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der Christian-Albrechts-Universität zu Kiel

**Estimating Intestinal Digestibility
of Feedstuffs for Ruminants Using Three-step
in Situ-in Vitro and in Vitro Procedures**

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DEDICATION

My unlimited gratitude is dedicated to the **Almighty Allah** for
everything in my life

So my parents who illuminate my life and promote me to
continue

So my family who love and encourage me

So my dear brothers and sisters

So everyone wished me to accomplish my intent

Zusammenfassung

Rabie Abdul-Hamaid Hafiz Irshaid

Schätzung der Verdaulichkeit des Proteins von Futtermitteln für Wiederkäuer mittels dreistufiger in situ-in vitro oder in vitro-Verfahren

Übergeordnetes Ziel dieser Arbeit war die Entwicklung und Etablierung einer vollständig laborbasierten in vitro-Methode zur Schätzung der (Dünn-) Darmverdaulichkeit (intestinal protein digestibility, IPD) von im Pansen unabgebautem Futterprotein (ruminally undegraded protein, RUP). Im ersten Versuch wurden 66 Futtermittel genutzt, um IPD-Werte sowohl mit einer dreistufigen, kombinierten in situ-in vitro-Methode (ISIVP) als auch einer sogenannten „mobile-bag“-Technik (MBT; Inkubation im Pansen mit anschließender Passage der Residuen in kleinen Kunststoffbeuteln durch den (Dünn-) Darm) zu ermitteln. Beide Methoden erfordern den Einsatz fistulierter Tiere. Die Futtermittel umfassten eine weite Spanne verschiedener Futtermitteltypen: Grobfutter mit sehr unterschiedlichen Rohprotein-(RP)-Gehalten, unbehandelte und pansenstabile Proteinfuttermittel sowie Getreidesamen als Vertreter energiereicher Futtermittel mit niedrigen bis mittleren RP-Gehalten. Die Futtermittel wurden zunächst für 16 Stunden (h) im Pansen fistulierter Rinder inkubiert. Die Residuen aus diesem Schritt wurden in einer Pepsin-HCl-Lösung (pH 1,9) für 1 h inkubiert und die Residuen danach für 24 h einer Inkubation in einer Pankreatin-Lösung unterzogen. Die Beziehung zwischen den beiden Methoden ließ sich mit einer linearen Regressionsgleichung mäßiger Güte beschreiben: $IPD_{MBT} = 1,113 IPD_{ISIVP} - 125,37$ ($n = 53$, $r^2 = 0,36$, $p < 0,0001$). Nachdem diejenigen Futtermittel ($n = 14$) ausgeschlossen wurden, deren Werte um mehr als 15 Prozenteinheiten zwischen den beiden Methoden differierten, wurde die Regressionsanalyse wiederholt und ergab die folgende Beziehung: $IPD_{MBT} = 1,062 IPD_{ISIVP} - 43,66$ ($n = 39$, $r^2 = 0,661$; $p < 0,0001$). Die ISIVP konnte erfolgreich genutzt werden, um die IPD-Werte von Grob- und Konzentratfutter unterschiedlichster Herkunft und mit stark variierenden RP-Gehalten zu schätzen. Im zweiten Versuch wurden 49 der zuvor untersuchten Futtermittel benutzt, um eine enzymatische, vollständig laborbasierte in vitro-Methode (enzymatic in vitro procedure, EIVP) zu entwickeln und zu etablieren. Die Vorgehensweise bestand in der Verdauung der Proben mit einer pilzlichen (*Streptomyces griseus*) Protease und anschließender Pepsin-Pankreatin-Verdauung mit nachfolgender Schätzung der IPD-Werte des RUP. Die Konzentration der verwendeten *S. griseus*-

Protease wurde auf den Gehalt an Reinprotein (true protein, TP) des Futtermittels standardisiert. Die Durchführung der Methode begann mit der Bestimmung des TP-Gehaltes. Die Inkubation der Futtermittel beinhaltete als ersten Schritt eine 18-stündige Verdauung in einer Pufferlösung, die ein Protease:TP-Verhältnis von 41 Einheiten/g TP aufwies. Die getrockneten Residuen dieses Schrittes wurden 1 h in einer Pepsin-HCl-Lösung inkubiert. Die Rückstände wurden anschließend 24 h mit Pankreatin verdaut. Die Beziehung der IPD-Werte der EIVP-Methode zu den mittels ISIVP oder MBT ermittelten Werten ließ sich mit linearen Regressionsgleichungen beschreiben: $IPD_{MBT} = 1,221 IPD_{EIVP} - 165,95$ ($n = 38$, $r^2 = 0,666$, $p < 0,0001$) and $IPD_{ISIVP} = 1,053 IPD_{EIVP} - 28,14$ ($n = 49$, $r^2 = 0,985$, $p < 0,0001$). Die Ergebnisse der rein enzymatischen EIVP-Methode spiegelten diejenigen der ISIVP-Methode sehr gut wider. Die im Rahmen dieser Arbeit an Futtermitteln heterogener Herkunft und Zusammensetzung entwickelte und etablierte, vollständig laborbasierte in vitro-Methode erscheint deshalb geeignet, die kombinierte in situ-in vitro-Methode (ISIVP) zur Schätzung der Dünndarmverdaulichkeit von RUP zu ersetzen.

Abstract

Rabie Abdul-Hamaid Hafiz Irshaid

Estimating Intestinal Protein Digestibility of Feedstuffs for Ruminants Using Three-step in Situ-in Vitro and in Vitro Procedures

The overall objective of this thesis was to develop and establish a completely laboratory-based in vitro procedure for estimating (small) intestinal digestibility of ruminally undegraded feed protein. In the first trial, sixty-six feed samples were used to estimate intestinal protein digestibility (IPD) with a three-step in situ-in vitro procedure (ISIVP) and a mobile-bag technique (MBT). Both methods require access to surgically prepared (“fistulated”) animals. The feed samples encompassed forages with varying crude protein (CP) contents, unprotected or rumen-protected protein supplements and cereal grains representing energy-rich feeds of low to medium CP concentration. Feed samples were first incubated in the rumen of fistulated cattle for 16 h, then the residues were incubated in pepsin-HCl solution (pH 1.9) for 1 h and the residues of this step were incubated in pancreatin solution for 24 h. The relationship between the two methods was described by a linear regression equation of moderate fit: $IPD_{MBT} = 1.113 IPD_{ISIVP} - 125.37$ ($n = 53$, $r^2 = 0.36$, $P < 0.0001$). After excluding feed samples showing differences in IPD between the two methods of more than 15 percentage units ($n = 14$), the regression analysis was repeated and revealed the following relationship: $IPD_{MBT} = 1.062 IPD_{ISIVP} - 43.66$ ($n = 39$, $r^2 = 0.661$; $P < 0.0001$). In conclusion, the ISIVP could be applied to estimate the IPD of forages and concentrates covering a wide range of sources and CP concentrations. In the second trial, forty-nine of the previously evaluated feed samples were utilized to develop and establish an enzymatic in vitro procedure (EIVP) involving the subsequent digestion of samples with a protease from *Streptomyces griseus* and pepsin-pancreatin for estimating the IPD of RUP. The concentration of the *S. griseus* protease was related to the true protein (TP) content of the feed sample. Briefly, the EIVP started with determination of true protein. Feeds were incubated for 18 h in a buffer solution at a constant ratio of *S. griseus* protease to feed TP (41 U/g). The dried residues from this step were incubated in pepsin-HCl solution for 1 h and residues from this step were incubated with pancreatin solution for 24 h. Again, feed samples encompassed forages with varying CP contents, unprotected or rumen-protected protein supplements and cereal grains. The relationships between IPD values estimated by EIVP and ISIVP or MBT were best described by linear regression equations: $IPD_{MBT} = 1.221 IPD_{EIVP} - 165.95$ ($n = 38$, $r^2 = 0.666$, $P < 0.0001$)

and $IPD_{ISIVP} = 1.053 IPD_{EIVP} - 28.14$ ($n = 49$, $r^2 = 0.985$, $P < 0.0001$). Results from the EIVP closely resembled those obtained with the ISIVP and thus, the completely laboratory-based, standardized EIVP can replace the more invasive ISIVP for estimating IPD of a wide range of feedstuffs for ruminants.

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LIST OF ABBREVIATIONS

ADF:	acid detergent fibre (inclusive residual ash)
ANC:	the actual N content of the feed sample
CP:	crude protein
DDGS:	dried distillers' grains plus solubles
DM:	dry matter
EIVP:	enzymatic in vitro procedure
ICP:	initial CP (g) in the feed sample
IPD:	intestinal protein digestibility
ISIVP:	three-step in situ-in vitro procedure
MBT:	mobile-bag technique
RCP:	residual CP in the residues after 16 h rumen incubation (g)
RCPD:	16 h rumen CP degradability
RNC:	the residual nitrogen (g) of the precipitate
RUP:	ruminally undegraded feed CP
TP:	true protein
TPD:	total CP digestibility

CHAPTER 1

Introduction

In contrast to other estimates of feed quality, chemical analysis is the only one that provides absolute values, e.g. the exact quantity of nitrogen presents (Mould, 2003). Therefore, the crude protein (CP; N x 6.25) content of feedstuffs is considered as an important factor in evaluating feeds for ruminants. However, the CP concentration alone does not provide any information on the fate of the CP during digestion in the rumen and postruminal digestive tract of the ruminant. The extent of simultaneous microbial CP degradation and synthesis in the forestomachs, in particular the rumen, and the postruminal (abomasum, small intestine) digestion by host enzymes determine the amount of amino acids that are released in and can be absorbed from the small intestine to satisfy the ruminants' requirements for maintenance and for production of milk, meat, wool, etc. Thus, feedstuffs of similar CP contents may vary considerably in respect of the amount of amino acids which they deliver to the ruminant. It has been shown for forages that in general the chemical composition is a poor indication of the duodenal flow of microbial N in ruminants fed all-forage diets (Gosselink et al., 2003) and thus, a poor predictor of amino acid supply to the ruminant.

Because in ruminants, CP consumed is either degraded (and synthesized) in the rumen, digested in the abomasum (true stomach) and/or digested in the small intestine, values of ruminal and post-ruminal CP digestibility should be estimated simultaneously to characterize the protein value of feeds. Improved knowledge on both variables would also greatly improve the quality of feeding standards that are used in the feed industry or extension services. Providing accurate estimates of ruminal and postruminal CP digestibility was a major challenge that faced researchers for several decades. Direct in vivo measurements would appear to be the most desirable way to determine both ruminal degradation and post-ruminal digestibility of consumed CP in ruminants (Broderick, 1994; GfE, 2001). However, results obtained using this methodology have not been completely satisfactory because the in vivo procedures require considerable technical investments and use of animals with cannulas in various sections of the gastrointestinal tract (Broderick, 1994). In addition, in vivo measurements of nutrient digestion are expensive, labour-intensive, time consuming and subject to error related to use of digesta flow rate markers,

microbial markers and inherent animal variation (Stern et al., 1997). Tamminga and Chen (2000), from a systematic review of animal-based techniques of protein value of forages, have concluded that, direct measurements in vivo of microbial CP and ruminally undegraded dietary CP (RUP) will probably not be developed much further but its application will remain when absolute protein value measurements are needed.

Therefore, alternative procedures which should – at least – allow a reliable ranking of feeds in terms of CP flow to the duodenum and CP digestibility within the small intestine were developed with the final goal to replace in vivo methods for estimating ruminal and intestinal CP digestibility. One of the major attempts, which were developed to estimate post-ruminal CP digestibility, was the mobile bag technique (MBT; Hvelplund, 1985; Hvelplund et al., 1992). It was considered being the most promising method for measuring intestinal CP digestibility of various feedstuffs such as forages and concentrates and for detecting the impact of, for example, treatment and extent of rumen degradation (see Kusumanti et al., 1996). Although the mobile bag method is much less laborious than the alternative in vivo method for estimating intestinal digestibility, it is still too work-consuming for routine feed evaluation. Moreover, it still requires access to intestinally fistulated animals. Therefore, it is important to develop laboratory methods for estimating protein quality variables such as intestinal CP digestibility. Many approaches have been developed and evaluated to supply researchers and, finally, extension people and the farmer, with in vitro procedures, mainly applying enzymatic methods that should be capable of simulating intestinal CP digestion as an alternative to in vivo or MBT methods (Antoniewicz et al., 1992; Van Straalen et al., 1993; Calsamiglia and Stern, 1995; Kopečný et al., 1998). Among these procedures the three-step in situ-in vitro procedure (ISIVP) of Calsamiglia and Stern (1995) was (1) thoroughly evaluated regarding factors that could affect the results, (2) standardized and (3) calibrated against in vivo digestibility values of CP in the small intestine. This procedure was developed to simulate and replace in vivo methods and also MBT but be still reliable, rapid, inexpensive and be applied to a wide variety of proteins with no need for intestinally fistulated animals. However, this technique was not applied so far to a wide range of forages and

concentrates in a strictly standardized protocol and still requires ruminally cannulated animals.

Therefore, the objectives of this thesis were:

(1) To evaluate the applicability of the ISIVP in comparison with the MBT to estimate intestinal CP digestibility when applied to feedstuffs encompassing forages with varying CP contents, unprotected or rumen-protected protein supplements and cereal grains representing energy-rich feeds of low to medium CP concentration;

(2) To further develop the ISIVP by replacing the rumen incubation step with an enzymatic step, i.e. incubating feed samples in a solution of a protease from *Streptomyces griseus*. This part of the research should help to avoid use of surgically modified animals in nutrition research, increase the degree of standardization of the procedure and also decrease the cost and labour requirements. Finally the modified procedure was applied to the same range of feedstuffs characterized above.

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grass and clover measured with mobile nylon bag and other methods. *J. Dairy Sci.* 76, 2970-2981.

