AN ABSTRACT OF THE THESIS OF

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Radish anthocyanin extract has potential as a natural colorant because of its pigment stability and attractive red hue. Presence of undesirable aroma compounds could limit its applications in foods. The pungent principle of radish, 4-methylthio-3-butenyl isothiocyanate (MTBI), is produced enzymatically upon cell injury from its glucosinolate precursor, 4-methylthio-3-butenyl glucosinolate (MTBG), and undergoes subsequent degradation to produce a number of volatiles. To evaluate the potential of flavor formation, juices were prepared from winter and spring radish cultivars. Whole radishes, peels and flesh, as well as radish extracts, were analyzed for glucosinolates and isothiocyanates. Aroma intensities of radish juice extracts were evaluated using sensory analysis.

MTBI was monitored by HPLC (detection level 160 ppb). MTBG was extracted from freeze-dried radish tissue with boiling methanol, purified by anion exchange and enzymatically desulfated. DesulfoMTBG was quantified by HPLC, using desulfosinigrin as internal standard. Identification was performed by fast atom bombardment and electrospray mass spectroscopy. MTBI formation was higher in winter than in spring cultivars (1.5-2.8 and 0.8-1.3 mg/100g fresh weight, respectively), and higher in flesh than in peels.

MTBG ranged from 30-65 mg (spring cultivars) to 260-320 mg/100g fresh weight (winter cultivars) with greater concentration in peels than in flesh. Isothiocyanates and glucosinolates were not detected in radish juices.

Overall aroma intensities of radish juice concentrates, diluted to 150, 300, 600 and 1200 mg anthocyanin/L in water, were rated using a 16-point scale. Radish concentrates from cultivars Fuego (pigmented peels) and Red Meat Takii (whole red flesh) were compared to commercial red cabbage and radish colorants. Aroma intensities followed first order relationships with anthocyanin concentrations. The commercial colorants were rated slight to moderate, while radish extracts (Fuego and Takii) were rated moderate to large. The aroma intensity of red flesh radish extract was more potent than those prepared from radish peels. Further work includes development of purification techniques which would provide an odorless aqueous extract.

Analysis of Flavor Precursors in Radish and Radish Color Extracts

by

Myriam M. Kucza

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Myriam M. Kucza, Author

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I. INTRODUCTION

The addition of flavors, colors, texturing agents, and preservatives to processed foods is usually necessary to provide consumers with appealing, microbial safe and tasty products. Consumer behavior is driven by the flavor, nutritional value and visual appearance of foods. The use of synthetic dyes in food products has seen a great success not only because of their color characteristics, efficiency and stability throughout the processing and storage of foods, but also because the synthetic colors were believed to be safe until a few decades ago (Gennaro, 1994). In the USA, coloring materials are regulated under Title 21 of the Code of Federal Regulations (Parts 70 to 82) by the US Food and Drug Administration (FDA).

The 1960 Color Additive Amendment to the Federal Food, Drug and Cosmetic Act of 1938 specified two groups of colorants: certified color additives and color additives exempt from certification (Dziezak, 1987). Certified colors include seven compounds that are chemically synthesized to a high degree of purity. Each batch of color is tested by FDA for compliance with chemical specifications established by the agency. In the process of certification, suitability of color additives is determined by scientific investigations which include toxicity testing on laboratory animals. Colors exempt from certification includes 26 colorants and are divided into (a) colorants extracted from natural sources such as vegetables, animals or minerals, and (b) 'nature-identical' compounds which are synthetic

counterparts of natural derivatives. FDA does not consider batch certification of these colorants necessary to protect public health. The word 'natural' is not recognized by FDA. However, generally speaking, this class of coloring materials is often referred to as natural colorants (Hendry et al, 1996).

Among color additives, red color has the greatest usage, finding wide-spread application in beverages, fruit drinks, candies, pie fillings and dairy products (Newsome, 1986). Sources of natural colorants are now attracting increasing attention as alternatives to the artificial compounds after the banning of color additives such as FD&C Red No. 2. Increasing consumer demand for more 'natural' products along with restrictive regulations tend to completely or selectively ban artificial color additives from some foods.

Anthocyanins are red-purple pigments, wide-spread in nature. Various plants have been suggested as potential commercial sources: cranberry, blueberry, bilberry, red cabbage, roselle flowers and miracle fruits (Hendry, 1996). A major source of anthocyanins used as food colorants is grapes extracted from waste material of wine and grape juice processing. In the USA, grape color extract and grape skin extract are listed by the FDA as colorant exempt from certification for food and beverage use. Also, elderberry extract and red cabbage colorant are available commercially, as fruit and vegetable juices which do not require certification. Other red pigments such as betacyanins from red beet are used as food colorants. Tinctorial value and pigment stability of these natural colorants are not entirely satisfactory for the food processors compared to artificial red dyes. In particular, maraschino cherry producers have been seeking for a natural bright red colorant that could be an alternative to the use of FD&C Red No. 40, the azo dye now in use.

Recently, a purified radish extract was prepared at Oregon State University by Giusti and Wrolstad (1996b) which showed promise because of its red hue and good stability (half life of six months) when used to color maraschino cherries. An aqueous extract of radish peels was purified by adsorption on C18 resin with subsequent alcohol extraction. While effective, this highly purified extract would not meet FDA approval as a vegetable juice and would require extensive safety and toxicity testing to be approved as a food additive. Research is now in progress for alternative methods of extraction involving water as the extraction solvent, and avoiding organic solvents. As an aqueous extract, the radish colorant would be approved by the FDA in the category of fruit and vegetable juices.

A disadvantage of many natural color extracts is that they may impart undesirable flavor characteristics that could limit their food applications. Isothiocyanates are responsible for the pungency of radishes. They are degradation compounds of sulfur-containing precursors, known as glucosinolates (Friis and Kjaer, 1966). The assessment of these flavor compounds in radish and radish extracts is of importance in the process of making a colored extract that would be suitable for industry uses and accepted by consumers. This project had therefore the following objectives:

- develop a methodology to extract, isolate and identify the major flavor components and precursors of radish and radish extracts, using preferentially high performance liquid chromatography (HPLC) or spectrophotometric techniques;

- monitor their concentration in radish tissue (peels vs. flesh; winter vs. spring cultivars) and in color extracts;

- apply sensory analysis in the evaluation of radish anthocyanin extracts.

II. LITERATURE REVIEW

1. Radish, a cruciferous plant

1.1. Botanical characteristics

Raphanus sativus, or radish, belongs to the Brassicacea or Cruciferae, a large family of the dicotyledonous angiospersms (order of Capparales) which is often referred to as the Mustard family. Members of this family are characterized by a floral structure of four sepals and four equal cruciform petals, hence the name of the family, Cruciferae. The flower consists of six stamens and an ovary with two parietal placenta. The fruit is a capsule with a false-septum, so called since it is not the wall between carpels. In these characteristics, the Cruciferae are close to the Umbelliferae such as carrot and celery. The structure of the flower and the fruit is basically homogenous with few exceptions. However, there is a large variation in the shape, number, arrangement and location of the fruit and flower components which allow further classification (Crisp, 1976).

1.2. Radish production and consumption

Egyptians, in the time of Pharaohs, and ancient Greek cultivated radishes quite extensively. Presently, radishes are eaten raw, cooked, brined, fermented or dried as vegetables and condiments. They are consumed in Europe and America mainly raw as salad radish and in Asia, cooked or fermented as daikon (Carlson, 1985). Some varieties can also be used as oilseed (*R. sativus* ssp. *oleifera*). Radishes contain moderate levels of vitamin C and potassium, are low in protein, and contain no vitamin A.

Radish cultivars are numerous and differ in their shape, size, color and flavor properties depending on the cultivar, harvest time (age), soil, and climate, among other agricultural factors. The European-American market supplies mostly salad radish cultivars such as Cherry Belle, Fuego, Comet, and Scarlet. Daikon radishes, which can weigh several pounds, are predominantly available in Japanese and Korean markets. Daikons produced in Florida can reach 20 pounds and more. Some radish cultivars are round, others can be cylindrical with roots 40 cm long and 5 cm in diameter (Carlson, 1985). Spring and summer radishes (salad types) are annuals while the winter types (daikon and Chinese) are either annuals or biennals. Radishes may be grown in any type of soil but for commercial purposes, a muck is preferred to insure uniform shape of the hypocotyls, the edible part often referred to as roots.

Although radishes can be grown almost anywhere, several states are predominant in their production: Florida, California and Michigan. In 1992, these three states accounted for 76% (22,550 acres) of the total surface harvested for radishes in the USA, with 20% (155) of the farms producing radishes (Appendix 1).

Florida alone accounted for more than half of the harvested acreage of radishes in the USA. During the 1994-95 crop season, 19,200 acres of radishes were planted and 15,700 were harvested. The production totaled 4 million 15-lb cartons (60 million lb) with a yield of 3,840 lb per acre. In value, the production translated to almost 24 million dollars. The peak of production is between February to April. Looking at the production trend from 1985 to 1994 (Appendix 2), we notice a reduction in planted acreage of almost 30% after 1993, leading to a lower volume in production. The 1994-95 season was

characterized by the lowest production of record since the 1965-66 season. Difficult weather conditions with tropical storms and freezes lowered the acreage harvested, yield and production (Florida Agricultural Statistics, 1996).

1.3. Importance of cruciferous crops

The Cruciferae family includes over 2,000 species, containing a considerable number and diversity of crop plants. Most of them are condiments, oil-seeds or are cultivated for use in salads, vegetables (raw or cooked) and animal feeds. Many of the cruciferous plants have been cultivated for substantial lengths of time, especially within the genus *Brassica*. Cruciferous crops have been developed where seed, leaf, root, inflorescence or stem can be used.

In Europe, *Brassica napus* L. is grown as a vegetable for animal feed with swollen root (Swede) or as an oil-seed crop. The cruciferous oil crops (*Brassica napus* oilseed rape and *Brassica campestris* oilseed turnip rape) are used for the production of an oil low in polyunsaturated fat and an industrial feed stock. The seed meal remaining after oil extraction is a valuable animal feed, rich in protein. Rapeseed is an important forage crop in crop rotation, especially with cereals. In the mustard industry, seeds of *Brassica juncea* (brown mustard) and *Sinapis alba* (white mustard) are the main sources (Crisp, 1976; Bones, 1996).

Other cruciferous plants and vegetables that are part of our diet include members of the species *Brassica oleracea* such as red/white cabbage (var. *capitata* L.), Brussels sprouts (var. *gemmifera*), cauliflower (var. *botrytis*), and belonging to other genera of the Cruciferae, water cress (*Nasturtium officinalis*) and horseradish (*Armoracia lapathiofolia*).

2. Glucosinolates, flavor precursors

The flavor components of cruciferous species are mainly degradation compounds of thioglycosides called glucosinolates. Upon enzymatic degradation, all glucosinolates produce corresponding isothiocyanates which have considerable importance in flavor attributes.

Glucosinolates are a class of sulfur and nitrogen containing compounds that occur in all species of the Cruciferae (Brassicaceae) and several other higher plant families of the dicotyledonous angiosperms, most of which belong to the order of Capparales. They are flavor precursors and are responsible for the typical flavor characteristics of vegetables, cole crops and condiments such as cabbage, cauliflower, broccoli, horseradish, mustard, radish, watercress (Fenwick, 1983) and have also been reported in papayas (Cairns et al., 1988). As reported by Fenwick and coworkers (1983), no member of the Cruciferae has been found to lack glucosinolates. Therefore, their presence has been used as a chemotaxonomic index for classification within the family.

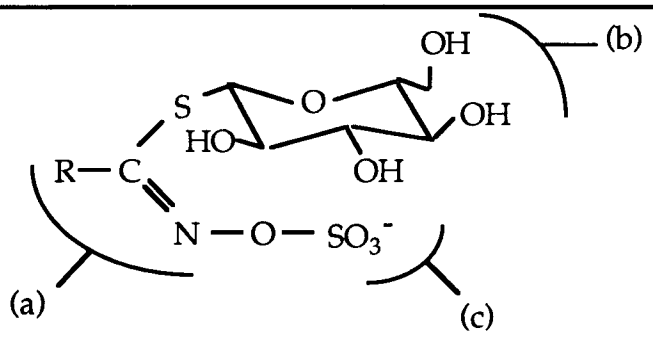
2.1. Chemical structure of glucosinolates

The basic structure of glucosinolates includes three main elements in the molecule (Figure 1):

(a) the R-C-N structure derived from an amino acid,

(b) a glucose moiety attached via a thioester link,

(c) the sulfate group linked to the nitrogen.



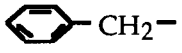
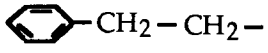
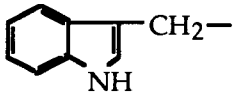
TRIVIAL NAME of Glucosinolate	STRUCTURE OF R GROUP	NAME
	ALIPHATIC SIDE CHAINS	
Sinigrin	$CH_2 = CH - CH_2 -$	2-Propenyl (or Allyl)
Gluconapin	$CH_2 = CH - (CH_2)_2 -$	3-Butenyl
Progoitrin	$CH_2 = CH - CHOH - CH_2 -$	2-Hydroxyl-3-butenyl
Glucoerucin	$CH_3 - S - (CH_2)_4 -$	4-Methylthiobutyl
	$CH_3 - S - CH = CH - (CH_2)_2 -$	4-Methylthio-3-butenyl
Glucoibervirin	$CH_3 - S - (CH_2)_3 -$	3-Methylthiopropyl
Glucoraphanin	$CH_3 - SO - (CH_2)_4 -$	4-Methylsulfinylbutyl
Glucoraphenin	$CH_3 - SO - CH = CH - (CH_2)_2 -$	4-Methylsulfinyl-3-butenyl
	HOMOAROMATIC SIDE CHAINS	
Glucotropaeolin		Benzyl
Gluconasturtiin		2-phenylethyl
Glucosinalbin		*p* -Hydroxybenzyl
	HETEROAROMATIC SIDE CHAINS	
Glucobrassicin		3-Indolylmethyl

Figure 1: General chemical structure of glucosinolates and possible structure for the R group (modified from Larsen, 1981; Fenwick, 1983; Falk, 1994).

All natural glucosinolates are anions containing β-D-thioglucoside. They generally show a Z configuration around the carbon nitrogen double bond between the R and sulfate groups (Larsen, 1981). Sinapyl-3-butenyl glucosinolate has been identified in fruits of *Boreava orientalis* by Sakushima and coworkers (1995). It contains a sinapic ester, sinapine which is attached to the sulfate group.

Well over 100 types of glucosinolates found in nature have been described (Fenwick, 1983). They can be divided into three groups according to the structure of the aglycone (Falk, 1994) : aliphatic (alkyl, methylthio, methylsulfinyl, methylsulfonyl), aromatic (homoaromatic) or indolyl (heteroaromatic) (Figure 1). Sinigrin and sinalbin were the first glucosinolates isolated by P. J. Robiquet and F. Boutron in 1831 (Fenwick, 1983). Before introduction of a systematic nomenclature, the glucosinolates were generally called mustard oil glucosides after the mustard oils (isothiocyanates) liberated by enzymatic degradation.

2.2. *Occurrence and distribution*

2.2.1. In cruciferous plants

Glucosinolates generally occur in all parts of a plant but in widely different concentrations depending upon the part of the plant examined. In cabbage, glucosinolates were found in lower amount in the outer leaves than in the inner leaves (Van Etten et al., 1976).

The level in fresh plants is usually about 0.1% or less based on fresh weight whereas levels in seeds may reach 10% of the dry weight. In seeds, the endospersm is the site of accumulation.

The total glucosinolate content can vary according to the variety, the cultivation conditions, the climate and the agronomic practices. Glucosinolate-containing plants may show only a few glucosinolate types up to more than twenty different ones in some plant species (Fenwick, 1983).

2.2.2. Glucosinolates in radish

In whole radish roots or hypocotyls (Table 1), 4-methylthio-3-butenyl was reported as the major glucosinolate, small amounts of 4-methylsulfinylbutyl, 4-methylsulfinyl-3-butenyl and 3-indolylmethyl glucosinolates have been also reported (Carlson et al, 1985). Sang and coworkers (1984) analyzed seed, root and leaf tissue of radishes and other different cruciferous vegetables. They found that radish seed meal contains mainly 4-methylsulfinyl-3-butenyl glucosinolate and 4-hydroxy-3-indolylmethyl glucosinolate whereas the major glucosinolate in radish leaf was 3-indolylmethyl glucosinolate.

2.3. Stability of glucosinolates

Because of their glucose moiety and ionic form, glucosinolates are hydrophilic, non-volatile compounds. In order to balance the negative charges on glucosinolate anions, cations must be present in the cell. In most species, potassium is the dominating counterion. Glucosinolates are fairly stable at neutral pH values but can be decomposed by the action of strong acids and strong bases (Larsen, 1981). As we will discuss, they can be hydrolyzed upon enzymatic action when the plant is wounded.

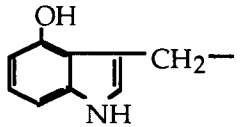
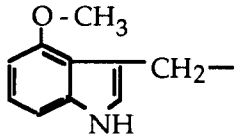
NAME OF GLUCOSINOLATE	STRUCTURE OF R GROUP	PARTS OF THE PLANT AND CONCENTRATION	REFERENCES
4-Methylthio-3-butenyl (MW= 418)	CH_3-S-CH=CH-$(CH_2)_2$-	intact hypocotyl (39-499 µmol/100 g fresh wt.) seed meal, hypocotyl	Carlson et al., 1985 Sang et al., 1984
4-Methylsulfinylbutyl *or Glucoraphanin* (MW= 436)	CH_3-SO-$(CH_2)_4$-	intact hypocotyl (0-5 µmol/100 g fresh wt.)	Carlson et al., 1985
4-Methylsulfinyl-3-butenyl *or Glucoraphenin* (MW= 434)	CH_3-SO-CH= CH-$(CH_2)_2$-	intact hypocotyl (0-28 µmol/100 g fresh wt.)	Carlson et al., 1985
3-Indolylmethyl *or Glucobrassicin* (MW= 447)		intact hypocotyl (0-18 µmol/100 g fresh wt.) seed meal, leaf, hypocotyl	Carlson et al., 1985 Sang et al., 1984
4-Hydroxy-3-Indolylmethyl *or 4-Hydroxyglucobrassicin* (MW= 459)		leaf, hypocotyl	Sang et al., 1984
4-Methoxy-3-Indolylmethyl *or 4-Methoxyglucobrassicin* (MW= 461)		leaf, hypocotyl	Sang et al., 1984
Total Glucosinolates (average MW = 457 g/mol)		hypocotyl 75-551 µmol (=34-251 mg)/100 g fresh wt. seed meal 14.9 mmol (=6.89 g)/100 g fresh wt.	Carlson et al, 1985 Daxenbichler, 1991

Table 1: Glucosinolates reported in radish seed, leaf and root.

2.4. Biosynthesis of glucosinolates

Until recently, the biosynthesis of glucosinolates was poorly understood and thought to have common features with the biosynthesis of cyanogenic glucosides. Glucosinolates, as well as the cyanogenic glucosides, are derived from amino acids and have some common intermediates in their biosynthetic pathway.

However, recent research has shown that the enzymes that catalyze the first step of glucosinolate and cyanogenic glycoside biosynthesis are different (Bennett et al, 1993 and 1995a). *In vivo*, labeled precursor studies (Haughn, 1991) have led to the proposal of a general glucosinolate biosynthetic pathway (Figure 2).

2.4.1. Amino acids as precursors

Amino acids are considered to be the precursors of all glucosinolates. The derivation from amino acid was first demonstrated in 1962 with the works of Kutacek et al., Underhill et al. and Benn et al, using radioactive ^{14}C labeled tryptophan (Wallsgrove, 1995). The aglycone part of the glucosinolate is derived from the amino acid methionine for aliphatic glucosinolates, tryptophan for indolyl glucosinolates, or phenylalanine for aromatic glucosinolates (Sorensen, 1991). In many cases, particularly with the methionine-derived compounds, there is a side-chain elongation which takes place before the amino-acid enter the biosynthetic pathway (Larsen, 1981). In oilseed rape, some examples are homophenylalanine, precursor of phenylethyl glucosinolate, and dihomo-methionine, precursor of but-3-enyl glucosinolate (Bennett, 1993). Both protein and non-protein amino acids serve as substrate for the biosynthesis of glucosinolates.

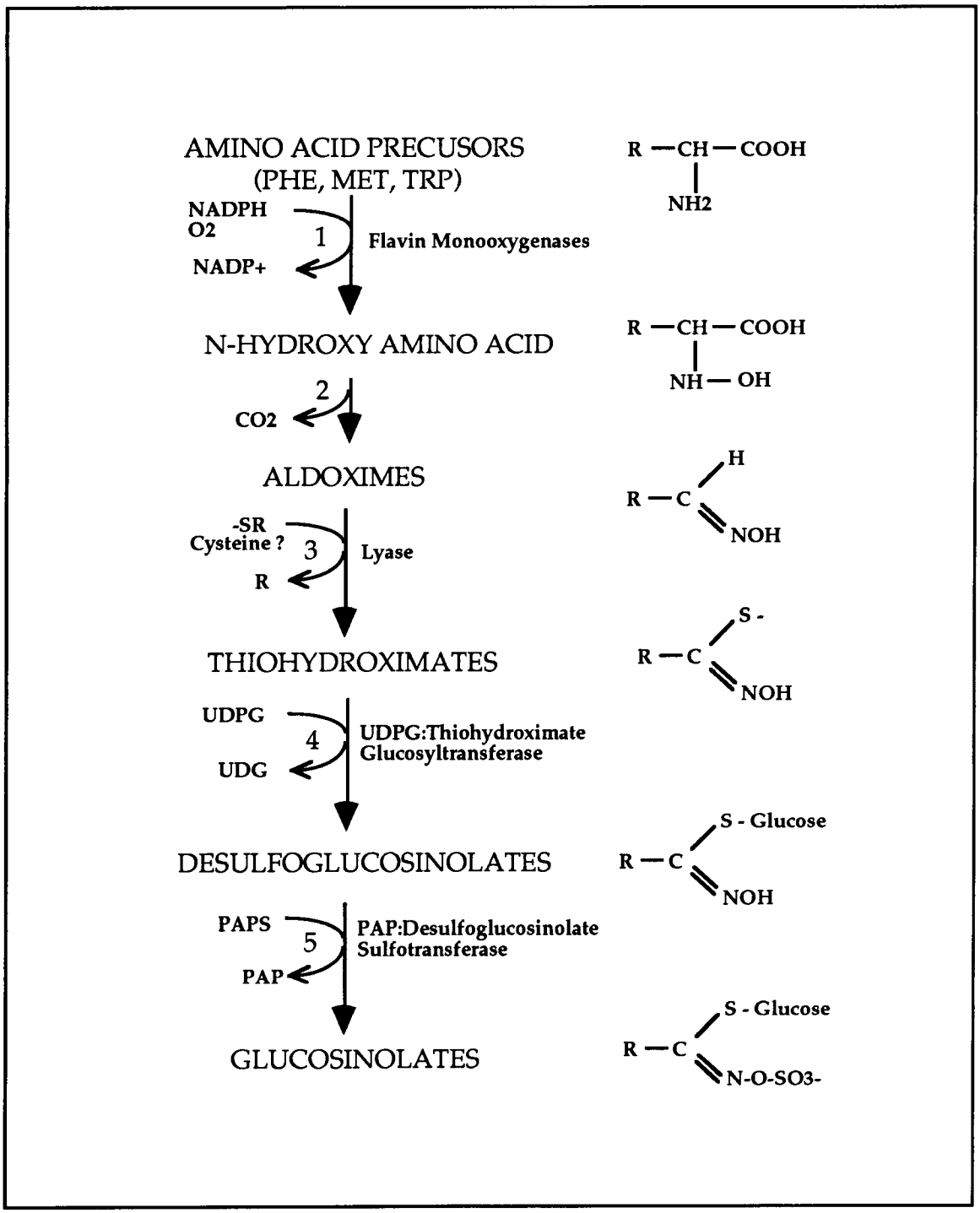


Figure 2: Proposed biosynthetic pathway for glucosinolates in Crucifers. Modified from Jain et al. (1989), Bennett et al. (1993) and Wallsgrove et al. (1995). PAP: 3' phosphoadenosine 5' phosphate, UDPG: UDP Glucose.

2.4.2. Conversion of amino acid precursors to aldoximes

The conversion from amino acid to aldoxime occurs in the microsomal fraction of crucifers (Larsen, 1981). Using ^{14}C labeled precursors, Bennett (1993, 1995a) and Dawson (1993) have shown that microsomal preparations from young green leaves of *Brassica napus* (oilseed rape) will catalyze the NADPH-dependent oxidative decarboxylation of methionine and phenylalanine homologues to their aldoximes. The reactions appear to be catalyzed by monooxygenases similar to the flavin-containing monooxygenases found in mammals and not by cytochrome P450-type enzymes.

Research has implied that there were common intermediates (N-hydroxy amino acids) and microsomal enzymes (cytochrome P450) involved in the early steps of both glucosinolates and cyanogenic glucosides biosynthesis (Poulton, 1993). However, no cytochrome P450 activities associated with the formation of aldoximes for glucosinolate biosynthesis could be detected in oilseed rape (Bennett, 1993). This is an evidence that cyanogenic glucosides and glucosinolates have distinct biosynthetic pathways.

The next steps of glucosinolate biosynthesis include the incorporation of a sulfur atom, a glucosyl and a sulfate moiety.

2.4.3. Formation of thiohydroximates from aldoximes

It has been suggested that the aldoxime is conjugated to cysteine or possibly methionine to form the thiohydroximates. The biosynthesis of thiohydroximates in oilseed rape and other crucifers is the least understood aspect of glucosinolate biosynthesis. Glucosides and sulfatoglucosides can be produced from aldoximes and not only in glucosinolate-containing plants (Wallsgrove, 1995). Aldoximes are ubiquitous in plants, acting as precursors

of many metabolites, including the plant growth hormone, indole 3-acetic acid (Conn, 1981).

The source of the thioglucosidic sulfur may vary depending on the plant species. The aldoxime-cysteine conjugate is thought to be cleaved by a cysteine-sulfoxide (CS) lyase to produce the thiohydroximate along with pyruvic acid and ammonia. The most common CS-lyase is cystathione which is involved in the methionine biosynthetic pathway (Wallsgrove, 1995).

2.4.4. Glycosylation and sulfation steps

Thiohydroximates are converted into desulfoglucosinolates by UDPglucose:thiohydroximate glucosyltransferase. This enzyme has been partially purified from *Tropaeolum majus* (Indian cress) and *Brassica juncea* (Brown mustard) and detected from a variety of other glucosinolate-producing plants. It is a soluble cytosolic protein with a molecular weight of 40 to 44 kDa (Wallsgrove, 1995; Jain, 1989). The analogy to cyanogenic glucoside biosynthesis is questioned again since for this pathway, microsomal enzymes (cytochrome P450-type activity) are involved whereas cytosolic enzymes catalyze the last steps of glucosinolate biosynthesis.

The conversion of desulfoglucosinolates to glucosinolates is then catalyzed by PAPS:desulfoglucosinolate sulfotransferase. Since this enzyme is very unstable, its purification has not been achieved. In *Brassica juncea* , its activity seem to be linked to that of the glucosyltransferase. This suggests that the two enzyme activities are either located on the same polypeptide or consist of two polypeptides in a functional complex. They have little specificity for the various side chain structures (Wallsgrove, 1995).

2.4.5. Side chain modifications in glucosinolates

Changes in side chain are assumed to take place after completion of the parent glucosinolate or at least at a very late stage in the biosynthetic sequence. Modifications can include hydroxylation, methoxylation, and, for the aliphatic glucosinolates, removal of the terminal methylthiol to produce alkenyl compounds (Wallsgrove, 1995). The initial products of aliphatic glucosinolate biosynthesis are probably methylthioalkyl glucosinolates (Mithen, 1995).

2.4.6. Regulation of the biosynthetic reactions

Very little is known about the control of glucosinolate biosynthesis. The enzymes involved in the late steps of the biosynthetic sequence have low specificity towards the substrates. Therefore, it is more likely that some sort of control is exerted in the first step of the pathway, especially during the activation of the amino acid to form the N-hydroxy amino acids (Larsen, 1981). The composition of glucosinolates in specific tissues could be controlled by the alteration of the substrate-specificity of the enzymes (flavin monooxygenases) initiating the biosynthesis by site-directed mutagenesis (Falk, 1994).

3. Enzymatic breakdown products of glucosinolates

Because most of the biological effects and flavor characteristics of cruciferous plants are not caused by glucosinolates themselves but by their degradation products, it is important to understand their hydrolytic events, which are mainly resulting from one enzyme action, myrosinase.

3.1. The glucosinolate-myrosinase system

The myrosinase-glucosinolate system has been investigated quite extensively because it is involved in the formation of biologically active compounds which can be toxic and would restrict the use of cruciferous crops for human consumption and as cattle feed. Plant breeding programs, particularly for rapeseed, have focused their efforts on reducing the glucosinolate content of the seeds such as progoitrin. An alternative approach would be to limit the amount of myrosinase available to reduce the hydrolysis of glucosinolates (Bones, 1996).

Glucosinolate-containing plants always carry the enzyme myrosinase (thioglucoside glucohydrolase E. C. 3:2:3:1) that can hydrolyze glucosinolates. In individual plant cells, glucosinolates are stored in the vacuoles (Helminger et al., 1983, Hoglund et al., 1992) but they are not in contact with the myrosinase enzyme. In the presence of moisture and when the structure of the plant material breaks down, glucosinolates are hydrolyzed by myrosinase which behaves as a β-thioglucosidase (Figure 3). After the cleavage of the thioester bond, glucose is released and the resulting intermediate, thiohydroximate-O-sulphonate, is unstable and will undergo a spontaneous rearrangement (Lossen-type) to give a variety of compounds including isothiocyanates, thiocyanates, nitriles, oxazolidine-2-thiones, hydroxynitriles, epithionitriles and epithioalkanes.

The formation of glucosinolate breakdown products depends on different factors: (a) the structure of the side chain R of the glucosinolate itself,

(b) the pH conditions (alkaline, acidic), and

(c) the presence of compounds which modify the action of the enzyme such as an epithiospecifier protein and ferrous ion.

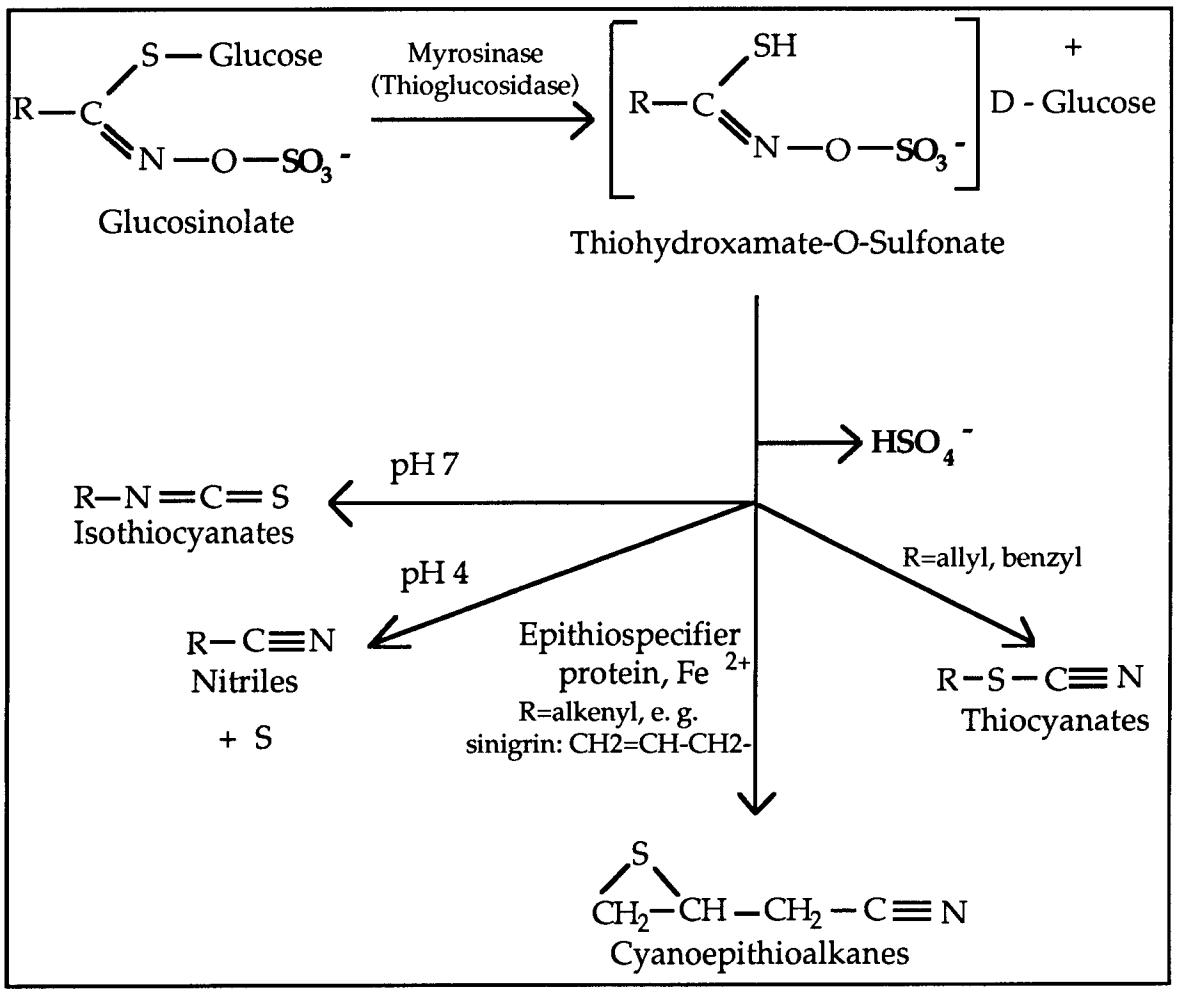


Figure 3: Degradation of glucosinolates by myrosinase, modified from Fenwick et al, (1983) and Bones (1996).

