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Neutral Detergent-Soluble Carbohydrates Nutritional Relevance and Analysis

A Laboratory Manual

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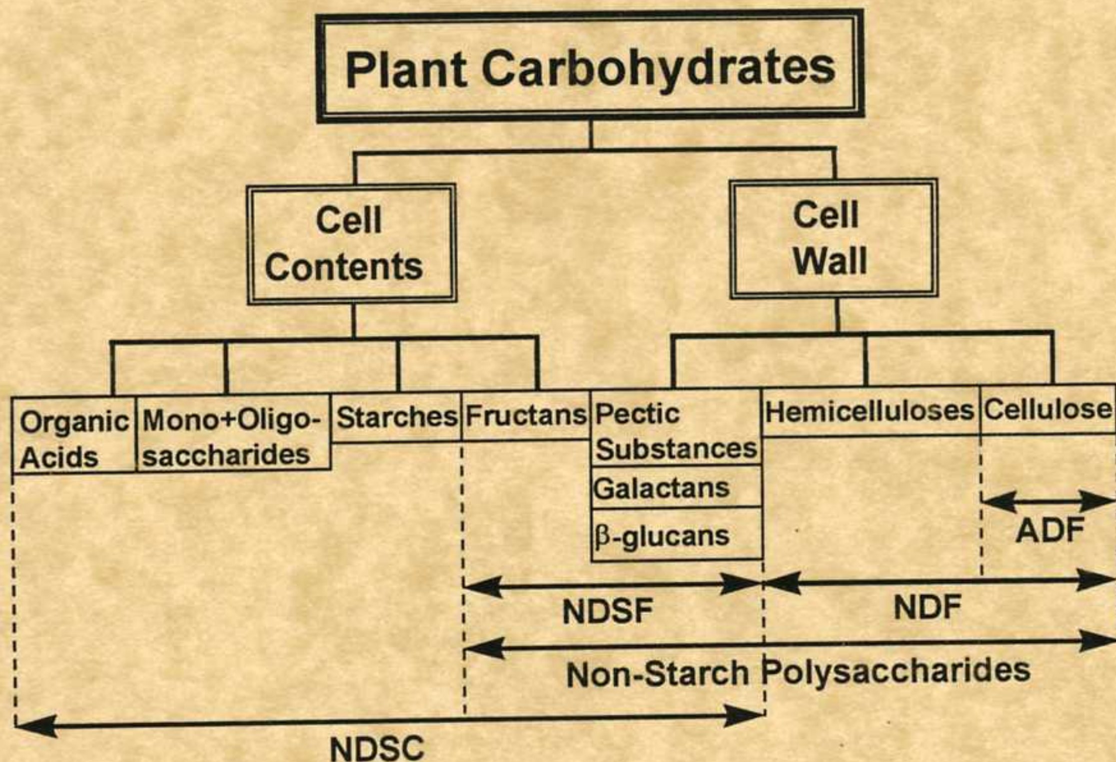

This publication was written for use as a laboratory manual by those involved in feed analysis, and those who wish to gain a better understanding of the relevance of those assays to ruminant nutrition. It describes the nutritional characteristics of neutral detergent-soluble carbohydrates (NDSC), and a practical, coherent system for their analysis. It is not a comprehensive discussion of all available analyses, but covers one system for partitioning NDSC and factors that can affect accuracy of the analyses.

Although there is information in the literature on animal responses to dietary NDSC, without a practical partitioning system, much of the work evaluated feed sources rather than different NDSC. The various carbohydrates in NDSC are not nutritionally equivalent. Changing their proportions in diets can change the metabolizable nutrient supply to the animal, and has implications for animal performance. With a system to determine amounts of NDSC fractions in rations, we can better understand how the different types affect animal performance, and develop recommendations for NDSC in ration formulation. Just as we understand that crude protein, or neutral detergent fiber from all sources do not function in exactly the same way in all diets, we need to bring the same view to NDSC. As with these other feed fractions, NDSC also require further investigation into differences in their digestive characteristics (e.g., fermentation rates and products) within NDSC type, by feed source and composition.

I would especially like to thank Drs. Betty Lewis and Dave Mertens for their reviews of this manual, and Jocelyn Jennings for her assistance with refining the methods.

M. B. Hall
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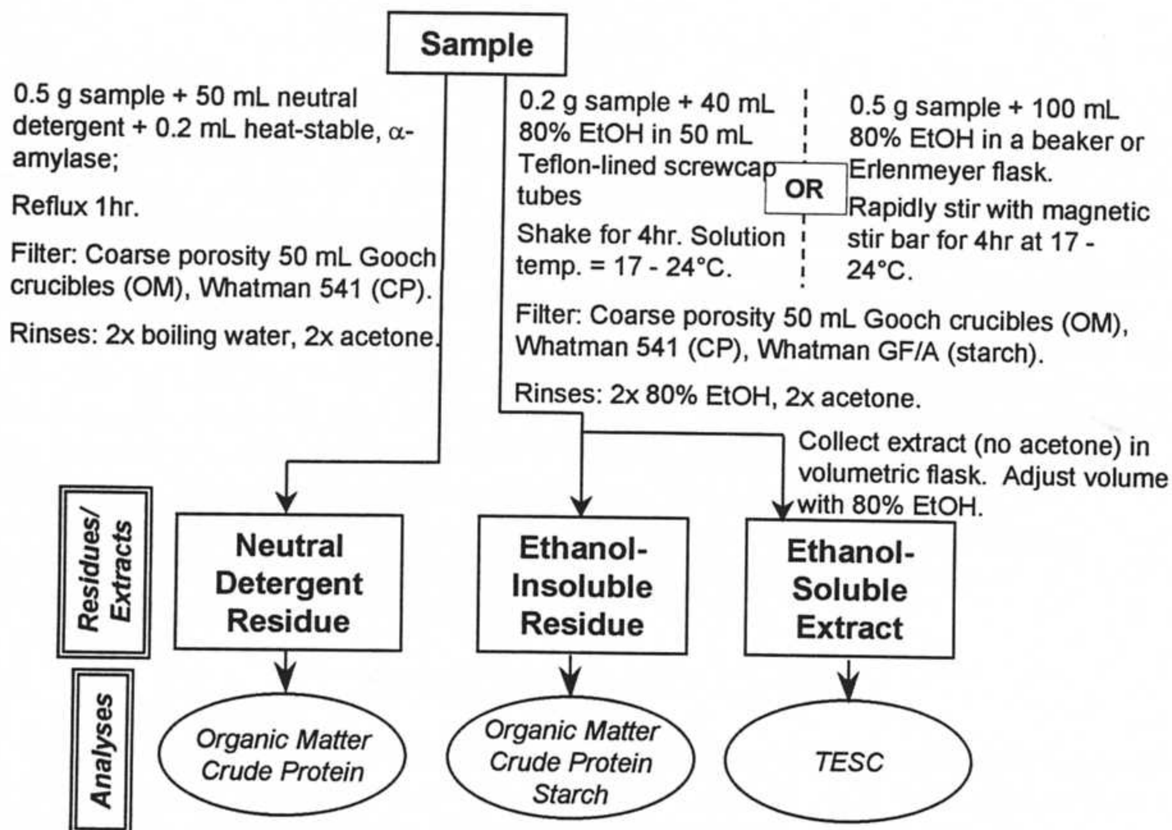
Abbreviations

A	Absorbance
ADF	Acid detergent fiber
CP	Crude protein
DM	Dry matter
dH_2O	Distilled water
EE	Ether extract
EIR	Ethanol-insoluble residue
EIRCP	Ethanol-insoluble residue crude protein
EIROM	Ethanol-insoluble residue organic matter
EtOH	Ethanol
80% EtOH	80:20 (v:v) ethanol:water solution
g	gram
GOP	Glucose oxidase-peroxidase reagent
L	liter
M	molar concentration (moles per liter)
μg	microgram
mL	milliliter
μL	microliter
NDF	Neutral detergent fiber
NDSC	Neutral detergent-soluble carbohydrates
NDSF	Neutral detergent-soluble fiber
NDR	Neutral detergent residue
NDROM	Neutral detergent residue organic matter
NDRCP	Neutral detergent residue crude protein
NFC	Non-fiber carbohydrates
nm	nanometers, for absorption wavelength
NSC	Non-structural carbohydrates
OM	Organic matter
TESC	Total 80% ethanol-soluble carbohydrates

Overview of Neutral Detergent-Soluble Carbohydrate Analysis

Analyze original sample for OM, ether extract, and CP.



Express all values as a percentage of the original sample's dry matter.

80:20 EtOH (v:v) (80% EtOH): 840 mL 95% ethanol + 160 mL high quality distilled water (dH2O).

Organic Matter (OM): Obtain dry weight of residue and Gooch crucible. Ash residue and crucible overnight at 512°C. Obtain dry weight of crucible and ash. OM = residue - ash

Crude Protein (CP): Analyze residue in Whatman 541 filter paper for total Kjeldahl nitrogen. CP = nitrogen x 6.25. Use a Whatman 541 filter paper blank in the analysis.

Total 80% Ethanol:Water-Soluble Carbohydrate (TESC): Analyze the 80% EtOH extract for total carbohydrates by phenol-sulfuric acid assay or other broad spectrum carbohydrate assay. Dilute the extracts 1 part extract:9 parts dH2O. Use the carbohydrate most likely to predominate in the extract as the assay's standard (sucrose, glucose, fructose, lactose, etc.).

Starch: Analyze the ethanol-insoluble residue for starch. Gelatinize the sample, hydrolyze the starch with amylases (α-amylase, amyloglucosidase), and measure released glucose. Preextraction with 80% EtOH removes low molecular weight carbohydrates that can interfere with the analysis.

Organic acids & Neutral detergent-soluble fiber (NDSF): These compositionally diverse fractions are calculated as the difference in mass among residues of known composition.

- Unextracted Sample: OM (organic matter), CP (crude protein), EE (ether extract)
- Ethanol-Insoluble Residue: EIROM (organic matter), EIRCP (crude protein), starch
- Neutral Detergent Residue: NDROM (organic matter), NDRCP (crude protein)

Calculations: **Organic Acids = (OM - CP) - (EIROM - EIRCP) - EE - TESC**

NDSF = (EIROM - EIRCP) - (NDROM - NDRCP) - Starch

Neutral Detergent-Soluble Carbohydrates

Nutritional Characteristics

Non-structural (NSC) or non-fiber (NFC) carbohydrates and neutral detergent fiber (NDF) comprise the two distinct classes of carbohydrates used in dairy ration formulation. Together, these two nutrient fractions can comprise more than 75% of ration dry matter, providing the majority of the energy that cattle derive from their diets. Neutral detergent fiber is commonly measured by chemical analysis. In contrast, the use of the terms "NSC" or "NFC" is often confused. The term NSC designates the non-structural or non-cell wall carbohydrates, and NFC, the non-NDF carbohydrates. However, NSC or NFC are often used interchangeably to describe a calculated value for the non-NDF carbohydrates. A single value does not adequately describe the compositionally and nutritionally diverse carbohydrates found in NSC. Our ability to predict how feeds will be digested and fermented by ruminants, and how rations may be formulated to support animal health and production depends upon our ability to accurately separate carbohydrate fractions based upon their digestion characteristics. An examination of their chemical and nutritional partitioning is in order.

Current Estimation of NFC

The calculated NFC value we use today is a direct descendant of the nitrogen-free extract (NFE) value calculated in the Weende system of proximate analysis, which was developed more than 100 years ago. The goals of both NFE and NFC were to obtain a crude estimate of the carbohydrate pool that differed in digestibility from crude fiber or NDF. As with NFE, the NFC content of feeds has been calculated rather than directly analyzed because of the many types of carbohydrates present in this fraction. The range of carbohydrates, combined with a lack of reliable methods for routine analysis prevented measurement and summing of individual NFC. Currently, the NFC content of feedstuffs is commonly calculated as:

$$NFC\% = 100\% - (CP\% + NDF\% + EE\% + Ash\%)$$

or

$$NFC\% = 100\% - [CP\% + (NDF\% - NDFCP\%) + EE\% + Ash\%]$$

where,
CP = crude protein,
NDF = neutral detergent fiber,
NDFCP = neutral detergent-insoluble crude protein, and
EE = ether extract (crude fat).

Although the first equation is most commonly used, the second equation is preferred because it corrects for CP in NDF (NDFCP) and avoids subtracting NDFCP twice (as part of CP and as NDFCP). Functionally, NFC include any carbohydrate soluble in neutral detergent.

Errors associated with each component in the equations, either technical errors or those inherent within the assay itself, shift the estimated NFC from its true value. Because it is calculated by difference, errors from each of the component analyses accumulate in NFC. In specific cases where NFC is underestimated because the mass of CP from non-protein nitrogen sources is overestimated, computing a more accurate NFC value may be possible (1). However, correcting for known errors in these equations still does not accurately describe the nutritional value of NFC.

Nutritional Characteristics of NFC

Carbohydrates soluble in neutral detergent are a very heterogeneous group, both compositionally and nutritionally. They are more accurately called neutral detergent-soluble carbohydrates (NDSC), rather than NSC or NFC, because they are measured on the basis of their solubility. The NDSC include both structural (cell wall) and non-structural (cell contents) carbohydrates, and fiber and non-fiber carbohydrates (Figures 1 & 2). "Fiber" in this case is defined nutritionally as carbohydrates not digestible by mammalian enzymes. The only carbohydrate linkages that mammalian enzymes hydrolyze are those in sucrose, starch, and lactose, leaving all other polymerized carbohydrates indigestible, except by microbes. Generally, NDSC are considered to be more rapidly and readily digested or fermented than NDF (6).

The carbohydrates, or carbohydrate derivatives in NDSC include organic acids, "sugars" (monosaccharides and oligosaccharides), starch, fructans, pectic substances (includes galactans), $(1\rightarrow3)(1\rightarrow4)$-β-glucans, and other carbohydrates of appropriate solubility (Table 1). Organic acids are not carbohydrates, but are often grouped with them for the purpose of describing feed components. Different NDSC tend to predominate in different feeds. Nutritionally, they may be partitioned in various ways (Figure 3). They can be divided based upon their digestion by the cow

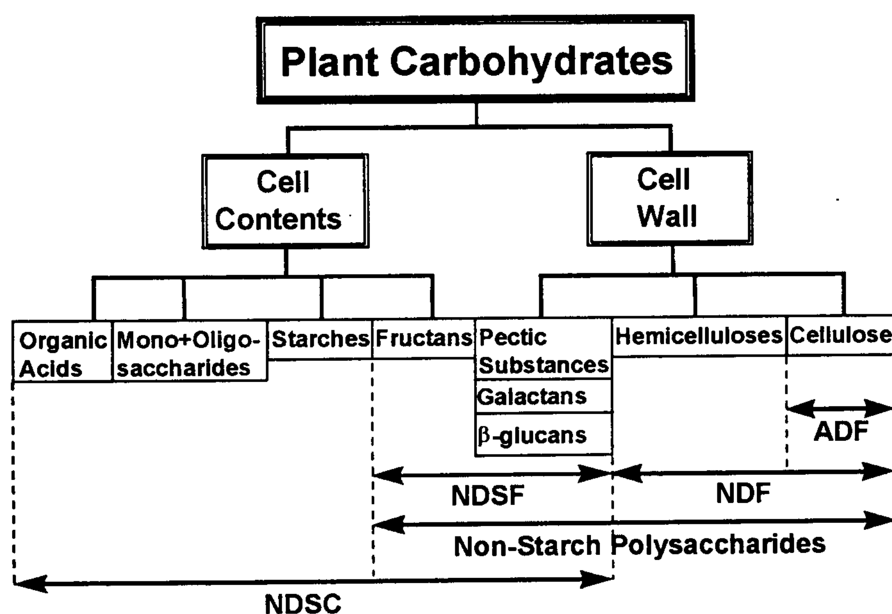


Figure 1. Plant carbohydrate fractions. ADF = acid detergent fiber, NDF = neutral detergent fiber, NDSC = neutral detergent-soluble carbohydrates, NDSF = neutral detergent-soluble fiber, Sugars = mono- and oligosaccharides. Lignin is present in ADF and NDF, but is not included here because it is not a carbohydrate.

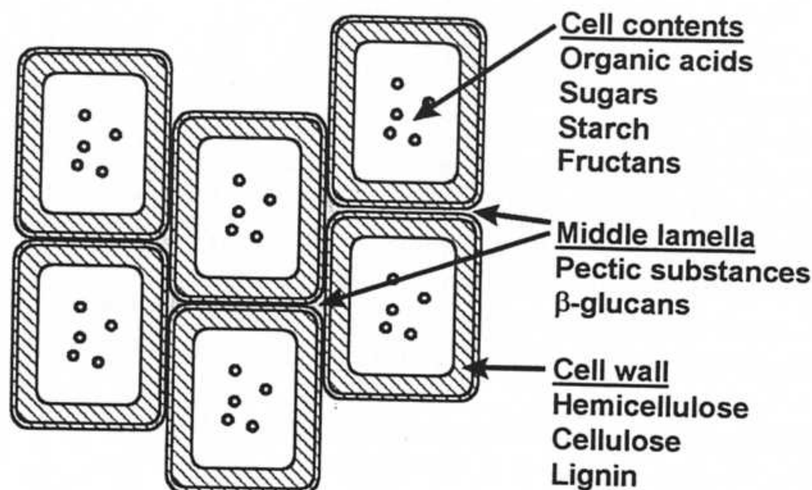


Figure 2. Primary locations of carbohydrates and lignin in plant cells.

or ruminal microbes, their ability to support microbial growth, their potential for fermentation to lactic acid in the rumen, and whether they suffer marked depression in fermentation at low rumen pH. The last two characteristics essentially define the NDSC by the types of microbes that ferment them. These microbes have been termed the "structural" and "non-structural" carbohydrate fermenting bacteria (4). When microbes ferment carbohydrates, they produce gases ($CO_2 + CH_4$), microbes, and organic acids such as acetate and propionate. Microbes tend to produce relatively more propionate when sugars and starches are fermented, or more acetate from pectic substances (5). Differences in fermentation products provide the animal with different profiles of metabolizable nutrients. The predominance of glucogenic (propionate) vs. lipogenic (acetate) nutrients may affect amount or composition of milk production and growth.

Based upon their digestion characteristics, NDSC should be partitioned into at least 4 fractions: organic acids, sugars (mono- and oligosaccharides), starch, and neutral detergent-soluble fiber (NDSF). Depending upon their digestion in, or passage from the rumen, fructans may be included with starch due to their potential to ferment to lactic acid, or with NDSF, because of the inability of mammals to digest them directly.

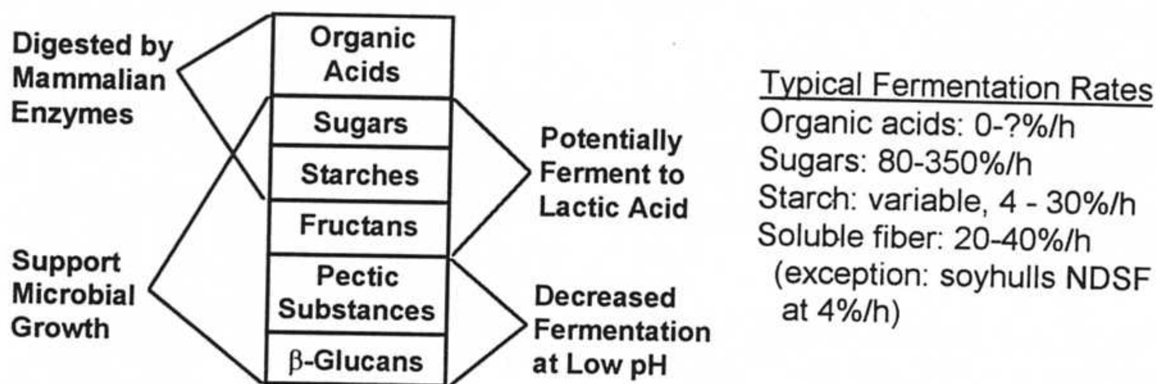


Figure 3. Nutritional characteristics of neutral detergent-soluble carbohydrates.