CHAPTER 2

Estimating intestinal protein digestibility of forages and concentrates with a three-step in situ-in vitro procedure

Summary

Sixty-six feed samples were used to estimate intestinal protein digestibility (IPD) with the three-step in situ-in vitro procedure (ISIVP) of Calsamiglia and Stern (1995; *J. Anim. Sci.* 73, 1459-1465). The feed samples encompassed forages with varying crude protein (CP) contents, unprotected or rumen-protected protein supplements and cereal grains representing energy-rich feeds of low to medium CP concentration. Feed samples were first incubated in the rumen for 16 h, then the residues were incubated in pepsin-HCl solution (pH 1.9) for 1 h and the residues of this step were incubated in pancreatin solution for 24 h. Both IPD and 16 h rumen crude protein degradability (RCPD) values were estimated, from which total CP digestibility (TPD) values were derived. All feed samples under investigation were processed previously at the Danish Institute of Agricultural Sciences applying a mobile bag technique (MBT) to determine TPD values and estimate IPD values. The IPD and TPD values from both methods were compared and the relationships between the two methods were best described by linear regression equations: $IPD_{MBT} = 1.113 IPD_{ISIVP} - 125.37$; $n = 53$, $r^2 = 0.36$, $P < 0.0001$; $TPD_{MBT} = 177.59 + 0.793 TPD_{ISIVP}$; $n = 57$; $r^2 = 0.708$, $P < 0.0001$). After excluding feed samples showing differences in IPD between the two methods of more than 15 percentage units ($n = 14$), the regression analysis was repeated and revealed the following relationship: $IPD_{MBT} = 1.062 IPD_{ISIVP} - 43.66$; $n = 39$, $r^2 = 0.661$; $P < 0.0001$). In conclusion, the ISIVP could be applied to estimate the IPD of forages and concentrates covering a wide range of sources and CP concentrations. The method was capable of evaluating untreated and rumen-protected protein supplements. Further studies should aim at replacing the rumen incubation step in the ISIVP with a standardized enzymatic procedure to further improve the standardization of the procedure and avoid the use of surgically prepared animals.

1. Introduction

Crude protein (CP), or more specifically amino acids, which flow from the forestomachs to the small intestine and are digested and absorbed therein, are the N sources used for meat and milk production in ruminants. They originate from three resources, i.e., microbial CP synthesized in the rumen, ruminally undegraded dietary CP (RUP) and endogenous CP. Although the small intestinal digestibility of amino acids in microbial CP is assumed to be high ($\geq 85\%$) and relatively constant (85 - 87%; Storm et al., 1983; Hvelplund, 1985; Tas et al., 1981), the digestibility of RUP can vary considerably depending on the type of feedstuff and also on the type and intensity of specific treatments during feedstuff processing (Kusumanti et al., 1996). Using the small intestinal digestibility of RUP as a constant factor will lead to errors in prediction of nutrient supply due to the variation of that factor among or between feeds, (Hvelplund et al., 1992). Therefore, intestinal digestibility of RUP has become an important variable in recent protein evaluation systems for ruminants (NRC, 2001; Hvelplund and Nørgaard, 2003).

Several methods have been developed and applied in order to estimate intestinal protein digestibility (IPD) of feedstuffs. The mobile-bag technique (MBT) has been suggested as being the most promising method for measuring IPD of a range of feedstuffs (Kusumanti et al., 1996). In this technique, feeds that have been pre-incubated in the rumen are placed in synthetic fibre bags and incubated in a pepsin-HCl solution. Then they are introduced directly into the duodenum via a cannula and subsequently collected from the ileum, or more typically from the faeces. Bags are then washed to remove endogenous and other contaminating proteins (Stern et al., 1997). In other words, IPD values based on the MBT are obtained as the disappearance of CP from the bags during passage through the post-ruminal intestinal tract (Hvelplund and Weisbjerg, 2000). However, many factors may influence the disappearance of CP, including bag-cloth characteristics, pepsin-HCl pretreatment of the sample and place of recovery (Hvelplund and Weisbjerg, 2000). Because the aforementioned factors cause variation among the reported results and due to the high costs of this technique, which needs surgically prepared animals, the development of in vitro techniques for estimating IPD is required.

Calsamiglia and Stern (1995) developed a three-step in situ-in vitro procedure (ISIVP) to estimate IPD in ruminants. The technique was developed to closely simulate physiological conditions in the ruminants' digestive tract (including potential effects of ruminal fermentation), be rapid, reliable and inexpensive, be applied to a wide variety of protein supplements and accurately reflect differences in protein digestion (Calsamiglia and Stern, 1995). This technique differs from the MBT in one major feature: Pancreatin is used as an intestinal enzyme mixture responsible for (CP) digestion opposed to inserting bags with feed samples into the proximal small intestine of surgically prepared animals. Therefore, the ISIVP is more suitable for routine work and the results obtained reflect abomasal and small intestinal in vivo digestion and are also close to that estimated by MBT (Calsamiglia and Stern, 1995). However, this technique was not applied so far to a wide range of forages and concentrates in a strictly standardized protocol.

Therefore, the objective of the present study was to apply the ISIVP procedure of Calsamiglia and Stern (1995) on a wide range of feedstuffs, encompassing forages with varying crude protein (CP) contents, unprotected or rumen-protected protein supplements and cereal grains representing energy-rich feeds having low to medium CP concentrations.

2. Materials and Methods

2.1. Animals

Four-year old German Red Pied steers, three, weighing 879 ± 68 kg were used. Each of the three steers was fitted with a 10 cm internal diameter ruminal cannula (Model 1 C, Bar Diamond, Parma, ID, USA) and housed in individual tie stalls with free access to water in a temperature controlled room (18°C) under continuous lighting. The steers received a mixed diet consisting of two thirds of long mixed grass-legume hay or silage and one third of mixed concentrates. The diet was supplemented with a commercial mineral and vitamin mixture. Animals were fed the diets according to the AFRC (1993) values for maintenance. The daily amount of feed was offered in two equal meals at 7:00 h and 19:00 h.

2.2. *Feedstuffs*

Sixty-six feed samples were used (Table 1). These feed samples encompassed twenty-one feedstuff types, which originated from different commercial sources and comprised a range of different feedstuff treatments, e.g. fresh or ensiled and solvent-extracted or expeller-extracted meal. These samples were evaluated previously at the Danish Institute of Agricultural Sciences by MBT to determine total CP digestibility (TPD) and estimate IPD.

2.3. *Mobile-bag technique*

The MBT was performed at the Danish Institute of Agricultural Sciences according to an established protocol (Hvelplund et al., 1992). About 1 g of feedstuff (0.8 g of bulky feed, e.g. roughage) is weighed to a small nylon bag (6 cm x 6 cm) with 11 µm pore. For each feedstuff, 6 bags are prepared and pre-incubated in the rumen of three cows for 16 h with 2 bags in each cow. The bags are then washed in a washing machine using cold water. They are then deep frozen (-18 °C) until next incubation step. After thawing, the nylon bags containing the samples are soaked for one hour in HCl (2.4 pH). The bags are then incubated for two hours in HCl-pepsin (2.4 pH) at 40 °C in a shaking water bath. The bags are inserted into the small intestine through a duodenal cannula. The intestinal incubations are performed using either three cows with two bags per feed in each cow or six cows with one bag per feed in each cow. Max. 12 bags are introduced in the duodenum per cow per day. After intestinal and hind gut passage (4-36 hours) the bags are recovered from the faeces and washed in a washing machine using cold water. The residue in each nylon bag is transferred into a N-free filter with de-mineralized water and very carefully wrapped up.

Table 1

Concentrations (g/kg DM unless stated) of dry matter (DM, g/kg), crude protein (CP), acid detergent fibre (ADF) and starch of the feed samples tested with a three-step in situ in vitro procedure (ISIVP).

No.	Feedstuff ^a	DM	CP	ADF	Starch
1	Barley, grain 1	922.2	135.6	56.0	535.0
2	Barley, grain 2	933.3	144.3	59.0	489.0
3	Barley, grain 3	930.3	137.5	52.0	441.0
4	Barley, grain 4	909.7	117.1	-	-
5	Barley, grain, treated ^b	918.7	111.1	-	-
6	Blood meal	921.8	938.1	7.0	-
7	Brewers' grain 1	937.7	348.1	181.0	45.0
8	Brewers' grain 2	935.3	300.6	183.0	101.0
9	Cocksfoot, fresh	931.3	165.6	315.0	-
10	Cocksfoot, grass	927.0	125.6	-	-
11	Cocksfoot, silage	897.7	220.6	291.0	-
12	Coconut meal, solvent-extracted	912.8	228.8	-	-
13	Commercial mixed concentrates	932.2	123.1	-	-
14	Fish meal	936.8	786.3	3.0	-
15	Fish meal, Norway, treated ^c	943.5	741.0	-	-
16	Fodder beets	936.9	56.3	101.0	-
17	Galega	929.8	177.5	-	-
18	Guar meal 1	937.2	408.1	-	-
19	Guar meal 2	941.4	392.6	163.0	14.0
20	Lucerne, artificially dried	938.6	186.3	414.0	-
21	Maize, grain	920.5	104.3	30.0	662.0
22	Maize, distillers' grain	915.2	286.9	164.0	62.0
23	Maize, grits 1	922.2	218.8	113.0	194.0
24	Maize, grits 2	928.4	226.3	107.0	219.0
25	Maize meal 1	924.5	116.9	72.0	439.0
26	Maize meal 2	926.0	123.1	117.0	301.0
27	Oat, grain 1	937.7	132.5	124.0	431.0
28	Oat, grain 2	922.6	134.3	116.0	465.0
29	Peas	919.7	235.6	61.0	431.0
30	Rapeseed cake, expeller-extracted 1	931.1	348.1	-	-
31	Rapeseed cake, expeller-extracted 2	919.0	398.8	-	-
32	Rapeseed cake, expeller-extracted 3	901.9	392.0	238.0	-
33	Rapeseed cake, expeller-extracted 4	903.5	391.9	238.0	-
34	Rapeseed cake, expeller-extracted 5	930.2	181.9	-	-
35	Rapeseed cake, expeller-extracted 6	926.7	334.4	222.0	-
36	Rapeseed cake, expeller-extracted 7	931.0	370.0	214.0	-
37	Rapeseed cake, expeller-extracted 8	925.3	348.1	242.0	-
38	Rapeseed cake, expeller-extracted 9	920.0	352.5	-	-
39	Rapeseed cake, expeller-extracted 10	916.5	398.1	188.0	-
40	Rapeseed meal, solvent-extracted 1	925.3	380.6	218.0	-
41	Rapeseed meal, solvent-extracted 2	920.8	375.0	213.0	-
42	Rapeseed meal, solvent-extracted 3	918.9	383.1	220.0	-
43	Rapeseed meal, solvent-extracted 4	908.8	378.8	234.0	-
44	Rapeseed meal, solvent-extracted 5	917.6	402.0	189.0	-
45	Rapeseed meal, solvent-extracted 6	923.1	401.9	204.0	-
46	Rapeseed meal, solvent-extracted 7	922.5	380.6	210.0	-
47	Rapeseed meal, solvent-extracted 8	931.7	372.1	-	-

Table 1 (continued)		DM	CP	ADF	Starch
48	Rapeseed meal, solvent-extracted 9	930.3	344.9	-	-
49	Rapeseed meal, solvent extracted (MervoBest) ^d	930.1	387.0	-	-
50	Perennial ryegrass, silage 1	866.5	130.0	272.0	-
51	Perennial ryegrass, silage 2	882.7	135.0	276.0	-
52	Rye, grain 1	904.5	91.7	-	-
53	Rye, grain 2	922.4	105.6	33.0	579.0
54	Rye, grain, treated ^b	915.5	91.4	-	-
55	Soybean hulls	926.5	130.0	468.0	-
56	Soybean meal (Bioprofin S) ^d	937.4	478.0	-	-
57	Soybean meal, solvent-extracted (MervoBest) ^d	933.2	530.0	-	-
58	Soybean meal, solvent-extracted (SoyPass) ^b	927.6	532.0	-	-
59	Sugar beet pulp, dried 1	927.5	106.9	166.0	-
60	Sugar beet pulp, dried 2	927.7	93.1	237.0	-
61	Triticale, grain	916.4	123.8	42.0	614.0
62	Triticale, straw	955.5	51.3	515.0	-
63	Wheat, grain 1	908.6	132.3	-	-
64	Wheat, grain 2	920.6	133.8	30.0	649.0
65	Wheat, grain, treated ^b	918.9	170.5	-	-
66	Wheat, straw	946.0	35.6	480.0	-

^a Figures following feed names indicate number of source of the feedstuff (1, 2, ..., n).

^b Treated with xylose in liginosulphonate solution to increase the ruminally undegraded crude protein fraction.

^c Treated with heat to increase the ruminally undegraded CP fraction.

^d Treated with formaldehyde to increase the ruminally undegraded CP fraction.

2.4. Rumen incubation

About 1.5 g feed sample, ground to pass a 1-mm screen, was weighed into small polyester bags (5 x 10 cm; R510, Ankom Technology, Macedon, NY, USA) with a pore size of 50 ± 15 µm. Nine bags were used for each feed. Bags were numbered, washed in a commercial washing machine at 55 – 60 °C and dried before use. Prior to incubation, the bags were soaked in warm water (40 °C) for 10 min. Bags were clamped to an 800 g cylindrical plastic weight, which was tied to an 80 cm long main line tied outside the fistula. All bags were inserted into the ventral sac of the rumen at 19:00 h immediately before the evening feeding. Immediately after removing the bags from the rumen, they were immersed in ice-water to stop or minimize microbial activity and then

washed with cold water in a washing machine for 45 min. After washing, all bags were dried in a forced-air oven at 55 °C for 48 h.

2.5. Laboratory procedure for intestinal digestion

The dried residues were collected from the bags. Dry matter (DM) and N contents were determined on each feedstuff residue. Three replicates of each feedstuff residue were weighed into a 50 ml centrifugation tube in an amount containing 15 mg N. Then 10 ml of a 0.1 *N* HCl solution (pH 1.9) containing 1g/l of pepsin were added to each centrifugation tube, which was shaken and then incubated for 1 h in a shaking water bath at 38 °C. After incubation, 0.5 ml of 1.0 *N* NaOH solution were added to each centrifugation tube to neutralize the solution, and then 13.5 ml of a pancreatin solution (pH 7.8) were added. The tubes were shaken and then incubated at 38 °C for 24 h in a shaking water bath. The tubes were vortexed approximately every 8 h. After incubation immediately 3 ml of trichloroacetic acid solution (100 % w/v) were added to each tube to stop enzymatic action and precipitate undigested proteins. After about 15 min, the samples were centrifuged at 10,000 × *g* for another 15 min at 5 °C. The precipitate was then filtrated through filter paper (no. 589¹, Schleicher and Schuell, Dassel, Germany) and the residue on the filter paper was analyzed for insoluble N by the Kjeldahl procedure.

2.6. General analytical procedures and calculations

The DM was estimated by oven-drying at 105 °C overnight and the N was determined using a standard Kjeldahl procedure using Cu²⁺ as a catalyst (method 4.1.1) according to “Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten” (VDLUFA; Bassler, 1993). The CP was calculated by multiplying N with 6.25. Starch content was measured by enzymatic hydrolysis of starch to glucose as described by Brandt et al. (1987) and acid detergent fibre (ADF) was analyzed according to the AOAC (1990) and expressed inclusive residual ash. Both IPD and 16 h rumen CP degradability (RCPD) were estimated for each feedstuff. For estimating RCPD, the dried empty bags and feed samples

used for rumen incubation were weighed, and after incubating the bags in the rumen of the three steers for 16 h, dry bags with residues were again weighed.