Isothiocyanates are produced at neutral pH whereas nitrile formation is favored under acidic conditions and accelerated by the presence of ferrous ion (Uda et al, 1986). Isothiocyanates are generally volatile compounds with a strong smell and taste and have cytotoxic activity. They are known to possess antifungal and antibacterial activities against a wide range of organisms. They have been found to be effective against postharvest fruit pathogens (Mari et al., 1996). Various isothiocyanates have been shown to inhibit the oxygen

uptake of yeast (Fenwick, 1983). They are unstable compounds and can be involved in the formation of other compounds. An example is the formation of goitrin derived from 2-hydroxy-3-butenyl glucosinolate. Isothiocyanates with a hydroxy group in the 2 position of their R group will undergo cyclization to form oxazolidine-2-thiones (Larsen, 1981; Fenwick, 1983; Bones, 1996).

In cabbage, the presence of an epithiospecifier protein (EPS) induces the formation of a cyanoepithioalkane, 1-cyano-2,3-epithiopropane from sinigrin, the major glucosinolate. This compound was reported as the primary hydrolysis product of fresh cabbage whereas low amount of allyl isothiocyanate was found (Kyung et al, 1995). The EPS is believed to block the Lossen rearrangement and thus the formation of isothiocyanates. A possible mechanism for the cyanoepithioalkane production is the formation of a bond between the nitrogen of the unstable intermediate and the ferrous ion of the EPS (Springett, 1988; Petroski et al, 1982).

3.2. Myrosinase enzymes

Myrosinase activity involves the action of isoenzymes which show diverse physico-chemical characteristics. Myrosinase enzymes have been extensively purified from various plants and shown to be glycoproteins containing SH groups essential for their activity. The glycosylation corresponds to 10-15% of the weight. Their molecular weight normally ranges from 125 to 150 kDa with two to four subunits depending upon plant sources (Falk, 1994).

Myrosin cells were first described in the late 1880's. Attempts to localize myrosinase in plants have been reported for more than 100 years. In tissue of

Brassica napus, myrosinase was localized in the vacuoles of certain cells called myrosin cells using immuno electron microscopy and immuno chemical techniques (Thangstad, 1990; Hoglund, 1992). Luthy and coworkers (1984) demonstrated that myrosinase from horseradish was a cytosolic enzyme while glucosinolates are localized in vacuoles. Therefore, the only requirements to start the reaction between myrosinase and glucosinolates are the disruption of the vacuole and the mixing of its content with the cytoplasm. This model is referred as the 'mustard oil bomb' with respect to its explosive character.

Myrosinase activity occurs probably only after mechanical injury of the plant. Isothiocyanates and other decomposition products have been detected at very low levels in intact plant but they are not normally present (Larsen, 1981). In addition to its function in plant defense (Mari, 1996), the myrosinase-glucosinate system has been suggested to have a role in the nutrient storage system in *Brassica* species, especially for the storage of sulfur (Falk, 1994).

Myrosinase from different plants seem to have the same specificity. Various glucosinolates are degraded at different rates but all naturally occurring glucosinolates are substrates. Some myrosinases require ascorbic acid for activation, whereas others are not affected by ascorbic acid. Both types are often present in the same plant. However, ascorbic acid does not participate in the reaction catalyzed by myrosinase. Its effect is not due to the oxidation-reduction properties of this compound but is probably allosteric; that is, caused by a conformational change of the enzyme when the binding sites are occupied by ascorbic acid (Bones, 1996). Wilkinson and coworkers (1984) studied myrosinase enzymes of several cruciferous vegetables. They reported that, in radish, the ascorbate-independent myrosinase activity

accounted for 30% of the maximal myrosinase activity. Also, the ascorbate concentration, over which greater than 90% maximal activity occurred, ranged from 0.8 to 2 mM in radish.

Optimum activity of myrosinase from radish roots has been recorded at pH 6-6.5 at 37°C. At pH 6, the Km for sinigrin was 0.47 mM. Inactivation of the enzyme was achieved above 45°C. Different glucosinolates could be hydrolyzed by radish myrosinase but *O*-glycosides, such as amylose, glycogen, sucrose, lactose, and maltose were not digested (Jwanny et al, 1995).

3.3. Plant growth hormones from glucosinolates

Indole-containing glucosinolates may be transformed into indole-3-acetic acid (IAA), also known as auxin, a plant growth hormone. A nitrilase, which has been detected in Crucifers, is responsible for this reaction (Bartling, 1992; Bestwick, 1993). IAA is believed to be synthesized from tryptophan via indole-3-acetonitrile and indole-3-acetaldoxime. The turnover of glucosinolates, which is reported to be quite important, may provide additional indole-3-acetonitrile (Larsen, 1981). The indole glucosinolate pool may act as a reserve of indole-3-acetonitrile which is an intermediate in auxin biosynthesis.

A nitrilase, capable of converting phenylpropionitrile to phenylpropionic acid, was purified from seedlings of *Brassica napus* (oilseed rape). This protein had a molecular weight of approximately 420 kDa, made of 38 kDa subunits. This enzyme is not present in seed but is rapidly synthesized over a period of about four days after which the activity declines (Bestwick, 1993). It might be involved in the *in vivo* degradation of glucosinolates to release glucose and sulfate for use in the biosynthetic pathway as well.

4. Flavor characteristics of Cruciferae

4.1. Glucosinolates and flavor

Glucosinolates are odorless, however, they are precursors of degradation compounds with flavor attributes typical of cruciferous plants. Among them, isothiocyanates, also know as mustard oils, are wide spread. Chin and coworkers (1996) reported that, in fresh cabbage, allyl isothiocyanate formed from its glucosinolate precursor, sinigrin, had a characteristic sharp mustard- and horseradish-like aroma. Its odor threshold in water was found to be 180 ppm. Allyl isothiocyanate was also described as pungent, lachrymatory and bitter (Buttery, 1976). Isothiocyanates and nitriles have low flavor threshold values: Buttery and coworkers (1976) reported odor threshold of isothiocyanates and nitriles identified in cooked *Brassica* in the range of 2 to 482 ppb. Pungency of Crucifer species is associated with the volatile 2-propenyl, 3-butenyl and 4-methylthio-3-butenyl isothiocyanates (Fenwick, 1983). The later one is the major glucosinolate degradation product of radish root (Friis and Kjaer, 1966).

The isolation of the pungent principle of radish roots has been attempted since the 1890's. After a large scale steam distillation, Bertram and Walbaum (1894) obtained an 'oil of an evil smell' but no further characterization was reported. The presence of a glucoside degrading into an isothiocyanate was suggested in 1948, but the isolation and structural characterization of the major radish isothiocyanate was performed by Friis and Kjaer (1966) using gas chromatography - mass spectrometry (GC-MS). Because of their volatility, isothiocyanates were difficult to extract in sufficient amount to be detected. Nuclear magnetic resonance and infra-red spectroscopy supported the

identification of 4-methylthio-3-butenyl isothiocyanate, predominantly in the *trans* form, as the pungent principle of radish roots (Friis and Kjaer, 1966).

In contrast to isothiocyanates, thiocyanate ion found in *Brassica* vegetables has a garlic-like odor and lacks pungency and lachrymatory character (MacLeod, 1976). Formation of nitriles is favored under acidic conditions and this aspect is important for fermented processed vegetables like sauerkraut, pickled cabbage and coleslaw (Fenwick, 1983; Wallsgrove, 1995).

4.2. Non-glucosinolate derivatives

Glucosinolate breakdown compounds are not the only flavor components produced by the Cruciferae. Cis-3-hexenol and trans-2-hexenal have been identified in the leaves of cabbage. They are also known as 'leaf alcohol' and 'leaf aldehyde' since they are common constituents of the aroma volatiles of green leaves. The alcohol is typical of green aroma such as freshly cut grass, whereas the aldehyde is the main active component of the defensive secretion of coackroach. These compounds are enzymatic oxidation products of lipids (MacLeod, 1976). Wallbank and coworkers (1976) reported that vapor from intact radish plants contain hexenyl acetate in small amount (40 ng/L). Much larger amounts were detected in vapor of disrupted plant tissue.

4.3. Effects of food processing on flavor of Crucifers

Production of isothiocyanates in vegetables and other food plants contribute to the characteristic spicy flavor we find in radish, mustard, and cabbage. Loss below the natural level results in unusually flat and dull products. Isothiocyanate formation is an enzymatic process and will occur

upon cell breakdown. As a consequence, isothiocyanate content will decrease in the foods after any process which, prior to consumption, destroys or inactivates the enzyme. For instance, blanching of Brussels sprouts reduces considerably the formation of isothiocyanates (MacLeod, 1976). The alteration of flavor by processing is a highly complex situation. Flavor volatiles which are formed may be lost or further hydrolyzed or can be involved in secondary reactions.

Van Langenhove and coworkers (1991) identified several volatiles in the head space of Brussels sprouts and cauliflower, during their blanching. Among the compounds reported were thiols, sulfides, polysulfides, isothiocyanates, nitriles, carbonyl compounds, furans, esters and terpenes. Breakdown compounds of glucosinolates (isothiocyanates, nitriles) were predominant in Brussels sprouts, while aldehydes were dominant in cauliflower. In both vegetables, dimethyl sulfide was the most abundant sulfur compound.

During the sulfiting process of mustard paste (*Brassica juncae*), isothiocyanates and bisulfite react chemically and form 2-propenylamino-thiocarbonyl sulfonate which decomposes in 2-propenyl mercaptan, di-2-propenyl sulfide and disulfide which are responsible for a fetid odor and a garlic-like off-flavor.

Methanethiol and its derivatives (Figure 4) are volatile sulfur compounds produced from *S*-methyl-L-cysteine sulfoxide, following cooking or *via* the action of cystein-sulfoxide lyase (Shankaranarayana et al., 1974). They were reported in broccoli florets stored under modified atmosphere and in sauerkraut (Chin et al., 1994). They are responsible for undesirable odors; methanethiol possesses a strong flavor and fecal-like odor, it can be readily

converted to unpleasant oxidized sulfurous off-flavors compounds with low detection threshold such as dimethyl disulfide and dimethyl trisulfide. Caraway seed extract, and the commonly used synthetic antioxidant, tertiary butylhydroquinone (TBHQ), were used to extend the shelf-life of processed cruciferous vegetables. Flavanoids in the caraway seed extract were found responsible for the suppression of the unpleasant flavors caused by methanethiol and its derivatives.

Twenty compounds have been identified in cooked radish with alkyl isothiocyanates being the major class (Whitfield, 1991). Volatiles from Japanese radish have been identified as mainly thioenol isothiocyanates, dimethyl disulfide from methanethiol (Kjaer, 1978a). In the same fermented radishes, isothiocyanates are rapidly catabolized to acetic acid, alcohols, carbonyl compounds and acetals with various sulfur-containing products (Kjaer, 1978b).

The hydrolysis of isothiocyanates (RNCS) can produce carbonyl sulfide (COS), hydrogen sulfide (H_2S) and carbon disulfide (CS_2). The latter can also be formed after reaction of isothiocyanates with hydrogen sulfide. Dimethyl disulfide, dimethyl trisulfide and allyl isothiocyanate contribute to the characteristic cabbage flavor (Shankaranarayana et al., 1974). Glucosinolates can produce a variety of flavor compounds which can be desirable or also objectionable. Modulation of the formation of the intermediates could reduce development of off-flavors in processed cruciferous vegetables.

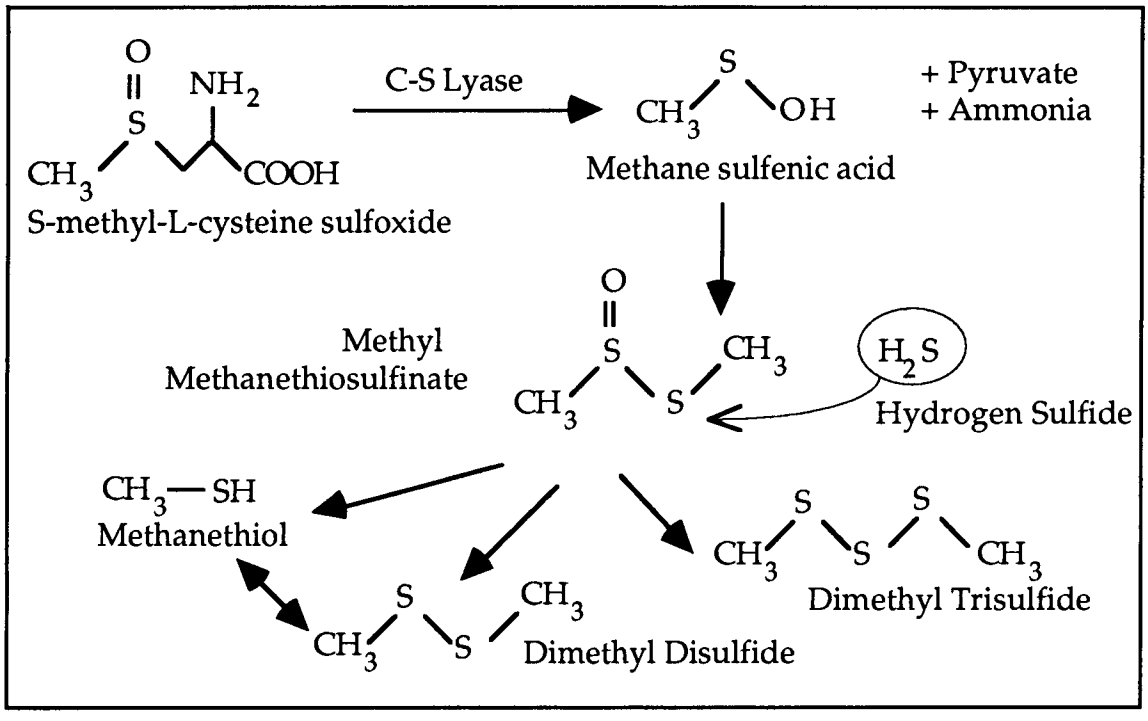


Figure 4: Formation of methanethiol-related volatile sulfur compounds in cruciferous vegetables. Modified from Chin and Lindsay (1996), and Stoewsand (1995).

4.4. Influence of agricultural practices on flavor

The glucosinolate content of cruciferous crops is dependent upon fertilization; especially, the application of sulfate increases glucosinolate synthesis in plants. Freeman and Mossadeghi (1972) demonstrated that in radish (cultivar Cherry Belle) isothiocyanate formation is well correlated with sulfur nutrition. Sensory evaluation showed that flavor intensity of radish roots increased with sulfur application.

A study (MacLeod, 1976), conducted on cabbage and Brussels sprouts, showed that crop spacing had an effect on the composition of the flavor volatiles at non-limiting sulfate concentrations in the soil. The closer the plants were grown, the greater the relative abundance of the glucosinolate

degradation products, particularly the isothiocyanates. Under harsh conditions limiting plant growth, like reduced crop spacing, the plant tends to increase some biosynthetic pathways, particularly amino acid synthesis which would lead to increasing glucosinolate biosynthesis.

5. Bioactivity of glucosinolates and their breakdown products

Degradation compounds of glucosinolates are responsible for the flavor characteristics of glucosinolate-containing vegetables but are also known for their biological activities, which can be beneficial or undesirable. Glucosinolates themselves exhibit low bioactivities. Higher bioactivity is attributed to their various breakdown products formed under the action of myrosinase. This enzyme, naturally present in the cruciferous plants, is released under disruption of the plant tissue such as cutting, chewing, chopping, crushing, etc. Myrosinase will then degrade glucosinolates into various products such as nitriles, thiocyanates and isothiocyanates as previously described.

5.1. Undesirable and toxicological effects

5.1.1. Responsible compounds

The first harmful effect due to *Brassica* vegetables was reported in the 1930's in rabbits fed with high levels of cabbage. It was found later on that the thiocyanate ion present in cabbage was responsible for the symptoms of goiter, (thyroid hypertrophy, reduced growth) and that its effects were dependent upon low dietary iodine intake (Astwood, 1943). Iodine supplementation of the diet as well as thyroxine intake were shown to reduce the symptoms. The

thiocyanate ion behaves as an iodine competitor by blocking or reducing iodine capture by the thyroid (Fenwick et al, 1989; Larsen, 1981).

Another active principle causing goiter is 5-vinyl-oxazolidinethione which interferes with thyroxine synthesis, a thyroid hormone (Fenwick, 1989). This compound, known as goitrin, is the product of cyclization of 2-hydroxy-3-butenyl isothiocyanate or progoitrin, formed from its glucosinolate precursor. Both indole- and β-hydroxyalkenyl glucosinolates can act as precursors for goitrogenic compounds by producing goitrin and thiocyanate ion. Goiter was observed in most animals (poultry, livestock, laboratory animals) when their diet contains approximately 2 to 5 mg glucosinolates/g diet (Fenwick, 1989; Stoewsand, 1995).

5.1.2. Detoxification of glucosinolate-containing foods and feed

The occurrence of antinutritional compounds like oxazolidine-2-thiones, (responsible for goiter) derived from glucosinolates in seed-meals of major oilseed *Brassica* crops has led to considerable efforts to reduce the level of aliphatic glucosinolates in the seeds of oilseed rape. The detoxification methods available are the following (Shahidi, 1994):

(a) genetic improvement,

(b) chemical degradation of glucosinolates and removal of their degradation products by oxidation, addition of metal salts or acids/bases,

(c) microbiological breakdown of glucosinolates by fungus, mold or bacteria and their removal,

(d) physical extraction of glucosinolates and/or their degradation products,

(e) enzymatic breakdown of glucosinolates by endogenous/exogenous myrosinase and removal of the resulting compounds by extraction or adsorption on carbon,

(f) combination methods such as diffusion extraction,

(g) enzymatic inactivation by heat, steaming or microwave,

(h) protein isolation.

An eight to ten fold reduction in the aliphatic glucosinolate levels of oilseed rape was achieved by Canadian breeders and led to the development of the "double zero cultivars", i.e. low in both erucic acid and glucosinolates in their seed (Falk, 1994). The reduction of glucosinolate was restricted to the propagative tissues, in order to preserve the ability of the plant to resist pests. Breeding to produce plants with low glucosinolate content in the seeds has been achieved; however, it was not possible to obtain varieties with no glucosinolates.

5.2. Anticarcinogenic activity

The anticarcinogenic properties of cruciferous vegetables have been mainly attributed to the degradation products of glucosinolates and indoles. More than 20 natural and synthetic isothiocyanates and several glucosinolates have been found to have blocking effect against chemically induced carcinogenesis in animal models (Zhang and Talalay, 1994). It is still not clear to what extent these phytochemicals have a chemopreventive action. However, diets rich in fruits and vegetables, cruciferous vegetables among them, have been associated with lower risk of developing cancer and were shown to have anti-tumorigenic activities in animal studies (Dragsted, 1993). Our daily intake is evaluated to contain milligram quantities of

isothiocyanates and glucosinolates (Fenwick et al., 1983). For instance, Brussels sprouts contain the highest level of glucosinolates with an average of 200 mg/100 g fresh weight (Heaney and Fenwick, 1980a). A daily consumption of two servings would provide about 3 mg glucosinolates/kg body weight.

The anticarcinogenic properties of isothiocyanates have been observed in rodents with a wide variety of chemical carcinogens including polycyclic aromatic hydrocarbons, azo dyes, ethionine, fluorenylacetamide and several nitrosamines (Talalay and Zhang, 1996). The mechanisms underlying the chemoprotective affects of isothiocyanates and their glucosinolate precursors involve modulation of carcinogen metabolism by:

(a) suppression of carcinogen activation by inhibition of phase I enzymes (oxidases, reductases, hydrolases, cytochrome P450);

(b) induction of phase II enzymes (glutathione-S-transferases, quinone reductase) which detoxify any residual electrophilic compounds generated by phase I enzymes. Carcinogens are transformed into less reactive and more easily excreted compounds.

Levels such as 3-4 μmol of organic isothiocyanates /g diet fed to rats and mice had significant effect on induction of phase 2 enzymes (Zhang and Talalay, 1994). Isothiocyanates are thought to induce an 'electrophilic counter-attack' process. The chemical reactivity of isothiocyanates (R-N=C=S) arises from its highly electrophilic central carbon atom (Figure 5). Carbamates, thiocarbamates or thiourea derivatives are formed by the reaction of isothiocyanates with oxygen-, sulfur-, or nitrogen-centered nucleophiles respectively. *In vivo*, conjugation of isothiocyanates with glutathione leads to the formation of dithiocarbamates, which are important products of

isothiocyanate metabolism. This reaction is accelerated by glutathione-S-transferases but also occurs non-enzymatically (Zhang and Talalay, 1994; Talalay and Zhang, 1996).

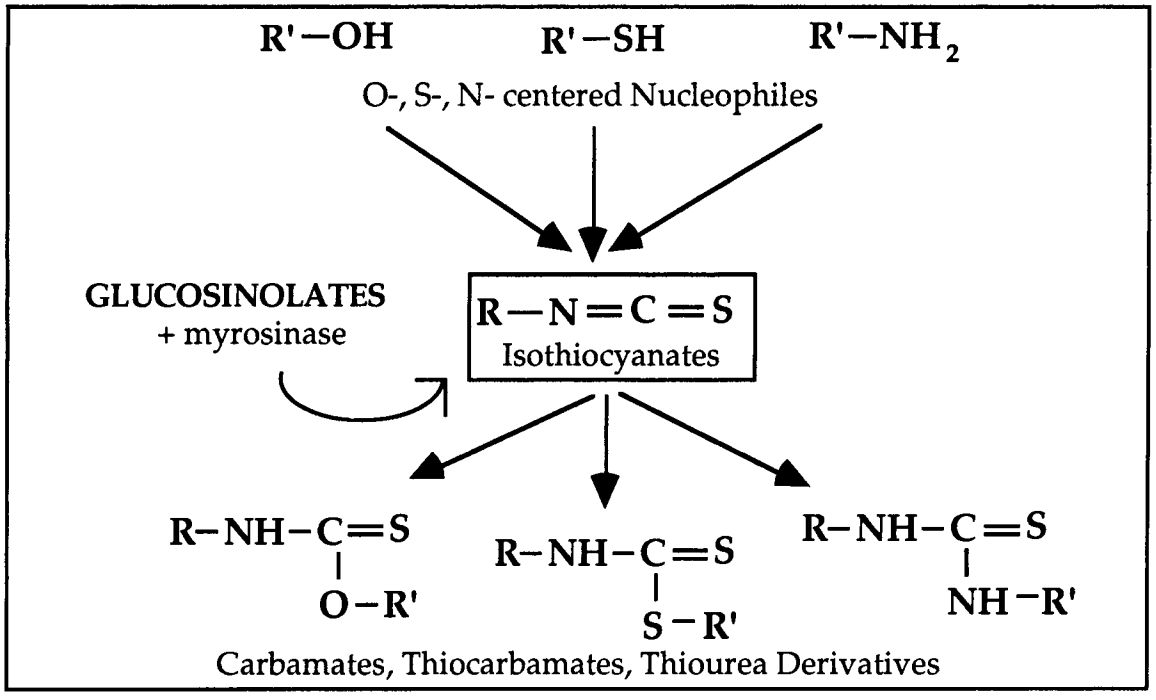


Figure 5: Chemical reactivity of isothiocyanates forming carbamates. Modified from Zhang and Talalay (1996).

The potency of the chemoprotective effects of isothiocyanates depends on the structure of the side chain. Sulforaphane [CH_3-SO-$(CH_2)_4$ - NCS] isolated from broccoli was found to be a potent phase II enzyme inducer (Talalay and Zhang, 1994). The indole glucosinolate glucobrassicin found in relatively high amount in cabbage leaves, has been shown to hydrolyze to non-volatile indoles such as indole-3-acetonitrile, indole-3-carbinol and 3,3' di-

indolylmethane. These compounds are involved in anticarcinogenic activities and have been studied quite extensively (MacDanell et al., 1988).

6. Analytical methods for detection of glucosinolates and their derivatives

Considerable progress in the analysis of glucosinolates and their breakdown products has been made during the last two decades. Byproducts of mustard and canola oil production are commonly used as animal feed and can contain high level of glucosinolates. At high dosage, these compounds were found responsible for toxic effects in the cattle such as goiter. Consequently, methods for the separation and analysis of glucosinolates were first developed for rapeseed and derived products (canola oil) as well as other *Brassica* vegetables. With the understanding of glucosinolate biosynthesis, breeding programs have led to the development of new cultivars of rape and turnip with low glucosinolate content (Heaney and Fenwick, 1987; Whatelet, 1987; Betz and Fox, 1995). More recently, potential anticarcinogenic properties of glucosinolate breakdown compounds have led to an increasing attention towards their biosynthetic pathway and their evaluation methods.

Because of the presence of the sulfate group having a pKa of 9, glucosinolates are usually encountered under the negatively charged form (Prestera, 1996a). The separation of these highly charged molecules is therefore difficult and requires the use of indirect methods of evaluation. A wide range of glucosinolate breakdown products have been investigated to evaluate the total or individual glucosinolates (Heaney and Fenwick, 1989). An outline of qualitative and quantitative methods used for the analysis of glucosinolates and their derived products is presented in Figure 6 and some of these techniques are further described and referenced.

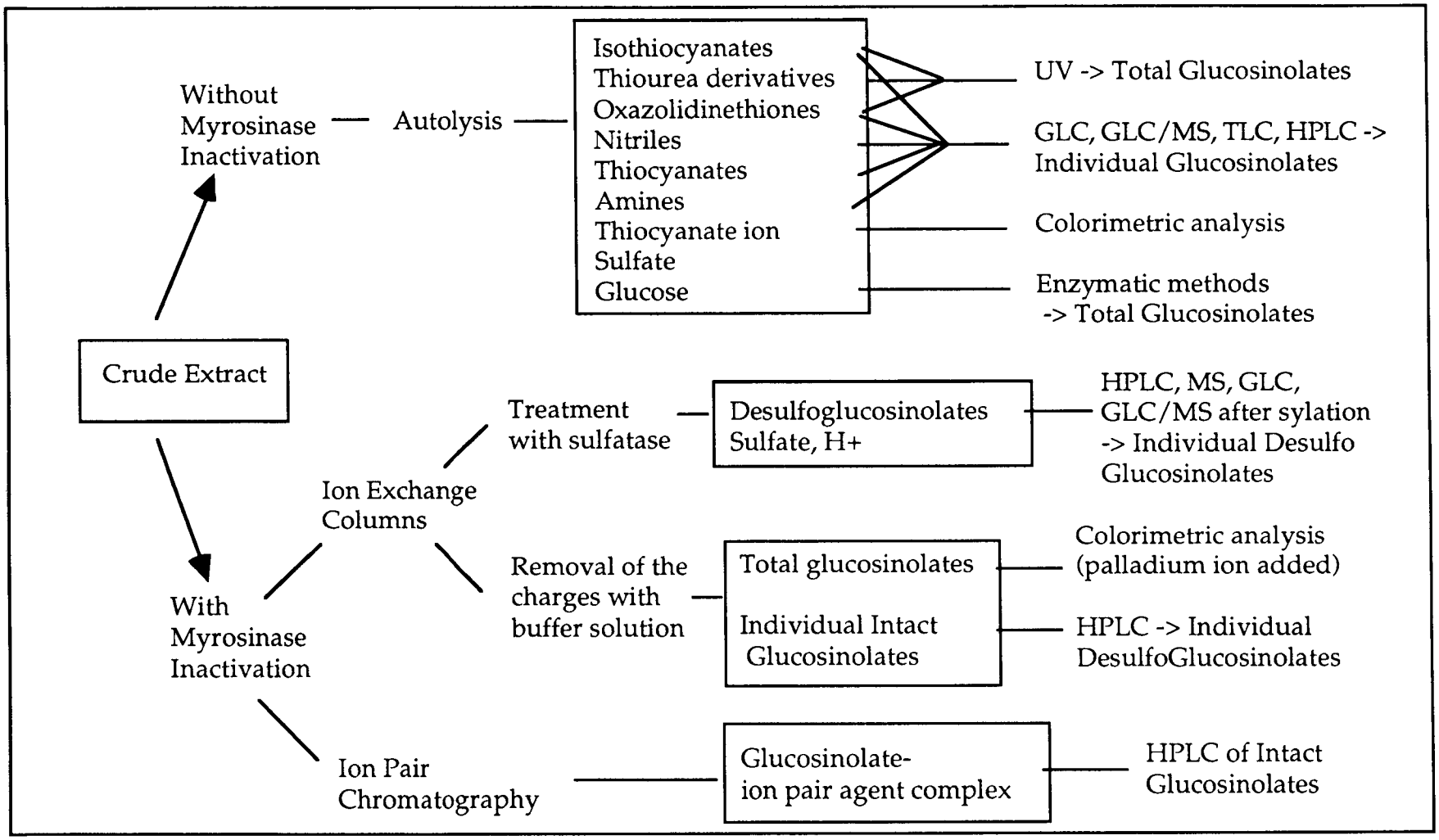


Figure 6: Overview of analytical methods used for analysis of glucosinolates and their derivatives. Modified from Sorensen, 1985; Betz et al, 1995.

6.1. Total glucosinolate content

Total glucosinolate content can be evaluated by measuring resulting enzymatic products of glucosinolates. Endogenous myrosinase is first inactivated, allowing extraction of intact glucosinolates which, under the action of exogenous myrosinase, are further hydrolyzed into detectable products. These products can be: glucose, volatile aglycones, sulfate and thiocyanate ion.

The most common compound used to evaluate total glucosinolate content is certainly glucose. It can be measured by gas-chromatography but now it is preferentially done using kits containing specific glucose-degrading enzymes which involve spectrophotometric measurements. Sample preparation is designed to eliminate potential interfering compounds that are colored or may inhibit color development of the glucose reagent. Methods involving a clean-up step were developed using an anion-exchange resin (Van Etten et al, 1977; Heaney et al, 1981). The crude plant extract containing intact glucosinolates is run through a resin on which glucosinolates will adsorb, since they are negatively charged. Exogenous myrosinase is then added and the eluting glucose, resulting from the selective enzymatic action (thioglucosidase), is collected.

6.2. Glucosinolate breakdown products

The breakdown products of glucosinolates are essentially compounds that are formed under the action of myrosinase (thioglucosidase) followed by rearrangement and further degradation. They include isothiocyanates, thiocyanate ion, oxazolidinethiones, nitriles, sulfate and glucose. It is not the glucosinolates but products of myrosinase action that are responsible for the

flavor and aroma characteristics as well as the biological effects of glucosinolate containing plants. It is therefore of considerable interest to evaluate myrosinase hydrolysis products. Intact glucosinolates are extracted after inactivation of the endogenous myrosinase in boiling methanol. Their aglycones are liberated into organic solvents after enzymatic hydrolysis by adding myrosinase enzyme to the glucosinolate extracts.

The volatile character of glucosinolate breakdown products such as isothiocyanates has led to an extensive use of gas chromatography (GC) for their analysis (Daxenbichler et al., 1977; Heaney and Fenwick, 1980b). On the other hand, the use of high performance liquid chromatography (HPLC) is limited. Detection of these molecules eluting from the chromatographic column is difficult: thiocyanates and nitriles are not detectable spectrophotometrically (Heaney and Fenwick, 1987). Also, some reactivity problems with the mobile phases containing methanol were reported by Mullin (1978). However, Maheshwari et al (1979) reported an HPLC method for the quantification of individual isothiocyanates employing a C18 column and an aqueous acetonitrile as mobile phase (60:40 to 40:60 v/v). The method was applied to evaluate glucosinolate content of rapeseed meal by assaying the isothiocyanates released after myrosinase action. Identification was performed using gas chromatography - mass spectrometry. Kim and Rhee (1986) used a similar procedure to measure the compounds responsible for the pungency of radish kimchi.

More recently, a UV spectroscopic method for quantitating isothiocyanates was developed by Zhang et al (1996) based on the formation of 1,3 benzodithiole-2-thione, a cyclic product formed under the action of a dithiol

reagent with isothiocyanates and detectable at 365 nm. The quantification was made possible at the nanomole level.

6.3. Individual glucosinolates

The first separation of glucosinolates was performed by gas-liquid chromatography of trimethylsilated derivatives of desulfoglucosinolates (Underhill, 1971). The sulfate group was removed enzymatically under the action of a sulfohydrolase.

The necessary derivatization of glucosinolates for GC analysis has led to investigation of analytical methods requiring shorter and easier sample preparation. The use of HPLC is usually preferred for the analysis of water-soluble, nonvolatile compounds. Several HPLC methods have been developed for desulfoglucosinolates (Minchinton, 1982; Spinks, 1984; Quinsac, 1991) and for intact glucosinolates (Helboe, 1980; Moller, 1985; Mellon, 1987; Bjorkqvist, 1988). The main advantage of HPLC methods over GC is that glucosinolates are detected by UV absorbance, rather than the destructive flame ionization detector (FID) used in GC. They can therefore be further collected and identified by mass-spectrometry and nuclear magnetic resonance (NMR) (Burke, 1988; Mellon, 1987).

