exposed to more than 70% full sunlight (FS) (Heinicke, 1966). Also, apples exposed to 90–100% FS had the highest content of soluble solids. In navel oranges, it was shown that fruit composition was associated with the available heat units (Jones et al., 1962) and that fruit from widely spaced trees colored faster and matured earlier than fruit from trees closely planted (Boswell et al., 1982). Higher minimum and maximum temperatures and better light penetration prevail in orchards with widely spaced trees.

When gibberellic acid (GA_3) is applied to navel oranges, it delays rind senescence and moderates compositional changes of epicuticular wax *n*-alkanes (El-Otmani and Coggins, 1985b). In particular, GA_3 moderates the shift from short to long chains during development and reduces the subsequent shift toward short chains during postmaturation and senescence. Also, when expressed as a percentage, wax composition of the GA_3-treated fruit had significantly lower *n*-alkane percentages than wax of untreated fruit. In addition, on an absolute basis ($\mu g/cm^2$), GA_3-treated fruit accumulated significantly less *n*-alkanes than did control fruit. Therefore, because SW-quadrant Valencia orange epicuticular wax contained a higher percentage of total *n*-alkanes (Figure 1) and of short-chain components (Figure 4) and a higher quantity ($\mu g/cm^2$) of *n*-alkanes (Figures 2, 6, and 7) from fruit color break onward, and because most of these differences appeared to increase over time, we suggest that SW quadrant fruit were physiologically more advanced as they approached maturity than fruit from the NE quadrant.

ACKNOWLEDGMENT

This research was conducted in partial fulfillment of the requirements of the Ph.D. degree by M.E.-O. and was supported by a Citrus Research Board grant. We thank N. O'Connell and J. Pehrson for their help with the fruit sampling.

LITERATURE CITED

Avato, P.; Mikkelsen, J. D.; von Wettstein-Knowles, P. *Carlsberg Res. Commun.* **1980**, *45*, 329.
Baker, E. A. In *The Plant Cuticle*; Cutler, D. F., Alvin, K. L., Price, C. E., Eds.; Academic: London, New York, 1982.
Baker, E. A.; Procopiou, J.; Hunt, G. M. *J. Sci. Food Agric.* **1975**, *26*, 1093.
Boswell, S. B.; Nauer, E. M.; Atkin, D. R. *J. Am. Soc. Hortic. Sci.* **1982**, *107*, 60.
El-Otmani, M. Ph.D. Dissertation, University of California, Riverside, CA, 1985.
El-Otmani, M.; Coggins, C. W., Jr. *J. Am. Soc. Hortic. Sci.* **1985a**, *110*, 371.
El-Otmani, M.; Coggins, C. W., Jr. *J. Agric. Food Chem.* **1985b**, *33*, 656.
Freeman, B. Ph.D. Dissertation, University of Florida, Gainesville, FL, 1978.
Freeman, B.; Albrigo, L. G.; Biggs, R. H. *J. Am. Soc. Hortic. Sci.* **1979**, *104*, 801.
Giese, B. N. *Phytochemistry* **1975**, *14*, 921.
Heinicke, D. R. *Proc. Am. Soc.* Hortic. *Sci.* **1966**, *89*, 10.
Jones, W. W.; Embleton, T. W.; Cree, C. B. *Calif. Citrogr.* **1962**, *47*, 132.
Macey, M. J. K. *Phytochemistry* **1970**, *9*, 757.
Nagy, S.; Nordby, H. E.; Lastinger, J. C. *Phytochemistry* **1975**, *14*, 2443.
Nordby, H. E.; Nagy, S. *Phytochemistry* **1977**, *16*, 1393.
Sites, J. W.; Reitz, H. J. *Proc. Am. Soc. Hortic. Sci.* **1949**, *54*, 1.
Turrell, F. M. *Tables of Surfaces and Volumes of Spheres and Prolate and Oblate Spheroids* and *Spheroidal Coefficients*, 1st ed.; University of California: Berkeley, Los Angeles, 1946.

Received for review August 12, 1985. Accepted July 29, 1986.

