Efforts to Quantify Changes in Near-Infrared Spectra Caused by the Influence of Water, pH, Ionic Strength, and Differences in Physical State

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The application of near-infrared spectroscopy to high-moisture samples has shown that the accuracy does not match that found for dried materials. The objective of this work was to attempt to quantify the effects of water, pH, ionic strength, and differences in physical state on near-infrared spectra with the use of model compounds. Spectra were compared by regression analysis of second derivatives after spectral subtraction of water. Spectra from 4900 to 4100 cm^{-1} at a resolution of 4 cm^{-1} were examined. Regression results showed spectra to be more similar among amorphous sugars and among dissolved sugars than among crystalline sugars. Also, spectra of amorphous sugars were statistically more similar to spectra of dissolved sugars than to spectra of crystalline sugars. While the spectra of one dissolved or amorphous sugar were statistically similar, this was not true for amino acids. Spectra of amorphous amino acids were similar to those of crystalline forms and neither were similar to those of dissolved forms. Spectrally, polymeric carbohydrates appeared very similar to one another when dry and behaved like amino acids when wet. Finally, efforts to directly relate these findings to near-IR spectroscopy calibration problems will require further research.

Index Headings: **Near-infrared spectra; Water; pH; Physical state; Ionic strength.**

INTRODUCTION

While near-infrared reflectance spectroscopy (NIRS) has been used extensively for the analysis of dried feedstuffs, with hundreds of references being available, less research has been done on the analysis of high-moisture agricultural materials such as silage.[1-3] Still, the application of near-IR spectroscopy to high-moisture samples has shown that results do not match those of dried materials,[1,3-5] with the *r*-square (R^2) and standard error (SE) values for dried materials consistently better than those for wet. With the use of dry ice to grind samples,[3] it was shown that near-IR spectroscopy could be used with undried silages to determine many components of interest, although the results were not as good as found for dried samples (lower R^2 and higher SE).

A second study[4] examined the question of how the method of sample presentation, the amount of sample scanned, the sample grind, the spectral region scanned (680–1234, 1100–2498, and 680–2498 nm), and the particular chemistry of interest (dry matter, fiber, protein, etc.) interacted to influence the quality of near-IR calibrations. Although the results showed that the accuracy of near-IR determinations on wet silages could be affected by these various parameters, again, the best results (highest R^2s, lowest SEs) were achieved with dried samples. Similar conclusions were drawn from a study using undried and dried *in situ* digested samples.[5]

The question then remained, Why were near-IR results for wet samples less accurate than those for the same samples in dry form? Since samples such as silages are very complex mixtures of monomers (i.e., volatile fatty acids, ethanol, etc.) and polymers (i.e., cellulose, proteins, etc.), and dealing with such a complex mixture is very difficult, a study was designed using model materials (i.e., mixtures of water and single compounds) to find the basis for the poorer performance of near-IR spectroscopy with wet materials.[6] Examination of organic acids, alcohols, ketones, amines, and amides showed the presence of water to cause shifts in spectral wavelengths not related to OH or NH groups. The most significant shifts were for alcohols and ketones (up to 10+ nm at 90% H_2O) and the least for acids. These peak shifts increased with increasing amounts of water and varied within individual spectra and among the compounds tested. Peak shifts, such as those seen in the near-infrared, have been long recognized in the mid-infrared and have been attributed to changes in hydrogen bonding.[7-9]

As solids, sugars and amino acids had many sharp peaks in their spectra; however, with these compounds as solutions, the sharp spectral features disappeared, resulting in large broad peaks. The spectra of polymers such as starch, cellulose, and casein did not appear to be significantly altered by the presence of water (0 to 50% w/w), although the polymers appeared to alter the water spectra. Similarly, in 1960 Kaye showed that the spectra of cellulose could be altered by solvents, although the results were for comparisons between differing solvents, such as tetrachloroethane vs. acetone.[10] Hirschfeld also showed that sodium chloride, which has no near-infrared spectra, can alter the spectra of water in a manner similar to that seen for starch, cellulose, and casein, because of ionic strength and hydrogen-bonding effects.[11]

