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Use of central composite design to optimize working conditions of *Streptomyces griseus* enzymatic method in estimating *in vitro* rumen undegraded crude protein of feedstuffs

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Abstract

The aim was to identify optimized combinations of *Streptomyces griseus* protease concentration (CONC), incubation length (TIME), or amount of crude protein (CP) incubated in buffered enzymatic solution (CPW) to predict the *in vitro* rumen-undegraded feed CP (RUP) of 26 different feeds (soybean, rapeseed or sunflower meals, wheat bran, distillers dried grains with solubles, maize co-products and alfalfa hay). Different levels of CONC (0.08, 0.19, 0.44, 0.69 and 0.80 enzymatic units [U] of *S. griseus* protease/ml), TIME (6, 10, 18, 26 and 30 h) and CPW (69, 118, 235, 353 and 401 mg CP) were tested in agreement with a central composite design (CCD) with four replications of the central point to calculate second-order polynomial equations of main tested effects. The RUP was estimated by incubating samples in a buffered rumen fluid for 16 h or by adopting different enzymatic approaches as planned *a priori* in CCD. Differences between rumen and enzymatic RUP (ΔRUP) were estimated and regression terms of second-order polynomial equations for estimating ΔRUP were calculated between and within feeds. These equations were optimized using the non-linear generalized reduced gradient method with the objective set at ΔRUP equal to 0. The adoption of CCD permitted identification of optimized enzymatic combinations of CONC (0.12 U of *S. griseus* protease/ml), TIME (18 h) and CPW (from 233 to 458 mg CP for distillers dried grains with solubles and soft white wheat bran, respectively) to predict RUP accurately in all feed categories except for soybean meal, where optimized combinations were 0.47 U of *S. griseus* protease/ml, 18 h and 435 mg CP.

Introduction

Rumen degradation of dietary feed crude protein (CP) influences rumen fermentation and amino acid supply to ruminants. In particular, rumen-undegraded feed CP (RUP) together with rumen-synthesized microbial CP and endogenous CP contributes to the passage of metabolizable protein (MP) to the small intestine (Hristov *et al.* 2004). Evaluation of the amount of dietary RUP is required by different feed evaluation systems (Broderick *et al.* 2004; Edmunds *et al.* 2014; Paz *et al.* 2014) to properly characterize feeds entering dairy cow diets.

The RUP could be estimated by adopting different *in situ* or rumen-based *in vitro* procedures, after correction for microbial nitrogen (N) colonization (Broderick 1987; Calsamiglia *et al.* 2000; Gargallo *et al.* 2006). More recently, Ross *et al.* (2013) proposed a two-step *in vitro* assay with rumen fluid to determine RUP as well as RUP intestinal digestibility in ruminant feeds (Spanghero *et al.* 2015; Fessenden *et al.* 2017; Giallongo *et al.* 2017). As discussed at length by Ross *et al.* (2013), the assay was proposed to reduce sample loss and variation among samples due to use of bags. Furthermore, a novel approach to estimate microbial contamination of samples was tested, and it consisted of incubating a substrate low in N content, i.e. the neutral detergent (ND) residue of maize silage, during the *in vitro* rumen assay. However, these methods are difficult to standardize and require the use of rumen-cannulated animals, which are expensive to maintain and rarely available for commercial laboratories (Madsen *et al.* 1997; Coblentz *et al.* 1999; Hippenstiel *et al.* 2015).

Alternatively, RUP values can be evaluated by adopting enzymatic methods based on the use of different commercially available bacterial, fungal, pancreatic or plant proteases: the literature (Luchini *et al.* 1996; Stern *et al.* 1997) provides a full description of these enzymes and their ability in predicting rate and extent of protein degradation. Among these, one of the most commonly used is the *Streptomyces griseus* protease (Krishnamoorthy *et al.* 1983; Nocek 1988; Michalet-Doreau & Ould-Bah 1992; Coblentz *et al.* 1999; Calsamiglia *et al.* 2000) and indications were that it was appropriate for differentiation of potential protein degradation of different feeds (Cone *et al.* 2004; Chaudhry 2005). Therefore, the enzymatic approach has been

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largely adopted by both research and commercial laboratories to evaluate the RUP content of feeds entering dairy cow diets (Nocek 1988; Jones & Theodorou 2000), despite poor relationships having been reported between rumen-based *v.* enzymatic-based RUP values (Luchini *et al.* 1996; Gosselink *et al.* 2004). The enzymatic approach was first proposed by Pichard & Van Soest (1977) to evaluate the rate of hydrolysis of the insoluble protein fraction in forages (i.e. alfalfa hay or alfalfa, grass and maize silages) and soybean meal. Different authors have modified the enzymatic conditions since the 1970s, aiming to find optimal *S. griseus* protease working conditions for predicting RUP. In particular, several methodological aspects, such as *S. griseus* protease concentration or enzyme to substrate ratio (Krishnamoorthy *et al.* 1983; Aufrère & Cartailler 1988; Coblentz *et al.* 1999), length of incubation (Mahadevan *et al.* 1980; Aufrère *et al.* 1991; Cone *et al.* 2002, 2004), buffer pH value (Cone *et al.* 1996; De Boever *et al.* 1996; Licitra *et al.* 1998), type of substrate (Calsamiglia *et al.* 1995; Mathis *et al.* 2001) and pre- or post-incubations with carbohydrase or amylase (Assoumani *et al.* 1992; Kohn & Allen 1995; Abdelgadir *et al.* 1997) have been evaluated. However, all these variations contributed to make the interpretation of results from different trials difficult (Klopfenstein *et al.* 2001; Edmunds *et al.* 2014).

