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بسم الله الرحمن الرحيم

" وقل ربي زدني علما" صدق الله العظيم

رمزى محمد كامل وسعاد مرسى ابو طالب. ابى وامى لولا حبهم وتشجيعهم لى ما كان لهذه الدراسة ان تكتمل. اليهم اهدى هذه الرسالة اعترافا وتقديرا.

This thesis is dedicated to my mother, Souad and to the memory of my father, Ramzy. Their love, encouragement and help have made my studies possible.

ABSTRACT

The possibility of producing a food-grade protein concentrate from forest foliage was investigated. Hybrid poplar leaves were extracted for soluble proteins. Studies were carried out to try to compare poplar protein with leaf proteins from plants which have already been shown to yield commercial protein products. Methods were developed for isolating poplar proteins with minimal interference from phenolic compounds. Polyvinylpyrrolidone (0.2%) and sulfite (1-5%) added to the extraction buffer allowed the optimal recovery of crude protein concentrates with minimal coloring. During the field studies a comparison between poplar leaf protein and alfalfa, tobacco and rapeseed protein was done. The results show that, the amount of protein recovered from poplar leaf was slightly lower than that from alfalfa and higher than that from tobacco. Also, poplar leaves contained higher amount of phenol compounds than those of alfalfa, rapeseed and tobacco leaves. Greater extractability of proteins was observed when fresh leaves were used for extraction. Freezing of leaves decreased the yield of both extractable protein and phenol. The effect of seasonal variation on the amount of both extractable protein and phenol

was studied to find the times of highest and lowest concentrations during the growing season. It was observed that the highest amount of protein was recovered in mid August whereas the lowest was by the end of September.

ACKNOWLEDGMENTS

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Dr. Aileen Urquhart, merits a very special acknowledgment in this thesis for her friendship and many useful discussions during the course of this work.

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I wish to thank Mr. A.J. Campbell, the superintendent of the OMNR Howard Ferguson Tree Nursery in Kemptville. I also wish to thank Mr. H.W. Anderson, research scientist at OMNR for his advice and for supplying most of the samples.

Special thanks to my husband, Emad-Eldin Aly. By his companionship and constant faith in the undertaking, he instilled me with patience and strength.

Soumia, Amira and Mohamed Ramzy, my sisters and brother, I wish to thank them for their encouragement and support throughout my undergraduate studies.

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ABBREVIATIONS

EDTA	Disodium Ethylenediaminetetraacetate
FAO	Food and Agriculture Organization
OMNR	Ontario Ministry of Natural Resources
PVP	Polyvinylpyrrolidone
Tris.	Hydroxymethyl Aminomethane
LPC	Leaf Protein Concentrate
BV	Ratio of protein consumed to the fraction that is excreted in urine and faeces.
PER	Measurement of weight gain per gram of protein eaten.
NPU	The proportion of nitrogen intake that is retained.
TD	The proportion of consumed food nitrogen that is absorbed.

CHAPTER I

INTRODUCTION

1. General

World population continues to increase at a rate that will double the population of the world in about 30 years. There is a world wide shortage of food energy and protein sources (Pirie, 1970; Thomas et al., 1976).

The total amount of protein in the world at the retail level was estimated by the Food and Agriculture Organization (FAO) to be 90 million tons per year. The Protein Advisory Group of the United Nations concluded that the world deficit was 20 million tons even on its rather ungenerous estimate that individual needs ranged, according to the climate and age distribution in different countries, from 40 to 50 g a day (Pirie, 1970). In 1974, 460 million people were suffering from hunger and malnourishment (estimated by FAO). There is a general agreement that malnourishment, particularly protein deficiency, in infancy restricts brain development.

A great need exists to accelerate both the production and yield of edible proteins from conventional sources, as

well as to develop procedures for producing proteins from unconventional sources, such as single-cell protein (e.g. yeast, bacteria, algae and fungi), fish protein concentrate and leaf protein.

Rapid growth rate, high yields and the high degree of control that can be imposed on growing conditions in a fermentor are attractive advantages when single-cell organisms are considered as a source of food protein.

Much research effort has been aimed toward greater utilization of fish species (hake, menhaden, etc.) not generally consumed by man directly.

The third category of unconventional protein sources is leafy plants, which is the subject of the present thesis.

Green leaves constitute the primary and largest source of protein in the world. Leaves supply protein to other plant tissues. However, leafy plants contain high levels of fibrous structural materials, which cannot be digested by man and other monogastric animals. Ruminant animals and animals with functional caeca can digest large amounts of cellulose due to the action of symbiotic micro-organisms in the rumen or caecum. Those animals play an important role in converting forage grasses into animal protein and will continue to be of great value to man in the future.

The existence of leaf protein has been known since

1773, when the first experimental leaf protein extracts were produced by Rouelle (Rouelle 1773). A great deal of research was carried out in England by Pirie and his group at Rothamsted in the 1940's with the primary objective of developing a simple low cost process for use in developing countries (Pirie, 1942, 1971). Pirie called the protein recovered from leaves, Leaf Protein Concentrate (LPC).

The primary problem of introducing leaf protein products into the human diet is one of acceptability. Acceptability of leaf protein refers to the product color, flavour and nutritional value (Bray et al.,1976). In terms of nutritional value, leaf protein has a good biological value (BV) [ratio of protein consumed to the fraction that is excreted in urine and faeces], true digestability (TD) [the proportion of consumed food nitrogen that is absorbed], net protein utilization (NPU) [the proportion of nitrogen intake that is retained] and protein efficiency ratio (PER) [measurement of weight gain per gram of protein eaten]. Also it has the correct amount of essential amino acids [the amino acids not made by the body but obtained from food).

The more purified leaf protein is usually tinted. It does not have a bland white color usually associated with purified proteins, such as caseinates or soy protein concentrates. Possibly the reason is that leaf protein

concentrate has been shown to have phenolic compounds, such as tannin and chlorogenic acid bound to its protein molecules. The presence of phenolic compounds in plant protein concentrates affects the final acceptability (primarily due to the color of foods containing the plant protein) and possibly lowers the digestibility of LPC by inhibiting the rate of trypsin attack on the protein. In 1971 Allison suggested that binding polyphenols and quinones to the ε-amino groups of leaf protein lysine and subsequent polymerization of the polyphenols into tannin-protein complexes renders large amounts of the leaf protein inaccessible to digestion by monogastrics (Allison, 1971). In 1975 Free and Satterlee demonstrated that, the attack of the proteolytic enzymes trypsin and chymotrypsin on alfalfa leaf protein was slower when chlorogenic acid was bound to the leaf protein. Removal of the chlorogenic acid which is bound to protein fractions enhanced the rate of attack by both proteolytic enzymes on leaf protein concentrates (Free and Satterlee, 1975).

Leaf protein concentrate has a plant taste similar to chewed grass, which increases the unacceptability of it as a human food ingredient.

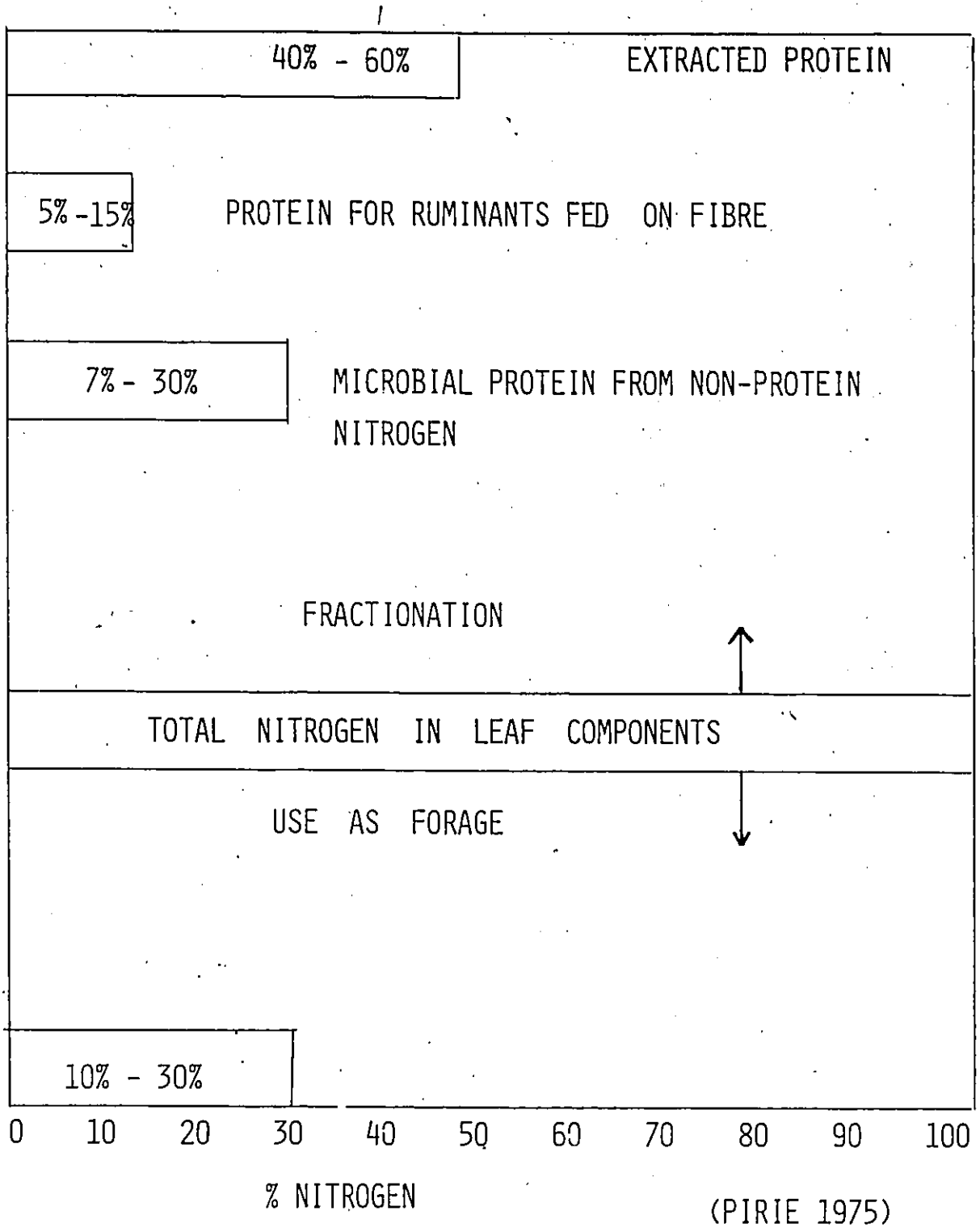
Leaf Protein Concentrate is one product resulting from the process of green crop fractionation. Two reasons for proposing to fractionate leafy crops instead of using

them as ruminant fodder are: the amount of fibre in some leaves makes them unacceptable as human food, and the conversion of forage into edible products by ruminants is inefficient. Fractionation of leafy crops can yield more edible protein than other forms of agriculture. The fibre residue which contains the protein that was not extracted, can be used in animal feeds. Moreover, the soluble leaf components can be used as a culture medium for the production of microbial protein (Pirie, 1975).

The differences in protein yield between a leafy crop used as forage or fractionated to extract the leaf protein concentrate is described in Figure 1. It shows that, between 10 and 30% of the protein in a forage is converted into human food by ruminants, whereas 40 to 60% of the protein can be extracted for human food. The fibrous residue containing the protein that was not extracted can be used for ruminant feeds. The clear brown juice obtained in the last step of the purification, after the precipitation of leaf protein concentrate, can be used as a culture for the production of single-cell protein (Figure 1).

In 1968 Pirie found that, protein extracts more readily from soft lush leaves than from those that are fibrous and dry (Pirie, 1968).

Figure 1 Shows differences in Protein Yield between a Crop Used as Forage or Fractionated to Extract the Protein


40% - 60%
EXTRACTED PROTEIN
5% -15%
PROTEIN FOR RUMINANTS FED ON FIBRE
7% - 30%
MICROBIAL PROTEIN FROM NON-PROTEIN NITROGEN
FRACTIONATION
TOTAL NITROGEN IN LEAF COMPONENTS
USE AS FORAGE
10% - 30%
0 10 20 30 40 50 60 70 80 90 100
% NITROGEN

(PIRIE 1975)

In large-scale work, re-extracting the fibre can give half as much protein again as a single extraction, but it would be difficult in the laboratory to get quantitative and repeatable results from a double extraction (Pirie, 1974).

2. Plant Sources for Leaf Protein Production:

A. Alfalfa Leaf Protein

There are a number of studies done on the extraction of leaf protein concentrate from alfalfa. This is because alfalfa has a few added advantages over other forage crops. These advantages are: it is a legume, perennial, grows fast and recovers quickly after harvesting and thrives in a variety of locations.

1. The Technique of Alfalfa LPC Extraction

The significance of the work by Pirie in the 1950's is that he extracted LPC containing from 40 to 50% protein with a PER of 1.7 (casein has a PER of 2.5). Machinery he developed ruptures the tough-fibered plant cells of alfalfa and presses out a green juice that has 25 to 35 percent protein. When the juice is heated, it separates into protein crudes and a clean brown liquid. The dried crudes become a dark green powder(leaf protein concentrate (LPC)(Figure 2)). Heat coagulation is the most satisfactory method for separating crude protein from the green-colored extract. Green

predominantly "Chloroplastic" protein coagulates at 50 °C-60 °C. It may be removed by filtration or centrifugation. If the now brown-colored extract is further heated to 70 °C, the colourless "cytoplasmic" protein coagulates. The protein can then be separated from the "whey" and dried. Whey would be used as a medium for culturing micro-organisms (Pirie, 1971).

Bawden and Pirie (1944) mentioned that after grinding the leaves to release the protein, some nitrogen remains attached to the fibre. Part of this is probably protein that was initially soluble but gets attached to the fibre as a result of the stresses during grinding (perhaps due to momentary local heating or by binding to polyphenols).

Commercial scale production of leaf protein concentrate from alfalfa was realized in the United States and Europe. LPC product contains high percentages of protein and low percentages of fibre. This product is an excellent feed ingredient for mongastric animals. The commercial processes which have been developed are based upon the work of U.S. Department of Agriculture researchers, who have named the animal feed LPC product Pro-Xan I (Kohler and Knuckles, 1977).

For more references as well as further discussions on alfalfa LPC refer to Kohler et al. (1973).

Pro-Xan II is a modified process which yields not only Pro-Xan I for animal feed but also a white protein product suitable for human food. Recently, the conditions

for the efficient separation of the green and white fractions of leaf protein were established on a laboratory scale (de Fremery et al., 1973). Present research is directed to produce Pro-Xan II in commercial scale (Figure 3).

2. Yields of Leaf Protein Concentrate

Yields of products of the two processes (Pro-Xan I and Pro-Xan II) are shown in Table I. In the Pro-Xan I process about 68% of the dry matter of the alfalfa was recovered in the press cake. About 32% was removed in the juice and about 17% was recovered in the soluble fraction. The remaining 15% was recovered as whole LPC (Pro-Xan I). In the Pro-Xan II process, 11% was recovered as green "chloroplastic" LPC and 3.3% as white "cytoplasmic" LPC. A look at the crude protein distribution column shows that the pressing removed 57% of the crude protein in the juice. Of this, 42% was recovered in the Pro-Xan I, the remaining 15% in the brown juice. When the split was made in the Pro-Xan II process, about 60% of the recoverable protein was in the green fraction, and about 40% in the white. The latter represents about 43% of the 7% soluble protein of the original alfalfa (Kohler, 1977).

Figure 2 Extraction of Green Leaf Protein Concentrate from Alfalfa Leaves (Pirie, 1971).

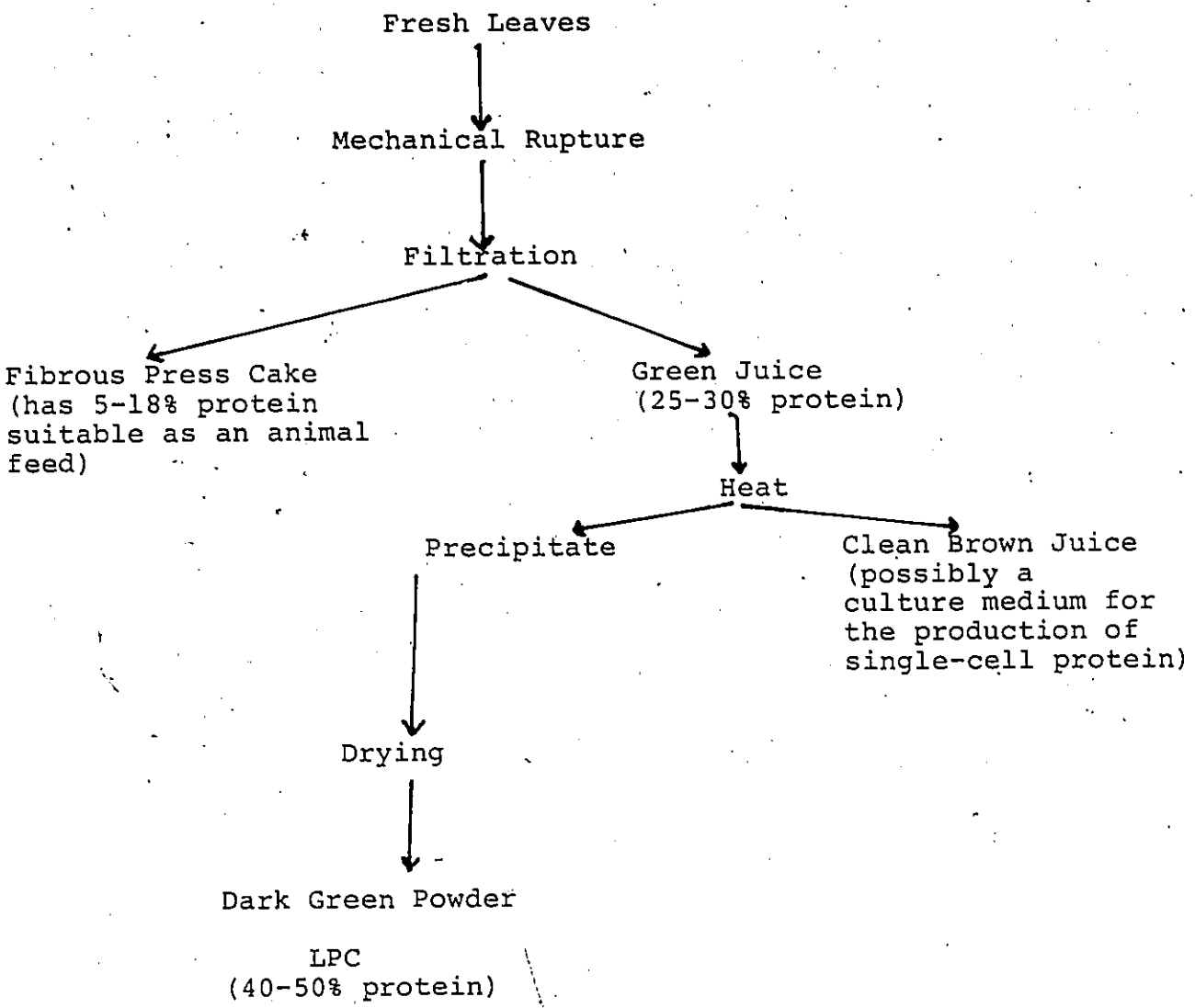

Fresh Leaves
Mechanical Rupture
Filtration
Fibrous Press Cake
(has 5-18% protein
suitable as an animal
feed)
Green Juice
(25-30% protein)
Heat
Precipitate
Clean Brown Juice
(possibly a
culture medium for
the production of
single-cell protein)
Drying
Dark Green Powder
LPC
(40-50% protein)

Figure 3 The Production of White Leaf Protein Concentrate. (Berkeley Method) (Kohler, 1968)

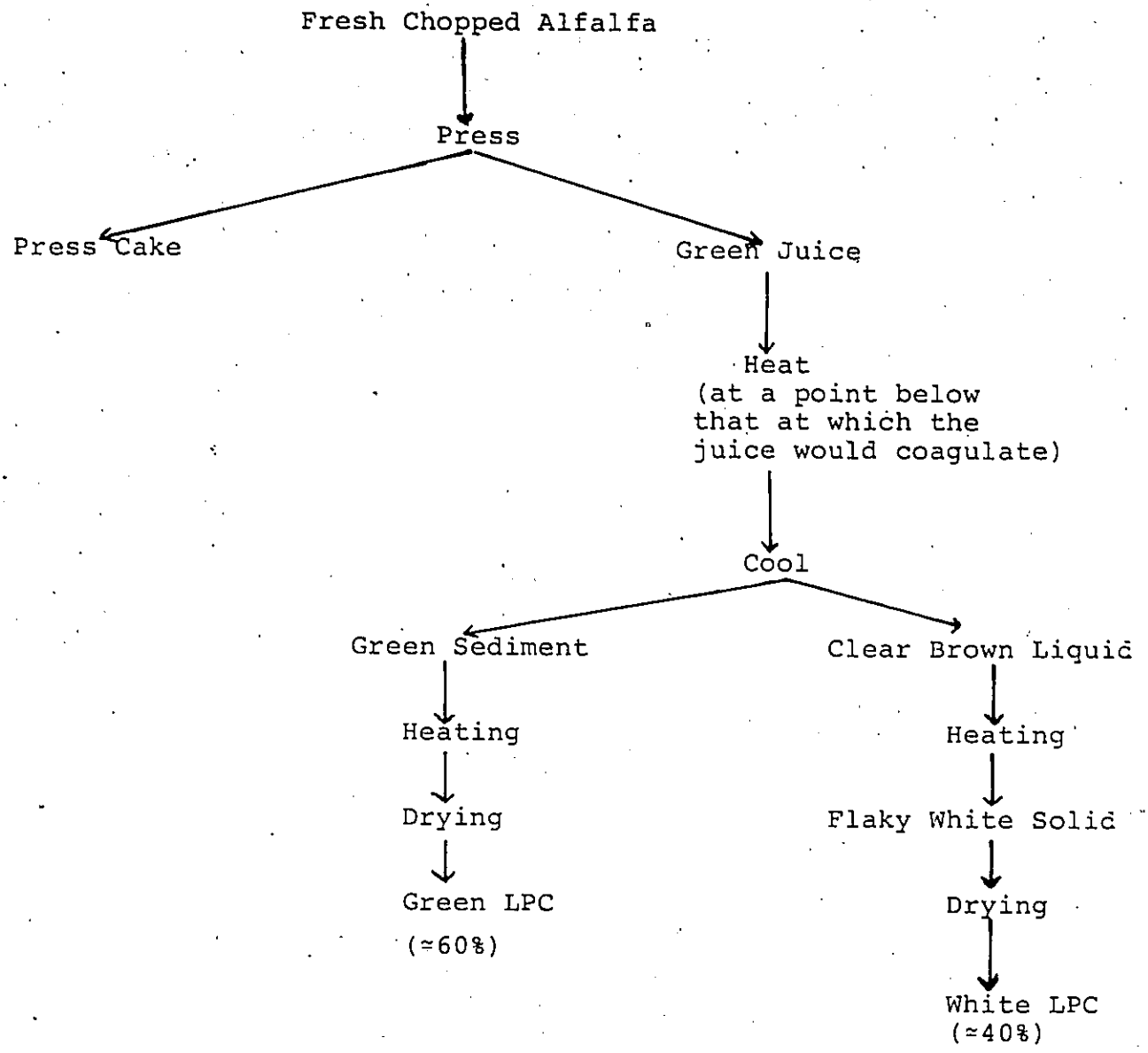

Fresh Chopped Alfalfa
Press
Press Cake
Green Juice
Heat
(at a point below
that at which the
juice would coagulate)
Cool
Green Sediment
Clear Brown Liquid
Heating
Drying
Green LPC
(≈60%)
Heating
Flaky White Solid
Drying
White LPC
(≈40%)

Table I

Distribution of Solids in the Fractionation of Alfalfa Leaf Protein Concentrate

Process Step	Dry Weight %	Crude Protein (% of original)	Soluble Protein (% of original)
Raw material (alfalfa leaves)			
Solids	100	1.0	
Crude Protein	21	100	—
Soluble Protein	7	33	100
Recovery in Pro-Xan I Products			
Whole LPC	15	42	
Press Cake	68	43	—
Brown Juice	17	15	—
			—
Recovery in Pro-Xan II Products			
White LPC	3.3	16	43
Green LPC	11	26	
			—

(Kohler, 1977).