A study on the effect of ionic strength, pH, and physical states showed that the spectra of materials commonly found in silages can be greatly influenced by pH and, in some cases, by the ionic strength of their watery environment.[12] Non-neutral materials, such as acids and amines, and materials with amino and carboxylic groups, like amino acids, were the most affected by pH, while neutral materials like acetone and ethanol were least affected. Finally, the physical state of compounds (crystalline, amorphous, etc.) also altered near-IR spectra to

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varying degrees. Although similar changes in spectra due to variation in and loss of crystallinity have been well documented in the mid-infrared,[13-15] the spectral alterations and differences do not appear to be as dramatic as those found in the near-infrared.[12]

While the previous studies provide evidence that may explain the near-IR spectroscopy results obtained with high-moisture materials, they were strictly qualitative in nature.[6,12] Also, while previous mid-infrared[7-9,13-15] and near-infrared studies[10-11] may provide the mechanisms for the effects seen in the previous two near-infrared studies,[6,12] they cannot estimate the quantitative effects on near-infrared calibrations. This study was therefore carried out in an effort to quantify the degree of information loss or change that occurs in near-IR spectra under the influence of water, pH, and physical state—the ultimate objective being to determine whether such spectral changes can account for the differences in performance of near-IR spectroscopy when calibrations for wet as opposed to dry forage-type materials are being developed.

EXPERIMENTAL

Spectroscopy. Near-infrared spectra were taken with the use of a dual-bench (near- and mid-infrared) Digilab FTS-65 Fourier transform spectrometer equipped with PbSe and TGS detectors (Bio-Rad, Cambridge, MA). Two methods were used to obtain spectra: diffuse reflectance (for solids and wet solids), with the use of sulfur (sublimed, 100 mesh) as the background spectrum, and transmission (for solutions), with an empty quartz cell as the background. Spectra were taken from 10,000 cm^{-1} (1000 nm) to 4000 cm^{-1} (2500 nm) at a resolution of 4 cm^{-1}, corresponding to 0.4 nm at 1000 nm, 1 nm at 1750 nm, and 2.5 nm at 2500 nm. Effects on the spectra of the model compounds were searched for in the spectral region from 4900 to 4100 cm^{-1}, where water does not absorb strongly but where organic molecules do. Spectral subtraction was used to remove the spectrum of water, and data were examined closely to be sure that effects noted were not due to spectral distortions caused by overlapping of the water and model compound spectra. Finally, second derivatives (after spectral subtraction of water) were taken of all spectra to mitigate baseline differences, and results from 4900 to 4100 cm^{-1} were compared with the use of regression analysis. Please note that the second-derivative spectra shown are inverted, with the positive peaks matching the nonderivatized spectral peaks.

Samples. Liquid solutions were prepared at volume-to-volume dilutions of 50%. For solids either mixtures containing 50% water on a weight basis or saturated solutions were investigated. Deionized water and reagent-grade (or better) chemicals were used. Sugars and amino acids were the biologically active forms. For studies on the effect of ionic strength, saturated sodium chloride solution was used in place of water. With pH studies, either water adjusted to pH 2 or 8 (for alcohols, sugars, and ketones) or direct pH adjustment of the material or solution in question (acids, amines, proteins, etc.) was employed, with the use of hydrochloric acid or sodium hydroxide solution. The alfalfa hay, and alfalfa, and corn silages used were typical high-quality materials produced at the Beltsville Agricultural Research Center, Beltsville, Maryland.