High-Performance Liquid Chromatographic Analysis of Anticarcinogenic Indoles in *Brassica oleracea*

Christopher A. Bradfield and Leonard F. Bjeldanes*

Vegetables of the *Brassica* genus contain an (indolylmethyl)glucosinolate, glucobrassicin (GB). The autolysis products of this glucosinolate include indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (I33′), all of which inhibit chemically induced neoplasia in rodents. Analytical methodology, utilizing RP-HPLC, was developed to quantify this potentially important series of indolylic metabolites in foods and to describe the thioglucosidase-mediated autolytic process. Our results demonstrate that I3C is the major GB metabolite generated when plant material is disrupted, although it is not stable in the autolytic milieu (84% conversion to other products in 24 h). Additionally, these results indicate that common cooking practices employed on cruciferous vegetables do not inactivate the thioglucosidase to a significant extent, and thus autolytic products are likely to occur in cooked as well as raw vegetables.

The National Research Council, Committee on Diet, Nutrition and Cancer, has recently recommended increased consumption of cruciferous vegetables as a measure to decrease human cancer incidence (National Research Council, 1982). This recommendation is based on epidemiological evidence (Graham, 1983) and results from animal experiments (Stoewsand et al., 1978; Wattenberg, 1983) that suggest that these vegetables possess cancer-modulating properties. The inhibitory effects may result from exposure to a number of nutritive and nonnutritive constituents known to inhibit chemically induced carcinogenesis in experimental animals [see Wattenberg (1983)].

Vegetables of the *Brassica* genus (family Cruciferae) contain an (indolylmethyl)glucosinolate, commonly known as glucobrassicin (GB). Levels of this thioglucoside are reported to be as high as 1100 $\mu g/g$ in some cultivars (Fenwick et al., 1983). Upon disruption of the plant material a thioglucosidase-mediated autolytic process ensues, yielding a series of 3-substituted indoles; among these are indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (I33′) (Virtanen, 1965; Figure 1). Rodents exposed to these indoles, via intubation or diet,

Department of Nutritional Sciences, University of California, Berkeley, California 94720.

0021-8561/87/1435-0046$01.50/0

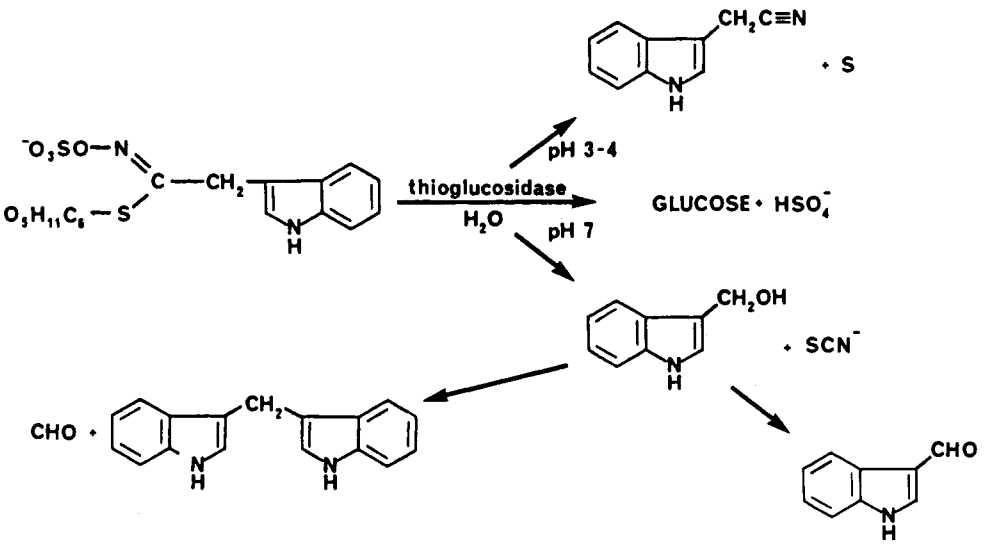

Figure 1. Autolytic products of glucobrassicin (adapted from Virtanen (1965) and experimental results included in this report). The pH-dependent, enzymatic hydrolysis of GB yields either the acetonitrile (IAN) or the alcohol (I3C). The resulting I3C is unstable in aqueous solutions and undergoes self-condensation to the dimer (I33′) or oxidation to the aldehyde (I3CHO).

exhibit increases in the activities of a variety of xenobiotic metabolizing enzymes including cytochrome P-448 dependent monooxygenases, cytosolic glutathione *S*-transferases, and microsomal epoxide hydrolase (Bradfield and Bjeldanes, 1984, 1985; Loub et al., 1975; Miller and Stoewsand, 1983; Shertzer, 1982; Sparnins et al., 1982). These indoles have also been demonstrated to be inhibitors of either dimethylbenzanthracene-induced mammary cancer in the rat or benzo[*a*]pyrene-induced cancer of the forestomach in mice (Wattenberg and Loub, 1978).

