Free and Bound Phenolic Acids Present in Rapeseed Meal Inhibitory Towards *Bacillus stearothermophilus*

by

Robert Michael Myhara

A thesis
presented to the faculty of graduate studies
in partial fulfillment of the
requirements for the degree of
Master of Science
in
The Department of Food Science

Winnipeg, Manitoba, 1983

FREE AND BOUND PHENOLIC ACIDS PRESENT IN RAPESEED MEAL INHIBITORY TOWARDS *BACILLUS STEAROTHERMOPHILUS*

BY

ROBERT MICHAEL MYHARA

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ABSTRACT

Myhara, Robert M. M.Sc. The University of Manitoba. February, 1983. Free and Bound Phenolic Acids Present in Rapeseed Meal Inhibitory Towards Bacillus stearothermophilus

Extracts of crude meal from Brassica species, B. napus (variety Tower and Turret) and B. campestris (var Candle and R500) isolated by methanol or methylene chloride extraction were shown to exhibit inhibitory activity on bioassay plates employing Bacillus stearothermophilus. The extract obtained using chloroform showed only slight activity. Preliminary investigations have shown that the components do not belong to the major group of mycotoxins. Partial purification by ultrafiltration indicated that the component(s) have a molecular weight between 1000 and 5000d. Six compounds isolated were found to display inhibitory activity. Three compounds: sinapine, 1-sinapoyl-glucose, and sinapic acid were identified. Analysis of rapeseed meal extract by reverse phase HPLC revealed the additional presence of gallic, chlorogenic, gentisic, protocatechuric, caffeic, and p-coumaric acids in either free or bound forms. Temperature and pH studies indicated that the inhibitory materials were unstable at temperatures above 60 °C. and at pH levels below 4 or above 6.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my appreciation and gratitude to my advisor Dr. G. Blank, for his guidance and supervision during the course of this study, and for his criticism and suggestions during preparation of this manuscript. The assistance and criticism of Dr. M. Ballance and Dr. E. Hoehn are truely appreciated.

My sincere thanks also goes out to Dr. E.D. Murray for allowing me the opportunity to carry out this research.

The author would like to gratefully thank Dr. D. Abramson, of the Canadian Department of Agriculture for mycotoxin analysis.

I am indebted to my parents who instilled in me the value of knowledge and initiative. My sincere thanks goes to my sister Patricia for help in preparation of this manuscript. To other staff members and students I thank for their help and guidance during my stay here.

This thesis is dedicated to my wife Brenda, who helped me through the good times as well as the bad.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Rapeseed is the oil producing seed of rape (Latin rapum, turnip) belonging to the genus Brassica of the family Cruciferae. The genus includes several closely related plants such as B. nigra (black mustard), B. carinata (Abbyssinian mustard), B. oleracea (cabbage, cauliflower, broccoli), B. napus (rutabaga) and B. campestris (turnip). These plants generally grow well in the cooler regions of the world's agricultural areas, such as western Canada. The oil producing rapeseed grown in Canada are derived from the Agentine rapeseed (B.napus), and the Polish rapeseed (B.campestris). Initially, seed stocks introduced from Argentina were grown in Canada as the source of an industrial oil. Through selective breeding the erucic acid content of the oil, and the glucosinolate content of the rapeseed meal (RSM) was reduced by succesive stages through the variety Turret (1970), to Tower (1974). Erucic acid compromised the nutritional value of the oil, while toxic glucosinolates limited the feeding value of the meal. Polish rape followed a similar pattern of development through R500, to Candle(1976). Seed, oil, or meal of varieties containing less than 0.3% glucosinolates and with 5% or less erucic acid are referred to by the Canadian rapeseed industry as canola. B.napus var Tower and B.campestris var Candle meet these standards while Turret and R500, do not (Downey, et al.,1975; Vaisey-Genser, and Eskin,1979).

The first domestic commercial production of edible rapeseed oil began in 1956. From a small beginning, Canada has become the largest exporter of rapeseed in the world, exporting ca 1.37 million tonnes (1980), which was 77% of total domestic production. Approximately 1.45 million hectares of rapeseed were planted in 1980 (Canadian Grains Council, 1981).

RSM the residue left after oil extraction used as the primary protein source in animal feed, has been shown to induce goiter, and hemmorhagic liver disease. Hydrolytic products of glucosinolates such as 1-cyano-2-hydroxy-3-butene (CHB), and 5-vinyloxazolidine-2-thione (OZT), were implicated (Bowland, 1965; Jackson, 1969; Cansfield and Campbell, 1980). Subsequently it has been shown that other component(s) of RSM may also be implicated (Israels et al, 1979). It had been suggested that mycotoxins, products of certain fungi known to promote hepatic hemmorhage, may be common to most rapeseed. Mycotoxins display a marked toxicity towards the bacteria Bacillus stearothermophilus, and as such has been used as an indicator organism to detect their presence. If mycotoxins were present, then extracts of RSM would display inhibitory activity. Preliminary investigation showed that although RSM extracts did show inhibitory activity the RSM samples used in this study were devoid of common mycotoxins. Subsequent analysis showed these inhibitors to be phenolic in nature. The object of this study then was to detect, isolate, and identify these phenolic-like inhibitory materials (IM) present in RSM, and to determine their stability at varying pH and temperature. These studies have been carried out with a view towards developing a simple, effective, and fast bioassay of phenolic-like substances in RSM.

LITERATURE REVIEW

Rapeseed Meal

Commercial Processing

Commercial processing of rapeseed is directed towards the maximum extraction of oil from the seed. The oil, ca 40% of the seed must be extracted without chemically altering either the oil or the meal protein (Simmons, 1970).

Oil extraction is first initiated by rolling the seeds between large rollers. The resulting flakes (ca 2.0 mm thick) are then heated for 15-20 min at 77-105°C, depending on the seed variety. The rolled and cooked rapeseed is then screw-pressed, releasing 20-25% of the oil. The remaining presscake which contains 12-18% oil and 4-6% moisture is then subjected to countercurrent hexane extraction at 50-55°C. A movement of presscake in one direction, with solvent/oil moving in the other, ensures maximum oil extraction. The spent cake emerging from such a solvent extraction contains <1% oil. Conversion of the cake to RSM is initiated by heating it with steam (100-103°C) which flashes off any residual solvent. Successive dry heating treatments yield a meal with a moisture content of 10-12% (Beach, 1975).

Nutritional Aspects of RSM

The efficient and successful rearing of livestock and poultry is dependent upon adequate supplies of protein to supplement and balance the normal protein availability from many major cereals and forages. In addition, proteins are quite frequently supplemented in human food stuff to improve the protein quality of the food or to fulfill some functional role regarding structure, texture or other physical characteristic (Holme, 1975).

The quality of a protein supplement partly depends upon the essential amino acids supplied by the supplement, and the biological availability of these amino acids. The biological value (BV) of a protein compares the amino acids present in that protein compared to those amino acids required by a growing animal. Amino acids not utilized by the animal for protein synthesis and body maintenance, are deaminated and the resulting nitrogen excreted. Net protein utilization (NPU) combines both digestibility and the BV, where the resultant NPU is a function of the availability of those amino acids, in that protein (Lloyd et al., 1978). RSM is a protein supplement of high BV, with values of 89.8% for Tower, and 90.4% for Candle. NPU values for the two varieties vary between 76.2-76.4% (Campbell and Eggum, 1980). These BV values indicate that the RSM have excellent protein quality, however, their value as protein sources as indicated by their NPU values, are somewhat low. Factors present in RSM which tend to interfere with protein utilization, such as high fibre content (Campbell and Eggum, 1980) and glucosinolates (Bowland, 1965) have been reduced somewhat by the introduction of newer low fibre, low glucosinolate varieties. Plant phenolics not only reduce

protein quality but also produce brown coloration and impart a bitter flavor to RSM (Sosulski, 1979). Plant breeders have had limited success in improving the phenolic make-up of newer rapeseed varieties, which partly hinders the use of RSM as a protein supplement.

Feeding Effects of RSM

Several studies have been undertaken to investigate the effect of RSM feeding on animals. Jackson (1969) supplied RSM to laying hens in nine 28-day periods at levels ranging from 4 to 20%. Results indicated considerable thyroid enlargement and a high mortality caused by massive liver hemorrhage. This mortality, ranging from 6 to 50%, was shown to vary between various strains of birds. The RSM also tended to reduce egg production. Summers et al. (1969) found that diets composed of 5-15% RSM did not induce mortality, but weight gains and egg weights were reduced. Similarily, rats and mice fed RSM at levels from 5 to 20% in their diets caused growth depression (Bell et al., 1971). It was concluded that hydrolytic products of glucosinolates were involved. In addition, Hobson-Frohock et al. (1977) found that laying hens supplied with a diet composed of 10% RSM, produced eggs with a fishy taint. It was suggested that the taint may have been caused by sinapine, the choline ester of sinapic acid. Lo and Hill (1971) determined that in rats, the toxicity of RSM was not confined to the thyroid gland, but rather the toxic factors were distinct from goitrogenic substances. Israels et al. (1979) studied RSM feeding trials of chickens and found hepatotoxicity was accompanied by elevated levels of cytochrome P-450. A large number of foreign compounds are known to be metabolized by a

mixed oxidase system of which cytochrome P-450 is one. Substances present in RSM were shown to enlarge the liver and induce production of the enzyme. It was postulated that through some unknown mechanism increased levels of cytochrome P-450 induced hepatic intravascular coagulation, resulting in necrosis. However, glucosinolates, or glucosinolate aglycones were not identified as the toxic agents. Since the pathogenesis of RSM remained unclear, it was suggested that some other factor could be present contributing to the hepatotoxicity. Among those factors postulated were mycotoxins.

Mycotoxins

Mycotoxins are products of certain fungi such as Penicillium, Aspergillus, and Alternaria spp. The mycotoxins produced by these fungi include aflatoxins, sterigmatocystin, ochratoxin A, zearalenone, penicillic acid, and patulin. Mills and Sinha (1980) found deterioration and mold growth in initially sound rapeseed during storage for 147 days at temperature and moisture levels typical of farm bins in Manitoba. In rapeseed, stored at 25°C and 12.4% moisture, Penicillium, and Aspergillus spp. were most frequently isolated. Abramson et al. (1980) stored various grains at temperatures ranging from 14 to 30°C at moisture levels ranging from 19 to 30%. Aspergillus, Penicillium, and Alternaria spp. all appeared. Ochratoxin A was found after the fourth week, and gradually increased to 3900 ppb after 20 weeks.

Mycotoxin Bioassay

Standard methods for mycotoxin analysis usually involve thin layer chromatography (TLC), and/or high performance liquid chromatography (HPLC), (Josefsson and Moller,1977; Wilson et al., 1976). However, the bioassay of mycotoxins using sensitive indicator microorganisms have been used as a screening technique. Paper disc bioassays employing Bacillus megaterium, B. cereus, and B. subtilis detected the presence of aflatoxin, ochratoxin A, and patulin respectively (Clements,1968; Broce et al. 1970). Reiss(1975) found growth inhibition of B. stearothermophilus in solutions containing 0.001mg of aflatoxins and patulin.

Bacillus stearothermophilus

B.stearothermophilus is a Gram positive, thermophilic, facultative aerobic, spore-forming rod. The microorganism was first isolated from heat processed cream-style corn by Donk (1920). Subsequent investigations reported B. stearothermophilus to be a typical flat-sour organism of low acid canned foods. The organism produces acid but no gas from the assimilation of a wide variety of food carbohydrates. The organism, thermophilic by nature, shows an optimum growth temperature of 50-60°C. The minimum temperature of growth has been shown to be partly dependent upon the nutritional quality of the substrate (Fields, 1963).

Phenols

Reactivity of Phenolic Compounds

Phenolic compounds represent a wide variety of substances possessing an aromatic ring bearing a hydroxyl substituent (Harborne, 1964). Most phenolic reactions can be divided on the basis of those which involve the hydroxyl group and those which involve the aromatic ring.

Phenols are weakly acidic and are converted into their salts by aqueous hydroxides, but not by bicarbonates. The salts are converted into free phenols by aqueous mineral acids, carboxylic acids, or carbonic acid. Substitution of a carboxylic acid group onto the aromatic ring (phenolic acids) enhances the acidic nature of phenolic compounds. Acetyl (benzoic acid derivatives) or acrylic (cinnamic acid derivatives) substituted phenols are considerably more acidic than their corresponding unsubstituted counterparts (Morrison and Boyd,1976).

All phenols take part in hydrogen bonding. Both intra- and intermolecular hydrogen bonding occurs producing conformational stability or polymeric substances. Simple phenolics are water-soluble due to the presence of the hydrophilic hydroxyl group. Increasing the number of phenolic groups in more complex compounds, however, increases intermolecular hydrogen bonding. As a consequence solubility decreases; the increase in hydroxyl bearing phenolic groups being offset by the tendency to form a strong crystal lattice with the maximum number of hydrogen bonds. This tendency to form strong crystalline structures results in the formation of large polymeric molecules. Generally hydrogen bonding is an important structural feature of phenols stabilizing conformations and large complexes (Ribereau-Gayon,1972).

Phenols, like alcohols, form esters with acids. Conversely phenolic acids form esters with compounds containing hydroxyl groups. Phenolic compounds may undergo intermolecular esterification with carbohydrates, forming glycosidic compounds of varying complexity, while o-hydroxycinnamic acids may undergo intramolecular condensations forming lactones. Phenolic esters undergo hydrolysis in acidic or alkaline conditions, to yield the parent phenolic compound, in the case of an intramolecular ester, or the parent phenolic compound (aglycone), and the original carbohydrate (glycone), in the case of an intermolecular ester. Acidic hydrolysis, unlike alkaline hydrolysis, is reversible and the ester may reform upon neutralization (Harborne,1964; Morrison and Boyd,1976).

Phenolic compounds may be oxidized either in the presence of a nucleophilic radical or by enzymatic action. In either case removal of a hydrogen atom precipitates the formation of a free radical bearing a singly-bound oxygen atom. The radical can then react with another radical forming an oxidized compound. Phenols exposed to oxygen in an alkaline environment will oxidize rapidly forming polymeric dark colored materials. As the number of hydroxyl groups increases, the potential for oxidation also increases (Ribereau-Gayon,1972). The presence of an acrylic acid group on the aromatic ring has been shown to facilitate the oxidation of cinnamic acids. The potential of this oxidation varies from the very easily oxidized trihydroxy, to the more stable methoxy compound (Sosulski,1979).

Plant Phenolics

Phenolic compounds are widely distributed in plants, occurring in the leaves, stems, and seeds. Phenolic compounds may be essential metabolites, precursors of larger molecules, or they may serve some unique function of a particular genera (Sosulski,1979). The plant phenols are arranged into four broad, general groups of compounds; the phenolic acids, coumarins, flavonoids, and tannins.

Phenolic Acids. It is known that most of the simple phenolic acids such as p-hydroxybenzoic acid, protocatechuric, vanillic, gallic, salicylic, and gentisic acids are widely distributed in the higher plants (Harborne,1964). These may occur as free phenolic acids, or in combined form with sugars or other aliphatic compounds. The cinnamic acids such as p-coumaric, caffeic, ferulic, and sinapic acids are found as esters of hydroxy compounds in the majority of higher plants. Two examples of these esters found extensively are chlorogenic acid (caffeic and quinic acid), and sinapine (sinapic acid and choline).

Coumarins. The coumarins are a common plant constituent formed by the intraesterification of o-coumaric acid, forming a lactone heterocycle. These phenolic compounds occur almost exclusively as glycosides, releasing coumarins or coumaric acid upon hydrolysis (Ribereau-Gayon, 1972).

Flavonoids. The flavonoids are a group of compounds including flavones, flavonols, and flavanones. The flavones and flavonols contain a double bond between the 2,3 carbon atoms, while flavanones have a single bond. Flavonols differ from flavones by the presence of a

hydroxyl group at the number 3 carbon atom. Flavonoids do not normally occur as glycosides, but rather tend to polymerize into condensed tannins (Harborne, 1964).

Antimicrobial Activity of Phenols

Mode of Action. Initiation of inhibition towards bacterial cells begins with adsorption of the phenolic compound onto the cell wall, in competition with solvent molecules or other adsorbed species. Techniques for studying the rate of adsorption employ measuring, over suitable time intervals, the uptake of phenols in solution by the bacterial cell. From such studies it was found that phenols in general adsorb to the cell wall more readily than competing molecules, and penetrate it at a constant rate. This penetration is enhanced by bacteria containing a high proportion of lipid in their cell wall (Hugo, 1976).

It has been shown (Hugo, 1976) that Eschericia coli streptococci, and staphylococci encountered cell wall damage when exposed to 0.032% phenol. Since protoplasts were not formed, it appeared that the phenol attacked the cell wall directly.

It is clear that certain antibacterial agents, including phenols, promote cellular membrane disruption with a concomitant leakage of ions, nucleic acids, pentoses, and inorganic phosphorus. Lethality was also attributed to inhibition of part of the electron transport chain. Whether cellular leakage was a secondary event brought about by this inhibition was not determined (Hugo,1976). Microscopic examination of rumen bacteria grown in the presence of several plant phenolic acids

displayed Gram variability, lysis, and leakage of cell contents. Evidence of structural damage was noted with vanillic, cinnamic, p-coumaric, 3-methoxycinnamic, ferulic, and sinapic acids (Chesson et al., 1982).

Factors Affecting Activity. Phenolic antagonism towards bacteria increases with increase in temperature while the organisms are in contact with the inhibitor. Increasing the temperature from 20 to 37°C., doubled the effectivness of phenol against E.coli, and pseudonomads (Bennett, 1959).

It is well known that phenols are most effective in an acid environment and that their antibacterial activity decreases as the pH becomes more alkaline. The unionized phenol is the active form and since phenols in alkaline solution tend to form the phenoxide ion, a subsequent reduction in inhibitory activity is observed (Hamilton, 1971).

Interaction of Phenols with Proteins and Amino Acids

Phenolic compounds in the presence of air, or through enzymatic action (such as polyphenol oxidase) are oxidized to form lactones, and peroxides. These compounds can subsequently react with specific amino acids, causing protein denaturation and/or enzyme inhibition. Enzyme inhibition by lactones and peroxides have been observed with trypsin and lipase (Sosulski, 1979). In rapeseed, the cinnamic acids are enzymatically oxidized to o-quinones which react non-enzymatically with amino, thio and methylene groups. The amino group of lysine and the methylthio group of methionine may be attacked, leading to

polymerization. Phenolic compounds have also been shown to bind, by hydrogen bonding, to enzymes and other proteins forming large insoluble complexes. The combined effect of quinone/protein interaction, hydrogen bonding and subsequent polymerization, has rendered large numbers of essential amino acids, nutritionally unavailable (Sosulski, 1979).