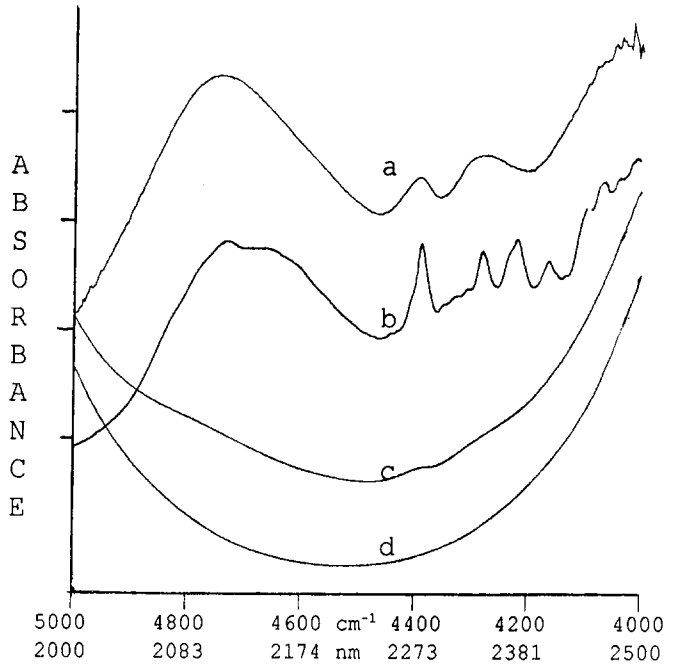

FIG. 1. Near-infrared spectra of (*a*) saturated glucose solution after water subtraction; (*b*) crystalline glucose; (*c*) saturated glucose solution; and (*d*) water.

Regression Analysis. Quantitative comparisons were performed by comparing two second-derivative spectra by regression analysis using SAS Proc Glm.[16] This analysis was performed by converting each spectrum into an ASCII file consisting of a list of wavenumbers and corresponding absorbances. All spectral comparisons for wet materials or solutions were performed with the use of derivatized spectra after spectral subtraction of the water spectrum. Two spectra (**A** and **B**) were then compared by regressing the absorbances at each wavenumber in spectrum **A** against the absorbances at the same wavenumbers in spectrum **B**. The results indicate the relative similarity between the two spectra in question, with an R^2 of 1.00 showing the two spectra to be identical. Comparing the results obtained with various spectra gives information on their relative similarity or differences. For example, if the regression of the spectra of crystalline **A** and crystalline **B** yields an R^2 of 0.3, and the regression of the spectra of **A** in solution and **B** in solution gives an R^2 of 0.8, then we can say that **A** and **B** are spectrally more similar when dissolved in water than as crystalline solids. Finally, from an information standpoint, the greater the differences (the lower the R^2) between two spectra of different materials, the better. The more similar the spectra, the more difficult it would be to find definitive differences upon which to base calibrations. The opposite is of course true for two spectra of the same material in different states (i.e., crystalline vs. amorphous or dissolved). Here it would generally be best to maintain similarity among spectra.

RESULTS AND DISCUSSION

Regressions for Sugars. In Fig. 1 are the spectra of water, glucose, and saturated glucose solution with and

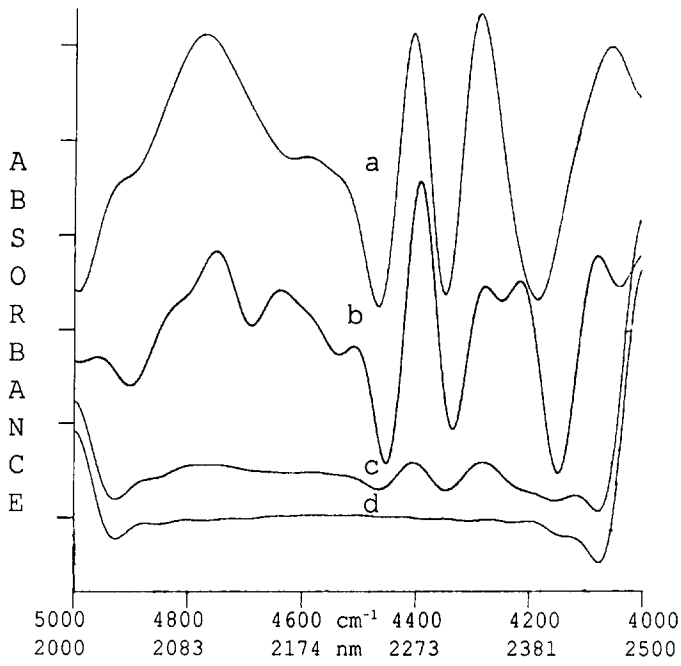

FIG. 2. Near-infrared derivatives (spectra inverted) of spectra: (*a*) saturated glucose solution after water subtraction; (*b*) crystalline glucose; (*c*) saturated glucose solution; and (*d*) water.