These methodological aspects were usually studied by adopting a 'one-factor-at-a-time' approach. This experimental approach consists of varying one factor at a time while keeping other factors fixed (Czitrom 1999), thus supposing that: (i) different factors influence enzymatic RUP determination linearity and (ii) no interactions exist among different factors. However, the effects of different factors may not be linear, thus meaning that they could influence response variables in a curvilinear manner or concomitantly with other tested factors if interactions exist (St-Pierre & Weiss 2009). Therefore, there is a need to design experiments in which tested conditions are changed simultaneously to quantify possible intra- (i.e. curvilinear) or inter- (i.e. interaction) non-linear relationships among different factors. As declared by Czitrom (1999), when testing the effects of two or more factors on a dependent variable, response surface methodology rather than a one-factor-at-a-time approach should be adopted, because: (i) it usually requires fewer resources (e.g. experiments, time, materials, etc.) to obtain the same or more information; (ii) estimates of the effect of each factor are more accurate, because a greater number of observations are tested; (iii) the interactions existing among factors can be systematically studied, whereas they are not estimable using the one-factor-at-a-time approach; and (iv) there is an opportunity to obtain information in a larger region of the factorial space. Despite the existence of several response surface methodologies, they can be assigned to two main categories: full or fractional factorial designs (Carley *et al.* 2004; Khuri & Mukhopadhyay 2010). As discussed by St-Pierre & Weiss (2009), among fractional factorial designs, the central composite design (CCD) appears able to reduce the number of treatments required to estimate all terms of a second-order polynomial equation considerably, without any loss of efficiency as compared with the full factorial design.

The aim of the current work was to identify, by adopting a CCD experimental design, the optimized combinations of three enzymatic methodological conditions, i.e. *S. griseus* protease concentration in the enzymatic working solution (CONC), length of enzymatic incubation (TIME), or total amount of sample CP incubated in the buffered enzymatic solution (CPW), to predict *in vitro* rumen evaluated RUP of different feed categories.

Materials and methods

Feeds and chemical analysis

A total of 26 samples consisting of solvent-extracted soybean meal (sSBM, $n = 4$), expeller-extruded soybean meal (eSBM, $n = 4$), solvent-extracted rapeseed meal (RM, $n = 3$), solvent-extracted sunflower meal (SFM, $n = 3$), soft white wheat bran (WB, $n = 2$), distillers dried grains with solubles (DDGS, $n = 2$), dried maize gluten feed (CGF, $n = 1$), dried maize gluten meal (CGM, $n = 1$) and alfalfa hay (AH, $n = 6$) were used in CCD. Both CGF and CGM were considered in the maize co-products (CCP) feed category. Among these, 18 samples (sSBM = 2, eSBM = 2, RM = 3, SFM = 3, WB = 2, DDGS = 2, CCP = 2, AH = 2) were re-used in the confirmatory test along with an additional set of 15 samples (SBM = 5, AH = 10) selected from samples sent to the laboratory of the Feed and Food Science and Nutrition Institute (Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, Piacenza, Italy) for routine analysis. Samples with a mean particle size >1 mm (Gallo *et al.* 2016a) were ground through a cutter mill (Pulverisette 19; Fritsch, Idar-Oberstein, Germany) equipped with a 1-mm screen. An aliquot (100 g) of AH samples was ground using a 2-mm screen to assay the *in vitro* RUP determination. All samples were analysed in duplicate as previously described by Gallo *et al.* (2016b), except for dry matter (DM) assay, which was analysed once. In particular, DM was determined by gravimetric loss of free water from heating at 105 °C for 3 h (AOAC 1995, method 945.15), ash was determined as gravimetric residue after incineration at 600 °C for 2 h (AOAC 1995; method 942.05), CP (N × 6.25) was determined using the Kjeldahl method (AOAC 1995; method 984.13), and ether extract (EE) by the method 920.29 (AOAC 1995). The soluble fraction of CP (solCP, expressed on a CP basis) was determined according to Licitra *et al.* (1996). The ND and the acid detergent (AD) fibre fractions were determined using the Ankom^{II} Fibre Analyser (Ankom Technology Corporation, Macedon, NY, USA) according to the method described by Van Soest *et al.* (1991). The ND solution contained sodium sulphite and a heat-stable amylase (activity of 17.400 Liquefon units/ml, Ankom Technology). All fibre fractions were corrected for residual ash (i.e. aNDFom and ADFom).

In vitro *rumen-undegraded feed crude protein determination*

The *in vitro* RUP (expressed on a CP basis) was carried out in accordance with the rumen step of the method proposed by Ross *et al.* (2013). In particular, fresh rumen fluid was obtained from two cannulated dry Holstein dairy cows (625 ± 10 kg of body weight, 38 ± 0.3 months old) fed at maintenance (NRC 2001) with a total mixed ration (120 g/kg CP and 550 g/kg aNDFom on a DM basis) composed of alfalfa hay, grass hay, maize silage, beet pulp and a protein vitamin-mineral supplement (250, 450, 150, 50 and 100 g/kg DM, respectively). The diet was administered to the cows twice a day, at 8.00 and 18.00 h. Collected rumen fluids were maintained in a warm, insulated carbon dioxide (CO_2) flask at 39 °C, filtered through two layers of cheesecloth and used within 20 min from the collection. For each sample tested, about 500 mg were weighed into 125 ml Pyrex glass Erlenmeyer flasks, then 10 ml of filtered rumen fluid plus 40 ml of Van Soest buffer were added (Spanghero *et al.* 2015). Blanks (buffered rumen fluid only) and maize silage previously treated with ND solution were incubated simultaneously to correct sample RUP for enzyme-derived and microbial

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N colonization (Ross *et al.* 2013). All samples were incubated in a water bath set at 39 °C. As indicated by Ross *et al.* (2013), the contents of the flasks were filtered carefully (Whatman 54 filter paper) and residues analysed for CP content after 16 h of rumen incubation. Each sample was tested in duplicate on two different days. Samples within-day were considered analytical repetitions, whereas samples between days were experimental replicates.

