Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Lehrstuhl für Tierernährung

Assessment of soy cake protein quality for broiler feeding through infrared spectroscopy calibration validated by in-vitro feed analyses and in-vivo feeding studies

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Agrarwissenschaften (Dr. agr.)

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. Heinz Bernhardt
Prüfende der Dissertation:	1. Prof. Dr. Wilhelm Windisch 2. Prof. Dr. Urs Schmidhalter

Die Dissertation wurde am 21.01.2019 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 20.05.2019 angenommen.

Finanzielle Förderung / Funding

Die Förderung des Vorhabens erfolgte aus Mitteln des Bundesministeriums für Ernährung und Landwirtschaft (BMEL) aufgrund eines Beschlusses des deutschen Bundestages. Die Projektträgerschaft erfolgte über die Bundesanstalt für Landwirtschaft und Ernährung (BLE) im Rahmen der Eiweißpflanzenstrategie. Fördernummer: 2814EPS022.

This research project was fully sponsored and supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program with grant number: 2814EPS022.

Zusammenfassung

Um Sojabohnen an Monogastrier verfüttern zu können, müssen diese, wegen ihres hohen Gehalts an antinutritiven Substanzen (z. B. Trypsininhibitoraktivität (TIA)) aufbereitet werden. Hierbei werden verschiedene Aufbereitungsmethoden angewendet, um diese Substanzen zu reduzieren, jedoch kann eine übermäßige Behandlung auch zu einer Reduktion der Futterqualität führen.

In dieser Dissertation wurden aktuell angewendete Sojaaufbereitungstechniken eingesetzt, um über-, optimal- und unterbehandelte Sojakuchenvarianten zu produzieren. Dadurch sollte eine große Spreizung an TIA Gehalten in den Sojapartien erzeugt werden. Weiterhin wurde die Tauglichkeit der Nahinfrarotspektroskopie zur schnellen TIA Schätzung getestet. In einem weiteren Schritt wurden die generierten Sojakuchenvarianten an Broiler verfüttert, um den Einfluss von TIA sowie der Proteinschädigung zu evaluieren. Zwei akzeptierte Publikationen behandeln die oben dargestellten Ziele dieser Arbeit.

Publikation 1 fokussiert sich auf die ersten beiden Ziele. Hierfür wurden zwei verschiedene Sorten Sojabohnen mittels vier verschiedenen Aufbereitungstechniken bearbeitet. Um eine robuste Nahinfrarot-Spektroskopie (NIRS) Kalibration erstellen zu können, wurde die Intensität der vier Aufbereitungstechniken variiert, um über-, optimal- und unterbehandelte Sojakuchenvarianten zu erhalten, um somit ein möglichst breit gefächertes Datenset zu erzielen. Die Futterqualität der Varianten wurde durch nasschemische Laboranalysen getestet. Die, während der Aufbereitung von Sojabohnen in teilentölten Sojakuchen aufgenommen, nahinfrarot Spektren, wurden mit den Laboranalysen kombiniert, um eine NIRS Kalibration für TIA in Sojakuchen zu erstellen. Für 50 Proben wurden 200 Spektren aufgenommen und analysiert. Nach der Datenvorbehandlung und der partial-least-square (PLS) Analyse wurde die Kalibration mittels einer leave-one-out Kreuzvalidierung geprüft. Die Ergebnisse zeigten, dass sich die Nahinfrarot-Spektroskopie mit Datenvorbehandlung und PLS gut für die Schätzung von TIA in Sojakuchen eignet (R^2 = 93.95 %), jedoch muss die Genauigkeit der Schätzgleichung für TIA Werte unter 4 mg/g durch den Einbau weiterer Spektren verbessert werden.

Publikation 2 behandelt das letzte Ziel dieser Arbeit. Hierfür wurde der Einfluss unterschiedlicher TIA- (0.25 - 23.6 mg/g), HDL- (hitze-geschädigtes Lysin) (1.40 - 8.60 g/kg) und KOH-XP Gehalte (Proteinlöslichkeit in Kalilauge) (65.5 - 97.6 %) in den im ersten Versuch produzierten 34 Sojakuchenvarianten auf die Wachstumsleistungen und das Pankreasgewicht von Broilern getestet. Die Sojakuchenvarianten, sowie eine Variante mit handelsüblichem Sojaextraktionsschrot wurden mit festen Zulagestufen (Grower: 35 %; Finisher: 25 %) in ein Grower- und Finisher-Futter für Broiler gemischt und während der 35 tägigen Mast in der zweiten und dritten Phase an 1680 Broiler verfüttert (Grower Phase: d

11 – d 24; Finisher Phase d 25 – d 35). Hierbei stellte sich TIA als dominierender Faktor hinsichtlich zootechnischer Leistungen und Pankreasgewicht heraus. TIA beeinflusste das Lebendgewicht (d 24 $P < 0.006$; d 35 $P = 0.026$), die Gewichtszunahme (d 24 $P < 0.006$) und die Futterverwertung (d 24 $P < 0.005$) negativ und bewirkte ein ansteigendes Pankreasgewicht (d 35 $P < 0.010$). Negative Effekte durch TIA konnten auch unterhalb derzeit empfohlener TIA Grenzwerte im Sojakuchen festgestellt werden. Daraus leitet sich die Notwendigkeit ab, TIA, soweit technisch möglich, in Broiler Rationen zu eliminieren.

Hinsichtlich der Ergebnisse beider Publikationen empfiehlt diese Dissertation derzeitige Ziele der Sojaaufbereitung mit Fokus auf die TIA Reduzierung, soweit technisch möglich, anzupassen. Hierbei kann die, bei übermäßiger Aufbereitung entstehende, Proteinschädigung vernachlässigt werden, da Futterrationen mit zusätzlichen Aminosäuren ergänzt werden können. Um eine kontinuierlich hohe Aufbereitungsqualität bei aufbereitetem Sojakuchen zu gewährleisten, wird die TIA Analytik mittels NIRS als Schnellanalysemethode empfohlen.

Summary

Soybeans must be processed to reduce their content of antinutritive factors (ANFs) before being fed to monogastric animals. Different processing methods are applied to reduce these substances and increase nutritional value, but excessive treatment might not only reduce ANFs but also nutritional quality.

Hence, this dissertation uses current soybean processing methods to produce over-, optimum-, and under-processed soy cake variants and generate soybean batches with a wide range of trypsin inhibitor activity (TIA). Furthermore, as TIA is the most common indicator used during soybean processing, near infrared spectroscopy (NIRS) is tested as a technique for fast estimation of TIA. In the last step, the generated soybean batches were used to evaluate the nutritional effects of TIA and protein degeneration by feeding soybeans to broiler chickens. Two accepted publications deal with the above-stated objectives.

Publication 1 deals with the first two objectives. Two different batches of soybeans where processed into partly de-oiled soy cakes using four different approaches. To produce a robust near infrared calibration, each method of processing was adapted in order to over treat, under treat, and optimally treat the soybeans. The feed quality of the soy cake variants was assessed using laboratory analyses. The near infrared spectra, recorded along with the processing of soybeans into partly de-oiled soy cakes, were combined with the laboratory analyses to be able to establish a NIRS calibration for the TIA in soy cakes. Therefore, for a sample size of 50 samples, 200 spectra were recorded and analyzed. After pre-treatment of the spectra and partial least square (PLS) regression analysis, the calibration was automatically tested with a leave-one-out validation. The results showed that the NIRS method, combined with pre-treatment and PLS, offered good accuracy (R^2 = 93.95 %) and allowed fast estimation of TIA in processed soy cakes, nevertheless, the calibration has to be improved for TIA values below 4 mg/g by implementing further samples.

In Publication 2, the last objective was focused on. The effects of varying TIA, heat-degraded lysine concentration (HDL), and protein solubility in potassium hydroxide (KOH-CP) on broiler performance and pancreas weight was tested using the 34 soy cake variants, widely varying in TIA (0.25 to 23.6 mg/g), HDL (1.40 to 8.60 g/kg), and KOH-CP (65.5 to 97.6 %), produced in Publication 1. These soy cake variants, as well as a commercial soybean meal extract, were included in a common grower and finisher diet for broiler chicks at fixed amounts (grower: 35 %; finisher: 25 %) and tested in a 35 d fattening experiment with 1680 broiler chicks (grower phase: d 11 – d 24; finisher phase d 25 – d 35). TIA was the dominant factor affecting zootechnical performance and pancreas weight at slaughter (d 35), depressing liveweight at d 24 ($P < 0.006$) and d 35 (0.026), reducing weight gain

(grower: $P < 0.006$) and feed:gain ratio during the grower phase ($P < 0.005$), and increasing pancreas weight ($P < 0.010$). The negative effects of TIA were also visible in soy cake variants with TIA below the recommended thresholds. This highlights the necessity of the complete elimination of TIA in broiler diets, as far as technically possible.

Considering the findings in both publications, this dissertation suggests updating the current aims of soybean processing with a focus on TIA elimination, as far as technically possible. Any resulting protein degeneration can be tolerated, since feed can be supplemented with amino acids. To obtain high quality in soybean processing, the fast detection of TIA in soybeans using NIRS is recommended.

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List of Abbreviations and Symbols

A_λ	Absorbance
ADL	Acid detergent lignin
ADF	Acid detergent fiber
ANF	Antinutritive factor
BW	Body weight
c	Concentration of solution
CKK	Cholecystokinin
CP	Crude protein
DM	Dry matter
ε_λ	Wavelength-dependent molar absorptivity coefficient
FCR	Feed conversion ratio
FIR	Far-infrared
HDL	Heat-degraded lysine
IR	Infrared
KOH	Potassium hydroxide
KOH-CP	Protein solubility in potassium hydroxide
l	Light's wavelength in the sample
LW	Live weight
ME	Metabolizable energy
MIR	Mid-infrared
MSC	Multiple scatter correction
NDF	Neutral detergent fiber
NIR	Near infrared
NIRS	Near infrared spectroscopy
NSP	Non-starch polysaccharides
PDI	Protein dispersibility index
PLS	Partial least square
R_λ	Reflection
RMSECV	Root mean square error of cross validation
T_λ	Transmission
TFI	Total feed intake
TI	Trypsin inhibitor
TIA	Activity of trypsin inhibitors
TWG	Total weight gain
UA	Urease activity

1 Introduction, Background Information, and Research Aims

Improving production efficiency and sustainability and maintaining a sustainable and competitive agri-food industry are two of the main agricultural challenges identified in the Horizon 2020 program (The Council of the European Union, 2013). Hence, the capacity of plant, animal, and production systems must be adapted to endure a rapidly changing climate with decreasing natural resources. Furthermore, these production systems must implement process controls to cope with social, environmental, and economic changes (The Council of the European Union, 2013). As a subfield of agricultural sciences, animal nutrition deals with the question of how to optimize the transformation of biomass into the precursors of human food, through the digestion and metabolism of agricultural livestock (TUM Chair of Animal Nutrition, 2017).

The soybean (*Glycine max* L.) is an important plant in both human and animal nutrition due to its protein and energy components (Clarke and Wiseman, 2005). However, raw soybeans contain antinutritive factors (ANFs, e.g. the activity of trypsin inhibitors (TIA)) that must be eliminated before they are fed to monogastric livestock to avoid harmful effects on the animals' health (Clarke and Wiseman, 2005). From a global perspective, most harvested soybeans are processed in hexane extraction plants. For an economic operating grade, the plants must process around 1,000 t/d (Seiler, 2006). Furthermore, hexane may be an environmental pollutant (Lee and Garlich, 1992).

Although in Germany the domestic production of soybeans is gaining importance regarding protein sources and organic farming, hexane extraction cannot be considered for local farmers due to the small cultivated and harvested area of soybeans. This has resulted in small, decentralized soybean processing plants that enable farmers to process their own harvests. Raw soybeans are processed into full fat or partly de-oiled soy cakes while inactivating the ANFs. However, processed soybean batches received from these decentralized processing plants in Bavaria, Germany, tend to show unintentional outliers in their TIA values, which can be up to double the recommended values (Zeindl, 2013).

Furthermore, exposing soybeans to over treatment could result in the glycosylation of amino acids, which impairs feed protein utilization. Therefore, the processing of soybeans into soy cakes needs to be controlled, which on the one hand requires up-to-date thresholds for the ANFs in soy cakes, and on the other hand requires a technique to control processing. Being able to control soybean processing in processing plants will help to produce a continuous supply of high quality feed for monogastric livestock and thus help to meet the goals set in Horizon 2020 (The Council of the European Union, 2013).

1.1 Background Information

This chapter presents a literature overview of the topics needed to understand this research. It is split into the following subchapters: “Nutritive aspects of soybeans”, “Antinutritive aspects of soybeans”, “Soybean processing”, and “Monitoring nutritional quality during soybean processing”. It concludes with the research aims of this dissertation.

History and purpose of cultivation

The soybean (*Glycine max* L.) is a species of Fabaceae (the legume family), deriving from East-Asia around 4000 years ago. Soybeans are cultivated for human and animal nutrition due to their high nutritional value (Fischbeck et al., 1985). Their high protein and energy content results in many possible applications. Soybean protein is often used as a substitute for milk, meat, or fish protein (Lütke Entrup and Schäfer, 2011) and soybean meal is a very important feed material for animal nutrition. Compared to other legumes, for example peas or lupines, soybeans contain more protein and fat and a very valuable amino acid composition, which has led to an increase in global soybean production (Lee et al., 2007). Soybeans are produced on around 6 % of global farmed fields (Hartman et al., 2011). Recently, the production of soybeans has increased. In 2014 more than 306 million metric tons were harvested, compared to approximately 26 million metric tons in 1961 (FAO, 2017). In Europe, especially in Germany, efforts are made to promote the use of locally grown legumes as a protein source for animal feed (Bundesanstalt für Landwirtschaft und Ernährung, 2015).

Interest in being economically independent of soybean imports, social demands, and costumer expectations regarding the absence of genetically modified feed are all factors that push local legume production (Rohe et al., 2017). Most harvested soybeans are used for animal nutrition, although they are also used industrially for glue, plastic, and textiles. Soybean oil is also an important component and its uses include cooking, paint, and bio diesel (Johnson and Myers, 1995). Soybean meal is the primary protein source in poultry diets worldwide (Lee and Garlich, 1992; de Coca-Sinova et al., 2010). Over the last few years the feed industry has shown increasing interest in full-fat soybeans as their high oil content enables the establishment of high-energy poultry diets (Heger et al., 2016).

Global, European, and German soybean cultivation

According to Goldhofer et al. (2017) 347.9 million tons of soybeans where produced in 2017/2018, which accounts for 60 % of global oilseed production. About 82 % of global soybean production occurs in three countries: the USA, Brazil, and Argentina. European soybean production only accounts for 7.2 % of global oilseed production. Nevertheless,

soybean production in Europe is showing a growing trend. In Europe, soybeans were harvested on 922,000 ha (Goldhofer et al., 2017). Italy, Romania, France, Croatia, Hungry, and Austria produce most of the soybeans in Europe (Goldhofer et al., 2017). In Germany, in 2017, soybeans were produced on only 19,100 ha with the largest harvested areas located in the free state of Bavaria (8,400 ha) and the state of Baden-Wuerttemberg (6,900 ha), due to climatic factors (DESTATIS, 2017). Nevertheless, in Germany, local soybean production is governmentally encouraged to increase local protein sources (Bundesanstalt für Landwirtschaft und Ernährung, 2015).

1.1.1 Nutritive aspects of soybeans

As mentioned earlier, soybeans are recognized for their high nutritive value due to their high oil and protein contents and their superior amino acid profile, compared to other plant proteins (Clarke and Wiseman, 2005, 2007). This results in many possible applications, including the use of soybean meal as a dominant component in animal feed mixtures, and has led to an increase in global soybean production (Lee et al., 2007).

Soybeans consist of about 40 % protein, 24 % fat, 31 % carbohydrates, 4 % ash, and 13 % water, which leads to multiple possibilities for feeding soybeans to livestock (Fischbeck et al., 1985; Liu, 1999). According to de Coca-Sinova et al. (2008) soybeans suit monogastric livestock diets particularly well due to their amino acid profiles (Table 1). Soybeans' nutritional values not only refer to their protein content and amino acid profiles, but also to their high fractions of fats and carbohydrates (non-starch polysaccharides). Non-starch polysaccharides can be digested easily due to their lack of cellulose, which can restrict protein digestibility. Soybeans show high digestibility and high energy content (15.59 MJ ME) as seen in Table 1 (Jeroch et al., 2016; USDA, 2016; LfL, 2017). Therefore, they can be used as a protein or energy component in livestock diets, unlike species such as rapeseed (e.g. canola) which can only be used for protein components.

As mentioned above, soybean meal is the primary protein source in poultry diets worldwide (Lee and Garlich, 1992; de Coca-Sinova et al., 2010) and the feed industry has shown increasing interest in full-fat soybeans for use in high-energy poultry diets (Heger et al., 2016). Nevertheless, soybeans also contain antinutritive substances which are explained below.

Table 1: Soybeans' nutritional value (Jeroch et al., 2016; USDA, 2016; LfL, 2017)

Nutrient	Unit	Values	Nutrient	Unit	Values
Water	g/100g	8.54	Albumins	% of CP	10
Energy	kJ/100g	1866	Globulins	% of CP	90
Met. Energy[1]	MJ ME	15.59	Glutelins	% of CP	0
CP[2]	g/kg DM	320	Lysine	% of CP	6.4
CL[3]	g/kg DM	190	Methionine	% of CP	1.3
CF[4]	g/kg DM	60	Cysteine	% of CP	1.3
NDF[5]	g/kg DM	117	Threonine	% of CP	4.8
ADF[6]	g/kg DM	95	Tryptophan	% of CP	1.6
ADL[7]	g/kg DM	26	Magnesium	g/kg DM	2.2
Cellulose	g/kg DM	69	Calcium	g/kg DM	2.4
NSP[8]	g/kg DM	47[9]	Phosphor	g/kg DM	5.6
Starch	g/kg DM	15	Potassium	g/kg DM	17.0

[1]Metabolizable energy; [2]Crude protein; [3]Crude fat; [4]Crude fiber; [5]Neutral detergent fiber; [6]Acid detergent fiber; [7]Acid detergent lignin; [8]Non starch polysaccharides; [9]De-oiled.

1.1.2 Antinutritive aspects of soybeans

Almost all plants contain ANFs to defend themselves against predators or diseases. ANFs are produced by plants' secondary metabolism and are slightly to highly toxic to humans and animals (Jeroch et al., 2013). Plant breeding methods try to reduce ANFs because nutritional potential is limited by their presence, which interferes with the intake, digestion, absorption, and metabolism of nutrients, as well as affecting the health status of the animal (Liener, 1994; Clarke and Wiseman, 2005). This shows the necessity of processing plant material before feeding to reduce ANFs.

The ANFs in soybeans can be split into heat-labile and heat-stable ANFs (Table 2). Following the table showing of the main ANFs present in soybeans, protease inhibitors are described in detail. Only minimal information has been given about lectins, tannins, phytate, and saponins as they are not the focus of this thesis.

Table 2: Heat labile and heat stabile ANFs in soybeans (Liener, 1994)

Heat labile	**Heat stable**
Protease inhibitors (e.g. trypsin inhibitors)	Saponins
Lectins	Tannins
Antivitamins	Estrogens
Goitrogens	Flatulence factors
	Lysinoalanine
	Phytate
	Allergens

Protease inhibitors

The presence of protease inhibitors in soybeans influences the use of soybeans in human and animal nutrition (Vollmann et al., 2003). Protease inhibitors are produced as part of the plants' secondary metabolism to fend off fungi and bacteria (Jeroch et al., 2013).

According to Liener (1994) protease inhibitors can be split into two groups: Group one consists of those that have a molecular weight of about 20,000 with two disulfide bridges, while group two consists of those that have a molecular weight of 6,000 to 10,000 with a high proportion of disulfide bonds and the capacity to inhibit chymotrypsin and trypsin at independent binding sites (Liener, 1994). Group two inhibitors are commonly referred to as Kunitz and Bowman-Birk inhibitors (Bowman, 1944; Kunitz, 1945; Birk, 1961; Vollmann et al., 2003).

Read and Haas (1938) first reported the ability of a soybean extract to inhibit trypsin. The protein fractions where purified by Bowman (1944) and Birk (1961) by crystallization of the Kunitz trypsin inhibitor (TI) (Kunitz, 1945; Liener, 1994). Kunitz TI-free soybeans contain 40 - 50 % less TIA than raw soybeans (Anderson-Hafermann et al., 1992). The remaining TIA in Kunitz TI-free soybeans is supposed to be traced back to Bowman-Birk protease inhibitors. The differences between Kunitz TIs and Bowman-Birk TIs are due to their amino acid sequences, electrophoretic mobility, and specificity (Clark et al., 1970; Obara et al., 1970; Hwang et al., 1977; Freed and Ryan, 1980; Kim et al., 1985; Tan-Wilson et al., 1985). The Kunitz TIs consists of 181 amino acid residues. To date, five related Bowman-Birk TIs have been isolated and characterized (PI-I to PI-V) (Hwang et al., 1977; Tan-Wilson et al., 1985).

Trypsin inhibitors are probably the best-known toxic inhibitors. Their name derives from their ability to inhibit the action of the enzyme trypsin, which is found in humans' and animals' digestive tracts (Liener, 1962). Trypsin is a leading enzyme in protein digestion. The inhibition of trypsin leads to incomplete protein digestion in the small intestine and to inactivation of pancreas proteases. This results in missing feedback regulations and increased enzyme production, which can consequently lead to increased losses of nitrogen and growth depression in humans and animals (Ahmed, 2001). The TIA content in raw soybeans ranges from 40 to 140 mg/g (Vollmann et al., 2003). These inhibitors are not necessarily restricted to trypsin but may also inhibit chymotrypsin, elastase, and serine proteases (Liener, 1994).

The antinutritional effects of protease inhibitors cause pancreatic hypertrophy and hyperplasia, due to the lack of enzymes, which results in growth depression when fed to monogastric animals (Chernick et al., 1948; Lyman and Lepkovsky, 1957; Liener, 1994; Grant et al., 1995; Heger et al., 2016). As early as 1948 and 1957 soybean TIs were

reported to cause stimulation of pancreatic secretions and to induct pancreatic hypertrophy in chickens and rats (Chernick et al., 1948; Lyman and Lepkovsky, 1957). Pancreatic secretion is controlled by a negative feedback mechanism. The level of intestinal trypsin is inversely related to the enzyme secretion of the pancreas. Therefore, when trypsin is depressed by TIs, the pancreas responds by producing more enzymes, which results in the growth of the pancreas (Liener, 1994). An enlarged pancreas leads to higher energy and nutrient demands and digestion becomes inefficient.

Lectins

Lectins are proteins that inhibit the enzymes of bacteria and fungi due to their chemical characteristics and therefore help to protect the plant (Liener, 1994). The antinutritional effects caused by lectins are the lowering of blood insulin levels and degenerative changes in the liver and kidneys (de Aizpurua and Russell-Jones, 1988). Lectins bind to carbohydrate-containing molecules, preferring the sugar component (Liener, 1994).
Protease inhibitors are seen as the main ANFs in soybeans, but as Liener (1994) suggests, they do not account for all of the growth inhibition observed with raw soybeans. About 60 % of lectins survive intestinal transit and bind to the intestinal epithelium, resulting in atrophy of the microvilli and reduced variability of the epithelial cells (Jindal et al., 1984; Ishiguro et al., 1992). According to Liener (1994) inactivation of soybean lectin due to moist heat treatment shows parallels to the destruction of TIs, but it was shown that they are denatured faster than TIs (Leeson and Atteh, 1996; Clarke and Wiseman, 2007; Furuse et al., 2009).

Phenolic compounds: Tannins

Tannins are defined as polyphenolic substances with a molecular weight over 500 and can be detected in most legumes. They decrease protein and carbohydrate digestibility and hence cause growth depression in humans and animals (Liener, 1994).
Tannin content ranges from as low as 0.45 mg/g in soybeans to 20 mg/g in faba beans (Narasinga Rao and Prabhavathi, 1982). According to Liener (1994) not much research on the effect of tannins in soybeans has been done due to their low content. Most studies have been conducted on sorghum and faba beans. Because the tannins are located in the shell, dehulling may be the simplest method to remove them (Liener, 1994).

Phytate

Phytate is a cyclic compound containing six phosphate groups binding to proteins and occurs in soybeans to the extent of 1.0 to 1.5 % of the dry matter (Liener, 1994). The antinutritional effects of phytate are, on the one hand, interference with the availability of proteins and, on the other hand, the inhibition of protease activity (Liener, 1994). Phytate

also inhibits several enzymes needed for digestion, such as pepsin, trypsin, and alpha amylase (Singh and Krikorian, 1982; Deshpande and Cheryan, 1984; Thompson and Yoon, 1984; Knuckles and Betschart, 1987; Vaintraub and Bulmaga, 1991; Caldwell, 1992).

Saponins

According to Liener (1994) saponins consist of a large family of structurally-related compounds and are responsible for the bitter taste and astringency of plant materials. Five saponins have already been characterized in soybeans and they are all linked to sugars (Liener, 1994). The saponins of some plants show negative effects on the growth performance of animals, but this was not supported by Ishaaya et al. (1969) when feeding chickens, rats, and mice with levels even three times higher than those found in soybeans (Liener, 1994). Soybeans' saponins are supposedly heat-stable. Therefore heat treatment does not change the saponin content of the plants and other processing methods must be applied (Birk et al., 1963).

