Extraction of Protein from Forages and Comparison of Two Methods To Determine Its Concentration†

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Twenty-three samples representing seven plant species were analyzed to evaluate a procedure to extract protein from forages. The method involved a borate–phosphate buffer (pH 9) containing 1% sodium dodecyl sulfate and sonication for 2 min. This method extracted an average of 85% of Kjeldahl crude protein (N × 6.25) and 81% of Kjeldahl true protein (tungstic acid precipitable N × 6.25). Extractable protein was measured as Kjeldahl crude protein, Kjeldahl true protein, and bicinchoninic acid protein (BCA protein). Concentrations of BCA protein in crude extracts were related linearly to both Kjeldahl crude and true protein. However, BCA was not an accurate or precise method of predicting Kjeldahl crude or true protein. When Kjeldahl crude protein and true protein, expressed as a percentage of dry matter, were regressed on BCA protein, relative ranking of crude and true protein in samples was improved over measurement in crude extracts.

INTRODUCTION

Extraction procedures that recover a high percentage of protein from forages are a prerequisite to characterization of the protein. Approximately 35–50% of the Kjeldahl crude protein (KJCP) in alfalfa has been extracted using buffers that did not contain detergents (Balestreri et al., 1989; Grum et al., 1991; Hood and Brunner, 1975). Fishman (1980) reported that adding sodium dodecyl sulfate (SDS) to an extraction buffer increased recovery of KJCP by 6% for ensiled and by 55% for fresh bermudagrass compared with buffer without SDS. Van Soest (1963) and Fishman et al. (1982) were able to extract 69 and 84% of KJCP from alfalfa and annual ryegrass, respectively. Extraction entailed refluxing (Van Soest, 1963) or stirring on a stir plate (Fishman et al., 1982) for 1 h in borate buffer that contained 2% SDS.

After extraction, the amount of protein in the extract must be quantified. Because of the hazardous and time-consuming nature of Kjeldahl analysis, alternative methods for protein determination are desirable. Although several colorimetric procedures to measure protein have been proposed [i.e., Lowry et al. (1951), Bradford (1976), Smith et al. (1985)], there are relatively few data comparing these procedures to KJCP or Kjeldahl true protein (KJTP) of plants. Protein assay using Bradford's method is unsuitable for buffers containing detergent (Bradford, 1976), and most buffers designed to extract a maximum amount of plant protein contain detergent. Lowry et al. (1951) reported good agreement between KJTP in mammalian organ tissue extracts and protein determined using the Lowry method. Kjeldahl protein in plant extracts has not been estimated accurately using the Lowry method (Khanna et al., 1969; Mattoo, 1969). The bicinchoninic (BCA) protein assay is less sensitive to compounds which interfere with the Lowry protein assay (Smith et al., 1985).

The objectives of this research were to develop a rapid method that could be used to extract a large proportion of the protein from forages and to compare protein values estimated using BCA to those obtained using Kjeldahl analysis.

MATERIALS AND METHODS

Twenty-three samples representing seven different species of plants were analyzed (Table I). All forages were grown outdoors. Fresh samples were frozen (−20 °C) within 1 h of cutting and subsequently lyophilized. Wilted forage was allowed to dry for 24–36 h in the field after cutting. This forage was then frozen and lyophilized. Hay was allowed to dry in the field until dry matter approached 85%, at which time the forage was baled. Silage, except crown vetch, was wilted in the field to 35–45% dry matter and ensiled in farm-scale silos; crown vetch was ensiled in glass jars. All silage samples were ensiled for at least 56 days and were lyophilized prior to grinding. All alfalfa samples, except hay, were harvested in the late bud stage of maturity. Alfalfa hay was harvested in the early bloom stage of maturity. Crown vetch samples from years 1 and 2 were harvested in the late bud stage of maturity. Chopped frozen spinach was purchased from a local grocer. Perennial ryegrass and the four fresh orchardgrass samples were fertilized with 0 or 78 kg/ha of N and harvested at either early anthesis or late anthesis. Wilted, hay, and silage samples of orchardgrass were fertilized with 375 kg/ha of N and harvested preanthesis. Corn plants were fertilized with 112 kg/ha of N and harvested at physiological maturity and ensiled. Pearl millet was fertilized with 56 kg/ha of N and harvested at anthesis.

