Netherlands Journal of Agricultural Science 45 (1997) 291-306

Potential of solubility, enzymatic methods and NIRS to predict *in situ* rumen escape protein

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Received 23 August 1996; accepted 2 January 1997

Abstract

The percentage of feed protein escaping rumen degradation was measured by the *in situ* method (%EP*situ*) for 29 compound feeds, untreated and formaldehyde-treated soybean meal and 12 forages: 3 grass silages, 2 maize silages, fresh grass, grass hay, fodder beets, fresh potatoes, ensiled beet pulp, chopped ear corn silage and brewers' grains. The loss of particles through the bag pores was determined as the difference between the washable fraction (W) and the fraction, soluble in borate-phosphate buffer at pH 6.7 (S). W-S was most pronounced for compound feeds (on average 14.4%-units), for brewers' grains and maize silages. A correction of %EP*situ*, assuming that W-S degrades like the potentially degradable fraction, appeared however not appropriate. Solubility in borate-phosphate buffer after 1 h, enzymatic degradability by protease from *Streptomyces griseus* or ficin after 1, 6 and 24 h and NIRS (for compound feeds alone) were examined as routine method to predict %EP*situ*. With the buffer and with *S. griseus* the effect of pH (6.7 vs. 8.0) and at pH 8.0 the effect of the amount of substrate (500 mg sample vs. 20 mg nitrogen (N)) was tested. With ficin, 500 mg samples were incubated at pH 6.7. Predictions were better when compound feeds and forages were considered separately. However, the best *in vitro* method was different for the two feed categories, being solubility in buffer for the compound feeds and enzymatic degradation of a constant amount of protein with *S. griseus* at pH 8.0 for forages. NIRS showed potential to predict %EP*situ* of compound feeds, but needs more reference samples. The Dutch feed tables appeared more accurate than the best *in vitro* method for compound feeds, but seemed too rough for some forages like fodder beets, maize silage and ear corn silage.

Keywords: rumen escape protein, *in situ*, solubility, enzymatic methods, NIRS, tables

Introduction

In the new Dutch protein evaluation system for cattle (Tamminga *et al.*, 1994) the percentage of feed protein escaping rumen degradation (%EP) has to be known. The *in situ* procedure, by which feed samples are incubated during different times in the

[1] Communication n° 993 of the institute.

rumen of cows or sheep, is accepted as reference method. Besides the need for fistulated animals, the method is laborious, time-consuming and expensive and not suited for feed evaluation in practice. Therefore, tables are available with mean values for most compound feed ingredients and forages and regression equations based on chemical composition and harvest day for grass products (Anonymous, 1992, 1996). However, due to the variability within feeds the use of tabular values may give rise to serious errors. As a solution in between, different *in vitro* methods, with either buffer solutions (Crooker *et al.*, 1978; Waldo & Goering, 1979; Krishnamoorthy *et al.*, 1982), rumen fluid (Broderick, 1987) or commercial enzymes (Poos-Floyd *et al.*, 1985; Aufrère & Cartailler, 1988) as inoculum, were developed. Solubility methods are very simple, but are poorly related to %EP*situ*, when feedstuffs of different nature are pooled (De Boever *et al.*, 1984). Rumen fluid and enzymatic methods offer the advantage that degradation can be followed in time and yield data suitable for modelling. However, methods based on rumen fluid are less suited for practice and suffer from problems with re-utilization of degration products when used fresh or with a substantial loss of proteolytic activity when making an enzyme preparation (Broderick, 1987; Mahadevan *et al.*, 1987). Feed evaluation in practice increasingly makes use of near infrared reflectance spectroscopy (NIRS) as a fast and ecologically sound technique. Up to now, experience with NIRS to predict protein degradability is limited to forages (Waters & Givens, 1992; Antoniewicz *et al.*, 1995).

This study aims to examine the accuracy of solubility, enzymatic methods, using either a bacterial or a vegetal protease and NIRS to predict the %EP*situ* of compound feeds and some forages. Besides, the effect of a correction for loss of particles when applying the bag technique was investigated.

Material and methods

Feeds

In totally 43 feeds were examined, comprising 29 compound feeds, untreated and formaldehyde treated soybean meal and 12 fresh or ensiled forages. The compound feeds were composed at the institute for experiments with dairy and finishing beef cattle. Their ingredient composition is given in a previous paper (De Boever *et al.*, 1995). The forages concerned three grass silages (A, B and C), fresh grass, grass hay, two maize silages (A and B), chopped ear corn silage, ensiled pressed beet pulp, ensiled brewers' grains, fresh potatoes and fodder beets. The grass products originated from mixed pastures with predominantly *Lolium perenne*. Grass for silage A was cut on 18 May 1992 and prewilted for one day. Grass for silages B and C as well as for hay was from the same parcel cut on 10 May 1993; one third was respectively ensiled directly (without preservative), ensiled after one day prewilting and pressed after 7 days drying in the field and further dried in the barn. Maize silage A was from cultivar Aladin and B from cultivar Magister, both harvested in 1993 at the mature grain stage.

Rumen in situ method

In situ protein degradation was measured in 1992 (16 compound feeds), in 1993 (13 compound feeds, 2 soybean meals, grass silage A, beet pulp and fodder beets) and in 1994 (other forages). In 1992 two and in 1993 and 1994 four Holstein-Friesian cows, with a moderate to high milk production were used. The animals were fed according to their requirements with maize silage and concentrates in a ratio of about 65/35 (DM-basis) and in two equal amounts at 8.00 a.m. and 8.00 p.m.