1.1.3 Soybean processing

Necessity of soybean processing

Processing is important to reduce the abovementioned ANFs before feeding soybeans to animals as ANFs interfere with the intake, digestion, absorption, and metabolism of nutrients, as well as the health status of the animal (Liener, 1994; Clarke and Wiseman, 2005). The most well-known ANF in soybeans is TIA, which is also the most common indicator considered during soybean processing. Therefore, different techniques have been established to decrease TIA and thus increase the nutritional value of the beans (Liener, 1962; Rohe et al., 2017).
This chapter will deal with the "Techniques of soybean processing", "Risk of collateral damage during soybean processing and applied quality parameters to control processing" and "Impact of differently processed soybeans on animal performance".

Techniques of soybean processing

The goal of all processing methods is to produce a uniform final product with a minimum ANF content. Processing methods all depend on duration, temperature, pressure, moisture content, and particle size (Marsman et al., 1997; Zeindl, 2013).
In the following section the processing methods needed to understand this dissertation will be briefly described, as they are defined in the feed processing industry:

Hydrothermal treatment

Hydrothermal processing includes autoclaving and cooking. Process duration, temperature, and moisture content are the main influences in this type of processing. A combination of heat and moisture leads to the gelatinization of starch. Vaporization during the process protects the soybeans and this method is believed to be less aggressive than the thermal treatment method (Liener, 1962; Liener, 1994; Ahmed, 2001; Zeindl, 2013).

- Autoclaving: Water is heated in a closed container using pressure. Depending on the pressure, duration, and temperature, this leads to ANF reduction (Liener, 1962; Araba and Dale, 1990b; Anderson-Hafermann et al., 1992; Parsons et al., 1992; Liener, 1994).
- Cooking: According to Petres et al. (1990) cooking is based on 100 °C water using normal pressure. Depending on duration, this leads to a reduction in ANFs (Ruiz et al., 2004).

Thermal treatment

During thermal processing (e.g. toasting) direct heat is applied to the product (Marsman et al., 1997). Processing conditions like temperature, moisture content, and shear forces determine the effectiveness of the ANF inactivation (Marsman et al., 1997). Thermal treatment tends to reduce moisture content as only heat, without moisture, is applied to the soybeans. Thermal processing is believed to be more aggressive than hydrothermal processing as no vaporization with cool water is implemented during processing (Liener, 1962; Liener, 1994; Ahmed, 2001; Zeindl, 2013).
The main type of thermal processing, toasting, is briefly described below:

- Toasting: Toasting is the most common type of heat treatment processing during which soybeans are horizontally moved through a toaster unit. The toaster unit is rotated around a horizontal axis and during rotation direct heat is applied to the soybeans. When leaving the toaster unit, the soybeans' temperature is about 110 °C (Faldet et al., 1992; Lee and Garlich, 1992; Zeindl, 2013). If mixing different soybean sizes, small beans might be over treated while large beans are inadequately processed. It is therefore recommended to sort soybeans before treatment (Faldet et al., 1992; Lee and Garlich, 1992; Qin et al., 1998).

Pressure and thermal treatment

Pressure and thermal treatment cover the techniques of extrusion and expansion. Heat and pressure are applied to the product.

The two techniques are briefly explained below:

- Extrusion: This technique uses the principle of high-temperature-short-time. Therefore, a high temperature (approximately 140 – 170 °C) is applied to the product for less than 90 s (Heger et al., 2016). Extruders are based on the design of worm shafts, that move the material forward and increase the pressure due to the narrowing of the shaft. Because of the high pressure and shear forces, the product is heated very fast. Extrusion depends on the soybeans' particle size, on the velocity of the extruder unit and therefore the duration of the process, and on the geometrical form of the extruder (Mateos et al., 2002; Clarke and Wiseman, 2007; Bandegan et al., 2010). Extrusion can be split into dry and moist extrusion. During moist extrusion, wet steam is added. Therefore, a dryer unit is needed at the end of moist extrusion.
- Expansion: The technique of expansion is very similar to that of (moist) extrusion and the process is also based on heat and pressure. The main difference between extrusion and expansion is the lower energy input needed for the expander due to a hydraulically controlled conus at the end of the expander (Zeindl, 2013). The soybeans are also pushed through a worm shaft, generating heat due to the narrowing of the worm shaft. Steam is added to the process. The use of an expander is believed to be less energy intensive than moist extrusion (Mateos et al., 2002; Ruiz et al., 2004).

Risk of collateral damage during soybean processing and applied quality parameters to control processing

As explained above, processing mostly depends on hydrothermal-, thermal- or pressure-treatment to reduce ANFs. Nevertheless, due to over treatment, proteins might be physically and chemically changed, which negatively effects the nutritional values of proteins and results in the derivatization of lysin, the oxidation of the sulfur in cysteine and methionine, and cross linkages at amides and carboxyl groups. The brown color of soybeans, resulting from thermal treatment, may be due to a Maillard reaction, in which lysine reacts with reducing sugars (Lee and Garlich, 1992).

Over-processing might lead to protein denaturation and agglutination, reducing protein solubility. It might also lead to specific bindings between proteins and molecules. In particular, if the essential amino acid lysine is bound to other molecules it can no longer be digested as free lysine, thus leading to an overall reduction in protein quality and decreased feed efficiency. This is because lysine is a primary limiting amino acid, confirmed by Lee and Garlich (1992) as well as Parsons et al. (1992).

However, if inadequately processed soybeans, may still contain ANFs which have negative effects on feeding efficiency and animal health (Liener, 1994; Clarke and Wiseman, 2005). Hence, quality parameters detect both ANFs, represented by TIA, and protein degeneration. Table 3 shows a summary of the quality parameters used for soybean processing.
In the following section the currently used quality parameters and currently applied thresholds for feeding practices are displayed:

Activity of trypsin inhibitor (TIA)

TIA is the most well-known ANF in soybeans and the most common indicator used in soybean processing due to its slow degradation (Clarke and Wiseman, 2007). TIA can be determined using a direct laboratory assay. Today, the most frequently used methods are based on Monari et al. (1993) and DIN (2001), however, both are based on the original works by Kakade et al. (1974) and Smith et al. (1980). Following these methods, trypsin is offered a substrate in the presence of, and without, a potential inhibiting material. The cleaving of this substrate produces a color change that can be detected with a photometer. Depending on the assessed optical density, the activity of trypsin in both reaction mixes can be estimated and the TIA can be calculated by comparing the loss in activity in the presence of the inhibiting material to that in the non-treated control. Results are interpreted with reference to the laboratory method used. Monari et al. (1993) also suggests that the associated raw soybeans be analyzed to assist in interpreting the results.
Currently, the defined upper limits for TIA in soybean products within the diets of fast growing animals are ≤4 mg/g for broiler chickens and ≤4.7 mg/g for pigs (Batterham et al., 1993; Clarke and Wiseman, 2005, 2007). The latest studies show that a decrease in the growth performance of fast growing monogastric animals fed on full fat soy cakes can be seen at a TIA ≤8 mg/g (Heger et al., 2016). However, the available data is quite limited and sometimes contradictory. For example, Huisman and Tolman (2001) recognized adverse effects on the fattening performance of pigs at TIA levels as low as ≥0.5 mg/g.

Activity of urease (UA)

UA is used to evaluate the protein quality of processed soybeans but is more suitable to detect under-processing (Ruiz et al., 2004). Soybeans contain TIs as well as the enzyme urease. Both are heat labile proteins and therefore UA is also suitable for calculating protein degeneration during heat treatment. Monari et al. (1993) suggest over treatment at a UA < 0.05 mg N/min at 30 °C, optimal processing at a UA between 0.1 and 0.3 mg N/min at 30 °C, and under-processing at a UA between 0.3 and 0.5 mg N/min at 30 °C. In pig trials, Lee et al. (2007) stated that UA is only capable of detecting under-processing in soybeans. UA values also decrease very fast below the level that is assumed to be that of optimal

processing. UA does not have a physiological impact but can be used to detect whether processing has led to protein denaturation.

Protein solubility in potassium hydroxide (KOH-CP)

KOH-CP is used to assess protein quality by determining protein solubility in KOH. As mentioned above, over-processing might lead to protein denaturation and agglutination, and therefore reduce protein solubility. Hence, with increased processing, the KOH-CP reduces due to extended protein agglutination. KOH-CP is a parameter used to state the extent of protein agglutination, because less protein is soluble in KOH with increasing protein agglutination.

KOH-CP is used as an indicator of soybean protein quality for feeding chickens and pigs. Parsons et al. (1991) found that a decrease in chickens' growth performance could be seen when KOH-CP was below 59 % and it generally seems to be critical at 70 %, according to Araba and Dale (1990a). Although Araba and Dale concluded in both of their studies (Araba and Dale, 1990a, b) that KOH-CP can be used to detect the over- and under-processing of soybean samples, Anderson-Hafermann et al. (1992) discovered that KOH-CP might not be sensitive enough to detect under-processed samples. In pig trials, Lee et al. (2007) confirmed the ability of KOH-CP to indicate over-processed samples. Hence, KOH-CP is a good indicator to detect the protein quality of over-processed soybeans (Araba and Dale, 1990a).

Protein Dispersibility Index (PDI)

The PDI was established for use in human nutrition to assess protein degradation and can be compared with the abovementioned KOH-CP as it is also a method of measuring protein solubility. In soybean processing, the PDI is used to evaluate the protein quality of processed soybeans. Batal et al. (2000) conducted growth performance experiments with broilers and concluded that the PDI is useful for determining processing quality, especially when already analyzed using UA and KOH-CP. Monari et al. (1993) suggest a PDI between 15 and 28 % as the optimal processing parameter. Lee et al. (2007) showed that, when feeding processed soybeans to pigs, the optimal PDI was seen between 21 and 22 %, but Batal et al. (2000), while conducting broiler experiments, concluded that the optimal PDI was seen between 40 and 45 %. Currently, the most valuable parameter to detect protein solubility is KOH-CP.

Reactive lysine

Reactive lysine is used to determine protein quality after soybean processing. Usually amino acids are analyzed using hydrolysis to separate them out, but during hydrolysis

bindings between lysine and carbohydrates are also dissolved. Hence, an amino acid analysis displays the total lysine content. Damage due to the Maillard reaction can thus not be determined. Therefore, a method of assessing reactive lysine was established and is used to transform unbound lysine to homoarginine to calculate the total contents of heat-degraded lysine (HDL) in soybean products (Fickler, 2005; Pahm et al., 2008). HDL reflects the degree of lysine degradation through Maillard reactions and hence is an accepted parameter of over-processing that indicates a decreasing availability of amino acids in soybean products (Faldet et al., 1992; Fontaine et al., 2007).

Heger et al. (2016) conducted experiments with soybeans, also measuring reactive lysine. The reactive lysine content in raw soybeans was 92.4 % of the total lysine. The values ranged from 91.4 % to 92.9 % which indicates no damaging effect of processing on lysine availability (Heger et al., 2016). Both total lysin and reactive lysin decreased with processing, but reactive lysin is a more sensitive indicator of lysine damage (Fontaine et al., 2007).

Table 3: Summary of quality parameters used for soybean processing. (++: highly suitable, +: suitable, -: less suitable, --: not suitable)

	TIA	UA	KOH-CP	PDI	Reactive Lysine
Under-processing	++	+	-	-	-
Over-processing	--	--	++	+	+
Values for optimal processing	< 4 – 5 mg/g	0.1 - 0.3 mg N/min at 30 °C	crit. < 70 %	15 – 28 %	> 90 % of total lysine
Literature	Clarke and Wiseman (2005)	Monari et al. (1993)	Araba and Dale (1990a)	Monari et al. (1993)	Heger et al. (2016)

Impact of differently processed soybeans on animal performance

Toasting/extrusion cooking

Most studies use laboratory autoclaves to simulate the conditions of commercial units. Lysin is the amino acid that is most vulnerable to heat (Adrian, 1974). Therefore Faldet et al. (1992) measured the nutritional availability of Lysin in heat-treated soybeans fed to rats. They found that lysin content decreased with higher temperatures and the duration of heat treatment. Marsman et al. (1997) tested the effect of the toasting and extrusion cooking of soybean meal using 520 female broiler chicks with 10 dietary treatments. As a parameter to measure protein quality, they used the PDI. Marsman et al. (1997) showed an improved

feed conversion ratio in broiler chicks using extrusion rather than toasting. Extrusion at different shear levels did not produce any differences in chickens' growth performance. Qin et al. (1998) also used a toaster at 102 °C, 118 °C, and 136 °C to conduct experiments with full-fat soybeans, originating from Argentina and China. They also showed that, with increasing heating time at various temperatures, the TIA and PDI decreased. Chinese soybeans required a longer processing time than Argentinian soybeans, which led to the conclusion that soybeans with different origins require different processing methods or parameters.

Lee et al. (2007) fed heat-treated soy flakes to pigs and examined the PDI and KOH-CP. With increasing heat treatment, the PDI (from 65 to 22 %) and KOH-CP (from 96 to 42 %) decreased. They found that bodyweight significantly increased with increasing heat treatment, except in the case of overheated samples. Heat treatment is required to destroy ANFs that are naturally present in soybeans, but excessive heat treatment might lower amino acid availability (Lee and Garlich, 1992). Therefore, it would be beneficial to reduce the temperature in the toaster units.

Cooking

Ruiz et al. (2004) conducted experiments with full fat soybeans treated with a thermal processor or expander-extruder cooker. In this section the results of the thermal processing are described. The results from the expander-extruder cooker are described in a later section. Raw soybeans were wet toasted at 113, 120, 130, 135, and 150 °C with durations from 3.0 to 9.5 min and fed to broiler chickens. Regarding bodyweight, raw soybeans and the 113 °C treatment were significantly different ($P < 0.05$). The highest bodyweights were recorded when fed with soybeans from the 130 °C and 120 °C treatments (Ruiz et al., 2004). TIA dropped sharply from the raw soybeans to the 113 °C treatment. Additional treatments showed a slow decrease in TIA (Ruiz et al., 2004). KOH-CP showed a consistent decrease in protein solubility with increasing temperature and time (Ruiz et al., 2004).

Expanding

Heger et al. (2016) conducted an experiment feeding full-fat soybeans, processed with a short-term conditioner, a long-term conditioner, and an expander, to chickens. Samples were analyzed for UA, KOH-CP, PDI, TIA, and homoarginine (reactive lysine). The lowest TIA was slightly above 4.0 mg/g (Heger et al., 2016). KOH-CP was suggested to be critical at 70 % by Araba and Dale (1990a), but Heger et al. (2016) reached values of 94 to 89 % before over-processing. Heger et al. (2016) also detected a close correlation between UA, PDI, and TIA with R^2 values ranging from 0.97 to 0.99. The reactive lysine content in raw soybeans was 92.4 % of the total lysine, and it ranged from 91.4 % to 92.9 % indicating that

lysine availability was not affected by the processing (Heger et al., 2016). Heger et al. (2016) detected decreased growth performance in chickens fed with raw or under-processed soybeans instead of soybean meal. Increasing the required amino acid concentration from 95 to 100 % improved growth rate, feed conversion ratio, and feed intake (Heger et al., 2016). This study confirms the accepted opinion that chicken growth performance and pancreatic hypertrophy is affected by the presence of TI in soybeans (Applegarth et al., 1964; Han and Parsons, 1991; Leeson and Atteh, 1996). A close correlation was found by Heger et al. (2016) between in-vivo chicken responses and in-vitro indicators in soybeans. In their study Heger et al. (2016) found TIA and PDI to be the best predictors of chicken growth performance. The best performance in terms of feed intake, growth rate, and feed conversion ratio was found when using expanding (15 s, 125 °C) followed by short term (1 min, 100 °C) and long term (5 min, 100 °C) conditioning (Heger et al., 2016). Heger et al. (2016) also switched from TI ratios to a TIA-free diet and detected a significant decrease in the relative pancreatic weight, but could not confirm whether pancreatic enlargement was fully reversed.

Autoclaving

Applegarth et al. (1964) fed raw soybeans that were autoclaved for 15 min, as well as supplemented autoclaved diets, to chickens. Chickens fed with the un-supplemented diet showed slower growth performance and increased pancreas weight compared to those fed the supplemented diets. Anderson-Hafermann et al. (1992) conducted chicken growth experiments to verify the effect of steam heating on raw conventional full-fat soybeans and on full-fat Kunitz TI-free soybeans. Growth performance was better for full-fat Kunitz TI-free soybeans. Samples where autoclaved at 121 °C for varying times. The experiment showed that an increase in autoclaving time (from 0 to 12 min) for raw soybeans, as well as for Kunitz TI-free soybeans, resulted in a linear increase ($P < 0.001$) in weight gain and feed efficiency. Higher autoclaving times showed no further improvements. Pancreas weight decreased as autoclaving time increased. KOH-CP did not change substantially as autoclaving time increased (Anderson-Hafermann et al., 1992). Anderson-Hafermann et al. (1992) confirmed Han and Parsons (1991)'s result that the nutritive value of full-fat Kunitz TI-free soybeans is greater than that of raw soybeans, but that heat treatment is required for both types to obtain their maximum nutritive value for broiler chicks. Although Kunitz TI-free soybeans contain 40 – 50 % less TIA than raw soybeans, the level is still higher than in soybean meal (Anderson-Hafermann et al., 1992). The remaining TIA in Kunitz TI-free soybeans can supposedly be traced back to Bowman-Birk protease inhibitors. Anderson-Hafermann et al. (1992) showed that KOH-CP was not a sensitive indicator to detect under-

processed soybean samples during autoclaving and thus could not confirm Araba and Dale (1990b)'s results.

Fontaine et al. (2007) used an autoclave to process soy products to test the suitability of the homoarginine reaction for determining reactive lysin in soy products. Two soybean meals differing in their protein content and one full-fat soybean sample were used to test lysine damage. Autoclaving was performed at 135 °C for varying times. Both total lysin and reactive lysin decreased but reactive lysin was found to be a more sensitive indicator of lysine damage (Fontaine et al., 2007).

Parsons et al. (1992) tested the effect of over-processing on the availability of amino acids in soybean meal. They conducted over-processing using an autoclave at 121 °C for 0, 20, 40, and 60 min. Increasing the autoclaving time reduced lysine and cystine, but it did not affect other amino acids. Growth performance was better when feeding 40 min autoclaved samples to broilers than when feeding them with unautoclaved samples. The results indicate that over-processing influences amino acid quality, especially lysine. It is assumed that advanced Maillard reaction products were formed during over-processing due to nonenzymic browning reactions between lysine and oligosaccharides (Parsons et al., 1992).

Araba and Dale (1990b) conducted experiments using different autoclaving times (experiment 1: 0, 15, 30, and 60 min; experiment 2: 0, 5, 10, 15, and 20 min) on soybean meal and feeding all samples to broiler chickens. They showed that the greatest TIA reduction during heating was seen during the first 15 min. Experiment 1 showed that significant improvements in chick weight gain and feed conversion were obtained from 15- and 30-min autoclaved samples. Experiment 2 showed that the best chicken performance was obtained for samples that where processed for 5, 10, and 15 min. Batal et al. (2000) showed that the KOH-CP values of soybean meal decrease with increasing autoclaving time. Soybean meal samples where autoclaved in different assays for varying times. They detected no further increase in weight gain after 18 min of processing. TIA levels decreased with increasing autoclaving time.

Extrusion

Extrusion processing followed by expelling is a relatively recent technology for soybean meal processing (Bandegan et al., 2010). This method produces a product with a higher fat content than extracted soybean meal. Clarke and Wiseman (2007) showed that water input facilitated the denaturation of TI at lower temperatures. Dry matter content increased with increasing extrusion temperature. TIA decreased with increasing heating temperature and was reduced from 28.4 mg/g in raw soybeans to 1.9 mg/g in soybeans heated to 160 °C. The four extrudates where used at three different inclusion levels and where fed to male

Ross broiler chicks from day 19 to day 26. Mean weight increased with increasing extrusion temperatures for full fat soybeans. Differences in weight gain could already be seen after a 3-day period. Pancreatic enlargement could be seen after 6 d of feeding with soybeans processed at lower temperatures. Clarke and Wiseman (2007)'s results emphasize the importance of controlled conditions during processing. Most procedures involve high temperatures, but they may then result in damage to amino acids. Therefore Leeson and Atteh (1996) tested the effect of low temperature extrusion on the TIA of soybeans. The extruded soybeans where fed to broilers. The study showed that, using low temperature extrusion, the TIA could not be reduced below 10 mg/g. The higher the extrusion temperature, the better the feed intake of the birds, which illustrates the need for high temperatures to process soybeans.

Ruiz et al. (2004) conducted experiments with full fat soybeans treated with a thermal processor and an expander-extruder cooker. The results from the expander-extruder cooker are mentioned here. The raw soybeans where wet extruded at 118, 120, 122, 126, and 140 °C, with a mean duration of 20 s, and fed to broiler chicken. The results showed that raw soybean treatments where significantly different from heat treatments (118 °C, 120 °C), confirming the improvement in animal performance due to heating of soybeans (Ruiz et al., 2004). This research also showed maximum bodyweight and feed conversion for treatments heated at 122, 126, and 140 °C (Ruiz et al., 2004). TIA dropped consistently as temperature increased. Raw soybeans also had the highest UA. KOH-CP, used to detect over-processing, was 90 % for the raw soybeans. All values were in the upper 80 % except in the treatment processed at 140 °C (79 %) (Ruiz et al., 2004).

1.1.4 Monitoring nutritional quality during soybean processing

The goal of soybean processing is to reduce TIA to above the displayed thresholds in monogastric livestock feeding. As described above, over-processing can lead to protein denaturation and agglutination and therefore reduce protein solubility, and under-processing does not reduce TIA levels as much as necessary (Liener, 1994; Clarke and Wiseman, 2005).

Due to multiple influences during processing, such as duration, temperature, pressure, moisture content, and particle size, varying TIA contents have been observed between different soybean batches after processing, especially when working with small soybean batches (Clarke and Wiseman, 2005; Zeindl, 2013). The quality parameters presented above (TIA, UA, KOH-CP, PDI, and reactive lysine) depend on time-consuming laboratory analyses, but the process of soybean processing has to be monitored to quickly respond

during processing to consistently meet the recommended thresholds in processed soybean batches.

This illustrates the necessity of establishing a technique to measure quality parameters in real time to control processing and hence receive consistent high feed quality. Controlling processing quality during soybean processing could be accomplished using the quality parameters above combined with near infrared spectroscopy (NIRS), as NIRS is often used for feed quality control and biogas processing control in the agricultural sector (Fontaine et al., 2001; Delwiche et al., 2006; Krapf et al., 2011).

The following chapter will briefly explain the principles of NIRS calibration and how it can be used in the agricultural sector. The chapter deals with “basic information about near infrared spectroscopy”, “measuring principles”, “near infrared spectra evaluation/multivariate calibration” and the “application of near infrared spectroscopy in agricultural sciences”.

Basic information about near infrared spectroscopy

In the 1800s Sir Friedrich Wilhelm Herschel conducted an experiment that set the basis for today’s infrared spectroscopy. He directed sunlight through a prism and split the generated rays onto different thermometers. He attempted to illustrate that the thermometer showing the highest temperature was the one irradiated by yellow-green light. However, the thermometer that was irradiated by non-visible light showed the highest temperature. Herschel thus concluded that there is light that is not visible to the human eye. Based on this conclusion, he conducted another experiment to show that the non-visible light also followed the rules of optics. Herschel called this kind of radiation “infrared radiation.” Based on these experiments, the development of spectral photometers was encouraged and can be applied in many different fields today (Gottwald, 1997; Baßler and Hauser, 2015).

The fundamental principle of NIRS is the Beer-Lambert Law, which relates to the attenuation of light to the properties of the material through which it is travelling. The Beer-Lambert Law can only be applied for fluid solutions; therefore, transmission (T_λ), used for fluids, has to be changed to diffuse reflection (R_λ) when dealing with solid matter. The Beer-Lambert Law reads as follows (Tillmann, 1996; Dolud, 2006):

$$A_\lambda = \log\left(\frac{1}{R_\lambda}\right) = \varepsilon_\lambda \, x \, c \, x \, l$$

With A_λ = absorbance

R_λ = reflection

c = concentration of solution

ε_λ = wavelength-dependent molar absorptivity coefficient

l = light's wavelength in the sample

The electromagnetic spectrum covers a wide range of wavelengths. This range spreads from the energetic, short-wavelength Roentgen (x) radiation (wavelength: 10^{-11} – 10^{-16} m), to the low energy, long wavelength Radio-waves (wavelength: - 10^{7} m). Only a small part of the electromagnetic spectrum can be seen by the human eye as visible light (400 – 750 nanometers (nm)). The visible light is followed by the infrared light, which can be separated into near infrared (NIR), mid-infrared, and far infrared radiation (Figure 1). NIR light ranges from 780 nm to 3 µm and can be divided into IR-A (shortwave NIR) and IR-B (longwave NIR). IR-B is limited due to the absorbance of water at 1400 nm (Stockl, 2013; Baßler and Hauser, 2015).