The RCPD values were calculated using the equation:

$$RCPD (g/kg) = ((ICP - RCP)/ICP) * 1000 \quad (1),$$

where ICP = initial CP (g) in the feed sample and RCP = residual CP in the residues after 16 h rumen incubation (g).

The IPD_{ISIVP} was calculated directly using the equation:

$$IPD_{ISIVP} (g/kg) = ((ANC - RNC)/ANC) * 1000 \quad (2),$$

where IPD_{ISIVP} = IPD estimated by ISIVP, ANC = the actual N content of the feed sample, which was the weight of the sample that should contain 15 mg N (g) x DM ((g/kg)/1000) x N ((g/kg)/1000) and RNC = the residual nitrogen (g) of the precipitate.

The IPD_{MBT} was calculated from TPD_{MBT} (g/kg) values determined at the Danish Institute of Agricultural Sciences using the equation:

$$IPD_{MBT} (g/kg) = ((TPD_{MBT} - RCPD)/(1000 - RCPD)) * 1000 \quad (3),$$

where IPD_{MBT} = IPD estimated by MBT, TPD_{MBT} = TPD determined at the Danish Institute of Agricultural Sciences and RCPD defined as in equation (1).

The TPD_{ISIVP} was also calculated for each feedstuff from values of RCPD and IPD_{ISIVP} as follows:

$$TPD_{ISIVP} (g/kg) = ((1000 - RCPD) * IPD_{ISIVP}/1000) + RCPD \quad (4),$$

with TPD_{ISIVP} , RCPD and IPD_{ISIVP} as defined above.

2.7. Testing the standardization of the ISIVP

The ISIVP was previously applied to a limited number of feedstuffs, in particular animal by-product proteins (Calsamiglia and Stern, 1995; Howie et al., 1996), which are no more allowed for ruminants in the European Union, and vegetable protein supplements (Woods et al., 2003). Therefore, the procedure was primary tested by using two feed samples with previously established IPD coefficients from one of the co-authors' (MDS) laboratory, namely rumen-protected soybean meal ("SoyPass") and dried distillers' grains plus solubles (DDGS). They were used in an attempt to improve the standardization of the laboratory procedure and to ensure that the present work accurately followed the steps and stages of the ISIVP (Calsamiglia and Stern, 1995).

2.8. Statistical methods

Relationships between the ISIVP and MBT were evaluated by generating simple linear regression equations. Relationships between chemical constituents and IPD values were evaluated using the Pearson correlation coefficient.

3. Results

3.1. Standardization of the ISIVP

The average IPD values for the soybean meal and DDGS samples estimated by Calsamiglia and Stern (1995) versus those in the present study were 0.789 versus 0.781 and 0.860 versus 0.851, respectively. The results obtained in the two laboratories were almost identical, which would indicate that the procedure yields reproducible values across laboratories. Unfortunately, no more than these two samples were available for substantiating our observation.

3.2. Chemical composition and IPD values of feedstuffs

Feedstuffs with their DM, CP, ADF and starch values are presented in Table 1. The DM contents ranged from 867 to 956 g/kg. These values simply reflect that feed samples were stored dry for further analytical feed evaluation. The CP values ranged from 36 to 938 g/kg DM, thus covering the widest possible range of CP concentrations of feedstuffs. Starch values ranged from 14 to 662 g/kg DM and ADF values ranged from 3 to 515 g/kg DM. This large variation of CP, starch and ADF values was helpful to improve the quality of the evaluation of the ISIVP and strengthen the recommendations as to the suitability of the procedure for estimating IPD values.

The IPD values estimated by ISIVP and MBT are summarized in Table 2. The IPD_{MBT} values were calculated from RCPD and TPD values determined at the Danish Institute of Agricultural Sciences (equation 3). The IPD_{ISIVP} values ranged from 278 to 868 g/kg CP, with an average of 664 g/kg CP, while the IPD_{MBT} values ranged from 151 to 941 g/kg CP, with an average of 628 g/kg CP. Variation of IPD values with either method (ISIVP or MBT) was particularly noticeable for expeller-extracted rapeseed cakes and solvent-extracted rapeseed meals from different commercial sources. Values ranged from low 278 g/kg CP to high 729 g/kg CP and might be related to intensity of moist or dry heat treatment of the commodities during and following oil extraction. The relationship between IPD_{ISIVP} and IPD_{MBT} was expressed as simple linear regression and is illustrated in Figure 1. The equation best describing the relationship was: $IPD_{MBT} = 1.113 IPD_{ISIVP} - 125.37$ ($n = 53$, $r^2 = 0.360$, $P < 0.0001$). Although the relationship was positive and significant, it was not satisfactory. After excluding feed samples showing differences in IPD between the two methods of more than 15 percentage units ($n = 14$; see Table 3 for data of these feeds), the regression analysis was repeated and revealed the following relationship (Figure 2): $IPD_{MBT} = 1.062 IPD_{ISIVP} - 43.66$ ($n = 39$, $r^2 = 0.661$, $P < 0.0001$). The coefficient of determination (r^2) almost doubled compared with the regression equation based on the whole dataset.

Table 2

Values for intestinal protein digestibility (IPD) estimated by a three-step in situ-in vitro procedure (IPD_{ISIVP}) and mobile-bag technique (IPD_{MBT}), ruminal CP degradability (RCPD), total CP digestibility estimated by ISIVP (TPD_{ISIVP}) and MBT (TPD_{MBT}).

No.	Feedstuff ^a	IPD _{ISIVP} ^b (g/kg CP)	IPD _{MBT} ^c (g/kg CP)	RCPD (g/kg CP)	TPD _{ISIVP} (g/kg CP)	TPD _{MBT} ^c (g/kg CP)	IPD _{MBT} - IPD _{ISIVP} (g/kg CP)	TPD _{MBT} - TPD _{ISIVP} (g/kg CP)
1	Barley, grain 1	764± 1.5	766	722	934	935	2	1
2	Barley, grain 2	608± 2.7	773	811	926	957	165	31
3	Barley, grain 3	786± 6.2	831	811	959	968	45	9
4	Barley, grain 4	741± 2.6	-	926	981	-	-	-
5	Barley, grain, treated ^d	794± 1.2	-	745	948	-	-	-
6	Blood meal	861± 0.1	941	4	861	941	80	80
7	Brewers' grain 1	666± 12.5	623	679	893	879	-43	-14
8	Brewers' grain 2	725± 0.4	650	651	904	878	-75	-26
9	Cocksfoot, fresh	634± 7.9	177	881	957	902	-457	-55
10	Cocksfoot, grass	655± 9.4	347	836	943	893	-308	-50
11	Cocksfoot, silage	661± 0.7	777	704	900	934	116	34
12	Coconut meal, solvent-extracted	745± 1.3	566	813	952	919	-179	-33
13	Commercial mixed concentrates	657± 16.0	617	681	891	878	-40	-13
14	Fish meal	868± 2.6	863	199	894	890	-5	-4
15	Fish meal, Norway, treated ^e	852± 1.5	822	730	960	952	-30	-8
16	Fodder beets	628± 5.8	344	872	952	916	-284	-36
17	Galega	491± 1.5	609	524	758	814	118	56
18	Guar meal 1	657± 4.7	604	947	982	979	-53	-3
19	Guar meal 2	761± 1.2	512	791	950	898	-249	-52
20	Lucerne, artificially dried	616± 4.0	591	351	751	735	-25	-16
21	Maize, grain	716± 1.8	732	344	814	824	16	10
22	Maize, distillers' grain	680± 10.9	753	551	856	889	73	33
23	Maize, grits 1	666± 8.5	646	644	881	874	-20	-7
24	Maize, grits 2	687± 8.0	743	615	879	901	56	22
25	Maize meal 1	674± 11.4	711	696	901	912	37	11
26	Maize meal 2	732± 7.0	547	574	886	807	-185	-79
27	Oat, grain 1	663± 13.8	799	672	889	934	136	45
28	Oat, grain 2	635± 7.9	-	742	906	-	-	-

Table 2 (continued)		IPD _{ISVP} ^b (g/kg CP)	IPD _{MBT} (g/kg CP)	RCPD (g/kg CP)	TPD _{ISVP} (g/kg CP)	TPD _{MBT} ^c (g/kg CP)	IPD _{MBT} - IPD _{ISVP} (g/kg CP)	TPD _{MBT} - TPD _{ISVP} (g/kg CP)
29	Peas	861±1.2	582	842	978	934	-279	-44
30	Rapeseed cake, expeller-extracted 1	455±7.6	207	876	933	902	-248	-31
31	Rapeseed cake, expeller-extracted 2	428±12.5	261	880	931	911	-167	-20
32	Rapeseed cake, expeller-extracted 3	652±18.0	702	691	893	908	50	15
33	Rapeseed cake, expeller-extracted 4	712±5.2	740	646	898	908	28	10
34	Rapeseed cake, expeller-extracted 5	624±6.8	519	786	920	897	-105	-23
35	Rapeseed cake, expeller-extracted 6	641±1.9	640	759	913	913	-1	0
36	Rapeseed cake, expeller-extracted 7	513±1.4	401	858	931	915	-112	-16
37	Rapeseed cake, expeller-extracted 8	729±12.3	818	496	864	908	89	44
38	Rapeseed cake, expeller-extracted 9	557±1.8	-	972	988	920	-	-68
39	Rapeseed cake, expeller-extracted 10	694±1.8	692	701	909	908	-2	-1
40	Rapeseed meal, solvent-extracted 1	368±3.5	-	930	956	903	-	-53
41	Rapeseed meal, solvent-extracted 2	278±0.4	-	933	951	915	-	-36
42	Rapeseed meal, solvent-extracted 3	300±1.1	-	920	944	907	-	-37
43	Rapeseed meal, solvent-extracted 4	708±1.2	822	468	845	905	114	60
44	Rapeseed meal, solvent-extracted 5	574±10.6	474	852	937	922	-100	-15
45	Rapeseed meal, solvent-extracted 6	543±0.3	490	832	923	914	-53	-9
46	Rapeseed meal, solvent-extracted 7	662±3.5	690	719	905	913	28	8
47	Rapeseed meal, solvent-extracted 8	716±0.8	-	633	896	-	-	-
48	Rapeseed meal, solvent-extracted 9	547±2.5	-	823	920	-	-	-
49	Rapeseed meal, solvent extracted (MervoBest) ^f	711±0.1	695	429	835	826	-16	-9
50	Perennial ryegrass, silage 1	641±7.3	151	841	943	865	-490	-78
51	Perennial ryegrass, silage 2	655±5.7	428	801	931	886	-227	-45
52	Rye, grain 1	800±1.2	-	896	979	-	-	-
53	Rye, grain 2	664±7.9	843	714	903	955	179	52
54	Rye, grain, treated ^d	733±2.3	-	702	920	-	-	-
55	Soybean hulls	671±9.9	556	520	842	787	-115	-55
56	Soybean meal, solvent-extracted (Bioprofin S) ^f	807±1.2	759	531	909	887	-48	-22
57	Soybean meal, solvent-extracted (MervoBest) ^f	810±1.5	720	208	849	778	-90	-71
58	Soybean meal, solvent-extracted (SoyPass) ^d	848±10.3	917	638	945	970	69	25

Table 2 (continued)

	IPD _{ISVP} ^b (g/kg CP)	IPD _{MBT} (g/kg CP)	RCPD (g/kg CP)	TPD _{ISVP} (g/kg CP)	TPD _{MBT} ^c (g/kg CP)	IPD _{MBT} - IPD _{ISVP} (g/kg CP)	TPD _{MBT} - TPD _{ISVP} (g/kg CP)
59	Sugar beet pulp, dried 1	553 ± 10.7	538	842	929	-15	-2
60	Sugar beet pulp, dried 2	783 ± 1.0	641	502	892	-142	-71
61	Triticale, grain	606 ± 14.3	561	918	968	-45	-4
62	Triticale, straw	662 ± 1.1	685	65	684	23	21
63	Wheat, grain 1	658 ± 0.9	-	946	982	-	-
64	Wheat, grain 2	605 ± 3.7	782	661	866	177	60
65	Wheat, grain, treated ^d	848 ± 0.8	-	423	912	-	-
66	Wheat, straw	603 ± 1.8	623	29	614	20	20

^a Figures following feed names indicate number of source of the respective feedstuff type (1, 2, ..., n).

^b Means ± SE, based on 3 replicates.

^c TPD_{MBT}: only means values were available of the data at the Danish Institute of Agricultural Sciences.

^d Treated with xylose in lignosulphonate solution to increase the ruminally undegraded CP fraction.

^e Treated with heat to increase the ruminally undegraded CP fraction.

^f Treated with formaldehyde to increase the ruminally undegraded CP fraction.

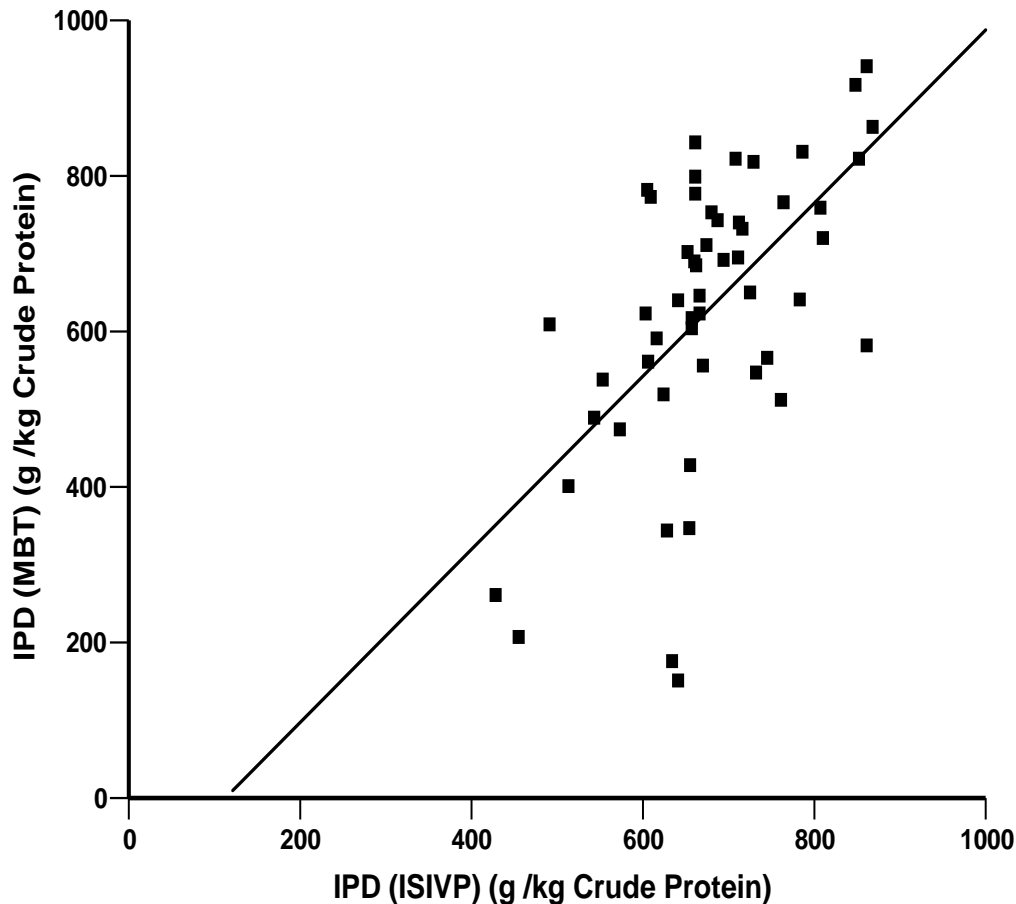


Figure 1

Relationship between intestinal protein digestibility (IPD) values estimated from a three-step in situ-in vitro procedure (ISIVP) and mobile-bag technique (MBT). The regression equation is: $IPD_{MBT} = 1.113 IPD_{ISIVP} - 125.37$ ($n = 53$, $r^2 = 0.36$, $P < 0.0001$).

