Scanning Microscopy

Volume 4 | Number 2 Article 19

4-17-1990

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Scanning Microscopy, Vol. 4, No. 2, 1990 (Pages 399-405)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

X-RAY MICROANALYSIS OF Ca AND K IN CORN BRAN AND OAT HULLS

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(Received for publication February 8, 1990, and in revised form April 17, 1990)

Abstract

Oat hulls and dry-milled corn bran were loaded with calcium or potassium and made into either sectioned bulk specimens of intact tissue embedded in resin or into non-sectioned bulk specimens made from powdered-compressed tissue formed into disks without resin. Regression lines of X-ray count versus mineral concentration were similar for both Ca and K. X-ray count versus mineral concentration relationships were similar for intact oat hulls and powdered-compressed specimens of either oat hulls or corn bran. However, the relationship for intact corn bran embedded in resin was significantly different. While the reason for this difference is not known, the result emphasizes the importance of using a proper "calibration" matrix to relate mineral concentration in biological material with X-ray count values. The standard error in slope of the regression lines, 0.07 and 0.08 for corn bran and oat hull, respectively, embedded in aged epoxy resin suggest that X-ray counts from these specimens allow one to estimate Ca or K concentration with a standard deviation of ± 10%. X-ray counts of Ca, K, and Cl in specimens embedded in epoxy resin decreased to stable values approximately four weeks after the resin was cured.

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Keywords: Corn bran, oat hulls, bulk specimens, calcium, potassium, powdered-compressed tissue, coefficient of variation, peak/background ratio, peak height

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Introduction

One of our research interests is to elucidate interactions between minerals in the diet and *in vivo* mineral binding by plant tissues. It seemed to us that energy-dispersive X-ray microanalysis could reveal significant information about such interactions by providing a quantitative measure of the mineral content of plant tissues retrieved from various locations within the gastrointestinal (GI) tract. The concept we wished to test was whether or not integrated X-ray counts obtained from relatively large areas of a plant tissue cross-section could be converted to a quantitative measure of mineral content. Previous studies (Dintzis et al., 1989a,b) revealed that significant differences in X-ray counts of Na, Ca, K, S, and P could be detected from plant tissues retrieved from the stomach, ileum and colon of pigs. The purpose of this study was to establish a model system to test some characteristics of measurements by X-ray microanalysis of the calcium and potassium content of corn bran (maize pericarp) and oat hull.

The model system consisted of specimens of oat hulls and dry-milled corn bran loaded *in vitro* with Ca or K. These two tissues were chosen because both generally maintained their integrity after GI tract passage: corn bran composition was not greatly altered after passage through the human GI tract (Dintzis et al., 1979) and intact particles of both corn bran and oat hulls were recovered after passage through the pig GI tract (Dintzis et al., 1989b). Specimens were examined in the same manner as particles of similar tissues would be when retrieved from the GI tract of killed pigs. The intent was to provide a quantitative relationship between X-ray count values and Ca or K concentrations in the loaded tissues as determined by atomic absorption measurements and regression analysis of the X-ray counts. It was important to determine how many specimens and how many observations on each specimen were needed to obtain a reasonable count value representing the mineral content of the tissue.

Materials and Methods

Preparation of loaded tissues

Whole oat hulls (donated by the Quaker Oats Co., Cedar Rapids, IA) were screened over a 20-mesh screen (0.86 mm openings) to remove dust and fines. A 10 g portion of dry-milled corn bran (donated by the Lauhoff Grain Co., Danville, IL) from degermer stream #2 was placed in a beaker with 1.0 L distilled water, stirred, and allowed to settle to the bottom. Floating germ particles were skimmed from the surface with a spoon and the liquid then decanted. The remaining corn bran, or 10 g of screened oat hulls, were loaded with Ca or K by placing them in 1.0 L of 10-200 mM solutions of $CaCl_2$, KCl or KH_2PO_4. The mixtures were stirred continuously for several hours, then stirred occasionally and allowed to remain overnight at room temperature without stirring.