III. MATERIALS AND METHODS

1. Plant Material

1.1. Whole radishes, peels and core

Radish seeds were planted during the second half of June 1996 at the Oregon State University Experimental Station, Corvallis. Spring cultivars (Fuego, Chinese Cherry radish, Wu Ying Shui and Chinese Red) were harvested on July 25 and August 8, 1996, five and seven weeks after the planting. Winter varieties (Chinese Red Meat Takii, Man Tang Hong and Jingqing) were harvested on August 20, 1996 at nine-ten weeks after planting. Immediately after harvest, radish roots were washed with cold water and refrigerated. Within one week, about two kilograms of whole radishes for each variety and harvest date, were frozen at -40°C and freeze-dried (freeze-dryer from Hull Corporation, Hatboro, PA) for 3 to 10 days according to the size of the roots. Also, 200 to 300 grams of peels and one to two kilograms of peeled roots (hand peeled) were lyophilized for each variety and harvest time. The freeze-dried material ranged from 6 to 15% dry matter of fresh weight.

1.2. Radish juices

Spring type radishes (Fuego), with pigment on the epidermal tissue, were processed into juice at a pilot plant scale with an abrasive peeler (Model 20B, Blakeslee and Co., Chicago, IL) and 10 to 15 kg of radishes at a time. Some adjustments on the equipment allowed collection of peels and reduction of the amount of cold water added. Approximately 100 kg of radishes for the Fuego cultivar were processed for each harvest time. Samples were taken

from the juice, as well as the peeled radishes and the resulting pulp from the peels. Red-flesh varieties (Red Meat type) were processed with a disintegrator M8A (Corenco). The resulting slurry was pressed mechanically to 500 psi with a mechanical press. The juice extracts were blanched for 10 min at 100°C and filtered through a cheese cloth. Samples were taken both before and after blanching.

1.3. Commercial color extracts

Red cabbage colorant (Warner-Jenkinson, St. Louis, MO) containing red cabbage juice, propylene glycol and citric acid and radish color extract SN Red-RR (Stange, Tokyo, Japan) containing radish pigment, ethanol and citric acid were included in the analyses.

2. Crude glucosinolate extract

A crude glucosinolate extract was prepared after modification of the procedure applied by Betz and Fox to broccoli (1995). Five grams of freeze-dried radish powder was mixed with 100 mL boiling methanol in water (70:30); 15 mg of sinigrin (Aldrich, Milwaukee, MI) was added as internal standard. After cooling at room temperature, the mixture was homogenized with a Waring blender and filtered through Whatman filter paper #1 on a Buchner funnel under vacuum. The residue was washed twice with 50 mL of methanol/water (70:30) and the filtrate (200 mL) was concentrated to approximately 20 mL on a Büchi rotavapor (Switzerland) at 35°C for 30 to 40 minutes. The concentrated extract was then taken up to 25 mL with deionized water and stored at -40°C until further analyses. For radish peels, extracts were

concentrated to 35-40 mL and made up to 50 mL, due to the viscosity of the concentrate.

3. Extraction of glucosinolates

3.1. Intact glucosinolates

Extraction and concentration of intact glucosinolates were done following the procedure described by Betz and Fox (1995) with some modification. One volume of crude glucosinolate extract or radish juice was passed through a C-18 Sep-Pak cartridge (Waters Assoc., Milford, MA), previously activated with methanol (5 mL) and conditioned with 5 mL 0.01 M aqueous tetrabutyl ammonium sulfate (Low UV IPC-A) (Alltech Associates, Deerfield, IL). The anions present in the extract were retained on the column as well as other compounds having an affinity for the C-18 resin, like anthocyanins. Water soluble compounds (sugars, acids) were washed away with one volume of deionized water and intact glucosinolates were eluted with two or three volumes of methanol in water (55:45).

3.2. Desulfoglucosinolates

Desulfoglucosinolates were extracted according following the procedures reported by Bjerg and Sorensen (1987) and Minchinton and coworkers (1982) with a few modifications. An anion exchange column was prepared from DEAE Sephadex A-25 (Sigma Chemical Co., St. Louis, MO). The resin material was stirred in excess 0.02 M acetate buffer pH 5.0 and resuspended in fresh acetate buffer to give a total volume twice that of the settled resin. The buffer was prepared as follows: 1.2 mL acetic acid mixed with ca. 990 mL deionized

water, the pH was adjusted to 5.0 with NaOH and the volume was taken up to 1 L with deionized water. One mL of the resin suspension in acetate buffer was added to a plastic pipette plugged with glass wool and washed with water after settling.

Two mL of radish juice or 0.5 mL of crude glucosinolate extract was transferred to the top of the column and allowed to drain. Negatively charged molecules like glucosinolates were retained on the resin while positively charged and neutral compounds were eluted. The column was then washed with water to eliminate water soluble compounds that are not strongly retained by the positive beads of the resin (sugars). After draining, 0.5 mL of 0.02 M acetate buffer is passed through the resin twice and allow to drain. The column was plugged with a plastic tip and sulfatase type H-1 from an edible snail *Helix pomatia* (Sigma Chemical Co., St. Louis, MO), containing 16,100 units/g solid, was added to the top of the column (100 µL of 0.3% w/v containing approximately 50 units/mL). The columns were allowed to stand overnight for 16-17 hours at 38°C. Desulfoglucosinolates were eluted with 2 x 1 mL of water and the eluate was made up to 3 mL water.

4. High performance liquid chromatography of glucosinolates

Chromatographic analyses were performed at room temperature using a Perkin-Elmer Series 400 liquid chromatograph equipped with a 1040A Hewlett-Packard photodiode array detector and a Hewlett-Packard ChemStation computer system. An autosampler model 501 (Beckman, San Ramon, CA) was coupled to the instrument. The flow rate was 1 mL/min and the injection volume 50 µL. Solvents and samples were filtered through a 0.45 µm Millipore filter (Millipore Corp., Bedford, MA). An analytical reverse

phase C-18 column Supelcosil (5 µm particle size, 25 cm x 4.6 mm id, Supelco Inc., Bellefonte, PN) was used combined with a guard column ODS-2 (5 µm, 1.5 cm x 4.6 mm i.d., Alltech Associates, Deerfield, IL). The spectra of all peaks, detected at a specific wavelength, were recorded between 200 and 600 nm.

4.1. Intact glucosinolates

Separation of individual intact glucosinolates in radish extracts and juices was performed by ion pair chromatography described by Betz and Fox (1995) with some modifications. A binary gradient was used composed of solution A (10% methanol) and solution B (98% methanol) both in 0.005 M aqueous tetrabutylammonium sulfate (IPC-A) (Alltech Associates, Deerfield, IL). The solvent program was as follows: 10 minutes, isocratic 28% B, 10 minutes, linear gradient from 28 to 44% B and 5 minutes at 44% B. The program returned to 28% of B in 5 minutes with a linear gradient and the system was allowed to equilibrate for 5 minutes. Detection was performed at 229 and 280 nm. The Supelcosil C-18 column used for these analyses was dedicated to ion pair chromatography.

4.2. Desulfoglucosinolates

Individual desulfoglucosinolates in radish extracts and juices were separated with reverse phase chromatography using a modification of Bjerg and Sorensen (1987) procedure. A binary gradient was used composed of 100% acetonitrile and 100% water. The solvent program was the following: from 0 to 25 minutes, linear gradient from 5 to 20% acetonitrile and 8 minutes at 20% acetonitrile. The program returned to 5% acetonitrile in 4

minutes with a linear gradient and the system was allowed to equilibrate for 4 minutes. Detection was performed at 230 and 280 nm.

5. 4-methylthio-3-butenyl isothiocyanate (MTBI)

5.1. Extraction of MTBI

Determination of 4-methylthio-3 butenyl isothiocyanate (MTBI), the pungent principle of radish, was performed following the method reported by Kim and Rhee (1986). Whole peeled radishes and epidermal tissue were analyzed within three days after harvesting. Radishes were peeled immediately before the analysis. Radish tissue (30 g) was blended with 0.1 M borate buffer pH 8.5 (60 mL) for 2 min at medium speed in a Waring blender. The buffer was made as follows: 6.18 g of boric acid, 990 mL deionized water, adjusted to pH 8.5 with potassium hydroxide and made up to 1 L with water. As an internal standard, 1.5 mg of 3-benzoyl pyridine (8.25 μmol) was added in the borate buffer before blending radish tissue. The mixture was allowed to stand for 1 min and was then filtered through paper filter Whatman #1. An aliquot of the filtrate (1.5 mL) was mixed with acetonitrile (3.5 mL).

5.2. High performance liquid chromatography of MTBI

The chromatographic separation was performed with 50 μL injected on a C18 reverse phase analytical column similar to the one described for the analysis of desulfoglucosinolates. MTBI was eluted with acetonitrile/water (65:35) at a flow rate of 1 mL/min. Detection was carried out at 254 nm. The response factor of 3-benzoyl pyridine used as an internal standard was

calculated for concentrations ranging from 5 to 300 nmol of 3-benzoyl pyridine/mL of water.

6. Determination of the total glucosinolate content

The total glucosinolate content was determined by the glucose released method (Heaney and Fenwick, 1981). An ion exchange column using Sephadex A-25 was used as described previously for the isolation of desulfoglucosinolates. One or two mL of crude glucosinolate extract or 3 mL of radish juice was transferred to the top of the anion exchange resin. The column was then rinsed with water, allowed to drain and washed twice with 0.5 mL 0.02 M acetate buffer pH 5, prepared as previously described. After draining, 250 µL of myrosinase (thioglucosidase EC 3:2:3:1 extracted from *Sinapis alba*, or white mustard, Sigma Chemical Co., St. Louis, MO) was added to the tipped column. A myrosinase solution of 3.6 units/ mL was made up as follows: myrosinase powder, containing 240 units/g solid, was diluted in acetate buffer pH 5 at a concentration of 15 mg/mL. A few insoluble particles were formed. The reaction was carried out for 2 hours at 38°C. Glucosinolates were then eluted with 2 x 0.5 mL deionized water.

Glucose concentration in the eluate was determined with a glucose kit (Boehringer Mannheim Corporation, Indianapolis, IN) by measuring spectrophotometrically the change in absorbance at 340 nm. Optical glass cells of 1 cm-pathlength were used for the spectrophotometric measurements on a Shimadzu 300 UV-visible spectrophotometer (Japan). An aliquot of 100 or 200 µL of eluate was mixed with 1 mL of solution 1 containing NADP/ATP reagents and the volume was made up to 3 mL with deionized water. A first reading was taken at 340 nm (A_1). Twenty µL of solution 2 containing

hexokinase and glucose-6-phosphate dehydrogenase was added to start the reaction. A reading was taken after 15 to 20 minutes (A_2). The glucose present in the solution reacts with ATP to form glucose-6-phosphate and ADP under the action of hexokinase. Glucose-6-phosphate is oxidized by $NADP^+$ to gluconate-6-phosphate with formation of NADPH in the presence of glucose-6-phosphate dehydrogenase. One mole of glucose induces the formation of one mole of NADPH. The $NADP^+$ to NADPH reduction in the presence of glucose was monitored at 340 nm.

7. Mass spectrometry analyses for glucosinolates

The same crude glucosinolate, concentrated intact glucosinolate and desulfoglucosinolate extracts analyzed by HPLC were used for mass spectrometry (MS).

Fast atom bombardment (FAB) was performed on Kratos MS-50 magnetic sector instrument (Manchester, UK) equipped with a Ion-Tech FAB-gun (Teddington, UK). Xenon was used as the bombarding gas with a beam energy of 7000 V. The ion source was at room temperature. Resolution was 1000 and the scan speed was fixed at 10 seconds/decade.

Low resolution electrospray MS was also performed on a Perkin-Elmer SCIEX API III^+ mass spectrometer equipped with an ion spray source (ISV = 4700, orifice voltage of 80) and loop injection. Coupled MS-MS was used to obtain further fragmentation of the main compound.

8. Sensory analysis

8.1. Sample preparation

Colored extracts of decreasing monomeric anthocyanin content (1200, 600, 300, 150 mg /L) were rated by 24 panelists for overall aroma intensities. The following samples were evaluated:

(1) - Radish juice concentrate from cultivar Fuego (red epidermal tissue), 4950 mg anthocyanins /L;

(2) - Radish juice concentrate from cultivar Red Meat Takii (red flesh), 2300 mg anthocyanins /L;

(3) - Red cabbage extract supplied by Warner - Jenkinson (St. Louis, MI), 19785 mg anthocyanins /L;

(4) - Radish extract supplied by Stange (Tokyo, Japan), 8530 mg anthocyanins /L.

Dilutions of the four concentrates were made in Aquacool spring water. A small volume (8 mL) of diluted colorant was transferred into a 60-mL, amber-colored glass bottle. Each of the 16 samples was covered with a teflon cap, providing a head space of more than 85%. A total of six sets of 16 samples was prepared for sensory evaluation which allowed rotation of the samples. Rotation was necessary so that the headspace could rebuild in between panelist usage.

8.2. Training session for rating overall aroma intensity

A total of 24 panelists completed the evaluation: training took part over two 30-min sessions. Half of the panelists attended the first session and the other half were trained in the second session. Panelists were student and

faculty volunteers from the Food Science and Technology Department of Oregon State University.

The training session had the following objectives:

- to become familiar with the proper smelling technique,

- to focus on overall aroma intensity or total impact, and not the individual notes of the aroma,

- to rate aroma intensity using a 16-point intensity scale (0 = none, 3 = slight, 7 = moderate, 11 = large and 15 = extreme). The scale was anchored with four aroma intensity standards.

Panelists were trained to evaluate the overall aroma intensity of the samples with reference to intensity standards. These standards were served in tulip-shaped wine glasses capped with aluminum foil (Table 2). The following products and rating were used: safflower oil = 3, orange drink = 7, grape juice = 10, cinnamon gum = 13.

Intensity (Scale Value)	**Intensity Standard**	**Preparation of Standard**
Slight (3)	Oil	30 mL (2 tablespoons) of Saffola, 100 % Safflower oil (Saffola Quality Foods Inc., Los Angeles, CA).
Moderate (7)	Orange	30 mL (2 tablespoons) of Hi-C Orange Drink (Coca Cola Foods Inc., Plymouth, FL).
Large (10)	Grape	30 mL (2 tablespoons) of Welch's 100 % Natural Grape Juice (Welch Foods Inc., Westfield, NY).
Large to Extreme (13)	Cinnamon	1stick Big Red chewing gum (Wrigley's Jr. Co. Chicago, IL), in its aluminum foil wrap.

Table 2: Intensity Standards used for overall aroma evaluation in wine glass.

To familiarize the panelists with the samples, three test samples, which represented a range of sample aromas and intensities, were presented during the training session. The panelists were asked to rate their overall aroma intensity and to give descriptors of the aroma as well. The panelists were not aware that these samples were going to be presented in the test. Samples were labeled with three-digit random numbers which differed from the ones used in the test.

8.3. Experimental design and sample presentation

A completely randomized block design was used with two replications, the blocks being the panelists. All 16 samples (four colored-extracts diluted to four increasing anthocyanin concentrations) were evaluated twice by each panelist in a random order during two different sessions. Samples were randomized across panelists within each replication. Samples were labeled with 3-digit random numbers. Different sets of 3-digit random numbers were used for each replication. The panelists were not aware that their second day of testing was a replication of the first day. Training of all 24 panelists and testing was done in 4 days (Table 3).

PANELIST 1 - 12		**PANELIST 13 - 24**	
Day 1 (Thursday)	**Day 2** (Friday)	**Day 3** (Tuesday)	**Day 4** (Wednesday)
Training session + Test Replicate 1	Test Replicate 2	Training session + Test Replicate 1	Test Replicate 2

Table 3: Organization of the sensory test and training sessions.

Samples were evaluated in individual sensory booths under red lighting, to mask color differences, at ambient temperature (~22°C). The four aroma intensity standards (oil=3, orange drink=7, grape juice=10, gum=13) were provided in each booth as references with their respective intensity rating marked on each glass. Four samples were presented together and panelists were instructed to evaluate them from left to right. A two-to-four-minute break was mandatory between each set of four samples. Each panelist evaluated a total of four trays (four samples per tray) per session. Intensity ratings and comments were typed by the panelists using a computerized sensory software system (CompuSense *Five* for Windows®, Compusense Inc., Guelph, Canada).

8.4. Statistical analyses

The data were collected using CompuSense Five software and were further analyzed by SAS for Windows (SAS Institute Inc., Cary, NC). Mean differences in overall aroma intensity scores were analyzed through analysis of variance. To indicate which samples were significantly different, a multiple comparison Tukey test (least significant difference) was employed .

9. Monomeric and polymeric anthocyanin measurements

The monomeric anthocyanin content of samples used in the sensory analysis was evaluated by the pH differential method (pH 1.0 and 4.5) as described by Wrolstad, 1976. Spectrophotometric measurements were taken on Shimadzu 300 UV-visible spectrophotometer, at the wavelength of maximum absorbance of the pigments: 510 nm for the radish extracts and 525 nm for red cabbage. Monomeric anthocyanin content was determined as

pelargonidin-3-glucoside for the radish extracts (extinction coefficient ε = 31,600 and molecular weight MW = 433.2) and for red cabbage colorant, as cyanidin-3-glucoside (ε = 29,600; MW = 445.2). Polymeric anthocyanin content was determined by recording the difference in absorbance at 520 and 420 nm between a sample in a citric acid pH 3.0 buffer and a bleached sample using potassium metabisulfite (Wrolstad, 1976).

10. Color measurements

Color parameters, CIELAB L*, a*, b* and haze, were monitored for colored solutions made up for the sensory analysis with a ColorQuest Hunter tristimulus colorimeter (HunterLab Assoc., Reston, VA.). The instrument was set up for total transmittance measurements with specular included, illuminant D65, observer angle 10°. The samples were placed in a 5-mm pathlength optical glass cell without any dilution. The cell was filled with about 5 ml of colored sample. Two readings were taken for each sample.

IV. RESULTS AND DISCUSSION

1. 4-MethylThio-3-Butenyl Isothiocyanate (MTBI)

1.1. Chromatographic separation

In this experiment, we measured the potential of fresh radish tissue to form 4-MTBI, the pungent compound of radish (Kjaer et al, 1966). The analysis does not reflect the amount of isothiocyanate present in the sample but how much 4-MTBI can be formed under optimal conditions. The procedure for extraction of isothiocyanates should permit the release of endogenous myrosinase segregated from its glucosinolate substrates and also favor the enzyme activity to form isothiocyanates (Kim and Rhee, 1993). Formation of 4-MTBI is favored under alkaline conditions after blending of the fresh material to release myrosinase. Kim and Rhee (1986) reported that, isothiocyanates hydrolyze slowly under alkaline conditions but are relatively stable in organic solvent (65% acetonitrile).

The HPLC separation of 4-MTBI was performed using 3-benzoyl pyridine as an internal standard for whole radish, peeled radishes, and peels (Figure 7). Identification of 4-MTBI was accomplished by comparison of retention times and UV absorption spectra (Figure 8) reported by Kim and Rhee (1986). In a similar chromatographic system, Kim and Rhee (1986) obtained retention times close to the ones we observed, with 4.3 and 6.1 min for 3-benzoyl pyridine and 4-MTBI, respectively, as compared to 4 and 7 min elution time obtained with our system (Figure 7).

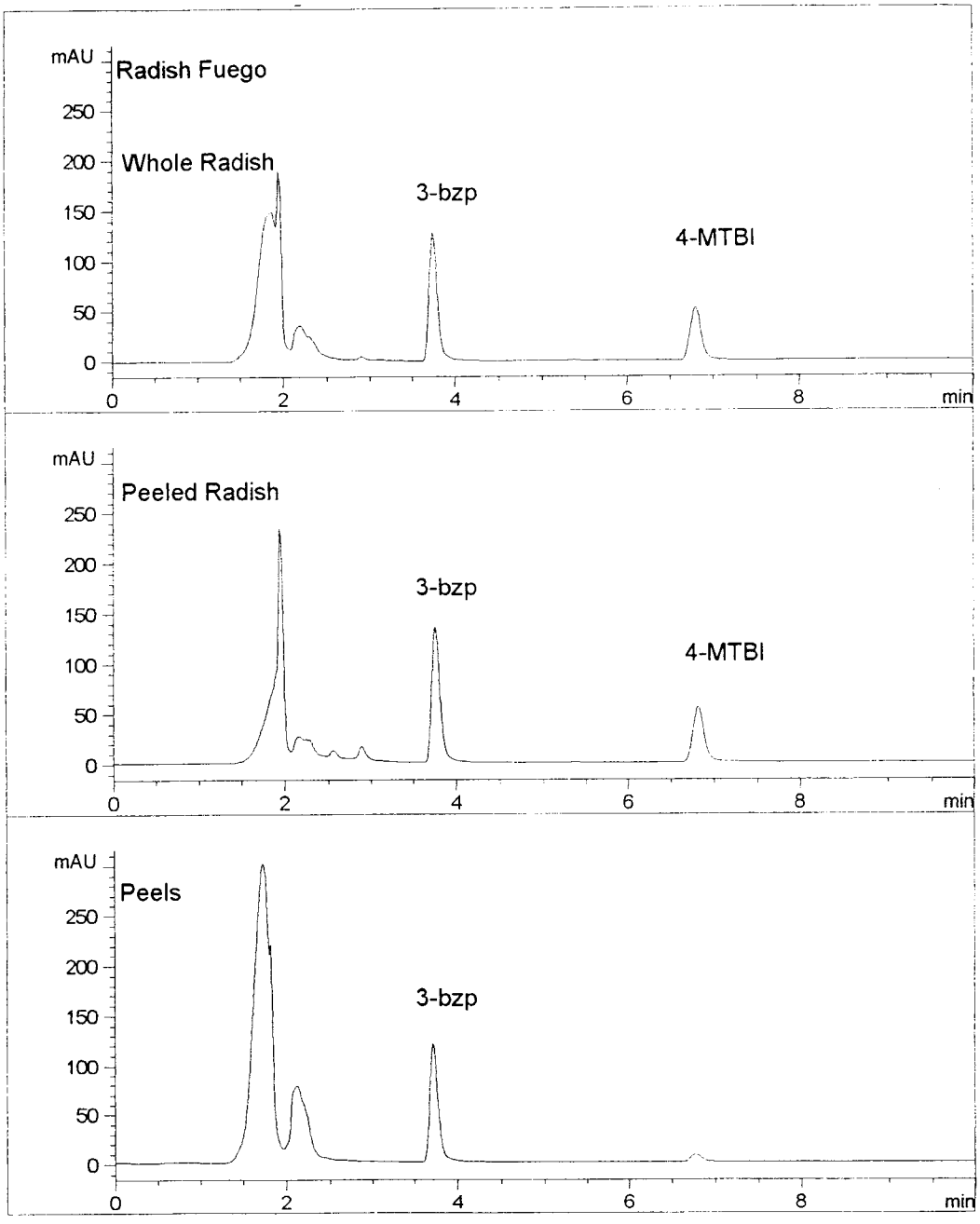


Figure 7: HPLC separation of 4-methylthio-3-butenyl isothiocyanate (4-MTBI) extracted from whole radish (cultivar Fuego), peeled radish and peels. 3-bzp = 3-benzoyl pyridine used as internal standard. HPLC conditions: Supelcosil C18 column (5 µm particle size, 25 cm x 4.6 mm id.); Isocratic 65% acetonitrile/water; Flow rate: 1 mL/min. Injection volume: 50 µL. Detection wavelength: 254 nm.

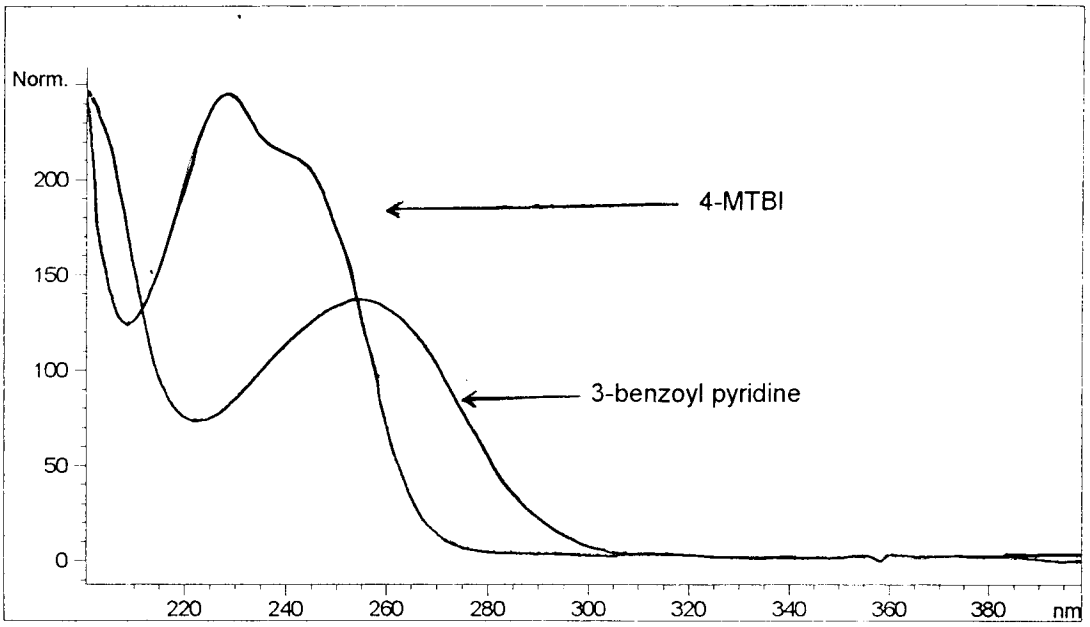


Figure 8: UV spectra of 4-methylthio-3-butenyl isothiocyanate (4-MTBI) extracted from whole radish cultivar Fuego and 3-benzoyl pyridine, internal standard.

1.2. Calibration curve

The calibration curve was constructed using concentrations of 3-benzoyl pyridine (3-bzp) in the range of 5 to 300 nmol/mL (Figure 9). The ratio of peak area of 3-bzp to its molar concentration was found to be 37 after construction of the curve.

Since pure MTBI was not available as an external standard, quantitation was done using the standard curve reported by Kim and Rhee (1986). They reported that the peak area ratios of 4-MTBI to 3-benzoyl pyridine was proportional to the molar concentration of 4-MTBI, ranging from 20 to 120 nmol/mL, by a factor of 0.027 (or 1/37). Therefore, the molar concentration of 4-MTBI can be expressed as:

$$[\text{4-MTBI}] \text{ in nmol/mL} = \frac{\text{Peak Area of 4-MTBI}}{[\text{3-bzp}] \text{ in nmol/mL}}$$

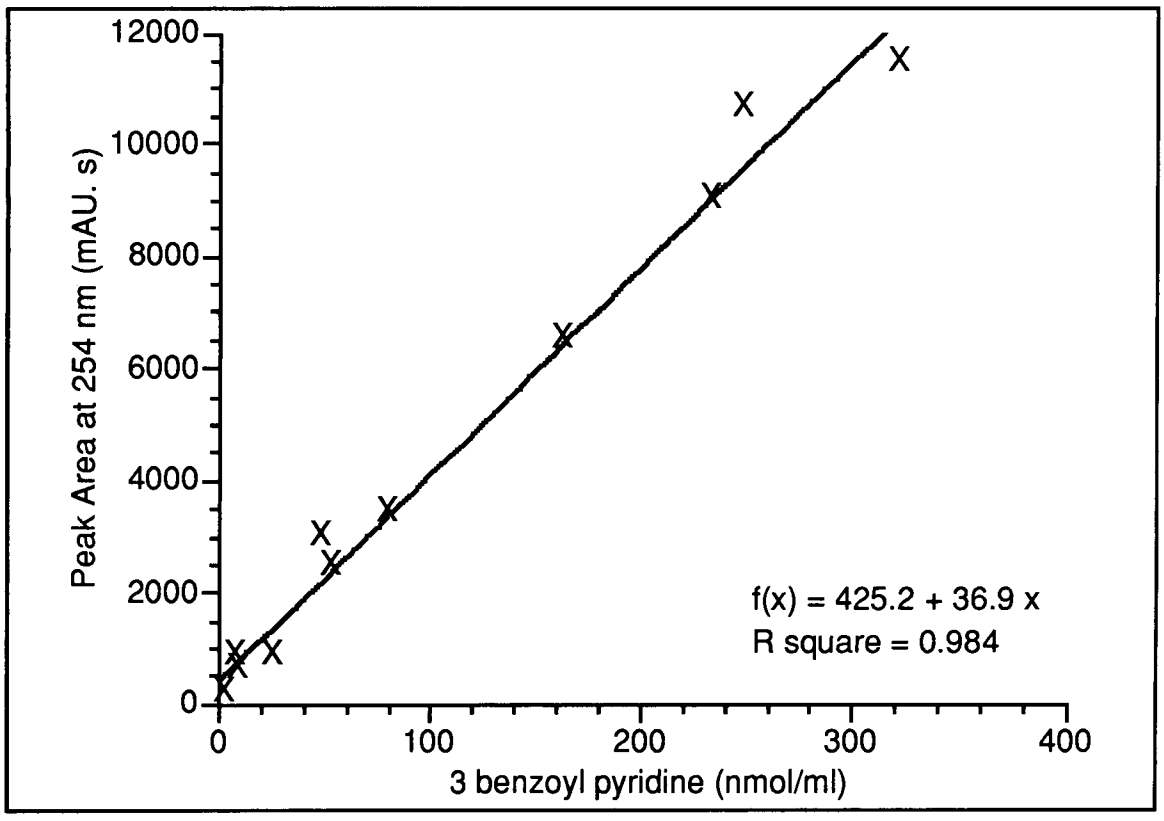


Figure 9: Standard curve for 3 benzoyl pyridine used as an internal standard in 4-methylthio-3-butenyl isothiocyanate separation

1.3. Quantitative analysis of 4-MTBI

An average content of MTBI of 1 mg/100g whole fresh radish (Figure 10) was obtained for the salad type radishes, Fuego and Spring Chinese "Cherry Radish" cultivars, whereas 1.5 to 2.5 mg was detected per 100 g of whole radishes of bigger size such as the cultivars Chinese Red (round shape), Wu Ying Shui (elongated shape) and the red meat cultivars (Takii and Man Tang Hong). The winter varieties were found to have a higher potential (2 fold difference) to form the pungent principle than the salad radishes.

The formation of MTBI was lower in the peels than in the peeled radishes, representing between 10 to 25% of the total MTBI formed in the whole

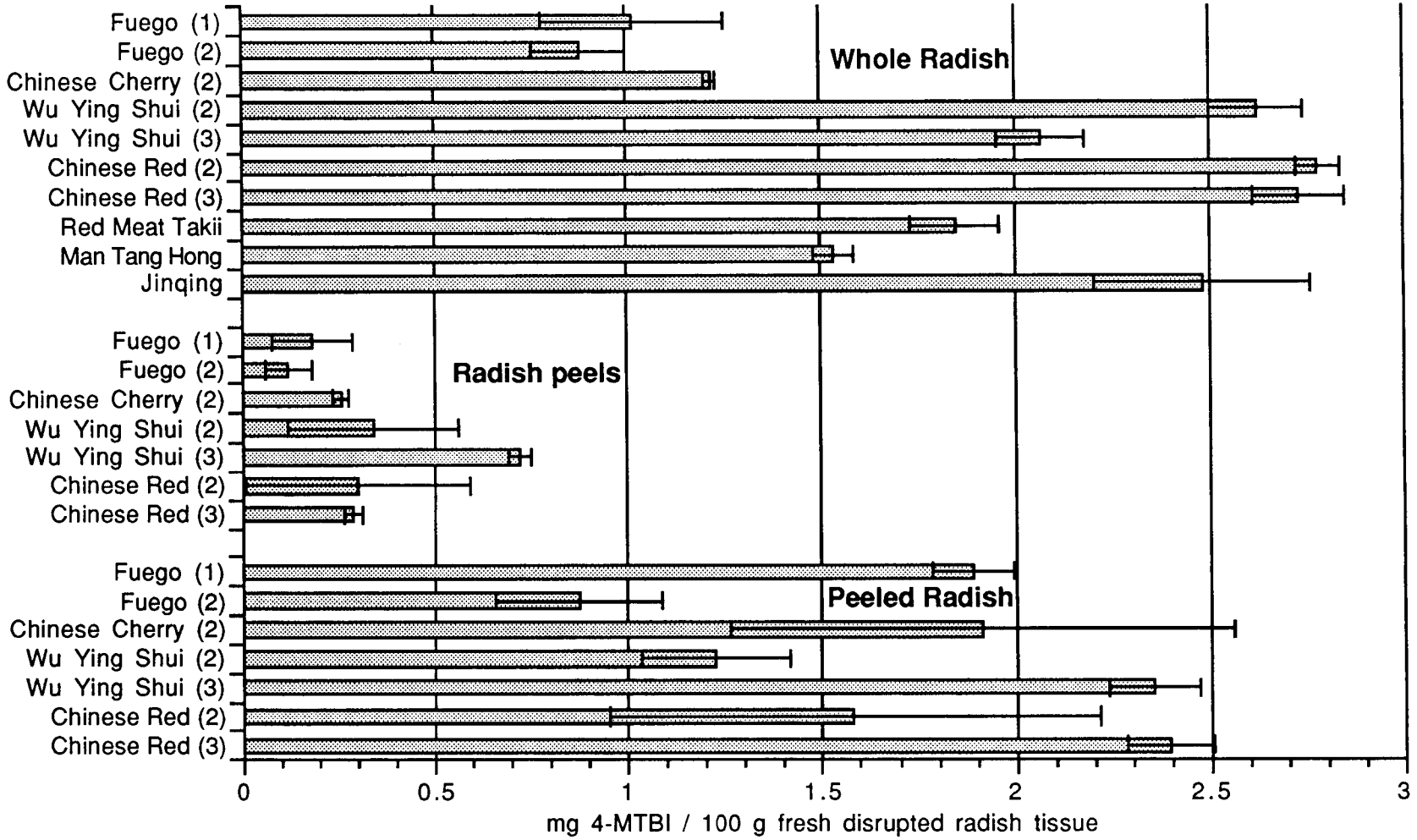


Figure 10: 4-methylthio-3-butenyl (4-MTBI) content in fresh disrupted radish tissues (whole, core and peels) of different radish cultivars harvested (1) five weeks, (2) seven weeks and (3) nine weeks after planting.

radishes. Low contribution of radish peels to MBTI formation could be explained by actual lower content in the peels than in the flesh but also by higher loss of MTBI during the peeling process.