3. The Nutritional Value of the Leaf Protein Concentrate

LPC products from different methods are well balanced and contain high levels of essential amino acids. There is a slight deficit of total sulfur amino acids (methionine and cystine) in the green LPC. The white LPC has higher levels of almost all essential amino acids as compared with green leaf protein concentrate(Table II).

Protein efficiency ratio (PER) of freeze-dried green LPC, when supplemented with 0.2% methionine was not significantly different from casein. The PER of white LPC, before adding 0.2% methionine was not significantly different from casein, whereas, after adding 0.2% methionine there was a significant increase (Bickoff et al.,1975).

Green LPC gave a good true digestability value, but the white LPC gave a very good value (Table III).

B. Tobacco Leaf Protein Concentrate

Tobacco is another agriculture crop whose foliar protein has come under considerable investigation. Changes in smoking patterns have forced the industry to re-evaluate the end-use of this vast resource. Also, changes in the economics of commercial protein isolates (e.g. from soybean) have caused much work to proceed on finding other vegetable protein sources. Since 1947, Wildman and his group at

University of California in Los Angeles, have been working on the recovery of leaf protein from tobacco foliage. They found from amino acid studies that, tobacco leaf protein is a superior source of supplemental protein in the human diet compared with leaf protein from other plants. Leaf protein can be easily separated from tobacco and the relatively pure protein can be recovered by crystallization.

Tso and his group at the United States Department of Agriculture, Maryland, developed a process known as Homogenized Leaf Curing (Ford, 1978). Protein is extracted from tobacco leaves before they are cured for cigarette manufacture. The process is based on the fact that, the compounds that enhance smoke and tobacco usability are non-proteinaceous. This makes it possible to separate and recover the protein before curing.

More than 50% of the protein extracted from tobacco leaves is Fraction-1 protein. Except methionine, the amount of essential amino acids in tobacco leaf protein either meet or exceed the standard patterns of the essential amino acids (FAO, 1973). It is possible to crystallize Fraction-1 protein from tobacco. It is a pure, tasteless, colorless and odorless product. Fraction-1 protein is suitable as an animal and human food (Wildman, 1974).

The other proteins recovered from tobacco leaves are a mixture of many proteins with small molecular weights.

Table II

Amino-Acid Composition (g/16 gN)

of Green LPC and White LPC

Amino Acid	FAO[1] Standard	Green LPC	White LPC
Isoleucine	4.00	4.94	5.46
Leucine	7.04	8.19	9.37
Lysine	5.44	6.18	6.54
Methionine+ Cystine	3.54	3.25	3.71
Phenylalanine+ Tyrosine	6.08	9.34	11.68
Threonine	4.00	4.81	5.76
Valine	4.96	6.25	7.19

FAO: Food and Agriculture Organisation (1973) (Bickoff et al.,1975).

Table III

Protein Efficiency Ratio (PER), True Digestibility (TD) and Biological Value (BV) of Green and White LPC

	Green LPC	White LPC	Casein
PER			
. with no additional methionine	1.84	2.40	2.5
. with 0.2% methionine	2.63	2.76	
TD	87.7	98.7	99.6
BV	79	80	83

(Bickoff, 1975).

C. Other Green Leaves

Spinach, pea, bean, rapeseed and grasses have been studied to a lesser degree than alfalfa and tobacco as a source for leaf protein extraction (Ellis, 1979; Haslett et al., 1976; Brown, 1975 and Bray, 1975). Spinach LPC has been characterized recently in England (Ellis, 1979).

There is not much difference between the process of extracting leaf protein from alfalfa, tobacco or any other green leaves. All the methods are based on rupturing and juicing the leaf tissues followed by recovery of protein from the juice. Different methods can be used to recover the protein from the juice: acid precipitation, heat coagulation and filtration. The amount of protein extracted depends on the recovery method. In 1971 Pirie concluded that, heat coagulation is the most satisfactory method for protein recovery from the leaf juice (Pirie, 1971).

3. Fraction-1 Protein

Fraction-1 protein was observed to be one of the major leaf proteins in terms of quality. Up to 65% of the total soluble protein in leaf extracts is Fraction-1 protein (Ellis, 1979). It was later found that Fraction-1 protein was identical to ribulose 1,5-bisphosphate carboxylase (RuBPCase). This enzyme catalyzes the first step of the

dark reactions in photosynthesis:

CO_2 + D-ribulose-1,5-bisphosphate + H_2O $\xrightarrow{Mg^{2+}}$ 2·3-phospho-D-glycerate.

In 1971 it was discovered that, this protein catalyzes an additional reaction, the first reaction in the process of photorespiration (Bowes, 1971);

O_2 + D-ribulose-1,5-bisphosphate $\xrightarrow{Mg^{2+}}$ 3-phospho-D-glycerate +2-phosphoglycolate.

Aqueous extracts of the leaves of higher plants contain 1-10 mg/g fresh weight of Fraction-1 protein. The actual amount of this protein depends on the stage of development of the plant, and also on the growth conditions (Ellis, 1979). The purification of Fraction-1 protein from the other soluble proteins was easy. The purification process included the following steps: ammonium sulfate precipitation (30 to 55% saturation); Sephadex G-200 gel filtration and ultracentrifugation on a sucrose step gradient (Wildner et al.,1980).

Fraction-1 protein from higher plants has a molecular weight of about 560,000. It is composed of eight large subunits (55,000 mw) and eight small subunits (12,500 mw). The large subunits are responsible for enzymatic activity. The role of the small subunits has yet to be determined, although there is some evidence for its role as a promotor in the synthesis of the large subunits (Haslett et al., 1976).

A. Use of Fraction-1 Protein as a Protein Source

Amino acid analysis and feeding trials have been carried out on Fraction-1 protein (Wildman, 1974). In terms of absolute amounts of essential amino acids (mg per g of protein) the concentration of each essential amino acid is greater than the recommended requirements. When the ratio of each essential amino acid is compared to the total essential amino acids, a deficiency of sulfur-containing amino acids appear. By adding 0.2% methionine, Fraction-1 protein can be used as complete protein source.

Unfortunately more work must be done before the problems of LPC as a human food can be overcome (the main component of LPC is Fraction-1 protein). For the most part these problems are sensory. The color, taste and smell of LPC, also the presence of some phenolic compounds associated with LPC increase the unacceptability of it as a novel food.

Fraction-1 protein has been obtained in a crystalline form (e.g. tobacco Fraction-1 protein). The crystalline form of Fraction-1 protein is a bland white material. From this it can be inferred that the sensory properties associated with LPC can be overcome. The properties of taste, smell, and color are not intrinsic to the protein but are due to contamination. Most of this contamination is due to the protein phenolic interaction. If this can be controlled or eliminated it should be possible to produce LPC from

almost any plant source with little detectable taste.

The control of phenolics can be accomplished in a number of ways. The addition of phenolase inhibitors or phenolic binding substances to the extraction buffer is an easy and fast strategy that might be used (e.g. sulfite and polyvinylpyrrolidone) (Loomis and Battaile, 1966).

4. Utilization of Poplar Leaf Protein

A breeding programme was developed in the early 1970's by the Ontario Ministry of Natural Resources (OMNR) to produce genetically selected hybrid poplars suitable for the eastern region of Ontario, where deficiencies have developed in the supply of materials for some wood industries. In 1977 Holder studied the chemistry of the wood of many hybrid poplar clones and he found that, in terms of quality, hybrid poplar wood is satisfactory for the fibre-based industries (Holder, 1977). Hybrid poplar trees have a large number of leaves. These leaves are high in protein content and may be used as a protein source. In 1970 Siren in Sweden was the first to point out that poplar may be considered as a good source of LPC.

Poplar leaf was chosen for this thesis after many practical observations. The growth rate of poplar trees is remarkably high. A poplar may increase in height by as much as three meters in a single year. This represents a

large amount of usable biomass for the production of leaf protein concentrate. Poplar is a fast growing species, grows in a wide variety of soils and climatic conditions (Anderson and Zsuffa, 1975).

In 1979 Chen studied the quality and quantity of protein derived from hybrid poplar leaves (Chen, 1979). He found that the amino acid composition of poplar leaf protein concentrate is similar to that found in other leaf protein concentrates and the essential amino acid content is higher than FAO's recommended values for protein. Also, Chen states that the true metabolic energy and digestible energy of poplar leaf protein concentrate are competitive with the reported values for other leaf protein concentrates such as alfalfa (metabolic energy of poplar leaf protein concentrate was 3.03, for alfalfa it was 2.58-3.18. The true digestible energy of poplar leaf protein concentrate was 67%, for alfalfa it was 64.5-67.8%).

Poplars have good characteristics for industrial biomass production. They can be cultivated in various ways in monoculture or in association with other tree species, and even farm crops (Anderson and Zsuffa, 1979).

In 1979 Anderson mentioned that, poplar plantations have shown significant potential in Ontario as alternative crops on marginal farmlands and as improved crops replacing low quality forests. Large yields have been obtained in

short rotations and the produced biomass was of a good quality for a variety of products, such as fibre, fuel, forage, food and fodder, the so-called "Five Fs" (Anderson and Zsuffa, 1979).

5. Plant Protein and Phenolic Compounds

It is well known that poplar is low in lignin content and is therefore the softest tree of the hardwoods or deciduous class of woody plants. The low lignin content may mean that its leaf tissue may also be low in phenolic compounds. The possibility merits some investigation for it would mean that poplar could be a good source of LPC since interfering phenolics would be lower in concentration than in other woody leaves.

The reaction between protein and free phenolics or their oxidation products have long been known. Such reactions are involved during the extraction of protein from plants (Swain, T., 1965). The presence of phenolic compounds often makes it impossible to isolate active enzymes by conventional techniques (Loomis and Battaile, 1966).

Phenolic compounds are present in all species of leaf. There is enough in some leaves to prevent protein extraction completely (Bawden and Kleezkowski, 1945). Besides interfering with protein extraction, it is reasonable to assume that some of these substances accompany what protein does extract, and thus contaminate the final leaf product.

In 1968 Jennings measured the absorption spectrum of bean (Vicia faba) leaf protein, compared it with the spectrum calculated from its tyrosine and tryptophan content, and showed that, the difference between the spectra resembled the typical spectrum of phenolic material (Jennings et al., 1968).

Phenolic binding may alter the nutritive value of protein extracted from plant tissue. Subba Rau et al., (1972) found that, there is no correlations between nutritive value and the amount of phenolics in eleven preparations from eight species used as sole protein sources for rats. However, when the results were expressed as the ratio of phenolics to nitrogen, it was clear that samples with the greater ratios were worse nutritionally.

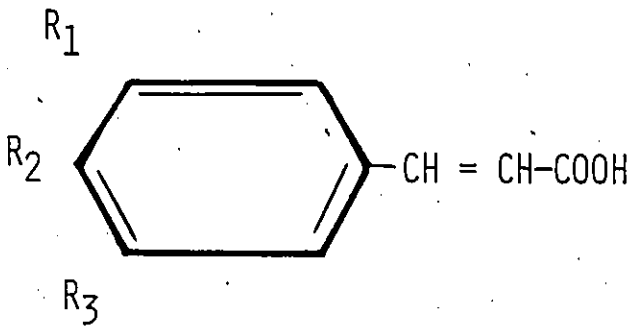
A. Possible Reactions Between Phenolics and Proteins

The phenols and biochemically related substances of natural origin in plants can be divided into two groups. The first group consists of approximately 25 simple phenols such as caffeic, ferulic, sinapic, p-coumaric, gallic acids (Figure 4) or their derivatives; flavonoids (e.g. flavone and flavonol) (see Figure 5); lignin and hydrolyzable and condensed tannins (Figure 6). The second group consists of highly toxic phenolic derivatives, heterogeneous compounds.

Phenols combine with proteins reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent

condensations (Pierpoint, 1970). In the case of tannins, weak ionic bonds between suitable charged anionic groups on the phenolics and cationic groups on the protein must be considered (Loomis and Battaile, 1966; Swain, 1965).

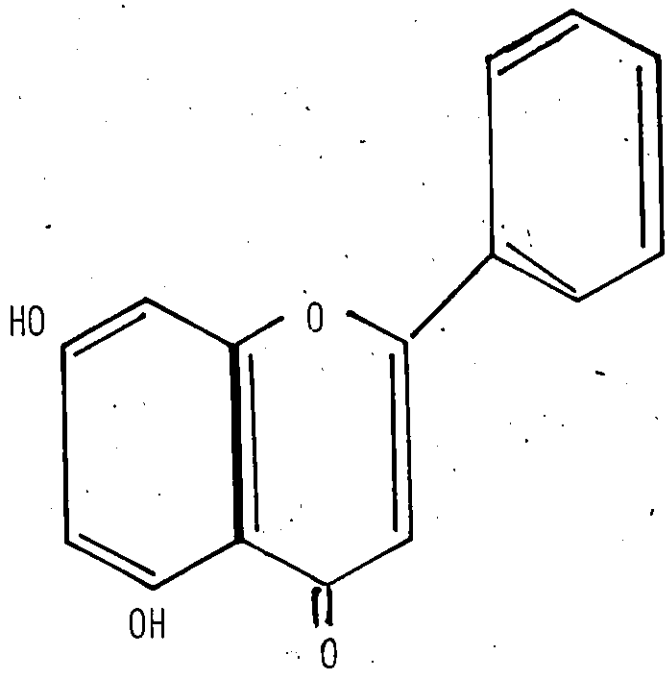
Figure 4 The Structures of Some Simple Phenolic Compounds Found in Plants



PHENOLIC ACID	R_1	R_2	R_3
COUMARIC ACID	H	OH	H
CAFFEIC ACID	OH	OH	H
FERULIC ACID	CH_3	OH	H
SINAPIC ACID	OCH_3	OH	OCH_3
GALLIC ACID	OH	OH	OH

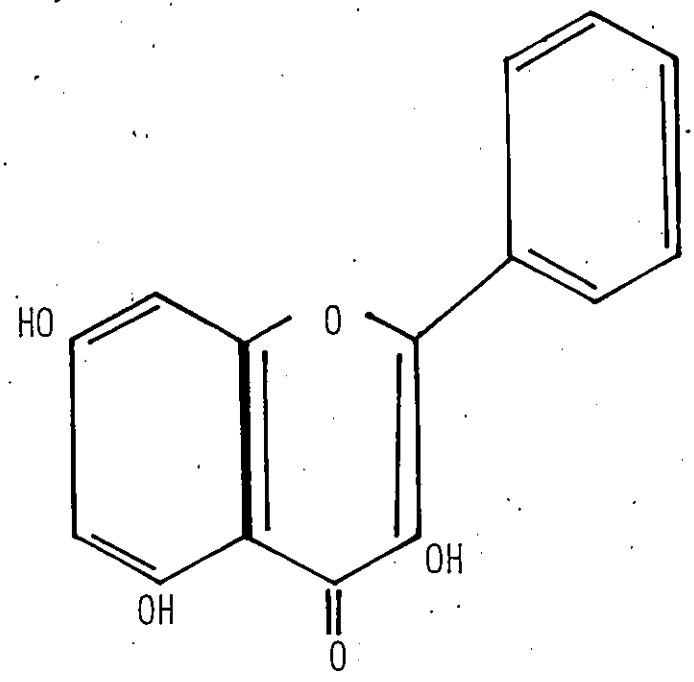
Figure 5 Examples of the Structure of Flavonols, a Common Group of Plant Phenolic Compounds. a) 5,7-dihydroxy flavone. b) quercetin.

(A)



5,7-DIHYDROXY FLAVONE

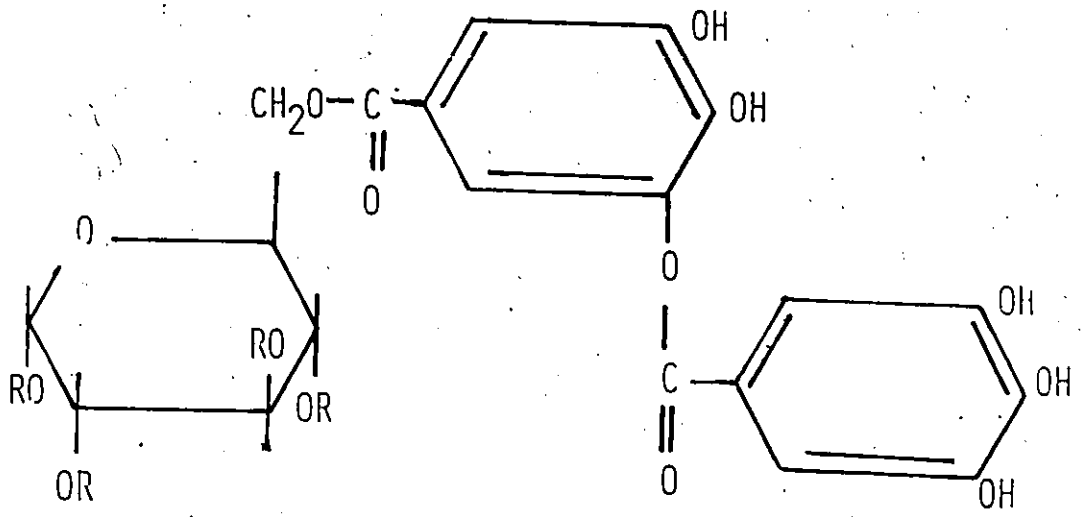
(B)



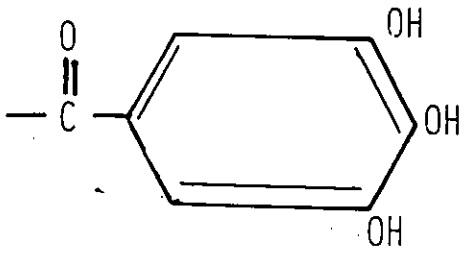
QUERCETIN (FLAVONOL)

Figure 6 The Structure of Tannin
a) Hydrolyzable Tannin
b) Condensed Tannin (Flavone)

(A)

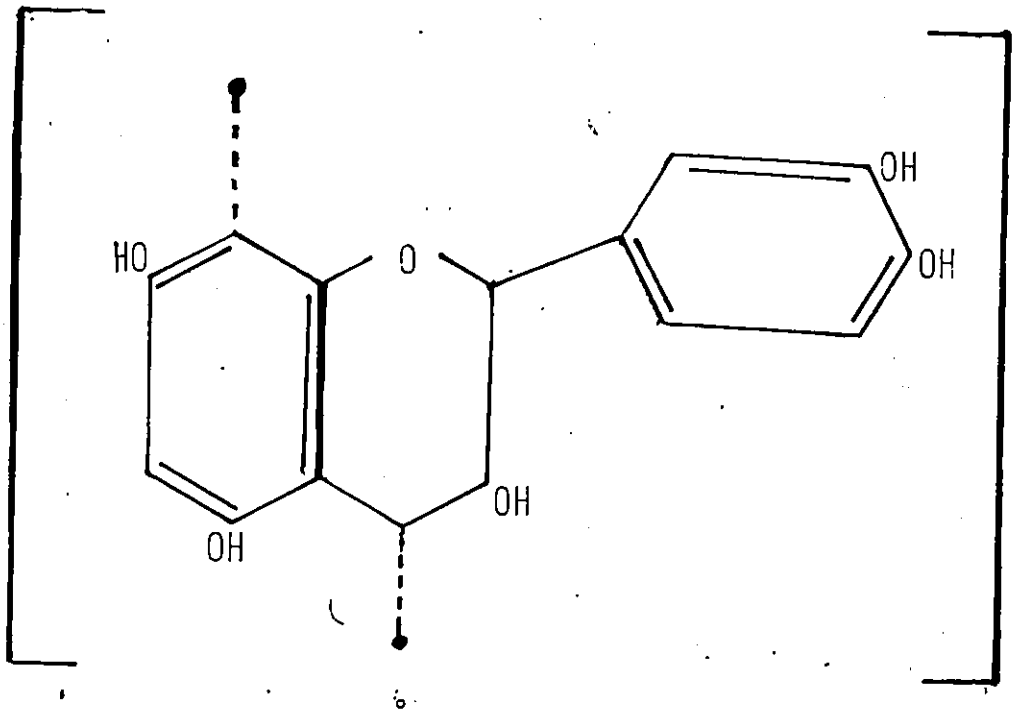


R =



GALLIC ACID

(B)



1. The Hydrogen Bond

In 1969 Loomis mentioned that the amount of phenolic material bound to protein by hydrogen bonding may be equal to more than 33% dry weight of the protein. Peptide links are the most powerful binding sites in the formation of hydrogen bonds between tannins and proteins (Loomis, 1969).