without the subtraction of water. In Fig. 2 are the spectra of the same solutions and materials, presented as second derivatives. As shown in Fig. 1, without the subtraction of water, few of the spectral details for glucose can be seen. Note also the apparent noise in the water-subtracted glucose spectrum between 4200 and 4000 cm^{-1}, particularly below 4100 cm^{-1}. The same thing occurs to a lesser degree from 5000 to 4900 cm^{-1}. With some materials, it was difficult to carry out water subtractions without adding erroneous inflections at the ends of the spectrum. This problem can be seen to a small degree in Fig. 2 for glucose. Notice that at each end (particularly at 4000 cm^{-1}) the inflection of the derivatized spectrum (Fig. 2a) for the water-subtracted sample was different from that for the pure glucose spectrum (Fig. 2b). This effect is due to the difficulty in properly subtracting the spectrum of water because of changes in the spectrum caused by the solute. This effect is very pronounced in some spectra and, because of this problem, derivative spectra from 4900 to 4100 cm^{-1} were used. Finally, the use of derivative spectra also mitigated baseline differences that sometimes occurred because of the subtraction process or because of the different methods used to collect spectra (transmission for liquids and reflectance for solids).

The regression results for various sugars are presented in Table I. In an attempt to assess the effects of water or other factors on the information content of spectra, there are several themes that can be examined.

Question I. Within a class of materials, such as sugars, are the spectra more similar for different compounds when the materials are examined in a particular state? For example, are solutions of sugars spectrally more similar than their crystalline counterparts? In this regard, from Table I we can see the following: (1) There was little similarity among the spectra of the crystalline sugars, with the highest R^2 being 0.46 (glucose spectrum regressed against that of maltose) and averages being 0.15 (average of all individual regressions of crystalline sugar spectra with other crystalline sugar spectra) and 0.20 (glucose, maltose, and sucrose spectra only). (2) Amorphous forms were much more similar, with the highest R^2 being 0.85 (for maltose vs. sucrose regression), and the average R^2 for regressions involving only glucose, maltose, and sucrose being 0.71. (3) Saturated solutions were the most similar, with the highest R^2 being 0.94 (for maltose vs. glucose regression), the average for all regressions involving only solutions being 0.62, and the average for regressions using only glucose, maltose, and sucrose being 0.79. From these results, one can conclude that spectra were more similar among amorphous and among dissolved sugars and were more distinct among crystalline sugars. Since two identical spectra offer no information of value in analytical efforts, the more similar the spectra (the higher the R^2), the less information there is available to discriminate between the two.

Question II. For the same material are the spectra more similar between some states than others? For example, are crystalline and amorphous forms of the same sugars more alike than crystalline and dissolved or amorphous and dissolved? With the use of data found in Table I for glucose, maltose, and sucrose regressions involving the same sugar, but in different states, the following average R^2 values were found: crystalline vs. amorphous, 0.40; crystalline vs. dissolved, 0.36; and amorphous vs. dissolved, 0.72. These values indicate that, for the three sugars in question, considerable differences in spectra exist between the crystalline and other states, but that dissolved and amorphous sugars are spectrally similar. This observation indicates that the central cause for information loss is the loss of crystallinity.

Question III. Within a class of materials, such as sugars or amino acids, do all materials behave similarly or does a particular compound stand out from all the others? While there were differences between the various sugars (i.e., the regression of the spectrum of amorphous glucose against that of dissolved glucose produced an R^2 that was less than half that found for similar comparisons for the same two forms of maltose or sucrose), the wide range of R^2s produced makes any simple conclusion about a single sugar versus the others impossible.