The principle of NIRS is based on the stimulation of molecular vibrations through electromagnetic radiation. The characteristic absorptions of certain molecules can thus be seen. Stretching-, bending- and deforming-vibrations, resulting from constant movements between atoms and molecules, set the basis for molecular absorptions at different wavelengths (Dolud, 2006; Stockl, 2013). O-H (e.g. water), N-H (e.g. protein), or C-H (e.g. carbohydrate) groups represent the most important molecular groups that absorb or reflect radiation in the IR range and can be measured in the NIR range (Baßler and Hauser, 2015). Figure 2 shows the typical absorption areas of these molecular groups. Depending on the mass of the atoms and their form of binding, different vibrations can occur. NIR cannot detect chemical substances like inert gasses, salts, or symmetric molecules, because they appear inactive in the IR range (Czeslik et al., 2007). The differences in frequencies, derived from the electromagnetic radiation and resulting in overtone- and combination vibrations, offer a classification of different molecular groups and therefore an indirect identification of substances via the recorded spectra (Stockl, 2013). Any recorded NIR spectra stores chemical and physical information about the recorded sample, as well as overlapping bands that result from multiple components or functional groups. In addition, each molecule has a specific NIR spectra. Hence, NIRS can be used to identify and quantify unknown substances (Baßler and Hauser, 2015).

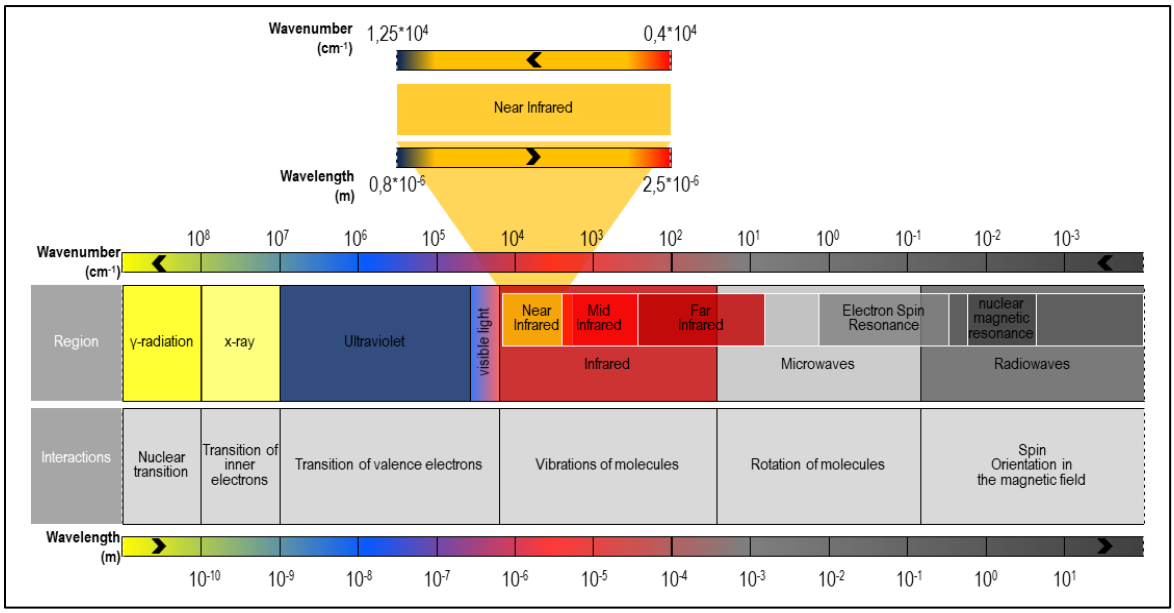

Figure 1: The electromagnetic range separated into regions and interactions (Baßler and Hauser, 2015) (modified).

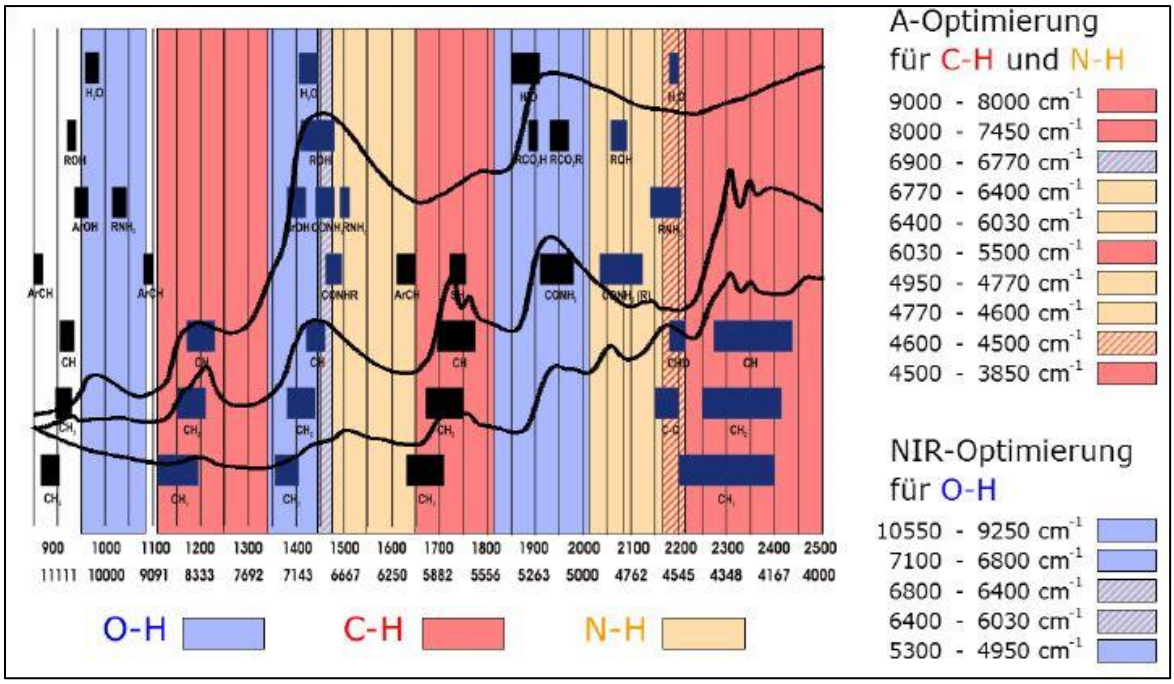

Figure 2: Typical absorption areas of organic substances or molecular groups (Baßler and Hauser, 2015).

Measuring principles

Spectral sensors operate either actively of passively. An active sensor provides its own source of light while passive sensors depend on sunlight. In both types of sensors, the light reflected by the measured product is turned into electromagnetic light. NIRSs can be split into three groups (Dolud, 2006; Erdle et al., 2011):

1. Dispersive Spectrometers: Generate spectra by optically dispersing the incoming radiation into its frequencies or spectral components. They consist of a light source, monochromator, and detector (Saptari, 2003).
2. Fourier-transform Spectrometers: During one scan the whole dataset is captured. An interferogram is established, using an interferometer with a movable mirror, that includes information on all the frequency regions of the NIR range. The Fourier-transformation transforms the data into NIR spectra (Saptari, 2003; Baßler and Hauser, 2015).
3. Diode-Array Spectrometer: The whole NIR spectra can be established using one diode-array (Dolud, 2006).

Both active and passive sensors use the technique explained above and can detect certain molecular groups (Figure 2).

Near infrared spectra evaluation / multivariate calibration

To establish a new NIRS calibration, samples of recorded NIR spectra must be combined with data from chemical analyses (Beebe and Kowalski, 1987). Most NIRSs are supplied with device-specific evaluation software. Most of this software uses multivariate calibration methods, because they store more information about the sample. Multivariate calibration combines spectral information with the information received from reference values. Partial least square (PLS) regression is the most common method used to generate multivariate calibrations (Brown, 1995; Martens and Naes, 2001; Haaland and Thomas, 2002; Conzen, 2005). PLS regression is explained in Haaland and Thomas (2002).

The following steps briefly explain how to establish an NIRS calibration:

1. Generating the spectra: Either a sample is placed on the NIRS sensor or the NIRS sensor is placed above a sample. Rotating sample cups are often used to screen a larger surface area of the sample (Bruker, 2017). The more samples recorded, the better it is for choosing the calibration spectra.
2. Selecting the spectra: The selection of the samples/spectra included in a calibration is a critical process that greatly affects the performance of the calibrations. The samples selected should include all possible variations encountered during prediction. Also, if the calibration is used on a population with large variability in chemical composition and source, the calibration databases should also include a larger number of samples which represent this variability. This will increase the robustness of the calibration, but it will often decrease the accuracy of prediction

(Berzaghi et al., 2017). The sample size should not be below 20 spectra (Conzen, 2005).

3. Combining the spectra and the results of chemical analyses: Once the chosen spectra are uploaded into the calibration software, they must be linked with the results from chemical analyses. For a robust calibration, it is crucial to work very precisely while combining spectra and chemical analyses.
4. Data pretreatment: The selected spectra are pretreated to produce better results during the optimization process. Pretreatment mostly includes the evaluation of the first derivatives or multiple scatter correction (Norris and Williams, 1984; Conzen, 2005; Hoffmann et al., 2017).
5. Selecting frequency areas: To obtain high-quality calibration, the selection of the frequency areas is indispensable. The frequency areas should obtain high correlations between the spectral data and the concentration data (Conzen, 2005).
6. Validation and optimization: The selection of the right data pretreatment is evaluated during the process of validation. Statistical parameters like the coefficient of determination (R^2) and the root mean square error (RMSECV) are calculated. Outliers are also examined after the first validation. Outliers are samples that are not representative of the calibration samples and therefore must be treated with caution (Haaland and Thomas, 2002). For establishing an NIR calibration, a reasonable combination of the statistical data must be selected (Conzen, 2005).
7. Calibration: After removing the outliers and selecting the statistical values, the final calibration will be calculated, most often using a PLS regression (Conzen, 2005; Locher et al., 2005; Lopez et al., 2013).
8. Verification: The calibration model is then validated using new samples that are scanned by the NIRS sensor. The samples are also chemically analyzed to compare the values obtained from chemical analyses to the values predicted by the NIRS sensor. This is called a “test-set” validation. Another possible method of verifying the calibration is “leave one out cross validation”. The software automatically removes one sample from the calibration data set and tests this sample in the calibration. The software performs this routine for every sample in the calibration (Conzen, 2005).

The NIRS calibration is now ready to use.

Application of near infrared spectroscopy in agricultural sciences

Agricultural goods inspection is generally performed by wet chemical analyses of different parameters. Compared to this classical method, NIRS generally requires no sample preparation, it enables easy and fast data collection using a non-destructive technique, and

it can be applied in real time (Berardo, 1997; Bouveresse and Campbell, 2008; Andueza et al., 2011; Krapf et al., 2011). Since NIRS sensors are easy-to-use tools that use fast, accurate methods, different scientific fields and companies apply them in their fields of research or for product screening (Park et al., 1998). For example, chemical companies use NIRS for incoming and outgoing goods inspection (Taris et al., 2015) and the medical sector uses NIRS to determine the oxygen (Jobsis, 1977) and sugar content in blood samples (Yamakoshi et al., 2007). NIRS is also already applied widely in the agricultural sector and the food industry (Williams, 2004). In the agricultural sector large numbers of samples often need to be analyzed quickly, which results in an increasing interest in fast analyzing methods like NIRS (Berardo, 1997).

Various parameters can be detected using NIRS. An NIRS calibration of quality parameters for white clover *(trifolium repens)* feed was established in 1997 to determine parameters like ash, crude protein, crude fiber, and neutral detergent fibers. The calibration was established using an NIR monochromator and by scanning 145 samples. The parameters predicted by the NIRS sensor where highly correlated (e.g. crude protein: $R^2 = 0.96$) with the values of the chemical analyses (Berardo, 1997). Focus has also been put on establishing NIRS calibration models for the chemical compositions of potatoes, silage, forage, grain, rice, meat, and soil (Park et al., 1998; Tillmann et al., 2000; Delwiche et al., 2006; Andueza et al., 2011; Berndt et al., 2011; Lopez et al., 2013; Bagchi et al., 2016; Berzaghi et al., 2017). Various parameters like dry-matter, crude protein, crude fiber, fat, and amylose have been calibrated, but substances such as phosphors could also be detected. As these studies show, NIRS is not only capable of detecting single parameters but can also estimate the percentages of different parameters in samples. Calibrations were also established for liquid manure and to estimate process parameters in biogas plants (Dolud, 2006; Krapf, 2013). NIRS is already used for oilseeds, pellets, and to monitor the legume content in multispecies mixtures (Locher et al., 2005; Berndt et al., 2011). Most of the different agricultural subfields have already tried NIRS. The studies mostly used PLS regression to establish an NIRS model. The studies showed that NIR sensors can detect various organic ingredients if there is an adequate sample size. The studies' reasons for establishing NIRS calibrations can be summarized as the following advantage: Once a calibration is established, NIRS offers a fast and cheap way to analyze products. Expensive wet chemical analyses can thus be reduced. The studies also showed that different temperatures, variations in breeding types, and variations in the particles of the scanned product could affect the final calibration (Martens and Martens, 2001; Martens and Naes, 2001; Kessler, 2007). Delwiche et al. (2006) established an NIRS calibration for inorganic phosphorus in soybeans. They used single beam transmittance on randomly drawn soybean samples and diffuse reflectance on ground soybean meal. Using PLS regression

they established NIR models for single beam transmittance spectra and diffuse reflection spectra. The diffuse reflection spectra of the ground soybeans showed the best performance.

As mentioned above, NIRS is often used in the agricultural sector, hence, it might also be possible to use it for monitoring soybean processing. Using NIR sensors detecting C-H, O-H, and N-H bindings, it should be possible to establish a calibration for the abovementioned quality parameters (TIA, UA, KOH-CP, PDI, and reactive lysine).

1.2 Research Aims

Soybeans must be processed before being fed to monogastric animals to reduce their contents of antinutritive substances. TIA is the most well-known ANF in soybeans and also the most common indicator used during soybean processing. Different feed processing methods, especially heat treatment, decrease the amount of TIA in feed and thus increase the nutritional value (Liener, 1962; Rohe et al., 2017). Nevertheless, Lee and Garlich (1992) as well as Parsons et al. (1992) reported that excessive heat treatment causes denaturation of amino acids, resulting in a loss of protein quality and decreased feed efficiency. However, if inadequately processed, soybeans may still contain ANFs (Heger et al., 2016). Processed soybean batches received from processing plants tend to show unintentional outliers in their TIA values (Clarke and Wiseman, 2005; Zeindl, 2013). The reasons above show the necessity of monitoring soybean processing in real time to control processing and hence produce continuously high quality feed.

Currently, the defined upper limits for soybean products within the diets of fast growing animals are ≤4 mg/g for broiler chickens and ≤4.7 mg/g for pigs (Batterham et al., 1993; Clarke and Wiseman, 2005, 2007). The latest studies show that a decrease in the growth performance of fast growing monogastric animals fed on full fat soy cakes can be seen at a TIA ≤8 mg/g (Heger et al., 2016). However, the available data is quite limited and sometimes contradictory. For example, Huisman and Tolman (2001) recognized adverse effects on the fattening performance of pigs at TIA levels of ≥0.5 mg/g. Therefore, the TIA limits for soybean products within the diets for fast growing monogastric animals should be reevaluated.

This leads to the aims of this dissertation:

- Using current soybean processing methods to produce over-, optimum-, and under-processed soy cake variants and generate soybean batches with varying TIA contents.
- Testing NIRS as a technique to detect TIA in processed soybeans as it is the most common indicator used during soybean processing.
- Using the generated soybean batches to evaluate the nutritional effects of TIA and protein degeneration when feeding soybeans to broiler chickens.

2 Methodological overview and main results

Due to the different goals, this dissertation can be divided into three steps that are all linked to each other to achieve an overall conclusion.

Step 1: Choosing soybean batches/soybean processing

To start, the goal is to process two homogenous batches of soybeans, derived from organic and conventional farming, into soy cakes. The batches will be chosen with regard to the content of TIA in the raw soybeans. The soybean processing should implement the following processing techniques to produce soy cakes: thermal treatment, hydrothermal treatment, and pressure and thermal treatment. Setting different processing parameters within the treatment methods will hopefully result in varying TIA contents in the soy cakes and therefore produce over-, under-, and optimal-treated soy cake variants.

Step 2: Laboratory analyses/near infrared spectroscopy calibration

In the second step the goal is to verify the soy cake's quality by in-vitro analyses. Therefore, the processed soy cake variants will be analyzed in the laboratory for TIA, HDL, and amino acids, as well undergoing Weender analysis. During the soybeans' processing into soy cakes (step 1) their NIR spectra will be recorded. Combined with the in-vitro results of the laboratory analysis an NIRS calibration model for TIA will be established. The more spectra available for the NIR calibration the more robust the calibration will be. Therefore, additional field samples from decentral soybean processing plants will be collected and added to the calibration. Possible influences of breeding or cultivation should thus be eliminated. The NIRS calibration should help to detect the TIA content in the processed soy cakes quickly and easily.

Step 3: in-vivo chicken studies

To test whether current processing parameters are still applicable for the processed soy cake samples, in-vivo broiler growth performance studies will be conducted to gain knowledge about modern broilers' growth performance. Therefore, all the processed soy cake variants will be fed to broilers to determine the growth performance tolerance of different soy cake processing techniques.

Main results

As explained above, the project can be divided into three steps. Step 1 and step 2 are covered by Publication 1 (Hoffmann et al., 2017) and step 3 is covered by Publication 2 (Hoffmann et al., 2019). Linkages between research goals and publications are displayed in Table 5, full content of both publications is displayed in the Appendix, main findings are displayed below.

Step 1: Choosing soybean batches/soybean processing

In a first step two homogenous batches of soybeans were chosen based on their TIA. The first batch, Sultana (conventional farming, harvested in Bavaria, Germany), showed a TIA of 37.3 mg/g and the second batch, Merlin (organic farming, harvested in Romania), 40.5 mg/g. TIA values were analyzed according to DIN (2001).

Both batches were processed using different types of processing with varying parameters, as displayed in Table 5. Additional field samples were collected during the processing of various batches of soybeans to generate a wide spread sample set, using processing methods described in Table 5. Further explanation of conducted processing trials can be found in Publication 1 in the Appendix. Processed soy cake variants were analyzed in the laboratory for TIA, HDL, and amino acids, as well undergoing WEENDER analysis. The processing of the two soybean batches led to a stepwise degradation of TIA which is supported by the results of the KOH analysis as displayed in Figure 3. Hence, under-, over- and optimum treated samples were received.

Table 4: Linkages between research goals and publications

Publication 1	○ Selection of soybean batches ○ Soybean processing ○ In-vitro analyses of soy cake batches ○ Generating NIR spectra ○ Testing the method of NIRS to detect TIA in processed soy cakes
Publication 2	○ Based on soybean processing parameters and in-vitro analyses of Publication 1 ○ Evaluating the influence of varying TIA, HDL, and KOH-CP in soy cake samples on chickens' growth performance during the grower and finisher phases

Table 5: Different types of processing per soybean batch Sultana and Merlin (ST: short time conditioning, LT: long time conditioning, EXP: expander). According to Hoffmann et al. (2017), see Appendix, Publication 1.

Thermal processing [°C; min]	Hydrothermal processing [ST: min, LT: min, Exp.: °C]	Pressure and thermal processing [ST: min, LT: min, Exp.: °C]	Kilning and thermal processing [°C; min]
115; 0.6	ST: 0; LT: 00; Exp.: 0	ST: 1; LT: 00; Exp.: 110	130; 40
120; 0.6	ST: 1; LT: 03; Exp.: 0	ST: 1; LT: 03; Exp.: 110	160; 30
	ST: 1; LT: 12; Exp.: 0	ST: 1; LT: 03; Exp.: 130	190; 20
	ST: 1; LT: 48; Exp.: 0	ST: 1; LT: 12; Exp.: 110	190; 30
		ST: 1; LT: 12; Exp.: 130	
		ST: 1; LT: 48; Exp.: 110	
		ST: 1; LT: 48; Exp.: 130	

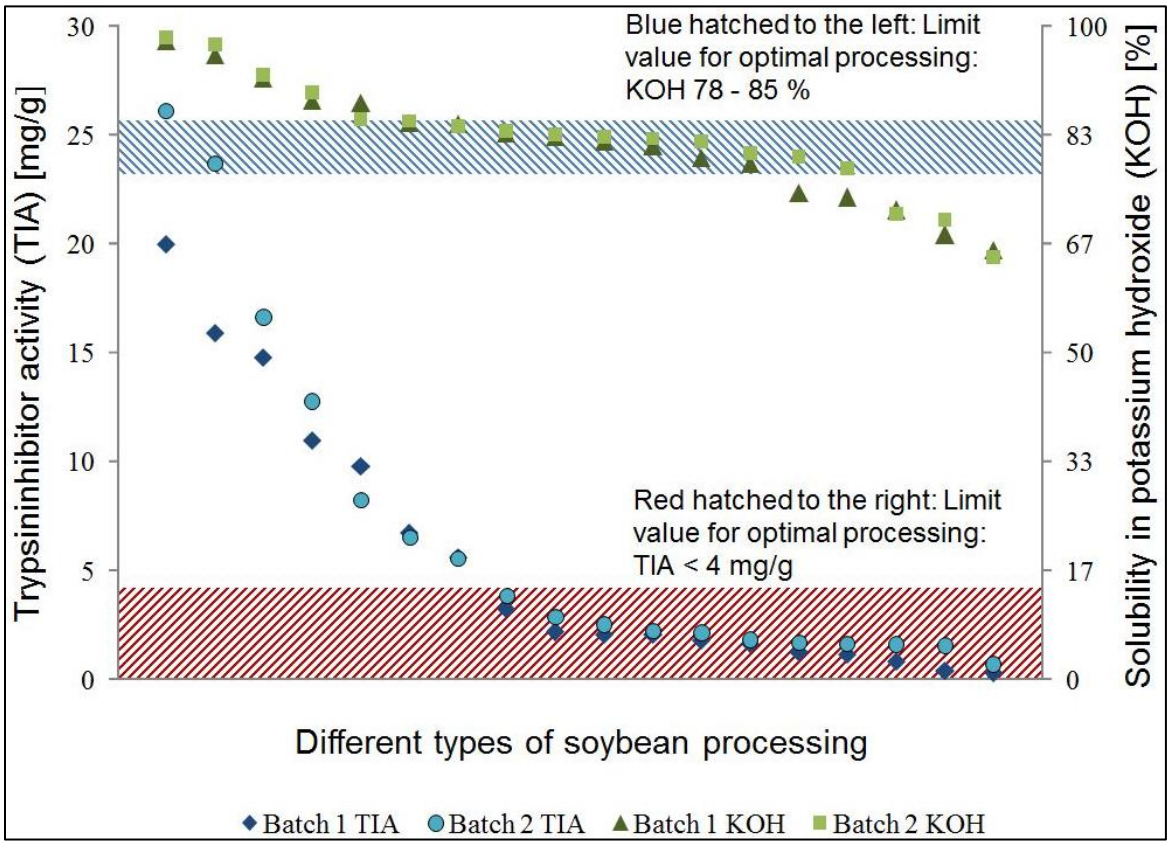

Figure 3: Reducing the activity of trypsin inhibitor (TIA) and the protein solubility in potassium hydroxide (KOH) using different processing types and two different soy batches (table 1). The processing resulted in a stepwise degradation of TIA and KOH. According to Hoffmann et al. (2017), see Appendix, Publication 1.

Step 2: Laboratory analyses/near infrared spectroscopy calibration

During processing trials near NIR spectra were recorded and combined with the laboratory analyses to establish a first NIRS calibration for TIA in processed soy cake as proof of concept. Statistical data of calibration and validation model can be seen below. Further explanation and information on calibration generation can be found in Publication 1 in the Appendix.

The final calibration consists of 151 calibration spectra, based on 200 recorded spectra and 50 analyzed TIA values. The calibration was tested with a leave-one-out cross validation (Martens and Martens, 2001; Martens and Naes, 2001; Conzen, 2005). A test set validation was not conducted at this time due to a lack of samples (Hoffmann et al., 2017). NIRS cross-validation and calibration model for TIA in processed soy cake show the following regression equations:

$$Cross - validation: y = 0.936x + 0.424$$
$$Calibration: y = 0.967x + 0.967$$

Considering the statistical values (Table 6), the established TIA calibration shows that the method of NIRS combined with pre-treatment and PLS offers a good accuracy and allows fast detection of TIA in processed soy cake. Nevertheless, this calibrations serves as proof of concept and further samples have to be integrated in the calibration and a calibration for lower TIA regions only should be considered (Hoffmann et al., 2017).

Table 6: Statistics of PLS models of TIA. Results for cross-validation and calibration contain 151 spectra from 50 samples. According to Hoffmann et al. (2017), see Appendix, Publication 1.

Component	Model	Pre-treatment	Region (cm^{-1})	RMSEE	RMSECV	R^2	BIAS	Offset	Slope	RPD
TIA (mg/g)	Cross-validation	1st+MSC	9400-7496 6104-5448		2.05	93.95	0.0487	0.424	0.936	4.07
	Calibration		4600-4248	1.56		96.74	0.0487	0.214	0.967	5.54

Abbreviations: 1st+MSC: first derivative plus multiple scatter correction (calculated with 17 smoothing points); RMSE: root means square error of cross validation (CV), estimation (E); RPD: ratio of standard deviation of the reference values to the standard error of prediction.

