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Grain Protein Quantity and Quality: A Rapid Non-destructive Means of Evaluating Cereal Grain is Provided by X-ray Photoelectron Spectroscopy*

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Introduction

The increasing world population has been accompanied by demands to maintain a commensurate food supply. The cereal grains, legumes and oil seeds provide the major protein sources in the less developed countries. It was thus natural to seek methods to improve the yields of these crops. The successes of Borlaug and his collaborators in these endeavors, acknowledged recently by the award of the Nobel Peace Prize, have led to the so-called "green revolution" (1). It is desirable that increased yields be accompanied by concomitant increases in the quantity of protein per grain, by the quality of the protein as measured by the content of essential amino acids and by the digestibility of the protein. This last property can be evaluated only by feeding trials; it lies outside our area of competence and will not be discussed further.

In order to assess the relative nutritional values of the massive number of seeds involved in any plant protein improvement program, it would be desirable for the agronomist and plant breeder to have available a convenient, rapid, economical, and non-destructive method for determining the quantity and quality of grain protein (2). We present some results of a new method which can now satisfy some of these requirements and offers the potential of satisfying them all.

Method

When a sample is irradiated with light of sufficient energy, electrons may be ejected and their kinetic energy is given by the well

known Einstein relation,

$$K.E. = h\nu - B.E.$$

where K.E. is the kinetic energy of the electrons, h is Planck's constant, ν is the frequency of the light quantum, and B.E. is the binding energy of the electrons in the sample. If the photon energy is increased sufficiently, by using X-rays, electrons may be ejected from the inner or core levels of the constituent atoms of the sample. Since photon or X-ray energies are known to high accuracy, the binding energies may be determined with precision by measurement of the kinetic energies. The measurements may be performed with a variety of devices, but most commonly magnetic and electrostatic deflection analyzers are employed. The overwhelming majority of the energy levels of the elements across the periodic table have been determined in this manner. Although the complementary method of X-ray emission contains the same information, the former method, called X-ray photoelectron spectroscopy, offers so many direct advantages that we will confine the present discussion thereto.

The method is outlined schematically in Fig. 1. In this figure are sketched the discrete energy levels of a particular atom in a compound as well as those levels which are a collective property of the compound as a whole and make up its valence band or molecular orbitals. An X-ray photon is shown lifting an electron from the 2s level of this atom into the continuum of energies or, equivalently, removing it to infinity. At this point the electron enters the spectrometer wherein it is brought to a focus and impinges on a detector when the electron has the correct energy (more rigorously the correct momentum). The

energy range is scanned by varying the strength of the magnetic field and thus a spectrum is traced out by recording the number of electrons reaching the detector at each value of magnetic field. This method was developed and brought to its present state of refinement by the group at the Institute of Physics, University of Uppsala, Sweden (3).

Since all atoms are constructed in the same manner, it is possible to photoeject their electrons, and by a suitable choice of exciting X-ray energy and particular atomic level one can distinguish among the different constituent atoms of the sample. It is apparent, then, that calibration against a sample of known elementary composition will permit a qualitative and quantitative analysis of the unknown sample. The absolute sensitivity of the method is very high although it is not especially suitable for detecting small amounts of one element in the presence of a large excess of other elements.

More recently it has been shown that a given element in different chemical configurations exhibits chemical shifts of its binding energies (4,5). These chemical shifts thus extend the utility of the method so that not only the total quantity of a given element may be determined, but also the type or types of chemical bonding situation in which the atom is situated. The origins and theoretical foundations for these chemical shifts are rather well understood (4,6,7).

Experimental Design

Since virtually all of the previous work in this field has been directed toward precision measurements of the energies of the photoelectrons, we were obliged to devise techniques to permit the quantitative

determinations of the relative numbers of relevant atoms contained in the samples. We were further interested in establishing the feasibility of measuring not only total protein by nitrogen, but also of assessing the amino nitrogen as a measure of lysine, arginine and histidine and the sulfur as a measure of cysteine, cystine, and methionine. Our principal research endeavors prior to our introduction to the present topic are related to iron and sulfur and the non-heme iron-sulfur proteins known as the ferredoxins. Accordingly, we had some prior knowledge of sulfur spectra but no current nitrogen data were pertinent to the present problem (8).

The first task undertaken was the observation of the nitrogen and sulfur spectra of relevant amino acids, simple peptides, and known proteins. Fig. 2 shows spectra of three such compounds. The cystine spectra show lines from both nitrogen and sulfur. The designations N1S and S2P indicate that the electrons arise from the 1S level of nitrogen and the 2P level of sulfur, respectively. The di-peptide, L-isoleucyl-L-alanine, exhibits a nitrogen peak considerably broader than that of the cystine. This peak may be decomposed into two peaks of width equal to the cystine peak; one component is of the same energy as the cystine peak and we conclude that it corresponds to the amino nitrogen. The other peak occurs at lower binding energy and is attributed to the amide nitrogen formed during the peptide bonding. The lower lines originate from a sample of hemoglobin. The nitrogen peak occurs at the position of lower binding energy and is therefore the amide nitrogen. The sulfur peak is shown magnified by 50-fold and exhibits an increasing shoulder at higher binding energies. We

believe this shoulder arises from sulfur as $SO_4^=$ and that it most probably entered the sample during ammonium sulfate precipitation of the protein (9).

Figure 3 shows the nitrogen and sulfur spectra from three protein samples, hemoglobin (repeated for continuity), equine cytochrome C, and the apo-ferredoxin and its native form from Clostridium pasteurianum. This latter protein has a molecular weight of 6000 and contains 8 iron atoms, 8 moles of cysteine, and 6-8 moles of acid labile sulfur (10). The cytochrome C spectrum is similar to that of hemoglobin but shows a higher sulfur content. It also contains $SO_4^=$. The apo-ferredoxin spectra are qualitatively similar to both the heme-proteins, except for the larger quantity of sulfur. The native ferredoxin spectra are significantly different from the heme-proteins; both lines are broader and exhibit rather obvious shoulders. The nitrogen shoulder at higher binding energy probably arises from nitrogen entering as NH_4^+ during ammonium sulfate precipitation of the protein. The lowenergy sulfur shoulder definitely arises from sulfur bonded to iron and is characteristic of such bonding (8).

