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Determination of Methionine in Peas by Near-Infrared Reflectance Spectroscopy (NIRS)

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Methionine and protein were determined in field peas (*Pisum sativum*) by standard chemical methods. Three different near-infrared reflectance techniques were employed to measure both constituents directly in ground peas without hydrolysis or any chemical treatment. Accuracy of prediction of methionine was ±0.011% and of protein ±0.76% of the whole peas with a commercially available near-infrared reflectance instrument. Time for testing each sample for both methionine and protein was 45 s.

Methionine, isoleucine, lysine, threonine, and tryptophan are the most frequently limiting amino acids in the plant materials used as human food. Of these, methionine and tryptophan are the most difficult to determine with accuracy, since both tend to become degraded during hydrolysis, which normally precedes the determination of amino acids by standard ion-exchange chromatography. Recently Finlayson and Mackenzie (1976) and Mackenzie (1977) announced improved techniques for the determination of methionine, both of which were faster and less destructive of methionine than the ion-exchange chromatography method. Their procedure involves gas/liquid chromatography following selective extraction of methionine and does not call for hydrolysis.

One of the most important applications of methionine determination is associated with the identification of genotypes of food legumes with higher methionine content. Although food legumes offer valuable sources of protein and lysine for the diets of peoples of developing countries they are often deficient in methionine (Ackroyd and Doughty, 1964; Kelly, 1972; Hulse, 1975). Table I compares the methionine content of several food legumes with the cereal staples and illustrates the generally lower methionine content of the legumes. The breeding of cultivars with enriched methionine content has been inhibited by the lack of a practicable method for the rapid, accurate determination of methionine. Since its first description as a spectroscopic method for the determination of moisture in soybeans (Ben-Gera and Norris, 1968) near-infrared reflectance spectroscopy (NIRS) has become a

Table I. Comparison of Methionine Content of Some Food Legumes with Cereal Staples

source	species	mg of methionine/g of total N	ref[a]
chickpea	*Cicer arietinum*	80	1
cowpea	*Vigna unguiculata*	120	1
broad bean	*Vicia faba*	30	1
lentil	*Lens esculenta*	50	1
pea	*Pisum sativum*	70	1
phaseolus bean	*Phaseolus vulgaris*	60	1
pigeon pea	*Cajanus cajans*	80	1
millet	*Panicum miliaceum*	117	2
rice (milled)	*Oryza sativa*	145	3
sorghum	*Sorghum vulgare*	100	2
wheat	*Triticum aestivum*	106	4

[a] 1. Ackroyd, W. R., and Doughty, J., 1964. 2. Hulse, J. H., et al., 1981. 3. Juliano, B. O., 1972. 4. Ksarda, D. D., et al., 1971.

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valuable tool in the measurement of oil, protein, moisture, and fiber in a wide range of commodities (e.g., Hymowitz et al., 1974, Rinne et al., 1975, Williams, 1975, Williams et al., 1978, Williams and Starkey, 1980). More recently NIRS has been applied to the determination of lysine (Rubenthaler and Bruinsma, 1978) and other amino acids (Norris et al., 1977, Williams et al., 1984) in cereals. This communication describes the application of NIRS to the determination of methionine in peas.

NIRS instruments receive the optical signals from samples in the form of the log 1/R, or apparent reflectance. Some instruments employ the signal directly in this form, which has the advantage that as long as a wavelength monitored by the instrument corresponds to a wavelength where the constituent to be measured causes an absorption, a measurement can be made. A second wavelength acts as a reference wavelength. The log $1/R$ signal itself, and consequently any instrument which employs this algorithm directly, is susceptible to variations in the surface of the sample caused by differences in mean particle size, particle size distribution, and bulk density. The principal effect of particle size/surface changes is on the optical signal which reaches the instrument detection system, which in turn affects the accuracy of measurement. Earlier work carried out during the development of the NIRS technique indicated that errors caused by the surface effects could be reduced by taking the 1st or 2nd derivatives of the log $1/R$ signal and using these variations of the optical response to compute constituent concentration (Norris and Williams, 1984).