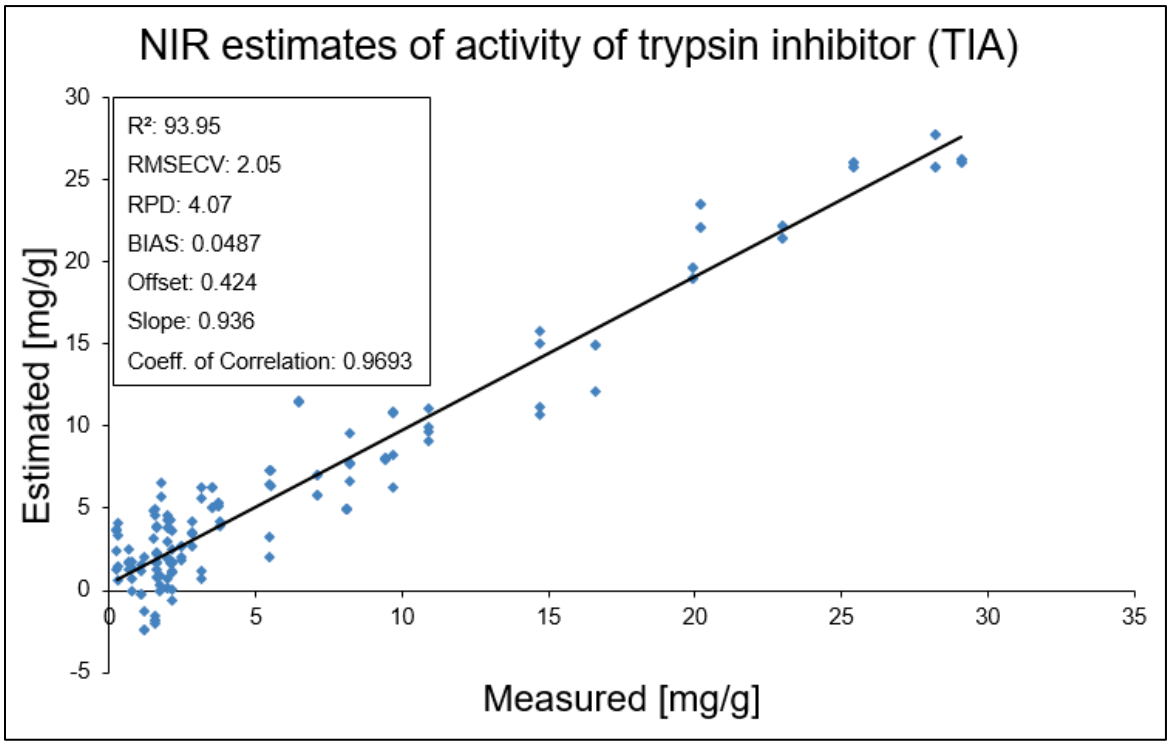

Figure 4: NIR estimates of activity of trypsin inhibitor (TIA) validated via leave-one-out cross validation. According to Hoffmann et al. (2017), see Appendix, Publication 1.

Step 3: in-vivo chicken studies

In a third step, the effect of varying TIA, HDL or KOH concentration in soy cake resulting from the processing trials, was tested on broiler performance and pancreas weight in a 35 d fattening experiment with 1680 broiler chicks (grower phase: d 11 – d 24; finisher phase d 25 – d 35) (Hoffmann et al., 2017; Hoffmann et al., 2019). Hence, the 34 soy cake variants as well as one batch of commercial soybean meal, were used to generate grower and finisher diets for broiler chicken. The inclusion of soybean products into grower and finisher diets was 35 % and 25 %, which resulted in a total of 70 feed batches meeting or exceeding regulations for male Ross 308 broilers with a final weight of 1.7 – 2.4 kg (Aviagen, 2014) (Hoffmann et al., 2019). Information on the animal study and on nutritional concentrations are presented in Publication 2 in the Appendix. The soy cake samples contained TIA contents ranging from 0.25 mg/g to 23.6 mg/g, KOH-CP ranging from 65.5 % to 97.6 %, and HDL contents ranging from 1.40 g/kg to 8.60 g/kg, which also led to a finely-graded range of TIA in complete feed (grower: 0.5 to 8.7 mg/g, finisher: 0.3 to 7.2 mg/g) and, at the same time, different degrees of heat damage to lysine (grower: 0.47 to 3.4 g/kg, finisher: 0.34 to 2.43 g/kg) and KOH (grower: 60.8 % to 84.9 %; finisher: 59.4 % to 88.4 %) (Hoffmann et al., 2019).

Figure 5 shows the relation of HDL and KOH to TIA in complete feed. A stepwise reduction of TIA until a threshold of 1.8 mg/g in grower and 1.4 mg/g in finisher diets did not lead to a correlation with HDL, however, below the thresholds HDL significantly increased with further declining TIA. Statistical data can be seen in Publication 2. Due to the potential for autocorrelation between dietary HDL and TIA below the aforementioned thresholds, all animal related data were divided into pre and post threshold subsets for subsequent regression analysis (Hoffmann et al., 2019).

The main influencing factor on growth performance was TIA. Growth performance was based on total feed intake, total weight gain, feed conversion ratio, live weight, and pancreas weight. Results were evaluated for the grower and finisher phases (Figure 6 and Figure 7). The trials showed that feed processing jointly modifies TIA, HDL, and KOH-CP, but TIA was revealed to be the most important parameter negatively affecting zootechnical performance and pancreas weight at slaughter (d 35), depressing liveweight at d 24 ($P < 0.006$), and d 35 (0.026), weight gain (grower: $P < 0.006$) and feed:gain ratio during grower phase ($P < 0.005$) and increasing pancreas weight ($P < 0.010$) at the time of slaughter. The effect of TIA on the birds' growth performance was constantly present over the whole range of dietary activities, including at very minute concentrations below the currently applied thresholds (≤4 mg/g for broiler chickens and ≤4.7 mg/g for pigs (Batterham et al., 1993; Clarke and Wiseman, 2005, 2007)). Negative effects of TIA were also visible in soy cake variants below recommended thresholds.

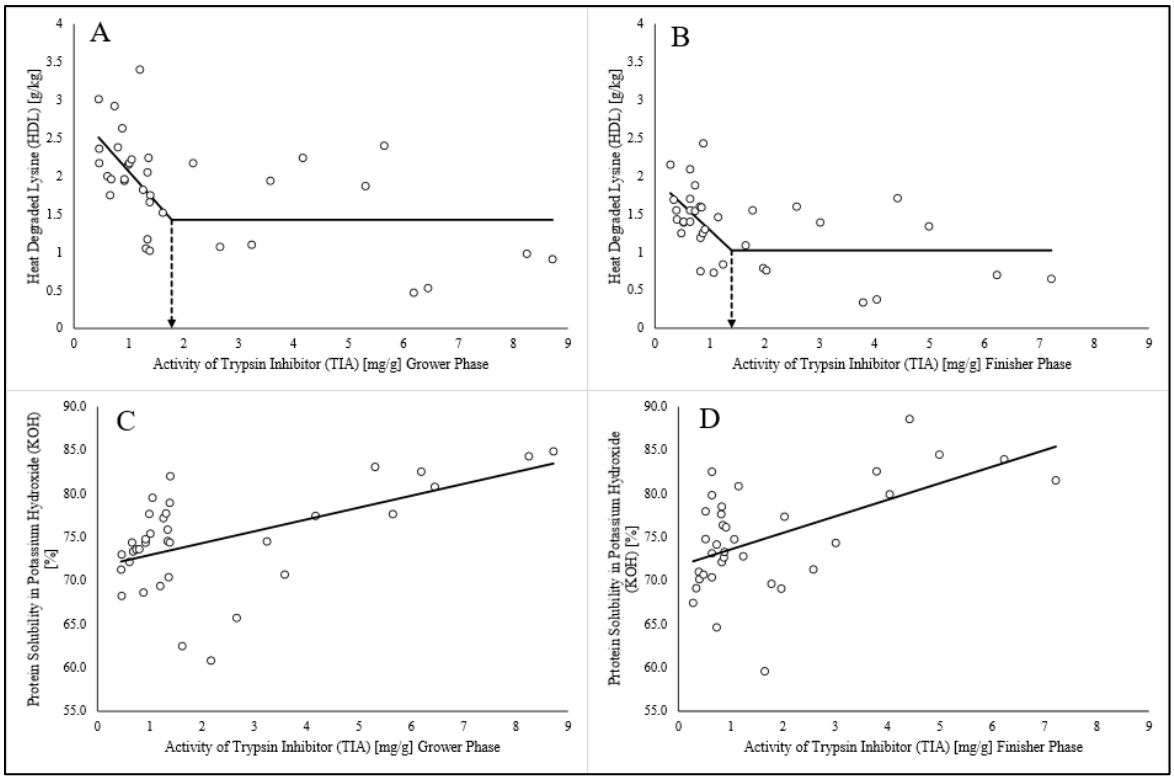

Figure 5: Broken-line regression analyses for data of grower (A) and finisher phase (B) displaying heat degraded lysine (HDL) in relation to trypsin inhibitor (TIA) and regression analyses for data of grower (C) and finisher phase (D) displaying protein solubility in potassium hydroxide (KOH) in relation to trypsin inhibitor (TIA). A breakpoint is detected for grower feed (1.8 mg/g TIA) and finisher phase (1.4 mg/g TIA) indicating a plateau for HDL above the breakpoint. Each dot represents the dietary analysis of each of the 35 feed mixtures. Statistical data see Appendix, Publication 2, Table 2. According to Hoffmann et al. (2019), see Appendix, Publication 2.

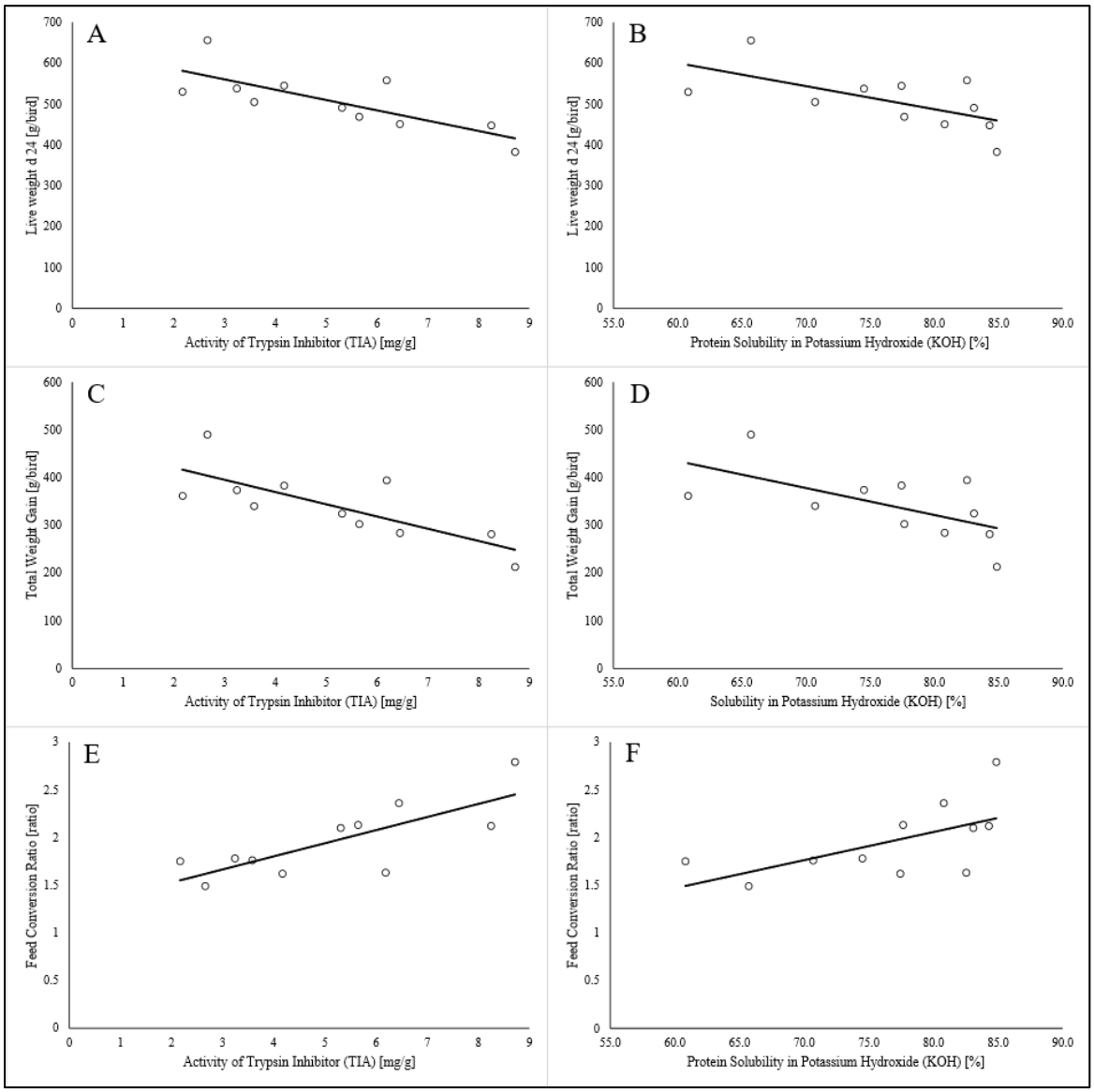

Figure 6: The effect of trypsin inhibitor activity (TIA) and protein solubility in potassium hydroxide (KOH) in processed soy cake fed to broiler chickens on live weight (LW) (A, B), total weight gain (TWG) (C, D) and feed conversion ratio (FCR) (E, F) for dietary treatments with above calculated breakpoints in the grower phase. Statistical data behind graphs see Appendix, Publication 2, Table 3. Each dot represents the mean value of each dietary treatment (n = 11). Treatment means of LW are each calculated on base of n = 48 individual values (bird-wise). Treatment means of TWG and FCR are each calculated on base of n = 6 individual values (cage-wise). According to Hoffmann et al. (2019), see Appendix, Publication 2.

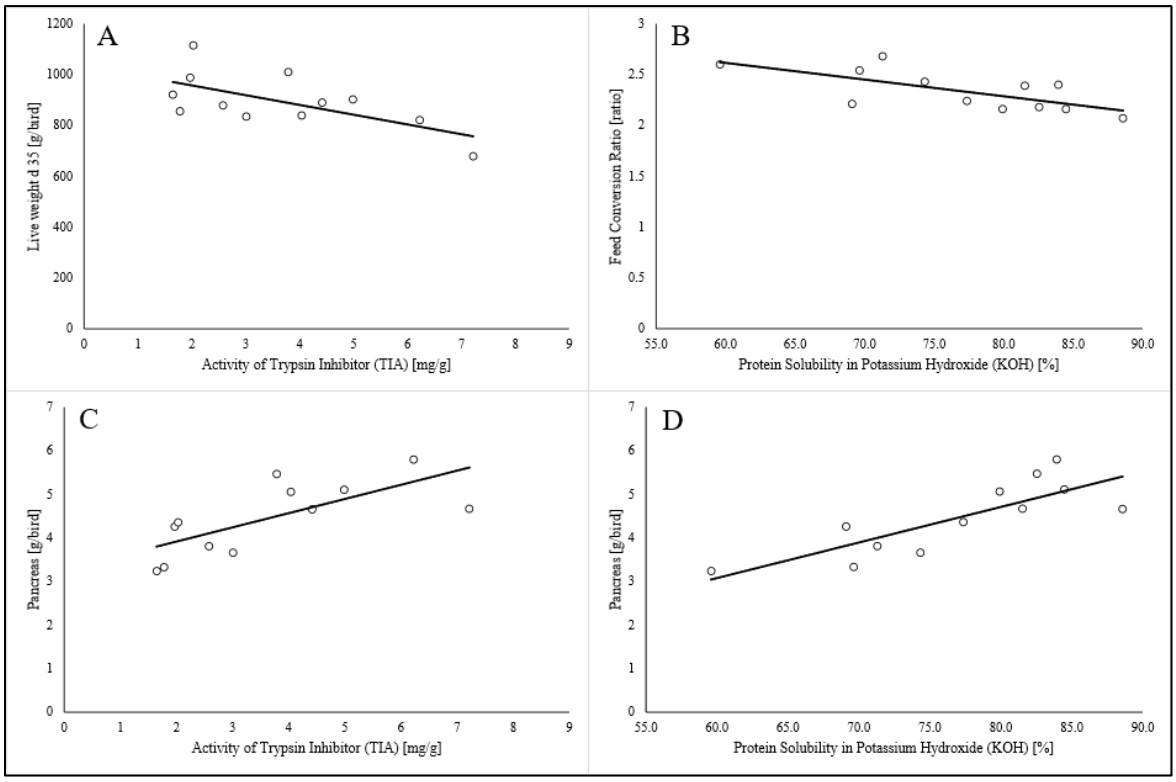

Figure 7: The effect of trypsin inhibitor activity (TIA) and protein solubility in potassium hydroxide (KOH) in processed soy cake fed to broiler chickens on live weight (LW) (A), feed conversion ratio (FCR) (B) and pancreas weight (C, D) for dietary treatments with above calculated breakpoint in finisher phase. Statistical data behind graphs see Appendix, Publication 2, Table 4. Each dot represents the mean value of each dietary treatment (n = 12). Treatment means of LW are each calculated on base of n = 48 individual values (bird-wise). Treatment means of FCR are each calculated on base of n = 6 individual values (cage-wise). According to Hoffmann et al. (2019), see Appendix, Publication 2.

3 General Discussion

Using soybeans in monogastric livestock feeding is gaining more importance due to soybeans' high nutritional value. Nevertheless, soybeans must be processed before being fed to monogastric animals to reduce their contents of antinutritive substances. The most well-known ANF in soybeans is TIA, which is also the most common indicator used in soybean processing. Different feed processing methods, especially heat treatment, decrease the amount of TIA in feed and thus increase the nutritional value (Liener, 1962; Rohe et al., 2017). Nevertheless, Lee and Garlich (1992) as well as Parsons et al. (1992) reported that excessive heat treatment causes the denaturation of amino acids, resulting in a loss of protein quality and decreased feed efficiency. However, under-processed soybeans may still contain ANFs (Heger et al., 2016). Processed soybean batches received from processing plants tend to show unintentional outliers in their TIA values (Clarke and Wiseman, 2005; Zeindl, 2013). The reasons above illustrate the necessity of monitoring soybean processing in real time to control processing and hence produce continuously high quality feed.

Currently, the defined upper limits for soybean products within the diets of fast growing animals are ≤4 mg/g for broiler chickens and ≤4.7 mg/g for pigs (Batterham et al., 1993; Clarke and Wiseman, 2005, 2007). The latest studies show that a decrease in the growth performance of fast growing monogastric animals fed full fat soy cakes can be seen at a TIA ≤8 mg/g (Heger et al., 2016). However, the available data is quite limited and sometimes contradictory. For example, Huisman and Tolman (2001) recognized adverse effects on the fattening performance of pigs at TIA levels of ≥0.5 mg/g. Therefore, the TIA limits for soybean products in the diets for fast growing monogastric animals should be reevaluated.

This leads to the overall aims of this dissertation:

- Using current soybean processing methods to produce over-, optimum-, and under-processed soy cake variants and generate soybean batches with varying TIA contents.
- Testing NIRS as a technique to detect TIA in processed soybeans as it is the most common indicator used during soybean processing.
- Using the generated soybean batches to evaluate the nutritional effects of TIA and protein degeneration when feeding soybeans to broiler chickens.

To meet the aims of this study, three different steps were performed, and are presented below in Publication 1 and Publication 2 in the Appendix. Main findings were summarized above in chapter 2. Step one dealt with soybean processing experiments, in step two

laboratory analyses (in-vitro) were evaluated and an NIRS calibration was established, and in step three processed soybeans were fed to broiler chickens for in-vivo verification. Table 7 shows the main findings of the publications. The findings were already discussed in the above publications' discussions. This general discussion aligns the findings of the two publications in an overall context.

Soybean processing

The ultimate goal of all processing methods is to produce a uniform final product with a minimum content of ANFs, without decreasing the protein quality. Clarke and Wiseman (2005) stated that, due to the wide variation in the processing conditions of soybeans, the amount of TI may vary considerably between batches. Zeindl (2013) supported this finding when testing decentral processing plants and applying the different processing techniques used in those plants. As shown in Publication 1 (Hoffmann et al., 2017) it is possible to process soybeans with different processing techniques and parameters to receive over, under, and optimum treatment, based on quality parameters received from in-vitro analyses. Increasing processing conditions confirmed TIA degradation, as shown by Clarke and Wiseman (2005), among others. In Publication 1 four different processing methods with varying parameters were used and effects on quality parameters could be achieved with all methods. Compared to other studies, over-processing could be reached at KOH-CP values below 70 % (Heger et al., 2016). Since it is possible to process soybeans and receive high quality material, a method that can be used for the fast detection of ANFs was prospected.

Near infrared spectroscopy calibration

NIRS was focused on, since it is a fast method compared to in-vitro laboratory analyses. NIRS generally requires no sample preparation, it enables easy and fast data collection while using a non-destructive technique, and it can be applied in real time (Berardo, 1997; Williams, 2004; Bouveresse and Campbell, 2008; Andueza et al., 2011; Krapf et al., 2011). Many different NIRS calibrations have already been established in agricultural fields (Williams, 2004).

The spectrometer used in this experiment was a Fourier-transform spectrometer, based on reflection measurement. This active sensor captured a whole dataset during one scan. Using a cup with a glass bottom, the sample rotated above the sensor and resulted in even more precise spectra due to the larger sample area that was captured (Saptari, 2003; Baßler and Hauser, 2015; Bruker, 2017). For a strong calibration, the samples selected should include all possible variations encountered during prediction. Also, if the calibration is used over a population with large variability in chemical composition and source, the calibration databases should also include a larger number of samples, which represent the

variability. This will result in increased robustness of the calibration, but it will also likely decrease the accuracy of prediction (Berzaghi et al., 2017). The sample size should not be below 20 spectra (Conzen, 2005).

Thus, receiving widespread quality parameter values during processing experiments, an NIRS calibration could be considered to be established. The more differing values the better, to establish a precise calibration. Therefore, external samples were also collected to obtain a high number of calibration spectra and samples, since the selection of samples/spectra included in a calibration is a critical process that greatly affects the performance of the calibrations.

As seen in Publication 1 a NIRS calibration that can be used to detect the main ANF (TIA) in processed soy cakes could be established. Nevertheless, further samples should be collected to improve the accuracy of the calibration for values below 4 mg/g TIA, which is also the area with the highest possibility of protein degeneration. As on the one hand TIA values should be below 4 mg/g, protein degeneration on the other hand has to be avoided. Taking both restrictions into account, the present calibration's equation is not accurate enough to predict precise values for low TIA regions.

Furthermore, NIRS calibrations for other quality parameters, such as HDL, KOH-CP, UA, and PDI, should be established to allow more detailed control of soybean processing. However, processing is influenced by multiple parameters such as duration, temperature, pressure, and moisture content and thus the NIRS calibration could also be influenced. Taken together, the NIRS technique, once it is established, is a fast and easy method of detecting ANFs in processed soybeans. This tool should be implemented in (decentral) soybean processing plants to guarantee optimum feed quality.

In-vivo chicken studies

According to de Coca-Sinova et al. (2008), one of the biggest problems faced by the feed compound industry is the lack of techniques to correctly evaluate the quality of commercial soybean meal since processing is influenced by many parameters. Therefore, in-vitro quality parameters should be verified with in-vivo chicken growth performance trials to gather updated information about broiler's tolerance to soybeans in different conditions, using different processing methods, as described above. In-vitro quality parameters are based on either the detection of ANFs, or the detection of protein degradation. Since poultry diets are mostly calculated on the basis of crude protein, this seems to be the right procedure. Processing must be examined, since on the one hand excessive heat treatment causes the denaturation of amino acids and on the other hand under-processing leads to a high content of ANFs, both resulting in growth retardation of animals and decreased feed efficiency (Parsons et al., 1992; de Coca-Sinova et al., 2010).

An in-vivo verification of the in-vitro results received in was conducted in Publication 1 and Publication 2 (Hoffmann et al., 2019). Thirty-four soy cake samples of two different soybean breeds, and one soybean meal sample, were analyzed in-vivo for growth performance parameters using male broiler chicks. The soy cake samples contained TIA contents ranging from 0.25 mg/g to 23.6 mg/g, KOH-CP ranging from 65.5 % to 97.6 %, and HDL contents ranging from 1.40 g/kg to 8.60 g/kg. This confirms the large variation in the results received due to different processing conditions.

As the results showed, the main influencing factor on growth performance was TIA. Growth performance was based on the total feed intake, total weight gain, feed conversion ratio, live weight, and pancreas weight. Results were evaluated for the grower and finisher phases. The trials showed that feed processing jointly modifies TIA, HDL, and KOH-CP, but TIA was revealed to be the most important parameter negatively affecting zootechnical performance and pancreas weight. Nevertheless, it must be kept in mind that the indicators of protein degeneration, HDL, and KOH-CP may affect performance only under conditions which induce a limitation in essential amino acids or due to decreasing KOH-CP. Since our experimental diets were composed according to recommendations, it may be assumed that limiting amino acids were provided with some safety margins that compensated for rising HDL and for the potentially depressing effects of KOH-CP on performance.

The effect of TIA on the birds' growth performance was constantly present over the whole range of dietary activities, including at very minute concentrations below the currently applied thresholds (≤4 mg/g for broiler chickens and ≤4.7 mg/g for pigs (Batterham et al., 1993; Clarke and Wiseman, 2005, 2007)). This leads to the conclusion that TIA should be completely eliminated as far as technically possible, even at the expense of protein degeneration.

Taken together, the in-vivo broiler trials showed that the current aims of soybean processing need to be adapted with a focus on TIA elimination. Protein degeneration can be tolerated, since feed can be supplemented with amino acids.

