Anti-nutritional Factors in Field Pea (*Pisum sativum* L.) and Grass Pea (*Lathyrus sativus* L.)

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

by

Xiaofang Wang

Faculty of Pharmacy

University of Manitoba

Winnipeg, Manitoba

October, 1996

The author claims copyright. Use shall not be made of the material contained herein without proper acknowledgement, as indicated on the following page.

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file *Votre référence*

Our file *Notre référence*

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-16363-6

Canada

Name XIAOFANG WANG

Dissertation Abstracts International and *Masters Abstracts International* are arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation or thesis. Enter the corresponding four-digit code in the spaces provided.

SUBJECT TERM

Food Science and Technology

SUBJECT CODE 0359

UMI

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
- Architecture 0729
- Art History 0377
- Cinema 0900
- Dance 0378
- Design and Decorative Arts 0389
- Fine Arts 0357
- Information Science 0723
- Journalism 0391
- Landscape Architecture 0390
- Library Science 0399
- Mass Communications 0708
- Music 0413
- Speech Communication 0459
- Theater 0465

EDUCATION
- General 0515
- Administration 0514
- Adult and Continuing 0516
- Agricultural 0517
- Art 0273
- Bilingual and Multicultural 0282
- Business 0688
- Community College 0275
- Curriculum and Instruction 0727
- Early Childhood 0518
- Elementary 0524
- Educational Psychology 0525
- Finance 0277
- Guidance and Counseling 0519
- Health 0680
- Higher 0745
- History of 0520
- Home Economics 0278
- Industrial 0521
- Language and Literature 0279
- Mathematics 0280
- Music 0522
- Philosophy of 0998
- Physical 0523
- Reading 0535
- Religious 0527
- Sciences 0714
- Secondary 0533
- Social Sciences 0534
- Sociology of 0340
- Special 0529
- Teacher Training 0530
- Technology 0710
- Tests and Measurements 0288
- Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
- Language
  - General 0679
  - Ancient 0289
  - Linguistics 0290
  - Modern 0291
  - Rhetoric and Composition 0681
- Literature
  - General 0401
  - Classical 0294
  - Comparative 0295
  - Medieval 0297
  - Modern 0298
  - African 0316
  - American 0591
  - Asian 0305
  - Canadian (English) 0352
  - Canadian (French) 0355
  - Caribbean 0360
  - English 0593
  - Germanic 0311
  - Latin American 0312
  - Middle Eastern 0315
  - Romance 0313
  - Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
- Philosophy 0422
- Religion
  - General 0318
  - Biblical Studies 0321
  - Clergy 0319
  - History of 0320
  - Philosophy of 0322
- Theology 0469

SOCIAL SCIENCES
- American Studies 0323
- Anthropology
  - Archaeology 0324
  - Cultural 0326
  - Physical 0327
- Business Administration
  - General 0310
  - Accounting 0272
  - Banking 0770
  - Management 0454
  - Marketing 0338
- Canadian Studies 0385
- Economics
  - General 0501
  - Agricultural 0503
  - Commerce-Business 0505
  - Finance 0508
  - History 0509
  - Labor 0510
  - Theory 0511
- Folklore 0358
- Geography 0366
- Gerontology 0351
- History
  - General 0578
  - Ancient 0579
  - Medieval 0581
  - Modern 0582
  - Church 0330
  - Black 0328
  - African 0331
  - Asia, Australia and Oceania 0332
  - Canadian 0334
  - European 0335
  - Latin American 0336
  - Middle Eastern 0333
  - United States 0337
- History of Science 0585
- Law 0398
- Political Science
  - General 0615
  - International Law and Relations 0616
  - Public Administration 0617
- Recreation 0814
- Social Work 0452
- Sociology
  - General 0626
  - Criminology and Penology 0627
  - Demography 0938
  - Ethnic and Racial Studies 0631
  - Individual and Family Studies 0628
  - Industrial and Labor Relations 0629
  - Public and Social Welfare 0630
  - Social Structure and Development 0700
  - Theory and Methods 0344
- Transportation 0709
- Urban and Regional Planning 0999
- Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
- Agriculture
  - General 0473
  - Agronomy 0285
  - Animal Culture and Nutrition 0475
  - Animal Pathology 0476
  - Fisheries and Aquaculture 0792
  - Food Science and Technology 0359
  - Forestry and Wildlife 0478
  - Plant Culture 0479
  - Plant Pathology 0480
  - Range Management 0777
  - Soil Science 0481
  - Wood Technology 0746
- Biology
  - General 0306
  - Anatomy 0287
  - Animal Physiology 0433
  - Biostatistics 0308
  - Botany 0309
  - Cell 0379
  - Ecology 0329
  - Entomology 0353
  - Genetics 0369
  - Limnology 0793
  - Microbiology 0410
  - Molecular 0307
  - Neuroscience 0317
  - Oceanography 0416
  - Plant Physiology 0817
  - Veterinary Science 0778
  - Zoology 0472
- Biophysics
  - General 0786
  - Medical 0760

EARTH SCIENCES
- Biogeochemistry 0425
- Geochemistry 0996
- Geodesy 0370
- Geology 0372
- Geophysics 0373
- Hydrology 0388
- Mineralogy 0411
- Paleobotany 0345
- Paleoecology 0426
- Paleontology 0418
- Paleozoology 0985
- Palynology 0427
- Physical Geography 0368
- Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
- Environmental Sciences 0768
- Health Sciences
  - General 0566
  - Audiology 0300
  - Dentistry 0567
  - Education 0350
  - Administration, Health Care 0769
  - Human Development 0758
  - Immunology 0982
  - Medicine and Surgery 0564
  - Mental Health 0347
  - Nursing 0569
  - Nutrition 0570
  - Obstetrics and Gynecology 0380
  - Occupational Health and Safety 0354
  - Oncology 0992
  - Ophthalmology 0381
  - Pathology 0571
  - Pharmacology 0419
  - Pharmacy 0572
  - Public Health 0573
  - Radiology 0574
  - Recreation 0575
  - Rehabilitaion and Therapy 0382
  - Speech Pathology 0460
  - Toxicology 0383
- Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences
- Chemistry
  - General 0485
  - Agricultural 0749
  - Analytical 0486
  - Biochemistry 0487
  - Inorganic 0488
  - Nuclear 0738
  - Organic 0490
  - Pharmaceutical 0491
  - Physical 0494
  - Polymer 0495
  - Radiation 0754
- Mathematics 0405
- Physics
  - General 0605
  - Acoustics 0986
  - Astronomy and Astrophysics 0606
  - Atmospheric Science 0608
  - Atomic 0748
  - Condensed Matter 0611
  - Electricity and Magnetism 0607
  - Elementary Particles and High Energy 0798
  - Fluid and Plasma 0759
  - Molecular 0609
  - Nuclear 0610
  - Optics 0752
  - Radiation 0756
- Statistics 0463

Applied Sciences
- Applied Mechanics 0346
- Computer Science 0984
- Engineering
  - General 0537
  - Aerospace 0538
  - Agricultural 0539
  - Automotive 0540
  - Biomedical 0541
  - Chemical 0542
  - Civil 0543
  - Electronics and Electrical 0544
  - Environmental 0775
  - Industrial 0546
  - Marine and Ocean 0547
  - Materials Science 0794
  - Mechanical 0548
  - Metallurgy 0743
  - Mining 0551
  - Nuclear 0552
  - Packaging 0549
  - Petroleum 0765
  - Sanitary and Municipal 0554
  - System Science 0790
- Geotechnology 0428
- Operations Research 0796
- Plastics Technology 0795
- Textile Technology 0994

PSYCHOLOGY
- General 0621
- Behavioral 0384
- Clinical 0622
- Cognitive 0633
- Developmental 0620
- Experimental 0623
- Industrial 0624
- Personality 0625
- Physiological 0989
- Psychobiology 0349
- Psychometrics 0632
- Social 0451

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

COPYRIGHT PERMISSION

ANTI-NUTRITIONAL FACTORS IN FIELD PEA (*Pisum sativum* L.) AND GRASS PEA (*Lathyrus sativus* L.)

BY

XIAOFANG WANG

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Xiaofang Wang © 1996

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this thesis/practicum, to the NATIONAL LIBRARY OF CANADA to microfilm this thesis/practicum and to lend or sell copies of the film, and to UNIVERSITY MICROFILMS INC. to publish an abstract of this thesis/practicum..

This reproduction or copy of this thesis has been made available by authority of the copyright owner solely for the purpose of private study and research, and may only be reproduced and copied as permitted by copyright laws or with express written authorization from the copyright owner.

ABSTRACT

Seed samples of seventeen field pea cultivars grown at five locations, and nine grass pea lines grown at two locations, in western Canada during 1993 and 1994 were analysed for total phenolics, condensed tannins and trypsin inhibitor activity (TIA). Total phenolics in field pea differed significantly among cultivars. The cultivar x year interaction was significant, whereas there were no significant differences between years or locations, and the cultivar x location interaction was not significant for total phenolics in field pea. Total phenolics in field pea ranged from 162 mg kg^{-1} DM (Dry matter) (CE) (Catechin equivalents) for AC Tamor to 325 mg kg^{-1} DM (CE) for Richmond. Field pea had barely detectable levels of condensed tannins which did not differ among cultivars, years, locations, and their interactions. TIA in field pea differed significantly among cultivars. The cultivar x location interaction for TIA in field pea was also significant, however, the effects of year, location, and the cultivar x year interaction for TIA in field pea were not significant. TIA in field pea ranged from 2.22 TIU mg^{-1} (DM) for Danto to 7.66 TIU mg^{-1} (DM) for Baroness. Total phenolics and condensed tannins in grass pea differed significantly among cultivars, whereas no significant differences existed between years, locations, or their interactions. Total phenolics in grass pea ranged from 868 mg kg^{-1} DM (CE) for L880388 to 2059 mg kg^{-1} DM (CE) for LS89110. Condensed tannins in grass pea ranged from 0.89 g kg^{-1} DM (CE) for L880388 to 5.18 g kg^{-1} DM (CE) for LS89125. TIA in grass pea did not differ among cultivars, years, locations, or their interactions. Mean TIA was 27.51 TIU mg^{-1} (DM) for the grass pea lines tested. The

correlations between the levels of total phenolics, condensed tannins and TIA with seed yield were near zero or negative in field peas and grass pea, thus, selection for high yield and low levels of these anti-nutrients will be possible. Levels of total phenolics and condensed tannins were positively correlated in grass pea. Grass pea seeds with darker seed coat colour contained higher levels of condensed tannins.

ACKNOWLEDGEMENTS

Funding was provided by the Manitoba Pulse Growers Association, Agriculture and Agri-Food Canada, and the Natural Sciences and Engineering Research Council of Canada. I acknowledge the guidance received from my supervisor Dr. Colin J. Briggs. Thank you also to the research group members of this project: Thomas D. Warkentin, Clayton G. Campbell and B. Dave Oomah. Thank you to Dean K. Wayne Hindmarsh for the comments and suggestions on this thesis. Technical expertise of E. Loewen and M. Hodgins is gratefully acknowledged. Field pea samples were generously provided by Manitoba Agriculture.

TABLE OF CONTENTS

ABSTRACT.....i

ACKNOWLEDGEMENTS.....iii

TABLE OF CONTENTS.....iv

LIST OF TABLES.....viii

LIST OF FIGURES.....ix

1. INTRODUCTION.....1

2. LITERATURE REVIEW.....4

2.1 Phenolics and condensed tannins.....4

2.1.1 General.....4

2.1.2 Classification and chemistry of phenolic compounds.....5

2.1.2.1 Simple phenolics.....5

2.1.2.2 Flavonoid compounds.....7

2.1.2.2.1 Anthocyanins.....7

2.1.2.2.2 Leucoanthocyanins.....8

2.1.2.2.3 Anthoxanthins.....9

2.1.2.2.4 Catechins.....11

2.1.2.2.5 Tannins.....12

2.1.2.3 Gossypol.....14

2.1.3 Occurrence, nature and composition of tannins.....15

2.1.4 Tannin interactions with proteins.....16

2.1.5 Anti-nutritional effects of tannins.....17

2.1.5.1 Effect on digestive enzymes.....18

2.1.5.2 Effect on digestibility.....19

2.1.5.3 Effect on intake of forage legumes.....20

2.1.5.4 Effect on vitamin and iron availability.....21

2.1.5.5 Effect on animals.....22

2.1.6 Plant protection by tannins.....23

2.1.7 Analytical aspects for total phenolics and condensed tannins.....24

2.1.7.1 Vanillin method.....25

2.1.7.2 Oxidation-reduction methods.....27

2.1.7.2.1 Prussian blue assay.....28

2.1.7.2.2 Folin-Denis method.....29

2.2 Protease inhibitors.....30

2.2.1 General.....30

2.2.2 Protease inhibitor reaction mechanisms.....30

2.2.3 Structure.....31

2.2.4 Properties.....35

2.2.4.1 Specificity.....35

2.2.4.2 Inhibition of human enzymes.....35

2.2.4.3 Stability.....38

2.2.5 Anti-nutritional effects.....38

2.2.5.1 Animals.....38

2.2.5.2 Humans....40
2.2.6 Plant protection by protease inhibitors....44
2.2.7 Protease inhibitor intake....45
2.2.8 Analytical aspects....47
2.2.8.1 Enzymatic methods....49
2.2.8.2 Immunochemical methods....52
2.3 Anti-nutritional factors in field pea and grass pea....53
2.3.1 Haemagglutinins (Lectins)....53
2.3.2 Trypsin inhibitors....54
2.3.3 Chymotrypsin inhibitors....55
2.3.4 Amylase inhibitors....56
2.3.5 Oxalate....56
2.3.6 Phytic acid....56
2.3.7 Tannins and phenolic acids....57
2.3.8 Lipoxygenase....58
2.3.9 Volatiles....58
2.3.10 Saponins....59
2.4 Field pea and grass pea in pig and poultry rations....60
2.5 Objective of this research....63
3. MATERIALS AND METHODS....64
3.1 Chemicals and equipment....64
3.2 Materials and statistical analyses....64

3.3 Moisture content and condensed tannin measurements.....67

3.4 Total phenolics and protein content measurements.....67

3.5 Trypsin inhibitor activity measurement.....69

3.5.1 Enzyme solution.....69

3.5.2 Substrate solution.....69

3.5.3 Extraction procedure.....70

3.5.4 Large scale Kakade assay.....70

3.5.5 Calculations.....71

3.6 Seed coat colour measurement for grass pea.....71

4. RESULTS AND DISCUSSION.....73

4.1 Field pea.....73

4.2 Grass pea.....78

5. CONCLUSION.....84

6. REFERENCES.....86

7. APPENDIX.....108

LIST OF TABLES

1. Protease inhibitor families from plant sources.....32

2. Protease inhibitors of plant origin.....36

3. Stability of inhibitors against pepsin at pH 2.....37

4. Effect of soybean trypsin inhibitors on growth of rats.....39

5. Destruction of trypsin inhibitors by heating.....39

6. Biological effects of inhibitor preparations from legumes in animals.....41

7. Inhibition of human enzymes by pulses.....46

8. Average daily inhibitor intake.....48

9. Chemical sources.....65

10. Equipment sources.....66

11. Total phenolics, condensed tannins and trypsin inhibitor activity (TIA) of field pea grown at five locations in Manitoba in 1993 and 1994.....74

12. Correlation coefficients for total phenolics, condensed tannins, trypsin inhibitor activity (TIA), seed yield, and seed protein content in field pea (n=340).....77

13. Total phenolics, condensed tannins, trypsin inhibitor activity (TIA) in grass pea lines grown at Morden and Portage la Prairie, Manitoba in 1993 and 1994.....79

14. Correlation coefficients for total phenolics, condensed tannins, trypsin inhibitor activity (TIA), seed yield, and seed protein content in grass pea (n=72).....82

15. Correlation coefficients between seed coat colour parameters (Hunterlab L, a, b) and total phenolics, condensed tannins, trypsin inhibitor activity (TIA), seed yield, and seed protein content in grass pea (n=72).....82

LIST OF FIGURES

1. Structures of common phenolic compounds.....6

2. C_6-C_3-C_6 skeleton of flavonoids.....7

3. Parent structure of anthocyanidins.....8

4. Flavan-3,4-diol structure.....9

5. Parent structure of anthoxanthins.....10

6. Various types of flavonoids.....10

7. Catechin structure.....11

8. Tannins and tannin fragments.....13

9. Gossypol structure.....14

10. Condensation reaction of vanillaldehyde with flavans.....26

11. Oxidation of a typical phenol - catechol.....28

12. Comparison of the amino acid sequences of the 'double headed' protease inhibitors from various members of the Leguminosae.....34

13. Scheme to explain the possible deleterious effects of protease inhibitors on the nutritive value of proteins.....42

1. INTRODUCTION

Field pea (*Pisum sativum* L.) is a cheap source of protein and energy and consequently is an important part of human diets and animal feeds in many parts of the world. There are two subspecies of field pea which are grown in Canada: *Pisum sativum* subsp. *hortense* has white flowers and is used mainly for human consumption and feed for monogastric animals, primarily pigs; *Pisum sativum* subsp. *arvense* which has dark colour seed coats has dark-coloured flowers and is primarily used as forage. *Pisum sativum* subsp. *hortense* which has a white-yellow or blue-green cotyledon colour, can be classified into wrinkled and smooth seeded and into spring and winter types. The latter are not grown in western Canada. Grass pea (*Lathyrus sativus* Linn, chickling vetch or khesari), an environment-tolerant pulse with high yield potential and drought tolerance, is an essential food crop for animals and humans in some countries in west Asia and north Africa (Spencer *et al.* 1986). Grass pea could be a significant contributor to sustainable agriculture, as its well nodulated root system fixes nitrogen, reducing the need for application of supplemental fertilizer. It could become a useful rotation crop in the brown soil zone of the Canadian prairies, an area that lacks an adapted annual legume alternative. The first grass pea cultivar in Canada, X850002, was recently released by the Morden Research Centre. Field pea has been grown to a limited extent in western Canada ever since farmers started plowing the prairies in the past century. Until the 1900's, Canadian pea production was concentrated in Ontario. Field pea production has since increased in western Canada, especially since 1985 with the opening of the

European feed pea market. Field pea planting area in western Canada increased from 73,600 ha in 1985 to 800,000 ha in 1995. Field pea is now a major pulse crop in western Canada, which diversifies cropping options for cereal growers. Currently, approximately two-thirds of the field pea production in Canada is exported for animal feed; the largest buyer is the European compound feed industry. In addition, field pea is sold to many countries for human consumption. Efforts are underway in Canada to develop new export markets and to develop the domestic feed market.

Field pea and grass pea contain a number of naturally occurring compounds which interfere with nutrient availability and are thus designated as anti-nutritional factors. These include tannins, alkaloids, saponins, glucosides, trypsin inhibitors (Liener and Kakade 1980), lipoxygenase, and antithyroid substances. In addition, grass pea contains a low-molecular-weight neurotoxin β-N-oxalyl-L-α, β-diaminopropionic acid, ODAP, also known as β-N-oxalyl-amino-L-alanine, BOAA which causes lathyrism in man and animals (Chowdhury 1988). The protein content of field pea and grass pea is high (field pea: 22-25%; grass pea: 20-30%), but their protein quality is not ideal. This has been mainly attributed to two factors. They have a deficiency of sulfur-containing amino acids (Bressani *et al.* 1973, Elias and Bressani 1974) and the presence of various anti-nutritional factors. These factors, such as condensed tannins, form complexes with dietary proteins and make them resistant to the action of digestive enzymes (Griffiths 1981) or inactivate digestive enzymes. Protease inhibitors (trypsin inhibitors and chymotrypsin inhibitors) are present which decrease the digestibility of protein and cause pancreatic hypertrophy.

So far, most work on grass pea has been confined to developing lines with reduced levels of the neurotoxin ODAP (Campbell 1989), and combined with selection for improved yield and early maturity (Ramanujan *et al.* 1980, Chandan *et al.* 1991). In comparison to research on ODAP, little information is available on the presence of condensed tannins, total phenolics, and enzyme inhibitors in grass pea. All of these anti-nutritional factors ultimately lower the feeding quality of animal rations containing grass pea. For field pea, several studies have been reported on the presence of condensed tannins, total phenolics and enzyme inhibitors, but few studies have examined the relative influence of genotype and the environment on these factors.

To assist in proportioning the relative contribution of various anti-nutritional factors to nutrient availability in field pea and grass pea and to facilitate the breeding of cultivars for improved nutritional quality for export and domestic use, it was necessary to survey existing germplasm for the levels of the relevant anti-nutritional compounds. Thus, the objectives of this study were to determine whether genotype and environment have significant influences on total phenolics, condensed tannins and trypsin inhibitors in field pea and grass pea, and to determine whether these compounds are correlated with seed protein and yield; and to determine whether the seed coat colour of grass pea is correlated with total phenolics, condensed tannins, seed protein content or seed yield.

2. LITERATURE REVIEW

Many legumes contain anti-nutritional factors which adversely affect the nutritive value of the seeds. These anti-nutritional factors are a group of unrelated chemical compounds with varying effects on metabolic processes. Anti-nutritional compounds are important in establishing the feeding quality of grain legume seeds. The major anti-nutritional factors in legumes are trypsin and chymotrypsin inhibitors, tannins, lectins, amylase inhibitors, and phenolics (Savage and Deo 1989). Of these anti-nutritional factors, Gatel and Grosjean (1990) suggested that the protease inhibitors and tannins are the most important. Consequently total phenolics, condensed tannins and trypsin inhibitors in field pea and grass pea were studied here.

2.1 Phenolics and condensed tannins

2.1.1 General

Living matter contains a very large number of phenolic and polyphenolic compounds which, although of widespread occurrence and considerable importance, do not account for more than a minute fraction of the organic substance present in plants and animals. Phenolic compounds, a group of secondary metabolites, embrace a large array of chemical compounds that have at least one aromatic ring bearing one or more hydroxyl groups, together with a number of other substituents. In recent years,

polyphenols in many edible plant products have received increased attention as a result of their influence on nutritional and aesthetic quality of foods, biochemical and physiological functions, and pharmacological implications. The changes that occur during post-harvest storage and processing of fresh fruit, produce, cereals, and legumes have been partly associated with reactions of phenolic substances.

Cereals and legumes contain proteins which are nutritionally complementary, occupy an important place in the diets in many countries throughout the world, and can be grown with relative ease. Polyphenols in cereals and legumes have been receiving considerable attention largely because of their adverse influence on colour, flavour, and nutritional quality. The majority of there compounds belong to the flavonoid and tannin groups.

2.1.2 Classification and chemistry of phenolic compounds

2.1.2.1 Simple phenolics

Phenolic substances containing a single benzene ring are simple phenolics which possess a range of substituted benzoic (C_6-C_1) acid derivatives and others derived from cinnamic (C_6-C_3) acid. Both types of phenolic acids usually occur in conjugated and esterified forms widely distributed in foods and living tissue.

The simpler types of benzoic acid derivatives include cinnamic acids (the main constituent of Styrax balsam used in medicine), *o*-coumaric acids, *p*-coumaric acids

(ortho- and para-hydroxy cinnamic acids), caffeic acids and gallic acids (Figure 1). Chlorogenic acid, which commonly occurs in sunflower seeds and coffee beans, is an ester of caffeic and quinic acids and is found in several isomeric and derivatized forms. The coumarins possess the same C_6-C_3 configuration of the cinnamic acids, but the C_3 chain is formed into an oxygen heterocycle. They are less widely distributed in seeds and occur primarily as glucosides. The aromatic amino acids, phenylalanine and tyrosine (C_6-C_3 pattern), occur in plants and animals where they are involved in the biosynthesis of a number of other phenolic compounds.