Kim and Rhee (1986) reported that the intact root of Korean radish contained 210-420 μmol of MTBI per 100 g fresh weight, which is equivalent to 33-67 mg/100 g, the molecular weight of MTBI being 159 g/mole. We found values more than 20 fold lower. Cultivation practices, maturity stages at harvest as well as cultivar differences might explain these differences (Fenwick et al, 1983).

Radish juices were also analyzed for MTBI with direct injection of the filtered juice on the HPLC. No peak was detected at the retention time of MTBI. It is likely that, during processing of the juice, MTBI was lost by volatilization and/or subsequent degradation. It is also possible that 4-MTBI might still be present in the sample but was not detected by HPLC. Detection level of 4-MTBI on HPLC was determined close to 1 nmol/mL in the injected sample, which is equivalent to 159 μg/L (or ppb). The aroma threshold of 4-MTBI has been reported to be 3.4 μg/L (Whitfield, 1991). Therefore, at very low concentrations, in the order of ppb, 4-MTBI is not detected by HPLC but may still be present and its characteristic pungent aroma could be detected by human subjects. Gas chromatography may be an alternative method, with lower detection limit and higher sensitivity, for monitoring 4-MTBI in radish juices since this compound is volatile. OSME, a method developed at Oregon State University, combines gas chromatography and olfactometry using a time-intensity scale (Osme means smell in greek). This method could be used to separate and detect individual aroma compounds using analytical tools and sensory analysis.

2. Intact glucosinolates

2.1. HPLC separation of intact glucosinolates in radish tissue

Intact glucosinolates, forming a neutral complex with the counter ion tetrabutylammonium (ion pair agent), were extracted from radish tissue and separated by reverse phase HPLC. Some gradient work was necessary to obtain the separation shown in Figure 11 based on conditions reported by Betz and Fox (1996) for broccoli. Sinigrin, the internal standard, eluted at about 34% methanol with 5 mM tetrabutylammonium and was detected at 4 min. Prestera and coworkers (1996) reported that 4-methylthio-3-butyl glucosinolate, close in structure to 4-methylthio-3-butenyl glucosinolate (4-MTB-GLS), eluted after sinigrin on reverse-phase ion pair chromatography. We therefore expected 4-MTB-GLS to elute after sinigrin.

In whole radish tissue, several peaks were detected after sinigrin. UV spectra (Figure 12) and retention time gave strong evidence that peak 5 at 7.8 minutes was 4-MTB-GLS. It was a major peak of the chromatogram, its UV spectrum showed a wavelength of maximum absorbance at 229 nm and no absorbance after 280 nm. Sinigrin spectra had a wavelength of maximum absorbance at 228 nm and a similar shape, with no absorbance beyond 260 nm. The UV spectrum obtained for sinigrin matched that reported by Thies (1988). Peak 2, at 3.7 min, also presented a similar UV spectrum but it was shifted to lower wavelengths with a wavelength of maximum absorbance at around 210 nm. This compound could be a glucosinolate since its structure is close to sinigrin (2-propenyl R group).

Peak 3 eluted at a similar retention time as sinigrin and showed absorption at 210 nm, 260 and 280-300 nm. It interfered with the elution of

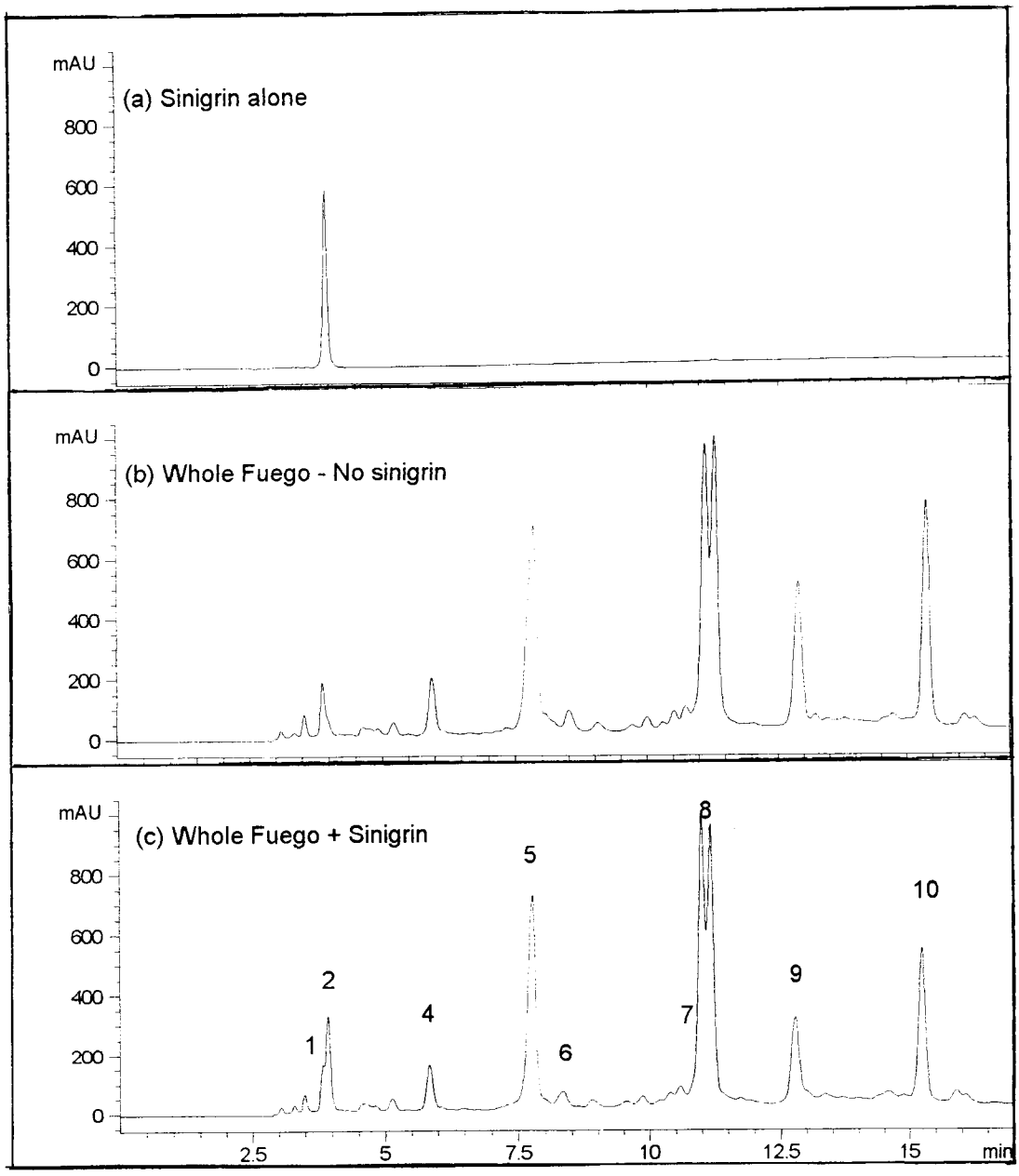


Figure 11: Reverse phase ion pair chromatography of intact glucosinolates: (a) sinigrin, (b) whole radish (cultivar Fuego), (c) whole radish spiked with sinigrin. HPLC conditions: Supelcosil C18 column (5 µm particle size, 25 cm x 4.6 mm id.); Mobile phase: gradient of A (10% methanol+IPA) and B (98% methanol+IPA) 10 min 28% B, 10 min 28% to 44% B; IPA = ion pair agent tetrabutylammonium sulfate 5 mM; Flow rate: 1 mL/min. Injection volume: 50 µL. Detection wavelength: 229 nm.

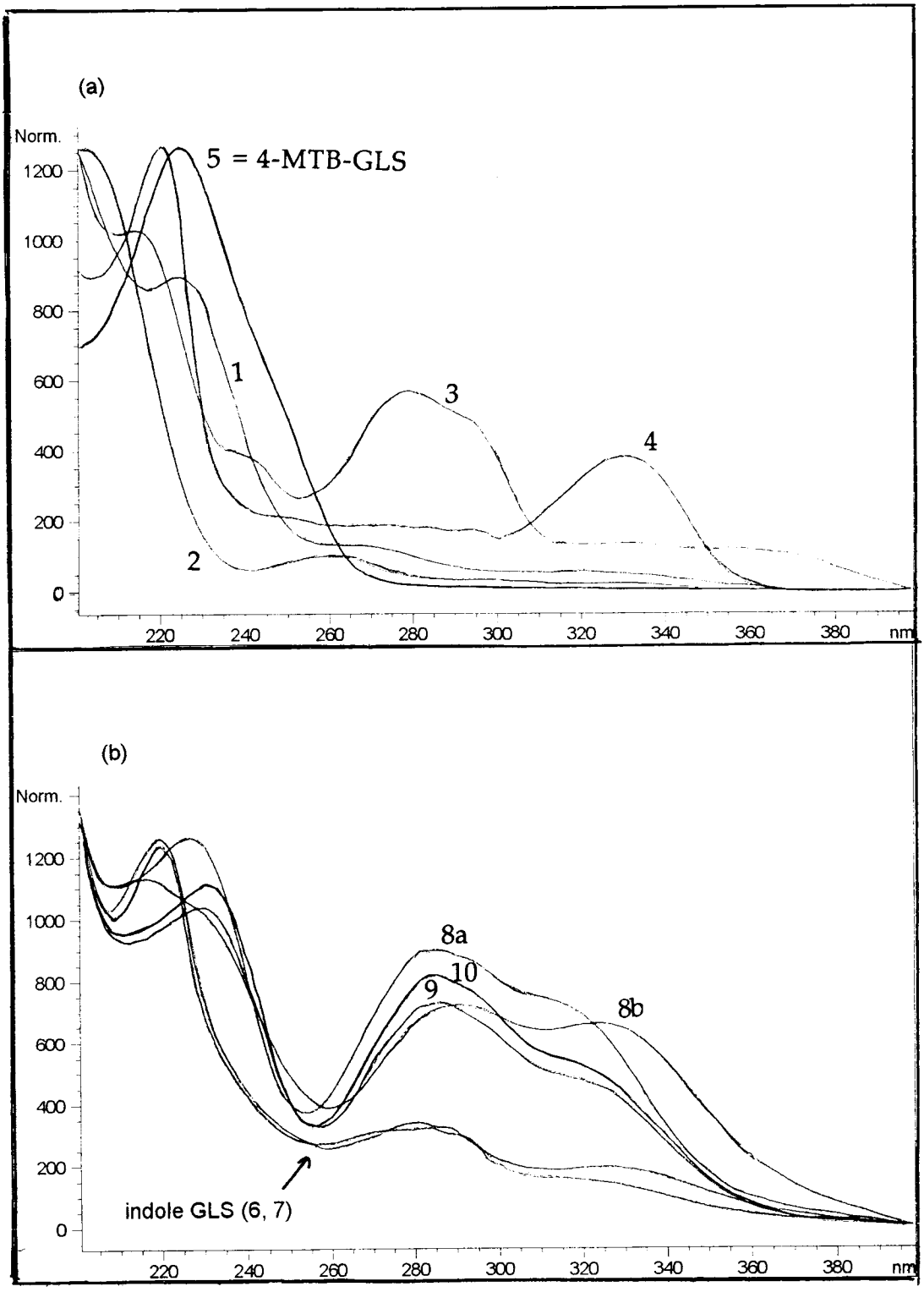


Figure 12: UV spectra of sinigrin, 4-MTB-GLS and other potential glucosinolates detected at 229 nm. (a) early eluting peaks, (b) late eluting peaks. Numbers refer to chromatographic signals of Figure 8.

sinigrin, no gradient or isocratic solvent program was able to separate peak 3 from sinigrin. Because of this coelution, it was not possible to quantitatively determine 4-MTB-GLS in the radish samples. The use of a different glucosinolate standard, such as glucotropaeolin (benzyl glucosinolate) or glucobarbarin (2-hydroxy-2-phenylethyl glucosinolate) may have been more appropriate as recommanded by Bjerg and Sorensen (1987) for the analysis of glucosinolates in rapeseed. However, at the time of the analysis, these standards were not available for our use. Commercial glucosinolates are rare, sinigrin is actually the only one readily available.

Peak 4, eluting around 6 min, showed a UV spectrum close to peak 3 except that its UV absorption at 260 nm was lower and a stronger absorption was detected in the 320-340 nm region, which peak 3 did not show.

Peaks 6 and 7, at 8.5 and 10.5 min respectively, could be indole derivatives since their UV spectra resemble glucobrassicin (3-indolylmethyl glucosinolate) as reported by Buchner (1987). Wathelet and coworkers (1987) reported that maximum wavelength of absorbance for intact indole glucosinolates was close to 220 nm, generally at a lower wavelength than the alkyl or alkenyl glucosinolates.

Peaks 8, 9 and 10 at 11.5, 13.3 and 16 min respectively had similar UV spectra with absorbance at 230 nm and in the 280-320 nm region. Their UV characteristics agreed with the typical spectra of phenolics such as *p*-coumaric, caffeic or ferulic acids. However, elution time for these compounds was much shorter (4 to 7 min) in our system. Peaks 8, 9 and 10 were detected as major compounds in whole radishes and in radish peels but were not found in flesh (Figure 13). There is evidence to associate these peaks to flavanoids since phenolic compounds are more abundant in external (skins) than in internal

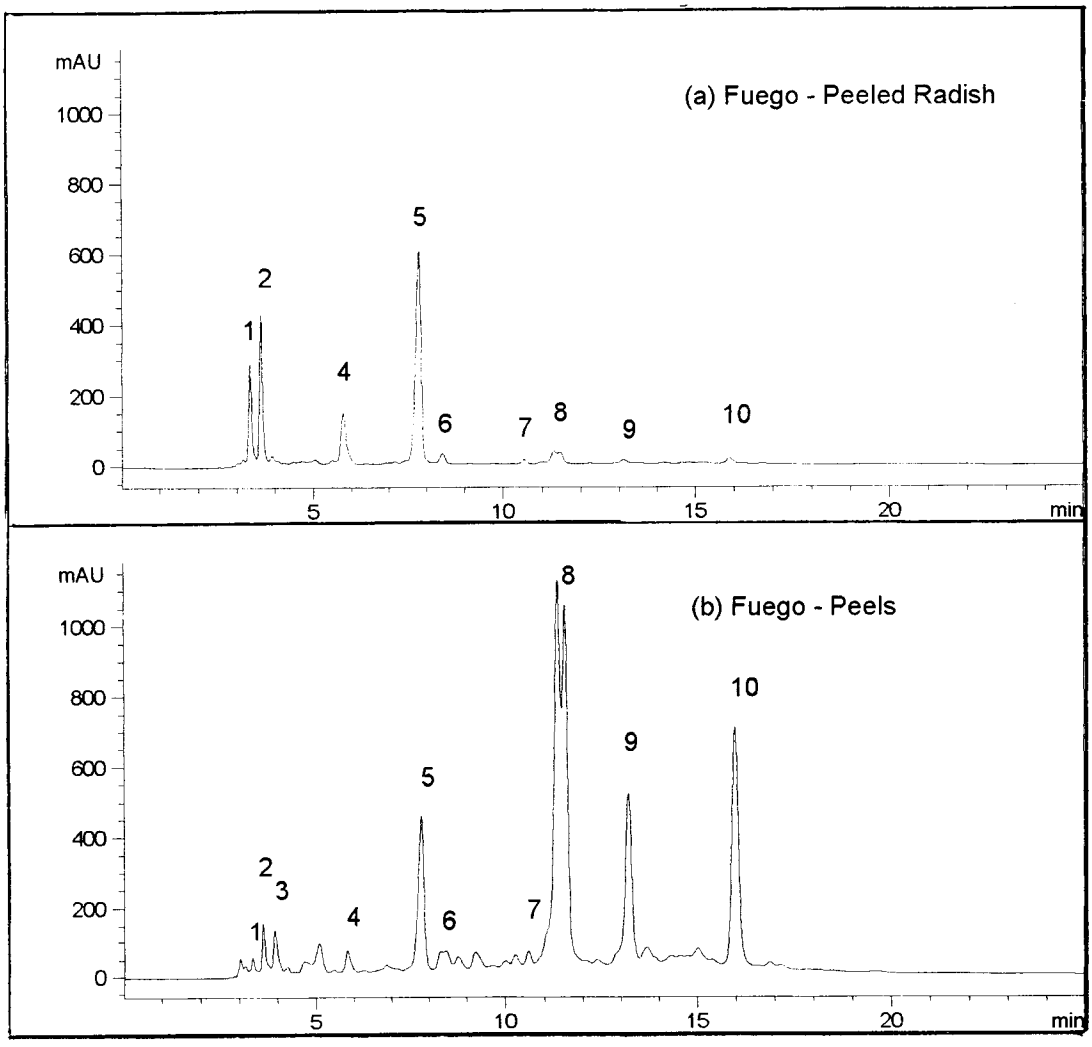


Figure 13: Reverse phase ion pair chromatography of intact glucosinolates from cultivar Fuego: (a) peeled radish, (b) radish peels. HPLC conditions: same as Figure 11.

tissue (flesh) (Macheix et al, 1990). Giusti and Wrolstad (1996a) reported reverse phase HPLC separation of radish anthocyanins with elution around 17-18% acetonitrile which, in solvent strength, corresponds to about 20 % methanol. Unknown peaks 8, 9 and 10 eluted during the gradient part of the program, between 37 and 48% methanol. This difference in solvent strength at the elution time is significant. In addition, peaks 8, 9 and 10 did not show any absorbance in the 520 nm region. It is therefore more likely that these peaks are phenolics but not anthocyanins. However, extraction of anthocyanins and glucosinolates is based on C18 affinity, coated in the case of glucosinolates, with an ion pair agent. We observed that the sample preparation used for glucosinolate extraction was also effective for anthocyanins since the HPLC samples were slightly red-colored.

2.2. HPLC intact glucosinolate profiles of radishes and radish juices

Radish cultivar Fuego gave similar HPLC profiles when comparing two harvest times, two weeks apart. We also obtained similar profiles for the other spring and winter cultivars. Peak 5 was present in much larger concentration in the red-fleshed cultivars, Red Meat Takii (Figure 14) and Man Tang Hong, relatively to the other peaks. It was tentatively identified as 4-MTB-GLS from spectral characteristics and HPLC retention time. Peak 6, a potential indole glucosinolate, also showed a large area relative to the other peaks and, also, compared to Fuego HPLC profile (Figure 11).

Radish juices from Fuego cultivars (Figure 15) did not show any peak at the retention time of 4-MTB-GLS, both before and after blanching. Peaks 2, 3, 8, 9 and 10 were detected but did not show spectral characteristics of glucosinolates. They could be due to phenolic compounds. HPLC separation

of glucosinolates in Red Meat Takii juice gave numerous peaks including peaks within the elution time of 4-MTB-GLS. However, all these peaks gave a spectrum with absorbance in the 280-320 nm region which is not typical of 4-MTB-GLS. Radish juices from red flesh cultivars were processed by crushing the whole radish to extract the pigment from the flesh whereas juices made from Fuego cultivar were made from pigmented peels only. Red flesh radish juice probably contained more compounds absorbing at 254 nm that interfered with glucosinolate detection.

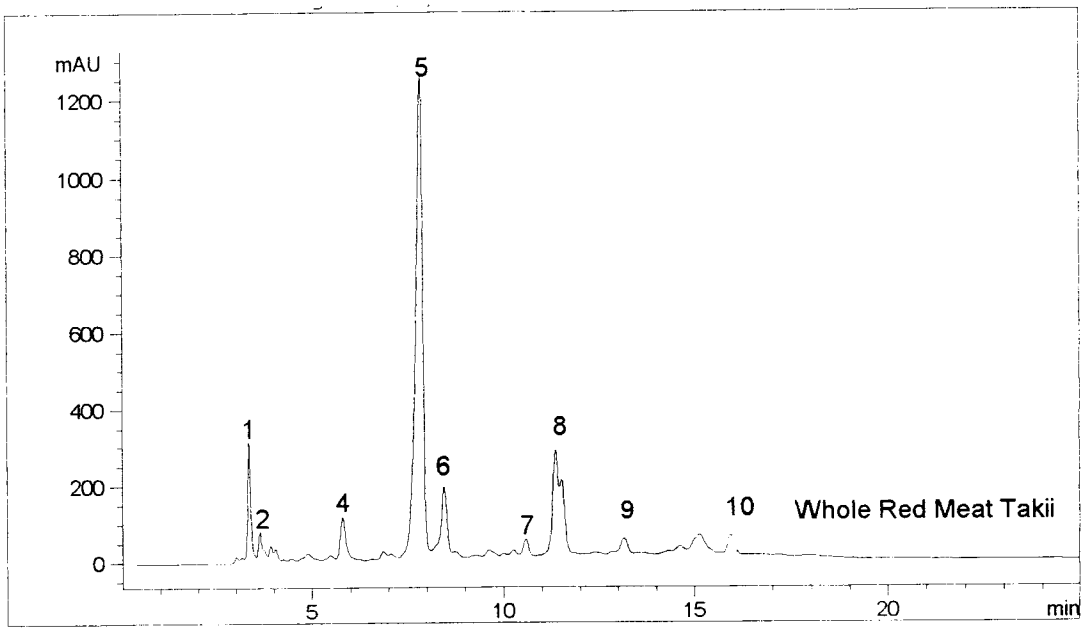


Figure 14: Reverse phase ion pair chromatography of intact glucosinolates: whole radish cultivar Red Meat Takii. HPLC conditions: same as Figure 11.

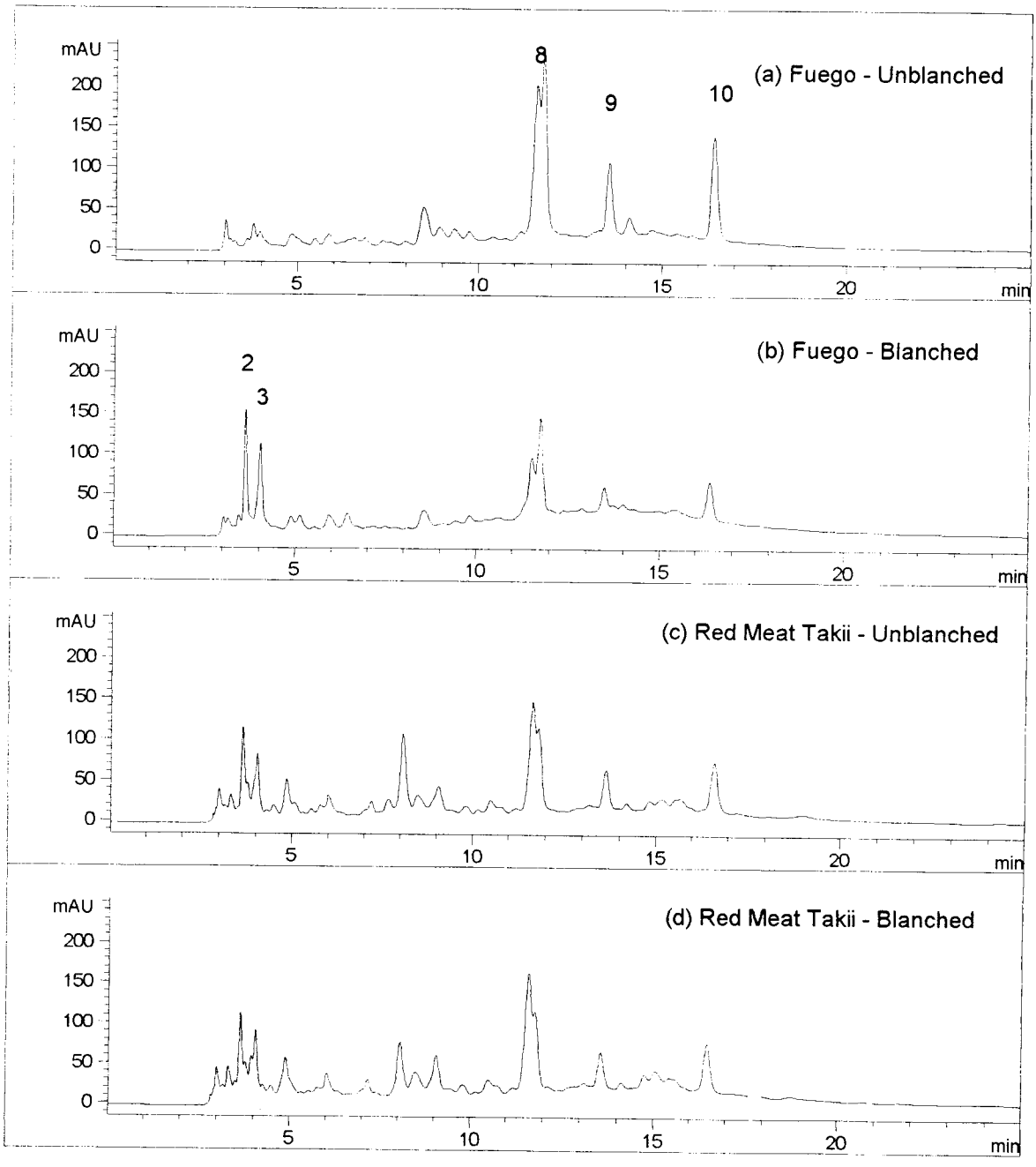


Figure 15: Reverse phase ion pair chromatography of intact glucosinolates: radish juices before and after blanching from cultivar Fuego (a, b) and Red Meat Takii (c, d). HPLC conditions: same as Figure 11.

Due to coelution with the glucosinolate standard, sinigrin, the quantification of intact 4-MTB-GLS, precursor of the pungent principle of radish, was not possible using ion pair chromatography. Also, identification of the peaks obtained by ion pair chromatography was difficult. The presence of the ion pair agent made difficult handling of eluted peaks. Mass spectrometry requires the elimination of the ion pair agent before injection in the mass spectrometer to avoid interference. Such sample preparation was not achieved successfully because it was not possible to dedicate a preparative column for the collection of large amounts of eluted peaks. Because of the presence of the ion pair agent which affects the stationary phase characteristics during the chromatographic separation, it is preferred to dedicate a column for this use, which could not be done in our laboratory.

An attempt was done for mass spectrometry analysis of a concentrated sample ready for HPLC injection. The ion pair agent was eliminated by solvent-solvent extraction after dilution of the glucosinolate extract in diluted ammonium hydroxide to regenerate the free acid form of glucosinolate. Dichloromethane was then added to dissolve the ion pair agent while the glucosinolates would be in the alkaline aqueous phase. Unfortunately, no significant peaks were detected after low resolution mass spectroscopy analysis. Therefore, a new approach was taken, still using HPLC as a tool to separate glucosinolates, but using different techniques to extract and isolate glucosinolates as desulfoglucosinolates.

3. Desulfoglucosinolates

Desulfoglucosinolates were obtained after enzymatic treatment by sulfatase of the glucosinolates isolated on an anion exchange resin (Figure 16).

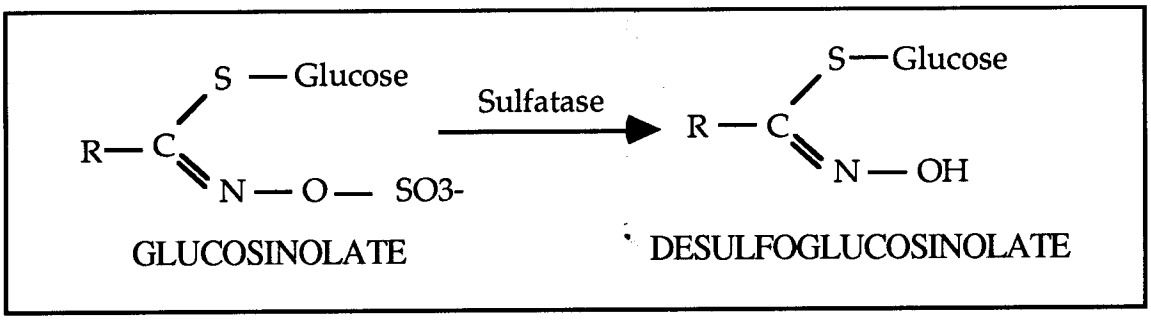


Figure 16: Enzymatic formation of desulfoglucosinolates.

3.1. Sulfatase activity

Sulfatase activity was tested on sinigrin, a commercial glucosinolate, at room temperature (Figures 17 and 18). A solution containing 0.5 mg sinigrin ion was treated with 125 µL sulfatase solution at 50 units/mL that is 6.25 units. During the first 160 minutes of the reaction, samples were run on reverse phase HPLC for detection of desulfosinigrin at 7.5 min elution (Figure 17). After 60 minutes, sinigrin was totally desulfated into desulfosinigrin (Figure 18). Therefore, one enzyme unit of sulfatase can desulfate 0.08 mg of sinigrin ion (or 0.223 µmol) into desulfosinigrin in one hour.

A standard curve (Figure 19) was then constructed with increasing sinigrin concentrations (0.01 to 0.5 mg sinigrin ion/mL injected). In further determinations of glucosinolate content, we assumed that other desulfoglucosinolates have a relative response factor of 1 compared to desulfosinigrin. Relative response factors were reported by Buchner (1987) for

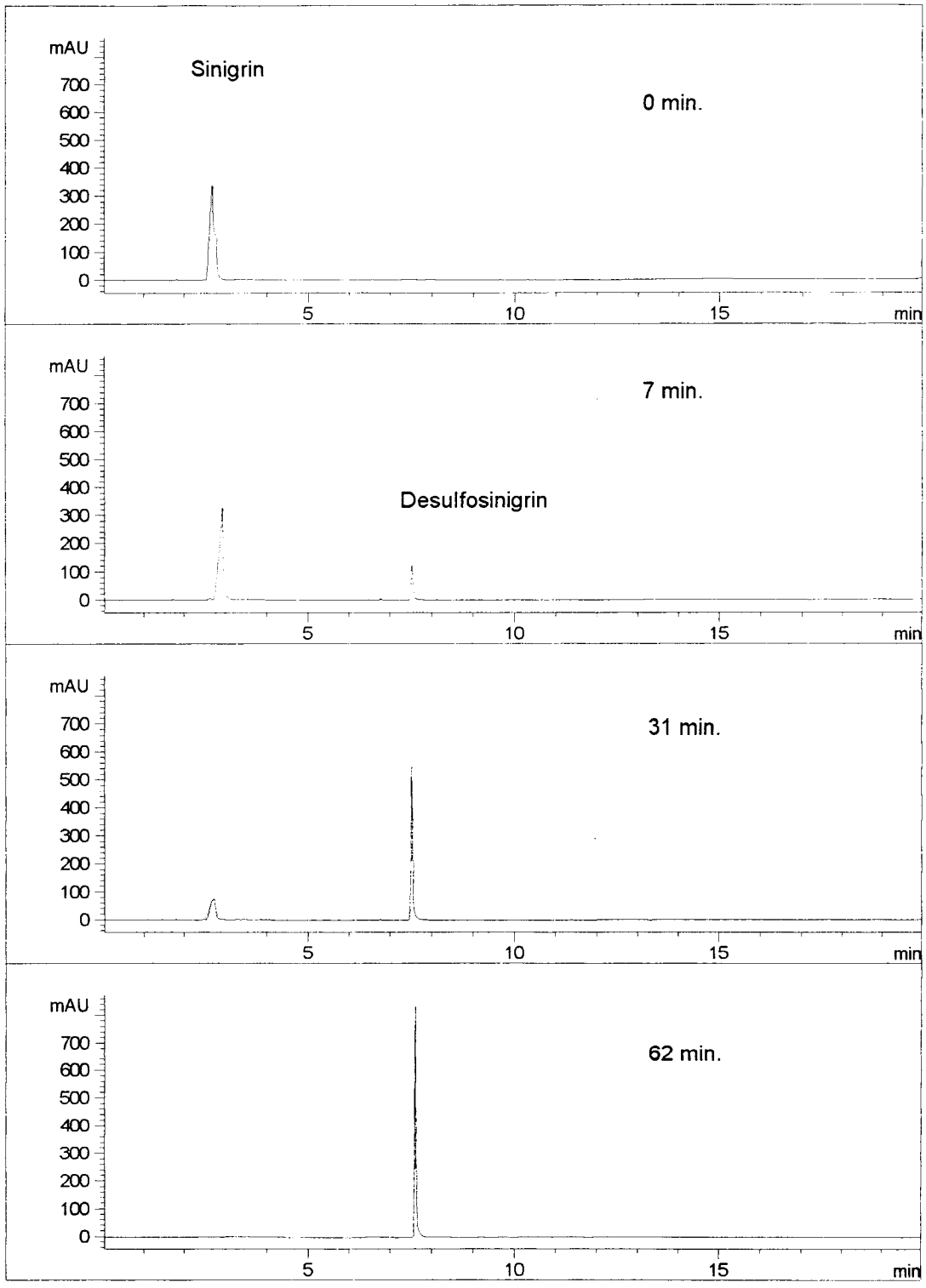


Figure 17: HPLC chromatograms of sinigrin and desulfosinigrin solutions at zero time and after 62 minute reaction. 5 mL of 0.1 mg/mL sinigrin in acetate buffer pH 5.0, at zero time addition of 125 µL of 50 units/mL sulfatase. Room temperature. HPLC conditions: Supelcosil C18 column 25 min 5-20% CH_3CN. Detection wavelength: 230 nm.