Enzymatic rumen-undegraded feed crude protein determination

Three methodological factors, CONC, TIME and CPW, were tested simultaneously in the CCD experiment. Details of the statistical approach are reported elsewhere (St-Pierre & Weiss 2009). In particular, 18 treatments were formulated, of which four were replications of the central point, combining the main tested effects at five different levels as planned *a priori* and detailed in Table 1. Codes used to test different levels for each factor were −1.4142 ($-\alpha$), −1, 0, 1 and 1.4142 (α) (St-Pierre & Weiss 2009), corresponding to 0.08, 0.19, 0.44, 0.69 and 0.80 U of *S. griseus* protease (P5147, Sigma-Aldrich, Milan, Italy)/ml of enzymatic working solution for CONC; 6, 10, 18, 26 and 30 h of incubation for TIME; and 69, 118, 235, 353 and 401 mg CP incubated for CPW. All other conditions were constant among treatments. In particular, samples were weighed into Pyrex glass Erlenmeyer flasks and 40 ml of a 0.1 M borate/phosphate solution at pH 8.0 was added (Aufrère & Cartailler 1988; Cone *et al.* 1996). Then, samples were pre-incubated in the buffer solution for 1 h at 39 °C. At the end of the pre-incubation, 10 ml of enzymatic working solution was added. Blanks were also included to correct enzymatic sample RUP values for enzyme-derived N. At the end of the incubation, flasks were emptied, rinsed with distilled water (2 × 25 ml) and residues were collected carefully by filtration (Whatman 54 filter paper). Then, water-washed residues were analysed for CP content as detailed previously. Each sample was tested in duplicate on two different days. As reported above, samples within-day were considered analytical repetitions, whereas samples between days were experimental replicates.

Statistical analysis and optimization process

Results from chemical assays and *in vitro* RUP determinations are presented descriptively (mean ± S.D.). Differences between *in vitro* rumen and enzymatic RUP (ΔRUP) or differences between experimental replicates of enzymatic method were analysed according to a CCD with four replications of central point (St-Pierre & Weiss 2009) by the Mixed procedure of SAS (2003). The fixed effects of the model were CONC, TIME and CPW, their squared terms (CONC × CONC, TIME × TIME and CPW × CPW) and their single interactions (CONC × TIME, CONC × CPW and TIME × CPW). In the first step of the analysis, the complete model was fitted using the code values ($-\alpha$, −1, 0, 1, α) for each of the three tested factors (Gallo *et al.* 2015). The absolute α value was estimated as reported by St-Pierre & Weiss (2009). Then, non-significant effects were removed from the model, whereas significant effects were expressed in their natural scale to obtain regression terms of second-order polynomial equations using the Mixed procedure of SAS (2003).

Differences between experimental replicates of the enzymatic method were not significant. Significance was declared at $P < 0.05$.

Then, the second-order polynomial equations were optimized by using the non-linear generalized reduced gradient method of

Table 1. Treatment layout of the three enzymatic methodological conditions[a] tested in the orthogonal central composite design (CCD) with four replications of central point

	Code values of tested conditions			Natural scale values of tested conditions		
Treatments	CONC	TIME	CPW	CONC	TIME	CPW
1	−1.4142	0	0	0.08	18	235
2	−1	−1	−1	0.19	10	118
3	−1	−1	1	0.19	10	353
4	−1	1	−1	0.19	26	118
5	−1	1	1	0.19	26	353
6	0	−1.4142	0	0.44	6	235
7	0	0	−1.4142	0.44	18	69
8, 9, 10, 11[b]	0	0	0	0.44	18	235
12	0	0	1.4142	0.44	18	401
13	0	1.4142	0	0.44	30	235
14	1	−1	−1	0.69	10	118
15	1	−1	1	0.69	10	353
16	1	1	−1	0.69	26	118
17	1	1	1	0.69	26	353
18	1.4142	0	0	0.80	18	235

[a]Enzymatic methodological conditions: *Streptomyces griseus* protease concentrations in the enzymatic working solution (CONC, U of protease/ml of enzymatic working solution), length of enzymatic incubation (TIME, h) or total amount of sample CP incubated in the buffered enzymatic solution (CPW, mg CP/sample incubated in enzymatic test).
[b]Central points of CCD characterized by having 0, 0, 0 codifications for the three tested factors.

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the Solver option of Excel (Microsoft Office Professional Plus 2010®, Microsoft Corporation, Seattle, WA, USA). The objective of optimization was set either between or within sample categories at ΔRUP equal to 0. The optimization was carried out by changing CONC, TIME and CPW values and opportune constraints were used on these terms to obtain suitable solutions. Solver options were: interactions equal to 1000, precision equal to 0.00001, convergence equal to 0.001, tangent estimates, forward derivatives and the Newton research method. In confirmatory tests, the REG procedure of SAS (2003) was used to verify relationships between *in vitro* rumen RUP (dependent variable) and enzymatic RUP values optimized for the tested working conditions (independent variables).

Results

The chemical composition of samples employed in both CCD and confirmatory tests is presented in Table 2, and appeared to be typical for the different feed categories. A wide range of *in vitro* RUP was measured among samples. In particular, values ranging from 251 to 913 g/kg CP were measured for sSBM 1 and eSBM 7, whereas values from 374 to 615 g/kg CP were obtained for AH 10 and AH 3. On average, *in vitro* RUP values of 583, 379, 370 and 678 g/kg CP were achieved for RM, SFM, WB and DDGS. Lastly, the RUP of CGF and CGM were 326 and 920 g/kg CP, respectively.