Tannin-Like Material in Rapeseed Products

Tannins are closely related phenolic compounds whose degree of hydroxylation and molecular size are large enough to form complexes with proteins and other polymers (Joslyn, 1970). Since most phenols, and all polyphenolic compounds can to a certain extent form these complexes, it may be more accurate to describe those phenols relevant to this study as "tannin-like" substances.

Clandinin and Heard (1968) found an average of 3.01% tannin-like material in solvent and pre-pressed solvent extracted RSM. It was proposed that this high level of tannin-like material may adversely affect chicken growth and the metabolizable energy value of rations when RSM was fed in high levels. Fenwick and Hoggan (1976) analyzed RSM and found it to contain 0.91% sinapine. It was suggested that the tannin-like material figure supplied by Clandinin and Heard (1968) should have been reduced by this amount since, it was argued, sinapine could not be considered a tannin. It was felt that this amount of sinapine present in a diet would not promote tannin induced metabolic disorders affecting chickens fed on a RSM diet not exceeding 10% of the ration (0.091% sinapine). Previously Clandinin (1961) showed that chickens fed a diet containing RSM supplemented with 0.39% sinapine was neither growth

depressing nor thyrotoxic. Joseffson and Uppstrom (1976) confirmed these results with mice, but found that at high levels sinapine severely affected meal palatability.

Isolation, Separation, and Identification of Phenolic Compounds

Extraction. Due to the nature of the hydroxyl group(s) attached to the aromatic ring, phenolic compounds are usually soluble in polar solvents. The hydrogen bonding exhibited by phenols as well as the types and numbers of alkyl and other ring substitution groups all affect the degree of solubility. Most phenolic compounds are extracted from rapeseed or RSM with aqueous solutions of alcohol or alcohol/acetone (Krygier et al., 1982a). Durkee and Thivierge (1975) extracted phenolic acids and esters from rapeseed with 70% boiling ethanol, while Krygier et al. (1982a) extracted these compounds with 70% methanol-70% acetone (1:1). It has been recommended (Ribereau-Gayon, 1972) that a pre-extraction of plant material with a non-polar solvent, such as hexane be employed to remove fats, waxes, chlorophyll, and carotenoids which may interfere with subsequent separation and identification of the phenolic compounds. Krygier et al. (1982a) found that rapeseed extracts obtained without a pre-extraction step, showed the presence of free fatty acids which interfered with the identification of phenolic acids. It was also shown that hexane did not extract significant amounts of phenolic compounds.

Purification. The alcoholic or aqueous solution prepared from rapeseed or RSM may be suitable for direct chromatography, but many

compounds such as sugars, organic acids, and proteinaceous material may present difficulties. In such cases purification has been necessary (Ribereau-Gayon 1972). Kozlowska et al. (1975) purified methanolic extracts of rapeseed by continuous extraction with diethyl ether for 19h. The resultant ether extract was evaporated under vacuum and redissolved in methanol. Ribereau-Gayon (1972) described a process by which activated carbon was used to eliminate interfering material from the extract. Carbon was added to the aqueous plant extract, adsorbing the phenolics. When the carbon was eluted with alcohol the phenolic material was desorbed. Desorption of the phenolic with alcohol was shown to be poor, with a large portion of the phenol remaining adsorbed to the carbon.

Liberation of Phenolic Compounds from Esters. Hydrolysis of phenolic compounds from their esters is performed using acid, base, or enzymes. Durkee and Thivierge (1975) treated an aqueous extract of rapeseed with 2N HCl or NaOH. Acid hydrolysis was performed at room temperature for 4h followed by acidification (pH2.0). Phenolic acids were extracted from the hydrolyzates with diethyl ether. Results indicated that alkaline hydrolysis was more effective in liberating phenolic acids than acid hydrolysis. Enzymatic hydrolysis was performed by these investigators by mixing a small amount of β-glucosidase with an aliquot of extract, followed by incubation at room temperature for 4h. Fewer numbers of phenolic acids were liberated by β-glucosidase than by either alkaline, or acidic hydrolysis.

Separation and Identification of Phenolic Compounds. Various methods for the separation and identification of phenolic compounds have been utilized: column chromatography, TLC, and HPLC. Lo and Hill (1972) utilized gel filtration chromatography to separate aqueous RSM extract into several fractions. The gel (Sephadex G25) was able to fractionate compounds with a molecular weight between 1000 and 5000d, which was the region where the greatest proportion of phenolic compounds appeared.

TLC utilizing silica gel G, or cellulose coatings have been employed successfully to separate both phenolic esters, and free phenolic acids (Durkee and Thivierge, 1975; Fenton et al., 1980). Butanol:acetic acid:water in various proportions are used for the separation of phenolic acids and their esters (Stahl, 1969). The more polar phenolic esters are easily separated with relatively polar solvent mixtures while the less polar tri-, di-, and monohydroxyl phenolic acids are more easily separated by increasingly less polar mixtures.

Recently use has been made of a reversed phase HPLC to separate and identify phenolic acids and their esters. A non-polar stationary phase with a polar mobile phase was employed by Melander and Horvath (1980). Partition of the phenolic compounds between the stationary and mobile phase was used to exploit the wide range of polarity exhibited by these compounds. Manipulation of the mobile phase polarity by altering the solvent mixture, enhanced the separation. Court (1977) employed an aqueous methanol solution ranging from 16 to 40% over 15 min to separate phenolic compounds found in tobacco, using this technique. Similarily Murphy and Stutte (1977) separated soybean phenols with a butanol:methanol:acetic acid:water mobile phase. The mobile phase was

allowed to range from a relatively polar mixture, to a relatively nonpolar mixture, which effectively separated polar phenolic esters from the less polar phenolic acids. Ammonium acetate buffer was added to the mobile phase in both studies to inhibit intra-molecular hydrogen bonding between hydroxylated compounds which caused abnormal retention times.

Phenolic Compostion of RSM Tannin

Lo and Hill (1972) separated aqueous extracts of RSM by gel filtration chromatography. Four fraction peaks were obtained. Fractions I and II contained most of the proteinaceous material, while fractions II and III contained most of the glucosinolates. Fraction IV contained only trace amounts of protein and glucosinolates, but contained considerable polyphenolic material. Partition column chromatography of fraction IV utilizing silicic acid revealed the presence of caffeic, and chlorogenic acids. Durkee and Thivierge (1975) utilizing TLC could not detect free phenolic acids in RSM; large amounts of sinapic and lesser quantities of protcatechuric, p-hydroxybenzoic, caffeic, p-coumaric, and ferulic acids were found after alkaline hydrolysis. Kozlowska et al. (1975) studying rapeseed flour found several free phenolic acids present including chlorogenic, p-hydroxybenzoic, cinnamic, caffeic, p-coumaric, ferulic, and sinapic acids. In response to these investigations Fenton et al (1980) reported that several of the phenolic acids found by Kozlowska et al. (1975) were long chain fatty acids. These fatty acids were not extracted by hexane. In addition caffeic acid, reported in the free form by Kozlowska et al. (1975) and in the ester form as chlorogenic acid (Durkee and Thivierge, 1975; Lo and Hill, 1972), cinnamic acid

(Kozlowska et al., 1975), and esters of p-coumaric acid (Durkee and Thivierge, 1975), were not present. Fenton et al. (1980) reported the presence of protcatechuric, p-hydroxybenzoic, vanillic, syringic, ferulic, and sinapic acids in the esterified form only. Of these esters sinapine, and 1-sinapoyl-glucose were the most abundant. The only free phenolic acid detected was sinapic acid.

In an attempt to clarify the situation, Krygier et al. (1982b) agreed with Lo and Hill (1972) and Kozlowska et al. (1975) that rapeseed does in fact contain free phenolic acids. The analysis showed the presence of chlorogenic, p-hydroxybenzoic, caffeic, p-coumaric, ferulic, vanillic, syringic, gentisic, and sinapic acids. These free phenolic acids cumulatively constituted 0.084% of the rapeseed. In addition, Krygier et al. (1982b) determined the free and esterified phenolic acid compostion of three common varieties of rapeseed. Brassica campestris var R500 was found to be lower than either Candle or B. napus var Tower. Candle, however was higher in total phenolic acids as compared to Tower despite its lighter seed coat.

MATERIALS & METHODS

Inhibitor Bioassay

Source of Organism and Maintenance

The organism used in this study was Bacillus stearothermophilus var. calidolactis , hence referred to as B. stearothermophilus. The organism was isolated from Delvotest P ampules purchased from the technical microbiology laboratory of The Technical University, Delft, Netherlands. Ampule contents were streaked directly onto trypticase soy agar (TSA, BBL) plates incubated at 55°C for 18-24 hours. Typical colonial isolates (Fields, 1963) were subcultured on the same medium and similarily incubated. Well isolated colonies were then streaked onto TSA slants, and incubated at 55°C for 18-24 hours. The stock culture slants were stored at 4-6°C and transferred every two weeks.

Cultural Conditions and Harvest

B.stearothermophilus was grown in 1 L Roux bottles each containing 200 ml TSA medium. Inocula were prepared by gently washing the surface of stock culture slants with 2.0 ml portions of sterile physiological saline which were then introduced into each Roux bottle. Growth was carried out at 55°C for 18-24 hours after which the resultant growth was gently washed off the surface of the slant with 20 ml sterile saline (stock cell suspension). Sterile glass beads (5 mm dia), added to each Roux bottle slant after agar solidification, allowed for uniform

distribution of the inocula and aided in dislodging the cellular growth from the slants.

Cell Standardization

The stock cell suspension was standardized, with additional sterile saline, to 25% transmittance (410 nm; Spectronic 20, Bausch & Lomb).

Bioassay Plates

Assay plates consisted of disposable (85 mm I.D.) petri dishes each containing 7.0 ml sterile TSA and 1.4 ml standardized stock cell suspension. The prepared plates contained a final concentration of approximately $1x10^6$ cells/ml assay medium. Assay plates were stored, at 4-6°C in plastic sleeve containers for a maximum of three days, until needed.

Activity of Inhibitor Substance

Disc Assay Plates. The activity of the IM was determined by disc assay using sterile blank discs (6.4 mm dia., Difco) placed onto the surface of previously prepared assay plates. The quantitative evaluation of inhibitor was performed by diluting the IM with sterile distilled water and recording the highest dilution yielding inhibition. This activity was expressed as arbitrary units (Au), where 1 Au equaled the reciprocal of the highest dilution yielding inhibition. The inhibitory activity of sinapine was determined by establishing the minimum inhibitory concentration of sinapine required to produce a zone. Twenty µL samples of the IM were pipetted directly onto the surface of a

blank disc using an automatic micropipettor with disposable plastic tips. All assays were performed in duplicate, including controls. Controls were performed with distilled water and with those solvents used to extract the IM. Assay plates were incubated at 55°C for 6 hours.

Zones of inhibition were also recorded qualitatively, and semi-quantitativly. Qualitative inhibition was recorded by the presence (+) or absence (-) of inhibition, while semi-quantitative inhibition was recorded by measuring the zone of inhibition perpendicular to both axes.

Inhibitor Activity Extracted by Various Organic Solvents. Four 50 g samples of the respective meal were thoughly wetted with 50 ml water, each contained in 1L flasks. To each flask 200 ml of either methanol, chloroform, or methylene chloride were added and the resulting slurries shaken and filtered without precipitation or partition as described by Pons (1979). The resultant extracts were reduced in volume to 10ml by evaporation under vacuum, and the inhibitory activity determined by disc assay.

Rapeseed Meal Source

The meals used in this study were from Brassica napus var Tower , B. campestris var Candle, B. napus var Turret, and B. campestris var R500. The crude meals were obtained from a commercial crushing plant where the oil seeds were subjected to crushing, cooking, and pressing followed by countercurrent hexane solvent extraction (Beach, 1975).

Extractions

Preliminary Extraction

Fifty g of the respective meal (Pons,1979) was thoughly wetted with 50ml distilled water contained in a 500-ml Erlenmeyer flask. Two hundred ml methanol was added and the flask was shaken for 30 min at room temperature using a Burrell wrist-type shaker set at 315 reciprocations min^{-1}. Ten g of filter aid (Celite 545) was added to the slurry and subsequently filtered under reduced pressure on a Buchner funnel fitted with an 11 cm circle of rapid filter paper (Whatman no.4). A 100 ml portion of the filtrate was then transferred into a zinc salt solution. The mixture was stirred and left to stand for ca. 5 min. Five g filter aid was then added and the mixture filtered under reduced pressure as previously described. A 100 ml portion of the resultant filtrate was then transferred into a 250-separatory funnel and 25 ml methylene chloride added. The funnel was shaken vigorously by hand for ca. 1 min and after phase separation, the methylene chloride (lower) phase was drained through a 2.0 cm bed of anhydrous sodium sulfate contained in a small (22x100 mm) chromatography column. The above partition was repeated with an additional 25 ml portion of methylene chloride, and a final 25 ml portion of methylene chloride was used to wash any remaining extract through the sodium sulfate bed. The total volume of extract was ca. 75 ml. The zinc-salt solution was prepared by dissolving 150 g zinc acetate dihydrate, and 150 ml sodium chloride in ca. 500 ml water. One ml glacial acetic acid was then added and the solution made to a final volume of 1 L with distilled water.

Finalized Methanol-Water Extraction

Fifty g of the respective meal was extracted with methanol without precipitation or partition as previously described (Pons, 1979). Prior to filtration under reduced pressure, the methanol extract was evaporated under vacuum at 40°C to dryness and redissolved with 250 ml distilled water. The resultant methanol candle extract (original MCE) was used as such or freeze-dried and subsequently reconstituted to a desired concentration by the addition of distilled water.

Separation Techniques

Ammonium Sulfate Treatment

Ammonium Sulfate Precipitation. Two hundred and fifty ml of original MCE was initially treated at room temperature with 60 g of ammonium sulfate (40% ammonium sulfate fraction). The mixture was stirred until the salt was completely dissolved, and left to stand ca. 5 min. The mixture was centrifuged (10400xg) for 20 min at 4°C. After centrifugation the supernatant was decanted and an additional 60 g of ammonium sulfate added (65% ammonium sulfate fraction). The mixture was stirred, allowed to stand and similarily centrifuged. The resultant supernatant was again decanted and treated with 70 g of ammonium sulfate (100% ammonium sulfate fraction).

Dialysis. The 40,65,and 100% ammonium sulfate fractions were separately dissolved in 150 ml of distilled water. The resultant solutions were placed into cellulose acetate dialysis tubing (31 mm dia. 12000 mol wt cut-off, Fisher). Dialysis was carried out against 7.5 L distilled water at 4 °C.

Ultrafiltration

Preliminary Filtration. Ultrafiltration membranes with 10000,5000,and 1000 molecular weight cut-off (UM 10,UM 5,UM 2, 62mm Amicon) were initially employed. These membranes were pretreated according to the manufacturers directions. Fifty ml aliquots of original MCE were placed into ultrafiltration stirred -cells (model 202,Amicon) each equipped with one of the membranes. The cells were placed onto magnetic stirrers and ultrafiltrated at room temperature using ca. 345 kPa air pressure. Approximately 25 ml of permeate was collected from each cell.

Ultrafiltration Purified Extracts. Two hundred and fifty ml of original MCE was made up to 500ml with distilled water. The extract was placed into stirred-cells equipped with a 5000 mol wt cut-off membrane and the permeate collected. Additional distilled water was added towards the end of filtration to wash the cell. Six hundred ml of permeate was collected and reconcentrated in a stirred-cell equipped with a 1000 mol wt cut-off membrane. Concentration continued until ca. 100 ml of retentate remained. The retentate was freeze-dried

Thin Layer Chromatography

Materials. Thin layer chromatography (TLC) plates used throughout this study were commercially prepared 20x20cm silica gel G plates (Fisher) which were developed in developing chambers. Visualization sprays were applied with a glass atomizer, utilizing compressed air as the propellant.

Chromatogram Development. TLC plates were spotted with 10 µL of sample 1.5 cm from the bottom of the plate. Sample solutions were applied in 1.0 µL increments using a 20 µL Hamilton type syringe. A hot air blower was used to evaporate the extract solvent during application. The TLC plates were placed into developing chambers, previously equilibriated for ca. 30 min, each containing 100 ml developing solvent. The solvent front was allowed to rise ca. 15 cm from the point of application. The TLC plates were then removed and dried at room temp. Drying time varied depending on the volatility of the solvents employed. Developing solvents were prepared fresh each day. When employing two-dimensional TLC, plates were spotted with 10µL of sample ca. 4 cm from the left edge and 1.5 cm from the bottom. After primary development, chromatograms were air dried as previously described and then placed into a developing chamber containing the secondary solvent arranged such that the secondary solvent front was at right angles to the primary solvent front.

Compound Separation and Visualization. The following compounds were separated with TLC:

Phenolic glycosides

Phenolic glycosides were separated with the following solvent systems (Fenton,et al., 1980):

1. n-butanol, acetic acid, water (BAW 8:2:2).
2. n-butanol, acetic acid, water (BAW 4:1:5).
3. n-butanol, acetic acid, water (BAW 5:2:5).

Phenolic glycosides were visualized with Folin-Ciocalteau spray (Stahl,1969).

Phenolic acids

Phenolic acids were separated with benzene:methanol:acetic acid (BMA 20:4:1) as described by Krygier, et al. (1982a). Visualization was performed with Folin-Ciocalteau spray.

Sugars

Sugars were visualized with an aniline/diphenylamine phosphoric acid spray as described by Stahl (1969).

Amino compounds

Compounds containing amino groups were visualized with a ninhydrin spray (Stahl, 1969).

Bioautography. Ultrafiltration partially purified MCE was separated on TLC plates utilizing BAW (8:2:2), followed by drying for 18 hr at room temp (Herbst, 1982). Bioautograms were prepared by placing the dry TLC plates onto a flat level surface, and a mask consisting of a picture-frame like form with a 16.5 cm square opening was placed over the TLC plate forming a well over the plate. Bioassay medium, consisting of 5 parts sterile TSA and 1 part standardized stock cell suspension, was poured into the well forming a gel layer over the plate ca. 1mm thick.

After solidification the mask was removed and the TLC plate-gel layer was placed into a hinged top plastic tray. The tray was incubated for 6 h at 55°C. Damp towels placed into these trays were included to delay agar dehydration. After incubation, the gel layer was sprayed with a solution consisting of 200 mg 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT, Sigma) dissolved in 10 ml methanol, and diluted to 100 ml with water. The tray was returned to the incubator for ca. 15 min. The spray-incubtion process was repeated 2 to 3 times

until yellow zones of inhibition were visible upon a red background. Two dimensional TLC was performed by separating raw MCE utilizing BAW (8:2:2) on the first dimension, followed by a brief drying period, and BAW (5:2:5) on the second.