Pearson correlation coefficients were computed to analyze the relationship between CP, starch or ADF contents and IPD values estimated by both methods (data not shown). The CP content was not significant ($P > 0.05$) related to IPD_{ISIVP} , whereas a significant ($P < 0.05$, $n = 53$, $r = 0.322$) and positive correlation between CP content and IPD_{MBT} was observed. Neither ADF nor starch contents were significantly related to IPD_{ISIVP} , whereas a significant ($P < 0.05$, $n = 53$, $r = -0.350$) and negative correlation between ADF content and IPD_{MBT} was observed.

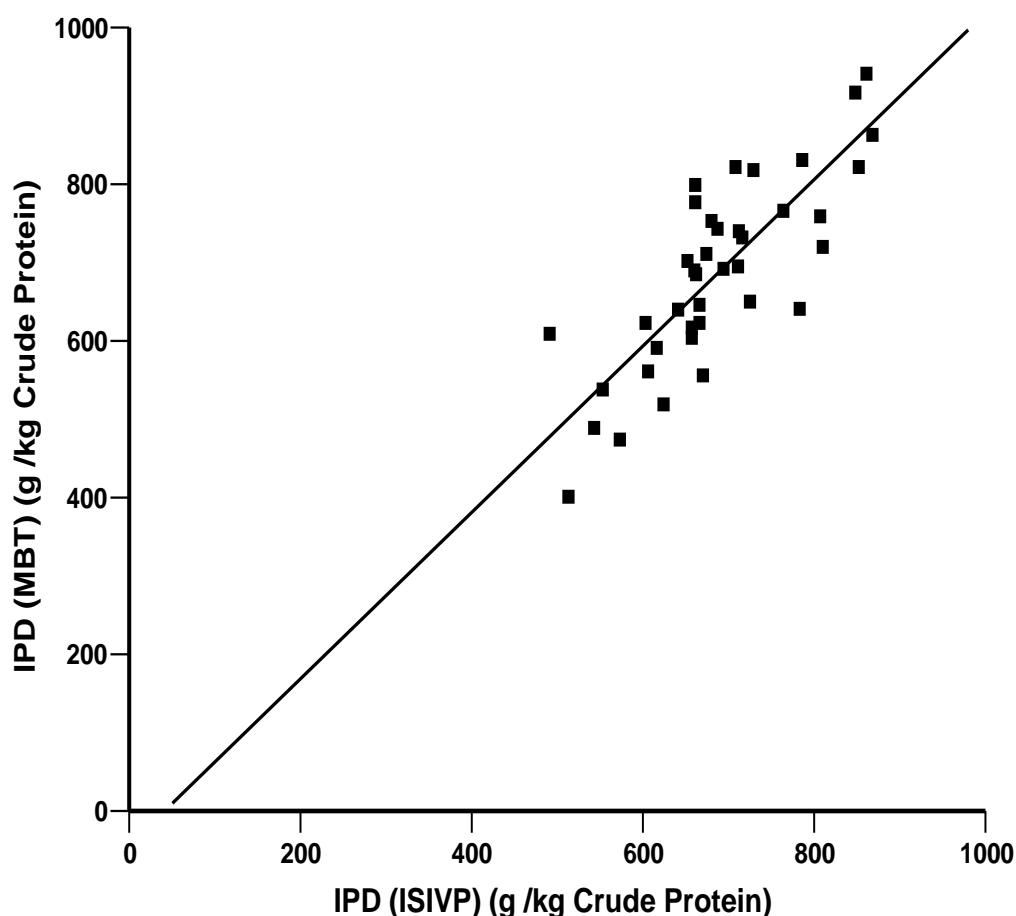


Figure 2

Relationship between intestinal protein digestibility (IPD) values estimated from a three-step in situ-in vitro procedure (ISIVP) and mobile-bag technique (MBT) after excluding feed samples showing differences in IPD between the two methods of more than 15 percentage units. The regression equation is: $IPD_{MBT} = 1.062 IPD_{ISIVP} - 43.66$ ($n = 39$, $r^2 = 0.661$, $P < 0.0001$).

3.3. Ruminal crude protein degradability and total crude protein digestibility values

Although rumen degradation values are not directly used for estimating IPD, incubation of feed samples in the rumen for 16 h was the preparatory step for the other stages of the ISIVP and MBT. Therefore, RCPD values are also presented in Table 2. Values for RCPD ranged from 4 to 972 g/kg CP. These values were combined with IPD_{ISIVP} to calculate TPD_{ISIVP} , which was then compared with TPD values determined with the MBT. Further, they served as a term in the

equation to calculate IPD_{MBT} from TPD_{MBT} , and those values were then compared with IPD_{ISIVP} .

Table 3

Intestinal protein digestibility (IPD) estimated by a three-step in situ-in vitro procedure (IPD_{ISIVP}) and mobile-bag technique (IPD_{MBT}) for feedstuffs, which were excluded from the general relationship^a.

Feedstuff^b	IPD_{ISIVP}^c (g/kg CP)	IPD_{MBT} (g/kg CP)	$IPD_{MBT} - IPD_{ISIVP}$ (g/kg CP)
Barley, grain 2	608± 2.7	773	165
Cocksfoot, fresh	634± 7.9	177	-457
Cocksfoot, grass	655± 9.4	347	-308
Coconut meal, solvent-extracted	745± 1.3	566	-179
Fodder beets	628± 5.8	344	-284
Guar meal 2	761± 1.2	512	-249
Maize, meal 2	732± 7.0	547	-185
Peas	861± 1.2	582	-279
Rapeseed cake, expeller-extracted 1	455± 7.6	207	-248
Rapeseed cake, expeller-extracted 2	428± 12.5	261	-167
Perennial ryegrass, silage 1	641± 7.3	151	-490
Perennial ryegrass, silage 2	655± 5.7	428	-227
Rye, grain 2	664± 7.9	843	179
Wheat, grain 2	605± 3.7	782	177

^a Data were excluded when differences in IPD between the two methods were > 150 g/kg units (15 percentage units).

^b Figures following feed names indicate number of source of the respective feedstuff type (1, 2, ..., n).

^c Mean ± SE, based on 3 replicates.

Values of TPD_{ISIVP} and TPD_{MBT} are shown in Table 2. In general, both the means and range of values were very similar for the two methods of estimating

TPD. The values of TPD_{ISIVP} ranged from 614 to 988 g/kg CP with an average of 904 g/kg CP, and the TPD_{MBT} values ranged from 634 to 979 g/kg CP with an average of 890 g/kg CP. The relationship between TPD_{ISIVP} and TPD_{MBT} is shown in Figure 3. It was best described by the linear regression equation: $TPD_{MBT} = 177.59 + 0.793 TPD_{ISIVP}$ ($n = 57$, $r^2 = 0.708$, $P < 0.0001$). This relationship is stronger than that between IPD_{ISIVP} and IPD_{MBT} . After excluding the same 14 feed samples as above, the relationship between TPD_{ISIVP} and TPD_{MBT} was only slightly improved (Figure 4): $TPD_{MBT} = 0.855 TPD_{ISIVP} + 126.94$ ($n = 43$, $r^2 = 0.797$, $P < 0.0001$). This observation can be explained by the impact of RCPD values on the calculated TPD numbers (equation 4). As RCPD increases, the proportional contribution IPD values to TPD numbers decreases. From the Pearson correlation coefficients between RCPD and both IPD_{ISIVP} and IPD_{MBT} ($r = -0.420$ ($n = 66$) and $r = -0.499$ ($n = 53$), respectively, $P < 0.01$), it becomes obvious that, as RCPD increases, IPD estimated by ISIVP or MBT will decrease.

Pearson correlation coefficients between TPD (ISIVP or MBT) and concentrations of ADF and starch were calculated. A negative ($P < 0.01$) relationship evolved between ADF and TPD_{ISIVP} ($r = -0.541$, $n = 44$) and TPD_{MBT} ($r = -0.705$, $n = 43$), whereas no ($P > 0.05$) significant relationship was found between starch contents and TPD values from either method. Thus, TPD values will decrease at increasing ADF contents, indicating an association between part of the CP with cellulose and/or lignified cellulose in forage and other fibrous feedstuffs. In addition, the TPD values in the current study were estimated based on a 16-h rumen incubation period, which is not enough for fibre to be extensively degraded and by this release most or all of the associated CP and by this increasing RCPD, which would in turn also increase TPD values.

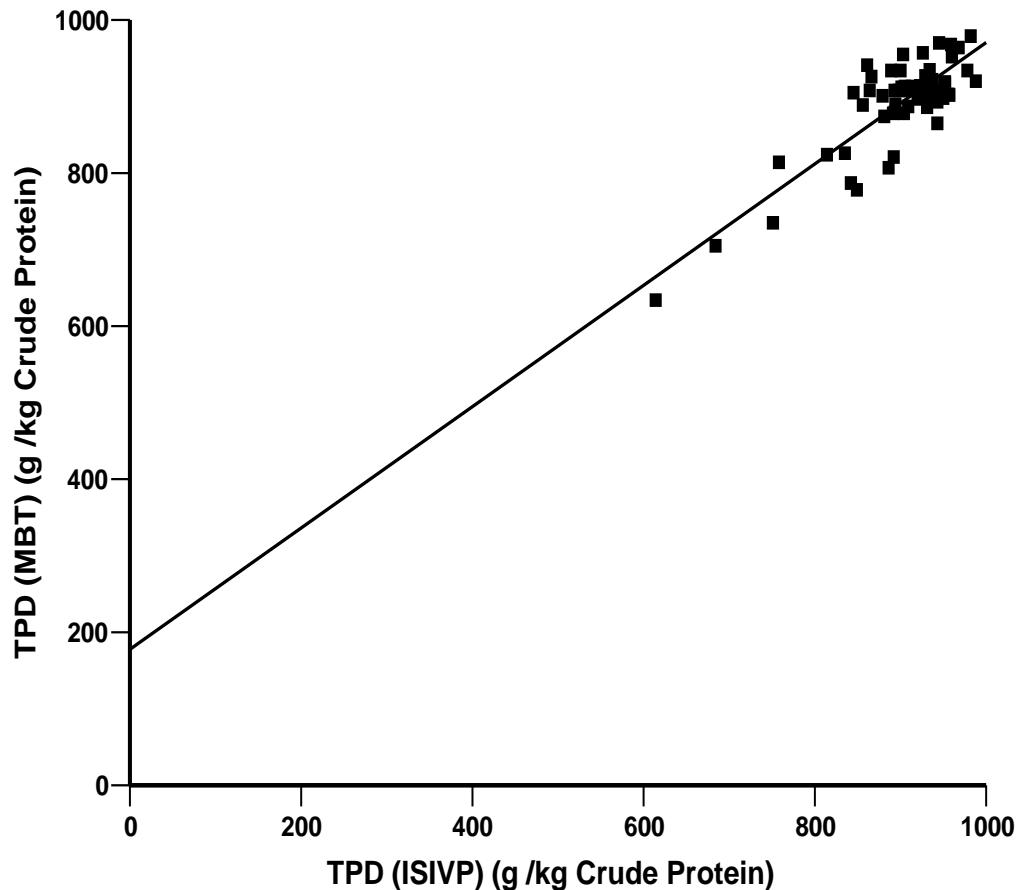


Figure 3

Relationship between total crude protein digestibility (TPD) values estimated from a three-step in situ-in vitro procedure (ISIVP) and mobile-bag technique (MBT). The regression equation is: $TPD_{MBT} = 177.59 + 0.793 TPD_{ISIVP}$ ($n = 57$, $r^2 = 0.708$, $P < 0.0001$).

3.4. Applying the three-step in situ-in vitro procedure to feed samples with elevated ruminally undegraded crude protein concentrations

Eight feed samples, which were specifically treated with heat, xylose in liginosulphonate solution or formaldehyde to increase concentrations of RUP, were compared for their IPD values estimated with ISIVP and MBT methods. The IPD_{ISIVP} versus IPD_{MBT} values (Table 2) for fish meal (Norway), solvent extracted rapeseed meal (MervoBest) and soybean meals (Bioprofin S, MervoBest, Soypass) were 852 versus 822, 711 versus 695, 807 versus 759, 810 versus 720 and 848 versus 917 g/kg CP, respectively. Both procedures ranked

IPD values similarly. Although most of the treated feed samples had low RCPD values, it was noticeable that their IPD values were in the upper range of values, indicating that no chemical or thermal damage to the CP or RUP had occurred. Moreover, the three treated soybean meals had similar IPD values, indicating that their RUP was similarly affected by the different treatment methods, albeit RCPD values were not the same across treated soybean meal samples.