Figure 4 shows the nitrogen spectra of Barker barley seed and of a dark red kidney bean. The barley spectrum is very similar to the other proteins; the kidney bean spectrum is considerably broader and exhibits a high energy shoulder.

Figure 5 shows the nitrogen spectrum of a light red kidney bean and the curve has been decomposed into two components by computer. The major peak, at lower binding energy, represents the amide nitrogen, while the smaller peak at higher binding energy represents the amino nitrogen. Figure 6 shows the sulfur region of this same sample.

Figure 7 shows the nitrogen spectrum of a sample of ramona wheat where again the experimental data have been fit by two peaks corresponding to the amide and amino nitrogen. Figure 8 shows the sulfur region of the wheat sample.

All of the foregoing spectra were excited by Mg Kα radiation at 1252 eV and analyzed in the Berkeley iron-free spectrometer (11).

Sample Preparation

The nature of photoelectron spectroscopy imposes constraints on the form of the samples. Since the kinetic energy of the photoelectrons is very low, usually less than 1.5 keV, they must be analyzed in vacuo to reduce scattering. Also, because of their low energy, they are able to penetrate a distance of only a few tens to a few hundreds of angstroms in solid material. Thus only those electrons originating within this distance from the surface of the sample escape elastically and give rise to discrete photoelectron lines. Only the surface of a given sample is of use.

Accordingly, for these studies the samples were prepared as powders by grinding in a ball mill. For the quantitative determinations the biochemicals were mixed with NaCl and then pulverized together. The amino acids, di-peptides, hemoglobin, and equine cytochrome C were obtained from commercial sources. The ferredoxin was prepared in the laboratory of Professor J. C. Rabinowitz in Berkeley. The seed samples were obtained from the seed station of the University of California, Davis, California.

Quantitative Determinations

The basic relative sensitivity for nitrogen and sulfur was obtained from a sample of cystine mixed with NaCl. The areas under the N1S, S2P, and Na2S peaks were integrated by computer and were treated as follows:

Sulfur

1. $\frac{\text{Area S2P}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{\text{Moles of compound}} = 14/1 \text{ atom equivalent}$

2. $\frac{\text{Weight of compound} \times \% \text{ S}}{32} = \text{Moles of compound}$

3. $\frac{\text{Area S2P}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{14} \times \frac{32}{\text{Weight of compound}} = \% \text{ S}$

Nitrogen

4. $\frac{\text{Area N1S}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{\text{Moles of compound}} = 9.4/1 \text{ atom equivalent}$

5. $\frac{\text{Weight of compound} \times \% \text{ N}}{14} = \text{Moles of compound}$

6. $\frac{\text{Area N1S}}{\text{Area Na2S}} \times \frac{\text{Moles NaCl}}{9.4} \times \frac{14}{\text{Weight of compound}} = \% \text{ N}$

These sensitivity factors are specific for our instrument and for the particular sample geometry and method of data analysis.

Table I gives the quantitative values for several samples determined by this method. The two cysteine samples were prepared independently and are shown as an indication of the reproducibility of the method. The cystine sample was different from that used as the calibration standard. For the seeds, the experimental weight percent nitrogen, determined from the photoelectron spectral amide nitrogen peak, was converted to percent protein by assuming a nitrogen:protein weight ratio of 0.15. Table I includes both nitrogen and sulfur analyses by both X-ray photoelectron spectroscopy

and by conventional wet chemical analysis. Except for the light red kidney bean, the results are comparable. The apparent discrepancy for the light red kidney bean sample may be attributed to the fact that this material is extremely hard and may not have been milled adequately to produce a sufficiently fine powder.

Also shown in Table I is the distribution of protein between the embryo and the total seed for the light red kidney bean. To ascertain the validity of the hypothesis that the component of the nitrogen photoelectron line ascribed to the amino nitrogen did in fact correspond to the quantity of basic residues, an amino acid analysis was performed. The results, derived from such an analysis on the total acid hydrolysate, are given in Table II wherein the fraction of the basic residues is shown to be 17.4%. This value equals that derived from the photoelectron data thus substantiating the assumption.

Conclusions

This report demonstrates that the quantity and quality of grain protein may be determined by X-ray photoelectron spectroscopy. The quantity of protein may be determined by quantitation of the amide nitrogen photoelectron line. Two measures of the quality of the protein are available: 1) The basic amino acids including lysine, arginine, and histidine may be determined by quantitation of the amino nitrogen photoelectrons, and 2) the sulfur-containing amino acids, cystine, cysteine, and methionine, may be quantitated by measuring the sulfur photoelectron line.

The quantity of material required to perform the measurements need

be only a few milligrams. Indeed, the active volume giving rise to the photoelectron lines is probably no greater than a few tens of micrograms of material of unit density. Thus, it is feasible to excise a small section of a seed for the analysis while retaining the remainder of the seed for future planting. We performed one such experiment and were able to bring the bulk of the seed to germination.

The experiments reported here were designed to test the feasibility of the method to measure protein quality and quantity. Thus, care was taken to measure the entire nitrogen and sulfur regions of the photoelectron spectrum. The times required for these nitrogen determinations were approximately 1 hour per sample. Such a large period of time clearly limits the utility of this method for mass screening operations. Were it satisfactory to perform only a total nitrogen--and thus total protein--analysis on a large number of samples, one could dispense with the high spectral resolution and measure only the integrated nitrogen intensity. Such a measurement could be accomplished in about 2 minutes per sample. This estimate is for the spectrometer observation time alone and does not include the time required for sample preparation. Since the seed material is analyzed in its native state, aside from the pulverizing operation, the time consuming preparative steps requisite to and employed in purely chemical methods are eliminated, as are the separate and independent procedures required for each element of interest. Newer instrumentation promises to offer a significant further saving in time.