All samples were ground through a 1-mm Wiley mill screen. Nitrogen in plant material was determined (in duplicate) using the Kjeldahl method (AOAC, 1984). Kjeldahl crude protein was calculated by multiplying nitrogen content by 6.25. Kjeldahl true protein was calculated by subtracting tungstic acid non-precipitable N (Winter et al., 1964) from total N and multiplying by 6.25.

Methods to extract plant protein using SDS buffer and sonication were tested. Plant samples (500 mg; ca. 95% dry matter) were sonicated at 180 W for 1, 2, or 5 min with a sonication probe (Ultrasonic 2000, Artek Systems Corp., Farmingdale, NY) in 75 mL of extraction buffer. Extraction buffer (pH 9) contained 41 mM sodium borate, 36 mM dibasic sodium phosphate, 10 mM EDTA, and variable concentrations of SDS (0.1, 1, 2, 3, and 10% w/v). Protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 0.5 μg/mL leupeptin, and 0.1 μg/mL pepstatin

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Table I. Concentration of Total Kjeldahl Crude and True Protein (Dry Matter Basis) and Recovery (R) of Extractable Kjeldahl Crude and True Protein

		KJCP[a]			KJTP[b]	
	n	%	R, %	SE[c]	%	R, %
alfalfa						
fresh	4	24.2	79.9	4.47	22.1	78.1
wilted	7	22.1	83.9	1.67	20.0	82.2
hay	7	18.9	73.8	5.45	11.8	69.3
silage	7	22.9	93.7	4.61	10.0	89.3
leaves	4	27.7	77.3	4.77	25.4	75.1
crown vetch						
fresh, year 1	7	24.8	75.8	2.10	20.7	71.2
fresh, year 2	4	27.5	72.6	1.85	23.3	67.7
wilted, year 2	4	27.7	70.4	1.45	23.0	68.4
silage, year 1	7	19.7	77.4	2.08	14.1	70.1
spinach						
fresh	4	30.4	79.1	2.29	27.1	76.6
perennial ryegrass						
fresh EA + N[d]	4	11.3	102.1	2.78	10.3	102.1
fresh EA[e]	4	10.3	76.3	6.29	9.6	74.8
fresh LA + N[f]	4	12.5	101.8	2.09	10.0	101.8
fresh LA[g]	4	6.1	103.1	4.03	5.6	103.1
orchardgrass						
fresh EA + N[d]	4	15.2	87.1	1.93	13.3	85.3
fresh EA[e]	4	11.1	87.1	4.48	10.1	85.9
fresh LA + N[f]	4	11.1	90.4	5.80	10.1	89.6
fresh LA[g]	4	8.8	83.6	2.21	8.1	81.9
wilted	7	20.2	71.4	3.04	17.4	66.8
hay	7	18.7	71.3	2.83	16.5	67.4
silage	7	19.7	86.4	3.92	12.3	78.2
corn plants						
silage	7	5.9	92.0	7.06	4.2	85.3
pearl millet						
fresh	7	13.2	100.6	3.16	11.8	100.6
mean SD			84.6			81.0
			10.27			12.27

[a] Kjeldahl crude protein. [b] Kjeldahl true protein. [c] Standard error associated with crude protein recovery. [d] Early anthesis plus N fertilization. [e] Early anthesis. [f] Late anthesis plus N fertilization. [g] Late anthesis.

A) were added to preserve the integrity of extracted proteins (Jervis and Pierpoint, 1989). Protease inhibitors were added to the sample immediately after addition of buffer, 2 min prior to sonication. After sonication, the homogenate was poured through four layers of cheesecloth and then filtered through Whatman No. 541 filter paper.