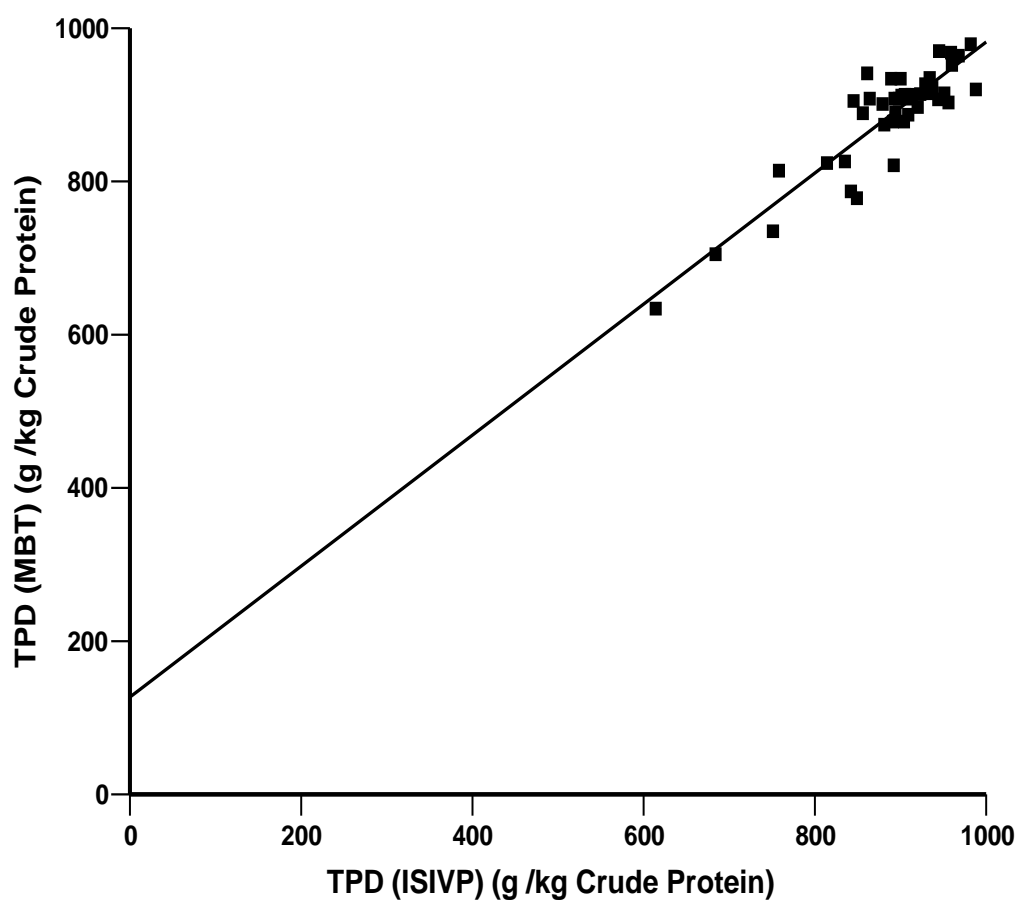


Figure 4

Relationship between total crude protein digestibility (TPD) values estimated from a three-step in situ-in vitro procedure (ISIVP) and mobile-bag technique (MBT) after excluding feed samples showing differences in IPD between the two methods of more than 15 percentage units. The regression equation is: $TPD_{MBT} = 126.94 + 0.855 TPD_{ISIVP}$ ($n = 43$, $r^2 = 0.797$, $P < 0.0001$).

4. Discussion

The present study focused on evaluating ISIVP as a combined in situ-in vitro procedure for estimating IPD. Calsamiglia and Stern (1995) attempted to mimic in vivo digestion in dairy cows when developing the ISIVP and delineated that this method potentially has less variation and within-method interference than the MBT. However, these authors examined the ISIVP with only few feed samples. Woods et al. (2003) conducted a comprehensive study to develop a database of small intestinal digestibility of protein concentrates applying both ISIVP and MBT methods. However, no forage or grain samples were included in the study of Woods et al. (2003).

Sixty-six feed samples of largely varying botanical and chemical composition with previously determined MBT values were examined in the present study to establish the ISIVP. The relationship between IPD estimates from ISIVP and MBT was weaker than that between TPD estimated by both methods. One major source of this difference could be the rumen incubation stage. Volden and Harstad (1995) found a negative correlation between ruminal CP degradation and true indigestibility of feed protein. They also found that using true indigestibility of feed protein for predicting true digestibility of RUP for peas, rapeseeds, lupin seeds and for barley and oat grains should be determined on feed samples after rumen incubation when using MBT as a procedure for estimating IPD. Calsamiglia and Stern (1995) tested the ISIVP method with or without rumen incubation. They reported that rumen incubation affected IPD values such that lower values were obtained for feed samples after ruminal pre-incubation. Therefore, they used 16-h rumen incubation as a primary stage of ISIVP, reflecting an average rumen residence time of the feed before passage to the abomasum and intestines. However, further work and clarification are still needed (Hvelplund and Weisbjerg, 2000).

DeBoer et al. (1987), Frydrych (1992) and Hvelplund et al. (1992) estimated IPD of different feedstuffs from the MBT method. These values are summarized in Table 4. Data indicates that IPD_{ISIVP} values are mostly lower than IPD_{MBT} values. This may be due to the length of the rumen incubation period, which was only 8 h in the work of DeBoer et al. (1987) and Hvelplund et al. (1992). As

rumen incubation period increases, rumen degradability also increases due to increasing time of exposure of feed material to the microorganisms. Hvelplund et al. (1992) found that as rumen degradability increased, IPD decreased. Therefore, these authors concluded that feed samples contain a CP fraction which is both undegradable in the rumen and indigestible in the intestines.

Table 4

Intestinal protein digestibility (IPD) estimated by a three-step in situ-in vitro procedure (ISIVP) and mobile-bag technique (MBT) for feedstuffs – overview of literature data.

Feedstuff	IPD _{ISIVP} (g/kg CP)	IPD _{MBT} ^a (g/kg CP)	IPD _{MBT} ^b (g/kg CP)	IPD _{MBT} ^c (g/kg CP)	IPD _{MBT} ^d (g/kg CP)
Barley, grain	786	-	-	887	-
Blood meal	861	-	-	898	-
Coconut meal, solvent-extracted	745	-	959	-	-
Cotton seed cake, expeller-extracted	-	-	950	949	889-964
Fish meal	868	828	-	945	-
Guar meal	761	-	-	-	919-943
Lucerne, artificially dried	616	712	-	739	-
Maize, grain	716	-	-	946	-
Meat and bone meal	-	730	-	724	-
Oat, grain	663	-	-	700	-
Peas	861	-	972	925	-
Rapeseed cake, expeller-extracted	557 ^e	-	-	571	855-905
Rye, grain	800	-	-	944	-
Soybean meal, solvent-extracted	-	993	991	990	-
Wheat, grain	658	-	-	886	-
Wheat, straw	603	-	-	615	-

^a DeBoer et al. (1987; 8 h rumen pre-incubation).

^b Hvelplund et al., (1992 ; 8 h rumen pre-incubation).

^c Frydrych (1992).

^d Kusumanti et al. (1996).

^e Rapeseed cake, expeller-extracted (feedstuff number 38 from Tables 1 and 2).

One might argue that excluding part of the data to improve the coefficient of determination for the relationship between the IPD_{ISIVP} and IPD_{MBT} has introduced an author-based bias into the relationship between the two methods. However, if values of IPD_{MBT} of the excluded feed samples are compared with IPD_{MBT} values from other studies (Table 4), it becomes obvious that most of the

respective IPD_{MBT} values in our study were much lower than that reported in other studies or when estimated by ISIVP. Therefore, for any unknown reason(s), the samples that were excluded from the final data evaluation obviously lay outside the typical range of values for such type of feedstuffs. Thus, the samples in the final dataset that was used to generate Figure 2 and the corresponding regression equation should reflect more representative numbers for IPD values estimated with the MBT.

Furthermore, results of the rumen incubation step are influenced by different factors, including bag porosity, bag material, bag size, sample processing, sample size, incubated substrate-to-bag surface area ratio, type of basal diet, concentrate level in the basal diet, bag insertion and removal procedures, location of bags within the rumen, washing procedure, incubation time and number of replicate animals and bags to obtain repeatable estimates of ruminal degradation, which may cause variations in data (Robinson et al., 1992; Vanzant et al., 1998; Noziere and Michalet-Doreau, 2000). In addition, Hvelplund and Weisbjerg (2000) clarified that differences observed between IPD values estimated by ISIVP and MBT could be due to the factors related to MBT, which includes bag-cloth characteristics of bags incubated in the intestine, pepsin-HCl pretreatment of the sample and place of recovery (ileum versus faeces) of the bags, which influence the disappearance of feed samples during passage through the intestines. However, Vanhatalo and Ketoja (1995) found that the site of collection of bags virtually does not affect small intestinal digestibility values of feed CP, unless the feed is rich in fibre. This however was the case with many feeds in the current study and might therefore have had an impact on the comparison between ISIVP and MBT.

Additional evidence that rumen incubation period has a major impact on differences between MBT and ISIVP is provided by Kopečný et al. (1998), who compared IPD measured by a pancreatin method with results obtained by MBT for 55 feed samples and a polycentric fungus. They pre-incubated the samples in the rumen for 16 h and continued with either MBT or pancreatin, thus eliminating the effect of rumen incubation as a source of variation between the two procedures. They found a close relationship between values from the two procedures ($r^2 = 0.861$). The IPD values of 55 feed samples estimated by

pancreatin method ranged from 500 to 972 g/kg CP and the MBT method gave values from 483 to 990 g/kg CP. In addition, Woods et al. (2003) estimated IPD of different concentrate ingredients with ISIVP and MBT methods and also reported a close relationship between results obtained with the two methods. The IPD_{ISIVP} values ranged from 447 to 840 g/kg CP and the IPD_{MBT} values ranged from 427 to 978 g/kg CP.

Regarding the sensitivity of the ISIVP, i.e. the ability of the procedure to differentiate between untreated or treated feed samples (e.g., to increase the RUP fraction of feeds), the results obtained from the present study are in agreement with that reported by Antoniewicz et al. (1992). These authors investigated feed samples treated with formaldehyde at 0, 10, 20, 30 and 40 g/kg CP with both an enzymatic (pepsin-pancreatin) in vitro method and the MBT and found a significant ($P < 0.001$) correlation between the two methods.

5. Conclusions

The ISIVP could be applied to a wide range of feedstuffs encompassing forages with varying CP contents, unprotected or rumen-protected protein supplements and cereal grains representing energy-rich feeds having low to medium CP concentrations. The method provided reliable estimates of IPD and, when combined with estimates of RPCD, also TPD. Moreover, the procedure allowed estimates to be made of IPD values of unprotected and rumen-protected protein supplements. Rumen incubation caused differences of IPD values for different batches of the same type of feedstuff. To further improve standardization and applicability of the procedure, the pre-incubation of the feeds in the rumen should be replaced with a standardized enzymatic procedure.

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CHAPTER 3

Development and establishment of an enzymatic in vitro procedure for estimating intestinal protein digestibility of feedstuffs for ruminants

Summary

This study utilized forty-nine feed samples to develop and establish a completely laboratory-based enzymatic in vitro procedure (EIVP) for estimating the intestinal protein digestibility (IPD) of rumen-undegradable protein (RUP) of forages and concentrates. Feed samples encompassed forages with varying CP contents, unprotected or rumen-protected protein supplements and cereal grains representing energy-rich feeds of low to medium CP concentration. The EIVP involved the subsequent digestion of samples with a protease from *Streptomyces griseus*, pepsin-HCl and pancreatin. The concentration of the *S. griseus* enzyme was related to the true protein (TP) content of the feed sample. Briefly, the EIVP started with determination of true protein. Feeds were incubated for 18 h in a buffer solution at a constant ratio (41 U/g) of *S. griseus* protease to feed TP. The dried residues were incubated in pepsin-HCl solution for 1 h and residues from this step were incubated with pancreatin solution for 24 h. Samples had previously been used for IPD estimates using a three-step in situ-in vitro procedure (ISIVP) and mobile-bag technique (MBT). The relationships between IPD values estimated by EIVP and ISIVP or MBT were best described by linear regression equations: $IPD_{MBT} \text{ (g/kg CP)} = 1.221 IPD_{EIVP} \text{ (g/kg CP)} - 165.95$ ($n = 38$, $r^2 = 0.666$, $P < 0.0001$) and $IPD_{ISIVP} \text{ (g/kg CP)} = 1.053 IPD_{EIVP} \text{ (g/kg CP)} - 28.14$ ($n = 49$, $r^2 = 0.985$, $P < 0.0001$). Results from the EIVP closely resembled those obtained with the ISIVP and thus, the completely laboratory-based, standardized EIVP can replace the more invasive ISIVP for estimating IPD of a wide range of feedstuffs for ruminants.

1. Introduction

The intestinal digestibility of ruminally undegraded dietary crude protein (RUP) has recently become an important variable in protein evaluation systems for ruminants (e.g. NRC, 2001; Hvelplund and Nørgaard, 2003). Inclusion of such a variable into a feed evaluation system requires reliable data be generated that can serve as a sound basis for predicting the amount of RUP, which can be digested in the small intestine and thus deliver absorbable amino acids to the host. In vivo measurement of nutrient digestion in the (small) intestine not only requires that animals be surgically prepared with cannulas in the abomasum or duodenum (and ileum). In addition, suitable markers are required for calculating flow rate of digesta and for differentiation between microbial and dietary nutrients flowing to the small intestine (Stern and Satter, 1982). Endogenous contributions of nutrients are difficult to measure but they should be assessed to obtain

accurate values of digestion; however, these data are limited. In vivo measurement of nutrient digestion is expensive, labour-intensive, time-consuming, and subject to error associated with use of digesta flow rate markers, microbial markers, and inherent animal variation (Stern et al., 1997). In addition, the use of invasive surgical procedures for nutritional research in general is becoming increasingly unacceptable to the public on animal welfare grounds. Therefore, invasive techniques are not suitable for routine estimation of protein values of feedstuffs on a wide range of feeds (Tamminga, 1979). According to that, there is a continuing need to develop and establish laboratory procedures, which can replace in vivo measurements that still provide accurate and reliable results.

Several methods have been developed and established in order to estimate intestinal protein digestibility (IPD) of feedstuffs. The mobile-bag technique (MBT) has been suggested as being the most promising method for measuring IPD of a range of feedstuffs (Kusumanti et al., 1996). In this technique, feeds that have been pre-incubated in the rumen are placed in synthetic fibre bags and incubated in a pepsin-HCl solution. Then they are introduced directly into the duodenum via a cannula and subsequently collected from the ileum, or more typically from the faeces. Bags are then washed to remove endogenous and other contaminating proteins (Stern et al., 1997). Because the MBT still needs surgically prepared animals, the development of in vitro techniques for estimating IPD is required.