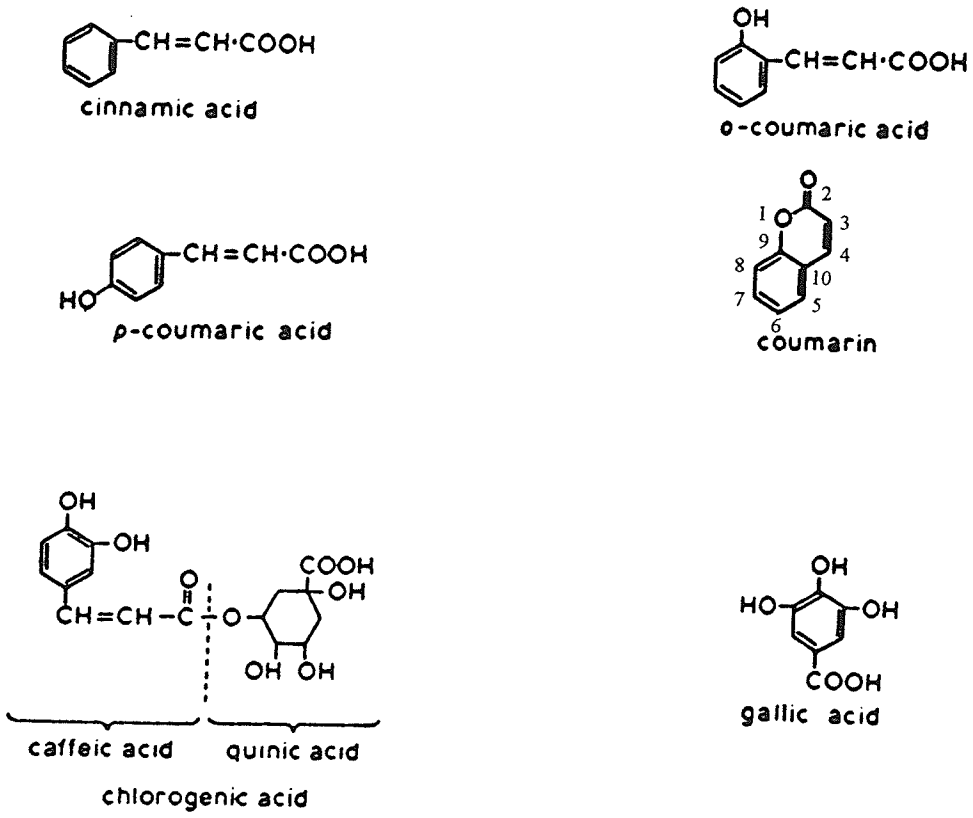


Figure 1. Structures of common phenolic compounds.

2.1.2.2 Flavonoid compounds

This large and important group encompasses the anthocyanins, anthoxanthins, leuco-anthocyanins and catechins. They all possess a basic structure consisting of a C_6-C_3-C_6 skeleton (Figure 2). Flavonoid compounds include by far the largest and most diverse range of plant phenolics. Most flavonoids occur as glycosides in which the C_6-C_3-C_6 aglycone part of the molecule is esterified with a number of different sugars. The flavanols are unique in that they do not occur as glycosides, but show reactivity through polymerization into "condensed tannins" (Figure 8).

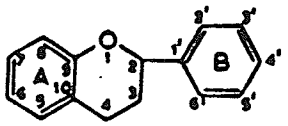


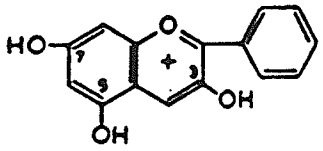
Figure 2. C_6-C_3-C_6 skeleton of flavonoids.

2.1.2.2.1 Anthocyanins

This is a very large group of plant phenolics which are the water soluble red, blue, purple pigments of flowers, fruits, fruit juices, wines and vegetables. The anthocyanin pigments change their colour with a change in pH. Anthocyanins are glycosides and

when hydrolysed, they yield a sugar and an aglycone, called anthocyanidine. The carbohydrate residues most frequently encountered are glucose, rhamnose, galactose and gentiobiose. The anthocyanidins have a common basic structure consisting of a benzopyrylium nucleus and a phenol ring, the two together being called flavylium (Figure 3).

Because of the trivalent position of the oxygen, flavylium is a cation. The position at carbon atoms 3, 5, and 7 are hydroxyl substituted, the sugar moiety being usually attached to the hydroxyl at position 3. From this parent structural formula, anthocyanidins are derived by diverse substitution in the phenol ring B.



flavylium ion

Figure 3. Parent structure of anthocyanidins.

2.1.2.2.2 Leucoanthocyanins

The name of this group of plant phenolics is due to the fact that they are colourless but produce anthocyanidins when treated with boiling hydrochloric acid. The basis of leucoanthocyanidins is flavan 3,4-diol. Leucoanthocyanidins (Figure 4), their

glycosides (leucoanthocyanins) and polymeric derivatives, are widely distributed in woody plants.

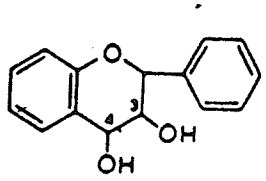


Figure 4. Flavan-3,4-diol structure.

2.1.2.2.3 Anthoxanthins

Anthoxanthins differ from anthocyanins and leucoanthocyanins in the degree of oxidation of the aliphatic fragment in the C_6-C_3-C_6 skeleton. The aglycones of the anthoxanthins consist of a benzopyrone nucleus (Figure 5).

The various types of flavonoids (R indicates the position of the sugar residue) are presented in Figure 6. The sugar moiety attached at position 7 of the A ring is the most frequently encountered position of the glycosidic bond. However, 3, 5 and 4'-glycosides are also found often.

In contrast to anthocyanins, most of the flavonoids are colourless or only very slightly coloured; however, their chalcones are yellow to strongly brown in colour. Many anthoxanthin glycosides are bitter.

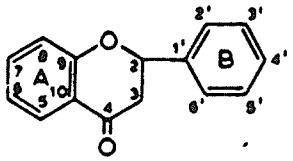


Figure 5. Parent structure of anthoxanthins.

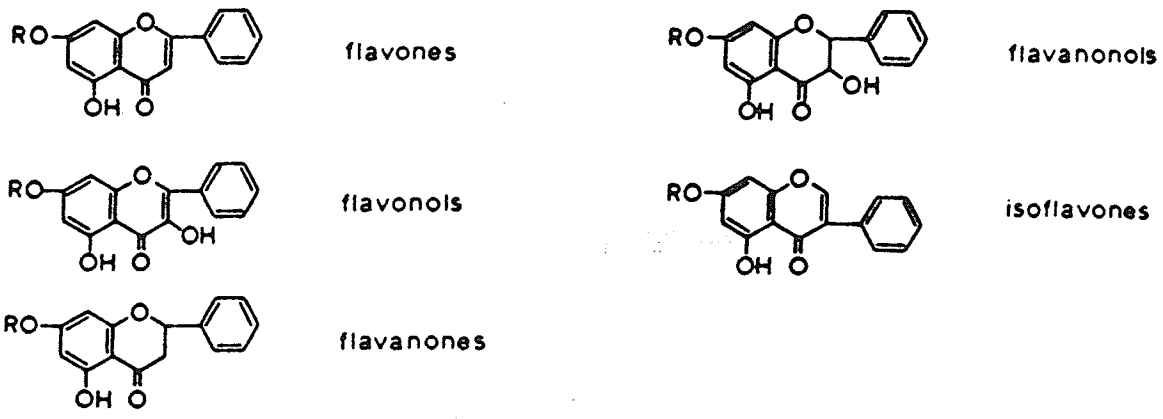


Figure 6. Various types of flavonoids.

2.1.2.2.4 Catechins

This class of flavonoids is characterized by a single hydroxyl group on the C_3 portion, at the 3-position. Catechins are therefore polyhydroxyflavan-3-ols (Figure 7). Catechin occurs in nature in two stereoisomeric forms (+) - catechin and (-) - epicatechin. The two stereoisomers differ in the relative direction of H and OH about the carbon at the 3-position.

Catechins take part in the enzymic browning process of many foods. Together with the flavan-3,4-diols (leucoanthocyanidins) they constitute the building blocks of " condensed tannins".

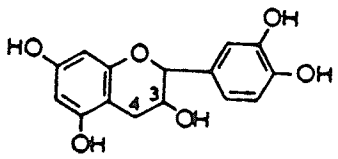
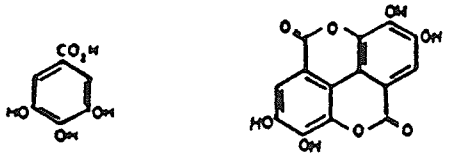


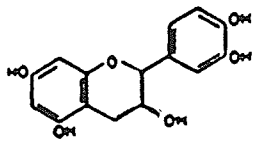
Figure 7. Catechin structure.

2.1.2.2.5 Tannins

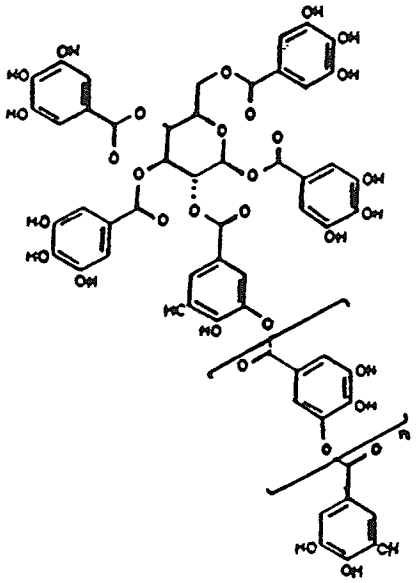
Polymeric phenols can be further distinguished into two broad groups: tannins and lignin, three-dimensional polymers of phenyl-propanoid C_6-C_3 units which encrust and penetrate the cellulose cell walls of higher plants. Tannins are a heterogenous group of complex polyphenolics found widely in the plant kingdom (Hagerman and Butler 1978). Tannins are water-soluble polymeric phenolics of relatively high molecular weight (mol. wt. 500 to 5000) which precipitate proteins (Haslam 1989). Tannins containing sufficient phenolic hydroxyl groups form stable cross-linkages between molecules of collagen (Swain 1965), especially in the amorphous regions of this protein. These linkages are believed to involve the hydroxyl groups of the polyphenol and the peptide bond of the protein. Hydrophobic binding may be an important contribution to the stability of the complex. Tannins have been classified into two groups based on structural types: the hydrolysable tannins and the condensed tannins (Figure 8). Hydrolysable tannins contain a central core of glucose or other polyhydric alcohol esterified with gallic acid (gallotannins), or ellagic acid (ellagitannins). Part or all of the five hydroxyl groups of the sugar may be esterified. These types of tannins are readily hydrolysed by acids, bases, or certain enzymes. Condensed tannins are formed by the polymerization of the flavan-3-ols (catechins) or flavan-3,4-diols (leucoanthocyanidins) linked through an interflavan carbon bond that is not susceptible to hydrolysis. Condensed tannins are the most common type of tannin found in forage legumes.



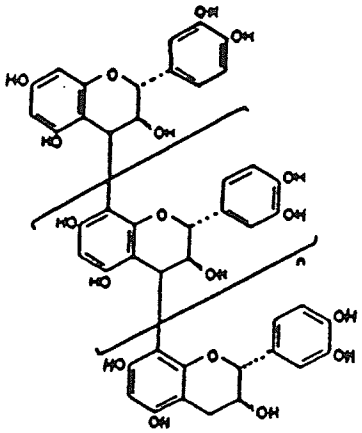
Gallic Acid Ellagic Acid



Flavan-3-ol



Hydrolysable Tannin



Condensed Tannin

Figure 8. Tannins and tannin fragments.

2.1.2.3 Gossypol

Gossypol is the characteristic pigment of cottonseed. Gossypol has a specific polyphenolic structure, not resembling any of the other groups of phenolic substances. It has four benzene rings fused in two symmetric naphthalene groups, each half carrying one aldehyde, three hydroxyl, one methyl and one isopropyl radicals (Figure 9).

Gossypol is mainly found in the cotyledons of cottonseed, concentrated in small bodies called "pigment glands" (Adams *et al.* 1960). It is intensely coloured, dark brownish red. Gossypol is a anti-nutritional substance which may cause inflammation of tissues, haemorrhage and nervous disorders (Aletor submitted). These properties of gossypol exclude the use of untreated cottonseed meal as a food for monogastric animals.

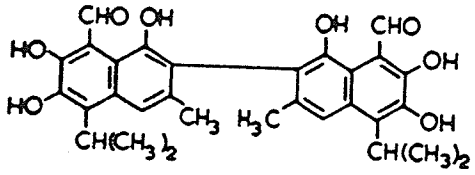


Figure 9. Gossypol structure.

2.1.3 Occurrence, nature and composition of tannins

Tannins (condensed and hydrolysable tannins) are widely distributed in the plant kingdom. Their presence in a variety of plants utilized as food and feed includes: fruits, barley, millet, sorghum, field pea, cowpea, pigeon pea, grass pea, dry bean, horse bean, faba bean, and forage legumes.

Several types of anthocyanidin generating compounds have been identified in different varieties of sorghum. Bate-Smith and Rasper (1969) reported that the principal tannin of sorghum is a leucoanthocyanidin. Strumeyer and Malin (1975) successfully isolated and characterized tannins present in grain sorghum as condensed tannins.

Tannins in sorghum grains are located in the seed coat, associated with the brown colour of the grains or dark seed coat (Maxson *et al.* 1973). Maxson *et al.* (1972) reported that tannin content was higher in grains with red pericarp than grains with white pericarp. They found that the total tannin content of grain sorghum is influenced by pericarp colour, presence or absence of a pigmented testa, the extent to which the pigmented testa is developed, and plant colour. Tannins in common bean (*Phaseolus vulgaris* L.) are located in the seed coat of the grain with low or negligible amounts in the cotyledons (Bressani and Elias 1979). They observed lower amounts of polyphenols in white bean than in black, red, and bronze varieties. Ma Yu and Bliss (1978) reported that white-seeded strains in common bean contained no detectable amounts of tannins. However, coloured seeds contained appreciable quantities. Tannins were mostly associated with testa layer, and black seeds were usually higher in tannin content than

other coloured seeds. Although black seeds contained high tannin concentrations, a few plants with non-black but coloured seeds also had high tannin content.

Variation in tannin content of grain sorghum from 2.7 to 10.2 percent catechin equivalents (CE) was reported (Harris and Burns 1970, Jambunathan and Mertz 1973). Khan *et al.* (1979) reported that chick pea (*Cicer arietinum* L.) contained 0.51 to 0.85% (CE) tannins in the seeds, mung bean (*Phaseolus aureus* L.) 1.32 to 1.55% (CE), cow pea (*Vigna sinensis* L.) 0.46 to 0.76% (CE). Pigeon pea (*Cajanus cajan* L.) contained 0.0 to 0.08% (CE) tannins in the seeds (Price *et al.* 1980) .

2.1.4 Tannin interactions with proteins

Tannins are known to interact with proteins to form tannin-protein complexes. Formation of such complexes leads to either inactivation of enzymes or makes the proteins insoluble. Tannins have a large number of free phenolic hydroxyl groups that form strong hydrogen bonds with proteins and carbohydrates (Haslam 1989). Tannins may also complex with proteins through hydrophobic bonding (Oh *et al.* 1980). In addition, tannins form covalent bonds with proteins through oxidative polymerization reactions as a result of heating, exposure to UV radiation, and the action of polyphenol oxidase. The ability of polyphenols to form complexes with casein as well as with the enzyme proteins depends upon the number of hydroxyl groups of the polyphenols and increases with the degree of polymerization (Endres and Hormann 1963). Zitko and Rosik (1962) reported that one tannin molecule binds two or more peptide groups with

the possible formation of cross-linkages between the protein chains. The degree of cross-linkage depends upon the number and accessibility of the peptide carbonyl groups per protein molecule as well as on the relative concentration of the reactants. The binding between tannins and proteins consists of hydrogen bond formation between the phenolic hydroxyl groups of the tannins and the carbonyl groups of the peptide bonds of enzyme proteins (Cannon 1955). The strength of these complexes depends on the characteristics of both the tannin and the protein (molecular weight, tertiary structure, isoelectric point, and compatibility of binding sites). Calderon *et al.* (1968) have shown that polyphenols form complexes with proteins producing turbidity and under appropriate conditions of the medium, coagulated precipitate. The degree of the tannin-protein complex formation depends on the ratio of protein to tannin concentration and the time of contact between the reactants.

Inhibition of different enzymes by tannins has been reported, and may be due to the protein binding nature of the tannins. Tamir and Alumot (1969) demonstrated that tannins extracted from carob bean inhibited digestive enzymes, including trypsin, amylase and lipase.

2.1.5 Anti-nutritional effects of tannins

Tannins have been reported to occur in appreciable amounts in some legume grains that are important as human food or animal feed. Tannins present in legume seeds are implicated in decreasing the activities of digestive enzymes, limiting energy release,

reducing protein and amino acid availability, inhibiting vitamin and mineral uptake, and other anti-nutritional effects.

2.1.5.1 Effect on digestive enzymes

Tannins isolated from green carob bean significantly inhibited the activities of digestive enzymes like trypsin, lipase, and amylase (Tamir and Alumot 1969). Nitsan (1971) reported that *Vicia faba* tannins lowered enzymatic activities, particularly reducing amylase levels in the pancreas and intestine. Griffiths and Jones (1977) observed that the presence of high levels of tannin in faba bean reduced *in vitro* digestibility and solubility of cotyledon proteins. Griffiths and Moseley (1980) found that the activities of both trypsin and amylase were significantly reduced in rats consuming diets containing testa from high tannin/coloured flower varieties of field bean over those of rats receiving diets containing testa from a tannin-free white flowered variety.

Tannins are known to form complexes with proteins through the formation of multiple hydrogen bonds between the phenolic hydroxyl groups of tannins and the carbonyl function of the peptide linkages of proteins (Gustavson 1954) and /or through hydrophobic bonding (Oh *et al.* 1980), causing precipitation. The size of the condensed tannin molecule has been shown to influence protein binding activity (Goldstein and Swain 1963). Thus, tannins can form complexes with digestive enzymes and affect the digestion and availability of dietary proteins.

2.1.5.2 Effect on digestibility

Tannins are well documented to depress the growth rate of rats and chicks, as well as protein and dry matter digestibilities. Ringrose and Morgan (1940) reported that tannic acid fed as 2% of the diet resulted in reduced growth of chicks. Fuller *et al.* (1966) reported that dietary levels of tannin, between 0.64 and 0.84% (from grain sorghum), are required to show a depressing effect on the growth rate of chicks.

Joslyn and Glick (1969) reported that tannic acid fed to rats at 5% of the diet reduced weight gains to approximately 50% of the control. Lease and Mitchell (1940) found that rats could tolerate up to 5% tannic acid mixed in a good ration, but higher levels caused marked growth depression and lower hemoglobin counts. Jambunathan and Mertz (1973) fed rats with diets containing equal amounts of proteins, oil, minerals, and fibre, but differing in tannin content. The high tannin diet resulted in a lower weight gain and reduced protein efficiency ratios when compared to the low tannin diets.

Tannin and tannic acid have been shown to reduce the digestibility of sorghum and other plant materials. Vohra *et al.* (1966) indicated that dietary tannic acid resulted in reduced nitrogen retention in chicks. Increased quantities of insoluble nitrogen in the digestive systems of rats fed with tannins have been reported by Tamir and Alumot (1970). They concluded that the increased insoluble nitrogen was due to nonspecific binding of proteins to tannins.

The digestibility of soybean proteins was significantly decreased when rats were fed soybean diets containing 1.5% tannins (Eggum and Christensen 1975). In addition, a

significant decrease in the availability of several amino acids occurred when from sorghum diets were fed with tannins to rats. Chicks fed a diet containing 3.0% purified condensed tannins as compared with those fed a controlled diet had markedly reduced feed intake, negative weight gains, and crude fibre retention (Marquardt *et al.* 1977). The consumption of tannin-rich faba beans in a longer trial was found to decrease the efficiency of food utilization and tended to increase mortality (Guillaume and Bellec 1977). Bressani and Elias (1979) observed that white-coated bean cultivars had the highest digestibility and the lowest tannic acid content. Red-coloured beans showed the lowest protein digestibility with the highest tannic acid content. It was suggested that coloured beans had a lower protein quality value than white beans. Tannins affect the utilization of proteins in beans through increasing faecal nitrogen output. It was suggested that polyphenol compounds in common beans decrease protein digestibility either by inhibiting digestive enzymes or by reacting with protein, reducing amino acid availability, or both. The decrease in protein digestibility in turn reduces absorption of amino acids.

2.1.5.3 Effect on intake of forage legumes

Tannins may reduce intake of forage legumes by decreasing palatability or by negatively affecting digestion. Astringency is the sensation caused by the formation of complexes between tannins and salivary glycoproteins (Aletor submitted). Astringency may increase salivation and decrease palatability. Some phenolic compounds such as

anthoxanthins are bitter.

2.1.5.4 Effect on vitamin and iron availability

Tannins can affect the utilisation of vitamins and minerals. Suschetet (1975) studied the influence of tannic acid on vitamin A content in the liver of rats. He found that there was a reduction in liver vitamin A as a result of the inclusion of 3.2% tannic acid in the diet. It was suggested that absorption of vitamin A was impaired in the presence of tannic acid. Ascorbic acid reaction is completely inhibited if tannic acid present at the beginning, and could partially reverse the reaction if added during the first 30 min (Rungruangsak *et al.* 1977). Vitamin B_{12} utilisation is also reduced by tannic acid. Carrera *et al.* (1973) found that the coefficient of digestive utilization of vitamin B_{12} was 42.0 in the control and 22.9 and 22.7 with two doses of tannic acid when tannic acid was given at the rate of 250 to 500 mg/kg body weight.

Tannins are known to form insoluble complexes with divalent metal ions rendering them less absorbable (Srikantia 1976). Studies on iron availability from sorghum with different tannin contents showed that bioavailability of iron from a variety of sorghum which had approximately 130 mg of tannin per 100 g of grain was much less than that from a variety of sorghum which contained only 20 mg of tannin.

2.1.5.5 Effect on animals

Tannins are well documented as anti-nutritional factors in cereals and legume seeds. These polyphenolic compounds exert severe negative effects on protein, fat, and carbohydrate utilization of food. There is general agreement that tannins in these grains exert anti-nutritional effects.

Ringrose and Morgan (1940) attributed reduced growth of chicks to the appetite-depressing effect of tannins in the diet. Lease and Mitchell (1940) noticed a marked decrease in blood hemoglobin of rats fed tannic acid. Chang and Fuller (1964) observed the production of fatty livers upon feeding tannic acid to chicks. Glick and Joslyn (1970) reported that weanling rats fed 8% tannic acid in the diet died within 4 to 6 days and such deaths were mainly due to severe depression in food intake. These observations suggested that the anti-nutritional effect of tannic acid, other than that of feed intake, is associated with low nitrogen retention. The reduced nitrogen retention can be improved by increasing the proportion of proteins in the diet.

Tannic acid and tannins are reported to cause ultrastructural changes in the liver of rabbits (Arhelger *et al.* 1965). Kapadia *et al.* (1978) have observed the production of local tumours in rats upon subcutaneous administration of tannins. The minimum amount of dietary tannin needed to elicit a negative growth response has not been established.