MATERIALS AND METHODS

Samples (20 g) of 80 cultivars of whole field peas (*Pisum sativum* v. Arvense), including smooth and wrinkled types, were ground in a Udy cyclone grinder with a 1.00-mm screen. This technique gives a fine meal with a mean particle size of about 190 μm (for peas). The ground samples were well-mixed and analyzed for protein by the Kjeldahl procedure (AACC Cereal Laboratory Methods, 1969) and methionine by the method of Mackenzie (1977). Subsamples of the same ground material were used for NIRS analysis, where the sample size was about 6 g. There is no need to weigh samples for NIRS analysis. Two series of samples were prepared, the first series of 60 for calibration, and the second of 20 for prediction. The second series of samples was not used in the calibration. Both series represented a uniform distribution of samples across the range of methionine and protein concentrations. Sample weights for total protein and methionine analysis by standard methods were respectively 0.5 g and 10 mg. A Pacific Scientific Model 6350 Research Composition Analyzer (RCA 6350) was interfaced to a Pacific Scientific Model 51A Feed Quality Analyzer (FQA 51A). The FQA 51A carries 6 near-infrared narrow band-pass filters, scanning the range from about 1600 to 2340 nm. The RCA 6350 includes a holographic grating spectrophotometer and scans from 1100 to 2500 nm. The spectrum is divided into 700 wavelength points, each of which represents 2 nm. Both instruments employ four lead sulfide detectors. The first series of 60 samples was read on the RCA 6350. The optical data were used to identify the optimum wavelengths and mathematical treatment, and to establish calibrations for the determination of methionine and protein. The samples were then used to record optical data with the FQA 51A interfaced to the RCA 6350. In this mode the samples are read by using the FQA 51A optics, and the log $1/R$ signals are recorded directly onto the flexible disk system of the RCA 6350, which enables the use of the more powerful computing of the RCA 6350 to select the optimum wavelengths and mathematical treatment for use with the FQA 51A optical signals.

Table II. Composition of Samples of Peas Used for Calibration and Analysis (Prediction)

	calibration		analysis	
	protein %	methionine %	protein %	methionine %
high	30.4	0.300	30.4	0.283
low	21.1	0.167	20.4	0.169
mean	25.64	0.229	25.42	0.225
std dev	2.34	0.034	3.05	0.034

All Pacific Scientific bench instruments employ wavelengths in the form of "pulse points". In the case of the FQA 51A, the six narrow band-pass filters are incorporated into a filter wheel which rotates in the vertical plane. As the plane of an interference filter "tilts", or passes through an arc of 90–0° relative to the source or irradiation the optimum absorbance changes. In the FQA 51A the relationship between the angle of the filter and the absorption efficiency is such that the operational range of each filter extends downward to about 10% from the "starting" wavelength of the filter. The starting wavelength is the wavelength of maximum absorption when the filter is at an angle of 90° to the light source. As an example, a filter with starting wavelength of 2192 nm can be used effectively as far down as 1973 nm. The range of each filter in the FQA 51A is divided into 128 pulse points, each of which corresponds to about 1.7 nm. A computerized encoder instructs the instrument to take a reading at specific pulse points, and these readings are taken for every revolution of the filter wheel. Since each pulse point corresponds to a single wavelength, optical data are recorded at the specific wavelengths for every rotation of the filter wheel. Wavelengths (as pulse points) can be entered into the FQA 51A by the operator by using a push-button sequence, and the operator can select the wavelengths to use by means of a computerized spectrophotometer, such as the RCA 6350.

The 60 calibration samples were then reused to calibrate the FQA 51A with the wavelengths (pulse points) which had been selected when the instrument was interfaced to the RCA 6350. When the FQA 51A is not interfaced, it uses its own microprocessor for recording and storing optical data, computing its calibration constants and analyzing samples, by translating the optical signals into composition information. To summarize, the same samples were read three times, once in the RCA 6350 to select wavelengths and mathematical treatments for use with the RCA 6350 itself, and twice in the FQA 51A, first in the interfaced mode to use the RCA 6350 computer for wavelength and mathematical treatment selection, and the second time to calibrate the FQA 51A with its own microprocessor and the wavelengths (pulse points) and mathematical treatment selected for it by the RCA 6350. Calibrations were generated for all three systems by using up to four primary wavelengths and three mathematical treatments, the log $1/R$ unprocessed and the first and second derivatives thereof. The three NIRS systems incorporating up to nine different calibrations were then used to determine methionine and protein in the second series of 20 samples.