Gel Filtration Chromatography

Gel filtration was carried out using Sephadex G10, and G25 (Pharmacia), and was prepared according to the manufacturers specifications. Expanded gel bed volume was 190 ml. Separation with G10 was performed in a 2.6x40 cm laboratory column (K26/40, Pharmacia) at room temperature. Samples of ultrafiltration partially purified freeze-dried MCE were prepared in distilled water at a concentration of 40 mg/ml. Five ml of sample was applied to the column. Distilled water was used as the eluent, at a flow rate of ca. 0.6 ml/min, controlled by gravity feed. Three ml fractions were collected using a spiral-type fraction collector (LKB). Separation using G25 was performed in a 1.6x100 cm laboratory column at room temperature. Distilled water was used as the eluent, at a flow rate of ca. 0.6 ml/min, controlled by a variable speed peristaltic pump (LKB). Ultrafiltration partially purified MCE was prepared in distilled water at a concentration of 100 mg/ml. Five ml of sample was applied to the column. Three ml fractions were collected.

Activated Carbon Treatment

Five ml of a 100mg/ml freeze-dried MCE solution was treated with 500 mg activated carbon(Darco HDC, Atlas Chemical Industries) at room temperature. The resultant slurry was thoughly mixed and allowed to stand, with occasional stirring, for ca. 30 min. The slurry was then centrifuged (27000xG) for 20 min, and the supernatant decanted. Five ml of methanol was then added to the activated carbon, and allowed to stand, with occasional mixing. The methanol desorbate was separated as above.

Optical and Chemical Analysis

Ultraviolet Absorbance

Samples were monitored for phenolics at 280 nm using a spectrophotometer (Spectronic 710, Bausch and Lomb).

Absorbance Spectra

Absorbance spectra of phenolic compounds were monitored from 190 to 450 nm using a scanning spectrophotometer (sp.800b, Unicam).

Total Sugars

Total sugars were determined by the phenol sulfuric acid colorimetric method as described by Dubois et al. (1956). A calibration curve was determined using glucose as the standard (500 nm).

Tannin-Like Material

Tannin-like material was determined by oxidation with Folin-Ciocalteau reagent. The resulting blue complex was measured colorimetrically at 725 nm (Swain and Hillis, 1959). A calibration curve was determined using tannin acid as the standard.

Total Tannin-Like Material

Extraction of total tannin-like material was performed as described in method 28.016 for cloves and allspice in the 10th edition of Offical Methods of Analysis of the AOAC (1965). The extract was diluted to 500ml as described and tannin-like material was determined with Folin-Ciocalteau reagent as previously described.

Total Reducing Sugars

Total reducing sugars were determined by the reduction of the cupric ion and subsequent formation of blue molybdic oxides from arsenomolybdate, as described by Joslyn (1970). The molybdate oxides were measured colorimetrically at 525 nm. A standard calibration curve was determined with gucose.

Sinapine

Sinapine was precipitated with Reinecke's salt followed by colorimetric determination as described by Tzagoloff (1963) as modified by Austin and Wolfe (1968).

Acidic Hydrolysis

Freeze-dried MCE, or peak B material, reconstituted with distilled water to 5 ml (1.0 mg/ml) was acid hydrolyzed with 2.0 N HCl (Durkee and Thivierge, 1975). Since the method outlined by these authors did not state the volume of acid used, HCl in this study was added to the solution until an arbitrary chosen pH (1.5) was obtained.

Alkaline Hydrolysis

Freeze-dried MCE, peak B material, or sinapine, reconstituted with distilled water to 5 ml (1.0 mg/ml) was alkaline hydrolyzed with 2.0 N NaOH (Durkee and Thivierge, 1975). Since the method outlined by these authors did not state the volume of alkaline used, NaOH in this study was added to the solution until an arbitrary chosen pH (10.0) was obtained.

Temperature Stability Studies

One ml samples of freeze-dried MCE (100 mg/ml),or test solution, were pipetted into 10x100 mm screw-cap test tubes and heated for 30 min at 50,60,70,80, and 100 °C using a thermostatically controlled water or oil bath. At each specified time period of heat treatment, tubes were removed and rapidly cooled. Residual inhibitor activity was determined.

pH Stability Studies

The pH stability of the IM was performed by pipetting 0.5 ml of a 200mg/ml MCE into 10x75 mm test tubes. The test tube contents were then adjusted to selected pH values by the addition of 2.0 N HCl, or 2.0 N NaOH. After pH adjustment each tube was made up to a final volume of 1.0ml, with distilled water and incubated at room temperature for 30 min. Following incubation residual inhibitor activity was assayed. The pH stability of sinapine was determined similarily.

High Preformance Liquid Chromatography

HPLC analysis of original MCE was performed according to the methods described by Murphy and Stutte(1977) using a Waters Associates HPLC equipped with a nonpolar 5.0 μm Bondpak/C18 column (4mmx30cm). Flow rate was 2.0 ml/min. Absorbance was measured at 280 nm. Phenolic acid standards (Sigma), and sample peaks were measured with a Hewlett Packard(Model 3390A) reporting integrator. The amounts of phenolic acids detected in MCE were expressed on a percent RSM basis.

RESULTS

Preliminary Studies

The presence of OZT and CHB in RSM, and their effects on chickens has been documented (Cansfield & Campbell, 1980). In order to determine their effect upon B. stearothermophilus, disc assays were performed with concentrations varying from 1.0 to 100 mg OZT/CHB/ml distilled water. No inhibitory activity was observed.

The RSM varieties investigated in this study (Brassica napus var Tower and Turret, and B. campestris var Candle and R500) were screened for the presence of aflatoxins, sterigmatocystin, ochratoxin A, citrinin, penicillic acid, patulin, and zearalenone by the methods described by Josefsson and Moller (1977) and Wilson et al. (1976). Results were negative for the presence of these mycotoxins. During the course of these preliminary investigations a methanol-water solution (Pons, 1979) was added to RSM, as part of the initial extraction method for the detection of aflatoxin. The methanol-water extract from RSM contained no aflatoxin, however, the extract did elicit a zone of inhibition on bioassay plates seeded with B.stearothermophilus, an organism sensitive to aflatoxin. The nature and distribution of this Inhibitory material in the RSM studied was then further investigated.

Extractions

In order to obtain sufficient quantities of the inhibitory material for analysis the efficacy of IM extraction by various solvents was investigated using methanol, chloroform, and methylene chloride (Figure 1). Results indicated that both methanol and methylene chloride were good solvents for IM extraction. Chloroform showed only slight extraction. In addition, methanol showed good extraction only with the Candle variety. Extraction levels of the IM with methanol from Tower and Turret with methanol were less than that that of Candle. The IM from R500 was poorly extracted with all solvents investigated. Methylene chloride showed greatest efficacy of IM extraction with Tower (Figure 1). Extraction of Tower gave an IM concentration approximately that of Candle, while Turret and R500 showed no IM when extracted with this solvent. Since the major groups of mycotoxins were shown to be absent in the samples investigated, it was thought that the IM in RSM extracted by various solvents could be either a minor mycotoxin or some other chemical substance protein or carbohydrate-like in nature.

With the view of further characterizing the chemical nature of the inhibitor ammonium sulfate fractionation was employed using a MCE. The MCE was chosen since it showed the greatest overall inhibitory activity. Ammonium sulfate precipitation fractions corresponding to 40,65, and 100% saturation, showed the presence of larger amounts of IM in the supernatant fractions, than in precipitates (Figure 2). Precipitates and the supernatants desalted by dialysis (12000 molecular weight cut-off dialysis tubing) against distilled water, showed an absence of activity, indicating a molecular weight smaller than 12000d.

Figure 1. Inhibitory Activity Extracted by Various Solvents.

Expressed as arbitrary units (Au)/50g RSM.

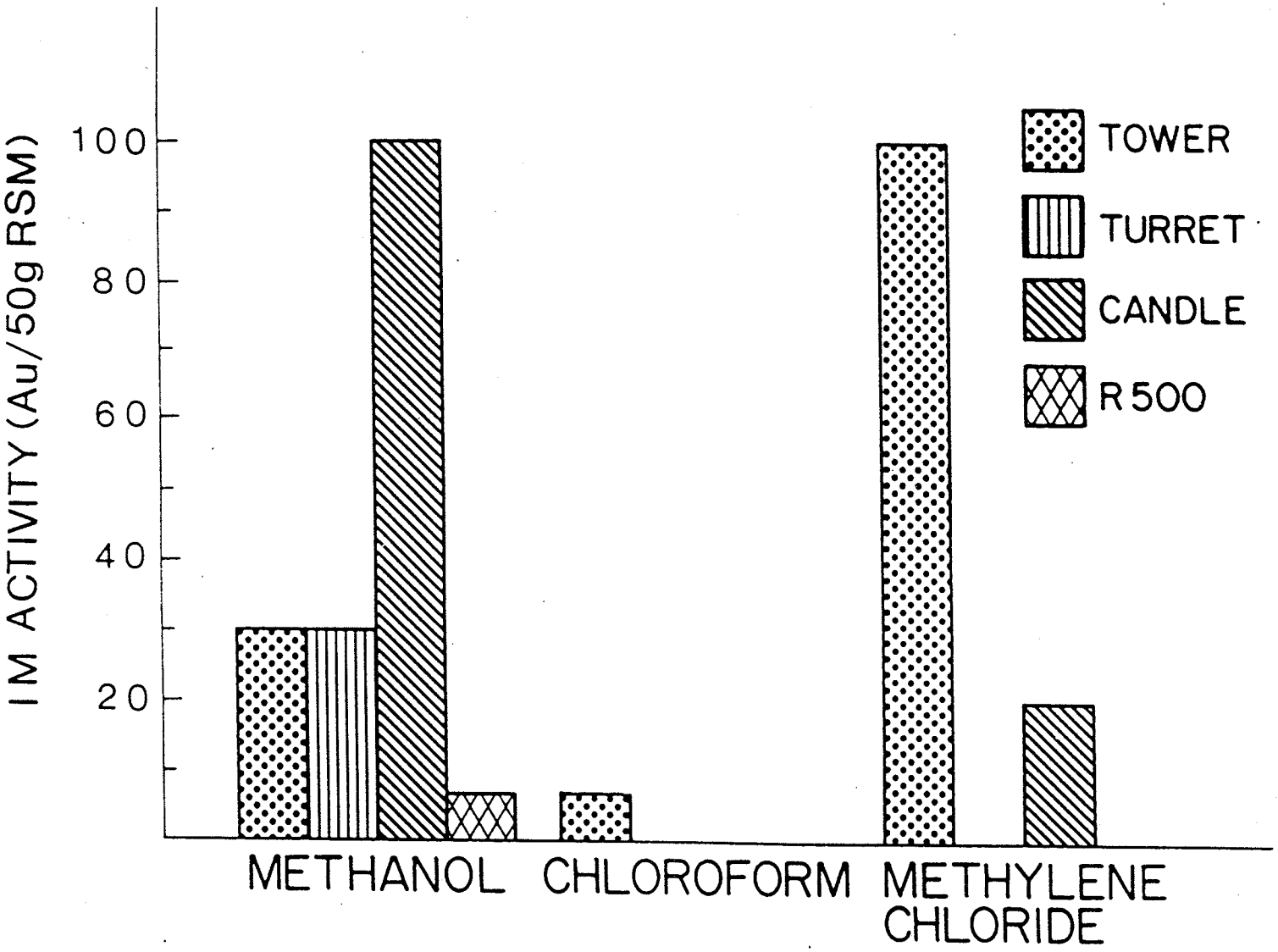

IM ACTIVITY (Au/50g RSM)
100
80
60
40
20
TOWER
TURRET
CANDLE
R500
METHANOL
CHLOROFORM
METHYLENE CHLORIDE

Figure 2. Bioassay of 100% Ammonium Sulfate Precipitate and Supernatant.

A, ammonium sulfate precipitation supernatant; B, ammonium sulfate precipitation precipitate.

B
A

Characterization of the IM

Preliminary Studies

MCE, concentrated and partially purified by ultrafiltration, showed inhibitory activity only from that fraction having an approximate molecular weight of 1000-5000 d. Ultrafiltration partially purified MCE was separated by column chromatography using Sephadex G10. The fractions were monitored for their absorbance at 280nm (Figure 3). Samples of these fractions were also analyzed for total carbohydrate. Several fractions displayed strong absorbance. Those fractions which absorbed the strongest at 280nm also contained the highest levels of carbohydrate (>1.4mg/ml). Fraction number 30 showing the highest absorption at 280 nm was subjected to UV scanning (190-450 nm). A typical scan indicated maximum absorbance at 280 and 317 nm (Figure 4). Addition of OH^- ions to this fraction shifted the absorbance maximum to 375 nm typical of phenolic-type substances. None of these fractions, however, displayed inhibitory activity.

MCE concentrated and partially purified by ultrafiltration, when separated by Sephadex G25, displayed several well defined peaks (fractions 40-90) which absorbed strongly at 280 nm (Figure 5). These fractions were analyzed for total carbohydrate, and for tannin-like substances. Fraction number 55 showing the highest absorption at 280 nm also displayed the largest amount of tannin-like material (ca 0.16 mg/ml expressed as tannic acid). In addition fraction number 55, was the only fraction showing inhibition towards B. stearothermophilus. Fraction number 55 which showed inhibitory activity, and fraction number 60 which

Figure 3. Separation of Ultrafiltration Partially Purified MCE With Sephadex G10.

Three ml fractions were collected at a flow rate of 0.6ml/min.

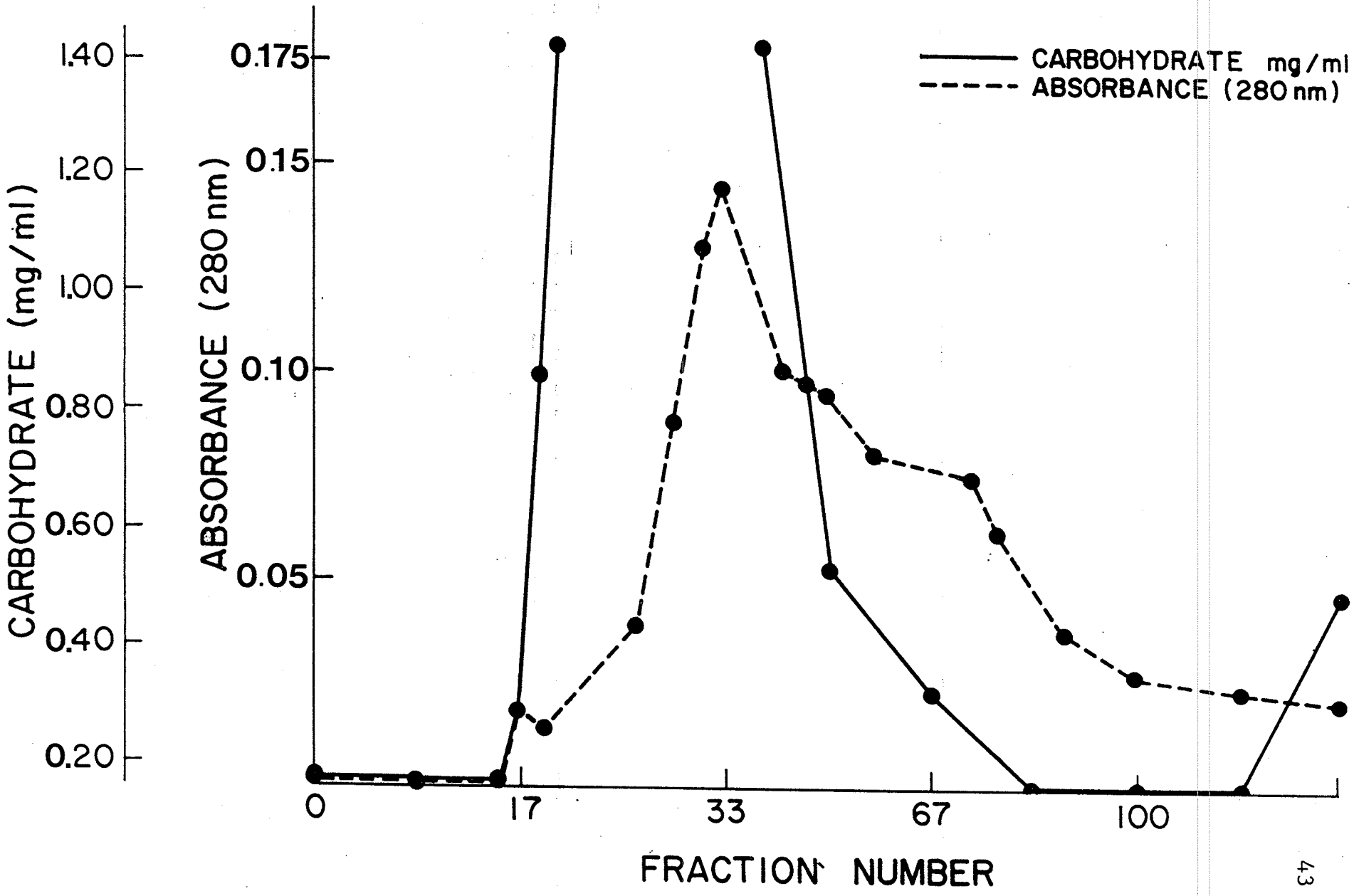

CARBOHYDRATE mg/ml
ABSORBANCE (280 nm)
CARBOHYDRATE (mg/ml)
ABSORBANCE (280 nm)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.175
0.15
0.10
0.05
0
17
33
67
100
FRACTION NUMBER

Figure 4. Absorbance Scan of Selected Fractions Separated with Sephadex G10.

Fraction 30,--------; fraction 30+ NaOH, _____.