The next morning, the mixture was swirled and the liquid decanted. A liter of fresh solution was added, the mixture was stirred for a few minutes to resuspend remaining fine particulates, and the liquid with suspended fines was then decanted. The remaining oat hulls or corn bran were drained of most of the residual aqueous salt solution and rinsed for about 10 seconds with 100 ml of 95% ethanol. The ethanol was decanted and drained from the tissues which were dried in the airstream of a hood and stored in glass jars until used. All batches were visually inspected under a low-power microscope to verify the absence of visible mineral precipitates on tissue surfaces.

Mineral content of loaded tissues

Concentrations of Ca and K in the loaded tissues were determined by atomic absorption (Garcia et al., 1972) with a Varian Techtron AA6. Approximately 1.0 g (dry wt.) portions of loaded corn bran or oat hulls were ashed in duplicate and made into 25 ml master solutions. Average values of the duplicates were considered to represent the concentrations of Ca and K in specimens for X-ray microanalysis.

Specimen preparation

Strips of corn pericarp (approximately 0.5 mm width X 1-2 mm length) were cut with a razor parallel to the longitudinal axis of the corn kernel. Oat hull particles (glume and lemma) were used without further treatment prior to embedding. Effapoxy resin (Ernest F. Fullam, Inc., Latham, NY) was prepared using the following components (weight in grams): Effapoxy (10.5), DDSA (3.0), NMA (8.0), DMP-30 (0.45). Well-mixed resin was poured directly over the specimens, subjected to a vacuum to remove air, and cured. All embedded specimens were cut to form transverse sections and, except for those listed in Table 3, were embedded in Effapoxy resin. Corn bran section dimensions were about 0.1-0.3 mm (approximate pericarp width) X < 1.0 mm length; oat hull section dimensions were frequently larger. Samples were dry-cut with a glass knife to obtain a relatively smooth top surface and mounted as bulk specimens (approximately 1 mm thick) as previously described (Dintzis et al., 1989a).

Powdered specimens were prepared by first milling about a gram of Ca- or K-loaded plant tissue in a Wiley mill to pass a 40-mesh screen (0.38 mm openings). About 0.3 g of milled material was then placed with two ball bearings in a small stainless steel cylinder which was closed and shaken vigorously until the plant tissue was battered to a powder (The device, called a "WIG-L-BUG," is sold by dental suppliers to make amalgams.). The powder was then compressed in a Model A Carver Laboratory Press at 9000 kg to form a disk of 13 mm diameter with a thickness of > 0.2 mm. The disks of powdered-compressed oat hulls or corn bran were fastened to carbon planchets with a paste of colloidal graphite and were coated with carbon. These disks were non-sectioned bulk specimens prepared without surrounding resin and with unknown amounts of the native plant cell structures destroyed.

Measurement and statistical analysis

Each embedded specimen was derived from a different flake of corn bran or oat hull which was randomly selected from the distribution of plant tissue particles loaded with a known concentration of mineral. For a given concentration of Ca or K, six embedded specimens were measured. Three different areas within the cross-section of each specimen were exposed to the electron beam and x-ray counts obtained. Thus, a total of n=18 observations were used per concentration of Ca or K. A measurement was made on the resin surrounding each specimen to obtain a "resin blank" value. The elements Na and Cl are constituents of the epoxy resin as well as being important ionic species in the digestive system. Counts of Na and Cl in the resin were routinely monitored as a measure of resin uniformity and for possible future use in establishing relationships between X-ray counts and concentrations in plant tissues retrieved from digesta.

Eight different areas on a disk of powdered-compressed oat hull or corn bran were measured to obtain an x-ray count mean (n=8).