Regressions for Amino Acids and Urea. *Question IV.* A final question concerns the comparisons between different classes of materials. Table II contains the same types of comparisons as Table I, but for amino acids and urea. While crystalline and amorphous forms of the same sugar were not similar, with an average R^2 of 0.40, amino acids and urea behaved differently. Thus, for crystalline vs. amorphous forms of the same amino acids or urea, the R^2 values were 0.96, 0.23, 1.00, and 0.69 for alanine, glycine, serine, and urea, respectively, for an average R^2 of 0.72. Also for amorphous vs. dissolved, the R^2 for the same three amino acids and urea was only 0.17, while it was 0.72 for sugars. Finally, while solutions of individual sugars were similar to solutions of other sugars (average R^2 of 0.62), this was not true for amino acids or urea (average R^2 of 0.10). Thus amino acids and urea, as a class, behave very differently from sugars. While for amino acids and urea information seems to be lost on dissolution, freeze drying to an amorphous state does not

TABLE I. Regression analysis results (R^2) for sugars[a] for the spectral region from 4900 to 4100 cm^{-1} using derivative spectra.

	Crystalline				Amorphous[b]				Solution[c]			
CR[d]	GA	GL	MA	SU	GL	MA	SU	FR	GA	GL	MA	SU
FR	0.12	0.04	0.01	0.39	0.02	0.04	0.04	0.13	0.05	0.02	0.03	0.03
GA		0.25	0.03	0.06	0.01	0.03	0.00	0.00	0.01	0.09	0.04	0.00
GL			0.46	0.00	0.47	0.50	0.50	0.35	0.20	0.45	0.37	0.41
MA				0.15	0.28	0.46	0.29	0.28	0.22	0.45	0.37	0.22
SU					0.15	0.33	0.26	0.24	0.17	0.31	0.40	0.27
Ave.		0.15 (0.20)[e]				0.23 (0.43)				0.20 (0.33)		
AM[f]						MA	SU	FR	GA	GL	MA	SU
GL						0.66	0.63	0.48	0.40	0.41	0.45	0.51
MA							0.85	0.60	0.62	0.80	0.84	0.71
SU								0.75	0.42	0.61	0.69	0.90
Ave.						0.71 (0.71)				0.61 (0.56)		
SO[g]									GA	GL	MA	SU
FR									0.31	0.59	0.61	0.86
GA										0.53	0.58	0.34
GL											0.94	0.68
MA												0.74
Ave.										0.62 (0.79)		

[a] FR = fructose; GA = galactose; GL = glucose; MA = maltose; SU = sucrose.
[b] Amorphous forms from freeze-dried solutions.
[c] Solutions = saturated solutions in water; water-subtracted spectra analyzed.
[d] CR = crystalline.
[e] Average of individual R^2s for block of comparison presented above (i.e., CR vs. crystalline); (X.XX) = R^2 involving glucose, maltose, and sucrose with each other only.
[f] AM = amorphous.
[g] SO = solutions.

seem to have as great an effect for amino acids as it does for sugars. Also, while sugars were more similar in the amorphous and dissolved states than in the crystalline forms, this sort of effect was not nearly as pronounced for the amino acids and urea. While dissolving amino acids or urea may produce spectra that are vastly different from their crystalline counterparts, the various compounds still have distinct spectra. Individual sugars, however, appeared very different from other sugars as crystalline solids, but largely alike in amorphous or dissolved forms.

Regressions for Carbohydrate Polymers. Another form of solids is comprised of carbohydrate polymers, for which results are presented in Table III. In general, the individ-

TABLE II. Regression analysis results (R^2) for amino acids and urea[a] for the spectral region from 4900 to 4100 cm^{-1} using derivative spectra.