Undried samples of the compound feeds and soybean meals were ground to pass a 5-mm sieve. Forages were preserved in a frozen state (except grass hay and fodder beets) after being minced (except beet pulp, ear corn silage and brewers' grains) following different procedures. Grass products were chopped with a paper-cutter in pieces of about 1 cm; frozen maize silage was further reduced in size with a dish-cutter (Hobart type 84415, Ohio, USA), frozen potatoes were mechanically smashed, lyophilized beets were ground with a Brabender mill without sieve. About 5 g dry matter (DM) (± 10 g DM for the prolonged incubation) was weighed in sealed nylon bags of 10 × 8 cm (Nybolt, polyamide, porosity 26%, mesh size 40 µm, Zurich, Switzerland). All the feeds were incubated during 0, 3, 6, 12, 18, 24 hours and 12–14 days; the forages were further incubated for 48 and 72 hours. To obtain enough residue, the number of bags increased from two for 3h to six for 24h and longer. All incubations started after the morning feeding with exception of the 18h-bags, which were inserted after the evening feeding. In 1992, all incubation intervals up to 24 hours were done for two feeds on the same day, whereas in 1993 and 1994 feeds were simultaneously incubated following a random distribution of intervals over days. The prolonged incubation occurred afterwards. After incubation, bags were washed in a washing machine with cold water during 50 min. (no spinning). Residues were dried in a ventilated oven at 60–70 °C and weighed. They were pooled per time and per cow, ground through a 1 mm-sieve and analyzed for DM, ash and N. The crude protein (CP) degradation characteristics were derived after averaging the *in situ* values over cows. The potentially degradable CP fraction was calculated as D = 100 – W – U, with W: the washable fraction and U: the undegradable fraction after prolonged incubation. The degradation rate (k_d) was estimated by iteration from the first order model of Ørskov & McDonald (1979). The %EP*situ* was calculated using equation (1) (Ørskov & McDonald, 1979).

$$\%EP = U + (k_p/(k_d + k_p)) \times D \quad (1)$$

In this equation, k_p is the rumen passage rate, assumed to be 4.5% h^{-1} for roughages and 6% h^{-1} for concentrates (Tamminga *et al.*, 1994); in our study a k_p of 4.5% h^{-1} was applied for the grass products and maize silages and a k_p of 6% h^{-1} for the remaining feeds. For the ensiled feeds 5% of W was supposed to be undegradable (Tamminga *et al.*, 1994). Because there are indications that W overestimates the real soluble fraction, particularly for concentrates (Cone *et al.*, 1995; De Boever *et al.*, 1995; Dewhurst *et al.*, 1995), %EP*situ* was also corrected for loss of particles. Therefore, the soluble fraction (S) was measured after soaking for 1h in a borate-phosphate buffer at pH 6.7 (see further). The corrected %EP*situ* was calculated with

equation (1), but using corrected values for D and U, obtained with equations (2) and (3), respectively (Lopez *et al.*, 1994):

$$Dc = D / (1 - \lambda) \text{ with } \lambda = W - S / (100 - S) \quad (2)$$

$$Uc = 100 - Dc - S \quad (3)$$

According to this correction, which does not affect the estimate of the degradation rate k_d, W – S is assumed to degrade with the same rate as D.

The residues in the bags after incubation were not corrected for possible contamination with microbial matter. More or a similar residu during the first hours of incubation than after simply washing was observed for the two maize silages, beet pulp and fodder beets. However, a correction, as proposed by Michalet-Doreau & Ould-Bah (1989) seemed not appropriate here because their washing procedure was less intensive than in this study.

Solubility and enzymatic methods

For the *in vitro* experiments, samples were dried and ground through a 1 mm screen. Nitrogen solubility was measured by incubation during 1h in 0.1 M borate-phosphate buffer at 40°C (Cone *et al.*, 1995). Using the same buffer, enzymatic degradability was determined after 1, 6 and 24 h incubation with either a bacterial protease from *Streptomyces griseus* (type XIV, Sigma P-5147, St Louis, MO, USA) or a vegetal protease ficin (from fig tree latex, Sigma EC 3.4.22.3, F-4165, St Louis, MO, USA), as described by Aufrère & Cartailler (1988) and adapted by Cone *et al.* (1995). With the buffer alone and with *S. griseus*, the effect of pH (6.7 vs. 8.0) and at pH 8.0 the effect of the amount of substrate (20 mg N vs. 500 mg sample) was tested. In the case of ficin, 500 mg samples were incubated and the pH of the buffer was 6.7. The amount of enzyme per sample corresponded to 1 mg for *S. griseus* and 5 mg for ficin. After incubation, tubes were centrifuged for 5 min at 1500 × *g* and N was determined in 10 ml of the supernatant. To correct for the enzyme residue in the supernatant, two blanks, containing 50 ml protease-buffer without sample, were incubated for 1 h in each run. All in vitro tests were carried out in duplicate in two runs; when disappearance values differed by more than 10%, a third determination was carried out. The repeatability of the *in vitro* tests was calculated as the mean standard error (SE) between replicates.

Similarly to %EP*situ*, %EP*vitro* was calculated for the four enzymatic procedures using equation (1) and D = 100 – S – U, with S being the corresponding buffer soluble fraction (0 h incubation) and U the residue after 24h incubation.

NIRS-analysis

Because NIRS-calibration needs sufficient samples preferably belonging to the same product group, analysis was only carried out for the 29 compound feeds. With an Infra-alyzer 500 spectrophotometer (Bran&Luebbe, Norderstedt, Germany) the spectrum was taken from 1100 to 2500 nm in steps of 4 nm. The air-dry samples

were scanned in closed cups on two days and the scans were averaged. A calibration for %EP*situ* was developed by means of partial least-squares regression (Unscrambler version 5.01, Camo, Trondheim, Norway). The calibration was tested by cross-validation.

Chemical analyses

The content of DM was determined by drying at 103 °C for 3h. Crude ash content was obtained after ignition in a furnace at 550 °C. N was analyzed with a Kjeltec-apparatus (Tecator, Höganäs, Sweden) and CP was calculated as N × 6.25. Crude fat (Cfat) was extracted with petroleum ether during 6h. Neutral detergent fibre (NDF) was determined according to Wainman *et al.* (1981), pretreating compound feeds and starch-containing forages with α-amylase. Starch (STA) was obtained after hydrolysis with amyloglucosidase (Van Gelder *et al.*, 1992).

Results

Chemical composition and in situ CP degradation characteristics

The chemical composition and protein degradation characteristics of the feeds are given in Table 1. The compound feeds showed a wide variation in chemical composition. Per kg DM, CP ranged from 156 to 357 g, Cfat from 11 to 87 g, ash from 68 to 117 g, NDF from 120 to 310 g and starch from 33 to 407 g. The treated soybean meal contained 4%-units more protein and 5%-units less cell-walls. CP-content of the grass silages and hay were similar but lower than that of fresh grass. The NDF-content of the hay was clearly higher than that of the other grass products. Despite a higher DM-content at harvesting, maize silage A contained more NDF and less starch than B.