The relationship between count and concentration was evaluated by a least-squares fit to a linear regression model. The software used was PROC REG from SAS (1987) version 6.03. Further analysis of the data was carried out to investigate the questions of how many specimens and how many observations per specimen were required to obtain an "acceptable" measure of mineral concentration in the loaded tissues. The original data set was divided into overlapping subsets of data, and regression parameters were estimated for each subset. The original data set for corn bran had three observations of each of six specimens (flakes) at seven concentrations (Fig. 1) for a total of 126 observations. Subsets were made up of combinations of 1, 2 or 3 observations on each of 1 to 6 specimens.

Samples of corn bran and oat hull, with 8700 and 8200 $\mu g/g$ of K, respectively, were milled to pass a 40-mesh screen and analyzed in

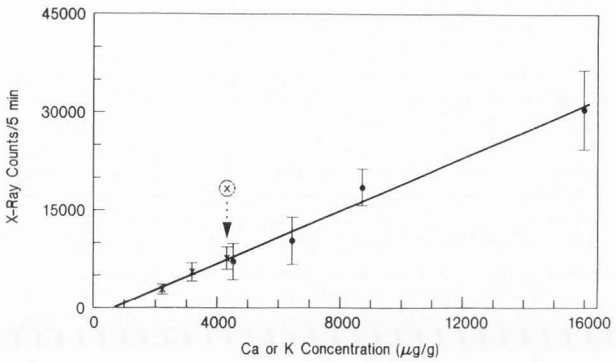

Figure 1. X-ray counts of Ca and K versus concentration in loaded, resin-embedded corn bran. X = Ca, • = K, ⊗ = mean count value from specimens embedded in resin cross-linked 5 days prior to measurement. Other data in this figure and in other figures are from "aged" specimens in which count values have stabilized.

duplicate for C, H, O and N. An estimate of "direct density" was obtained from samples of corn bran and oat hull with 4300 μg/g Ca and 5300 μg/g K, respectively. These samples were milled to pass a 20-mesh screen and placed into a 5-ml graduated cylinder. The cylinder was tapped vigorously several times against a laboratory bench top until the sample level remained constant. The weight and volume occupied by the sample were then recorded.

X-ray microanalysis

An ISI-SS130 scanning electron microscope with a microprobe detector was used to measure X-rays through a 12-mm² beryllium window. Counts were acquired using an electron beam voltage of 15 kv, a beam current of 1.0 nA (determined by focusing the beam into a Faraday cup and measuring current), a count rate of approximately 1000 counts/sec, a dead time of about 8%, a sample tilt angle of 45 deg, a take-off angle of 47 deg between planchet surface and detector, a working distance of 20 mm from sample to detector, and a 5-min counting period, and were processed with a Princeton Gamma Tech System 4 operating at 20 V per channel. A copper-aluminum standard was used in conjunction with the calibration sequence supplied by the manufacturer to set and verify proper voltage and off-set values. Window widths were set at 1.2 X peak width at half maximum height. Measurements on powdered and nonpowdered specimens generally were made at magnifications of about 2000-4500. This range of magnifications corresponded to a range of probe areas of about 1000-9000 μm². All listed count values were background subtracted manually in conjunction with manufacturer-supplied software. Except for the example in Figure 1, X-ray count averages from resin-embedded specimens were obtained more than 30 days after the resin was cured. The electron beam was positioned to scan areas within the cross-section of a specimen in such a manner as to avoid specimen edges and to encompass at least half the width of the cross-section. Specimens retrieved from the GI tract may have adhering digesta that contain high concentrations of minerals. Thus, in the application of this technique, the precaution of avoiding specimen edges prevents exterior digesta from contributing false counts.

Results

A graph of mineral concentration versus calcium and potassium counts from resin-embedded corn bran specimens is presented in Figure 1 and from resin-embedded oat hulls in Figure 2. The two elements yielded similar regression lines from the respective embedded plant tissue. The embedding resin was free of Ca and K counts, but yielded significant counts for Na and Cl.