	Crystalline				Amorphous[b]				Solution[c]				
CR[d]	CY	GY	SR	UR	AL	GY	SR	UR	AL	CY	GY	SR	UR
AL	0.34	0.17	0.03	0.00	0.96	0.02	0.03	0.00	0.33	0.17	0.50	0.02	0.02
CY		0.04	0.01	0.06	0.45	0.11	0.01	0.02	0.21	0.07	0.14	0.05	0.00
GY			0.27	0.00	0.14	0.23	0.29	0.02	0.00	0.00	0.64	0.10	0.00
SR				0.19	0.04	0.03	1.00	0.26	0.04	0.06	0.40	0.28	0.19
UR					0.02	0.00	0.18	0.69	0.00	0.16	0.01	0.16	0.26
Ave.		0.11 (0.11)[e]				0.23 (0.10)				0.15 (0.14)			
AM[f]						GY	SR	UR	AL	CY	GY	SR	UR
AL						0.03	0.04	0.01	0.33	0.20	0.51	0.03	0.02
GY							0.04	0.00	0.00	0.03	0.03	0.01	0.01
SR								0.24	0.04	0.06	0.41	0.27	0.10
UR									0.00	0.10	0.04	0.19	0.06
Ave.						0.06 (0.06)				0.12 (0.11)			
SO[g]										CY	GY	SR	UR
AL										0.09	0.02	0.11	0.12
CY											0.01	0.42	0.01
GY												0.11	0.08
SR													0.04
Ave.										0.10 (0.08)			

[a] AL = alanine; CY = cysteine; GY = glycine; SR = serine; UR = urea.
[b] Amorphous forms from freeze-dried solutions.
[c] Solutions = saturated solutions in water; water-subtracted spectra analyzed.
[d] CR = crystalline.
[e] (X.XX) = average R^2 involving alanine, glycine, serine, and urea only.
[f] AM = amorphous.
[g] SO = solutions.

TABLE III. Regression analysis results (R^2) for polysaccharides[a] for the spectral region from 4900 to 4100 cm^{-1} using derivative spectra.

	Solid						Solution[b]					
CR[c]	CE	LO	PE	ST	TR	XA	AR	CE	LO	PE	ST	TR	XA
AR	0.60	0.92	0.04	0.82	0.75	0.78	0.42	0.46	0.75	0.00	0.81	0.42	0.27
CE		0.61	0.02	0.44	0.36	0.48	0.27	0.92	0.66	0.01	0.42	0.14	0.19
LO			0.04	0.81	0.63	0.60	0.36	0.48	0.80	0.00	0.79	0.32	0.16
PE				0.00	0.35	0.19	0.06	0.00	0.00	0.84	0.00	0.26	0.06
ST					0.48	0.43	0.48	0.39	0.81	0.03	1.00	0.38	0.14
TR						0.79	0.16	0.22	0.34	0.11	0.46	0.66	0.28
XA							0.28	0.35	0.44	0.03	0.42	0.47	0.55
Ave.			0.48							0.36			
SO[d]								CE	LO	PE	ST	TR	XA
AR								0.39	0.59	0.09	0.50	0.34	0.49
CE									0.68	0.03	0.38	0.14	0.26
LO										0.04	0.81	0.27	0.23
PE											0.03	0.19	0.04
ST												0.38	0.14
TR													0.47
Ave.										0.31			

[a] AR = gum arabic; CE = crystalline cellulose; LO = locust bean gum; PE = pectin; ST = soluble starch; TR = gum tragacanth; XA = xanthan gum.
[b] Solutions = saturated solutions in water; water-subtracted spectra analyzed.
[c] CR = crystalline.
[d] SO = solutions.

ual polymeric carbohydrates have a closer similarity to other polymeric carbohydrates in the solid form than do the sugars or nitrogen compounds (amino acids and urea) (average R^2 of 0.48 vs. 0.15 for crystalline sugars and 0.11 for crystalline nitrogen compounds). The presence of water, however, decreased the similarity between different polymers. This behavior was unlike that of the simple crystalline sugars but like that of the nitrogen compounds.