Other researches have therefore focused on simulating the MBT by replacing the duodenum-ileum incubation step with pancreatin (CAS registry number 8049-47-6), an enzyme mixture from the pancreas containing amylase, protease and lipase (Antoniewicz et al., 1992; van Straalen et al., 1993; Calsamiglia and Stern, 1995; Kopečný et al., 1998). The method can be summarized as follows: Bags containing feed samples are suspended in the rumen for 12 - 16 h, and the residues are then incubated in pepsin-HCl solution for 1 - 2 h, after which the residues of this step are digested in pancreatin solution for 12 - 24 h. Calsamiglia and Stern (1995) further developed and standardized this method by determining optimum level of pancreatin, optimum buffer type, buffer molar concentration, pH, incubation time and mixing protocol for maximum simulation of MBT. Finally, these authors concluded that their technique is closely simulating physiological conditions of ruminants, reliable, rapid and inexpensive (Calsamiglia and Stern, 1995). However, animals with ruminal cannula are still required for the rumen incubation step, which warrants further time and effort be invested in developing

techniques that allows protein evaluation of feedstuffs be conducted without use of surgically prepared animals.

The primary objective of the present study was to develop an enzymatic in vitro procedure (EIVP) based on the three-step in situ-in vitro procedure (ISIVP) of Calsamiglia and Stern (1995). The second and the third steps of the Calsamiglia and Stern (1995) procedure were completely adopted based on results of a concomitant study which confirmed the applicability of the technique using a wide range of forages and concentrates (Chapter 2 of this thesis). However, the rumen incubation step of the Calsamiglia and Stern (1995) procedure was replaced with an enzymatic treatment using a protease from *Streptomyces griseus* to mimic ruminal degradation of crude protein (CP).

The second objective of this study was to establish the EIVP by applying the method to a wide range of feedstuffs for ruminants, encompassing forages with varying CP contents, unprotected or rumen-protected protein supplements and cereal grains representing energy-rich feeds having low to medium CP concentrations.

2. Materials and Methods

2.1. Feedstuffs

Forty-nine feed samples were used (Table 5). These feed samples encompassed sixteen feedstuff types, which originated from different commercial sources and comprised a range of different feedstuff treatments, e.g. fresh or ensiled and solvent-extracted or expeller-extracted meal. These samples formed were a part of previously utilized ones used in a comparison of two combined animal-laboratory methods to estimate IPD, namely MBT and ISIVP (see Chapter 2).

Table 5

Concentrations (g/kg DM unless stated) of dry matter (DM, g/kg), crude protein (CP), true protein (TP), acid detergent fibre (ADF) and starch of the feed samples tested with enzymatic in vitro procedure (EIVP).

No.	Feedstuff ^a	DM	CP	TP	ADF	Starch
1	Barley, grain 1	922.2	135.6	107.5	56.0	535.0
2	Barley, grain 2	933.3	144.3	121.6	59.0	489.0
3	Barley, grain 3	930.3	137.5	109.0	52.0	441.0
4	Barley, grain 4	909.7	117.1	91.5	-	-
5	Barley, grain, treated ^b	918.7	111.1	97.1	-	-
6	Blood meal	921.8	938.1	884.2	7.0	-
7	Brewers' grain 1	937.7	348.1	285.4	181.0	45.0
8	Brewers' grain 2	935.3	300.6	250.7	183.0	101.0
9	Coconut meal, solvent-extracted	912.8	228.8	184.7	-	-
10	Commercial mixed concentrates	932.2	123.1	94.4	-	-
12	Fish meal	936.8	786.3	620.4	3.0	-
13	Fish meal, Norway, treated ^c	943.5	741.0	550.2	-	-
14	Galega	929.8	177.5	135.1	-	-
15	Guar meal	937.2	408.1	329.3	-	-
16	Maize, grain	920.5	104.3	101.7	30.0	662.0
11	Maize, distillers' grain	915.2	286.9	208.5	164.0	62.0
19	Maize, grits 1	922.2	218.8	132.2	113.0	194.0
20	Maize, grits 2	928.4	226.3	145.4	107.0	219.0
17	Maize meal 1	924.5	116.9	95.1	72.0	439.0
18	Maize meal 2	926.0	123.1	88.7	117.0	301.0
21	Rapeseed cake, expeller-extracted 1	931.1	348.1	273.5	-	-
22	Rapeseed cake, expeller-extracted 2	919.0	398.8	292.1	-	-
23	Rapeseed cake, expeller-extracted 3	901.9	392.0	355.2	238.0	-
24	Rapeseed cake, expeller-extracted 4	930.2	181.9	114.6	-	-
25	Rapeseed cake, expeller-extracted 5	926.7	334.4	290.3	222.0	-
26	Rapeseed cake, expeller-extracted 6	931.0	370.0	269.7	214.0	-
27	Rapeseed cake, expeller-extracted 7	920.0	352.5	89.6	-	-
28	Rapeseed cake, expeller-extracted 8	916.5	398.1	332.6	188.0	-
29	Rapeseed meal, solvent-extracted 1	925.3	380.6	289.8	218.0	-
30	Rapeseed meal, solvent-extracted 2	920.8	375.0	279.7	213.0	-
31	Rapeseed meal, solvent-extracted 3	918.9	383.1	293.8	220.0	-
32	Rapeseed meal, solvent-extracted 4	917.6	402.0	332.7	189.0	-
33	Rapeseed meal, solvent-extracted 5	923.1	401.9	316.5	204.0	-
34	Rapeseed meal, solvent-extracted 6	922.5	380.6	300.0	210.0	-
35	Rapeseed meal, solvent-extracted 7	931.7	372.1	318.0	-	-
36	Rapeseed meal, solvent-extracted 8	930.3	344.9	315.3	-	-
37	Rapeseed meal, solvent extracted (MervoBest) ^d	930.1	387.0	323.9	-	-
38	Rye, grain 1	904.5	91.7	67.6	-	-
39	Rye, grain 2	922.4	105.6	69.7	33.0	579.0
40	Rye, grain, treated ^b	915.5	91.4	72.7	-	-
41	Soybean hulls	926.5	130.0	101.7	468.0	-
42	Soybean meal (Bioprofin S) ^d	937.4	478.0	402.7	-	-
43	Soybean meal, solvent-extracted (MervoBest) ^d	933.2	530.0	438.1	-	-
44	Soybean meal, solvent-extracted (SoyPass) ^b	927.6	532.0	454.2	-	-
45	Sugar beet pulp, dried 1	927.5	106.9	55.0	166.0	-
46	Sugar beet pulp, dried 2	927.7	93.1	70.6	237.0	-

Table 5 (continued)		DM	CP	TP	ADF	Starch
47	Triticale, grain	916.4	123.8	91.7	42.0	614.0
48	Wheat, grain, treated ^b	918.9	170.5	141.8	-	-
49	Wheat, straw	946.0	35.6	29.2	480.0	-

^a Figures following feed names indicate number of source of the feedstuff (1, 2, ..., n).

^b Treated with xylose in liginosulphonate solution to increase the ruminally undegraded crude protein fraction.

^c Treated with heat to increase the ruminally undegraded CP fraction.

^d Treated with formaldehyde to increase the ruminally undegraded CP fraction.

2.2. Laboratory procedure for rumen incubation

Feed samples were ground to pass a 1-mm screen. The true protein (TP) contents of all samples were determined using trichloroacetic acid (10% in water, w/v) as precipitating agent (Licitra et al., 1996). For the next step, solutions were prepared as follows:

- 1 l of borate-phosphate buffer, pH 6.7 - 6.8: 12.20 g of monosodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and 8.91 g of sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) per 1 l of deionized water.
- 1 l protease solution (protease type XIV from *S. griseus*; Catalogue No. P5147, 4.6 U/mg or 5.2 U/mg; Sigma, Munich, Germany;) containing 1.0 U/ml of borate-phosphate buffer at pH 6.7 - 6.8: Dissolve 218 or 193 mg protease (depending on U/mg) to 1 l borate-phosphate buffer and filtrate the solution through Whatman #54 or 541 filter paper (25µm pore size) before use.

Based on the TP concentration of the feed sample, the addition of the *S. griseus* protease solution was adjusted to the ratio of 41 U/g TP (Licitra et al., 1998; 1999). The *S. griseus* procedure followed the standardized protocol of Licitra et al. (1998) with a different enzymatic concentration (1.0 U/ml instead of 0.33 U/ml) and slight technical modifications, which became necessary during establishment of the procedure. Feed samples (2.5 g) were weighed into 500-ml Erlenmeyer flasks and 200 ml of borate-phosphate buffer (pH 6.7 - 6.8) were added. Flasks were then kept in a shaking water bath at 39 °C for 1 h. The required amount of fresh protease solution (see above) was added to the flask. The flasks were removed after 18 h and the whole contents filtered through fibre filter bag (38 µm pore size). Mild vacuum was used to facilitate the filtration. Laboratory experience during establishment of the protocol showed that Whatman #541

or #54 filter paper as in Licitra et al. (1998) were not suitable for the purpose of this work because of the large sample volume that had to be filtrated and the time filtration required. The residues were washed with 1.25 l deionized water and then dried in a forced-air oven at 55 °C for 48 h. The dried residues from 6 flasks per feed were combined to provide enough material for the subsequent analytical steps.

2.3. Laboratory procedure for intestinal digestion

Dry matter (DM) and N contents were determined. Three replicates of each feedstuff residue were weighed into a 50 ml centrifugation tube in an amount corresponding to 15 mg N. Then 10 ml of a 0.1 N HCl solution (pH 1.9) containing 1g/l of pepsin were added to each centrifugation tube, which was shaken and then incubated for 1 h in a shaking water bath at 38 °C. After incubation, 0.5 ml of 1.0 N NaOH solution were added to each centrifugation tube to neutralize the solution, and then 13.5 ml of a pancreatin solution (pH 7.8) were added. The tubes were shaken and then incubated at 38 °C for 24 h in a shaking water bath. The tubes were vortexed approximately every 8 h. After incubation immediately 3 ml of trichloroacetic acid solution (100 % w/v) were added to each tube to stop enzymatic action and precipitate undigested proteins. After about 15 min, the samples were centrifuged at 10,000 x g for another 15 min at 5 °C. The precipitate was then filtrated through filter paper (no. 589¹, Schleicher and Schuell, Dassel, Germany) and the residue on the filter paper was analyzed for insoluble N by the Kjeldahl procedure.

2.4. General analytical procedures and calculations

The DM was estimated by oven-drying at 105 °C overnight and the N was determined using a standard Kjeldahl procedure using Cu²⁺ as a catalyst (method 4.1.1) according to “Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten” (VDLUFA; Bassler, 1993). The CP was calculated by multiplying N with 6.25. Starch content was measured by enzymatic hydrolysis of starch to glucose as described by Brandt et al. (1987) and acid detergent fibre (ADF) was analyzed according to the AOAC (1990) and expressed inclusive residual ash.

The IPD_{EIVP} was calculated directly for each feedstuff by using the following formula:

$$IPD_{EIVP} = (ANC - RNC / ANC) * 1000,$$

where IPD_{EIVP} = IPD estimated by EIVP (g/kg), ANC = the actual N content of the feed sample, which was the weight of the sample that should contain 15 mg N ($g \times DM$ ((g/kg)/1000) \times N ((g/kg)/1000)), and RNC = the residual nitrogen (g) of the precipitate.

The IPD values estimated from ISIVP (IPD_{ISIVP}) and MBT (IPD_{MBT}) were available from the concomitant comparison of these two procedures (see Chapter 2 for experimental methods and calculations).

2.5. Statistical methods

Differences between the EIVP and ISIVP or MBT were evaluated by generating linear regression equations. Relationships between chemical constituents and IPD values were evaluated using the Pearson correlation coefficient.

3. Results

3.1. Feedstuffs chemical composition and intestinal protein digestibility values

Feedstuffs with their DM, CP, ADF and starch values are presented in Table 5. The DM contents ranged from 902 to 946 g/kg. These values simply reflect that feed samples were stored dry for further analytical feed evaluation. The CP values ranged from 36 to 938 g/kg DM, with TP concentrations ranging from 29.2 to 884 g/kg DM, thus covering a very wide range of CP and TP concentrations of feedstuffs. The feedstuffs of this study had a close relationship between CP and TP values ($n = 49$, $r = 0.975$, $P < 0.01$). Starch values ranged from 45 to 662 g/kg DM and ADF values ranged from 3 to 480 g/kg DM. This large variation of CP, TP, starch and ADF values was helpful to improve the quality of the establishment of the EIVP and strengthen the recommendations as to the suitability of the procedure for estimating IPD values.

The IPD values estimated by EIVP, ISIVP and MBT are summarized in Table 6. The IPD_{EIVP} values ranged from 29.7 to 841 g/kg CP (mean, 654 g/kg CP), the IPD_{ISIVP} values ranged from 278 to 868 g/kg CP (mean 660 g/kg CP) and the IPD_{MBT} values ranged from 207 to 941 g/kg CP (mean 651 g/kg CP).

Table 6

Values of intestinal protein digestibility estimated by a three-step in situ-in vitro procedure (IPD_{ISIVP}), enzymatic in vitro procedure (IPD_{EIVP}) and mobile-bag technique (IPD_{MBT}).