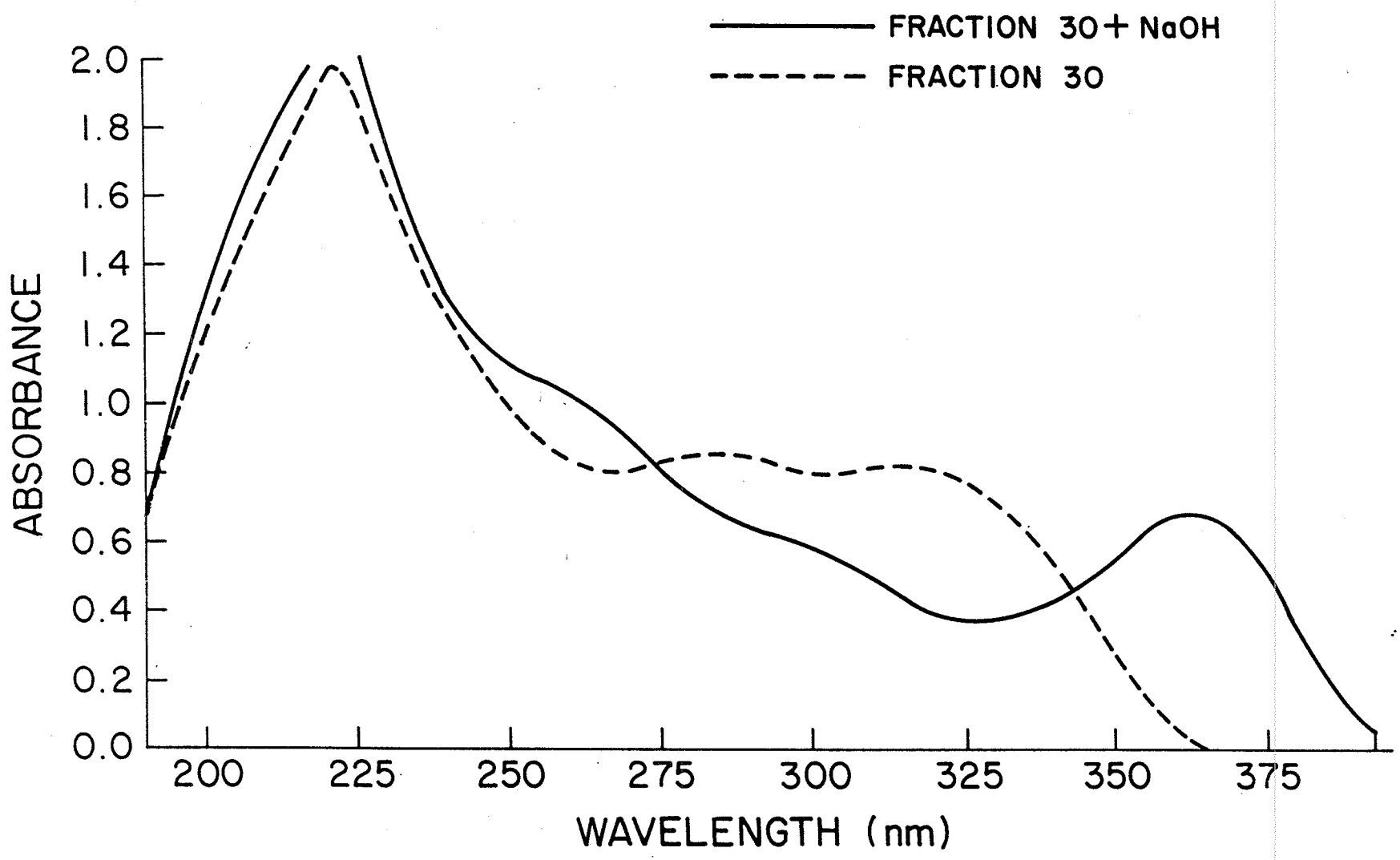
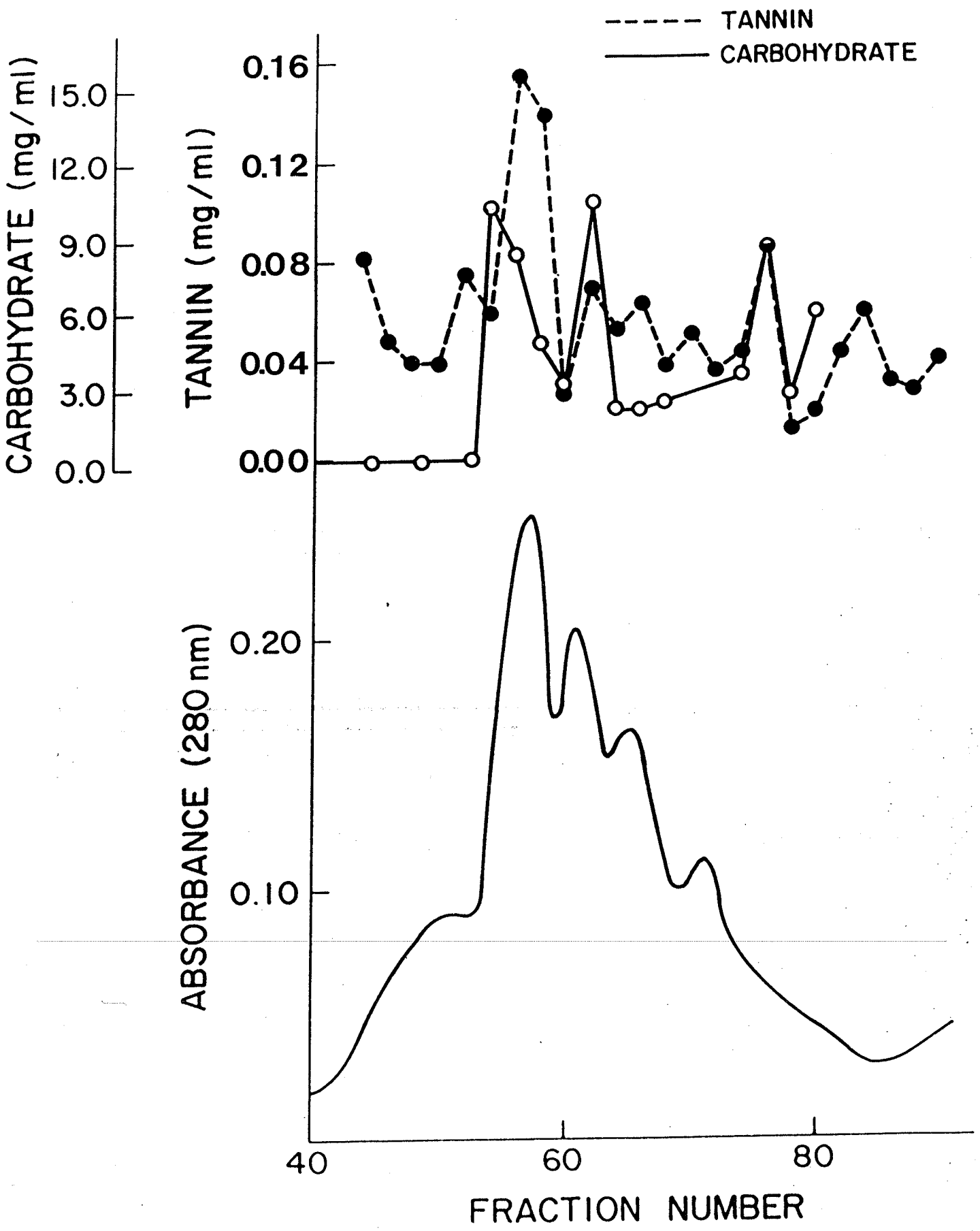

FRACTION 30 + NaOH
FRACTION 30
ABSORBANCE
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
200
225
250
275
300
325
350
375
WAVELENGTH (nm)

Figure 5. Separation of Ultrafiltration Partially Purified MCE with Sephadex G25.

Three ml fractions were collected at a flow rate of 0.6ml/min.


TANNIN
CARBOHYDRATE
CARBOHYDRATE (mg/ml)
15.0
12.0
9.0
6.0
3.0
0.0
TANNIN (mg/ml)
0.16
0.12
0.08
0.04
0.00
ABSORBANCE (280 nm)
0.20
0.10
40
60
80
FRACTION NUMBER

did not show inhibitory activity were separated by TLC utilizing BAW(8:2:2), and visualized with ninhydrin (Figure 6). Three spots (Rf values 15,30,and 58) present in MCE and fraction 55, were absent in the fraction which did not display inhibitory activity.

When all fractions were freeze-dried the color of the dried material ranged from yellow (fractions 1-40), to white-brown (40-80), to yellow (>80).

Tannin-Like Material in RSM

Analysis of the four RSM varieties for total tannin-like material showed a mean content of 2.42% (Figure 7). Tower contained the highest tannin-like material (3.03%), followed by R500 (2.72%), Candle (2.72%), and Turret (1.21%). Analysis of methanol extracted RSM showed a mean tannin-like content of 1.04%. MCE contained the most tannin-like material (1.46%), followed by Tower (0.97%), Turret (0.94%), and R500 (0.80%). The mean level of methylene chloride extracted tannin-like material was 0.14%. Tower extract had the highest tannin content (0.28%), followed by Candle (0.14%), Turret (0.07%), and R500 (0.07%).

Identification of the Tannin-Like Material

Preliminary Studies

Separation of the tannin-like compounds in MCE by TLC was investigated using various solvent systems. Separation of MCE with benzene-methanol-acetic acid (20:4:1) failed. Little, if any, material migrated from the origin with this strongly non-polar solvent system. Separation of MCE

Figure 6. Thin Layer Chromatogram of Sephadex G25 Separated MCE.

A, ultrafiltration partially purified MCE; B, fraction number 55; C, fraction number 60. Solvent used BAW(8:2:2).

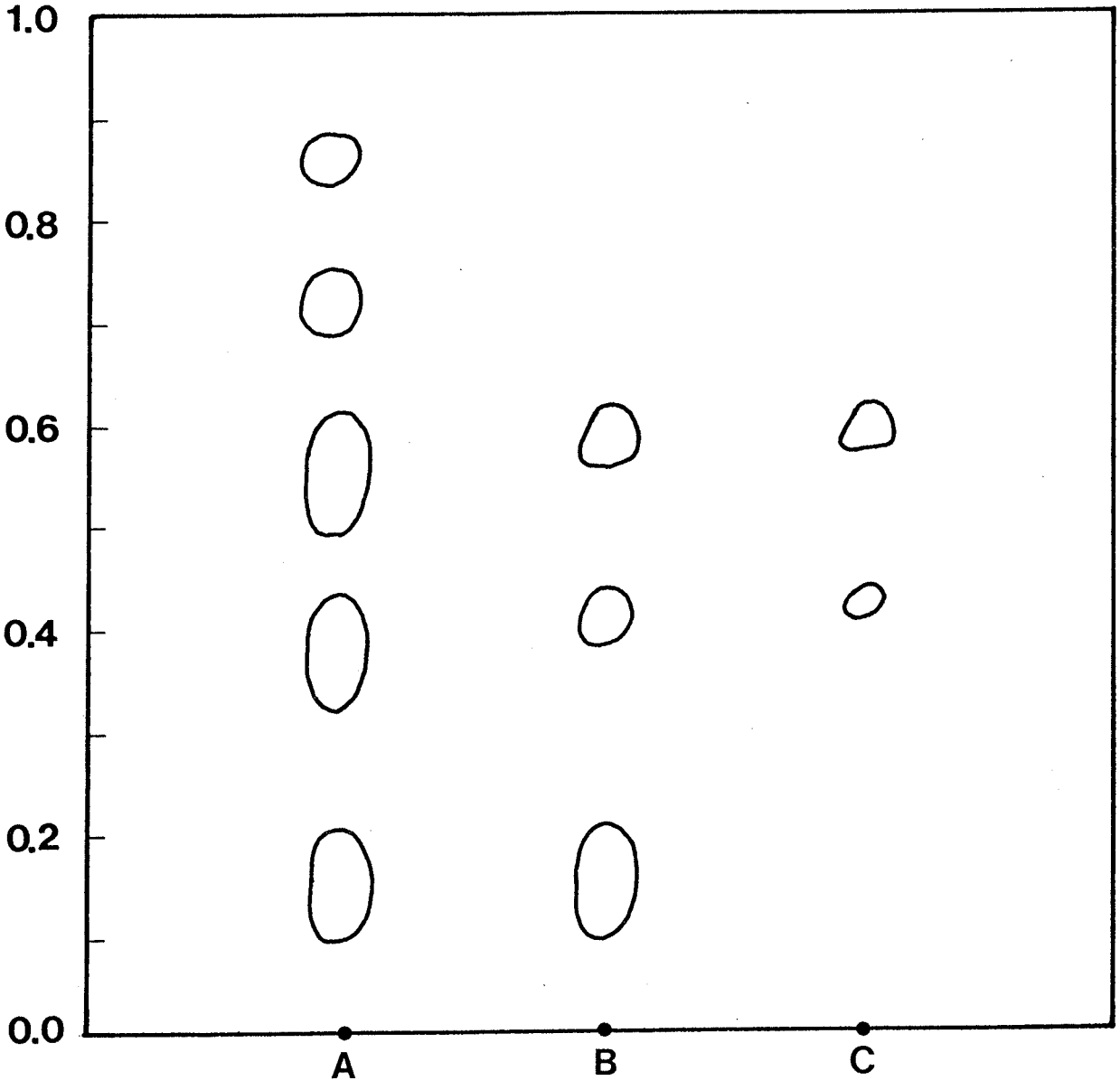
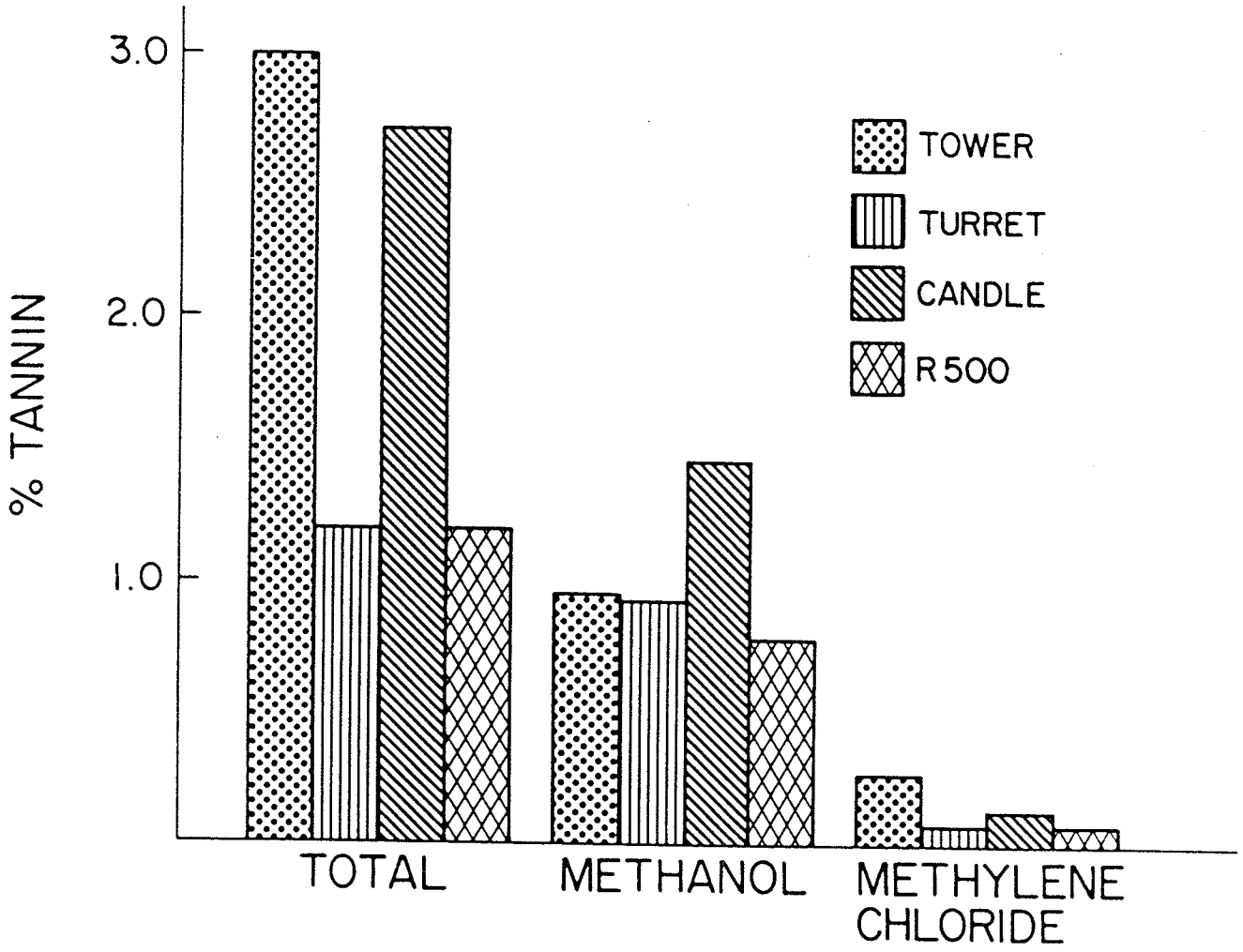

1.0
0.8
0.6
0.4
0.2
0.0
A
B
C

Figure 7. Percent Tannin in RSM Extracted with Selected Solvents.


3.0
2.0
1.0
% TANNIN
TOWER
TURRET
CANDLE
R500
TOTAL
METHANOL
METHYLENE CHLORIDE

into polar and non-polar constituents was achieved utilizing BAW (4:1:5). This solvent system, however, was too polar in nature and failed to separate individual polar compounds. A satisfactory separation was achieved utilizing BAW (8:2:2). A typical chromatogram of partially purified MCE visualized with Folin reagent, revealed five spots (Figure 8). Spots one and five, found on the chromatogram were identified as sinapine and sinapic acid, respectively, from Rf values of authentic compounds (Table 1). Spot two was tentatively identified as 1-sinapoyl-glucose from Rf values found in the literature (Fenton et al., 1980).

Bioautography

In order to determine which of these compounds displayed inhibitory activity ultrafiltration partially purified MCE was separated by two dimensional TLC employing BAW (8:2:2) on the first dimension, and BAW(5:2:5) on the second dimension. Visualization by bioautography revealed the presence of six areas of inhibition (Figure 9). Comparison of the two-dimensional bioautogram with a chromatogram visualized with Folin reagent (Table 2) showed spots with Rf values of 15,38, and 86(Folin) corresponded to sinapine, 1-sinapoyl-glucose, and sinapic acid respectively (Table 2). Spots with Rf values of 55 folin (58 bioautogram), 72 (with no corresponding bioautogram spot), 21, and 43(bioautogram) could not be identified.

Figure 8. Thin Layer Chromatogram of Ultrafiltration Partially Purified MCE, and Sinapine.

A, ultrafiltration partially purified MCE; B, sinapine; C, sinapic acid. Solvent used BAW(8:2:2).