As demonstrated above, the most common ANF in soybeans is TIA. To reduce TIA in soybeans, heat or pressure is most commonly applied. Therefore, not only TIA but also protein degeneration is an important factor to consider in soybean processing, as heat treatment is applied to soybeans. However, this dissertation shows that the reduction of TIA as far as technically possible should be achieved, even at the expense of protein degeneration. TIA can only be eliminated using different processing methods, but degenerated proteins can be supplemented with additional amino acids. Hence, TIA should remain the most important indicator in soybean processing.

Table 7: Main findings in Publications 1 and 2

Publication 1	○ Different processing types can be used to process soybeans and to reach targeted quality parameters ○ Wide variation in the relevant parameters was achieved during processing ○ A proof of concept NIRS calibration was established for TIA in processed soybeans and could be verified
Publication 2	○ Differently processed soy cake samples fed to broiler chickens showed variation in results regarding growth performance parameters ○ The effect of TIA on birds' growth performance was constantly present over the whole range of dietary activities ○ TIA reduction as far as technically possible, even at the expense of protein degeneration, should be achieved

4 General Conclusion

Due to the different goals, this dissertation was divided into three steps that are all linked to achieve overall conclusions. This dissertation showed that soybean processing and the in-vitro verification of processing is crucial before feeding soybeans to fast growing monogastric animals to reduce TIA content. Under-processed soybeans may still contain high amounts of TIA, but over-processing might have led to protein degeneration, resulting in reduced growth performance (Lee and Garlich, 1992; Heger et al., 2016). However, this dissertation showed that the reduction of TIA is more important for broilers' growth performance than the influence of protein degradation. The processing methods used in this dissertation led to the over-, optimum-, and under-processing of soybeans, which could be verified in-vitro. NIRS, with an implemented calibration for TIA, showed that it is possible to detect ANFs in processed soy cakes, but further samples have to be included in the calibration to predict TIA values below 4 mg/g more precisely. For soybean processing, fast detection of ANFs is important to be able to interact with the process of processing. Hence, NIRS should be implemented in processing plants to obtain consistent processing parameters and thus lead to higher quality animal nutrition.

This leads to three overall conclusions:

- The current aims of soybean processing should be adapted with a focus on TIA elimination as far as technically possible.
- Protein degeneration can be tolerated, since feed can be supplemented with amino acids.
- Fast detection of TIA in soybeans using NIRS is recommended to obtain high quality in soybean processing.

4.1 Outlook for Further Research

This dissertation leads to multiple new questions. Therefore, further research should be conducted using different techniques on various topics. Soybean processing was tested using multiple methods and parameters. The established NIRS calibration was able to detect TIA in the processed soy cake samples, but further samples should be collected to improve the accuracy of the calibration for values below 4 mg/g TIA, which is also the area with the highest possibility of protein degeneration. Furthermore, NIRS calibrations for other quality parameters, e.g. HDL, KOH-CP, UA, and PDI, should be established to allow more detailed control of soybean processing with a focus on protein degeneration at low TIA

values. NIRS is a useful tool for the fast control of soybean processing. However, samples need to be placed onto the NIRS sensor by hand. Integration of the NIRS sensor into a processing plant and controlling the plant in real time would be an even faster solution to obtain maximum feed quality.

This study tested processed soy cake samples with in-vivo broiler trials. The animals are monogastric, fast growing, and only live for 35 days. Thus, feeding the samples to either other animal species or to other types of chicken (e.g. laying hens) might lead to different results. Further studies should thus be conducted to feed similar samples to different animals. The study also showed the major influence of TIA on monogastric animals. Different in-vivo trials with added amino acids should be conducted to verify this effect even more precisely.

The quality parameters used today are either designed to detect ANFs or to determine protein quality. This can be referenced, as most poultry diets worldwide are calculated based on crude protein. However, the question arises if there are parameters other than ANFs and protein degeneration that could be used to verify soybean processing.

5 Summary of Publications and Authors' Contributions

Publication 1: Soybean processing and near infrared spectroscopy calibration

Title: Calibration Model for a Near Infrared Spectroscopy (NIRS) System to Control Feed Quality of Soy Cake Based on Feed Value Assessments In-Vitro.

Journal: Chemical Engineering Transactions

DOI: 10.3303/CET1758064

Citation: **Hoffmann, D.[1]**, D. Brugger[2], W. Windisch[2], and S. Thurner[1]. 2017. Calibration Model for a Near Infrared Spectroscopy (NIRS) System to Control Feed Quality of Soy Cake Based on Feed Value Assessments In-Vitro. Chemical Engineering Transactions 58:379-384. doi: 10.3303/CET1758064

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Authors' contributions

Stefan Thurner contributed the idea for the project and helped to decide what soybean processing methods and parameters should be used. He also helped to verify the established NIRS calibration. Daniel Brugger and Wilhelm Windisch contributed with ideas regarding the soybean processing and the chemical analyses and parameters. Dominik Hoffmann managed the processing trials, generated the NIR spectra, established the NIRS calibration, and wrote the journal publication.

Summary

This publication deals with step 1 and step 2 of this dissertation, described in chapter 2. When harvested from the field, soybeans and the products derived thereof may contain significant amounts of TIs. To reduce these substances in livestock feeding, different

processes (e. g. heat treatment) may be used. However, exposing soybeans to excessive over treatment could result in glycosylation of amino acids (especially lysine) which impairs feed protein utilization.

The goal of the present project was to optimize the treatment of soybeans in decentral processing plants by implementing an NIR calibration system. Therefore, two different batches of soybeans were chosen based on their content of TI. The soybeans were processed into partly de-oiled soy cakes using four different approaches. To receive a robust NIR calibration, each method of processing was adapted to produce over treatment, under treatment, and optimal treatment of soybeans. The feed quality of the soy cake variants was assessed using laboratory analyses. The NIR spectra, recorded along with the processing of soybeans into partly de-oiled soy cakes, were combined with the laboratory analyses to be able to establish an NIRS calibration for the TIA in soy cakes. The more spectra available for an NIR calibration the more robust the calibration will be. Therefore, additional field samples from decentral soybean processing plants were collected and added to the calibration. Possible influences of breeding or cultivation should thus have been eliminated. For a sample size of 50 samples, 200 spectra were recorded and analyzed. After pre-treatment of the spectra and PLS regression analysis, the calibration was automatically tested with leave-one-out validation. The result showed that the NIRS combined with pre-treatment and PLS offered a good accuracy (R^2 = 93.95 %) and allowed fast detection of TIA in processed soy cakes. The study also showed that it is possible to establish an NIRS calibration for TIA in soy products processed at decentral processing plants. Nevertheless, more samples should be implemented into the calibration to make the calibration model more stable. Moreover, further calibration factors like crude protein, oil content, and water content, as well as KOH-CP should be integrated into the main calibration to be able to predict more information during soybean processing. Further steps would include implementing an NIRS sensor in a decentral processing plant to realize real time process control in order to guarantee optimum feed quality.

Publication 2: Chicken growth performance trials

Title: Chickens' growth performance and pancreas development exposed to soy cake varying in trypsin inhibitor activity, heat-degraded lysine concentration, and protein solubility in potassium hydroxide

Journal: Poultry Science

DOI: 10.3382/ps/pey592

Citation: **Hoffmann, D[1].**, S. Thurner[2], D. Ankerst[3], K. Damme[4], W. Windisch[1], and D. Brugger[1]. 2019. Chickens' growth performance and pancreas development exposed to soy cake varying in trypsin inhibitor activity, heat-degraded lysine concentration, and protein solubility in potassium hydroxide. Poultry Science. doi: 10.3382/ps/pey592

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Authors' contributions

Stefan Thurner contributed the idea for the project and helped to set up the feeding trials. Daniel Brugger and Wilhelm Windisch helped design the growth performance experiments. Wilhelm Windisch explained how to establish scientific feed ratios to receive mixed feeding variants. He also controlled the calculated feed ratios. Klaus Damme provided the facilities to conduct the chicken growth performance trials. He also helped to plan the trials and the barn arrangement. Donna Ankerst established the statistical certificate to allow the study to

be approved by the District Government in the Free State of Bavaria, Germany. She also rechecked the statistical analyses shown in the publication. Daniel Brugger and Dominik Hoffmann conducted the statistical analyses shown in this study. Dominik Hoffmann established the feed ratios, planned and managed the chicken trials and data generation, managed the data after the trial, and wrote the publication.

Summary

This publication deals with step 3 of this dissertation, described in Chapter 2 Methodological overview and. The goal of this study was to show the influence of differently treated soy cakes on broiler chickens' growth performance to determine which factor should be focused on during processing. Soy cake samples were received from the experiments described in Publication 1.

This study focused on the effect of varying trypsin inhibitor activity, heat-degraded lysine concentration and protein solubility in potassium hydroxide on broiler performance and pancreas weight. Two soybean breeds were subject to varying thermal, hydrothermal, and pressure and kilning processing. This resulted in a total of 34 soy cake variants, widely varying in their trypsin inhibitor activity (0.25 to 23.6 mg/g), heat degraded lysine (1.40 to 8.60 g/kg), and protein solubility in potassium hydroxide (65.5 to 97.6 %). These soy cake variants, as well as a commercial soybean meal extract, were included into a common grower and finisher diet for broiler chicks at fixed amounts (grower: 35 %; finisher: 25 %) and tested in a 35 d fattening experiment with 1680 broiler chicks (grower phase: d 11 – d 24; finisher phase d 25 – d 35). TIA was the dominant factor affecting zootechnical performance and pancreas weight at slaughter (d 35), depressing liveweight at d 24 ($P < 0.006$) and d 35 (0.026), reducing weight gain (grower: $P < 0.006$) and feed:gain ratio during grower phase ($P < 0.005$), and increasing pancreas weight ($P < 0.010$). The negative effects of TIA were also visible in soy cake variants with TIA below the recommended thresholds. This highlights the necessity of complete elimination of TIA in broiler diets, as far as technically possible.

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Appendix

Both publications are pasted below, as published in respective journals.

Publication 1

Calibration Model for a Near Infrared Spectroscopy (NIRS) System to Control Feed Quality of Soy Cake Based on Feed Value Assessments In-Vitro

This study was accepted and published as a peer-reviewed journal article in the Journal Chemical Engineering Transactions. Full content is displayed below.

Hoffmann, D., D. Brugger, W. Windisch, and S. Thurner. 2017. Calibration Model for a Near Infrared Spectroscopy (NIRS) System to Control Feed Quality of Soy Cake Based on Feed Value Assessments In-Vitro. Chemical Engineering Transactions 58:379-384.
DOI: 10.3303/CET1758064

CHEMICAL ENGINEERING TRANSACTIONS
VOL. 58, 2017
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Remigio Berruto, Pietro Catania, Mariangela Vallone

ISBN 978-88-95608-52-5; **ISSN** 2283-9216
DOI: 10.3303/CET1758064

Calibration Model for a Near Infrared Spectroscopy (NIRS) System to Control Feed Quality of Soy Cake Based on Feed Value Assessments in-Vitro

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When harvested from the field, soybeans and products derived thereof may contain significant amounts of trypsin inhibitors. In order to reduce these substances in livestock feeding different processes (e. g. heat treatment) may be used. However, exposing the soybean to an excessive over treatment could result in glycosylation of amino acids (especially lysine) which impairs feed protein utilization. The goal of the present project was to optimize the treatment of soybeans in decentral processing plants by implementing a near infrared calibration system. Therefore, two different batches of soybeans where processed into partly de-oiled soy cake using four different approaches. To receive a robust near infrared calibration, each way of processing was adapted in order to produce over treatment, under treatment and optimal treatment of soybeans. The feed quality of the soy cake variants was assessed using laboratory analyses. The near infrared spectra, recorded along with the processing of soybeans into partly de-oiled soy cake, were combined with the laboratory analyses to be able to establish a near infrared spectroscopy (NIRS) calibration for the trypsin inhibitor activity (TIA) in soy cake. Therefore, for a sample size of 50 samples, 200 spectra were recorded and analyzed. After pre-treatment of the spectra and partial least square (PLS) regression analysis the calibration was automatically tested with a leave-one-out validation. The result showed that the method of NIRS combined with pre-treatment and PLS offered a good accuracy (R^2 = 93.95 %) and allowed fast detection of TIA in processed soy cake.

1. Introduction

Locally produced feed is gaining more importance in monogastric livestock feeding especially when regarding protein sources. Due to its amino acid composition with high amounts of essential amino acids like lysine, soy is a very valuable component in monogastric livestock feeding. Nevertheless, the raw untreated soybean contains significant amounts of trypsin inhibitors. Heat treatment is commonly used to reduce the activity of these substances to a minimum tolerable (Kraft et al. 2013). It was shown by (Ahmed 2001) and (Heger et al. 2016) that the intensity of heat treatment had a significant influence on the feed's digestibility and therefore also on the quality of the feed. Soybeans fed to fast growing monogastric animals should contain a trypsin inhibitor activity (TIA) not higher than 5 mg/g (Monari et al. 1993; Clarke and Wiseman 2005, 2007; Batterham et al. 1993). The latest studies show that a decrease in growth performance of fast growing monogastric animals for full fat soy cake can be seen at a TIA below 8 mg/g (Heger et al. 2016). If heat treatment is applied in excess it may also lower the feed's quality due to amino acid glycosylation (Faldet et al. 1992; Adrian 1974). Therefore heat treatment of raw soybeans and products derived thereof like full fat soy cake or partly de-oiled soy cake needs to be optimized. Near infrared spectroscopy (NIRS) is often used in the agricultural sector especially for feed quality control or biogas processes control (Delwiche et al. 2006; Krapf et al. 2011; Fontaine et al. 2001). Different soybean batches, different temperatures and different sizes of soybean particles might affect the final calibration (Kessler 2007; Martens und Martens 2001; Martens and Naes 2001; Zhang and Zhang 2015). Evonik Industries AG already established a NIR calibration for grounded soy

Please cite this article as: Hoffmann D., Brugger D., Windisch W., Thurner S., 2017, Calibration model for a near infrared spectroscopy (nirs) system to control feed quality of soy cake based on feed value assessments in-vitro, Chemical Engineering Transactions, 58, 379-384
DOI: 10.3303/CET1758064

products in laboratory use. Therefore, the aim of this study is to test a first calibration for processed soybeans that can be used for optimizing the heat treatment at decentral soybean processing plants.

2. Material and Methods

2.1 Soybean processing / sample generation

At the beginning of the project the focus was put on the different processing techniques for soybeans. Therefore, two homogenous batches of soybeans Sultana (conventional farming, harvested in Bavaria, Germany) and Merlin (organic farming, harvested in Romania) where chosen based on their TIA (Sultana: 37.3 mg/g; Merlin: 40.5 mg/g; (Deutsches Institut für Normung - Normenausschuss Lebensmittel und landwirtschaftliche Produkte 2002). Both batches were processed as follows (table 1):
Thermal treatment: The raw untreated soybeans were slightly moistened and then toasted (temperature 115 °C, duration 40 s). Following the thermal treatment the soybeans were cooled down and de-oiled by mechanical force. The thermal treatment was conducted at the facilities of Gerauer OHG (Kirchham, Germany), the deoiling at MEIKA Tierernährung GmbH (Großaitingen, Germany). Four final samples and 16 NIR spectra could be established.
Hydrothermal treatment: The raw, untreated soybeans were treated with steam (temperature 103 °C, duration 40 min). After the hydrothermal treatment the soybeans were cooled down and de-oiled by mechanical force. The hydrothermal treatment was conducted at the facilities of Amandus Kahl GmbH & Co. KG (Reinbeck, Germany), the de-oiling at MEIKA. Eight final samples and 32 NIR spectra could be established.
Pressure and thermal treatment: At first the raw untreated soybeans were mechanically de-oiled. Afterwards they were treated with steam (temperature 103 °C, duration 10 min) and extruded with an expander (temperature 130 °C, duration 1 – 5 s). The soy cake was then cooled down after processing. The pressure and thermal treatment was conducted at the facilities of Amandus Kahl, the de-oiling at MEIKA. 14 final samples and 56 NIR spectra could be established.
Kilning and thermal treatment: The raw, untreated soybeans were first treated with heat. During this process the revoked steam was circulated around the soybeans (temperature 160 °C, duration 30 min). The soybeans were de-oiled by mechanical force after this process. The kilning and thermal treatment as well as the de-oiling was conducted at the facilities of EST GmbH (Mühlberg, Austria). Eight final samples and 32 NIR spectra could be established.
Field samples: Since the new calibration should be used at decentral processing plants, different field samples were collected during the processing of various batches of soybeans. 14 samples and 56 NIR spectra could be established at the facilities of Rieder Asamhof GmbH & Co. KG (Kissing, Germany) using hydrothermal treatment parameters as described above.

Table 1: Different types of processing per soybean batch Sultana and Merlin (ST: short time conditioning, LT: long time conditioning, Exp: expander)

Thermal processing [°C; min]	Hydrothermal processing [ST: min, LT: min, Exp.: °C]	Pressure and thermal processing [ST: min, LT: min, Exp.: °C]	Kilning and thermal processing [°C; min]
115; 0.6	ST: 0; LT: 00; Exp.: 0	ST: 1; LT: 00; Exp.: 110	130; 40
120; 0.6	ST: 1; LT: 03; Exp.: 0	ST: 1; LT: 03; Exp.: 110	160; 30
	ST: 1; LT: 12; Exp.: 0	ST: 1; LT: 03; Exp.: 130	190; 20
	ST: 1; LT: 48; Exp.: 0	ST: 1; LT: 12; Exp.: 110	190; 30
		ST: 1; LT: 12; Exp.: 130	
		ST: 1; LT: 48; Exp.: 110	
		ST: 1; LT: 48; Exp.: 130	

Parameters chosen for laboratory analysis were TIA, solubility in potassium hydroxide (KOH) and the WEENDER analysis. For the first calibration, presented in this paper, only the TIA was chosen.

2.2 Near infrared spectroscopy calibration

During each step of the soybeans' processing into soy cake NIR spectra were recorded. In combination with the results of the laboratory analysis (TIA) a calibration model with the calibration software OPUS 7.5 by Bruker Optics GmbH was established.

2.2.1 Near infrared spectrometer

The processed soybeans were recorded by a TANGO FT-NIR Spectrometer (Bruker Optics GmbH 2017). The NIR sensor used in this experiment is capable of recording spectra by reflection measurements at a spectral range between 11500 and 4000 cm^{-1}. A cup, also provided by Bruker Optics GmbH, with a glass bottom was fully filled with processed soybeans. Once the soybean was processed, two warm and two cold NIR spectra were recorded. Therefore the temperature was measured with an infrared thermometer (Testo, 830-T2) and the cup was rotating on the NIR sensor in order to record a higher quality spectra.

2.2.2 Near infrared spectra evaluation

All processed soybeans were chemically analyzed for TIA at the laboratory of the Chair of Animal Nutrition (Technical University of Munich) following DIN EN ISO 14902:2002-02. Since there were two warm and two cold spectra per analysed sample, four spectra per processing step were available for the evaluation using the software OPUS. In total, 200 spectra were available for the calibration (Figure 2, left). At first, data pre-treatment was conducted which included the evaluation of the first derivative, multiple scatter correction (MSC) and 17 smoothing points (Figure 2, right). A Partial Least Square (PLS) regression was performed with OPUS as described by Conzen (2005). The calibration was automatically tested by a leave-one-out cross validation (Martens and Martens 2001). The final calibration was determined from an optimisation routine of OPUS after the removal of the outliers. During the optimisation step, various frequency regions and also spectral pre-treatments were systemically tested to determine the optimal calibration (Krapf et al. 2011). During the optimisation process the maximum number of PLS Components was restricted to 10. The model performance was assessed by the following statistical parameters (Conzen 2005):

Coefficient of determination (R^2): R^2 indicates the proportion of the variance in Y, that is accounted for by the model. The higher the value for R^2, the better the correlation between the variance of the concentration and the spectral data.

Root Mean Square Error of Cross Validation (RMSECV): The RMSECV indicates the square root of the mean square error of the cross validation. This indicates how precise the value of the samples is presumed during the internal validation.

BIAS: Indicates the ordinate of a regression line. The closer the bias gets to 0, the better the calibration.

RPD value: This value indicates the suitability of the calibration for the prediction. With a higher RPD value the calibration will more likely be able to predict the right sample values.

Offset: The offset variable is the log of the time period under study with a regression coefficient of 1.

Slope: The slope indicates the steepness of a line. The greater the magnitude, the steeper the line.

Coefficient of Correlation: The coefficient of correlation predicts the degree to which changes to the value of one variable predict change to the value of another.

3. Results and discussion

3.1 Soybean processing / sample generation

The processing of the two batches of soybeans resulted in a stepwise degradation of the TIA. As shown in Figure 1, samples with over, under and optimum treatment could be generated. The results of the KOH analysis of the processed batches Merlin and Sultana support the gradient from over to under and optimum treatment. A NIR spectra could be recorded for every different step during processing. Soybeans fed to fast growing monogastric animals should contain a TIA not higher than 5 mg/g (Monari et al. 1993; Clarke and Wiseman 2005, 2007). New studies suggest a possible TIA as high as 8 mg/g before growth depressions occur (Heger et al. 2016). The protein dispersibility (KOH) reduces while intensifying the heat treatment (Faldet et al. 1992). KOH should therefore be kept between 78 and 85 % (Van Eys, 2012).

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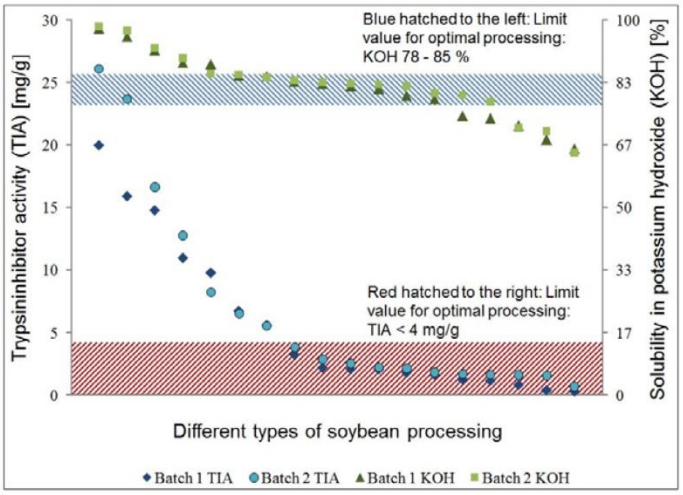

Figure 1: Reducing the activity of trypsin inhibitor (TIA) and the protein solubility in potassium hydroxide (KOH) using different processing types and two different soy batches (table 1). The processing resulted in a stepwise degradation of TIA and KOH.

3.2 Near infrared spectroscopy calibration

Figure 2 (left) shows all of the recorded spectra measured from 11000 to 4000 cm^{-1}. A very similar progression of the spectra can be seen. The final calibration consists of 151 calibration spectra, based on 200 recorded spectra and 50 analyzed TIA values. The data pre-treatment included the evaluation of the first derivative, multiple scatter correction (MSC) and 17 smoothing points (figure 2, right). After pre-treatment the following regions were used for optimisation as indicated by vertical lines in figure 2 (middle): 9400 - 7496 cm^{-1}, 6104 - 5448 cm^{-1}, 4600 - 4248 cm^{-1}. Those regions were chosen, based on the software's optimization routine. The white areas show the areas included in the calibration (figure 2, middle). The calibration was tested with a leave-one-out cross validation (Conzen 2005; Martens and Martens 2001, Martens and Naes 2001). A test set validation was, not conducted at this time due to a lack of samples.

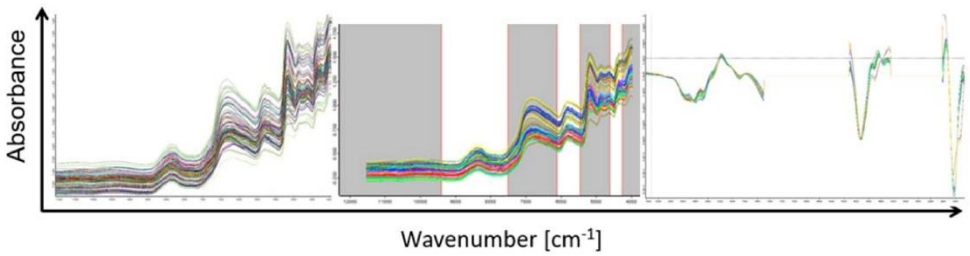

Figure 2: Left: the untreated spectra as recorded by the NIRS Sensor in the 11000 cm^{-1} to 4000 cm^{-1} region. Middle: For PLS regression the following regions were used for the optimisation as indicated by vertical lines: 9400 - 7496 cm^{-1}, 6104 - 5448 cm^{-1}, 4600 – 4248 cm^{-1}. The grey areas indicate those areas excluded from the calibration. Right: the spectra after pre-treatment with the first derivative, multiple scatter correction (MSC) and 17 smoothing points.

Considering the statistical values (R^2: 93.95; RMSECV 2.05; BIAS: 0.0487; RPD 4.07; slope: 0.788; offset 0.424; coefficient of correlation 0.9693, figure 3) the established TIA calibration shows that the method of NIRS combined with pre-treatment and PLS offers a good accuracy and allows fast detection of TIA in processed soy cake (figure 3). Also comparing the statistical values to other soybean calibrations or other calibrations in general, the established statistical parameters of this calibration show good results (Delwiche et al. 2006; Krapf et al. 2011; Zhang and Zhang 2015). The RPD value (RPD: 4.07) is solid but could still be

higher for a robust calibration but this may also be due to the multiple constitutions of the soybeans after the different processing steps. All spectra were recorded right after the soybeans processing. The soybeans were not grounded but used as they turned out after the process. Different sizes in soybean particles, different batches in general and different processing parameters might have also affected the outcome of the calibration (Fontaine et al. 2001; Kessler 2007; Martens und Naes 2001; Haaland and Thomas 1988). In order to generate a more stable calibration (higher R^2 and better RPD value) more processed soybean samples need to be recorded. Furthermore, a test set validation should be undertaken for the established calibration for external verification.