Accuracy was expressed in terms of the "standard error of performance" (SEP) which is the standard deviation of differences between NIRS and standard chemical analyses. The mean of the deviations ($\bar{d}$), and the coefficient of correlation (r) between NIRS and standard analysis are also quoted, as well as the coefficient of variability (CV), which expresses the SEP in terms of the mean standard

Table III. Wavelengths and Accuracy of Prediction of Methionine and Protein in Peas with a Model 6350 Research Composition Analyzer and Three Mathematical Treatments

order of terms	wavelengths selected for analysis by					
	log $1/R$		1st derivative		2nd derivative	
	methionine, nm	protein, nm	methionine, nm	protein, nm	methionine, nm	protein, nm
1[a]	1426	1426	1662	2154	2352	2158
2	1720	2218	1103	2324	1352	1996
3	1572	1438	1762	1466	2438	1986
4	1710	1508	1774	2010	1142	2272
parameter	results of statistical analysis of predictions					
SEP[b]	0.013	0.53	0.018	0.31	0.025	0.80
$\bar{d}$	0.014	1.38	0.020	0.22	0.014	0.56
r	0.99	0.98	0.98	0.99	0.95	0.99
CV	5.8	2.1	8.0	1.2	11.1	3.1

[a] Order of wavelength selection by computer. [b] SEP = standard error of performance; $\bar{d}$ = mean difference; r = coefficient of correlation; CV = coefficient of variability.

chemical result for methionine or protein. The time-tested method of verifying accuracy by "spiking" samples with additional amino acids (for example) is not applicable to NIRS work, since the configurations in which the amino acids actually exist in the protein complex are those which cause the molecular vibrations responsible for the absorbances. These absorbances are not exactly the same as absorbances generated by the amino acids in their pure forms. Precision of the determinations was assessed by 12 repeated analyses of a single check sample of peas.

RESULTS AND DISCUSSION

The range in concentration, mean, and standard deviation for methionine and protein in the 60 samples used in calibration and the 20 used for prediction are given in Table II. The results of methionine and protein determination with the RCA 6350 are summarized in Table III, which also gives the wavelengths selected for analysis with the RCA 6350 alone. The underivatized log $1/R$ algorithm gave the most accurate prediction for methionine but not for protein. the mean of deviations between standard and NIRS analyses is normally referred to as bias, in the field of NIR technology. The large bias of 1.38% protein which occurred with the log $1/R$ algorithm is not serious, since biases can be adjusted out in any NIR instrument. When the log $1/R$ algorithm was used, the primary wavelength points were identical for methionine and protein. This is not uncommon since most of the absorbers carried by proteins arise from amino acid side chains, and any individual amino acid and the protein to which it belongs may have a common absorber. It is the 2nd and subsequent wavelengths which enable the differentiation between the amino acids from themselves and from protein (Williams et al., 1984).

Table IV illustrates the principal absorption bands of the NIR spectra of methionine and protein. The protein was a wheat gluten preparation containing 96% "protein" (N × 5.7 dry basis). Earlier work at the USDA, Beltsville, MD (Norris, K. H. and Williams, P. C., unpublished), had revealed that the principal bands for a number of isolated plant and animal proteins were remarkably similar. The wavelengths selected for methionine and protein by the 1st and 2nd derivative algorithms were clearly different from each other. This indicated that the NIRS measurement of methionine was distinct from that of protein with the RCA 6350, by either 1st or 2nd derivative, but possibly not by the underivatized log $1/R$.

The RCA 6350 was then used to select wavelengths (as "pulse points") directly for the interfaced FQA 51A. The computer again selected a common wavelength (pulse point) for both methionine and protein by using the log

Table IV. Principal Absorption Bands in Methionine and Protein

methionine[a]			protein			
1145*†	1760*†	2225	1065	1415	1840	2265
1190*	1790	2285*†	1085	1460†	1865	2278†
1365*	1810	2305*	1100	1495	1915	2310†
1390*	1845*	2335}†[b]	1110	1515	1940	2345
1400	1875	2370}	1140	1540	1478	2385
1450	1915*	2385*	1185	1575	2055	2415
1495*	2025	2410*	1230	1625	2105	2470
1630}*†[b]	2060*	2435†	1260	1695	2120	2510
1680}†	2085	2485	1280	1735	2145}†[b]	2535
1695*†	2100*	2510*	1320	1755	2170}	
1730*†	2145*	2550	1365	1800	2185†	
1740*†	2175*	2590	1390	1815	2195	

[a] All wavelengths in nanometers. Bands for methionine with asterisk are coincident ±5 nm with bands for protein. Bands with "dagger" were used for calibration. [b] Primary bands selected for measurement were broad and encompassed several wavelengths.

$1/R$ signal directly. Different pulse point sets were selected for methionine and protein by using first or second derivative mathematics. The FQA 51A can be used to analyze samples, while interfaced to the RCa 6350 by reading the samples on the FQA 51A and using optical signals at the pulse points set into the FQA 51A but using the more powerful computer of the RCA 6350 to compute the signals taken at these pulse points. The accuracy of prediction of both protein and methionine with log $1/R$ or first or second derivative was similar, although the first derivative data were very slightly superior (Table V).