2.1.6 Plant protection by tannins

The production and storage of legume grains have suffered significantly due to attack by birds, microorganisms, and preharvest seed germination. High tannin content is the characteristic most often associated with bird resistance in sorghum (McMillian *et al.* 1972). Prine *et al.* (1967) observed significantly reduced damage by birds in high tannin cultivars during the milk stage when birds are most attracted to sorghum. Bird resistance in grain sorghum can be attributed to the tactile astringent response (Bullard and Elias 1979).

Scalbert (1991) identified three mechanisms of tannin negative effects in microorganisms: enzyme inhibition and substrate deprivation, action on membranes, and metal ion deprivation. Enzyme inhibition and substrate deprivation are characteristic of tannin/protein interactions. Tannins induce changes in morphology of several species of ruminal bacteria (Jones *et al.* 1994). Some possible mechanisms of microbial defence of tannins are secretion of tannin-binding polymers, synthesis of tannin-resistant enzymes, and the biodegradation of tannins (Scalbert 1991).

Brown-seeded sorghum hybrids have shown a marked degree of resistance to preharvest seed germination (Harris and Burns 1970). It is well known that brown- and reddish brown-coloured sorghum grains contain higher amounts of tannins (Rooney *et al.* 1979). Tannins were responsible for retarding seedling growth by decreasing the rate of starch and protein degradation in germinating high tannin seeds (Chavan *et al.* 1981). Thus, tannins in high concentrations during the milk stage of grain development deter

bird attack, while tannins in matured grains are responsible for decreased preharvest seed germination in sorghum.

2.1.7 Analytical aspects for measurement of total phenolics and condensed tannins

Methods for the determination of phenolic compounds in plant products are mostly based on the polarity of the phenolic hydroxyl groups. They can be classified as either those which measure all phenols as a group or those which assay specific individual substances or classes of phenols.

The most common methods for determination of total phenolics and condensed tannins of legumes and cereals include the vanillin test (Burns 1963), the Prussian blue test (Price and Butler 1977), and the Folin-Denis test (Burns 1963). The Prussian blue and Folin-Denis procedures are based on the reducing power of phenolic hydroxyl groups. They are not very specific and detect all phenols (phenolic acids, flavonoids, and tannins) with variable sensitivity. Hence, when screening for condensed tannins in plants, the vanillin test is generally preferred, because of its sensitivity, specificity and simplicity. Although this reagent detects monomeric as well as polymeric flavonoids, it is quite specific to a narrow range of flavanols and dihydrochalcones which have a single bond at 2,3 position and free meta-oriented hydroxy groups on the B ring (Sarkar and Howarth 1976).

2.1.7.1 Vanillin method

The substitution reaction (replacement of H by a substituent) is an essential characteristic of substances possessing a benzene ring. All these substitutions are the result of attachment to the benzene ring of an electrophilic reagent, i.e., one deficient in electrons, constituting a positively charged group. A carbonium ion (or carbonation) is formed by a carbon atom losing an electron and in consequence gaining a positive charge. The reactions of the vanillin test are summarized in Figure 10. The electrophilic radical of vanillin in an acid solution reacts only on those benzene rings which are "activated", i.e., have a resorcinol or phloroglucinol nuclei. Thus, vanillin reacts with the A ring of leucocyanidin (5,6,3',4'-tetrahydroxyflavan-3,4-diol) at one of the positions 6 or 8 activated by the presence of several OH groups. It does not react with the B ring. The radical resulting from the protonation of the aldehyde is substituted exclusively in positions 6 and 8 to give an intermediate which is readily dehydrated to the end product of the reaction. This condensation product is red in colour with an absorbance maximum at 480 to 550 nm, depending on the peak absorbance of the carbonium ion formed.

As stated earlier, the vanillin test is the preferred method for screening condensed tannins. Several variations of the method have been developed to improve specificity and reproducibility. In the modified vanillin-hydrochloric acid (MV-HCl) method suggested by Maxson and Rooney (1972), extractions are carried out with 1% HCl in methanol rather than absolute methanol. Recently, Price *et al.* (1978) critically evaluated the vanillin reaction by examining several factors that influenced the assay. Some of these

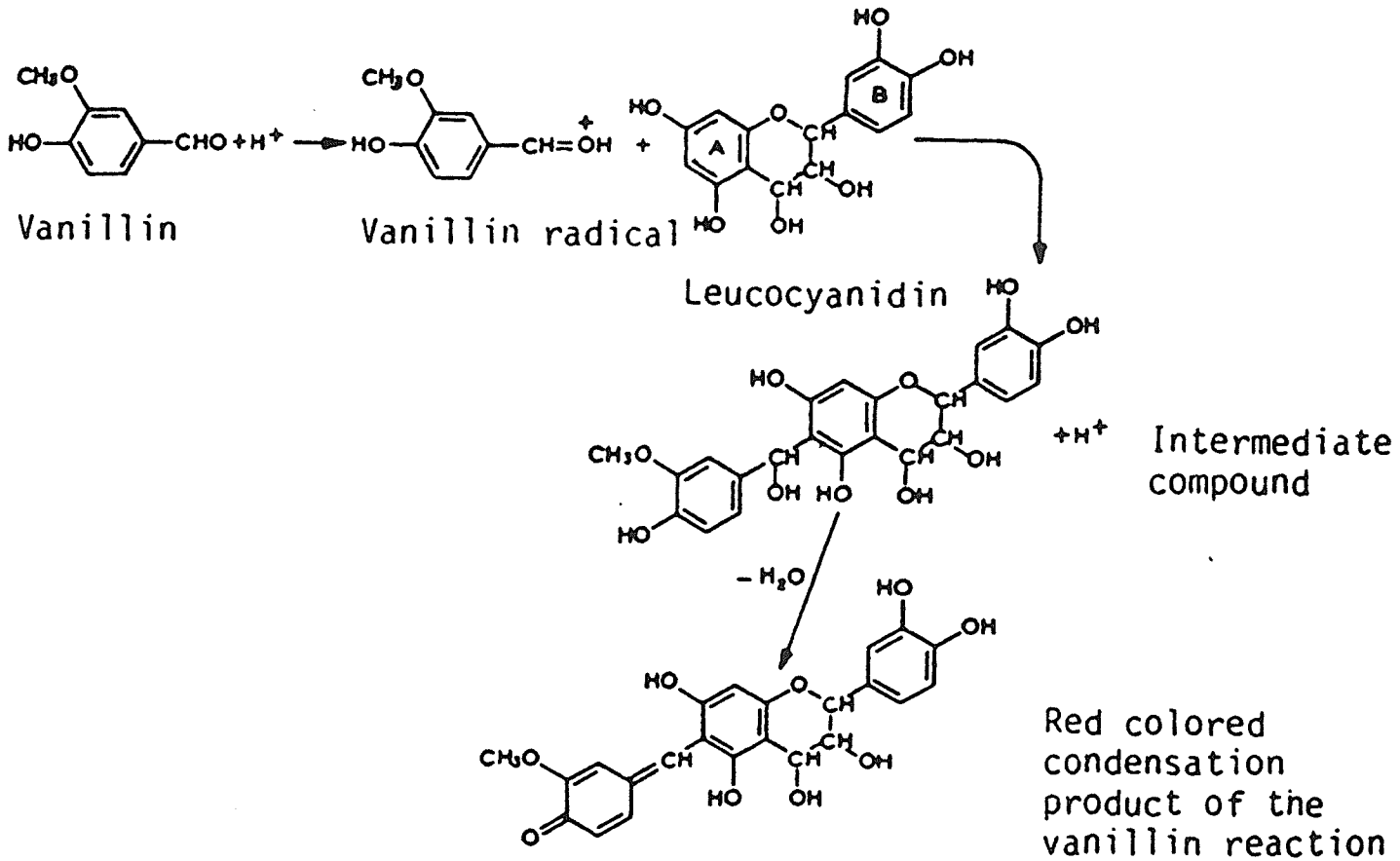


Figure 10. Condensation reactions of vanillaldehyde with flavans.

included time elapsed between grinding and analysis, the extraction time and medium, background sample colour, adequacy of catechin as reference standard, vanillin and HCl concentration, temperature, humidity, the sensitivity and reproducibility of the assay. Within the narrow specificity of the reagent and with certain restrictions in regard to the choice of reaction conditions, the vanillin assay appears to be a workable and reproducible procedure.

For the vanillin test, separate blanks are read for each sample and subtracted from the results of the regular vanillin test. The blanks are run under conditions identical with the regular vanillin test except that vanillin is omitted from the 4% HCl in methanol (Price and Butler 1977).

2.1.7.2 Oxidation-reduction methods

The oxidation of phenols involves chemical reactions of extraordinary complexity. The first step in the oxidation process is the removal of a hydrogen atom from the phenolic hydroxyl. The free radical with a singly bound oxygen atom carries an unpaired electron. Being highly unstable, it rapidly changes into one which has, in the *ortho* or *para* position, a trivalent carbon atom carrying an unpaired electron. The radical so formed can either dimerize or react with another radical, forming, in order of importance, C-C, C-O, or O-O bonds. Forsyth and Quesnel (1957a and 1957b) showed that in the case of catechol, these two types of oxidation lead through intermediate radicals to a mixture containing several tetrahydroxybiphenyls and a quinone (Figure 11).

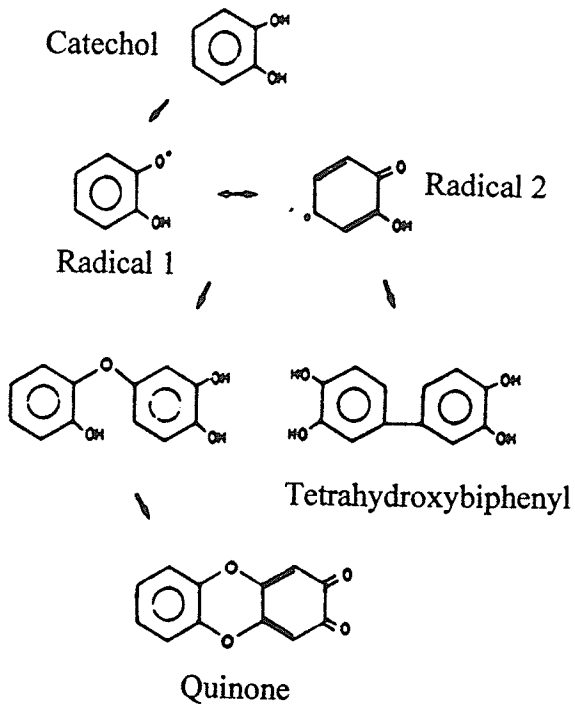


Figure 11. Oxidation of a typical phenol - catechol.

2.1.7.2.1 Prussian blue assay

Price and Butler (1977) suggested a qualitative as well as quantitative method for the condensed tannins of grain sorghum based on the formation of the Prussian blue complex. It involves the reduction by tannin and other phenolics of ferric ion to ferrous ion, followed by the formation of a ferricyanide-ferrous ion complex. The coloured product, commonly known as Prussian blue, absorbs maximally at 720 nm.

In common with other methods of total phenol estimations, the Prussian blue

assay makes no distinction between condensed tannins and other phenolic compounds. Also, polyphenolics with varying hydroxylation patterns and degrees of polymerization react differently to the reagents. The formation of the complex appears to be rate limiting, the redox reaction apparently reaching completion in seconds. The absorbance increases with time. The A_{720} readings are extremely sensitive to the slit width of the spectrophotometer (Price and Butler 1977).

2.1.7.2.2 Folin-Denis method

The Folin-Denis colorimetric method is perhaps the most widely used method for the determination of total phenols in a wide range of plant products and beverages. The intensity of the blue colour produced on reduction of the Folin-Denis reagent by phenols is measured in a spectrophotometer at 725 to 760 nm.

Goldstein and Swain (1963) reported that unlike the vanillin reagent, the Folin-Denis reagent does not react in a stoichiometric manner with phenolic compounds, even when the number of hydroxyl groups are taken into account. One of the major drawbacks of the Folin-Denis reagent is that it also reacts with other plant constituents, i.e., xanthine, amino acids, and proteins (Lowry *et al.* 1951). The Folin-Denis reagent is often substituted by the Folin-Ciocalteu (Folin and Ciocalteu 1927) reagent. In this latter reagent, the sensitivity of the reagent to reduction is improved by increasing the molybdate content and by the addition of lithium sulfate to prevent precipitation of sodium complex salts.

2.2 Protease inhibitors

2.2.1 General

Proteins with the property of forming stoichiometric protein-protein complexes with various enzymes resulting in the competitive inhibition of their catalytic functions are known to be extremely widespread in the plant kingdom. The best known of these protein inhibitors are those affecting the activities of the protease enzymes. Protease inhibitors occur in most legumes and cereals, in certain vegetables, such as cabbage, cucumbers, potatoes, tomatoes and spinach, and in fruits, such as apples, bananas, pineapples and raisins. The quantity of inhibitor depends on the variety, the physiological status of the plant and on levels of insect infestations or damage (Richardson 1977).

From a nutritional perspective the inhibition of the mammalian serine protease trypsin and chymotrypsin is most important. These protein inhibitors have been particularly well studied in important crops such as the cereals, legumes and potatoes.

2.2.2 Protease inhibitor reaction mechanisms

The inhibition of proteases by inhibitors is strictly competitive, and in the 1:1 complexes formed, all of the activities of the enzyme are completely abolished. Crystallographic, nuclear magnetic resonance (NMR) and mechanistic studies all indicate that the inhibitors act as highly specific substrates for the enzyme they inhibit at a unique

peptide bond called the reactive site peptide bond. Unlike normal enzyme-substrate and enzyme-product complexes, which dissociate very readily, the enzyme-inhibitor complexes are very stable ($K_a = 10^8$-10^{13} M^{-1}) (Laskowski and Kato 1980).

The results of X-ray crystallographic (McPhalen *et al.* 1985, Read and James 1986) studies indicate that as few as 10-15 residues of the inhibitor are in contact with the enzyme forming an almost lock-and-key type interaction, confirming that there is no tetrahedral carbon atom in the reactive site peptide carbonyl of the inhibitor. While those portions of the inhibitor molecule which are in enzyme-inhibitor contact tend to have very similar conformations the contact residues tend to be hypervariable, i.e. not strongly conserved (Read and James 1986).

2.2.3 Structure

Many inhibitors have been isolated in pure form and characterized by the elucidation of their amino acid composition, amino acid sequence, conformation, and chemistry of the reactive sites. Molecular weights are normally in the range of 6,000-50,000, with most of plant inhibitors lying between 8,000 and 25,000. A list of the ten most common families of plant enzyme inhibitors is shown in Table 1. This tabulation is not restricted to the inhibitors of the serine proteases, as certain of the families include proteins which are inhibitors of other classes of enzymes (e.g. α-amylase) or have no known inhibitory activity, although they are structurally related to known inhibitors.

Inspection of the primary sequences of several of the proteases inhibitors reveals

Table 1. Protease inhibitor families from plant sources[1].

Family	Enzymes inhibited	Distribution
1. Bowman-Birk	Trypsin[a]	Leguminosae
	Chymotrypsin	Gramineae
	Elastase	
2. Kunitz	Trypsin[b]	Leguminosae
	Chymotrypsin	Gramineae
	Subtilisin	Araceae
	Kallikrein	Alismataceae
	Amylase	
3. Potato I	Chymotrypsin	Solanaceae
	Trypsin	Gramineae
	Subtilisin	Leguminosae
		Polygonaceae
		Cucurbitaceae
		Hirudo edicinalis[c]
4. Potato II	Trypsin	Solanaceae
	Chymotrypsin	
5. Cucurbit	Trypsin	Cucurbitaceae
	Hageman factor	
6. Cereal superfamily	Amylase[b]	Gramineae
	Trypsin	(CM, 2S storage
	Hageman factor	Proteins in
		Cruciferae
		Euphorbiaceae
		Lecythidaceae
		Leguminosae)
7. Ragi AI2/barley,	Amylase[b]	Gramineae
Rice PAPI	Protease	
8. Thaumatin-PR like	Amylase[b]	Gramineae
	Trypsin	Solanaceae
9. Carboxy-peptidase	Carboxy-peptidase	Solanaceae
10. Cystatin-like	Cysteine-proteases	Gramineae animals

[a] Double-headed; [b] Bifunctional (α-amylase/protease); [c] Leech (animal source).
[1] From Laskowski and Kato (1980).

a striking degree of structural homology between them. Figure 12 illustrates this homology and demonstrates the fact that several of the plant protease inhibitors contain repetitive sequences or regions of internal homology. Another feature of these separate but homologous regions within the same molecule is that they very often contain the reactive (or inhibitory) sites which interact with the proteinases affected. Thus it can be seen in Figure 12 that several of the inhibitors are 'double-headed' proteins contain two separate peptide bond reactive sites, for example, two sites for trypsin or one site for trypsin and another site for chymotrypsin. On the other hand, some inhibitors have a single peptide bond which serves as the reactive site for a single inhibited protease or acts against several enzymes. Such inhibition is referred to as 'single-headed' inhibitors.

The sequence of the 'double-headed' garden bean inhibitor shown in Figure 12 clearly differs from the other related sequences shown by having replacement of amino acids at both reactive sites. The usual Lys (lysine) residue at position 26 is replaced by an Ala (alanine) residue. The site thus loses its ability to bind trypsin, and binds instead the related protease elastase. Similarly the replacement of the usual Leu (leucine) (Position 53) by an Arg (arginine) at the second reactive site makes it inactive towards chymotrypsin and converts it to a trypsin binding unit.

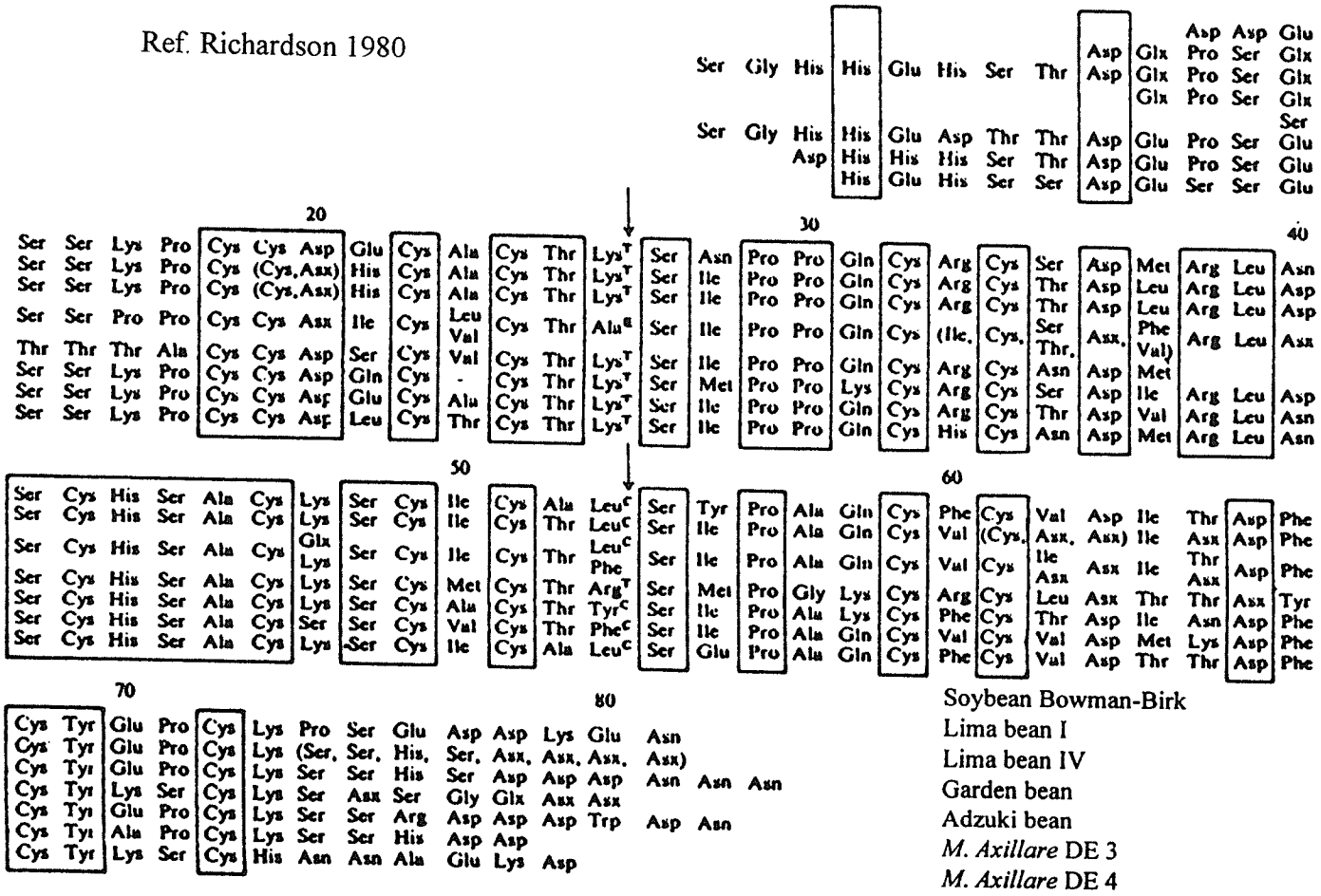


Figure. 12. Comparison of the amino acid sequences of the 'double-headed' protease inhibitors from various members of the Leguminosae. Segments of identical (homologous) sequences in the proteins are enclosed in boxes. The reactive (inhibitory) sites of each protein are indicated by the arrows; T, site reacting with trypsin; C, Chymotrypsin; E, elastase. The sequences are arranged to facilitate comparison of the regions of internal homology (repetitive sequences) around the reactive sites (e.g. residues 14 36 and 41 63).

2.2.4 Properties

2.2.4.1 Specificity

The specificity of inhibitors differs widely. Table 2 shows that while some of the compounds inhibit only trypsin, most of them are active against trypsin and chymotrypsin, and others additionally inhibit microbial or plant proteases, for example, subtilisin or papain.

2.2.4.2 Inhibition of human enzymes

The activity of the inhibitors is usually estimated with commercial enzymes, that is, bovine trypsin or bovine chymotrypsin. However, an evaluation of foodstuffs for potential deleterious effects on human health must be based on the inhibition of human enzymes.

