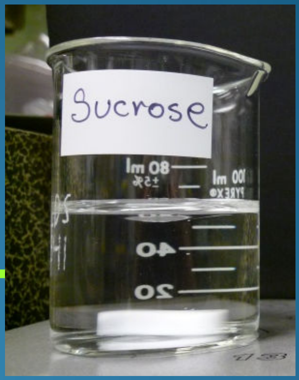
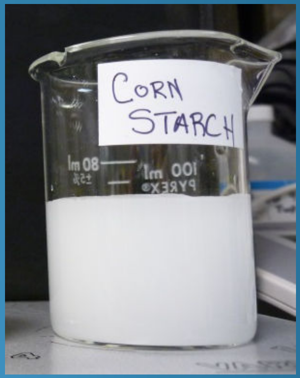
Success and Continuing Challenges in Analyzing Nonfiber Carbohydrates





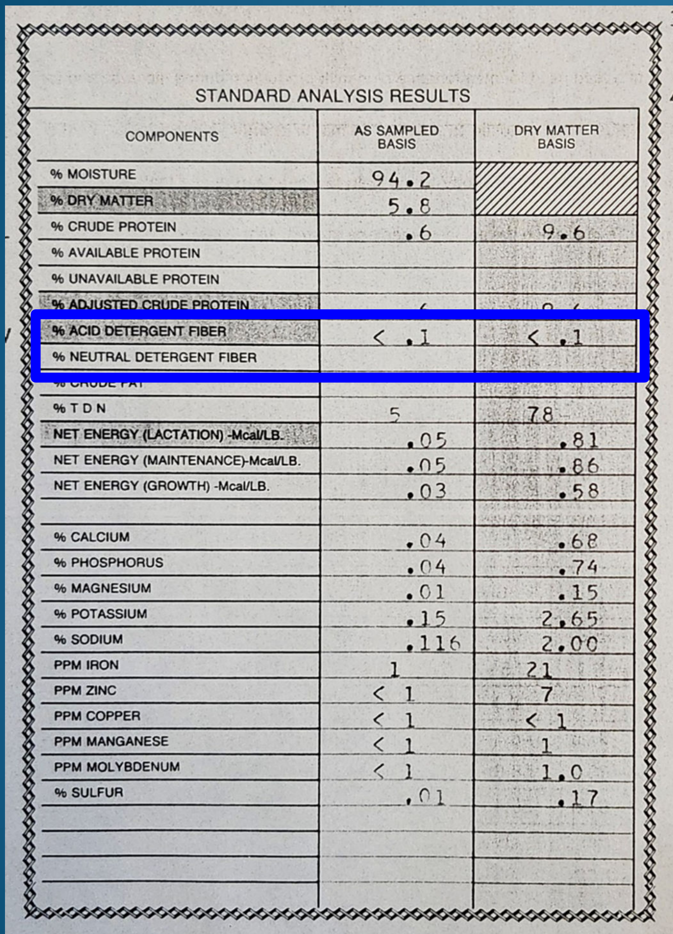
Mary Beth Hall, PhD, PAS

The Cows Are Always Right LLC

TCAAR

ADSA 2025

1980s: Carbohydrates

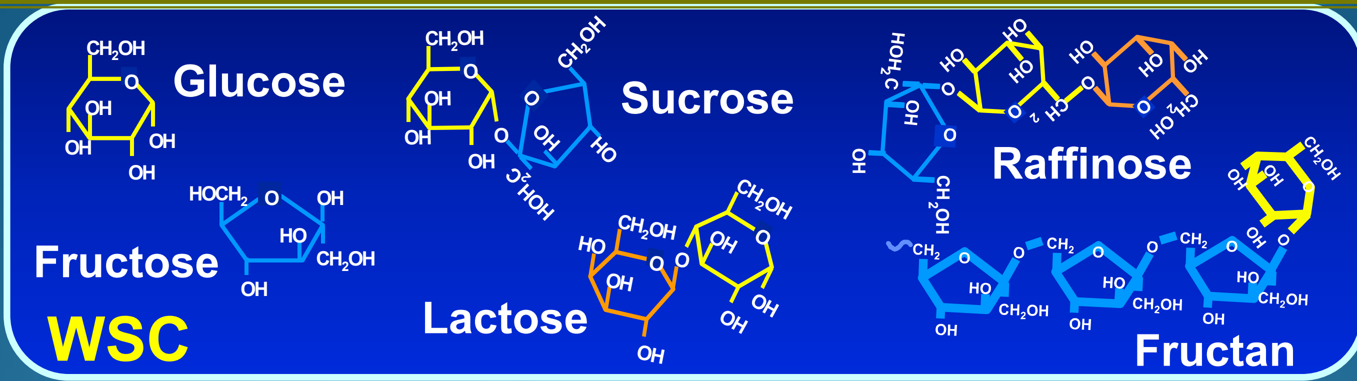


STANDARD ANALYSIS RESULTS

COMPONENTS	AS SAMPLED BASIS	DRY MATTER BASIS
% MOISTURE	94.2	
% DRY MATTER	5.8	
% CRUDE PROTEIN	.6	9.6
% AVAILABLE PROTEIN		
% UNAVAILABLE PROTEIN		
% ADJUSTED CRUDE PROTEIN	[illegible]	[illegible]
% ACID DETERGENT FIBER	< .1	< .1
% NEUTRAL DETERGENT FIBER		
% CRUDE FAT		
% T D N	5	78
NET ENERGY (LACTATION) -Mcal/LB.	.05	.81
NET ENERGY (MAINTENANCE)-Mcal/LB.	.05	.86
NET ENERGY (GROWTH) -Mcal/LB.	.03	.58
% CALCIUM	.04	.68
% PHOSPHORUS	.04	.74
% MAGNESIUM	.01	.15
% POTASSIUM	.15	2.65
% SODIUM	.116	2.00
PPM IRON	1	21
PPM ZINC	< 1	7
PPM COPPER	< 1	< 1
PPM MANGANESE	< 1	1
PPM MOLYBDENUM	< 1	1.0
% SULFUR	.01	.17