As consumption of *Brassica* vegetables is considerable in both western and eastern cultures (Fenwick et al., 1983; Prescott, 1983), investigations into the levels of exposure to these indoles are appropriate. Information regarding the relative levels of GB metabolites in the diet should prove useful in understanding their relevance to the anticarcinogenic characteristics of *Brassica* vegetables. This paper presents a simple and sensitive method for the determination of GB autolysis products in food samples and describes the autolytic process in plant material and in a reconstituted system.

MATERIALS AND METHODS

Chemicals. Organic solvents were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). GB (as the tetramethylammonium salt) was a most generous gift from Dr. G. R. Fenwick (Agricultural Research Council Food Research Institute, Norwich, U.K.). I3C, IAN, and indole-3-carboxaldehyde (I3CHO) were purchased from Aldrich Chemical Co. (Milwaukee, WI). I33′ was synthesized by the base reflux method described by Leete and Marion (1953). I3C, I3CHO, and I33′ were recrystallized from toluene: mp 98–100, 193–195, and 161–163 °C, respectively. IAN was purified by vacuum distillation to a light yellow oil that yielded only one spot on TLC (mobile phase CH_2Cl_2; visualized with *p*-(dimethylamino)benzaldehyde; Aldrich Chemical Co., Milwaukee, WI). Vegetable samples were purchased at local supermarkets. Kirby yellow mustard seed (*Brassica hirta* or *Sinapis alba*) was a generous gift from MINN-DAK Growers Association, Grand Forks, ND.

Extraction of GB Autolysis Products from *Brassica oleracea*. Fresh plant material (100 g) was blended with 200 mL of distilled H_2O for 60 s in a Waring blendor, followed by homogenization for 30 s with a Polytron homogenizer at medium speed (Brinkman Instruments, Westbury, NY). Thirty milliliters of homogenate was filtered through Whatman filter paper (No. 41). The residue was rinsed with 20 mL of H_2O and homogenized in 30 mL of acetonitrile (ACN). The ACN extract was filtered and combined with the aqueous fraction. The residue was then homogenized three times in 33 mL of CH_2Cl_2. The CH_2Cl_2 extracts were filtered and pooled with the aqueous/ACN fraction. The organic/aqueous phases were partitioned in a separatory funnel with vigorous shaking for 30 s. The organic phase was collected and the aqueous phase partitioned three more times with 75 mL each of CH_2Cl_2. The organic phases were pooled, and the volume was measured. A portion of the organic phase (100 mL) was then evaporated with a Rotorvapor-R (Brinkman Instruments, Westbury, NY) at 22 °C. The residue was dissolved in 1.6 mL of ACN with sonication. Phosphate buffer, pH 7.0 (0.4 mL, 5 mM), was added, and a 1.0-mL aliquot was loaded onto a sample cleanup column (C-18) previously equilibrated with 8:2 ACN–phosphate buffer (Spice C-18 sample preparation cartridge; Analtech Inc., Newark, DE). The indoles were eluted with a total volume of 5.0 mL.

HPLC Analysis of GB Autolysis Products. Separation of GB autolysis products was performed at room temperature on a Beckman Model 332 HPLC (Beckman Instruments, Fullerton, CA) fitted with an Ultrasphere 4.6 mm × 250 mm ODS column (particle size 5 μm; Altex Scientific, Inc., Berkeley, CA). Indoles were eluted in a reversed-phase gradient system, with the percentage of ACN in 5 mM phosphate buffer (pH 7.0) increasing from 5 to 80% over a period of 60 min (solvent flow rate 1.0 mL/min). Indoles were detected either by their absorption at 280 nm (Beckman Model 155-40 UV detector; Beckman Instruments, Fullerton, CA) or by their fluorescence (excitation 280 nm, emission 350 nm; Perkin-Elmer LS-4 spectrofluorometer; Perkin-Elmer, Norwalk, CN). Indoles were quantified by comparison with external standards. For fluorescence, 3-methylindole has proven to be an effective internal standard.