Human trypsin is generally inhibited to the same extent or somewhat less than bovine trypsin by the inhibitors of grain legumes. On the other hand, human chymotrypsin is inhibited significantly more than the bovine enzyme by most inhibitors. The bovine pancreatic trypsin inhibitor (Kunitz) affects human trypsin (Feeney *et al.* 1969) but does not influence human chymotrypsin (Belitz *et al.* 1982). The stability of the inhibitors while passing through the stomach is also important (Table 3). The Kunitz inhibitor from soybeans is completely inactivated by human gastric juice within 24 h,

Table 2. Protease inhibitors of plant origin[a]

Source/inhibitor	MW (M_r)	T	CT	P	Bs	AP	Sg	PP
Cruciferae								
Raphanus sativus[b]	8,000-11,200	+	+-	-	+		+	
Brassica juncea	10,000-20,000	+	+-					
Leguminosae								
Arachis hypogaea[b]	7,500-17,000	+	+					
Cicer arietinum	12,000	+	+					
Dolichos lablab[b]	9,500-23,500	+	+	-				
Glycine max								
Kunitz inhibitor	21,500	+	+	-	-			
Bowman-Birk inhibitor	8,000	+	+	-			+	
Lathyrus odoratus	11,800	+	+					
L. Sativus[b]		+						
Phaseolus aureus[b]	8,000-18,000	+	+-	+				
P. Coccineus[b]	8,800-10,700	+	+					
P. lunatus[b]	8,300-16,200	+	+	-	-		+-	
P. vulgaris[b]	8,000-10,000	+	+	-	-			
Pisum sativum[b]	8,000-12,800	+	+					
Vicia faba[b]	23,000	+	+					
Vigna sinensis	9,500-13,300	+	+					
Convolvulaceae								
Ipomoea batatas[b]	23,000-24,000	+	-	-	-	-		
Solanaceae								
Solanum tuberosum[b]	22,000-42,000	+-	+-	-	+-	+-	+-	+-
Bromeliaceae								
Ananas comosus[b]	5,500	+	+					
Gramineae								
Hordeum vulgare[b]	14,000-25,000	+-	-	-	+-	+-	+-	
Oryza sativa[b]		+-	-		+			
Secale cereale[b]	10,000-18,700	+	+	-				
Triticum aestivum[b]	12,000-18,500	+-	-	-				
Zea mays[b]	7,000-18,500	+	+	-				

[a] Kassell and Williams (1976). [b]Properties of various inhibitors are compiled.
T: trypsin; CT: α-chymotrypsin; P: papain; Bs: *Bacillus subtilis* proteases; AP: *Aspergillus* spp. proteases; Sg: *Streptomyces griseus* proteases; PP: *Penicillium* spp. Proteases; +: inhibited; - not inhibited; +-: inhibited by some inhibitors of this source, not inhibited by others.

Table 3. Stability of inhibitors against pepsin at pH 2[a]

Source/inhibitor	Remaining activity[b] (%)
Soybean	
Kunitz inhibitor	0
Bowman-Birk inhibitor (BBI)	100
Extract	30-40
Lima bean: BBI-type inhibitor	70-93
Kidney bean: BBI-type inhibitor	100
Kintoki bean: BBI-type inhibitor	100
Lentil: BBI-type inhibitor	83-100
Chickpea: inhibitor	100
Horse gram: trypsin-chymotrypsin inhibitor	100
Moth bean: trypsin inhibitor	91
Broad bean: trypsin inhibitor	100

[a] From Weder (1986) (Incubation times vary).
[b] Against bovine and human trypsins and chymotrypsins.

while the Bowman-Birk inhibitor remains active (Birk 1961, Yavelow *et al.* 1983).

2.2.4.3 Stability

Generally, the inhibitors are thermolabile and are more or less completely inactivated by appropriate thermal procedures. Soybeans support the growth of rats as well as does casein when about 90 % of the proteases inhibitors are eliminated (Table 4). The differences in thermostability of inhibitors from different sources are shown in Table 5. The processing parameters (mainly time, temperature, pressure, moisture content of the sample) are of great importance.

2.2.5 Anti-nutritional effects

2.2.5.1 Animals

One of the major reasons why the enzyme inhibitors in seeds have long attracted so much attention is their potential to cause nutritional disorders and negative effect when ingested by animals and humans as components of plant foods. The exact nutritional significance of plant enzyme inhibitors in the diets of animals and man is difficult to assess. The first implication of these proteins is the cause of certain nutritional disorders, growth depression, pancreatic hypertrophy and/or hyperplasia (Liener and Kakade 1980)

Table 4. Effect of soybean trypsin inhibitors on growth of rats[a]

Trypsin inhibitor		Body weight (g)	Protein efficiency ratio
Amount (mg/100 g diet)	Destruction (%)		
887	0	79	1.59
532	40	111	2.37
282	68	121	2.78
119	87	148	3.08
Control (casein)		145	3.35

[a] Rackis *et al.* (1975).

Table 5. Destruction of trypsin inhibitors by heating

Sample	Procedure	Inhibitor destruction (%)	Ref.
Soybean	10% $Ca(OH)_2$, 80 °C, 1h	100	(Cravioto *et al.* 1951)
Kidney bean	Quick-cooking, 15 min	89	(Iyer *et al.* 1980)
Chickpea	Autoclaving	54	(Kao & Robinson 1978)
Horse bean	Autoclaving	100	(Kao & Robinson 1978)
Black gram	Cooking, 100 °C, 10 min	15	(Padhye & Salunkhe 1980)
Cow pea	Cooking, 90-95 °C, 45 min	52	(Elias *et al.* 1976)

when animals such as rats, mice and chickens were fed with plant products known to contain high levels of protease inhibitors. In rodents and birds, growth depression is accompanied by enlargement of the pancreas due to cell hypertrophy and / or hyperplasia. Kakade *et al.* (1973) demonstrated that protease inhibitors are responsible for about 40 % of the growth depression and pancreas enlargement observed in rats while the remainder was caused by the refractory nature of the soy protein. Examples for the response of animals to inhibitor preparations from other legumes are compiled in Table 6. Growth depression and pancreas enlargement were observed in most of the cases. It was also noticed that the inhibitors caused a metabolic disturbance in the utilisation of the sulphur amino acids methionine and cysteine. Some of the suggested explanation of these disorders are illustrated in Figure 13.

2.2.5.2 Humans

It is known that many plant protease inhibitors can inactivate the digestive enzymes of humans if they reach the small intestine unaltered (Belitz *et al.* 1982). Lewis and Taylor (1947) observed 20 % less nitrogen retention, but still positive nitrogen balance, when raw soybean flour was given to two adult humans, compared with autoclaved flour given to the same people. Gunn *et al.* (1980) reported an outbreak of gastrointestinal illness in humans who had consumed under-processed soy protein extender for a tunafish salad. Linscheer *et al.* (1980) showed that raw soy protein infused into the duodenum caused pancreatic trypsin inhibition, resulting in impaired

Table 6. Biological effects of inhibitor preparations from legumes in animals

Legume species	Animal species	Growth depression	Pancreas enlargement
Broad bean	Chicken	0 (Wilson *et al.* 1972)	+ (Wilson *et al.* 1972)
Grass pea	Rat	+ (Roy 1972)	+ (Roy 1972)
Lima bean	Mouse	+ (Tauber *et al.* 1949)	-
Navy bean	Rat	+ (Kakade & Evans 1965)	-
Winged bean	Rat	+ (Chan & de Lumen 1982)	+ (Chan & de Lumen 1982)

+: effect; 0: no effect; -: no data. References as indicated.

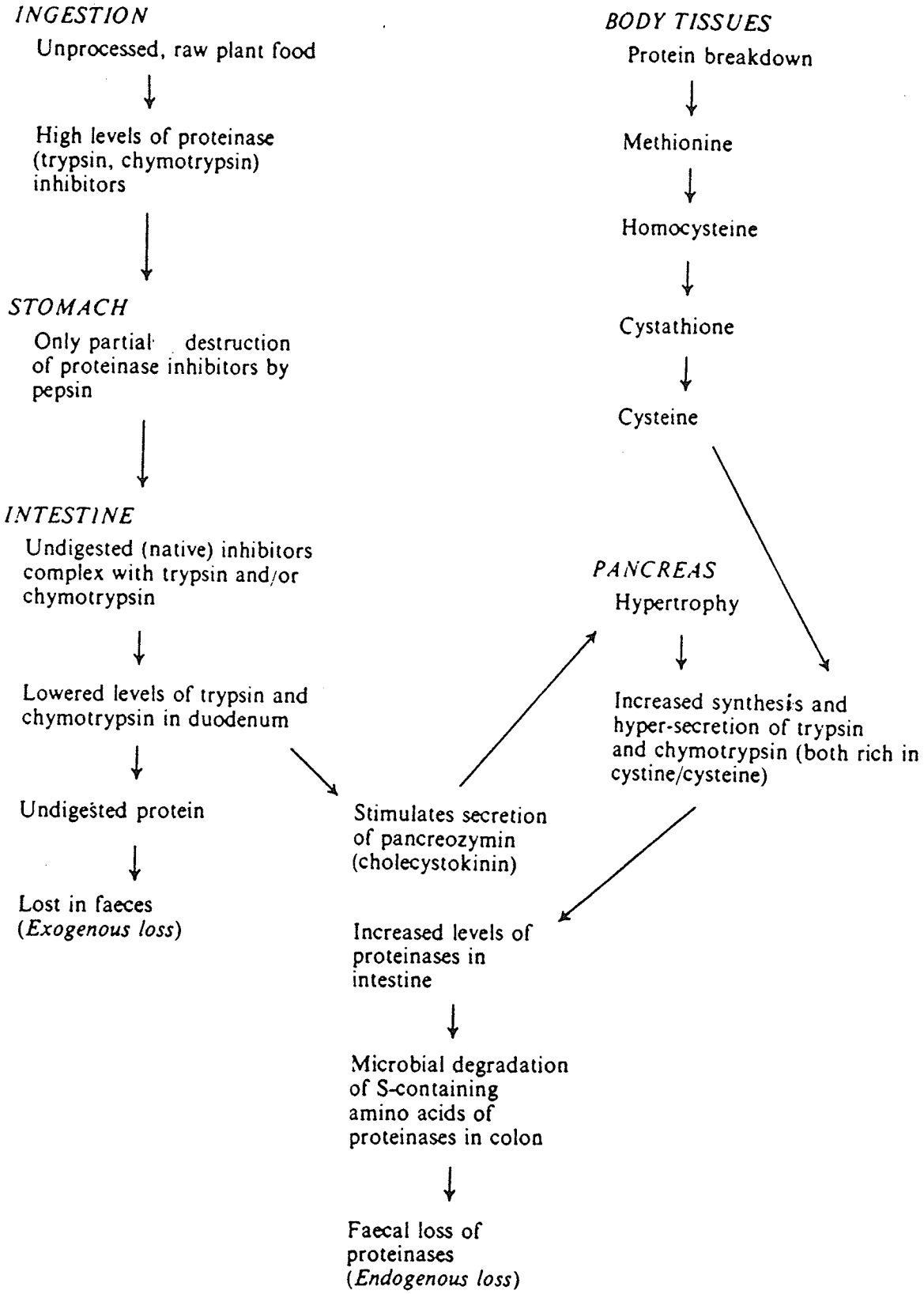


Figure. 13. Scheme to explain the possible deleterious effects of protease inhibitors on the nutritive value of proteins.

protein digestion and adsorption in nine healthy volunteers.

The consumption of improperly heated flakes from white and some spotted garden beans caused vomiting, diarrhea, and often headache, vertigo, and fever in a considerable number of cases after World War 2 in Berlin (Griebel 1950). Generally the same symptoms were recorded after consumption of African vetch (*Lathyrus tingitanus*), which was bought on the black market as lentils in Berlin after the war (Griebel 1950). Recent work has shown that the purified inhibitors as well as crude extracts of raw soybeans completely inhibit *in vitro* human trypsin and chymotrypsin activity (Krogdahl & Holm 1979).

There is a need for detailed studies on the ability of the plant inhibitors to survive in an active form during passage through the stomach. The likelihood of a particular inhibitor causing adverse physiological effects in the intestine depends to a considerable extent on its stability under acid (pH 2-3) conditions, and its resistance to digestion by pepsin. Many inhibitors are known to be very stable under those conditions but a few are quickly destroyed (Richardson 1977). The results lead to the conclusion that unusually high doses of protease inhibitors are deleterious to humans while moderate inhibitor content will not cause problems in single intakes. Fortunately for humans, most methods of cooking and processing largely destroy these inhibitors (Liener and Kakade 1980, Sathe and Salunkhe 1984, Rackis *et al.* 1986).

2.2.6 Plant protection by protease inhibitors

Enzyme inhibitors might be part of a general plant defence mechanism against tissue damage, environment stress, invasion by pathogens, or consumption by predators such as insect and microbial pests. The effects of protease inhibitors on insects have been investigated. The inhibitors caused a significant elevation of the levels of trypsin in the insect guts, causing pernicious hyperproduction of trypsin, which in turn leads to depletion of the sulphur-containing amino acids, this being detrimental to the insects (Gatehouse and Boulter 1983). A possible involvement of these inhibitors in the protection of barley against grasshoppers was postulated because leaf extracts of a less sensitive variety tested had a considerably higher anti-chymotrypsin activity (Weiel and Hapner 1976). Growth retardation and delayed pupation of corn borer larvae have been observed in feeding experiments with the Kunitz soybean trypsin inhibitors, while the maize trypsin inhibitor had no effect (Steffens *et al.* 1978). These and similar studies suggest but do not prove a protective role for the inhibitors. More conclusive evidence has been obtained for the resistance of the cowpea (*Vigna unguiculata*) to the bruchid beetle *Callosobruchus maculatus*. The only resistant variety found in a screening of over five thousand varieties had a higher level of protease inhibitors, whose antimetabolic nature was demonstrated in feeding trials (Gatehouse *et al.* 1979). In comparison, soybean trypsin inhibitor and lima bean trypsin inhibitor were relatively ineffective (Gatehouse and Boulter 1983).

Since many of the seed protein inhibitors are active *in vitro* against microbially

produced proteases, the inhibitors act in the defence of the seeds against these organisms. Wilson (1980) demonstrated that legume seeds secrete some of their protease inhibitors into the external medium during germination. This release plays a role in establishing a normal microbial complement in the rhizosphere of the legumes, by the inhibition of pathogenic microorganisms.

2.2.7 Protease inhibitor intake

A first comprehensive attempt to directly compare trypsin inhibitor activities of various foodstuffs was made by Rackis *et al.* (1986). Table 7 shows that 100 g of raw seeds of a number of pulses have potential to inhibit the amounts of trypsin and/or chymotrypsin produced daily by humans (1-2 g of each). Other species which may display similar activities against the human enzymes are cow pea, field bean, pigeon pea, and tepary bean. Usually, these seeds are not eaten raw, and processing will inactivate the inhibitors to some degree. Even processed foods may display trypsin inhibitor levels that lie between 14% and 45% of that of raw soybeans on sample weigh basis (Rackis *et al.* 1986). Soy-based instant formulas are reported to cause inhibition of 35-75 mg bovine trypsin per day, assuming a daily intake of 850 mL of formula for a 7-kg infant at 3 months of age (Harwood *et al.* 1986). This equals inhibition of 50-100 mg of human trypsin and 10-20 mg of human chymotrypsin per day.

Table 7. Inhibition of human enzymes by pulses[a]

Common name	RTIA (%, based on bovine trypsin)	Human enzymes inhibited by 100 g raw seeds	
		Trypsin	Chymotrypsin
Soybean	100	3.0	0.7
Chick pea	33	0.6	1.6
Grass pea	84	1.4	2.2
Lima bean	42	1.4	0.8
Winged bean	56	1.6	3.5

[a] From Belitz *et al.* (1982). RTIA: relative trypsin inhibitor activity.

Another approach to evaluation of the exposure of humans to proteases inhibitors in foods was made by Doell *et al.* (1981). They estimated the inhibition of bovine trypsin by ordinary western foods and calculated the daily intake of trypsin inhibitor activity from the average British diet. The activities of the individual items are listed in Table 8, transformed into human protease inhibitor activities using conversion factors from the literature. The contribution of the different groups of foods varies considerably, i.e., diets containing substantial amounts of milk, potatoes, and legumes will contain more inhibitor activity than those containing higher amounts of meat, fish, and eggs.

2.2.8 Analytical aspects

Appropriate assay methods are needed to estimate the inhibitor content of raw foods and to follow the effects of various food processing techniques on inhibitor levels. Most of the procedures described in the literature are enzymatic methods using the target enzyme and an appropriate substrate. These methods are special emphasis on inhibitors of digestive proteolytic enzymes. Besides enzymatic methods, immunochemical techniques have been used to determine inhibitor content.

Table 8. Average daily inhibitor intake[1]

Food	Inhibitor activity[a] against BT[b]	HT[c]	HCT[c]
Milk and milk products	56.8	45.4[d]	113.6[d]
Meat	19.8	34.5	0.2
Fish	1.5	1.2[d]	3.0[d]
Eggs	93.6	5.2	11.0
Vegetables			
Potatoes	42.5	30.6	173.3
Others	37.8	30.2[d]	75.6[d]
Fruit and nuts	11.8	9.4[d]	23.6[d]
Bread and cereals	25.3	25.3	12.6
Other foods	5.5	4.4[d]	11.0[d]
Total	294.6	186.2	423.9

[a] Uptake/person/day (mg enzyme inhibited). [b] BT: Bovine trypsin; data from Doell *et al.* (1981). [c] HT: Human trypsin; HCT: Human chymotrypsin; values calculated from those for BT with individual or general (d) conversion factors (0.8 for HT/BT, 2.0 for HCT/BT).
[1] From Belitz *et al.* (1982).

2.2.8.1 Enzymatic methods

The general principle of all enzymatic inhibitor determinations is based on measuring the decrease in enzymatic activity cause by the sample under investigation. Basically, any method for quantifying enzyme activities can be employed to determine inhibitor content. For a particular problem, the procedure must be chosen according to specificity and sensitivity. A calibration curve for the enzyme assay should be established in order to check the relationship between response and amount of enzyme present, and to select the range within which the assay is sufficiently sensitive to be used for determining the activity of the inhibitors.

For inhibitor determinations, equal amounts of the enzyme, producing a response in the upper part of this range, are mixed with increasing amounts of a sample solution containing the inhibitor and treated in the same manner as the enzyme assay. The amount of enzyme still active or inhibited are plotted versus the amounts of sample producing that effect to give the so-called inhibition curve. From this curve the amount of enzyme inhibited by a given amount of the sample is determined directly, either by extrapolating the initial slope up to 100% inhibition or by duplicating the amount of inhibitor necessary to inactivate half of the enzyme present in the test (Fritz *et al.* 1984). The prime relevance of the calculations depends on the aim of the study. In order to determine the stoichiometry of the reaction between enzyme and inhibitor, the former is generally used; to evaluate the nutritional consequences of inhibitors present in food, the latter method is normally used. The latter method requires that the exact concentration of the enzyme

used is known. This is best achieved for serine proteinases by titration of the active site (Chase and Shaw 1970). While strong inhibitors will give the same results with the two methods, greater differences will be obtained with weak inhibitors.

Several parameters may influence the results of inhibitor activity determinations including: the type of enzyme preparation used, the substrate used, and the reaction conditions. Proteases from different species, including humans, are affected differently by plant extracts and inhibitor preparations (Rao and Pattabiraman 1985, Rascon *et al.* 1985, Krogdahl and Holm 1983, Struthers and MacDonald 1983). In addition, even if enzyme preparations from the same species are used, different results may be obtained because individual enzyme forms (isoenzymes or different molecular species) can be present at varying ratios that are inhibited differently by the same inhibitor preparation. For instance, human trypsin and human chymotrypsin are not single compounds, but mixtures of at least two components (Coan *et al.* 1971, Coan and Travis 1972, Feinstein *et al.* 1974, Mallory and Travis 1975, Figarella *et al.* 1975). Therefore, some authors prefer to use pancreatic preparations instead of purified enzymes, approximating more closely the situation *in vivo* and being more meaningful nutritionally. Variation in isoenzyme composition of the individual samples can not be overcome by this method and specific substrates must be used to discriminate between the different activities.

Secondly, the substrates used may influence the results of inhibitor activity determinations. Generally, natural substrates (i.e., substrates which the enzymes transform *in vivo*) are suited best for evaluating the influence of inhibitors present in foods on the utilization of nutrients. However, other reasons such as selectivity and

greater sensitivity may render necessary the use of synthetic substrates. Substrates used to determine proteases can be classified into proteins, synthetic amino acid amides, and synthetic amino acid esters. For the assay of the protease inhibitors, the decrease in the hydrolysis of protein substrates such as casein, azocasein or haemoglobin, or of synthetic substrates such as α-N-benzoyl-L-arginine-ρ-nitroanilide (BAPNA) for trypsin, α-N-benzoyl-L-tyrosine ethyl ester (BTEE) for chymotrypsin, and hippuryl-L-phenylalanine and hippuryl-L-lysine for carboxypeptidases, may be monitored spectrophotometrically and titrimetrically (Kassell 1970). Proteins are suited best to simulate *in vivo* digestion, but they can only be used to determine individual activities if purified enzymes are studied. In the case of digestive juice (e.g., gastric, pancreatic or intestinal juice), specific synthetic substrates must be used to differentiate between the individual activities , for example, between trypsin and chymotrypsin in pancreatic juice.

The specific substrates for trypsin determinations are amides, detecting what is called aminolytic activity, while others are esters detecting esterolytic activity. With respect to sensitivity, the substrates yielding fluorescent products are usually much more sensitive than those yielding coloured products.

Thirdly, the influence of reaction conditions is important. Preincubation of the enzyme with the inhibitor before the substrate is added will be necessary if the reaction between enzyme and inhibitor is slow. Another important factor is the inhibitor to enzyme ratio used in the assay. In some cases special reaction conditions must be met. For example, when soybean samples are being assayed for trypsin inhibitor content, the presence of adequate calcium ions is imperative. Calcium is an essential cofactor for

trypsin. It can be complexed by phytate, which is present in most soy preparations, thus decreasing the enzyme activity and stimulating inhibitors (Lehnhardt and Dills 1984). For samples with low protease inhibitor content, large quantities must be added in the assay to obtain measurable responses. However high protein levels may compete with synthetic substrates for the proteases, resulting in falsely high trypsin inhibitor activity determinations.

Enzyme units related to the absorbance of the amount of product formed under standardized conditions have been defined, for example, the Kunitz trypsin unit (Kunitz 1947) and the trypsin unit of Kakade *et al.* (1974). Although these units have been introduced into official inhibitor determination methods (AACC 1976), they can only be used for comparing different samples or studying the relative influence of processing techniques. For nutritional evaluations, the specific activity of the enzyme must be known. Some conversion factors have been compiled elsewhere (Rackis 1986).

2.2.8.2 Immunochemical methods

The general principle of all immunochemical methods is the binding of an antigen to its specific antibody. Therefore, an antiserum has to be prepared first by injection of the antigen (i.e., the inhibitor) into experimental animals (e.g., rabbits). In order to obtain highly specific antisera, pure inhibitor preparations are needed. This restricts the method to inhibitors that have already been isolated in a pure state. Then, the specificity of the antiserum has to be tested (e.g. by the Ouchterloney double-diffussion assay 1949), in

order to ensure that only the target protein is detected. The high specificity of the antigen antibody reaction offers both advantages and disadvantages depending on the problem to be studied. It can be used to discriminate between different inhibitors present in the same sample (Pearce *et al.* 1979). On the other hand, the method cannot be used to determine the amount of inhibitors active against one particular enzyme if different inhibitors participate in this action.

The most commonly used immunological method in inhibitor research is the radial diffusion technique (Ryan 1967). Aspects of quantifying these results have been discussed by Trautmann *et al.* (1971) and Berne (1974). More recently, immunoelectrophoresis (rocket electrophoresis, Freed and Ryan 1978) and the enzyme-linked immunosorbent assay (ELISA, Brown and Ryan 1984) were introduced into inhibitor research. More general aspects of immunochemical methods can be found elsewhere (Clausen 1969).

2.3 Anti-nutritional factors in field pea and grass pea

2.3.1 Haemagglutinins (Lectins)

Haemagglutinin is a protein with the remarkable property of agglutinating red blood cells. In addition, it precipitates polysaccharides and glycoproteins, and is known to agglutinate malignant cells and induce mitosis in lymphocytes (Sharon and Lis 1972). Haemagglutinins are found in a wide range of legumes including field pea. The

haemagglutinin content of field pea ranged from 5100 to 15060 U/g (Valdebouze *et al.* 1980). Manage *et al.* (1972) showed that haemagglutinins in field pea were essentially non-toxic when incorporated in the diet at a level of 1%. Tannous and Ullah (1969) showed that the haemagglutinin activity of field pea was completely eliminated by autoclaving at 121 ^{0}C for 5 minutes. Bender (1983) reported removal of 65% of haemagglutinin activity after soaking the field pea for 18 hours.