The washable CP-fraction (W) of the compound feeds varied considerably from 23.2 to 63.6%. The W-fraction of the two soybean meals was relatively low and comparable. Within the forages, W was lowest (24.7%) for beet pulp and highest for fodder beets (79.7%). Of the grass products, W was clearly lower for hay and fresh grass than for the silages. Concerning the maize products, W increased from ear corn silage over maize silage A to silage B. The undegradable CP-fraction (U) of all but two compound feeds and the two soybean meals was lower than 3%. The highest U was found for the two maize silages, while that of the other forages varied between 4.4% for fodder beets and brewers' grains and 9.2% for beet pulp. The degradation rate of the potentially degradable CP-fraction (k_d) ranged from 0.8% h^{-1} for the treated soybean meal to 10.6% h^{-1} for the fresh grass. %EP*situ* varied from 17.7 to 43.1% for the compound feeds and from 10.8 (fodder beets) to 49.6% (beet pulp) for the forages. The formaldehyde treatment almost doubled %EP*situ* of soybean meal.

To correct for the loss of particles during the washing procedure, the solubility of CP in borate-phosphate buffer at pH 6.7 (Table 2, 0 h) was measured. This method

Table 1. Chemical composition, *in situ* CP degradation characteristics and rumen escape protein.

Feed	DM g/kg	CP[1]	Fat	Ash	NDF[2]	STA[3]	W[4] %	U[5] %	k_d[6] %/h	EP[7] %	EPc[8] %	EP_{tab}[9] %
		g/kg DM										
F88–19	882	189	45	92	310	72	37.9	1.2	6.6	30.2	34.6	37.9
F88–21	878	193	31	82	152	361	44.7	0.9	7.1	25.9	34.8	34.7
F88–24	870	357	53	115	156	62	23.2	1.3	9.0	31.4	32.4	37.3
F90–21	873	200	53	87	304	128	37.7	2.4	6.8	30.4	35.5	35.2
F90–22	887	213	35	85	269	135	39.6	1.0	5.3	32.5	42.4	41.1
F90–23	889	252	40	93	275	33	24.1	1.3	6.7	36.7	39.5	42.6
F90–24	897	211	87	89	291	81	36.4	1.6	5.6	33.6	39.9	41.5
F91–21	876	239	11	74	121	340	31.2	0.5	6.1	34.3	37.4	35.9
F91–22	871	241	21	68	120	353	29.5	0.0	4.6	40.0	44.2	40.7
F91–23	878	225	30	80	221	43	35.4	0.3	4.2	38.1	45.4	37.8
F91–24	894	278	51	93	276	37	31.3	1.5	5.2	37.5	43.0	38.6
F91–25	871	276	54	79	291	64	36.3	1.2	5.7	33.2	40.1	38.9
F91–26	871	245	36	73	207	226	35.9	1.0	5.7	33.5	39.9	37.8
F91–27	888	156	40	97	258	218	53.3	2.6	5.4	25.7	34.0	31.0
F91–28	885	179	43	96	263	193	52.7	2.3	6.4	24.0	31.5	31.1
F91–29	877	210	43	98	224	213	43.6	1.9	7.2	26.7	31.5	31.8
F85–08	884	187	38	94	241	145	50.5	2.0	4.5	29.2	42.2	33.4
F92–05	879	235	53	91	245	92	45.5	2.6	5.2	30.3	39.3	33.9
F92–27	870	348	48	117	173	50	28.7	0.5	6.3	35.0	42.3	40.1
F92–46	869	163	22	81	282	233	60.7	4.8	5.2	23.2	34.3	28.6
F92–47	868	205	34	90	297	138	44.7	4.1	3.8	35.6	46.7	37.8
F92–48	863	232	24	90	256	123	40.8	1.5	2.3	43.1	56.3	43.3
F92–49	863	163	35	71	155	407	63.6	2.4	7.3	17.7	26.5	27.1
F92–50	874	188	61	76	297	150	49.0	2.4	4.7	29.5	39.5	38.5
F92–51	876	224	60	81	301	94	44.2	2.1	3.2	37.3	49.8	44.7
F92–52	888	263	42	101	175	141	49.1	2.0	5.0	28.7	41.0	34.4
F92–53	891	241	45	102	197	111	45.3	2.0	4.8	31.2	42.4	36.8
F92–54	897	221	44	101	203	71	35.3	2.0	5.2	35.8	44.0	39.2
F92–55	897	197	45	100	246	85	50.8	2.2	2.3	36.2	52.8	44.5
Soybean meal	860	483	17	72	131	13	11.4	1.0	6.1	45.0	44.1	39.0
Soyb. m. form.	866	521	14	75	83	8	11.7	1.0	0.8	78.5	84.4	67.0
Beet pulp	203	116	7	69	431		24.7	9.2	4.1	49.6	52.7	54.0
Fodder beets	130	85	5	87	111		79.7	4.4	8.8	10.8	6.3	20.0
Grass silage A	376	144	39	119	487		66.4	6.2	4.8	22.7	20.7	28.5
Grass silage B	204	144	43	267	426		52.7	8.9	5.4	28.9	26.0	23.3
Grass silage C	366	167	38	160	468		55.6	8.5	5.5	27.5	23.5	25.8
Grass hay	914	168	25	116	572		33.9	6.5	5.7	32.8	29.6	36.9
Grass fresh	165	218	27	138	463		32.0	8.0	10.6	25.9	23.0	31.7
Maize silage A	383	65	21	51	420	309	49.1	18.5	2.8	40.9	41.4	28.0
Maize silage B	357	70	24	46	363	362	60.2	15.5	2.6	34.0	41.1	28.0
Potatoes	201	109	2	107	287	641	59.3	5.1	8.1	20.2	13.6	20.0
Ear corn silage	614	87	30	24	222	536	33.2	7.4	3.1	48.2	47.0	30.0
Brewers' grains	240	302	83	42	559	15	39.6	4.4	6.1	34.1	49.4	35.0

[1] crude protein ; [2] neutral detergent fibre ; [3] starch
[4] washable fraction ; [5] undegradable fraction ; [6] degradation rate ;
[7] % escape protein calculated with equation (1)
[8] % escape protein, corrected for loss of particles following equations (2) and (3)
[9] % escape protein, calculated with tables for concentrates (Anonymous, 1996) and forages (Anonymous, 1992).

was found most appropriate by Krishnamoorthy *et al.* (1982). The so-called real soluble CP-fraction S was clearly lower than W for the compound feeds. The difference amounted on average to 14.4%-units, varying from 2.5 (F88-24) to 22.6% (F92-55). When %EP*situ* was corrected for the loss of particles (%EPc in Table 1), values increased on average 8.2%-units, with a minimum of 1.0% and a maximum of 16.6%. W equalled S for soybean meal, but was 7.8%-units higher for the treated meal. Within the forages, the difference W – S was only positive for beet pulp, the two maize silages and particularly for brewers' grains (+ 31.7%-units), whereas more CP was solubilised than washed out for the other feeds.