Extractions were replicated either four or seven times for each plant sample. Some samples were replicated seven times because part of the experiment had been completed when it was discovered that the EDTA concentration in the original buffer interfered with the BCA assay. The EDTA concentration of the extraction buffer was reduced, and those samples were extracted again. Because the EDTA concentration did not affect recovery, all extraction data were retained in the data set.

The crude extracts were analyzed for total KJCP in duplicate. Recovery of extractable KJCP was calculated as [(total N in crude extract)/(total N in initial sample)] × 100. Recovery of extractable KJTP was measured as [(total N in crude extract − nonprotein N of initial sample)/(total N in initial sample − nonprotein N in initial sample)] × 100. Recovery of nonprotein N was assumed to be 100% (Bell, 1963); therefore, total nonprotein N was subtracted from extracted nitrogen.

The crude extracts were analyzed for BCA protein in triplicate (Smith et al., 1985) with bovine serum albumen (BSA) as the standard. The BSA standard was analyzed for Kjeldahl N and then multiplied by 6.25 to obtain its KJCP concentration. This correction was necessary since the nitrogen to protein conversion factor for BSA is not 6.25. Wiechelman et al. (1988) found that compounds with sulfhydryl and aromatic functional groups interfere with determination of protein (BSA) using the BCA assay. Brown et al. (1989) reported that deoxycholate and trichloroacetic acid (DOC/TCA) was a rapid and selective method to precipitate protein (BSA) from interference caused by ampholines and reducing compounds. Acetone precipitation is another method used to remove protein from interfering compounds. Precipitation of protein by DOC/TCA was carried out on a 1-mL aliquot of crude extract by addition of 0.1 mL of 0.15% deoxycholate (w/v) and 0.1 mL of 72% (w/v) trichloroacetic acid (TCA; Brown et al., 1989). The TCA precipitate was solubilized in 1 mL of 0.2 N NaOH and assayed for protein using the BCA method. In addition, a 2-mL aliquot of crude extract was precipitated with 18 mL of cold (−20 °C for 24 h) acetone. Acetone-precipitated protein was centrifuged at 5000*g* for 20 min at −15 °C. The resulting pellet was resuspended in 2 mL of extraction buffer and assayed for protein using BCA.

The accuracy and precision of using BCA to estimate concentrations of extractable and total KJCP and KJTP were tested using regression. Regression equations and associated statistics were calculated according to the method of Neter et al. (1989) using SAS (1988).

RESULTS AND DISCUSSION

Extraction Procedure. The effect of sonication time upon recovery of protein was tested using orchardgrass (17% KJCP) and alfalfa (24% KJCP). There was a linear increase ($P < 0.01$; SE = 1.17) in crude protein recovery as samples were sonicated for 1, 2, and 5 min (73, 80, and 85%, respectively). Sonication for 5 min caused the instrument to malfunction (overheated the electronics). The increase in recovery of extractable KJCP between sonication for 2 and 5 min was marginal (ca. 6%); therefore, we chose to sonicate samples for 2 min.

Concentration of SDS in the extraction buffer had no effect on KJCP recovery ($P > 0.44$). Using the average binding constant of 1.4 g of SDS/g of protein (Reynolds and Tanford, 1970), all SDS concentrations that were tested were sufficient to interact with all protein in the extract. We chose 1% SDS in the buffer to ensure enough detergent to solubilize plant lipid membranes.