Finally, the FQA 51A was calibrated with its own autocal software and the pulse points were selected by the RCA 6350. The Pacific Scientific system for computing the first derivative for their FQA 51A uses a 10-pulse-point smoothing and a derivative or gap size of 13 points. For the first derivative the operator must enter two pulse points into the instrument, so that for four first derivative terms eight pulse points must be entered. For the second derivative the FQA 51A computes the derivative automatically from the primary wavelength, so that the operator has only to insert one pulse point for each term point. This explains the eight pulse points for the first derivative in Table VI, and the four points for the second derivative algorithm. Table VI indicates that both derivatized treatments gave excellent predictions of methionine with again the first derivative being slightly the better. The predictions using the log $1/R$ algorithm in the FQA 51A were not satisfactory, and the standard deviation of difference between standard and NIR methods for methionine determination was ±0.22, about 20 times higher than that of the 1st derivative algorithm. Table VII gives the

Table V. Wavelength (Pulse Points)[a] and Accuracy of Prediction of Methionine and Protein in Peas with FQA 51A Interfaced to RCA 6350 and with Three Mathematical Treatments

order of terms	wavelengths in nm and (pulse points) selected for analysis by: log 1/R, methionine, nm	(pp)	log 1/R, protein, nm	(pp)	1st derivative, methionine, nm	(pp)	1st derivative, protein, nm	(pp)	2nd derivative, methionine, nm	(pp)	2nd derivative, protein, nm	(pp)
1	1599	(1)	1599	(1)	1751	(228)	2137	(403)	2302	(609)*	2180	(443)*
2	1711	(165)	2216	(503)	1675	(129)*	1633	(37)	2138	(404)	2137	(403)
3	1757	(248)*[c]	2115	(386)*	1749	(223)	2314	(663)*	1603	(6)	2026	(279)
4	1675	(129)*	1658	(72)	1732	(193)*	1634	(38)	2257	(523)	1609	(11)

parameter	results of statistical analysis of predictions					
SEP[b]	0.013	0.48	0.011	0.55	0.012	0.58
$\bar{d}$	0.017	0.65	0.006	−0.31	0.01	−0.40
r	0.93	0.99	0.95	0.98	0.93	0.98
CV	5.8	1.9	4.9	2.2	5.3	2.3

[a] See text for explanation of pulse points. [b] SEP = standard error of performance = standard deviations of differences between NIR and standard analysis; $\bar{d}$ = mean difference between NIR and standard analysis; r = coefficient of correlation; CV = coefficient of variability. [c] Asterisked wavelengths correspond approximately with absorption bands on the spectra of methionine or protein.

Table VI. Wavelengths (Pulse Points) and Accuracy of Prediction of Methionine and Protein in Peas with FQA 51A Alone and with Two Mathematical Treatments

order of terms	wavelengths in nm and (pulse points) selected for analysis by: 1st derivative, methionine, nm	(pp)	1st derivative, protein, nm	(pp)	2nd derivative, methionine, nm	(pp)[a]	2nd derivative, protein, nm	(pp)
1	1751	(228)	2137	(403)	2318	(669)	2180	(443)*
2	1758	(251)*[b]	2204	(426)	2138	(404)	2137	(403)
3	1675	(129)*	1633	(37)	1603	(6)	2073	(279)
4	1699	(152)	1650	(60)	2257	(523)	1609	(11)
5	1749	(223)	2314	(663)*				
6	1756	(246)*	2329	(686)				
7	1732	(193)*	1634	(38)				
8	1745	(216)*	165	(61)				

parameter	results of statistical analysis of predictions			
SEP[c]	0.011	0.76	0.013	0.88
$\bar{d}$	−0.044	0.41	−0.080	0.31
r	0.94	0.97	0.93	0.97
CV	4.9	3.0	5.8	3.5

[a] These pulse points represent the central points of individual groups of 3 pulse points used in each 2nd derivative term. [b] Asterisked wavelengths correspond approximately with absorption bands on the spectra of methionine or protein. [c] SEP = standard error of performance = standard deviation of differences between NIR and standard results; $\bar{d}$ = mean difference between NIR and standard analysis; r = coefficient of correlation; CV = coefficient of variability.