Table 3 shows the ΔRUP values obtained by incubating samples adopting different combinations of enzymatic methodological conditions. Differences between experimental replicates of the enzymatic method were not reported, as none of the tested effects were significant. When all samples were considered, the lowest and the highest ΔRUP values were observed for Treatment 1 (i.e. CONC of 0.08 U/ml working solution, TIME of 18 h and CPW of 235 mg CP incubated in enzymatic test) and Treatment 16 (i.e. CONC of 0.69 U/ml working solution, TIME of 26 h and CPW of 118 mg CP incubated in enzymatic test), respectively. Similarly, the highest ΔRUP values were measured for the different sample categories in Treatment 16, being 298, 393, 288, 218, 288, 192 and 234 g/kg CP for SBM, RM, SFM, WB, DDGS, CCP and AH, respectively. The lowest ΔRUP values were measured in Treatment 1 for SBM, RM, SFM, DDGS and AH (i.e. −79, −16, 28, −34 and −16 g/kg CP, respectively). In WB, the lowest ΔRUP value was obtained in Treatment 12 (i.e. CONC of 0.69 U/ml working solution, TIME of 26 h and CPW of 118 mg CP incubated in the enzymatic test), being −41 g/kg CP. For CCP, the lowest ΔRUP values (i.e. −3 g/kg CP) were observed in both Treatment 1 and 3, the latter being characterized by CONC of 0.19 U/ml working solution, TIME of 10 h and CPW of 353 mg CP incubated in the enzymatic test.

The regression terms of significant linear (i.e. CONC, TIME and CPW) and quadratic effects (i.e. CONC × CONC, TIME × TIME and CPW × CPW), as well as the interactions among main tested factors (i.e. CONC × TIME, CONC × CPW and TIME × CPW), are reported both as the average of all tested samples and within sample categories. In all developed models, the intercepts were maintained with values ranging from −536.8 to 175.0 for SBM and SFM. The three linear components influenced final ΔRUP values, with the sole exception of WB, in which the developed model did not include linear TIME component. Overall, greater ΔRUP values were obtained by increasing CONC and TIME or by decreasing CPW. The quadratic term CONC × CONC decreased ΔRUP values when all samples were considered, or in SBM, RM, SFM and DDGS, with regression coefficients ranging from −577.3 to −294.1 (U^2/ml^2 working solution) for SFM or all samples. The quadratic term TIME × TIME decreased in SBM and increased in WB the ΔRUP values, regression coefficients being equal to -484.2×10^{-3} or 83.9×10^{-3} (h^2), respectively. The quadratic term CPW × CPW increased the ΔRUP values in all samples, SFM and AH, regression coefficients being 14.7×10^{-4}, 1.0×10^{-4} and 27.5×10^{-4} (mg^2 CP). The interaction CONC × TIME increased ΔRUP values only in CCP, whereas interaction TIME × CPW decreased ΔRUP values in all samples, SBM and AH. The interaction term CONC × CPW decreased ΔRUP values in all samples and SBM, whereas it increased ΔRUP in RM and SFM.

Four non-linear generalized reduced gradient solutions are presented in Table 4, both as the average of all tested samples and within sample categories. Within each enzymatic solution, both CONC and TIME were unchanged to avoid an excessive number of possible solutions. Therefore, the main factor changed during the optimization processes was CPW. Solution 1 was characterized by CONC of 0.47 U of *S. griseus* protease/ml working solution and TIME of 18 h, whereas CPW ranged from 301 mg CP in AH to 724 mg CP in DDGS. The attempted solution ΔRUP equal to 0 was obtained in all sample categories, except for RM and AH. For solution 2, CONC was set at 0.12 U/ml working solution, TIME at 18 h, and CPW ranged from 233 mg CP in DDGS to 495 mg CP in SBM. The condition ΔRUP equal to 0 was calculated for all sample categories, except for a slight difference from 0 estimated in SBM and RM (i.e. 6 or 3 g/kg CP, respectively). The TIME was reduced to 6 h in solution 3 and CONC was equal to 0.14 U/ml working solution. The CPW ranged from 195 mg CP in DDGS to 579 mg CP in RM, and a ΔRUP equal to 0 was obtained for all feed categories. For solution 4, CONC was set at 0.08 U/ml working solution, TIME at 24 h, and CPW ranged from 253 mg CP in SBM to 510 mg CP in WB. A ΔRUP value different from 0 was estimated exclusively in AH.

In Fig. 1, results of the confirmatory test carried out by employing the four optimized methodological conditions are reported. Very high coefficients of determination as well as low prediction errors were obtained when samples were analysed by both solution 1 [*in vitro* rumen RUP (g/kg CP) = 121.6 (S.E. 32.5) + 8.2 (S.E. 0.7) × enzymatic RUP (g/kg CP), RMSE = 64.5, $R^2 = 0.82$, $P < 0.001$] and solution 2 [*in vitro* rumen RUP (g/kg CP) = −25.6 (S.E. 36.9) + 9.5 (S.E. 0.7) × enzymatic RUP (g/kg CP), RMSE = 65.2, $R^2 = 0.86$, $P < 0.001$]. However, underestimated *in vitro* rumen RUP values were observed by carrying out solution 1 in RM and SFM. On the contrary, a slight overestimation of *in vitro* rumen RUP was observed for most SBM employed when applying solution 2. Both solutions 3 and 4 showed lower coefficients of determination ($R^2 = 0.59$ or $R^2 = 0.58$, respectively) as well as greater errors of prediction (RMSE = 100.2 or RMSE = 101.8, respectively) than those previously presented.