The final extraction buffer was a borate–phosphate buffer, pH 9, containing 1% SDS. The sample was sonicated for 2 min. Recovery averaged 85% (SD = 10.3%) for KJCP and 81% (SD = 12.3%) for KJTP (Table I). Within a sample, the standard error for recovery of KJCP ranged from about 1.5 to 7%. Recovery of extractable KJCP was negatively related ($P < 0.01$) to concentration of total KJCP in the plant tissue (recovery = 99.7 − 0.9 × total KJCP). Although this relationship was statistically significant ($P < 0.01$), the relationship was weak ($r^2 = 0.18$) and was not a major factor affecting protein recovery. Recovery of extractable KJCP in the present experiment is similar to those reported by Van Soest (1963) and Fishman et al. (1982). Buffers used in all three experiments were similar (except for pH), but the Van Soest (1963) and Fishman et al. (1982) methods required extraction for 1 h, while our method requires sonication for 2 min. This method represents a considerable time savings. Substantially more protein was extracted using our method than was reported in many other studies (Grum et al., 1990; Hood and Brunner, 1975; Fishman, 1980; Balestreri et al., 1989). Singh et al. (1990) extracted about 100% of wheat flour protein by sonication for 30 s in a 2% SDS buffer. Recovery values presented here are lower than those reported for cereal grains (Singh et al., 1990). This difference would be expected since the cell wall matrix acts as an impediment to extraction of green plant protein.

Protein Assay. Brown et al. (1989) found that DOC/TCA precipitation was a quantitative, repeatable, and rapid procedure to separation protein (BSA) from soluble compounds that interfered with determination of protein using the BCA assay. Since crude extracts of plant material would be expected to contain many compounds that interfere with the BCA protein assay, concentrations of

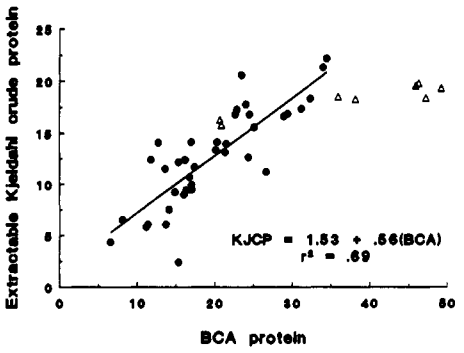

Figure 1. Relationship between BCA and extractable Kjeldahl crude protein (percent of dry matter). Crown vetch samples (△) are not included in the regression model.

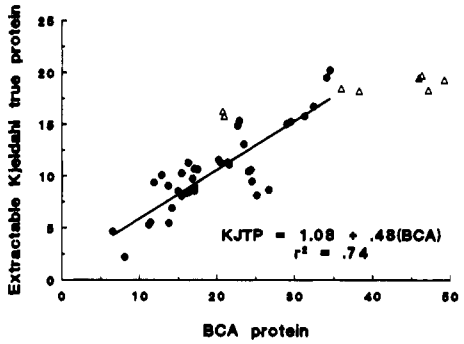

Figure 2. Relationship between BCA and extractable Kjeldahl true protein (percent of dry matter). Crown vetch samples (△) are not included in the regression model.

BCA protein in resuspended DOC/TCA and acetone precipitates were regressed against extractable KJCP and KJTP concentrations. Concentrations of BCA protein in extracts precipitated with either DOC/TCA or acetone were not related to KJCP (DOC/TCA, $p < 0.94$; acetone, $P < 0.36$) or KJTP (DOC/TCA, $P < 0.34$; acetone, $P < 0.37$) concentrations. Mahuran et al. (1983) reported that between 40 and 80% of various protein standards dissolved in water were recovered after DOC/TCA precipitation (protein was determined by electrophoresis followed by densitometry). Plant extracts contain a wide array of proteins. One possible reason for the poor relationship between extractable Kjeldahl (KJCP and KJTP) and BCA protein values for precipitated extracts was low recovery of protein by the precipitating agents. We have found that stain intensity of Coomassie blue on electrophoretic gels is less for extracts precipitated with DOC/TCA than for extracts not precipitated.

