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Repeatability and reproducibility of the Cornell Net Carbohydrate and Protein System analytical determinations

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ABSTRACT

The increasing use in Italy of the Cornell Net Carbohydrate and Protein System (CNCPS) led researchers of five Italian universities to carry out a collaborative study to evaluate the precision of the CNCPS chemical analysis and derivative fractions. Each laboratory conducted in duplicate the chemical analyses according to the Weende (dry matter; crude protein; ether extract; crude fibre; ash), Van Soest (neutral and acid detergent fibre, NDF and ADF; acid detergent lignin; ADL) and CNCPS (soluble proteins, SP; non-protein nitrogen, NPN; neutral and acid detergent insoluble protein, NDIP and ADIP; starch, ST) schemes on the same five feeds (barley meal, wheat straw, maize silage, dried lucerne and field beans). Anomalous analytical data were identified and corrected by the "box-plot" graphic tool before the calculation of the CNCPS protein (B1, B2 and B3) and carbohydrate (A, B2 and C) fractions. Finally, repeatability (chemical analysis) and reproducibility (chemical analysis and fractions) were calculated and expressed as relative values (repeatability and reproducibility standard deviation as percentage of the corresponding mean, RSDr and RSDR, respectively). Chemical analyses of the Weende scheme, together with NDF, ADF and ST analyses, have satisfactory repeatability (0.3-6.2%) and reproducibility (0.3-11.2%) values. On the contrary the ADL, NPN, NDIP and ADIP analyses showed high variability, both within and between laboratories (RSDr and RSDR between 20 and 45%). The SP analysis had an intermediate value of precision (RSDr=10.6%; RSDR=16.4%).

Finally, since different combinations of several chemical analyses with scarce (ADL, NPN, NDIP, ADIP, SP) or average precision (e.g. NDF and starch) are used to calculate CNCPS fractions (excluding B3 protein fraction), also the reproducibilities of these fractions are poor and range from 10 to 20%.

Key words: CNCPS, Analytical determinations, Repeatability, Reproducibility.

RIASSUNTO

RIPETIBILITÀ E RIPRODUCTION DELLE DETERMINAZIONI ANALITICHE PREVISTE DAL CNCPS (CORNELL NET CARBOHYDRATE AND PROTEIN SYSTEM).

Il diffondersi in Italia del sistema di valutazione di alimenti per ruminanti CNCPS ha indotto alcuni ricercatori di 5 Università italiane ad effettuare una prova collaborativa per verificare la precisione delle determinazioni previste da que-
sto sistema. Pertanto, su 5 alimenti (farina di orzo, paglia di frumento, insilato di mais, farina di erba medica e favino) ciascun laboratorio ha effettuato in doppio le analisi chimiche previste dagli schemi Weende (sostanza secca; proteina grezza; estratto etereo; fibra grezza; ceneri), Van Soest (fibra neutro e acido detergente, NDF e ADF; lignina acido detergente, ADL) e CNCPS (proteine solubili, SP; azoto non proteico, NPN; proteina insolubile al detergente neutro e acido, NDIP e ADIP; amido, ST ). Inizialmente i dati sono stati analizzati mediante lo strumento grafico "box-plot" allo scopo di individuare e correggere i dati anomali e quindi sono state calcolate le frazioni proteiche (B₁, B₂ e B₃) e glucidiche (A, B₂ e C) previste dal CNCPS. Infine, sono stati calcolati i parametri statistici di precisione di ripetibilità (analisi chimiche) e di riproducibilità (analisi chimiche e frazioni) che sono stati espressi in termini relativi (deviazione standard della ripetibilità e della riproducibilità come percentuale del corrispondente valore medio, RSDr e RSDR, rispettivamente). Per le analisi chimiche dello schema Weende assieme alle analisi di NDF, ADF e amido, sono stati calcolati soddisfacenti valori di ripetibilità (0.3-6.2%) e riproducibilità (0.3-11.2%). Al contrario le analisi di ADL, NPN e ADIP hanno presentato variabilità elevate, sia entro che tra laboratori (RSDr e RSDR tra 20 e 45%), mentre l’analisi delle proteine solubili sì è collocata ad un livello intermedio di precisione analitica (RSDr=10.6%; RSDR=16.4%). Infine, poiché nel calcolo delle frazioni CNCPS (ad esclusione della frazione proteica B₂) vengono utilizzate combinazioni di diverse analisi caratterizzate da scarsa (ADL, NPN, NDIP, ADIP, SP) o intermedia precisione (e.g. NDF e amido) anche la riproducibilità delle frazioni CNCPS è risultata piuttosto bassa e compresa tra 10 e 20%.