2.3.2 Trypsin Inhibitors

Trypsin is a proteolytic enzyme secreted by the pancreas. Trypsin inhibitors are present in many legumes in varying amounts. Field pea trypsin inhibitors belong to the Bowman-Birk class (a member of the serine protease family, molecular wt 11,000-20,000) (Domoney *et al.* 1993). Grass pea trypsin inhibitor is "LSTI-B" type (molecular wt. 22,000) (Roy 1980). When trypsin is inhibited, proteins are not digested adequately and fewer amino acids are available for growth.

The trypsin inhibitor content of field pea (150-10800 U/g) is one-tenth the level found in soybeans (*Glycine max*) and is similar to that of the field bean (*Vicia faba*) (Hove and King 1979, Valdebouze *et al.* 1980). Domoney and Welhan (1992) reported that TIA levels of five field pea genotypes were 3.0 to 16.3 TIU mg^{-1} (DM). Leterme *et al.* (1990) reported that TIA levels ranged from 1.71 to 8.40 TIU mg^{-1} (DM) in 33 European spring field pea varieties. Mean values of TIA were 0.93 to 2.55 TIU mg^{-1} (DM) in 11 field pea cultivars grown at seven European locations during the 1990 season

(Bacon *et al.* 1995). The trypsin inhibitor content depends on the type of field pea. Wrinkled-seeded varieties have less TIA than smooth-seeded types and spring types on average have less than winter types (Valdebouze *et al.* 1980). Valdebouze *et al.* (1980) stated that 90% of the trypsin inhibitor activity was found in the kernel and 10% in the testa.

Deshpande and Campbell (1992) reported that the ranges for TIA in 100 lines of grass pea germplasm were 133- 174 TIU mg^{-1} (DM). Roy and Bhat (1975) reported that TIA levels in 10 grass pea cultivars ranged from 11.1 to 28.5 TIU mg^{-1} (protein). Aletor *et al.* (1994) reported that levels of TIA were 13.88 to 23.22 g kg^{-1} (DM) in 36 grass pea lines maintained in the germplasm collection at International Center for Agricultural Research in the Dry Areas (ICARDA).

Griffiths (1984) showed that trypsin inhibitors were stable at or below 80 ^{0}C. However, at 100 ^{0}C, small but significant reductions in trypsin inhibitors were observed. Autoclaving (121 ^{0}C, 170 kPa, 10 minutes) almost completely destroyed trypsin inhibitors.

2.3.3 Chymotrypsin Inhibitors

Chymotrypsin is a proteolytic enzyme similar to trypsin. The mode of action of chymotrypsin inhibitor is very similar to that of the trypsin inhibitor. Griffiths (1984) showed that the chymotrypsin inhibitor content of field pea cultivars ranged from 740 to 10240 U/g. He showed that the effect of heat treatment on chymotrypsin inhibitors was

similar to that on trypsin inhibitors. Chymotrypsin inhibitors were stable and their inhibitory properties were unaffected at or below 80 ^{0}C. At 100 ^{0}C, a small but significant reduction in activity was observed. However, autoclaving (121 ^{0}C, 170 kPa, 10 minutes) almost completely destroyed all inhibitor activity.

2.3.4 Amylase Inhibitors

Amylase inhibitors are inhibitors of pancreatic and salivary amylase found in a wide range of legume seeds (Jaffè *et al.* 1973). The highest levels of amylase inhibitors are found in kidney beans, while field pea contains relatively low levels (14-80 U/g). Amylase inhibitors are readily inactivated at 100 ^{0}C (Jaffè *et al.* 1973) so they are unlikely to pose any problems in well-cooked human food.

2.3.5 Oxalate

The oxalate content of field pea is reported to be 6.67 g/kg (Gad *et al.* 1982). They concluded that both cooking and dehusking pea reduced the oxalate content.

2.3.6 Phytic Acid

Phytic acid is an important storage form of phosphorus in field pea seeds and is also an anti-nutritive factor in field pea. Phytic acid is a powerful chelating agent for

divalent cations and has the potential to interfere with mineral availability (Erdman and Forbes 1977). Phytic acid can also affect digestibility by chelating with calcium or by binding with substrate or proteolytic enzymes. The phytic acid content of field pea ranges from 4.7 to 8.2 g/kg (Elkowicz and Sosulski 1982, Kumar and Kapoor 1983). Cooking field pea results in 82% reduction in the phytic acid content of Pakistani varieties without any loss of total phosphorus (Manan *et al.* 1987).

2.3.7 Tannins and Phenolic Acids

The tannin content of field pea seeds ranges from 0 to 13 g/kg while the phenolic acid content of field pea ranges from 13 to 27 mg/kg (Sosulski and Dabrowski 1984). White-flower field pea usually contains negligible amounts of tannins, whereas coloured-flower field pea contains more tannins. Since most of the tannins and the phenolic acid is located in the testa, dehusking can reduce the tannin content of the seeds.

The regression coefficient linking tannin content and the digestible crude protein in hens suggests that 1% reduction of tannin content in field pea would result in 5% increase of crude protein digestibility (Lindgren 1975). Growth rates of rats and chicks are greatly depressed when fed diets containing high-tannin sorghum (Rostagno *et al.* 1973, Price *et al.* 1979). The high tannin-containing field pea also contains high levels of total polyphenols.

Deshpande and Campbell (1992) reported that the ranges for some anti-nutrients in 100 lines of grass pea germplasm were as follows: total phenolics 86 - 891 mg kg^{-1}

(DM), condensed tannins 0.0 - 4.38 g kg^{-1} (DM). Aletor *et al.* (1994) reported that levels of condensed tannins were 0 to 5.50 g kg^{-1} (DM) in 36 grass pea lines maintained in the germplasm collection at International Center for Agricultural Research in the Dry Areas (ICARDA).

2.3.8 Lipoxygenase

Lipoxygenase is a enzyme which can catalyse lipid oxidation. Although the levels of lipids in field pea are low, if field pea is not processed correctly lipoxygenase activity may become important. Deterioration in field pea quality may be a result of microbiological, enzymic and non-enzymic changes. The last two are of particular importance in the preservation of fresh and processed field pea. In both cases deterioration occurs mainly via lipid oxidation resulting in off-flavour development (Eriksson 1967). Linoleic and linolenic acids are extremely susceptible to oxidation which is responsible for the development of rancid flavours. Eriksson (1967) showed that for peas 5 to 8% of the total lipoxidase content is located in the testa, 80% in the outer and 12% in the inner tissue of the kernel.

2.3.9 Volatiles

Field peas are an important food product because of their protein content and their good storage stability. Lovegren *et al.* (1979) showed that dried split field pea contained

a wide range of volatile constituents. Volatile constituents are presumably the products of microbial metabolism and may contribute to the development of off-aromas and off-flavours. The three compounds found in the highest amounts in split peas are methanol 3.6 mg/kg, ethanol 4.9 mg/kg and acetaldehyde 4.3 mg/kg. Aromatic hydrocarbons are found at extremely low levels in dry split field pea. The levels of volatile constituents found in field pea are insignificant and are largely eliminated on cooking.

2.3.10 Saponins

Saponins are secondary metabolites containing carbohydrate moieties attached to an aglycone, which may either be steroidal or triterpenoid in structure (Aletor submitted). Saponins are found in a relatively small number of food plants, primarily legumes. The dietary presence of saponins can either be beneficial or deleterious. The nutritional significance of saponins stems largely from their hypocholesterolaemic action, leading to the belief that they could prove useful in the control of human cardiovascular disease (Oakenfull and Sidhu 1983). It is believed that the hypocholesterolaemic activity of dietary saponins may be due to the formation of some complexes with dietary cholesterol or their bile salt precursors which are then made unavailable for absorption. Recent studies with dietary saponins (Johnson *et al.* 1986, Gee and Johnson 1988) have shown that besides lowering serum cholesterol, they also readily increase the permeability of the mucosal cells of the small intestine, thereby inhibiting active nutrient transport. This permeability effect of saponins may not be beneficial. The saponin content of peas ranges

from 1.1 g/kg for yellow peas to 2.5 g/kg for green field pea (Fenwick and Oakenfull 1983, Price *et al.* 1986) which is lower than the levels found in lentils (3.7 to 4.6 g/kg).

2.4 Field pea and grass pea in pig and poultry rations

Field peas are considered by feed compounders to be a high protein and a high energy ingredient in the diets of pigs and poultry. Taverner and Curic (1983) showed that when field pea comprised 30% of the total diet fed to pigs, the digestibility of protein in the field pea was 82%. The digestibility of total energy in field pea was 85% which was comparable to the other protein sources evaluated. The high digestibility of field pea protein in poultry has been confirmed in the experiments of Nguyen and Kermorgant (1983). They showed that availability of the essential amino acids in raw field pea ranged from 80 to 95% in caecectomized cockerels.

Field pea meal is reported to be highly palatable to pigs, and recommended at levels of 25 to 30% of the ration (Bell and Wilson 1970, Vogt 1983). Gatel *et al.* (1989) and Grosjean *et al.* (1991) showed that a diet based on a cereal and field pea or a more complex diet containing 40 to 45% raw field pea are equally effective in terms of growth rate for growing/finishing pigs. The prime nutritional advantages of field pea meal are the relatively high lysine content and favourable essential amino acid balance. The disadvantage of field pea meal is its low levels of sulphur amino acids and tryptophan (Carrouée and Gatel 1995).

Field pea is an under-utilized resource in animal feeding. They have a high

protein content and good dietary energy value which allows them to replace soybean meal and cereals which form the mainstay of many diet formulations. They also contain relatively low levels of anti-nutritive factors allowing them to be incorporated raw into feeds, although some heat treatment yields beneficial effects. Amino acid deficiencies limit the total field pea content that can be used in dietary formulations, although amino acid supplements are often used in animal rations.

Studies of chemical composition and nutritive value of field pea show that their pattern of nutrients is similar to that found in other grain legumes. The protein content in field pea is relatively high and superior to that of cereals (Ali-Khan and Youngs 1973). Field pea contains low levels of fat (Haydar and Hadziyev 1973). The nutritive value of field pea seeds is limited by anti-nutritive factors, however, most of these can be reduced or eliminated by processing. The increase in digestibility of the field pea seeds on cooking is probably due to the elimination of trypsin and chymotrypsin inhibitors.

The seed proteins of field pea are deficient in sulphur amino acids, particularly methionine. Some field pea cultivars may also be deficient in tryptophan (Carrouée and Gatel 1995). Field pea is rich in lysine (Carrouée and Gatel 1995), therefore cereal and field pea protein exert a considerable complementary effect; the protein value of mixtures exceeds those of the components given separately. Field pea is a potential protein concentrate and could be processed to produce many products similar to those based on soybean. An increase in field pea consumption would increase the protein content of the diet of many at-risk groups in society.

Although grass pea is grown in many countries its acceptance as a major crop has

been reduced due to its potential to cause neurotoxicity (Roy 1981). When consumption exceeds 35% of diet for 3-4 months, neurolathyrism may develop. It is caused by the neurotoxin β-N-oxalyl-L-α-β diaminopropionic acid (ODAP or BOAA) (Spencer *et al.* 1986). The mechanism for this toxicity is not fully understood, but it is associated with binding of ODAP to the quisqualate glutamate receptors of the brain (Ross and Spencer 1989).

Pig-feeding studies have shown that grass pea can be used as a component of diet, but when levels exceed 20%, performance is reduced (Castell *et al.* 1994). Anti-nutritional factors, such as trypsin inhibitors and chymotrypsin inhibitors, rather than ODAP, limit the potential of grass pea as a feedstuff for swine. Residues of ODAP in the meat were insufficient to cause a problem, as they were not detected at the 100 ng/g level. Lower limits on the inclusion rate could be justified to reduce the likelihood of adverse effects resulting from deficiencies of methionine and tryptophan or presence of anti-nutritional factors. It was apparent that there are anti-nutritional factors in the grass pea seed used in these studies confirming results obtained in chicks (Rotter *et al.* 1991).

Proteins and polypeptides in grass pea constitute about 20 to 40% of dry seeds and they have various biological functions, such as enzymic, hormonal, structural, reserve functions, etc. Grass pea contains adequate concentrations of most amino acids, especially rich in lysine, compared to cereal grains, but deficient in methionine. Grass pea is regarded as a useful source of protein in regions where it is used as a staple food and has the potential as a feed ingredient for swine, but the presence of anti-protease factors appears to limit its usefulness in an unprocessed form (Castell *et al.* 1994).

2.5 Objective of this research

Field pea and grass pea seeds have been shown to contain total phenolics, condensed tannins, and trypsin inhibitors. These anti-nutritional factors should be evaluated in field pea and grass pea. Total phenolics will be measured using Prussian blue assay. Condensed tannins will be determined by the vanillin test. It is important to distinguish between condensed tannins and low molecular weight phenolic compounds, since the former contribute to anti-nutritional properties, the latter primarily affect palatability unless present in high concentrations (Deshpande and Campbell 1992). TIA will be determined according to the method of Kakade *et al.* using the large scale Kakade assay.

To determine whether genotype and environment have a significant influence on these anti-nutritional factors, seed samples of seventeen field pea cultivars grown at five locations, and grass pea lines grown at two locations, in western Canada during 1993 and 1994 were selected. Correlation between these anti-nutritional factors and seed protein and yield in field pea and grass pea and between seed coat colour with total phenolics, condensed tannins, TIA, seed protein or yield in grass pea will be analysed.

The proposed research will quantify anti-nutritional components of field pea and grass pea lines potentially useful in breeding programs, enabling selection for optimum feed potential. This research will also lead to recommendation for new pulse crop lines for introduction as alternatives for cultivation in the prairies as well as increase field pea and grass pea production.

3. MATERIALS AND METHODS

3.1 Chemicals and equipment

All chemicals are listed in table 9. All chemicals used were of reagent grade. All equipment used is listed in table 10.

3.2 Materials and statistical analyses

Seed samples of seventeen field pea cultivars commonly grown in western Canada were obtained from regional adaptation trials conducted at five locations in Manitoba (Arborg, Dauphin, Minto, Rosebank and Thornhill) during 1993 and 1994. For grass pea, seed samples of nine breeding lines were obtained from the Agriculture and Agri-Food Canada, Morden breeding program which had been grown in preliminary tests in Morden and Portage la Prairie, Manitoba during 1993 and 1994. Field pea cultivars used included yellow and green cotyledon types; all cultivars had white seed coats. Samples of grass pea had a range of seed coat colours from white through dark brown. Whole grain samples of all field pea and grass pea seeds were ground to pass through a 20-mesh screen for total phenolics and condensed tannins and a 80 mesh screen for trypsin inhibitor activity (TIA) in a Thomas-Wiley Intermediate Mill equipped with stainless steel blades.

Analysis of variance, means comparison by Duncan's Multiple Range Test, and

Table 9. Chemical sources

Name	Catalogue number	Company
Acetic acid	AC-135	Anachemia
BAPNA[1]	B-4875	Sigma
Calcium chloride[2]	C-3881	Sigma
Catechin (+)[3]	C-1251	Sigma
Dimethyl Sulfoxide	D-8779	Sigma
Ferric chloride	UN-1773	Fisher Scientific
Hydrochloric acid	C9800-10	Baxter
Methanol	AC-5730	Anachemia
Potassium ferricyanide	P-232	Fisher Scientific
Tris[4]	T-1378	Sigma
Trypsin	3703	Worthington
Vanillin	V-2375	Sigma

[1] N α-BENZOYL-DL-ARGININE-p-NITROANILIDE Hydrochloride.
[2] $CaCl_2 . 2H_2O$.
[3] 3.5 moles of H_2O per mole of catechin.
[4] Tris (hydroxymethyl) amino-methane. 7-9.

Table 10. Equipment sources

Name	Model	Company
Balance	AE160	Mettler
Centrifuge	Biofuge 13	Baxter, Herraeus Instruments
Centrifuge	IEC Centra-8	International Equipment Company
Hematology/chemistry mixer	346	Fisher Scientific
Hunterlab tristimulus colorimeter	D25L-9	Hunter Associated Laboratory Inc.
Laboratory oven	30F	Fisher Econotemp
Multipoint Mixter	HP 15	Variomag Electronicruhrer
Orbit shaker	3520	Lab-Line Instruments Inc.
pH meter	50	Fisher Scientific
Spectrophotometer	DU 50	Beckman Instruments Inc. U.S.A.
Spectrophotometer	DU 640B	Beckman Instruments Inc. U.S.A.
Spectrophotometer	Spectronic 501	Milton Roy Company
Superspeed centrifuge	RC 2-8	Sorvall
Thomas-Wiley laboratory mill	072-397	Anachemia Science
Water bath	Haake W13	West Germany
Wrist action shaker	AC	Eberbach Corporation, Michigan

correlation analysis were conducted using SAS system (SAS Institute, Inc.1990). All chemical analyses were performed at least in duplicate.

3.3 Moisture contents and condensed tannins measurements

Moisture contents of sample flours of field pea and grass pea were determined by the AOAC method (1990). Condensed tannin contents of ground samples of field pea and grass pea were determined according to the method of Burns (1971) as modified by Price *et al.* (1978) using 1% HCl in methanol as an extracting solvent and vanillin (0.5%) hydrochloride (4%) in methanol as a chromogen. Appropriate sample blanks were used to eliminate interference by seed pigments. Sample size was 2.0 g for field pea and 0.1 g for grass pea. Smaller samples were used for grass pea since concentrations of tannins in grass pea were greater than in field pea. Sample flours were extracted with 10 mL extracting solvent and shaken on a reciprocating shaker for 20 min at room temperature. A water bath at 30 ^{0}C was used for the 20-min interval between mixing reagents and reading the absorbance. Absorbance at 500 nm was read in 1-cm cuvettes on a Beckman Model DU 50 spectrophotometer zeroed against a reagent blank.

3.4 Total phenolics and protein content measurements

Total phenolics of sample flours of field pea and grass pea were determined as described by Price and Butler (1977) using the Prussian blue assay. Sample size was 1 g

for field pea and 0.1 g for grass pea. Smaller samples were used for grass pea since concentrations of total phenolics in grass pea were greater than in field pea. Sample flours were extracted with 5 mL of absolute methanol and shaken on a reciprocating shaker for 30 min. An aliquot was centrifuged at 14,926 g for 10 min (Biofuge, Baxter Diagnostic Corp., Ontario, Canada). The supernatant was diluted 100 times with distilled water mixed with 3 mL of 0.1 M $FeCl_3$ in 0.1 N HCl for 3 min, followed by the timed addition of 3 mL of 0.008 M K_3 $Fe(CN)_6$. The absorbance was read after 10 min at 720 nm on a Spectronic 501 spectrophotometer which had been zeroed with water (Oomah *et al.* 1995). A blank of identical composition, but omitting the sample extract, was analysed and subtracted from all other readings. All samples were extracted within three days of milling into flour. The standard curves for total phenolics and condensed tannins were prepared with catechin (+) (3.5 moles of H_2O per mole of catechin, Sigma Chemical Co.), and the results were expressed in terms of catechin equivalents on a dry weight basis. Condensed tannins were expressed as gram catechin per kilogram dry flour, total phenolics was expressed as milligram catechin per kilogram dry flour. Total phenolics standard curves for field pea and grass pea were individually made to various dilutions from 0.3 mg catechin (+) / 5 mL methanol. Condensed tannin standard curve for field pea was made to various dilutions from 2 mg catechin (+) / 50 mL 1% HCl in methanol. Condensed tannin standard curve for grass pea was made to various dilutions from 5 mg catechin (+) / 50 mL 1% HCl in methanol. Protein contents (N X 6.25) in field pea and grass pea were determined according to the Kjeldahl method with a Tecator digester and a Kjeltec (System 1002) distillation unit (Tecator AB, Höganäs, Sweden).

3.5 Trypsin inhibitor activity measurement

TIA of sample flours of field pea and grass pea was determined according to the method of Kakade *et al.* (1974) using the large scale Kakade assay. Sample size was 50 mg for field pea and 5 mg for grass pea; smaller samples were used for grass pea since TIA in grass pea was greater than that in field pea.

3.5.1 Enzyme solution

Forty mg of bovine trypsin (Worthington Biochemical Corporation, 3703) was dissolved in 2000 mL of 0.001 M HCl. This solution can be stored at 4 ^{0}C for 2 or 3 weeks without appreciable loss in activity (Kakade *et al.* 1974).

3.5.2 Substrate solution

Four hundred mg of Nα benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) hydrochloride (Sigma, Cat. No. B-4875) was dissolved in 20 mL dimethyl sulphoxide and allowed to stand for at least 30 min. One mL aliquots of the resulting solution were then stored at -20 ^{0}C for future use. As required, 1 mL of BAPNA solution was diluted to 50 mL with freshly prepared 20 mM $CaCl_2$, 50 mM Tris-HCl pH 8.2, preheated to 37 ^{0}C. The final substrate solution was then stored at 37 ^{0}C and discarded after 4 h. Larger quantities (600 mL) were prepared for the large scale assay.

3.5.3 Extraction procedure

Extracts were prepared fresh daily as described by An *et al.* (1993). Fifty mg field pea flour and 5 mg grass pea flour were extracted on an orbital shaker (Lab-Line Instruments Inc.) with 20 mL of 0.009 M HCl for 1 h at room temperature. The extract was then centrifuged (Sorvall superspeed RC 2-B) at 10,000 g for 20 min at room temperature.

3.5.4 Large scale Kakade assay

Five aliquots of between 0 and 2.0 mL of supernatant solution were pipetted into test tubes. Sufficient distilled water was added to give a total volume of 2.0 mL. Two mL trypsin solution was added to each tube. To two other tubes, 2 mL of sample supernatant (Sample Blank) or 2 mL of water (Substrate Blank) was added. All the tubes were placed in a water bath at 37 ^{0}C to equilibrate. After 10 min, 5.0 mL of substrate solution was added to every tube. After a further 10 min, the reaction was stopped by the addition of 1.0 mL of 30% v/v acetic acid to each tube. Two mL of trypsin solution was added to both the Substrate and Sample Blanks. The A_{410} of the tube contents were measured in a spectrophotometer (Beckman DU 640B) using the Substrate Blank as reference. Several sets of tubes were analysed at once, starting each, in sequence, at timed intervals with the addition of the enzyme solution.

3.5.5 Calculations

All sample data values were corrected for sample blank as follows:

Corrected Sample (A_{410}) = Sample (A_{410}) - (Sample blank (A_{410}) x Sample volume/2.0 mL).

The corrected Sample (A_{410}) values were plotted vs Sample volume. Trypsin TIA was calculated from the slope of the linear part of the plot as follows:

TIA (TIU)/ mg sample = SLOPE x dilution factor/(0.01 x (total assay volume (mL)/10) x sample concentration (mg mL^{-1})). Trypsin inhibitor units were calculated from data where trypsin inhibition was in the range 40 -60% (Bacon *et al.* 1995). One TIU is defined as a decrease in A_{410} by 0.01 in 10 min using the large scale assay (Kakade *et al.* 1974, Stauffer 1990). TIA was expressed in units of trypsin inhibited (TIU) per milligram of dry matter of the sample (Kakade *et al.* 1974).