Analysis Methods

The challenges posed by NDSC analysis revolve around the analyses themselves, and arriving at values that are nutritionally relevant. Although detergent fiber analyses rely upon weighing of samples after extraction, soluble carbohydrate methods typically rely upon differential extractions and digestions with colorimetric analysis of filtrates (Table 1). The usefulness of some analyses is reduced by the requirement for sequential extractions, or by poor specificity (they measure carbohydrates other than those of interest). Assays vary in their ease of use, in their potential analytical accuracy, and in how well they accommodate other limitations (infrastructure, labor) of a laboratory. The following assays (2, 3) are offered as tools to partition NDSC.

See Appendix "Making Sense of Non-Structural Carbohydrates for further discussion of individual neutral detergent-soluble carbohydrates.

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Table 1. Neutral detergent-soluble carbohydrate composition, analysis methods & pitfalls, and sources.

Carbohydrate	Composition	Analysis method	Analytical Pitfalls	Common Sources
Organic acids Carboxylic acids	Diverse; acetate, propionate, lactate, malate, quinate, oxalate, shikimate, etc.	Extraction followed by chromatography (GC or HPLC), or various colorimetric analyses of extract.	Organic acids are sufficiently diverse as to preclude practical analysis for all acids.	Silage, plant materials.
Sugars Mono- and oligosaccharides	Glucose, fructose, and sucrose predominate in plants, lactose in milk. Raffinose, stachyose and others possible.	Extraction in water or aqueous ethanol followed by chromatography (GC or HPLC), or various colorimetric analyses of filtrate.	Colorimetric methods differ in their sensitivities for different sugars. The sugar used as a reference standard affects the accuracy of results.	Molasses, citrus pulp, almond hulls, sugar beet pulp, bakery waste, fresh forages or hays.
Starch Polysaccharide	Glucose in α-(1→4)- and α-(1→6)-linkages	Gelatinization, enzymatic hydrolysis, and measurement of released glucose. Starch = glucose x 0.9.	Contaminating enzymes in the amylase hydrolyze non-starch carbohydrates. Free glucose in the sample may be accounted as starch. Incomplete hydrolysis reduces starch estimate. Measurement of reducing sugars may include non-glucose monosaccharides.	Grain products, bakery waste, corn silage, potatoes.
Fructans or Fructosans Non-starch polysaccharides	Fructose with a reducing end glucose	Pre-extraction of sample with acetone or ethanol, followed by extraction with water, and analysis of second extract for fructose via colorimetric assay.	Multiple extractions required. Incomplete extraction.	Cool season temperate grasses, Jerusalem artichoke.
Pectic substances Non-starch polysaccharides	Diverse; galacturonic acid backbone with rhamnose inserts, arabinose (arabinan side chains), galactose (galactan side chains), etc.	Pre-extraction of sample with acetone or ethanol, followed by extraction with chelating agents, weak acids, weak alkali, or water. Second extract analyzed for carbohydrates (colorimetric or chromatographic analysis).	Multiple extractions required. Incomplete extraction. Neutral sugar interference in galacturonic acid assay. Requires uronic acid and neutral sugar analyses.	Citrus pulp, sugar beet pulp, soybean hulls, almond hulls, legume forages.
(1→3)(1→4)-β-Glucans Non-starch polysaccharide	Glucose in β-(1→3)- or β-(1→4)-linkages	Extraction with alkali followed by enzymatic hydrolysis of extract and measurement of glucose.	Incomplete extraction. Impure enzymes hydrolyze non-β-glucan carbohydrates.	Barley, oats, small grains, grasses.

Partitioning Neutral Detergent – Soluble Carbohydrates

The system for partitioning neutral detergent-soluble carbohydrates (NDSC) is designed to separate them into four nutritionally relevant fractions: 1) organic acids, 2) sugars, 3) starch, and 4) neutral detergent-soluble fiber (NDSF). The system follows the same logic as a method previously described for NDSF (2) which estimates a feed's content of a fraction by accounting for differences in mass between residues of known composition (Figure 1).

The NDSC system partitions carbohydrates by their solubility in 80% ethanol/water solution, or in neutral detergent with heat-stable α-amylase (Figures 1 and 2). The analysis of ethanol extracts and residues with colorimetric or enzymatic assays allows direct measurement of carbohydrate in the 80:20 ethanol:water (v:v) (80%) ethanol extract (TESC), and starch in the ethanol-insoluble residue (EIR). The organic acids and NDSF are estimated as the mass that is not protein, ash, or ether extract, and not accounted for by the TESC and starch analyses.

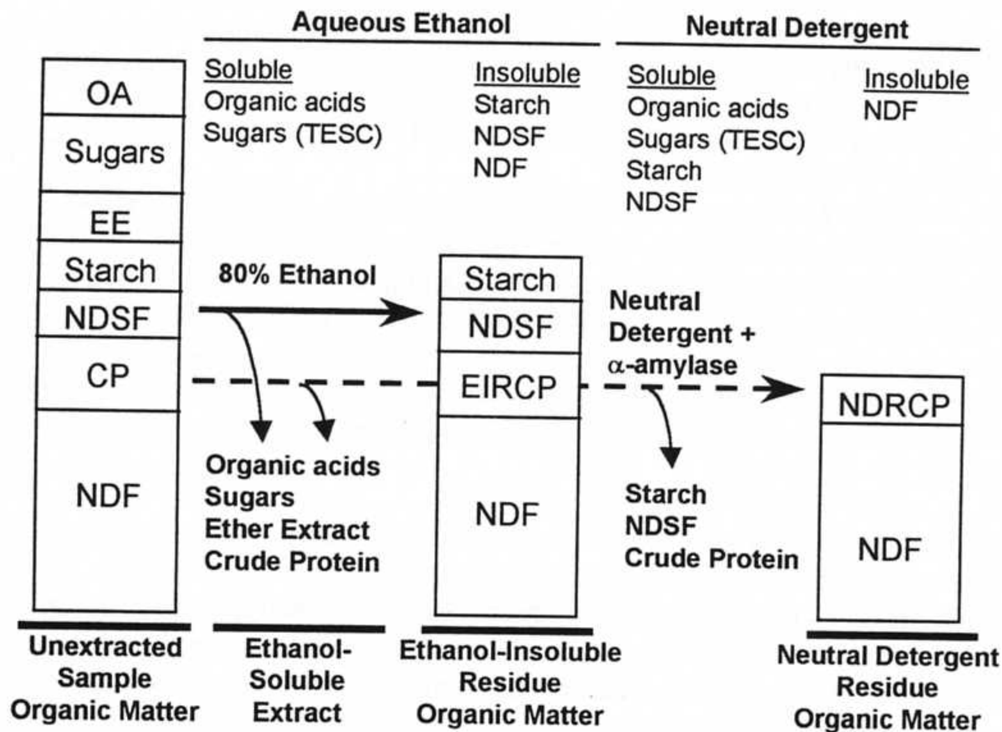

Figure 1. Composition of residues and extracted materials in the neutral detergent-soluble fiber estimation (Hall *et al.*, 1997, Hall et al., 1999). CP = crude protein, EE = ether extract, EIRCP = ethanol-insoluble residue crude protein, EIROM = ethanol-insoluble residue organic matter, NDF = neutral detergent fiber, NDRCP = neutral detergent residue crude protein, NDSF = neutral detergent-soluble fiber, OA = organic acids, Sugars = mono- and oligosaccharides = TESC (total 80% ethanol-soluble carbohydrates).

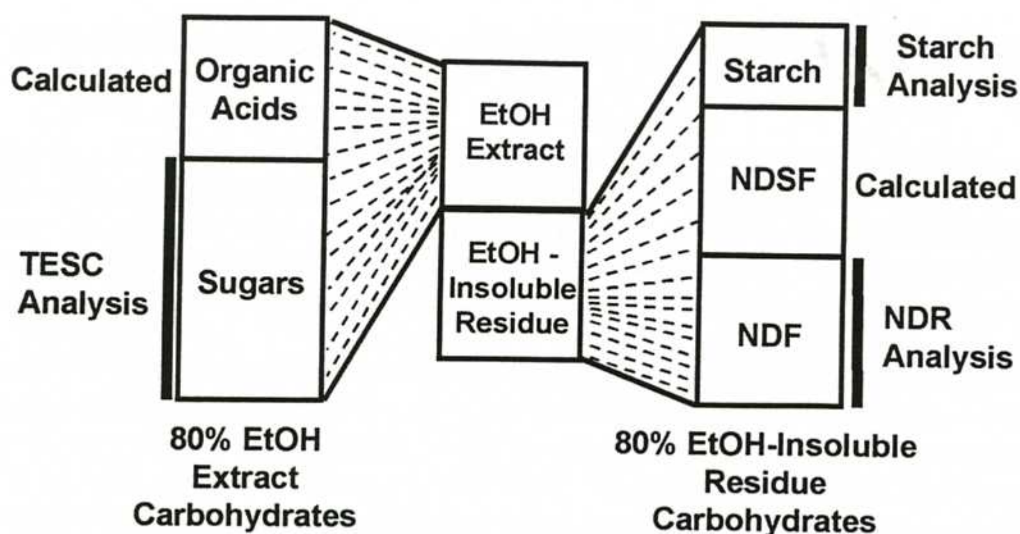

Figure 2. Partitioning NDSC with 80% ethanol, direct analyses, and calculated estimates. 80% ETOH = 80:20 ethanol:water (v:v), NDF = neutral detergent fiber, NDR = neutral detergent residue, NDSF = neutral detergent-soluble fiber, TESC = total 80% ethanol-soluble carbohydrates.

The NDSC system requires several analyses:

- Analysis of the original sample for organic matter (OM) and crude protein (CP)
- Extraction with 80% ethanol
  - Ethanol-Insoluble Residue analysis for OM (EIROM), CP (EIRCP), and starch
  - Ethanol-Soluble Extract analysis for total 80% ethanol-soluble carbohydrates (TESC)
- Extraction with neutral detergent and heat-stable α-amylase
  - Neutral Detergent Residue analysis for OM (NDROM) and CP (NDRCP)

All values are expressed on the basis of the original sample dry matter. All extraction and carbohydrate analyses are described previously in this publication, and presented in the schematic at the end of this section.

Organic acids and NDSF are the most heterogeneous fractions defined by the NDSC procedure. The organic acid pool may contain lactate, citric acid cycle components, and plant secondary compounds such as oxalate and shikimate. The volatile fatty acids are not included in this fraction because they are lost during drying of the sample. Fructans, pectic substances, β-glucans and other non-starch polysaccharides of the appropriate solubility are included in NDSF. Their compositional diversity rules out the use of simple procedures to directly measure them. In the NDSC partitioning system, their content in feeds is estimated as the difference in OM mass among extracted residues and original sample, with appropriate corrections for CP, EE, TESC, and starch. The NDSF is contained in the EIROM. It is calculated as :

NDSF = (EIROM – EIRCP) – (NDROM – NDRCP) – EIR Starch

Organic acids are soluble in aqueous ethanol, and are therefore included in the ethanol extract. The majority of the EE and some CP will be extracted, as well. (Pre-extraction with acetone of samples containing greater than 10% EE reduces the EE content of the EIROM. This does not appear to be necessary with samples lower in EE.) With sample OM minus EIROM setting boundaries on the basis of weight to the amount of dry matter in the ethanol extract, organic acids can be calculated as:

Organic Acids = (Sample OM – CP) – (EIROM –EIRCP) – EE – TESC

The estimation of these organic acid and NDSF fractions by difference makes them prone to the same errors suffered by NFE and NFC. It would seem likely that the error in NDSF is the comparatively smaller of the two. The majority of low molecular weight nitrogenous compounds that invalidates the use of a 6.25 factor for CP estimation are not present in the EIROM, because they are extracted into the ethanol extract. The lack of certainty that CP accurately defines the mass of protein in the extract, may make the organic acid estimate prone to error.

It appears that use of NDSC analyses on digesta or samples fermented in vitro will provide values for apparent rather than true digestibilities because of the NDSC content of microbes. Microbes can store a variety of hydrolyzed carbohydrates as α-glucan that analyzes as starch. Microbes also contain material that analyzes as NDSF. Two analyses done in our laboratory on mixed ruminal microbes isolated from continuous culture fermentations (supplied by M. Stern, University of Minnesota) indicate that microbes contain approximately 6 to 8% of dry matter as NDSF.

The proposed system for partitioning the neutral detergent-soluble carbohydrate pool is simple and has good precision. In this procedure, sugars and starches are measured directly, and the more compositionally diverse fractions, the organic acids and neutral detergent-soluble fiber, are estimated by difference. The NDSC system values are consistent with those previously reported for the different fractions (Figure 3a and b). The component methods do not require sequential extractions as is often the case in the direct analyses for NDSC components, uses common lab equipment (shaker, water baths, spectrophotometer, balance) and requires relatively safe, inexpensive reagents.

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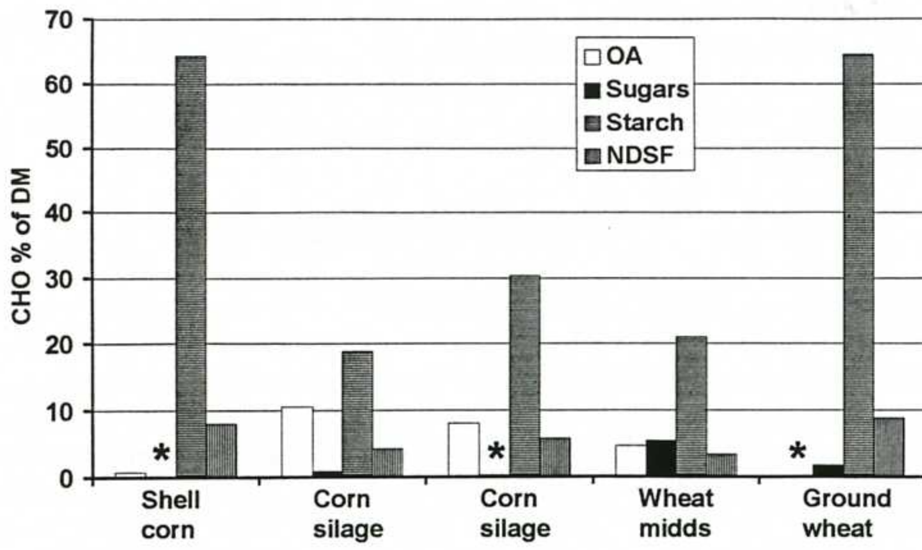
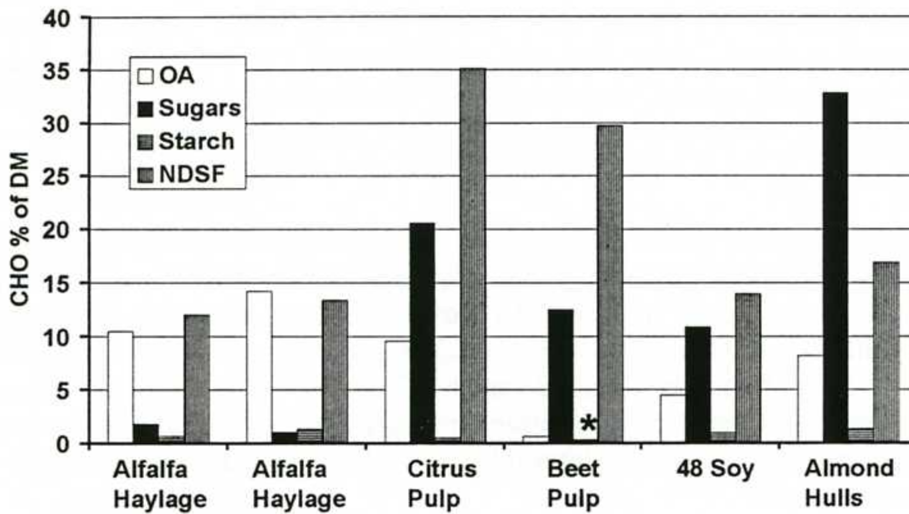
Hall, M.B., W.H. Hoover, J.P. Jennings, and T.K. Miller Webster. 1999. A method for partitioning neutral detergent-soluble carbohydrates. J. Sci. Food Agric. 79:2079.



* ND or trace.



* ND or trace.

Figures 3 a and b. Neutral detergent-soluble carbohydrate composition of feedstuffs as determined with the proposed NDSC partitioning system (Hall et al., 1999).

Feed and microbe composition values for analyses performed at the University of Florida through February, 2000. Values are presented as a percentage of sample dry matter.

Feed	Ash	CP	NDF	NDFCP	Organic Acids	Sugars	Starch	Soluble Fiber
Alfalfa Hay					6	8	3	14
Alfalfa hay, CV						12.1	1.7	
Alfalfa hay, CV						8.2	4.5	
Alfalfa hay, FL 4/99	9.8	21.0	37.8	4.4		5.8	1.9	16.8
Alfalfa hay, average						10	3	14-17
Alfalfa silage, WH	9.5	19.1	45.5	2.1	10.4	1.8	0.7	12.1
Alfalfa silage, WH	11.3	18.1	38.1	2.0	14.2	1.1	1.4	13.3
Alfalfa silage, CV						7.7	1.1	
Alfalfa silage, CV						7.3	4.9	
Alfalfa silage average					12	2	1	12.5
Alfalfa stem, mature	7.8	12.4	58.0	2.3	4.6	7.2	0.3	10.8
Alfalfa stem, immature	14.0	18.5	32.9	1.3			0.4	16.9
Alfalfa leaf mature	10.5	31.5	22.2	3.1			1.0	18.4
Alfalfa leaf, immature	9.2	29.3	18.6	1.6	9.1	10.2	3.4	19.4
Almond hulls, WH	5.0	7.1	26.0	1.2	8.2	32.8	1.4	16.9
Broccoli	8.8	30.4	14.3	0.5	5.0	17.7	0.7	18.9
Citrus pulp, FL 4/99	8.5	8.7	24.1	4.2		13.4	1.4	37.5
Citrus pulp, FL 4/99	8.5	8.2	24.4	4.2		18.4	1.6	34.5
***Citrus pulp, average*[1]**	6.7	7.2	22.1	2.9	9	26.5	1	32.9
Citrus pulp, ranges[1]	4.4-8.7	4.1-9.4	17.8-29.4	1.6 - 4.5		12.5-40.2		25.2-43.7
Corn distillers, ethanol						14.5	6.6	
Corn distillers, whiskey						6.2	4.2	
Corn distillers, FL 4/99	4.31	31.3	58.2	15.2		11.0	2.0	8.9
Corn distillers, FL 5/99	4.75	28.3	54.4	14.8		5.4	3.1	7.8
Corn gluten feed						5.9	16.4	
Corn grain, WH	1.5	9.0	12.6	0.7	0.7	0	64	8.1
Corn grain, CV						5.2	60.8	
Corn meal, FL 4/99	1.6	8.9	20.5	3.6		0	66.2	6.4
Corn meal, FL 5/99	3.3	9.0	15.7	3.8		4.5	55.9	10.6
Corn silage, WH	4.9	7.5	50.9	0.9	10.6	0.9	18.9	4.3
Corn silage, WH	3.8	7.0	41.8	0.6	7.9	0.3	30.4	5.8
Corn silage, CV						3.4	14.4	
Corn silage, CV						4.7	29.9	
Corn silage, FL 4/99	4.8	10.2	51.1	4.4		0.5	19.4	7.0
Corn silage, FL 5/99	3.8	10.4	51.1	3.4		4.6	23.6	3.2
Corn silage, average					8	2	14-30	5
Cottonseed, whole							1	8.5
Ctsd whole, FL 4/99	4.2	24.2	48.3	5.3		6.2	1.6	6.8
Ctsd whole, FL 5/99	4.1	23.3	47.6	5.6		5.9	0.8	10.8

Feed	Ash	CP	NDF	NDFCP	Organic Acids	Sugars	Starch	Soluble Fiber
Cottonseed hulls							< 1	4
Green peas (frozen)	3.2	25.9	18.2	0.4	1.7	25.0	20.6	2.4
Potatoes						4.8	57.5	
Soybean meal (48%)	6.5	52.7	10.9	1.4	4.2	10.9	1.0	14.0
SBM (48%) FL 4/99	7.3	56.9	14.4	8.6		11.9	2.4	18.8
SBM (48%) FL 5/99	7.0	56.3	16.0	8.5		11.6	2.1	14.0
Soybean hulls	4.2	9.8	69.0	4.0	< 1	< 1	1	17.4
Sugar beet pulp	8.9	8.0	44.6	5.1	0.4	12.8	0	30.0
Timothy hay	5.0	8.2	67.3	1.8	4.4	9.1	0.4	6.4
Ground wheat	1.7	10.9	12.1	1.1	0	1.8	64.6	8.8
Wheat middlings	5.5	19.0	42.3	3.4	4.6	5.4	21	3.4
Mixed rumen microbes[2]		46.2 - 46.9					8.8 - 7.6	6.0 - 8.5

[1] Results from analyses of 79 dried citrus pulp samples.