There are two types of tannin, condensed and hydrolyzable. They show very different pH responses in their reactions with polypeptides. Loomis and Battaile, (1966) reviewed the chemistry of these reactions and the use of polyvinylpyrrolidone (PVP) to compete for phenolics. PVP adsorbs phenols from plant tissue extracts and forms stable insoluble complexes with tannin material. Using this compound, they were able to obtain active, soluble enzymes from leaves of peppermint, thistle and apple (Figure 7).

To minimize browning and increase protein extractability, many other protective agents have been used besides PVP. Woodham has proposed the use of sulfite for inhibiting the oxidation of phenolic compounds in leaves and their subsequent attack on proteins (e.g., Na_2SO_3). He also showed that, sulfite treatment increased the quantity of both methionine and cystine in the protein products (Figure 8). Mercaptoethanol is believed to act as a quinone-removing substance (Welander, 1978). In higher concentrations, it

will react with proteins by disrupting disulfide bridges. For this reason, it is important to investigate the effect of mercaptoethanol on the activity of different enzymes (Welander, 1978).

2. The Covalent Bond

Phenols can be also combined with proteins irreversibly by oxidation followed by covalent condensations (Loomis and Battaile, 1966).

For example the o-dihydroxy phenols, especially caffeic and chlorogenic acids, are oxidized to o-quinones by a copper-containing enzyme, phenol-oxidase. O-quinones (e.g., chlorogenoquinone) react nonenzymatically to polymerize, reduce or bond covalently to amino, thiol and methylene groups (e.g., ε-amino group of lysine and thio-ether group of methionine). This is illustrated in Figure 9.

The combined reaction of quinones, polyphenols and tannins on the ε-amino groups of lysine and their subsequent polymerization into tannin-protein complexes may make large amounts of lysine and other essential amino acids unavailable for nutritional utilization. Thus a low level of oxidation can result in a substantial decrease in protein nutritive value. It can also cause inactivation and precipitation of the enzyme during its purification.

Figure 7 Postulated Hydrogen Bonding of Plant Phenol to Polyvinylpyrrolidone

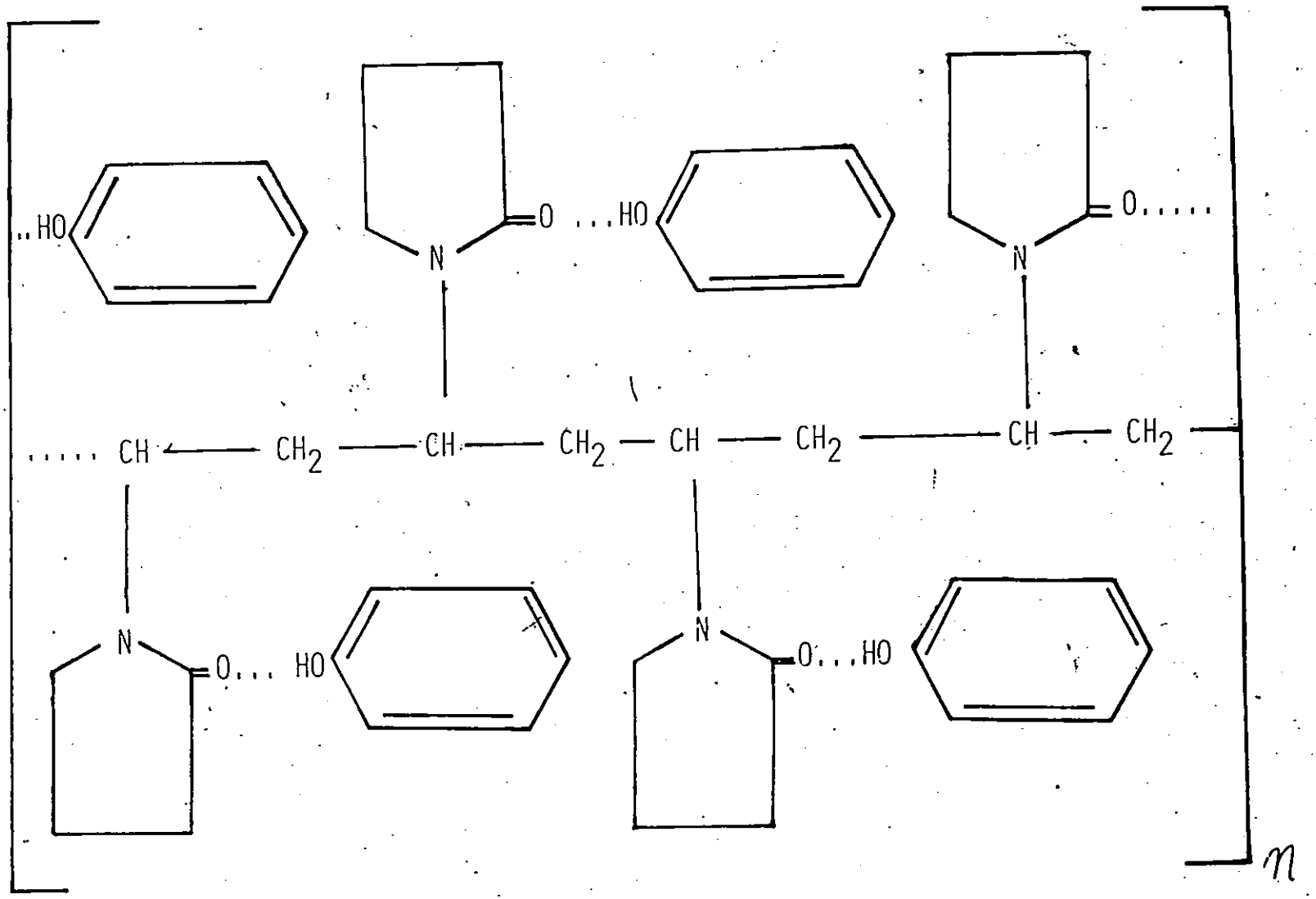
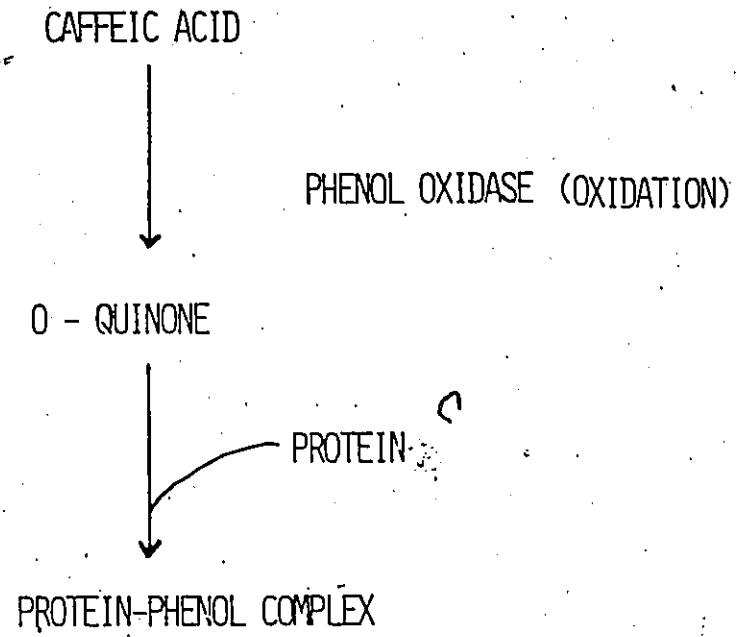

HO
O
HO
O
N
N
N
N
CH
CH2
CH
CH2
CH
CH2
CH
CH2
O
HO
O
HO
n

Figure 8 The Use of Sodium Sulfite in Inhibiting Phenol Protein Interaction During LPC Extraction



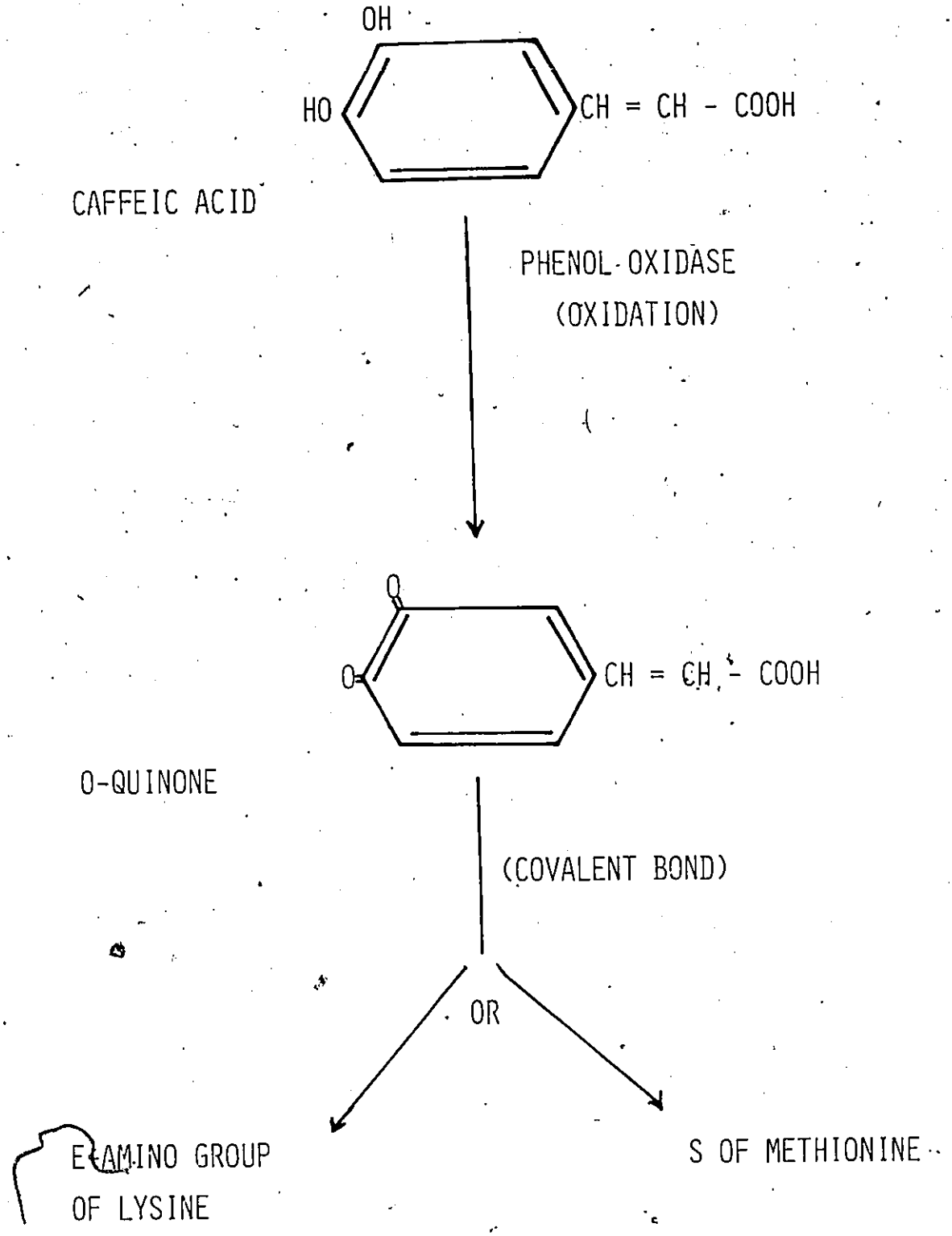
OR

SODIUM SULFITE

Na_2SO_3

$(2Na^{+})\ (SO_3^{-2})$ —OXIDATION→ (SO_4^{-2})

Figure 9 Enzyme-Catalyzed Oxidation of Caffeic Acid to Caffeoquinone Followed by Autolytic Bonding to Amino and Thiol Groups in Protein


OH
HO
CH = CH - COOH
CAFFEIC ACID
PHENOL-OXIDASE
(OXIDATION)
O
O
CH = CH - COOH
O-QUINONE
(COVALENT BOND)
OR
E-AMINO GROUP
OF LYSINE
S OF METHIONINE

6. Aims of Present Research

I have been interested in improving LPC extraction from hybird poplar foliage. The focus has been on two different problems. First, purification of crude protein recovery from hybrid poplar leaves may be optimized by using sulfite and/or polyvinylpyrrolidone. Secondly, the concentrations of inhibitory phenols and subsequent protein extractability should be compared between two hybrid poplar clones and other well studied systems like alfalfa, tobacco and rapeseed. Then the biochemical and economical feasibility of producing food grade protein isolate from hybrid poplar may be evaluated. I have also studied the effect of seasonal variation on the amounts of both extractable protein and phenol. The latter study was carried out in order to find the times of highest and lowest concentrations and to check whether there is any relationship between the concentration of phenol and the extractable protein from the leaves.

CHAPTER II

EXPERIMENTAL PROCEDURE

1. Materials

A. Leaf Tissue

Hybrid poplar leaves were chosen for this study after many practical observations. Hybrid poplars grow rapidly and in a wide variety of soils and climatic conditions. The Ontario Ministry of Natural Resources (OMNR) has been studying hybrid poplar for possible harvesting of fibre, fuel, forage, fodder and food. For a study of poplar leaf protein concentrate as a possible food source, a ready supply of leaf tissue was available. Clones $I_{45/51}$ (*Populus euramericana* cl. $I_{45/51}$ Italy) and DN113 (*P. deltoides* x. *P. nigra*) were chosen from the OMNR poplar clone bank. $I_{45/51}$ is the internal standard used throughout the OMNR breeding program so it was included as a reference material. DN113 is a new fast-growing hybrid with large leaves. It produces a large amount of foliage biomass and may be a good potential source of leaf protein concentrate.

Alfalfa, tobacco and rapeseed have already been shown

to yield commercial protein products from leaf tissue (Pirie, 1971; Wildman, 1974; Brown et al., 1975). Extractability has been shown to be dependent on inhibitory phenols and other factors such as moisture content, crude soluble protein levels and total nitrogen content. To evaluate the economic feasibility of producing a food-grade protein isolate from hybrid poplar, the concentration of phenols, moisture and protein were compared with those of the more common agricultural crops, alfalfa, tobacco and rapeseed.

Rapeseed (Brassica napus var. Altex), tobacco (Nicotiana tabacum cv. Delhi 34) and alfalfa (Medicago sativa L.) were supplied by Ottawa Research Station, Agriculture Canada.

B. Field Site and Design

The experimental trial of agricultural crops and poplars was established at the OMNR Howard Ferguson Tree Nursery in Kemptville in 1980. An area of land was chosen in the north-east corner of compartment 6 (Figure 9a). The site is a loamy sand, high in humus and was prepared to a depth of 6" by roto-tiller immediately before planting and hand raked. A Randomized Block Experiment (RBE) was used.

Two hybrid poplar clones, $I_{45/51}$ and DN113, were randomly planted (30 of May 1980) in four replications along with three agricultural crops, alfalfa, tobacco and rapeseed.

The field trial was repeated for a second growing season in 1981. The two poplar clones and alfalfa plants, being perennial, all survived the winter and sprouted well in the spring. New tobacco and rapeseed were planted in the same experimental design on May 21, 1981.

The trial was oriented north north-west to minimize any shading effect of hybrid poplars on agricultural crops. Irrigation was readily available if required. Four replicates running south to north were comprised of five random plots each, two poplar and three agricultural, separated by a dummy plot. Each plot was 2.0 m wide by 90.0 cm long. Each replicate was 4.5 m long giving a total experimental site length, including dummy plots of 20.7 m (Figure 9b).

C. Chemicals

Chemical materials were purchased from the following suppliers: Sephadex, Sepharose and Sephacryl gel filtration column chromatographic media: Pharmacia Chemicals, Uppsala, Sweden, Pharmacia (Canada) Ltd., Montreal, P.Q.; Polyvinyl-pyrrolidone (phenol adsorbent): Sigma Chemical Co., St. Louis, Mo., U.S.A.; Electrophoresis chemicals and protein molecular weight standards: Bio-Rad Laboratories, Toronto, Ontario. All other chemicals were from Canlab Supplies Ltd., Ottawa, Ontario.

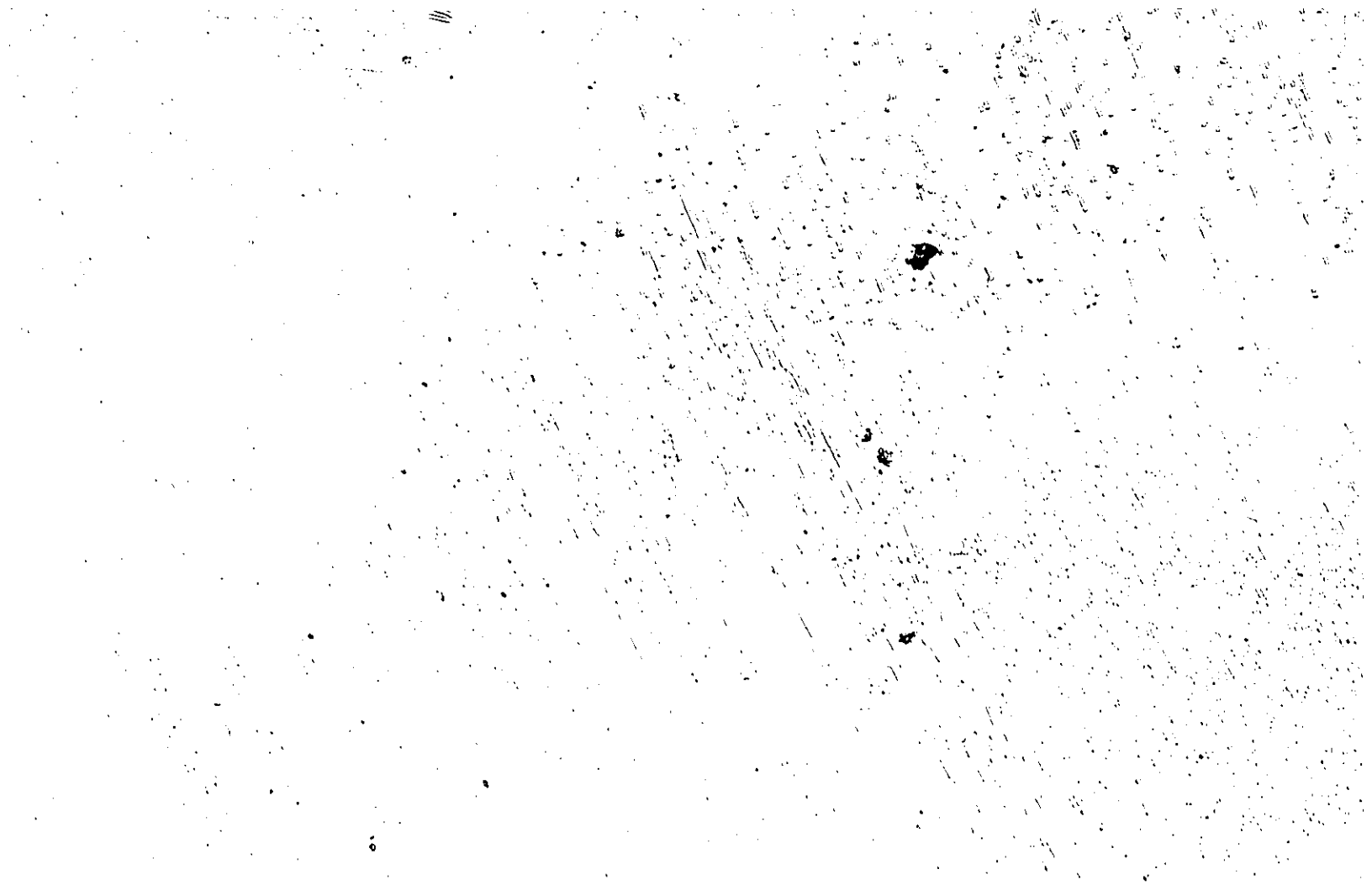


Figure 9a " Field Site at OMNR Howard Ferguson Tree Nursery in Kemptville. Plants were Grown in Compartment 6 Near Highway 43.