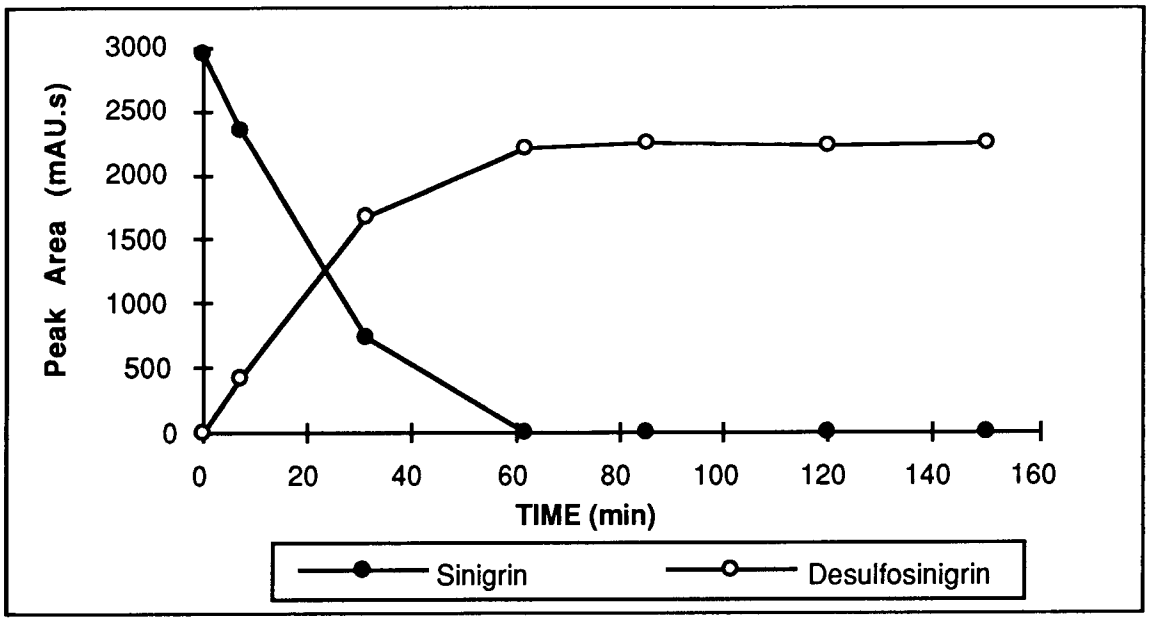


Figure 18: Sulfatase activity tested on sinigrin: 5 mL of 0.1 mg/mL sinigrin in acetate buffer pH 5.0, at zero time addition of 125 µL of 50 units/mL sulfatase. Room temperature. Desulfosinigrin monitored by HPLC.

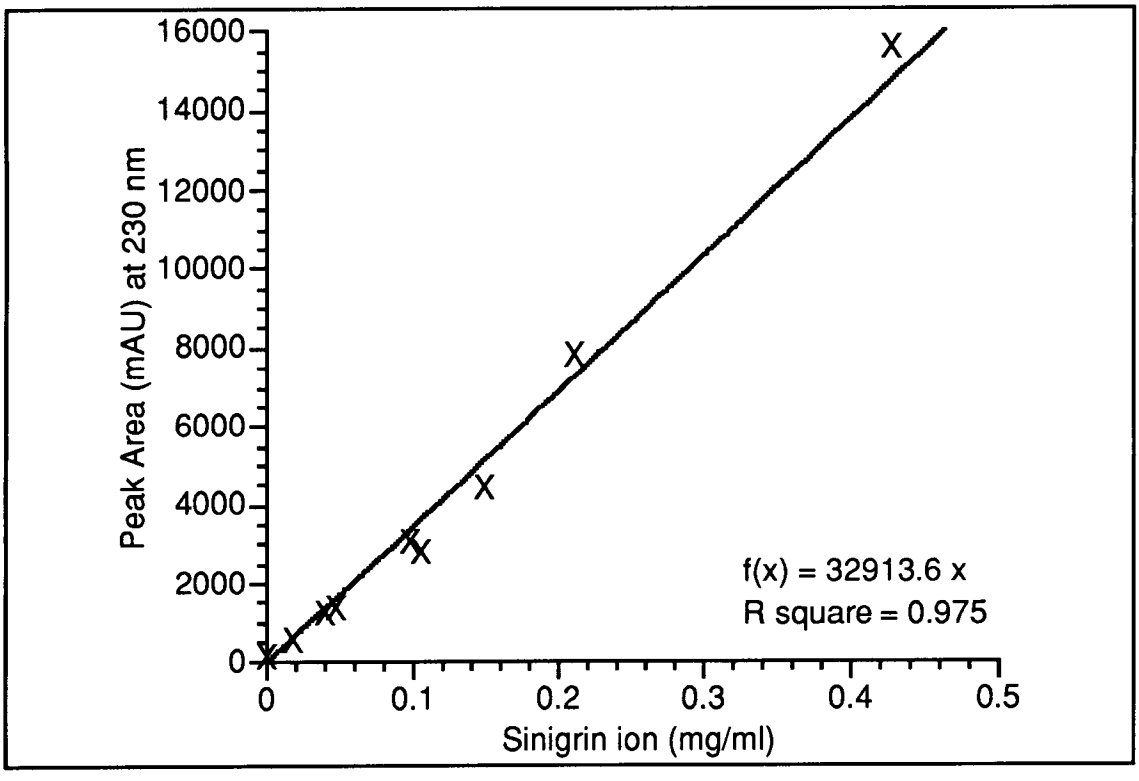


Figure 19: Standard curve for desulfosinigrin as detected at 230 nm on reverse phase HPLC.

several desulfoglucosinolates and all were close to 1.00, except for indole glucosinolate having lower values. No value was reported for 4-methylthio-3-butenyl desulfoglucosinolate (4-MTB-DFGLS).

3.2. HPLC separation of desulfoglucosinolates from radish tissue

Reverse phase HPLC separation was performed on sinigrin and crude glucosinolate radish extract after desulfatation (Figure 20). Desulfosinigrin (4.8 min) was well resolved from the other peaks of the radish extract. The main radish glucosinolate, detected as 4-methylthio-3-butenyl desulfo-glucosinolate (4-MTB-DFGLS) eluted at 16.5 min. Identification was achieved by comparison of HPLC retention time and elution order to that reported by Sang and coworkers (1984) for radish hypocotyls under similar conditions. Further characterization was also performed using mass spectroscopy analysis and will be discussed later. UV spectra (Figure 21) of the detected peaks also agreed with its identification as 4-MTB-DFGLS.

Sang and coworkers (1984) also found three minor glucosinolates in radish: identified as 4-hydroxy-3-indolylmethyl desulfoglucosinolate (4-OH-desulfoglucobrassicin) eluting before 4-MTB-DFGLS and, eluting after 4-MTB-DFGLS, indole glucosinolates identified as 3-indolylmethyl desulfoglucosinolate (desulfoglucobrassicin) and 4-methoxy-3-indolylmethyl desulfoglucosinolate (4-methoxy-desulfoglucobrassicin). These compounds could be respectively peaks 2, 4 and 5 in radish extract (Figure 20) which showed UV spectra (Figure 21) typical of indole glucosinolates. Peak 3 did not show a typical glucosinolate UV spectrum with absorption in the 280-320 nm range. Also, they obtained a broad peak between 4-OH-desulfoglucobrassicin

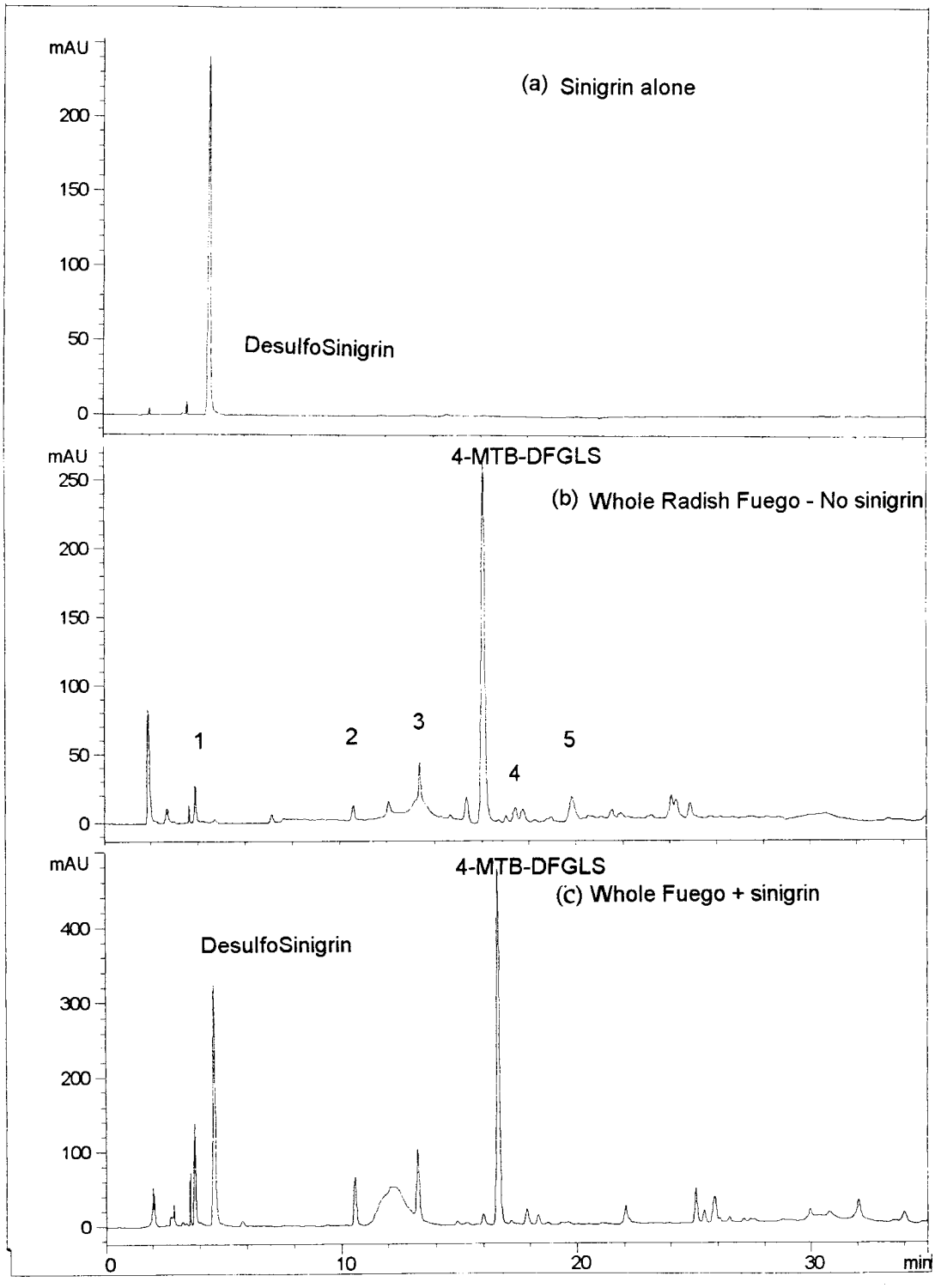


Figure 20: HPLC chromatograms of (a) desulfosinigrin, (b) desulfoglucosinolates in whole radish Fuego extracts and (c) radish spiked with sinigrin. HPLC conditions: Supelcosil C18 column; 25 min gradient 5-20% acetonitrile; flow rate: 1 ml/min; detection wavelength: 230 nm. 4-MTB-DFGLS: 4-methylthio-3-butenyl desulfoglucosinolate.

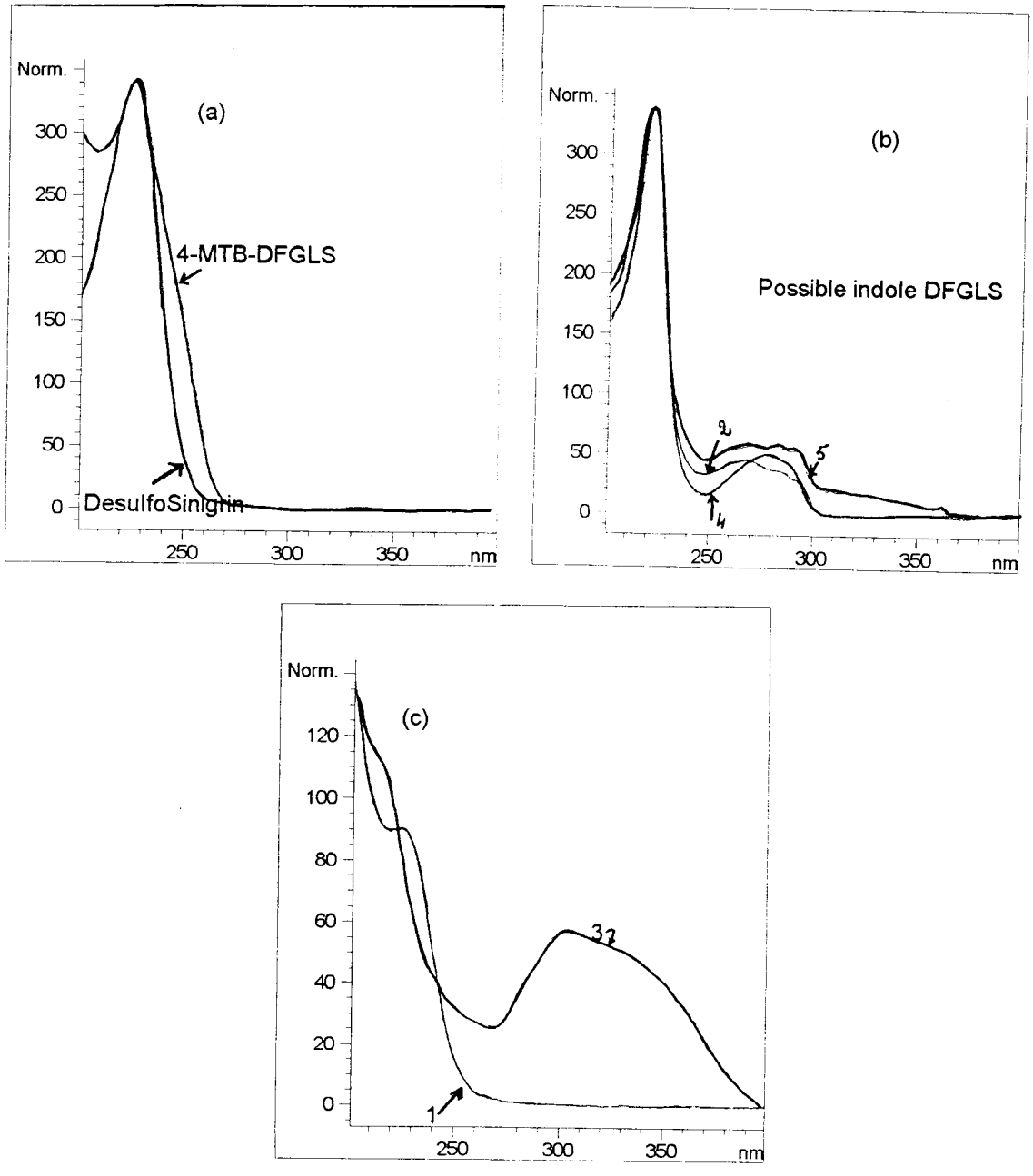


Figure 21: UV spectra of (a) desulfosinigrin and 4-MTB-DFGLS, (b) potential indole glucosinolates and (c) other peaks. Numbers refer to peaks Figure 20.

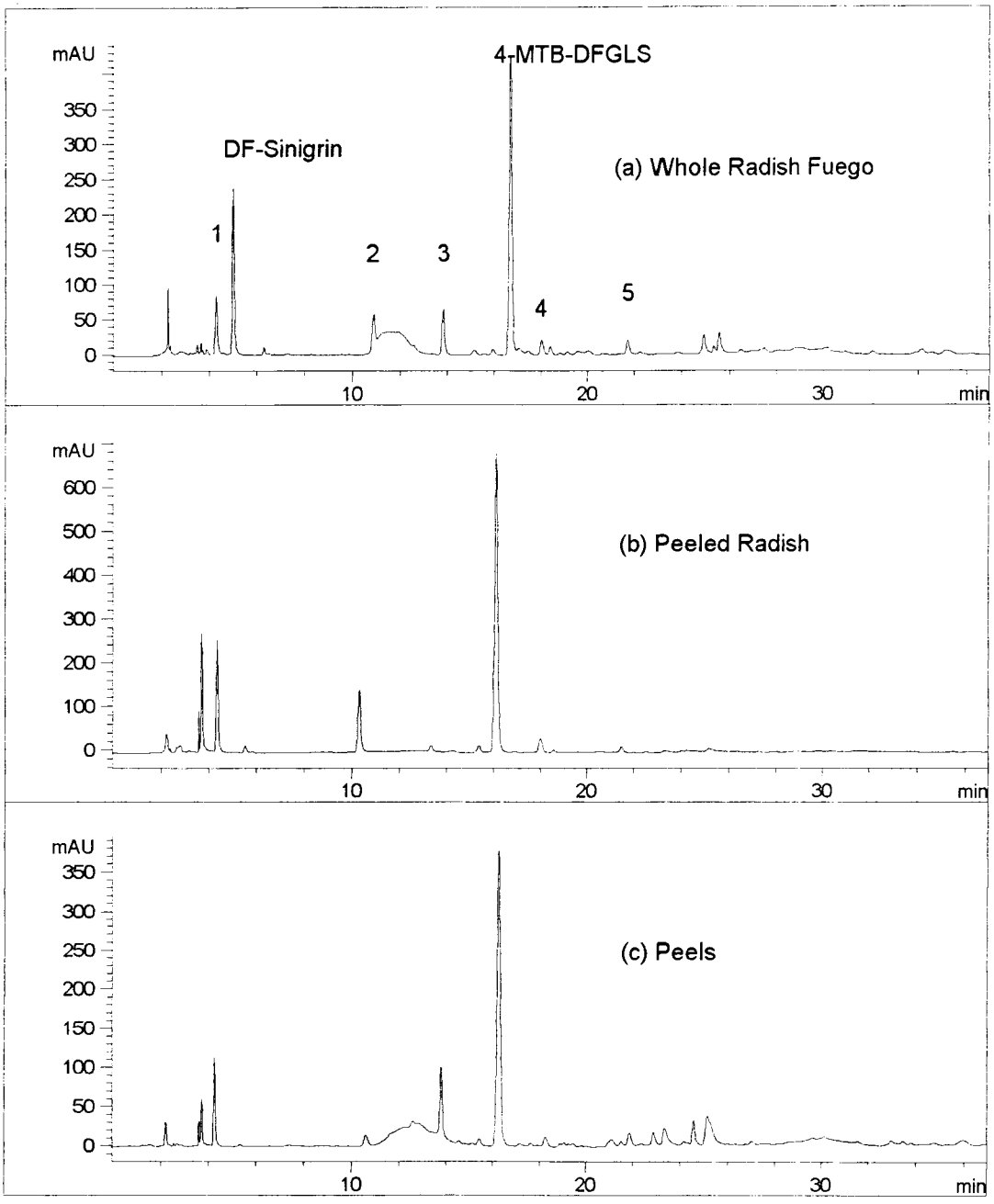


Figure 22: HPLC chromatograms of desulfated (a) whole radish cultivar Fuego, (b) peeled radish and (c) radish peels. HPLC conditions: same as in Figure 20. 4-MTB-DFGLS: 4-methylthio-3-butenyl desulfoglucosinolate.

and 4-MTB-DFGLS, which did not show the glucosinolate backbone structure after mass spectroscopy. Such a peak was observed between peaks 2 and 3 in many colored samples and was especially wide in radish peel extract (Figure 22) whereas it was not detected in peeled radishes, suggesting that this broad peak was due to anthocyanins. HPLC profiles of whole radish, peeled radish and radish peels were very similar for the different harvest times.

3.3. HPLC separation of desulfoglucosinolates in radish juice

Single strength radish juices, from both Fuego and Red Meat Takii, did not show any peak with a typical glucosinolate UV spectrum at 4-MTB-DFGLS retention time (Figure 23). It is more likely that in the juices, very low amount of the major glucosinolate remain. The HPLC profiles showed some unidentified peaks, present in similar amounts and profiles in both juices. We could notice a broad peak before peak 3, which could be related to the presence of anthocyanins or phenolic compounds. Also late eluting peaks, between 22 and 26 min, were major peaks and showed a large UV absorption in the 280-320 nm region.

Chromatographic analyses of commercial red cabbage colorant and Japanese radish extract were also performed (Figure 24) but did not present identified peaks. Both polar molecules eluting before 4 min and late eluting peaks between 22 and 26 min were detected. Recorded signals from the chromatographic separation were however much lower for commercial extracts than for the experimental radishes, although the former ones were analyzed at greater concentration. Fresh red cabbage contains glucosinolates as a member of cruciferous vegetables like radish. In the red cabbage colorant we used, no glucosinolates were detected. Sapers (1982) reported a deodorization

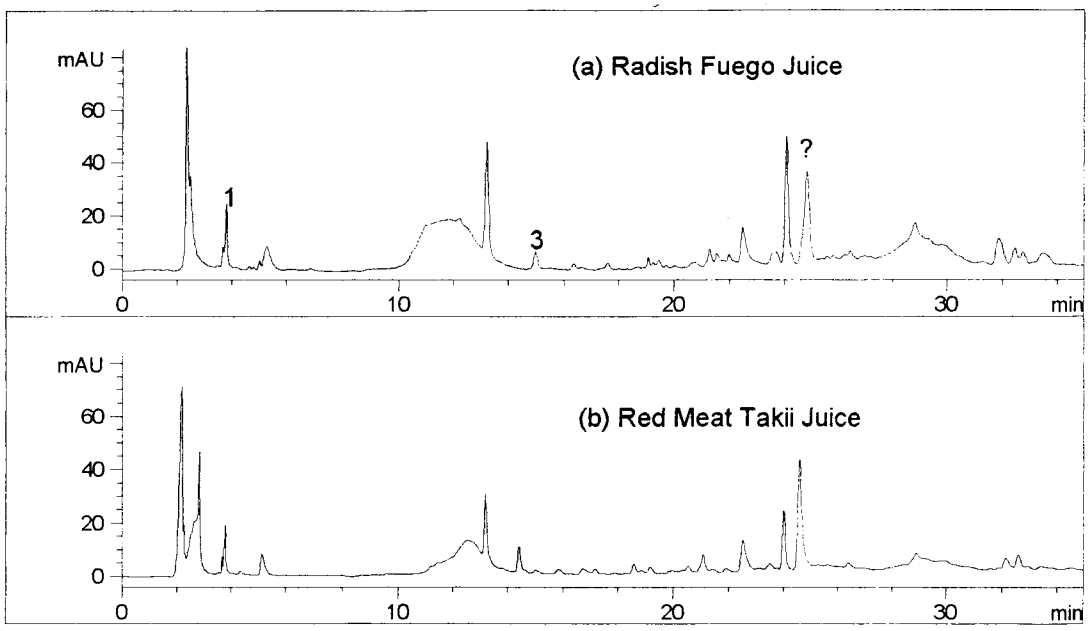


Figure 23: HPLC chromatograms of desulfated single strength radish juices (a) radish cultivar Fuego, (b) Red Meat Takii. HPLC conditions: same as in Figure 20.

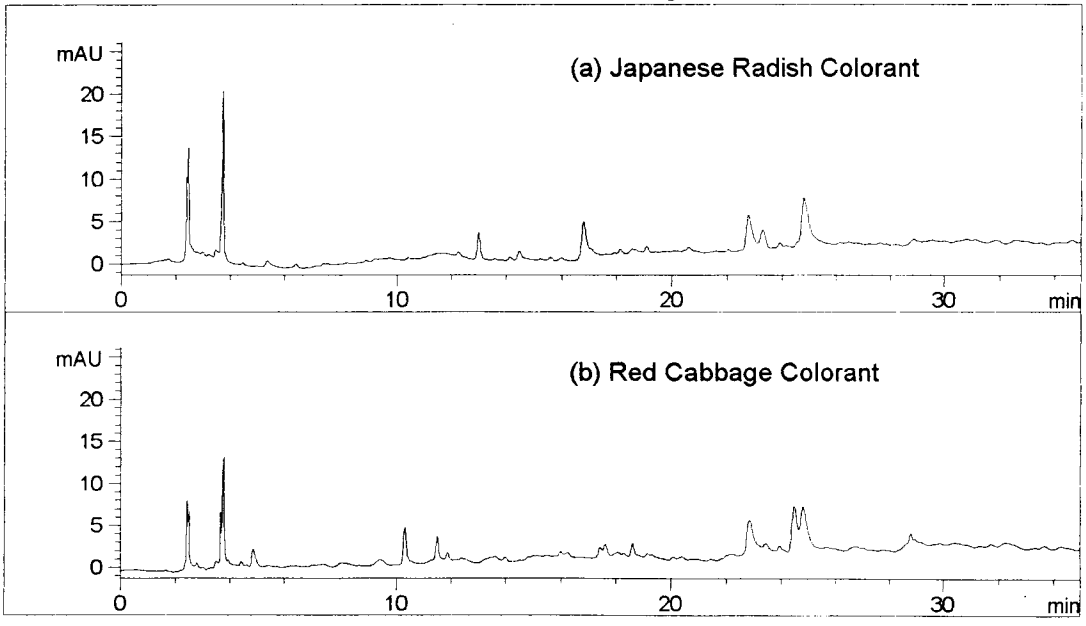


Figure 24: HPLC chromatograms of desulfated commercial samples (a) Japanese Radish extract, (b) Red Cabbage colorant HPLC conditions: same as in Figure 20.

process for red cabbage colorant involving absorption onto an Amberlite resin. Pigment recovery exceeded 90% and, stability and color properties were not significantly altered. Polystyrene resin such as Amberlite adsorbs various hydrophobic materials, such as phenolics, quinones, terpenes and organic isothiocyanates (Loomis et al., 1979). Similar physical purification techniques may have been utilized to prepare the red cabbage colorant we used.

3.4. Quantification of 4-MTB-GLS in radish tissue

The whole salad-type radish cultivar (Fuego and Cherry Radish), were found to contain 4-methylthio-3-butenyl glucosinolate (4-MTB-GLS) in the range of 30 to 65 mg/100g fresh radish weight, whereas winter-type red flesh cultivars (Red Meat Takii, Man Tan Hong) and the non-pigmented cultivar Jinqing contained five to ten times this amount ranging from 260 to 320 mg/ 100 g fresh weight (Figure 25). Radish peels contained more glucosinolates than peeled radish for a same weight.

Carlson and coworkers (1985) analyzed different radish cultivars for their glucosinolate content in the roots using gas-liquid chromatographic analyses. They found that the main glucosinolate was 4-methylthio-3-butenyl glucosinolate, representing more than 70% of the total glucosinolates. The 4-MTB-GLS content reported in this study ranged from 64-187 μmole/100 g fresh weight for red European-American cultivars, equivalent to 27-78 mg/100 g, the molecular weight of 4-MTB-GLS being 418 g/mole. In Japanese cultivars, 4-MTB-GLS represented more than 80% of the total glucosinolates, with content in the range of 109-488 μmole/100 g fresh weight, equivalent to 46-204 mg/100 g. They also analyzed a Takii cultivar which showed the highest 4-MTB-GLS content of the Japanese cultivars in the study.

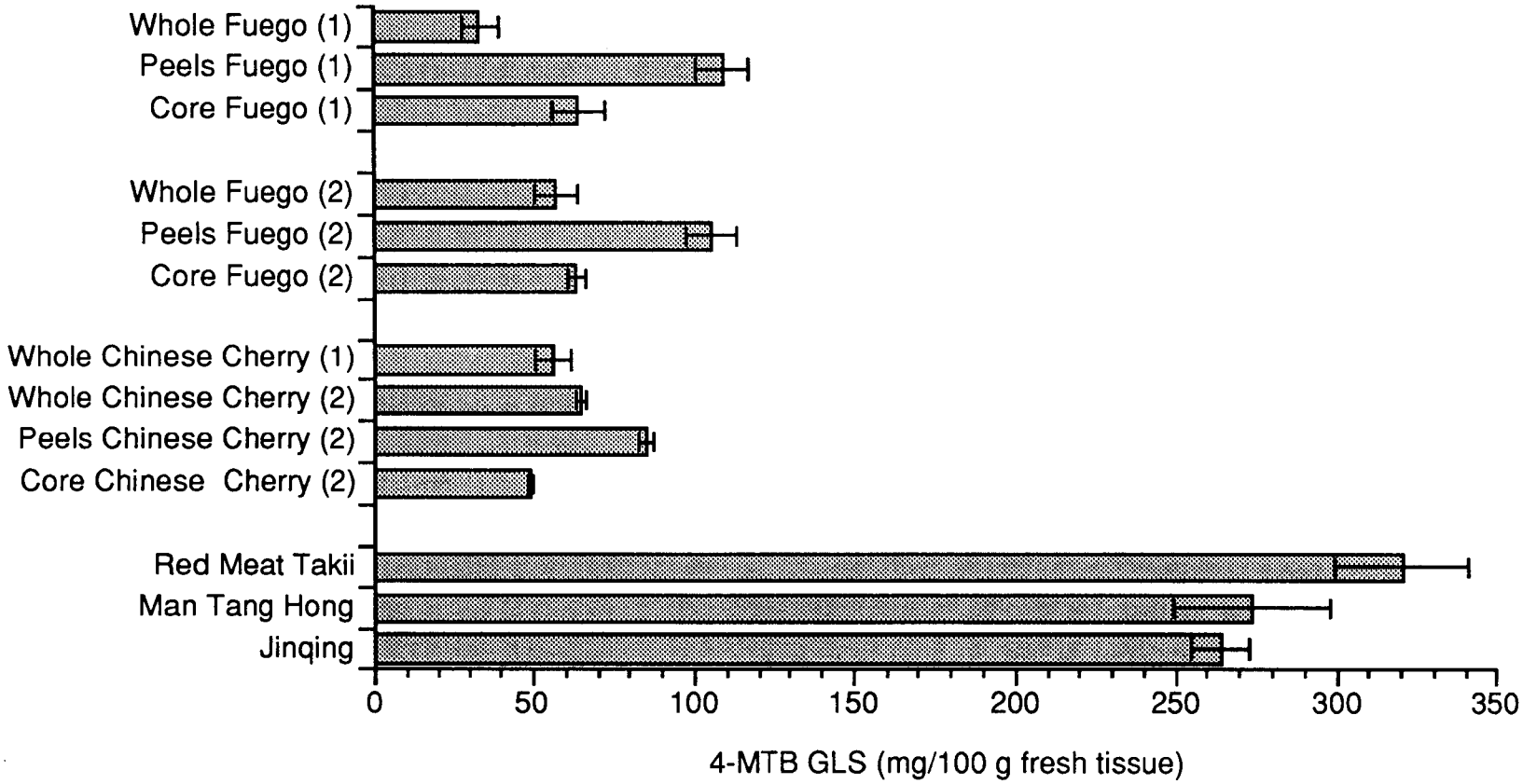


Figure 25: 4-methylthio-3-butenyl glucosinolate (4-MTB-GLS) content in fresh disrupted radish tissues (whole, core and peels) of different radish cultivars and different harvest times (1) five weeks and (2) seven weeks after planting.

Carlson and coworkers (1985) reported small amounts of 4-methyl sulfinylbutyl, 4-methyl sulfinyl-3-butenyl and 3-indolylmethyl glucosinolates in radish roots. In another study involving liquid chromatography of desulfoglucosinolates, Sang and coworkers (1984) detected the previously enumerated glucosinolates and 4-hydroxy-3-indolylmethyl glucosinolate as well. These compounds were not identified with certainty in our radish samples.

Glucosinolates in peeled radish roots, peelings and intact roots of Japanese cultivars were also reported (Carlson et al, 1985) and revealed significantly greater concentration in the peels than in the peeled radish or intact root. We saw a similar pattern in radish tissue from Fuego and Red Chinese cultivars.

4. Total glucosinolate content

Total glucosinolate content was determined in radish tissue from different cultivars and two harvest times for the salad-type radishes (Figure 26). Whole salad-type radishes ranged from 30 to 45 mg total glucosinolate/100g fresh weight whereas red flesh cultivars (Red Meat Takii, Man Tang hong) and Jingqing had much higher glucosinolate content ranging from 230-370 mg/100g fresh weight. These figures were close to the ones previously found for 4-methylthio-3-butenyl glucosinolate determined as desulfoglucosinolate. Using a similar glucose released method reported by Van Etten and coworkers (1977), Carlson and coworkers (1985) found that red European-American radish cultivars contained 100 to 199 μmol (or 42-84 mg) of total glucosinolates per 100g of root, while the Japanese cultivars had 200 to 399 μmol (or 85-128 mg)/100g fresh weight.