[2] Results from two analyses performed on mixed rumen microbes isolated from a continuous culture fermentation. Isolated microbes were provided by P. Ariza and M. Stern, University of Minnesota.

80% Ethanol Extraction for NDSF and Starch Analyses

I. Citation

Extraction of carbohydrates with aqueous ethanol has been in common use for decades. The procedure below is described in Hall, M.B., W.H. Hoover, J.P. Jennings, and T.K. Miller Webster. 1999. A method for partitioning neutral detergent-soluble carbohydrates. J. Sci. Food Agric. 79:2079.

II. Comments

Functionally, 78 - 80% EtOH is used to separate mono- and oligosaccharides from polysaccharides (Asp, 1993). Eighty percent ethanol (80% EtOH) extracts the low molecular weight carbohydrates leaving the polysaccharides in the residue.

Asp, N-G 1993. Nutritional importance and classification of food carbohydrates. In: *Plant Polymeric Carbohydrates*, eds Meuser F, Manners D J, & Seibel W. Royal Society of Chemistry, Cambridge, UK, pp 121 - 126.

III. Notes / Warnings

- Maintaining the solution temperature between 17 and 24°C is crucial. Higher or lower temperatures may cause more or less material to be extracted.
- Samples should be thoroughly and continuously agitated for best extraction.
- Use a wash bottle with a tip that produces a very fine stream to rinse glassware and sample with 80% EtOH.
- To quickly tell if an 80% EtOH solution was made properly, pour some of a known 80% EtOH solution into a clear, clean beaker. Using a transfer pipette, add the questionable solution dropwise to the known 80% EtOH and observe them closely. If no mixing is evident, the solutions are equivalent.
- Mark the 50 mL extraction tubes at the level of the 80% EtOH. This will offer a quick indicator of solution loss during the extraction due to cracked or loose screw caps.
- Take appropriate precautionary measures (safety glasses, gloves, lab coat) for working with the chemicals in this assay. Check the MSDS pages in your laboratory for further details.

IV. Sample Preparation

Dry moist samples in a 55°C forced air-oven to a constant weight. Grind dry samples through the 1 mm screen of a Wiley mill or UDI mill.

V. Reagents & Equipment

Reagents

95% ethanol, reagent grade
Distilled water (dH_2O)
Acetone, reagent grade

Equipment

50 mL screw-cap tubes w/ Teflon lined caps
Shaker or magnetic stir plate
Gooch crucibles, coarse porosity
Whatman 541filter paper
Whatman GF/A glass fiber filter paper

I. Reagent Preparation

80:20 Ethanol:water (80% EtOH)

1. Measure 840 mL 95% EtOH and 160 mL dH_2O with graduated cylinders.

2. Pour together in a flask and thoroughly mix. Volume contracts to provide ~ 990 mL 80% EtOH.

3. Store in a tightly capped non-reactive bottle at ambient temperature.

VII. 80% EtOH Extraction Procedure

1. Weigh dry, ground samples into extraction containers: 0.2 ± 0.01 g in 50 mL screw cap tubes (caps with Teflon liners), 0.5 ± 0.02 g in 250 mL Erlenmeyer flasks. One sample plus duplicate is required for each OM, EIRCP, and starch assay.

2. Add 80% EtOH to maintain a sample to solution ratio of 1.0 g : 200 mL. For example, 0.5 g samples require 100 ± 2 mL 80% EtOH, and 0.2 g samples require 40 ± 2 mL 80% EtOH.

3. Tightly cap vessels, and stir or shake for 4 h at room temperature (17 - 22°C).

4. a. Filter under vacuum through
   - 50 mL coarse porosity Gooch crucibles for OM determination
   - Whatman 54 or 541 filter paper for Kjeldahl N determination
   - Whatman GF/A glass fiber filter paper set in a Buchner funnel for starch analysis

   b. Capture extract from filtration under gravity or under vacuum in a volumetric flask for total 80% ethanol-soluble carbohydrate (TESC) assay of the extract.

5. Thoroughly rinse extraction vessel with 80% EtOH. Clean both vessel and cap (on tubes) with a rubber policeman to recover all residue.

Note: Vacuum may be on at start of filtration as the sample is poured into crucible or filter paper. The vacuum **must be** on at the start of filtration through GF/A. These samples filter very easily.

6. Rinse 2x with 80% EtOH.

7. Rinse samples 2x with acetone.

Note: DO NOT include acetone rinses in the extract reserved for TESC analysis.

8. Process samples as appropriate for subsequent organic matter, crude protein, or starch analyses.

Note: Stirring or shaking should be vigorous enough to keep the sample suspended in the solution for effective extraction. On a magnetic stir plate, the rpm needed for this will vary with the density of the sample. Make certain the agitation does not throw sample up on the walls of the flask. With 50 mL tubes, placing them lengthwise in a horizontal shaker allows the solution to

agitate the whole length of the tube. It's a good idea to mark the solution level on the side of the tube (held vertically) to verify that the tube did not leak during the extraction.

Stir plates generate heat. Placing a thin piece of styrofoam under the flask, and if necessary, directing a fan at the flask will help to hold the temperature within the desired range. Multiple place stir plates are helpful. Shakers can also generate heat, and may require a fan directed at the shaker to maintain the correct temperature. Solution temperature of at least one flask or tube should be taken immediately at the end of an extraction. Final temperature of solution: 17 - 24°C.

The EIR for starch and nitrogen analyses may be dried at (at 55°C) after extraction. Samples for starch analyses may be stored in the vessels in which they will be gelatinized.

Allow residues collected on filter paper to dry thoroughly before determining Kjeldahl nitrogen. With some Kjeldahl N procedures, the presence of acetone can generate artificially high nitrogen values.

Processing of 80% Ethanol-Insoluble Residues (EIR)

Organic matter analysis (EIROM). Use samples filtered through Gooch crucibles.

1. Dry the EIR+crucible and determine their dry weight according to an AOAC method.
2. Ash the sample by an AOAC procedure and determine the weight of ash+crucible.

Crude protein analysis (EIRCP). Use samples filtered through Whatman 541.

1. Determine nitrogen content of the EIR+filter paper. Do not subsample.
2. Use a filter paper plus Kjeldahl reagents as a blank.

Starch analysis. Use samples filtered through Whatman GF/A filter paper. Do not subsample.

Pre-extraction of lipids for EIR and EIROM

If a sample contains more than 10% of dry matter as lipid, pre-extraction with acetone is recommended. The pre-extraction will remove lipid that the 80% EtOH may not.

1. Weigh 0.5 g of air dry sample into a 100 mL beaker.

2. Add 50 mL reagent grade acetone to sample. Swirl once 5 minutes after addition. Allow to extract at ambient temperature (17 – 21°C).

3. After 20 min, filter sample under gravity through Whatman 541 hardened filter paper.

4. Quantitatively transfer all residues from the beaker to the filter paper using acetone rinses. Rinse residue and filter paper at least 2 x with acetone.

5. Air dry residue + filter paper overnight.

6. Carefully scrape residue from filter paper and transfer into a 250 mL Erlenmeyer flask or beaker. Take care to transfer all residue, but not the filter paper.

7. Proceed with 80% EtOH extraction using 100 mL 80% EtOH.

VIII. Calculations

Weights (Wt.) are expressed in grams.
All values are calculated as a percentage of the original sample dry matter.
"Sample Wt." is the amount of the original sample weighed out for the 80% ethanol extraction.
"Dry" indicates a dry matter weight.
DM% is the dry matter percent of the original sample.
In the development of the procedures, dry matters were determined after drying at 105°C overnight. Ashing was performed at 512°C for a minimum of 8 hours.

EIR Organic Matter (EIROM) % of Sample Dry Matter:
= [((Wt. of dry crucible + residue) - (Wt. of dry crucible + ash))/(Sample Wt. x DM%)] x 100

EIR Crude Protein (EIRCP) % of Sample Dry Matter:
= [(Nitrogen in EIR, g) x 6.25)/(Sample Wt. x DM%)] x 100

Analysis for Total 80% Ethanol-Soluble Carbohydrate (TESC) (mono- & oligosaccharides)

I. Citation

Dubois, M., K. A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350.

II. Comments

The phenol – sulfuric acid assay is a broad spectrum method for carbohydrates, measuring both mono- and polysaccharides. Functionally, 78 - 80% EtOH is used to separate mono- and oligosaccharides from polysaccharides (Asp, 1993). Eighty percent ethanol (80% EtOH) extracts the low molecular weight carbohydrates leaving the polysaccharides in the residue. By analyzing the 80% ethanol extract for total carbohydrate, the mono- and oligosaccharide content of the sample can be measured.

See Asp, N-G 1993. Nutritional importance and classification of food carbohydrates. In: *Plant Polymeric Carbohydrates*, eds Meuser F, Manners D J, & Seibel W. Royal Society of Chemistry, Cambridge, UK, pp 121 - 126.

III. Notes / Warnings

- For a standard, select a carbohydrate that is likely to predominate in the samples tested. Sucrose is a reasonable choice for most plant samples, lactose for samples containing milk products.
- 80% EtOH shows a greater change in volume with changing temperature than does water. Therefore, do the analyses at the same temperature at which the extracts were adjusted to volume.
- Thorough mixing is essential to the success of this assay. Pay attention to the mixing recommended in the method.
- It is crucial that the sulfuric acid be added in a consistent fashion to all phenol + sample solutions. A repipetter can be helpful in accomplishing this. Do not add the sulfuric acid by allowing it to flow down the side of the test tube. The rapid mixing of sulfuric acid with the other reagents is essential to the success of the assay.
- A standard curve must be run with each group of samples. Prepare standard stock solution and individual standard solutions fresh on the day they are to be used. Microbes may readily degrade sucrose, glucose, and other carbohydrates soluble in water solutions.
- This assay is sensitive to carbohydrate from all sources, including cellulosic lint which is nearly everywhere. Check that test tubes are free of lint or dust prior to starting the analysis.
- Sample extracts should be prepared from duplicate extractions, and the extracts and standards analyzed and read in triplicate because of the potential for contaminating carbohydrate (including cellulosic dust). Data from tubes with very high readings relative to the other replicates may be discarded (contamination suspected).
- Take appropriate precautionary measures (safety glasses, gloves, lab coat, saturated sodium bicarbonate solution) for working with the chemicals in this assay. Check the MSDS pages in your laboratory for further details.

- This colorimetric assay requires the use of acid resistant cuvettes. Acid resistant disposable cuvettes are available from Laboratory Products Sales (LPS), 1665 Buffalo Rd. Rochester, NY 14624, 800-388-0166. Catalogue # 2400.

IV. Sample Preparation

Samples: 80% EtOH extracts from 4 h extractions of samples at ambient temperature:

1. Filter extracts through Whatman 541 filter paper to remove any remaining fine particles.
2. Pipette 1 mL of filtered extract into a 10 mL volumetric flask.
3. Bring to 10 mL volume with dH_2O.

Comments on Sample Preparation

- Extracts for TESC analysis should not be pre-extracted with acetone – some monosaccharides are sparingly soluble in the acetone and will be lost.
- Do not include acetone rinses with extracts destined for TESC analysis.
- Extract the samples with 80% ethanol, and filter through Whatman 541 filter paper or through coarse porosity Gooch crucibles, collecting all filtrate in an appropriately sized volumetric flask (100 mL for extractions with 40 mL 80% EtOH, 250 mL for extractions with 100 mL 80% EtOH. Samples should be brought to volume after filtration with 80% EtOH.
- Use a wash bottle with a tip that produces a very fine stream to rinse glassware and sample with 80% EtOH. The fine stream allows better control over the amount of 80% EtOH used – you will be less likely to overfill the volumetric flask.
- Stopper the flasks and cover with Parafilm to reduce losses due to evaporation.
- Analyze samples within a few days of extraction – the sooner the better. Extended storage of the 80% EtOH extracts in plastic containers results in sample evaporation and erroneous values as the sugars become concentrated in the smaller volume.
- Dilute samples the day of the TESC analysis. Do not dilute them beforehand or microbes may consume some of the carbohydrate. The additional filtration is to assure that no fine particles of plant material contaminate the extract. The particles contain carbohydrates and will increase the TESC values.

V. Reagents and Equipment

Reagents

80% Ethanol
Phenol, solid, certified ACS
Sucrose, reagent grade
Sulfuric acid, concentrated, ACS

Equipment

Repipetter (0 - 5 or 10 mL)
Vortex
16 x 150 mm disposable borosilicate tubes
Acid resistant cuvettes for spectrophotometer
10 and 100 mL volumetric flasks
Spectrophotometer

VI. Reagent Preparation

1. **5% Phenol** (w/v):
   1. Weigh 10 grams of solid phenol certified ACS into a 200 mL volumetric flask.
   2. Add 150 mL of dH_2O.
   3. Swirl to bring phenol into solution; bring to 200 mL volume with dH_2O.
   4. Store in properly labeled amber bottle at 4°C.

2. **Sulfuric Acid ACS grade (concentrated H_2SO_4).** Most safely and accurately dispensed with a repipetter.

3. **Preparation of Standard Solutions**

Weigh 1.0000 grams of ACS grade sucrose (or other appropriate carbohydrate) and record weight to the fourth decimal. Transfer sucrose to a 100 mL volumetric flask. Add dH_2O, mix, and bring to volume with dH_2O. Calculate sucrose concentration in stock solution as:

Sucrose stock μg/mL = [(Sucrose g) x (DM% of Sucrose) x (1,000,000 μg/g)]/100 mL

Sucrose dry matter % is determined by the AOAC method.

<u>Prepare a serial dilution of the sucrose standard.</u>

1. Add 10 mL of 80% EtOH to each 100 mL flask.
2. Transfer aliquots of the sucrose standard solution to the 100 mL volumetric flasks.
3. Bring the flasks to 100mL volume with dH_2O.

Each of the standards will contain sucrose, 10 mL 80% EtOH and 90 mL dH_2O so that they contain the same proportion of ethanol as the samples (i.e., 1 = mL 80% EtOH in each 10 mL volumetric flask).

<u>Dilutions for Standard Solutions</u>

1. 0 μg/mL = 0 mL stock solution/100 mL dilution = blank.
2. ~25 μg/mL = 0.250 mL stock solution/100 mL dilution
3. ~50 μg/mL = 0.500 mL stock solution/100 mL dilution
4. ~75 μg/mL = 0.750 mL stock solution/100 mL dilution
5. ~100 μg/mL = 1.000 mL stock solution/100 mL dilution

VII. Phenol – Sulfuric Acid Procedure

Prepare samples and standards as follows (do in triplicate):

1. For each replicate, pipette 0.5 mL of sample into a 16 x 150 mm test tube.

2. Add 0.5 mL of 5% phenol.

3. Add 2.5 mL of concentrated H_2SO_4 -- do not let run down sides of tube (the reproducibility of this assay strongly depends upon the manner of and consistency in the addition of the acid -- see notes).

4. Vortex to mix. This is done to capture any sample and acid that splashed up on the walls of the test tube.

5. Vortex again after all samples are pipetted.

6. Cover test tubes with glass marbles. Place test tubes in rack in 30°C water bath for 20 minutes.

7. Remove samples from water bath, vortex, and allow to sit on the bench at ambient temperature for 30 minutes (this allows bubbles to clear after last vortexing and before reading the absorbance).

8. Read absorbance at $\lambda = 490$ nm in a spectrophotometer. Zero the spectrophotometer using the 0 μg/mL standard. Acid resistant cuvettes are needed.

Note: **Keep samples out of sunlight** until read in a spectrophotometer. Sunlight degrades the chromogen. Fluorescent lights in the lab do not appear to have the same effect.

VIII. Calculations

Standard Curve

1. Calculate to 4 decimals the concentration of sucrose in the standard solutions.

Sucrose µg/mL in standard solutions = [(Sucrose stock solution µg/ml) x Va] / Vs

where,

Va = aliquot volume of stock solution (0, 0.25, 0.50, 0.75, or 1.00 mL in above example)
Vs = the final dilution volume that the Va is diluted into (100 mL in above example)

Graph sucrose concentrations of the standard solutions (µg/mL) (y axis) against their absorbance values (A 490 nm) (x axis). Plot all three replicates for each standard. Calculate the regression line through the data and use the equation to calculate the TESC content of the samples. The R^2 of the regression line should be 0.96 or greater.
The regression line should take the form of:

Sucrose µg/mL = Value of slope x Absorbance + Value of the intercept

The line should be linear, and the intercept should cross the y axis close to 0.

Sample TESC Content

For the samples, calculate the average absorbance for the three replicates.
Using the regression equation, calculate the amount of sucrose or other carbohydrate (µg/mL) in the sample.
Correct for dilutions, and calculate TESC as a percentage of sample dry matter.

Example Calculations of TESC

Calculate the average absorbance of the three replicate tubes for a sample.
Using the regression equation, calculate the amount of carbohydrate (TESC) µg/mL in the sample aliquot.
Correct for dilutions, and calculate TESC as a percentage of sample dry matter.

Example
Standard curve regression: Sucrose µg/mL = 123.58 x Absorbance + 0.4843, R^2 = 0.9992
Sample weight: 0.1968 g
Sample DM%: 88.54%
Sample dilution volumes: 100 mL of 80% ethanol extract, and 1 mL of 80% ethanol extract diluted to 10 mL with the dH_2O.
Sample absorbance replicate values: 0.152, 0.152, 0.146

Absorbance: sample average
= [(0.152 + 0.152 + 0.146)/3] = 0.150 absorbance

Carbohydrate (TESC) (µg/mL) content of solution
= 123.58 x (0.150) + 0.4843 = 19.02 µg carbohydrate (TESC)/ mL sample solution

TESC µg in sample (dilution correction)
= (19.02 µg / mL) x (100 mL) x (10 mL / 1 mL) = 19,020 µg TESC in sample

Conversion of TESC µg to g
= 19,020 µg x (1 g/1,000,000 µg) = 0.0190 g TESC in sample

TESC% of Sample Dry Matter
= [(0.0190 g TESC) / (0.1968 g sample x 88.54%)] x 100
= [(0.0190 g TESC) / (0.1742 g sample dry matter)] x 100
= 10.91% TESC (dry matter basis)

Note: Generally speaking, do not round numbers until the final calculation is reached. Values above were rounded for ease in showing the example.

Starch Analysis: Overview

Most starch analyses are enzymatic, relying on the specificity of the enzymes to distinguish starch from other glucose-containing carbohydrates. The steps in a starch assay are generally gelatinization, hydrolysis, and measurement of end products. Critical elements in an accurate starch analysis are:

1) Complete gelatinization of starch.
2) Specificity and purity of enzymes.
3) Complete hydrolysis of starch to glucose.
4) Measurement of glucose produced from hydrolyzed starch.
5) Minimization of interference.