Summary

X-ray photoelectron spectroscopy offers a method of high potential for determining the quantity and quality of grain protein. In this method a small quantity of material is irradiated with X-rays from a suitable target material and electrons from all constituent atoms may be photo-ejected. The kinetic energy of these photoemitted electrons is equal to the photon energy of the incident X-rays minus the binding energy of the level from which the electrons are emitted, K.E. = $h\nu$ - B.E. This kinetic energy, and hence the binding energy, are determined by a magnetic or electrostatic momentum analyzer, and are characteristic of the atom and level whence the electrons originated. All elements across the periodic table may be investigated. Calibration against compounds of known elementary composition permits a quantitative determination of the amount of each atomic species contained in the sample. The binding energies exhibit chemical shifts which permit a distinction between a given element in different chemical groupings. These experiments have demonstrated: 1) Total protein may be evaluated by quantitative determination of the amide nitrogen peak; 2) amino nitrogen may be distinguished from amide nitrogen, thus providing a measure of the basic amino acids lysine, arginine and histidine; and 3) the sulfur content may be determined by observing the sulfur photoelectrons.

Instrumentation currently under development promises to reduce the time per sample from hours to minutes and thus render possible large-scale screening.

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TABLE I

QUANTITATIVE DETERMINATION OF NITROGEN AND SULFUR CONTENT

Compound	Element	Experimental Weight %	Calculated Weight %
Cysteine* (1)	Nitrogen	8.2	8.2
	Sulfur	18.0	19.3
Cysteine* (2)	Nitrogen	8.5	8.5
	Sulfur	20.0	19.3
Cystine (1)	Nitrogen	13.0	11.7
	Sulfur	26.3	26.7

*Cysteine-hydrochloride-monohydrate

ELEMENTAL ANALYSIS

Seed	% N(XPS)	% N(anal)	% S(XPS)	% S(anal)
Barker Barley	1.5 ± 0.2	1.5 ± 0.2	$.05 \pm .02$	$.01 \pm .01$
Ramona Wheat	1.6 ± 0.2	1.7 ± 0.2	$.05 \pm .02$	$.06 \pm .03$
Rapida Oats	2.2 ± 0.2	1.9 ± 0.2	$.1 \pm .03$	$.02 \pm .02$
Light Red Kidney Bean	3.2 ± 0.3	4.1 ± 0.3	$.08 \pm .04$	$.13 \pm .06$

SEED PROTEIN DISTRIBUTION (LIGHT RED KIDNEY BEAN)

Sample	% N(XPS)	% N(anal)	% Basic A.A.(XPS)	% Basic A.A.(anal)
Endoplasm	3.1 ± 0.3	4.0 ± 0.3	17 ± 5	17.4
Embryo and Endoplasm	3.1 ± 0.3	4.2 ± 0.3	18 ± 5	

TABLE II

AMINO ACID ANALYSIS - LIGHT RED KIDNEY BEAN

Amino Acid	Quantity (μmole)	Amino Acid	Quantity (μmole)
Aspartic Acid	.163	Allo-isoleucine	.007
Threonine	.030	Isoleucine	.060
Serine	.016	Leucine	.109
Glutamic Acid	.203	Tyrosine	.011
Proline and Cystine	.063	Phenylalanine	.057
Glycine	.099	NH_3	.369
Alanine	.087	Basic Amino Acids	
Cystine	.001	Lysine	.093
Valine	.086	Histidine	.039
Methionine	.005	Arginine	.078

Total A.A. content = 1.207 μmole

$$\% \text{ Basic A.A.} = \frac{.210}{1.207} \times 100 = \underline{17.4\%}$$

FIGURE CAPTIONS

Figure 1. Energetics and analysis of X-ray photoelectrons.

Figure 2. X-ray photoelectron spectra of three compounds showing nitrogen and sulfur lines. The dipeptide nitrogen line is decomposed into two components of equal width. One component is isoenergetic with the cystine nitrogen and is assigned to the amino nitrogen. The other component, isoenergetic with the hemoglobin, is assigned to the amide nitrogen.

Figure 3. Nitrogen and sulfur X-ray photoelectron spectra of three proteins.

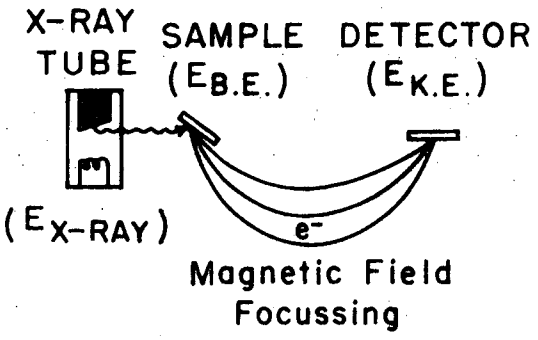
Figure 4. Nitrogen X-ray photoelectron spectra of two seeds.

Figure 5. Nitrogen X-ray photoelectron spectrum of light red kidney bean decomposed into two components corresponding to the amide nitrogen at lower binding energy and amino nitrogen at higher binding energy.

Figure 6. Sulfur X-ray photoelectron spectrum of light red kidney bean.

Figure 7. Nitrogen X-ray photoelectron spectrum of ramona wheat decomposed into two components corresponding to the amide nitrogen at lower binding energy and the amino nitrogen at higher binding energy.

Figure 8. Sulfur X-ray photoelectron spectrum of ramona wheat seed.



$$E_{B.E.} = E_{X\text{-}RAY} - E_{K.E.}$$
(Known) (Measured)