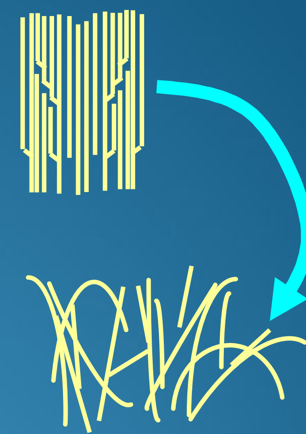
- 2001 Dairy NRC was the first to reference carbohydrates other than fiber.
- **NFC by-difference**:
100% of DM - crude protein - crude fat - NDF - ash

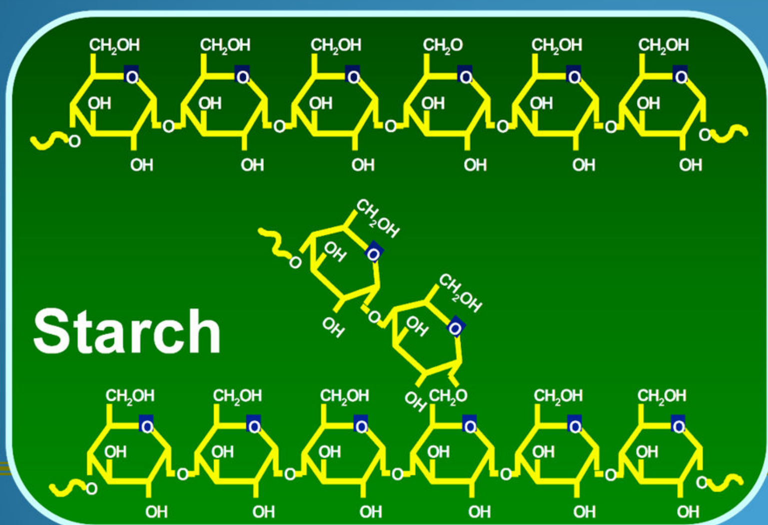
What we strive for is for analyses to be nutritionally relevant and reflect the value to and effect on the animal.

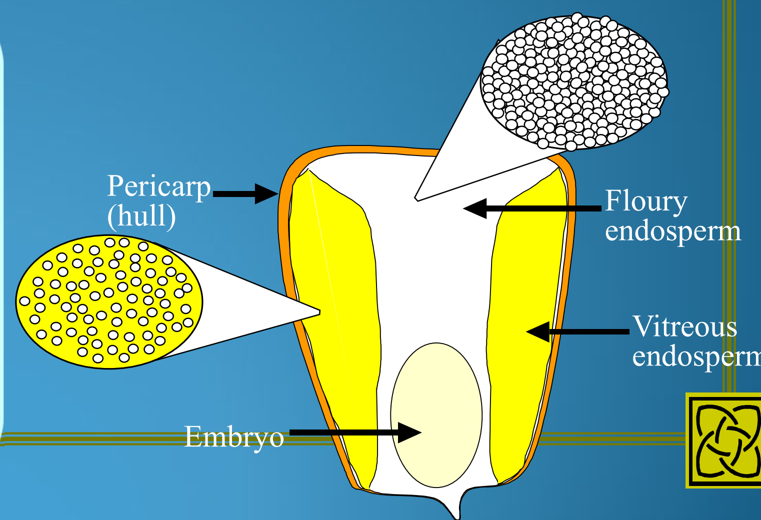


- Water-soluble carbohydrates are not “Sugars”
- “Sugars” (mono-, di-), oligo-saccharides, fructans
- Digestible by microbes; some in small intestine
- Readily available to the microbes
- Can ferment to lactic acid
- May give microbial glycogen
- Give a greater molar proportion of butyric acid

- Glucose in α-(1-4) and α-(1-6) linkages
- Digestible by microbes and cow
- Can ferment to lactic acid
- Digestion affected by:
  - Protein matrix around granules
  - Gelatinization or damage to starch
  - Particle size, moisture content





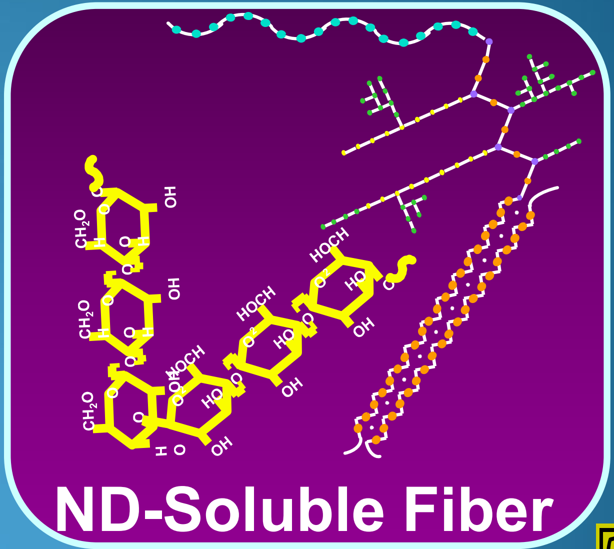


- Pectins, mixed linkage beta-glucans, gums, other
- Polysaccharides (?) soluble in neutral detergent
- Complex, diverse compositions
- Only digestible by microbes
- Behave like fiber?