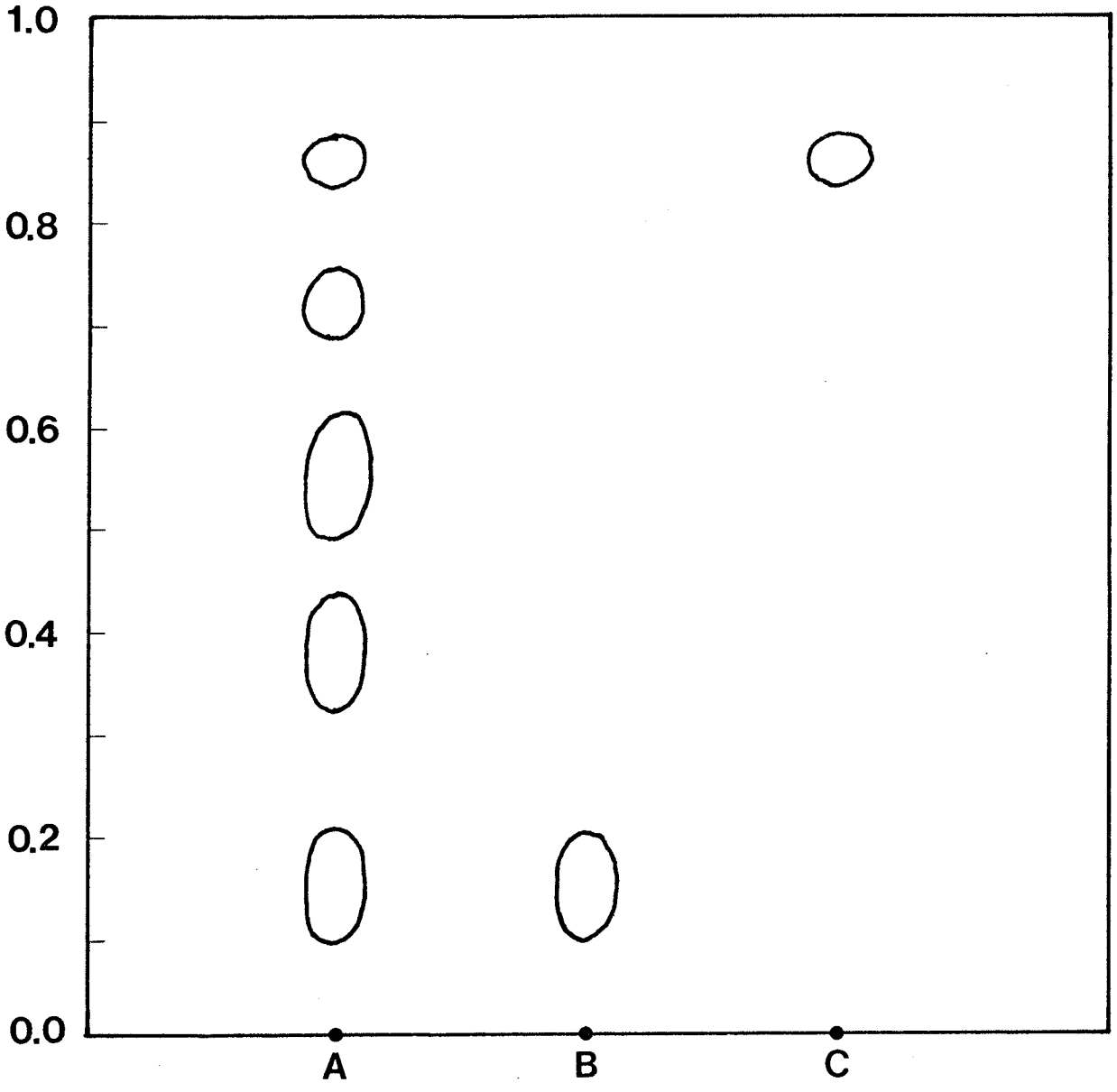

1.0
0.8
0.6
0.4
0.2
0.0
A
B
C

TABLE 1

Rf Values for Figure 8

Spots	Approx Rf Values	Tentative Identification
1	15	sinapine
2	38	1-sinapoyl-glucose
3	55	unknown
4	72	unknown
5	86	sinapic acid

Results shown are derived from Figure 8

Figure 9. Two Dimensional Bioautogram of Freeze-Dried MCE.

1, sinapine; 2, unknown; 3, 1-sinapoyl-glucose; 4, unknown; 5, unknown; 6, sinapic acid. Solvent used BAW(8:2:2) on the first dimension, and BAW(5:2:5) on the second.

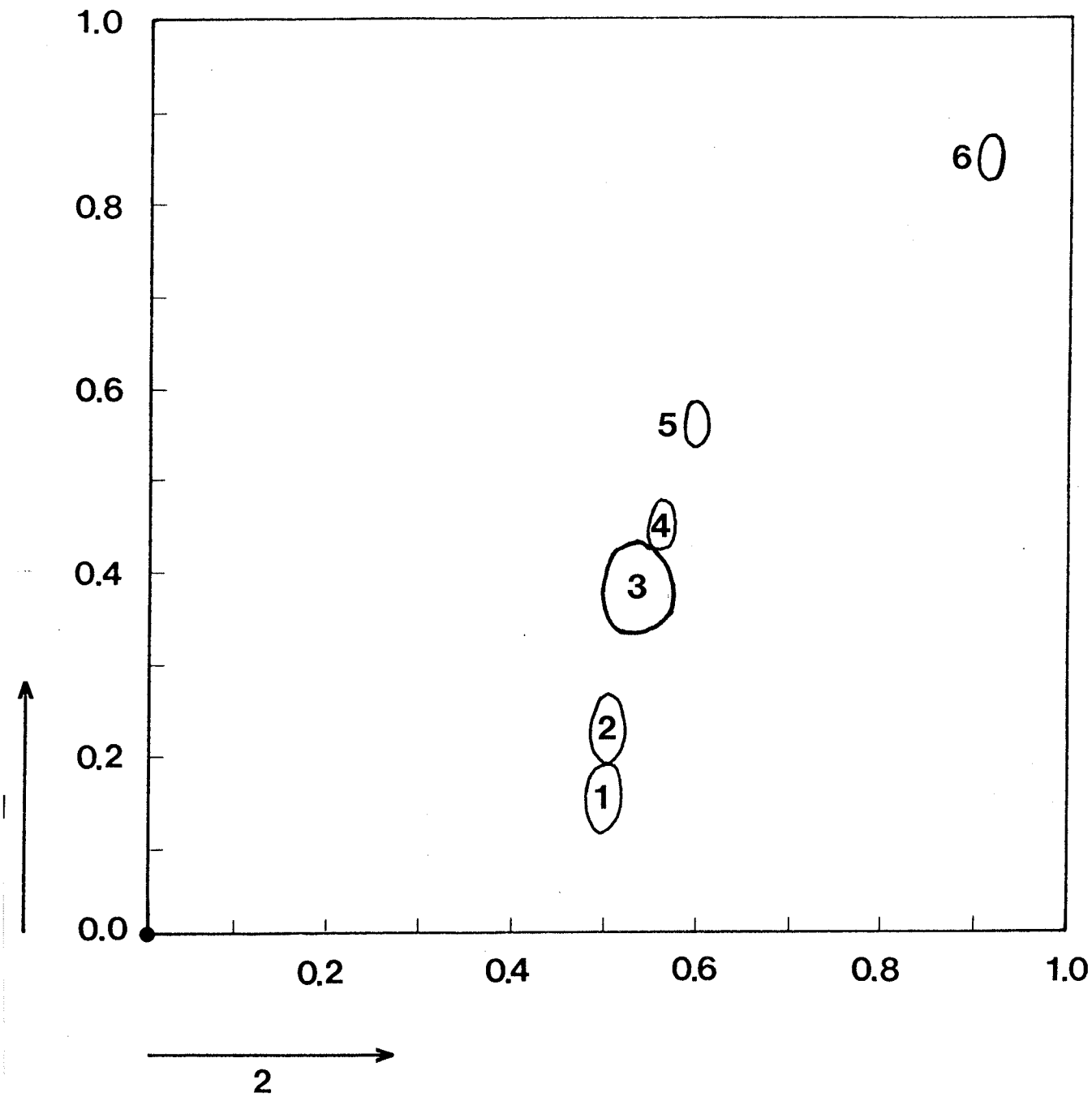

1.0
0.8
0.6
0.4
0.2
0.0
0.2
0.4
0.6
0.8
1.0
6
5
4
3
2
1
2

TABLE 2

TLC Rf Values of MCE Visualized with Folin-Ciocalteau and Bioautography

Folin Chromatogram	Bioautogram	Identification
15	15	Sinapine
--	21	Unknown
38	38	1-Sinapoyl-glucose
--	43	Unknown
55	58	Unknown
72	--	Unknown
86	85	Sinapic Acid

Results shown are derived from Figure 9, and Figure 8

TABLE 3

Sinapine Content and Tannin Content of RSM Varieties

	Tower	Turret	Candle	R500
Percent total tannin	3.03	1.21	2.72	2.72
Percent sinapine	0.68	0.87	1.15	0.59
Sinapine mg/ml MCE	6.80	8.70	11.50	5.90

Specific Inhibitory Substances

Sinapine. Methanol extracts of the four RSM varieties were analyzed for their sinapine content. Candle contained the highest sinapine level followed by Turret, Tower, and R500 (Table 3). Sinapine concentrations in methanol extracts varied from 5.90 mg/ml (R500), to 11.50 mg/ml (Candle). The inhibitory activity of sinapine to B.stearothermophilus, performed by disc assay, showed it to have a minimum inhibitory concentration of 7.0 mg/ml.

Destruction of sinapine in partially purified MCE, occurred when subjected to alkaline hydrolysis, resulting in a 95.7% reduction, while acidic hydrolysis did not affect it as greatly (Table 4). The effect of alkaline hydrolysis was also demonstrated to destroy inhibitory activity of partially purified MCE. Thin layer chromatography of alkaline hydrolyzed sinapine and MCE showed the destruction of sinapine along with the release of sinapic acid and choline (Figure 10). Acidic hydrolysis however showed no obvious effect.

1-Sinapoyl-Glucose. Freeze-dried MCE treated with activated carbon and separated by gel filtration chromatography with Sephadex G25, gave rise to fractions which absorbed strongly at 280 nm. Those fractions giving the strongest absorption were retained and rechromatographed with Sephadex G25. The chromatogram indicated the presence of two fractions designated peak A and B (Figure 11).

Peaks A and B were subjected to U.V. scanning from 190 to 450 nm. A typical scan of peak B displayed maximum absorbance at 280 and 317 nm (Figure 12). Peak A did not show this pattern of absorbance. The

TABLE 4

Effect of Alkaline and Acidic Hydrolysis on Sinapine and IM Content in MCE

	MCE	Acidic	Alkaline
sinapine[1]	1.15	1.06	0.054
inhibition[2]	12.00	11.50	0.000

(1). percent RSM

(2). The inhibitory activity was determined qualitatively by measuring the zone of inhibition perpendicular to both axes.

compounds contained in peak B showed inhibitory activity towards B. stearothermophilus, while those in peak A were negative for IM.

Material from peak B was separated on TLC (Figure 13). Only one spot was detected (Rf=38) which matched that of a substance found in MCE, tentatively identified as 1-sinapoyl-glucose. Analysis of peak B material showed the presence of 85% total sugar. Acidic hydrolysis however, resulted in the liberation of 56.7% reducing sugar.

Alkaline hydrolyzed peak B material, separated by TLC utilizing (BAW 8:2:2) visualized with Folin reagent, could not be detected. Sinapic acid however was observed (Figure 14). Peak B material separated by TLC under the same conditions, visualized with analine-diphenylamine-phosphoric acid reagent showed the presence of glucose (Figure 15).

Temperature Stability Studies

The inhibitory activity of freeze-dried MCE towards B. stearothermophilus was assayed at temperatures ranging from 50 to 100 °C. Residual activity was shown to decrease, particularily at temperatures greater than 70 °C (Figure 16;Table 5). Sinapine in aqueous solution did not undergo significant decomposition when subjected to temperatures ranging from 60 to 100°C.

pH Stabilty

The activity of MCE at various pH(2-10) expressed in Au of remaining residual activity, was constant over a pH4-6 (Figure 17). The IM appeared to be more stable under alkaline pH than at acidic pH. Readjustment of the IM from pH2 to the original pH(5.5) restored

Figure 10. Thin Layer Chromatogram of Alkaline Hydrolyzed MCE and Sinapine.

A, ultrafiltration partially purified MCE; B, ultrafiltration partially purified MCE hydrolyzate; C, sinapine; D. alkaline hydrolyzed sinapine; E, choline ; F, sinapic acid. Solvent used BAW (8:2:2).

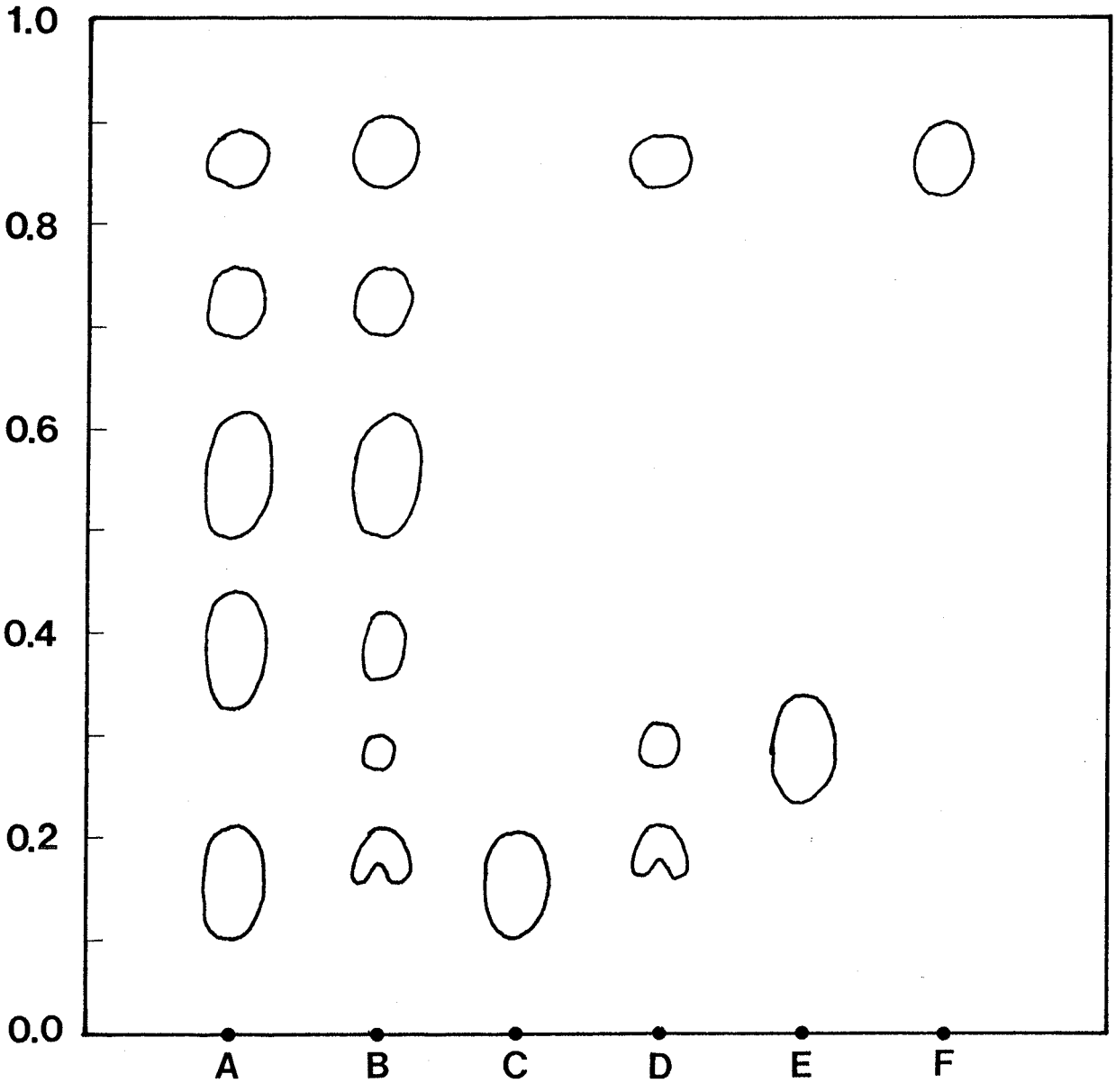

1.0
0.8
0.6
0.4
0.2
0.0
A
B
C
D
E
F

Figure 11. Activated Carbon Treated Freeze-Dried MCE Rechromatographed with Sephadex G25.

Freeze-dried MCE was initially separated with Sephadex G25. Fractions absorbing strongly at 280nm were concentrated and rechromatographed with Sephadex G25. Three ml fractions were collected at a flow rate of 0.6ml/min.

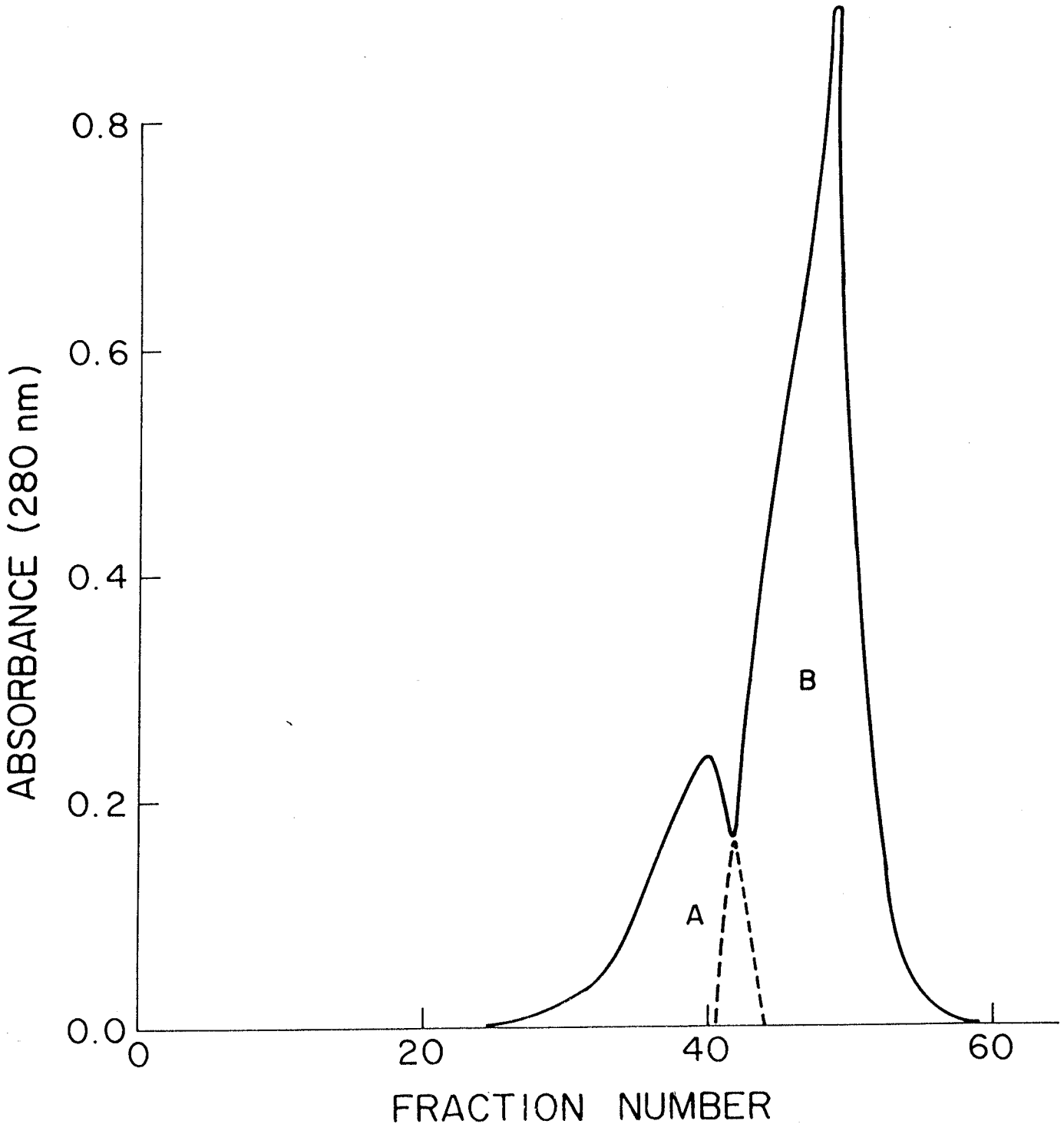

0.8
0.6
0.4
0.2
0.0
ABSORBANCE (280 nm)
0
20
40
60
FRACTION NUMBER
A
B

Figure 12. Absorbance Scan of Fractions A and B.