Regression of Sugars versus Amino Acids and Urea. In results not shown, the spectra of five sugars (fructose, glucose, galactose, maltose, and sucrose) were compared by regression to four amino acids (alanine, cysteine, glycine, and serine) and urea. Overall, little similarity was found between spectra for the two classes of compounds, except when serine in solution was involved. For example, the average R^2 for the regressions of spectra of amorphous sugars (glucose, maltose, and sucrose) against spectra for solutions of alanine, cysteine, glycine, and urea was 0.06, but for serine it was 0.59. Comparing spectra of solutions of the five sugars to spectra of solutions of the same four nitrogen compounds resulted in an average R^2 of 0.10, but for serine (as a solution) 0.65, with two R^2s of 0.80 or better (serine vs. fructose and sucrose). In the crystalline state, the average R^2 for serine vs. the five sugars was 0.12, and the highest was 0.24 with fructose. Thus, while not generally true for cross comparisons between sugars and amino acids, spectrally serine was very similar in amorphous and particularly in dissolved form to sugars in similar states.

Regressions for Silages and Hay. Table IV contains the results for two sets of more complex samples, wet and dried alfalfa and corn silages, and alfalfa hay at various moisture contents. Examining the alfalfa hay data, one can see that the addition of increasing amounts of water resulted in decreasing similarity to the dried sample (A0), but that the overall similarities were always quite high. The values shown are more in line with the types of decreases seen in calibration R^2 values for wet silages, when compared to those found for dry materials, and indicate that for real forage samples the severity of the effects seen in individual materials (sugars, amino acids, etc.) is somehow moderated. Finally, the data for silages show that, when dried, silages are more like each other than when wet.

Effects of pH and Ionic Strength. Two other factors that vary in silages are pH and ionic strength (as reflected in the amounts of soluble moments such as volatile fatty acids, ammonia, and ethanol). In Table IV, some results are presented for the effect of these factors on spectra, which, as shown, vary widely. For acetone and ethanol, differences due to pH variation were nonexistent (R^2 for pH 2 vs. pH 8 = 1.00). The decreases in R^2 for acetone vs. 50% acetone (pH 2 or 8) were thus due to the water present. Interestingly, saturated NaCl did not alter the spectra of acetone very much (acetone vs. 50% in saturated NaCl, R^2 = 0.98), while for ethanol the effect was closer to that of water alone. Cellulose, glucose, and urea behaved much like acetone and ethanol, with water, and not pH differences, having the most effect. The greatest effects of pH were seen for acidic and basic materials such as acetic acid vs. sodium acetate (R^2 = 0.08); *n*-butylamine (100 and 50%) vs. 50% at pH 1 (R^2 of 0.11 and 0.19); and, for casein, pH 2 vs. pH 8 (R^2 = 0.21). Interestingly, trypticase, a casein digest, did not show the pH effect (R^2 = 0.93). The difference between casein and trypticase may be due to the state of the materials; trypticase was a solution at both pH levels, while casein was a wet solid at pH 2 and a solution at pH 8.

Relations to Near-Infrared Spectroscopy Calibrations. This study was carried out in an effort to quantify the degree of information loss or change that occurs in near-IR spectra under the influence of water, pH, ionic strength, and physical state. As for quantifying the spectral changes, the results presented were an improvement over the qualitative visual examination of spectra.[6,12] Visually the overall shape of a spectrum may remain similar, and thus from a qualitative sense may appear similar; but in reality, because of shifts in the overall spectrum, there may

be considerable differences in a quantitative sense. However, relating these spectral differences to the decrease in performance of near-IR calibrations when one is dealing with high-moisture materials does not appear to be so easy. Typically, near-IR calibrations for wet materials result in R^2s that are a few hundredths less than those found for the same assay for dried materials. Examining the effect of water on many of the samples studied here, one sees much larger quantitative differences in spectra.