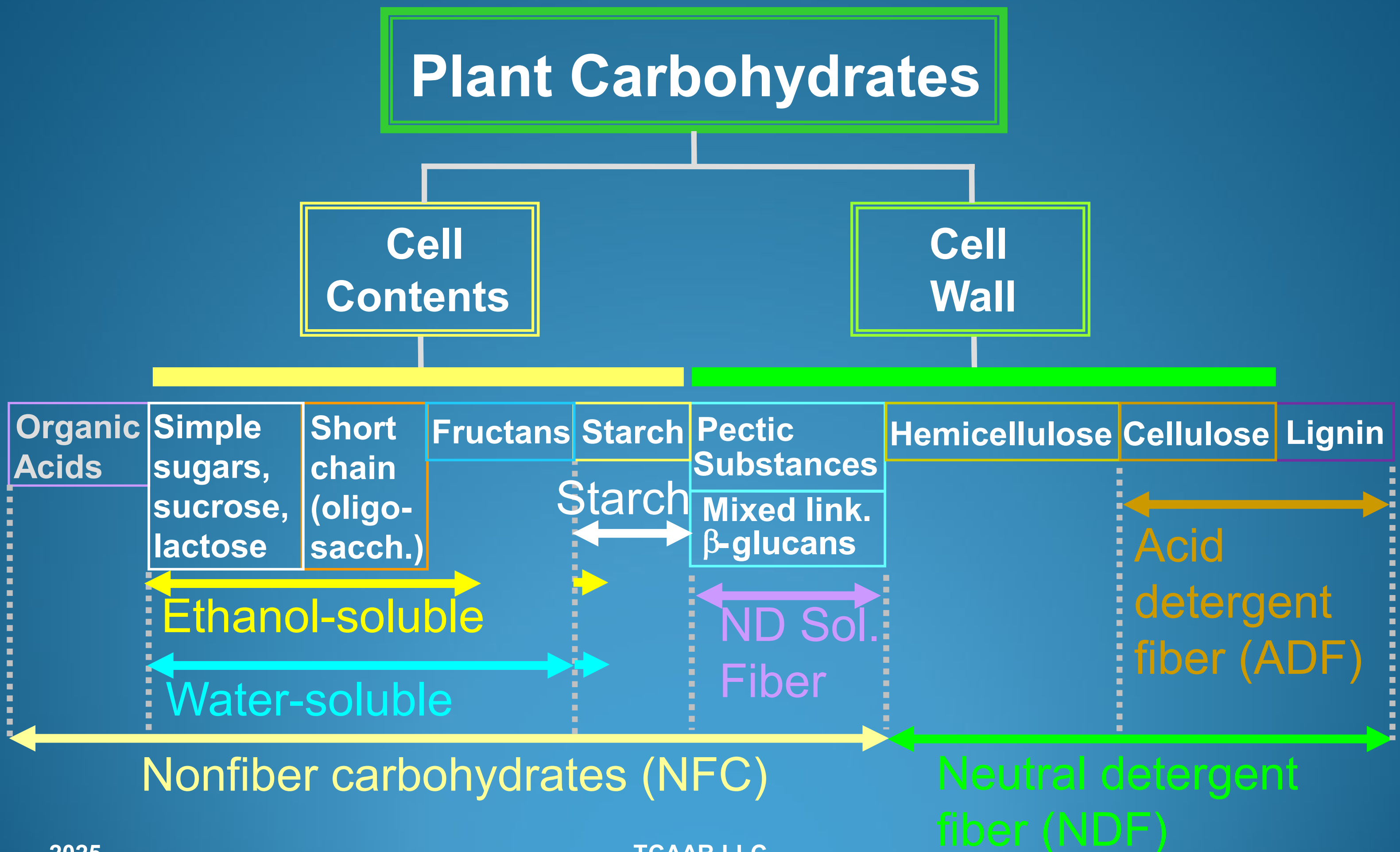
Pectin Fermentation:

- Low pH reduced fermentation
- No lactic acid
- Highest acetate (10.1:1.3)

Strobel and Russell, 1986



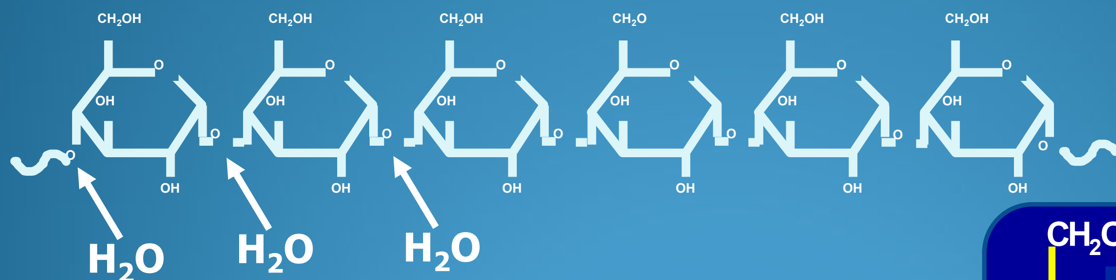
Carbohydrate Fractions

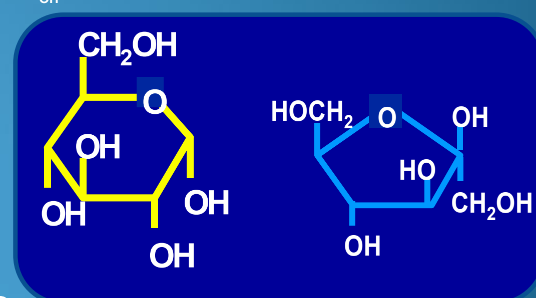


Dietary Starch: Enzymatic Analysis

- **Gelatinization**
  - Disrupting the hydrogen bonding/ crystalline structure of starch chains

- **Hydrolysis**
  - α-amylase (α-(1-4) endoamylase)
  - amyloglucosidase [α-(1-6), α-(1-4), non reducing end]





- **End product detection**
  - Glucose, reducing sugars
  - Glucose x ((180-18)/180) = Starch

Dietary Starch Analysis

100°C, 1 h
Vortex 3x

- Volumetric additions.
- Run w/ & w/o enzymes.