Precipitation prior to measuring BCA protein was unreliable; therefore, BCA protein was measured in crude extracts. Concentrations of BCA protein were linearly related to concentrations of extractable KJCP ($P < 0.01$; Figure 1) and extractable KJTP ($P < 0.01$; Figure 2) in most samples. Crown vetch samples did not react with BCA reagent in a manner similar to other samples within this data set; therefore, those samples were not included in the data used to derive regression equations (Table II). Sarkar et al. (1976) reported that crown vetch contained more phenolic compounds than many other legumes, including alfalfa. Compounds with aromatic functional groups have been shown to increase the reduction of copper which produces superfluous results in BCA assay (Wiechelman et al., 1988). Regression models (Table II) for predicting both extractable KJCP and KJTP had intercepts that were not different from zero ($P < 0.25$, $P < 0.29$, respectively); however, slopes for each model were different

Table II. Regression Statistics[a]

	KJCP[b]		KJTP[c]	
item	extractable[d]	total[d]	extractable[d]	total[d]
slope	0.56	0.90	0.48	0.80
slope SE	0.06	0.07	0.08	0.07
intercept	1.53	−1.49	1.08	−2.28
intercept SE	1.34	1.50	1.00	1.38
SE(NEW)[e]	2.74	3.81	2.07	3.42
PRESS[f]	289	375	168	316
mean	12.7	16.3	10.5	13.5

[a] Statistics for the model: Kjeldahl = $a + b$(BCA); crown vetch not included. [b] KJCP, Kjeldahl crude protein. [c] KJTP, Kjeldahl true protein. [d] Expressed as percent of dry matter. [e] Standard error of prediction. [f] Prediction sum of squares (PRESS statistic).

from unity ($P < 0.01$, $P < 0.01$), which indicates that BCA did not accurately measure extractable KJCP or KJTP.

Concentrations of extractable protein measured using Kjeldahl (KJCP and KJTP) and BCA differed for several reasons. First, plant pigments, peptides, and amino acids containing aromatic or sulfhydryl functional groups can increase color intensity developed during the BCA assay (Wiechelman et al., 1988). Second, the BCA color reaction depends on the amino acid composition of the protein (Smith et al., 1985; Wiechelman et al., 1988). Third, a constant N to protein conversion factor was used; however, these factors vary depending on sample source (Sosulski and Imafidon, 1990).

Although extractable KJCP and KJTP were not measured accurately using BCA, if precision was acceptable, an equation could be used to correct for inaccuracy. Residual deviations (BCA − extractable KJCP; BCA − extractable KJTP) ranged from −8 to 5 (extractable KJCP) and from −5 to 3 (extractable KJTP) percentage units. These residual values are relatively high considering the average concentrations of extractable KJCP and KJTP (12.7 and 10.5%, respectively; Table I). For a given BCA protein value there was, at times, a large amount of variation in extractable KJCP or KJTP values (Figures 1 and 2). Within a feed, the relative ranking for extractable KJCP and KJTP in crude extracts was not consistent with the ranking obtained with the BCA assay (Table III). Standard errors of prediction were 20–22% of the mean of extractable KJCP and KJTP concentration. Analytical error associated with both Kjeldahl and BCA assays was less than or equal to 5%; therefore, the use of BCA to predict extractable KJCP and KJTP was about 4 times more variable than direct measurement of extractable KJCP or KJTP. Relatively high residual deviations and a poor predictive relationship indicate that extractable KJCP and KJTP could not be predicted precisely from BCA. The prediction sum of squares (PRESS) was smaller when BCA was used to predict extractable KJTP than when it was used to predict extractable KJCP (Table III). The difference in PRESS between extractable KJCP and KJTP signified that for certain samples nonprotein N added greatly to the error of using BCA to predict extractable KJCP.

The BCA assay was used to predict concentrations of total KJCP and KJTP in the samples (Figures 3 and 4). Intercepts of regression equations used to predict total KJCP and KJTP on a dry matter basis were not different ($P < 0.05$) from 0 (Table II), and the slope for predicting total KJCP was not different ($P < 0.05$) from 1 (the slope for total KJTP was statistically different from 1). Much of the difference between the regression models derived to predict total KJCP and KJTP and those derived to predict extractable KJCP and KJTP is accounted for by protein recovery, i.e., total KJCP and KJTP are about