Discussion

When researchers move to develop a method, or optimize related working conditions, two different approaches can be employed. In particular, a one-factor-at-a-time experiment, which consists of varying only one factor at a time and keeping all others fixed, can be adopted. Accordingly, Aufrère & Cartailler (1988) verified how several enzymatic working conditions separately influenced the prediction of rumen CP degradability of 12 feeds. As a result,

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Table 2. Chemical composition and *in vitro* rumen undegraded feed crude protein (RUP) of samples

	Chemicals[a]					
Items	DM g/kg as fed	CP g/kg DM	solCP g/kg CP	Ash g/kg DM	$aNDF_{om}$ g/kg DM	*in vitro* RUP[b] g/kg CP
Samples[c] used in central composite design (CCD)						
Soybean, meal						
sSBM 1	891	473	234	64	138	251 ± 12
sSBM 2	895	480	164	63	152	298 ± 24
sSBM 3	904	447	158	59	168	390 ± 18
sSBM 4	889	443	197	58	147	540 ± 32
eSBM 1	921	453	120	61	149	804 ± 11
eSBM 2	894	448	145	58	151	623 ± 9
eSBM 3	892	445	156	60	138	570 ± 34
eSBM 4	891	423	85	58	146	565 ± 12
Rapeseed, meal						
RM 1	898	340	125	48	188	599 ± 23
RM 2	899	329	136	52	191	602 ± 22
RM 3	902	349	281	47	169	549 ± 10
Sunflower, meal						
SFM 1	898	357	243	62	357	348 ± 20
SFM 2	922	272	274	58	387	360 ± 30
SFM 3	889	244	185	59	432	429 ± 21
Bran, wheat						
WB 1	899	165	319	58	522	397 ± 25
WB 2	891	175	402	52	459	343 ± 24
DDGS 1	884	324	230	54	388	626 ± 11
DDGS 2	875	303	146	49	384	729 ± 4
Corn, co-products						
CGF	896	206	638	58	343	326 ± 16
CGM	865	615	40	35	134	920 ± 2
Alfalfa, hays						
AH 1	884	197	226	82	496	389 ± 34
AH 2	889	187	268	94	520	461 ± 25
AH 3	894	206	232	104	485	615 ± 40
AH 4	874	154	234	124	543	523 ± 12
AH 5	860	179	308	87	524	456 ± 19
AH 6	875	233	273	101	442	401 ± 35
Additional samples[c] used only in the confirmatory test[d]						
Soybean, meal						
sSBM 5	911	444	201	63	140	440 ± 32
sSBM 6	895	459	220	59	141	392 ± 10
eSBM 5	913	454	172	60	146	701 ± 22
eSBM 6	895	443	197	62	141	594 ± 12
eSBM 7	911	489	134	63	163	913 ± 12

(*Continued*)

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Table 2. (*Continued.*)

Items	Chemicals[a]					*in vitro* RUP[b]
	DM	CP	solCP	Ash	$aNDF_{om}$	
	g/kg as fed	g/kg DM	g/kg CP	g/kg DM	g/kg DM	g/kg CP
Alfalfa, hays						
AH 7	892	204	292	96	478	465 ± 15
AH 8	871	179	235	86	457	451 ± 32
AH 9	890	201	287	125	476	570 ± 31
AH 10	893	161	245	91	528	374 ± 33
AH 11	889	212	241	112	442	447 ± 37
AH 12	883	188	297	108	437	533 ± 38
AH 13	885	204	279	96	454	555 ± 42
AH 14	875	184	260	103	474	428 ± 34
AH 15	875	167	278	94	511	460 ± 37
AH 16	861	184	258	79	451	523 ± 31

[a]Dry matter (DM), crude protein (CP), soluble crude protein (solCP), neutral detergent fibre fraction corrected for residual ash (aNDFom), acid detergent fibre fraction corrected for residual ash (ADFom).
[b]The *in vitro* RUP determinations were carried out in agreement to the rumen step of the method proposed by Ross *et al.* (2013).
[c]Solvent-extracted soybean meal (sSBM), expeller-extruded soybean meal (eSBM), solvent-extracted rapeseed meal (RM), solvent-extracted sunflower meal (SFM), soft white wheat bran (WB), distillers dried grain with solubles (DDGS), dried maize gluten feed (CGF), dried maize gluten meal (CGM), alfalfa hay (AH).
[d]All samples used in the CCD were successively employed in the confirmatory test, with the exception of sSBM 3, sSBM 4, eSBM 1, eSBM 2, AH 3, AH 4, AH 5 and AH 6 due to the limited amount of available substrate.