In vitro methods to predict % EPsitu

In Table 2, the percentage CP solubilised in buffer (0 h) and after incubation with protease (during 1, 6 and 24 h), according to respectively three and four procedures, is presented. Generally, solubility of 500 mg samples in buffer at pH 8.0 resulted in higher values than in buffer at pH 6.7 (on average 1.5%-units) or compared with incubating 20 mg N (on average 1.9%-units). With *S. griseus* more protein was solubilised than with ficin, amounting on average to 2.3, 14.4 and 20.8%-units after 1, 6 and 24 h, respectively. Measuring solubility in buffer alone was better repeatable than when protease was added. For treated soybean meal and some forages, CP-disappearance after 1 h incubation in buffer with protease was sometimes lower than in buffer alone, particularly at pH 8.0. This discrepancy can be ascribed to the repeatability error of the method and of the blanks. Repeatability was highest for the procedure with *S. griseus* at pH 6.7 and a 500 mg sample and lowest for that with *S. griseus* at pH 8 and a 20 mg N sample.

To find the most accurate *in vitro* test, the percentage CP solubilised in buffer and in buffer-protease after 1, 6 and 24 h of incubation as well as the calculated %EP*vitro* were linearly regressed to %EP*situ*. Regressions were calculated for all feeds and separately for the compound feeds and the forages. Because brewers' grains heavily disturbed the regressions, it was excluded. In Table 3, the determination coefficient (R^2) and the residual standard deviation (RSD) of the relationships are given. Besides, all *in vitro* tests were regressed to %EP*situ*, corrected for loss of particles. These regressions had in a few cases similar but mostly higher RSD's than the corresponding regressions to the uncorrected %EP*situ* and are therefore not shown. As an example, the RSD of the regression of %EP*vitro* for *S. griseus* (pH 8.0 – 500 mg sample) to %EP*situ* and corrected %EPsitu amounted to 5.0 and 6.8%, respectively.

Considering all feeds, enzymatic degradability was better related to %EP*situ* than solubility in buffer. The % degraded protein after 6 h incubation gave a lower prediction error than that after 1 or 24 h, but the best result was obtained when %EP*vitro* was calculated from degradation and passage rate. Incubation of 500 mg samples with *S. griseus* at pH 8.0 gave the best results (RSD=5.0%), followed by the version with *S. griseus* at pH 6.7 (RSD=5.3%), then incubation with ficin (RSD=6.1%) and finally the version with *S. griseus* and a constant amount of feed protein (RSD=6.6%). The relationship between %EP*vitro* for *S. griseus* at pH 8.0 – 500 mg sample and %EP*situ* is also presented in Figure 1. The percentage EP*vitro* agrees

Table 2. Mean %CP solubilised after incubation in protease during 0 (1 h in buffer alone), 1, 6 and 24h.