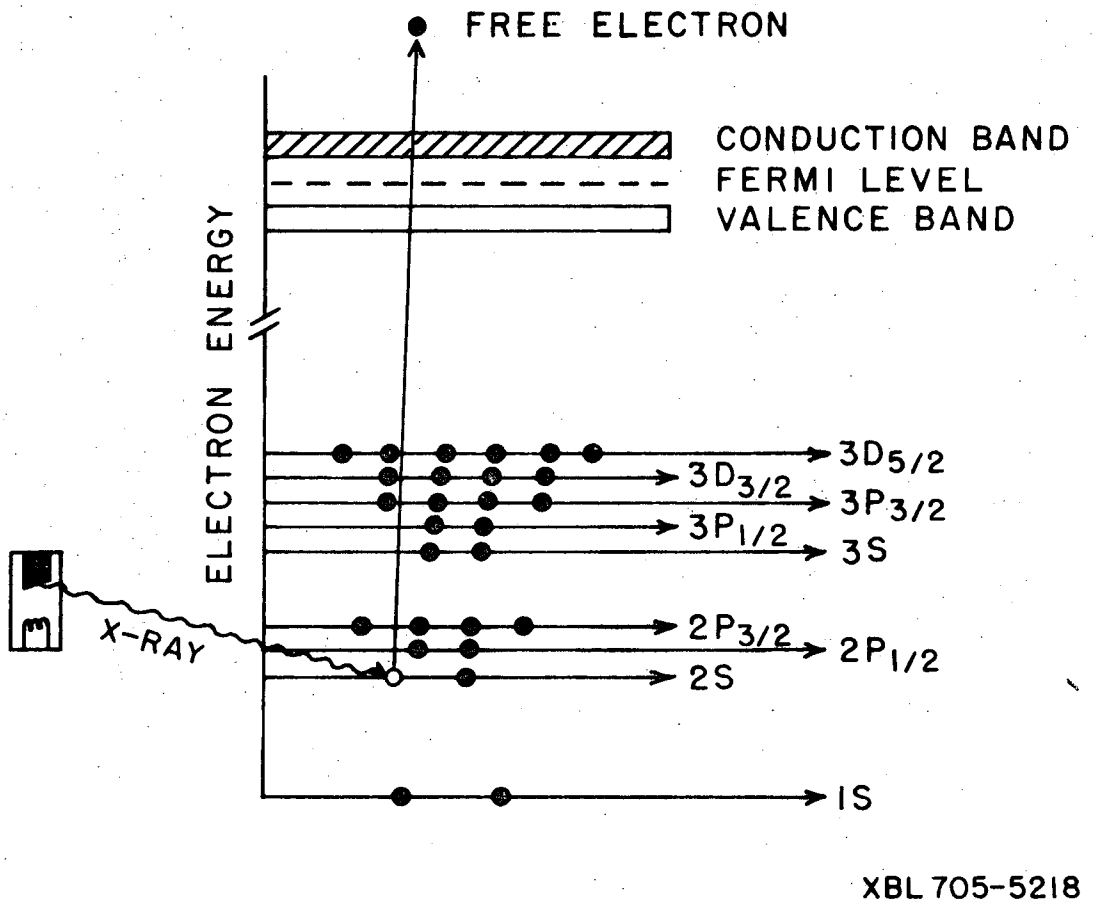


Fig. 1

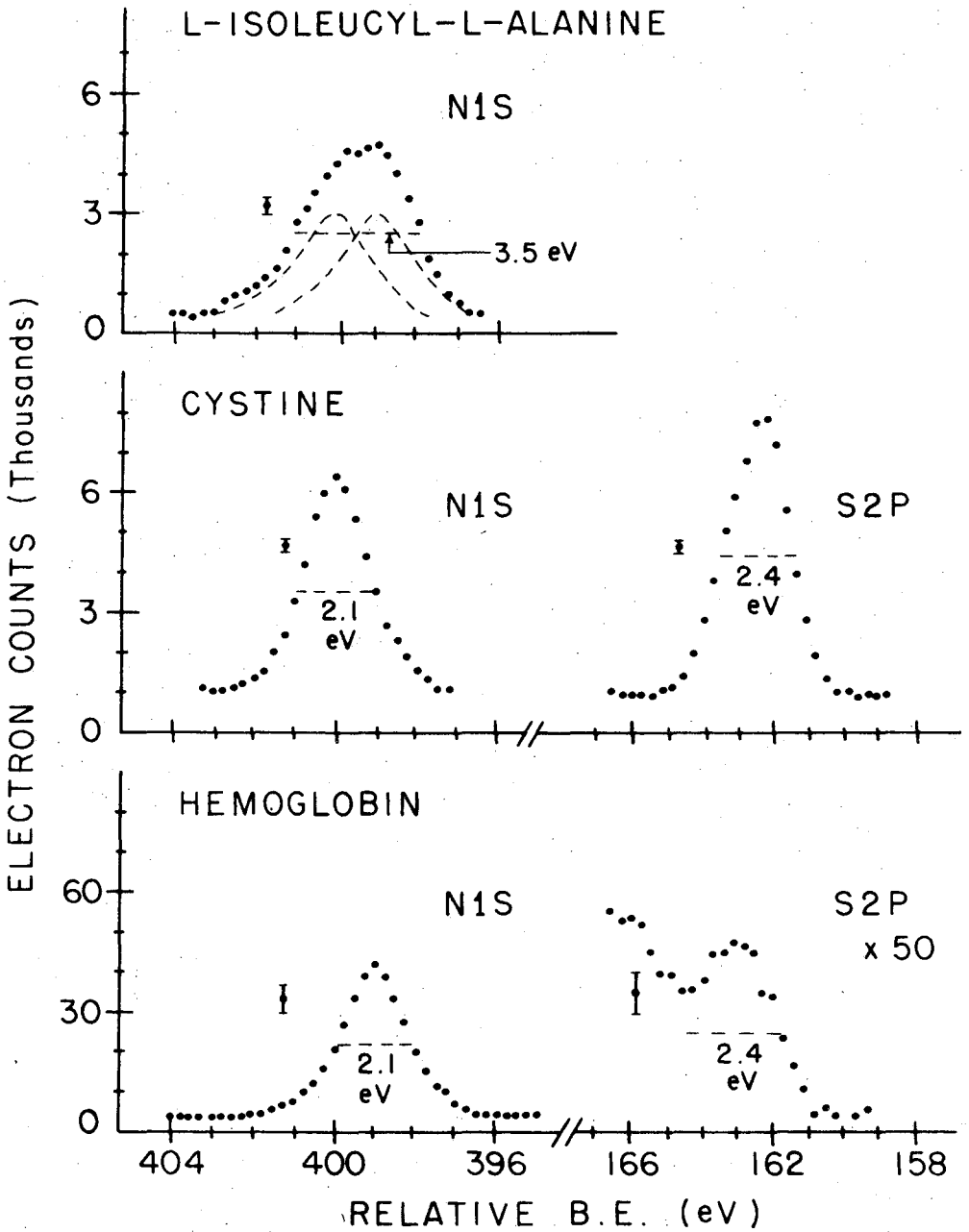

L-ISOLEUCYL-L-ALANINE
N1S
3.5 eV
CYSTINE
N1S
2.1 eV
S2P
2.4 eV
HEMOGLOBIN
N1S
2.1 eV
S2P
x 50
2.4 eV
ELECTRON COUNTS (Thousands)
RELATIVE B.E. (eV)
404
400
396
166
162
158
XBL705-5216