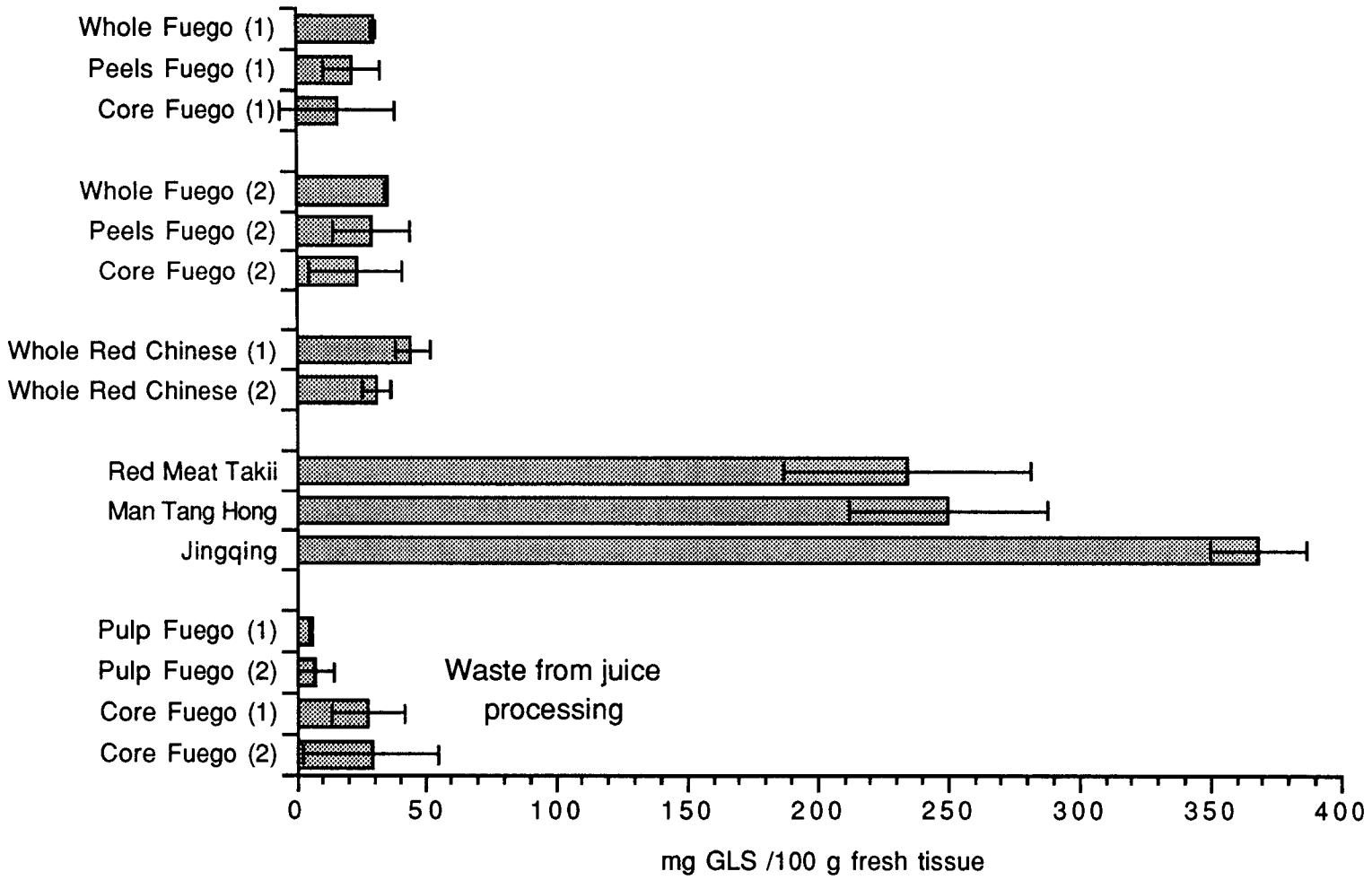


Figure 26: Total glucosinolate content as determined with the glucose released method on radish tissues (whole, core and peels) of different radish cultivars and different harvest times (1) five weeks and (2) seven weeks after planting.

For the radish juices, it was not possible to obtain consistent results. There was considerable fluctuation from one reading to another and this could be attributed to interfering materials.

5. Mass spectroscopy analyses

5.1. Fast atom bombardment mass spectrometry

Desulfated forms of glucosinolates from whole radish cultivar Fuego and desulfosinigrin were injected directly on the fast atom bombardment (FAB) mass spectrometer. Burke and coworkers (1988) identified several desulfoglucosinolates by FAB mass spectrometry (MS). Desulfoglucosinolate molecules were referred to as "M". They found that desulfoglucosinolate molecular ions $[M+H]^+$ were always formed and some fragment ions were also obtained from the side chain "R" of the glucosinolate in a consistent pattern according to the type of the side chain (aliphatic or aromatic). The aglycone fragment ion $[RCSNH_2OH]^+$, designated "***a***" by Burke and coworkers (1988) resulted from the cleavage of the glucose-sulfur bond with hydrogen rearrangement and loss of the resulting glucopyranose molecule ($C_6H_{10}O_5$), a process (Figure 27) often observed in FAB mass spectra of glycosides and carbohydrates (Reinhold et al., 1983).

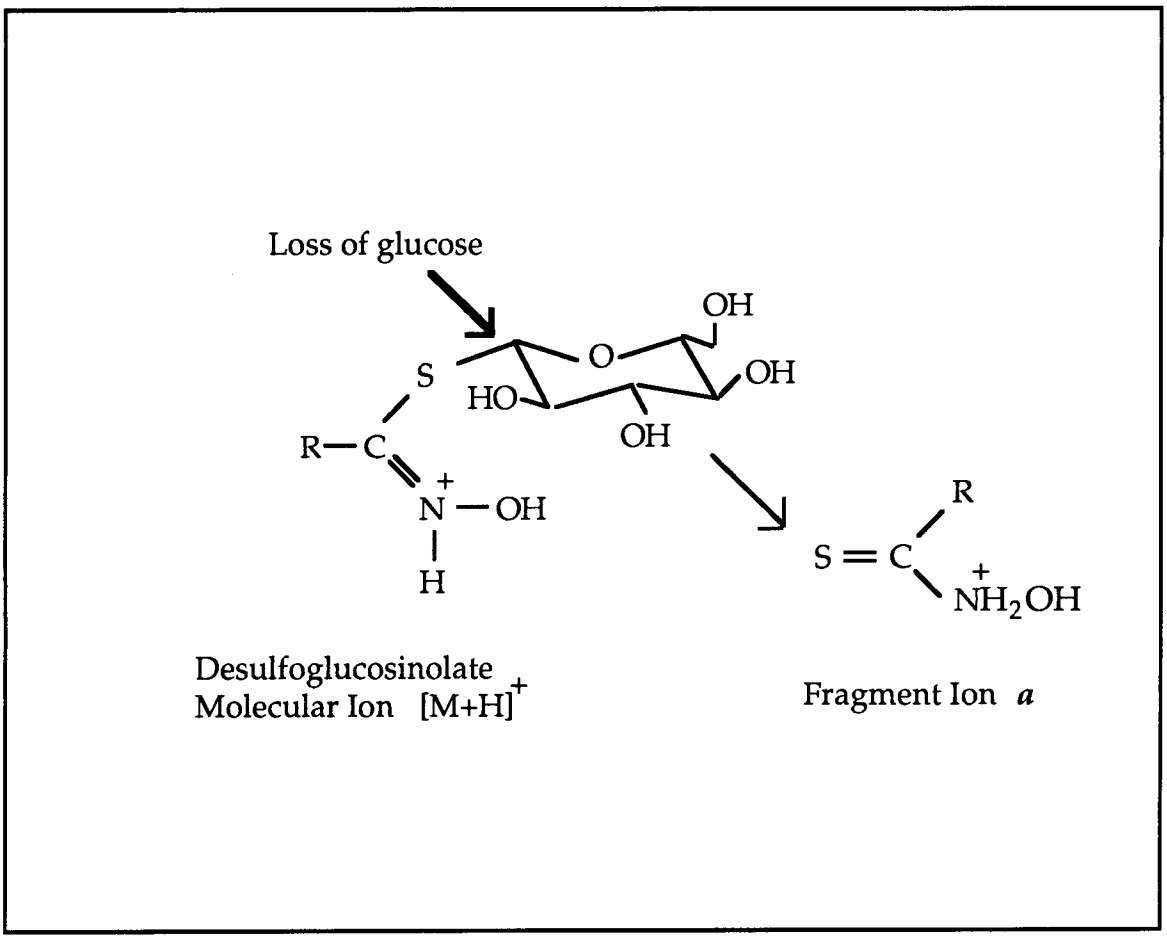


Figure 27: Fragmentation pathway for desulfoglucosinolates occurring by FAB mass spectrometry. Modified from Burke et al. (1988).

FAB MS of the standard desulfosinigrin (Figure 28) showed the molecular ion $[M+H]^+$ at *m/z* 280 as a major peak. An ion at *m/z* 118 was also detected, which corresponded to the aglycone moiety or "***a***" fragment $[RCSNH_2OH]^+$. The $[M+Na]^+$ ion at *m/z* 302 confirmed also the identification of the R group of sinigrin. Glycerol and glycerol-sodium ions were also detected at *m/z* 93, 115, 185 and 207.

Radish cultivars Fuego (Figure 29) and Red Meat Takii both gave

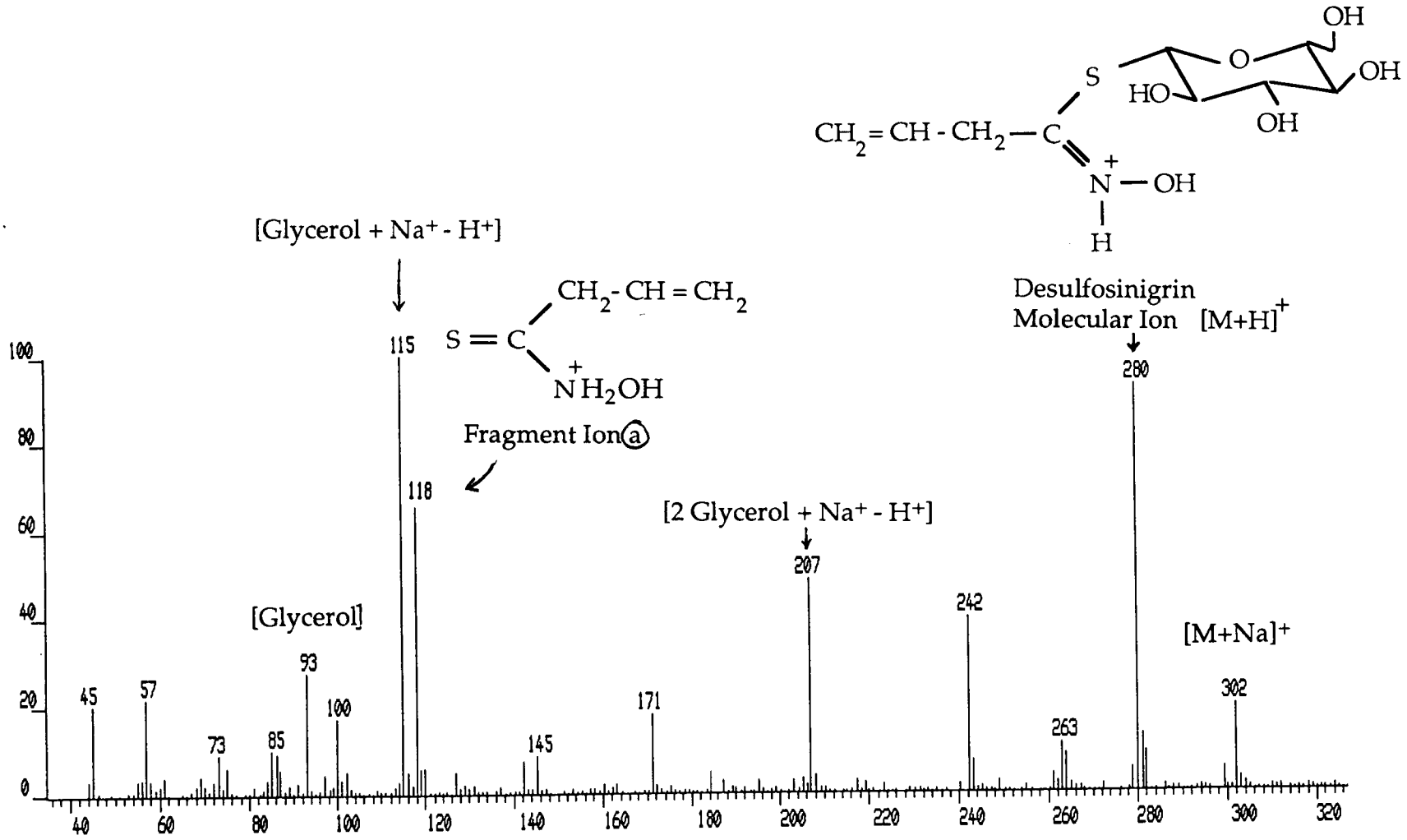


Figure 28: Positive-ion FAB mass spectrum of desulfosinigrin. Glycerol used as the matrix.

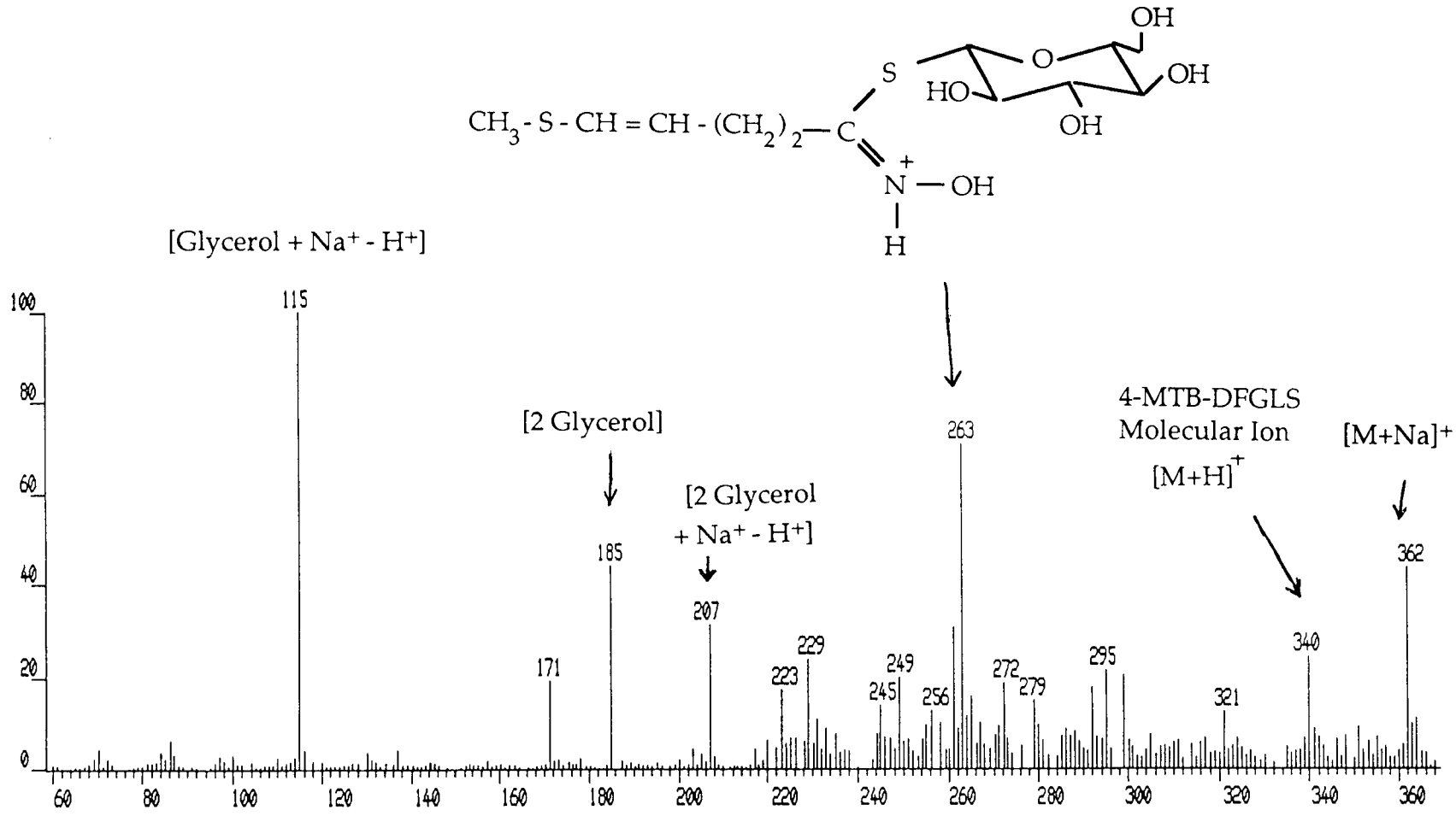


Figure 29: Positive-ion FAB mass spectrum of desulfated glucosinolate from radish cultivar Fuego. Glycerol used as the matrix.

molecular ion $[M + H]^+$ at *m/z* 340, corresponding to 4-methylthio-3-butenyl desulfoglucosinolate. $[M+Na]^+$ ion at *m/z* 362 was found for the radish Fuego extract. The sodium-desulfoglucosinolate ion was not detected in Red Meat Takii extract but this could be due to a lower sodium concentration in the sample. Fragment ion ***a*** expected at *m/z* 178 was not detected in the radish extracts. Peaks at *m/z* 171 and 263, present in both sinigrin and radishsamples, as well as peak at *m/z* 242 obtained in radish, did not match potential compounds found in the samples.

FAB mass spectra of radish samples gave evidence that 4-methylthio-3-butenyl glucosinolate is present in radish. The disadvantage of this technique is mainly that the sample needs to be dissolved in glycerol. Therefore, the compounds of interest have to be in high concentration in order to obtain good signals.

Burke and coworkers (1988) used desulfoglucosinolate concentration of 15 µg/µl whereas we used lower desulfoglucosinolate concentration. Sinigrin sample after treatment with sulfatase was assayed on HPLC and determined to contain 5 mg desulfosinigrin/ml water while radish samples were found to have 0.5 to 1 mg 4-methylthio-3-butenyl desulfoglucosinolate/ml of extract. Low resolution electrospray MS was therefore attempted on desulfoglucosinolates of the same samples to confirm the results obtained with FAB MS.

5.2. Electrospray mass spectrometry

Electrospray MS of desulfosinigrin (Figure 30) showed a major peak at *m/z* 302 which corresponded to $[M+Na]^+$, M being the desulfoglucosinolate molecule. Other peaks confirmed the presence of desulfosinigrin: $[M+H]^+$ at *m/z* 280, fragment ion ***a*** $[RCSNH_2OH]^+$ at *m/z* 117.6 and $[RCNOH_2]^+$ at *m/z* 84.8. The major peak at *m/z* 302 was further fragmented by a consecutive second MS run (MS/MS experiment).

The daughter profile of peak 302 showed a major peaks at *m/z* 218.8 corresponding to the fragment ion thioglucose-sodium $[S\text{-}C_6H_{11}O_5+Na+H]^+$, and at *m/z* 184.8 corresponding to glucose sodium $[C_6H_{10}O_5+Na]^+$ (Figure 31). Two other peaks resulting from the fragmentation of the parent molecular ion were also detected at *m/z* 140.0, matching $[a+Na]^+$, and at *m/z* 85.0, matching $[RCNOH_2]^+$. Increased sensitivity of desulfosinigrin was observed with positive ion electrospray MS compared to FAB MS.

The mass spectrum of whole radish cultivar Fuego (Figure 32) gave a major peak at *m/z* 361.6, corresponding to $[M+Na]^+$, M being 4-methylthio-3-butenyl desulfoglucosinolate (4-MTB-DFGLS). The molecular ion $[M+H]^+$, obtained in the case of sinigrin and expected at *m/z* 340 for 4-MTB-DFGLS, was not detected. However, we obtained several fragmentation ions of M as $[S\text{-}C_6H_{11}O_5+Na+H]^+$ at *m/z* 218.8, $[R]^+$ at *m/z* 101, and fragment ion ***a*** $[RCSNH_2OH]^+$ at *m/z* 178.0.

The daughter MS profile of the parent ion $[M+Na]^+$ (Figure 33) gave the same peaks as obtained from the parent ion $[M+Na]^+$ of sinigrin, matching glucose-sodium and sulfur-glucose-sodium at *m/z* 218.8 and 184.8. We also obtained $[a+Na]^+$ at *m/z* 199.8, confirming the R group of 4-MTB-DFGLS.

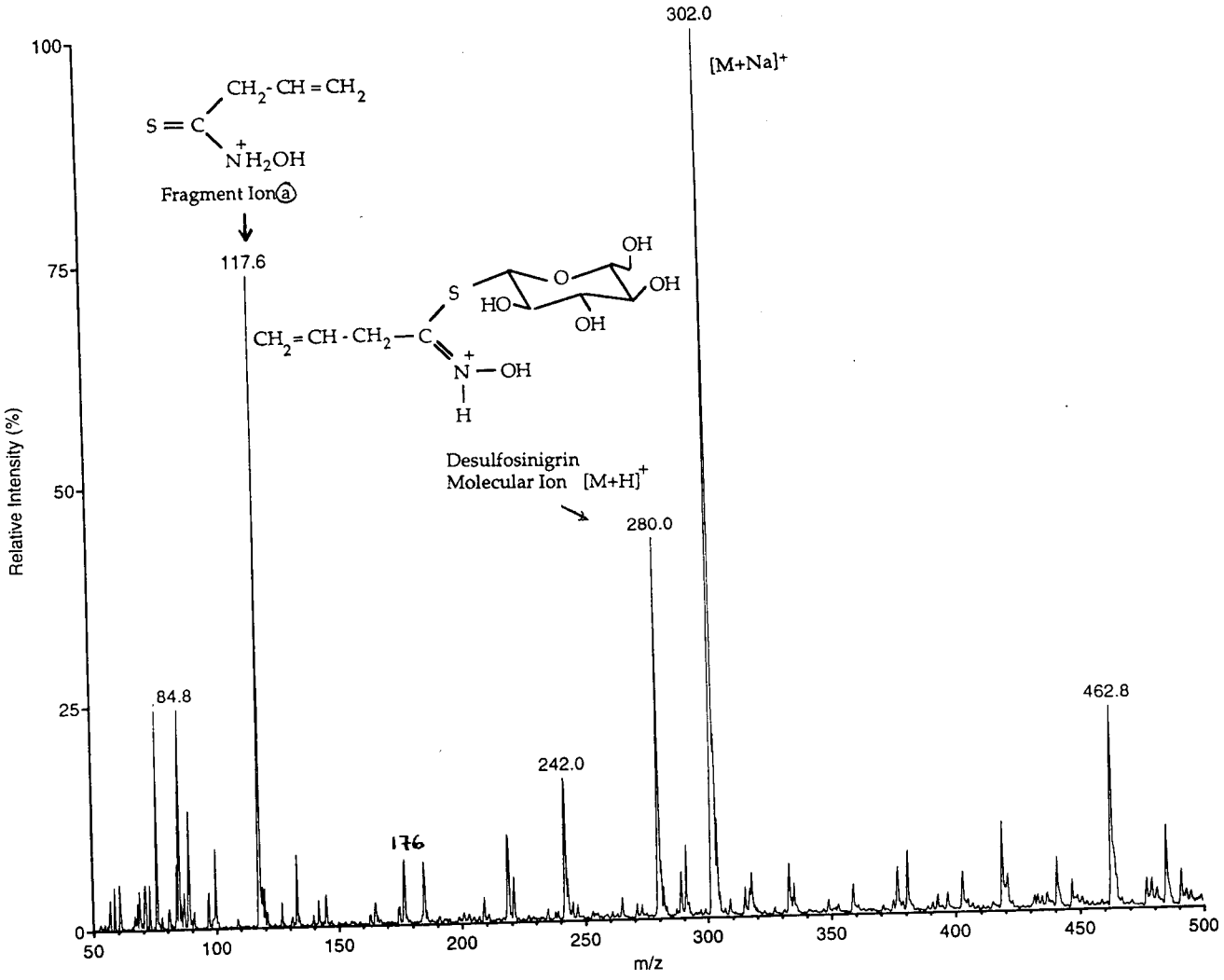


Figure 30: Positive-ion electrospray mass spectrum of desulfosinigrin.

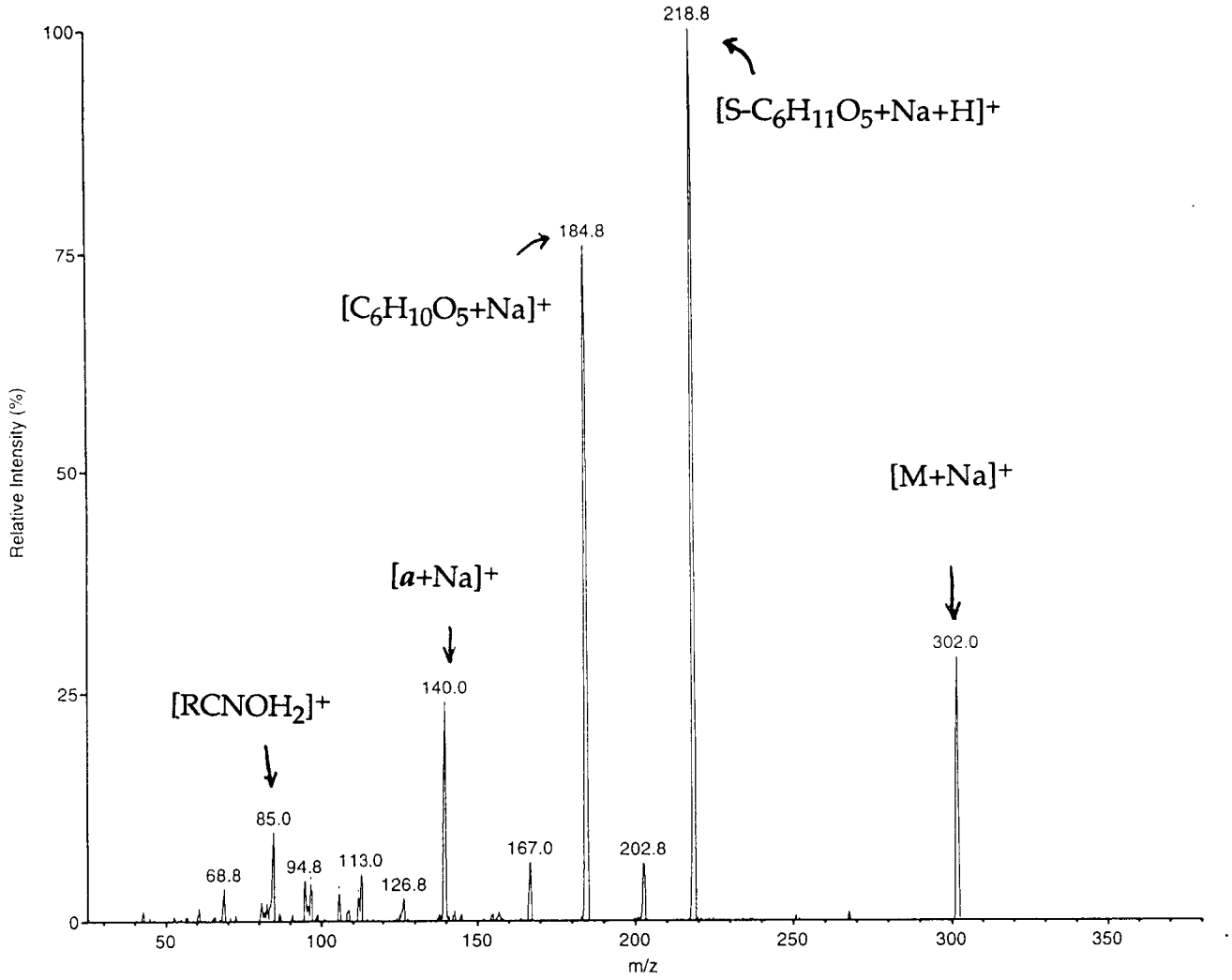


Figure 31: Positive-ion electrospray mass spectrum of parent peak *m/z* 302 from desulfosinigrin.

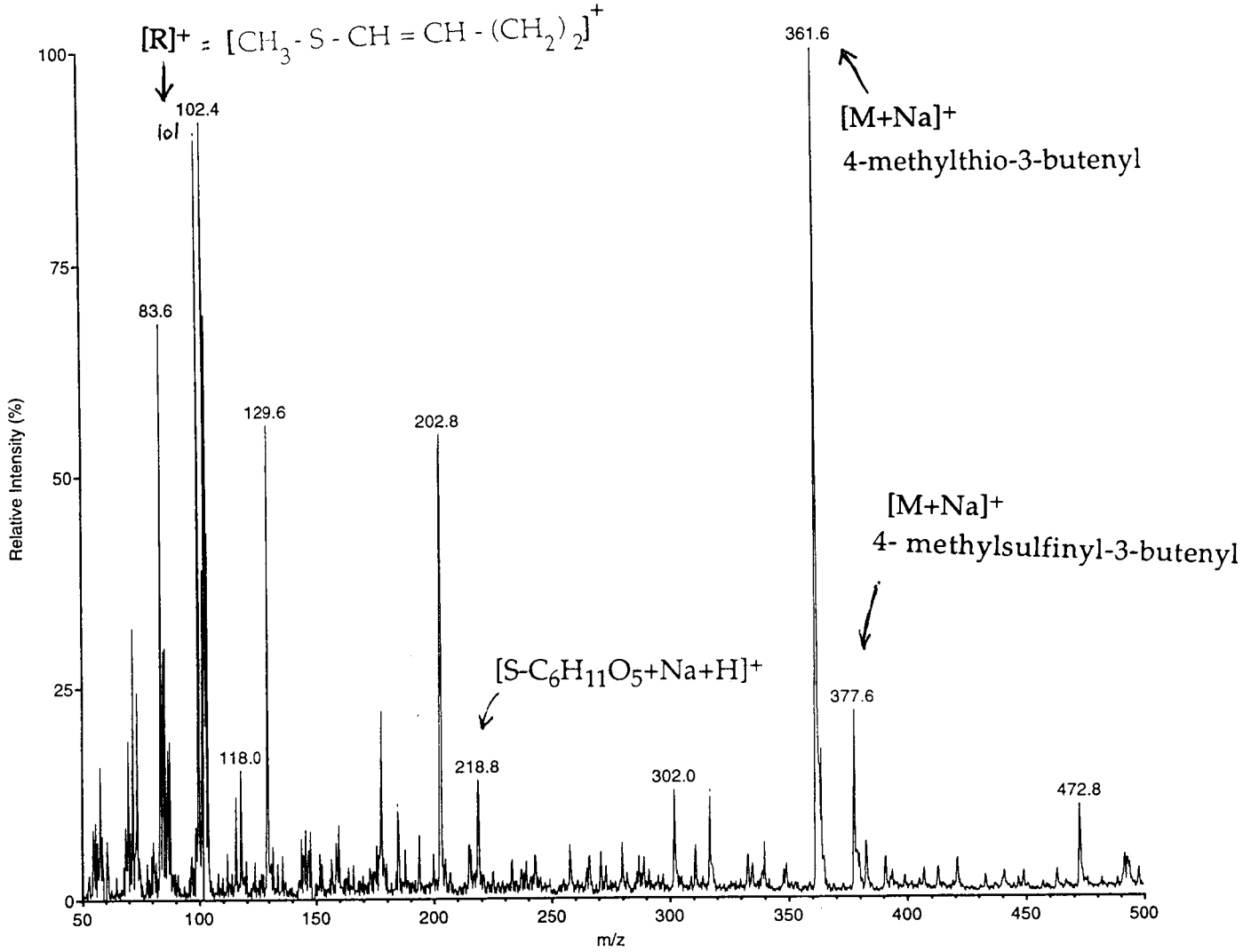


Figure 32: Positive-ion electrospray mass spectrum of desulfated whole radish cultivar Fuego.

