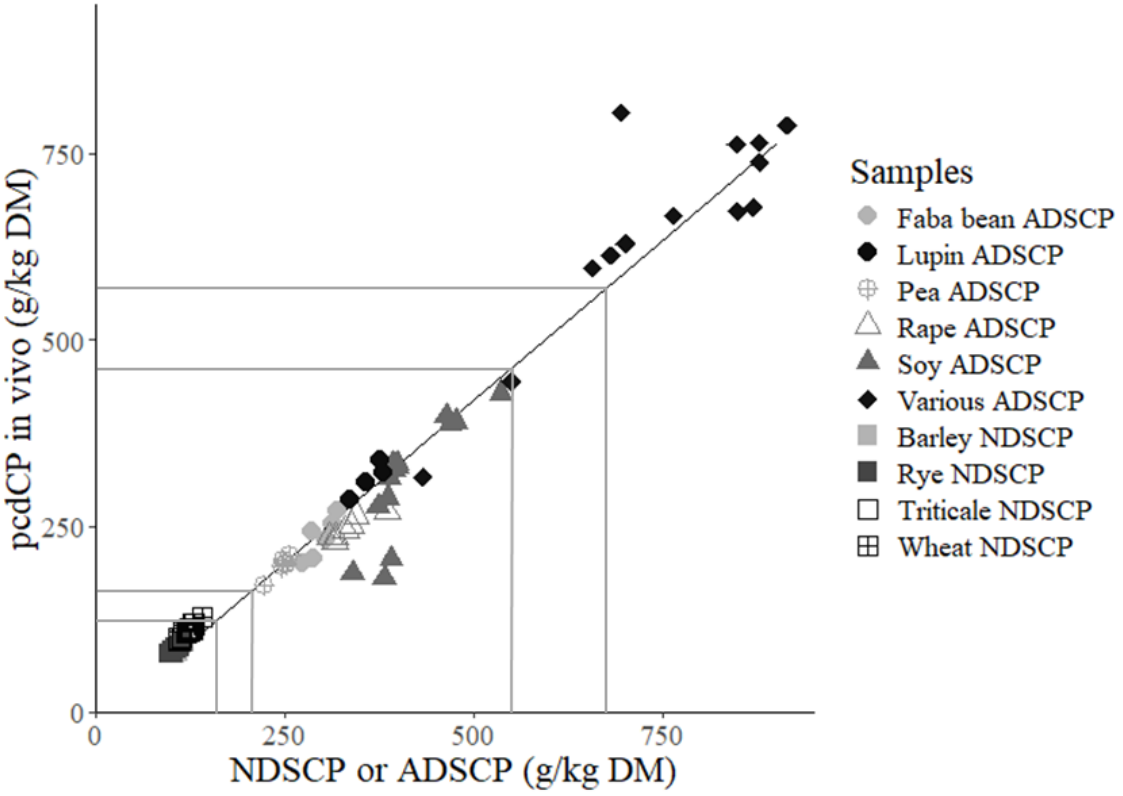

Figure 1: Relationship between neutral-detergent-soluble crude protein (NDSCP) and acid-detergent-soluble crude protein (ADSCP) data from the laboratory method (X) and *in vivo* pcdCP (Y) for cereal grains and protein feedstuffs for all 82 samples. The highlighted sections represent the gaps in the lower and middle part und middle and upper part.

The distinct relationship between *in vivo* and laboratory values means, for instance, that a pcdCP between 300 and 600 g/kg DM at an assumed pc digestibility of 80% is associated with a CP content of 375 to 750 g/kg DM. Feed ingredients such as maize gluten feed, peanut meal, sesame meal, sesame cake, sunflower meal could be considered. However, these components are either not at all or not commonly used in Germany and, to the best of our knowledge, no *in vivo* data are available.

One potential solution to fill the values gaps without necessitating further *in vivo* experiments would be to prepare mixtures from feed ingredients with low and high concentrations of CP and pcdCP which would then represent medium concentrations of each variable, for which lack of *in vivo* and *in vitro* data exist. For example, with a combination of components with a low pcdCP content (cereal grains wheat, rye, barley and triticale) and a medium pcdCP content (e.g.

faba bean, lupin pea, rape seed meal) it would be possible to create data to fill the gap in the low range (Table 1). And with a combination of feed ingredients with a medium pcdCP value (e.g., faba bean, lupin, pea, rape seed meal, soy bean meal) and a high pcdCP or pcdAA value (soy protein concentrate, wheat gluten, soy bean meal and fishmeal) it would be possible to create data which could fill the gap in the upper range values (Table 1). This procedure assumes that in mixed feeds, additivity of values of feed ingredients exist which is a general assumption, e.g., in standard *in vivo* digestibility trials on farm animal species (see, e.g., Dhanoa et al., 2008). Jansman et al. (2002) applied this concept also on the standardised pcdAA for pig feeeds. Deviations from additivity, also named interaction or associative effects (Huhtanen, 1991) may occur when feed ingredients rich in phytase (rye, triticale, wheat) (Rodehutscord et al., 2016) and phytates (peas, rape seed meal, soy bean meal) (Düngelhoef and Rodehutscord, 1995) are mixed. These combinations could cause a degradation of phytate-protein complexes by phytase, which may impact on the results such that higher digestibility values are observed.