Fig. 2

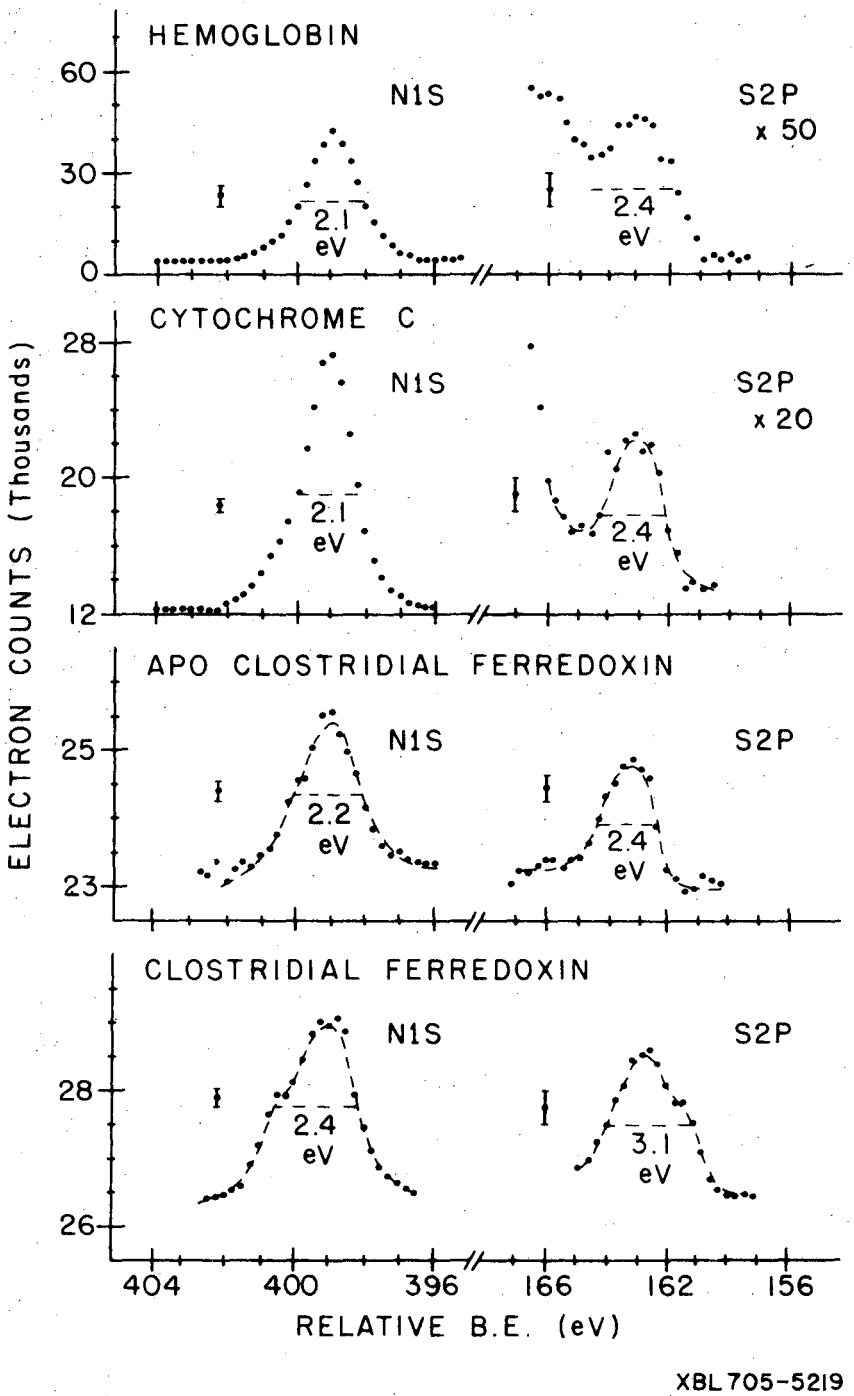

HEMOGLOBIN
N1S
S2P
x 50
2.1 eV
2.4 eV
CYTOCHROME C
N1S
S2P
x 20
2.1 eV
2.4 eV
APO CLOSTRIDIAL FERREDOXIN
N1S
S2P
2.2 eV
2.4 eV
CLOSTRIDIAL FERREDOXIN
N1S
S2P
2.4 eV
3.1 eV
ELECTRON COUNTS (Thousands)
RELATIVE B.E. (eV)
XBL705-5219