Table III. Concentrations (Percent of Dry Matter) of Extractable Kjeldahl and Bicinchoninic Acid Protein (BCA)

forage	extractable KJCP[a]	extractable KJTP[b]	BCA[c]
alfalfa			
fresh	16.7	15.2	29.2
wilted	17.0	15.1	22.7
hay	13.2	9.7	12.3
silage	18.1	10.6	24.3
leaves	17.8	11.3	31.8
crown vetch			
fresh, year 1	18.8	13.8	37.0
fresh, year 2	16.0	15.4	46.0
wilted, year 2	18.9	16.4	48.1
silage, year 1	19.6	10.7	20.7
spinach			
fresh	21.7	19.9	34.2
perennial ryegrass			
fresh EA + N[d]	11.2	10.2	17.1
fresh EA[e]	7.5	6.9	14.9
fresh LA + N[f]	11.8	9.7	14.5
fresh LA[g]	6.0	5.4	11.3
orchardgrass			
fresh EA + N[d]	13.3	11.4	20.6
fresh EA[e]	9.4	8.3	16.2
fresh LA + N[f]	9.7	8.7	16.2
fresh LA[g]	8.4	7.7	14.5
wilted	14.4	11.2	20.9
hay	11.9	9.6	25.5
silage	17.3	10.0	24.4
corn plants			
silage	5.5	3.4	7.3
pearl millet			
fresh	13.3	11.2	16.5

[a] Kjeldahl crude protein. [b] Kjeldahl true protein. [c] Bicinchoninic acid protein. [d] Early anthesis plus N fertilization. [e] Early anthesis. [f] Late anthesis plus N fertilization. [g] Late anthesis.

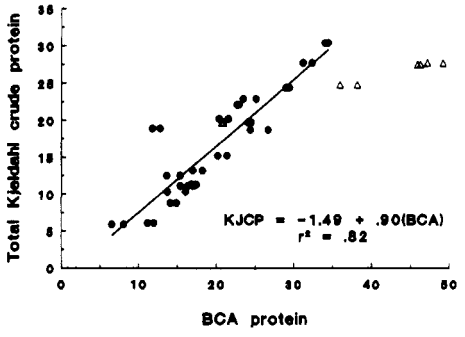

Figure 3. Relationship between BCA and total Kjeldahl crude protein (percent of dry matter). Crown vetch samples (△) are not included in the regression model.

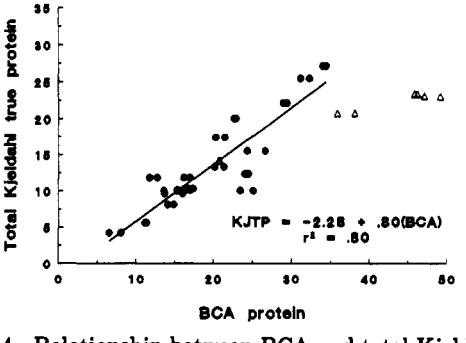

Figure 4. Relationship between BCA and total Kjeldahl true protein (percent of dry matter). Crown vetch samples (△) are not included in the regression model.

17% (recovery averaged about 83%) higher than extractable KJCP and KJTP. The precision of regression equations that were derived to predict total KJCP and KJTP is similar to that for predicting extractable KJCP and KJTP; however, relative ranking of concentrations of total KJCP and KJTP (dry matter basis) by BCA assay was satisfactory. Assay for protein by BCA represents a considerable time savings over Kjeldahl and may be a viable alternative to Kjeldahl in circumstances where a large number of plant samples need to be screened for relative protein concentration (e.g., genetic selection for plant protein content). Caution must be exercised when using BCA to rank protein concentration of forage samples. All forages do not react with the BCA reagent in a similar manner. For example, alfalfa and crown vetch are both forage legumes, but at a given total KJCP concentration their BCA protein concentrations were considerably different.

In conclusion, we have outlined a high-yielding protein extraction procedure for forage samples. On average, 85% of KJCP and 81% KJTP were recovered after 2 min of sonication. Neither extractable KJCP nor KJTP was accurately or precisely predicted by BCA assay. Using concentrations of BCA protein in crude extracts to predict total KJCP and KJTP concentrations (dry matter basis) proved to be satisfactory for ranking forages according to protein concentration.

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