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Allyl Isothiocyanate and Allyl Cyanide Production in Cell-Free Cabbage Leaf Extracts, Shredded Cabbage, and Cole Slaw

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Using cell-free extracts, it was demonstrated that the myrosinase in cabbage leaves (*Brassica oleracea* L. var. *capitata L.*), responsible for hydrolyzing sinigrin to allyl isothiocyanate and allyl cyanide, had a pH optimum near neutrality; was activated by *l*-ascorbic acid by factors of 13.6 at pH 7.0 and 10.3 at pH 4.45 (the pH of cole slaw); and was activated by *d*-ascorbic acid and ascorbigen by factors of 6.0 and 2.9 at pH 4.45, respectively. Activation by *l*-ascorbic acid was terminated by oxidation with ferric ion. Cole slaw was studied for 22 days and allyl isothiocyanate concentration was found to be almost six times greater in the food product than in the shredded cabbage control. Allyl cyanide concentration in cole slaw was approximately one-tenth of the concentration of allyl isothiocyanate. Vitamin C levels in cole slaw were sufficiently high initially to activate the myrosinase, but they declined rapidly. The concentration of allyl isothiocyanate and allyl cyanide was found to be equal in shredded cabbage.

The effects of food processing on the enzymatic hydrolysis of glucosinolates present in cabbage to yield different types and abnormal amounts of natural flavorants and toxicants has received renewed interest (Daxenbichler et al., 1977). Such studies are of importance since the usual environment for the enzymatic formation of these secondary metabolites is considerably altered by the addition of the necessary ingredients to make food products.

As reported in this paper, procedures were developed to study cell-free cabbage leaf extracts to determine the properties of the cabbage myrosinase which hydrolyzes the natural glucosinolate, sinigrin; the concentrations of allyl isothiocyanate and allyl cyanide were quantitated in shredded cabbage and cole slaw. This work was initially prompted by customer complaints about variation in the flavor of commercially prepared cole slaw, and the well-known flavoring properties of these volatile components suggested closer examination of their formation.

EXPERIMENTAL SECTION

Preparation of Cell-Free Extracts. Fifty grams of dried and pulverized cabbage leaves (white cabbage purchased from local supermarkets and freeze-dried at 0 °C) were extracted with ice-cold acetone until no more color was removed. The defatted plant material was homogenized with 600 mL of pH 6.5 citrate phosphate buffer for 40 s in a Waring blender. The extract was expressed through tissue paper and centrifuged at 23 500*g* for 30 min. Control samples consisting of only the supernatant cell-free preparation or sinigrin in buffer revealed no hydrolysis products upon GLC analysis.

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Procedure for Conducting Cell-Free Extract Experiments. One milligram-sinigrin samples were weighed using a Cahn Microbalance. Samples were transferred quantitatively to a microvial; 250 μL of cell-free extract was added. The vial was shaken gently to dissolve the sinigrin, and incubation was begun. When room temperature incubation for the desired time was complete, a minute quantity of the liquid was quickly removed with a small capillary, and the pH was checked using narrow range pH paper. Any sample in which the pH had changed during incubation was rejected.

Methylene chloride (250 μL) was added to the vial followed by either 50 μL or 100 μL (depending upon the particular experiment) of a known quantity of freshly distilled ethyl isothiocyanate (Aldrich Chemical Co.) in methylene chloride as the internal standard. The vials were all shaken at constant agitation for 3 min. After centrifugation for 5 min, the methylene chloride layer was removed with a glass syringe and placed in a microvial at 0 °C until GLC analysis.

For those experiments involving enzyme activators, aqueous solutions of the suspected activator were prepared in concentrations such that 50 μL added to the 250 μL of cell-free solution resulted in the desired concentration of activator without exceeding the buffering capacity of the extract.

GLC Analysis for Allyl Isothiocyanate and Allyl Cyanide. Two microliter portions of the methylene chloride extracts were subjected to GLC with a 6 ft × 0.25 in. × 2 mm glass column packed with 20% FFAP on Chromosorb W, H/P, 100/120 mesh size (Varian Instruments). A Varian Model 1400 gas chromatograph equipped with a flame ionization detector was used for the chromatography. The detector and injector temperatures were at 160 °C, and the column temperature was programmed starting at 60 °C and rising to 110 °C at a rate of 2 °C/min. The column was allowed to cool after

reaching the maximum temperature. The nitrogen carrier gas flow was 72 mL/min.

Cell-Free Experiments. *Determination of pH Optimum and Control Experiments.* Five solutions each with a different pH were prepared from the pH 6.5 cell-free extract by titrating with either 0.2 dibasic sodium phosphate or 0.1 M citric acid. Precipitates were removed by centrifugation, and the amount of protein in each sample was determined (Lowry et al., 1951). The samples were incubated with sinigrin for 2 h and analyzed as previously described.