Fig. 3

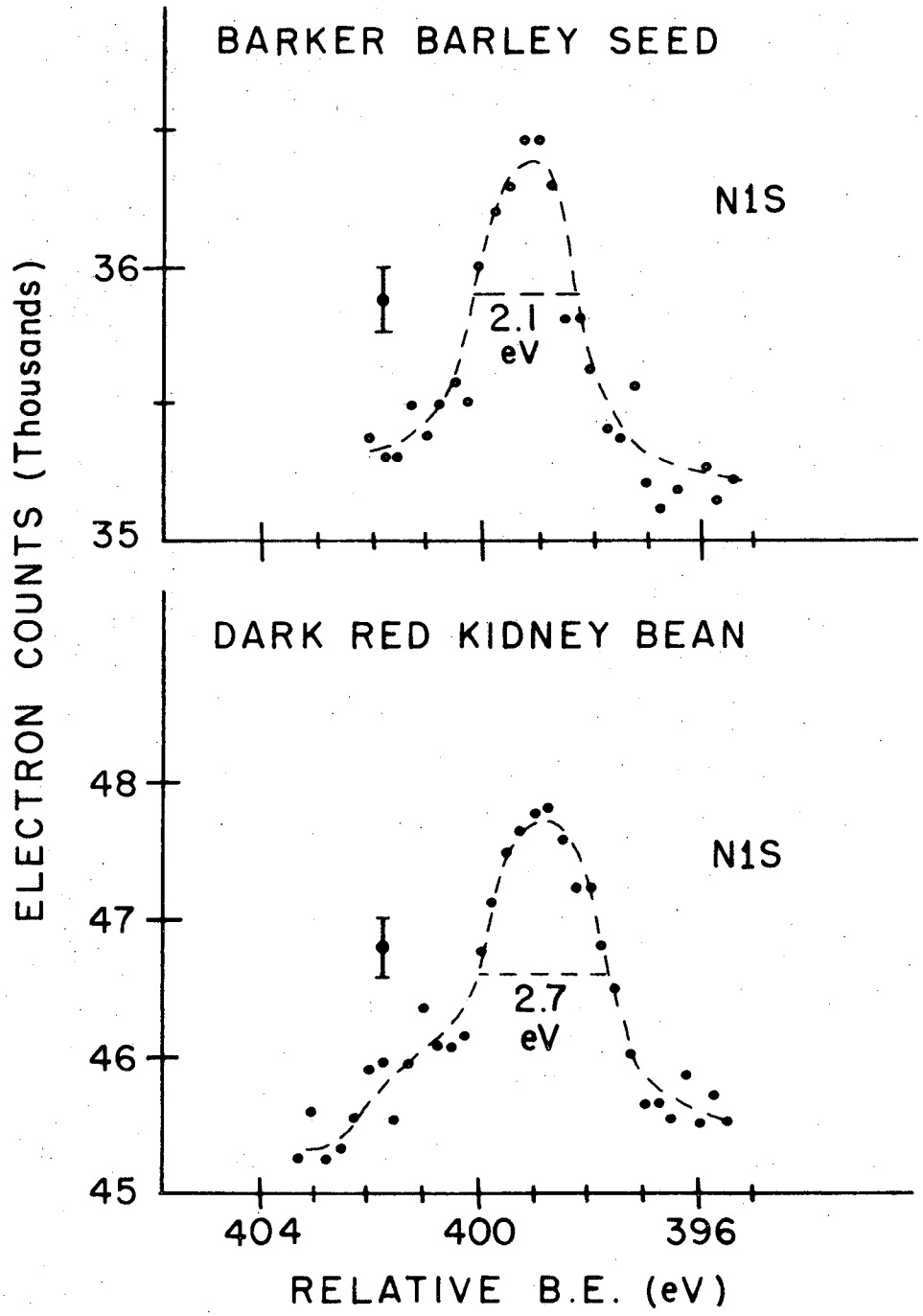

BARKER BARLEY SEED
N1S
2.1
eV
36
35
DARK RED KIDNEY BEAN
N1S
2.7
eV
48
47
46
45
ELECTRON COUNTS (Thousands)
404
400
396
RELATIVE B.E. (eV)
XBL 705-5217