There are at least two possible explanations for this discrepancy. First, while spectra may be altered by differences in pH, water content, etc., this does not mean that sufficient information does not still exist upon which to develop a calibration. For example, if one were to develop a calibration for various concentrations of material "X" in water, even if 9 out of 10 peaks vanished in the presence of water, and therefore R^2s between spectra of dry and wet forms of "X" were very low, the remaining peak might be used quite satisfactorily. Second, in most forage-based samples, most of the sample is made up of polymers such as cellulose, gums (hemicelluloses and pectins), protein, and lignin, and, as seen, these materials are often the least affected by water, pH, etc. Examining the variations in the quality of near-IR calibrations with wet materials, one also sees that calibrations are often best for measures that are based on exactly these polymeric materials, such as fiber determinations.[3-5] The exception is protein, where casein showed large spectral changes, but near-IR calibrations are quite good even for wet silages.[3-5] This result may be due to casein in particular, because of the different states (solid vs. solution) or the extremes of pH used. In an actual calibration set, good silages would range only from pH 4 to 4.5, and thus pH effects would be minimal for most of the samples. However, the difference between "most" and "all" of the samples might be exactly the reason for the decrease in near-IR performance.

TABLE IV. Regression analysis results (R^2) for miscellaneous materials[a] and materials at different pH values and in the presence or absence of saturated NaCl for the spectral region from 4900 to 4100 cm^{-1} using derivative spectra after subtraction of the spectra of water.

Spectra of	Regressed against spectra of			
	AW	CD	CW	
AD	0.57	0.90	0.68	
AW		0.49	0.74	
CD			0.75	
	A2	A4	A6	A8
A0	0.97	0.93	0.86	0.88
50% Acetone	50% Acetone pH 2 or 8			0.84
50% Acetone	50% Acetone in sat. NaCl			0.98
Acetone pH 2	50% Acetone pH 8			1.00
Acetone pH 2 or 8	50% Acetone in Sat. NaCl			0.92
Acetic acid	Sat. Sodium acetate sol.			0.08
n-Butylamine	50% *n*-Butylamine in water			0.71
n-Butylamine	50% *n*-Butylamine pH 1			0.11
50% *n*-Butylamine in water	50% *n*-Butylamine pH 1			0.19
Casein pH 2	Casein pH 8			0.21
Cellulose	50% Cellulose pH 2			0.90
Cellulose	50% Cellulose pH 8			0.87
50% Cellulose pH 2	50% Cellulose pH 8			1.00
Ethanol	50% Ethanol pH 2 or 8			0.80
Ethanol	50% Ethanol in sat. NaCl			0.85
50% Ethanol pH 2	50% Ethanol pH 8			1.00
50% Ethanol pH 2 or 8	50% Ethanol in sat. NaCl			0.99
Glucose sol. pH 2	Glucose sol. pH 8			1.00
Glucose sol. pH 2	Glucose in sat. NaCl sol.			0.80
Trypticase sol. pH 2	Trypticase sol. pH 8			0.93
Urea sol. pH 2	Urea sol. pH 8			0.99

[a] AD = dried alfalfa silage; AW = wet alfalfa silage; CD = dried corn silage; A0–A8 = alfalfa hay at 0, 20, 40, 60, and 80% water by weight.

CONCLUSION

Results showed that changes in spectra caused by water, pH, etc., can be quantified by regression analysis. Thus, conclusions can be drawn about the behavior of both classes of materials (sugars vs. amino acids, monomers vs. polymers, etc.) and specific single compounds. While these quantitative results can be related in a qualitative manner to near-IR calibration problems with wet materials, by relating compositional knowledge to the degree of spectral changes for specific materials, efforts to relate in a more absolute manner the quantitative changes in individual compound spectra to the decrease in the performance of near-IR spectroscopy with high-moisture samples will require more work with the use of more complex mixtures of materials.

Finally, the question remains, What if anything can be done to eliminate or avoid the potential problems caused by these effects when one is trying to develop near-IR calibrations? Since the effects are due to changes in the materials involved, and not to the instrumentation or statistical procedures used in data treatment, the only option would appear to be to avoid the effects in the first place. Except for working with dried samples, avoidance would appear to require finding a spectral region where the effects are reduced. Comparing published and some preliminary unpublished efforts in the mid-infrared indicates that the effects are less severe in the mid-infrared than in the near-infrared. The region from 600 nm (16,667 cm^{-1}) to 1000 nm (10,000 cm^{-1}) is another possibility, although this region has not yet been examined for these effects. Further research is therefore needed to determine whether a method can be found to reduce, avoid, or eliminate the problems discussed in this paper.

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