Method	*S. griseus* pH 8 500 mg sample				*S. griseus* pH 8 20 mg N				*S. griseus* pH 6.7 500 mg sample				Ficin pH 6.7 500 mg sample		
Time in h	0	1	6	24	0	1	6	24	0	1	6	24	1	6	24
F88–19	29.9	37.1	63.7	80.2	27.2	33.2	55.6	72.8	29.0	36.5	62.2	79.1	33.0	41.1	48.1
F88–21	27.6	36.8	61.1	82.1	26.1	29.6	49.1	72.5	25.7	34.4	60.6	79.1	32.4	45.0	51.6
F88–24	22.4	30.3	61.6	84.5	22.1	29.1	59.7	79.8	20.7	30.9	60.7	80.6	27.4	41.7	53.5
F90–21	28.5	34.5	57.4	78.2	26.9	35.2	56.0	75.2	27.4	36.8	64.7	76.3	37.7	50.0	55.4
F90–22	24.4	34.4	66.1	87.1	25.1	35.8	66.3	85.1	21.1	38.4	73.3	88.0	35.4	47.1	55.5
F90–23	18.4	36.9	67.3	88.2	20.4	34.2	67.9	84.0	18.3	33.3	72.4	79.5	27.9	43.8	54.3
F90–24	25.8	36.6	69.4	87.7	24.5	36.0	70.4	90.3	24.6	37.8	72.9	86.3	28.8	42.9	47.8
F91–21	26.2	32.3	59.8	85.9	26.0	31.0	57.8	80.8	25.0	33.5	68.0	83.5	33.3	48.0	57.5
F91–22	23.6	28.9	53.4	76.7	24.6	28.3	54.7	77.0	22.1	30.0	61.9	77.8	27.6	41.0	50.6
F91–23	22.9	32.9	63.5	88.1	24.8	32.2	66.1	86.0	22.9	35.0	70.9	83.8	28.2	39.5	49.7
F91–24	22.6	27.6	54.7	82.1	22.7	29.2	59.3	80.3	21.3	32.2	65.0	80.8	22.5	30.5	41.6
F91–25	23.2	27.2	48.6	75.4	24.1	27.7	50.6	78.5	23.0	30.9	53.4	71.0	27.9	37.1	46.5
F91–26	22.5	27.9	51.6	78.7	23.6	28.5	52.9	76.3	23.5	30.4	56.3	75.1	27.9	38.7	49.2
F91–27	37.0	45.5	66.6	83.0	35.3	38.6	54.5	69.1	38.2	45.6	63.9	73.8	44.0	50.8	58.2
F91–28	38.9	47.2	64.8	82.2	36.1	39.0	56.4	74.0	38.0	46.3	64.3	79.5	44.0	52.6	53.9
F91–29	34.1	42.2	62.7	81.3	32.7	36.8	53.8	71.7	33.5	41.9	62.1	77.8	41.6	50.4	57.2
F85–08	31.9	39.7	57.1	76.8	29.0	33.1	52.4	67.5	28.4	38.2	56.6	74.0	30.7	42.5	50.3
F92–05	31.4	36.9	54.7	77.5	30.1	34.5	53.9	72.1	29.4	35.8	55.6	72.3	32.6	44.1	50.6
F92–27	15.9	24.5	48.8	83.5	16.1	24.0	54.8	82.1	13.9	25.6	53.6	81.3	17.1	29.6	42.4
F92–46	42.3	50.0	59.9	76.8	38.9	41.5	51.0	62.0	42.0	48.7	62.6	73.2	48.6	56.5	57.7
F92–47	29.8	33.8	51.3	68.4	27.7	31.0	41.6	58.3	27.4	33.8	49.3	62.5	33.7	39.5	47.4
F92–48	23.4	28.9	43.7	65.5	23.4	26.1	39.2	56.2	22.8	28.3	44.2	62.6	29.4	34.6	40.2
F92–49	44.6	53.4	69.9	86.4	40.9	45.0	59.9	70.0	45.5	56.5	69.7	80.9	56.6	63.0	66.0
F92–50	32.7	45.4	73.8	83.1	30.1	38.1	60.2	80.5	31.9	43.2	66.8	80.3	40.8	49.1	52.9
F92–51	27.9	38.5	63.1	78.0	24.8	33.1	53.7	75.5	25.5	36.9	60.7	74.8	32.3	38.9	44.9
F92–52	29.6	37.1	56.4	78.4	28.4	32.0	55.2	71.8	27.3	36.2	59.4	78.3	34.7	48.0	55.6
F92–53	27.3	37.4	61.6	82.8	26.5	34.7	59.5	75.6	25.6	35.6	63.0	74.6	31.7	45.7	55.2
F92–54	24.0	35.9	63.0	79.8	22.1	30.5	60.4	78.2	20.3	34.3	63.5	71.5	26.2	43.8	51.4
F92–55	29.7	40.7	58.9	77.9	27.5	35.5	56.2	72.0	28.2	38.0	58.9	77.0	33.0	39.9	45.0
Soybean meal	13.8	18.6	42.0	76.9	13.1	18.4	45.7	75.3	11.4	17.9	41.8	74.0	17.1	37.0	56.0
Soyb. m. for.	4.6	4.6	7.4	16.2	4.6	3.6	8.4	17.6	3.9	4.6	8.8	19.5	4.5	6.3	11.0
Beet pulp	22.2	24.0	39.3	65.8	17.6	18.5	34.4	52.4	19.5	23.0	38.9	61.7	19.8	25.3	28.1
Fodder beets	88.3	94.5	97.2	98.8	87.0	89.6	92.5	91.8	88.2	93.0	100.0	99.2	96.1	98.2	99.8
Grass sil. A	72.7	74.7	84.4	85.1	69.5	74.4	81.3	85.2	70.3	73.4	80.4	85.2	72.8	76.6	79.6
Grass sil. B	62.1	61.6	73.1	81.4	57.6	59.8	73.5	77.8	58.5	62.5	69.4	77.3	60.1	62.3	65.7
Grass sil. C	66.8	68.1	77.8	81.5	64.6	67.2	75.2	81.8	63.5	67.4	73.0	79.0	66.2	66.8	73.9
Grass hay	41.3	48.7	62.1	68.3	39.1	44.2	57.9	68.2	40.4	46.6	60.5	66.6	46.6	49.7	59.7
Grass fresh	41.3	43.9	60.0	69.2	40.4	44.3	58.4	70.0	39.7	43.1	56.8	67.1	47.3	54.0	60.8
Maize sil. A	52.8	52.6	64.0	76.5	45.6	48.6	56.8	64.1	48.5	53.0	64.5	73.2	51.8	54.8	58.6
Maize sil. B	52.2	53.3	66.2	80.5	45.7	48.4	59.2	68.8	50.5	54.4	65.2	73.5	49.8	52.7	56.0
Potatoes	73.9	71.3	76.8	82.8	66.9	70.4	77.7	79.5	72.6	72.6	80.1	78.4	77.6	80.4	91.7
Ear corn sil.	39.1	36.7	48.9	61.1	31.9	33.7	42.3	52.6	35.2	32.7	41.6	54.4	34.8	34.6	40.9
Brewers' gr.	8.7	12.0	23.8	45.8	9.5	12.7	22.6	48.3	7.9	11.8	22.0	37.2	9.9	13.7	18.5
Mean SE[1]	3.1	4.2	3.1	3.2	3.3	5.5	3.5	3.3	3.3	3.5	2.0	2.2	4.6	3.2	2.7