Gelatinization is the breaking of hydrogen bonds among and within starch molecules that opens the granules to hydration and enzymatic hydrolysis. Before gelatinization, starch, especially unprocessed starch, is partially crystalline. The linear portions of starch molecules are partially aligned and hydrogen bonded to each other in such a fashion that they exclude water and resist enzymatic activity. That crystalline structure must be disrupted for complete enzymatic hydrolysis of the starch to take place in a reasonable amount of time. Gelatinization is typically accomplished with heating (90 – 100°C) in water, or, alternatively, with use of a base (e.g., potassium hydroxide) followed by neutralization. Incomplete gelatinization can lead to incomplete hydrolysis of starch to glucose.

The enzymes must release glucose by specifically and completely hydrolyzing only the bonds between glucose molecules in starch. Since there are many glucose-containing carbohydrates in plants, specific hydrolysis of the α-(1→4) (linear chain) and α-(1→6) (branch) linkages in starch is required to make a method specific for starch (Figure 1). Heat-stable α-amylase, which can be added during the gelatinization step, and amyloglucosidase, which hydrolyzes starch to glucose, are commonly used. Care must be taken to ensure that a given enzyme is incubated at the correct pH and temperature to optimize its effectiveness. Since starch is estimated as the glucose released by enzymatic hydrolysis, the hydrolysis must be complete, or starch content will be underestimated. Presence of other enzymes such as invertase (digests sucrose), or cellulase (digests cellulose) that release glucose through hydrolysis will inflate the calculated starch value.

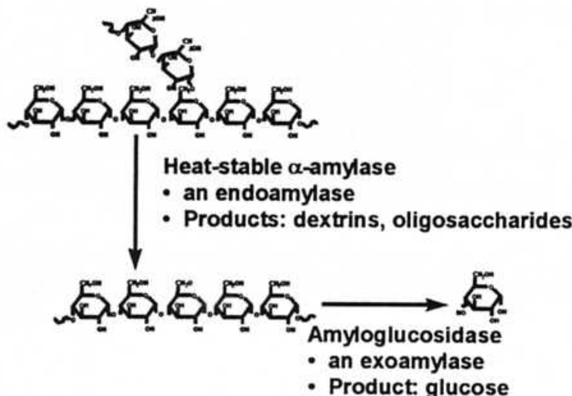


Figure 1. Enzymic action on starch.

Measurement of the glucose from starch hydrolysis is the final step for calculation of a starch value. Starch is computed as glucose times 0.9, because the total weight of glucose released is higher than the weight of the starch. One molecule of water (M.W. = 18) is required for every covalent bond hydrolyzed, which adds the weight of water used for hydrolysis to the weight of the starch in the sample. Commonly, glucose is measured with a glucose-specific assay such as the glucose oxidase-peroxidase assay (Karkalas, 1985). Although either purified starch carried through the gelatinization and hydrolysis procedures or glucose may be used as standards for the end product assay, glucose is preferred. Use of starch as the standard relies upon its complete hydrolysis to glucose, and presumes similar recoveries for starch from all sources. Use of glucose as a standard removes the question of percent recovery. Including starch as a reference sample in starch analyses allows assessment of recovery and enzyme efficacy.

An alternative method for measuring glucose resulting from starch hydrolysis is the reducing sugar assay. This second approach carries a greater risk of including monosaccharides not derived from starch. Such sugars may be present in the sample as mono- or disaccharides, or released by hydrolysis of non-starch carbohydrates. Particularly when the enzyme preparations used contain invertase, fructanase, or other contaminating enzymes, simultaneous measurement of hydrolysis products from sucrose, fructans, and other carbohydrates can significantly inflate starch estimates.

Interfering substances include any substance that increases or decreases the starch estimate. The method of glucose measurement used, either for glucose or reducing sugars, determines what substances are measured. Some commercial amyloglucosidase preparations contain glucose and should not be used for starch analysis. Non-carbohydrate substances that absorb at the appropriate wavelength in colorimetric analyses can unduly alter starch values. Interference from low molecular weight carbohydrates can be eliminated by pre-extracting them with 80% ethanol:water (v:v) before starch analysis. The effect of glucose, sucrose, and oligosaccharides on starch analysis values can be seen in figures 3, 6, 8, 9, and 11. In this comparison of laboratories, starch values from samples such as confectioners sugar, starch+glucose, soybean meal, and citrus pulp were inflated by the inclusion of the nonstarch carbohydrates in the starch value. Samples from the UF lab were extracted with 90% EtOH prior to starch analysis. Another method of accounting for free glucose or reducing sugars, is to measure a sample blank untreated with enzymes. This approach requires that the enzymes used be of sufficient purity so that they do not hydrolyze non-starch carbohydrates to any appreciable extent and thereby add to the free monosaccharide pool. The extent to which one should be concerned about interfering substances will depend upon the type of sample. Mature grain samples and silage samples will likely have little sugar remaining to interfere with starch analysis. Interfering carbohydrates may be an issue in by-product feeds such as bakery waste, almond hulls, and citrus pulp.

References

Hall, M. B. 1998. Analyzing nonstructural carbohydrates: methods and relevance. Proc. National Forage Testing Association Workshop, Midwest Section AOAC International Meeting, June 8 – 10, 1998, Madison, WI.

Karkalas, J.J. 1985. An improved enzymatic method for the determination of native and modified starch. J. Sci. Food Agric. 36:1016

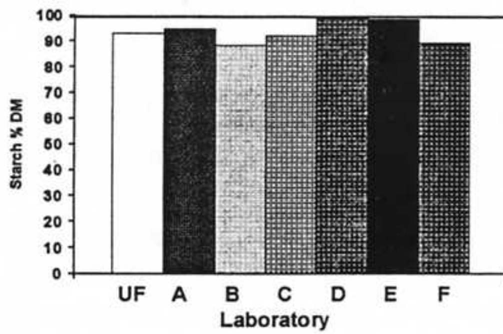


Figure 2. Starch assay of corn starch.

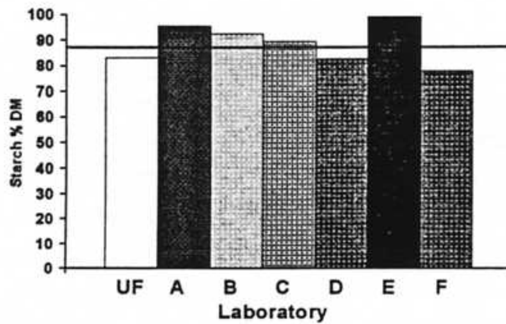


Figure 3. Starch assay of corn starch+glucose.

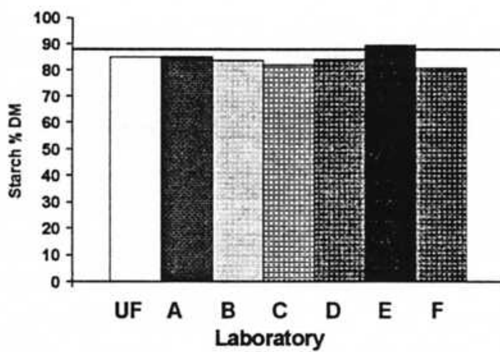


Figure 4. Starch assay of corn starch+fructose.

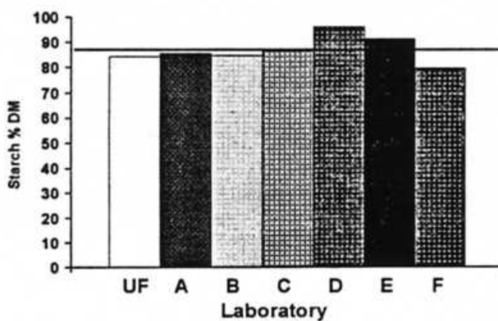


Figure 5. Starch assay of corn starch+cellobiose.

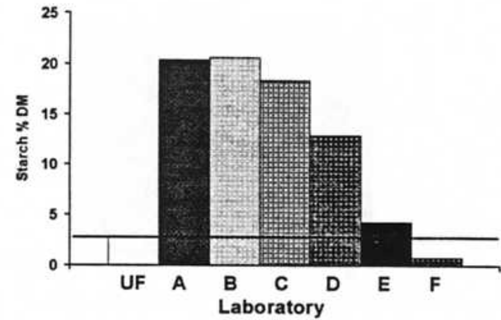


Figure 6. Starch assay of confectioner's sugar.

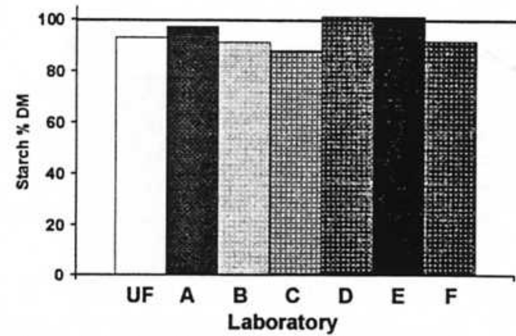


Figure 7. Starch assay of potato starch.

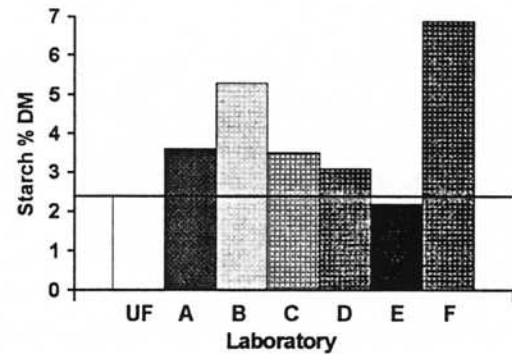


Figure 8. Starch assay of 48% soybean meal.

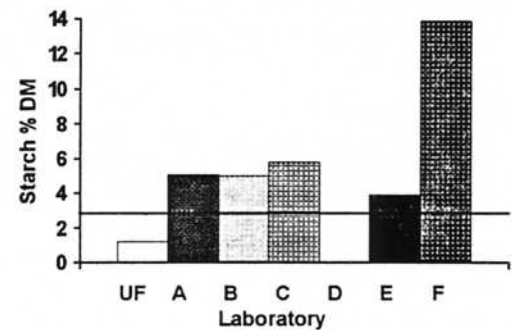


Figure 9. Starch assay of citrus pulp.

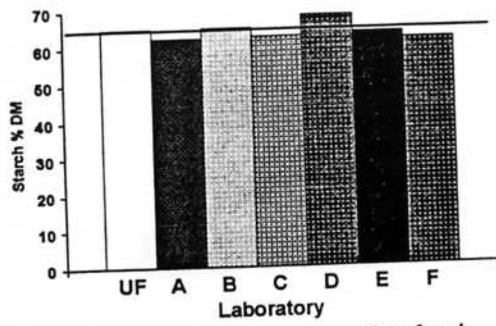


Figure 10. Starch assay of hominy feed.

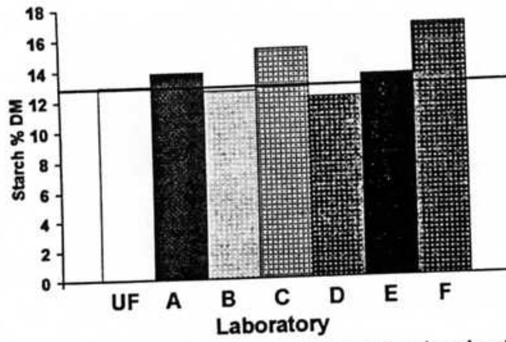


Figure 11. Starch assay of a total mixed ration containing citrus pulp.

Starch Gelatinization & Hydrolysis Method

I. Citation

This procedure is a modification of Holm, J., I. Björck, A. Drews, and N.-G. Asp. 1986. A rapid method for the analysis of starch. Starch/die starke 7:224-226.

II. Comments

This simple method for gelatinizing and hydrolyzing starch gives a similar recovery (starch measured/actual starch) as gelatinization with potassium hydroxide for both unextracted and 80% ethanol extracted samples. We have typically obtained recoveries of 97% on corn starch samples using this method.

III. Notes / Warnings

- Once begun, all steps in starch analysis should be completed without delay (from gelatinization through reading of the glucose concentration) on the same day. This typically means setting aside a 6 hour day, depending upon the number of samples.
- Do not use the commercial preparation of amyloglucosidase used for total dietary fiber analysis – it typically contains glucose.
- The α-amylase preparations may contain any of a variety of contaminants including invertase (sucrase). This enzyme will release glucose from sucrose, inflating the measured starch value. Analyze a sample such as sucrose or confectioners' sugar (contains ~3% starch, and 97% sucrose) with the starch method to determine whether the enzymes used will hydrolyze sucrose.
- Some samples contain significant amounts of low molecular weight carbohydrates, such as sucrose or glucose, that may interfere (non-starch materials that may analyze as glucose). Pre-extraction with 80% ethanol for 4 hours at room temperature (17 – 24 °C) before starch analysis of the residue should largely remove the problem (see 80% ethanol extraction instructions).
- If the sample solution destined for glucose analysis in step 10 is cloudy or contains fine particulate matter, the absorbance in colorimetric assays will be inappropriately increased. The solution should be clear. Take an aliquot of this solution and filter it through filter paper, or alternatively (preferably), centrifuge the solution to provide the aliquots for glucose analysis. Microcentrifugation of 1 – 2 mL works well for this.
- Take appropriate precautionary measures (safety glasses, gloves, lab coat) for working with the chemicals in this assay. Check the MSDS pages in your laboratory for further details.

IV. Sample Preparation

Dry moist samples in a 55°C forced air-oven to a constant weight.
Grind dry samples through the 1 mm screen of a Wiley mill or UDI mill.

V. Reagents and Equipment

Reagents

Heat-stable α-amylase, (Termamyl 120L, Novo Nordisk Biochem, 77 Perry Chapel Church Rd., Box 576, Franklinton, NC 27525-0576; 919-496-3038), or A-3306 α-amylase, Sigma-Aldrich Company, St. Louis, MO)

0.1 M sodium acetate buffer, (pH ~ 4.5) (see attached sheet on preparation)

Amyloglucosidase, (Sigma, A-3514 from A. niger in ammonium sulfate. Sigma-Aldrich Co.)

Equipment

Water baths (90°C and 60°C)
Funnels
100 mL beakers
Glass wool
50 and 100 mL volumetric flasks

VI. Reagent Preparation

See attached sheet on preparation of sodium acetate buffer.

VII. Starch Gelatinization & Hydrolysis Procedure

1. Accurately weigh 75 – 100 mg samples (weigh to the nearest 0.001 g, record weight to 0.0001g) in duplicate into 100 mL beakers. Samples should contain up to 100 mg starch. Alternatively, filter 80% EtOH extracted 0.2 g samples through Whatman GF/A (70 mm diameter) and transfer entire filter paper to beaker. Besides samples, include an empty beaker to which all reagents will be added that will act as your reagent blank.

2. Add 20 mL of high quality dH_2O to the sample and stir with a magnetic stir bar.

3. Add 0.1 mL heat-stable α-amylase to sample and water and stir with a magnetic stir bar.

4. Cover beaker with aluminum foil and place in a 92 - 93°C water bath for 1 hour. Remove beakers from water bath and cool on bench for 15 minutes.

5. Filter samples through glass wool plugs in funnels into 100 mL volumetric flasks. Rinse beakers, then funnels and glass wool thoroughly with dH_2O. Adjust filtered solutions to volume with dH_2O. Mix solutions thoroughly through repeated inversion and shaking of capped or stoppered flask.

   Note: If filtering through the glass wool plug becomes difficult, the plug may be too tightly packed into the neck of the funnel. Take a clean probe and use it to carefully draw the plug out of the funnel neck just far enough to allow more rapid filtering, but without allowing free passage of all material. Rinse the probe into the funnel with dH_2O.

6. Pipette a 1 mL aliquot of each sample into individual 50 mL volumetric flasks.

   Note: The size of volumetric can be varied depending upon the concentration of starch in the sample so that the glucose concentration falls within the range of the standard curve.

7. Add 8 mL of 0.1 M sodium acetate buffer (pH ~ 4.5) to each flask

8. Add 50 µL of amyloglucosidase. Gently swirl flask to mix.

9. Incubate flasks in 60°C water bath for 30 minutes, gently swirling every 10 minutes.

10. Bring samples to volume with dH_2O.

11. Assay the hydrolyzed sample for glucose to determine starch content.

Preparing Sodium Acetate Buffer for Starch Analysis

0.1 M Sodium Acetate buffer Adjusted to pH 4.5

Reagents

Sodium acetate trihydrate, FW = 136.1 (if anhydrous sodium acetate is used, adjust weight for different MW)
Hydrochloric acid (HCl), both concentrated and dilute
Dilute sodium hydroxide (NaOH) solution
High quality distilled water (dH_2O)

Equipment

A 4 place (after decimal) balance	pH meter (calibrated between pH 7.0 and 4.0)
Beaker (100 mL)	1 liter graduated cylinder
Magnetic stir bar	Transfer pipettes for addition of acid or base
Magnetic stir plate	1 liter plastic or glass sealable container

Calculations:

Na acetate g needed for desired amount of buffer =

(X g Na acetate /(136.1 g/mole)) x (0.1 mole/liter) x (# liters)

eg. to make 750 mL of 0.1 M buffer with Na acetate trihydrate (136.1 g/mole)

(136.1 g/mole) x (0.1 mole/liter) x (0.75 liters) = 10.21 g of Na acetate trihydrate

For 1 liter of buffer:

1. Weigh 13.61 g of sodium acetate trihydrate into a glass beaker (100 mL).
2. Add a magnetic stir bar and enough distilled water (30-40 mL) to dissolve sodium acetate with stirring (no heat).
3. After calibrating the pH meter, rinse the electrode with dH_2O and check the pH of the sodium acetate solution.
4. Carefully add concentrated HCl dropwise to adjust the pH of the solution to 4.5 to 4.6 while stirring on a stir plate. When the solution comes close to the desired pH, dropwise addition of dilute HCl may be used to carefully adjust pH. If pH is too acidic, use dilute sodium hydroxide to raise pH to desired level. Avoid excessive correcting with acid and base.
5. When the desired pH is achieved, rinse the electrode into the beaker, and carefully pour the solution into a 1 liter graduated cylinder. Rinse the beaker repeatedly into the cylinder to assure transfer of all chemicals.
6. Add dH_2O to adjust the solution volume to 1 liter.
7. Store in a plastic or glass container and label with the date, name of solution, (0.1 M sodium acetate buffer), and pH.
8. Recheck the pH of the buffer before use, particularly if it is stored for an extended period. Acetate can degrade (microbial action) which will cause changes in the buffering capacity of the solution.

Glucose Oxidase – Peroxidase Reagent and Glucose Analysis

I. Citation

Karkalas, J.J. 1985. An improved enzymatic method for the determination of native and modified starch. J. Sci. Food Agric. 36:1016.

II. Comments

This assay is specific for glucose and is appropriate for starch analysis.

III. Notes/Warnings

- With each new batch of enzyme purchased, check the bottle for the units/g of solid. Adjust the amount of enzyme used to provide the desired number of units.
- Remove the glucose oxidase and peroxidase from the freezer and allow the bottles to sit on the bench to equilibrate to room temperature while the other materials are being weighed. If containers are opened while cold, moisture will condense on the enzymes.
- Prepare the standard glucose solution fresh on the day of analysis. Glucose is readily degraded by microorganisms.
- A standard curve is run when the GOP is first made and the same curve can be used for subsequent runs.
- As the GOP reagent ages, its absorbance increases. Check this in each run by measuring the absorbance of the zero glucose blank after zeroing the spectrophotometer against dH_2O. It may be wise to run new standard curve towards the end of the GOP reagent's month life.
- Take appropriate precautionary measures (safety glasses, gloves, lab coat) for working with the chemicals in this assay. Check the MSDS pages in your laboratory for further details.