Fig. 4

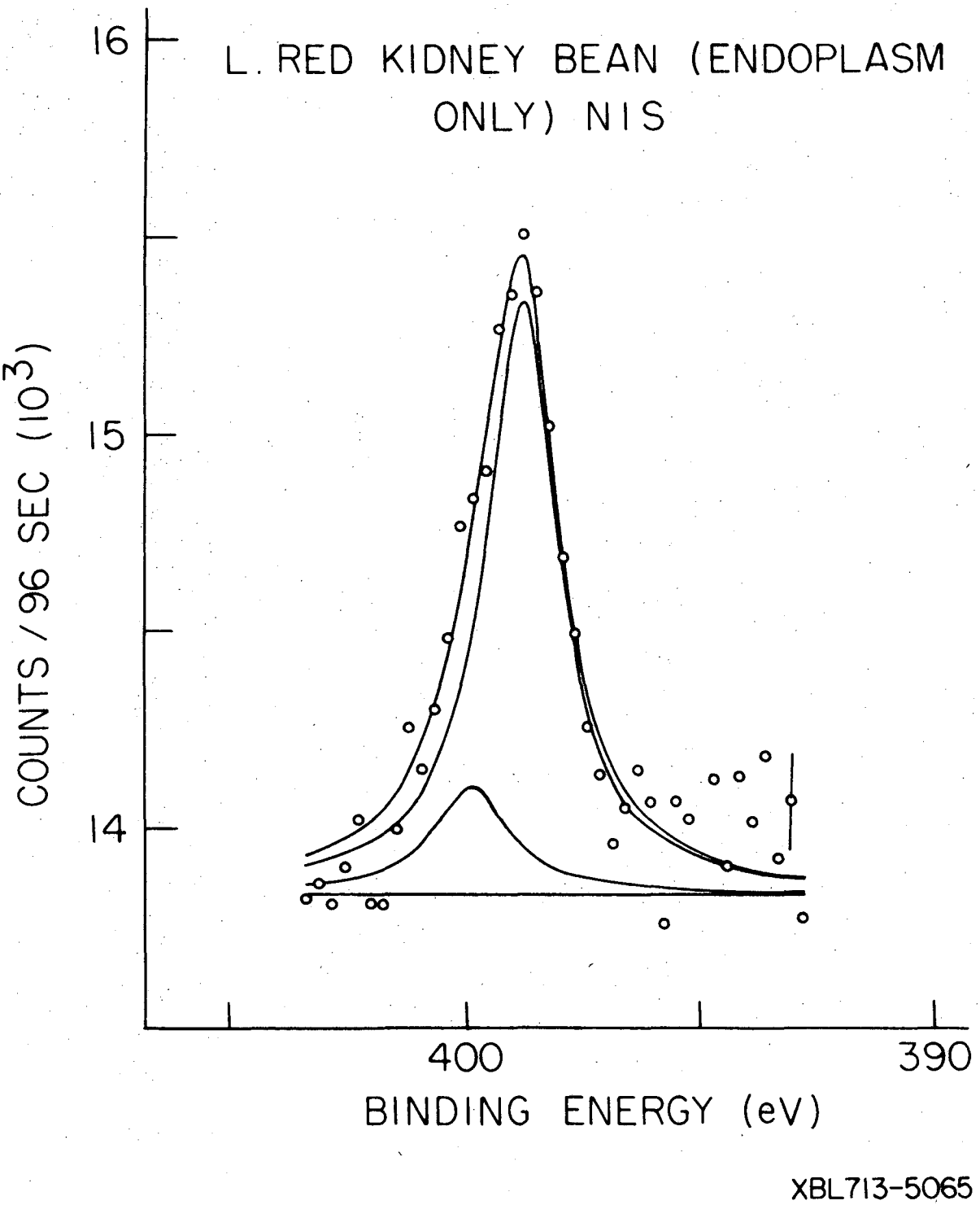

L. RED KIDNEY BEAN (ENDOPLASM ONLY) N1S
16
15
14
COUNTS / 96 SEC (10^3)
400
390
BINDING ENERGY (eV)
XBL713-5065

Fig. 5

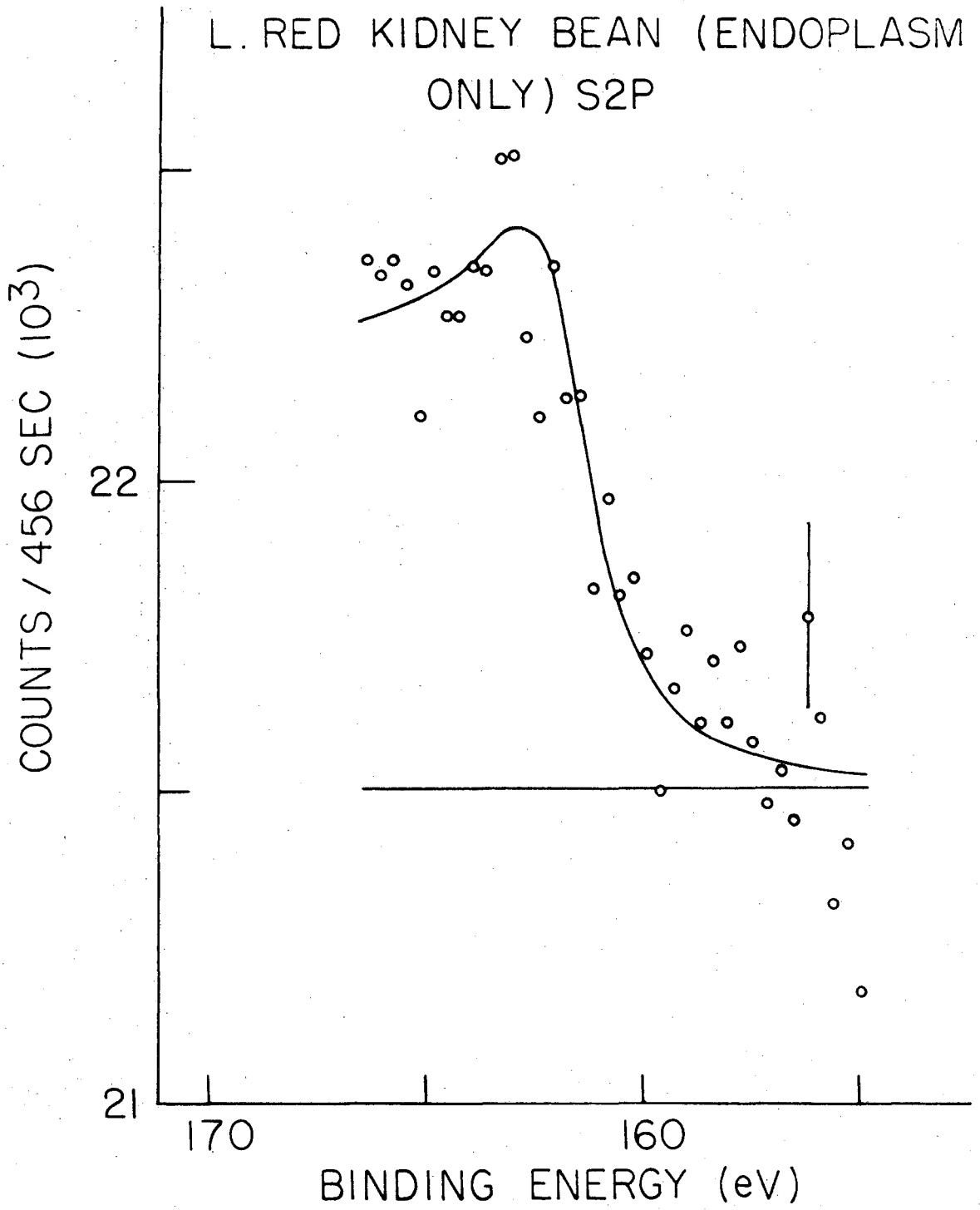

L. RED KIDNEY BEAN (ENDOPLASM ONLY) S2P
COUNTS / 456 SEC (10³)
22
21
170
160
BINDING ENERGY (eV)
XBL713-5068

Fig. 6