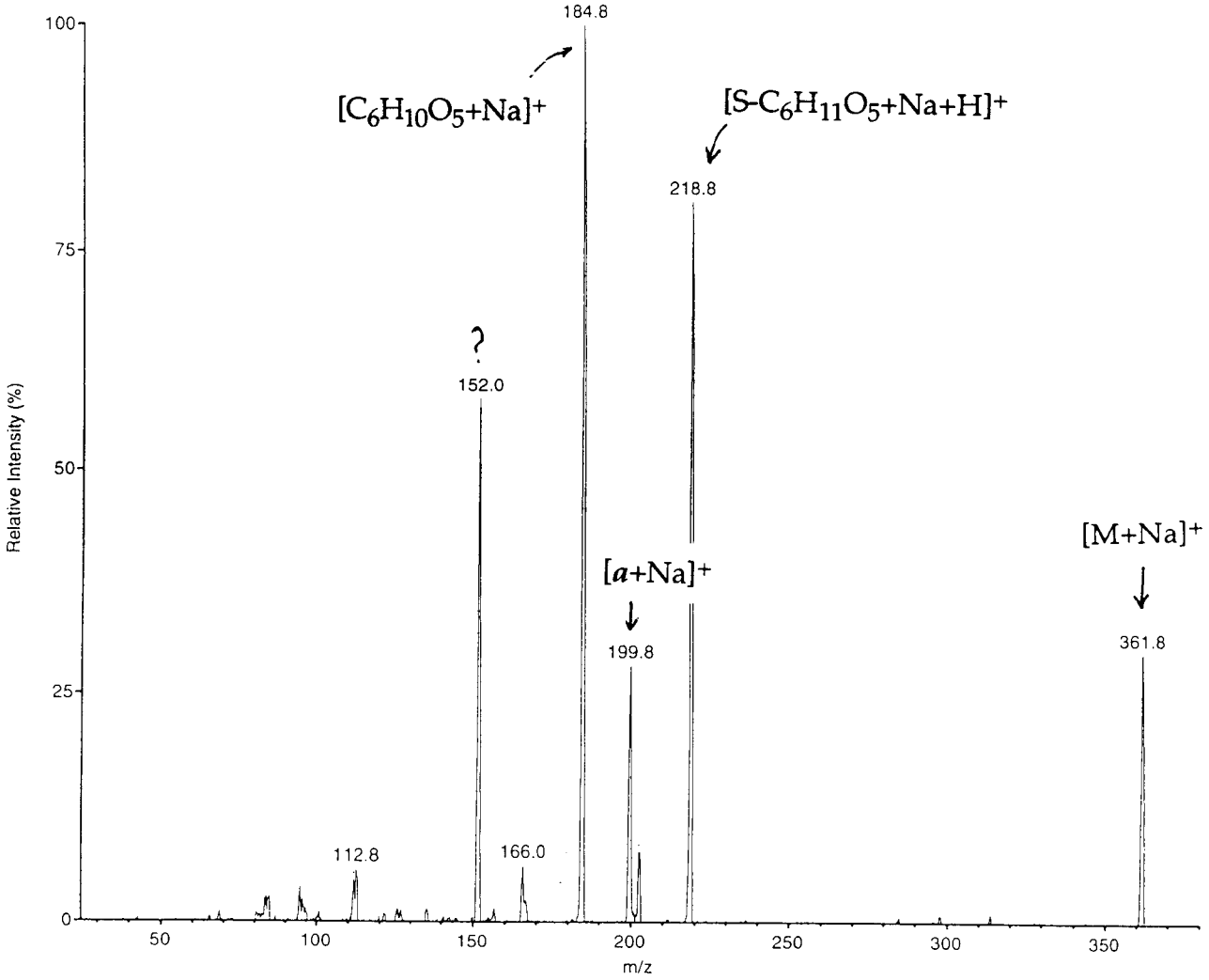


Figure 33: Positive-ion electrospray mass spectrum of parent peak *m/z* 361 from desulfated whole radish cv. Fuego.

These results confirm the presence of desulfoglucosinolates in the radish sample and more specifically 4-methylthio-3-butenyl glucosinolate.

There is some evidence that another glucosinolate, reported by Carlson and coworkers (1985) in small amounts in radish, was also present in the radish sample. Peaks at *m/z* 377.6 and 117 matched $[M+Na]^+$ and $[R]^+$ of 4-methylsulfinyl-3-butenyl desulfoglucosinolate. The fragment ions $[M+Na]^+$ and $[R]^+$ were detected as the major peaks from 4-MTB-DFGLS in mass spectrometry of whole radish. The secondary radish glucosinolate with 4-methylsulfinyl-3-butenyl for R group may therefore be detected by mass spectrometry in whole radish Fuego. Further isolation and collection of reverse phase HPLC fractions could be helpful in identification of this compound.

6. Comparison of analytical methods for glucosinolates

Glucosinolates were examined with three procedures:

(1) as individual intact glucosinolates by ion pair chromatography (IPC),

(2) as individual desulfoglucosinolates on HPLC after enzymatic treatment with sulfatase and,

(3) as total glucosinolates by spectrophotometric measurements of glucose released after myrosinase treatment.

The analytical procedure involving desulfoglucosinolates appeared to be the most effective from the standpoint of sample preparation and reproducibility. Although IPC of intact glucosinolates was the least demanding technique in sample preparation, it did not allow quantification of glucosinolates due to coelution with sinigrin, glucosinolate used as an internal standard. Also, the presence of the counter ion interfered with

further identification by mass spectroscopy. Use of a different glucosinolate standard such as glucotropaeolin or glucobarbarin could be of interest. However, the availability of these compounds is limited and can be an important factor to consider in the choice of a procedure.

In all cases, regarding both intact and desulfoglucosinolate analytical techniques, the internal standard must be a glucosinolate since insufficient myrosinase inactivation is a critical step in glucosinolate analysis and because purification is based on glucosinolate affinity for resin material (C-18 or anion exchange).

Desulfoglucosinolates analysis allowed quantification by HPLC and identification by mass spectroscopy of the main isothiocyanate precursor, 4-methylthio-3-butenyl glucosinolate, within reasonable analysis time on HPLC (less than 20 min). Total glucosinolate determination was as time consuming and demanding in sample preparation as the HPLC methods and relied also on enzyme activity. Variations within measurements were larger with this method than with the desulfoglucosinolate technique.

High performance liquid chromatography (HPLC) also allowed detection of the pungent principle of disrupted fresh radish tissue, 4-methylthio-3-butenyl isothiocyanate (MTBI). This technique was used to evaluate isothiocyanate formation in fresh radish. It gave an indication of the activity of endogenous myrosinase and of the glucosinolate content, precursor for isothiocyanates.

Sensory evaluation was also used to evaluate aroma intensity of radish juices.

7. Sensory evaluation of radish juice concentrates

7.1. Overall aroma intensity

The overall aroma intensity of four color extracts diluted with increasing anthocyanin concentrations were evaluated by 24 panelists over two replications. Juice concentrate obtained from winter-type radish cultivar Red Meat Takii was judged as the highest in aroma intensity over all concentrations (Figure 34). Its overall aroma intensity was rated on average from 8.9 (moderate to large) to 11.2 (large). Radish cultivar Fuego followed with aroma intensity ranging from 7.6 to 10.2 on average.

The commercial samples, red cabbage and Japanese radish extracts, were not significantly different from each other and had lower overall aroma intensities than Fuego and Red Meat Takii radish color extracts. Average ratings for commercial samples ranged from 5.4 (slight to moderate) to 7.4 (moderate). Red cabbage extract is approved by the Food and Drug Administration and meets the specification of the Code of Federal Regulations (Title 21, Parts 73.260). Radish extract has Japanese regulating approval, but is not permitted in the US.

All colorants followed a similar increasing trend in overall aroma intensity with increasing anthocyanin concentrations. A first order relationship was found for the radish color extracts Fuego and Red Meat Takii (Figure 34). For the commercial samples, adaptation occurred at the two highest concentrations with ratings close to large. The overall aroma intensity was similar for red cabbage and Japanese radish color extracts containing 600 and 1200 mg anthocyanin/L. The ratings were no longer dependent on the color strength.

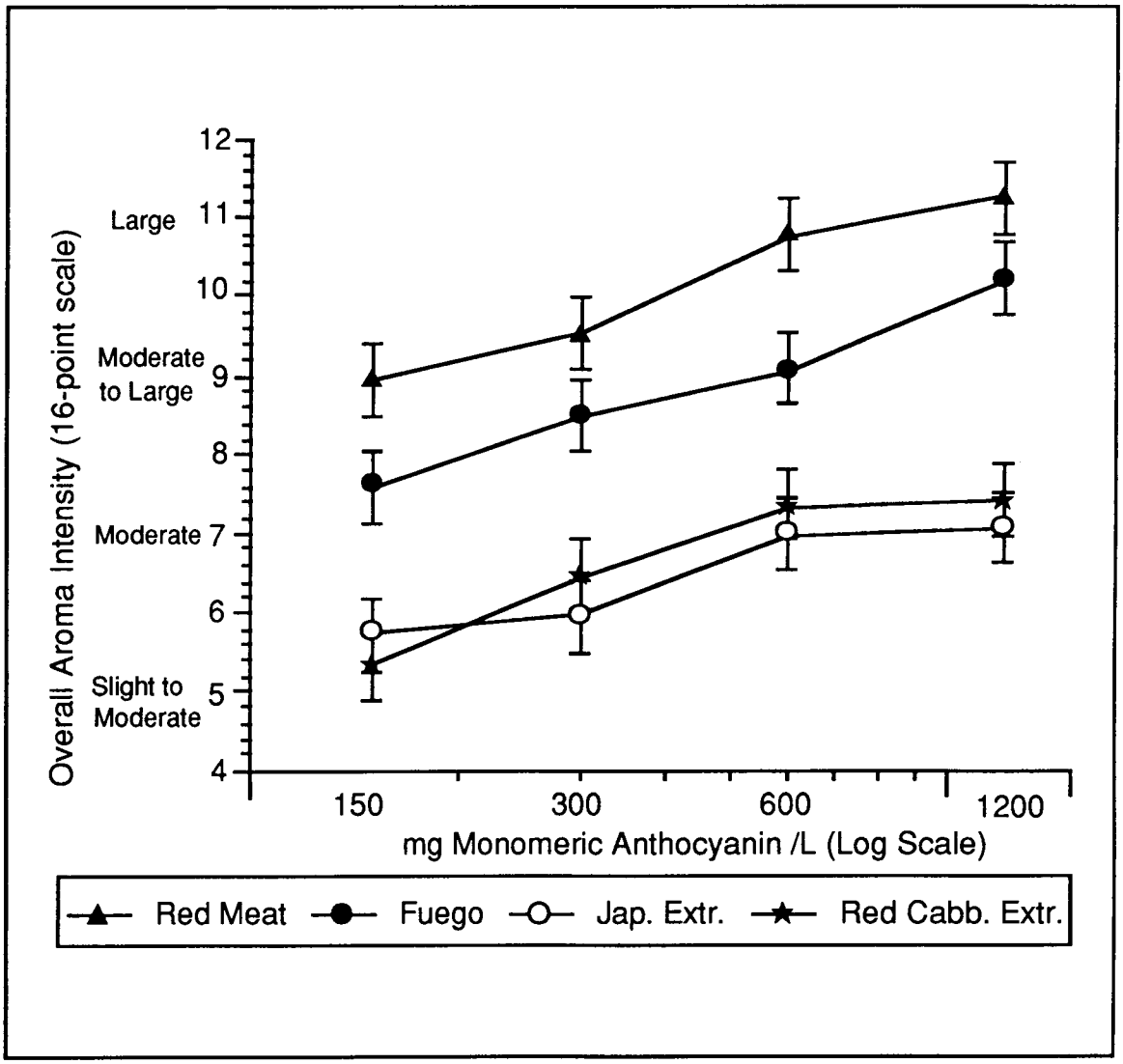


Figure 34: Overall aroma intensity of color extracts with increasing anthocyanin content.

Samples: Radish juice concentrates cultivars Red Meat Takii and Fuego, radish color extract (Stange, Japan), red cabbage colorant (Warner Jenkinson). 16 point intensity scale from 0 = none to 15 = extreme. Each data point represents 48 judgments, standard error is indicated with error bars.

Panelist judgments were submitted to analysis of variance using overall aroma intensity as dependent variable and four independent variables which were: Panelists, Colorant, Concentration and Replication. Interaction effects between the judges and the samples were also tested (Appendix 3, Table A).

The sample variables 'Colorant' and 'Concentration' were found significant ($p<0.0001$), indicating that there was a relationship between the overall aroma intensity and both the colorant nature and anthocyanin concentration (strength of the color). The overall aroma intensity of the color extracts was dependent on their anthocyanin concentration and the colorant type.

The Tukey test separated the colorants in three distinct groups according to colorant types and three groups according to anthocyanin concentrations (Table 4). Regarding the colorant nature, the overall aroma intensity of both commercial samples (red cabbage colorant and radish extract from Japan) were found non significantly different from one another at the 5% confidence level across all levels of anthocyanin concentrations. The radish juice concentrates were each found in a separate group. Looking only at concentrations for all colorant types, the two highest anthocyanin concentrations (600 and 1200 mg/L) were grouped together while the two lowest were significantly different from one another at the 5% confidence level. This suggest that adaptation tends to occur at higher anthocyanin content (>600 mg/L): the panelists did not see any difference between the two highest color strengths.

REPLICATE	T Grouping	Mean	Observations
2	a	8.07	384
1	a	7.94	384

DF = 383, MSE = 3.77, Critical value of T = 1.97, LSD = 0.2755

COLORANT	T Grouping	Mean	Observations
Radish Red Meat Takii	a	10.12	192
Radish Fuego	b	8.84	192
Red Cabbage Extract	c	6.64	192
Radish Color Extr. (Japan)	c	6.41	192

DF = 383, MSE = 3.77, Critical value of T = 1.97, LSD = 0.3896

CONCENTRATION (Anthocyanin mg/L)	T Grouping	Mean	Observations
1200	a	8.99	192
600	a	8.53	192
300	b	7.61	192
150	c	6.88	192

DF = 383, MSE = 3.77, Critical value of T = 1.97, LSD = 0.3896

Table 4: Multiple Comparison Tukey test for overall aroma intensity on the replicates and the samples. Alpha= 0.05, means with the same letter are not significantly different.

The variable 'Panelist' was found significant at the 5% level (p=0.0001) which illustrates the fact that the panelists did not use the same part of the scale to rate the samples. This is commonly encountered in sensory analyses, involving minimum training session. In our study, the overall aroma intensity means ranged from 5.9 to 10.5 within the panelists.

However, good replication was obtained. Within the main effects, only the variable 'Replication' came up non-significant at the 5% level. There is therefore no significant differences between the replicates, which demonstrates the overall consistency of the panelists. A multiple comparison Tukey test, performed on the panelists (Appendix 3, Table B), showed that panelists with an overall mean between 8 and 9 represented more than half of the panel. They also formed a fairly uniform group. Consequently, the panel was centered around a majority of people who rated moderately and in a similar way. Panelists who were familiar with the aroma of the samples had a tendency to use the lower part of the scale.

First-order interaction effects were found non significant for Panelist x Concentration and Colorant x Concentration as well as the second-order interaction Panelist x Concentration x Colorant. Consequently, no difference was found in overall aroma intensity for each level of anthocyanin concentration across the panelists, and for all types of colorant. There was an interaction effect of the samples with panelists, meaning that the same samples were rated differently by the panelists. However, the F-values for these interaction effects are low compared to the F-value obtained for the main effect of the samples, colorant types and concentrations. The effect of the samples on the rating can be considered predominant and interaction effects of the samples on the panelists were minor.

The samples were grouped by a multiple comparison Tukey test (Table 5). Commercial samples were grouped while the radish extracts we had prepared were clustered together on another part of the scale. An interesting finding was that the overall aroma intensity of radish juice concentrate from cultivar Fuego, diluted to 150 mg anthocyanin /L was close to red cabbage extract at 600 and 1200 mg/L.

SAMPLES	T Grouping	Mean	Observations
Takii 1200	a	11.25	48
Takii 600	a b	10.77	48
Fuego 1200	b c	10.21	48
Takii 300	c d	9.52	48
Fuego 600	d e	9.06	48
Takii 150	d e	8.94	48
Fuego 300	e	8.52	48
Fuego 150	f	7.58	48
Red Cabb. 1200	f	7.42	48
Red Cabb. 600	f	7.31	48
Jap. Radish 1200	f g	7.08	48
Jap. Radish 600	f g	6.98	48
Red Cabb. 300	g h	6.46	48
Jap. Radish 300	h i	5.96	48
Jap. Radish 150	i	5.63	48
Red Cabb. 150	i	5.35	48

DF = 383, MSE = 3.770, Critical value of T =1.97, LSD = 0.7793

Table 5: Multiple Comparison Tukey test for overall aroma intensity on the samples. Alpha= 0.05, means with the same letter are not significantly different.

7.2. Sample descriptors

During the training session panelists were asked to write a few words that could describe the aroma of the samples and the descriptors were discussed. Three samples were evaluated and described with the following words in order of importance:

(a) Radish cultivar Fuego: cooked vegetable, herbal, sauerkraut, cauliflower, broccoli, horseradish, fermented, kimchi, cheesy;

(b) Radish cultivar Red Meat Takii: cooked cabbage, sulfur, burnt matches, smoky, oak, sauerkraut, kimchi;

(c) Red Cabbage Color Extract: honey, tea leaves, spicy, fruity, alcoholic, sauerkraut, spicy, medicinal, earthy, musty, anise, cabbage, chrysanthemum.

During the test, panelists were also asked to give aroma descriptors (Table 6). We noticed that the radish extracts were readily associated with cruciferous vegetables and sulfur compounds. A spoiled vegetable note was attributed to radish Red Meat Takii juice concentrate and not for radish Fuego extract. This could be attributed to the color extraction process. In the case of cultivar Red Meat Takii, whole radishes were crushed since the color was located in the flesh whereas only the pigmented peels were extracted for Fuego. Commercial samples had more fruity/herbal and sweet notes, with some alcoholic aroma. Purification processes and formulation of these extracts can probably explain these notes and the fact that the cruciferous origin of these extract was hardly recognized by the panelists. Some sulfur aromas were detected in the commercial samples but not as often as in the radish juices.

Juice Concentrate or Extract	Monomeric anthocyanin content in mg / L			
	150	300	600	1200
Radish cultivar Fuego	cooked cabbage /vegetable, sauerkraut, kimchi, sulfur, beefy, horseradish, shitake mushroom, metallic	sauerkraut, sulfur, spicy, vinegar, pungent, cooked cabbage, burnt match	sauerkraut, sulfur, cooked cabbage, cooked meat, burnt match, fermented	sulfur, spicy, vinegar, pungent, acid, onion, burnt, sauerkraut, fermented
Radish cultivar Red Meat Takii	cooked cabbage /vegetable, sauerkraut, sulfur	cooked cabbage, sauerkraut, vinegar, kimchi,	sauerkraut, putrid, rotten, burnt match, cooked/spoiled vegetable	sauerkraut, kimchi, sulfur, burnt match, acidic, cooked/spoiled vegetable
Radish Japanese Color Extract	medicinal/cough syrup, alcohol, sweet, slightly sulfur	honey, medicine, alcohol, slightly spicy, tea, vanilla, iodine, anise, cardboard	alcohol, sulfur, acetic, pungent, sweet, old apple, honey, anise, cardboard	alcohol, fermented, vegetable, fruit, earthy, spicy, sulfur, honey, cardboard
Red Cabbage Colorant	honey, tea leaves, medicine, herbal, sweet, floral, musty, pepper	honey, tea leaves, sweet, floral, herbal, cooked vegetable	honey, tea, medicine, floral, burnt, spicy	honey, tea, floral, spicy, medicine, floral, burnt, sulfur

Table 6: Descriptors of colored extracts with different anthocyanin concentrations as suggested by the panelists

7.3. Overall aroma intensity and appearance of the samples

Anthocyanin concentrations used in this study were chosen according to levels of radish anthocyanin extract used by Giusti and Wrolstad (1996) in a previous study to dye maraschino cherries. A pigment content of the syrup between 150-300 mg anthocyanin/L gave color characteristics close to the commercial samples dyed with FD&C Red No. 40. These treatments were found to be a good compromise between desirable color and acceptable pigment stability.

We found that the overall aroma intensity of 300 mg anthocyanin /L was rated in average 7.6 for cultivar Fuego, 9.5 for Red Meat Takii, 6.0 for Japanese extract and 6.5 for red cabbage extract, that is between moderate and moderate to large.

However, the color (hue) and intensity of each were slightly different (Figure 35) at pH 3.0. The Japanese radish extract presented a darker red color than the radish juice concentrated at the same anthocyanin content. A color strength closer to the radish samples Fuego was obtained with half the anthocyanin content of Japanese radish extract that is 150 mg/L. At this concentration, Japanese radish extract was rated 5.6. Red Cabbage extract gave also a darker color but in a purple hue. A comparable color strength was obtained with an anthocyanin content of 150 mg/L which was rated 5.4 (slight to moderate). Fuego and Red Meat Takii were close in color characteristics. However, Red Meat Takii was more hazy and much harder to filter. At 300 mg/L, panelists gave a mean overall aroma intensity of 7.5 for Fuego and 9.5 for Red Meat Takii. Considering a similar usage level in term of color intensity, the difference would be about 3 points higher for the radish juices compared to the commercial color extracts.

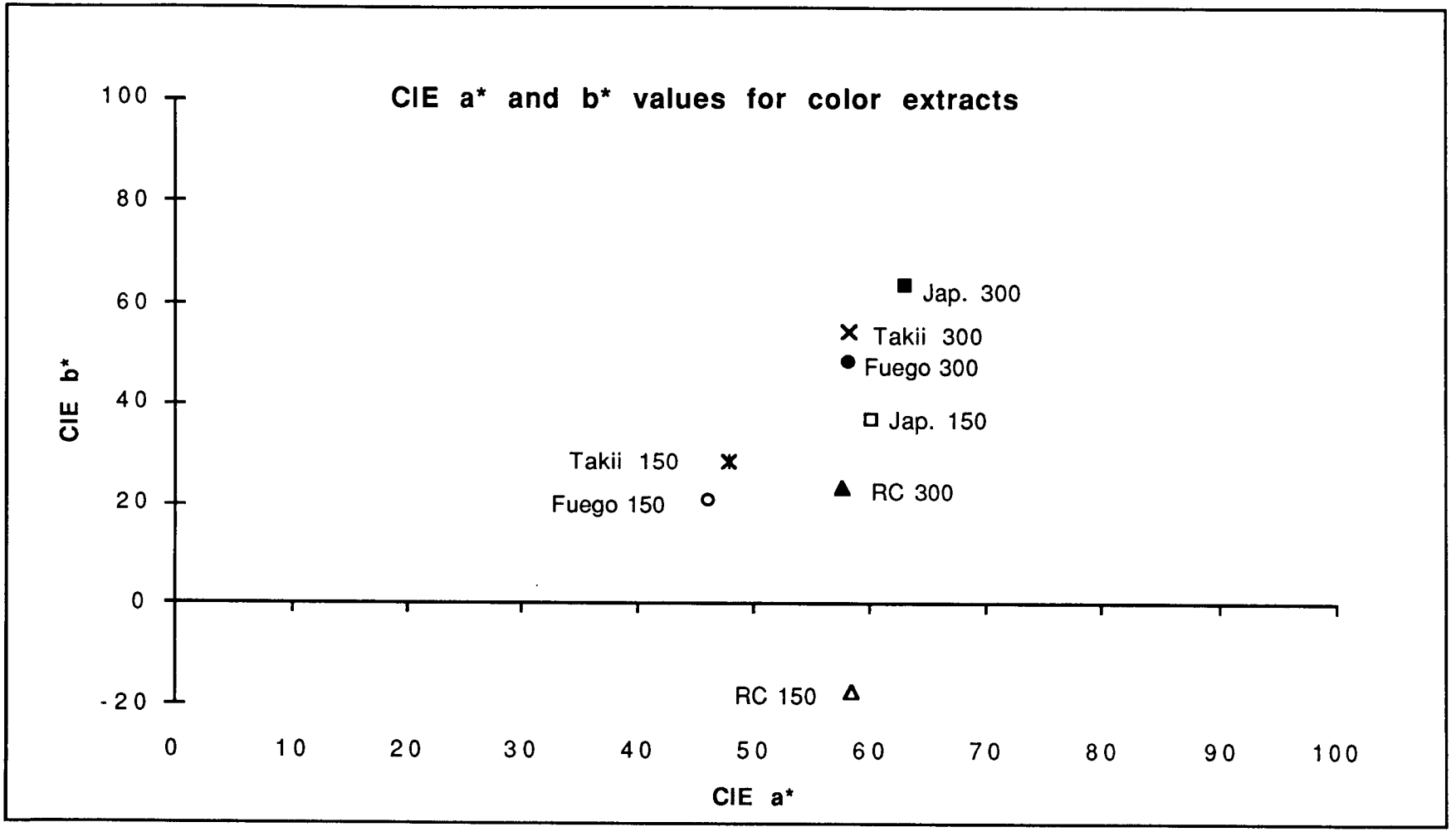


Figure 35: CIE a* and b* values for radish juice concentrates (Fuego and Red Meat Takii), Japanese radish extracts and red cabbage colorant at 150 and 300 mg anthocyanin /L in water pH 3.0. Length of the line from the origin to each point is *chroma* or *color intensity*. Angle of this line formed with the X axis is *hue angle*.

Although sensory analyses showed that overall aroma intensity of radish juice concentrates ranged from moderate to large with increasing color strength, the following considerations must be accounted before further conclusions:

(a) The level of usage in term of color appearance is close to 150-300 mg anthocyanin/L or lower depending on the pigment characteristics and the application. For these concentrations, we observed a decreasing trend in overall aroma with decreasing anthocyanin content.

(b) The juice concentrates were diluted in water in this study. The use of red color is usually associated with products of a much more complex flavor system. Natural or artificial flavorings added to the product may play a role in masking the aroma of the colorant. In maraschino cherries for instance, benzaldehyde is added as a flavoring agent.

(c) The radish juice concentrates were prepared by mechanical injury of fresh radishes and water extraction, blanched, concentrated and filtered, but no further purification step was included in the process. These extracts could be considered as the most potent in aroma character. Their sensory characteristics could be improved by susequent filtration and concentration techniques.

V. CONCLUSIONS

Qualitative and quantitative determination of the major flavor precursors (isothiocyanates and glucosinolates) were carried out using different analytical methods, for both fresh tissue and radish color extracts of winter (red flesh) and spring (pigmented peels) radish cultivars. Aroma intensity of concentrated radish juices was compared to that of commercial red cabbage and radish colorants.

The major pungent principle of disrupted radish tissue, 4-methylthio-3-butenyl isothiocyanate (MTBI), was analyzed by HPLC. The analytical method permitted a measurement of MTBI formation in fresh radish and also gave an indication of the activity of the endogenous myrosinase enzyme, responsible for the breakdown of glucosinolate upon cell injury. MTBI precursor, 4-methylthio-3-butenyl glucosinolate (MTBG) was quantified by HPLC analyses as desulfoMTBG and identified using mass spectroscopy techniques. HPLC of intact MTBG was performed, however quantification was not possible due to coelution with the internal standard.

Isothiocyanates and glucosinolates were found in higher amounts in red fleshed radish cultivars (winter types) than in pigmented peel cultivars (spring types). In color extracts prepared from both radish types, isothiocyanates and glucosinolates were not detected by analytical procedures. MTBI was not detected in radish color extracts but sensory analyses showed that overall aroma intensity of these extracts was rated moderate to large and described as sulfury and pungent, a characteristic aroma note of MTBI.

Sensory evaluation also demonstrated that overall aroma intensity was significantly higher in radish color extract made from red flesh cultivars than that of the colorant prepared from peels of spring cultivars. The salad radish cultivars such as Fuego appeared therefore to be advantageous over the winter cultivars from the standpoint of aroma intensity. Fuego contained less flavor precursors and since the skins only were used for colorant preparation, the resulting colored extract was less potent than the one made from red fleshed radishes.

Compared to commercial red cabbage and radish colorant, experimental radish extracts were rated significantly higher in aroma intensity. However commercial colorants were not odorfree when studied at similar anthocyanin content. The aroma of the radish juice concentrates was described as cooked/fermented cruciferous vegetables whereas the commercial extracts had more pleasant notes (honey, tea leaves). Red cabbage and radish belong to the same botanic family and therefore, both contain glucosinolates and isothiocyanates. Red cabbage colorant processors have been confronted to similar challenges to reduce flavor compounds in their final product.

Blanching, filtration and concentration by rotoevaporation of radish juices were the only processing steps used and did not permit to obtain odorfree extracts. Extraction, concentration and subsequent purification techniques as well as their impact on color and pigment properties are now being investigated in an effort to reduce the levels of unwanted flavors. An odorfree extract may not be required depending on the application for the colorant; especially flavor attributes of the natural colorant could be masked by other flavor compounds in the final product. A light fruit drink would

require almost odorless extract whereas other products such as maraschino cherries could mask some of the aroma and taste from the food colorant.

Further analytical work is required to detect isothiocyanates and glucosinolates in radish juices and color extracts. Sensitive techniques such as gas chromatography coupled to mass spectroscopy could be relevant in this investigation and could allow analysis of headspace volatiles of radish extracts. Such a technique may be relevant for the analysis of other flavor compounds such as sulfides. Sensory testing comparing colored extracts at different purification steps is also of interest. Color and flavor evaluation of final products colored with radish extract by both sensory and analytical techniques may be decisive in the development of a commercial colorant.

BIBLIOGRAPHY

Abramski W., Chmielewski M., 1996, Practical synthesis of sinigrin, *Journal of Carbohydrate Chemistry,* Vol. 15 (1), pp. 109-113.

Acree T.E., Teranishi R., 1993, Flavor Science, American Chemical Society, Washington DC, 351 pp.

Adams H., Vaughan J. G., 1989, The use of glucosinolates for cultivar identification in Swede*Brassica napus* L var *napobrassica* (L) Peterm, *Journal of the Science of Food and Agriculture,* Vol. 46, pp. 319-324.

Astwood E. B., 1943, The chemical nature of compounds which inhibit function of the thyroid gland, *Journal of Pharmacology and Experimental Therapeutics,* Vol. 78, pp. 79-89.

Bartling D., Seedorf M., Mithofer A., Weiler E. W., 1992, Cloning and expression of an *Arabidopsis* nitrilase which can convert indole-3-acetonitrile to the plant hormone growth, indole-3-acetic acid, *European Journal of Biochemistry,* Vol. 205, pp. 417-424.

Bennett R. N., Donald A., Dawson G. W., Hick A. J., Wallsgrove R. M., 1993, Aldoxime-forming microsomal enzyme systems involved in the biosynthesis of glucosinolates in oilseed rape *(Brassica napus)* leaves, *Plant Physiology,* Vol. 102, pp. 1307-1312.

Bennett R. N., Hick A. J., Dawson G. W., Wallsgrove R. M., 1995a, Glucosinolates biosynthesis: further characterization of the aldoxime-forming microsomal monooxygenases in oilseed rape leaves, *Plant Physiology,* Vol. 109, pp. 299-305.

Bennett R. N., Ludwig-Muller J., Kiddle G., Hilgenberg W., Wallsgrove R. M., 1995b, Developmental regulation of aldoxime formation in seedlings and mature plants of Chinese cabbage *(Brassica campestris* ssp. *pekinensis)* and oilseed rape *(Brassica napus)* : glucosinolate and IAA biosynthetic enzymes, *Planta,* Vol. 196, pp. 239-244.

Bestwick L., Gronning L. M., James D. C., Bones A., Rossiter J. T., 1993, Purification and characterization of a nitrilase from *Brassica napus, Physiologia Pantarum,* Vol. 89, pp. 811-816.

Betz J. M., Fox W. D., 1995, Chapter 14: High-Performance Liquid Chromatographic determination of glucosinolates in *Brassica* vegetables, In Huang M. T., Osawa T., Ho C. T., Rosen R. T., Food Phytochemicals for Cancer Prevention I: Fruits and vegetables, , ACS Symposium Series #546, American Chemical Society, Washington DC, pp. 181-196.

Bjerg B., Sorensen H., 1987, Quantitative analysis of glucosinolates in oilseed rape based on HPLC of desulphoglucosinolates and HPLC of intact glucosinolates, In Whatelet J-P., Glucosinolates in Rapeseeds: Analytical Aspects, Martinus Nijhoff Publishers, pp. 125-150.

Bjorkman R., 1976, Properties and function of plant myrosinase, In Vaughan J. G., MacLeod A. J., Jones B. M. G., The Biology and Chemistry of the *Cruciferae*, Academic Press, London, pp. 191-203.

Bjorkqvist B., Hase A., 1988, Separation and determination of intact glucosinolates by high-performance liquid chromatography, *Journal of Chromatography*, Vol. 435, pp. 501-507.

Bones A. M., Rossiter J. T., 1996, The myrosinase-glucosinolate system, its organisation and biochemistry, *Physiologia Plantarum*, Vol. 97, pp. 194-208.

Bradfield C. A., Bjeldanes L. F., 1987, High-performance liquid chromatographic analysis of anticarcinogenic indoles in *Brassica oleracea*, *Journal of Agricultural and Food Chemistry*, Vol. 35, pp. 46-49.

Buchner R., 1987, Approach to determination of HPLC response factors for glucosinolates, In Whatelet J-P., Glucosinolates in Rapeseeds: Analytical Aspects, Martinus Nijhoff Publishers, pp. 50-58.

Burke D. G., Cominos X., 1988, Identification of desulfoglucosinolates using fast atom bombardment mass spectrometry, *Journal of Agricultural and Food Chemistry*, Vol. 36, pp. 1184-1187.

Buttery R. G., Guadagni D. G., Ling L. C., et al., 1976, Additional volatile components of cabbage, broccoli and cauliflower, *Journal of Agricultural and Food Chemistry*, Vol. 24, pp. 829.

Cairns T., Siegmund E. G., Samp J. J., Jacobs R., 1988, Characterization of benzyl isothiocyanate and phenyl acetonitrile from papayas by mass spectrometry, *Journal of the Association of Official Analytical Chemists*, Vol. 71 (3), pp. 547-550.

Carlson D. G., Daxenbichler M. E., Van Etten C. H., Hill C. B., Williams P. H., 1985, Glucosinolates in radish cultivars, *Journal of American Society of Horticultural Science*, Vol. 110 (5), pp. 634-638.

Carlson D. G., Daxenbichler M. E., Van Etten C. H., Tookey H. L., Williams P. H., 1981, Glucosinolates in crucifer vegetables: turnips and rutabagas, *Journal of Agricultural and Food Chemistry*, Vol. 29, pp. 1235-1239.