Parole chiave: CNCPS, Determinazioni analitiche, Ripetibilità, Riproducibilità.

Introduction

An innovative system for the expression of the requirements and the energy and protein value of feeds for ruminants was proposed a decade ago. Some researchers of the Cornell University (Russell et al., 1992; Sniffen et al., 1992; Fox et al., 1992; O’Connor et al., 1993), defined it as the “Cornell Net Carbohydrate and Protein System” (CNCPS). This system evaluates feedstuffs on the basis of a set of four carbohydrate and five protein fractions obtained by the combination of the classic determinations of the Weende scheme (dry matter, DM; crude protein, CP; ether extract, EE; crude fibre, CF; ash), the fractions of structural carbohydrates defined by Van Soest neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL, Van Soest et al., 1991) with other chemical analyses. These include measures of starch (ST), non protein nitrogen (NPN), protein solubility (soluble protein, SP, Krishnamoorty et al., 1983) and analyses aimed at determining the amount of protein rendered unavailable because linked to fibrous components (neutral detergent insoluble protein, NDIP; and acid detergent insoluble protein, ADIP; Sniffen et al., 1992).

The results of NPN and ADIP determinations (barley meal; wheat straw; maize silage; dried lucerne; field beans). Each research group was asked to select and send to the others a sample (500 g) of one of the feeds, ground with a 1 mm
sieve. If the moisture content was higher than 14%, the sample had to be dried in a forced air oven (63°C) and ground through a 1 mm screen. After checking the operative conditions of their equipment, each laboratory (identified as V, W, X, Y and Z) carried out analytical determinations in duplicate.

**Analytical determinations**

For each feed sample the following analyses were carried out:

- **DM**, **CP** and **EE** (ASPA, 1980, Martillotti et al., 1987); the latter procedure was followed avoiding the initial acid hydrolysis;
- **CF**, corrected for the ash content of the residue, was determined by the Fibertec apparatus by labs X, Y and Z and by Ankom Fiber Analyser (Ankom Technology Corporation, Fairport, NY) by labs V and W;
- **NDF**, corrected in any case for the ash content of the residue, was determined, with previous treatment with α-amylase (Van Soest et al., 1991), by the Fibertec apparatus by labs X and Y or by Ankom by the others;
- **ADF** and **ADL** were determined sequentially either with Fibertec or Ankom, according to the apparatus used for the determination of NDF;
- **ST**, all the five laboratories utilised the polarimetric method described by Martillotti et al., 1987;
- **NPN**, **SP**, **NDIP** and **ADIP** were determined after the indications provided by Licitra et al., 1996.

**Statistical analysis**

Explorative and confirmative analyses were performed on each parameter. The graphical tool “box plot” (SPSS, 2000) was used for the first

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**Table 1.** DM content (%), chemical composition (% DM) and CNCPS protein and carbohydrate fractions (%) of the feeds tested in the five laboratories.

| Chemical composition: | Barley | Wheat straw | Maize silage | Dried lucerne | Field beans |
|------------------------|--------|-------------|--------------|---------------|-------------|
| DM                     | 87.13  | 91.54       | 92.18        | 93.43         | 87.86       |
| Crude Protein          | 12.86  | 4.47        | 7.69         | 15.99         | 30.78       |
| Ether Extract          | 2.13   | 0.55        | 3.05         | 1.46          | 0.95        |
| Crude Fibre            | 4.99   | 41.91       | 18.22        | 30.89         | 6.58        |
| Ash                    | 3.28   | 8.42        | 4.48         | 9.80          | 3.64        |
| NDF                    | 21.26  | 80.09       | 37.93        | 47.75         | 16.93       |
| ADF                    | 6.38   | 52.30       | 22.20        | 36.31         | 8.94        |
| ADL                    | 1.24   | 6.40        | 2.09         | 8.15          | 0.62        |
| Starch                 | 53.81  | 1.72        | 36.99        | 3.30          | 46.19       |
| Soluble proteins       | 2.42   | 1.62        | 4.27         | 4.81          | 15.14       |
| NPN                    | 0.12   | 0.12        | 0.62         | 068           | 0.62        |
| NDIP                   | 1.72   | 1.48        | 1.08         | 3.26          | 1.49        |
| ADIP                   | 0.60   | 1.25        | 0.77         | 1.56          | 0.74        |
| Protein fractions:     |        |             |              |               |             |
| B₁                     | 1.70   | 0.91        | 0.50         | 0.64          | 11.24       |
| B₂                     | 8.72   | 1.65        | 2.35         | 7.92          | 14.15       |
| B₃                     | 1.12   | 0.42        | 0.31         | 1.70          | 0.76        |
| Carbohydrate fractions:|        |             |              |               |             |
| A                      | 8.38   | 6.24        | 10.93        | 24.96         | 5.11        |
| B₁₀                    | 16.57  | 63.26       | 31.85        | 24.92         | 11.84       |
| B₂₁                    | 2.97   | 15.35       | 5.00         | 19.56         | 1.50        |
approach. This feature allows to visualise the main data distribution characteristics starting from the median and from the interquartile difference (ID). This parameter is calculated as the distance between the third quartile (X75%) and the first (X25%), where X25% is the value which separates the lower 25% of observations from the rest and X75% is the value which separates the lower 75% of observations.