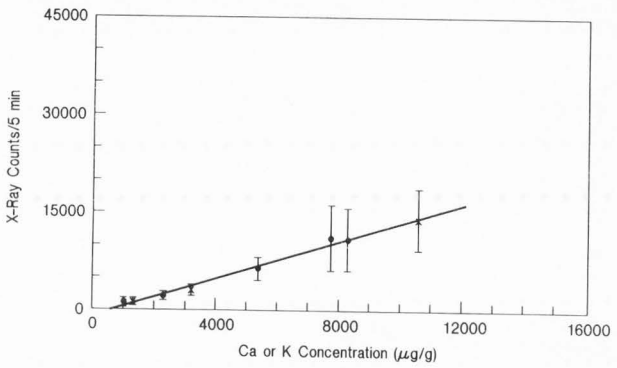

Figure 2. X-ray counts of Ca and K versus concentration in loaded, resin-embedded oat hulls. X = Ca, • = K .

An effect of resin age upon X-ray count values was observed. An example of this is shown for one data point in Figure 1. In this example, the initial calcium count in the corn bran specimens embedded five days previously decreased from 17.3 x 10^3 to 7.8 x 10^3 counts/5 min when measured four months later. During this time, the chloride count intrinsic to the resin surrounding the specimen decreased from 14.1 x 10^4 to 5.8 x 10^4 counts/5 min. Similar effects of resin age were observed in specimens loaded with potassium: counts of K in the specimen and Cl in the resin were significantly greater when the resin was freshly cured.

Measurements made on powdered-compressed tissues yielded a relationship different from resin-embedded corn bran. Counts of Ca and K from either of the powdered-compressed tissues followed similar linear functions. An analysis of covariance was used to determine that fitting individual slopes for each tissue was a better ($P<.05$, as tested by full vs. reduced model F test) fit than a simple overall regression across all four tissues, i.e., embedded corn bran, embedded oat hull and the two powdered-compressed samples. A t-test analysis of the slopes revealed that the slope

for embedded corn bran was significantly greater than for embedded oat hull ($P<0.001$). There was no significant difference in slope for embedded oat hulls versus that of powdered-compressed corn bran and oat hull tissues. All three regression lines are displayed in Figure 3 for visual comparison. The equation for each line (± standard error) is listed in Table 1.

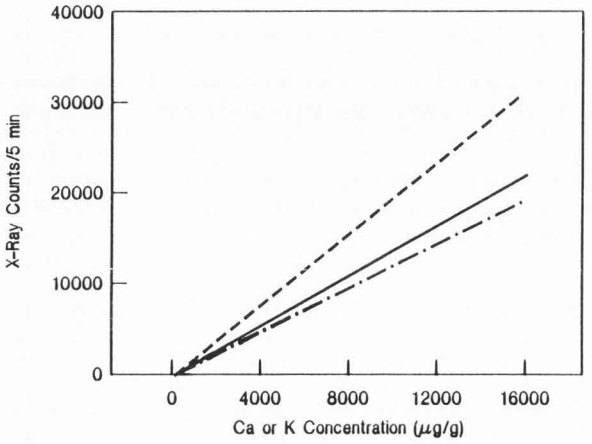

Figure 3. Comparison of regression lines fitted through means of X-ray counts versus concentration of Ca and K. - - - - - - = resin-embedded corn bran, ———— = resin-embedded oat hulls, —·—·— = powdered-compressed specimens of corn bran or oat hulls. Effapoxy resin used with embedded specimens.