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KEMPTVILLE PROTEIN - PHENOL TRIAL 30/5/80

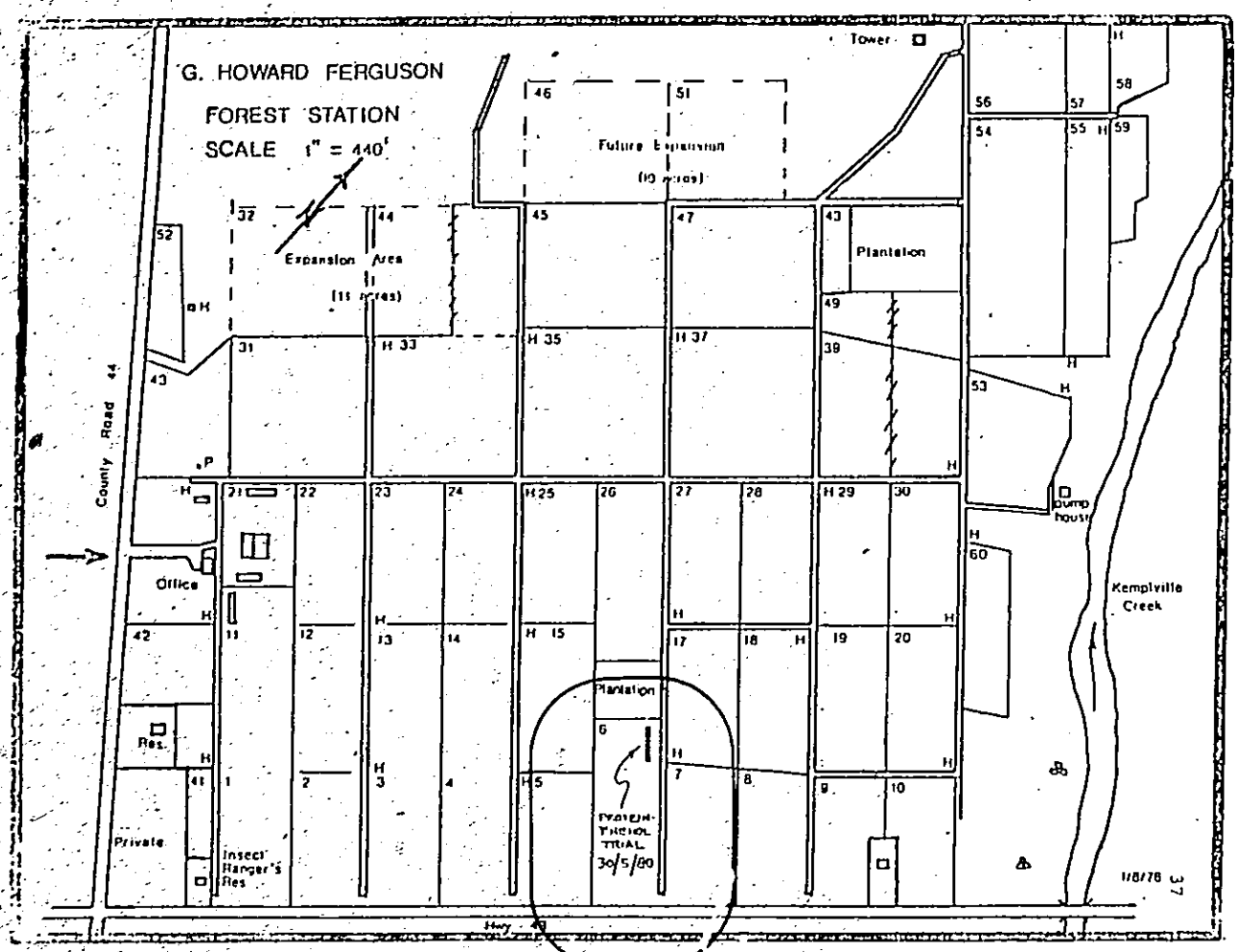
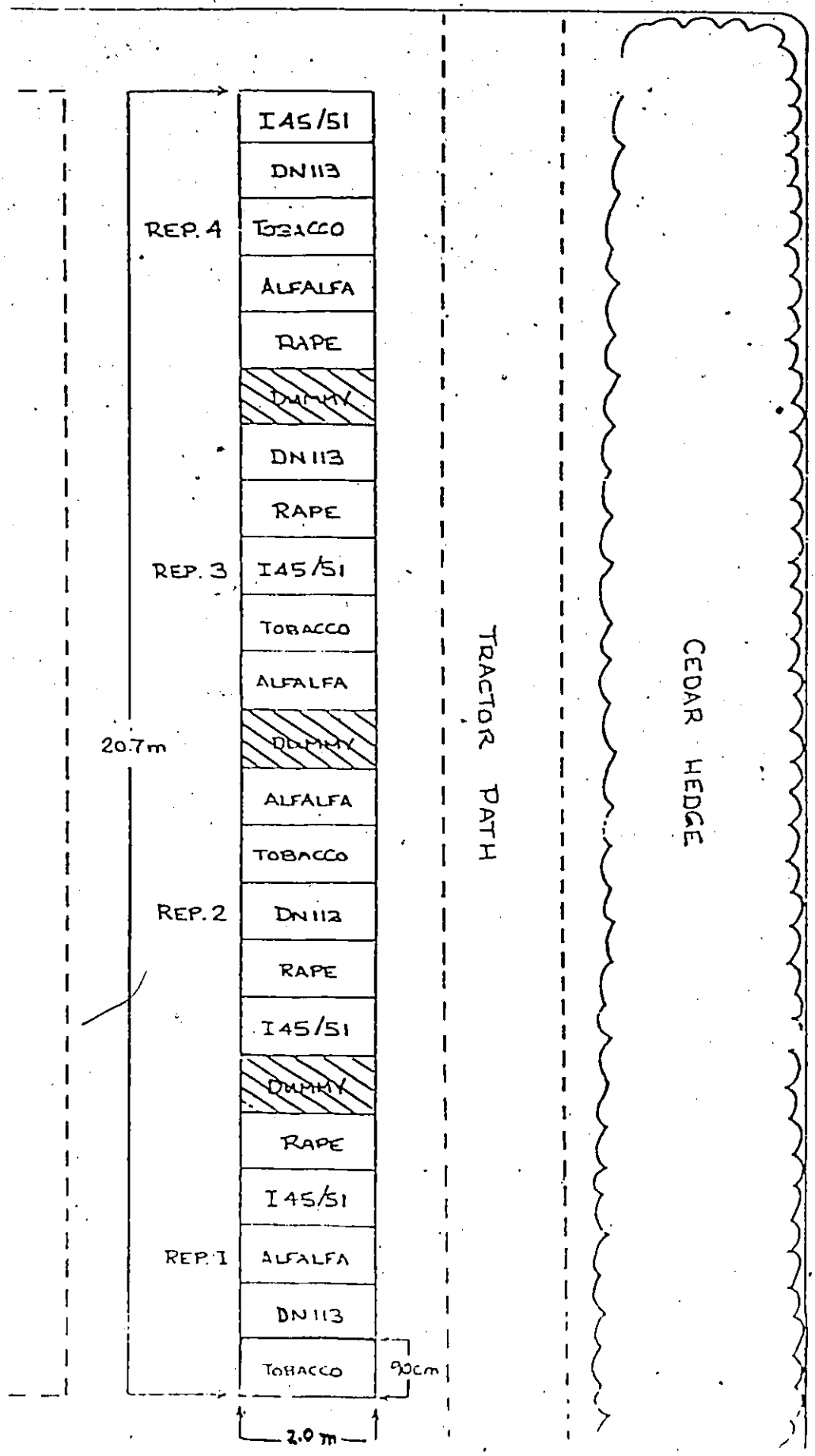


Figure 9b Field Design of Hybrid Poplar Clones, Tobacco, Alfalfa and Rapeseed in Compartment 6 of OMNR Nursery, Kemptville, Ontario. In this Randomized Block Experiment, each block or plot contained nine plants spaced at 30 cm x 67 cm intervals.


NURSERY ACCESS ROAD
REP. 4
REP. 3
REP. 2
REP. 1
20.7m
I45/51
DN113
TOBACCO
ALFALFA
RAPE
DUMMY
DN113
RAPE
I45/51
TOBACCO
ALFALFA
DUMMY
ALFALFA
TOBACCO
DN113
RAPE
I45/51
DUMMY
RAPE
I45/51
ALFALFA
DN113
TOBACCO
90cm
2.0 m
TRACTOR PATH
CEDAR HEDGE
SECONDARY ROAD

2. Methods

A. Optimization of Protein Extraction

1. Adsorption of Phenolics with Polyvinylpyrrolidone

The use of polyvinylpyrrolidone (PVP) was studied by Loomis and Battaile for the isolation of protein from plants (Loomis and Battaile, 1966). PVP acts as a competitive binder for polyphenols which would otherwise complex with plant proteins during extraction and make them partly or wholly insoluble or bind them irreversibly to the leaf fibre residue.

2. Inhibition of Phenolics with Sulfite

The use of sulfite for inhibiting the oxidation of phenolic compounds in leaves and their subsequent attack on proteins was suggested by Woodham (Woodham, 1974). He also showed that sulfite treatment increased the quantity of both methionine and cystine in the leaf protein concentrate product.

3. Optimization Trials

Poplar leaves (5 g) were weighed, quickly crushed by hand, homogenized for 2 min. in normal extraction buffer (25 ml) containing 0.05 M Tris-HCl pH 7.4, 1.0 M NaCl, 0.001 M EDTA, 0.002 M $MgCl_2$ and 0.08 M β-mercaptoethanol

and quickly squeezed through cheesecloth. Leaf protein solutions were centrifuged at 15,000 X g for 30 min. and the amounts of protein in the supernatant were measured using the Bio-Rad Protein Assay*. Hybrid poplar clone $I_{45/51}$ was used in this study.

To study the effect of PVP on poplar protein extraction, leaf samples were homogenized in normal extraction buffer with various amounts of PVP (0, 0.05, 0.1, 0.2, 0.5 and 1%) at pH 7.4.

To test the effect of sulfite on protein extraction, leaf samples were homogenized in the same buffer using the following concentrations (0, 1, 2, 5, 8 and 10%) at pH 7.4 and pH 9.0.

B. Characterization of Leaf Tissues

1. Harvest

Leaf tissue was collected six times during the period July to the end of September, 1980. Table IIIA shows the harvest number and the collection date for the 1980 harvests. Dates from 1981 analyses are reported in chapter IV where appropriate.

Random samples were collected in each harvest from four different replicates for each species (poplar $I_{45/51}$ and DN113, alfalfa, tobacco and rapeseed). Only green

* See page 45.

Table III A

Collection Dates of Leaf Material 1980

Harvest No.	Harvest Date
1	30 July
2	6 August
3	14 August
4	25 August
5	4 September
6	17 September

leaves with no obvious signs of infection were used. In the beginning of the season the minimum number of leaves necessary were collected to minimize any effect on the plant growth rates.

The rapeseed crop exhibited a very short growing season making it difficult to compare it to the other plants.

2. Moisture Content of Leaves

The moisture content may influence the mechanical properties of leaves during homogenization and filtration through cheesecloth.

Random samples of leaf tissue (1 g) from each collection were dried in an oven for at least 24 hrs at 110 °C. Moisture content was calculated as follows:

$$\text{Moisture Content \% (FW)} = \frac{\text{fresh weight - dry weight}}{\text{fresh weight}} \times 100$$

3. Total Protein Content

Fresh leaf samples were ground and analysed using the micro-Kjeldahl method as modified by Gunning and Arnold (Triebold, Aurand, 1963) to determine the total nitrogen content (TNC). Total protein content (TPC) was assumed to equal the total nitrogen content multiplied by a factor of 6.25. This factor has been used before to calculate leaf protein concentration (Lu et al., 1972).

Micro-Kjeldahl Method:

The method consists of: first, the wet oxidation of sample and the conversion of protein nitrogen into ammonium sulfate; second, the decomposition of the ammonium sulfate with strong alkali and the distillation of the ammonia evolved into saturated boric acid solution which holds the ammonia; third, the titration of the ammonia with standard acid; and fourth, the calculation of the percentage protein in the sample from its weight and the volume of standard acid required to titrate the ammonia.

Procedure:

The wet oxidation of the sample was accomplished by heating 0.5 g of fresh leaf sample with 5 ml of concentrated sulfuric acid in the presence of 50 mg of an oxidizing catalyst (mercuric oxide) and 2.0 g of salt, either potassium or sodium sulfate. The mixture was heated over low heat in a Kjeldahl digestion apparatus until the mixture no longer frothed. Then the heat was increased. At this point the digest solution became clear. The heating was continued for an additional 20 - 30 min. to ensure complete oxidation. The minimum volume of distilled water required to dissolve any solids was added. The cool digest was transferred to the flask of the distillation apparatus. A 125 ml erlenmeyer

flask containing 5.0 ml saturated boric acid and 2-4 drops of indicator solution (methyl red-methyl blue 2:1) was placed under the condenser with condenser tip below the surface of the receiving solution. Twenty millilitres of 50% NaOH/25% $Na_2S_2O_3$ (4:1) solution was added to the digest solution and distilled until 20 ml of distillate had been collected in the flask. The indicator color changed from purple to green. The distillate was titrated against hydrochloric acid to the gray end point.

The total nitrogen content was calculated from the expression:

$$\% \text{ TNC} = \frac{\text{(sample titre-blank titre) x normality of HCl x 14.007}}{\text{gram dry leaf material x 10}}$$

The total nitrogen values obtained using this method are refered to in this thesis as total protein content (TPC).

4. Protein Extraction

The method of Stroback and Gibbons (1976) was used for leaf protein extraction. The basic procedures for the extraction involved leaf grinding with ice cold buffer (0.05 M Tris-HCl buffer pH 7.4, 1.0 M NaCl, 0.001 M EDTA, 0.002 M $MgCl_2$, 0.08 M β-mercaptoethanol, and 0.2% polyvinyl-pyrrolidone) in a Waring blender, of 1 L. capacity. After grinding, the homogenate was filtered through 8 layers of cheesecloth to remove most of the leaf fibres. Finally

the material was centrifuged at 15,000 x g for 30 min. to obtain the extractable crude protein (ECP) in the supernatant. This ECP value was obtained by using the Bio-Rad assay detailed in section 5 below.

The efficiency of protein extraction was calculated by comparing extractable crude protein (ECP) to the total protein content (TPC) of the material in question:

$$\text{Ratio} = \frac{\text{extractable crude protein}}{\text{total protein content}}$$

It is understood that this expression of extraction efficiency is based on two different analytical methods, Bio-Rad and Micro- Kjeldahl. However, this ratio did allow a quick comparison of LPC extraction from poplar leaves and other species.

5. Protein Determination

To determine the protein concentration in the crude extracts (15,000 x g supernatant) a method much faster than micro-Kjeldahl was used. The Bio-Rad Protein Assay is a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein (Bradford, 1976).

Dye Reagent:	One volume of dye reagent concentrate (phosphoric acid and methanol) was diluted with four volumes of distilled water.
Protein Standard:	Several dilutions of protein standard (Lypophilized bovine gamma globulin) containing from 0.2 to about 1.4 mg/ml were prepared.
Sample Preparation:	Generally samples did not require any dilution of concentration.
Assay Procedure:	Diluted dye reagent (5.0 ml) was added to sample and standard tubes (100 µl). After Vortex mixing, absorptivity at 595 mµ was measured in a Bausch-Lomb Spectrophotometer. Blank consisted of 100 µl of buffer and 5 ml diluted dye reagent only.

6. Determination of Phenolics Concentration:

The methods of Swain and Hillis (1959) and Eskin et al. (1978) were used with modification. The basic procedure for phenolics extraction and determination involved extraction by refluxing the leaf material twice with 80% ethanol at pH 4.0 for 30 min. After the extraction the material was centrifuged

at 10,000 x g for 30 min. to obtain the phenolics in the ethanol extract. An aliquot (0.5 ml) of the ethanol extract was diluted with water to 7 ml. The contents were well mixed with 0.5 ml of the Folin reagent (Folin and Ciocalteu's phenol reagent). Exactly 3 min. later 1.0 ml of saturated sodium carbonate solution was added and the mixture made up to 10 mls with good mixing. After 1 hr, the absorptivity was determined at 720 mμ using only water and reagents as a blank.

Preparation of Phenolic Standard:

Chlorogenic acid was used as a phenolic standard because it is the principle color-forming flavonoid found in leaf protein concentrate (LPC). LPC has been shown to have chlorogenic acid bound to its protein molecules (Lahiry et al.,1977). Several dilutions containing 0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 μg/ml were prepared.

The phenolics concentration of the various leaves was expressed as yield:

$$\text{yield (mg/g)} = \frac{\text{total phenolics extracted}}{\text{fresh or dry weight of leaf material}}$$

CHAPTER III

RESULTS

A. Optimization of Protein Extraction

1. Adsorption of Phenolics with Polyvinylpyrrolidone

Leaf samples were homogenized in the appropriate extraction buffers with various amounts of polyvinylpyrrolidone. Both soluble and insoluble polyvinylpyrrolidone was studied and no differences were obtained.

At a neutral pH of 7.4, 0.2% polyvinylpyrrolidone in the homogenization buffer yields the optimum production of crude protein (Figure 10).

2. Inhibition of Phenolics with Sulfite

The protein extraction method outlined in chapter II was carried out with various concentrations of sulfite at different pH values. Leaf samples were homogenized in the appropriate extraction buffers with various amounts of sodium sulfite, a phenol oxidase inhibitor. The yields of extracted crude protein are reported in Figure 11.

At neutral pH the optimum protein production was

Figure 10 The Effect of Polyvinylpyrrolidone on Protein Extraction from Hybrid Poplar Leaves.
The Values are the Means of the Results of Three Experiments.

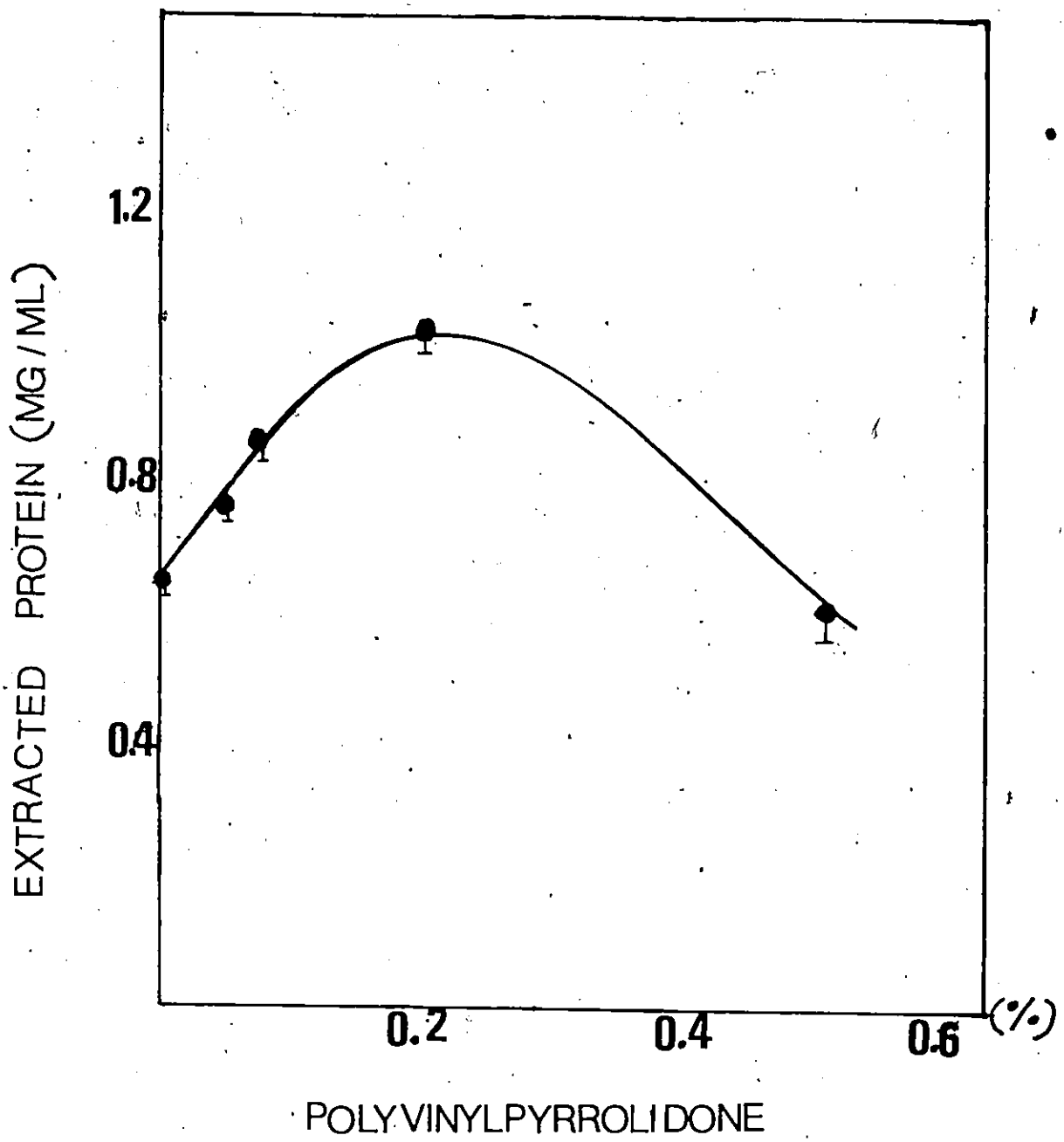
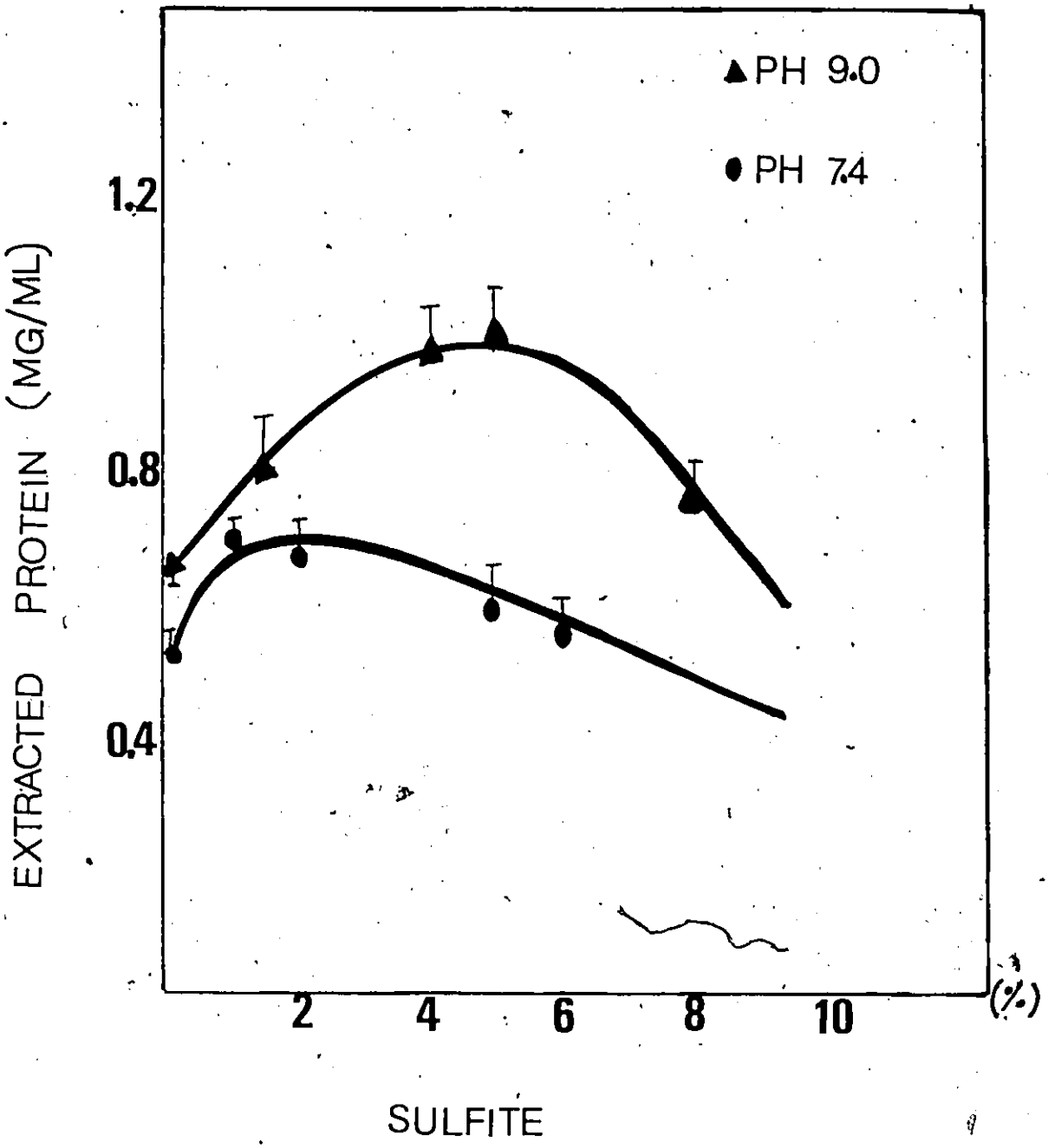

EXTRACTED PROTEIN (MG / ML)
1.2
0.8
0.4
0.2
0.4
0.6
(%)
POLY VINYL PYRROLI DONE

Figure 11 The Effect of Sulfite on Protein Extracted from Hybrid Poplar Leaves. The Values are the Means of the Results of Three Experiments.