Peak A material,--------; Peak B material,_____.

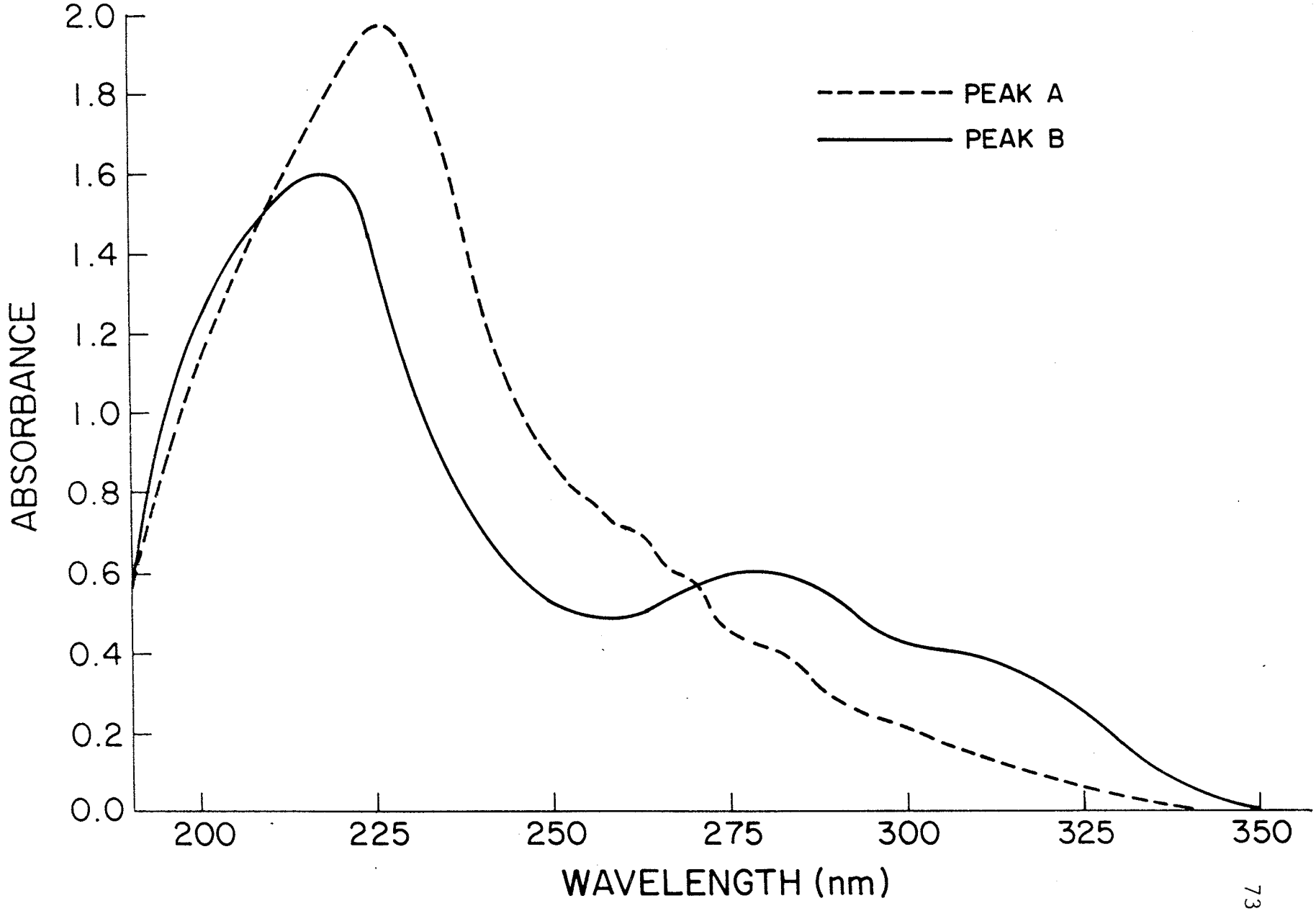
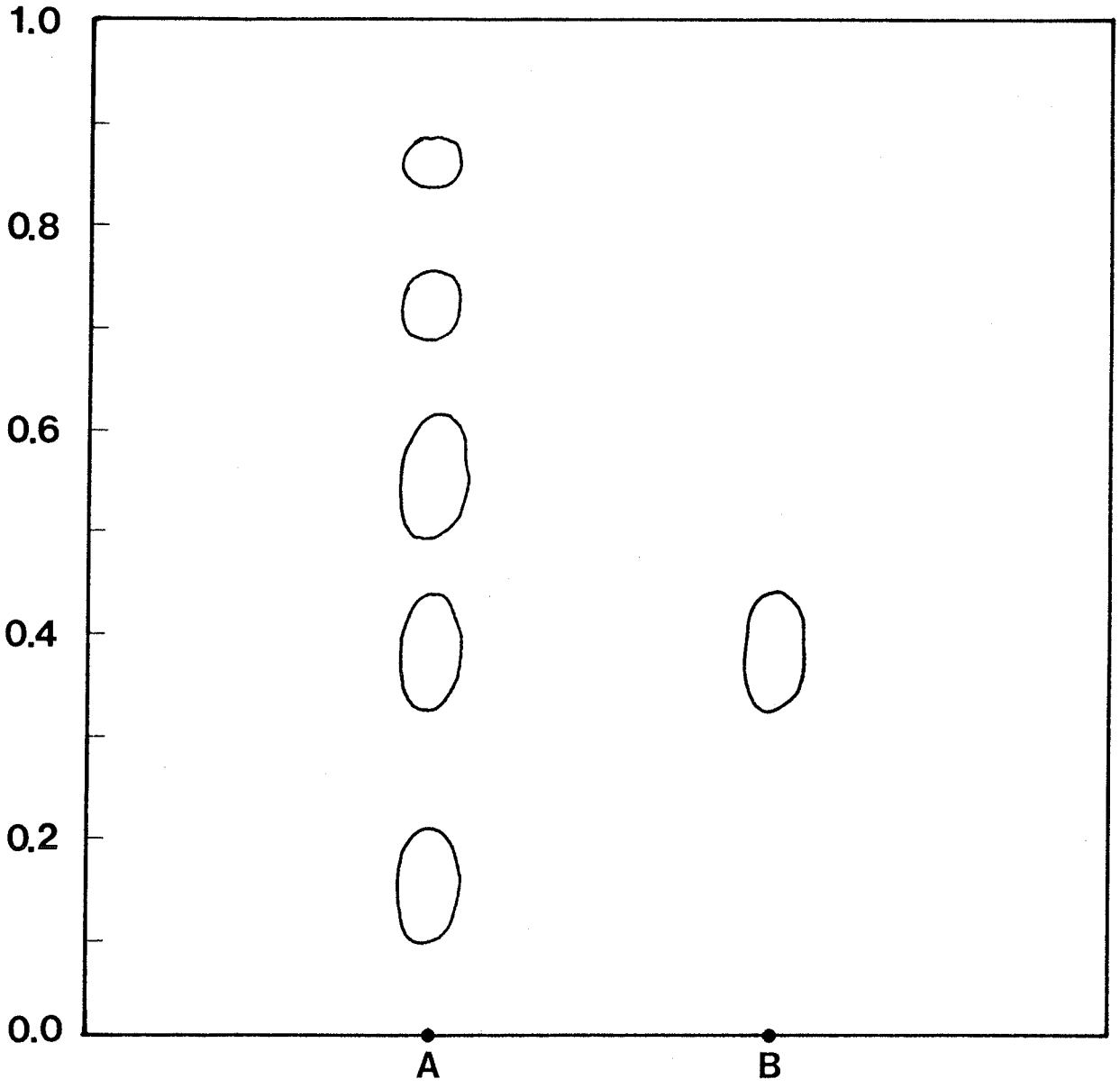

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE
200
225
250
275
300
325
350
WAVELENGTH (nm)
PEAK A
PEAK B

Figure 13. Thin Layer Chromatogram of Peak B Material.

A, ultrafiltration partially purified MCE; B, peak B material. Solvent used BAW(8:2:2).


1.0
0.8
0.6
0.4
0.2
0.0
A
B

complete activity of the inhibitor. Readjustment from alkaline conditions (pH8-10), however, resulted in poor activity recovery. Distilled water adjusted to pH2-10 did not display inhibitory activity. Disc assays of MCE, typically displayed an opaque zone immediately surrounding the paper disc. MCE adjusted to alkaline pH gradually lost this inner zone. MCE inhibition at pH2-6 exhibited well defined opaque zones, gradually disappearing at pH 8, with complete absence at pH10 (Figure 18). Sterile blank discs saturated with MCE, placed onto TSA devoid of B.stearothermophilus also displayed these opaque zones.

Identification of Free and Bound Phenolic Acids in RSM

In an attempt to identify other phenoic acids or their esters present in MCE which may have shown inhibitory activity, analysis of free phenolic acids, and those released by alkaline and acidic hydrolysis, was carried out by HPLC. Typical of reverse phase HPLC, polar compounds were retained the least. Retention times varied from 1.61 min for gallic acid, to >30 min for cinnamic acid. A lag of approximately 1.31 min occurred before the solvent gradient reached the absorbance detector. MCE contained 0.15% free phenolic acids. Phenolic acids detected were gallic, chlorgenic, gentisic, protocatechuric, and sinapic (Table 6). Acidic hydrolysis of MCE reduced or eliminated the amount and presence of some phenolic acids. Sinapic acid, for example could not be detected after acid hydrolysis. Alkaline hydrolylsis also reduced the amount of some phenolic acids. In addition to the five free phenolic acids detected in MCE, caffeic and p-coumaric acids appeared. An increase in the amount of sinapic acid was also detected.

Figure 14. Thin Layer Chromatogram of Hydrolyzed Peak B Material-Folin.

A, peak B material; B, peak B material alkaline hydrolyzed. Solvent used BAW (8:2:2).

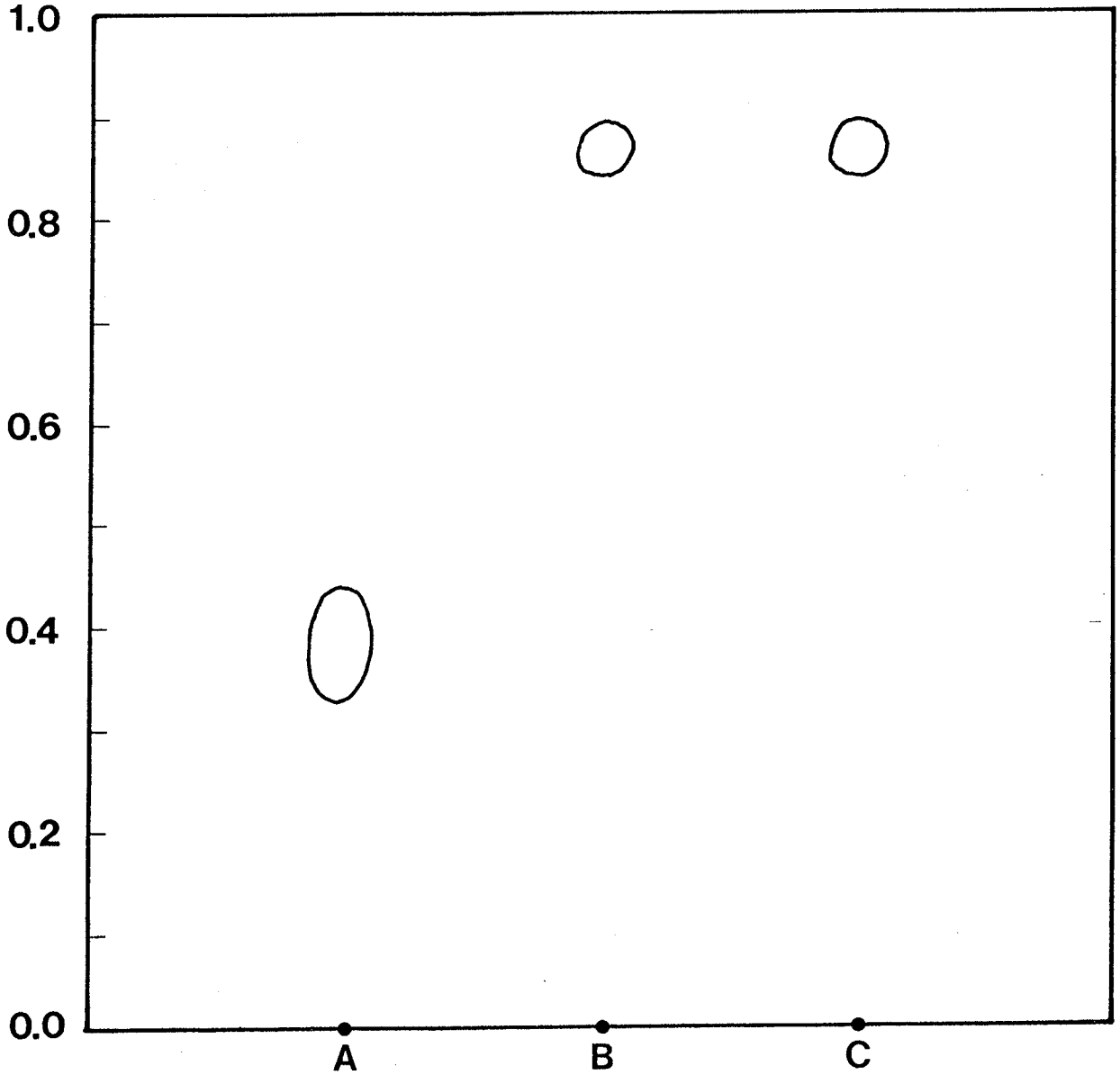

1.0
0.8
0.6
0.4
0.2
0.0
A
B
C

Figure 15. Thin Layer Chromatogram of Hydrolyzed Peak B Material-Glucose.

A, peak B material; B, peak B material alkaline hydrolyzed. Solvent used BAW (8:2:2).

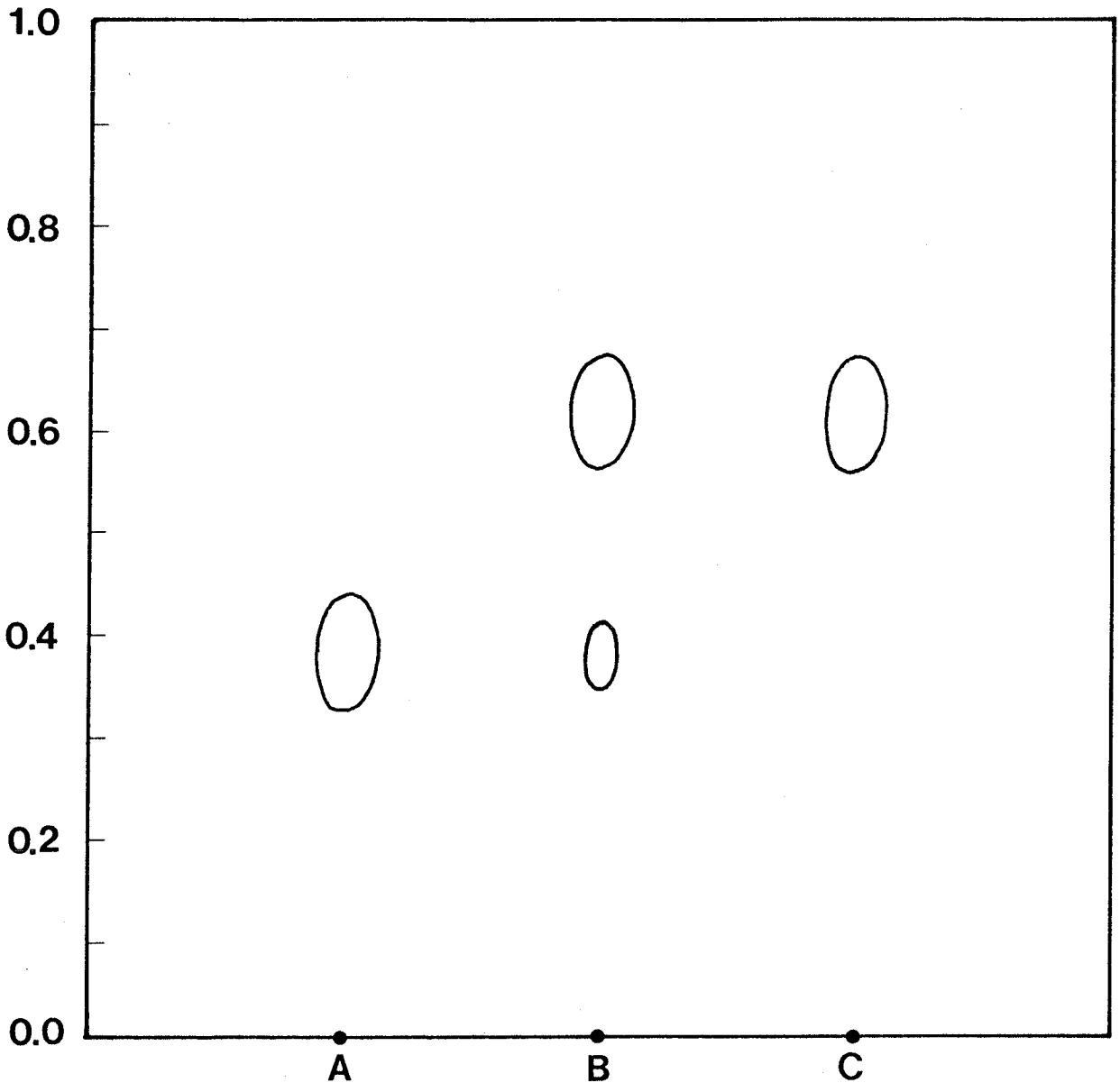

1.0
0.8
0.6
0.4
0.2
0.0
A
B
C

Figure 16. Temperature Stability of IM.