Table 1. Count vs. Concentration

Sample	Equation
Corn bran, embedded:	Y= (-1203±527) + (2.04±0.07)X CC: 0.94
Oat hull, embedded:	Y= (-632±482) + (1.41±0.08)X CC: 0.84
Powdered & compressed tissue:	Y= (-178±462) + (1.21±0.05)X CC: 0.96

Y: Counts of Ca or K above background/5 min.
X: Concentration of Ca or K in μg/g.
CC: Correlation Coefficient

In these experiments, in which known concentrations were used to predict counts, a standard error (S.E.) of ± 0.07 in a slope would translate to a 95% confidence interval (C.I.) of 1646 counts at a concentration of 6000 μg/g. At an S.E. of ± 0.10, the 95% C.I. is 2350 counts and, at an S.E. of ± 0.15, the 95% C.I. is 3528 counts. Thus, for corn bran (Fig. 1), the slope standard error would result in having a count of 11000 counts/5 min represent 6000 ± 450 μg/g.

The difference in count values from embedded corn bran vs oat hull loaded with equivalent contents of Ca or K surprised us and led to further measurements on the samples. The overlap of the potassium K beta X-ray signal into the Ca channel was measured for embedded and powdered-compressed specimens of corn bran loaded with about 15900 μg/g K. The difference between respective overlap values of 6.1% and 6.3% was not significant.

Counts from embedded specimens were more ($P<.01$) variable than counts from the powdered-compressed specimens. Residual mean squares obtained in the regression analysis (i.e., the amount of variation in count left over after fitting a regression of counts on concentration) were compared by F-test. Variation in counts from corn bran was similar ($P=.13$) to variation in counts from oat hulls. Residual mean squares for corn bran, oat hulls and powdered-compressed tissue were 13.48×10^6, 10.82×10^6 and 2.76×10^6, respectively.

Results of elemental analysis and density values of corn bran and oat hulls are presented in Table 2. The direct densities were similar to values of 0.46 g/ml and 0.36g/ml reported for corn bran and oat hull flour, respectively, that were ground to pass a 35-mesh screen (Schimberni et al., 1982).

Table 2. Comparison of elemental contents and direct density of potassium loaded corn bran and oat hulls ground to pass 20-mesh screen.

Element	Sample	
	Corn bran	Oat Hulls
	%	%
C	42.8	44.3
H	6.2	5.8
O	43.9	42.1
N	0.3	0.6
K	0.9	0.8
Total	94.1	93.6
Density (g/ml)	0.49	0.43

Figure 4 displays average standard error of slopes fit to a random selection of subsets at each observation-specimen combination.

Discussion

Our analysis of errors involved in establishing these "calibration" relationships indicates that a standard deviation of 10% should be expected as a practical lower limit to the precision of measuring mineral concentrations in corn bran and oat hulls by this X-ray technique. With this lower limit to data precision, an earlier suggested non-linear relationship for oat hulls (Dintzis et al., 1989b) is not supportable and for both corn bran and oat hulls a linear relationship exists, as shown in Figures 1 and 2.

The dependence of count values upon age of the cross-linked epoxy resin was not expected.

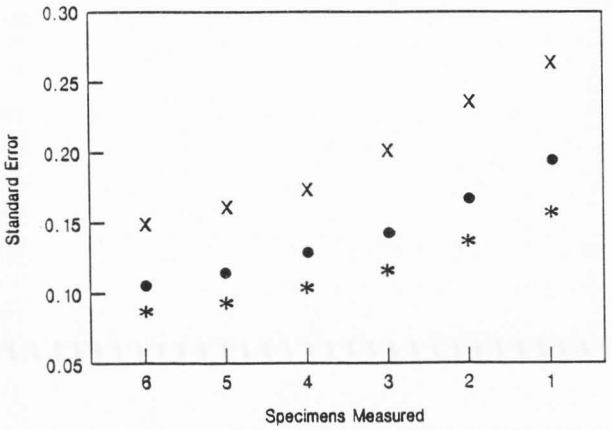

Figure 4. Standard error (S.E.) comparisons of data subsets. S.E. dependence of slopes of regression lines of mean X-ray counts from resin-embedded corn bran vs concentration upon measurement parameters. X = 1 measurement per specimen, • = 2 measurements per specimen, * = 3 measurements per specimen.