Experiment 1: Effect of Time of Autolysis on Product Yield. A 100-g sample of Brussels sprouts (*B. oleracea* var. gemmifera) was autolyzed, and a 30-mL aliquot of the autolysate was assayed at 10 min, 1.0 h, 6.0 h, 12.0 h, and 24 h. background levels (zero time) were determined by carrying out the autolysis step in ACN. The autolysis was carried out at room temperature, and the

Table I. Recovery of GB Autolysis Products from *B. oleracea*

product	% rec ± SEM[a]	product	% rec ± SEM[a]
I3C	86 ± 4	IAN	100 ± 5
I3CHO	96 ± 8	I33′	84 ± 3

[a] Values are means from three recovery experiments.

results are from three separate samples for each time point.

Experiment 2: Effect of Cooking on Autolysis Product Yield. Whole cauliflower (*B. oleracea* var. botrytis) was broken by hand into 20-g flowers that were boiled in 40 mL of H_2O for 10 min (100 °C). Water lost during cooking was added as ice, and the boiling flask was cooled on ice for 10 min. The sample was then homogenized and analyzed for autolysis products as described.

Note: Cauliflower was used due to the seasonal unavailabiity of Brussels sprouts at the time of this experiment.

Experiment 3: Identification of GB/Thioglucosidase Products in a Purified System. Thioglucosidase (also known as myrosinase) was prepared from yellow mustard seed as described by Appelqvist and Josefsson (1967). GB (100 μg in 50 μL of H_2O) was added to 450 μL of 0.05 M citrate–phosphate buffer (pH 3, 4, 5, 6, 7) containing 45 μg of the thioglucosidase preparation. The reaction was allowed to proceed at room temperature for 60 min at which time 500 μL of H_2O was added. The solution was filtered through a 0.45-μm nylon filter (Micron Separations Inc., Honeoye Falls, NY), and the sample was immediately injected onto the HPLC system described above.

RESULTS AND DISCUSSION

Recovery of all indoles was greater than 80% (Table I), with a coefficient of variation of no greater than 15%. The assay has been employed with success on Brussels sprouts, cabbage, and cauliflower. The combination of fluorescence- and ultraviolet-based detection, linked to a RP-HPLC separation system, allows confirmation of indole identity and flexibility in the determination of these biologically important 3-substituted indoles. I3CHO is a notable exception because it is nonfluorescent but has the greatest absorbance at 280 nm (Figure 2).

Although I3C has been demonstrated to be the main product of GB autolysis at neutral pH, in vitro (Virtanen, 1965), isolation of indoleglucosinolate autolysis products from plant material yields primarily nitriles and carboxaldehydes (Bradfield and Bjeldanes, 1986a; Jones and Taylor, 1957; Loub et al., 1975; Nomoto and Tamura, 1970). This fact has led to the suggestion that IAN is the most abundant GB metabolite and, thus, potentially of most dietary importance (National Research Council, 1982). Figure 3 may give a indication as to why IAN and I3CHO have been isolated with success relative to I3C. After 24 h, I3C levels dropped 84% from peak levels immediately following initiation of autolysis, while levels of I33′ and I3CHO increased and levels of IAN remained constant. Thus, results from isolation studies may not be indicative of relative levels of these indoles in the Cruciferae. Additionally, Figure 3 illustrates the generation of I3CHO and I33′ from I3C. Although our data do not rule out enzymatic generation of I3CHO or I33′, nonenzymatic generation of these compounds from I3C has been described previously (Fetizon et al., 1976; Leete and Marion, 1953). In this regard it is important to note that I3CHO was not generated in our in vitro system.