[1] Mean standard error between replicates

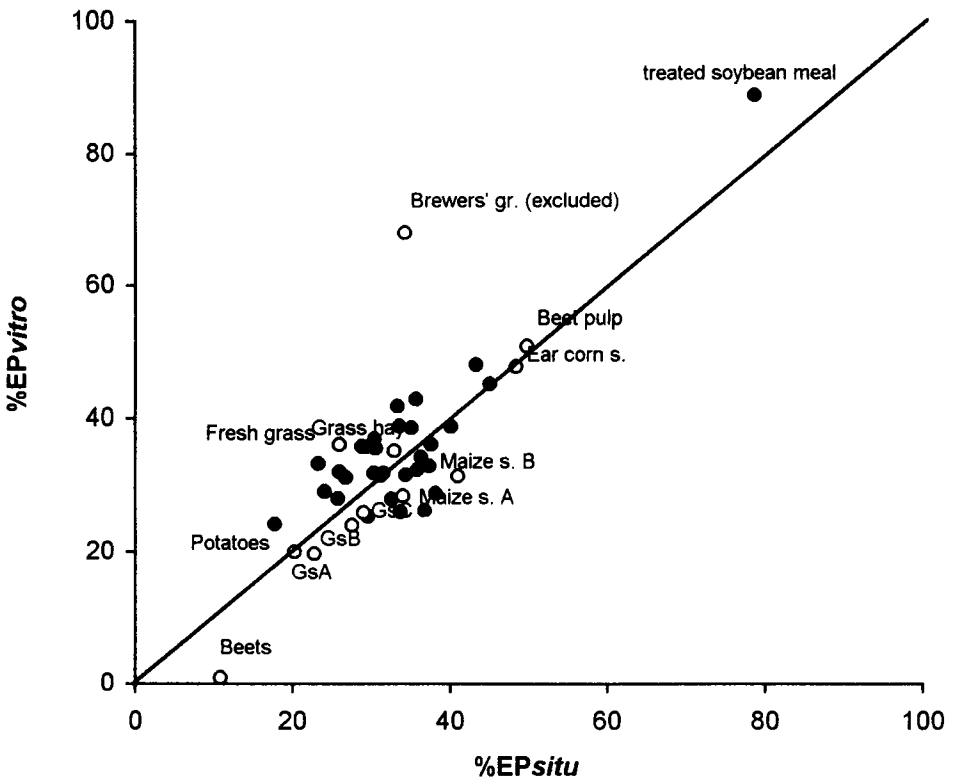

Figure 1. Relationship between %EP*situ* and %EP*vitro* for *S. griseus* at pH 8.0–500 mg sample (concentrates: ●; forages: ○).
%EP*situ* = 0.755 × EP*vitro* + 7.6 (R^2 = 77.3; RSD = 5.0%).

fairly well with that *in situ*, as most feeds with no distinction between concentrates and forages are closely situated around the bisector. Fodder beets with lowest and treated soybean meal with highest %EP*situ* was respectively underestimated and overestimated by %EP*vitro*. Protein of brewers' grains, not taken into account for the regression, appears twice as resistant *in vitro* than *in situ*. In Figure 2, the relationship between %EP*vitro* with ficin and %EP*situ* is presented. With exception of fodder beets and potatoes, %EP*vitro* of all other feeds is higher than *in situ*. Moreover, %EP*situ* of compound feeds tends to be more overestimated than that of forages.

Splitting the feeds into compound feeds and forages decreased RSD for both categories. For compound feeds, all three versions of solubility gave a good indication of %EP*situ* with an equal RSD of 3.4%. The CP-solubility (S) of a 500 mg sample in borate-phosphate buffer at pH 6.7 was related to %EP*situ* following the equation: %EP*situ* = 48.80 – 0.624 x S. The relationships did not improve when protease was added. Compared with solubility, the RSD was similar with ficin at all incubation times and with *S. griseus* after 1 h incubation. However, at longer incubation with *S. griseus* the relationship even became not significant (P>0.05).

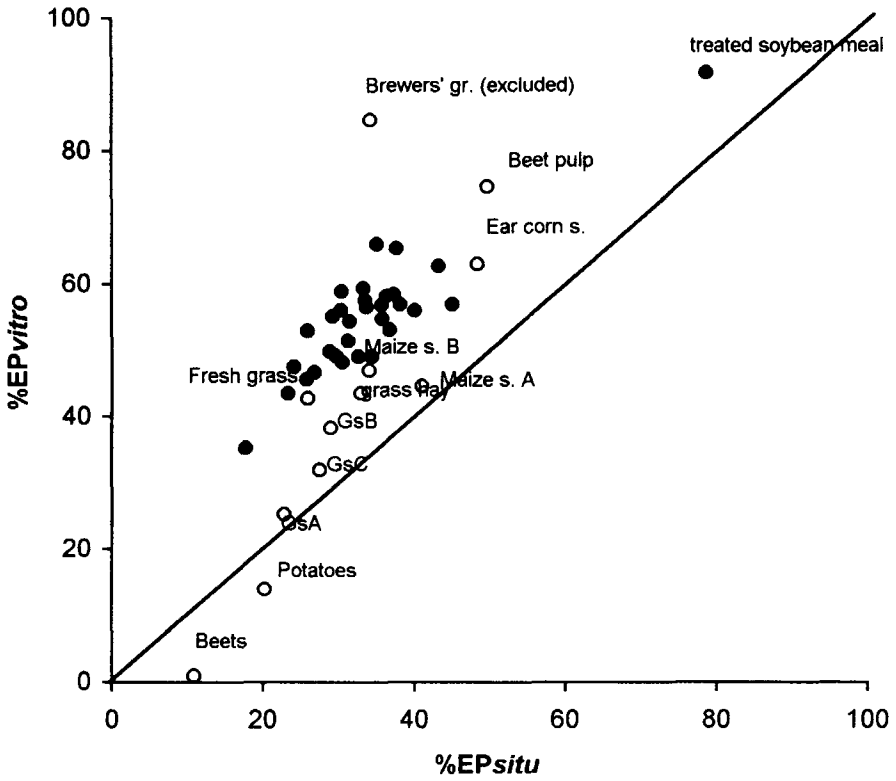

Figure 2. Relationship between %EP*situ* and %EP*vitro* for ficin at pH 6.7 sample (concentrates: ●; forages: ○).
%EP*situ* = 0.580 × EP*vitro* + 3.8 (R^2 = 66.1; RSD = 6.1%).

Similarly as to the pool of all feeds, enzymatic degradability was better related to %EP*situ* than solubility for forages. For the procedures with a 500 mg sample at pH 8.0 and 6.7, an incubation time of 6 h seemed optimal, whereas for the other two procedures RSD decreased with longer incubation up to 24 h. However, for all four enzymatic procedures a still better relationship was obtained when %EP*vitro* was calculated, the best being the incubation of a constant amount of 20 mg N with *S. griseus* at pH 8.0, followed by the procedure with ficin. For the former procedure the relationship is: %EP*situ* = 4.97 + 0.764 x %EP*vitro* with a RSD of 3.3%.

The use of tables and NIRS to predict % EPsitu

To look for the reliability of the studied *in vitro* methods in practice, their accuracy was compared with that of the current use of tabular values. The tabular %EP of the compound feeds was calculated from that of the ingredients incorporated (Anonymous, 1996), whereas it was taken or calculated from the Dutch feeding table

(Anonymous, 1992) for forages (Table 1). Compared with %EP*situ*, the use of tables resulted on average in a 5.1%-units higher value for the compound feeds and a 1.4%-units lower value for the forages (Table 3). In an earlier study with the same compound feeds, De Boever *et al.* (1995) showed that the differences were partly of systematic nature. Therefore, the tabular values were regressed to %EP*situ* (Table 3). For the pool of all feeds, the regression of the tabular %EP to %EP*situ* resulted in a RSD of 6.3%. The error was higher than that obtained with %EP*vitro* according to the procedures with *S. griseus* – 500 mg sample at pH 8.0 or 6.7. Considering the compounds separately, the tabular %EP was better related to %EP*situ* than any of the tested *in vitro* methods with a RSD of 2.8%. For the forages alone, the use of tabular values gave clearly worse results (RSD: 8.9%), mainly caused by an underestimation

Table 3. Mean %CP solubilised after incubation in protease during 0 (1 h in buffer alone), 1, 6 and 24 h and % escape protein calculated with equation (1), with NIRS (only compound feeds) and with tables and linear relationship with %EP*situ*.