No.	Feedstuff ^a	IPD_{ISIVP} ^b (g/kg CP)	IPD_{EIVP} ^b (g/kg CP)	IPD_{MBT} ^c (g/kg CP)
1	Barley, grain 1	764± 1.5	775± 4.6	766
2	Barley, grain 2	608± 2.7	635± 3.6	773
3	Barley, grain 3	786± 6.2	787± 5.6	831
4	Barley, grain 4	741± 2.6	731± 1.2	-
5	Barley, grain, treated ^d	794± 1.2	777± 5.1	-
6	Blood meal	861± 0.1	841± 1.3	941
7	Brewers' grain 1	666± 2.5	670± 10.1	623
8	Brewers' grain 2	725± 0.4	709± 8.6	650
9	Coconut meal, solvent-extracted	745± 1.3	730± 7.3	566
10	Commercial mixed concentrates	657± 6.0	670± 8.6	617
11	Fish meal	868± 2.6	841± 7.7	863
12	Fish meal, Norway, treated ^e	852± 1.5	805± 7.5	822
13	Galega	491± 1.5	480± 17.2	609
14	Guar meal	657± 4.7	661± 8.4	604
15	Maize, grain	716± 1.8	732± 6.1	732
16	Maize, distillers' grain	680± 0.9	670± 2.4	753
17	Maize, grits 1	666± 8.5	672± 13.8	646
18	Maize, grits 2	687± 8.0	694± 17.4	743
19	Maize meal 1	674± 1.4	694± 2.1	711
20	Maize meal 2	732± 7.0	709± 4.8	547
21	Rapeseed cake, expeller-extracted 1	455± 7.6	456± 10.2	207
22	Rapeseed cake, expeller-extracted 2	428± 2.5	430± 15.7	261
23	Rapeseed cake, expeller-extracted 3	652± 8.0	624± 7.4	702
24	Rapeseed cake, expeller-extracted 4	624± 6.8	613± 21.2	519
25	Rapeseed cake, expeller-extracted 5	641± 1.9	631± 8.2	640
26	Rapeseed cake, expeller-extracted 6	513± 1.4	517± 9.0	401
27	Rapeseed cake, expeller-extracted 7	557± 1.8	580± 8.5	-
28	Rapeseed cake, expeller-extracted 8	694± 1.8	663± 8.2	692
29	Rapeseed meal, solvent-extracted 1	368± 3.5	379± 20.4	-
30	Rapeseed meal, solvent-extracted 2	278± 0.4	297± 6.7	-
31	Rapeseed meal, solvent-extracted 3	300± 1.1	311± 36.0	-
32	Rapeseed meal, solvent-extracted 4	574± 10.6	533± 38.6	474
33	Rapeseed meal, solvent-extracted 5	543± 0.3	544± 2.7	490
34	Rapeseed meal, solvent-extracted 6	662± 3.5	638± 8.4	690
35	Rapeseed meal, solvent-extracted 7	716± 0.8	710± 9.3	-
36	Rapeseed meal, solvent-extracted 8	547± 2.5	539± 18.8	-
37	Rapeseed meal, solvent extracted (MervoBest) ^f	711± 0.1	705± 10.1	695
38	Rye, grain 1	800± 1.2	755± 3.1	-
39	Rye, grain 2	664± 7.9	654± 5.1	843
40	Rye, grain, treated ^d	733± 2.3	746± 9.9	-
41	Soybean hulls	671± 9.9	662± 11.4	556
42	Soybean meal (Bioprofin S) ^f	807± 1.2	793± 6.6	759
43	Soybean meal, solvent-extracted (MervoBest) ^f	810± 1.5	801± 8.1	720
44	Soybean meal, solvent-extracted (SoyPass) ^d	848± 10.3	822± 5.0	917
45	Sugar beet pulp, dried 1	553± 10.7	564± 11.7	538
46	Sugar beet pulp, dried 2	783± 1.0	785± 11.3	641

Table 6 (continued)		IPD _{ISIVP} ^b (g/kg CP)	IPD _{EIVP} ^b (g/kg CP)	IPD _{MBT} ^c (g/kg CP)
47	Triticale, grain	606± 14.3	586± 7.5	561
48	Wheat, grain, treated ^d	848± 0.8	802± 3.7	-
49	Wheat, straw	603± 1.8	616± 5.0	623

^a Figures following feed names indicate number of source of the feedstuff (1, 2, ..., n).

^b Means ± SE, based on 3 replicates.

^c TPD_{MBT}: only means values were available of the data at the Danish Institute of Agricultural Sciences.

^d Treated with xylose to increase the undegradable crude protein fraction.

^e Treated with heat to increase the undegradable crude protein fraction.

^f Treated with formaldehyde to increase the undegradable crude protein fraction.

The relationships between IPD_{EIVP}, and either IPD_{ISIVP} or IPD_{MBT} were evaluated by computing simple linear regression equations. The equation best describing the relationship between IPD_{ISIVP} and IPD_{EIVP} was (Figure 5): $IPD_{ISIVP} = 1.053 IPD_{EIVP} - 28.14$ ($n = 49$, $r^2 = 0.985$, $P < 0.0001$), whereas the following relationship evolved between IPD_{MBT} and IPD_{EIVP} (Figure 6): $IPD_{MBT} = 1.221 IPD_{EIVP} - 165.95$ ($n = 38$, $r^2 = 0.666$, $P < 0.0001$). The coefficient of determination (r^2) of > 0.98 for the relationship between the IPD_{ISIVP} and IPD_{EIVP} and the overall appearance of the graph (Figure 5) indicate that the enzymatic procedure (EIVP) performed as well as the combined in situ-in vitro procedure (ISIVP). As expected, the relationship between IPD values estimated by ISIVP and EIVP was closer than that between the EIVP and MBT.

Pearson correlation coefficients were computed to analyze the relationship between CP, TP, starch or ADF contents and IPD_{EIVP} values (data not shown). The CP, TP or starch contents were not ($P > 0.05$) related to IPD_{EIVP} values. However, a significant and negative correlation was observed between ADF concentrations contents and IPD_{EIVP} values ($n = 29$, $r = -0.382$, $P < 0.05$), indicating that feeds having higher lignocellulose concentrations generally will have lower IPD_{EIVP} values.

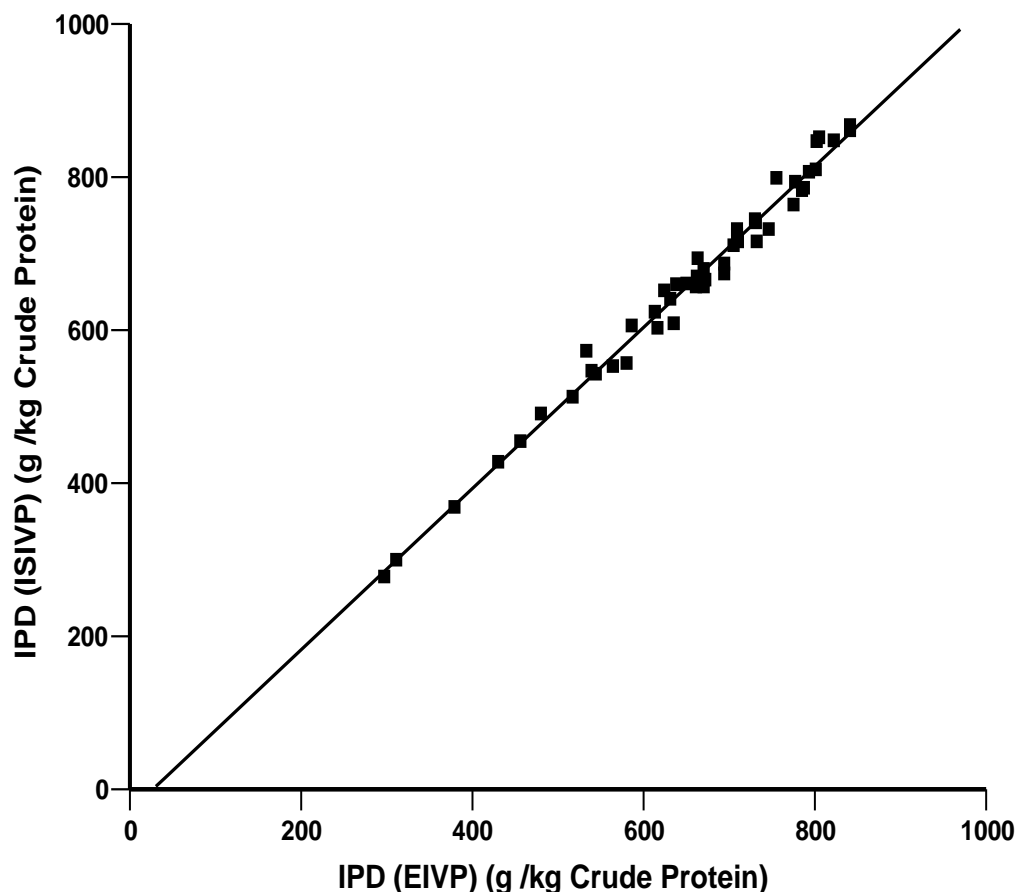


Figure 5

Relationship between intestinal protein digestibility (IPD) values estimated by a three-step in situ-in vitro procedure (ISIVP) and enzymatic in vitro procedure (EIVP). The regression equation is: $IPD_{ISIVP} = 1.053 IPD_{EIVP} - 28.14$ ($n = 49$, $r^2 = 0.985$, $P < 0.0001$).

3.2. Applying the enzymatic procedure to feed samples with elevated ruminally undegraded crude protein concentrations

Eight feed samples, which were specifically treated with heat, xylose in lignosulphonate solution or formaldehyde to increase concentrations of RUP, were compared for their IPD values estimated with EIVP and ISIVP. The IPD_{EIVP} vs. IPD_{ISIVP} values (Table 6) for barley grain, fish meal (Norway), solvent extracted rapeseed meal (MervoBest), rye grain, soybean meals (Bioprofin S, MervoBest, Soypass) and wheat grain were 777 versus 794, 805 versus 852, 705 versus 711, 746 versus 733, 793 versus 807, 801 versus 810, 822 versus 848 and 802 versus 848 g/kg CP, respectively. Both procedures ranked IPD values similarly. Although these treated feed samples had elevated RUP contents (see Chapter 2), it was noticeable that their IPD values were in the upper range of values, indicating that no chemical or thermal damage to the CP and particularly

the RUP had occurred. Moreover, the three treated soybean meals had similar IPD values, indicating that their RUP was similarly affected by the different treatment methods, albeit RUP values were not the same across treated soybean meal samples.

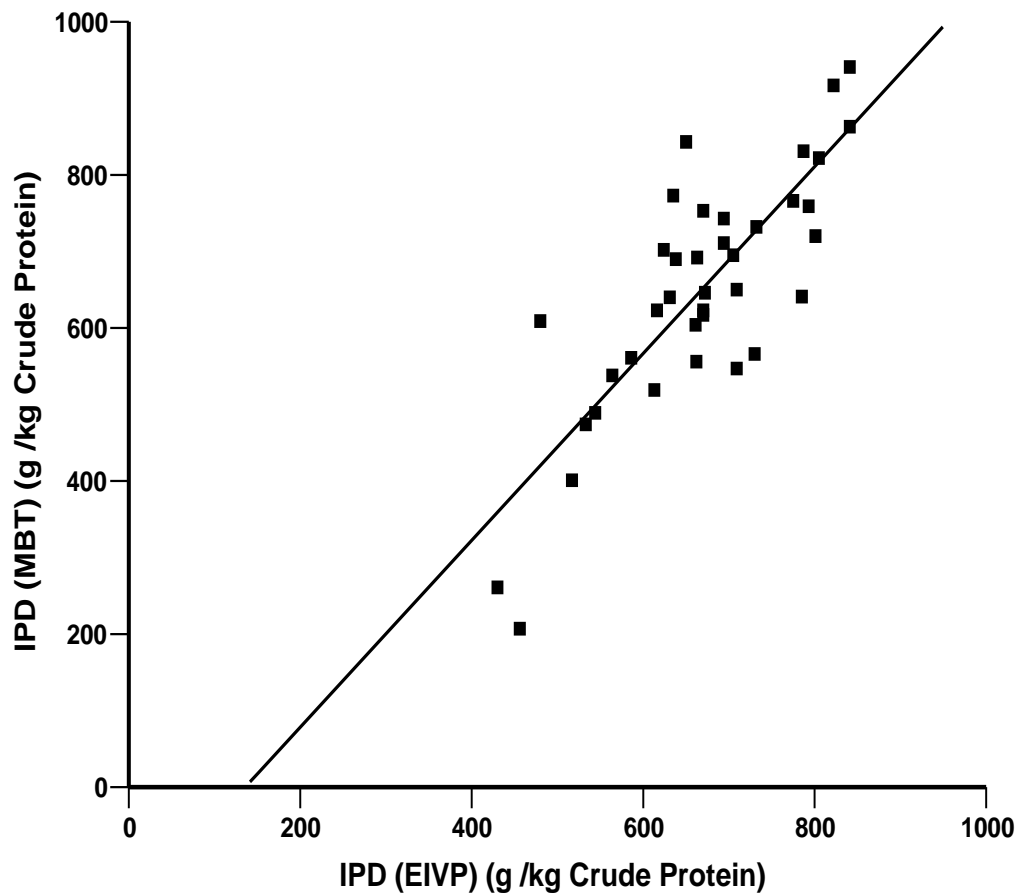


Figure 6

Relationship between intestinal protein digestibility (IPD) values estimated by enzymatic in vitro procedure (EIVP) and mobile-bag technique (MBT). The regression equation is: $IPD_{MBT} = 1.221 IPD_{EIVP} - 165.95$ ($n = 38$, $r^2 = 0.666$, $P < 0.0001$).

4. Discussion

The MBT, as a procedure for estimating IPD, has two inherent major sources of variation when repeated measurements are achieved. These sources are rumen incubation and (small) intestinal incubation and therefore, biologically caused. Table 7 provides an overview of IPD_{MBT} values for a range of feedstuffs taken from 10 different studies. Some of the feedstuffs show a much larger variation of values than would be assigned to a normal within-feedstuff variation. This observation can thus be considered as being caused by within-method variation, which is also likely because different authors used different protocols when conducting the MBT.

Therefore, a stricter standardization across laboratories of the MBT would be required to control the inevitable biological variation and widen the applicability of MBT data across different protein evaluation systems. An alternative approach which was followed in this study is to focus on development and standardization of procedures from which protein value estimates of feedstuffs can be obtained without animal-based protocols.

Comparisons between MBT and enzymatic (pepsin-pancreatin) in vitro methods as a replacement for the (small) intestinal incubation step have been conducted (van Straalen et al., 1993; Calsamiglia and Stern, 1995; Kopečný et al., 1998). Although based on only few feeds (Calsamiglia and Stern, 1995), forage as the sole feed (van Straalen et al., 1993) or using a less standardized in vitro protocol than was used in this study (Kopečný et al., 1998), those authors demonstrated the feasibility of enzymatic in vitro procedures as an alternative to MBT for estimating IPD. Antoniewicz et al. (1992) estimated IPD from either MBT or an enzymatic in vitro procedure for lupins, peas, field beans and rapeseed meals feeds which were treated with different concentrations (0, 10, 20, 30 and 40 g/kg CP) of formaldehyde to increase the concentration of RUP. They found that MBT digestibility values were significantly ($P < 0.001$) correlated with in vitro values (except for the samples of formaldehyde-treated rapeseed meal).

It should be noted, however, that the enzymatic (pepsin-pancreatin) in vitro procedure, which was used (Antoniewicz et al. 1992; van Straalen et al., 1993; Calsamiglia and Stern, 1995; Kopečný et al., 1998), still requires rumen incubation as the first stage. Therefore, rumen incubation was the final obstacle for a wider application – including routine laboratory measurements of IPD – of the pepsin-pancreatin methods.

Table 7

Literature values of intestinal protein digestibility (g/kg crude protein) of feedstuffs estimated by mobile-bag technique.