Table 1: Possible combinations of feed ingredients with high and low pcdCP values to fill the described gaps in the graph.

pcdCP estimated target value [g/kg DM]	Feed ingredients	pcdCP *in vivo* [g/kg DM]	Proportion in the mixture [%]	Calculated pcdCP* [g/kg DM]	Analysed ADICP [g/kg DM]	pcdCP estimated by regression from ADICP** [g/kg DM]
200	Rye (21)	80	50	201	?	?***
	Lupin (42)	322	50			
200	Triticale (9)	96	25	206	?	?
	Faba bean (44)	243	75			
500- 550	Rapeseed meal (69)	264	50	529	?	?
	Soy protein isolate hydrolised (76)	788	50			
500-550	Rapeseed meal 93 (66)	229	25	526	?	?
	Soy protein concentrate b coarse (74)	629	75			

pcdCP, precaecal digestible crude protein; ADICP, acid-detergent-insoluble crude protein; DM, day matter.
Numbers in parentheses refer to Table 1 in chapter 4; (Schumacher et al., 2025).
*Calculated from pcdCP *in vivo* [g/kg DM] and the proportion in the mixture [%].
**pcdCP estimated by the regression equation y=0.86 x -13.38 (Chapter 4, Equation 4), where x represented the analysed ADICP and y represented the estimated pcdCP.
***The calculated pcdCP and the pcdCP estimated by the regression equation from the analysed ADICP should be similar.

6.2 Methods

6.2.1 Selection criteria for assigning samples to the neutral-detergent- or acid-detergent-insoluble crude protein procedure

The feed ingredients of this study were assigned to ND or AD fibre fraction procedures, depending on their N-compounds, before CP and AA analyses (Schumacher et al., 2025; Chapter 4.) Feeds known for Maillard products or/and tannin-protein complexes, which are insoluble in AD but not in ND, were assigned to the determination of ADICP or ADIAA, whereas on all other samples NDICP or NDIAA were analysed. Therefore, the ND fractionation procedure was analysed on all cereal grain samples and AD fractionation procedure on all other samples, labelled protein feeds. The purpose of this assignment was to use as few analytical steps as possible to generate reliable results.

However, during the course of the project both NDICP and ADICP was analysed for all samples. Comparing the results of NDICP and ADICP analyses with the *in vivo* pcdCP values, it became evident that for most protein feeds the ADICP values were closer to the *in vivo* values than NDICP, supporting the predefined assignment of protein feeds to the ADICP determination. Only for grain legumes (field beans, peas and lupins) the results for NDICP and ADICP both were close to the *in vivo* data, it would thus be possible to also apply the NIDCP procedure. With a focus on practicability and routine application it appears advisable, to use the general assignment of cereal grains to NDICP and all other feed ingredients (designated protein feeds) to ADICP analysis. In a routine laboratory setting, also samples with ambiguous labels should undergo the ADICP analysis.

6.2.2 Analytical procedures

Depending on the mass recovered after isolation of ND or AD residues, the bags were reused one to four times for each sample to obtain sufficient amounts of material for AA analysis. It cannot be excluded that small amounts of material have been boiled several times, thus impacting negatively on the property of the residues. The weight of fresh bags and a minimum. of 5 reused bags were recorded for every boiling circle and deviated on average 5 mg which is about 1.2% of the bag weight. However, these deviations were also recognised for the blind values after the boiling procedure, which may suggest that the deviations are depending on the bags itself and not necessarily on the residues.

Another critical point is that it cannot be excluded that the one-hour boiling cycle had an influence on heat-induced reactions in the feed samples such as Maillard reactions (Ames et al., 1999) resulting in higher ND- and AD-insoluble residue values.

6.2.3 Data evaluation

The results of this study suggest that the estimation of pcdCP and pcdAA based on procedures for isolating ND and AD residues can be carried out reliably using equations developed for the entire dataset which consisted mainly of cereal grains and protein feeds. These equations are recommended currently for routine analysis of feed ingredients. Specific equations for particular groups of feed ingredients may improve the quality of pcdAA estimation which was exemplarily shown for cereal grains. To avoid bias, however, it appears advisable to use the general equations until specific estimates for other particular groups of feed ingredients have been developed. This was not possible in this study due to limited sample size.