Method	All feeds (n=42)			Compound feeds (n=29)			Forages (n=11)		
	Mean	R^2	RSD	Mean	R^2	RSD	Mean	R^2	RSD
%EP*situ*	33.1	–	–	32.0	–	–	31.1	–	–
S. griseus pH 8.0 – 500 mg sample									
0h	34.5	37.1	8.3	28.2	62.9	3.4	55.7	71.4	6.3
1h	40.8	52.1	7.3	36.6	56.3	3.6	57.2	78.4	5.5
6h	60.3	70.6	5.7	59.8	19.0	5.0	68.2	81.3	5.1
24h	78.1	63.6	6.3	80.6	NS[1]	–	77.4	66.0	6.9
%EP*vitro*	33.8	77.3	5.0	33.3	21.7	4.9	29.1	81.8	5.0
S. griseus pH 8.0 – 20 mg N									
0h	32.6	40.8	8.1	27.2	62.3	3.4	51.4	79.2	5.4
1h	37.7	48.0	7.6	33.3	48.7	4.0	54.5	81.4	5.1
6h	57.0	57.7	6.8	56.2	NS	–	64.5	86.9	4.3
24h	72.9	44.5	7.8	75.0	NS	–	71.8	87.9	4.1
%EP*vitro*	38.2	60.7	6.6	37.8	NS	–	34.1	92.1	3.3
S. griseus pH 6.7– 500 mg sample									
0h	33.0	39.5	8.2	27.0	62.3	3.4	53.4	76.0	5.8
1h	40.7	54.3	7.1	36.7	63.1	3.4	56.5	78.8	5.4
6h	61.4	67.1	6.0	61.9	NS	–	66.4	82.4	4.9
24h	74.9	61.3	6.5	77.1	NS	–	74.2	71.7	6.3
%EP*vitro*	34.9	74.1	5.3	33.9	NS	–	31.6	82.1	5.0
Ficin pH 6.7 – 500 mg sample									
1h	38.4	49.7	7.5	33.4	61.3	3.4	56.6	83.7	4.8
6h	47.1	65.7	6.2	44.0	64.4	3.3	59.8	88.6	4.0
24h	54.1	65.1	6.2	51.4	54.0	3.7	65.0	88.8	4.0
%EP*vitro*	50.6	66.1	6.1	53.5	60.6	3.5	38.7	89.5	3.8
%EP – NIRS	–	–	–	32.0	57.0	3.8	–	–	–
%EP – table[2]	35.9	64.2	6.3	37.1	74.6	2.8	29.7	42.5	8.9

[1] not significant ($P > 0.05$)

[2] % escape protein, calculated with tables for concentrates (Anonymous, 1996) and forages (Anonymous, 1992).

for ear corn silage (–18.2%) and maize silage A (–12.9%) and an overestimation for fodder beets (+ 9.2%).

The possibility of NIRS to predict %EP*situ* was investigated for compound feeds (Table 3). A PLS-calibration based on 10 factors appeared less accurate (RSD: 3.8%) than measuring solubility.

Discussion

In situ CP degradation and correction for loss of particles

Although the *in situ* incubation of feeds in the rumen is accepted as the reference method to determine %EP, the procedure has some deficiencies. Among them the loss of particles through the bag pores and microbial contamination of the residue are the most important (Van Straalen & Tamminga, 1990).

Following the Dutch DVE/OEB-system (Tamminga *et al.*, 1994), the soluble CP-fraction was determined as the loss of CP from bags after a rigorous washing procedure. In a previous study with compound feeds (De Boever *et al.*, 1995) serious doubts were risen if all washout is really soluble. Therefore, the washable fraction was compared with the fraction soluble in borate-phosphate buffer at pH 6.7. It has to be remarked that this comparison was not pure, because samples were not dried and coursely ground (concentrates) or chopped (forages) for the washing procedure, while they were air-dried and finely ground for measuring solubility. The difference in sample preparation was thus more pronounced for forages than for concentrates. For all compound feeds, W was higher than S, and the difference showed a large variation. W-S was not related to starch or NDF-content, whereas a weak significant relationship was found for CP-content ($r = -0.46$). Further examination revealed that the difference decreased with increasing inclusion percentage of soybean meal ($r = -0.61$), for which W equalled S. On the other hand, W-S increased when more maize gluten feed was incorporated ($r=0.40$). Besides the nature of the proteins in relation to solubility, the variability in W-S between feeds could be caused by the difference in particle size after grinding. Because grinding ruptures more cell walls and thus frees more protein than chopping, the lower W than S fraction, found for the grass products, fodder beets, potatoes and ear corn silage is understandable. However, this effect was not consistent for all forages as more protein washed out than solubised for beet pulp, the maize silages and particularly brewers' grains. In the case of brewers' grains, one may admit that W strongly overestimates the real soluble fraction and that %EP*situ* is seriously underestimated, considering the in vitro and in vivo trials of Firkins *et al.* (1984) and Armentano *et al.* (1986).

To correct for the loss of particles, it was assumed that W-S degrades like the potentially degradable fraction, as suggested by Lopez *et al.* (1994) and Madsen *et al.* (1995). However, the relationships of all in vitro tests with the corrected %EP*situ* did not improve compared with the uncorrected %EP*situ*, even when compound feeds, for which a correction seems more justified than for forages, were considered separately. On average for all feeds, the correction increased %EP*situ* with 17%

from 33.2 to 38.7%. This is appreciably more than the overall correction made in the Dutch protein system (factor 1.11) to convert %EP *in situ* to *in vivo* level (Tamminga *et al.*, 1994). So, the assumption that particles lost from the bags degrade like those in the bags is pobably not correct. According to Dewhurst *et al.* (1995), the difference W-S would mainly be caused by a rapidly fermented fraction, which is lost from the bags before it is fermented, but also by loss of undegradable particles. How to correct for that, is not evident and should be further investigated.