IV. Sample Preparation

For starch analysis, the samples are the hydrolysates from starch gelatinization and hydrolysis.

V. Reagents and Equipment

Reagents

Glucose oxidase-peroxidase reagent, see table in section VI.
Glucose, ACS reagent grade
Distilled water (dH_2O)

Equipment

Vortex
100 mL volumetric flasks
16 x 150 mm test tubes
Parafilm or stoppers
Water bath (35 °C)
Spectrophotometer

VI. Reagent Preparation

(Reagent information after version in B.A. Lewis lab, Cornell Univ.)

Amount of GOP	500 mL	1 L	2 L
Sodium phosphate, dibasic andhydrous Na_2HPO_4, FW = 141.96	4.55 g	9.1 g	18.2 g
Potassium phosphate, monobasic KH_2PO_4, FW = 136.09	2.5 g	5.0 g	10.0 g
Phenol, solid loose, crystals, ACS C_6H_5OH	0.5 g	1.0 g	2.0 g
4-Aminoantipyrine, desiccated @ room temp. (Sigma, A-4382) $C_{11}H_{13}N_3O$, FW = 203.2	0.075 g	0.150 g	0.300 g
Glucose oxidase, desiccated @ -20°C	3,500 units	7,000 units	14,000 units
Sigma, G-6125 23,900 units/g solid	0.1464 g	0.30 g	0.60 g
Sigma G-7016 165,000 units/g solid	0.0212 g	0.0424 g	0.0848 g
Peroxidase, desiccated @ -20°C	3,500 units	7,000 units	14,000 units
Sigma, P-8125 96 purpurogallin units/mg solid	0.036 g	0.073 g	0.146 g

1. Add about 1/3 the volume of high quality distilled water (dH_2O) to the appropriate volumetric flask. Weigh and transfer to the flask the sodium phosphate dibasic and potassium phosphate monobasic, swirling to dissolve. Mix until the chemicals are completely dissolved.

2. Weigh and add phenol and 4-aminoantipyrine (this is light sensitive) and dissolve.

3. Weigh and add the glucose oxidase and peroxidase and dissolve with gentle mixing.

4. Bring the solution to volume, mix and filter through Whatman GF/A microfiber glass filter paper into an amber bottle.

5. Store at 4°C. GOP reagent can be stored for ~ 1 month.

Preparation of Glucose Standard Solutions

Preparation of Stock Solution

1. Weigh 1.0000 grams of ACS grade glucose and record weight to the fourth decimal.
2. Transfer glucose to a 100 mL volumetric flask.
3. Add dH_2O, dissolve, and adjust to volume with dH_2O.
4. Calculate glucose concentration in stock solution as:

Glucose stock µg/mL = [(Glucose g) x (DM% of Glucose) x (1,000,000 µg/g)]/100 mL

Glucose dry matter % is determined by the AOAC method.

Note: Unless stock solution is prepared with saturated benzoic acid solution and stored at cool temperatures (4°C), it should be used the day it is made. Microbes devour glucose and will reduce the glucose content of the unpreserved solution during storage.

Prepare a serial dilution of the glucose standard.

1. Transfer aliquots of the glucose standard solution to the 100 mL volumetric flasks.
2. Bring the flasks to 100mL volume with dH_2O.

Dilutions for Glucose Standard Solutions

1. 0 µg/mL = dH_2O = standard blank (as opposed to reagent blank)
2. 25 µg/mL = 0.250 mL stock solution/100 mL dilution
3. 50 µg/mL = 0.500 mL stock solution/100 mL dilution
4. 75 µg/mL = 0.750 mL stock solution/100 mL dilution
5. 100 µg/mL = 1.000 mL stock solution/100 mL dilution

VII. GOP Procedure for Glucose Analysis

Note: Including a reagent blank from the starch gelatinization procedure allows samples to be corrected for the absorbance attributable to the enzymes, acetate buffer, and dH_2O.

1. Pipette 1.0 mL aliquots of dH_2O (0 µg glucose/mL = blank), sample, or standard solutions in duplicate into 15 mm glass tubes (16 mm x 150 mm works well). Samples should contain between 10 and 100 µg glucose/mL to fall within the standard curve. Standard curves up to 160 µg glucose/mL are possible. The dH_2O is used as a blank with each run.

Note:_ Reduction of aliquot size to 0.5 mL and GOP to 2.5 mL provides the same analytical values as the volumes in the original procedure.

2. Dispense 5 mL (or 2.5 mL, see note above) GOP reagent into each tube. Vortex.

3. Place tubes in a rack. Cover tops of tubes with a single sheet of Parafilm, or stopper each tube.

4. Place tubes in a 35 – 40°C water bath for 45 minutes.

5. Cool tubes to room temperature for 10 min in the dark.

6. Absorbance is measured at $\lambda = 505$ nm. Karkalas notes that absorbance readings were taken within 30 min. The spectrophotometer should be zeroed using dH_2O, and the blank tube (0µg/mL standard solution) read against this zero (this is done to check for age related changes in the GOP reagent; see p.30). Next, zero the spectrophotometer with the blank tube and read the samples.

VIII. Calculations

Standard Curve

1. Calculate to 4 decimals the concentration of glucose in the standard solutions.

   Glucose μg/mL = [(Glucose stock solution, μg/ml) x Va] / Vs

   where,

   Va = aliquot volume of stock solution (0, 0.25, 0.50, 0.75, or 1.00 mL in above example)
   Vs = the final dilution volume that the Va is diluted into (100 mL in above example)

   Graph glucose concentrations of the standard solutions (μg/mL) (y axis) against their absorbance values (A 505 nm) (x axis). Plot all three replicates for each standard. Calculate the regression line through the data and use the equation to calculate the glucose content of the samples. The R^2 of the regression line should be 0.96 or greater. An R^2 of 1.0 is possible with this assay.
   The regression line should take the form of:

   Glucose μg/mL = Value of slope x Absorbance + Value of the intercept

   The line should be linear, and the intercept should cross the y axis close to 0.

Sample Starch Content

For the samples, calculate the average absorbance for the three replicates.
Using the regression equation, calculate the amount of glucose (μg/mL) in the sample.
Correct for dilutions, and calculate glucose * 0.9 = starch as a percentage of sample dry matter.

Example Glucose/Starch Calculations

Take the average absorbance of the two replicate tubes and subtract the absorbance value of the reagent blank. The reagent blank contains all chemicals and enzymes from the gelatinization and hydrolysis procedures.
Using the regression equation, calculate the amount of glucose µg/mL in the sample.
Correct for dilutions, and calculate glucose as a percentage of sample dry matter.
Starch content is calculated as 0.9 x glucose content.

Example

Standard curve regression: Glucose µg/mL = 169.83 x Absorbance + 0.364, $R^2 = 0.9999$
Sample weight: 0.1968 g
Sample DM%: 88.54%
Sample dilution volumes: 100 mL after gelatinization, and 1 mL sample diluted to 50 mL after amyloglucosidase step.
Duplicate sample absorbance values: 0.144, 0.146
Reagent blank absorbance: 0.001

Absorbance: sample average – reagent blank
= [(0.144 + 0.146)/2] – 0.001
= 0.145 – 0.001 = 0.144 absorbance

Glucose (µg/mL) content of solution
= 169.83 x (0.144) + 0.364 = 24.82 µg glucose / mL sample solution

Glucose µg in sample (dilution correction)
= (24.82 µg / mL) x (100 mL) x (50 mL / 1 mL) = 124,100 µg glucose in sample

Conversion of Glucose µg to g
= 124,100 µg x (1 g/1,000,000 µg) = 0.1241 g glucose in sample

Conversion of Glucose to Starch
= 0.1241 g glucose x 0.9 = 0.1117 g starch in sample

Starch % of Sample Dry Matter
= [(0.1117 g starch) / (0.1968 g sample x 88.54%)] x 100
= [(0.1117 g starch) / (0.1742 g sample dry matter)] x 100
= 64.12% starch (dry matter basis)

Note: Generally speaking, do not round numbers until the final answer is reached. Values above were rounded for ease in showing the example.

Neutral Detergent Residue Method

I. Citations

Goering, H. K. and P. J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). Agriculture Handbook No. 379. Agricultural Research Service, United States Department of Agriculture, Washington, DC.

Van Soest, P. J. and J. B Robertson. 1985. Analysis of forages and fibrous foods: a laboratory manual for Animal Science 613. Cornell University.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583.

II.Comments

There are many variations on the method for preparing neutral detergent residue for organic matter and crude protein determinations. In the version used for NDSC partitioning, only the use of a heat-stable α-amylase has been investigated.

III. Notes / Warnings

- The use of heat-stable α-amylase is essential to assure the removal of starch from the neutral detergent residue (NDR).
- For high starch feeds such as corn meal, addition of another 0.1 to 0.2 mL of α-amylase late during the hour of refluxing, or during filtration may be necessary to further hydrolyze starch and increase ease of filtration.
- Samples should be discarded if refluxed for more than 10 min beyond 1 hour.
- Use of a rubber policeman on a Teflon rod with a metal core is much more rugged than using a glass stir rod for the purpose.
- Chemicals begin to precipitate out of neutral detergent solution at temperatures < 20.6°C. If this occurs, warm the entire solution (a drying oven is adequate) to dissolve the chemicals. Mix before use.
- Take appropriate precautionary measures (safety glasses, gloves, lab coat) for working with the chemicals in this assay. Check the MSDS pages in your laboratory for further details.

IV. Sample Preparation

Dry moist samples in a 55°C forced air-oven to a constant weight.
Grind dry samples through the 1 mm screen of a Wiley mill or UDI mill.

V. Reagents and Equipment

Reagent	Amount
Distilled water (dH_2O), high quality	18 liters
Sodium lauryl sulfate, laboratory grade	540.0 grams
Ethylenediaminetetraacetic acid (EDTA), A. C. S.	263.0 grams
Sodium hydroxide, NaOH, A. R.	72.0 grams
Sodium borate decahydrate, $Na_2B_4O_7 \bullet 10H_2O$, A. R.	122.6 grams
Disodium hydrogen phosphate, anhydrous, A. R. (Na phosphate dibasic, Na_2HPO_4)	82.1 grams
Triethylene glycol, purified grade	180.0 mL
Acetone, reagent grade	

Heat-stable α-amylase, (Termamyl 120L, Novo Nordisk Biochem, 77 Perry Chapel Church Rd., Box 576, Franklinton, NC 27525-0576; 919-496-3038), or A-3306 α-amylase, Sigma-Aldrich Company, St. Louis, MO)

Boiling distilled water

Equipment

Fiber analysis / reflux apparatus

Filtration manifold for crucibles and filter paper

Gooch crucibles, coarse porosity

Whatman 541 filter paper

VI. Preparation of Neutral Detergent

1. Dissolve 72.0 g of sodium hydroxide in 3 liters of dH_2O. Best done on a magnetic stir plate.

2. Add 263.0 g of EDTA and 122.6 g of sodium borate. Stir to dissolve.

3. In a separate beaker, dissolve the 82.1 g of disodium hydrogen phosphate in approximately 400 mL dH_2O over heat.

   Note: Have water hot/boiling when the chemical is added, or it will solidify and become more difficult to work with.

4. Add the dissolved components to a 20 liter container. Rinse the beakers into the container for complete transfer of chemicals.

5. Dissolve the sodium lauryl sulfate (540 g in ~ 8 liters of dH_2O) and add to the 20 liter container. Alternatively, add the sodium lauryl sulfate to the 20 liter container directly.

6. Add the triethylene glycol to the 20 liter container to control foaming.

7. Add the remaining dH_2O to the container and mix well. If not previously dissolved, sodium lauryl sulfate should be in solution by the next day.

8. The next day, verify that all chemicals are in solution and check the pH. The pH should be within the range of 6.9 to 7.1. Adjust the pH with NaOH or HCl if necessary.

VII. Neutral Detergent Residue Procedure

1. Accurately weigh 1 g samples in duplicate into 600 mL Berzelius beakers.

2. Add 100 mL neutral detergent and 0.2 mL heat-stable α-amylase.

3. Reflux for 1 hour. Should be brought to a rolling boil within 5 minutes of putting on the reflux apparatus.

   Note: After the foaming has ceased, use a fine stream of neutral detergent to rinse the sample down the walls of the beaker and into the solution. Foaming will be excessive if the reflux apparatus is not cool and functioning properly.

4. After 1 hr measured from the time boiling began, filter samples.
   Filtering:
   Preheat coarse porosity 50 mL Gooch crucible or Whatman 541 filter paper with boiling dH_2O for greater ease in filtration. Swirl the samples to suspend the residue and pour into the crucible/filter paper without vacuum. Allow the fiber a moment to settle in the bottom of the crucible, turn on the vacuum, and begin pouring the rest of the sample + solution into the beaker. (The fiber forms a mat to retain small particles to help prevent clogging of the filter). Stop pouring only if the solution is no longer flowing through the filter. Continue filtering & pouring until all detergent solution has passed through the filter.

   Note: The detergent solution filters best when hot. If filtration is a problem with high starch feeds, add 0.2 mL heat-stable α-amylase to the crucible while filled with solution. If the crucible becomes clogged, and if filled to within no more than 1 cm of the top of the crucible, turn off the vacuum, remove the crucible from the manifold, then press it back in to the manifold to force air through the glass frit and remove the clog. Take care not to lose sample!

5. Thoroughly rinse the beaker into the filter with boiling dH_2O. Use a rubber policeman to clean fiber from the beaker, and rinse into the filter using a fine stream of boiling dH_2O.

6. Rinse the residue 2x with boiling dH_2O.

7. Rinse the residue 2x with acetone.

Note: The NDR for nitrogen analyses may be dried at 55°C (vacuum or forced air oven) after extraction.

Processing of Neutral Detergent Residues (NDR)

Organic matter analyses (NDROM). Use samples filtered through Gooch crucibles.

1. Dry NDR+crucible and determine their dry weight according to an AOAC method.
2. Ash the sample by an AOAC procedure and determine weight of ash+crucible.

Crude protein analysis (NDRCP). Use samples filtered through Whatman 541.

1. Determine nitrogen content of the EIR+filter paper. Do not subsample.
2. Use a filter paper plus Kjeldahl reagents as a blank.

VIII. Calculations

Weights (Wt.) are expressed in grams.
All values are calculated as a percentage of the original sample dry matter.
"Sample Wt." is the amount of the original sample weighed out for the neutral detergent extraction. "Dry" indicates a dry matter weight.
DM% is the dry matter percent of the original sample.
In the development of the procedures, dry matters were determined after drying at 105°C overnight. Ashing was performed at 512°C for a minimum of 8 hours.

NDR Organic Matter (NDROM) % of Sample Dry Matter:
= [((Wt. of dry crucible + residue) - (Wt. of dry crucible + ash))/(Sample Wt. x DM%)] x 100

NDR Crude Protein (NDRCP) % of Sample Dry Matter:
= [(Nitrogen in NDR, g) x 6.25)/(Sample Wt. x DM%)] x 100

APPENDIX

The following papers are included to provide further information on working with the neutral detergent-soluble carbohydrates and feed analysis. Some information is repeated across the papers, however, each contains additional details that may be of use.

9th Ann. Florida Ruminant Nutr. Symp. Jan. 15-16, 1998
Gainesville, FL

MAKING NUTRITIONAL SENSE OF NONSTRUCTURAL CARBOHYDRATES

Mary Beth Hall
University of Florida, Gainesville 32611

Introduction

The nonstructural carbohydrates (NSC) have been an important but vexing part of ration formulation. Feeding NSC has been related to both high production and health problems. These carbohydrates can constitute up to 45% of ration dry matter, yet the number used to describe them has been a calculated value. The carbohydrates found in NSC are sufficiently diverse in their nutritional characteristics and presence in feedstuffs that their partitioning and individual examination is in order.

Current NSC Estimation

The NSC include all carbohydrates not found in neutral detergent fiber (NDF). Their content in feeds has been a calculated rather than directly analyzed value because of the many types of carbohydrates included in this fraction. The lack of methods or problems with the assays has prevented individually measuring NSC and summing the components. Currently, the NSC content of feedstuff dry matter (DM) is a calculated value based upon nutrient percentages subtracted from 100% of feed DM:

$$NSC\% = 100\% - (CP\% + NDF\% + EE\% + Ash\%)$$

or

$$NSC\% = 100\% - [CP\% + (NDF\% - NDFCP\%) + EE\% + Ash\%]$$

where,
CP = crude protein,
NDF = neutral detergent fiber,
NDFCP = neutral detergent-insoluble crude protein, and
EE = ether extract (crude fat).

Although the first equation is most commonly used, the second equation is preferable because it corrects for CP in NDF (NDFCP) and so avoids subtracting NDFCP twice (as part of CP and as NDFCP).

Errors that are associated with each component in the equations, either with how the assay was carried out or inherent within the assay itself, shift the estimated NSC from its true value. Because it is calculated by difference, the errors from each of the

component analyses accumulate in NSC. In specific cases where NSC is underestimated because the mass of CP from non-protein nitrogen sources is overestimated, computing a more accurate NSC value may be possible (21). However, correcting for known errors in these equations still does not accurately describe NSC's nutritional value.

Carbohydrates in NSC

A great variety of carbohydrates are soluble in neutral detergent. Based upon their locations in the plant cell and THEIR nutritional characteristics, they should be called neutral detergent-soluble carbohydrates (NDSC), rather than NSC or NFC (non-fiber carbohydrates). The NDSC include both structural and non-structural, and fiber and non-fiber carbohydrates (Figures 1 & 2). Overall, NDSC are considered to be more rapidly and readily digested or fermented than NDF, but their nutritional characteristics and compositions are far from uniform.

The carbohydrates, or carbohydrate derivatives included in NDSC are organic acids, sugars, oligosaccharides, starch, fructans, pectic substances, (1→3)(1→4)-β-glucans, and other carbohydrates of the appropriate solubility. One way they can be partitioned is based on their digestion by the cow vs. ruminal microbes. The only carbohydrate linkages that mammalian enzymes can hydrolyze are those in sucrose and starch, leaving all other polymerized carbohydrates indigestible, except by microbes. "Fiber" is the nutritional term applied to carbohydrates not digestible by mammalian enzymes. Accordingly, NDSC can be allocated into non-fiber, and fiber components. The non-fiber carbohydrates include organic acids, sugars, and starches. The fiber fraction includes fructans, pectic substances, and (1→3)(1→4)-β-glucans. Classification of oligosaccharides depends upon their composition and linkages.

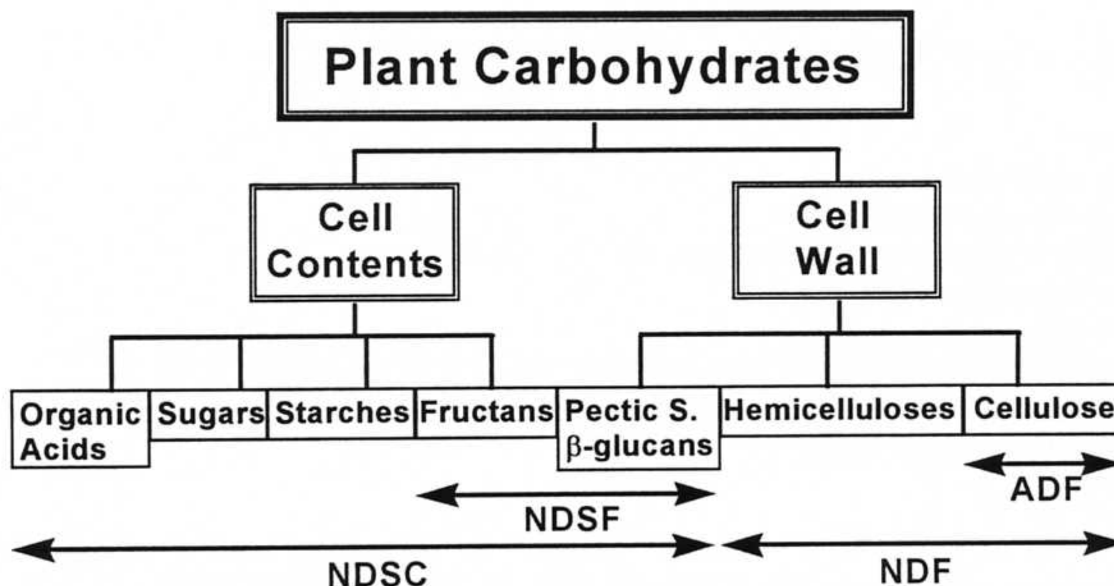


Figure 1. Plant carbohydrate fractions.