50°C, 2 h
Vortex 1x

Sample
0.1 *M* Acetate buffer, pH 5.0
Thermostable α-amylase

Amyloglucosidase

+20 mL H_2O. Mix. Clarify. Dilute, centrifuge, & analyze for glucose

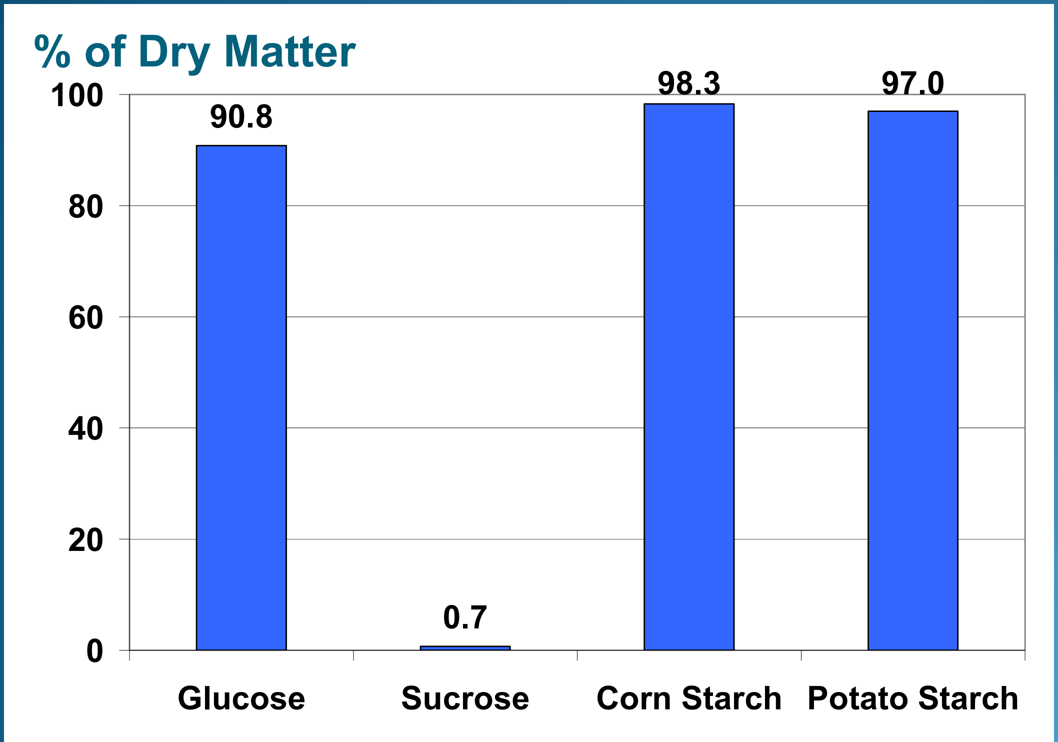
Dietary Starch = (Glc – free Glc) x 0.9

Danish Institute of Agricultural Sciences

Based on Bach Knudsen, 1997

Hall, 2009; Hall and Keuler, 2009; Hall, 2015; AOAC Official Method 2014.10

Dietary Starch Analysis



Repeatability: S_r = 0.5%

Limit of Determination: 0.3%

- Include Glc and starch as controls.
- Use sucrose in initial tests of reagents and assay.
- Ok for mass except does not include resistant starch.

7 Feeds

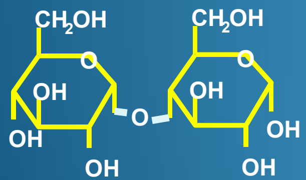
1-80% DS

CV% = 1.55

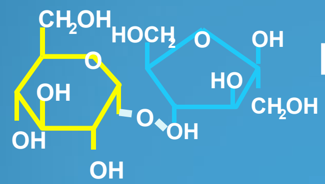
Hall, 2009, JAOACI 92:42
Hall, 2015, JAOACI 98:397

Sources of Error

- Non-acidic solutions
- Inaccurate standard curve
- Glucose standard purity
- Incomplete hydrolysis
- Microbial predation
- Volumetric accuracy
- Interfering substances: Anti-oxidants, non-specific enzymes & run conditions
- Accurate glucose measurement