Figure 1. Distribution of results of the analytical determinations of the Weende scheme.

BG = Barley grain; WS = Wheat straw; MS = Maize silage; DL = Dried lucerne; FB = Field beans
º = anomalous value; * = extreme value
observed value and the difference $X_{75\%} + 1.5 \times ID$, while the higher one coincides with the smaller between the greatest observed value and the sum $X_{75\%} + 1.5 \times ID$. The two break-off values are then united to the box by means of vertical segments, called “moustache”. Values outside the moustache were considered anomalous and were substituted with “corrected duplicate” according to the following procedure (Youden and Steiner 1975): the sums of each two replications were computed and an average value was calculated. From this latter value the single replication resulted from the outlier exclusion was subtracted, giving a “corrected duplicate”.

The average data from the required analytical determinations were used to calculate protein and carbohydrate fractions according to Sniffen et al. (1992).

The results of analytical determinations and CNCPS fractions calculated (protein B1, B2 and B3 and carbohydrate A, B2 and C fractions) were processed using, respectively, the following two linear models (PROC GLM, SAS 2000), according to Youden and Stern (1975):

Figure 2. Distribution of results of the analytical determinations of structural carbohydrate fractions.

BG = Barley grain; WS = Wheat straw; MS = Maize silage; DL = Dried lucerne; FB = Field beans
º = anomalous value
**Bovera et al.**

Y\_ijk = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk} \\
Y\_ij = \mu + \alpha_i + \beta_j + \epsilon_{ij}

where:

- Y\_ijk = single observation (replication number k=1,2);
- \mu = general mean;
- \alpha = laboratory effect (i = 1,..5);
- \beta = feed effect (j = 1,..5);
- \epsilon\_{ij} = interaction between laboratory and feed effects;
- \epsilon = residual error.

**Figure 3.** Distribution of results of the analytical determinations of starch, soluble proteins, NPN, NDIP and ADIP.

BG = Barley grain; WS = Wheat straw; MS = Maize silage; DL = Dried lucerne; FB = Field beans

° = anomalous value; * = extreme value
The variance components, relative to laboratory effect ($MS_{\alpha} = (j \times k) \sigma_{\alpha}^2 + k \sigma_{\alpha} \sigma_{\beta} + \sigma^2$), its interaction with the feed effect ($MS_{\alpha\beta} = k \sigma_{\alpha} \sigma_{\beta}$) and error variance ($MS_{e} = \sigma^2$) were used to calculate the standard deviation of repeatability and reproducibility ($s_r$ and $s_e$, respectively) using the following formulae suggested by AOAC (Youden and Stern, 1975):

$$s_r = \frac{MS_e}{\sqrt{\frac{MS_{\alpha} - MS_{\alpha\beta}}{j \times k} + \frac{MS_{\alpha\beta}}{k} + \frac{MS_{\alpha\beta} - MS_e}{j \times k}}}$$

Protein and carbohydrate fractions were calculated from average values and only reproducibility could be calculated, using the following equation:

**Table 2.** Results of analysis of variance for the analytical parameters (n = 50; 5 laboratories, 5 feeds, 2 replicates) and the CNCPS fractions (n = 25; 5 laboratories, 5 feeds).