3.6 Seed coat colour measurement for grass pea

Seed coat colour of grass pea was determined in triplicate by using a Hunterlab Tristimulus Colorimeter Model D25L-9 (Hunter Associated Laboratory, Inc., McLean, Virginia) which was calibrated by a standard white plate (L = 90.67; a = -0.80; b= 1.70). Three readings were measured on each 25 g sample. Seed coat colour was expressed as mean Hunter L, a, b values. "L" represents lightness and varies from 100 for perfect white to 0 for black. The chromaticity dimension "a" represents redness when positive,

gray when zero and greenness when negative. The chromaticity dimension "b" represents yellowness when positive, gray when zero and blueness when negative.

4. RESULTS AND DISCUSSION

4.1 Field pea

Total phenolic levels in field pea differed significantly among the 17 cultivars tested (P=0.05) (Table 11). Mean values of total phenolics ranged from 162 mg kg^{-1} DM (Dry matter) (CE, catechin equivalents) for AC Tamor to 325 mg kg^{-1} DM (CE) for Richmond. The cultivars Danto Express, Orb, Patriot, and Richmond contained relatively high levels of total phenolics (between 247 to 325 mg kg^{-1} DM, CE). Baroness, Highlight, Miko, and Trump contained intermediate levels of total phenolics (between 201 to 228 mg kg^{-1} DM, CE). The other eight cultivars contained the lowest levels of total phenolics (between 162 to 193 mg kg^{-1} DM, CE). The level of total phenolics in a cultivar did not appear to be associated with the breeding program that developed it. For example, the Svalöf-Weibull cultivar Richmond had the highest level, while another Svalöf-Weibull cultivar, Carneval, was among the lowest. The cultivar x year interaction for total phenolics was significant (P=0.05). Five cultivars (AC Tamor, Baroness, Spring D, Titan, and Trump) had greater levels of total phenolics in 1993 than in 1994, while the other ten cultivars had greater levels in 1994. The effects of year, location, and the cultivar x location interaction for total phenolics were not significant (P=0.05).

Condensed tannin levels in field pea were barely detectable and did not differ among cultivars, years, locations, or their interactions (P=0.05) (Table 11). The mean value of condensed tannins in field pea was 0.013 g kg^{-1} DM (CE). Gatel and Grosjean

Table 11. Total phenolics, condensed tannins and trypsin inhibitor activity (TIA) of field pea grown at five locations in Manitoba in 1993 and 1994.

Cultivar	Breeder	Total phenolics CE (mg kg^{-1} DM)	Condensed tannins CE (g kg^{-1} DM)	TIA ($TIU\ mg^{-1}$) (DM)
AC Tamor	Agriculture Canada	162^{g}	0.014	3.31^{cd}
Baroness	Sharpes, UK	201^{defg}	0.012	7.66^{a}
Bohatyr	Selgen, Czech	191^{efg}	0.014	2.94^{cde}
Carneval	Svalof-Weibull, Sweden	192^{efg}	0.006	2.51^{efg}
Celeste	Nickerson, France	182^{efg}	0.019	2.39^{efg}
Danto	Prodana, Denmark	257^{bc}	0.010	2.22^{g}
Express	Svalof-Weibull, Sweden	280^{b}	0.008	2.76^{defg}
Fluo	Blondeau, France	167^{fg}	0.022	2.33^{fg}
Highlight	Svalof-Weibull, Sweden	211^{def}	0.011	2.95^{cde}
Miko	IHAR, Poland	217^{cde}	0.019	2.81^{cdef}
Montana	Cebeco, Netherlands	193^{efg}	0.008	2.56^{efg}
Orb	Sharpes, UK	248^{bcd}	0.010	3.35^{c}
Patriot	Svalof-Weibull, Sweden	247^{bcd}	0.027	4.08^{b}
Richmond	Svalof-Weibull, Sweden	325^{a}	0.012	3.32^{cd}
Spring D	Danisco, Denmark	167^{fg}	0.021	2.52^{efg}
Titan	Agriculture Canada	193^{efg}	0.013	3.27^{cd}
Trump	Agriculture Canada	228^{cde}	0.003	2.92^{cde}
Mean		215	0.013	3.17
CV		11.5	73.8	7.2

[a-g] Means followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P=0.05). CE catechin equivalents. DM dry matter.

(1990) reported similar results in a literature review of this topic. Condensed tannin levels were 0 to 2.6 g kg^{-1} DM in white-flowered field pea cultivars and 2.3 to 13 g kg^{-1} DM in coloured-flowered field pea cultivars. White-flowered field pea cultivars almost always contained negligible amounts of condensed tannins. Griffiths (1981) reported that levels of total phenolics in three field pea cultivars were 0.50 to 1.05% (DM) and levels of condensed tannins were 0.06 to 0.35% (DM).

Trypsin inhibitor activity (TIA) in field pea differed significantly among the 17 cultivars tested (P=0.05) (Table 11). Mean values of TIA ranged from 2.22 TIU mg^{-1} (DM) for Danto to 7.66 TIU mg^{-1} (DM) for Baroness. Baroness had significantly higher levels of TIA (7.66 TIU mg^{-1}, DM) than all other cultivars. Patriot had significantly higher levels of TIA (4.08 TIU mg^{-1}, DM) than all other cultivars except Baroness. Orb, Richmond, AC Tamor, Titan, Highlight, Bohatyr, Trump, and Miko had intermediate levels of TIA (between 2.81 to 3.35 TIU mg^{-1}, DM). The other seven cultivars had lower levels of TIA (between 2.22 to 2.76 TIU mg^{-1}, DM). The level of TIA in a cultivar did not appear to be associated with the breeding program that developed it. For instance, the Sharpes cultivar Baroness had the highest level, while another Sharpes cultivar, Orb, had a lower level of TIA than that of Baroness. The cultivar x location interaction for TIA was significant (P=0.05). Baroness had greater levels of TIA in Thornhill, Rosebank and Dauphin than in Minto and Arborg. Danto had lower levels of TIA in Arborg than in Rosebank, Dauphin, Minto and Thornhill. The effects of year, location, and the cultivar x year interaction for TIA were not significant (P=0.05).

Gatel and Grosjean (1990) summarizing the literature on this topic reported that

TIA in field pea ranged from 0.52 to 12.5 TIU mg^{-1} (DM). Carrouée (Union Nationale Interprofessionnelle Des Plantes Riches En Proteines, France, unpublished data) reported that TIA levels of Baroness, Express, Montana and Carneval were 11.1, 3.7, 3.5 and 2.9 TIU mg^{-1} (DM) which were similar to our results. Slinkard and Tyler (University of Saskatchewan, unpublished data) reported that TIA levels of Baroness and Express were 9.6 and 4.4 TIU mg^{-1} (DM) which were also similar to our results. Domoney and Welham (1992) reported that TIA levels of five field pea genotypes were 3.0 to 16.3 TIU mg^{-1} (DM). Leterme *et al.* (1990) determined TIA levels of 33 European spring field pea varieties. TIA levels ranged from 1.71 to 8.40 TIU mg^{-1} (DM). TIA level of Danto was 1.94 TIU mg^{-1} (DM) which was close to our results. TIA levels were determined in seeds from 11 field pea cultivars grown at seven European locations during the 1990 season by Bacon *et al.* (1995). Mean values of TIA were 0.93 to 2.55 TIU mg^{-1} DM. Griffiths (1984) reported that TIA levels ranged from 0.15 to 4.62 TIU mg^{-1} (DM) in 18 field pea cultivars grown at the Welsh Plant Breeding Station in the 1981 season. The reported levels of TIA of field pea are variable due to genotype, but varied methods of analysis also contribute to the variation. Considering the variability of TIA, especially for field pea, and despite the existence of accurate and reliable laboratory methods of analysis, it would be beneficial for the feed industry to have a standard rapid assay to characterize batches according to their TIA.

The correlation coefficients for total phenolics, condensed tannins, TIA, seed yield, and seed protein content in field pea are summarized in Table 12. Levels of TIA showed weakly positive correlation with seed yield, but were not correlated with total

Table 12. Correlation coefficients for total phenolics, condensed tannins, trypsin inhibitor activity (TIA), seed yield, and seed protein content in field pea (n=340).

	Total phenolics	Condensed tannins	Seed yield	Seed protein content
TIA	0.065	-0.035	0.195[a]	-0.036
Total phenolics		-0.064	-0.001	0.036
Condensed tannins			-0.420[a]	0.116[b]
Seed yield				-0.475[a]

[a] $P < 0.01$ [b] $P < 0.05$

phenolics, condensed tannins, and seed protein content. Levels of total phenolics and condensed tannins were not correlated, nor were total phenolic levels and yield, nor total phenolics and protein content. Thus levels of total phenolics and TIA can be reduced while yield is increased through breeding. Condensed tannin levels showed a negative correlation with yield, although condensed tannin levels were very low in field peas. There was a weak positive correlation between condensed tannin levels and protein content. There was a significant negative correlation between protein content and yield in field peas, i.e., an increase in seed yield leads to a decrease in seed protein content in field peas.

All cultivars of field pea tested had white flowers and white seed coats and contained very low tannin levels, similar to results summarized by Gatel and Grosjean (1990) for European cultivars. The dark seeded cultivar Sirius, which was used as an internal standard in these analyses, had higher levels (3.32 g kg^{-1} DM, CE), similar to those reported by Carrouée and Gatel (1995).

4.2 Grass pea

The means of total phenolics and condensed tannins in grass pea differed significantly among nine lines grown at two locations in 1993 and 1994 (P=0.05) (Table 13). The effects of year, location, and year or location x genotype interaction were not significant (P=0.05). The mean values of total phenolics varied widely, ranging from 868 mg kg^{-1} DM (CE) for L880388 to 2059 mg kg^{-1} DM (CE) for LS89110. The lines

Table 13. Total phenolics, condensed tannins, trypsin inhibitor activity (TIA) in grass pea lines grown at Morden and Portage la Prairie, Manitoba in 1993 and 1994.

Line	Total phenolics CE (mg kg^{-1} DM)	Condensed tannins CE (g kg^{-1} DM)	TIA (TIU mg^{-1} DM)
L880294	878^{c}	2.00^{cd}	30.79
L880388	868^{c}	0.89^{d}	28.35
L900431	1385^{bc}	3.09^{bc}	28.74
LS89026	1200^{bc}	2.15^{cd}	23.78
LS89110	2059^{a}	4.27^{ab}	26.53
LS89125	1623^{ab}	5.18^{a}	29.38
LS90040	1547^{ab}	5.06^{a}	24.36
LS90043	1603^{ab}	4.37^{ab}	27.62
LS90045	1464^{abc}	3.93^{ab}	28.07
Mean	1403	3.44	27.51
CV	9.4	2.5	9.0

[a-e] Means within the same column followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P=0.05).
CE catechin equivalents.
DM dry matter.

LS89110, LS89125, LS90040, LS90043, and LS90045 had high levels of total phenolics (between 1464 to 2059 mg kg^{-1} DM, CE). The lines L880294, L880388, L900431, and LS89026 had lower levels of total phenolics (between 868 to 1385 mg kg^{-1} DM, CE).

The mean values of condensed tannins also varied widely, ranging from 0.89 g kg^{-1} DM (CE) for L880388 to 5.18 g kg^{-1} DM (CE) for LS89125 (Table 13). The lines LS89110, LS89125, LS90040, LS90043 and LS90045 had high levels of condensed tannins (between 3.93 to 5.18 g kg^{-1} DM, CE), the lines L880294, L 880388, L900431, and LS89026 contained lower levels of condensed tannins (between 0.89 to 3.09 g kg^{-1} DM, CE).

TIA in grass pea did not differ among cultivars, years, locations, or their interactions (P=0.05) (Table 13). The mean value of TIA in grass pea was 27.51 TIU mg^{-1} (DM). Deshpande and Campbell (1992) reported that the ranges for some anti-nutrients in 100 lines of grass pea germplasm were as follows: total phenolics 86 - 891 mg kg^{-1} (DM), condensed tannins 0.0 - 4.38 g kg^{-1} (DM), TIA 133 - 174 TIU mg^{-1} (DM). Roy and Bhat (1975) reported that TIA levels in 10 grass pea cultivars ranged from 11.1 to 28.5 TIU mg^{-1} (protein). Aletor *et al.* (1994) studied 36 grass pea lines maintained in the germplasm collection at International Center for Agricultural Research in the Dry Areas (ICARDA). The reported levels of condensed tannins were 0 to 5.50 g kg^{-1} (DM) and the levels of TIA were 13.88 to 23.22 g kg^{-1} (DM). It is difficult to directly compare our results to others reported in the literature since methods of analysis and calculation of TIA differ.

The correlation coefficients for total phenolics, condensed tannins, TIA, seed

yield, and seed protein content in grass pea are summarized in Table 14. Levels of TIA in grass pea were not correlated with total phenolics, condensed tannins, seed yield, and seed protein content. Reducing TIA in grass pea should not have much effect on yield. The relationship between yield and both total phenolics and condensed tannins in grass pea showed poor correlation. The weak association between them suggests that changes in total phenolics or condensed tannins in grass pea should have very little effect on yield. Total phenolics and condensed tannin levels were positively correlated. A similar observation was made by Deshpande and Campbell (1992). Protein contents in grass pea showed poor correlation with total phenolics, condensed tannins and yield.

The correlation coefficients between seed coat colour parameters (Hunterlab L, a, b) with total phenolics, condensed tannins, TIA, seed yield, and seed protein content are summarized in Table 15. There was a highly significant negative correlation between condensed tannins and lightness of seed coat colour, i.e., grass pea lines with darker seed coats contained higher levels of condensed tannins. A similar observation was made by Deshpande and Campbell (1992). Condensed tannins in grass pea showed poor correlation with "a", but showed a weak negative correlation with "b". Total phenolics, TIA, and protein contents of grass pea seeds showed poor correlation with Hunterlab L, a and b values, except that "L" and total phenolics were negatively correlated. Seed coat colour of grass pea had very little effect on TIA and protein contents. Seed yield of grass pea showed a highly significant positive correlation with "a", a weak negative correlation with "b", and a poor correlation with "L".

Table 14. Correlation coefficients for total phenolics, condensed tannins, trypsin inhibitor activity (TIA), seed yield, and seed protein content in grass pea (n=72).

	Total phenolics	Condensed tannins	Seed yield	Seed protein content
TIA	-0.158	-0.173	-0.185	-0.096
Total phenolics		0.501[a]	-0.195	0.134
Condensed tannins			-0.266[b]	0.264[b]
Seed yield				-0.197

[a] $P < 0.01$ [b] $P < 0.05$

Table 15. Correlation coefficients between seed coat colour parameters (Hunterlab L, a, b) and total phenolics, condensed tannins, trypsin inhibitor activity (TIA), seed yield, and seed protein content in grass pea (n=72).

Hunter	Total phenolics	Condensed tannins	TIA	Seed yield	Seed protein content
L	-0.375[a]	-0.612[a]	-0.007	-0.058	-0.025
a	0.143	0.178	-0.048	0.623[a]	0.026
b	-0.147	-0.378[a]	-0.045	-0.380[a]	0.179

[a] $P<0.01$

The initial emphasis of the grass pea breeding program at Morden has centred on the reduction or elimination of the neurotoxin, β-N-oxalyl-L-α,β-diaminopropanoic acid (ODAP) from seeds. Improvement of other traits, including purification of flower and seed coat colour, received lower priority. For this reason, all grass pea lines analysed in this study were non-uniform for seed coat colour. Those with the greatest proportion of dark coloured seed coats (>95%) had high levels of condensed tannins and total phenolics. Similar observations were made in common bean (Bressani and Elias 1980) and grass pea (Deshpande and Campbell 1992). In addition to the nine grass pea lines described in the initial analysis, seed samples of six grass pea lines from the Morden breeding program grown in co-operative tests in Morden during 1993-1994 were also tested. Line LS87099 had lower levels of total phenolics and condensed tannins (219 mg kg^{-1} DM, CE and 0.20 g kg^{-1} DM ,CE respectively) than lines in the initial analysis. LS87099 had about 98% of seeds with white seed coats.

5. CONCLUSION

In summary, field pea cultivars grown in western Canada had negligible tannin contents and low levels of total phenolics which should not cause adverse nutritional effects in humans or animals. Mean condensed tannin levels of grass pea lines analysed were 265 times greater than those of the field peas tested, while total phenolic levels of grass pea were 6.5 times greater than those of field peas. These levels would be sufficiently high to be of concern in nutrition. However, grass pea lines with a high proportion of white seed coats, such as LS87099, had a similar level of total phenolics to field pea, and only 15 times greater tannin level. Levels of condensed tannins in grass pea was highly significant negative correlated with lightness of seed coat colour. Grass pea lines with darker seed coat colour contained higher levels of condensed tannins. The most recent breeding lines have uniformly white seed coats and should have tannin levels lower than LS87099.

The contents of condensed tannins and total phenolics in field pea and grass pea were primarily dependent on genotype; the effect of environment was relatively small. The correlation between the levels of these compounds and seed yield were near zero or weakly negative, thus, selection for high yield and low levels of tannins and phenolics will be possible. In addition, variability in levels of these compounds among genotypes will allow selection for lower levels of total phenolics in both crops, and lower levels of condensed tannins in grass pea.

Field pea cultivars grown in western Canada had low levels of TIA, except for

Baroness and Patriot. Mean levels of TIA in grass pea lines analysed were 8.7 times greater than those of the field pea cultivars tested. The TIA levels in grass pea are of more concern in nutrition than those in field pea. TIA in grass pea did not differ among cultivars tested. The levels of TIA in field pea were mainly dependent on genotype; the effect of environment was relatively small. The correlation between the levels of TIA and seed yield in field pea were weakly positive, thus selection of field pea cultivars for high yield and low levels of TIA will also be possible.

Field pea cultivars tested had negligible condensed tannin contents and low levels of total phenolics. Field pea with white seed coat is suitable for breeding for the animal feeding industry. Its condensed tannin and total phenolics concentrations should not cause any negative effects in humans and animals. Field pea cultivars had low levels of TIA. Field pea cultivars with low TIA levels should be used as parents to breed for low TIA cultivars. Field pea cultivars with relative high TIA levels, such as Baroness and Patriot, should be avoided.

Grass pea cultivars tested had high levels of total phenolic, condensed tannins and TIA. But grass pea with white seed coat colour had low total phenolics and condensed tannins. Grass pea with white seed coat is suitable for breeding for the animal feeding industry. Surveying germplasm collections of grass pea to identify accessions with low TIA is necessary to breed low TIA cultivars.

6. REFERENCES

Adams, R., Geissman, T. A., & Edward, J. D., 1960. Gossypol, a pigment of cottonseed. Chem. Rev. 60: 555-574.

Aletor, V. A., Allelochemicals in plant foods and feedingstuffs. Part 1. Nutritional, biochemical and physiopathological aspects in animal production. Food Chem. (Submitted).

Aletor, V. A., El-Moneim, A. A., & Goodchild, A. V., 1994. Evaluation of the seeds of selected lines of three *Lathyrus* spp for β-*N*-oxalylamino-L-alanine (BOAA), tannins, trypsin inhibitor activity and certain In-vitro characteristics. J. Sci. Food. Agric. 65: 143-151.

Ali-Khan, S. T., & Youngs, C. G., 1973. Variation in protein content of field peas. Can. J. Plant Sci. 53: 37-41.

American Association of Cereal Chemists. 1976. Determination of trypsin inhibitor activity of soy products. In " Approved Methods of the AACC." AACC Method 71-10. The Association: St. Paul. MN.

An, J., Bacon J. R., & Fenwick G. R., 1993. Standardisation of the extraction procedure for the analysis of trypsin inhibitors in pea seeds. Proc. 2nd Workshop on Antinutritional Factors in Legume Seeds. p. 31-35. Wageningen. The Netherlands.

AOAC, 1990. Official Methods of Analysis (15th Ed). The Association of Official Analytical Chemists. p. 777. Arlington. VA.

Arhelger, R. B., Broom, J. S., & Boler, R. K., 1965. Ultrastructural hepatic alterations following tannic acid administration to rabbits. Am. J. Pathol. 46: 409.

Bacon, J. R., Lambert, N., Matthews, P., Arthur, A. E., & Duchene, C. 1995. Genotype, environment and genotype x environment effects on trypsin inhibitor in peas. J. Sci. Food Agric. 67: 101-108.

Bacon, J. R., S. C. D. R. Wanigatunga, J. An, & Fenwick, G. R., 1995. A microassay for the analysis of trypsin inhibitor activity in peas. Food Chem. 52: 77-80.

Bate-Smith, E. C. & Rasper, V., 1969. Tannins of grain sorghum. Luteoforol (Leucoluteolinidin), 3', 4, 4', 5, 7 pentahydroxyflavan. J. Food Sci. 34: 203-209.

Belitz, H. -D., Lynen, F., & Weder, J. K. P., 1982. Comparative studies on the inhibitory action of some legume seeds, potato tubers, and bran against human and bovine proteinases. Z. Lebensm. Unters. Forsch. 174: 442-446.

Bell, J. M. and Wilson, A. G. 1970. An evaluation of field peas as a protein and energy source for swine rations. Can. J. Anim. Sci. 50: 15-23.

Bender, A. E. 1983. Haemagglutinins (lectins) in beans. Food Chem. 11: 309-320.

Berne, B. H., 1974. Differing methodology and equations used in quantitating immunoglobulins by radial immunodiffusion - a comparative evaluation of reported and commercial techniques. Clin. Chem. 20: 61-69.

Birk, Y., 1961. Purification and some properties of a highly active inhibitor of trypsin and alpha-chymotrypsin from soybeans. Biochim. Biophys. Acta. 54: 378-381.

Bressani, R. & Elias, L. G., 1979. The nutritional role of polyphenols in beans. In Proc. Symp. Polyphenols in Cereals and Legumes. p. 61. 35th. IFT. St. Louis. Missouri.

Bressani, R. & L. G. Elias, 1980. The nutritional role of polyphenols in beans. In: Polyphenols in Cereals and Legumes. Proc. Symp. p. 61. J. H. Hulse Ed. 36th Annual Meeting Inst. Food Technologists. St. Louis. MO.

Bressani, R., M. Flores, & L. G. Elias, 1973. Acceptability and value of food legumes in the human diet. In: Potentials of Field Beans and Other Food Legumes in Latin America. p. 17. D. Wall Ed. Centro Internacional de Agricultura Tropical. Cali. Colombia. Series Seminars No. 2E.

Brown, W. E. & Ryan, C. A., 1984. Isolation and characterization of a wound-induced trypsin inhibitor from alfalfa leaves. Biochem. 23: 3418-3422.

Bullard, R. W. & Elias, D. J., 1979. Sorghum polyphenols and bird resistance. In Proc. Symp. Polyphenols in Cereals and Legumes. p. 43. IFT. St. Louis. Missouri.

Burns, R. E. 1971. Method for estimation of tannin in grain sorghum. Agron. J. 63: 511-512.

Burns, R. E., 1963. Methods of tannin analysis for forage crop evaluation. Ga. Agric. Exp. Stn. Tech. Bull. N. S. 32: 5.

Calderon, P., Van Buren, J., & Robinson, W. B., 1968. Factors influencing the formation of precipitates and hazes by gelatin on condensed and hydrolysable tannins. J. Agric. Food Chem. 16: 479-482.

Campbell, C. G. 1989. Improvement of *Lathyrus sativus*. In: The grass pea: Threat and promise. p. 139-146. P. S. Spencer. Ed. Third World Medical Research Foundation. New York. NY.

Cannon, C. G., 1955. The interactions and structure of the CONH-group in amides and

polyamides. Mikrochim. Acta. 555.

Carrera, S., Mitjavila, S., & Derache, R., 1973. Effect of tannic acid on the digestive availability of vit. B_{12} in rats. Ann. Nutr. Aliment. 27: 73.