Aufrère *et al.* (1991) proposed a method successively validated on a larger dataset of about 100 samples, which has been used as reference method in the French intestinal digestible protein system. In the current study, the adoption of CCD permitted examination of both linear and quadratic effects of the three combined methodological factors concomitantly, as well as their first order interactions. As expected, the estimates of ΔRUP within all sample categories were increased linearly with CONC or TIME and decreased linearly with CPW. However, quadratic terms of the main effects tested also influenced the enzymatic RUP determination within different feed categories. Concerning CONC, it was decided to test increasing concentrations of *S. griseus* protease and to express them as U for ml in 10 ml working solution, in line with previous approaches (Krishnamoorthy *et al.* 1983; Licitra *et al.* 1998, 1999; Coblentz *et al.* 1999). Thus, final enzyme concentrations in 50 ml buffer solution ranged from 0.016 to 0.160 U/ml. To develop enzymatic tests, different enzyme concentrations were tested with values ranging from approximately 0.020 (Krishnamoorthy *et al.* 1983) up to 6.6 U/ml of buffer solution (Coblentz *et al.* 1999). In addition, variable optimal enzyme concentrations as a function of experimental conditions have been suggested, with values ranging from 0.050 U/ml (Aufrère & Cartailler 1988) to 0.8–1.0 (Licitra *et al.* 1999) or 2.7 U/ml (Cone *et al.* 1996). Consequently, the aforementioned approaches did not converge on a unique enzyme concentration, suggesting that there is no constant enzyme to substrate specificity or that it is difficult to mimic the activity of rumen fluid using commercial enzymes (Aufrère & Cartailler 1988; Luchini *et al.* 1996; Velasquez & Pichard 2010). In particular, as Licitra *et al.* (1999) discussed, the problem of the 'true' enzymatic concentration could be overcome by using a proteolytic activity similar to that measurable in the rumen microbiota. However, several difficulties usually arise in rumen proteolysis simulations, including fluctuations in the rumen fluid proteolytic activity during the day, that could follow zero-, first- or second-order kinetics as a function of the amount of rumen-available protein substrate at a given time (Krishnamoorthy *et al.* 1983). In addition, De Boever *et al.* (1996) suggested that rumen fluid proteolytic activity could differ among feed categories, being greater when degrading CP of forages than that of concentrates, thus introducing a substrate-dependent effect. Lastly, Licitra *et al.* (1999) hypothesized that the different mean retention time of feeds in the rumen compartment could also be an aspect influencing rumen fluid proteolytic activity. Probably due to these aspects, very different rumen fluid proteolytic activities, equal to 0.066 U/ml as suggested by Krishnamoorthy *et al.* (1983) or 1.5 U/ml as proposed by Licitra *et al.* (1999), have been reported. In the current experimental conditions, the optimized enzymatic concentrations ranged from 0.016 to 0.094 U/ml, thus being comparable with values reported by Krishnamoorthy *et al.* (1983). Other authors have reported good agreement between *in situ* RUP measurements and enzymatic values, adopting similar enzymatic concentrations (Aufrère & Cartailler 1988; Mathis *et al.* 2001; Cone *et al.* 2004; Irshaid 2007). In the current study, enzymatic results were compared with an *in vitro* rumen-based method (Ross *et al.* 2013) used in the Cornell Net Carbohydrate and Protein System and recently proposed as a reference method for the evolution version of the NRC (2001) protein system (Schwab 2015). This method was developed to overcome some issues related to the use of *in vitro* (Gargallo *et al.* 2006) or *in situ* (Cone *et al.* 2002) rumen-based assays, such as loss of small particles in the bags (Fessenden *et al.* 2017). Furthermore, it proposed an easy-to-employ approach for estimating microbial contamination of samples, as discussed exhaustively by Ross *et al.* (2013).

Other than CONC, discrepancies among different approaches exist concerning the duration of the enzymatic incubation and

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Table 3. Effects of three enzymatic methodological conditions[a] on the differences between *in vitro* rumen and enzymatic evaluated undegraded CP (ΔRUP, g/kg CP) values both among and within sample categories

Natural scale values of tested conditions[b]			Samples categories[c]							
CONC	TIME	CPW	All feeds	SBM	RM	SFM	WB	DDGS	CCP	AH
0.08	18	235	−30	−79	−16	28	−3	−339	−3	−16
0.19	10	118	60	−30	116	219	129	14	36	70
0.19	10	353	59	13	32	142	65	−11	−3	136
0.19	26	118	157	123	240	264	134	103	68	161
0.19	26	353	66	26	79	145	174	52	16	61
0.44	6	235	4	−74	68	175	70	43	33	−55
0.44	18	69	240	250	348	283	192	204	133	216
0.44	18	235	136	125	222	248	80	126	69	93
0.44	18	401	73	26	168	208	−41	76	31	70
0.44	30	235	200	160	342	273	144	209	119	187
0.69	10	118	196	177	312	274	187	147	92	180
0.69	10	353	110	13	225	246	157	96	58	118
0.69	26	118	278	298	393	288	218	288	192	234
0.69	26	353	119	−46	299	255	140	194	103	154
0.80	18	235	188	182	310	260	69	188	110	167
Standard errors of the mean			19.7	37.6	16.1	18.6	48.8	56.5	23.0	33.9
Regression terms[d] of the significant ($P < 0.05$) fixed-effect factors										
Intercept			−99.6 (53.6)	−536.8 (103.8)	18.8 (37.5)	175.0 (49.8)	110.5 (47.9)	−117.7 (63.2)	47.2 (35.9)	41.5 (71.1)
CONC			636.4 (114.6)	1088.6 (217.7)	836.8 (105.1)	577.1 (131.5)	100.4 (62.6)	618.8 (238.5)	54.0 (70.0)	175.4 (36.5)
TIME			9.9 (1.9)	32.4 (7.6)	7.4 (0.7)	2.2 (0.8)	ns	6.4 (1.7)	0.7 (1.8)	10.5 (3.0)
CPW			−0.42 (0.30)	0.93 (0.33)	−1.29 (0.26)	−0.99 (0.31)	−0.33 (0.13)	−0.28 (0.11)	−0.25 (0.05)	−1.11 (0.40)
CONC × CONC			−294.1 (110.3)	−476.8 (209.8)	−491.0 (116.3)	−577.3 (126.5)	ns	−371.7 (263.8)	ns	ns
TIME × TIME (values are × 10^{-3})			Ns	−484.2 (188.8)	ns	ns	83.9 (52.6)	ns	ns	ns
CPW × CPW (values are × 10^{-4})			14.7 (5.1)	ns	ns	10.0 (5.9)	ns	ns	ns	27.5 (8.2)
CONC × TIME			Ns	ns	ns	ns	ns	ns	5.9 (3.7)	ns
CONC × CPW			−0.70 (0.21)	−1.90 (0.45)	0.01 (0.01)	0.58 (0.28)	ns	ns	ns	ns
TIME × CPW (values are × 10^{-3})			−21.6 (7.4)	−40.2 (13.6)	Ns	ns	ns	ns	ns	−24.1 (12.0)

ns, not significant.