| Analytical Parameters | Between laboratories | Interaction lab. x feed | Error variance |
|-----------------------|----------------------|-------------------------|----------------|
| DF = 4                | DF = 16              | DF = 25                 |
| Dry Matter            | 0.1300 ns            | 0.1124 ns               | 0.068          |
| Crude Protein         | 2.3177 **            | 0.6278 **               | 0.1370         |
| Ether Extract         | 0.0714 **            | 0.0527 **               | 0.0101         |
| Crude Fibre           | 12.3972 **           | 2.4262 ns               | 1.2886         |
| Ash                   | 0.0497 ns            | 0.0260 ns               | 0.0199         |
| NDF                   | 64.9164 **           | 18.2597 **              | 1.9509         |
| ADF                   | 14.7071 **           | 3.7263 *                | 1.5524         |
| ADL                   | 2.4337 *             | 0.4475 ns               | 0.6051         |
| Starch                | 21.1715 **           | 8.6344 **               | 1.0090         |
| Soluble proteins      | 1.1794 *             | 1.4065 **               | 0.3654         |
| NPN                   | 0.0147 ns            | 0.0152 ns               | 0.0271         |
| NDIP                  | 0.3501 ns            | 0.1717 ns               | 0.3324         |
| ADIP                  | 0.4860 **            | 0.2816 **               | 0.0673         |
| Protein fractions:    |                      |                        |                |
| B1                    | 0.4363 ns            | .                       | 1.1526a        |
| B2                    | 0.4097 ns            | .                       | 0.3859a        |
| B3                    | 0.0958 ns            | .                       | 0.1131a        |
| Carbohydrate fractions:|                     |                        |                |
| A                     | 22.5324 ns           | .                       | 11.0980a       |
| B                    | 40.1204 *            | .                       | 9.9987a        |
| C                     | 7.0203 *             | .                       | 1.2894a        |

ns = not significant; * = P < 0.05; ** = P < 0.01; a: DF = 16
Finally, relating \( s_r \) and \( s_R \) values to the corresponding general means of tested parameters, the percent variability coefficients were obtained, named relative repeatability and relative reproducibility (RSD\(_r\) and RSD\(_R\) respectively) by AOAC (2000).

Results and discussion

Figures 1, 2 and 3 show box-plots of the five tested feeds relative to the analytical determinations of Weende, Van Soest and CNCPS schemes. The data distributions were in most cases asymmetric and their dispersion, fairly small for DM, CP, EE, CF and ash determinations, tended to increase for the analyses of structural carbohydrate and the nitrogen fractions. The “box plot” graphical tool allowed to identify some outliers (located outside the “moustache”), with a limited incidence for the analyses of the Weende scheme, together with NDF and ADF analyses (5-6% of total observations, figures 1 and 2). Dataset for ADL showed a higher percentage of outliers (6 out of 50). SP and NPN data were affected by around a 10% proportion of outliers, which reached a top of 16% for NDIP (figure 3). No outliers were observed for starch analysis results, while their absence in ADIP data was probably due the high dispersion of the measures.

Average values for the data supplied by the

| Table 3. Means of analytical determinations and CNCPS fractions and values of repeatability (analytical parameters) and reproducibility (analytical parameters and CNCPS fractions) of the 5 feeds. |
|-------------------------------------------------|
| **Mean** | **Repeatability** | **Reproducibility** |
| **SD** | **Relative** | **SD** | **Relative** |
| Dry Matter | 90.43 % | 0.26 | 0.29 | 0.30 | 0.34 |
| Crude Protein | 14.36 % DM | 0.37 | 2.58 | 0.74 | 5.17 |
| Ether Extract | 1.63 " | 0.10 | 6.17 | 0.18 | 11.19 |
| Crude Fibre | 20.52 " | 1.14 | 5.53 | 1.69 | 8.23 |
| Ash | 5.92 " | 0.14 | 2.38 | 0.16 | 2.60 |
| NDF | 40.79 " | 1.40 | 3.42 | 3.84 | 9.42 |
| ADF | 25.23 " | 1.25 | 4.94 | 1.93 | 7.66 |
| ADL | 3.70 " | 0.78 | 21.02 | 0.85 | 23.01 |
| Starch | 28.40 " | 1.00 | 3.54 | 2.46 | 8.68 |
| Soluble proteins | 5.65 " | 0.60 | 10.60 | 0.93 | 16.44 |
| NPN | 0.43 " | 0.16 | 38.28 | 0.15 | 33.78 |
| NDIP | 1.81 " | 0.58 | 31.85 | 0.52 | 28.70 |
| ADIP | 0.98 " | 0.27 | 26.47 | 0.44 | 45.05 |
| Protein fractions: | | | |
| B1 | 3.00 " | . | . | 0.30 | 9.85 |
| B2 | 6.96 " | . | . | 0.29 | 4.11 |
| B3 | 8.86 " | . | . | 0.14 | 16.10 |
| Carbohydrate fractions: | | | |
| A | 10.70 " | . | . | 2.12 | 19.84 |
| B1 | 30.11 " | . | . | 2.83 | 9.41 |
| C | 8.88 " | . | . | 1.18 | 13.34 |
five laboratories involved in this study are shown in Table 1 where means for each feed were corrected according to explorative analysis, as well as the values of protein and carbohydrate fractions. Results from analysis of variance on analytical determinations and CNCPS fractions are reported in Table 2. Significant differences (almost always for $P < 0.01$) between laboratories were found for most analyses (with the exception of DM, ash, NPN and NDIP), while the laboratory x feed interaction was significant for about half of the chemical analysis considered. Finally, only B$_2$ and C carbohydrate fractions differed significantly between laboratories ($P < 0.05$). Therefore confirmative analysis suggests that the results supplied by the different laboratories do not appear reliable even when applying the same equipment and methods.