Carrouée, B. & Gatel, F., 1995. Peas: utilization in animal feeding. Interprofessional national union for protein rich crops. p. 7. Paris. France.

Castell, A., Cliplef, R. L., Briggs, C. J., Campbell, C. G., & Bruni, J. E., 1994. Evaluation of *Lathyrus sativus* as an ingredient in pig starter and grower diets. Can. J. Anim. Sci. 74: 529-539.

Chan, J. & de Lumen, B. O., 1982. Biological effects of isolated trypsin inhibitor from winged bean (*Psophocarpus tetragonolobus*) on rats. J. Agric. Food Chem. 30: 46-50.

Chandna, M., Sood, A. & Matta, N. K. 1991. Variation and inheritance studies on seed proteins of *Lathyrus sativus* L. In Proc. Golden Jubilee Celebrations Symposium on Grain Legumes. 9-11 Feb. Indian Society of Genetics and Plant Breeding. New Delhi. India.

Chang, S. I. & Fuller, H. L., 1964. Effect of tannin content of grain sorghum on their feeding value on growing chicks. Poult. Sci. 43: 30.

Chase, T. Jr. & Shaw, E., 1970. Titration of trypsin, plasmin, and thrombin with p-nitro-phenyl-p'-guanidinobenzoate HCl. In Methods in Enzymology. Vol. XIX. p. 20-27. Colowick, S. P. and Kaplan, N. O. Ed. Proteolytic Enzymes. Academic Press. New York.

Chavan, J. K., Kadam, S. S., and Salunkhe, D. K., 1981. Changes in tannin, free amino

acids, reducing sugars, and starch during seed germination of low and high tannin cultivars of sorghum. J. Food Sci. 46: 638-639.

Chowdhury, S. D. 1988. Lathyrism in poultry - A review. World's Poult. Sci. 44: 7-16.

Clausen, J., 1969. Immunochemical techniques for the identification and estimation of chromolecules. In " Laboratory Techniques in Biochemistry and Molecular Biology". Vol. 1. p. 399-572. Work, T. S., Work, E., Ed. North-Holland: Amsterdam.

Coan, M. H., Roberts, R. C., & Travis, J., 1971. Human pancreatic enzymes. Isolation and properties of a major form of chymotrypsin. Biochem. 10: 2711-2717.

Coan, M. H. & Travis, J., 1972. Human pancreatic enzymes: properties of two minor forms of chymotrypsin. Biochim. Biophys. Acta. 268: 207-211.

Cravioto B. R. O., Guzman, G. J., & Massieu H. G., 1951. Mejoramiento del valor nutritivo de la tortilla. III. Efecto del calentamiento con hidroxido de calcio sobre la actividad antitripsica de la soja. Ciencia (Mexico City). 11: 81-82.

Deshpande, S. S. & C. G. Campbell, 1992. Genotype variation in BOAA, condensed tannins, phenolics and enzyme inhibitors of grass pea (*Lathyrus sativus*). Can. J. Plant Sci. 72: 1037-1047.

Doell, B. H., Ebden, C. J., & Smith, C. A., 1981. Trypsin inhibitor activity of conventional foods which are part of the British diet and some soya products. Qual. Plant Foods Hum. Nutr. 31: 139-150.

Domoney, C. & Welham, T., 1992. Trypsin inhibitors in *Pisum*: variation in amount and pattern of accumulation in developing seed. Seed Sci. Res. 2: 147-154.

Domoney, C., Welham, T., & Sidebottom, C., 1993. Purification and characterization of *Pisum* seed trypsin inhibitors. J. Exp. Bot. 44: 701-709.

Eggum, B. O. & Christensen, K. D., 1975. Influence of tannin on protein utilization in feed stuffs with special reference to barley. In Breeding for Seed Protein Improvement Using Nuclear Techniques. p. 135-143. IAEA. Vienna.

Elias, L. G., Hernandez, M., & Bressani, R., 1976. The nutritive value of precooked legume flours processed by different methods. Nutr. Rep. Int. 14: 385-403.

Elias, L. G. & R. Bressani, 1974. Nutritional factors affecting the consumption of leguminous seeds. Arch. Latinoamer. Nutr. 24: 365.

Elkowicz, K. and Sosulski, F. W. 1982. Anti-nutritive factors in eleven legumes and their air-classified protein and starch fractions. J. Food Sci. 47: 1301-1304.

Endres, H. & Hormann, H., 1963. Preparative and analytical separation of organic compounds by means of chromatography on polyamide. Angew. Chem. Int. Edit. 2: 254.

Erdman, J. W. Jr. and Forbes, R. M. 1977. Mineral bioavailability from phytate-containing foods. Food Prod. Dev. 11: 46-48.

Eriksson, C. E. 1967. Pea lipoxidase, distribution of enzyme and substrate in green peas. J. Food Sci. 32: 438-441.

Feeney, R. E., Means, G. E., & Bigler, J. C., 1969. Inhibition of human trypsin, plasmin and thrombin by naturally occurring inhibitors of proteolytic enzymes. J. Biol. Chem. 244: 1957-1960.

Feinstein, G., Hofstein, R., Koifmann, J., & Sokolovsky, M., 1974. Human pancreatic

proteolytic enzymes and protein inhibitors. Isolation and molecular properties. Eur. J. Biochem. 43: 569-581.

Fenwick, D. E. and Oakenfull, D. 1983. Saponin content of food plants and some prepared foods. J. Sci. Food Agric. 34: 186-191.

Figarella, C., Negri, G. A., & Guy, O., 1975. The two human trypsinogens. Inhibition spectra of the two human trypsins derived from their purified zymogens. Eur. J. Biochem. 53: 457-463.

Folin, O. & Ciocalteu, V., 1927. On tyrosine and tryptophan determinations in proteins. J. Biol. Chem. 73: 627.

Forsyth, W. G. C. & Quesnel, V. C., 1957a. Cacao polyphenolic substances. IV. The anthocyanin pigments. Biochem J. 65: 177.

Forsyth, W. G. C. & Quesnel, V. C., 1957b. Intermediates in the enzymic oxidation of catechol. Biochem. Biophys. Acta. 25: 155.

Freed, R. C. & Ryan, D. S., 1978. Changes in Kunitz trypsin inhibitor during germination of soybeans: an immunoelectrophoresis assay system. J. Food Sci. 43: 1316-1319.

Fritz, H., Seemueller, H., & Tschesche, H., 1984. Proteinases and their inhibitors, general review. In: Methods of Enzymatic Analysis. Enzymes 3: Peptidases, Proteinases and Their Inhibitors. Vol. V. p. 74-98. Verlag Chemie Ed. 3rd Ed. Weinheim. FRG.

Fuller, H. L., Potter, D. K., and Brown, A. R., 1966. The feeding value of grain sorghum in relationship to their tannin content. Tech. Bull. N. S. 65th. p176. Georgia Agric. Exp. Sta. Athens.

Gad, S. S., El-Zalaki, M. E., Mohamed, M. S., and Mohasseb, S. Z. 1982. Oxalate content of some leafy vegetables and dry legumes consumed widely in Egypt. Food Chem. 8: 169-177.

Gatehouse, A. M. R. & Boulter, D. 1983. Assessment of the antimetabolic effects of trypsin inhibitors from cowpea (*Vigna unguiculata*) and other legumes on development of the bruchid beetle *callosobruchus maculatus.* J. Sci. Food Agric. 34: 345-350.

Gatehouse, A. M. R., Gatehouse, J. A., Dobie, P., Kilminster, A. M. & Boulter, D. 1979. Biochemical basis of insect resistance in *Vigna unguiculata.* J. Sci. Food Agric. 30: 948-958.

Gatel, F. & Grosjean, F., 1990. Composition and nutritive value of peas for pigs: a review of European results. Livestock Prod. Sci. 26:155-175.

Gatel, F., Grosjean, F., and Castaing, J. 1989. Utilisation par le porc charcutier de régimes à teneur élevée en pois de printemps. Journées Rech. Porcine en France 21: 69-74.

Gee, J. M. and Johnson, I. T. 1988. Interactions between haemolytic saponins, bile salts and small intestinal mucosa in the rat. J. Nutr. 118: 1391-1397.

Glick, Z. & Joslyn, M. A., 1970. Food intake, depression, and other metabolic effects of tannic acid in the rat. J. Nutr. 100: 509.

Goldstein, J. L. & Swain , T. 1963. Changes in tannins in ripening fruits. Phytochem. 2: 371.

Griebel, C., 1950. Erkrankungen durch Bohnenflocken (*Phaseolus vulgaris* L.) und Platterbsen (*Lathyrus tingitanus* L.). Z. Lebensm. Unters. Forsch. 90: 191-197.

Griffiths, D. W., 1981. The polyphenolic content and enzyme inhibitory activity of testas from bean (*Vicia faba*) and pea (*Pisum spp.*) varieties. J. Sci. Food. Agric. 32: 797-804.

Griffiths, D. W. 1984. The trypsin and chymotrypsin inhibitor activities of various pea (*Pisum spp.*) and field bean (*Vicia faba*) cultivars. J. Sci. Food Agric. 35: 481-486.

Griffiths, D. W. & Jones, D. I. H., 1977. Cellulase inhibition by tannins in the testa of field beans (*Vicia faba*). J. Sci. Food Agric. 28: 983-989.

Griffiths, D. W. & Moseley, G., 1980. The effects of diets containing field beans of high or low polyphenolic content on the activity of digestive enzymes in the intestines of rats. J. Sci. Food Agric. 31: 255-259.

Grosjean, F., Bourdon, D., Kiener, T., Castaing, J., and Gatel, F. 1991. Valeur alimentaire pour les porcs des pois français et importés. Journées Rech. Porcine en France. 23: 53-60.

Guillaume, J. & Bellec, R., 1977. Use of field beans (*Vicia faba* L.) in diets for laying hens. Br. Poult. Sci. 18: 573.

Gunn, R. A., Taylor, P. R., & Gangarosa, E. J., 1980. Gastrointestinal illness associated with consumption of a soy protein extender. J. Food Prot. 43: 525-527, 529.

Gustavson, K. H., 1954. Interaction of vegetable tannins with polyamides as proof of the dominant function of the peptide bond of collagen for its binding of tannin. J. Polymer Sci. 12: 317.

Hagerman , A. E., & Butler, L. G. 1978. Protein precipitation method for the quantitative

determination of tannins. J. Agric. Food. Chem. 26: 809.

Harris, H. B. & Burns, R. E., 1970. Influence of tannin content on preharvest seed germination in sorghum. Agron J. 62: 835-836.

Harwood, J. P., Ausman, L. M., King, N. W., Sehgal, P. K., Nicolosi, R. J., Liener, I. E., Donatucci, D., & Tarcza, J., 1986. Effect of long-term feeding of soy-based diets on the pancreas of *Cebus* monkeys. In Nutritional and Toxicological Significance of Enzyme Inhibitors in Food. 199th. p.223-237. Friedman, M. Ed. Advances in Experimental Medicine and Biology. Plenum Press. New York.

Haslam, E. 1989. Plant Polyphenols-Vegetable Tannins Revisited. Cambridge University Press. Cambridge. U. K.

Haydar, M., & Hadziyev, D., 1973. Pea lipids and their oxidation on carbohydrate and protein matrices. J. Food Sci. 38: 772-778.

Hove, E. L. and King, S. 1979. Trypsin inhibitor contents of lupin seeds and other grain legumes. New Zealand J. Agric. Res. 22: 41-42.

Iyer, V., Salunkhe, D. K., Sathe, S. K., & Rockland, L. B., 1980. Quick-cooking beans (*Phaseolus vulgaris* L.): II. Phytates, oligosaccharides, and antienzymes. Qual. Plant. Plant Foods Hum. Nutr. 30: 45-52.

Jaffé, W. G., Moreno, R., and Wallis, V. 1973. Amylase inhibitors in legume seeds. Nutr. Rep. Int. 7: 169-174.

Jambunathan, R. & Mertz, E. T. , 1973. Relationship between tannin levels, rat growth, and distribution of proteins in sorghum. J. Agric. Food Chem. 21: 692-696.

Johnson, I. T., Gee, J. M., Price, K., Curl, C., and Fenwick, G. R. 1986. Influence of

saponin on gut permeability and active nutrient transport *in vitro*. J Nutr. 116: 2270-2277.

Jones, G. A., T. A. McAllister, A. D. Muir, & K. J. Cheng, 1994. Effects of sainfoin (*Onobrychis viciifolia* Scop) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Appl. Environ. Microbiol. 60: 1374.

Joslyn, M. A. & Glick, Z., 1969. Comparative effects of gallotannic acid and related phenolics on the growth of rats. J. Nutr. 98: 119.

Kakade, M. L. & Evans, R. J., 1965. Growth inhibition of rats fed navy bean fractions. J. Agric. Food Chem. 13: 450-452.

Kakade, M. L., Hoffa, D. E., & Liener, I. E., 1973. Contribution of trypsin inhibitors to the deleterious effects of unheated soybeans fed to rats. J. Nutr. 103:1772-1778.

Kakade, M. L., Rackis, J. J., McGhee, J. E. and Puski, G., 1974. Determination of trypsin inhibitor activity of soy products: a collaborative analysis of an improved procedure. Cereal Chem. 51: 376-382.

Kao, C. & Robinson, R. J. 1978. Nutritional aspects of fermented foods from chickpea, horsebean, and soybean. Cereal Chem. 55: 512-517.

Kapadia, G. J., Chung, E. B., Ghosh, B., Shukla, Y. N., Basale, S. P., Morton, J. F., & Pradhan., S. N., 1978. Carcinogenicity of some folk medicinal herbs in rats. J. Natl. Cancer Inst. 60: 683.

Kassell, B., 1970. Proteolytic enzyme inhibitors from *Ascaris lumbricoides*. In Methods in Enzymology. Vol. XIX. p. 872-878. G. E. Perlman and L. Lorand. Ed. Academic Press. Inc. New York. N. Y.

Kassell, B. & Williams, M. J., 1976. Specificities and some properties of plant protease inhibitors. In Handbook of Biochemistry and Molecular Biology. Proteins-Vol. II. p. 586-604. Fasman, G. D., Ed. 3rd ed. CRC Press: Cleveland. Ohio.

Khan, M. A., Jacobsen, I. & Eggum, B. O., 1979. Nutritive value of some improved varieties of legumes. J. Sci. Food Agric. 30: 395-400.

Krogdahl, A. & Holm, H., 1979. Inhibition of human and rat pancreatic proteinases by crude and purified soybean proteinase inhibitors. J. Nutr. 109: 551-558.

Krogdahl, A. & Holm, H., 1983. Pancreatic proteinases from man, trout, rat, pig, cow, chicken, mink and fox. Enzyme activities and inhibition by soybean and lima bean proteinase inhibitors. Comp. Biochem. Physiol. 74B: 403-409.

Kumar, V. and Kapoor, A. C. 1983. Availability of zinc as affected by phytate. Nutr. Rep. Int. 28: 103-111.

Kunitz, M., 1947. Crystalline soybean trypsin inhibitor. II. General properties. J. Gen. Physiol. 30: 291-310.

Laskowski, M., & Kato, I., 1980. Protein inhibitors of proteinases. Ann. Rev. Biochem. 49: 593-626.

Lease, E. J. & Mitchell, J. H., 1940. A study of the tannins of *Lespedaza sericea*. So. Car. Agric. Exp. Sta. Ann. Rep. 53: 710.

Lehnhardt, W. L. & Dills, H G., 1984. Analysis of trypsin inhibitors in soy products: evaluation of methodology and improvements. J. Am. Oil Chem. Soc. 61: 691 (Abstr. 225).

Leterme, P., Beckers, Y., & Thewis, A., 1990. Trypsin inhibitors in peas: varietal effect

and influence on digestibility of crude protein by growing pigs. Anim. Feed Sci. Tech. 29: 45-55.

Lewis, J. H. & Taylor, F. H. L., 1947. Comparative utilization of raw and autoclaved soy bean protein by the human. Proc. Soc. Exp. Biol. Med. 64: 85-87.

Liener, I. E. & Kakade, M. L., 1980. Protease inhibitors. In " Toxic Constituents of Plant Foodstuffs". p. 7-71. Liener, I. E., Ed. 2nd ed. Academic Press. New York.

Lindgren, E. 1975. The nutritive value of peas and field beans for hens. Swedish J. Agric. Res. 5: 159-161.

Linscheer, W. G., Smith, N., & Mahany, D., 1980. Physiologic effects of a trypsin inhibitor in soya protein on trypsin activity, and on digestion and absorption of protein in man. Gastr. 78: 1209 (Abstr.).

Lovegren, N. V., Fisher, G. S., Legendre, M. G., and Schuller, W. H. 1979. Volatile constituents of dried legumes. J. Agric. Food Chem. 27: 851-853.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Mallory, P. A. & Travis, J., 1975. Inhibition spectra of the human pancreatic endopeptidases. Am. J. Clin. Nutr. 28: 823-830.

Manage, C., Joshi, A., and Sohonie, K. 1972. Toxicity to rats and mice of purified haemagglutinins from four Indian legumes. Toxicon. 10: 89-90.

Manan, F., Hussain, T., Alli, I., and Iqbal, P. 1987. Effect of cooking on phytic acid content and nutritive value of Pakistani peas and lentils. Food Chem. 23: 81-87.

Marquardt, R. R., Ward, T., Campbell, L. D., & Cansfield, P. E., 1977. Purification,

identification, and characterization of growth inhibitor in faba beans (*Vicia faba* L. Var minor). J. Nutr. 107: 1313.

Maxson, E. D., Clark, L. E., Rooney, L. W. & Johnson, J. W. 1972. Factors affecting the tannin content of sorghum grain as determined by two methods of tannin analysis. Crop. Sci. 12: 233-235.

Maxson, E. D. & Rooney, L. W., 1972. Evaluation of methods for tannin analysis in sorghum grain. Cereal Chem. 49: 719-729.

Maxson, E. D., Rooney, L. W., Lewis, R. W., Clark, L. E. & Johnson, J. W., 1973. The relationship between tannin content, enzyme inhibition, rat performance, and characteristics of sorghum grain. Nutr. Rep. Int. 8: 145.

Ma Yu, & Bliss, F. A., 1978. Tannin content and inheritance in common bean. Crop. Sci. 18: 201-204.

McMillian, W. W., Wiseman, B. R., Burns, R. E., Harris, H. B., & Greene, G. L., 1972. Bird resistance in diverse germplasm of sorghum. Agron. J. 64: 821-822.

McPhalen, C. A., Svendsen, I., Jonassen, I., & James, M. N. G., 1985. Crystal and molecular structure of chymotrypsin inhibitor 2 from barley seeds in complex with subtilisin. Novo. Proc. Natl. Acad. Sci. USA. 82: 7242-7246.

Nguyen, T. H. and Kermorgant, J. 1983. "True" amino acid availability (T.A.A.A.) of peas and soybean oil meals in cecectomized cockerels. In Proceedings of the 4th European Symposium on Poultry Nutrition Tours. p. 164. Larbier, M. Ed. France. World's Poultry Science Association.

Nitsan, Z., 1971. *Vicia faba* beans vs. soybean meal as a source of protein. J. Sci. Food

Agric. 22: 252-255.

Oakenful, D. G. and Sidhu, G. S. 1983. A physico-chemical explanation for the effects of dietary saponins on cholesterol and bile salt metabolism. Nutr. Rep. Int. 27: 1253-1259.

Oh, H. I., Hoff, J. E. , Armstrong, G. S. and Lawrence, A. H., 1980. Hydrophobic interaction in tannin protein complexes. J. Agric. Food Chem. 28: 394-398.

Oomah, B. D., E. O. Kenaschuk, & G. Mazza, 1995. Phenolic acids in flaxseed. J. Agric. Food Chem. 43: 2016-2019.

Ouchterlony, O., 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. 26: 507-515.

Padhye, V. W. & Salunkhe, D. K., 1980. Studies on black gram (*Phaseolus mungo* L.) trypsin inhibitor. J. Food Biochem. 4: 119-138.

Pearce, G., McGinnis, J., & Ryan, C. A., 1979. Utilization by chicks of half-cystine from native and denatured proteinase inhibitor protein from potatoes. Proc. Soc. Exp. Biol. Med. 160: 180-184.

Price, K. R., Curl, C. L., and Fenwick, G. R. 1986. The saponin content and sapogenol composition of the seed of 13 varieties of legume. J. Sci. Food Agric. 37: 1185-1191.

Price, M. L. & Butler, L. G., 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. J. Agric. Food Chem. 25: 1268-1273.

Price, M. L., Butler, L. G., Rogler, J. G., and Featherston, W. R. 1979. Overcoming the

nutritionally harmful effects of tannin in sorghum grain by treatment with inexpensive chemicals. J. Agric. Food Chem. 27: 441-445.

Price, M. L., Hagerman, A. E., & Butler, L. G., 1980. Tannin content of cow peas, chick peas, pigeon peas, and mung beans. J. Agric. Food Chem. 28: 459-461.

Price, M. L., Van Scoyoc, S., & Butler, L. G. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem. 26: 1214-1218.

Prine, G. M., Lutrick, M. C., & Lipscomb, R. W., 1967. Old crops have new outlook. Sunshine St. Agric. 12: 12.

Ramanujan, S., Sethi, K. L., & Rao, S. L. N. 1980. Stability of neurotoxin in khesari. Indian J. Genet. 40: 300-304.

Rackis, J. J., McGhee, J. E., & Booth, A. N., 1975. Biological threshold levels of soybean inhibitors by rat bioassay. Cereal Chem. 52: 85-92.

Rackis, J. J., Wolf, W. J., & Baker, E. C., 1986. Protease inhibitors in plant foods: content and inactivation. In Nutritional and Toxicological Significance of Enzyme Inhibitors in Food. 199th. p. 299-347. Friedman, M. Ed. Advances in Experimental Medicine and Biology. Plenum Press. New York.

Rao, N. M. & Pattabiraman, T. N., 1985. Natural plant enzyme inhibitors: comparison of the action of tuber protease inhibitors on animal pancreatic systems. Indian J. Biochem. Biophys. 22: 373-375.

Rascon, A., Seidl, D. S., Jaffe, W. G., & Aizman, A., 1985. Inhibition of trypsins and chymotrypsins from different animal species: a comparative study. Comp.

Biochem. Physiol. 82B: 375-378.

Read, R. J. & James, M. N. G., 1986. Introduction to the protein Inhibitors: X-ray crystallography. In Proteinase Inhibitors. p. 301-336. Barrett, A. J. and Salvesen, G. S. Ed. Elsevier: Amsterdam.

Richardson, M., 1980. Protein inhibitors of enzymes. Food Chem. 6: 235-253.

Richardson, M., 1977. The proteinase inhibitors of plants and micro-organisms. Phytochem. 16: 159-169.

Ringrose, R. C. & Morgan, C. L., 1940. The nutritive value of lespedeza for poultry feeding. South Car. Exp. Sta. Ann. Rep. 53: 91.

Rooney, L. W., Blakely, M. E., Miller, F. R., & Rosenow, D. T., 1979. Factors affecting the polyphenols of sorghum and their development, and location in the sorghum kernel. In Proc. Symp. Polyphenols in Cereals and Legumes. p. 25. IFT. St. Louis. Mo.

Ross, S. M., & Spencer, P. S. 1989. Beta-N oxalylamino-L-alanine: potential mechanisms of neurotoxicity. In The Grass Pea: Threat and Promise. p. 97-108. Spencer, P. S. Ed. New York. Third World Medical Research Foundation.