[a]Enzymatic methodological conditions: *Streptomyces griseus* protease concentrations in the enzymatic working solution (CONC, U of protease/ml of enzymatic working solution), length of enzymatic incubation (TIME, h) or total amount of sample CP incubated in the buffered enzymatic solution (CPW, mg CP/sample incubated in enzymatic test).

[b]The three enzymatic methodological conditions (CONC, TIME and CPW) were presented in their natural scale values as tested in the orthogonal central composite design (CCD). The code values of three tested conditions associated to specific treatment are described in Table 1.

[c]Soybean meals (SBM) consisting of four solvent-extracted soybean meals and four expeller-extruded soybean meals, three solvent-extracted rapeseed meals (RM), three solvent-extracted sunflower meals (SFM), two soft white wheat brans (WB), two distillers dried grains with soluble (DDGS), corn co-products (CCP) consisting of a dried maize gluten feed and a dried maize gluten meal, six alfalfa hays (AH).

[d]Values in brackets are the standard error (S.E.) of significant regression coefficients.

Table 4. Nonlinear generalized reduced gradient solutions both among and within samples obtained by optimizing the second-order polynomial equations resulted by testing the three tested enzymatic methodological conditions[a] in the central composite design

	Optimized nonlinear generalized reduced gradient solutions															
	Optimized solution 1				Optimized solution 2				Optimized solution 3				Optimized solution 4			
Sample categories[b]	CONC	TIME	CPW	ΔRUP	CONC	TIME	CPW	ΔRUP	CONC	TIME	CPW	ΔRUP	CONC	TIME	CPW	ΔRUP
All	0.47	18	381	100	0.12	18	302	16	0.14	6	353	0	0.08	24	337	21
SBM	0.47	18	435	0	0.12	18	495	5	0.14	6	505	0	0.08	24	253	0
RM	0.47	18	375	195	0.12	18	375	3	0.14	6	579	0	0.08	24	425	0
SFM	0.47	18	503	0	0.12	18	301	0	0.14	6	285	0	0.08	24	287	0
WB	0.47	18	565	0	0.12	18	458	0	0.14	6	390	0	0.08	24	510	0
DDGS	0.47	18	724	0	0.12	18	233	0	0.14	6	195	0	0.08	24	292	0
CCP	0.47	18	530	0	0.12	18	308	0	0.14	6	251	0	0.08	24	310	0
AH	0.47	18	301	60	0.12	18	298	0	0.14	6	353	0	0.08	24	309	45

[a]Enzymatic methodological conditions: *Streptomyces griseus* protease concentrations in the enzymatic working solution (CONC, U of protease/ml of enzymatic working solution), length of enzymatic incubation (TIME, h) or total amount of sample CP incubated in the buffered enzymatic solution (CPW, mg CP/sample incubated in enzymatic test).

[b]Soybean meals (SBM) consisting of four solvent-extracted soybean meals and four expeller-extruded soybean meals, three solvent-extracted rapeseed meals (RM), three solvent-extracted sunflower meals (SFM), two soft white wheat brans (WB), two distillers dried grains with soluble (DDGS), corn co-products (CCP) consisting of a dried maize gluten feed and a dried maize gluten meal, six alfalfa hays (AH). The objective of optimizations was set at the difference between *in vitro* rumen and enzymatic undegraded feed CP (ΔRUP, g/kg CP) equal to 0.

the amount of substrate or CP weighed. In particular, Krishnamoorthy *et al.* (1983) suggested incubating grain mixtures and forages for 18 and 48 h, respectively, thus mimicking appropriate rumen mean retention time. Accordingly, a 48-h incubation time was adopted by several authors when determining enzymatic RUP of different forages (Madsen & Hvelplund 1994; Coblentz *et al.* 1999; Mathis *et al.* 2001), whereas other authors adopted a shorter incubation time of 24 h (Aufrère & Cartailler 1988; Cone *et al.* 1996; 2004; Licitra *et al.* 1998; 1999; Edmunds *et al.* 2014). In addition, very short incubation times have been suggested (Assoumani *et al.* 1992; Susmel *et al.* 1993; Coblentz *et al.* 1999; Cone *et al.* 2002), aiming to make the enzymatic method less time-consuming. During the optimization processes, ΔRUP equal to zero was fitted by limiting possible solutions to short (i.e. 4–8 h) or medium (i.e. 18 and 24 h) enzymatic incubation time, thus excluding incubation times useless (i.e. from 8 to 16 h) or too long (i.e. >30 h) for practical laboratory purposes. Some authors weighed samples independently of the amount of enzyme (Krishnamoorthy *et al.* 1983; Aufrère & Cartailler 1988; Cone *et al.* 1996; 2002), whereas others adopted a fixed enzyme to sample CP ratio (Licitra *et al.* 1998; 1999; Coblentz *et al.* 1999; Mathis *et al.* 2001). De Boever *et al.* (1996), testing this effect in a pH 8 buffer enzymatic solution, suggested maintaining the enzyme to CP ratio constant for different feeds. This finding was in line with the current results, even if, as noted, a fixed ratio could be adopted within, but not between, different feed categories.