Table VII. Precision of NIR Analysis of Peas for Methionine and Protein with FQA 51A

	methionine, 1st derivative	methionine, 2nd derivative	protein, 1st derivative	protein, 2nd derivative
SD[a]	0.005	0.003	0.184	0.405
$\bar{x}$	0.224	0.224	26.54	26.54
CV	2.23	1.34	0.69	1.53

[a] SD = standard deviation (standard error of a single test); $\bar{x}$ = mean result; CV = coefficient of variability.

precision of the FQA 51A for measurement of methionine and protein by two mathematical treatments of the log 1/R data. Although the precision of methionine measurement by 2nd derivative mathematics was slightly better, when taking both protein and methionine into consideration the 1st derivative precision was generally superior, since its precision of protein measurement was more than twice as good as the precision by the second derivative treatment.

The accuracy of NIRS methionine determinations by all three systems (RCA 6350 and FQA 51A interfaced and alone) was considered adequate for use in breeding programs, or in the formulation of diets and food mixes of specific methionine content. Underivatized and derivatized treatments of the log 1/R signal gave acceptable results for methionine and protein, but the derivatized treatments were preferred due to the superior wavelength specificity.

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Varietal Differences in the Vitamin E Content of Corn

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The relationship of maturation time of corn grain to its α-tocopherol and γ-tocopherol contents was examined. Whether expressed as α-tocopherol/g of fat or α-tocopherol/g of dry weight of corn, there was no correlation between α-tocopherol content and the time required to reach maturation. γ-Tocopherol, however, declined, as time to maturity increased. The amount of γ-tocopherol was 1–4 times that of α-tocopherol. However, because of the reportedly low biological activity of γ-tocopherol, the calculated vitamin E activity in corn was independent of the time required to reach maturity.

Corn breeding has produced many changes in corn grain which have been beneficial to both the corn grower and livestock producer, such as pest resistance, hardiness for particular climatic conditions, increased protein quality, and shorter maturation times. Introduced with some of these improvements may be some other changes which affect the nutrient value of the corn in positive or negative ways. Since corn is a major constituent of many diets for livestock and is a primary provider of vitamin E in those diets before supplementation, we are concerned with the vitamin E content of corn and the potential effects of genetic alteration of the plant on the vitamin E content of corn grain.

Within a variety of corn, the vitamin E content increases as the corn matures (Contreras-Guzman et al., 1982). This raises the question of whether the vitamin E content of varieties requiring less time to reach maturity is less than that of those requiring longer times. Since the faster maturing varieties are popular in some regions, such an effect could be expected to result in reduced vitamin E levels in formulated feeds. To test this hypothesis, we analyzed the α-tocopherol and γ-tocopherol levels of different varieties of corn grain with maturation times ranging from 97 to 138 days.

MATERIALS AND METHODS

Corn. Triplicate samples of 42 varieties of corn were obtained from a major corn breeding company. All of the corn was grown in 1983 in Iowa on the company's test plots. The harvested corn was dried at 35 °C for 5 days, whereupon it was shelled. The dried grain was packaged and mailed to our laboratory where it was stored at −4 °C until it was analyzed. Thus, the observed differences in the vitamin E contents of the varieties should not be attributable to differences in conditions of growing, handling, or storage. Immediately before analysis, samples of corn grain were finely ground in a laboratory sample mill.

Determination of α- and γ-Tocopherols. Samples of ground corn were saponified by addition of equal volumes of 50% KOH and ethanol containing 10% pyrogallol (antioxidant) and heating to 70 °C for 15 min. Tocopherols were extracted from the saponified samples with hexane containing 0.2% BHT. The hexane extracts were evaporated to dryness under nitrogen and reconstituted in ethanol.

Tocopherols were separated by HPLC with a C-18 column with methanol–water (96:4, v:v) as the mobile phase. α- and γ-tocopherols were detected by fluorescence (excitation 291 nm, emission 330 nm) with a flow cell attachment and were quantitated by comparison to commercially available standards.

Fat. Fat was extracted from ground corn by ether reflux in a Goldfisch apparatus and was quantitated gravimetrically.

RESULTS AND DISCUSSION

The relationships of the α- and γ-tocopherol contents of the grain to maturation time are shown in Figure 1. Whether the results are expressed per unit dry matter or per unit fat, the α-tocopherol level did not vary significantly ($P > 0.05$) as a function of maturation time. γ-Tocopherol, on the other hand, decreased significantly ($P < 0.01$) as maturation time increased. Although the level of γ-tocopherol in most samples was much higher than that of α-tocopherol, the total vitamin E activity was largely attributable to the α-tocopherol content, as the γ form is much less biologically active (Bieri and Evarts, 1974). In order to consider the biological relevance of these physical measurements of tocopherol contents, they were converted to USP units of vitamin E activity (1 USP unit being equal to the formerly used International Unit). This was done by multiplying the γ-tocopherol values by 0.1 to convert them to equivalents of α-tocopherol and multiplying the

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