▲PH 9.0
● PH 7.4
1.2
0.8
0.4
EXTRACTED PROTEIN (MG/ML)
2
4
6
8
10
(%)
SULFITE

achieved with 1% sulfite present. At an alkaline pH of 9.0 similar amounts of protein were obtained with 1% sulfite. However, when the reducing agent was increased to 5% the extractability and recovery was much higher.

B. Characterization of Leaf Tissue

1. Moisture Content

The study of the percentage moisture content (MC) was very important in the experimental trial for two reasons. First, the moisture content is an important factor in the processing of both protein and phenol. The amount of water in the leaf cells acts as the extraction medium of both the protein and the deleterious phenols. Secondly, to compare the amount of protein and phenol in the various plant materials the main components should be expressed in terms of dry and fresh weight. Moisture contents for alfalfa, rapeseed, tobacco and poplar ($I_{45/51}$ and DN113) are represented in Tables IV, V, VI, VII and VIII. The effect of seasonal variation in the moisture content was also observed (Figure 12). The oven method was easy and gave reproducible results.

Table IV

Percentage Moisture Content of Alfalfa Leaves

(g per 100 g fresh weight)

Harvest	Replicate	Moisture Content	Average ±Range	Standard Deviation
H1	1	80	80 ±0.0	0.0
	2	80		
H2	1	79	80 ±1.0	0.8
	2	81		
	3	80		
	4	80		
H3	1	78	80 ±2.0	1.6
	2	80		
	3	82		
	4	80		
H4	1	80	78.5 ±1.5	1.3
	2	79		
	3	77		
	4	78		
H5	1	77	77.5 ±1.5	1.3
	2	79		
	3	76		
	4	78		
H6	1	75	75.5 ±3.5	2.9
	2	76		
	3	72		
	4	79		

Table V

Percentage Moisture Content of Rapeseed Leaves

(g per 100 g fresh weight)

Harvest	Replicate	Moisture Content	Average ±Range	Standard Deviation
H1	1	89	89.5 ±0.5	0.7
	2	90		
H2	1	90	88.8 ±2.8	2.2
	2	91		
	3	88		
	4	86		
H3	1	86	89.5 ±3.5	2.6
	2	92		
	3	89		
	4	91		
H4	1	84	86.8 ±3.2	2.5
	2	86		
	3	90		
	4	87		
H5	1	89	87.3 ±2.3	1.7
	2	85		
	3	88		
	4	87		
H6	1	87	87.5 ±0.5	0.7
	2	N.A		
	3	N.A		
	4	88		

N.A = Not Available

Table VI

Percentage Moisture-Content of Tobacco Leaves

(g per 100 g fresh weight)

Harvest	Replicate	Moisture Content	Average ±Range	Standard Deviation
H1	1	90	90.5 ±0.5	0.7
	2	91		
H2	1	92	90.0 ±2.0	1.6
	2	90		
	3	90		
	4	88		
H3	1	91	90.3 ±1.3	0.9
	2	90		
	3	89		
	4	91		
H4	1	89	88.8 ±1.2	0.9
	2	88		
	3	90		
	4	88		
H5	1	88	87.0 ±3.0	1.8
	2	86		
	3	85		
	4	89		
H6	1	83	82.0 ±1.0	0.8
	2	82		
	3	81		
	4	82		

Table VII

Percentage Moisture Content of Poplar $I_{45/51}$ Leaves

(g per 100 g fresh weight)

Harvest	Replicate	Moisture Content	Average ±Range	Standard Deviation
H1	1	75	75.0 ±0.0	0.0
	2	75		
H2	1	76	75.3 ±1.3	0.9
	2	76		
	3	74		
	4	75		
H3	1	77	74.5 ±2.5	2.5
	2	74		
	3	75		
	4	72		
H4	1	77	74.0 ±3.0	2.1
	2	72		
	3	73		
	4	74		
H5	1	73	71.5 ±1.5	1.2
	2	71		
	3	72		
	4	70		
H6	1	68	68.5 ±1.5	1.2
	2	70		
	3	69		
	4	67		

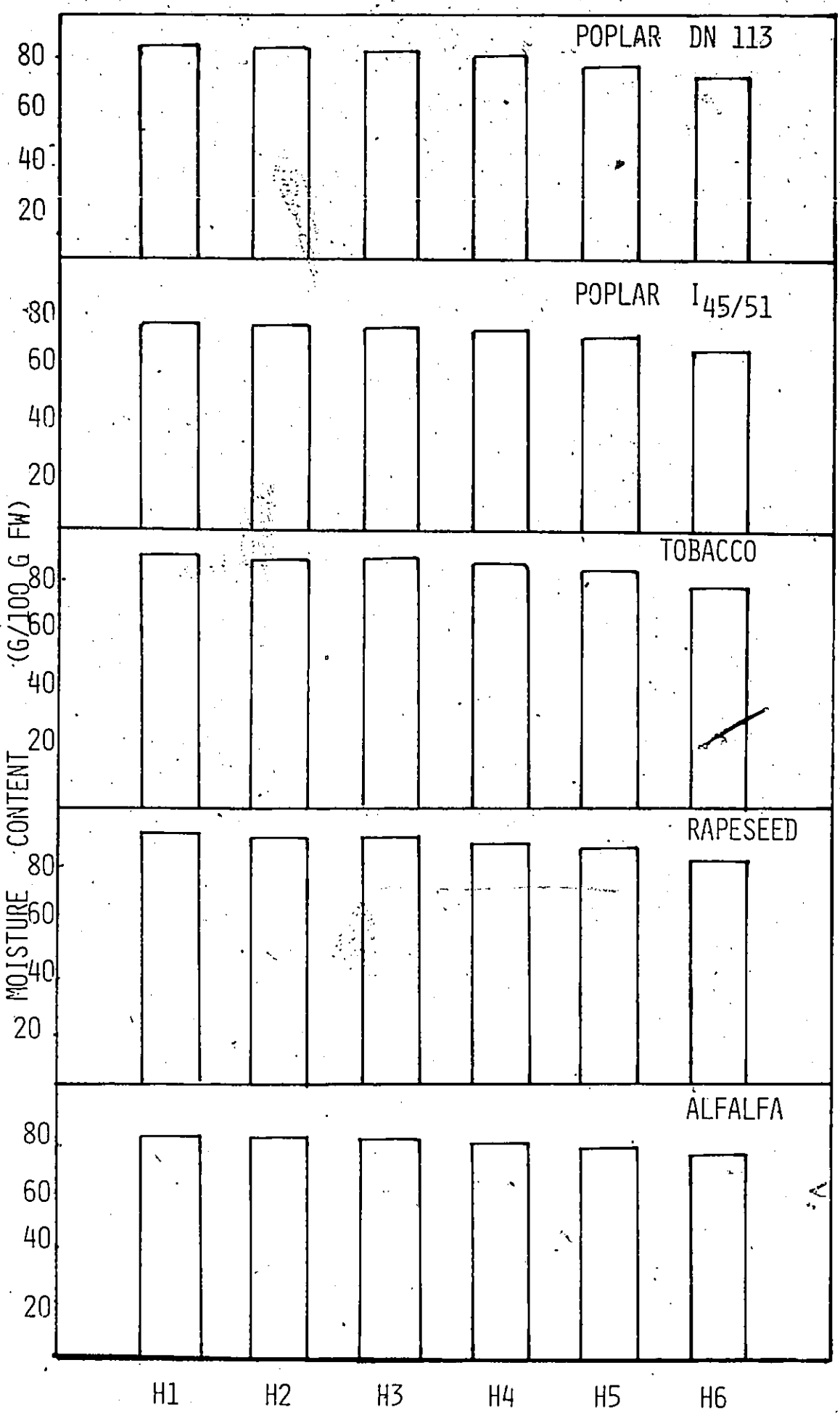
Table VIII

Percentage Moisture Content of Poplar DN113 Leaves

(g per 100 g fresh weight)

Harvest	Replicate	Moisture Content	Average ±Range	Standard Deviation
H1	1	76	76.0 ±0.0	0.0
	2	76		
H2	1	76	75.5 ±1.5	1.2
	2	77		
	3	75		
	4	74		
H3	1	75	75.3 ±1.7	1.2
	2	77		
	3	74		
	4	75		
H4	1	73	73.5 ±0.5	0.6
	2	73		
	3	74		
	4	74		
H5	1	69	71.0 ±2.0	1.8
	2	70		
	3	73		
	4	72		
H6	1	68	67.0 ±1.0	0.8
	2	66		
	3	67		
	4	67		

Figure 12 The Effect of Seasonal Variation on the Percentage of the Moisture Content Obtained from Alfalfa, Rapeseed, Tobacco and Two Poplar Clones ($I_{45/51}$ and DN113)
(H = Harvest No.)


POPLAR DN 113
POPLAR I45/51
TOBACCO
RAPESEED
ALFALFA
MOISTURE CONTENT (G/100 G FW)
80
60
40
20
H1
H2
H3
H4
H5
H6

2. Total Protein Content of Leaf Tissues

To understand the efficiency of leaf protein extraction under different conditions, the total protein contents in the starting leaf materials were determined. The Micro-Kjeldahl method was efficient and precise. Glycine was used as the standard (nitrogen content = 18.66%). In Tables IX, X, XI, XII and XIII the total protein contents obtained from alfalfa, rapeseed, tobacco and both poplar clones are given. Figure 13 shows the effect of seasonal variation on the different species. For all leaves maximum protein content occured in late summer.

3. Extractable Crude Protein (Bio-Rad Value)

The method used in the determination of extractable protein has proved to be good in the sense that it gave very good results. Indeed, applying Bio-Rad assay there was no practical observation of phenol binding to the extractable protein.

The protein extracted was expressed as yield (mg of crude protein per g of leaves once in terms of fresh weight, and another in terms of oven-dried weight). The ratio of the extracted crude protein to the total protein content was also calculated (Tables XIV, XV, XVI, XVII and XVIII).

The yield was calculated in case of fresh and frozen leaves in the six different harvests (Tables XIX, XX, XXI, XXII and XXIII). Figure 14 and 15 show the effect of seasonal

Table IX

Total Protein Content in Alfalfa Leaves

(g per 100 g dry weight)

(% Kjeldahl N_2 x 6.25)

Harvest	Replicate	Protein /(fresh weight)	Protein /(dry weight)	Average ± Range	Standard Deviation
H1	1	5.00	25.00	26.57 ±1.5	1.6
	2	5.88	28.13		
H2	1	5.90	29.70	30.5 ±0.8	0.9
	2	6.25	31.25		
	3	6.25	31.25		
	4	5.90	29.70		
H3	1	6.88	34.40	34.6 ±0.4	0.3
	2	6.88	34.40		
	3	6.99	35.00		
	4	6.88	34.40		
H4	1	6.88	32.70	33.8 ±2.8	1.9
	2	7.70	36.60		
	3	7.00	33.10		
	4	6.88	32.70		
H5	1	7.00	30.63	29.5 ±1.5	1.5
	2	7.10	31.00		
	3	6.50	28.25		
	4	6.50	28.25		
H6	1	5.90	24.75	25.2 ±1.9	1.2
	2	5.90	24.75		
	3	5.90	24.75		
	4	6.50	27.10		

Table X

Total Protein Content in Rapeseed Leaves

(g per 100 g dry weight)

(% Kjeldahl N_2 x 6.25)

Harvest	Replicate	Protein / (fresh weight)	Protein /(dry weight)	Average ±Range	Standard Deviation
H1	1	2.1	19.4	19.4 ±0	0
	2	2.1	19.4		
H2	1	2.1	19.4	19.4 ±0	0
	2	2.1	19.4		
	3	2.1	19.4		
	4	2.1	19.4		
H3	1	2.4	21.3	22.7 ±2.9	2.1
	2	2.4	21.3		
	3	2.8	25.6		
	4	2.4	22.4		
H4	1	2.9	21.3	19.6 ±1.7	1.3
	2	2.7	19.4		
	3	2.7	19.4		
	4	2.5	18.1		
H5	1	2.8	17.5	16.6 ±1.0	0.9
	2	2.8	17.5		
	3	2.5	15.6		
	4	2.5	15.6		
H6	1	2.8	17.5	17.5 ±0	0
	2	N.A.			
	3	N.A.			
	4	2.8	17.5		

N.A. = Not Available

Table XI

Total Protein Content in Tobacco Leaves

(g per 100 g dry weight)

(% Kjeldahl N_2 x 6.25)

Harvest	Replicate	Protein /(fresh weight)	Protein /(dry weight)	Average ±Range	Standard Deviation
H1	1	3.1	31.2	29.7 ±1.6	1.6
	2	2.8	28.1		
H2	1	3.1	31.2	33.1 ±1.9	1.9
	2	3.1	31.2		
	3	3.5	35.0		
	4	3.5	35.0		
H3	1	4.2	41.9	39.5 ±2.4	1.6
	2	3.9	38.8		
	3	3.9	38.8		
	4	3.9	38.8		
H4	1	3.9	35.2	33.5 ±2.4	2.0
	2	3.5	31.8		
	3	3.9	35.2		
	4	3.5	31.8		
H5	1	4.4	32.2	30.4 ±1.8	1.2
	2	3.9	29.8		
	3	3.9	29.8		
	4	3.9	29.8		
H6	1	4.4	24.7	26.2 ±1.5	1.7
	2	5.0	27.7		
	3	4.4	24.7		
	4	5.0	27.7		

Table XII

Total Protein Content in Poplar $I_{45/51}$ Leaves

(g per 100 g dry weight)

(% Kjeldahl N_2 x 6.25)

Harvest	Replicate	Protein /(fresh weight)	Protein /(dry weight)	Average ±Range	Standard Deviation
H1	1	5.6	22.5	23.2 ±0.7	0.65
	2	5.9	23.8		
H2	1	6.4	25.5	25.2 ±2.3	1.8
	2	6.9	27.5		
	3	5.9	23.8		
	4	5.9	23.8		
H3	1	8.1	31.3	29.9 ±1.4	1.0
	2	7.5	28.9		
	3	7.7	29.6		
	4	7.7	29.6		
H4	1	6.3	22.3	24.0 ±2.8	2.2
	2	6.9	24.6		
	3	7.5	26.8		
	4	6.3	22.3		
H5	1	5.6	19.4	20.0 ±1.6	1.1
	2	6.3	21.6		
	3	5.6	19.4		
	4	5.6	19.4		
H6	1	6.3	19.5	18.6 ±1.0	1.1
	2	6.3	19.5		
	3	5.6	17.6		
	4	5.6	17.6		

Table XIII

Total Protein Content in Poplar DN113 Leaves

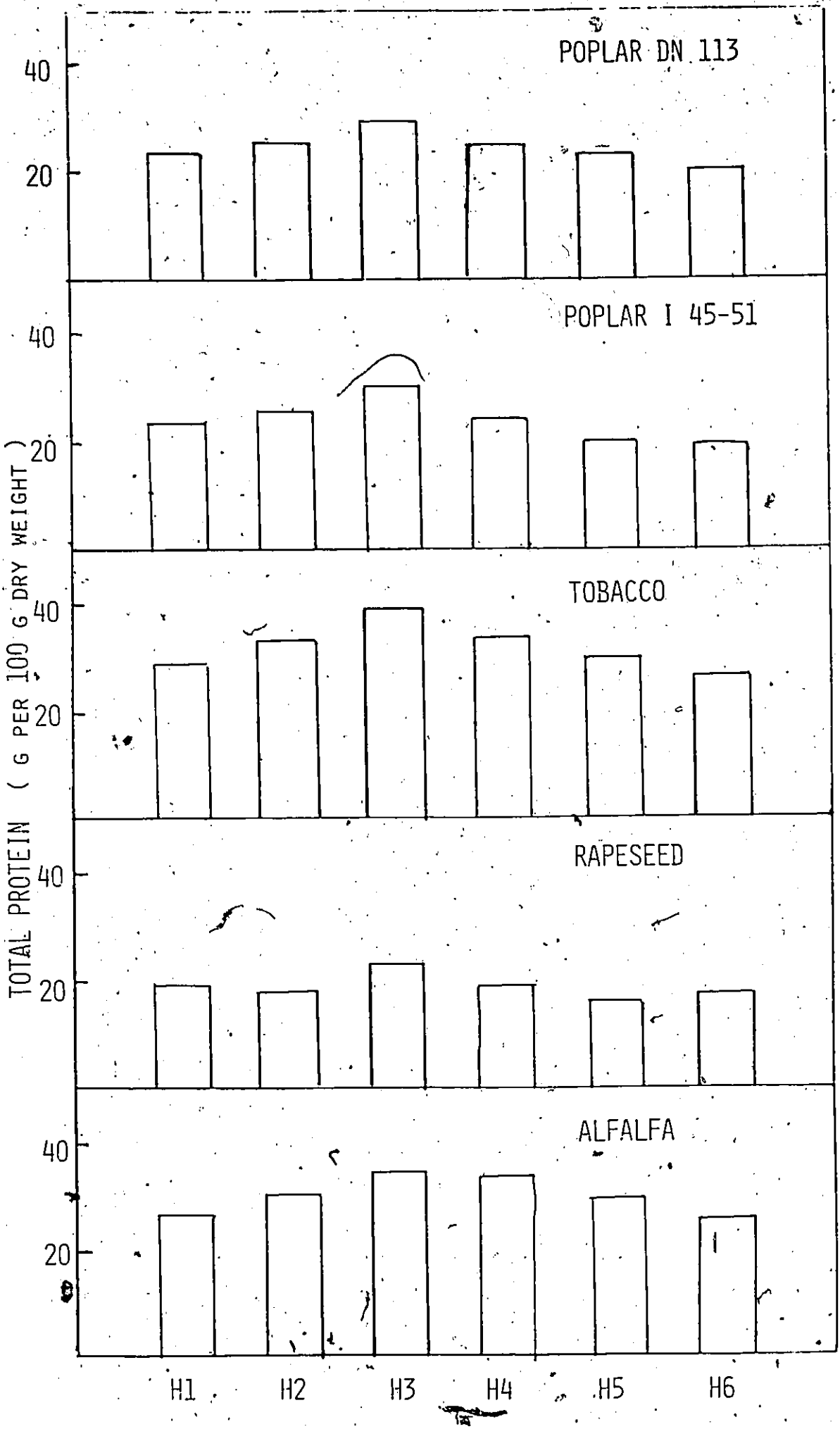
(g per 100 g dry weight)

(% Kjeldahl N_2 x 6.25)

Harvest	Replicate	Protein /(fresh weight)	Protein /(dry weight)	Average ±Range	Standard Deviation
H1	1	5.6	23.4	23.4 ±0	0
	2	5.6	23.4		
H2	1	5.6	23.4	25.0 ±1.6	1.3
	2	6.3	26.0		
	3	6.3	26.0		
	4	5.6	24.7		
H3	1	7.0	28.0	29.1 ±1.7	1.6
	2	6.9	27.5		
	3	7.5	30.0		
	4	7.7	30.8		
H4	1	6.9	25.5	24.4 ±2.4	1.5
	2	7.0	25.9		
	3	5.9	22.0		
	4	6.4	24.1		
H5	1	6.6	22.8	22.5 ±.3	1.4
	2	6.4	22.1		
	3	6.4	22.1		
	4	6.6	22.8		
H6	1	6.3	18.9	19.5 ±0.6	0.64
	2	6.3	18.9		
	3	6.6	20.0		
	4	6.6	20.0		

Figure 13 The Effect of Seasonal Variation on the Percentage of Total Protein Contained in Alfalfa, Rapeseed, Tobacco, and Two Poplar Clones ($I_{45/51}$ and DN113) Leaves

(H = Harvest No.)