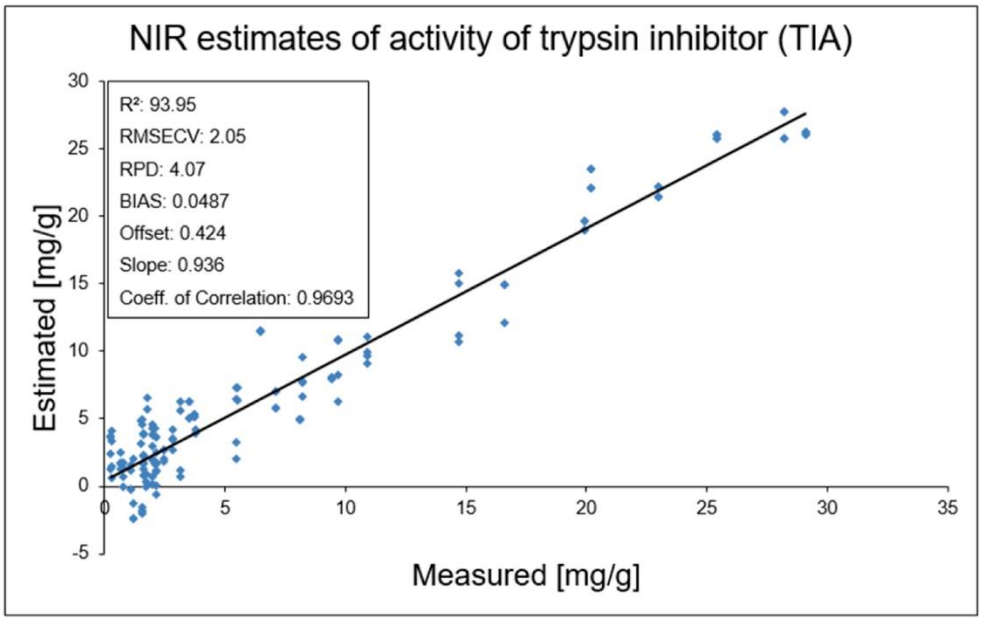

Figure 3: NIR estimates of activity of trypsin inhibitor (TIA) validated via leave-one-out cross validation.

4. Conclusions

All different processing types used in this experiment can be applied to process soybeans and to reach the targeted goals for optimum processing (especially for TIA and KOH). In order to reliably reach those goals in decentral processing plants an online process control should be established. The study also showed that it is possible to establish a NIRS calibration for TIA in soy products processed at decentral processing plants. Nevertheless, more samples should be implemented in the calibration to make the calibration model more stable. Therefore, 60 more samples are being chemically analysed at the moment and further samples will be collected at decentral processing plants. Moreover, further calibration factors like crude protein, oil content, water content as well as solubility in potassium hydroxide will be integrated in the main calibration. A further step of this project will be to implement a NIRS sensor in a decentral processing plant and to realize online process control in order to guarantee optimum feed quality.

Acknowledgments

The project was supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program. Thanks to Georg Peter who acquired the field samples within the scope of his Bachelor Thesis.

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Publication 2

Chickens' growth performance and pancreas development exposed to soy cake varying in trypsin inhibitor activity, heat degraded lysine concentration, and protein solubility in potassium hydroxide

This study was accepted and published as a peer-reviewed journal article in the Journal of Poultry Science. Full content is displayed below.

Hoffmann, D., S. Thurner, D. Ankerst, K. Damme, W. Windisch, and D. Brugger. 2019. Chickens' growth performance and pancreas development exposed to soy cake varying in trypsin inhibitor activity, heat-degraded lysine concentration, and protein solubility in potassium hydroxide. Poultry Science.
DOI: 10.3382/ps/pey592

Chickens' growth performance and pancreas development exposed to soy cake varying in trypsin inhibitor activity, heat-degraded lysine concentration, and protein solubility in potassium hydroxide

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ABSTRACT This study focused on the effect of varying trypsin inhibitor activity (**TIA**), heat-degraded lysine concentration and protein solubility in potassium hydroxide on broiler performance and pancreas weight. Two soybean breeds were subject to varying thermal, hydrothermal, pressure, and kilning processing. This resulted in a total of 34 soy cake variants, widely varying in TIA (0.25 to 23.6 mg/g), heat-degraded lysine (1.40 to 8.60 g/kg), and potassium hydroxide (65.5 to 97.6%), respectively. These soy cake variants as well as a commercial soybean meal extract were included into a common grower and finisher diet for broiler chicks at fixed amounts (grower: 35%; finisher: 25%) and tested in a 35 d fattening experiment with 1680 broiler chicks (grower phase: day 11 to 24; finisher phase day 25 to 35). TIA was the dominant factor affecting zootechnical performance and pancreas weight at slaughter (day 35), depressing liveweight at day 24 ($P < 0.006$), and day 35 (0.026), weight gain (grower: $P < 0.006$) and feed: gain ratio during grower phase ($P < 0.005$) and increasing pancreas weight ($P < 0.010$) at the time of slaughter. Negative effects of TIA were also visible in soy cake variants below recommended thresholds. This highlights the necessity of complete elimination of TIA in broiler diets as far as technically possible.

Key words: soybean, heat degraded lysine, trypsin inhibitor, broiler, growth performance

2019 Poultry Science 0:1–14
http://dx.doi.org/10.3382/ps/pey592

INTRODUCTION

Globally, soybean is the most important feed protein compound in the diets of monogastric livestock (Lee et al., 2007). Nevertheless, the utilization of raw soybeans for feed is mainly limited by the presence of protease inhibitors, which interfere with intake, digestion, absorption, and metabolism of nutrients as well as with health status of the animal (Liener, 1994; Clarke and Wiseman, 2005). In this context, pancreatic hypertrophy and pancreatitis have been recognized in growing animals in response to activities of protease inhibitors (Applegarth et al., 1964; Kakade et al., 1973; Clarke and Wiseman, 2005; Clarke and Wiseman, 2007). These inhibitors and in particular trypsin inhibitors (**TI**) require heat treatment in order to become inactivated (Liener, 1994). The trypsin inhibitor activity (**TIA**) varies among different soybean breeds and ranges around 70 to > 100 mg/g (Vollmann et al., 2003). During growth of the soybean plant, TIA is further affected by environmental and genetic factors (Vollmann et al., 2003).

Different feed processing methods, especially a combination of heat and pressure, decrease TIA and thus increase the nutritional value of respective feedstuffs (Liener, 1962; Rohe et al., 2017). On the other hand, Parsons et al. (1992) reported that excessive heat treatment causes denaturation of amino acids, resulting in reduced protein quality and lower feed efficiency. In this context, lysine appears to be the amino acid that is most vulnerable to heat due to Maillard reaction (e.g., epsilon-fructose lysine) (Adrian, 1974). Therefore, indicators used to refer to protein degeneration are protein solubility in potassium hydroxide (**KOH**) or heat-degraded lysine (**HDL**). With an increase in processing, KOH decreases and HDL increases, respectively.

Current upper limits tolerable for TIA in soybean products fed to monogastric livestock are considered to be ≤ 4 mg/g for broiler chickens and ≤ 4.7 mg/g for pigs (Batterham et al., 1993; Clarke

Received August 15, 2018.
Accepted December 18, 2018.
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and Wiseman, 2005). However, available data on the matter are quite limited and sometimes contradictory. For example, Huisman and Tolman (2001) already recognized adverse effects in fattening performance of pigs at TIA levels of ≥0.5 mg/g. Furthermore, there has been to our knowledge only one investigation by Heger et al. (2016) on the interaction between heat degraded amino acids and TIA on animal performance. Parsons et al. (1991) found that a decrease in chickens' growth performance could be seen when KOH was below 59% and generally seems to be critical below 70% according to Araba and Dale (1990).

Therefore, the goal of this study was to assess the dose-response relationship between TIA, HDL, and KOH, respectively, and zootechnical performance and pancreas weight of broilers in high resolution under practical feeding conditions.

MATERIALS AND METHODS

Soybean Processing

Two homogenous batches of each soybean breed (breed 1: Sultana; origin: conventional farming, harvested in Bavaria, Germany; breed 2: Merlin; origin: organic farming, harvested in Romania) were subject to a total of 17 different combinations of thermal, hydrothermal, pressure, and kilning processing variants in order to vary levels of TIA, HDL, and KOH, respectively. Treatments comprised over-, under- and optimum treatment of soy cake. Table A1 gives an overview of processing parameters as published earlier in detail by Hoffmann et al. (2017). In addition to the 34 soy cake variants, 1 batch of commercial soybean meal was included into the study, thus resulting in a total of 35 differentially processed soybean products. Respective concentrations of nutrients, TIA, HDL, and KOH are presented in Table A2.

Experimental Diets

The 35 soybean products were used to compose grower and finisher feeds for boiler chicks meeting or exceeding the feeding recommendations for male Ross 308 broilers with a final weight of 1.7 to 2.4 kg (Aviagen, 2014) (Table 1). Inclusion of soybean products into grower and finisher diets accounted for 35 and 25% of final feed, respectively. In total, 70 feed batches were produced (35 dietary variants within grower feed and finisher feed, respectively). Basal dietary components without soybean products were purchased from a commercial feed mixer (Likra West, Ingolstadt, Germany) and final feeds were completed at the experimental feed mixing plant of University of Hohenheim (Germany). Concentrations of nutrients, TIA, HDL, and KOH of the final grower and finisher feeds are presented in Table A3 and A4, respectively.

Table 1. Feed composition of grower and finisher diets and analyzed nutrient contents of grower and finisher basal components.

Fed ratio	Grower diet	Finisher diet
Soy cake sample/soybean meal reference (%)	35.0	25.0
Basal components (%)	65.0	75.0
Ingredients (%)		
Soy cake sample/soybean meal reference (%)	35.0	25.0
Maize	50.7	45.4
Wheat	11.1	26.3
Monocalcium phosphate	1.17	1.35
Calcium carbonate	0.83	0.89
Premix[2]	0.63	0.50
Sodium bicarbonate	0.36	0.41
Soybean oil	0.20	0.23
Sodium chloride	0.07	
Analyzed nutrients of basal components (%)		
KOH-CP[3]	56.4	66.3
ME[4] (MJ/kg)	13.2	13.0
Crude protein	8.80	9.30
Crude ash	5.50	5.50
Crude fat	3.90	3.50
Crude fiber	1.10	1.90
Calcium	0.91	0.91
Phosphor	0.76	0.76
Threonine	0.30	0.30
Lysine	0.25	0.30
TIA[5] (mg/g)	0.20	0.20
Sodium	0.18	0.15
Methionine	0.15	0.18
Tryptophan	0.08	0.10

[1]Analyzed nutrient concentrations and TIA of soy products and fed diets are displayed in Tables A2 to 4.

[2]Content per kg base diet: 12,500 IU vit. A; 5500 IU vit. D3; 125 mg vit E (α-tocopherol acetate); 62.5 mg L-Carnitine; 50 mg Fe (iron-(II)- sulphate); 125 mg Zn (zinc sulphate); 150 mg Mn (manganese-(II) sulphate); 20 mg Cu (copper-(II)-sulphate); 1.6 mg I (calcium iodate); 0.4 mg Se (sodium selenite); propyl gallate (E310); fumaric acid (E-297); calcium formate (E238); citric acid (E330); calcium lactate (E327); orthophosphoric acid (E338); propionic acid (E-280); 20,000 BXU Endo-1.4-ß-Xylanase; 625 FTU 3-phytase.

[3]KOH-CP: amount of potassium hydroxide soluble crude protein.

[4]ME (MJ/kg): Metabolizable Energy (ME) concentration was estimated on base of feed table information (DLG, 2018).

[5]TIA: trypsin inhibitor activity.

Animals and Housing

The animal study was reviewed and approved by responsible welfare authorities of the District Government of Lower Frankonia, Federal State of Bavaria, Germany (registered case number: 55.2–2532-2–331). The animal field trials were conducted at the Department for Education and Poultry Research, Bavarian State Research Center for Agriculture (Lower Frankonia, Germany).

Two consecutive and identical experimental runs with all 35 mixtures were conducted to provide an appropriate sample size. In each experimental run, a total of 840 male broiler chicks (1680 birds in total) (Ross 308; Aviagen Group, Huntsville, AL, USA) were obtained from a commercial hatchery (Brüterei Süd, Regenstauf, Germany). The mean BW at d 0 after hatching during the first experimental run was 44.0 (SD = 2.3) g and during the second run 40.9 (SD = 1.8) g. Birds were weighed and raised for the first 10 days in 3 big cages of 280 chicks/cage. Cages were bedded with

straw pellets. All birds were fed a commercial soybean meal based starter diet containing 12.4 MJ ME/kg and 21.5% crude protein from day 0 to 10, which met or exceeded all the nutrient requirements of ROSS 308 broilers according to Aviagen (2014).

At day 11 birds were individually weighed, wing tagged, and assigned to 35 individual grower diets in a balanced block design with BW as blocking factor yielding 105 separate blocks of 8 animals each. The mean initial BW at day 11 during the first experimental run was 174.5 g (SD = 21.8) and during the second run 157.8 g (SD = 17.4). Birds were fed the assigned experimental grower diet from day 11 to 24 and the experimental finisher diet from day 25 to 35. The mean initial BW at day 25 was 645.4 g (SD = 183.3) and 614.9 g (SD = 160.4) for the first and second experimental run, respectively. Cages (1.6 m^2/cage) were bedded with straw pellets. Birds had ad libitum access to feed and water during all stages of the study. Light program and ventilation were adjusted due to recommendations for Ross broilers by Aviagen (Aviagen, 2015). From day 11 to 35 birds received additional vitamin supplementation via the drinking water supply (Bela-Multivit AD3E forte: 0.1 ml/bird/d, Vechta, Germany; Biozink soluble: 1 g/l water, Vechta, Germany). Both sets of birds were vaccinated on day 16 for Newcastle Disease (Avipro ND LASOTA, Elanco Deutschland GmbH, Germany) and Infectious Bronchitis (Nobilis IB Ma5, MSD Tiergesundheit, Germany). On day 35 all birds were killed using electrical stunning and cervical dislocation.

In summary, the study comprised 35 different feeds, each of them represented by 6 cages (3 per experimental run) with 8 birds/cage.

Parameters of Zootechnical Performance

At day 1, 10, 24, and 35, birds were individually weighed to evaluate the liveweights (**LW**) at given time points as well as changes in total weight gain (**TWG**) over time. Furthermore, health conditions were evaluated according to Knierim et al. (2016). Total feed intake (**TFI**) was recorded per cage and production phase (grower, finisher), to estimate the feed conversion ratio as feed: gain (**FCR**). At day 35, 6 of 8 birds per cage were dissected after slaughtering and the pancreas of each bird was weighed.

Chemical Analyses

All chemical analyses were carried out in duplicates. TIA, KOH-soluble crude protein (KOH-CP) and crude nutrient analyses were determined for raw soybeans, processed soy cake samples, as well as in the experimental diets according to published standard procedures (DIN, 2001; VDLUFA, 2012). The amount of reactive (= non-glycosylated) lysine in soybean products was analyzed by the homoarginine method according to Pahm et al. (2008) and used to calculate the total contents of HDL in soybean products and final feed mixtures, respectively. Amino acids (except for tryptophan and tyrosine) were analyzed by ion-exchange chromatography according to Brugger et al. (2016).

Statistical Analyses

Data of experimental run 1 and 2 were merged to one common dataset. Individual animal data were pooled within cage and the mean values per cage were considered as the statistical replicate (n = 6 per feed variant). A preceding multi-factorial ANOVA analysis (data not shown) did not indicate any significant effect of the soybean breed; therefore, we excluded this aspect from all subsequent data analysis.

Data of the 35 variants of grower and finisher diets were statistically analyzed using descriptive statistics, linear regression analysis (y = a + bx), and, if applicable, broken-line regression analysis. Since broken-line regression analysis distinguished 2 ranges of dietary TIA with and without presence of correlation to HDL, the complete dataset was split into 2 subsets, each one comprising data derived from diets containing TIA below and above the respective breakpoint. Each of this subsets ('below breakpoint', 'above breakpoint') was individually analysed using linear regression. The threshold of significance was considered to be $\alpha \leq 0.05$ for all statistical procedures.

RESULTS

Dietary Parameters

Differential feed processing yielded a finely-graded range of TIA in complete feed (grower: 0.5 to 8.7 mg/g, finisher: 0.3 to 7.2 mg/g) and, at the same time, different degrees of heat damage to lysine (grower: 0.47 to 3.4 g/kg, finisher: 0.34 to 2.43 g/kg) and KOH (grower: 60.8 to 84.9%; finisher: 59.4 to 88.4%) (Table A2).

Figure 1 shows the relationship of HDL and KOH, respectively, to TIA in complete feed. Table 2 presents the statistical measures of the regression models in addition to Figure 1. Stepwise reduction in dietary TIA until a threshold of 1.8 mg/g in grower diets and 1.4 mg/g in finisher diets did not correlate to HDL. However, below the respective thresholds, HDL significantly increased with further declining TIA (slopes: -0.81 ± 0.33, $P < 0.02$ for grower and slopes: -0.67 ± 0.34, $P = 0.05$ for finisher diets, respectively). The latter was statistically evident for the grower diets ($P = 0.02$) and showed a similar trend in finisher diets ($P = 0.05$). It was not possible to estimate a broken-line regression for the relationship between KOH and TIA in complete feed. Therefore, we analyzed it using linear regression, which expressed a significant direct relationship in grower (slope = 1.37 ± 0.36, $P = 0.0006$) and finisher diets (slope = 1.94 ± 0.50, $P = 0.0005$), respectively (Figure 1, Table 2).

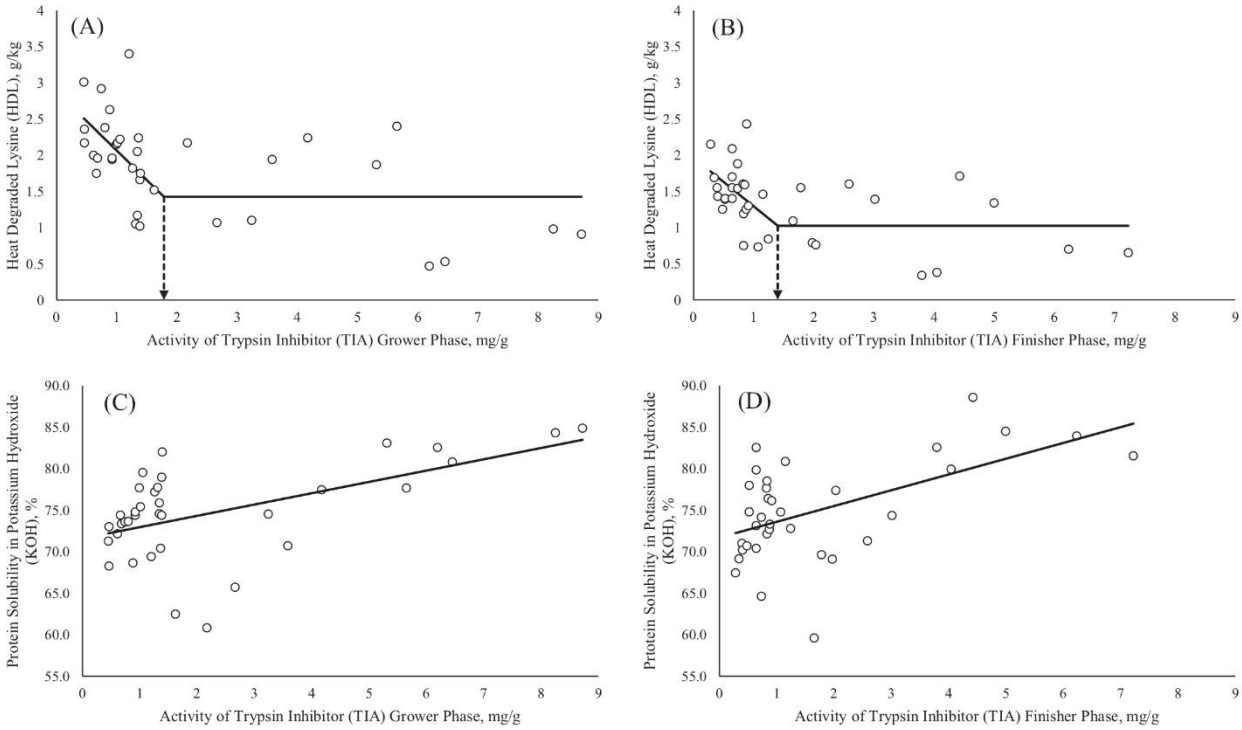

Figure 1. Broken-line regression analyses for data of grower (A) and finisher phase (B) displaying heat-degraded lysine (HDL) in relation to trypsin inhibitor (TIA) and regression analyses for data of grower (C) and finisher phase (D) displaying protein solubility in potassium hydroxide (KOH) in relation to trypsin inhibitor (TIA). A breakpoint is detected for grower feed (1.8 mg/g TIA) and finisher phase (1.4 mg/g TIA) indicating a plateau for HDL above the breakpoint. Each dot represents the dietary analysis of each of the 35 feed mixtures. Statistical data can be seen in Table 2.

Table 2. Broken-line regression analyses to examine the correlation between trypsin inhibitor activity (TIA) and heat-degraded lysine (HDL) in grower and finisher phase and regression analyses to examine the correlation between activity of TIA and protein solubility in potassium hydroxide (KOH).

	Regression models	Parameter estimates	*P* values	$^1R^2$
Heat-degraded lysine (HDL), g/kg, grower Phase	$y = a + bx$ for $x \leq X_B$ $y = Y_B$ for $x > X_B$	a, 2.87 ± 0.17 b, −0.81 ± 0.33 X_B, 1.78 ± 0.40	<0.0001 0.0200 <0.0001	0.31
Heat-degraded lysine (HDL), g/kg, finisher Phase	$y = a + bx$ for $x \leq X_B$ $y = Y_B$ for $x > X_B$	a, 1.96 ± 0.12 b, −0.67 ± 0.34 X_B, 1.40 ± 0.41	<0.0001 0.0547 0.0015	0.29
Solubility in potassium hydroxide (KOH), %, grower phase	$y = a + bx$	a, 1.37 ± 71.6 b, 1.37 ± 0.30	<0.0001 0.0006	0.30
Solubility in potassium hydroxide (KOH), %, finisher phase	$y = a + bx$	a, 1.94 ± 71.6 b, 1.94 ± 0.50	<0.0001 0.0005	0.31

a: intercept; b: regression factor x and y.
[1]Coefficient of determination of the respective regression model.

Due to the potential for autocorrelation between dietary HDL and TIA below the aforementioned thresholds, all animal related data were divided into pre- and post-threshold subsets for subsequent regression analysis.

Zootechnical Performance and Pancreas Weight

All animals performed well, and no veterinary interventions were necessary during the whole trial. Overall mortality rate for the first and second experimental run was 1.4% and 1.3%, respectively.

Ranges of LW, TWG, TFI, and FCR at the end of the grower phase revealed a high degree of variation, from 383 to 798 g/bird, 213 to 633 g/bird, 550 to 759 g/bird, and 1.10 to 2.79, respectively (Table A5).

In groups fed with diets containing TIA of 1.8 mg/g and below during the grower phase, linear regression analyses revealed no significant relationship between zootechnical performance and TIA, HDL, or KOH, respectively (Table 3). In strong contrast, animals

Table 3. Regression models of live weight (LW), total weight gain (TWG), total feed intake (TFI), and feed conversion ratio (FCR) of broilers as affected by varying ratios of dietary trypsin inhibitor activity (TIA), heat-degraded lysine (HDL) concentrations, and solubility in potassium hydroxide (KOH) during grower feed.