A common component in epoxy-resin formulations is an epoxide that contains chlorine, such as 1-chloro-2,3-epoxypropane (epichlorohydrin), which, upon cross-linking with other constituents, yields HCl as one of the by-products of the base-catalyzed condensation reaction (Dawes, 1971). We believe this HCl initially is entrapped within the bulk resin and slowly diffuses out. Some of the chloride from epichlorohydrin may be covalently bonded to the resin by other side reactions. These mechanisms would be compatible with our observed decrease in resin chloride count with time to a relatively constant value. We did not realize initially that the presumed presence of HCl in the resin, and therefore in the plant tissue, would be reflected in an increased count. This difficulty could be circumvented by allowing the embedded specimens to age several weeks prior to measurement. We now avoid this problem by using a different resin, medium grade "LR White" (London Resin Company Ltd., Basingstroke, Hampshire RG225AS, England). This acrylic resin does not contain chlorine and thus has the additional advantage of allowing chloride content of the specimen to be determined quantitatively. Preliminary results indicate similar X-ray means with specimens embedded in LR white versus aged Effapoxy resin.

The other unexpected result was the finding that there were significant differences in counts vs concentration from embedded corn bran and oat hulls. We are aware of only one major difference in composition between the two tissues: oat hulls contain approximately 1.5% silicon, corn bran <0.3%. Values of "direct density" of the two tissues are similar and, therefore, there should not be gross differences in density between the two tissues. However, this measurement does not provide an estimate of void volumes within the intact tissues, and significant differences in target density could influence X-ray counts. Because the powdered-compressed specimens of the two loaded tissues yielded similar regression lines, we infer that elemental composition, *per se*, was not involved in this phenomena. We suspect that significant differences in morphology and/or asymmetry of mineral distribution, in addition to target density, might cause the observed differences in counts. One consequence of this finding is that different calibration equations will be needed if this method of examining bulk specimen cross-sections is applied to different tissues. This finding also emphasizes the difficulties in attempting to use a synthetic matrix containing known concentrations of minerals as a calibration standard for the purpose of determining mineral concentrations in tissues removed from the GI tract.

A linear relationship can be fitted well to a comparison of X-ray counts versus Ca and K concentrations in these specimens. Therefore, we infer that the resin-embedded tissue matrix (corn pericarp or oat hull) is similar at all mineral loadings examined. Application of this method to samples retrieved from the GI tract would require limited digestibility of the tissue in order that properties of the matrix not be significantly altered as a function of location within the GI tract. Variation in count values caused by non-linear effects of specimen thickness was limited by use of specimens infinitely thick to the electron beam.

The procedure of embedding tissue directly in the resin is simple. However, we believe the major advantage is that translocation of labile ions, such as Na^+ and K^+, within the tissue cross-section is minimized--and possibly negligible. Some evidence for this is reflected in the result that there was no significant difference between slopes of regression lines of data from embedded oat hulls vs powdered-compressed oat hulls. In addition, we observed esentially no K counts in either Effapoxy or LR White resin blanks or Cl counts in LR White resin blanks with specimens loaded with KCl. Although we consider resin penetration into specimens to be limited (Dintzis et al., 1989b), we do not know the extent or whether penetration into oat hull differs from penetration into corn pericarp.

Gross cracks or fissures in specimens were detectable. In Effapoxy resin-invaded tissues, the chloride count was greatly increased by contributions from the resin. In samples retrieved from the GI tract, digesta allowed into a fissure significantly increased counts of other minerals relative to non-penetrated areas of the specimen.