POPLAR DN 113
POPLAR I 45-51
TOBACCO
RAPESEED
ALFALFA
TOTAL PROTEIN (G PER 100 G DRY WEIGHT)
40
20
H1
H2
H3
H4
H5
H6

variation on the yield of protein once in terms of fresh weight and another in terms of dry weight, while Figure 16, shows the effect of seasonal variation on the ratio of extracted leaf protein to the total protein content (Harvest 3).

Table XIV

The Ratio of Extractable Crude Protein to Total Protein Content per Dry Weight for Alfalfa

Harvest	Extractable Protein[a] (mg/g)	Total Protein[b] (%)	Ratio x 100
H 1	48.7	26.5	18.3
H 2	50.3	30.5	16.5
H 3	80.5	34.6	23.3
H 4	74.3	33.8	22.6
H 5	47.8	29.5	16.2
H 6	33.3	25.2	13.2

a Extractable Crude Protein values are averages from Table XIX

b Total Protein Content are averages from Table IX

Table XV

The Ratio of Extractable Crude Protein to Total Protein Content per Dry Weight for Rapeseed

Harvest	Extractable Protein[a] (mg/g)	Total Protein[b] (%)	Ratio x100
H 1	75.0	19.4	38.7
H 2	58.4	19.4	30.1
H 3	88.3	22.7	38.9
H 4	74.5	19.6	38.0
H 5	25.6	16.6	15.4
H 6	38.4	17.5	21.9

a Extractable Crude Protein values are averages from Table XX

b Total Protein Content are averages from Table X

Table XVI

The Ratio of Extractable Crude Protein to Total Protein Content per Dry Weight for Tobacco

Harvest	Extractable Protein[a] (mg/g)	Total Protein[b] (%)	Ratio x100
H 1	71.3	29.7	24.0
H 2	78.0	33.1	23.6
H 3	123.0	39.5	31.1
H 4	120.0	33.5	35.8
H 5	66.2	30.4	21.8
H 6	35.6	26.2	13.6

a Extractable Crude Protein values are averages from Table XXI

b Total Protein Content are averages from Table XI

Table XVII

The Ratio of Extractable Crude Protein to Total Protein Content per Dry Weight for Poplar $I_{45/51}$

Harvest	Extractable Protein[a] (mg/g)	Total Protein[b] (%)	Ratio x100
H 1	30.0	23.2	12.9
H 2	38.0	25.2	15.1
H 3	60.0	29.9	20.1
H 4	45.4	24.0	18.9
H 5	32.5	20.0	16.3
H 6	21.9	18.6	11.8

a Extractable Crude Protein values are averages from Table XXII

b Total Protein Content are averages from Table XII

Table XVIII

The Ratio of Extractable Crude Protein to Total Protein Content per Dry Weight for Poplar DN113

Harvest	Extractable Protein[a] (mg/g)	Total Protein[b] (%)	Ratio x100
H 1	32.8	23.4	14.0
H 2	39.0	25.0	13.6
H 3	61.6	29.1	21.2
H 4	47.4	24.4	19.4
H 5	32.5	22.5	14.4
H 6	21.5	19.5	11.0

a Extractable Crude Protein values are averages from Table XXIII

b Total Protein Content are averages from Table XIII

Table XIX

Extractable Crude Protein from Alfalfa Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	10.0	9.7 ±.25	0.25	50.0	48.7 ±1.2	1.76
		2	9.5			47.5		
	frozen	1	9.0	9.1 ±.1	0.1	45.0	45.5 ±.5	0.71
		2	9.2			46.0		
H2	fresh	1	10.5			52.5		
		2	10.0	10 ±.5	0.32	50.0	50.3 ±2.2	1.57
		3	9.7			48.7		
		4	10.0			50.0		
	frozen	1	9.5			47.5	47.4 ±3.9	3.06
		2	9.5	9.5 ±0.8	0.56	47.5		
		3	8.7			43.5		
		4	10.2			51.0		
H3	fresh	1	16.0			80		
		2	15.6	16.1 ±0.7	0.5	78	80.5 ±3.5	2.51
		3	16.0			80		
		4	16.8			84		
	frozen	1	16.0			80		
		2	14.8	15.3 ±0.9	1.0	74	76.5 ±4.5	2.10
		3	16.0			80		
		4	14.4			72		

Table XIX continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average Range	Standard Deviation	Yield (dry weight)	Average Range	Standard Deviation
H4	fresh	1	15.2			72.4		
		2	15.2	15.5 ±.5	0.38	72.4	74.3 ±1.9	1.81
		3	15.6			74.3		
		4	16.0			76.2		
	frozen	1	14.8			70.6		
		2	16.0	15.4 ±.6	0.69	76.2	73.4 ±2.8	3.23
		3	14.8			70.6		
		4	16.0			76.2		
H5	fresh	1	10.0			43.5		
		2	12.0	11 ±1.0	1.2	52.2	47.8 ±4.4	5.02
		3	12.0			52.2		
		4	10.0			43.5		
	frozen	1	10.0			43.5		
		2	10.0	9.5 ±1.5	1.0	43.4	41.3 ±6.5	4.35
		3	10.0			43.5		
		4	8.0			34.8		
H6	fresh	1	8.0			33.3		
		2	8.4	8 ±.4	0.33	35.0	33.3 ±1.7	1.35
		3	7.6			31.7		
		4	8.0			33.3		
	frozen	1	8.0			33.3		
		2	7.2	7.5 ±.5	0.38	30.0	31.3 ±2.0	1.58
		3	7.6			31.7		
		4	7.2			30.0		

Table XX

Extractable Crude Protein from Rapeseed Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	8.5			77.3		
		2	8.0	8.25 ±.25	.25	72.7	75 ±2.3	3.25
	frozen	1	7.5	7.6 ±.1	.1	68.2		
		2	7.7			70	69.1 ±.9	1.27
H2	fresh	1	7.0			58.3		
		2	7.2	7.0 ±.75	.32	60.4	58.4 ±6.3	4.49
		3	7.5			62.5		
		4	6.2			52.1		
	frozen	1	6.7			56.3		
		2	6.5	6.5 ±.5	.35	54.2	54.2 ±4.2	2.96
		3	6.7			56.3		
		4	6.0			50.0		
H3	fresh	1	9.6			87.3		
		2	9.6	9.7 ±.3	.2	87.3	88.3 ±2.6	1.80
		3	10.0			90.9		
		4	9.6			87.3		
	frozen	1	8.4			76.4		
		2	8.8	8.5 ±.5	.36	80.0	77.3 ±4.6	3.49
		3	8.0			72.7		
		4	8.8			80.0		

Table XX continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	10.8			75.0		
		2	10.4	10.5 ±.3	.2	74.3	74.5 ±.5	0.35
		3	10.4			74.3		
		4	10.4			74.3		
	frozen	1	10.4			74.3		
		2	10.0	10 ±.4	.33	71.4	71.4 ±2.9	2.33
		3	10.0			71.4		
		4	9.6			68.6		
H5	fresh	1	4.0			25.0		
		2	4.8	4.1 ±.9	.52	30.0	25.6 ±5.6	4.26
		3	4.4			27.5		
		4	3.2			20.0		
	frozen	1	4.0			25.0		
		2	3.2	3.4 ±.6	.52	20.0	21.4 ±3.8	3.23
		3	3.6			22.5		
		4	2.8			17.5		
H6	fresh	1	4.8	4.6 ±.2	.2	40.0		
		2	N.A.			—	38.4 ±1.7	2.33
		3	N.A.					
		4	4.4			36.7		
	frozen	1	3.6			30.0		
		2	N.A.	3.4 ±.2	.2	—	28.4 ±1.7	2.33
		3	N.A.					
		4	3.2			26.7		

N.A.= Not Available

Table XXI

Extractable Crude Protein from Tobacco Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	7.00	7.13 ±.12	.13	70.0	71.3 ±1.3	1.77
		2	7.25			72.5		
	frozen	1	6.50	6.4 ±.15	.13	65.0	63.8 ±1.3	1.77
		2	6.25			62.5		
H2	fresh	1	7.25			72.5		
		2	8.25	7.8 ±.55	.58	82.5	78 ±5.5	5.77
		3	8.25			82.5		
		4	7.25			72.5		
	frozen	1	6.75			67.5		
		2	7.25	6.7 ±0.55	.58	72.5	67 ±5.5	4.78
		3	7.25			72.5		
		4	6.25			62.5		
H3	fresh	1	12.80			128.0		
		2	12.40	12.3 ±0.70	.50	124.0	123 ±7.0	5.03
		3	12.40			124.0		
		4	11.60			116.0		
	frozen	1	10.40			104.0		
		2	11.20	10.5 ±0.7	.50	112.0	105 ±7.0	5.03
		3	10.40			104.0		
		4	10.00			100.0		

Table XXI continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	12.8			116.4		
		2	12.8	13.2 ±.4	.65	116.4	120 ±3.6	4.16
		3	13.6			123.6		
		4	13.6			123.6		
	frozen	1	12.0			109.1		
		2	10.4	11.6 ±1.2	.76	94.6	104.6 ±10.0	6.86
		3	11.6			105.5		
		4	12.0			109.1		
H5	fresh	1	8.4			64.6		
		2	8.8	8.6 ±0.6	.52	67.7	66.2 ±4.7	4.00
		3	9.2			70.8		
		4	8.0			61.5		
	frozen	1	8.0			61.5		
		2	7.6	8.0 ±.8	.38	58.5	61.6 ±6.1	4.34
		3	8.8			67.7		
		4	7.6			58.5		
H6	fresh	1	6.4			35.6		
		2	6.8	6.4 ±0.8	.57	37.8	35.6 ±4.5	3.16
		3	6.8			37.8		
		4	5.6			31.1		
	frozen	1	6.0			33.4		
		2	6.4	5.7 ±0.9	.68	35.6	31.7 ±5.0	3.81
		3	5.6			31.1		
		4	4.8			26.7		

Table XXII

Extractable Crude Protein from Poplar $I_{45/51}$ Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	7.75	7.4 ±1.25	.13	31.0	30.5 ±0.5	0.70
		2	7.50			30.0		
	frozen	1	5.00	4.75 ±.25	.13	20.0	19 ±1.0	1.41
		2	4.50			18.0		
H2	fresh	1	10.00			40.0		
		2	9.50	9.5 ±0.8	.56	38.0	38 ±3.2	2.25
		3	9.75			39.0		
		4	8.70			34.8		
	frozen	1	7.25			29.0		
		2	6.50	6.3 ±.95	.72	26.0	25.3 ±3.7	2.87
		3	5.75			23.0		
		4	5.75			23.0		
H3	fresh	1	15.20			58.5		
		2	15.60	15.6 ±.4	.33	60.0	60 ±1.6	1.26
		3	16.00			61.6		
		4	15.60			60.0		
	frozen	1	9.60			36.9		
		2	8.80	9.3 ±0.5	.26	33.9	35.8 ±2.9	1.44
		3	9.20			35.4		
		4	9.60			36.9		

Table XXII continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	13.6			48.6		
		2	13.2	12.7 ±0.9	.83	47.1	45.4 ±3.2	2.92
		3	12.0			42.9		
		4	12.0			42.9		
	frozen	1	8.8			31.4		
		2	9.6	9.2 ±0.8	.73	34.3	32.9 ±2.9	2.61
		3	10.0			35.7		
		4	8.4			30.0		
H5	fresh	1	8.8			30.4		
		2	10.0	9.4 ±0.6	.69	34.5	32.5 ±2.1	2.37
		3	10.0			34.5		
		4	8.8			30.4		
	frozen	1	7.6			26.2		
		2	7.2	7.4 ±.2	.23	24.8	25.5 ±1.7	0.80
		3	7.2			24.8		
		4	7.6			26.2		
H6	fresh	1	6.8			21.3		
		2	7.2	7 ±0.6	.52	22.5	21.9 ±1.9	1.63
		3	6.4			20.0		
		4	7.6			23.8		
	frozen	1	6.0			18.8		
		2	4.4	5 ±1.0	.69	13.8	15.7 ±3.1	2.17
		3	4.8			15.0		
		4	4.8			15.0		

Table XXIII

Extractable Crude Protein from Poplar DN113 Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	7.5	7.75 ±.25	.25	32.3	32.8 ±0.5	0.70
		2	8.0			33.3		
	frozen	1	4.2	4.5 ±.25	.25	17.7	18.8 ±1.1	1.48
		2	4.7			19.8		
H2	fresh	1	9.5			39.6		
		2	8.7	9.4 ±1.1	.85	36.3	39 ±4.8	3.56
		3	10.5			43.8		
		4	8.7			36.3		
	frozen	1	5.2			21.9		
		2	6.0	6.1 ±0.85	.63	25.0	25.3 ±3.4	2.58
		3	6.2			26.0		
		4	6.7			28.1		
H3	fresh	1	15.6			62.4		
		2	15.2	15.4 ±0.6	.52	60.8	61.6 ±2.4	2.06
		3	14.8			59.2		
		4	16.0			64.0		
	frozen	1	9.6			38.4		
		2	9.6	9.5 ±0.3	.83	38.4	38.0 ±1.2	0.80
		3	9.6			38.4		
		4	9.2			36.8		

Table XXIII continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	12.4			45.9		
		2	13.6	12.8 ±0.8	.57	50.4	47.4 ±3.0	2.12
		3	12.4			45.9		
		4	12.8			47.4		
	frozen	1	8.4			31.1		
		2	9.2	9.1 ±0.9	.68	34.1	33.7 ±3.6	2.52
		3	8.8			32.6		
		4	10.0			37.0		
H5	fresh	1	8.8			30.4		
		2	10.0	9.4 ±0.6	.69	34.5	32.5 ±2.1	2.36
		3	10.0			34.5		
		4	8.8			30.4		
	frozen	1	7.6			26.2		
		2	8.0	7.2 ±0.8	.72	27.8	24.9 ±2.9	2.57
		3	6.8			23.5		
		4	6.4			22.1		
H6	fresh	1	7.6			23.0		
		2	7.2	7.1 ±0.7	.5	21.8	21.5 ±2.1	1.50
		3	6.4			19.4		
		4	7.2			21.8		
	frozen	1	5.2			15.8		
		2	5.2	5.2 ±0	0	15.8	15.8 ±0	0
		3	5.2			15.8		
		4	5.2			15.8		

Figure 14 The Effect of Seasonal Variation on Extractable Crude Protein Obtained from Alfalfa, Rapeseed, Tobacco and Two Poplar Clones (I45/51 and DN113). Data are expressed per g fresh weight.

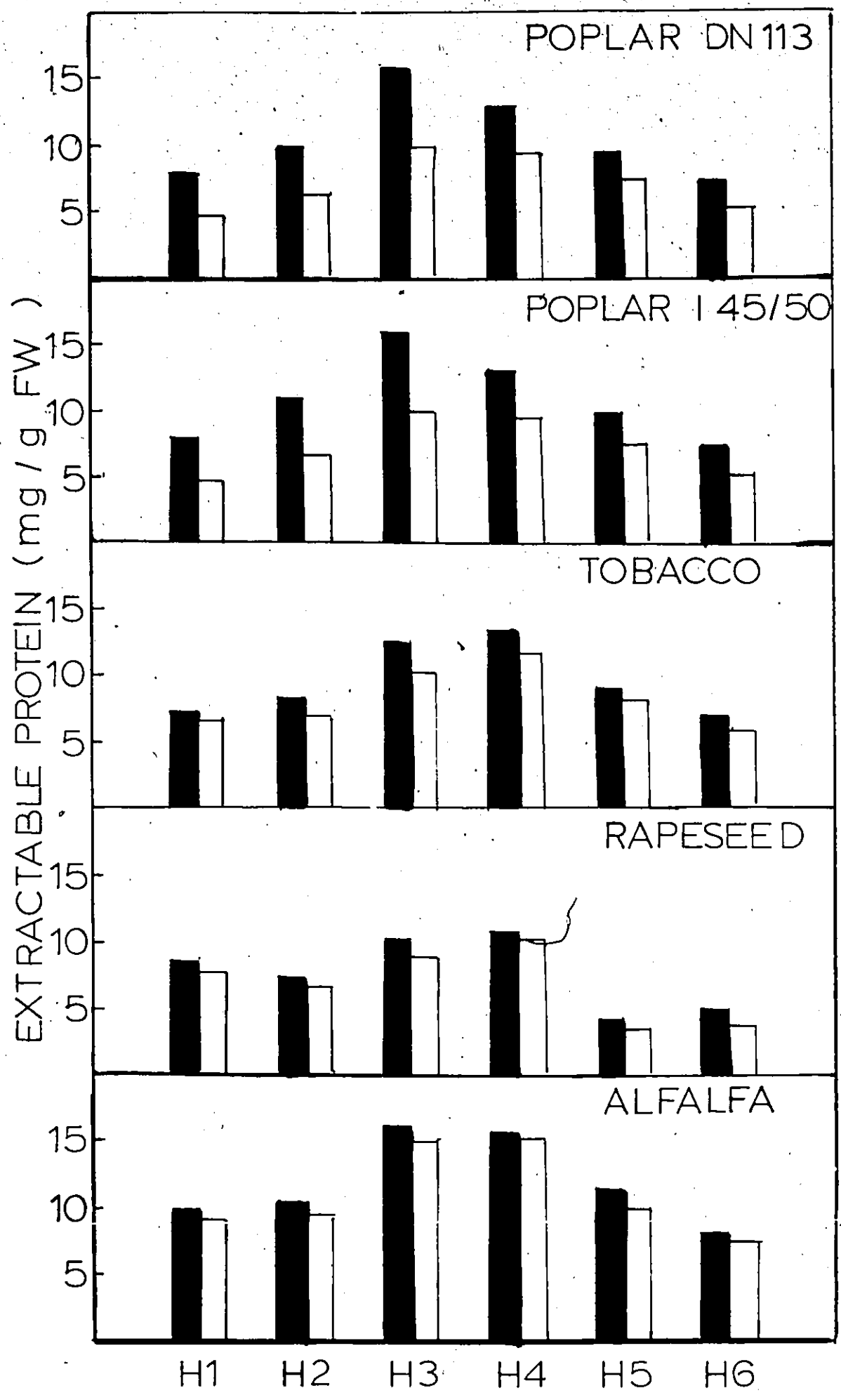
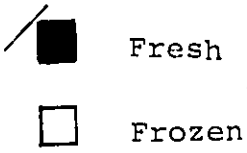

POPLAR DN113
POPLAR I 45/50
TOBACCO
RAPESEED
ALFALFA
EXTRACTABLE PROTEIN (mg / g FW)
15
10
5
H1
H2
H3
H4
H5
H6

Figure 15 The Effect of Seasonal Variation on Extractable Crude Protein Obtained from Alfalfa, Rapeseed, Tobacco and Two Poplar Clones ($I_{45/51}$ and DN113). Data are expressed per g dry weight.