Temperatures are as indicated. X, blank disc; W, distilled water.

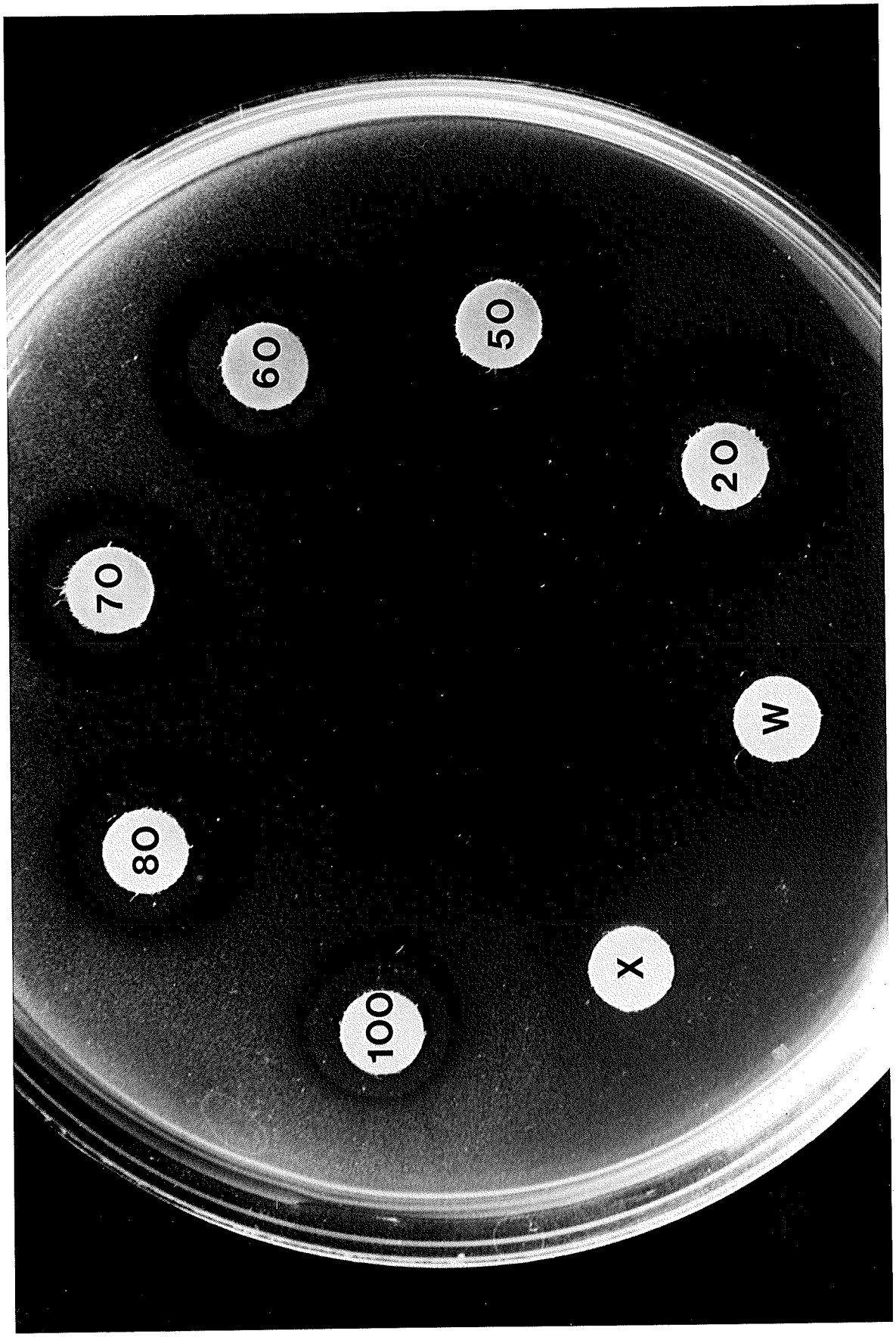

60
50
70
20
80
W
100
X

TABLE 5

Effect of Heat on Sinapine and IM Content of MCE

Temperature °C	Sinapine[1]	Inhibition[2]
20	0.10	12.0
60	0.12	11.35
70	0.12	10.05
80	0.12	9.41
100	0.12	7.46

(1). mg/ml

(2). The inhibitory activity was determined qualitatively by measuring the zone of inhibition perpendicular to both axes.

Figure 17. pH Stability of MCE Residual Activity.

Units indicated are arbitrary units (Au) of residual activity.

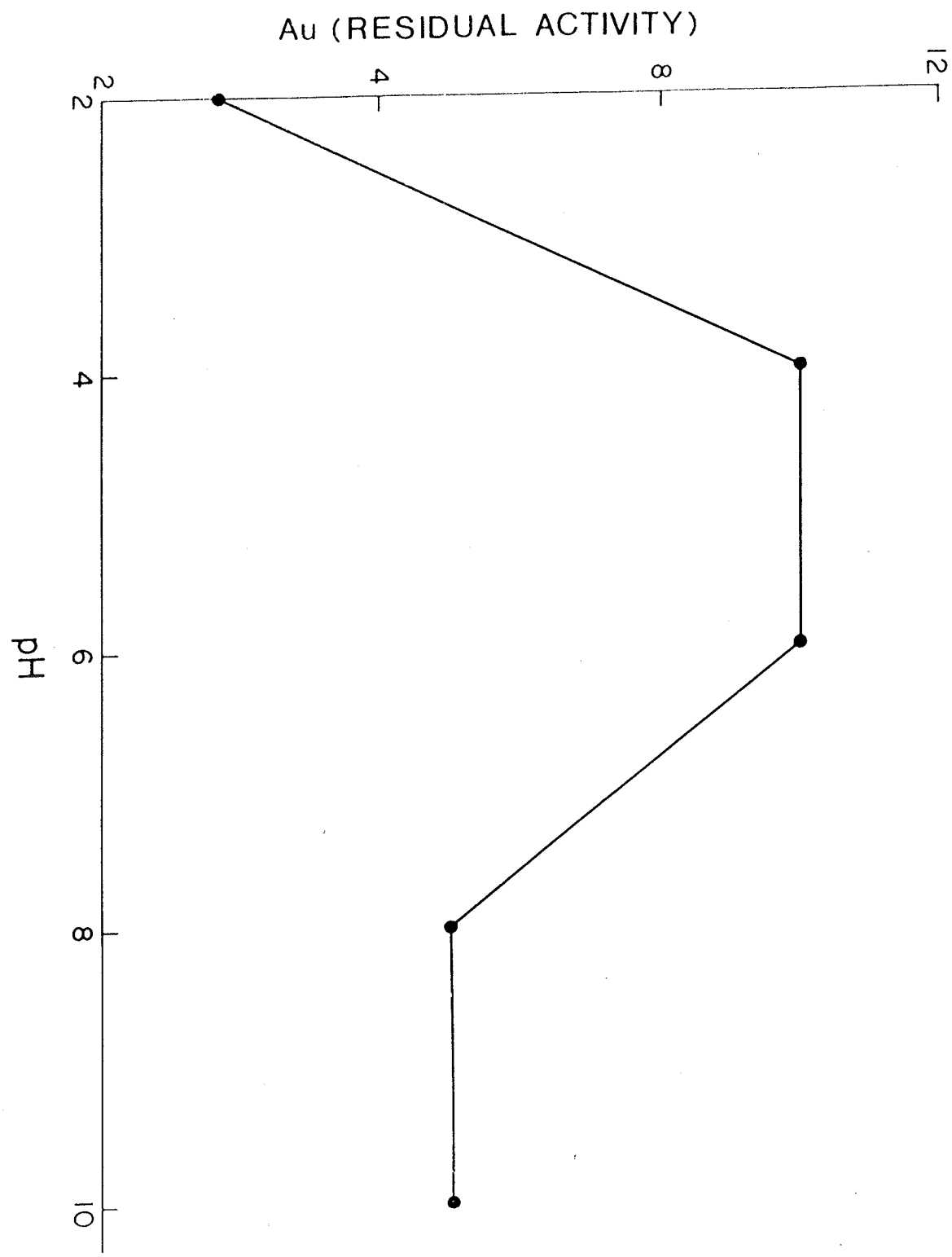

Au (RESIDUAL ACTIVITY)
2
4
8
12
pH
2
4
6
8
10

Figure 18. Effect of the Inner Zone at Various pH Levels.

pH of MCE as indicated. X, MCE unadjusted (pH5.5).

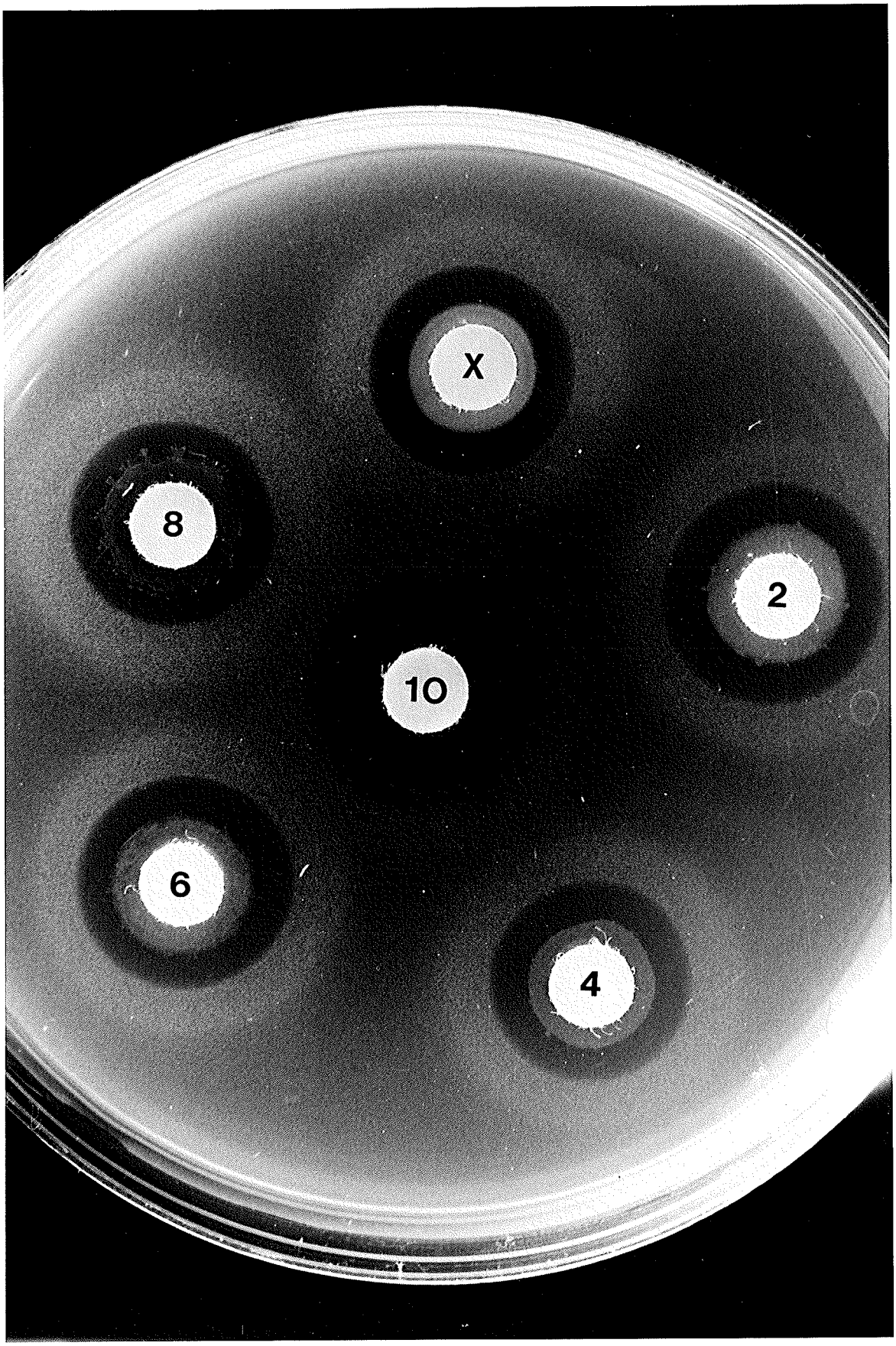

X
8
2
10
6
4

TABLE 6

HPLC Analysis of Phenolic Acids in MCE

Phenolic acid	Percent acid in RSM		
	Raw Extract	Acid Hydro	Alkaline Hydro
Gallic	0.0199	0.0328	0.0204
Chlorogenic	0.0110	0.0077	0.0075
Gentisic	0.1072	0.0718	0.0424
Protocatechuric	0.0113	0.0104	0.0171
Caffeic	------	------	0.0038
Sinapic	0.0018	------	0.3500
p-Coumaric	------	------	0.0010

Results shown are derived from HPLC analysis of MCE.

DISCUSSION

Feeding trials conducted at the University of Manitoba, using broiler chickens particularly susceptible to hemorhagic liver disease, failed to produce unequivocal results that glucosinolates and/or their hydrolytic products were involved (Israels et at,1979). Since many mycotoxins are also known to produce hemorrhagic liver disease (Allcroft,1969) it was of interest, initially, to investigate their presence in several commonly used RSM varieties. Preliminary studies showed that the 4 RSM varieties Tower, Turret, Candle, and R500 investigated in this study did not contain the mycotoxins aflatoxins, sterigmatocystin, ochratoxin A, citrinin, penicillic acid, patulin, and zearalenone and as such could not have been responsible for the inhibitory activity RSM displayed towards B. stearothermophilus. Since RSM did, however, show inhibitory activity to B.stearothermophilus, an organism used for the bioassay of mycotoxins, it was of interest to identify these inhibitory components. If these components, once identified, were novel/unique or nutritionally important it was hoped that their presence could in future be detected through a rapid bioassay screening technique. The object of this study was in part, then to develop a simple effective bioassay of toxic substance(s) present in RSM. Since OZT and CHB are commonly found in RSM, a bioassay of these toxins utilizing B. stearothermophilus was performed. The results indicated, that neither OZT or CHB displayed inhibitory activity to the test organism with the concentration employed.

The initial step towards identification of the IM was to determine its nature and distribution in RSM. Preliminary extractions of the RSM with methanol-water indicated, through bioassay, that an inhibitor was present. Dilution of extracts obtained from various organic solvents showed that MCE contained ca 3 times the amount of IM than did Tower or Turret, and ca 15 times that of R500. Methylene chloride on the other hand extracted ca 5 times the IM from Tower than it did from Candle. These results indicated that Candle contained large amounts of an IM readily soluble in methanol, while Tower contained essentially the same amount of another IM readily soluble in methylene chloride.

Ammonium sulfate treatment of MCE was performed in an attempt to determine the chemical nature of the IM. The resulting precipitates obtained from the various ammonium sulfate treatments showed only slight activity. In addition the largest proportion of activity resided in the supernatants even with 100% ammonium sulfate treatment, indicating that the bulk of the IM remained soluble in saturated ammonium sulfate. The slight activity exhibited by the ammonium sulfate precipitate may have been due to minor protein components exhibiting inhibition and/or co-precipitated phenolics. Dialysis of the 100% ammonium sulfate supernatant indicated that the molecular weight was less than 12000d.

Separation of MCE with Sephadex G10 yielded fractions which absorbed strongly at 280nm. Material contained in fractions showing the highest absorption also contained the largest amount of carbohydrate-like material. Fraction 30 showing the highest absorbance at 280nm also displayed strong absorption at 317nm. This pattern of absorption is typical of phenolic-like compounds (Ribereau-Gayon, 1972). Addition of

OH^- ions produced a bathochromic shift with a magnitude of ca. 77 nm. Harborne (1964) described the formation of the phenoxide ion from phenols in alkaline solution, and its corresponding shift of absorbance, the extent to which was determined by the degree of hydroxylization. At this point it was suspected that the IM was phenolic-ike in nature. None of the fractions separated with Sephadex G10 displayed inhibitory activity.

Partial purification and concentration of the IM in MCE was performed using ultrafiltration. During these purification steps it was established that the IM has a molecular weight of 1000-5000d. Since Sephadex G10 has a molecular weight range less than 1000d, the MCE was not partitioned, and was poorly resolved. As a consequence the phenolic-like materials were not concentrated in a few individual fractions, but rather were distributed over several fractions. This dilution effect reduced the inhibitor concentration below the resolution ability of the disc assay. Lo and Hill (1972) employing Sephadex G25 (molecular weight resolution of 1000-5000d) separated aqueous extracts of RSM into four fractions. Fraction I was found to contain large amounts of protein, fraction II contained glucosinolates, while fractions III and IV contained large amounts of polyphenolics. In this study MCE partially purified by ultrafiltration, was also chromatographed using Sephadex G25. Several compound peaks which absorbed strongly at 280nm resulted. Analysis of those fractions displaying strong absorbance peaks at 280nm also showed the presence of large amounts of phenolic and carbohydrate material. Only one fraction (55) exhibited inhibitory activity. This fraction also showed the

highest level of tannin-like material and strongest absorption at 280nm. It is known that polyphenolic compounds in RSM are primarily present not as free phenolic acids, but as esters of various simple and complex sugars (Durkee and Thivierge, 1975). As such it was concluded that the substances eluted in this fraction, corresponding to that portion of the chromatogram described by Lo and Hill (1972), contained phenolic glycosides. Since material with a molecular weight >5000d had been eliminated from the MCE separated by ultrafiltration the area of the chromatogram corresponding to fractions I and II (Lo and Hill, 1972), showed negligible material and was eliminated (Figure 5). TLC of the inhibitory fraction(55), showed the presence of substances (Rf=15,43,58) which were shown to be absent in a non-inhibitory fraction(60).