The precision of analytical procedures was evaluated in terms of repeatability and reproducibility; for CNCPS fractions, calculated from the average of duplicate analyses it was only possible to estimate reproducibility (Table 3).

Repeatability in the laboratory is the variability in the analytical results obtained in the same conditions (same operator, same laboratory, same time) and is expressed in terms of standard deviation ($s$) or, relating to the general mean of corresponding determination, as variability coefficient (RSD), allowing, in the latter case, comparison of determinations with different means. Reproducibility is, instead, the variability of results obtained in different conditions (different operators, different laboratories, different times) and is expressed like repeatability ($s_R$ and RSD$_R$). The analytical determinations indicated by the Weende scheme showed, in general, good precision in the same laboratory even if there were an ample range of variation for the RSD parameter from to the lowest value for DM (0.29%) to the highest values of CF and EE (5.53 and 6.17%, respectively). Starch, NDF and ADF determinations were also acceptable (3.54, 3.42 and 4.94% respectively) while ADL determinations and all the analysis for protein fractions had precision indexes higher than 20%, with the exception of SP values (10.6%). As expected, reproducibility values were higher than those of repeatability because, in addition to analytical variability in the laboratory, the formers are also affected by different operative conditions. Nevertheless, the reproducibility/repeatability ratio is not homogeneous between the different determinations tested: in particular, for the Weende scheme, the CP and EE determinations show values of reproducibility which doubled those of the corresponding repeatability. This situation indicates an important effect due to operative differences between laboratories. NDF and starch had even higher reproducibility/repeatability ratios (2.8 and 2.5, respectively). This is due to the use of different apparatus (two laboratories used the Fibertec and the remaining three the Ankom) or instruments with different sensitivity (polarimeter) by the five research groups.

In a national ring test (18 laboratories and four feeds) described by Lanari et al. (1991) the EE and ADL determinations showed, as in present study, the worsted repeatability. For reproducibility the results of two ring tests show non-homogeneous values (some determinations are more reproducible in one and others have higher reproducibility in the other test), excluding EE and NDF determinations for which the values are very similar (RSD$_R$ 11.2 vs 12.6% and 9.42 vs 7.65%, respectively) and ADL determinations that showed for both studies poor reproducibility (23.0 vs 41.7%).

Finally, the protein fraction determinations were scarcely reproducible due to insufficient repeatability discussed above. In particular, given the satisfactory results obtained from determinations of structural carbohydrate fractions, we suppose that the variability of results from the five laboratories regarding NDIP and ADIP contents could have originated from the use of a different apparatus (Fibertec vs Ankom), but also from the methodological difficulties involved. Regarding ADIP determination, our opinion is that their high variability may be imputed to the use of some reagents containing large quantities of nitrogen whose removal would require standard rinsing procedures.
Since in calculating CNCPS fractions also analytical determinations with scarce or average RSDs (e.g. NDF and starch) are used, for almost all the fractions (excluding B2 protein fraction) the RSDs values are poor and range from 10 to 20%.

Conclusions

The present investigation indicates that the procedures to fractionate the protein and carbohydrates of ruminant feeds proposed by the CNCPS use some analyses that, within the group of participant laboratories, showed low precision and poor reliability of subsequent estimations. Part of non-homogeneous values is attributable to the usage of non identical apparatus (i.e. for fiber fractions). However the adopted level of standardization and harmonisation of procedures between laboratories, which allowed repeatable and reproducible values for some procedures (i.e. part of Weende and Van Soest analysis), appears insufficient to guarantee acceptable precision standards for other analysis (i.e. ADL, SP, NPN, NDIN, ADIN).

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