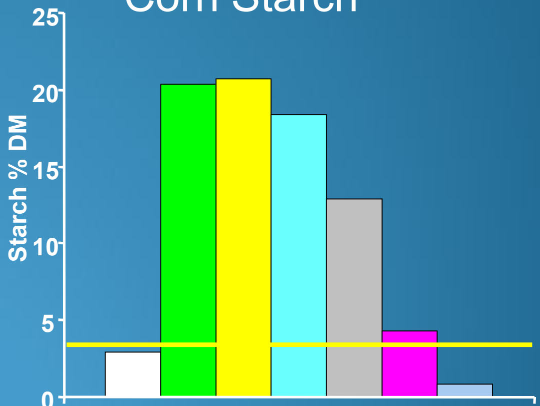
Maltose



Maltulose

Dias and Panchal, 1987

Corn Starch

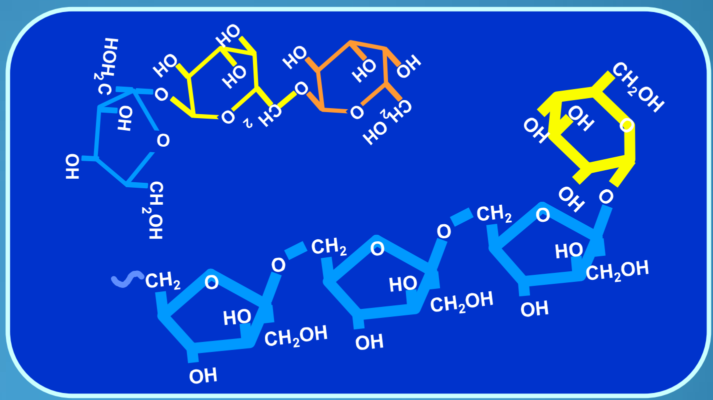


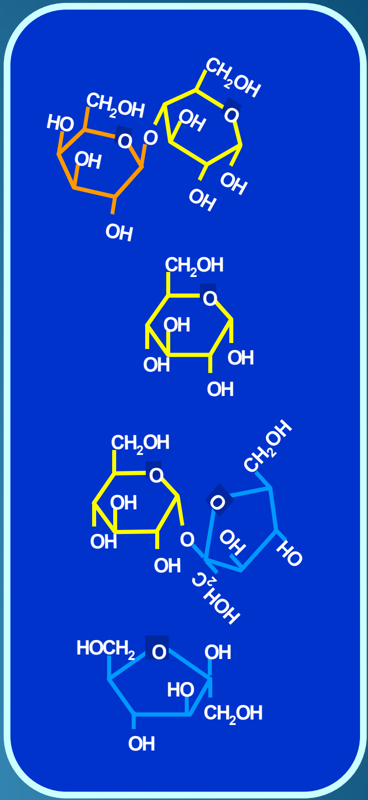
Confectioner's Sugar

Hall et al., 1999

Soluble Carbohydrate Methods

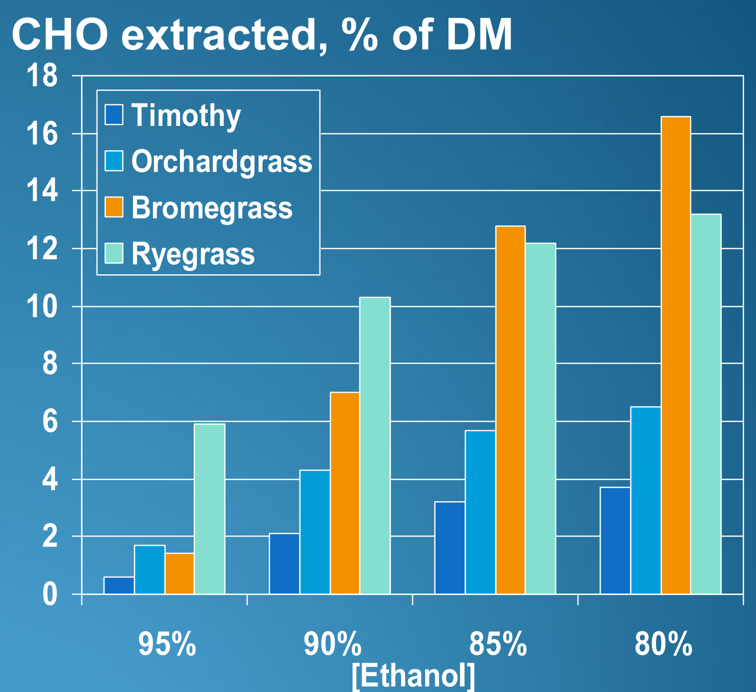
- Soluble in What? Water or ethanol
- Colorimetric: Phenol-sulfuric acid, reducing sugar
- Chromatography: needs standards for specific carbohydrates. Hydrolysis?
- Total sugars as invert: official method for sucrose in molasses, ≠ WSC or ESC
- Polarimetry: official method, various interferences





Aqueous Alcohol Extractions

- Materials extracted vary with [EtOH]
- Fructans are variably soluble in 70-90% EtOH, presumably based on degree of polymerization (DP)
- May extract a small amount of uronic acid (Bailey et al., 1978)
- Lactose is insoluble in 80% ethanol, becomes soluble at ~50% ethanol
- Excludes CHO that can ferment to lactic acid.
- Soluble starch? DP?



80% EtOH ESC omits lactose and long chain fructans.

Smith and Grotelueschen, 1966

Issues with WSC Analysis

- The carbohydrate standard used alters results.
- Use sucrose, or the carbohydrate that best represents the WSC in the samples. Lactose for milk products. With grasses, fructose or inulin? Glucose for soluble starch?