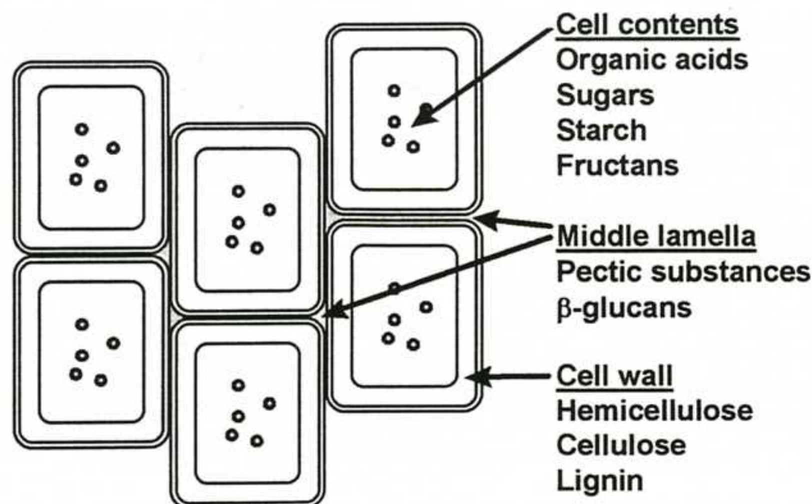


Figure 2. Locations of carbohydrates and lignin in plant cells.

Neutral Detergent-Soluble Carbohydrates: Non-Fiber

Organic Acids

Organic acids are not carbohydrates per se, but derivatives or precursors of carbohydrate. They generally come from two main sources: those within growing plants, and fermentation acids. In unfermented plant material, the organic acids are generally intermediates of the citric acid cycle, or plant defensive compounds. They include citrate, malate, quinate, succinate, fumarate, oxalate, shikimate, *trans*-aconitate, and malonate among others. The range of organic acids present in forage has made complete analysis for them difficult, a reason that there are relatively few values available for them. The concentrations of organic acids tend to decrease as plants age (7,34). Total levels in plant dry matter reported in cool season grasses are 1.3 to 4.5% (34), 5.8 to 9.8% for alfalfa, 2.8 to 3.8% in red clover, and 3.0 to 3.5% in white clover (10). Bermudagrass, contains little water-soluble carbohydrate (3 to 4% of DM) (65), but has been reported to contain low levels of oxalic acid (0.02 to 0.16% of DM)(10), and higher levels of malate (1.9 to 4.5% of DM) (7). Alfalfa varieties may contain 2.9 to 7.5% malate, depending upon variety and age (7). Citrus peel contains 3 to 4% of DM as organic acids (67).

Fermented feeds contain a different complement of organic acids than does fresh plant material. In the ensiling process, bacteria ferment sugars, starch, organic acids (42), and fructans (44) to a number of compounds including lactate, acetate, and other organic acids. Lactate (12.1%) and acetate (3.6%) can represent more than 15% of the dry matter in direct cut silage, however, the levels are typically lower in wilted material (42). Silage made from bermudagrass tends to have lower concentrations of organic acids than temperate forages, largely due to the low levels of carbohydrates available to be fermented (2 to 4% of DM). In a comparison of wilted and direct-cut bermudagrass silages, wilted silages contained more lactic acid and less acetic acid than direct-cut materials (65)

Table 1. Lactic and acetic acid contents of direct cut or wilted bermudagrass silage with or without lactic acid bacteria inoculation 114 days after ensiling (% of DM) (65).

Harvest method	Control Lactic acid	Acetic Acid	Inoculated Lactic acid	Acetic Acid
Direct cut	2.95	4.54	1.56	4.69
Wilted	2.97	0.67	3.62	0.93

(Table1). It should be noted that volatile fatty acids (VFA) such as acetate, propionate, and butyrate are not represented in feed analyses because they volatilize and are lost during the estimation of dry matter. Lactic acid is included in the NDSC.

The nutritional value of organic acids to the cow and microbes depends upon the particular organic acid. The cow can digest and absorb organic acids directly. Volatile fatty acids such as acetate, propionate, and butyrate have already been digested to their endpoint by microbes, and are not fermented to any great extent in the rumen. These VFA are available to the cow but not to rumen microbes.

The non-VFA organic acids are likely fermented in the rumen. Acetate is the primary fermentation product for the organic acids common in forage (citrate, *trans*-aconitate, malate, malonate, quinate, shikimate)(53). Among these acids, malate has been reported to enhance the uptake of lactate by certain rumen microbes, and may have a role in reducing the incidence of lactic acidosis (41). Lactic acid is fermented largely to propionate or to acetate (3,12,18,29,31). One study indicated that the ratio of acetate to propionate produced from the fermentation of lactate was related to the ration an animal was consuming. Fermentations performed with rumen inoculum from a cow fed a high grain ration produced a lower acetate to propionate ratio from lactate than did those from rations higher in forage (3). Although it is fermentable, lactate supports little microbial yield from the rumen (29).

In terms of the energy that lactate offers to the animal, a study with sheep (18) indicated that 90% of the lactate fed was fermented in the rumen, with little being absorbed directly by the animal. The molar ratios of VFA derived from fermentation of lactate were 1.0:0.57:0.08 for acetate:propionate:butyrate, respectively. Although lactate itself provided no direct contribution to gluconeogenesis, 10% of total lactate was converted to glucose through propionate which was derived from fermented lactate (18). In all probability, factors such as rumen pH, rate of passage, ration composition, and rumen microbial population affect the amount and type of organic acids digested by the ruminal microbes or cow.

Sugars and Oligosaccharides

Simple sugars are comprised of single sugar molecules with glucose and fructose being the most common in plants. Oligosaccharides are short chains of sugars, from 2 to 20 sugar residues in length, with sucrose, a disaccharide, the most prevalent. Together,

glucose, fructose, and sucrose are the predominant low molecular weight carbohydrates in forages, and will be referred to in this paper as "sugar(s)". These water-soluble cell contents are reported to account for 1 to 3% of forage DM for the simple sugars, and 2 to 8% for sucrose in "field grown herbage" (temperate forages) (57). Citrus pulp may contain 20% or more of DM as sugar (R. DeStefano, personal communication), but the sugar content will vary with the amount of citrus molasses applied and the citrus variety used to produce the pulp (61). Cane molasses may contain approximately 60% of DM as sugars (expressed as invert sugars; U.S. Sugar, Clewiston, FL), however, there is variation among molasses sources. Almond hulls contain 19 to 34% soluble sugars (1), varying by variety. An interesting note on almond hulls is that the sugars can be "washed out" when the hulls are rained upon, decreasing their content in the feed (1). This same scenario conceivably applies to sugars in other feedstuffs.

Glucose, fructose and sucrose are readily digested directly by the cow, if they reach the small intestine. By virtue of their chemical composition and high solubility, simple sugars and oligosaccharides are also among the most rapidly fermented carbohydrates. The fermentation of sugars is similar to that of starch in that both can ferment to lactic acid. Fermentation of sucrose by rumen microbes resulted in similar concentrations of microbial protein, acetate, and propionate as compared to starch, but more butyrate and lactate at pH 6.7 (59). Although we usually think of depressions in fiber digestion at low pH, changes also occur in the digestion characteristics of the NDSC. At a more acidic pH (5.5), fermentation of sucrose produced more lactate than did that of starch, and microbial protein yield from sucrose was reduced by 34% (59). More lactate, less acetate and butyrate, and the same amount of propionate were produced from sucrose fermented at pH 5.5 vs. 6.7 (59).

The rapid fermentation of sugars yields acid, which can rapidly decrease rumen pH. In cattle fed diets containing grass silage, sugar beet pulp or barley, minerals, and no or 17.4% of DM as molasses (26), rumen pH decreased more rapidly and went lower (rumen pH ≤ 6.0) than with diets without molasses. With molasses feeding, molar proportions of propionate and butyrate in the rumen tended to increase, which agrees with in vitro results (59). As for effects on production, inclusion of molasses (0, 4 or 8% of DM) in lactating cow rations varied in having positive or negative effects on milk yield, milk components, and DM intake. The effect appeared to depend upon the level of molasses offered and the type and amount of roughage in the diet (43). If they are added to the point that they depress pH, sugars can depress fiber digestion in the rumen, however, it appears that they may also be capable of enhancing it. Addition of molasses can decrease the lag time of silage and hay DM fermentation (26), possibly due to increasing the total microbial numbers available to ferment feeds (25).

Starch

Starch is the main storage polysaccharide in forage legumes, tropical grasses, and grass and legume seeds (58). Consisting entirely of glucose, it is arranged in two types of polymers: amylose, a linear molecule with α-(1$\rightarrow$4) linkages, and amylopectin, an α-(1$\rightarrow$4)-linked glucose polymer with α-(1$\rightarrow$6)-linked branches. Because of its α-linkages,

starch is digestible by mammalian enzymes, whereas cellulose, which consists entirely of β-(1→4)-linked glucose, is not. The simple change in the type of bond entirely changes a glucose polymer's susceptibility to enzymes. The difference in bonding is the reason we cannot simply analyze for glucose in a feedstuff and expect it to have nutritional relevance.

Fermentation of starch by rumen microbes has a variety of similarities to that of sugars. Starch may ferment to lactate (59), and tends to produce a lower acetate to propionate ratio than cell wall carbohydrates (40,59). Although starch fermenting bacteria are more tolerant of acidic conditions than are fiber digesters, growth of starch digesting microbes declines as pH declines (52,60). At acidic vs. neutral pH (5.8 vs. 6.7), yield of microbial protein decreased by 35% when starch was fermented by mixed rumen microbes (59). Consequently, microbial protein available to the cow likely decreases with decreasing ruminal pH.

The rate and extent of starch digestion is affected by a variety of factors. Particle size, grain type, steam flaking, preservation method (dry or ensiled) all affect the availability of starch. In feeds such as corn, the smooth covering of the seed offers the first barrier to digestion, and the protein matrix that surrounds the starch granules the second (37). For whole grain, approximately 30% may pass undigested into the manure in cattle (48). But as particle size in whole corn decreases, ruminal starch disappearance generally increases (16,17). Processing methods which disrupt the protein matrix around the starch granules have been shown to increase grain digestibility (28,63). Subjecting starch to heat and moisture gelatinizes it, destroying the crystalline structure of starch granules, and increasing digestibility (19,22,28,33,63) in the rumen and total tract (49). It has been suggested that overall metabolizable energy yield to the cow is best when starch is fermented in the rumen, due to possible limitations on its digestion in the small intestine (27). However, if digestion of starch in the small intestine were enhanced, there are possibilities for improving the animal's capture of glucose from starch (27).

Neutral Detergent-Soluble Carbohydrates: Fiber

Fructans (Fructosans)

Fructans are water-soluble chains of fructose found in the cell contents of plants. These carbohydrates may have β-(2→1) linkages as in inulin found in Jerusalem artichokes, or β-(2→6) linkages with some β-(2→1) branches as in levan found in temperate grass species (57). They are the principal storage carbohydrates of temperate cool season grasses (58). Depending upon the species and environmental conditions, temperate grass forage has been reported to contain less than 1% and up to 30% fructan (57).

Although mammals can utilize fructose, they do not have the enzymes to digest fructans (46,47). In the rumen, both bacteria and protozoa ferment fructan (68). Fructans can be fermented to lactic acid during ensiling (44,45) and in the rumen (68). Additionally, rumen microbes can degrade fructan and store it as "microbial starch"

(glucose polymers with the same bonding as starch) and utilize it at a later time when other nutrients are no longer available (68).

(1→3)(1→4)-β-Glucans

The (1→3)(1→4)-β-glucans are found in the endosperm and cell walls of grasses (66). They have the same β- (1→4) linkage between glucose molecules that cellulose does, but the β- (1→3) linkages create bends in the chain. These bends prevent the molecules from achieving the linearity and crystallinity of cellulose. Barley and oats are major sources of β-glucans, containing from 4 to 12% by weight (32).

Just as mammals cannot digest cellulose, neither can they digest (1→3)(1→4)-β-glucans. However, this carbohydrate appears to be very rapidly fermented. In steers fed barley, ruminal in sacco disappearance of β-glucans varied by lot of barley, but was between 61.4 and 70.4% of DM at time 0, and 93.8 to 96.2% of DM by 8 hours. Disappearance of β-glucan was greater from dry rolled than from steam rolled barley (13). The fermentation of β-glucans does not appear to give rise to lactic acid (66).

Pectic Substances

'Diverse' describes both the concentrations and compositions of pectic substances in plants. Pectic substances are found chiefly in the middle lamella of the plant cell wall. They have been operationally defined as non-starch polysaccharides soluble in water, in chemicals which remove divalent cations (eg., Ca^{++}, Mg^{++}), and in dilute acids or bases that break covalent bonds (11,14,50). In terms of sugar composition, they contain a galacturonic acid backbone with rhamnose inserts that is the portion we think of as pectin, plus neutral sugar side chains made up largely of arabinose and galactose (30). There are differences among plants and plant parts in the content and composition of pectic substances (2,20,50,54,56). Because they are very complex carbohydrates, analysis for pectic substances has been difficult, and there are few values available. By-products such as citrus pulp, sugar beet pulp, and soybean hulls are reported to contain 29, 33.7, and 20% pectin, respectively (36,51,55). Among forages, grasses are low in pectic substances (2 to 5%) (15), while legumes contain higher quantities (7 to 14%) (8,15). Pectic substances decline with increasing plant maturity in alfalfa stems (23).

Due to their carbohydrate composition and bonding, pectic substances are not digestible by mammalian enzymes. However, they are rapidly and extensively degraded by rumen microbes. In vitro fermentation rates of 30 to 40% per hour for pectin have been reported (24). The extent to which they are fermented does not appear to be affected by lignification (35) or, in alfalfa, by plant part (62). Pectin fermentation tends to produce high acetate to propionate ratios and relatively little or no lactate (9,24,40,59). Still, the organic acid contribution from the fermentation of pectic substances depends upon its sugar composition (24). The yield of microbes from pectin or pectic substances is not different from starch (38,39,59). However, the fermentation of pectin at low pH (5.8) is reduced, resulting in a lower extent of degradation, and up to70% less microbial protein produced (59). A more acidic ruminal pH translates into decreased amounts of pectin

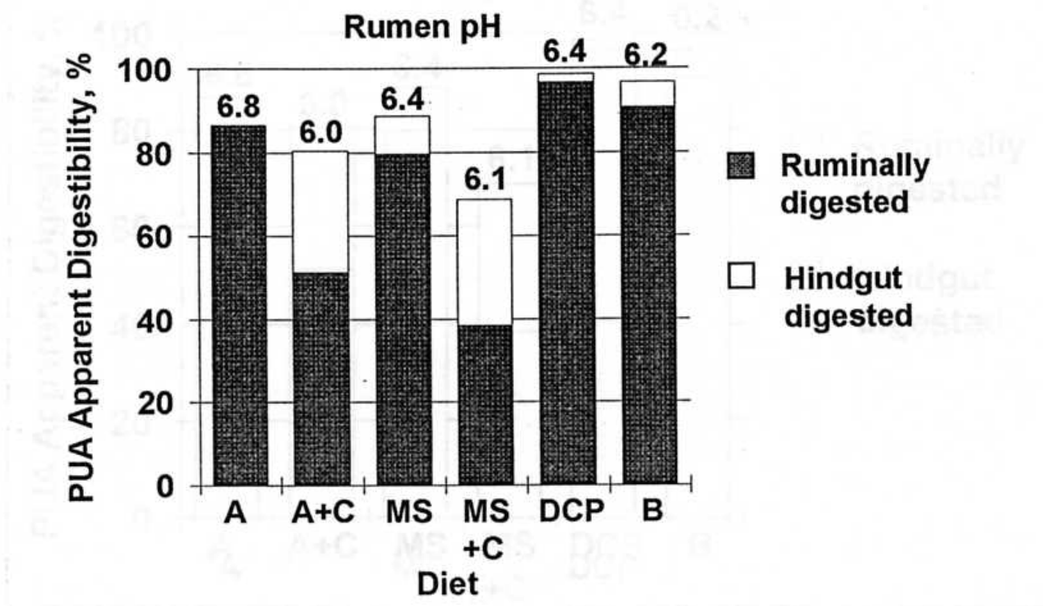


Figure 3. Changes in pectic uronic acid (pectin) digestibility in the rumen and hindgut, and changes in rumen pH when starchy concentrate is fed. Note how digestion of pectin is shifted out of the rumen and to the hindgut as rumen pH decreases. Experiment 1: A = alfalfa diet, A+C = alfalfa + concentrate; Experiment 2: MS = maize (corn) silage, MS+C = maize (corn) silage + concentrate; Experiment 3: DCP = dried citrus pulp, B = barley concentrate source (4, 5, 6).

fermented in the rumen (6) (Figure 3), and a decrease in the amounts of microbial protein and VFA available to the animal. Unlike starch, the cow cannot digest pectic substances that escape the rumen.

Uses of Metabolizable Nutrients From Carbohydrates

The digestion or fermentation of carbohydrates provides the animal with amino acids from rumen microbes, various VFA, lactate, and glucose. Since different carbohydrate sources offer different profiles of nutrients to the animal, it is worthwhile to consider the nutritional implications of their feeding.

The energy systems that we use are proxies to match animal requirements and nutrient efficiencies to estimated supplies of nutrients. For instance, animals do not use net energy or metabolizable energy, per se. They metabolize absorbed nutrients such as amino acids, acetate, propionate, butyrate, glucose, and fatty acids to generate energy to

do work, or meet specific nutrient needs. In the same fashion, animals do not use crude protein, which is the nitrogen content of feed times 6.25, but they do use amino acids. A reason that energy systems are needed, is the difficulty we have in accurately predicting how much of each nutrient the animal actually absorbs from the ration relative to their specific requirements. Despite the difficulty of prediction, it is important that we recognize the differences in how absorbed nutrients are used. Understanding these differences may help us to interpret the responses we see on farms.

Energy

To meet energy requirements, the animal uses substrates that can be oxidized and converted to energy, or incorporated into specific compounds. In gross terms, the cow needs glucogenic or ketogenic compounds to meet these needs. Glucogenic compounds are those absorbed nutrients that can be used to meet oxidative or synthetic demands for glucose. They include glucose, and gluconeogenic precursors such as the odd carbon organic acids (propionate, lactate), glycerol from triglycerides, and certain amino acids. "Gluconeogenesis" means "making glucose anew", which is refers to the conversion of the gluconeogenic precursors into glucose in the liver. Since the glucose absorbed directly by the cow is not sufficient to met her requirements, gluconeogenesis provides much of the glucose that the cow requires. Ketogenic compounds cannot be converted to glucose, but can give rise to ketone bodies (acetoacetate, β-hydroxy-butyrate, etc.), may be oxidized to provide energy, or incorporated into lipids in the animal. Ketogenic compounds include the even carbon organic acids (acetate, butyrate), some amino acids, and fatty acids. Although non-ruminants can convert glucose to fat, glucose is typically at a premium in ruminants, and they lack the enzyme for this conversion. Protein or fats incorporated into body tissues contribute to total energy accretion by the animal. Glucose may be stored as glycogen in the liver, and sugars may become associated with proteins.