	Trypsin inhibitor activity (TIA)	Regression models	Estimates of a	Estimates of b	$^1R^2$
Data of grower feed below breakpoint (1.8 mg/g TIA)	Live weight (LW) at day 24, g/bird	$y = a + bx$	a, 717 ± 42.8 ($P < 0.0001$)	b, −30.4 ± 40.2 ($P = 0.4581$)	0.03
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 546 ± 42.3 ($P < 0.0001$)	b, −31.2 ± 39.8 ($P = 0.4406$)	0.03
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 693 ± 29.9 ($P < 0.0001$)	b, −12.5 ± 28.0 ($P = 0.6596$)	0.01
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 1.29 ± 0.09 ($P < 0.0001$)	b, 0.08 ± 0.09 ($P = 0.3948$)	0.03
Data of grower feed above breakpoint (1.8 mg/g TIA)	Live weight (LW) at day 24, g/bird	$y = a + bx$	a, 636 ± 38.9 ($P < 0.0001$)	b, −25.2 ± 7.03 ($P = 0.0059$)	0.59
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 472 ± 39.8 ($P < 0.0001$)	b, −25.6 ± 7.19 ($P = 0.0061$)	0.59
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 674 ± 28.4 ($P < 0.0001$)	b, −7.22 ± 5.13 ($P = 0.1925$)	0.18
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 1.25 ± 0.21 ($P < 0.0002$)	b, 0.14 ± 0.04 ($P = 0.0049$)	0.60
	Heat-degraded lysine (HDL)				
Data of grower feed below breakpoint (1.8 mg/g TIA)	Live weight (LW) at day 24, g/bird	$y = a + bx$	a, 599 ± 48.5 ($P < 0.0001$)	b, 42.5 ± 22.8 ($P = 0.0750$)	0.13
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 431 ± 48.3 ($P < 0.0001$)	b, 40.5 ± 22.7 ($P = 0.0876$)	0.13
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 643 ± 16.5 ($P < 0.0001$)	b, 18.0 ± 16.5 ($P = 0.2866$)	0.05
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 1.55 ± 0.11 ($P < 0.0001$)	b, -0.09 ± 0.05 ($P = 0.0787$)	0.14
Data of grower feed above breakpoint (1.8 mg/g TIA)	Live weight (LW) at day 24, g/bird	$y = a + bx$	a, 498 ± 53.0 ($P < 0.0001$)	b, 6.65 ± 33.6 ($P = 0.8568$)	0.01
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 6.24 ± 54.0 ($P < 0.0002$)	b, 6.65 ± 34.22 ($P = 0.8502$)	0.01
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 654 ± 26.75 ($P < 0.0001$)	b, -11.7 ± 17.0 ($P = 0.5075$)	0.05
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 2.13 ± 0.28 ($P < 0.0001$)	b, −0.12 ± 0.18 ($P = 0.4995$)	0.05
	Solubility in Potassium Hydroxide (KOH)				
Data of grower feed below breakpoint (1.8 mg/g TIA)	Live weight (LW) at day 24, g/bird	$y = a + bx$	a, 439 ± 245 ($P < 0.0863$)	b, 3.35 ± 3.31 ($P = 0.3218$)	0.05
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 304 ± 243.7 ($P < 0.2260$)	b, 2.86 ± 3.29 ($P = 0.3948$)	0.03
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 440 ± 165 ($P < 0.0142$)	b, 3.25 ± 2.24 ($P = 0.1603$)	0.09
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 1.46 ± 0.55 ($P < 0.0145$)	b, −0.01 ± 0.01 ($P = 0.8703$)	0.01
Data of grower feed above breakpoint (1.8 mg/g TIA)	Live weight (LW) at day 24, g/bird	$y = a + bx$	a, 936 ± 180 ($P < 0.0006$)	b, −5.60 ± 2.34 ($P = 0.0402$)	0.39
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 770 ± 185 ($P < 0.0025$)	b, 5.61 ± 2.41 ($P = 0.0449$)	0.38
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 742 ± 113.8 ($P < 0.0001$)	b, 1.37 ± 1.48 ($P = 0.3794$)	0.09
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, -0.03 ± 0.98 ($P < 0.7765$)	b, 0.03 ± 0.01 ($P = 0.0475$)	0.37

a: intercept; b: slope of model.
[1]Coefficient of determination of the respective regression model.

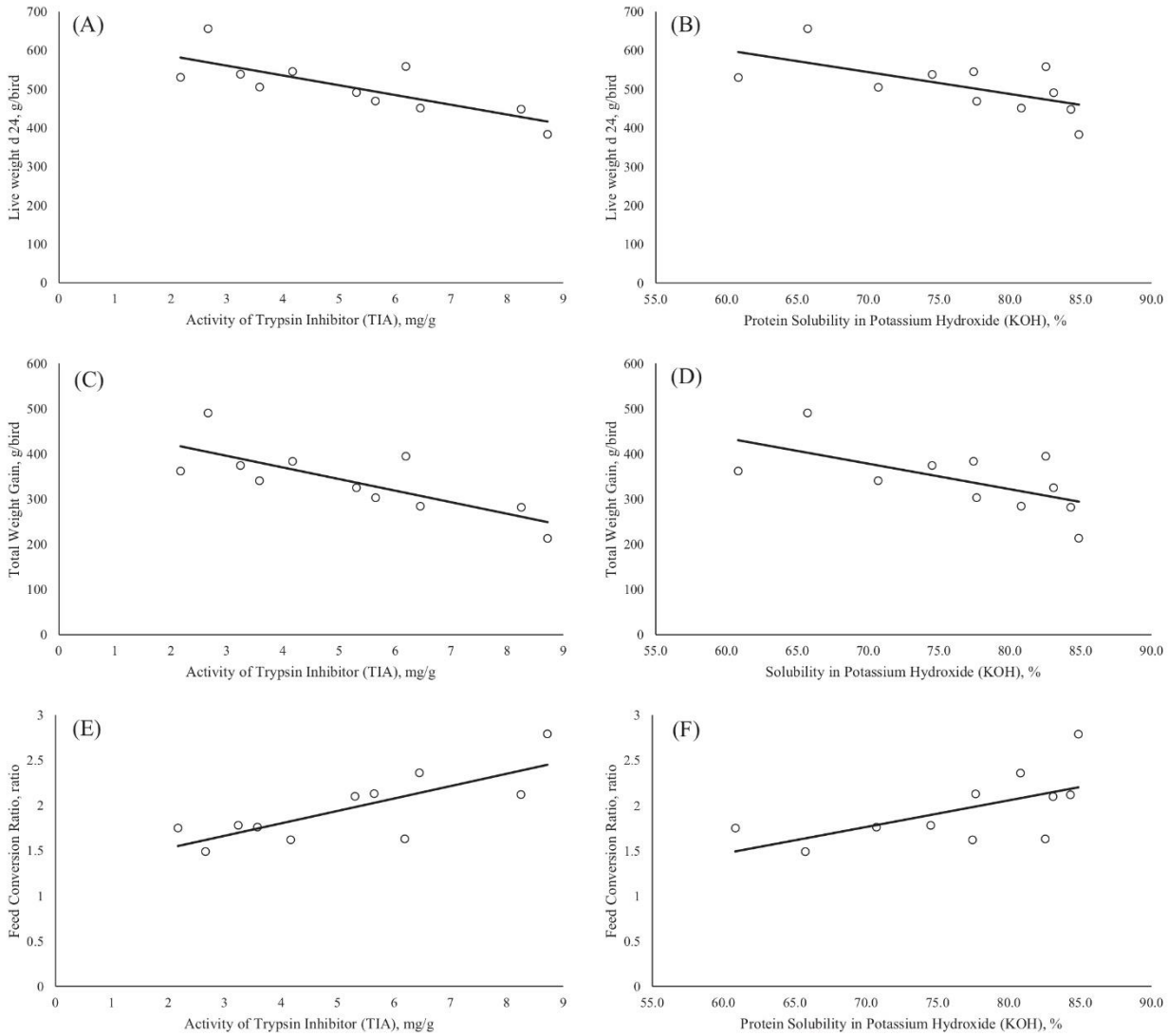

Figure 2. The effect of trypsin inhibitor activity (TIA) and protein solubility in potassium hydroxide (KOH) in processed soy cake fed to broiler chickens on live weight (LW) (A, B), total weight gain (TWG) (C, D), and feed conversion ratio (FCR) (E, F) for dietary treatments with above calculated breakpoints in the grower phase. Statistical data behind graphs can be seen in Table 3. Each dot represents the mean value of each dietary treatment (n = 11). Treatment means of LW are each calculated on base of n = 48 individual values (bird-wise). Treatment means of TWG and FCR are each calculated on base of n = 6 individual values (cage-wise).

challenged with TIA above 1.8 mg/g experienced an inverse linear response of LW (slope = −25.2 ± 7.03, $P = 0.0059$) and TWG (slope = −25.6 ± 7.19, $P < 0.0061$) (Figures 2 A, C, Table 3) and a direct response of FCR (slope = 0.14 ± 0.04, $P < 0.0049$) with decreasing TIA (Figure 2 E, Table 3), whereas TFI was not affected whatsoever (Table 3). Comparable response patterns were also evident in these groups as demonstrated in the relationship between dietary KOH and LW (slope = −5.60 ± 2.34, $P < 0.0402$) and TWG (slope = 5.61 ± 2.41, $P < 0.0449$) as well as for FCR (slope = 0.03 ± 0.01, $P < 0.0475$) (Figures 2 B, D, F, Table 3), but not for TFI. HDL did not affect zootechnical performance in grower phase within groups receiving dietary TIA >1.8 mg/g (Table 3).

At the end of the finisher phase, LW, TWG, TFI, and FCR exhibited ranges from 679 to 1317 g/bird, 290 to 590 g/bird, 699 to 1201 g/bird, and 1.98 to 3.33, respectively. Groups receiving dietary TIA 1.4 mg/g and below during finisher phase exhibited no significant changes of zootechnical parameters in response to dietary TIA, HDL, and KOH, respectively (Table 4). In contrast, above this threshold LW was negatively and significantly correlated to TIA (slope = 35.4 ± 14.6, $P < 0,0255$) (Figure 3 A, Table 4), whereas FCR directly responded to KOH (slope = −0.02 ± 0.01, $P < 0.014$) in a linear fashion (Figure 3 B, Table 4). All other parameters were neither significantly affected by TIA nor HDL (Table 4).

Table 4. Regression models of live weight (LW), total weight gain (TWG), total feed intake (TFI), feed conversion ratio (FCR), and pancreas weight (at day of slaughter) of broilers as affected by varying ratios of dietary trypsin inhibitor activity (TIA), heat-degraded lysine (HDL) concentrations, and solubility in potassium hydroxide (KOH) during finisher feed.

	Trypsin inhibitor activity (TIA)	Regression models	Estimates of a	Estimates of b	$^1R^2$
Data of finisher feed below breakpoint (1.4 mg/g TIA)	Live weight (LW) at day 35, g/bird	$y = a + bx$	a, 1199 ± 72.3 ($P < 0.0001$)	b, −33.7 ± 95.8 ($P = 0.7208$)	0.01
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 488 ± 41.4 ($P < 0.0001$)	b, −18.97 ± 54.8 ($P = 0.7325$)	0.01
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 1089 ± 66.47 ($P < 0.0001$)	b, −30.4 ± 88.0 ($P = 0.7331$)	0.01
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 2.26 ± 0.20 ($P < 0.0001$)	b, 0.20 ± 0.27 ($P = 0.4662$)	0.03
	Pancreas weight, g/bird	$y = a + bx$	a, 3.85 ± 0.26 ($P < 0.0001$)	b, 0.24 ± 0.35 ($P = 0.5045$)	0.02
Data of finisher feed above breakpoint (1.4 mg/g TIA)	Live weight (LW) at d35, g/bird	$y = a + bx$	a, 1034 ± 59.1 ($P < 0.0001$)	b, −38.4 ± 14.6 ($P = 0.0255$)	0.41
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 402 ± 40.8 ($P < 0.0001$)	b, −6.74 ± 10.1 ($P = 0.5198$)	0.04
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 949 ± 59.4 ($P < 0.0001$)	b, −23.5 ± 14.7 ($P = 0.1409$)	0.20
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 2.45 ± 0.13 ($P < 0.0001$)	b, −0.03 ± 0.03 ($P = 0.3612$)	0.08
	Pancreas weight, g/bird	$y = a + bx$	a, 3.27 ± 0.41 ($P < 0.0001$)	b, 0.33 ± 0.10 ($P = 0.0090$)	0.51
	Heat-degraded lysine (HDL)				
Data of finisher feed below breakpoint (1.4 mg/g TIA)	Live weight (LW) at d35, g/bird	$y = a + bx$	a, 1038 ± 85.6 ($P < 0.0001$)	b, 91.6 ± 55.6 ($P = 0.1145$)	0.11
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 408 ± 45.0 ($P < 0.0001$)	b, 45.2 ± 32.3 ($P = 0.1770$)	0.09
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 950 ± 79.2 ($P < 0.0001$)	b, 79.4 ± 51.5 ($P = 0.1377$)	0.10
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 2.74 ± 0.25 ($P < 0.0001$)	b, −0.23 ± 0.16 ($P = 0.1654$)	0.09
	Pancreas weight, g/bird	$y = a + bx$	a, 3.80 ± 0.33 ($P < 0.0001$)	b, 0.14 ± 0.21 ($P = 0.5067$)	0.02
Data of finisher feed above breakpoint (1.4 mg/g TIA)	Live weight (LW) at d35, g/bird	$y = a + bx$	a, 922 ± 79.9 ($P < 0.0001$)	b, −26.8 ± 71.1 ($P = 0.7140$)	0.01
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 416 ± 41.7 ($P < 0.0001$)	b, −37.1 ± 37.1 ($P = 0.3407$)	0.09
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 919 ± 67.1 ($P < 0.0001$)	b, −54.1 ± 59.7 ($P = 0.3860$)	0.08
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 2.20 ± 0.14 ($P < 0.0001$)	b, 0.14 ± 0.12 ($P = 0.2842$)	0.11
	Pancreas weight, g/bird	$y = a + bx$	a, 5.46 ± 0.50 ($P < 0.0001$)	b, −0.98 ± 0.45 ($P = 0.0525$)	0.33
	Solubility in Potassium Hydroxide (KOH)				
Data of finisher feed below breakpoint (1.4 mg/g TIA)	Live weight (LW) at d35, g/bird	$y = a + bx$	a, 806 ± 401 ($P < 0.0573$)	b, 4.98 ± 5.41 ($P = 0.3676$)	0.04
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 73.3 ± 217 ($P < 0.7382$)	b, 5.43 ± 2.92 ($P = 0.0773$)	0.14
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 896 ± 374 ($P < 0.0258$)	b, 2.32 ± 5.05 ($P = 0.6507$)	0.01
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 4.52 ± 1.06 ($P < 0.0004$)	b, −0.03 ± 0.01 ($P = 0.0584$)	0.16
	Pancreas weight, g/bird	$y = a + bx$	a, 1.23 ± 1.35 ($P < 0.3745$)	b, 0.04 ± 0.02 ($P = 0.0511$)	0.17
Data of finisher feed above breakpoint (1.4 mg/g TIA)	Live weight (LW) at d35, g/bird	$y = a + bx$	a, 1062 ± 318 ($P < 0.0075$)	b, −2.17 ± 4.11 ($P = 0.6091$)	0.03
	Total weight gain (TWG), g/bird	$y = a + bx$	a, 208 ± 167 ($P < 0.2400$)	b, 2.20 ± 2.16 ($P = 0.3309$)	0.09
	Total feed intake (TFI), g/bird	$y = a + bx$	a, 906 ± 279 ($P < 0.0088$)	b, −0.55 ± 3.61 ($P = 0.8831$)	0.01
	Feed conversion ratio (FCR), ratio	$y = a + bx$	a, 3.59 ± 0.42 ($P < 0.0001$)	b, −0.02 ± 0.01 ($P = 0.0140$)	0.47
	Pancreas weight, g/bird	$y = a + bx$	a, −1.83 ± 1.41 ($P < 0.2237$)	b, 0.08 ± 0.02 ($P = 0.0012$)	0.67

a: intercept; b: slope of model.
[1]Coefficient of determination of the respective regression model.

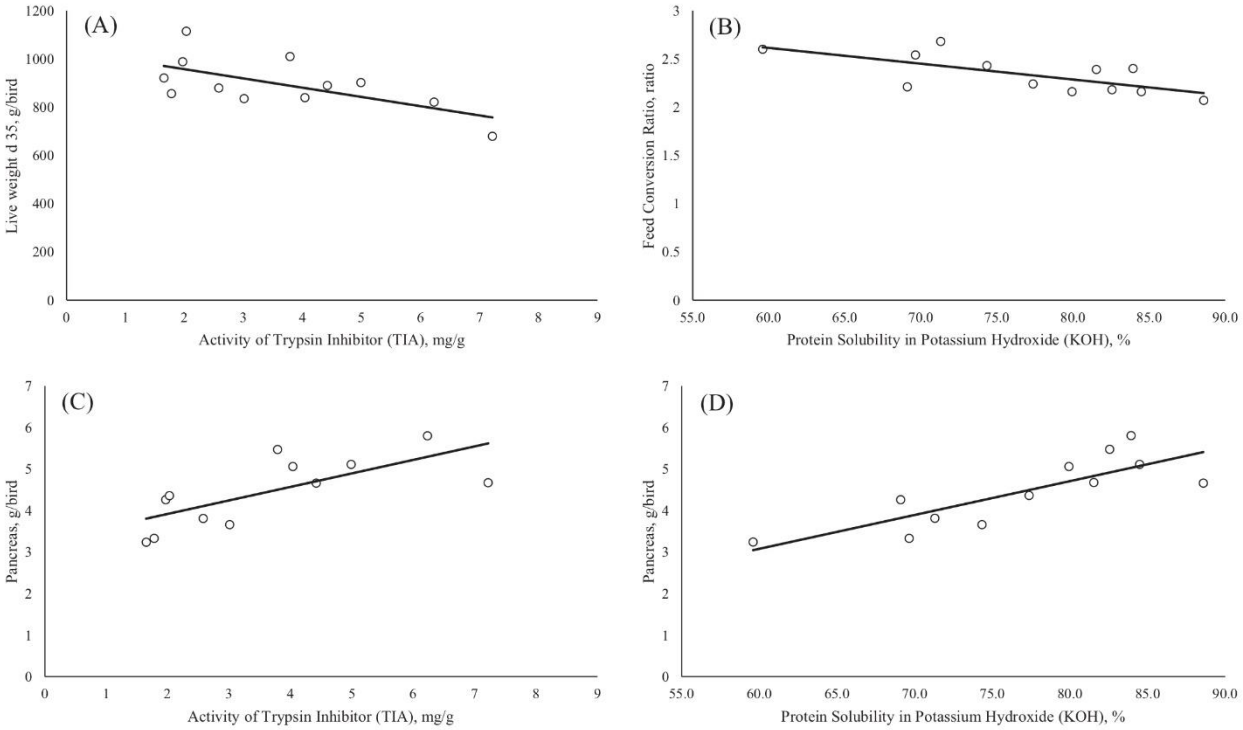

Figure 3. The effect of trypsin inhibitor activity (TIA) and protein solubility in potassium hydroxide (KOH) in processed soy cake fed to broiler chickens on live weight (LW) (A), feed conversion ratio (FCR) (B), and pancreas weight (C, D) for dietary treatments with above calculated breakpoint in finisher phase. Statistical data behind graphs can be seen in Table 4. Each dot represents the mean value of each dietary treatment (n = 12). Treatment means of LW are each calculated on base of n = 48 individual values (bird-wise). Treatment means of FCR are each calculated on base of n = 6 individual values (cage-wise).

Pancreas weight ranged from 3.24 to 5.80 g/bird at the time point of slaughtering. Below the TIA threshold of 1.4 mg/g in finisher feed, regression analyses did not reveal significant relationships between pancreas weight and dietary TIA, HDL, or KOH, respectively (Table 4). In groups fed diets with TIA above this threshold, pancreas weight responded in a straight linear fashion to TIA (slope = 0.33 ± 0.10, $P < 0.009$) and KOH (slope = 0.08 ± 0.02, $P < 0.0012$) (Figure 3 C, D, Table 4) but not HDL (Table 4).

Because intercepts and slopes derived from separate linear regression analyses of the 2 subsets of data (below and above TIA threshold, Figure 1) were quite similar, we performed a linear regression analyses over the entire dietary range. We analyzed the reaction of FCR during the grower phase in relation to dietary TIA over all treatment groups (Figure 4, Table 5) because FCR exhibited the strongest correlation to TIA ($R^2 = 0.77$) and exhibited a straight linear increase of FCR with increasing TIA (slope = 0.14 ± 0.01, $P < 0.0001$). Implementation of a broken-line model was not possible for this dataset.

DISCUSSION

This study investigated the response of zootechnical performance and pancreas weight of broiler chicks to varying dietary TIA, HDL, and KOH, as applied by different processing techniques of soybean cake. Consequently, we were able to generate a wide range of these parameters in the final grower and finisher feed mixtures. This applied particularly to low TIA levels. For example, a recent feeding study with broiler chicks conducted by Heger et al. (2016) reached TIA minimum levels of 1.01 mg/g and 0.88 mg/g in final grower and finisher feeds, respectively. Currently, the upper limits tolerable for TIA in full-fat soybeans are considered to be below 4 mg/g when used for broiler chickens (Clarke and Wiseman, 2005; Clarke and Wiseman, 2007). By increasing the range of TIA well below and above the practical threshold of TIA in broiler diets, this study allowed greater conclusions to be drawn about the birds' response.

High processing intensities depressed TIA to very low values but also elevated HDL. This increase occurred below comparably small TIA values of 1.8 mg/g (grower feed) and 1.4 mg/g (finisher feed), respectively. HDL reflects the degree of lysine degradation through Maillard reactions and hence is an accepted parameter of overprocessing that indicates a decreasing availability of amino acids in soybean products (Faldet et al., 1992; Fontaine et al., 2007). Indeed, responses of zootechnical performance and pancreas weight as observed in the present study at low TIA levels might have been

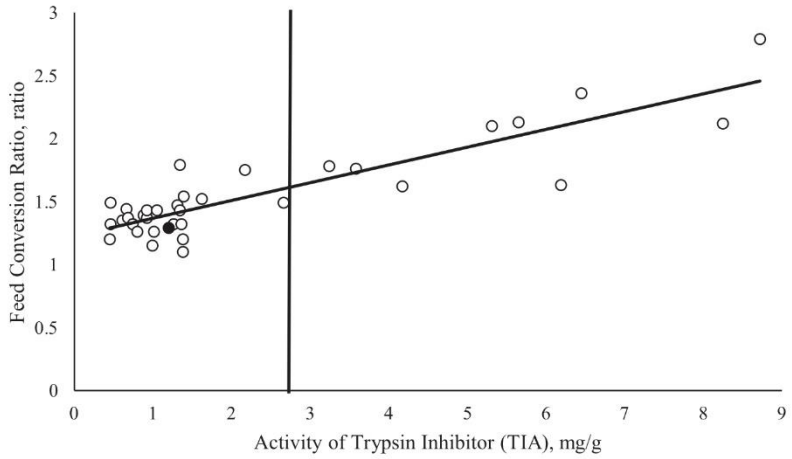

Figure 4. The effect of trypsin inhibitor activity (TIA) in processed soy cake fed to broiler chickens on feed conversion ratio (FCR) for all dietary treatments in grower phase. Statistical data provided in Table 5. Each dot represents the mean value of each dietary treatment (n = 35). Treatment means of FCR are each calculated on base of n = 6 individual values (cage-wise). Black dot represents commercial soybean meal. Black vertical line shows adapted TIA threshold for soy cake in fed diet (2.6 mg/g). Threshold in fed ratio was calculated using the accepted threshold of 4 mg/g for full fat soybeans (as published by Clarke and Wiseman 2005), estimating 15% oil content and 35% of soy cake in grower feed.

Table 5. Regression model of feed conversion ratio (FCR) of broilers as affected by varying ratios of dietary trypsin inhibitor activity (TIA) during grower phase.

	Regression models	Estimates of a	Estimates of b	$^1R^2$
Feed conversion ratio (FCR), ratio	y = a + bx	a, 1.22 ± 0.04 ($P < 0.0001$)	b, 0.14 ± 0.01 ($P < 0.0001$)	0.77

a: intercept; b: slope of model.
[1]Coefficient of determination of the respective regression model.

affected at least theoretically by additive effects of both TIA and HDL. For this reason, the statistical evaluations were performed separately for subsets of data below and above the respective TIA thresholds. Nevertheless, it must be kept in mind that rising HDL may affect performance only under the condition of inducing a limitation in essential amino acids, namely lysine. Since our experimental diets were composed according to recommendations, it may be assumed that limiting amino acids were provided with some safety margins that compensated for rising HDL. Therefore, it may be concluded that TIA had the most significant impact on zootechnical performance compared to HDL under the present experimental conditions.

Another indicator for protein degradation through feed processing is decreasing protein solubility in KOH. According to Araba and Dale (1990), KOH levels below 70% indicate over-processing. Indeed, our study reached dietary KOH levels of 60%. Again, diet formulations of our experimental feeds may be assumed to provide some safety margins in protein supply compensating potentially depressing effects of KOH on performance. Furthermore, since dietary KOH was correlated to TIA (r = 0.55 and 0.56 for grower and finisher, respectively), some effects statistically attributable to KOH might have descended from corresponding changes in TIA.

There is plenty of evidence that processing of soybeans improves their feed value (Osborne and Meldel, 1917; Liener, 1962; Van Der Poel et al., 1990; Heger et al., 2016; Rohe et al., 2017), thereby increasing the utilization of feed within the gastrointestinal tract through a decrease in antinutritive substances (Liener, 1962; Rohe et al., 2017). In the present study, untreated soybeans caused the lowest growth performance in the grower and finisher phases and a stepwise reduction of dietary TIA improved zootechnical performance and reduced pancreatic weight accordingly, which is in line previous results (e.g., Heger et al., 2016). Lower pancreas weights are considered to reflect relief from pancreatic hypertrophy induced specifically by dietary presence of TI (Applegarth et al., 1964; Kakade et al., 1973; Han and Parsons, 1991; Leeson and Atteh, 1996; Clarke and Wiseman, 2007). However, KOH was also inversely correlated with pancreas weight and associated with a reduction in FCR. This somewhat contradicts the current stage of knowledge of reduced KOH (below ~ 70%) and its adverse effects on animal performance (Araba and Dale, 1990). Given the aforementioned significant interaction between dietary TIA and KOH, we conclude this observation reflects an indirect TIA effect due to autocorrelation with dietary KOH. In summary, zootechnical performance and pancreas weights were mainly affected by dietary TIA rather than HDL or KOH during the present study.