Chin H., Lindsay R. C., 1994, Chapter 8: Modulation of volatile sulfur compounds in cruciferous vegetables, in Mussinan C. J., Keelan M.E., Sulfur Compounds in Foods, American Chemical Society, Washington DC.

Chin H., Zeng Q., Lindsay R. C., 1996, Occurrence and flavor properties of sinigrin hydrolysis products in fresh cabbage, *Journal of Food Science*, Vol. 61 (1), pp. 101-104.

Cole R.A., 1976, Isothiocyanates, nitriles and thiocyanates as products of autolysis of glucosinolates in *Cruciferae, Phytochemistry*, Vol. 15, pp. 759-762.

Conn E., 1981, Chapter 16: Cyanogenic glucosides, In The Biochemistry of Plants, Vol. 7: Secondary plant metabolites, Academic Press, New York, pp. 479-501.

Crisp P., 1976, Trends in the breeding and cultivation of cruciferous crops, In Vaughan J. G., MacLeod A. J., Jones B. M. G., The Biology and Chemistry of the Cruciferae, Academic Press, London-New York, pp. 307-328.

Dawson G. W., Hick A. J., Bennett R. N., Donald A., Pickett J. A., Wallsgrove R. M., 1993, Synthesis of glucosinolate precursors and investigations into the biosynthesis of phenylalkyl- and methylthio-alkylglucosinolates, *The Journal of Biological Chemistry*, Vol. 268 (36), pp. 27154-27159.

Daxenbichler M. E., Spencer G. F., Rose G. B., Brinker A. M., Powell R. G., 1991, Glucosinolate composition of seeds from 297 species of wild plants, *Phytochemistry*, Vol. 30 (8), pp. 2623-2638.

Daxenbichler M. E., Van Etten C. H., 1977, Glucosinolates and derived products in cruciferous vegetables: gas-liquid chromatographic determination of the aglucon derivatives from cabbage, *Journal of the Association of Official Analytical Chemists*, Vol. 60 (4), pp. 950-953.

Delaquis P. J., Mazza G., 1995, Antimicrobial properties of isothiocyanates in food preservation, *Food Technology*, pp. 73-85.

Dragsted L. O., Strube M., Larsen J. C., 1993, Cancer-protective factors in fruits and vegetables: biochemical and biological background, *Pharmacology and Toxicology*, Vol. 72 Suppl. 1, pp. 2116-134.

Dziezak J. D., 1987, Applications of food colorants, *Food Technology*, Vol. 4 (4), p. 78-80.

Elfakir C., Dreux M., 1996, Simultaneous analysis of intact and desulfated glucosinolates with a porous graphitized carbon column, *Journal of Chromatography*, Vol. 727, pp. 71-82.

Eskin M. N. A., 1979, Part 4: Sulfur Compounds - Biogenesis, In Plant pigments, flavors and textures, pp. 60-64, Academic Press, New York.

Falk A., 1994, Structure and expression of β-glucosidases and their binding proteins in *Brassica napus* L, Dissertation, Uppsala Genetic Center, Department of Cell Research, Swedish University of Agricultural Sciences.

FDA, 1986, Code of Federal Regulations, Title 21, Part 82.51, Washington DC.

Fenwick R. G., Heaney R. K., Mawson R., 1989, Chapter 1: Glucosinolates, In Cheeke P. R., Toxicants of Plant Origin, Volume II Glycosides, pp. 1-42, CRC Press, Boca Raton, Florida.

Fenwick R. G., Heaney R. K., Mullin J. W., 1983, Glucosinolates and their breakdown products in food and food plants, *Critical Review of Food Science and Nutrition*, Vol. 18 (2), pp. 123-199.

Florida Agricultural Statistics, 1996, Vegetable Summary 1994-95, Florida Department of Agriculture and Consumer Services, Orlando, FL, 72 pp.

Freeman G. G., Mossadeghi N., 1972, Influence of sulfate nutrition in flavor components of three cruciferous plants: radish (*Raphanus sativus*), cabbage (*Brassica oleracea capitata*), and white mustard (*Sinapis alba*), *Journal of the Science of Food and Agriculture*, Vol. 23 , pp. 387-395.

Friis P., Kjaer A., 1966, 4-(methylthio)-3-butenyl isothiocyanate, the pungent principle of radish roots, *Acta Chemica Scandinavica*, Vol. 20 (3), pp. 698-705.

Gennaro M. C., Abrigo C., Cipolla G., 1994, HPLC of food colours and its relevance in forensic chemistry, *Journal of Chromatography*, Vol. 674, pp. 281-299.

Giusti M. M., Wrolstad R. E., 1996a, Characterization of red radish anthocyanins, *Journal of Food Science*, Vol. 61 (2), pp. 322-326.

Giusti M. M., Wrolstad R. E., 1996b, Radish anthocyanin extract as a natural red colorant for maraschino cherries, *Journal of Food Science*, Vol. 61 (4), pp. 688-694.

Grootwassink J. W. D., Reed D., W., Kolenovsky A. D., 1994, Immunopurification and immunocharacterization of the glucosinolate biosynthetic enzyme thiohydroximate S-glucosyltransferase, *Plant Physiology*, Vol. 105, pp. 425-433.

Hanley A. B., Parsley K. R., 1990, Identification of 1-methoxyindolyl-3-methyl isothiocyanate as an indole glucosinolate breakdown product, *Phytochemistry*, Vol. 29 (3), pp. 769-771.

Hansen M., Moller P., Sorensen H., Trejo (de) M. C., 1995, Glucosinolates in Broccoli stored under controlled atmosphere, *Journal of the American Society for Horticultural Science*, Vol. 120 (6), pp. 1069-1074.

Haughn G. W., Davin L., Giblin M., Underhill E. W., 1991, Biochemical genetics of plants secondary metabolites in*Arabidopsis thaliana*, *Plant Physiology*, Vol. 97, pp. 217-226.

Heaney R. K., Fenwick R. G., 1980a, Glucosinolates in *Brassica* vegetables: analysis of 22 varieties of Brussels sprouts (*Brassica oleracea* var. *gemmifera*), *Journal of the Science of Food and Agriculture*, Vol. 31 , pp. 785-793.

Heaney R. K., Fenwick R. G., 1980b, The analysis of glucosinolates in *Brassica* species using gas chromatography. Direct determination of the thiocyanate ion precursors, glucobrassicin and neoglucobrassicin *Journal of the Science of Food and Agriculture*, Vol. 31 , pp. 593-599.

Heaney R. K., Fenwick R. G., 1981, A micro-column method for the rapid determination of total glucosinolates content of cruciferous material, *Z. Pflanzenzüchtg.*, Vol. 87 , pp. 89-95.

Heeremans E. M, VanderHoven R. A. M., et al., 1989, Specific screening of glucosinolates in sprout extracts using on-line thermospray liquid chromatography-tandem mass spectrometry, *Journal of Chromatography*, Vol. 472, pp. 219-229.

Helboe P., Olsen O., Sorensen H., 1980, Separation of glucosinolates by high-performance liquid chromatography, *Journal of Chromatography*, Vol. 197, pp. 199-205.

Helminger J., Rausch T., Hilgenberg W., 1983, Localization of newly synthesized indole-3-methylglucosinolate (= glucobrassicin) in vacuoles from horseradish *(Armoracia rusticana), Physiologia Plantarum,* Vol. 58, pp. 302-310.

Hendry G. A. F., Houghton J. D., 1996, Natural Food Colorants, 2nd Edition, Blackie Academic and Professional, Glasgow, Great Britain.

Hoglund A. S., Lenman M., Rask L., 1992, Myrosinase is localized to the interior of myrosin grains and is not associated to the surrounding tonoplast membrane, *Plant Science,* Vol. 85, pp. 165-170.

Jain J. C., Michayluk M. R., Grootwasssink J. W. D., Underhill E. W., 1989, Distribution of enzymes catalyzing the glucosylation and sulfation steps of glucosinolates biosynthesis in *Brassica juncea* seedlings and cultured cells, *Plant Science,* Vol. 64, pp.25-29.

Jwanny E. W., El-Sayed S. T., Rahad M. M., Mahmoud A. E., Abdallah N. M., 1995, Myrosinase from roots of *Raphanus sativus, Phytochemistry,* Vol.39 (6), pp.1301-1303.

Kim M. R., Rhee H. S., 1986, Quantitative determination of 4-methylthio-3-butenyl isothiocyanate in radish root by RP-HPLC, *Korean Journal of Food Science,* Vol. 18 (6), pp. 475-478.

Kim M. R., Rhee H. S., 1993, Decrease of pungency in "Radish Kimchi" during fermentation, *Journal of Food Science,* Vol. 58 (1), pp. 128-131.

Kjaer A., 1976, Glucosinolates in the *Cruciferae,* In Vaughan J. G., MacLeod A. J., Jones B. M. G., The Biology and Chemistry of the Cruciferae, Academic Press, London-New York, pp. 207-217.

Kjaer A., Madsen J. O., Maeda Y., Ozawa Y., Uda Y., 1978a, Volatiles in distillates of fresh radish of Japanese and Kenyan origin, *Agric. Biol. Chem.*, Vol 42 (11), pp. 1715-1721.

Kjaer A., Madsen J. O., Maeda Y., Ozawa Y., Uda Y., 1978b, Volatiles in distillates of processed radish of Japanese origin, *Agricultural and Biological Chemistry.*, Vol 42 (11), pp. 1989-1996.

Kore A. M., Gayland F. S., Wallig M. A., 1993, Purification of the ω-(methylsulfinyl) alkyl glucosinolate hydrolysis products: 1-isothiocyanato-3-(methylsulfinyl) propane, and 1-isothiocyanato-4-(methylsulfinyl) butane, 4-(methylsulfinyl) butanenitrile and 5-(methylsulfinyl) pentanenitrile from Broccoli and *Lesquerella fendleri*, *Journal of Agricultural and Food Chemistry*, Vol. 41, pp. 89-95.

Kyung K. H., Fleming H. P., Young C. T., Haney C. A., 1995, 1-cyano-2,3-epithiopropane as the primary sinigrin hydrolysis product of fresh cabbage, *Journal of Food Science*, Vol. 60 (1), pp. 157-159.

Larsen P. O., 1981, Chapter 17: Glucosinolates, In Conn E. E., The Biochemistry of Plants, Volume 7: Secondary plant metabolites, Academic Press, New York, pp. 502-526.

Leoni O., Iori R., Palmieri S., 1991, Immobilization of myrosinase on membrane for determining the glucosinolate content of cruciferous material, *Journal of Agricultural and Food Chemistry*, Vol. 39, pp. 2322-2326.

Lewis J. A., Fenwick G. R., Gray R. A., 1991, Glucosinolates in Brassica vegetables: green-curded cauliflowers (*Brassica oleracea* L. Botrytis group) and purple-headed broccoli (*B. oleracea* L. Italica group), *Lebensm. Wiss. u.- Technol.*, Vol. 24, pp. 361-636.

Loomis D. W., Lile J. D., Sandstrom R. P., Burbott A. J., 1979, Adsorbent polystyrene as an aid in plant enzyme isolation, *Phytochemistry*, Vol.18, pp.1049-1054.

MacDanell R., MacLean A. E. M., Hanley A. B., Heaney R. K., Fenwick G. R., 1988, Chemical and biological properties of indole glucosinolates (glucobrassicins): a review, *Food Chemistry and Toxicology*, Vol. 26 (1), pp. 59-70.

MacGregor D. I., Mullin W. J., Fenwick G. R., 1983, Review of analysis of glucosinolates: analytical methodology for determining glucosinolate composition and content, *Journal of the Association of Official Analytical Chemists*, Vol. 66 (4), pp. 825-849.

Macheix J. J, Fleuriet A., Billot J., 1990, Fruit Phenolics, CRC Press, Boca Raton, FL., pp. 98.

MacLeod A. J., 1976, Volatile flavour compounds in the Cruciferae, In Vaughan J. G., MacLeod A. J., Jones B. M. G., The Biology and Chemistry of the Cruciferae, Academic Press, London-New York, pp. 307-328.

Maheshwari P. N., Stanley D. W., Gray J. I., Van de Voort F. R., 1979, An HPLC method for simultaneous quantitation of individual isothiocyanates and oxazolidinethione in myrosinase digests of rapeseed meal, *Journal of the American Oil Chemists' Society,* Vol. 56, pp. 837-841.

Mari M, Iori R., Leoni O., Marchi A., 1996, Bioassays of glucosinolate-derived isothiocyanates against postharvest pear pathogens, *Plant Pathology,* Vol. 45, pp. 753-760.

Mellon F. A., Chapman J. R., Pratt J. A. E., 1987, Thermospray liquid chromatography-mass spectrometry in food and agricultural research, *Journal of Chromatography,* Vol. 394, pp. 209-222.

Minchinton I., Sang J., Burke D., Truscott R. J. W., 1982, Separation of desulphoglucosinolates by reverse-phase high performance liquid chromatography, *Journal of Chromatography,* Vol. 247, pp. 141-148.

Mithen R., Toroser D., 1995, Biochemical genetics of aliphatic glucosinolates in Brassica and Arabidopsis, In Wallsgrove R. M., Amino Acids and their Derivatives in Higher Plants, Cambridge University Press, pp. 261-276.

Moller P., Ploger A., Sorensen H., 1985, In Sorensen H., Advances in the Production and Utilization of Cruciferous Crops, Martinus Nijohff Publishers, Hingham, MA, pp. 97-110.

Mullin W. J., 1978, High performance liquid chromatography and gas chromatography of organic isothiocyanates and their methanol-isothiocyanate addition compounds, *Journal of Chromatography,* Vol. 155 (1), pp. 198-202.

Newsome R. L., 1986, Food colors, *Food Technology,* Vol. 40 (7), pp. 49-56.

Nijhoff W. A., Grubben J. A. L., Nagengast F. M. et al., 1995, Effects of consumption of Brussels sprouts on intestinal and lymphocytic glutathione S-transferases in humans, *Carcinogenesis,* Vol. 16 (9), pp. 2125-2128.

Olsson K., Theander O., Aman P., 1977, H-N.M.R., C-N.M.R. and mass spectra of glucosinolates and related compounds, *Carbohydrate Research,* Vol. 58, pp. 1-8.

Petroski R. J., Tookey H. L., 1982, Interactions of thioglucoside glucohydrolase and epithiospecifier protein of cruciferous plants to form 1-cyanoepothioalkanes, *Phytochemistry,* Vol. 21 (8), pp. 1903-1905.

Poulton J. E., 1990, Review: Cyanogenesis in plants, *Plant Physiology*, Vol. 94, pp. 401-405.

Poulton J. E., Moller B. L., 1993, Glucosinolates, In Lea P. J., Methods in Plant Biochemistry, Volume 9, Academic Press London, pp. 209-238.

Prestera T., Fahey J. W., Holtzclaw D. W., et al, 1996a, Comprehensive chromatographic and spectroscopic methods for the separation and identification of intact glucosinolates, *Analytical Biochemistry*, Vol. 239, pp. 168-179.

Prestera T., Holtzclaw D. W., Zhang Y., Talalay P., 1996b, Chemical and molecular regulation of enzymes that detoxify carcinogens, *Processings of the National Academy of Sciences*, Vol. 90, pp. 2965-2969.

Quinsac A., Ribaillier D., El-Fakir C., Lafosse M., Dreux M., 1991, A new approach to the study of glucosinolates by isocratic liquid chromatography: Part I - Rapid determination of desulfated derivatives of rapeseed glucosinolates, *Journal of the Association of Official Analytical Chemists*, Vol. 74 (6), pp. 932-939.

Reinhold V. N., Carr S. A., 1983, New mass spectrometry approaches to complex carbohydrate structure, *Mass Spectrometry Reviews*, Vol. 2, pp. 153-221.

Robinson T., 1991, Chapter 15: Miscellaneous nitrogen and sulfur compounds, In The Organic Constituents of Higher Plants, pp. 300-327, Cordus Press, Sixth Edition, North Amherst.

Sakushima A., Coskun M., Maoka T., 1995, Sinapyl but-3-enylglucosinolate from *Boreava orientalis*, *Phytochemistry*, Vol. 40 (2), pp. 483-485.

Sang J. P., Minchinton I. R., Johnstone P. K., Truscott R. J. W., 1984, Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede, *Canadian Journal of Plant Science*, Vol. 64, pp. 77-93.

Sang J. P., Truscott R. J. W., 1984, Liquid chromatographic analyses of glucosinolates in rapeseed as desulfoglucosinolates, *Journal of the Association of Official Analytical Chemists*, Vol. 67 (4), pp. 829-833.

Sapers G.M., 1982, Deodorization of a colorant prepared from red cabbage, *Journal of Food Science*, Vol. 47 (3), pp. 972-976.

Searle L. M., CHamberlain K., Rausch T., Butcher D., 1982, The conversion of 3-indolylmethyl-glucosinolate to 3-indolylacetonitrile by myrosinase and its relevance to the clubroot disease of the Cruciferae, *Journal of Experimental Botany*, Vol.33 (136), pp.935-942.

Shahidi F., 1994, Chapter 9: Thioglucosides of *Brassica* oilseeds and their process-induced chemical transformations, in MUSSINAN C. J., Keelan M.E., Sulfur compounds in foods, American Chemical Society, Washington DC, pp. 107-126.

Shankaranarayana M. L., Raghavan B., Abraham K. O., et al, 1974, Volatile sulfur compounds in food flavors, *CRC Critical Review of Food Technology*, Vol. January, pp. 395-429.

Slominski B. A., Campbell L. D., 1989, Formation of indole glucosinolate breakdown products in autolyzed steamed and cooked *Brassica* vegetables, *Journal of Agricultural and Food Chemistry*, Vol. 37, pp. 1297-1302.

Sorensen H., 1985, Limitations and possibilities of different methods suitable to quantitative analyis of glucosinolates occurring in double low rapeseed and product thereof, In Advances in the Production and Utilization of Cruciferous Crops, Martinus Nijohff Publishers, Hingham, MA, pp. 73-84.

Spencer G. F., Daxenbichler M. E., 1980, Gas chromatography - Mass spectrometry of nitriles, isothiocyanates and oxazolidinethiones derived from cruciferous glucosinolates, *Journal of the Science of Food and Agriculture*, Vol. 31 , pp. 359-367.

Spinks A. E., Sones K., Fenwick G. R., 1984, The quantitative analysis of glucosinolates in cruciferous vegetables, oilseeds and forage crops using high performance liquid chromatography, *Fette Seifen Anstrichmittel*, Vol. 89 (6) , pp. 228-231.

Springett M. B., Adams J. B., 1988, Identification of 1-cyano-2,3-epithiopropane in volatiles of a model system from Brussels sprouts, *Journal of the Science of Food and Agriculture*, Vol. 46 (1), pp. 211-219.

Stoewsand G. S., 1995, Bioactive organosulfur phytochemicals in *Brassica oleracea* vegetables - A review, *Food and Chemical Toxicology*, Vol. 33 (6), pp. 537-543.

Talalay P., Zhang Y., 1996, Chemoprotection against cancer by isothiocyanates and glucosinolates, *Biochemical Society Transactions*, Vol. 24 (3), pp. 806-809.

Tawfiq N., Heaney R. K., Plumb J. A., Fenwick G. R., Musk S. R. R., Williamson G., 1995, Dietary glucosinolates as blocking agents against carcinogenesis: glucosinolate breakdown products assessed by induction of quinone reductase activity in murine hep1c1c7 cells, *Carcinogenesis*, Vol. 16 (5), pp. 1191-1194.

Teranishi R., Buttery R. G., Stern D. J., Takeoka G., 1991, Use of odor thresholds in aroma research, *Lebensm. Wiss. u.- Technol.*, Vol. 24, pp. 1-5.

Thangstad O. P., Iversen T. H., Slupphaug G., Bones A., 1990, Immunocytochemical localization of myrosinase in *Brassica napus* L., *Planta*, Vol. 180, pp. 245-248.

Thangstad O. P., Winge P., Husebye H., Bones A., 1993, The myrosinase activity (thioglucoside glucohydrolase) gene family in Brassicaceae, *Plant Molecular Biology*, Vol. 23, pp. 511-524.

Thies W., 1988, Isolation of sinigrin and glucotropaeolin from cruciferous seeds, *Fat Science Technology*, Vol. 90 (8), pp. 311-314.

Tiedink H. G. M., Malingre C. E., et al., 1991, Roles of glucosinolates in the formation of *N*-nitroso compounds, *Journal of Agricultural and Food Chemistry*, Vol. 39, pp. 922-926.

Tookey H.L., Van Etten C. H., Daxenbichler M. E., 1980, Glucosinolates, In Liener I. E., Toxic Constituents of Plant Foodstuffs, Academic Press, Second Edition, New York, pp. 103-136.

Uda Y., Kurata T., Arakawa N., 1986, Effects of pH and ferrous ion on the degradation of glucosinolates by myrosinase, *Agricultural and Biological Chemistry.*, Vol 50 (11), pp. 2735-3740.

Underhill E. W., Kirkland D. F., 1971, Gas chromatography of trimethylsilyl derivatives of glucosinolates, *Journal of Chromatography*, Vol. 57, pp. 47-54.

Van Etten C.H., Daxenbichler M. E., 1977, Glucosinolates and derived products in Cruciferous vegetables : total glucosinolates by retention on anion exchange resin and enzymatic hydrolysis to measure released glucose, *Journal of the Association of Official Analytical Chemists*, Vol. 60 (4), pp. 946-949.

Van Etten C.H., Daxenbichler M. E., Williams P. H., Kwolek W. F., 1976, Gucosinolates and derived products in cruciferous vegetables. Analysis of the edile part from twenty two varieties of cabbage, *Journal of Agricultural and Food Chemistry*, Vol. 24 (3), pp. 452-455.

Van Langenhove H. J., Cornelis C. P., SchampP N. M., 1991, Identification of volatiles emitted during the blanching process of Brussels sprouts and Cauliflower, *Journal of the Science of Food and Agriculture*, Vol. 55 (3), pp. 483-487.

Visenti M., Tava A., Iori R., Palmieri S., 1992, Isolation of *trans*-4-(methylthio)-3-butenyl glucosinolate from radish roots (*Raphanus sativus* L.), *Journal of Agricultural and Food Chemistry*, Vol. 40 (9), pp. 1687-1691.

Wallbank B. E., Wheatley G. A., 1976, Volatiles constituents from cauliflower and other crucifers, *Phytochemistry*, Vol. 15, pp. 763-766.

Wallsgrove R. M., Bennett R. N., 1995, The biosynthesis of glucosinolates in Brassicas, In Wallsgrove R. M., Amino Acids and their Derivatives in Higher Plants, Cambridge University Press, pp. 243-260.

Whatelet J-P., Biston R., Marlier M., Severin M., 1987, Analysis of individual glucosinolates rapeseeds: comparison between different methods, In Whatelet J-P., Glucosinolates in Rapeseeds: Analytical Aspects, Martinus Nijhoff Publishers, pp. 125-150.

Whitfield F. B., Last J. H., 1991, Part 7: Vegetables, In Maarse E H., Volatile Compounds in Foods and Beverages, pp. 203-275, Marcel Dekker, New York.

Wilkinson A. P., Rhodes M. J. C., Fenwick R. G., 1984, Myrosinase activity of cruciferous vegetables, *Journal of the Science of Food and Agriculture*, Vol. 35, pp. 543-552.

Williams P. H., Wang H., Griffiths S., 1996, Glucosinolates as bioactive components of Brassica vegetables: induction of cytochrome P450 1A1 in Hep G2 cells as assessed using transient transfection, *Biochemical Society Transactions*, Vol. 24 (3), pp. 383s.

Wrolstad R. E., 1976, Color and pigment analyses in fruit products, Oregon State University, Agricultural Experimental Station, Bulletin No. 624.

Yen G. C., Wei Q. K., 1993, Myrosinase activity and total glucosinolate content of cruciferous vegetables and some properties of cabbage myrosinase in Taiwan, *Journal of the Science of Food and Agriculture*, Vol. 61, pp. 471-475.

Zhang C. S., Smith T. J., Hong J. Y., 1994, Cytochrome P-450 enzymes as targets for chemopreventives against chemical carcinogenesis and toxicity: opportunities and limitations, *Cancer Research (Supplement)*, Vol. 54, pp. 1982s-1986s.

Zhang Y., Talalay P., 1994, Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms, *Cancer Research (Supplement)*, Vol. 54, pp. 1976s-1981s.

Zhang Y., Wade K. L., Prestera T., Talalay P., 1996, Quantitative determination of isothiocyanates dithiocarbamates, carbon disulfide and related thiocarbamates compounds by cyclocondensation with 1,2-benzenedithiol, *Analytical Biochemistry*, Vol. 239, pp. 160-167.

APPENDICES

	Crop Season	Number of Farms	Acres Harvested
USA	1982	671	41480
	1987	636	45888
	1992	761	29893
FLORIDA	1982	21	23222
	1987	25	30501
	1992	30 (4%)	17177 (57%)
CALIFORNIA	1982	73	3575
	1987	73	2532
	1992	87 (11%)	2675 (9%)
MICHIGAN	1982	51	9527
	1987	37	5206
	1992	38 (5%)	2967 (10%)
OREGON	1982	31	382
	1987	31	629
	1992	24 (3%)	399 (1%)

Appendix 1: Harvested acreage of radishes in the USA, Florida, California, Michigan and Oregon from 1982 to 1992 (Census of Agriculture, 1992, Table 29: Vegetables, Sweet Corn and Melons).

RADISHES: Acreage, production, and value, Florida, crop years 1985-86 through 1994-95

Crop year	Acreage		Yield per acre	Production	Value per carton	Total value
	Planted	Harvested				
	Acres		15-lb carton	1,000 cartons	Dollars	1,000 dollars
1985-86	29,400	26,100	216	5,638	3.78	21,312
1986-87	29,700	27,000	207	5,589	3.15	17,605
1987-88	28,000	26,300	192	5,050	3.70	18,685
1988-89	28,200	27,000	235	6,345	3.15	19,987
1989-90	29,000	23,000	350	8,050	3.54	28,497
1990-91	26,100	25,000	285	7,125	4.95	35,269
1991-92	24,900	22,800	269	6,130	3.52	21,578
1992-93	24,900	23,800	261	6,212	5.95	36,961
1993-94	18,000	17,400	273	4,750	5.45	25,888
1994-95	19,200	15,700	256	4,019	5.94	23,873

RADISHES: Production sold, monthly, Florida, crop years 1990-91 through 1994-95

Crop year	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Total
	1,000 15-lb cartons									
1990-91	371	974	1,206	952	644	933	1,042	847	156	7,125
1991-92	213	853	931	673	707	787	905	922	139	6,130
1992-93	112	826	1,006	727	683	895	982	882	99	6,212
1993-94	90	499	755	689	708	969	608	266	166	4,750
1994-95	40	414	181	422	804	868	752	498	40	4,019
	Percent									
1990-91	5.2	13.7	16.9	13.4	9.0	13.1	14.6	11.9	2.2	100.0
1991-92	3.5	13.9	15.2	11.0	11.5	12.8	14.8	15.0	2.3	100.0
1992-93	1.8	13.3	16.2	11.7	11.0	14.4	15.8	14.2	1.6	100.0
1993-94	1.9	10.5	15.9	14.5	14.9	20.4	12.8	5.6	3.5	100.0
1994-95	1.0	10.3	4.5	10.5	20.0	21.6	18.7	12.4	1.0	100.0

RADISHES: Average value per carton for fresh market sales, monthly, Florida, crop years 1990-91 through 1994-95

Crop year	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Average
	Dollars									
1990-91	3.80	3.40	2.80	4.65	8.35	7.25	6.35	4.15	3.00	4.95
1991-92	4.00	3.65	3.00	3.35	3.35	4.05	3.40	3.75	3.35	3.52
1992-93	7.00	4.95	5.90	6.30	8.00	5.70	6.10	5.20	4.70	5.95
1993-94	8.20	7.30	5.35	4.95	4.30	4.25	4.75	8.50	10.70	5.45
1994-95	4.70	6.20	16.70	12.30	5.80	3.70	3.40	4.20	9.50	5.94

Appendix 2: Production of radishes, in volume and value, in Florida during the crop years 1985-86 through 1994-95. Source: Florida Agricultural Statistics, 1996, Vegetable Summary 1994-95, Florida Department of Agriculture and Consumer Services, Orlando, FL, p. 36.

Dependent Variable: OVERALL AROMA INTENSITY

Model: Overall Aroma Intensity =
{Panelist, Colorant, Concentration, one way and two way interactions}

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	384	5535.99	26.47	6.81	0.0001
Error	383	1444.00	3.889		
Corrected Total	767	6979.99			

R-Square	Coeff.Variation	Root MSE	INTENSITY Mean
0.793	21.57	1.942	9.003

ANALYSIS OF VARIANCE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Main effects					
PANELIST	23	1275.74	55.47	14.71	0.0001
COLORANT	3	1841.46	613.82	162.81	0.0001
CONCENTRATION	3	513.72	171.24	45.42	0.0001
REPLICATE	1	3.00	3.00	0.80	0.3729
Interactions					
PANELIST*CONC.	69	326.28	4.73	1.25	0.1230
PANELIST*COLORANT	69	691.54	10.02	2.66	0.0001
COLORANT*CONC.	9	33.47	3.712	0.99	0.4755
PANELIST*COLOR.*CONC.	207	850.78	4.110	1.09	0.2352

Appendix 3 Table A: Analysis of variance with overall aroma intensity as dependent variable (768 observations = 16 samples x 2 replicates x 24 panelists).

PANELIST #	T Grouping	Mean	SD	SE	N
12	a	10.47	3.12	0.55	32
2	b a	10.38	2.98	0.53	32
20	b a c	10.09	3.79	0.67	32
5	b d a c	9.50	3.34	0.59	32
9	e b d a c	9.31	2.64	0.47	32
6	e b d a c	9.09	2.76	0.49	32
11	e b d a c f	8.59	3.81	0.67	32
4	e b d c f	8.50	2.49	0.44	32
13	e d c f	8.34	3.05	0.54	32
15	e d g c f	8.19	2.15	0.38	32
3	e h d g f	7.88	2.27	0.40	32
8	e h d g i f	7.84	2.78	0.49	32
23	e h d g i f	7.81	2.10	0.37	32
19	e h d g i f	7.81	3.16	0.56	32
24	e h d g i f	7.72	2.22	0.39	32
16	e h d g i f	7.59	2.66	0.47	32
17	e h g i f	7.53	2.45	0.43	32
14	h g i f	7.13	1.95	0.34	32
18	h g i f	6.88	2.71	0.48	32
21	h g i f	6.78	2.96	0.52	32
7	h g i	6.38	1.93	0.34	32
10	h g i	6.28	3.44	0.61	32
1	h i	6.06	2.18	0.39	32
22	i	5.91	2.26	0.40	32

DF = 383, MSE = 3.77, Critical value of T = 1.97, LSD = 0.9544

Appendix 3 Table B: Multiple comparison Tukey test for overall aroma intensity on the panelists. Alpha= 0.05, N = number of observations, SD = standard deviation, SE = standard error. Means with the same letter are not significantly different.

REPLICATE	T Grouping	Mean	SD	SE	N
2	a	8.07	2.88	0.15	384
1	a	7.94	3.15	0.16	384

DF = 383, MSE = 3.77, Critical value of T = 1.97, LSD = 0.2755

COLORANT	T Grouping	Mean	SD	SE	N
Radish Red Meat Takii	a	10.12	2.75	0.20	192
Radish Fuego	b	8.84	3.00	0.22	192
Red Cabbage Extract	c	6.64	2.21	0.16	192
Radish Color Extr. (Japan)	c	6.41	2.33	0.17	192

DF = 383, MSE = 3.77, Critical value of T = 1.97, LSD = 0.3896

CONCENTRATION (Anthocyanin mg/L)	T Grouping	Mean	SD	SE	N
1200	a	8.99	3.05	0.22	192
600	a	8.53	2.90	0.21	192
300	b	7.61	2.84	0.21	192
150	c	6.88	2.83	0.20	192

DF = 383, MSE = 3.77, Critical value of T = 1.97, LSD = 0.3896

Appendix 3 Table C: Multiple comparison Tukey test for overall aroma intensity on the replicates and the samples. Alpha= 0.05, N = number of observations, SD = standard deviation, SE = standard error. Means with the same letter are not significantly different.

SAMPLES	T Grouping	Mean	SD	SE	N
Takii 1200	a	11.25	2.38	0.34	48
Takii 600	a b	10.77	2.63	0.38	48
Fuego 1200	b c	10.21	3.04	0.44	48
Takii 300	c d	9.52	2.77	0.40	48
Fuego 600	d e	9.06	2.88	0.42	48
Takii 150	d e	8.94	2.63	0.38	48
Fuego 300	e	8.52	2.85	0.41	48
Fuego 150	f	7.58	2.71	0.39	48
Red Cabb. 1200	f	7.42	1.99	0.29	48
Red Cabb. 600	f	7.31	2.40	0.35	48
Jap. Radish 1200	f g	7.08	2.47	0.36	48
Jap. Radish 600	f g	6.98	1.99	0.29	48
Red Cabb. 300	g h	6.46	1.92	0.28	48
Jap. Radish 300	h i	5.96	2.14	0.31	48
Jap. Radish 150	i	5.63	2.40	0.35	48
Red Cabb. 150	i	5.35	1.93	0.28	48

DF = 383, MSE = 3.770, Critical value of T =1.97, LSD = 0.7793

Appendix 3 Table D : Multiple comparison Tukey test for overall aroma intensity on the samples. Alpha= 0.05, N = number of observations, SD = standard deviation, SE = standard error. Means with the same letter are not significantly different.