Protein

Animals use absorbed amino acids from rumen microbes and rumen escape feed sources. The non-protein nitrogen fed to the cow is only made useful to her when the microbes convert it to protein, which occurs given enough fermentable carbohydrate and appropriate minerals. All of the proteins that animals produce, such as milk protein, lean tissue, and enzymes, each have a specific amino acid composition. Accordingly, animals have requirements for specific amino acids. Provision of amino acids that are most limiting can improve animal performance and feed efficiency.

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Interpreting Feed Analyses: Uses, Abuses, and Artifacts

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Successful ration formulation depends upon using accurate feed analyses. We rely upon the nutrient values that laboratories provide us with the implicit beliefs that they are nutritionally relevant and can be used across a wide array of feedstuffs with equal confidence. For the most part, when using reputable laboratories, these beliefs are reasonably well-founded. But, the assays used are based largely on differential solubilities of feed components in chemicals, not directly on biological bases. The caveat is that feed analyses are only as accurate as the methods used to measure them and their correlation to what occurs in the animal. Chemical analyses have made possible the evaluation of large numbers of feed samples, but their nutritional relevance varies.

Fiber Analysis

Fiber is the carbohydrate fraction in feedstuffs that is not digestible by mammalian enzymes. The insoluble fiber fraction as measured by the crude fiber or detergent fiber systems is intended to describe the less or more slowly digestible portions of the plant carbohydrate. These analyses achieve this goal with varying levels of success.

Crude Fiber

Crude fiber has long been used to assess fiber content of feeds for nutritional and regulatory purposes. Although the assay is repeatable, its nutritional relevance is questionable. The sulfuric acid and sodium hydroxide extractions used for crude fiber analysis solubilize some of the cellulose, hemicelluloses, and lignin in the sample (Van Soest, 1994). Consequently, these cell wall components are included in the nitrogen-free extract (NFE) which was intended to describe the water-soluble carbohydrates and starches. The extent to which the cell wall materials are extracted into the NFE varies by feedstuff. Because crude fiber does not accurately isolate the less digestible cell wall constituents, it is not among the best choices for a feed analysis.

Neutral Detergent and Acid Detergent Fibers

Starch Contamination of NDF

Wet chemistry feed analyses, such as those for neutral detergent fiber (NDF) and acid detergent fiber (ADF), are based on the differential solubility of plant components in boiling solutions of neutral, or acid detergents (Figure 1). Neutral detergent fiber represents the cell wall

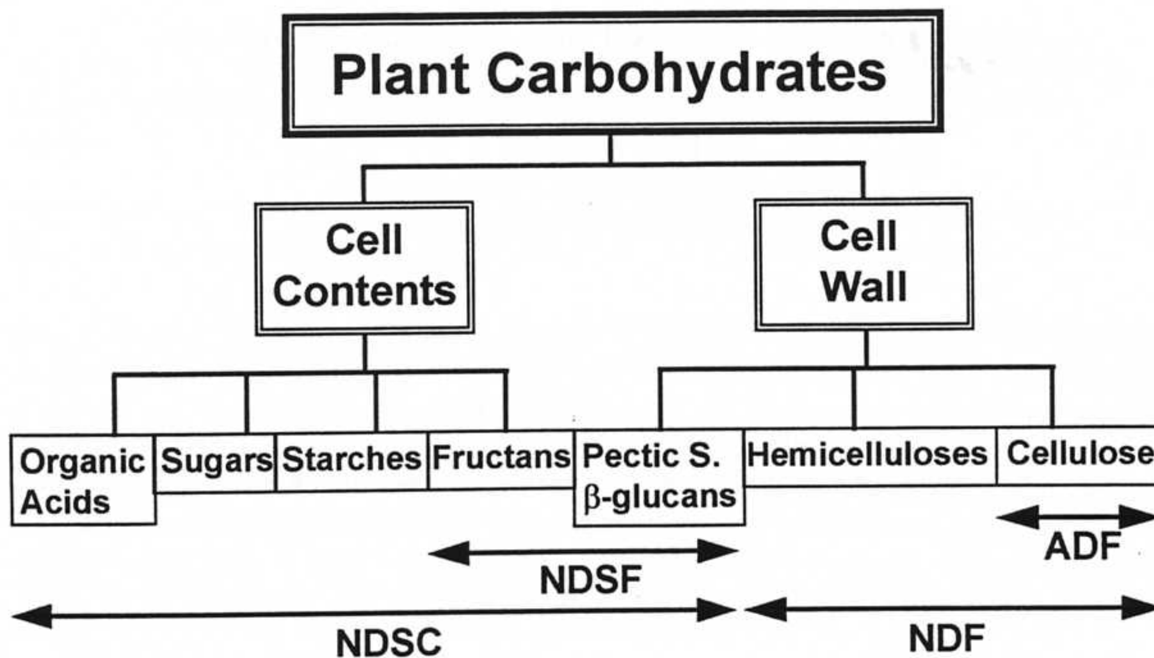

Figure 1. Carbohydrate composition of chemically analyzed fractions. (Pectic S. = pectic substances; ADF = acid detergent fiber; NDF = neutral detergent fiber; NDSC = neutral detergent-soluble carbohydrates; NDSF = neutral detergent-soluble fiber).

fraction, including hemicelluloses, cellulose, lignin, and neutral detergent-insoluble nitrogen. Acid detergent fiber represents a cell wall fraction consisting of cellulose, lignin, and acid detergent-insoluble nitrogen (bound protein). Either of these common analyses can suffer from contamination by other carbohydrates. When analyzed without using a heat-stable α-amylase, NDF values can be elevated by residual starch (Van Soest and Robertson, 1985). This offers one explanation for the 55% NDF reported for corn hominy feed in the NRC (1989). In comparison, Mertens (1992) arrived at an NDF for corn hominy feed of 23% using α-amylase. Even with tropical grasses which are not perceived to have high starch contents, NDF values were reduced by 0.5% of dry matter ($P < .01$) when amylase was used (R. Fethiere, personal communication). Presence of starch in NDF is problematic because the carbohydrates involved have very different fermentation and digestion characteristics. Starch may be fermented to lactic acid, or may be digested directly by the cow, whereas neither occurs with NDF. The use of heat-stable α-amylase in NDF analysis is recommended.

Pectin Contamination of ADF

Acid detergent fiber values can be inflated by contamination with pectin. Pectin is a carbohydrate found in the middle lamella between the cell walls of plants. It is a type of "neutral detergent-soluble fiber" that is indigestible by mammalian enzymes, but may be very rapidly fermented by rumen microbes. In acid detergent, some of the pectin remains unextracted or is precipitated under the acid conditions (Bailey and Ulyatt, 1970). Consequently, fiber fractions of

very different digestibilities are included together. This can be a particular problem in high pectin feeds such as citrus pulp and beet pulp, although other feeds containing pectin such as legume forages are not immune. A striking example of apparent pectin contamination is an analysis from a commercial lab reported the NDF of citrus pulp as 25.1% and the ADF as 30.6% of dry matter. A common calculation for hemicellulose (NDF% - ADF%) would tell us that this feed has a negative 5.5% hemicellulose content, which certainly isn't the case. The retention of pectin in ADF does not appear to be quantitative, thereby eliminating the possibility of a simple calculation for its correction.

The method that achieves a more accurate value for ADF in pectin-containing feeds is sequential analysis for NDF and then ADF performed on the same sample (Bailey and Ulyatt, 1970). The neutral detergent removes the pectin and the acid detergent removes the hemicelluloses. Such sequential analyses are labor intensive and have not been available commercially. Practically speaking, the need for sequential analysis is likely not a major issue for most feedstuffs.

Mineral Contamination

Both NDF and ADF should be expressed on an "ash-free", or "organic matter", basis. Organic matter (OM) includes the carbohydrates, proteins, fats and other carbon- or nitrogen-containing components, and excludes ash or minerals. Contamination with minerals unduly increases fiber values and does not allow accurate assessment of the fiber available for microbial use as a carbon source. Soil is often the biggest culprit in mineral contamination. Neither neutral, nor acid detergents remove soil contamination from feed samples. The error caused by soil contamination varies with harvesting method and soil types.

Unlike silica from soil contamination, biogenic silica responds differently to neutral and acid detergents. Biogenic silica is that silica which is absorbed by plants from the soil and deposited in their tissues. Neutral detergent dissolves this silica, whereas it is quantitatively recovered in ADF (Van Soest, 1994). Accordingly, biogenic silica is measured as a part of ADF, unless ADF is expressed on an OM basis. This form of silica is present in materials such as rice straw, sugarcane bagasse, and a variety of grasses.

Fermentation Characteristics

Common perception has it that NDF is always slowly fermented, but this is not always true. Neither is NDF uniform in composition within or among feedstuffs. This is can lead to variation in fiber digestion characteristics which must be considered on a feed by feed basis. Citrus pulp NDF fermented in vitro with mixed rumen microbes had exponential fermentation rates of 18 to 28%/h, and was 85% fermented in 24 h (Hall, 1996). These rates were similar to the rates of soluble fiber fermentation for the same feed. In the same study, dried sugar beet pulp NDF fermented at a rate of 17 to 19%/h with 78% of the NDF digested in 24 h. These rates are in contrast to the 3 - 4%/h typically reported for digestible NDF. The fermentation lag times were significantly longer for NDF than for soluble fiber for both feedstuffs. The National Research Council beef cattle nutrient recommendations (1996) contains feed tables that list fermentation rates for digestible NDF, as well as other carbohydrate and protein fractions.

Application to Animal Products

Neutral and acid detergent analyses were originally developed to measure fiber fractions in plant materials, and as a preparative step for lignin analysis. With fiber defined as carbohydrate that is not digestible by mammalian enzymes, animal products cannot contain fiber (excepting improbable and extensive contamination in the abattoir). The NDF and ADF values for animal products are used to identify fractions that are believed to be more slowly degraded (neutral detergent-insoluble nitrogen; NDIN), or unavailable (acid detergent-insoluble nitrogen; bound protein; ADIN) to ruminal or mammalian digestion (Van Soest, 1994). Still, the analytical values simply reflect the solubilities of feed components in acid and neutral detergents that may be correlated to digestion characteristics, not "true" fiber values for animal products.

Neutral Detergent-Soluble Carbohydrates

Estimation

The carbohydrates soluble in neutral detergent include the most digestible portion of the plant and are the most problematic to describe nutritionally. As opposed to non-structural carbohydrates (NSC) or non-fiber carbohydrates (NFC), the carbohydrates in question are actually "NDSC": "neutral detergent-soluble carbohydrates". The NDSC include both structural and fiber carbohydrates (Figure 1). As a class, NDSC are reported to be 98% digestible (Van Soest, 1967) and rapidly fermented, but they are a compositionally diverse group, which has tended to preclude their direct measurement by chemical analysis.

The equations for estimating NDSC provide the first difficulty for using this fraction. The NDSC is calculated as the difference between NDF and non-carbohydrate fractions by the equations:

100 - (Crude Protein + NDF + Ether Extract + Ash)

or

100 - (Crude Protein + (NDF - NDIN) + Ether Extract + Ash)

The second equation corrects for protein in the NDF (NDIN) and so avoids subtracting the protein twice. Because it is calculated by difference, all of the errors from the component analyses accumulate in NDSC.

The source of crude protein within a feed may be a source of error in the NDSC calculation. Crude protein is simply an estimation of protein mass arrived at by multiplying nitrogen content by 6.25. When the nitrogenous compounds present are not one-sixteenth nitrogen, factors other than 6.25 may be appropriate (Table 2), but there is often no practical way to determine the correct multiplier. The effect of miscalculation of crude protein mass on NDSC calculation is of especial concern with feeds high in non-protein nitrogen. For example, pure urea is 291% crude protein, with a 2.14 multiplier to calculate mass from nitrogen content. Multiplying urea nitrogen by 6.25 results in the mass of urea in a feed being overestimated by nearly three-fold, and NDSC being underestimated. Typically, ration formulation programs that calculate NDSC do not correct for changes in nitrogen content of crude protein. Due to such mathematical artifacts, NDSC estimates are prone to error.

Table 2. Factors for conversion of nitrogen to protein for foods and feeds (adapted from Jones, 1931) [1] and non-protein nitrogen sources.

Food	Factor	Food	Factor
Eggs	6.25	Navy beans	6.25
Gelatin	5.55	Lima beans	6.25
Meat	6.25	Soy beans	5.71
Milk	6.38	Peanut	5.46
Barley	5.83	Almonds	5.18
Corn	6.25	Cottonseed	5.30
Rice	5.95	Sesame	5.30
Urea[2]	2.14		
Nitrate, NO_3^- [2]	4.43		

[1] Factors determined on isolated protein fractions.
[2] Calculated by M.B. Hall as 1 /(N mass in compound / molecular weight of compound).

NDSC Variation

The greatest challenge to using NDSC in ration formulation is its diversity of components. The NDSC includes organic acids, sugars, disaccharides, oligosaccharides, starches, fructans, pectic substances, (1→3)(1→4)-ß-glucans, and other carbohydrates soluble in neutral detergent. Different carbohydrates predominate in the NDSC of different feeds. Beyond their composition, these carbohydrates also vary in their digestion and fermentation characteristics (Table 3). Organic acids do not support microbial growth to the extent that the other carbohydrates do (Figure 2). Acetate, propionate, butyrate are the end-products of ruminal fermentation and do not support additional microbial production. Lactate provides approximately half as much energy in the form of ATP as glucose to lactate-utilizing microbes (Russell and Wallace, 1988). Accordingly, lactate may support only half as much microbial synthesis as does glucose (S. Martin, personal communication). Infusion of lactate into the rumens of steers did not increase microbial protein flow to the duodenum over the basal diet (Jaakkola and Huhtanen, 1992) (Table 4). The rates and extents of starch fermentation in the rumen are highly variable, changing with processing method (Mertens, 1992), source (Rooney and Pflugfelder, 1986), and other feedstuffs in the ration. Pectic substances support a microbial yield similar to starch (Mansfield *et al.*, 1994), but their fermentation is depressed at low pH (Strobel and Russell, 1986).

Thus far, differences in NDSC among feeds have been used in a qualitative fashion for ration formulation because there was no practical way to measure the component carbohydrates. Hoover and Miller (1995) suggested that NDSC from high starch sources could comprise 35 to 40% of ration dry matter, and 40 to 45% when supplied by sources low in starch. Recent work (Hall et al., Accepted) offers a way of analyzing feeds to separate neutral detergent-soluble fiber (pectic substances, (1→3)(1→4)-ß-glucans, fructans) from starches, sugars, and organic acids. Although this improves upon the current situation, more work needs to be done to determine how

Table 3. Characteristics of neutral detergent-soluble carbohydrates.

NDSC Fraction	Predominant Composition	Digestible by Mammalian Enzymes[1,2]	May Ferment to Lactic Acid[1]	Fermentation Depressed at Low pH[1,2]	Common Sources
Organic Acids	Acetate, propionate, lactate, butyrate, etc.	+	-	-	Silage, feed additives, whey
Sugars & Disaccharides	Glucose, fructose, sucrose (glucose + fructose)	+	+	-	Molasses, citrus pulp, sugar beet pulp
Starch	Glucose	+	+	0	Corn and small grain products, bakery waste, potatoes
Fructans	Fructose	-	+	?	Temperate cool season grasses, Jerusalem artichoke
Pectic Substances	Galacturonic acid, arabinose, galactose, rhamnose, etc	-	-	+	Legume forages, citrus pulp, beet pulp, soybean hulls
(1→3)(1→4)-β-glucans	Glucose	-	-	+/?	Small grains

[1] + = yes, - = no, 0 = no difference, ? = unknown.
[2] Relative to starch.

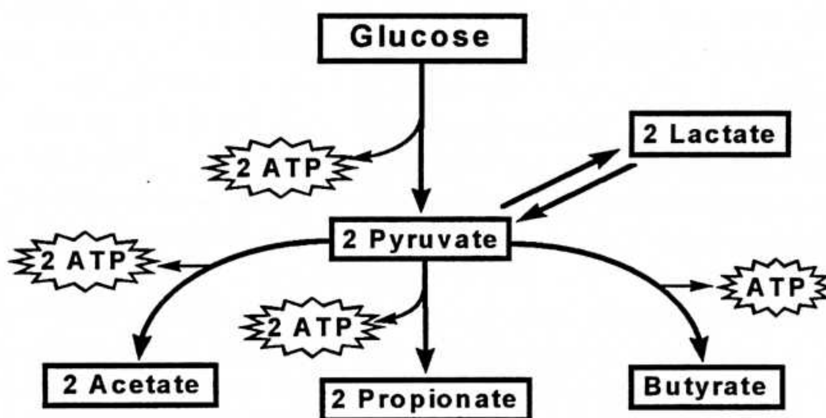

Figure 2. Energy yield from the utilization of glucose and lactate by rumen microbes (adapted from Russell and Wallace, 1988).

Table 4. Effects of lactate infusion on rumen variables, digestibility, and protein outflow in 1220 lb Friesian bulls (adapted from Jaakkola and Huhtanen, 1992) [1].

	Lactate ingested or infused, g/day			
	280	550	820	1090
Rumen				
pH	6.22	6.64	6.36	5.92
Apparent OM Digestibility	0.620	0.679	0.691	0.686
Lactic Acid (mmol/l)	1.64	3.79	8.63	23.16
Total VFA (mmol/l)	109.3	100.4	105.2	108.2
Acetate:Propionate	3.60	3.18	2.75	2.36
NDF digestibility	0.802	0.802	0.814	0.828
Duodenum				
Microbial Nitrogen (g/24 h)	85.8	70.8	72.4	71.1
Non-Ammonia Nitrogen (g/24 h)	141.6	119.5	128.6	132.8
Microbial Nitrogen g/100g of OMADR [2]	20.4	15.2	14.7	13.4

[1] Basal diet (15.6 lb DM/d) = 70% grass silage, 24% barley, and 6% rapeseed meal. Variation in basal diet intake due to changes in silage dry matter.
[2] Organic matter apparently digested in the rumen.

to optimally formulate rations using the different fractions, and how to separate organic acids from sugars and starches to better predict nutrients available to the animal.

Starch Analysis

The accuracy of starch analysis hinges upon two key factors: specificity of enzymes used and complete hydrolysis of starch to glucose. Cellulose and starch are both composed entirely of glucose, but can be differentiated on the basis of the covalent bonds between their molecules. Cellulose contain β-linkages, and starch, α-linkages. To separate the two analytically, amylases and amyloglucosidase which hydrolyze α-, but not β-linkages are used to convert the starch to glucose. The released glucose is then measured, and the starch content calculated (starch = glucose x 0.9). Specificity of the enzymes for starch, or extraction of interfering carbohydrates help to assure that other carbohydrates are not hydrolyzed and analyzed as starch. A case where lack of enzyme specificity affected starch estimation occurred when an amylase was contaminated with invertase (sucrase) (Will Hoover, personal communication). Invertase hydrolyzes sucrose to fructose and glucose in samples that have not had simple sugars and disaccharides previously extracted. The sugars liberated from sucrose measured as starch. In dried citrus pulp, this resulted in a starch value of 25.8%. Starch content of the same pulp analyzed as 2% of dry matter when sugars and disaccharides were pre-extracted (Hall et al., Accepted).

Complete hydrolysis of starch to glucose is the second critical factor. Starch granules are a naturally crystalline material which require an "opening up" of its structure to allow water and enzymes access. Gelatinization accomplished using heat and moisture, or an alkali, breaks hydrogen bonds among the glucose chains, reduces the crystallinity of starch, and opens the starch molecules to enzymatic attack. If gelatinization is incomplete, or the amount of enzyme and

accompanying digestion conditions are inadequate, the amount of starch in a sample may be underestimated because of incomplete digestion of starch to glucose.

Summary

The assays generally used for feed analysis generally tend to be robust and reliable, but none are perfect. Nutritionists must use their skills to first ensure that accurate samples are taken, and then use their knowledge of the feedstuffs to discern the validity and usefulness of the results.