Since cruciferous vegetables are consumed either raw or cooked, we investigated the effect of boiling-water treatment (10 min) on the autolysis process. Autolysis of

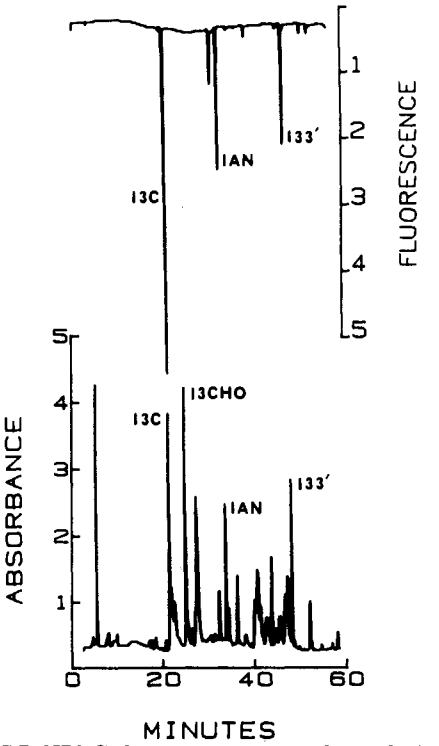

Figure 2. RP-HPLC chromatogram of 12-h autolysis. Sample is from Brussels sprouts (as described in Materials and Methods). Ordinates: absorbance (×1000); fluorescence (relative).

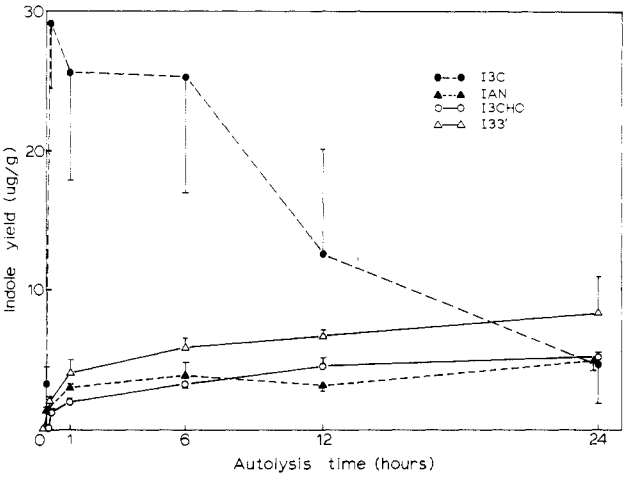

Figure 3. Profile of glucobrassicin autolysis products, over time, from Brussels sprouts (in vivo).

previously cooked vegetables yielded 80% of the autolytic products as were produced from raw vegetables.

The results of the in vitro thioglucosidase/GB experiment (Figure 4) indicate that I3C was the major indole produced at above pH 3. At pH 3 IAN was apparently the major indole produced, although the instability of I3C in acidic solution may not allow its detection at this pH. These results are in agreement with those of Virtanen (1965). In the in vitro system I3C was produced in at least 400 times greater quantity than IAN at pH 5 or above. In the autolysis study of the whole plant, only 10 times more I3C was produced than IAN. Since the pH did not drop below 6 during the autolysis study, it would appear either that the thioglucosidase required unavailable cofactors for the production of IAN in vitro or that there are thioglucosidase isozymes specific for nitrile production that are not isolated from mustard seed. Preliminary results from this laboratory confirm the ability of ascorbic acid

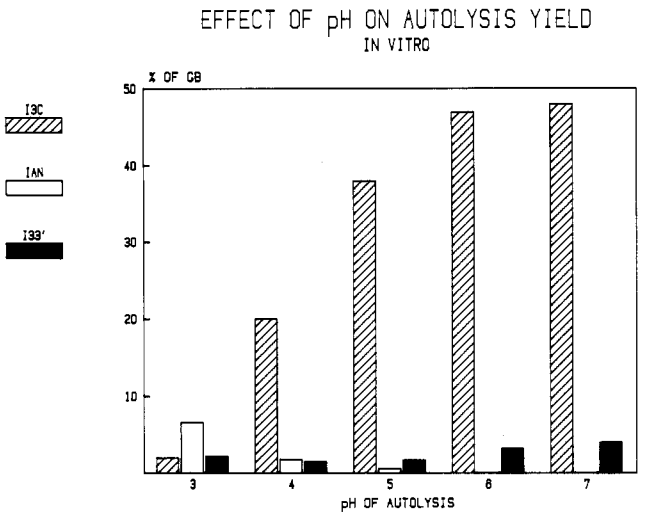

Figure 4. Effect of pH on glucobrassicin–thioglucosidase products in vitro.

to potentiate this thioglucosidase-mediated hydrolysis as reported by Ettlinger et al. (1961), although no relative increase in nitrile production was observed (Bradfield and Bjeldanes, 1986b).