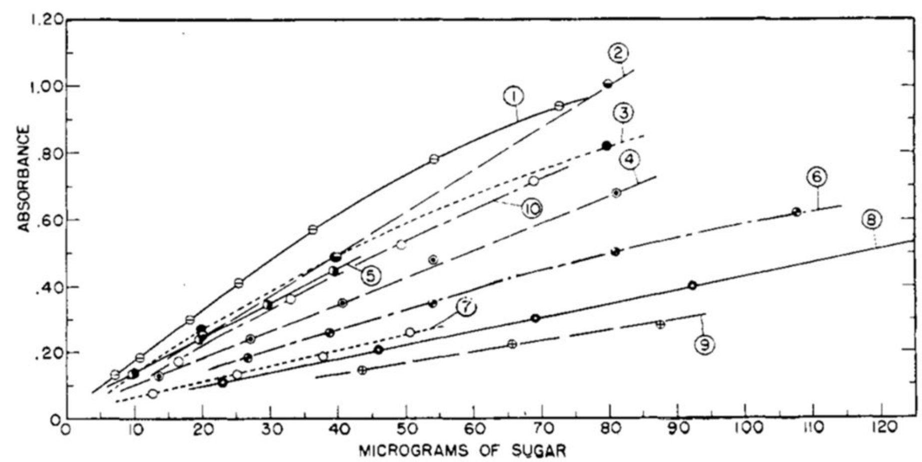
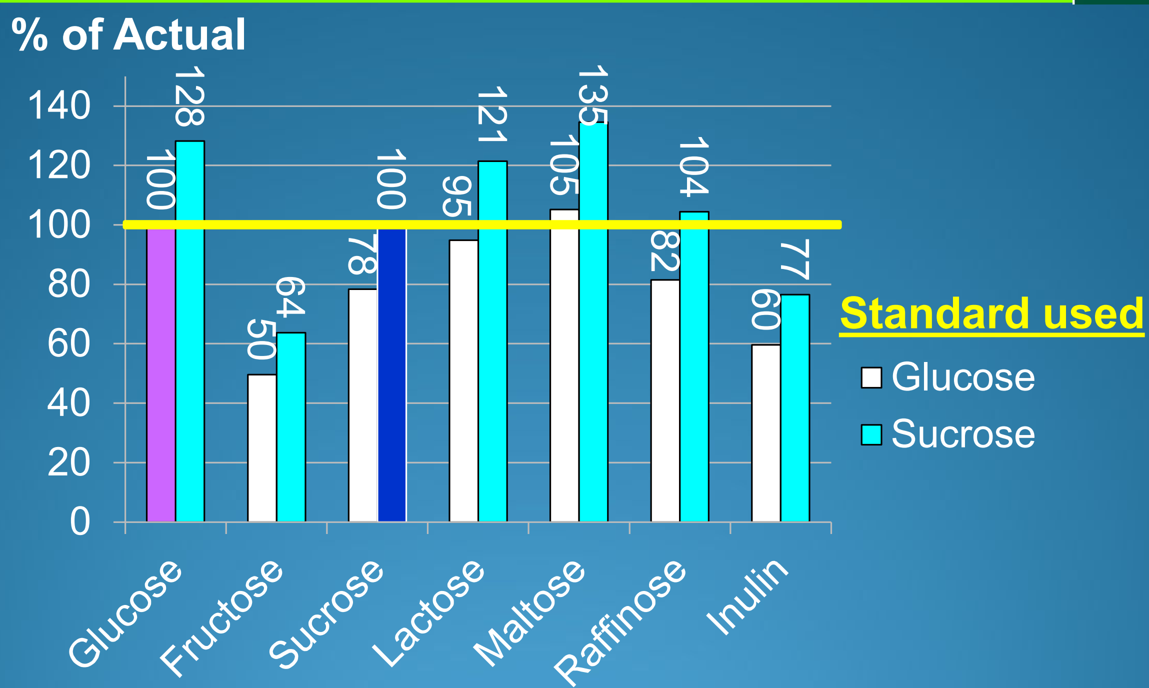


Figure 1. Standard curves

1. D-Xylose, Coleman Jr., 480 mμ, 17 mg. of phenol
2. D-Mannose, Beckman Model DU, 490 mμ, 40 mg. of phenol
3. D-Mannose, Evelyn, filter No. 490, 40 mg. of phenol
4. D-Galactose, Coleman Jr., 490 mμ, 33 mg. of phenol
5. L-Arabinose, Coleman Jr., 480 mμ, 17 mg. of phenol
6. D-Galacturonic acid, Coleman Jr., 485 mμ, 17 mg. of phenol
7. L-Fucose, Coleman Jr., 480 mμ, 40 mg. of phenol
8. D-Glucurone, Coleman Jr., 485 mμ, 17 mg. of phenol
9. 2,3,4,6-Tetra-o-methyl-D-glucose, Coleman Jr., 485 mμ, 17 mg. of phenol
10. D-Glucose, Beckman Model DU, 490 mμ, 100 mg. of phenol

Dubois et al., 1956

WSC like CP?: Standards and Mass

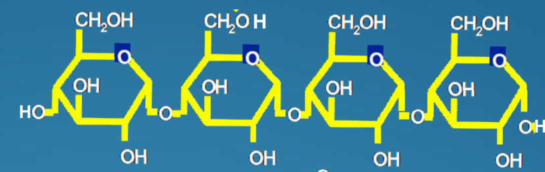


- No carbohydrate standard perfectly describes the mass amount of the diverse carbohydrates in WSC.
- We calibrate to an answer like we calibrate to N x 6.25?

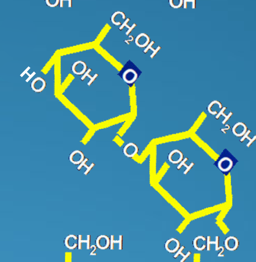
Hall, 2013

Starch in WSC?

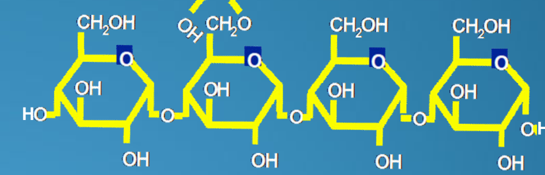
Amylose
Amylopectin



Baking
Some processing
Enzymes
Crop traits



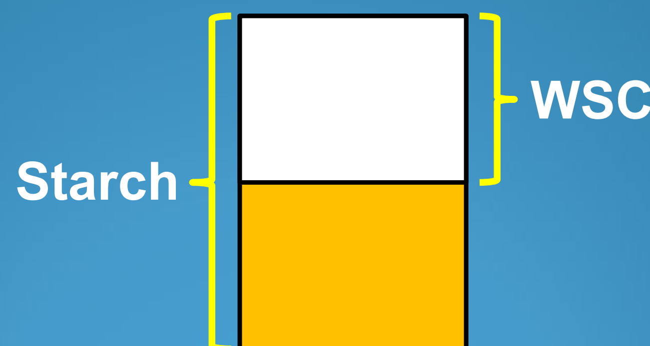
Starch "fragments" (dextrins, oligosaccharides)



- Partially hydrolyzed starch can be water-soluble
- Starch value looks normal, WSC looks high, >100% of DM?
- WSC starch is also in "Starch": double accounting
- Soluble starch in ESC?