Rostagno, H. S., Featherston, W. R., and Rogler, J. C. 1973. Studies on the nutritional value of sorghum grains with varying tannin contents for chicks. Poult. Sci. 52: 765-772.

Rotter, R. G., Marquardt, R. R., & Campbell, C. G., 1991. The nutritional value of low lathrogenic lathyrus (*Lathyrus sativus*) for growing chicks. British Poult. Sci. 32: 1055-1067.

Roy, D. N., 1972. Biological effects of the trypsin inhibitor isolated from *Lathyrus sativus* seeds. Curr. Sci. 41: 180-181.

Roy, D. N., 1981. Toxic amino acids and proteins from lathyrus plants and other leguminous species. A Literature Review. Nat. Abst. Rev. Series A. 51: 691-707.

Roy, D. N., 1980. Trypsin inhibitor from *Lathyrus sativus* seeds: final purification, separation of protein components, properties, and characterization. J. Agric. Food Chem. 28: 48-54.

Roy, D. N. & Bhat, R. V., 1975. Variation in neurotoxin, trypsin inhibitors and susceptibility to insect attack in varieties of *Lathyrus sativus* seeds. Environ. Physiol. Biochem. 5: 172-177.

Rungruangsak, K., Tosukhowong, P., Panijpan, B., & Vimokesant, S. L. 1977. Chemical interactions between thiamins and tannic acid. I. Kinetics, oxygen dependence, and inhibition by ascorbic acid. Am. J. Clin. Nutr. 30: 1680.

Ryan, C. A., 1967. Quantitative determination of soluble cellular proteins by radial diffusion in agar gels containing antibodies. Anal. Biochem. 19: 434-440.

Sarkar, S. K. & Howarth, R. E., 1976. Specificity of the vanillin test for flavanols. J. Agric. Food Chem. 24: 317-320.

SAS Institute, Inc., 1990. SAS/STAT User's Guide, version 6, 4th ed.; SAS Institute: Cary, NC. USA.

Sathe, S. K. & Salunkhe, D. K. 1984. Technology of removal of unwanted components of dry beans. CRC Crit. Rev. Food Sci. Nutr. 21: 263-287.

Savage, G. P., & Deo, S., 1989. The nutritional value of peas (*Pisum sativum*). A

literature review. Nutr. Abst. Rev. Series A: 65-87.

Scalbert, A., 1991. Antimicrobial properties of tannins. Phytochem. 30: 3875.

Sharon, W. and Lis, H. 1972. Lectin: Cell-agglutinating and sugar-specific proteins. Sci. 177: 949-959.

Sosulski, F. W. 1979. Organoleptic and nutritional effects of phenolic compounds on oilseed protein products: A review. J. Am. Oil Chem. Soc. 56: 711-715.

Sosulski, F. W. & Dabrowski, K. J., 1984. Composition of free and hydrolyzable phenolic acids in the flours and hulls of ten legume species. J. Agric. Food Chem. 32: 131-133.

Spencer, P. S., Roy, D. N., Ludolph, D, Hugon, J., Dwivedi, M. P., & Schaumber, H. H., 1986. Lathyrism: Evidence for the role of the neuroexcitatory amino acid BOAA. Lancet. II. 1066-1067.

Spencer, P. S., D. N. Roy, V. S. Palmer, & M. P. Dwivedi, 1986. *Lathyrus sativus*: The need for a strain lacking human and animal neurotoxic properties. In: Lathyrus and Lathyrism. Kaul, A K. and Combes, D. Ed. Third World Medical Research Foundation. New York. NY.

Srikantia, S. G., 1976. Chemistry and nutritive value of sorghum, maize, and millets. Proc. Symp. Production. Processing and Utilization of Maize, Sorghum, and Other Millets. p. 31. CFTRI. Mysore. India.

Stauffer, C. E., 1990. Measuring trypsin inhibitor in soy meal: suggested improvements in the standard method. Cereal Chem. 67: 296-302.

Steffens, R., Fox, F. R., & Kassell, B. 1978. Effect of trypsin inhibitors on growth and

metamorphosis of corn borer larvae *Ostrinia nubilalis* (Hubner). J. Agric. Food Chem. 26: 170-174.

Strumeyer, D. H. & Malin, , M. J., 1975. Condensed tannins in grain sorghum. Isolation, fractionation, and characterization. J. Agric. Food Chem. 23: 909-914.

Struthers, B. J. & MacDonald, J. R., 1983. Comparative inhibition of trypsins from several species by soybean trypsin inhibitors. J. Nutr. 113: 800-804.

Suschetet, M., 1975. Influence of tannic acid on the hepatic content of Vit. A in rats fed a Vitamin A-containing diet or a vitamin A-deficient diet. C. R. Seances Soc. Biol. Fil. 169: 970.

Swain, T., 1965. The tannins. In Plant Biochemistry. Bonner, J. and Varner, E. V. Ed. Academic Press, New York.

Tamir, M. & Alumot, E., 1969. Inhibition of digestive enzymes by condensed tannins from green and ripe carobs. J. Sci. Food Agric. 20: 199-202.

Tamir, M. & Alumot, E., 1970. Carob tannins-growth depression, and levels of insoluble nitrogen in the digestive tract of rats. J. Nutr. 100: 573.

Tannous, R. I. and Ullah, M. 1969. Effects of autoclaving on nutritional factors in legume seeds. Trop. Agric. 46: 123-129.

Tauber, H., Kershaw, B. B., & Wright, R. D., 1949. Studies on the growth inhibitor fraction of lima beans and isolation of a crystalline heat-stable trypsin inhibitor. J. Biol. Chem. 179: 1155-1161.

Taverner, M. R. and Curic, D. M. 1983. The influence of hind-gut digestion on measures of nutrient availability in pig feeds. In Feed information and animal production.

Proceedings of the 2nd Symposium of the International Network of Feed Information Centres. p. 295-298. Farnham Royal. Slough. UK. Commonwealth Agricultural Bureaux.

Trautmann, R., Cowan, K. M., & Wagner, G. G., 1971. Data processing for radial immunodiffusion. Immunochem. 8: 901-916.

Valdebouze, P., Bergeron, E., Gaborit, T., & Delort-Laval, J., 1980. Content and distribution of trypsin inhibitors and hemagglutinins in some legume seeds. Can. J. Plant Sci. 60: 695-701.

Vogt, H. 1983. Nutritional values of peas in chickens. In Proceedings of the 4th European Symposium on Poultry Nutrition Tours. p.132-137. Larbier, M. Ed. France. World's Poultry Science Association.

Vohra, P., Kratzer, F. H., & Joslyn, M. A. 1966. The growth depressing and toxic effects of tannins on chicks. Poult. Sci. 45: 135.

Walker, R. C., Ed. 1983. Trypsin inhibitor activity. In " Official Methods and Recommended Practices of the American Oil Chemists' Society". 34th ed. AOCS: Champaign. IL. A. O. C. S. Official Method Ba 12-75.

Weder, J. K. P., 1986. Inhibition of human proteinases by grain legumes. In Nutritional and Toxicological Significance of Enzyme Inhibitors in Food. 199th. p. 239-279. Friedman, M. Ed. Advances in Experimental Medicine and Biology. Plenum Press. New York.

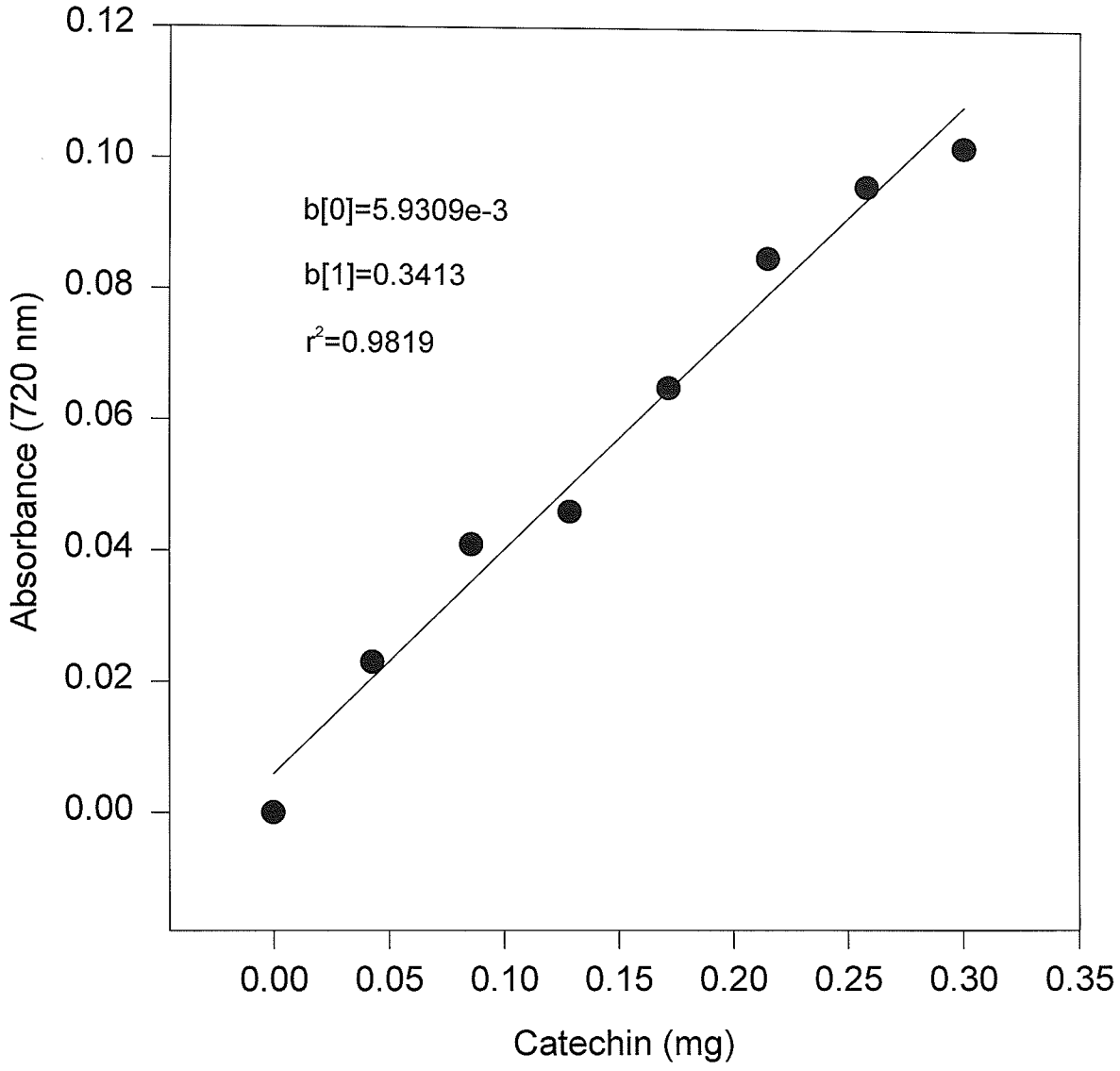
Weiel, J. & Hapner, K. D., 1976. Barley protease inhibitors - a possible role in grasshopper control. Phytochem. 15: 1885-1887.

Wilson, B. J., McNab, J. M., & Bentley, H., 1972. The effect on chick growth of a trypsin inhibitor isolate from the field bean (*Vicia faba* L.). Br. Poult. Sci. 13: 521-523.

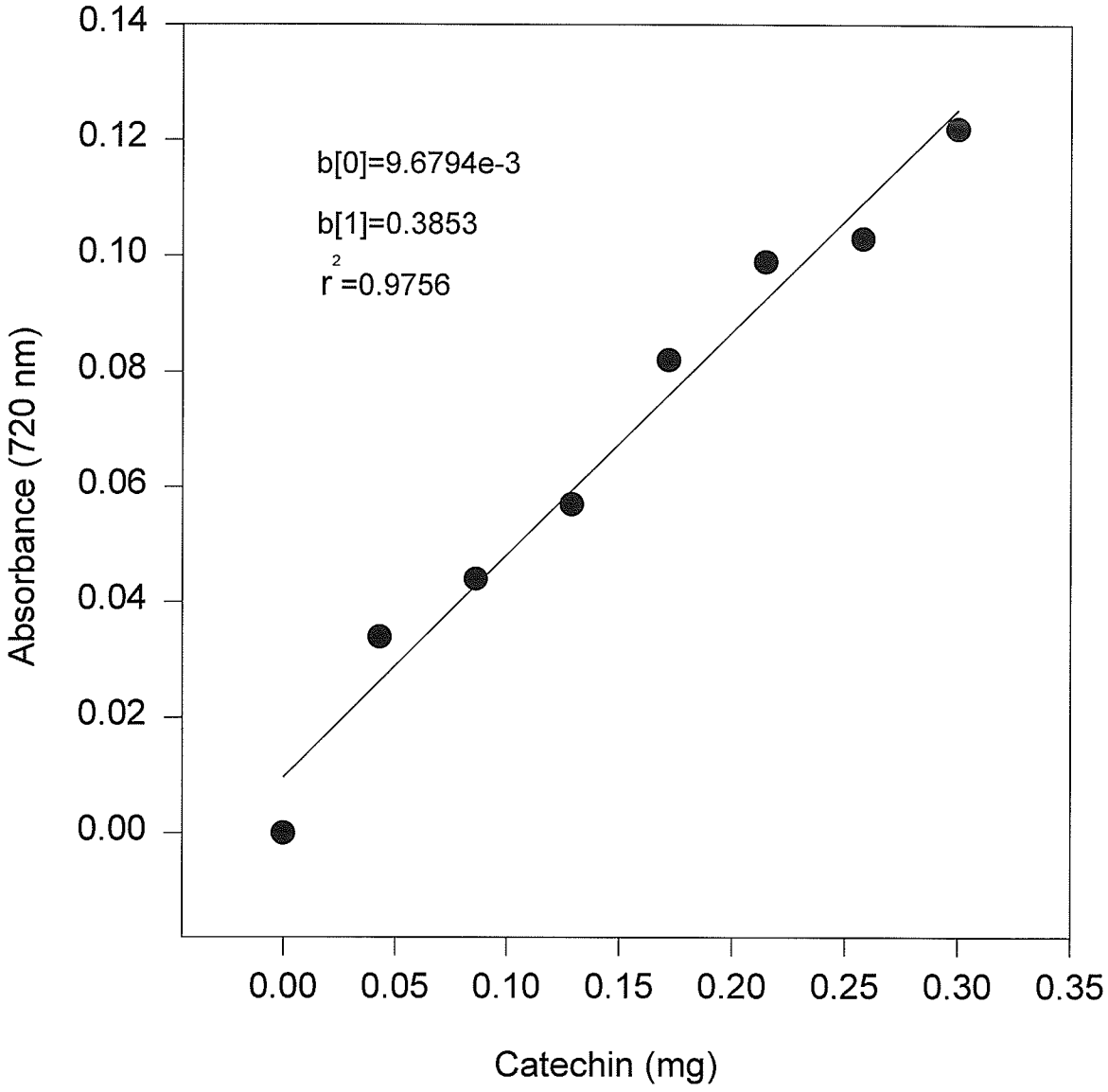
Wilson, K. A., 1980. The release of protease inhibitors from legume seeds during germination. Phytochemistry. 19: 2517-2519.

Yavelow, J. Finlay, T. H., Kennedy, A. R., & Troll, W., 1983. Bowman-Birk soybean protease inhibitor as an anticarcinogen. Cancer Res. (Suppl.). 43: 2454s-2459s.

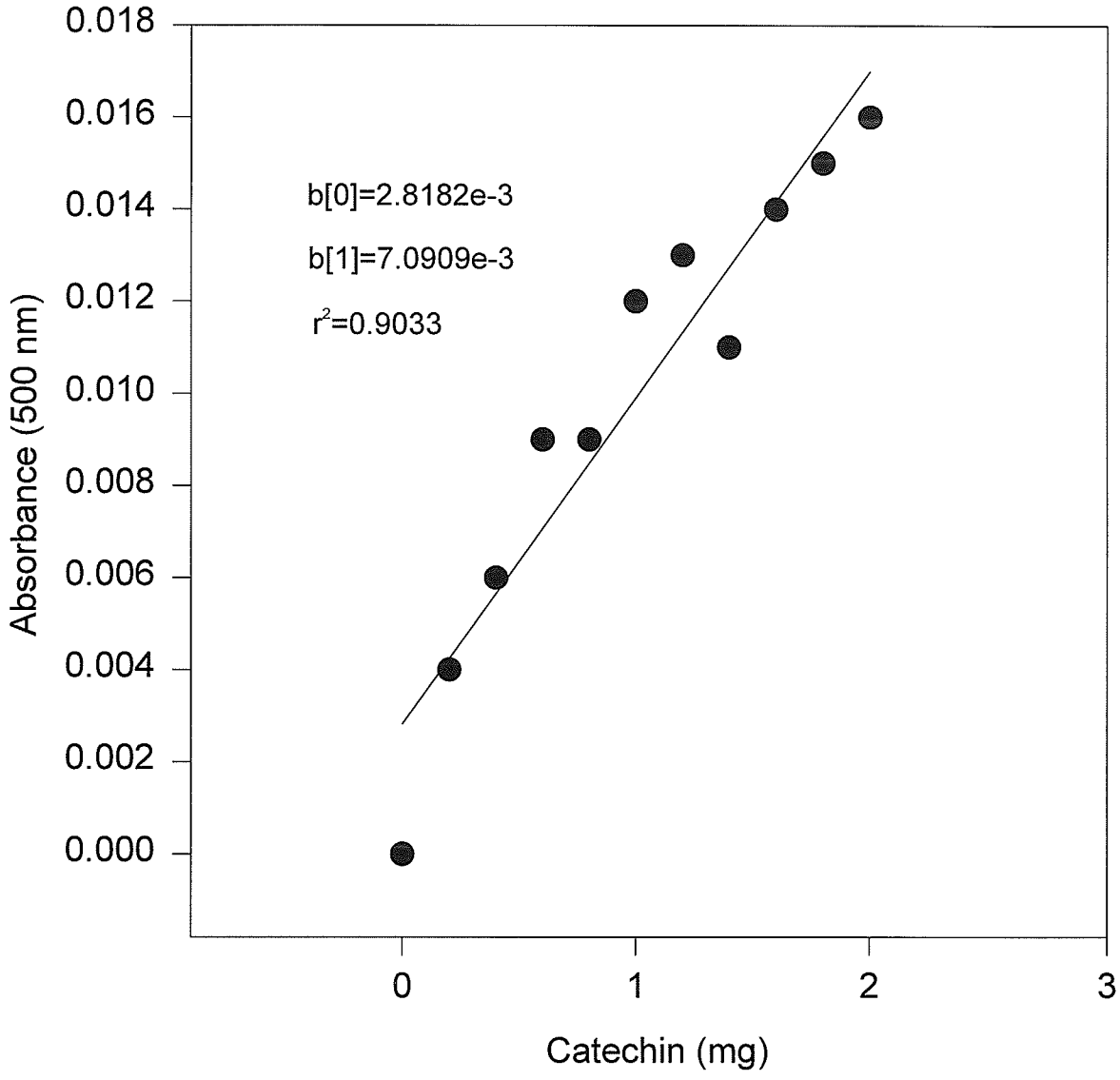
Zitko, V. & Rosik, J., 1962. Tannin-Gelatin-reaktion im Lichte der Theorie der Reaktionen Kleiner Molekule mit Makromolekulen. Collection Czech. Chem. Commun. 27: 2058.



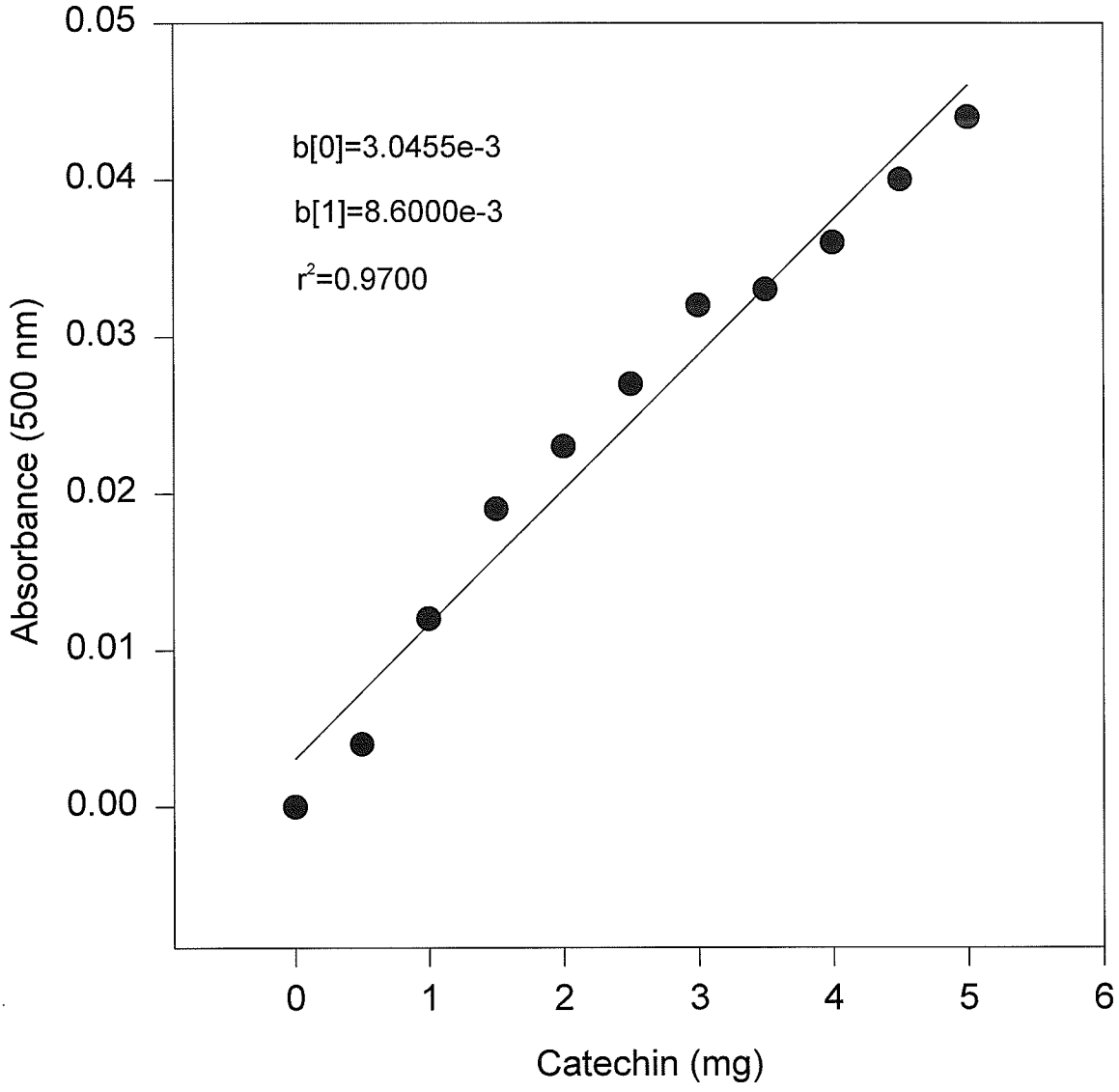
APPENDIX FIGURE 1. Total phenolics standard curve for field pea.



APPENDIX FIGURE 2. Total phenolics standard curve for grass pea.



APPENDIX FIGURE 3. Condensed tannin standard curve for field pea.



APPENDIX FIGURE 4. Condensed tannin standard curve for grass pea.