These results demonstrate that I3C is the major GB autolysis product generated when plant material is disrupted such as in chewing or food preparation. Of this indole series, I3C is the most potent inducer of cytochrome P-450 dependent monooxygenases and the most potent inhibitor of chemically initiated carcinogenesis in experimental animals (Loub et al., 1975; Wattenberg and Loub, 1978). I3C is also an inducer of glutathione *S*-transferases (Bradfield and Bjeldanes, 1985; Sparnins et al., 1982). In the rat, the no-effect level of dietary I3C exposure on intestinal monooxygenases has been estimated as between 16 and 25 ppm (Bradfield and Bjeldanes, 1984), a level well below the level of I3C observed in vegetables in this experiment.

Registry No. I3C, 700-06-1; I3CHO, 487-89-8; I33′, 1968-05-4; IAN, 771-51-7.

LITERATURE CITED

Appelqvist, L. A.; Josefsson, E. *J. Sci. Food. Agric.* **1967**, *18*, 510–519.
Bradfield, C. A.; Bjeldanes, L. F. *Food Chem. Toxicol.* **1984**, *22*, 977–982.
Bradfield, C. A.; Bjeldanes, L. F. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1985**, *44*, 7378.
Bradfield, C. A.; Bjeldanes, L. F., submitted for publication in *J. Agric. Food Chem.* 1986a.
Bradfield, C. A.; Bjeldanes, L. F. University of California, Berkeley, CA, unpublished results, 1986b.
Ettlinger, M. G.; Dateo, G. P.; Harrison, B. W.; Mabry, T. J.; Thompson, C. P. *Proc. Natl. Acad. Sci. U.S.A.* **1961**, *12*, 1875–1880.
Fenwick, G. R.; Heaney, R. K.; Mullin, W. J. *CRC Crit. Rev. Food Sci. Nutr.* **1983**, *18*, 123–201.
Fetizon, M.; Gomez-Parra, F.; Louis, J. *J. Heterocycl. Chem.* **1976**, *13*, 525–528.
Graham, S. *Epidemiol. Rev.* **1983**, *5*, 38–50.
Jones, E. R. H.; Taylor, W. C. *Nature (London)* **1957**, *179*, 1138.
Leete, E.; Marion, L. *Can. J. Chem.* **1953**, *31*, 775–784.
Loub, W. D.; Wattenberg, L. W.; Davis, D. W. *J. Natl. Cancer Inst. (U.S.)* **1975**, *54*, 985–988.
Miller, J. W.; Stoewsand, G. S. *J. Plant Foods* **1983**, *5*, 67–74.
National Research Council *Diet, Nutrition and Cancer*; National Academy Press: Washington, DC, 1982.
Nomoto, M.; Tamura, T. *Agric. Biol. Chem.* **1970**, *34*, 1590–1592.
Prescott, R. Economic Research Service Report No. AGE S821124; U.. Department of Agriculture: Washington, DC, 1983.
Shertzer, H. G. *Toxicol. Appl. Pharmacol.* **1982**, *64*, 353–361.
Sparnins, V. L.; Venegas, P. L.; Wattenberg, L. W. *JNCI, J. Natl. Cancer Inst.* **1982**, *68*, 493–496.
Stoewsand, G. S.; Babish, J. B.; Wimberly, H. C. *J. Environ. Pathol. Toxicol.* **1978**, *2*, 399–406.
Virtanen, A. I. *Phytochemistry* **1965**, *4*, 207–228.
Wattenberg, L. W. *Cancer Res.* **1983**, *43*, 2448s–2453s.
Wattenberg, L. W.; Loub, W. D. *Cancer Res.* **1978**, *38*, 1410–1413.

Received for review February 26, 1986. Revised manuscript received August 6, 1986. Accepted October 10, 1986.