Nutritionally, where does this fraction belong? Starch or WSC?

That dictates the analysis.



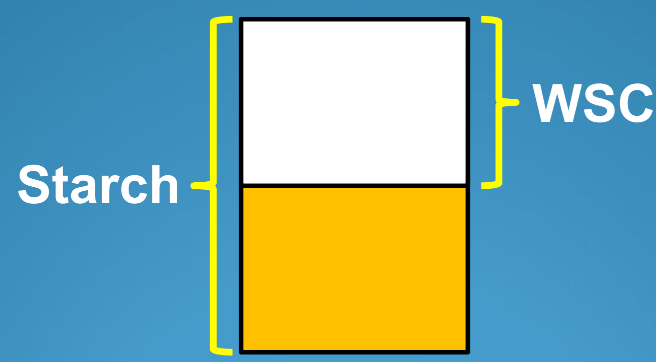
Maltodextrins & Cows

Not much information available.

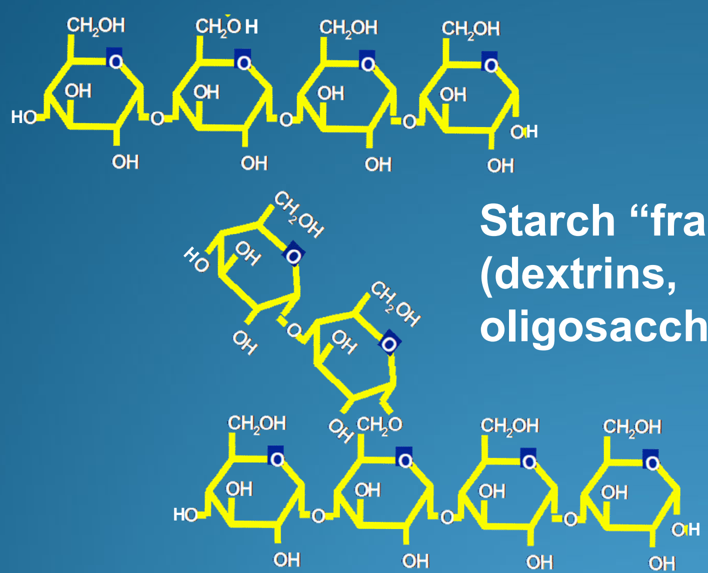
- 2 studies showed increases in butyrate molar %, tendency for increase in milk fat when maltodextrins were fed / made with enzyme.
  - Kim et al., 1999, J. Sci. Food Agric. 79:1441-1447
  - Tricarico et al., 2005, Animal Science. 81:365-374
- Butyrate and milk fat typically increase when sugars/WSC are fed.

Suggests putting maltodextrins with WSC to fit nutritionally.

Problem: Can't just subtract WSC from total starch. The phenol-sulfuric acid assay and starch assays are not on the same mass basis.



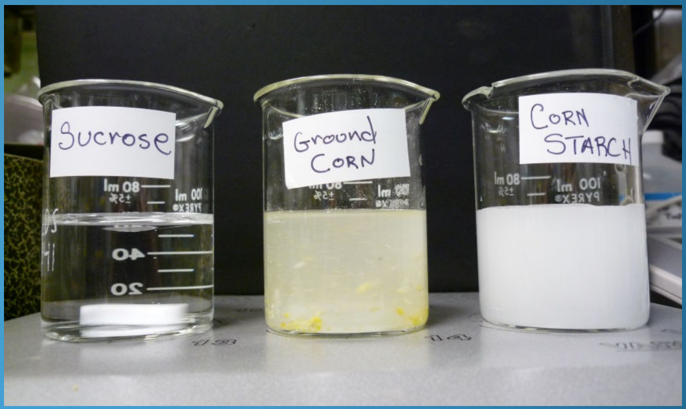
Water-Soluble Starch Analysis



Starch "fragments" (dextrins, oligosaccharides)

- Water-soluble starch analyzed as starch can be subtracted from dietary starch because they are on the same basis.

- Damaged starch fragments in water extract (cloudy extract):
  - May pass through filters
  - Handled by centrifugation, fine filters (?)



WSC Analysis

40°C, 1 h
Agitation or vortex 3x

Centrifuge to clarify (12,000 *x g*), or filter (Whatman 54)

Sample 0.2 g
Water, 35 mL

Dilute (usually 1 in 10 in sufficient).
Analyze by phenol – sulfuric acid assay.

0.5 mL sample soln
0.5 mL 5% phenol
2.5 mL conc. H_2SO_4
4 sucrose standards, 0–100 µg/mL in 0.2% benzoic acid

30°C 20 min → R.T. 30 min →

Read absorbance at 490 nm

Hall, 2014
Dubois et al., 1956

Analysis: Soluble Starch

40°C, 1 h
Agitation or vortex 3x

Centrifuge to clarify (12,000 *x g*), or filter (GFA?)

Sample 0.2 g
Water, 35 mL

Analyze supernatant for free glucose & for starch using amyloglucosidase.

Need clear supernatant.
No need to gelatinize.

Hall, 2020

By-Difference Fractions

"The greatest obstacle to discovery is not ignorance - it is the illusion of knowledge. "
-- Daniel J. Boorstin

Assumption: All analytes should sum to 100% of DM.

- 1864: Nitrogen-Free Extract (stickstofffreien Extraktstoffe) as 100% of DM minus CP, CFiber, CFat, ash, and possibly CP in crude fiber (Henneberg and Stohmann, 1864; Tollens, 1897)
- Revised to use NDF instead of crude fiber: **N**on**F**iber**C**HO
- Residual OM also subtracts starch (NASEM, 2021)
- NDF – ADF = Hemicellulose (?)