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Calculation of Non-Structural Carbohydrate Content of Feeds That Contain Non-Protein Nitrogen

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What is the cost of making a 2 to 5% error in the nutrient content of a ration? How does this affect animal production or our ability to predict it? What does it do to costs? The obvious answer is that it depends on which nutrient is involved. The likelihood of making a 2 to 5 % of dry matter error is high with current methods of calculating non-fiber or non-structural carbohydrates (NSC). The NSC are among the most digestible nutrients, consisting of organic acids (a breakdown product of carbohydrates), sugars, starches, pectins, and any carbohydrate soluble in neutral detergent solution. Together with digestible NDF, NSC comprise the main energy sources available in the rumen.

NSC in Dairy Rations

Nutritionists evaluate the level and type of NSC in rations in an effort to assure that the cow is receiving enough ruminally available and total energy, while limiting the overfeeding of NSC with its associated risks of low ruminal pH and acidosis. Accurately assessing NSC in the ration helps nutritionists determine the need for additional energy or undegradable protein supplements to meet cow requirements. Errors in estimating NSC can result in supplementing too much undegradable protein because microbial protein yield is underestimated, or feeding too much energy when the ration was in fact adequate, or in excess of the desired NSC levels.

The types of carbohydrates in a feedstuff's NSC dictate the effect of NSC accounting errors. Because different NSC have different digestion characteristics and predominate in different feedstuffs, the effects vary. There may be changes in the energy provided to the cow or microbes, decreases in microbial yield, decreases in ruminal pH, or all of the above. Understanding the nutritional characteristics of the NSC can help to predict the response to miscalculations in their amounts.

Organic acids in rations for ruminants are comprised largely of partially fermented carbohydrates, as found in silages. Unlike the other NSC, organic acids provide relatively little or no energy for the production of microbial protein in the rumen (Jaakkola and Huhtanen, 1992), although they are an energy source for the cow. Based on their digestion characteristics, they should be handled similarly to fat in determining energy contributions from the ration. Using a ruminal degradation rate near 0%/h with a high intestinal digestibility (high availability to the cow) would appear to accurately describe organic acids' nutritional value.

The remaining NSC support microbial growth and protein production in the rumen, although excesses of certain NSC may contribute to severe declines in ruminal pH through their fermentation to lactic acid under conditions of low ruminal pH. Sugars and starches are digested

by microbes or cow, and may be fermented to lactic acid by rumen microbes (Strobel and Russell, 1986). The cow does not produce the enzymes that digest pectic substances, $(1\rightarrow3)(1\rightarrow4)$-β-glucans, and fructans, so these carbohydrates must be fermented by microbes to be utilized. These "soluble fiber" carbohydrates differ from one another in that fructans in cool season grasses may be fermented to lactic acid (Müller and Steller, 1995), whereas pectic substance and β-glucan fermentations do not produce lactic acid to any appreciable extent (Strobel and Russel, 1986, Van Soest, 1994). As with NDF, the fermentation of pectic substances and β-glucans is reduced at low ruminal pH (Strobel and Russell, 1986, Van Soest, 1994). Current recommendations to maximize milk production and minimize the likelihood of ruminal upset are that NSC provide 35 to 40% of ration DM when ration ingredients are high in sugar and starch, and 40 to 45% when ingredients are low in sugar and starch (Hoover and Miller, 1995). Common sources of the various carbohydrates include: Organic acids: silage; Sugars: molasses, sugar beet and citrus pulps; Starch: Corn and small grain products, potatoes; Fructans: Temperate cool season grasses; Pectic substances: Citrus and sugar beet pulps, legume forages; and $(1\rightarrow3)(1\rightarrow4)$-β-glucans: Small grains, grasses.

Calculating NSC

The errors inherent in the values used to calculate NSC can underestimate its content in feedstuffs, and thereby the nutritional value of the feed. Depending upon the source of the error, computing a more accurate NSC value may be possible. The NSC content of feedstuff dry matter (DM) is a calculated value based upon nutrient percentages subtracted from 100% of feed DM by the equations:

Eqn. 1
NSC% =
100% - (CP% + NDF% + EE% + Ash%)

or

Eqn. 2
NSC% =
100% - [CP% + (NDF% - NDIN%) + EE% + Ash%]

where,
CP = crude protein,
NDF = neutral detergent fiber,
NDIN = neutral detergent-insoluble crude protein, and
EE = ether extract (crude fat).

Although equation 1 is the most commonly used calculation for NSC, the second equation is preferred because it corrects for CP in NDF (NDIN) and so avoids subtracting NDIN twice (as part of CP and as NDIN). Because it is calculated by difference, the errors from the component analyses accumulate in NSC.

Correcting NSC for NPN: Method 1

One error in particular that can cause gross underestimation of feed NSC content, irrespective of how well the component assays are run, is the calculation of mass allocated to CP. Here, "mass" refers to the proportional weight of a component within a feed. For use in ruminant rations, the CP mass of feedstuffs are generally calculated as the nitrogen content (N) x 6.25. This assumes that the combined nitrogenous fractions in a feed contain an average of 16% nitrogen (1 / 0.16 = 6.25). This is not true for all feedstuffs (Table 1) and can be far off the mark for feeds containing appreciable amounts of non-protein nitrogen compounds (NPN) (Table 2, Figure 1). For example, using equation 2, the calculated NSC of a feed that contains 5% urea, 10% ash, 4% EE, 20% CP, 20% NDF, and 2% NDIN on a DM basis, would equal:

100 - [20 CP + (20 NDF - 2 NDIN) + 4 EE + 10 Ash] = 48.0%

In reality, because the urea provides 14.05% CP in the feed (281% CP in urea x 0.05 of feed DM = 14.05% CP of feed from urea), but only takes up 5% of the mass of the feed, a corrected NSC value is calculated as:

$100 - [(20\ CP - 14.05\ CP_{urea} + 5_{urea\ mass}) + (20\ NDF - 2\ NDIN) + 4\ EE + 10\ Ash] = 57.05$

In this case, the corrected NSC value is 9 percentage units of DM more than the commonly calculated version (also see Example Box). This may significantly affect the estimated amount of NSC in the ration, depending upon how much of that particular feed is fed. The magnitude of the effect of NPN on NSC varies with quantity and type of NPN in the feed. The errors inherent in calculating NSC in NPN-containing feeds have the potential to affect the desired outcome of the formulation, whether providing adequate energy to the cow, avoiding ruminal acidosis, or predicting microbial yield.

As shown, the NSC content of feeds containing NPN can be calculated more accurately by replacing the CP term in the NSC calculation with a corrected term for CP mass:

Eqn. 3
Corrected CP Mass% = Total CP % - CP% from NPN + Mass % of NPN Compound

Substituting corrected CP mass for total CP in equation 2, corrected NSC is calculated as:

Eqn. 4
Corrected NSC% = 100% - [Corr. CP mass % + (NDF% - NDIN%) + EE% + Ash%]

When the mass of NPN in the feed is not known, it may be calculated as:

Eqn. 5
Mass% of NPN = (CP% in feed from NPN / CP% of NPN compound) x 100

The source of NPN (urea, ammonium, etc.) must be known so the appropriate CP% for the NPN compound may be used (Table 3).

Combining equations 3 and 5, the corrected CP mass % in a feed in which the amount of NPN compound is not given, is calculated by the equation:

Eqn. 6
Corrected CP Mass % =
Total CP% - CP% from NPN + [(CP% from NPN / CP% of NPN compound) x 100]

Corrected NSC values for feedstuffs can be entered in ration balancing programs to provide a more accurate estimate of ration NSC. The Spartan ration balancing program (Michigan State University) automatically calculates an NSC value that contains the NPN-related errors. To calculate a corrected NSC for the entire ration using Spartan, one of the blank columns in the program can be designated as "CorrNSC" and the corrected NSC values for feeds entered there. Spartan will then calculate the "CorrNSC" content of the ration.

Correcting NSC for NPN: Method 2

Ration formulation/evaluation programs such as the Cornell Net Carbohydrate and Protein System (CNCPS; Cornell University) or the 1996 Beef National Research Council (NRC, 1996) computer models which automatically calculate NSC content of feedstuffs do not allow the input of adjusted NSC values. With these programs, an approach that will achieve NPN-corrected NSC estimates is to partition a feedstuff containing NPN and treat it as if it contained two separate feeds: one that contains nitrogen ($Feed_N$), and one that contains the carbohydrate, fat, and ash ($Feed_C$). The two "subfeeds" are used proportionally so that the sum of their pounds of DM equals that of the original feed.

The first step is to calculate the corrected CP mass % as shown in equation 6. Then, the corrected non-CP mass % (carbohydrate, fat, ash) of the feed DM is calculated as:

Eqn. 7
Corrected Non-CP Mass % = 100% DM - Corr. CP mass %

To allocate the feed into nitrogenous ($Feed_N$) and non-nitrogenous ($Feed_C$) portions, the corrected CP mass % and non-CP mass % are used to proportionally divide the feed components. For all nitrogenous components, their proportion of $Feed_N$ equals the concentration of the component in the original feedstuff DM divided by the corrected CP mass % for that feed. Just as urea contains 281% CP, the components of $Feed_N$ will add up to more than 100%, which simply reflects the difference between the CP value (N x 6.25), and the true mass of the CP.

Eqn. 8
CP% of $Feed_N$ = (CP% of original feed / Corr. CP mass %) x 100

Eqn. 9
NDIN% of $Feed_N$ = (NDIN% of original feed / Corr. CP mass %) x 100

The proportions of soluble, degradable, and undegradable proteins as a percentage of CP remain the same in $Feed_N$ as in the original feedstuff.

For all carbohydrate, mineral, and fat components, their proportions in $Feed_C$ equal the concentration of that component in the original feedstuff divided by the corrected non-CP mass % for that feed. $Feed_C$ should contain 0% CP, since all CP is included in $Feed_N$. Accordingly, NDIN is subtracted from NDF so that only the non-CP portion of NDF is included in $Feed_C$. The components included in $Feed_C$ add up to 100%.

Eqn. 10
Corr. NSC% of $Feed_C$ = Corr. NSC% / Corr. non-CP mass %

Eqn. 11
NDF% of $Feed_C$ = (NDF% - NDIN%) / Corr. non-CP mass %

Eqn. 12
EE% of $Feed_C$ = EE% / Corr. non-CP mass %

Eqn. 13
Ash% of $Feed_C$ = Ash% / Corr. non-CP mass %

Eqn. 14
Mineral Ca % of $Feed_C$ = Mineral Ca% / Corr. non-CP mass %

The same energy values given for the original feed are entered in the analyses for both $Feed_N$ and $Feed_C$. In this way, the same total number of Mcal of NE or pounds of TDN in the original feed will be provided by the combination of $Feed_N$ and $Feed_C$. For instance, if a feed has an NEL, Mcal/lb of 0.78, the NEL of both $Feed_N$ and $Feed_C$ are 0.78.

To calculate the number of pounds of DM from the original feed to allocate to $Feed_N$ and $Feed_C$, multiply the corrected mass percentages times the pounds of DM of the original feed:

Eqn. 15
$Feed_N$ DM lb = Original Feed DM lb x Corr. CP mass %

Eqn. 16
$Feed_C$ DM lb = Original Feed DM lb x Corr. non-CP mass %

In summary, to use this approach with a ration balancing program,

1. Calculate corrected CP mass % and non-CP mass %.
2. Calculate the compositions of $Feed_N$ and $Feed_C$.
3. Enter the compositions of $Feed_N$ and $Feed_C$ into the ration balancing program.
4. Take the pounds of the original feed DM and multiply it by the corrected CP mass % to use as the number of pounds of $Feed_N$, and multiply by the corrected non-CP mass % to use as the number of pounds of $Feed_C$.
5. Enter all protein solubility and degradability values (% of CP) under the analysis for $Feed_N$ as the same percentages given for the original feed.
6. Enter energy values (NEL, TDN, ME) for both $Feed_N$ and $Feed_C$ as the same energy value given for the original feed.

(See Example Box for sample calculations and Table 4 for equations.)

Recalculation of NSC based on NPN content allows for more accurate accounting of the nutrient content of feedstuffs. Examples of differences in calculated NSC content of commercial feeds is given in Table 4. The impact that the correct calculation of NSC has on ration formulation using the standard and NPN-modified methods of calculation is a function of the degree of inaccuracy of the NSC value, the quantity of the NPN-containing feed that is fed, and the types of carbohydrate in the NSC. One example is a case where 6.5 lb (4.0 lb DM) of a liquid feed containing ammonium lactate (analysis in Table 3) was included in a milking herd ration for cows producing approximately 80 lb milk and consuming 56 lb of DM. Both Spartan and CNCPS calculated the feed's NSC as 19.6% of DM. After correcting for NPN, the feed's NSC content was given as 67.3% of DM, and ration NSC rose from 38% to 42% of DM. In CNCPS, the combined A and B1 carbohydrate pool (A = sugars and organic acids, B1 = starch and pectin) contribution from the liquid feed rose from 355 g to 1236 g after the division of the feed into nitrogen-containing and nitrogen-free components.

Summary

Non-structural carbohydrates (NSC) are a very digestible, diverse group that vary in their digestion characteristics. Because NSC content is calculated by difference, the accuracy of the value is affected by errors in its component analyses. One error in particular that can cause gross underestimation of NSC is due to miscalculation of the mass contributed by non-protein nitrogen (NPN). Correction for the NPN-related error provides a more accurate NSC value for use in ration formulation. Similar corrective calculations may also find application in the estimation of TDN and digestible energy (J. E. Moore, personal communication). In terms of predicting energy supply to the cow, or energy to support ruminal microbe production, accurate accounting of all carbohydrate fractions is essential.

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Table 1. Factors for conversion of nitrogen to protein for foods and feeds (adapted from Jones, 1931) [1].

Food	Factor	Food	Factor
Eggs	6.25	Navy beans	6.25
Gelatin	5.55	Lima beans	6.25
Meat	6.25	Soy beans	5.71
Milk	6.38	Peanut	5.46
Barley	5.83	Almonds	5.18
Corn	6.25	Cottonseed	5.30
Rice	5.95	Sesame	5.30

[1] Factors determined on isolated protein fractions.

Table 2. Nitrogen and crude protein contents of non-protein nitrogen sources.

NPN Source	N% of DM	CP% as N x 6.25
Ammonia	82.35	514.7
Ammonium (NH_4^+)	77.78	486.1
Ammonium chloride	26.19	163.7
Ammonium Phos (Mono)	11.10	69.4
Ammonium Phos (Dibasic)	18.06	112.9
Ammonium Sulfate	21.46	134.1
Nitrate	22.56	141.0
Urea, 45% N	44.96	281.0
Urea, 46.5% N	46.56	291.0

Table 3. Corrected NSC content of example commercial feeds containing NPN (all values as % of feed dry matter).

Nutrient (% of DM)[1]	Liquid whey w/ ammonium lactate	40% CP molasses supplement	20% CP dairy feed, 2% urea	32% CP dairy feed, 5% urea
CP	71.5	62.9	22.2	35.6
NDF	0.0	0.0	8.0	11.0
NDIN	0.0	0.0	1.0	2.0
EE	1.0	0.0	3.3	3.3
Ash	7.9	14.3	7.8	7.8
CP from NPN	60.2	57.1	6.2	15.6
Type of NPN	ammonium	urea	urea	urea
Corrected CP mass	22.9	25.4	18.2	25.6
Corrected non-CP mass	77.1	74.6	81.8	74.4
Calculated NSC	19.6	22.8	59.7	44.3
Corrected NSC	67.3	60.3	63.7	54.3

[1] CP = crude protein, NDF = neutral detergent fiber, NDIN = CP in NDF, EE = ether extract, NSC = non-structural carbohydrate.

Table 4. Equations for correcting NSC for NPN

	Eqn.
Correction Method 1	
$NSC\% = 100\% - (CP\% + NDF\% + EE\% + Ash\%)$	1
$NSC\% = 100\% - [CP\% + (NDF\% - NDIN\%) + EE\% + Ash\%]$	2
Corrected CP Mass% = Total CP % - CP% from NPN + Mass % of NPN Compound	3
Corrected NSC% = 100% - [Corr. CP mass % + (NDF% - NDIN%) + EE% + Ash%]	4
Mass% of NPN = (CP% in feed from NPN / CP% of NPN compound) x 100	5
Corrected CP Mass % =	
Total CP% - CP% from NPN + [(CP% from NPN / CP% of NPN compound) x 100]	6
Correction Method 2	
Corrected Non-CP Mass % = 100% DM - Corr. CP mass %	7
CP% of $Feed_N$ = (CP% of original feed / Corr. CP mass %) x 100	8
NDIN% of $Feed_N$ = (NDIN% of original feed / Corr. CP mass %) x 100	9
Corr. NSC% of $Feed_C$ = (Corr. NSC% / Corr. non-CP mass %) x 100	10
NDF% of $Feed_C$ = [(NDF% - NDIN%) / Corr. non-CP mass %] x 100	11
EE% of $Feed_C$ = (EE% / Corr. non-CP mass %) x 100	12
Ash% of $Feed_C$ = (Ash% / Corr. non-CP mass %) x 100	13
Mineral Ca % of $Feed_C$ = (Mineral Ca% / Corr. non-CP mass %) x 100	14
$Feed_N$ DM lb = Original Feed DM lb x Corr. CP mass %	15
$Feed_C$ DM lb = Original Feed DM lb x Corr. non-CP mass %	16

Examples Correcting NSC for NPN

Feed X Analysis (DM basis)

CP%	71.5%
CP% from NPN	60.2%
NDF%	5.0%
NDIN%	0.5%
EE %	1.0%
Ash %	7.9%
NPN source	urea

Uncorrected NSC% of DM for Feed X — Equation

= 100 - (71.5 CP + (5.0 NDF - 0.5 NDIN) + 1.0 EE + 7.9 ash) = 15.1% NSC (2)

Method 1. To calculate a corrected NSC for feed X:

Corrected CP Mass%

= 71.5% CP - 60.2% CP_{urea} + [(60.2% CP_{urea} / 281% CP_{urea}) x 100] (6)

= 11.3% non-urea CP + 21.4% mass from urea

= 32.7% corrected CP mass %

Corrected NSC% of DM

= 100 - (32.7 Corr. CP mass + (5.0 NDF - 0.5 NDIN) + 1.0 EE + 7.9 Ash) (4)

= 53.9% corrected NSC %

Method 2. To calculate CP-containing ($Feed_N$) and CP-free ($Feed_C$) fractions for use in ration formulation programs that automatically calculate NSC.

Corrected CP Mass% from above, equation 6 = 32.7

Corrected Non-CP Mass % = 100 - 32.7 = 67.3 (7)

Calculated compositions of $Feed_N$ and $Feed_C$ (DM basis):

Feed Fraction	% in Original Feed X on a DM basis		Corrected CP Mass %		Fraction % Feed X_N	Equation
CP	(71.5	/	32.7) x 100	=	218.7	(8)
NDIN	(0.5	/	32.7) x 100	=	1.5	(9)
Feed Fraction	**% in Original Feed X**		**Corrected Non-CP Mass %**		**Fraction % Feed X_C**	
NDF - NDIN	(5.0 - 0.5 = 4.5	/	67.3) x 100	=	6.7	(11)
EE	(1.0	/	67.3) x 100	=	1.5	(12)
Ash	(7.9	/	67.3) x 100	=	11.7	(13)
Corr. NSC	(53.9	/	67.3) x 100	=	80.1	(10)

Calculation of pounds allocated to $Feed_N$ and $Feed_C$ from 10 pounds of the original feed DM:

Feed X_N DM lb = 10 lb Feed X DM x 32.7% Corr. CP Mass = 3.27 lb (15)

Feed X_C DM lb = 10 lb Feed X DM x 67.3% Corr. Non-CP Mass = 6.73 lb (16)