Feedstuff	1	2	3	4	5	6	7	8	9	10
Barley, grain	-	-	887	-	-	790	955	-	-	766-831
Beet pulp, dried	-	-	-	-	738-855	-	-	-	670-727	538-641
Blood meal	-	-	-	898	-	-	-	-	-	941
Clover	-	-	-	773-858	-	-	-	914	-	-
Coconut meal, solvent-extracted	-	959	-	-	-	-	-	-	-	566
Cotton seed meal, expeller-extracted	-	950	949	949	889-964	800	-	-	737-904	-
Fish meal	828	-	945	945	-	930	-	-	-	863
Guar meal	-	-	-	-	919-943	-	-	-	-	604
Lucerne hay	712	-	739	739	-	730	-	-	-	591
Maize, grain	-	-	946	-	-	890	923	-	-	731
Maize distillers' grain	-	-	-	-	881-918	-	-	-	750-935	753
Maize gluten feed	950	-	-	-	924	-	-	-	550-721	-
Maize silage	-	-	483	483	-	890	-	-	-	-
Meat and bone meal	730	-	724	724	-	-	-	-	-	-
Oat, grain	-	-	700	-	-	-	720	-	-	798
Peas	-	972	925	-	-	-	-	-	-	582
Rapeseed meal, expeller-extracted	-	-	571	748-804	855-905	-	745	-	646-765	207-822
Rye, grain	-	-	944	-	-	-	865	-	-	843
Soybean meal, solvent-extracted	993	991	990	970-990	-	860	973	-	966-985	-
Sunflower meal, solvent-extracted	-	-	-	-	950-961	-	-	-	428-854	-
Wheat, grain	-	-	886	-	-	-	946	-	-	782
Wheat, bran	-	-	709	709	-	300	-	-	-	-
Wheat, straw	-	-	615	-	-	-	-	-	-	623

¹ DeBoer et al., 1987.

⁶ Taghizadeh et al., 2005.

² Hvelplund et al., 1992.

⁷ Tománková and Homolka, 2002.

³ Frydrych, 1992.

⁸ van Straalen et al., 1993.

⁴ Kopečný et al., 1998.

⁹ Woods et al., 2003.

⁵ Kusumanti et al., 1996.

¹⁰ Chapter 2 (this thesis).

In attempts to standardize the rumen degradation procedure with in vitro procedure, previous studies have investigated the feasibility of replacing the rumen incubation step with incubation of feed samples with mixed rumen microorganisms or proteolytic enzymes extracted from rumen contents (Kohn and Allen, 1995; Luchini et al., 1996a, b). The results showed the ability of these procedures to mimic in situ rumen incubation. However, ruminally fistulated animals are still needed. Alternatively, feed samples can be incubated in a solution containing a protease from *S. griseus* instead of rumen fluid or

proteolytic enzymes extracted from rumen contents. This approach was widely tested in different studies (Kopečný et al., 1989; Roe et al., 1991; Aufrère et al., 1991; Assoumani et al., 1992; Cone et al., 1996; Coblenz et al., 1998). Licitra et al. (1998, 1999) largely improved the quality of this technique by determining the activity of protease, which most closely reflected ruminal in situ CP degradability values. They also used variable ratio of enzyme units for each tested feed sample depending on TP content instead of using a constant ratio for all types of feedstuffs.

In the present study, every effort was undertaken to identify the most accurate, standardized and still practically applicable procedure for replacing the rumen incubation step of the ISIVP. Previously, Tománková and Homolka (2002) tested a combined enzymatic method for determination of IPD of selected concentrates and solvent-extracted oil meals. They used bromelain, a mixture of sulphur-containing protease and several other substances in smaller quantities including: peroxidase, acid phosphatase, protease inhibitors, and calcium (review by Gregory and Kelly, 1996), instead of rumen incubation for 1- or 16-h intervals followed by incubation of undegraded residues with pancreatin for 24 h instead of small intestinal incubation. Statistical analysis revealed that the combined enzymatic method using 1 h pre-incubation was more suitable for estimation of IPD compared with MBT. McNiven et al. (2002) also examined a modification of the Calsamiglia and Stern (1995) procedure by replacing the rumen incubation step with a *S. griseus* protease. The investigated samples were barley, oats, wheat and soybeans, heat-treated for various lengths of times. Contrary to the current study, McNiven et al. (2002) used a constant concentration of protease for all types of feeds and found a significant relationship ($r^2 = 0.95$, $P < 0.0001$) between IPD estimated by MBT and their modified procedure for all grain samples, whereas the relationship was only very weak ($r^2 = 0.02$) when soybean samples were included in the statistical analysis. Mesgaran and Stern (2005) evaluated the ruminal, post-ruminal and total tract CP disappearance of various feeds originating from Iranian plant varieties. They used MBT, ISIVP and the in vitro procedure of McNiven et al. (2002). Mesgaran and Stern (2005) found that the best correlation was observed when results of total tract CP disappearance determined by the procedure of McNiven et al. (2002) were compared with those obtained from ISIVP ($r^2 = 0.66$), while the correlations between MBT and ISIVP or the procedure of McNiven et al. (2002) were slightly lower (0.55 and 0.61, respectively). These results are in accordance with those observed in the current study where the relationship between ISIVP and EIVP was very close and significant ($n = 49$,

$r^2 = 0.98$, $P < 0.0001$) and also (but less pronounced) a significant correlation between EIVP and MBT was observed ($n = 38$, $r^2 = 0.66$, $P < 0.0001$).

5. Conclusions

A strictly standardized enzymatic procedure (EIVP) based on the three-step in situ-in vitro procedure of Calsamiglia and Stern (1995) was developed and established using a wide range of feedstuffs for ruminants, encompassing forages with varying CP contents, unprotected or rumen-protected protein supplements and cereal grains. The IPD values estimated from the new procedure compared well with data derived from ISIVP and MBT. The EIVP is more standardized than the ISIVP due to replacing the incubation of the feed samples in the rumen with a more standardized and thus more repeatable enzymatic procedure.

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CHAPTER 4

General Discussion

The overall objective of this thesis was to develop and establish a completely laboratory-based *in vitro* procedure for estimating (small) intestinal digestibility of ruminally undegraded feed protein. This goal was achieved with an enzymatic *in vitro* procedure (EIVP) that could be used effectively to estimate intestinal protein digestibility (IPD). The mobile-bag technique (MBT) was the reference method for evaluating the EIVP. In addition, the three-step *in situ-in vitro* procedure (ISIVP) developed by Calsamiglia and Stern (1995) was used as a previously established alternative for the MBT. In the ISIVP, pepsin-pancreatin incubation steps replace the small intestinal (duodenum to ileum) incubation protocol of the MBT and, therefore, no animals are required carrying cannula in the small intestine. However, as ruminal incubation of samples is still necessary, ruminally cannulated animals must be used.

Previous research was directed to assess the suitability of the ISIVP as a replacement for the more invasive MBT for estimating IPD, yet this was done with only few feed samples in the study by Calsamiglia and Stern (1995). Although Woods et al. (2003) conducted a comprehensive study to develop a database of small intestinal digestibility of protein concentrates applying both ISIVP and MBT, these authors had no forage or grain samples included in their study. Woods et al. (2003) found a close relationship between the two methods, ($r = 0.91$) and concluded that the ISIVP offers a quick and reliable method to assess IPD. The results obtained in this study are in general accordance with previous researches (Calsamiglia and Stern, 1995; Woods et al., 2003) as to the suitability of the ISIVP to estimate. In the current research, feedstuff types covered a wider range including forages and grains and can thus be considered as being more representative than the feedstuffs which provided the bases of previous studies. Thus, the comparatively weak relationship between IPD values estimated from ISIVP and MBT methods ($r^2 = 0.36$ which was improved to $r^2 = 0.661$ after excluding 14 feed samples) might at least partly be due to inclusion of very different feedstuffs into the investigation.

Further, rumen incubation has very likely played an important role in the weakening of the relationship between the two methods. Both MBT and ISIVP involved ruminal incubation of the samples as a preliminary stage but this was done at different places using cows with the MBT and steers with the ISIVP,

creating different rumen environments. Therefore, the role of rumen incubation in affecting the IPD values needs to be considered. Volden and Harstad (1995) found a negative correlation between ruminal CP degradation and true indigestibility of feed protein. By utilizing peas, rapeseeds, lupin, barley and oat grains, they also found that when values of true indigestibility of feed protein are used for predicting the true digestibility of ruminally undegraded feed CP (RUP) with the MBT, these values should be determined on feed samples after ruminal incubation and not on original feed samples. Calsamiglia and Stern (1995) evaluated whether rumen incubation had an influence on IPD values in the ISIVP and reported that rumen incubation affected IPD values such that they were lower for feed samples after pre-incubation in the rumen. As rumen incubation period increased, rumen degradability also increased due to increasing time for exposure of feed material to the microorganisms. Hvelplund et al. (1992) found that as rumen degradability increased, IPD decreased and concluded that feed samples contain a CP fraction which is both undegradable in the rumen and indigestible in the intestine. Furthermore, rumen incubation can be affected by numerous factors, including bag porosity, bag material, bag size, sample processing, sample size, incubated substrate-to-bag surface area ratio, type of basal diet, concentrate level in the basal diet, bag insertion and removal procedures, location of bags within the rumen, washing procedure, incubation time and number of replicate animals and bags to obtain repeatable estimates of ruminal degradation, which may cause variations in rumen degradation (Robinson et al., 1992; Vanzant et al., 1998; Nozière and Michalet-Doreau, 2000).

Additional evidence that rumen incubation period has a major impact on differences between MBT and ISIVP is provided by Kopečný et al. (1998), who compared IPD measured by a pancreatin method with results obtained by MBT for 55 feed samples and a polycentric fungus. Kopečný et al. (1998) pre-incubated the samples in the rumen for 16 h and continued with either MBT or pancreatin, thus eliminating the effect of rumen incubation as a source of variation between the two procedures and found a close relationship between values from the two procedures ($r^2 = 0.861$).

According to the above observations on the large possible effects of rumen incubation on IPD estimates and because of the difficulties in standardizing ruminal incubation of feedstuffs (confer e.g. Südekum, 2005) a widely accepted, accurate, practical, reliable and more standardized procedure is needed to replace ruminal incubation as a preliminary step in IPD estimates. One theoretically attractive and promising way to replace rumen incubation with more standardized in vitro procedures was to utilize mixed rumen microorganisms or proteolytic enzymes extracted from rumen contents (Kohn and Allen, 1995; Luchini et al., 1996a, b), as these procedures closely mimic ruminal degradation yet under more easily standardized conditions in the laboratory. Although the results of the studies by Kohn and Allen (1995) and Luchini et al. (1996a; b) demonstrated the ability of these procedures to mimic in situ rumen incubation, the major disadvantage is that ruminally cannulated animals are still needed.

Another more widely used approach to estimate ruminal CP degradation utilizes an incubation of feed samples with a protease from *Streptomyces griseus* instead of rumen fluid or proteolytic enzymes extracted from rumen contents (Kopečný et al., 1989; Roe et al., 1991; Aufrère et al., 1991; Assoumani et al., 1992; Cone et al., 1996; Coblenz et al., 1998). Licitra et al. (1998, 1999) largely improved and standardized this technique by determining the suitable activities of protease which closely resembled the CP degradability values estimated in situ. They also used variable ratio of enzyme units for each tested feed sample depending on true protein (TP) content instead of using a constant ratio for all types of feedstuffs.

In the current study the in vitro procedure of Licitra et al. (1998; 1999) was chosen and adopted in the EIVP as a substitute for rumen incubation of feed samples as used in the ISIVP. Regarding the estimation of IPD values, the relationship between ISIVP and EIVP was very strong ($n = 49$, $r^2 = 0.98$, $P < 0.0001$) and – although being a little weaker – was accompanied by a significant relationship between EIVP and MBT ($n = 38$, $r^2 = 0.66$, $P < 0.0001$).

In the present study, every effort was undertaken to identify the most accurate, standardized and still practically applicable procedure for replacing the rumen incubation step of the ISIVP. Previously, Tománková and Homolka

(2002) tested a combined enzymatic method for determination of IPD of selected concentrates and solvent-extracted oil meals. They used bromelain, a mixture protease and several other enzymes in smaller quantities, instead of rumen incubation for 1- or 16-h intervals followed by incubation of undegraded residues with pancreatin for 24 h instead of small intestinal incubation. The statistical analysis conducted by Tománková and Homolka (2002) revealed that the combined enzymatic method using 1 h pre-incubation was more suitable for estimation of IPD compared with MBT.

McNiven et al. (2002) examined a modification of the Calsamiglia and Stern (1995) procedure by replacing the rumen incubation step with a *S. griseus* protease. The investigated samples comprised barley, oats, wheat and soybeans, heat-treated for various lengths of times. Contrary to the current study, McNiven et al. (2002) used a constant concentration of protease for all types of feeds and found a significant relationship ($r^2 = 0.95$, $P < 0.0001$) between IPD estimated by MBT and their modified procedure for all grain samples, whereas the relationship was only very weak ($r^2 = 0.02$) when soybean samples were included in the statistical analysis. This observation again would points out that enzyme concentrations or – more precisely – activities should be used relative to the TP contents of the samples under investigation.

Mesgaran and Stern (2005) evaluated the ruminal, post-ruminal and total tract CP disappearance of various feeds originating from Iranian plant varieties. They used MBT, ISIVP and the in vitro procedure of McNiven et al. (2002). Mesgaran and Stern (2005) found that the best correlation was observed when results of total tract CP disappearance determined by the procedure of McNiven et al. (2002) were compared with those obtained from ISIVP ($r^2 = 0.66$), while the correlations between MBT and ISIVP or the procedure of McNiven et al. (2002) were slightly lower (0.55 and 0.61, respectively). These results are in accordance with those observed in the current study where the relationship between ISIVP and EIVP was very close and significant ($n = 49$, $r^2 = 0.98$, $P < 0.0001$) and also (but less pronounced) a significant correlation between EIVP and MBT was observed ($n = 38$, $r^2 = 0.66$, $P < 0.0001$).

Conclusions

From this study, three major conclusions can be drawn:

1. The three-step in situ-in vitro procedure (ISIVP) of Calsamiglia and Stern (1995) could be successfully applied to a wide range of feedstuffs encompassing forages with varying CP contents, unprotected or rumen-protected protein supplements and cereal grains representing energy-rich feeds having low to medium CP concentrations. The method provided reliable estimates of IPD.
2. A strictly standardized enzymatic procedure (EIVP) based on the ISIVP was developed and established using a wide range of feedstuffs for ruminants, encompassing forages with varying CP contents, unprotected or rumen-protected protein supplements and cereal grains. The IPD values estimated from the new procedure compared well with data derived from ISIVP and MBT. The EIVP is more standardized than the ISIVP due to replacing the incubation of the feed samples in the rumen with a more standardized and thus more repeatable enzymatic procedure.
3. The EIVP in its current, strictly standardized form can be applied to develop a database that can be used across protein evaluation systems for establishing tabular values of IPD.

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