By-Difference: Estimating Mass Is Hard

- **Crude protein factors**: based on N content of the predominant **true protein**, does not consider & overestimates nonprotein nitrogen mass. 6.25 does not always apply.
- Component assays: gravimetric, colorimetric, HPIC…
- Does not account for things we do not measure.
- Errors from all analyses reside in this term.
- Variance of a by-difference fraction is the sum of the variances of the analytes subtracted.

CP Factors

Corn grain	**6.25**
Soybean meal	**5.71**
Milk	**6.38**
Wheat	**5.83**
Barley	**5.83**

NPN not included.

Jones, 1941
Merrill and Watt, 1973

True Protein% of N

Grass meal	**79.5**
Barley	**90.0**
Soybean meal	**97.3**
Fish meal	**83.7**

Boisen et al., 1987

By-Difference: A Bad Idea

- Plant organic acids (Hall et al., 1999)

Plant organic acids = (OM – CP) – (EIROM – EIRCP) – EE – TESC

Assumes:

- 6.25 CP factor is correct and equivalent across fractions.
- TESC gives the correct mass.
- Extraction of crude fat with 80% EtOH tested only on few, low fat samples could be applied more broadly.
- Nothing else besides plant organic acids is in the residual fraction.

By-Difference: Smaller Fraction Issues

- CNCPS Soluble fiber (Lanzas et al., 2007; Higgs et al., 2015)

100% of Dry Matter (DM) =
CP + EE + ash + aNDFom + acetic + propionic + isobutyric + butyric + lactic + other organic acids + WSC + starch + soluble fiber

NFC = 100% of DM – (CP + EE + ash + aNDFom)

Soluble Fiber = 100% of DM – (CP + EE + ash + aNDFom) – (acetic + propionic + isobutyric + butyric + lactic + other organic acids + WSC + starch)

By-Difference: Smaller Fraction Issues

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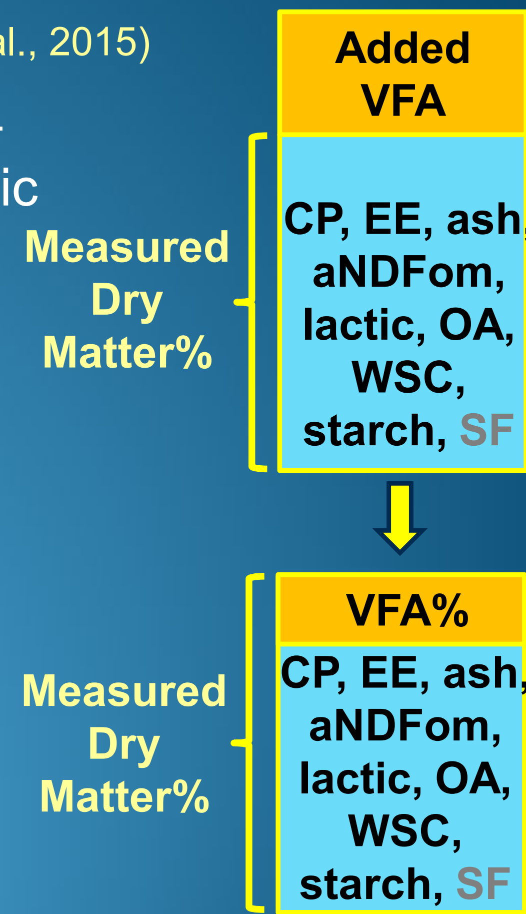
Soluble Fiber = 100% of DM – (CP + EE + ash + aNDFom) – (acetic + propionic + isobutyric + butyric + lactic + other organic acids + WSC + starch)

Volatile VFA: added to DM where they are not.

- Acetate losses on drying: 83% corn silage, 57% grass silage (80°C; Sørenson, 2004); grass silage, mean 87.9%, 72.7 to 98.4% (100°C; McDonald and Dewar, 1960). Average 1-3% of DM (Kung et al., 2018)
- No indication that measured DM was adjusted for added VFA in the calculations.
- Added VFA in measured DM reduces calculated soluble fiber.



By-Difference: Smaller Fraction Issues

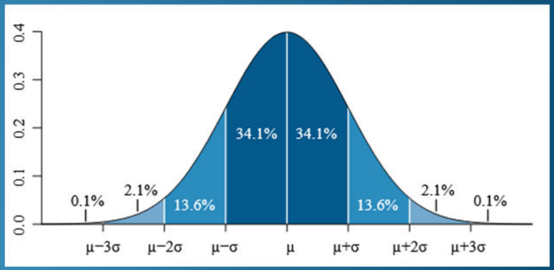
- CNCPS Soluble fiber (Lanzas et al., 2007; Higgs et al., 2015)

Soluble Fiber = 100% of DM – (CP + EE + ash + aNDFom) – (acetic + propionic + isobutyric + butyric + lactic + other organic acids + WSC + starch)

The by-difference calculated soluble fiber would be:

- Decreased by volatilized VFA added to, but not in, DM.
- Affected by mass accuracy of **WSC** and **CP** fractions.
- Affected by errors & variability in assays (e.g., aNDFom).
- Assumed to not include other unmeasured fractions.
- A smaller value is prone to going negative.

It would have a variance equal to the sum of the variances of the 9 or 13 analytes used in its calculation.



Summary

We've come a long way, but have more to do.

- **Dietary Starch**: In good shape. Ok for mass.
- **Soluble Starch**: Verify its nutritional home.
- **WSC**: Something we calibrate to? Alternatives that cover the carbohydrate ground?
- **By-Difference Fractions**: Recognize that the way they are currently assessed, they are nutritional black boxes to which we calibrate our nutritional models. They are affected by diverse errors and assumptions. Is accounting for 100% of DM realistic?
- If a fraction matters nutritionally, define it and come up with a way to measure it so we can work with it.


Questions?