This assumption is further supported by the fact that intercepts and slopes derived from separate linear

regression analysis of the 2 subsets of data (below and above TIA threshold as depicted in Figure 1) were quite similar. Consequently, it seems to be justified to perform linear regression analysis over the entire range of dietary TIA. As an example, we analyzed the reaction of FCR during the grower phase in relation to dietary TIA over all treatment groups. We chose this parameter because it exhibited the strongest correlation to TIA ($R^2 = 0.77$) and thus would be the best parameter of our dataset to derive a critical threshold of dietary TIA. As demonstrated, FCR responded directly in a linear fashion to dietary TIA without any indication of a plateau that would point towards a certain threshold of upper tolerable activities within complete feed. Compared to the threshold of TIA commonly used in practice (4 mg/g in full-fat soybeans (Clarke and Wiseman, 2005; Clarke and Wiseman, 2007), corresponding to around 2.6 mg/g assuming the inclusion levels used in the present study), further reductions in dietary TIA continued to improve FCR (the lowest FCR was evident in a group with TIA = 1.38 mg/g, HDL = 1.66 g/kg, KOH = 74.4%).

In conclusion, feed processing jointly modifies TIA, HDL, and KOH, but TIA was revealed to be the most important parameter negatively affecting zootechnical performance and pancreas weight. Since effectiveness of TIA was demonstrated over the whole range of dietary activities, including very minute concentrations below currently applied thresholds, it is recommended to completely eliminate dietary TIA under practical dietary conditions as far as technically possible with generous safety margins in terms of essential amino acid supply.

ACKNOWLEDGMENTS

The project was supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program (Grant number: 2814EPS022). We are grateful to Konstanze Kraft (DSM Nutritional Products, Kaiseraugust, Switzerland) and Markus Rodehutscord (University of Hohenheim, Institute of Animal Science, Animal Nutrition, Hohenheim, Germany) for their support and advice regarding production of experimental diets. Furthermore, we thank the staff of the Department for Education and Poultry Research, Bavarian State Research Center for Agriculture (LVFZ Kitzingen, Kitzingen, Germany) for advice and help during the experimental trials. Special gratitude is extended to Brett Boden for valuable comments on the manuscript and to Michael Gertitschke, Andrea Reichlmeir, and Magdalena Janovsky for excellent technical assistance.

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APPENDIX

Table A1. Overview of processing techniques and parameters used to process soybeans in soybean cake (as published in Hoffmann et al., 2017)

Thermal processing (1)	2: Hydrothermal processing (2)	3: Pressure and thermal processing (3)	Kilning and thermal processing (4)
[°C; min]	[ST: min, LT: min, Exp.:°C][1]		[°C; min]
A: 115; 0.6	A: ST: 0; LT: 00; Exp.: 0	A: ST: 1; LT: 00; Exp.: 110	A: 130; 40
B: 120; 0.6	B: ST: 1; LT: 03; Exp.: 0	B: ST: 1; LT: 03; Exp.: 110	B: 160; 30
	C: ST: 1; LT: 12; Exp.: 0	C: ST: 1; LT: 03; Exp.: 130	C: 190; 20
	D: ST: 1; LT: 48; Exp.: 0	D: ST: 1; LT: 12; Exp.: 110	D: 190; 30
		E. ST: 1; LT: 12; Exp.: 130	
		F: ST: 1; LT: 48; Exp.: 110	
		G: ST: 1; LT: 48; Exp.: 130	

[1]ST: short time conditioning (90°C), LT: long time conditioning (100°C), Exp.: expander.
Numbers (1 to 4) and letters (A to G) indicate processing techniques in Table A2.

Table A2. Analyzed nutrient concentrations and TIA of soy cake variants presented in order of ascending TIA levels. To be continued below.

Soy cake variants	SC11	SC13	SC28	SC12	SC10	SC8	SC22	SC27	SC9	SC30	SC29	SC7	SC19	SC2	SC5	SC24	SC6	SC25
DM[1]	84.8	87.3	86.3	87.1	87.1	84	83.9	88.8	87	89	88.5	87	97.8	97	86	87.7	88	86.2
Crude protein[2]	49.3	48.3	45.7	47	48	49	46.2	46.9	49	46.3	46.3	49	47.8	52	48	45.4	47	45.7
KOH-CP[2,3]	73.6	71.7	79.8	74.2	78.8	82	82.2	80.3	81	82.9	83.2	80	70.1	83	88	83.8	85	82.6
Crude ash[2]	6.33	6.26	6.19	6.72	6.42	6.4	6.44	6.28	6.5	6.36	6.31	6.6	6.38	6.5	6.6	6.1	6.4	6.11
Crude fat[2]	9.5	10.8	13.7	10.7	10.8	10	10.1	10.2	10	11.7	6.4	9.3	10.6	8.9	11	9.7	11	11.7
Crude fiber[2]	7.32	6.76	8.61	6.8	6.91	7.7	6.86	7.97	6.9	8.01	7.56	8.1	6.28	10	6.5	7.17	6.3	6.96
Total lysine[4]	23.1	23.3	22.9	22.8	22.9	23	22	24.2	24	23.6	23.3	24	25.2	26	24	23.1	25	23.1
Reactive lysine[4]	16.4	14.7	16.7	17.1	17.9	17	16.2	17.4	18	15.3	17.7	19	17.7	20	21	18.4	18	18.1
Heat-degraded lysine[4,5]	6.8	8.6	6.2	5.7	5	5.6	5.9	6.8	6.2	8.4	5.6	5.6	7.5	6.2	3	4.8	6.4	5
TIA[6]	0.25	0.31	0.65	0.77	1.10	1.19	1.50	1.56	1.58	1.60	1.64	1.73	1.78	1.99	2.01	2.10	2.14	2.16
Breed[7]	1	1	2	1	1	1	2	2	1	2	2	1	2	1	1	2	1	2
Processing method[8]	2D	3G	2D	3F	3E	2C	2B	3E	3D	3G	3F	3C	1B	1B	2B	3C	3B	2C

[1]DM; %.
[2]% DM.
[3]KOH-CP: amount of potassium hydroxide-soluble crude protein
[4] g/kg diet/product
[5]Heat-degraded lysine was calculated on base of total and reactive lysine in soy cake samples
[6]TIA: trypsin inhibitor activity; mg inhibited trypsin per g; mg/g diet
[7]Breed: 1: Sultana, 2 Merlin
[8]Processing Methods displayed in Table A1
SC: Soybean Cake.

Table A2. (continued): Analyzed nutrient concentrations and TIA of soy cake variants presented in order of ascending TIA levels

Soy cake variants	SC26	SC23	SC1	SC18	SC34	SC17	SC31	SC33	SC16	SC14	SC21	SC15	SC4	SC32	SC3	SC20	SM
DM[1]	87.9	88.5	96	96.8	96.5	96.9	96.7	95.2	93.9	95.1	87.9	95.4	89	95	89	90.4	91
Crude protein[2]	45.6	46.1	53	45.9	46.1	46.2	45.1	43	44.2	46.9	46.6	47.7	48	46.5	49	46.3	50
KOH-CP[2,3]	84.5	85.5	88	85.3	64.5	65.5	78	71.1	84.8	83.4	89.7	95.4	92	92.3	98	97	42
Crude ash[2]	6.24	6.43	6.3	6.27	5.97	6.36	6.12	5.91	6.21	6.19	6.22	6.65	6.5	6.2	6.4	6.53	6.8
Crude fat[2]	11.1	9.59	8.6	11.9	12.5	12.4	11.9	15.4	14.6	10.5	10.5	9.77	11	10.1	12	13.1	2.6
Crude fiber[2]	8.11	7.23	6.8	7.71	10	8.75	9.94	11.5	7.7	7.81	7.38	7.26	6.5	7.81	6.7	7.38	6.5
Total lysine[4]	23.6	23.5	28	24.4	22.2	23.2	23.8	21.4	23.8	25.2	23.4	24.2	24	25.7	25	24.5	28
Reactive lysine[4]	17.3	18.3	25	21.1	16	18.9	20.8	15.9	17.4	22.1	22.1	18.9	22	18.9	22	21.9	18
Heat-degraded lysine[4,5]	6.4	5.2	2.9	3.4	6.2	4.4	3.1	5.6	6.4	3.2	1.4	5.4	1.5	6.9	2.8	2.6	9.8
TIA[6]	2.47	2.83	3.16	3.79	5.49	5.52	6.47	8.21	9.71	10.9	12.7	14.7	15.9	16.6	19.9	23.6	2.1
Breed[7]	2	2	1	2	2	1	2	2	1	1	2	1	1	2	1	2	
Processing method[8]	3D	3B	1A	1A	4D	4D	4A	4C	4C	4A	3A	4B	3A	4B	2A	2A	

[1]DM; %
[2]% DM
[3]KOH-CP: amount of potassium hydroxide-soluble crude protein
[4] g/kg diet/product
[5]Heat-degraded lysine was calculated on base of total and reactive lysine in soy cake samples
[6]TIA: trypsin inhibitor activity; mg inhibited trypsin per g; mg/g diet
[7]Breed: 1: Sultana, 2 Merlin
[8]Processing Methods displayed in Table A1
SC: Soybean Cake
SM: Soybean Meal.

Table A3. Nutrient concentrations and TIA of final grower diets fed to broilers from day 11 to 24 presented in order of ascending TIA levels. To be continued below.

Grower Diets	G13[1]	G28	G11	G12	G10	G29	G30	G27	G19	G8	G7	G2	G9	G26	G23	G5	G22	G18
DM[2]	90.3	90.7	89.7	90.1	90.3	91.3	91.0	91.6	92.8	89.7	90.0	91.8	89.4	91.2	91.2	89.6	91.3	92.8
Crude protein[3]	24.7	24.0	24.2	23.1	23.6	23.3	25.3	27.0	22.6	23.5	23.3	23.1	24.0	23.0	23.3	22.6	22.4	22.3
KOH-CP[3,4]	71.3	72.2	68.3	72.1	74.4	73.4	73.6	73.6	68.6	74.8	74.4	77.7	75.4	79.5	87.2	77.7	75.9	74.6
Crude ash[3]	5.24	5.36	4.97	4.96	4.99	5.32	5.54	5.66	4.95	4.68	4.56	4.06	4.71	5.03	5.16	4.36	5.8	4.93
Crude fat[3]	6.57	8.17	6.52	6.52	7.00	6.70	6.69	7.16	7.19	5.96	5.80	5.18	6.10	6.49	6.63	5.80	5.98	6.38
Neutral detergent fiber[3]	10.7	12.6	11.9	10.5	10.3	11.6	11.9	11.7	13.7	10.6	10.0	11.5	10.3	10.1	10.5	10.8	10.7	12.7
Acid detergent fiber[3]	4.28	4.22	3.56	4.29	3.45	5.13	4.74	4.45	5.95	3.55	3.48	3.86	3.49	3.85	4.53	4.11	4.52	6.02
Acid detergent lignin[3]	0.56	0.61	0.79	0.84	0.77	0.59	0.67	0.38	0.52	0.71	0.82	0.25	0.87	0.79	0.67	0.33	0.35	0.77
Lysine[5]	10.0	12.0	12.0	12.0	11.0	12.0	13.0	12.0	9.30	13.0	12.0	11.0	12.0	12.0	12.0	12.0	12.0	9.90
Heat-degraded lysine[5,6]	3.01	2.17	2.36	2.00	1.75	1.96	2.92	2.38	2.63	1.96	1.94	2.15	2.17	2.22	1.82	1.05	2.05	1.17
Methionine[5]	3.11	3.20	3.30	3.50	3.10	3.20	3.30	3.10	2.80	3.40	3.20	3.10	3.30	3.20	3.30	3.40	3.20	2.90
Cysteine[5]	3.80	3.60	3.80	4.20	3.70	3.90	4.00	3.70	3.70	4.10	4.00	3.80	3.90	4.00	3.80	3.80	3.80	3.30
Threonine[5]	7.80	8.70	8.60	9.10	8.40	8.40	8.90	8.40	7.10	9.20	8.40	8.20	8.50	8.50	9.00	8.70	8.40	7.40
TIA[7]	0.45	0.46	0.46	0.61	0.66	0.68	0.74	0.80	0.88	0.92	0.92	0.99	1.01	1.05	1.26	1.31	1.34	1.34

[1]Numbers are linked to numbers of soybean cake variants in Table A2.
[2]DM; %
[3]% DM
[4]KOH-CP: amount of potassium hydroxide-soluble crude protein
[5] g/kg diet/product
[6]Heat-degraded lysine was calculated on base of total and reactive lysine in soy cake samples. HDL in final rations was calculated in relation to soy cake sample content (grower phase: 35% of soy cake sample) and is displayed as g/kg.
[7]TIA: trypsin inhibitor activity; mg inhibited trypsin per g; mg/g diet
Metabolizable Energy (ME) concentration of final grower products is estimated 13.5 MJ/kg. Estimation is based on ME-MJ concentration in basal diet (65%) (Table 1) mixed with 35% soybean cake. Soybean cake ME-MJ concentration is based on soybean cake parameters in DLG (2018).

Table A3. (continued): Nutrient concentrations and TIA of final grower diets fed to broilers from day 11 to 24 presented in order of ascending TIA levels including Reference Grower (RG)

Grower Diets	G6[1]	G1	G24	G25	G17	G34	G31	G14	G33	G16	G15	G32	G21	G4	G3	G20	RG
DM[2]	89.8	91.6	91.1	90.9	91.9	92.6	92.1	91.4	92.2	91.5	91.4	91.9	91.2	90.2	90.6	91.5	91.0
Crude protein[3]	23.9	25.4	26.4	22.8	23.6	23.2	23.3	24.0	22.8	24.4	24.0	24.0	22.9	23.6	23.1	23.2	25.5
KOH-CP[3,4]	70.4	79.0	74.4	82.0	62.5	60.8	65.7	74.5	70.7	77.4	83.1	77.7	82.5	80.8	84.3	84.9	69.4
Crude ash[3]	4.78	4.80	5.79	5.11	4.96	4.61	5.37	4.85	4.65	4.62	4.90	4.85	4.47	4.95	5.10	5.11	5.12
Crude fat[3]	6.83	5.53	7.61	7.20	7.26	6.90	7.02	6.60	7.48	8.00	6.33	5.91	6.22	6.64	6.34	7.47	3.34
Neutral detergent fiber[3]	10.7	11.1	10.4	10.2	15.5	15.9	14.2	12.2	14.7	12.1	10.5	11.4	10.1	9.90	11.4	10.7	11.0
Acid detergent fiber[3]	3.40	3.83	4.42	4.06	4.86	4.39	4.86	4.11	4.37	4.23	4.04	4.64	4.59	3.85	4.42	5.79	4.82
Acid detergent lignin[3]	0.98	0.21	0.59	0.69	0.61	0.99	0.70	0.69	0.79	0.91	0.74	0.99	1.40	0.43	0.59	1.13	0.90
Lysine[5]	12.0	12.0	12.0	12.0	12.0	11.0	12.0	13.0	11.0	12.0	14.0	13.0	1.20	13.0	13.0	11.0	12.0
Heat-degraded lysine[5,6]	2.24	1.02	1.66	1.75	1.52	2.17	1.07	1.10	1.94	2.24	1.87	2.40	0.47	0.53	0.98	0.91	3.40
Methionine[5]	3.40	3.40	3.20	3.30	3.40	3.30	3.30	3.20	3.00	3.40	3.70	3.30	3.30	3.30	3.30	3.30	3.30
Cysteine[5]	3.90	4.10	3.50	3.90	3.80	3.60	3.60	4.20	3.60	4.00	4.20	4.00	4.20	4.10	4.20	3.80	3.80
Threonine[5]	8.40	9.00	8.20	8.80	8.90	8.70	8.70	8.90	8.20	8.70	9.60	9.20	8.20	9.30	9.50	8.00	9.00
TIA[7]	1.36	1.38	1.38	1.39	1.62	2.17	2.66	3.24	3.58	4.17	5.31	5.65	6.19	6.45	8.25	8.72	1.20

[1]Numbers are linked to numbers of soybean cake variants in Table A2.
[2]DM; %
[3]% DM
[4]KOH-CP: amount of potassium hydroxide-soluble crude protein
[5] g/kg diet/product
[6]Heat-degraded lysine was calculated on base of total and reactive lysine in soy cake samples. HDL in final rations was calculated in relation to soy cake sample content (grower phase: 35% of soy cake sample) and is displayed as g/kg.
[7]TIA: trypsin inhibitor activity; mg inhibited trypsin per g; mg/g diet
Metabolizable Energy (ME) concentration of final grower products is estimated 13.5 MJ/kg. Estimation is based on ME-MJ concentration in basal diet (65%) (Table 1) mixed with 35% soybean cake. Soybean cake ME-MJ concentration is based on soybean cake parameters in DLG (2018).

Table A4. Nutrient concentrations and TIA of final finisher diets fed to broilers from day 25 to 34 presented in order of ascending TIA levels. To be continued below.

Finisher Diets	F13[1]	F11	F28	F12	F10	F8	F7	F29	F27	F30	F9	F2	F19	F6	F5	F24	F26	F25
DM[2]	90.6	89.7	90.9	90.4	90.1	89.9	90.2	91.9	91.3	92.0	90.3	92.0	92.6	90.0	90.2	91.1	90.7	91.0
Crude protein[3]	20.6	19.7	20.1	19.7	19.9	19.4	19.9	20.9	20.0	18.6	20.9	21.9	21.1	20.4	19.8	20.2	18.6	19.3
KOH-CP[3,4]	67.5	69.1	71.0	70.2	70.7	74.8	78.0	70.4	73.1	79.8	82.5	74.1	64.6	77.6	78.5	72.1	76.4	72.7
Crude ash[3]	5.07	4.36	5.24	5.16	4.92	5.42	4.55	5.62	5.23	4.70	5.25	5.12	5.77	4.56	5.43	5.20	4.32	5.27
Crude fat[3]	5.04	4.94	6.49	4.98	5.16	5.10	4.84	5.45	5.29	4.82	5.23	5.12	6.35	5.63	5.22	5.54	4.76	5.85
Neutral detergent fiber[3]	11.7	11.8	12.1	12.0	11.7	11.5	10.3	12.4	11.5	10.6	11.7	11.2	13.6	10.6	10.7	11.3	11.0	11.1
Acid detergent fiber[3]	3.91	3.70	4.16	3.82	3.87	3.82	3.35	4.31	4.02	3.78	3.81	3.76	5.15	3.72	3.96	4.15	3.85	4.07
Acid detergent lignin[3]	1.07	1.53	0.66	1.26	0.63	0.63	1.50	0.80	0.81	1.14	0.77	1.25	1.36	0.86	1.06	0.68	1.13	0.77
Lysine[5]	9.50	9.40	9.10	9.50	8.70	9.30	9.50	9.50	7.80	9.10	10.0	11.0	9.00	9.10	10.0	9.80	8.80	8.90
Heat-degraded lysine[5,6]	2.15	1.69	1.55	1.43	1.25	1.40	1.39	1.40	1.70	2.09	1.55	1.54	1.88	1.60	0.75	1.19	1.59	1.25
Methionine[5]	3.00	2.90	2.70	3.20	2.90	2.90	3.10	3.10	2.50	2.90	3.10	3.30	3.20	2.90	3.00	2.90	2.80	2.60
Cysteine[5]	3.40	3.40	3.50	3.50	3.20	3.20	3.40	3.60	3.50	3.30	3.60	3.60	3.30	3.40	3.60	3.90	3.20	3.20
Threonine[5]	7.50	8.00	6.70	8.10	7.50	7.80	8.00	6.90	5.90	6.80	8.40	9.30	7.60	7.70	8.40	7.20	6.60	6.60
TIA[7]	0.28	0.34	0.39	0.40	0.48	0.52	0.52	0.64	0.64	0.64	0.64	0.73	0.73	0.82	0.83	0.83	0.85	0.87

[1]Numbers are linked to numbers of soybean cake samples in Table A2.
[2]DM; %
[3]% DM
[4]KOH-CP: amount of potassium hydroxide-soluble crude protein
[5] g/kg diet/product
[6]Heat-degraded lysine was calculated on base of total and reactive lysine in soy cake samples. HDL in final rations was calculated in relation to soy cake sample content (finisher phase: 25% of soy cake sample) and is displayed as g/kg.
[7]TIA: trypsin inhibitor activity; mg inhibited trypsin per g; mg/g diet
Metabolizable Energy (ME) concentration of final grower products is estimated 13.3 MJ/kg. Estimation is based on ME-MJ concentration in basal diet (65%) (Table 1) mixed with 35% soybean cake. Soybean cake ME-MJ concentration is based on soybean cake parameters in DLG (2018).

Table A4. (continued): Nutrient concentrations and TIA of final finisher diets fed to broilers from day 25 to 34 presented in order of ascending TIA levels including Reference Finisher (RF)

Finisher Diets	F23[1]	F1	F22	F18	F17	F34	F14	F31	F16	F33	F21	F4	F32	F15	F3	F20	RF
DM[2]	91.5	91.8	90.5	92.7	91.8	93.2	91.6	92.7	91.6	93.2	91.6	91.0	92.8	91.6	90.9	91.4	92.6
Crude protein[3]	20.2	22.7	21.0	22.7	20.2	19.9	20.4	19.2	19.4	19.1	19.6	19.3	19.8	20.1	20.6	19.3	21.2
KOH-CP[3,4]	76.1	74.8	80.9	72.8	59.6	69.6	69.1	77.4	71.3	74.3	82.6	79.9	88.6	84.5	83.9	81.5	73.3
Crude ash[3]	6.06	5.28	4.08	6.57	6.00	5.02	5.41	4.84	5.82	5.30	5.83	5.08	5.14	5.29	5.35	6.17	5.27
Crude fat[3]	5.68	4.74	5.18	6.33	6.56	5.94	5.92	5.21	6.85	6.39	5.77	4.80	5.39	5.54	5.18	6.75	3.15
Neutral detergent fiber[3]	12.9	10.9	10.7	14.0	15.5	13.2	13.4	12.3	14.0	12.6	12.0	11.3	10.3	11.3	10.2	13.4	11.4
Acid detergent fiber[3]	3.97	3.86	3.13	5.33	4.31	3.84	4.06	4.21	4.08	4.15	4.01	3.95	3.89	4.00	3.85	3.93	4.23
Acid detergent lignin[3]	1.12	1.09	0.97	1.82	1.33	0.93	0.84	1.04	0.99	1.24	0.96	1.11	1.08	1.41	1.17	1.43	1.20
Lysine[5]	9.70	10.0	10.0	8.10	8.90	9.10	9.20	10.0	8.70	8.70	9.10	10.0	9.80	9.70	9.50	11.0	10.0
Heat-degraded lysine[5,6]	1.30	0.73	1.46	0.84	1.09	1.55	0.79	0.76	1.60	1.39	0.34	0.38	1.71	1.34	0.70	0.65	2.43
Methionine[5]	2.80	3.20	3.20	2.70	3.00	3.30	3.10	3.20	2.90	2.80	2.90	3.20	3.10	3.10	3.00	3.20	3.10
Cysteine[5]	3.50	3.60	3.60	3.30	3.50	3.50	3.50	3.60	3.70	3.30	3.30	3.50	3.40	3.60	3.40	3.80	3.50
Threonine[5]	6.90	8.60	8.20	7.00	7.90	7.20	7.70	7.50	7.30	6.80	7.30	8.40	7.20	8.10	8.00	8.40	7.90
TIA[7]	0.91	1.07	1.15	1.24	1.65	1.78	1.97	2.03	2.58	3.01	3.79	4.04	4.42	4.99	6.23	7.22	0.88

[1]Numbers are linked to numbers of soybean cake samples in Table A2.
[2]DM; %
[3]% DM
[4]KOH-CP: amount of potassium hydroxide-soluble crude protein
[5] g/kg diet/product
[6]Heat-degraded lysine was calculated on base of total and reactive lysine in soy cake samples. HDL in final rations was calculated in relation to soy cake sample content (finisher phase: 25% of soy cake sample) and is displayed as g/kg
[7]TIA: trypsin inhibitor activity; mg inhibited trypsin per g; mg/g diet
Metabolizable Energy (ME) concentration of final grower products is estimated 13.3 MJ/kg. Estimation is based on ME-MJ concentration in basal diet (65%) (Table 1) mixed with 35% soybean cake. Soybean cake ME-MJ concentration is based on soybean cake parameters in DLG (2018).

Table A5. Descriptive statistics of total weight gain (TWG), total feed intake (TFI), feed conversion ratio (FCR), live and pancreas weight of broilers as affected by varying ratios of dietary trypsin inhibitor activity, and heat-degraded lysine concentrations during the production cycle

		Mean	Standard deviation	Median	25% quantil	75% quantile	Minimum	Maximum
Grower phase	Live weight at d24, g/bird	630	108	656	538	700	383	798
	Total weight gain (TWG), g/bird	460	106	487	374	530	213	633
	Total feed intake (TFI), g/bird	668	48	671	631.25	706.25	550	759
	Feed conversion ratio (FCR), ratio	1.55	0.37	1.44	1.31	1.75	1.10	2.79
Finisher phase	Live weight at d35, g/bird	1078	173	1142	902	1208	679	1317
	Total weight gain (TWG), g/bird	442	76	459	387	504	290	590
	Total feed intake (TFI), g/bird	998	138	1030	876	1106	699	1201
	Feed conversion ratio (FCR), ratio	2.38	0.28	2.27	2.16	2.58	1.98	3.33
	Pancreas weight, g/bird	4.17	0.61	4.20	3.75	4.54	3.24	5.80