The information in Figure 4 is useful as a guide to determine how many specimens should be examined. The data points form a family of curves which approach asymptotic limits. The difference in the standard error of the slopes between 1 and 2 specimems per concentration is greater than the standard-error difference between 5 and 6 specimens. Little would be

gained by measuring 7 rather than 6 specimens per concentration. The gain in precision from 1 to 2 observations per specimen was greater than from 2 to 3 specimens. Although we did not make 4 observations per specimen, we infer from comparisons of standard error versus number of observations per specimen at constant number of specimens that the gain in precision by making 4 observations would not be worth the effort.

Note also in Figure 4 that 2 observations on 1 specimen appear to offer greater precision than 1 observation on each of 2 specimens. Three observations on 1 specimen appear more precise than 1 observation on each of 3 specimens. Such differences are diminished at higher total n; i.e., three observations per specimen on 4 specimens (n=12 per concentration) have a similar average standard error to 2 observations on each of 6 specimens (n=12 per concentration).

Conclusions

This study has demonstrated that quantitative measurements of Ca and K in corn bran and oat hulls may be obtained using X-ray microanalysis calibrated with measurements on *in vitro* loaded tissues. The relationship between X-ray count and element concentration is linear within the concentration ranges studied. With a specific tissue, the same regression equation may be used for Ca and K. The observation that X-ray count values may have a strong dependence upon the type of plant tissue examined, despite similarities in elemental composition, should be taken as a warning that careful attention to proper "calibration" methods is required for successful application of this methodology proposed for examining mineral contents of plant tissues retrieved from the GI tract. The observation that powdered-compressed samples of corn bran or oat hull, and embedded oat hull, loaded with the same concentration of Ca or K exhibit similar X-ray counts, while embedded corn bran does not, is worthy of further study.

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Table 3. Comparison of background subtracted peak height values with peak-to-background ratios.

Element	Peak Ht.	P/B
A. $CaCl_2$-loaded corn pericarp, 4 specimens, 3 observations/specimen.		
Ca	9728	1.10
	(1393)	(0.154)
	[14.3]	[14.0]
Cl	8644	0.740
	(1834)	(0.143)
	[21.2]	[19.3]
4300 ppm Ca, 2900 ppm Cl		
B. Corn pericarp from pig jejunum, 4 specimens, 3 observations/specimen.		
Ca	5140	0.574
	(1200)	(0.123)
	[23.3]	[21.4]
Cl	17547	1.49
	(3006)	(0.214)
	[17.3]	[14.4]
Na	4243	1.69
	(581)	(0.154)
	[13.7]	[9.1]
S	3735	0.302
	(791)	(0.056)
	[21.1]	[18.7]
P	3421	0.287
	(686)	(0.055)
	[20.1]	[19.0]
K	10343	1.12
	(2191)	(0.219)
	[21.1]	[19.6]

Peak Ht. = counts above background/5 min.
(Standard deviation), [CV]

The CVs usually are greater for the peak height data, thus indicating less variation in the P/B measurement versus the peak height measurement. This result has been observed for all six elements measured in corn bran specimens retrieved from five GI tract locations in each of two pigs. The greatest CV differences between the two types of data occurred for sodium.

Discussion with Reviewers

Reviewer #1: It would have been better to express the data as P/B ratios (the background under the peak) rather than as peak intensities. Use of P/B automatically corrects for the fluctuations in beam current and absorption due to local variations in surface texture of the specimen.

Authors: There is valid reason to suggest this for biological samples as indicated by the works of Statham (1981) and Echlin et al. (1982). Boekestein et al. (1984) concluded that "a peak to local background ratio" is to be preferred with bulk bio-organic specimens of poorly defined local tilt and takeoff angles. We tested this concept with two types of specimens: corn pericarp loaded in 0.05M calcium chloride and corn pericarp retrieved from the mid-jejunum of a pig. These specimens were embedded in acrylic resin (LR White) and prepared as previously described. Differences between use of background-subtracted peak height values and P/B (peak to the background under the peak) ratios were tested (Table 3) by comparison of percent coefficient of variation [CV] associated with the two types of data.