The four RSM varieties under investigation in this study contained a mean level of 2.42% tannin-like substances, with a maximum of 3.03% (Tower) and a minimum of 1.21% (Turret). These figures agreed closely with those reported in the literature, confirming that tannin content was species independant (Krygier et al., 1982b). Analysis of methanol extracted, tannin-like material yielded a mean value of 1.04% for the four RSM varieties investigated with a maximum of 1.46% (Candle) and a minimum of 0.79% (R500). A trend was apparent between methanol extracted tannin-like material and methanol extracted IM levels. As methanol extracted tannin-like material increased, a corresponding increase in methanol extracted IM was observed. The mean level of methylene chloride extracted tannin-like material was 0.14%, with a maximum of 0.28% (Tower) and a mimimum of 0.07% (Turret and R500). The level of methylene chloride extracted tannin-like material also

influenced the IM extracted with this solvent. Previously it had been determined that Candle and Tower contained essentially the same amounts of at least two IM(s), which differed in their solubilities. These results indicate that the pattern of inhibition methylene chloride and methanol RSM extracts displayed, followed the pattern of tannin-like material extracted by the respective solvent. Neither methanol extracts, nor methylene chloride extracts followed the levels of total tannin-like substances found in RSM.

Krygier et al. (1982b) found that sinapine represented the major phenolic ester in RSM. The presence of sinapine had been detected in this study by TLC of MCE. Sinapine was also shown to be present in inhibitory fractions separated by Sephadex G25. Sinapine levels for the four RSM varieties investigated in this study averaged ca 34% of total tannin-like material. A trend was observed not only between sinapine content and methanol extracted IM but also between sinapine content, methanol extracted IM, and methanol extracted tannin-like material. As sinapine levels increased, levels of methanol extracted IM, and methanol extracted tannin-like material also increased. Significantly the concentration of sinapine in MCE (11.5 mg/ml), Turret (7.7 mg/ml), and Tower (6.8 mg/ml), were near to or exceeded the minimum inhibitory concentration (7.0 mg/ml) required for inhibition of B. stearothermophilus on disc assay plates. It was concluded that sinapine was one of the major inhibitors present in methanol extracts.

Fenton et al. (1980) reported that seven compounds present in RSM, which upon hydrolysis, yielded sinapic acid. The study concluded that one compound found to release sinapic acid and glucose through alkaline

hydrolysis was 1-sinapoyl-glucose. In this study a compound shown by TLC to be present in MCE, was adsorbed by activated carbon treatment and subsequently fractionated by gel filtration chromatography. This compound which also released sinapic acid and glucose and exhibited inhibitory activity towards B.stearothermophilus. was tentitavely identified as 1-sinapoyl-glucose. This compound was composed of ca 85% non-reducing carbohydrate. Upon acidic hydrolysis the substance released considerable reducing sugar (56.7%). Glucose, bound to sinapic acid through a 1-ester linkage would not display such reducing activity since the reducing group would be involved in bond formation. These results confirmed that the isolated compound was 1-sinapoyl-glucose.

Ultrafiltration and gel filtration chromatography studies established that the IM had a molecular weight larger than 1000d. Since neither sinapine or 1-sinapoyl-glucose approach this size, it is thought that both compounds may exist in RSM as aggregates, or in combination with other substances. Since 1-sinapoyl-glucose isolated from MCE was composed of ca 85% carbohydrate, but released only 56.7% reducing sugar, under these conditions, it was concluded that other substances of a non-reducing carbohydrate nature remained.

Both sinapine and 1-sinapoyl-glucose were shown to be present in RSM. Direct evidence that these compounds were present in sufficient quantities to inhibit B. stearothermophilus was provided by bioautography. Two dimensional bioautography, resolved MCE into six substances showing inhibitory activity. A comparison of the resultant inhibition spots with compounds separated from MCE using conventional TLC confirmed that sinapine and 1-sinapoyl-glucose were inhibitory. In

addition it was shown through bioautography that sinapic acid also showed inhibitory activity. Alkaline hydrolysis of MCE destroyed inhibitory activity, and sinapine content. With the exception of 1-sinapoyl-glucose and sinapine spots, which were diminished, TLC of hydrolyzed MCE continued to display three spots of a phenolic nature. Two of these substances (Rf=58 and 85) were shown by bioautography to be inhibitory. Since alkaline hydrolysis of MCE destroyed its inhibitory activity the question arose as to why these two substances, found in hydrolyzed MCE, did not elicit a zone of inhibition on disc assay plates? Hewitt(1977) summarized the factors affecting zone size on agar diffusion disc assays. Those factors affecting the zone size included density of seeded inoculum, and thickness of the agar medium: an increase in both tended to decrease zone size and, hence, sensitivity of the assay. The agar layer used for bioautography (ca. 1mm) was not as thick as that used for disc assay. Consequently the technique of bioautography was more sensitive to inhibitory materials than the disc assay.

During the course of this study it was observed that hot solvent extracts of RSM were devoid of inhibitory activity. Subsequent investigations showed that part of the IM contained in MCE was unstable at temperatures >70°C. Sinapine ,however, did not undergo decompostion at these temperatures. The mechanism by which MCE lost its activity is not known.

The phenolic group is weakly acidic and will undergo reduction in alkaline conditions, forming the phenoxide ion. Since the unified phenolic group is the active inhibitor of bacteria, its acitivity

generally is greatest under acidic pH, decreasing in alkaline solutions. The pattern of inhibitory activity from MCE followed this rule from pH4 to pH10. At pH levels <4 activity, however, decreased. Sinapine and 1-sinapoyl-glucose are both esters of sinapic acid, and as such would undergo partial hydrolysis at pH<4, liberating sinapic acid (Harborne,1964). Readjustment of MCE from pH2 to pH5.5 resulted in complete recovery of inhibitory activity. Since the hydronium ion acts as a catalyst for both esterification as well as ester hydrolysis, the acid hydrolyzed esters were free to reform themselves and once more become active (Morrison and Boyd,1976). Readjustment of MCE from pH10 to pH5.5 resulted in poor activity recovery due, it is thought, to autoxidation of the liberated sinapic acid (Harborne,1964).

Five free phenolic acids were found to be present in MCE, with an additonal two appearing after alkaline hydrolsis. This conflicted with the results of Durkee and Thivierge (1975) and Fenton et al. (1980) who showed the absence of free phenolic acids in rapeseed or RSM. This study, in agreement with Kozlowska et al. (1975) and Krygier et al. (1982b) detected chlorogenic, gentisic, caffeic, sinapic, and p-coumaric acids in free or in bound forms. In addition the presence of protocatechuric, and gallic acids were detected. Fenton et al. (1980) reported that gallic acid was absent in RSM. Ferulic, cinnamic, vanillic, and p-hydroxybenzoic acids, however, were not detected. The alkaline hydrolyzate of MCE was shown to contain more sinapic acid than MCE (ca 194 times as much), released from sinapine and 1-sinapoyl-glucose. This level of sinapic acid, however, was much lower than would have been expected considering the large quantities of sinapine present

in MCE. Krygier et al. (1982a), however, found a 92% loss of sinapic acid under acid, and a 37% loss under alkaline conditions, due to oxidation.

It has been shown that several phenolic compounds present in either the free or the bound form inhibit the growth of B. stearothermophilus. The possibility remains that these compounds may also produce toxic effects in animals. By linking the toxic effect of these compounds from bacteria to animals, the bioassay described in this study may prove useful in selecting new toxin free varieties. Little is known of the interactions between phenolics and other constituents of RSM. Sinapine and 1-sinapoyl-glucose may be present in a polymeric state, or they may form bound complexes with carbohydrates, or other compounds. Confusion exists as to the composition of free or bound phenolic acids in RSM. An aggrement should be reached as to the extraction, purifiction, and analysis of rapeseed phenolics, since there is much disagreement in the literature.

Conclusion

As far as food technology is concerned, the signifigance of inhibitory substances present in rapeseed products cannot be ignored. Several attempts at isolation of rapeseed protein from rapeseed meal for use as a protein supplement in human food have met with some success. However the presence of unpalatable and potentially toxic substances have as of yet limited its use. As an animal feed, rapeseed meal continues to be used in limited amounts, despite its availability, and low cost. Plant breeding and improved methods of oil extraction have virtually

eliminated the glucosinolate problem, yet nutritional problems are still encountered especially when fed to non ruminants.

The use of B.stearothermophilus as a simple, effective, and fast bioassay for toxic substances in RSM has met with limited success. There are at least six compounds present in RSM which cause inhibition. In order to use the technique successfully, each of the substances would have to be isolated, and its individual inhibitory activity determined. The quantitative extraction of these compounds from RSM would be difficult since they differ greatly in their solubilities. Finally, IM present in RSM, which were identified in this study, have been isolated and identified in previous studies, and were not found to be toxic towards animals in the amounts commonly found in their diets.

SUMMARY

1. Rapeseed meal contains substances toxic towards B. stearothermophilus.
2. Preliminary investigation showed that no common mycotoxins were present in the RSM under investigation.
3. Hydrolytic products of glucosinolates were not inhibitory towards B. stearothermophilus.
4. Isolation of the IM established a molecular weight of 1000-5000d.
5. The IM was of a phenolic nature.
6. Characterization of the IM revealed sinapine, 1-sinapoyl-glucose, and sinapic acid to be involved.
7. These compounds existed as polymeric complexes, of themselves, or in combination with carbohydrates.
8. At least three other substance of unknown phenolic composition were also implicated.
9. Temperature stability studies revealed that the IM was unstable at temperatures >60°C.
10. The IM was active at pH4-6, but lost most activity at pH<4 and >6.
11. Free and bound phenolic acids are present in RSM.

REFERENCES

Abramson, D., Sinha, R., and Mills, J. 1980. Mycotoxin and odor formation in moist cereal grain during granary storage. Cereal Chem. 57(5):346-351

Allcroft, R. 1969. Aflatoxicosis in farm animals "aflatoxin-scientific background, control, and implications". Ed. Goldblatt, L.A. Academic Press, New york.

Association of Offical Agricultural Chemists. 1965. "Methods of Analysis". 10th. Ed. Washington, D.C.

Austin, F. and Wolfe, I. 1968. Sinapine and related esters in seed meal of Crambe abyssinica. J. Agr. Food Chem. 16(1):132-135.

Beach, D.L. 1975. Primary processing of vegetable oils. "Oilseed and Pulse Crops in Western Canada- A Symposium". Western Co-operative Fertilizers Ltd. Calgary, Alberta.

Bell, J.M., Youngs, C.G., and Downey, R.K. 1971. Nutritional comparison of various rapeseed and mustard seed solvent-extracted meals of different glucosinolate composition. Can. J. Anim. Sci. 51(2):259-269.

Bennett, E.O. 1959. Factors affecting the antimicrobial activity of phenols. Adv. Appl. Microbiol. 1:123-140.

Bowland, J.P. 1965. Feeding value of rapeseed meal for swine. "Rapeseed Meal for Livestock and Poultry- A Review" Canadian Dept. Agric. Publ. 1257.

Broce, D., Grodner, R.M., Killebrew, R.L., and Bonner, F.L. 1970. Ochratoxins A and B confirmation by microbiological assay using Bacillus cereus mycoides. J.A.O.A.C. 53:616-619.

Campbell, L.D., and Eggum, B.O. 1980. Protein quality of canola meal. "Research on Canola Meal, Oil and Seed". Canola Council of Canada. Winnipeg, Manitoba.

Canadian Grains Council. 1981. "Canadian Grains Industry Statistical handbook 81" Canadian grains council Winnipeg, Man. R3C-3Z3.

Cansfield, P.E., and Campbell, L.D. 1980. Toxicity to poultry of 1-cyano-2- hydroxy-3-butene (CHB), and 5-vinyloxazolidine-2-thione (OZT), hydrolysis products of rapeseed glucosinolates. "Research on Canola Meal, Oil and Seed". Canola Council of Canada. Winnipeg, Manitoba.

Chesson, A., Stewart, C., and Wallace, R. 1982. Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria. Appl. Environ. Microbiol. 44(3):597-603.

Clandinin, D. 1961. Rapeseed oil meal studies. 4. effect of sinapin the bitter substance in rapeseed oil meal, on the growth of chickens. Poult. Sci. 40(2):484-487.

Clandinin, D.R., and Heard, J. 1968. Tannins in prepress-solvent and solvent processed rapeseed meal. Poult. Sci. 47(2):688.

Clements, N.L. 1968. Note on a microbiological assay for aflatoxin B1: A rapid confirmatory test by effects on growth of Bacillus megaterium. J.A.O.A.C. 51:611-612.

Court, W. 1977. High performance liquid chromatography reversed phase liquid chromatography of naturally occurring phenolic compounds. J. Chromatography. 130:287-291.

Donk, P.J., 1920. A highly resistant thermophilic organism J. Bacteriol. 5:373-374.

Downey, R.K., Stefansson, B.R., Stringham, G.R., and McGregor, D.I. 1975. Breeding rapeseed and mustard crops. "Oilseed and Pulse Crops in Western Canada- A Symposium". Western Co-operative Fertilizers Ltd. Calgary, Alberta.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Ana. Chem. 8:350-354.

Durkee, A., and Thivierge, P. 1975. Bound phenolic acids in Brassica and Sinapis oilseeds. J. Food Sci. 40:820-822.

Fenton, T., Leung, J., and Clandinin, D.R. 1980. Phenolic components of rapeseed meal. J. Food Sci. 45:1702-1705.

Fenwick, R., and Hoggan, S. 1976. The tannin content of rapeseed meals. B. Poult. Sci. 17:59-62.

Fields, M. 1963. Effect of heat on spores of rough and smooth variants of Bacillus stearothermophilus. Appl. Microbiol. 11(2):100-104.

Hamilton, W.A. 1971. "Inhibition and Destruction of the Microbial Cell". Ed. Hugo, W.B. Academic Press, New York.

Harborne, J.B. 1964. "Biochemistry of Phenolic Compounds". Academic Press. New York.

Herbst, D. 1982. Identification and determination of four β-lactam antibiotics in milk. J. Food Protection. 45(5):450-451.

Hewitt, W. 1977. "Microbiological Assay: An Introduction to Quantitative Principles and Evaluation". Academic Press, New York.

Hobson-Frohock, A., Fenwick, R., Heaney, R., Land, D., and Curtis, R. 1977. Rapeseed meal and egg taint: association with sinapine. B. Poult. Sci. 18:539-541.

Holme, J. 1975. Utilization of protein supplements in human food. "Oilseed and Pulse Crops in Western Canada- A Symposium". Western Co-perative Fertilizers Ltd. Calgary, Alberta.

Hugo, W.B. 1976. The survival of vegatative microbes. Ed. Gray, T.R.G. and Postgate, J.R. "Twenty Sixth Symposium of the Society for General Microbiology". University of Cambridge.

Israels, E., Papas, A., Campbell, L., and Israels, L. 1979. Prevention by menadione of the hepatotoxic effects in chickens fed rapeseed meal. Gastroenterology 76(3):584-589.

Jackson, N. 1969. Toxicity of rapeseed meal and its use as a protein supplement in the diet of two hybrid strains of caged laying hens. J. Sci. Fd. Agric. 20:734-740.

Josefsson, E., and Moller, T. 1977. Screening method for the detection of aflatoxins, ochratoxin, patulin, sterigmatocystin, and Zearalenone in cereals. J.A.O.A.C. 60(6):1369-1371.

Josefsson, E., and, Uppstrom, B. 1976. Influence of sinapine and p-hydroxylbenzylglucosinolate on the nutritional value of rapeseed and white mustard meals. J. Sce Fd. Agric. 27:438-442.

Joslyn, M.A. 1970. "Methods in Food Analysis". Academic Press. New York.

Kozlowska, H.S., Sabir, M., Sosulski, F., and Coxworth, E. 1975. Phenolic constituents in Rapeseed flour. Can. J. Food Sci. Tech. 8(3):160-163.

Krygier, K., Sosulski, F., and Hogge, L. 1982a. Free, esterified, and insoluble bound phenolic acids: (a) extraction and purification procedure. J. Agric. Food Chem. 30:330-334.

Krygier, K., Sosulski, F., and Hogge, L. 1982b. Free, esterified, and insoluble bound phenolic acids: (b) composition of phenolic acids in rapeseed flour and hulls. J. Agric. Food Chem. 30:334-336.

Lloyd, L.E., McDonald, B.E., and Crampton, E.W. 1978. "Fundamentals of Nutrition". 2nd Ed. W.H. Freeman & Co. San Fransisco.

Lo, M.T., and Hill, D.C. 1971. Effect of dietary rapeseed meal on the serum proteins of rats. Can. J. Physiol. Pharmacol. 49(12):1100-1105.

Lo, M.T., and Hill, D.C. 1972. Composition of aqueous extracts of rapeseed meals. J. Sci. Fd. Agric. 23:823-830.

Melander, W.R., and Horvath, C. 1980 Reversed phase chromatography. "High-Performance Liquid Chromatography: Advances and Perspectives" Ed. Horvath, C. Vol.2. Academic Press, New York.

Mills, J., and Sinha, H. 1980. Safe storage periods for farm stored rapeseed based on mycological and biochemical assessment. Phytopathology. 70(6):541-547.

Morrison, R.T., and Boyd, R.N. 1976. "Organic Chemistry" Allyn and Bacon Inc. Boston.

Murphy, B.J., and Stutte, C. 1977. Analysis for substituted benzoic and cinnamic acids using high pressure liquid chromatography. Analyt. Biochem. 86:220-228.

Pons, W.A. jr. 1979. High pressure liquid chromatographic determination of aflatoxins in corn. J.A.O.A.C. 62(3):586-594.

Reiss, J. 1975. Mycotoxin bioassay using Bacillus stearothermophilus. J.A.O.A.C. 58(3):624-625.

Ribereau-Gayon, P. 1972. "Plant Phenolics". Oliver and Boyd, Edinburgh.

Simmons, R. 1970. The effect of rapeseed quality on processing procedures. "Internernational Conference on Scientific Technology and Marketing of Rapeseed and Rapeseed Products" Rapeseed Association of Canada. Winnipeg, Manitoba.

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

Stahl, E. 1969. "Thin-Layer Chromatography". Springer-Verlag, New York Inc. New York.

Summers, J.D., Bayley, H.S., Pepper, W.F., and Slinger, S.J. 1969. Value of rapeseed meal for growing pullets and laying hens. Can. J. Anim. Sci. 49:97-103.

Swain, T., and Hillis, W. 1959. The phenolic constituents of Prunis domestica. (1) The quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10:63-68.

Tzagoloff, A. 1963. Metabolism of sinapine in mustard plants (1) Degradation of sinapine into sinapic acid and choline. Plant Physiol. 38:202-206.

Vaisey-Genser, M., and Eskin, N.A.M. 1979. "Canola Oil: Properties, Processes and Food Quality". Rapeseed Assoc of Canada Winnipeg, Man.

Wilson, D.M., Tabor, W.H., and Trucksess, M.W. 1976. Screening method for the detection of aflatoxin, ochratoxin, zearalenone, penicillic acid, and citrinin. J.A.O.A.C. 59:125-127.