To the best of our knowledge, this is the first time a study was planned to investigate possible interactions among different methodological factors on the determination of enzymatic RUP, with the only exception being the experiment carried out by Coblentz *et al.* (1999). In particular, these authors studied the interaction between enzyme concentrations (i.e. 0.066, 0.66 and 6.6 U/ml) and incubation times (i.e. 2, 4 and 48 h), indicating that interactions were found for only one of two tested forages (i.e. alfalfa and prairie hays): they suggested that a different resistance to enzymatic attack could exist among tested forages. From the foregoing results, interactions among the main tested factors influenced the evaluations of enzymatic RUP, without consistency among different sample categories. As a matter of fact, solution 2 (CONC of 0.12 U of protease/ml of enzymatic working solution, 18 h time incubation and CPW ranging from 233 to 495 mg CP, respectively, for DDGS and SBM) guaranteed the best prediction of *in vitro* rumen RUP in all tested feed categories, except in SBM samples, where an overestimation of RUP values was observed. For these feeds, solution 1 (CONC of 0.47 U of protease/ml of enzymatic working solution, 18 h time incubation and CPW of 435 mg CP) seemed to be more precise than solution 2. These discrepancies could be attributed to the differing capacity of *S. griseus* protease to hydrolyse CP of different substrates (Aufrère & Cartailler 1988; De Boever *et al.* 1996; Velasquez & Pichard 2010). Consequently, the current results seemed to support the idea that the same methodological conditions should not be applied within different feed categories.

Conclusions

The application of a CCD experimental design permitted the study of both linear and quadratic effects of CONC, TIME and CPW, as well as their interactions and to develop second-order polynomial equations for each tested feed category. Using these equations in the optimization processes, the CONC, TIME and

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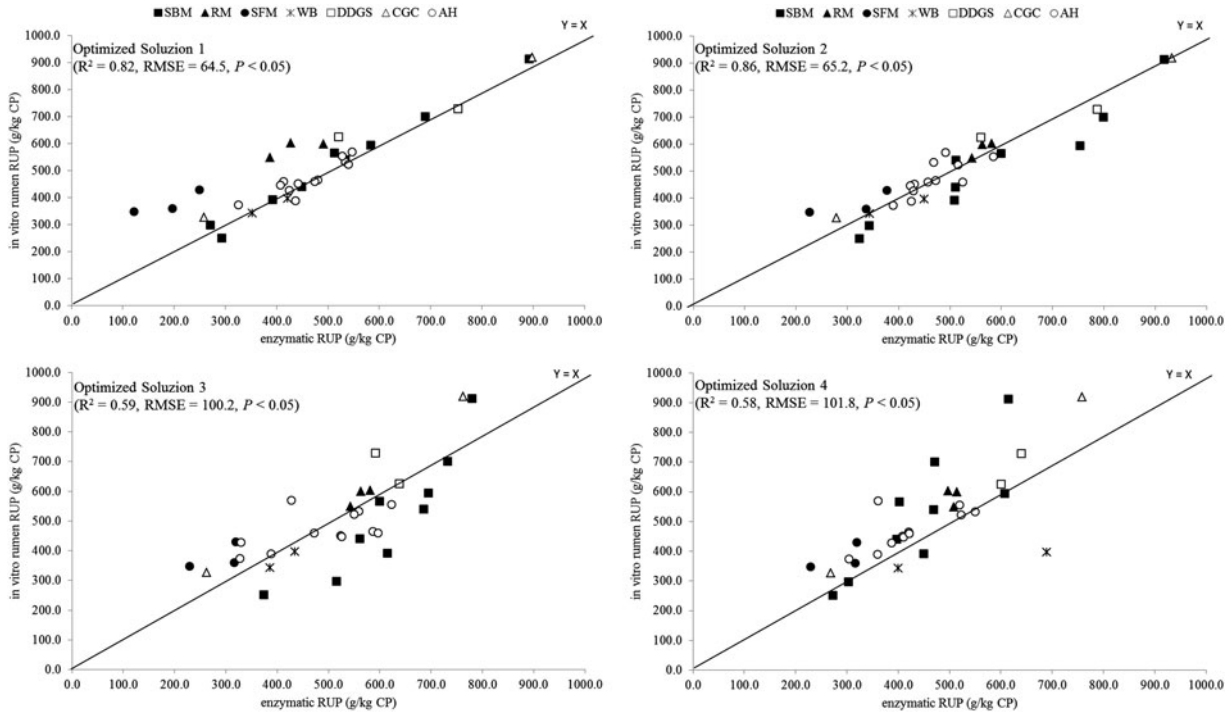

Fig. 1. Results of four confirmatory tests carried out by employing the four optimized enzymatic methodological solutions reported in Table 4 on 33 samples. Symbols in figure referred to soybean meal (SBM), solvent-extracted rapeseed meal (RM), solvent-extracted sunflower meal (SFM), soft white wheat bran (WB), distillers dried grain with solubles (DDGS), maize co-products (CCP) and alfalfa hay (AH).

CPW conditions guaranteeing that mathematical solution ΔRUP is equal to 0 could reasonably be obtained. When optimized methodological conditions were employed on a cohort of samples, a good agreement between *in vitro* rumen and enzymatic RUP values was reported. The results presented in the current manuscript compared results from an *in vitro* rumen-based assay to those obtained by using different enzymatic approaches. Since the *in vivo* conditions can never be exactly reproduced by *in vitro* methods, further investigations, comparing RUP evaluations obtained by the enzymatic method to RUP of feedstuffs obtained through *in vivo* trials, are warranted.

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