6.3 Near Infrared Spectroscopy (NIRS)

Initial approaches have been conducted to create a NIRS calibration with the sample pool. All 82 feed ingredients were measured successfully by the project partner VDLUFA Qualitätssicherung NIRS GmbH (Kassel, Germany; Dr. Peter Tillmann) to create and validate NIRS models. The quality parameters standard error of calibration in cross-validation (RMSECV) and standard error of calibration based on the validation samples (RMSEP) showed promising results. However, because numbers for NIRS measurements were comparatively low, the parameter values are currently of only limited significance. More samples should be analysed with the laboratory and NIRS methods to improve the quality of calibration and validation. Furthermore, samples for the NIRS calibration of ADICP and NDICP for feed ingredients of poultry feeds are still pending. As a result, the use of the estimation equation [4] described in chapter 4 ($y = 0.864$ (standard error [SE] 0.019) $x - 13.38$ (SE 7.479) could be simplified whereby a wider use is made possible.

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Chapter 7. Conclusion and outlook

Conclusion

In vivo determined standardised pcdCP and pcdAA of cereal grains and protein feeds was predicted accurately by a rapid chemical procedure. The procedure encompassed the determination of NDICP and NDIAA on cereal grains and ADICP and ADIAA on other feedstuffs, mainly protein feeds. The soluble fractions were used to predict *in vivo* determined standardised pcdCP and pcdAA by linear regression. This procedure can be used in any laboratory equipped for standard feedstuff analysis, is based on established analytical methods and does not involve multiple analytical steps. The analytical procedure for AA is the same as for CP, because the residues obtained with the ND or AD can also undergo AA analysis. The hypothesis could thus be maintained that the method developed and established to estimate pcdCP (Schumacher et al., 2025) is similarly suitable to estimate *in vivo* determined pcdAA of cereal grains and protein feeds. A major limitation of this study is that the validation of pcdCP values was only feasible on samples of cereal grains and grain co-products which generally had low concentration of CP. Future studies, therefore, should include validation on feed ingredients with higher CP content. For pcdAA an independent validation of the regression equations was impossible because no independent *in vivo* data set could be identified. An extension of the database of *in vivo* pcdCP and pcdAA values may yield predictions for specific types of feeds which would aid in further improving the accuracy of the prediction of pcdCP and pcdAA without compromising robustness. The applicability of the method to poultry feeds is also conceivable and would be worth studying.

Until now *in vivo* approaches on animals play an essential role for the precise determination of pcdCP and pcdAA in pigs. In Europe the 3-R-concept has to be implemented consistently in invasive animal experiments (Santos-Sánches et al., 2024). The 3-R-concept: Replace, Reduce, Refine, after Russell and Burch (1992) forms an important guideline for the realisation of animal experiments. However, with regard to animal welfare, low public acceptance and increasingly difficult authorisation and realisation they should be kept to the indispensable minimum. As described in Moughan et al. (2014) and Santos-Sánchez et al. (2024) rapid *in vitro* methods are needed as an alternative to *in vivo* experiments. The laboratory chemical method conducted and evaluated in this study can help to realise the 3-R-concept by replacing considerably the number of complex *in vivo* experiments with the rapid and relatively simple laboratory method. This also reduces the number of animals as *in vivo* data is only required for the further validation of a laboratory method.

Outlook

- Exclusively feed ingredients were analysed in this work. For future research an examination of mixed diets would be recommended to test if the laboratory method can also be applied to determine the pcdCP and pcAA not only in feed ingredients but also in mixed rations. This would extend the possible range of applications for the method in feed evaluation in practice.
- Grain legumes could be analysed using preparation of ND residues instead of AD residues before CP and AA analyses. However, a general use of AD for protein feeds would be easier to apply on routine analysis even if this means a slight loss of accuracy.
- A similar procedure could be performed to estimate the precaecal digestible organic matter and, potentially, pc digestible P. However, this would first require studies to clarify if phytate goes into solution although it is indigestible, which means that P digestibility would be overestimated.
- Malfermentation occurs in the large intestine when there is not enough fibre and protein is fermented by the microbes for the energy production The laboratory method could help to examine the ratio of N to fibre, how much N is bound to fibre and how much fermentable fibre gets into the large intestine (N from endogenous losses has to be included).

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Conference contributions and publications

CONFERENCE CONTRIBUTIONS

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Schumacher, V.; Kehraus S.; Südekum K.H.:
A simple laboratory method for estimating the standardised precaecal digestibility of crude protein and amino acids in pigs. (Poster)
15th International Symposium on Digestive Physiology of Pigs; Rotterdam (The Netherlands), 17.-20. May 2022 (DPP2022)

Schumacher, V., Kehraus, S., Südekum, K.-H.:
Schätzung des standardisiert praecaecal verdaulichen Proteins bzw. Aminosäuren mittels einer einfachen Labormethode.
133. VDLUFA-Kongress; Halle (Saale), 13.-15. September 2022

2023

Schumacher, V., Kehraus, S., Südekum, K.-H.:
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