Prediction of % EPsitu

To find a routine method to predict %EP*situ*, which is more reliable than the current use of tabular values, a comparison was made of solubility, enzymatic degradability and NIRS (only for compound feeds). From the above it is clear that the prediction error of any routine method will be the result of imperfections of the examined as well as the reference method. Moreover, the contribution of both may vary according to the feed or product group, but is not measurable. Therefore, the criterion for prediction accuracy was a minimal RSD.

From a literature study, the bacterial protease from *S. griseus* (Aufrère *et al.*, 1991) and the vegetal protease ficin (Kosmala *et al.*, 1996) appeared promising alternatives for rumen proteolytic activity. In order to derive degradation rate and calculate %EP*vitro*, enzymatic degradability was measured at 1, 6 and 24 h of incubation. The procedure with *S. griseus*, described by Aufrère & Cartailler (1988), uses 1 mg of enzyme for a 500 mg sample at pH 8.0. As possible improvements suggested by Cone *et al.* (1995), the effect of two modifications was studied: a pH, similar as in the rumen and a constant enzyme-protein ratio. Because in the procedure with ficin (Kosmala *et al.*, 1996) a too high enzyme concentration made modelling of the degradation dynamics impossible, the enzyme-substrate ratio was reduced to 5 mg per 500 mg sample.

The linear relationship between all *in vitro* tests and %EP*situ* was heavily disturbed by the sample of brewers' grains (Figures 1 and 2) and was therefore excluded from the regression calculations. The different behaviour of brewers' grains was not only the result of a very low solubility but also of a much higher undegradable residue after 24 h incubation with enzymes as compared with the *in situ* results. Cone *et al.* (1995) observed the same and were not able to reduce the U-fraction by longer incubation (70 h) with *S. griseus* nor by a pretreatment with cellulase. This could be an indication that undegradable protein leaves the bags or that the proteolytic activity of the proteases used is insufficient.

Considering all feeds, solubility was less well related to %EP*situ* than enzymatic degradability, which is in agreement with other studies (Aufrère *et al.*, 1991; Cone *et al.*, 1996). The calculated %EP*vitro* gave a better prediction of %EP*situ* than enzymatic degradability on distinct incubation times, mainly because the different passage rate for concentrates and forages could be taken into account. The rate of degradation by enzymes seems less determining, as the variance in %EP*situ* explained by one incubation time could not significantly be improved by a second one. The method with *S. griseus* at pH 8.0 was better than that with ficin at pH 6.7. The for-

mer could not be improved by using a rumen similar pH. Optimizing pH for the activity of *S. griseus* is obviously more important than simulating proteolytic circumstances in the rumen. Notwithstanding CP-content showed a wide variation, no advantage could be obtained by incubating a constant protein quantity. From Figure 1, it appears that concentrates and forages, including the extremes, fodder beets and treated soybean meal, are fairly close situated near the bisector. This is not the case with ficin (Figure 2), where %EP*situ* of concentrates tends to be more overestimated than that of forages. With tabular values the error was higher than with the best enzymatic procedure (6.3 vs. 5.0%).

By considering compound feeds and forages separately the prediction could be improved, but the best *in vitro* method was not the same for the two feed categories. For the compound feeds, the use of enzymes appeared not advantageous compared with solubility in borate-phosphate buffer, on the contrary. The relationship with enzymatic degradability worsened or even disappeared with longer incubation time, suggesting that degradation of insoluble proteins by commercial proteases does not simulate rumen proteolysis. This was recently confirmed by Luchini *et al.* (1996). On the other hand, a two-term regression with solubility (S) and enzymatic degradation by *S. griseus* at pH 8.0 after 24 h (D_{24}) could lower the RSD further to 2.9%. This equation is: %EP*situ* = 78.37 – 0.684 × %S – 0.337 x %D_{24}. All other combinations examined brought no significant (P>0.05) improvement. Considering effective degradabilities of 10 concentrate ingredients calculated at an outflow rate of 5% h^{-1}, Susmel *et al.* (1993) obtained similar correlation coefficients with *in situ* data for phosphate buffer (r=0.84) and *S. griseus* (r=0.85), which were clearly better than those for centrifuged (r=0.77) or whole rumen fluid (r=0.78). In contrast, Aufrère *et al.* (1991) found for the *in situ* effective degradability of 49 commercial compound feeds a lower RSD with *S. griseus* during 1 h incubation than with solubility in bicarbonate-phosphate buffer: 2.5 vs 4.9%. In the French study more mixtures contained protected-protein ingredients, of which %EP is underestimated by the solubility method. Another explanation could be the lower pH-stability of bicarbonate-phosphate buffer as compared with borate-phosphate buffer (Krishnamoorthy *et al.*, 1982). NIRS showed some potential to predict %EP*situ* of compound feeds as 57% of the variance could be explained. However, considering the complexity of both the parameter and the feed category much more samples are needed to derive a robust calibration. Apart from a certain systematic error, tabular values (Anonymous, 1996) appeared better related to %EP*situ* than any of the studied *in vitro* tests (RSD: 2.8%). This is somewhat understandable as tables are commonly based on nylon bag incubations. On the other hand, tables are only usable when the ingredient composition is known. For unknown mixtures, a solubility test is preferable to enzymatic degradation by *S. griseus* or ficin.

For the rather limited but heterogeneous group of 11 forages, best prediction was obtained by incubating a constant protein quantity with *S. griseus* at pH 8.0, followed by the procedure with ficin. The use of a constant enzyme-protein ratio mainly affected %EP*vitro* of the forages poor in protein like beet pulp, fodder beets, ear corn silage and the two maize silages, which obviously is underestimated by incubating the same sample weight. The use of the Dutch tables (Anonymous, 1992) result-

ed in a high error of 8.9%. The lack of agreement was mainly caused by fodder beets, the maize silages and ear corn silage, for which a mean value is probably to rough. So, Klop & De Visser (1995) found for 24 maize silages a mean %EP*situ* of 40%, varying from 32 to 53% and for 6 ear corn silages a mean value of 37%, ranging from 24 to 55%.

Acknowledgements

The authors are grateful to the personnel of the laboratory for the careful assessment of the in situ and in vitro determinations.

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