A control experiment was conducted using solutions of 2 μL (representing a theoretical 68% conversion of sinigrin to allyl isothiocyanate) of freshly distilled allyl isothiocyanate (Aldrich Chemical Co.) added to 3 mL of cell-free extracts in three of the buffer pHs, namely 4.2, 6.1, and 7.5. After thorough shaking, 250 μL of each solution was removed, extracted, and analyzed. Another 250-μL portion of each solution was held for 2 h before extraction and analysis.

A 100% extraction value was determined by adding 2 μL of allyl isothiocyanate to 3 mL of methylene chloride instead of an aqueous system. Using 250 μL of the dilution, the usual procedure was followed.

Nonenzyme-containing control buffer solutions of pH 4.2, 6.1, and 7.5 were prepared, and 250 μL of each was added to sinigrin samples. At the end of 2 h, the samples were extracted and analyzed for hydrolysis products.

Myrosinase Activators and Control Experiments. The procedures described are for pH 7.0 cell-free extracts using *l*-ascorbic acid. However, they are identical with procedures used in all activator experiments. Cell-free extract at pH 7.0 was added to five sinigrin samples, and *l*-ascorbic acid solutions were added to produce final *l*-ascorbate concentrations of 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M, and 5×10^{-2} M. Samples were incubated in the dark and at room temperature for 30 min. A 100% sinigrin to allyl isothiocyanate conversion control was prepared and used for comparison to insure that hydrolysis in the experimental samples was not complete during the incubation period. All samples were extracted and analyzed as before.

A control experiment was conducted by preparing solutions consisting of 2 μL of freshly distilled allyl isothiocyanate dissolved in 3 mL of pH 7.0 cell-free extract. After thorough shaking, 250 μL of the solution was placed in each of four microvials, and 50 μL of *l*-ascorbic acid solutions were added to three of the vials to give final concentrations of 10^{-5} M, 10^{-3} M, and 5×10^{-2} M *l*-ascorbate. Deionized water was added to the remaining vial, and all vials were held for 30 min prior to extraction and analysis. The three concentrations of *l*-ascorbate were prepared in sinigrin samples dissolved in pH 7.0 buffer which were then allowed to stand 30 min before analysis.

The same experiments were repeated at pH 4.45 using *l*-ascorbic acid, *d*-ascorbic acid, and ascorbigen as possible activators. Ascorbigen was synthesized from *l*-ascorbic acid and 3-indolecarbinol (Kiss and Neukom, 1966) and analyzed for residual ascorbic acid (Strohecker and Henning, 1965).

Myrosinase activation by the oxidation products of *l*-ascorbic acid was determined using cell-free extract at pH 7.0 and *l*-ascorbic acid solution (to give a final *l*-ascorbate concentration of 10^{-2} M) added to each of two sinigrin samples. Immediately after dissolution of the sinigrin, methylene chloride and internal standard were added. After a 5-min incubation period, the vials were agitated and analyzed for hydrolysis products. As soon as the 2-μL samples for GLC were removed, 10 μL of ferric sulfate solution (to give a final ferric ion concentration of 1 mM) was added to one vial, while the other received 10 μL of deionized water. Both samples were allowed to incubate for an additional 25 min and then were reanalyzed. Two nonascorbate-containing control samples were prepared and analyzed identically as described, except that the analysis after 5 min was omitted.

Shredded Cabbage and Cole Slaw Analysis. Cole slaw consisted of 2 parts dry slaw mix to 1 part cole slaw dressing. Dry slaw mix consisted of 18 parts chopped cabbage, 5 parts chopped carrots, and 2 parts celery seed. Cole slaw dressing consisted of a mixture of salad dressing, sugar, mustard, horseradish, vinegar, and stabilizer mix.

Cole slaw samples were prepared by accurately weighing 6.00 g of dry slaw into glass vials equipped with polyethylene cap-liners. Four milliliters of cole slaw dressing (amounting to 3 g) was injected onto the dry slaw mix and agitated to form a consistent food product. All samples were reweighed on an analytical balance and found to have a relative standard deviation of ±1.0%.

Samples were prepared in the following groups: (1) cole slaw for allyl isothiocyanate and allyl cyanide analysis, (2) dry slaw mix (without dressing) for allyl isothiocyanate and allyl cyanide analysis, (3) cole slaw for vitamin C analysis, and (4) cole slaw dressing (without dry slaw) for allyl isothiocyanate and allyl cyanide analysis.

Samples of cole slaw, dry slaw mix, and cole slaw dressing were analyzed by pipetting 10 mL of methylene chloride into each vial. Appropriate vial sizes were chosen to keep residual air space in each vial constant and minimal. After a 5-min agitation, the phases were allowed to separate for at least 15 min before removing approximately 3 μL of the organic layer and injecting onto the GLC column using the conditions previously described.