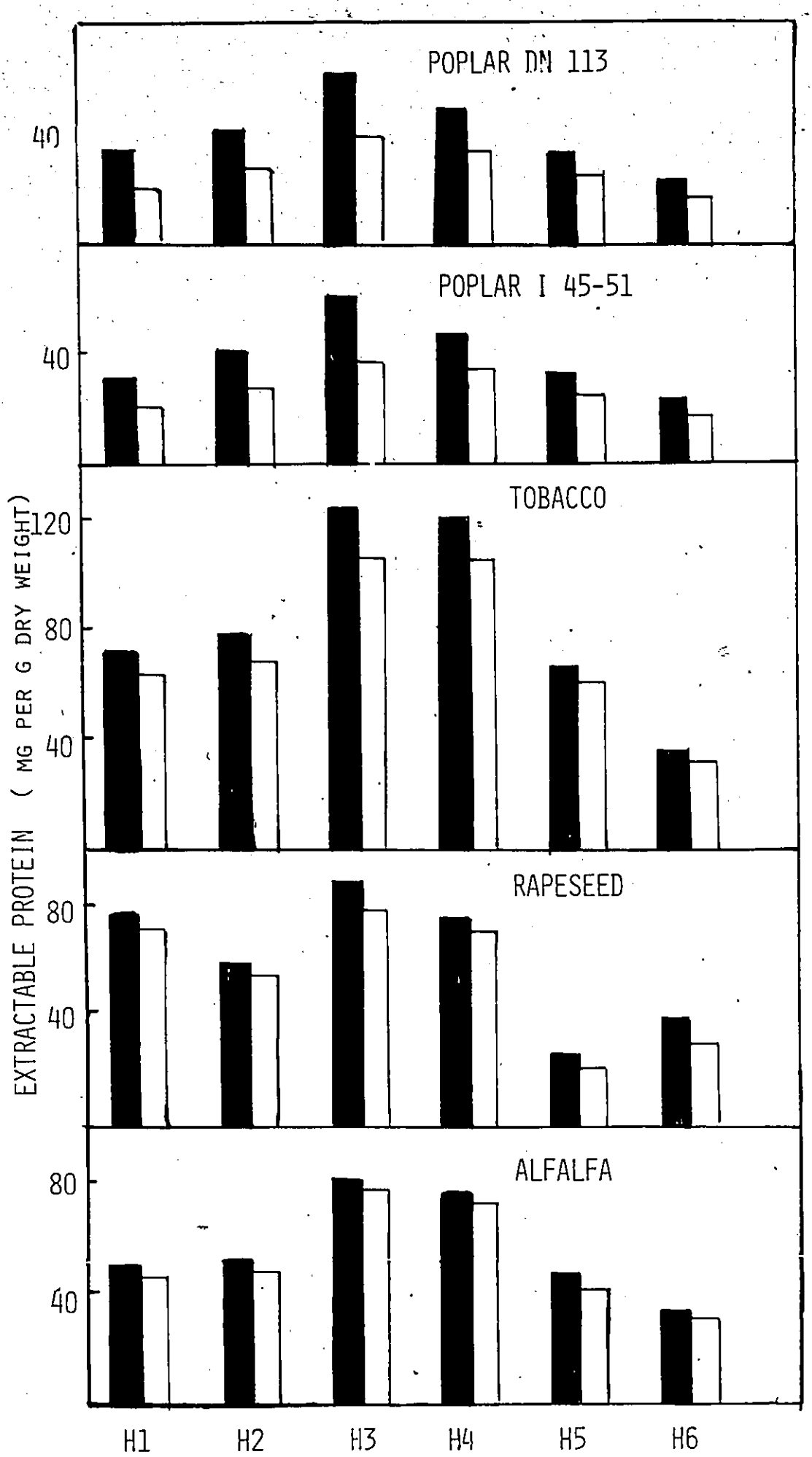
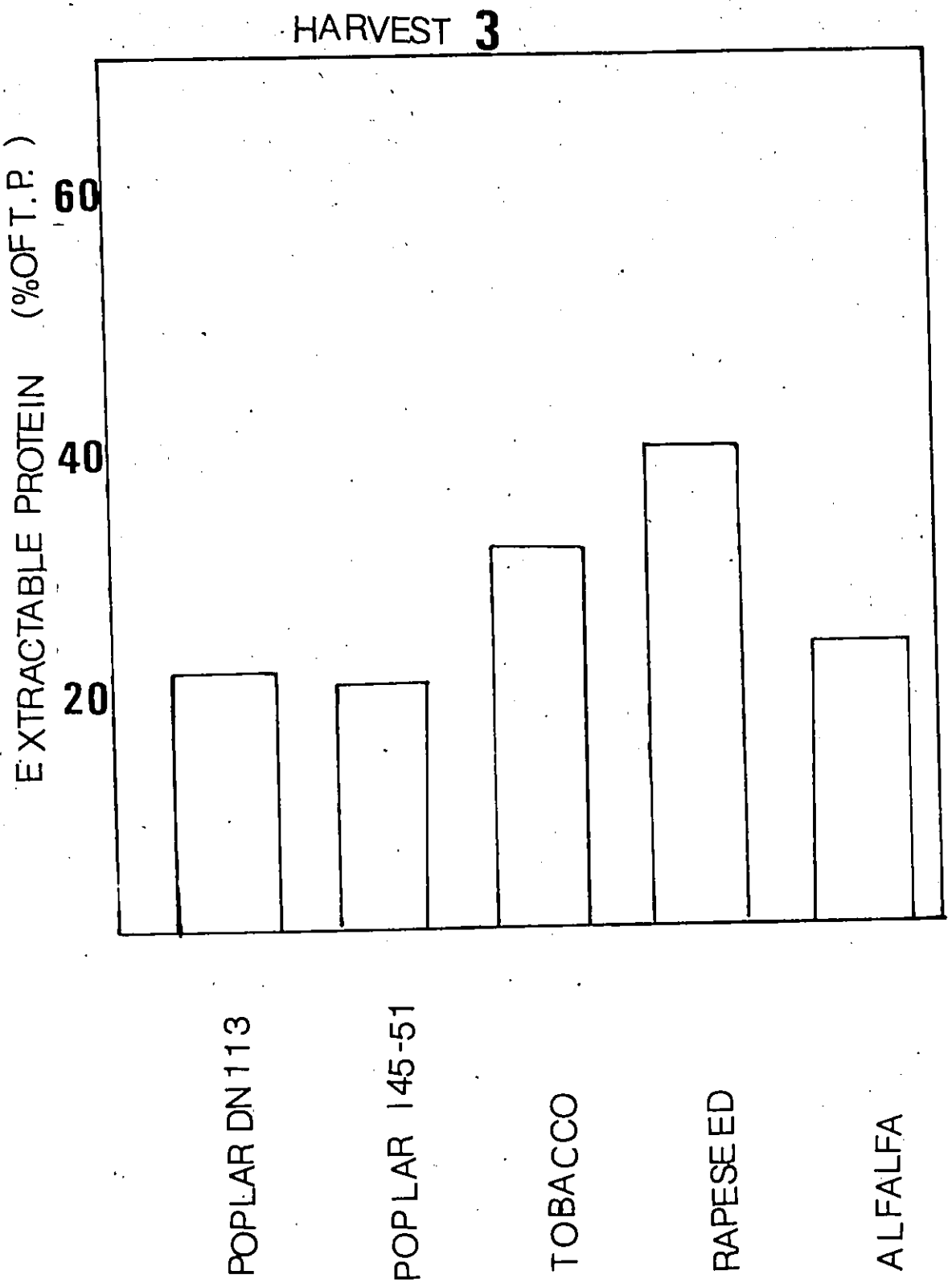

POPLAR DN 113
POPLAR I 45-51
TOBACCO
RAPESEED
ALFALFA
EXTRACTABLE PROTEIN (MG PER G DRY WEIGHT)
120
80
40
H1
H2
H3
H4
H5
H6

Figure 16 The Effect of Seasonal Variation on the Ratio of Extracted crude Protein to the Total Protein Content.


HARVEST 3
EXTRACTABLE PROTEIN (%OF T.P.)
60
40
20
POPLAR DN113
POPLAR 145-51
TOBACCO
RAPESEED
ALFALFA

4. Extractable Phenol

Refluxing with ethanol was used to extract the leaf phenols. The Folin-Ciocalteu was straightforward and it gave reproducible results in each of the four replicates of each species. Tables XXIV, XXV, XXVI, XXVII and XXVIII report the yields of phenol per fresh and dry weight of the five different types of leaves. The yield of phenol was calculated for both fresh and frozen leaves. The effect of seasonal variation on the amount of extractable phenols was also studied to find the times of highest and lowest concentration (Figures 17 and 18).

Table XXIV

Extractable Phenol in Alfalfa Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	4.0	3.9 ±0.1	0.14	20	19.5 ±0.5	0.7
		2	3.8			19		
	frozen	1	3.2	3.0 ±0.2	0.28	16	15.0 ±1.0	1.4
		2	2.8			14		
H2	fresh	1	8.0	7.4 ±1.6	0.95	40	37 ±7.0	4.7
		2	7.6			38		
		3	8.0			40		
		4	6.0			30		
	frozen	1	6.0	6.0 ±2.0	0.58	30	30 ±10.0	8.2
		2	4.0			20		
		3	6.0			30		
		4	8.0			40		
H3	fresh	1	10.0	11 ±2.0	1.0	50	55 ±5.0	5.7
		2	12.0			60		
		3	12.0			60		
		4	10.4			50		
	frozen	1	8.0	8.4 ±1.6	1.0	40	42 ±8.0	5.4
		2	7.6			38		
		3	8.0			40		
		4	10.0			50		

Table XXIV continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	12.4	13.2 ±0.8	0.57	59	63 ±4.0	2.8
		2	13.6			65		
		3	13.6			65		
		4	13.2			63		
	frozen	1	10.0	9.0 ±1.0	0.88	48	40 ±8.0	4.1
		2	8.8			42		
		3	8.0			38		
		4	8.8			42		
H5	fresh	1	10.0	9.0 ±1.0	1.0	44	40 ±5.0	5.1
		2	8.0			35		
		3	8.0			35		
		4	10.0			44		
	frozen	1	8.0	7.4 ±1.6	1.1	35	33 ±7.0	4.4
		2	7.6			34		
		3	6.0			26		
		4	8.0			35		
H6	fresh	1	4.0	3.9 ±0.3	0.1	17	16.5 ±1.5	1.0
		2	3.6			15		
		3	4.0			17		
		4	4.0			17		
	frozen	1	3.6	3.5 ±0.5	0.3	11	13.5 ±3.5	2.5
		2	3.2			13		
		3	3.2			13		
		4	4.0			17		

Table XXV

Extractable Phenol in Rapeseed Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	2.0	2 ±0	0	18	18 ±0	0
		2	2.0			18		
	frozen	1	1.6	1.7 ±0.1	0.1	15	15.5 ±0.5	0.7
		2	1.8			16		
H2	fresh	1	3.2	3.2 ±0.4	0.3	27	26 ±4.0	3.4
		2	3.6			30		
		3	2.8			23		
		4	2.8			23		
	frozen	1	2.8	2.5 ±0.5	0.3	23	21 ±4.0	2.8
		2	2.4			20		
		3	2.8			23		
		4	2.0			17		
H3	fresh	1	4.0	4.5 ±1.5	1.0	36	41 ±14	9.5
		2	6.0			55		
		3	4.0			36		
		4	4.0			36		
	frozen	1	3.8	4.0 ±0.8	0.5	35	37 ±7.0	4.5
		2	3.8			35		
		3	3.8			35		
		4	4.8			44		

Table XXV continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	10.0	10.4 ±0.8	0.6	71	74 ±6.0	4.2
		2	11.2			80		
		3	10.4			74		
		4	10.0			71		
	frozen	1	6.4	6.4 ±0.4	0.3	46	45 ±4.0	2.4
		2	6.0			43		
		3	6.8			49		
		4	6.4			46		
H5	fresh	1	6.8	6.6 ±1.0	0.74	43	42 ±9.0	4.8
		2	6.0			38		
		3	7.6			48		
		4	6.0			38		
	frozen	1	6.0	4.7 ±1.3	0.94	38	29.5 ±8.5	6.1
		2	4.0			28		
		3	4.8			30		
		4	4.0			25		
H6	fresh	1	12.0	11 ±1.0	1.0	100	92 ±9.0	12.0
		4	10.0			83		
	frozen	1	8.0	8 ±0	0	67	67 ±0	0
		4	8.0			67		

Table XXVI

Extractable Phenol in Tobacco Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	16	16 ±0	0	160	160 ±0	0
		2	16			160		
	frozen	1	12	13 ±1.0	1.0	120	130 ±10	14.1
		2	14			140		
H2	fresh	1	16	18.5 ±2.5	2.0	160	185 ±25	19.1
		2	20			200		
		3	18			180		
		4	20			200		
	frozen	1	12	13.5 ±2.5	2.0	120	135 ±25	19.1
		2	14			140		
		3	12			120		
		4	16			160		
H3	fresh	1	20	21.5 ±2.5	2.0	200	215 ±25	19.1
		2	24			240		
		3	20			200		
		4	22			220		
	frozen	1	16	17 ±3.0	2.0	160	170 ±30	20.0
		2	16			160		
		3	20			200		
		4	16			160		

Table XXVI continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	20	20 ±0	0	182	182 ±0	0
		2	20			182		
		3	20			182		
		4	20			182		
	frozen	1	12	12 ±0.4	0.3	109	109 ±4.0	2.9
		2	11			106		
		3	12			113		
		4	12			109		
H5	fresh	1	8	9.5 ±2.5	4.0	62	73 ±19.0	14.3
		2	10			77		
		3	12			92		
		4	8			62		
	frozen	1	6	7 ±1.0	1.2	46	54 ±8.0	9.2
		2	6			46		
		3	8			62		
		4	8			62		
H6	fresh	1	8	7 ±1.0	1.2	44	38.5 ±5.5	6.4
		2	6			33		
		3	6			33		
		4	8			44		
	frozen	1	4	4.5 ±1.5	1.0	22	25 ±8.0	5.5
		2	4			22		
		3	6			33		
		4	4			22		

Table XXVII

Extractable Phenol in Poplar $I_{45/51}$ Leaves

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	28	30 ±2	1.5	115	122 ±7	9.2
		2	32			128		
	frozen	1	24	25 ±1	1.0	96	100 ±4	5.6
		2	26			104		
H2	fresh	1	35	35 ±3	3.0	140	141 ±13	10.0
		2	36			144		
		3	32			128		
		4	38			152		
	frozen	1	30	30 ±2	2.0	120	120 ±8	6.5
		2	32			128		
		3	28			112		
		4	30			120		
H3	fresh	1	40	46 ±6	3.3	154	177 ±23	15.5
		2	48			185		
		3	48			185		
		4	48			185		
	frozen	1	36	36 ±4	3.3	139	141 ±18	13.2
		2	40			154		
		3	38			146		
		4	32			123		

Table XXVII continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	44	47 ±3	2	157	166 ±9	6.7
		2	48			171		
		3	46			164		
		4	48			171		
	frozen	1	40	38 ±2	2	143	136 ±7	8.1
		2	40			143		
		3	36			129		
		4	36			129		
H5	fresh	1	32	29 ±5	3	110	100 ±17	12.9
		2	32			110		
		3	28			97		
		4	24			83		
	frozen	1	24	22 ±2	2	83	76 ±7	8.1
		2	24			83		
		3	20			69		
		4	20			69		
H6	fresh	1	24	24 ±4	3.3	75	75.5 ±13.5	10.6
		2	28			89		
		3	20			63		
		4	24			75		
	frozen	1	20	21 ±3	2	63	66 ±9	6
		2	20			63		
		3	24			75		
		4	20			63		

Table XXVIII

Extractable Phenol in Poplar DN113

(Yield mg/g)

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H1	fresh	1	30	30 ±0	0	125	125 ±0	0
		2	30			125		
	frozen	1	26	27 ±1	1	108	112 ±5	6.4
		2	28			117		
H2	fresh	1	34	35 ±3	2	142	144 ±11	8.1
		2	36			150		
		3	32			133		
		4	36			150		
	frozen	1	30	30 ±2	1.2	125	126 ±7	5.0
		2	30			125		
		3	32			133		
		4	29			121		
H3	fresh	1	48	47 ±3	2	192	188 ±12	8.0
		2	44			176		
		3	48			192		
		4	48			192		
	frozen	1	40	36 ±4	3.3	160	144 ±16	13.0
		2	36			144		
		3	32			128		
		4	36			144		

Table XXVIII continued

Harvest	Fresh or Frozen	Replicate	Yield (fresh weight)	Average ±Range	Standard Deviation	Yield (dry weight)	Average ±Range	Standard Deviation
H4	fresh	1	44	46 ±2	2	163	168 ±10	7.1
		2	46			170		
		3	44			163		
		4	48			178		
	frozen	1	40	40 ±4	3.3	148	149 ±16	12.4
		2	36			133		
		3	44			163		
		4	41			152		
H5	fresh	1	32	29 ±3	2	110	100 ±10	5.8
		2	28			97		
		3	28			97		
		4	28			97		
	frozen	1	24	24 ±4	3.3	83	83 ±14	11.4
		2	20			69		
		3	28			97		
		4	24			83		
H6	fresh	1	24	24 ±4	3.3	73	73 ±1.3	9.8
		2	20			61		
		3	24			73		
		4	28			85		
	frozen	1	16	20 ±4	3.3	49	61 ±1.2	9.8
		2	20			61		
		3	24			73		
		4	20			61		

Figure 17 The Effect of Seasonal Variation on the Amount of Extractable Leaf Phenol from Alfalfa, Rapeseed, Tobacco and Poplar ($I_{45/51}$ and DN113) . Concentration in mg phenol per g fresh weight leaf.

Fresh

Frozen

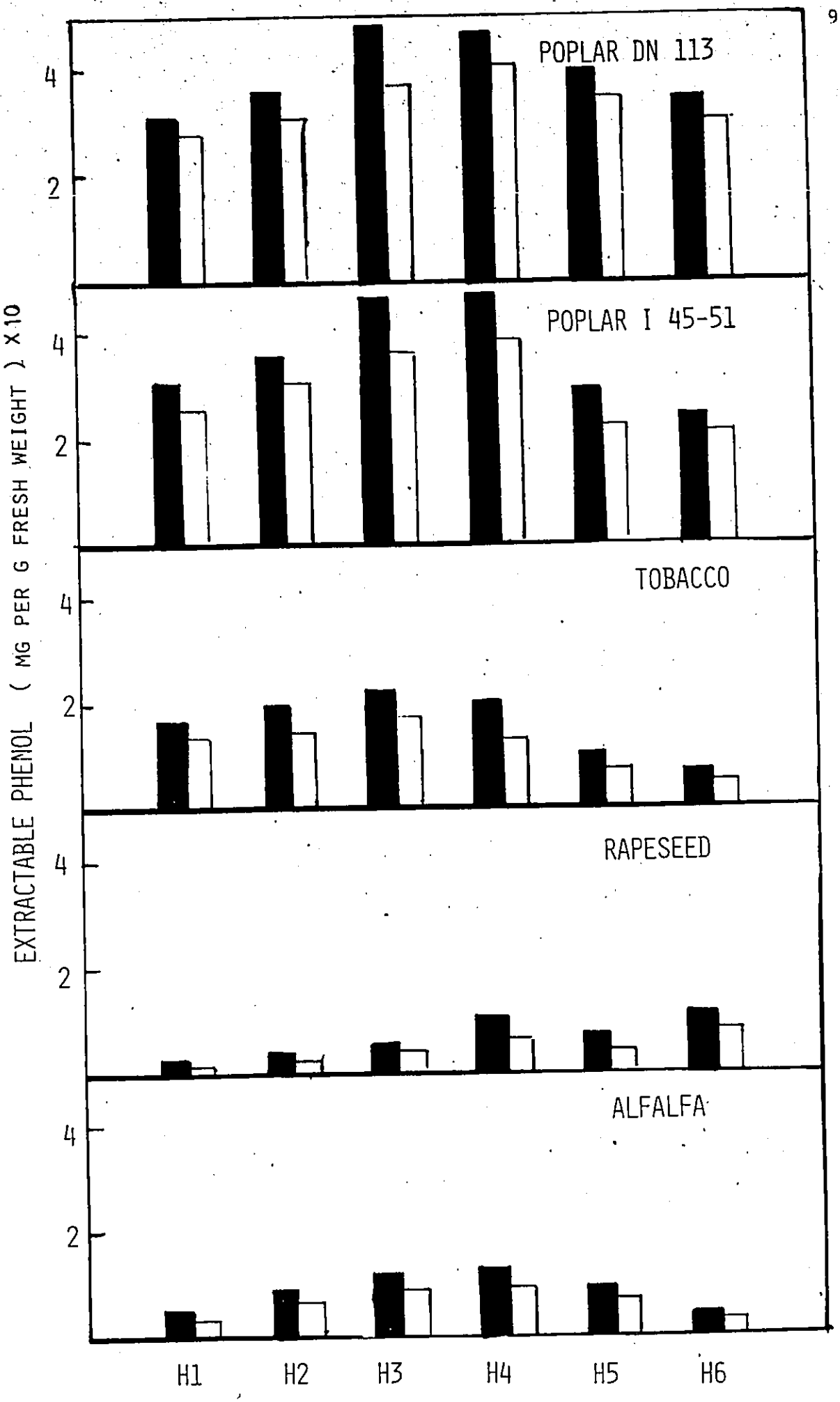
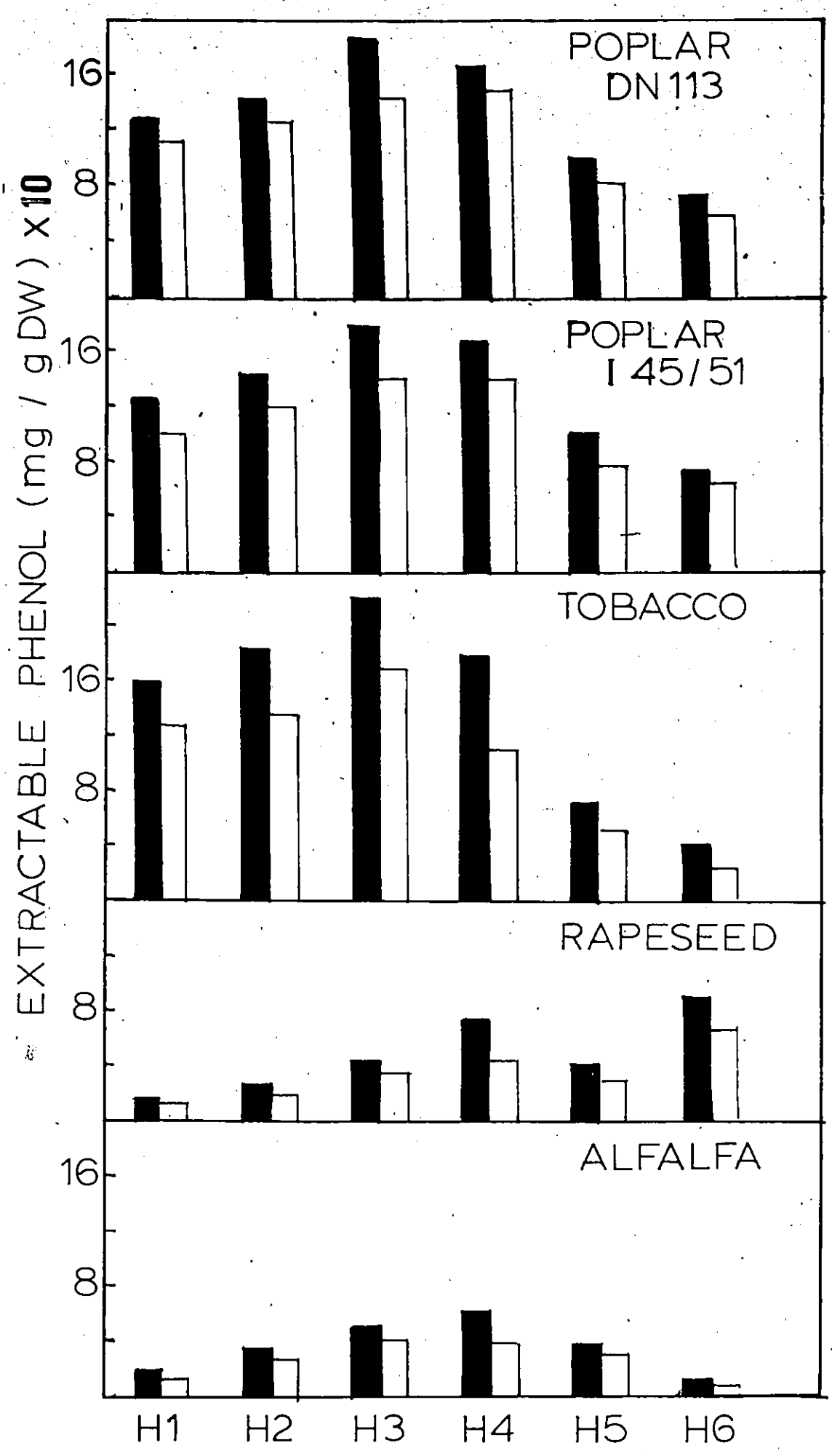

POPLAR DN 113
POPLAR I 45-51
TOBACCO
RAPESEED
ALFALFA
EXTRACTABLE PHENOL (MG PER G FRESH WEIGHT) X10
4
2
H1
H2
H3
H4
H5
H6

Figure 18 The Effect of Seasonal Variation on the Amount of Extractable Leaf Phenol from Alfalfa, Rapeseed, Tobacco and Poplar ($I_{45/51}$ and DN113). Concentration in mg phenol per g dry weight leaf.