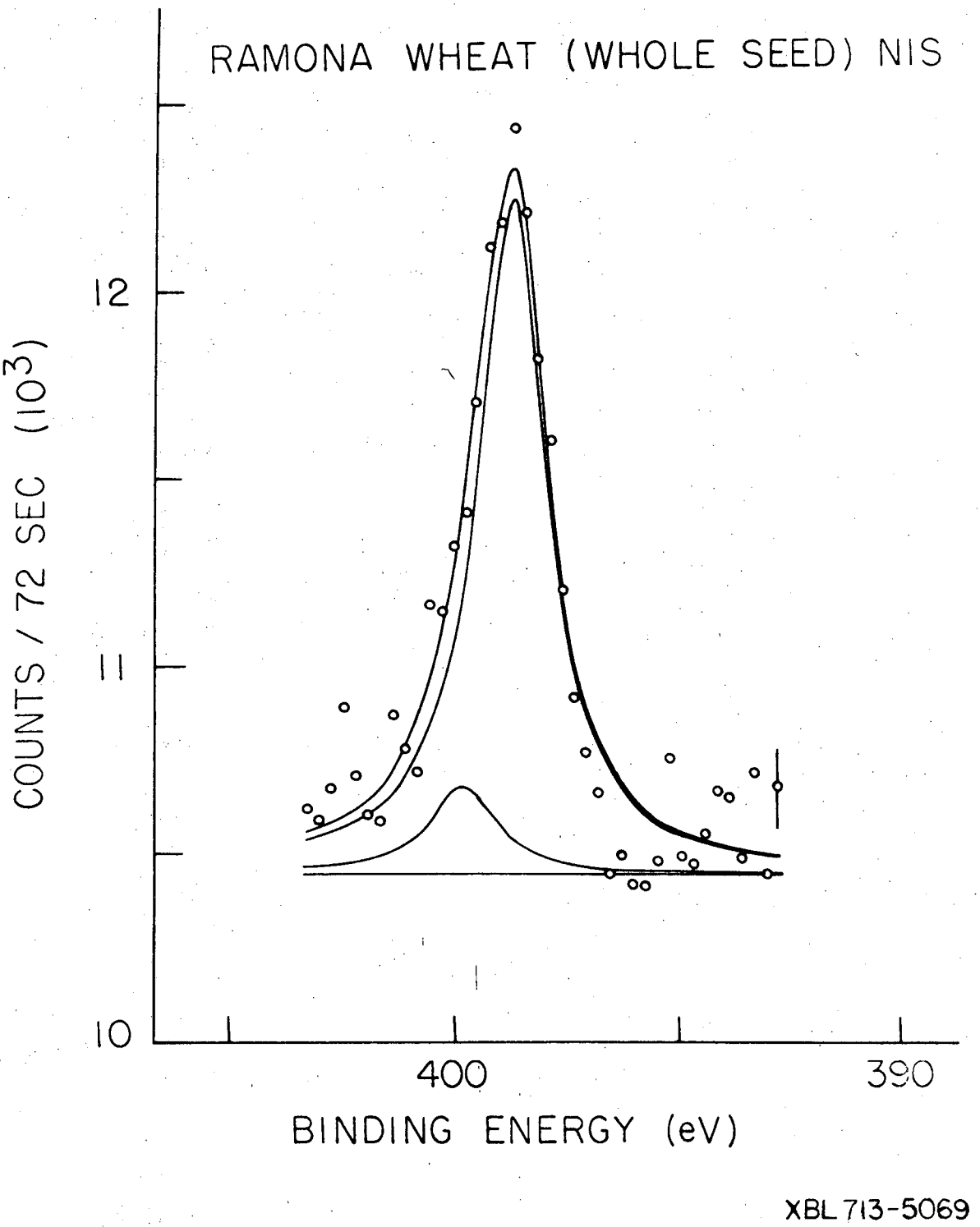

RAMONA WHEAT (WHOLE SEED) NIS
COUNTS / 72 SEC (10^3)
12
11
10
400
390
BINDING ENERGY (eV)
XBL 713-5069

Fig. 7

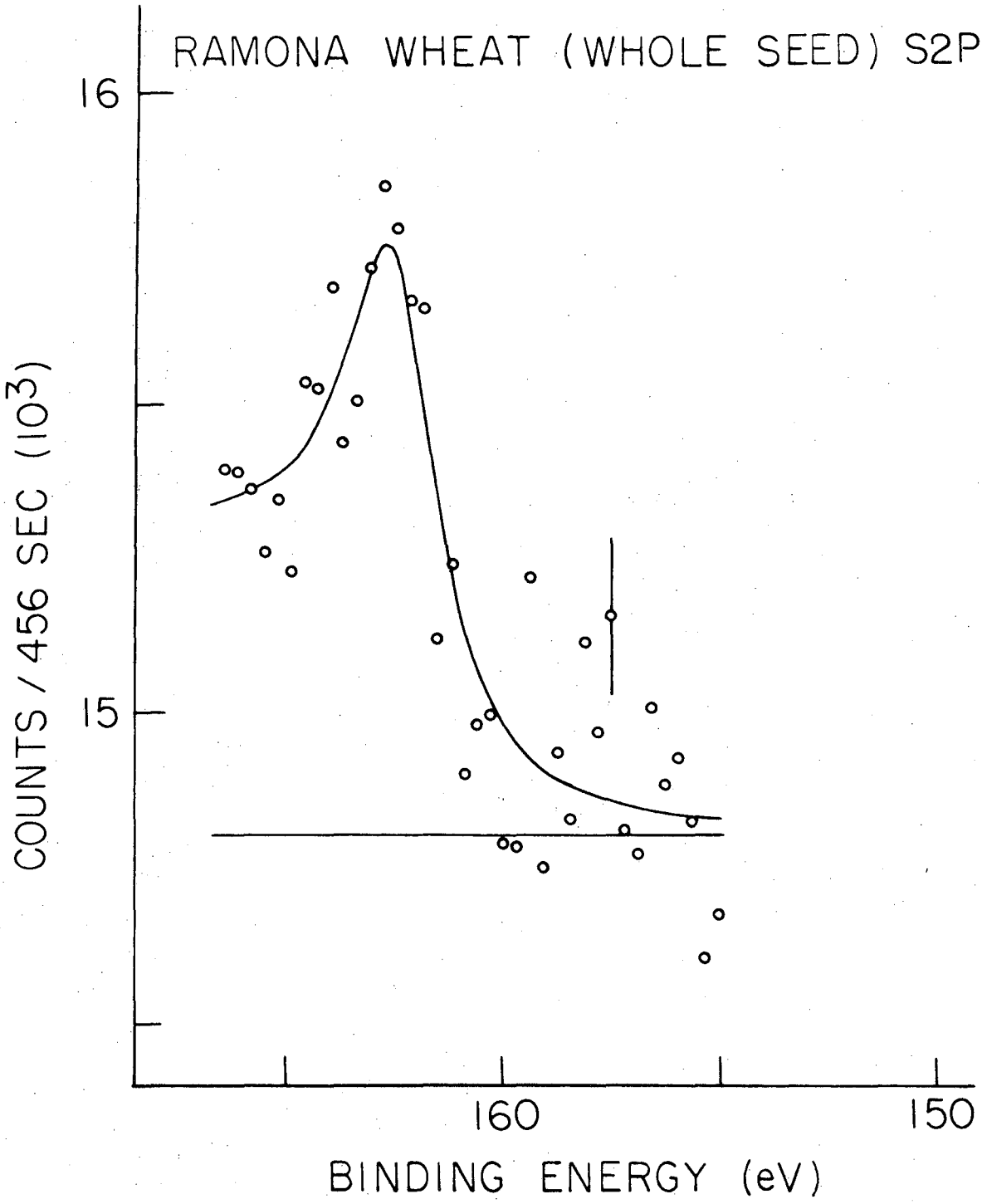

RAMONA WHEAT (WHOLE SEED) S2P
16
15
COUNTS / 456 SEC (10³)
160
150
BINDING ENERGY (eV)
XBL 713-5067

Fig. 8

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