Fresh

Frozen


POPLAR
DN 113
POPLAR
I 45/51
TOBACCO
RAPESEED
ALFALFA
16
8
EXTRACTABLE PHENOL (mg / g DW) x10
H1
H2
H3
H4
H5
H6

5. Summer 1981

In the summer of 1981 we were concentrating mainly on poplar leaves. This is due to the following reasons; the tobacco did not grow well, the alfalfa had a virus disease and the rapeseed exhibited a very short growing season making it difficult to compare it to poplar plant in its second growing season. In chapter IV we will discuss the results obtained from poplar leaves in the second growing season.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

A. Optimization of Protein Extraction

The presence of phenolic compounds and high activity of phenol oxidases in plant tissues results in browning of extracts and prevents isolation of active enzymes. To increase protein extractability and decrease browning of the protein extract, many protective agents have been used. Polyvinylpyrrolidone is believed to act as a phenol-adsorbing substance (Loomis and Battaile, 1966). Sulfite was found to inhibit the oxidation of phenolic compounds in leaves and their subsequent attack on proteins (Woodham, 1974). Mercaptoethanol seems to inhibit phenol oxidase and stimulates the activity of some leaf enzymes (Welander, 1978).

1. Adsorption of Phenolics with Polyvinylpyrrolidone (PVP)

Figure 10 shows the effect of adding polyvinyl-pyrrolidone to the extraction medium on recovery of crude protein. The use of 0.2% PVP in the homogenization buffer yields the optimum production of crude protein (1.02 mg protein/ml of leaf press juice supernatant, while without

adding PVP it is equal to 0.65 mg/ml). In 1976 Stroback and Gibbons refer in their extraction method of Fraction-1 protein (up to 65% of the total soluble leaf protein) to the use of 0.2% PVP in the extraction buffer.

2. Inhibition of Phenolics with Sulfite

The effect of adding sulfite in the extraction buffer on recovery of crude protein is shown in Figure 11. At pH of 7.4 the optimum protein production occurred with 1% sulfite present (0.7 mg/ml). At an alkaline pH of 9.0 similar amounts of protein were recovered at 1% sulfite. When the reducing agent was 5% the extractability and recovery was much higher, at about 1.0 mg/ml of leaf press juice supernatant. Free and Satterlee concluded that, sulfite increasesthe quality and quantity of the protein extracted from leaf tissues (Free and Satterlee, 1975).

B. Characterization of Leaf Tissue

Plant leaves were collected during the growing season July to September in summer 1980 and from June to August in summer 1981. Four species were chosen: alfalfa, rapeseed, tobacco and hybrid poplar (two poplar clones were used, $I_{45/51}$ and DN113).

To study the potential value of purified hybrid poplar leaf protein isolates, it would be most useful to

compare the extractable protein to the crude protein extracted from alfalfa, tobacco and rapeseed. The latter three protein isolates have been already well characterized by other workers (Pirie, 1971; Wildman, 1974; Brown et al., 1975). The present study also attempted to study the effect of phenol on the amount of protein extracted from forest foliage.

Comparison of the amount and characteristics of protein extracted from poplar with those of alfalfa, tobacco and rapeseed was very useful. The available information concerning the preparation of colorless, tasteless and acceptable protein products from alfalfa, tobacco and rapeseed leaves can be used in producing similar protein products from hybrid poplar leaves.

1. Moisture Content (MC)

The effect of seasonal variation on percentage moisture and comparison between the moisture content in hybrid poplar leaves and alfalfa, tobacco and rapeseed leaves was observed (Figure 12).

Poplar leaves are drier ($I_{45/51}$ average MC = 73%, DN113 MC = 73.1%) than alfalfa (MC = 78.6%), tobacco (MC = 88.1%) and rapeseed (MC = 88.2%). This may make the extraction of protein and phenol from poplar leaf more difficult than from other leaves. Moreover, poplar leaves may

need more extraction buffer than the others. Figure 12 shows that no big variation was observed in the moisture content at the beginning of the season compared with that at the end of it (e.g. poplar $I_{45/51}$ MC in H1 = 75% and in H6 = 68.5%).

In summer 1981 moisture content of poplar $I_{45/51}$ was 73% and DN113 was 72.1%. Those values were close to those found in the first growing season. In 1977 Anelli reported that alfalfa has 81% moisture content. Wildman mentioned that, tobacco leaf has from 80% to 90% water (Wildman, 1977). According to Chen, hybrid poplar leaves ($I_{45/51}$) have 75% moisture content in July and 64% in August (calculation based on g of dry leaves per g of fresh leaves) (Chen, 1979). Therefor there was no significant difference between the moisture content of poplar leaves grown in Kemptville in 1980-81 and those grown in Orono in 1975-76.

2. Total Protein Content

To study the ratio of the extractable protein to the total protein in leaf materials, it was necessary to determine first the total protein content. The Micro-Kjeldahl method was efficient and there was good correlation of results.

The results in tables IX, X, XI, XII and XIII show that, the total protein content in poplar leaf ($I_{45/51}$

average = 23.5% and DN113 = 24%) is slightly lower than in alfalfa leaf (TPC = 30%), but much lower than in tobacco leaf (TPC = 32.1%).

The effect of seasonal variation on the total protein content is illustrated in Figure 13. It is seen that, the quantity of total protein content from poplar ($I_{45/51}$ and DN113), alfalfa and tobacco leaves increased from July to mid August (e.g. $I_{45/51}$ had TPC in July = 23.2% and in mid August = 29.9%), but then decreased to the end of September ($I_{45/51}$ had TPC in H6 = 18.6%). Harvest 3 gave the highest quantity of total protein in all species. The total protein contents were calculated in terms of oven dried weight. The total protein content in poplar leaves in 1981 ($I_{45/51}$ = 21% and DN113 = 20%) was slightly lower than that in 1980 ($I_{45/51}$ 23.5% and DN113 24%).

Bojracharya et al. (1979) concluded that fresh alfalfa leaf has 30% protein (basis dry weight). Also, tobacco leaf has about 31.6% protein (basis dry weight) (calculated from Kung et al., 1980 and Wildman, 1977). Those results are in agreement with the Kemptville field study. It also means that, the method followed for total protein determination was accurate (Micro-Kjeldahl method).

3. Yield of Extractable Protein

Stroback and Gibbons mentioned a satisfactory method for the homogenization of leaf tissue with buffer containing phenol inhibitors at neutral pH of 7.4. The efficiency of these inhibitors was tested on poplar material in this study and discussed earlier in this chapter. In our analysis we used Stroback and Gibbons' method (Stroback and Gibbons 1978).

The seasonal variation and the difference in the yield of crude protein among two hybrid poplar clones and alfalfa, tobacco and rapeseed were studied in fresh leaves.

In this research it was important to study the effect of freezing on the yield of extractable crude protein from leaves. This research started over more than one year ago. Therefore, leaves had to be stored frozen for study during winter time. Freezing is known to disrupt cellular structures, and possibly frozen leaves would encounter increased protein-phenol interference on extraction.

The effect of seasonal variation on the yield of protein from 5 different plants studied here is illustrated in Figure 14. Working with poplar $I_{45/51}$ and DN113, alfalfa, and tobacco, the yield figures increased from the end of July to mid August and then decreased to the end of September. The same results were obtained from poplar $I_{45/51}$ in 1976 by Chen (Chen, 1979).

Figure 14 shows the yield of crude protein obtained from the fresh and frozen leaves of the four different species. Considering fresh leaves only, it was observed that the yield of extractable crude protein from poplar leaves (in H3 $I_{45/51}$ gave 15.6 mg/g and DN113 gave 15.4 mg/g) was lower than that of alfalfa (16.1 mg/g) and higher than tobacco (12.3 mg/g). Expressing the data on a dry weight basis, the yield of extractable crude protein from poplar leaves ($I_{45/51}$ gave 60 mg/g and DN113 gave 61.6 mg/g) was lower than that of alfalfa (80.5 mg/g) or tobacco (123 mg/g) (Figure 15). The amount of protein recovered from poplar leaves in 1981 ($I_{45/51}$ = 14.4 mg/g fresh weight and DN113 = 14.8 mg/g) was slightly lower than in 1980. Although tobacco and rapeseed protein is greater on dry weight basis, they are similar to poplar on fresh basis. This suggested that the harvesting costs (and perhaps growing costs) would be similar on a per tonne or hectare basis.

It was also observed from Figure 14, that freezing of leaves decreases the yield of extractable crude protein. The decrease in the yield of extractable crude protein recovered from poplar leaves ($I_{45/51}$ gave 9.3 mg/g and DN113 gave 9.5 mg/g) was larger than that in alfalfa (15.3 mg/g) and tobacco (10.5 mg/g). In 1972 Lu et al. mentioned that, greater extractability of undenatured proteins was observed when fresh leaves were used for extraction (Lu et al., 1972).

As was found for extraction efficiency, the lower values recovered from poplar leaves, when compared with that of alfalfa and tobacco are probably due to the inefficient extraction resulting from relatively high fibre

content and low moisture content in the poplar leaves and the interference of other chemical components in the leaf tissues (e.g. phenol compounds) as discussed in the next section.This should be tested in further research.

Kohler et al. (1978) reported that 90 mg/g crude protein could be extracted from alfalfa leaves (basis dry weight). About 150 mg/g crude protein was extracted from tobacco leaves (calculated from Wildman, 1977 and Kung et al., 1980). In 1979 Chen concluded that in the first growing season of 6 different clones of hybrid poplar the yield values from the leaves ranged from 14.0 mg/g to 50.8 mg/g (organic solvent used for extraction) and from 6.1 mg/g to 41.2 mg/g (heat coagulation used).

4. Ratio of Extractable Protein to the Total Protein Content

The effect of seasonal variation on the ratio of the extractable crude protein to the total was studied. As shown in Tables XIV, XV, XVI, XVII and XVIII the ratio of extractable crude protein to the total increased from July to mid August and then decreased to the end of September. Also the ratio in case of tobacco leaves (H3 = 31.1%) was higher than that of alfalfa (=23.3%) and poplar leaves ($I_{45/51}$ = 20% and DN113 = 21%) (Figure 16).

In 1972 Lu and Kinsella reported that the yield of protein extracted under standard procedure was approximately

30% of the total protein content. About 48% protein extracted out of the total in tobacco leaves (calculated from Kung, 1980 and Wildman, 1977). Chen reported that, when using coagulation techniques for protein recovery from the leaves of 6 different poplar clones, the crude protein extracted ranged from 4.34% to 33.52% of the total protein with heat coagulation and from 10% to 41.33% of the total protein with organic solvent. The lower values provided here, when compared with that reported in the literature are probably due to the simple and fast methods followed in the extraction (homogenization, filteration and centrifugation) and determination of the crude protein (Bio-Rad).

In order to increase the extraction efficiency one should be aware of the fact that the cell walls should be well-ruptured so as to release protein contained in cellular material for elution from plant tissue. Boyd (1968) pointed out that the extraction efficiency depends on some other factors such as leaf species and stage of maturity. Two other factors are the chemical composition and the pH of the extraction as mentioned by Lu and Kinsella (1972).

Lugg (1939) was the first to notice that, when macerating plant materials, fibrous debris tend to act as a filter and hold back the chloroplasts which contain up to 80% of the total leaf protein (Kohler et al., 1978).

5. Yield of Extractable Phenolics

A successful method modified from the methods of Swain and Hillis (1959) and Eskin et al. (1978) was used for the extraction of phenol from poplar, alfalfa, rapeseed and tobacco. The basic procedure for this method involved extraction by refluxing the leaf material twice with 80% ethanol at pH of 4.0. In 1977 Lahiry concluded that LPC has been shown to have chlorogenic acid bound to its protein molecules. Chlorogenic acid was therefore used as a phenolic standard in the present assays.

In order to study the effect of freezing condition on the yield of phenol, the difference on the yield of phenol among poplar ($I_{45/51}$ and DN113), alfalfa, tobacco and rapeseed was observed in the fresh and frozen leaves. Also, it was very important to observe the effect of seasonal variation on the yield of phenol in order to find the times of highest and lowest concentrations of phenol.

The results in Figure 17 show that the yield of extractable phenol from poplar leaves basis fresh weight (in H3 $I_{45/51}$ gave 46 mg/g and DN113 gave 47 mg/g) was much higher than that of alfalfa (11.0 mg/g) or rapeseed (4.5 mg/g) and higher than that of tobacco (21.5 mg/g). The yield of phenol recovered from poplar leaves basis dry weight (in H3 $I_{45/51}$ = 177 and DN113 188 mg/g) was higher than that of alfalfa (55 mg/g) and rapeseed (41 mg/g), but it was lower

than that of tobacco (215 mg/g), Figure 18. Poplar, being a woody plant, would be expected to be richer in phenolic materials. However, tobacco may have been selected as a smoking substance because of its high dry weight proportion of phenolics.

The amount of phenol extracted in the second growing season ($I_{45/51}$ = 30 mg/g fresh weight and DN113 = 29 mg/g) was lower than that of the first growing season.

In the study of the effect of freezing of leaves on the yield of phenol, it was observed that, freezing of leaves decreases the yield of phenol. The decrease in the yield of phenol recovered from poplar leaves (in H3 $I_{45/51}$ gave 36 and DN113 gave 36 mg/g) was larger than that of alfalfa (8.4 mg/g), rapeseed (4.0 mg/g) and tobacco (17.0 mg/g). Possibly more phenol is bound to protein making both less extractable.

The Effect of Seasonal Variation on the Yield of Phenol

As shown in Figure 17, the yield values recovered from both poplar clones $I_{45/51}$ and DN113, alfalfa, rapeseed and tobacco, increased slightly from July to mid August and then decreased to the end of September. As far as is known, little work has been done on the extraction and determination of phenols from poplar leaves.

C. Conclusions

1. The correct conditions have been developed for isolating poplar proteins with minimal interference from phenolic compounds. Sulfite (1-5%) and polyvinylpyrrolidone (0.2%) in the extraction medium or added to the press juice allow the optimal recovery of crude protein concentrates with minimal coloring. Then we can produce a pure white protein isolate for use not only in animal feeding but as a component of the human diet.

2. Seasonal variation, extraction techniques, leaf materials and species variation have significant influence on both yield of extractable protein and the ratio of the extractable protein to the total protein.

3. Plant leaves such as those of alfalfa, tobacco, rapeseed and poplar leaves contain from 75 to 90% water and from 10 to 25% solid matter.

4. Poplar leaves are drier than other leaves which may make the extraction of poplar leaf protein and phenol more difficult than from other species with leaves of higher moisture content. On the average, poplar leaves are 12.3% drier than the other leaves.

5. The greatest percentage of protein is extractable in mid August.

6. Freezing of leaves significantly decreases the yield of both extractable protein (by average of 32% in case of poplar $I_{45/51}$) and phenol (by average of 18.5% in case of poplar $I_{45/51}$) most probably due to the interaction between the phenol and protein caused by the rupture of vacuolar membranes. Use of fresh leaves increase the efficiency of protein extraction.

7. The amounts of protein and phenol recovered from poplar leaves in the second growing season were slightly lower than those recovered in the first growing season. This is probably due to the use of different equipment in the process of extraction and determination.

8. Field studies were carried out to try to compare poplar protein with leaf proteins from other plants. Some of them already have been shown to yield commercial protein products (e.g. alfalfa leaf protein concentrate). The results show that protein recovered from poplar leaf (in H3 $I_{45/51}$, clone gave 15.4 mg/g basis fresh weight) was slightly lower than that of alfalfa (it gave 16.1 mg/g) and higher than that of tobacco (it gave 12.3 mg/g).

9. The present study is the first comprehensive field study to detail the protein and phenol contents in poplar leaves in comparison to those of other leaf protein species grown in the same field site.

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APPENDIX I

THE AMINO ACID COMPOSITION OF PROTEIN RECOVERED FROM HYBRID POPLAR LEAVES (CLONE $I_{45/51}$)

1. Introduction

Except for methionine, the amount of amino acids in leaf protein either meet or exceed the standards of the provisional pattern of essential amino acids deemed optimal for human nutrition by the UN Food and Agricultural Organization (Wildman, 1977).

In 1979 Chen reported that the amino acid composition of poplar leaf protein concentrate (PLPC) was similar to those found in other leaf protein concentrates and lower in methionine, lysine and isoleusine than those of animal proteins. He also found that the essential amino acid content of poplar leaf protein concentrate is higher than the minimum values recommended by the FAO for human nutrition (Chen, 1979). Chen used different techniques in the coagulation process of poplar leaf protein concentrate, acid, organic solvent and heat precipitation techniques. However, in the present study poplar leaf protein was recovered in an aqueous extract. To

compare the quality of this crude poplar protein, the amino acid composition was determined.

2. Experimental Procedure

The amino acid composition of poplar leaf protein was determined on the Durrum automatic amino acid analyzer according to the method of Spackman, Stein and Moore (Spackman et al., 1958). Freeze-dried poplar leaf protein (1 mg) was dissolved in distilled water (100 ml). One hundred microlitre samples were hydrolyzed with 100 µl of concentrated HCl (Baker Analyzed) in evacuated sealed tubes at 104-108 °C. Duplicate samples were hydrolyzed for 24 hours. After hydrolysis the tubes were cooled, opened, and dried over NaOH pellets and P_2O_5 in a desiccator with oil pump vacuum. The dried hydrolyzates were dissolved in (200 µl) sodium citrate buffer, pH 2.2, according to standard procedure. Twenty microlitre aliquots of each hydrolyzate were analyzed on a Durrum amino acid analyzer. The individual amino acids were expressed as grams per 100 grams recovered amino acids.

3. Results

Poplar leaves were homogenized in the necessary buffer, then the homogenate was filtered and centrifuged. Finally the supernatant freeze-dried to produce powder of poplar leaf protein for use in amino acid analysis. During the

acid hydrolysis, cystine and tryptophan were destroyed and were omitted from the calculations. Also, some of the methionine was converted to methionine sulfoxide and some was destroyed. Because of analysis costs other preparation methods were not used to obtain the values of those amino acids (cystine, tryptophan and methionine).

The results of the amino acid analyses of poplar leaf protein are given in Table XXIV. Two samples of protein were hydrolyzed with 6NHCl for 24 hr.

Table XXIV

Amino Acid Analyses of Poplar Leaf Protein

(g amino acid/100 g recovered amino acids)

Amino Acid	Average of Two Samples	FAO[a] (e.a.a)	PLPC[b]
Aspartic acid	12.4		8.96-12.92
Threonine	6.2	2.8	4.44-5.14
Serine	9.0		4.4 -5.72
Glutamic acid	12.4		11.08-30.76
Proline	5.1		4.29-6.11
Glycine	10.9		4.49-7.10
Alanine	9.4		4.08-7.20
Valine	4.9	4.2	5.7 -7.08
Methionine	.4	2.2	1.21-2.10
Isoleucine	4.3	4.2	4.09-5.17
Leucine	7.8	4.8	7.48-10.06
Tyrosine	3.0	2.8	3.32-4.51
Phenylalanine	3.6	2.8	5.73-6.67
Histidine	1.4		1.24-3.53
Lysine	6.0	4.2	4.17-6.84
Arginine	3.6		4.38-11.85
Tryptophan	——	1.4	1.17-2.65
Total of Essential Amino Acids	37.5	29.4	37.30-50.27

a Food and Agricultural Organization (The provisional pattern of essential amino acids)

b Poplar Leaf Protein Concentrate (Chen, 1979)

4. Discussion and Conclusion

Table XXIV shows that the amino acid composition of leaf protein extracted by our simple and rapid aqueous extraction method was approximately the same as that reported in 1979 by Chen. The amounts of serine, glycine and alanine were higher than Chen's results. Also, the amount of methionine was lower, probably some of it was converted to methionine sulfoxide or destroyed during the acid hydrolysis. In addition, the same Table shows that the amounts of the essential amino acids found in poplar leaf protein (except methionine) were higher than the minimum values recommended by the FAO. These results are in agreement with Chen's results concerning essential amino acids.