The results were quantitated by preparing standard curves using known concentrations of freshly distilled allyl isothiocyanate, allyl cyanide, and ethyl isothiocyanate. Representative chromatograms of reference compounds and cole slaw extract are presented in Figure 1. The lowest concentration of either compound which could be observed at maximum attenuation approached 5×10^{-4} mg/mL. GLC peak areas were measured using the "height times width at half-height" technique. Values presented are the average of three determinations.

Vitamin C concentration was determined by extracting the cole slaw sample with 1% oxalic acid, using paper chromatography to separate ascorbic acid from other reducing substances, and titrating the vitamin eluted from the chromatogram with 2,6-dichlorophenolindophenol (Strohecker and Henning, 1965).

The pH of the food product was determined by tightly packing the cole slaw around a pH electrode before taking a reading. The validity of this technique was verified by homogenizing a cole slaw sample in a Waring blender and comparing the value obtained by the direct method with the pH of the homogenate.

RESULTS AND DISCUSSION

The pH optimum for the cell-free cabbage myrosinase preparation was near neutrality (Figure 2). Similar behavior has been reported for myrosinase from other Crucifers (Björkman and Lönnerdal, 1973), and this observation suggests no abnormally high enzymatic activity for the cabbage leaf enzyme in the pH 4–5 range; the pH of the cole slaw used in the later experiments was found to be 4.45. Control experiments revealed no significant loss of allyl isothiocyanate during the 2-h incubation time, from either hydrolysis or possible addition reactions (Kawakishi and Namiki, 1969; Tapper and Reay, 1973).

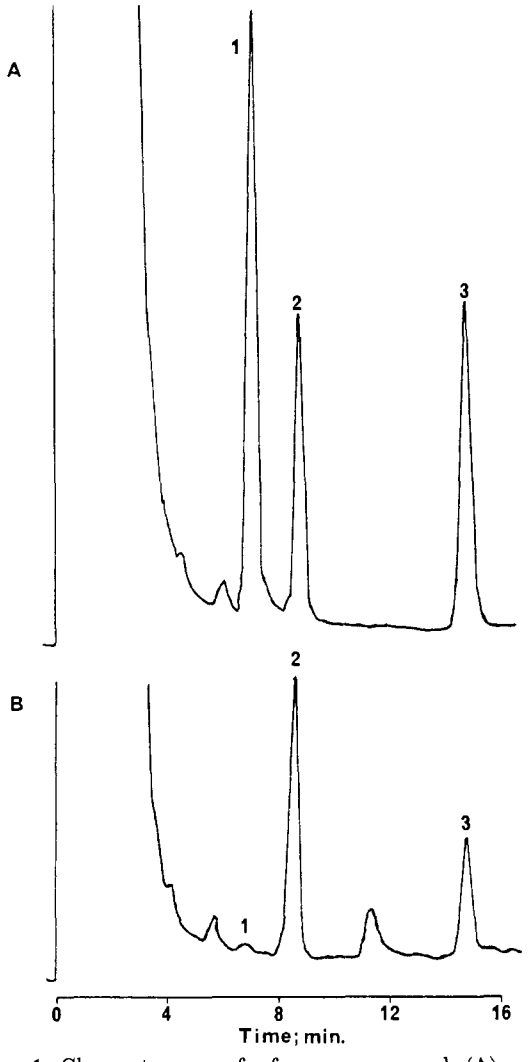

Figure 1. Chromatograms of reference compounds (A) and cole slaw extract on day 16 (B): (1) allyl cyanide, (2) ethyl isothiocyanate (internal standard), and (3) allyl isothiocyanate.

As shown by the maximum extraction control, approximately 74% of the allyl isothiocyanate was extracted in any single experiment. Sinigrin was not converted to hydrolysis products in buffer solutions without the enzyme, and its relative stability at varying pH has been documented (Schwimmer, 1961).

Clearly, cabbage leaves contain myrosinase which is capable of being activated by ascorbate derivatives (Figure 3). At 10^{-2} M *l*-ascorbic acid, the cabbage cell-free extract at pH 7.0 was activated 13.6 times as compared to the control. The activation maximum at pH 4.45 was shifted to 10^{-3} M and was slightly lower (10.3 times). The decrease in ascorbate activation at pH 4.45 may be the result of several factors.

First, the myrosinase is no longer functioning at its optimum pH, and one might expect a decrease in activity. Second, it has been proposed that a singly ionized ascorbate species activates more strongly than nonionized form (Ettlinger et al., 1961), and the ratio of ionized to unionized *l*-ascorbate is approximately 650 to 1 at pH 7.0, but only 2 to 1 at pH 4.45.

Third, one must consider the effect of pH on the intermediate released from the enzyme as it undergoes a spontaneous and nonenzymatic Lossen rearrangement to form the hydrolysis products. Previous work using myrosinase from yellow mustard seed has shown that as the environment is made more acidic, the amount of allyl

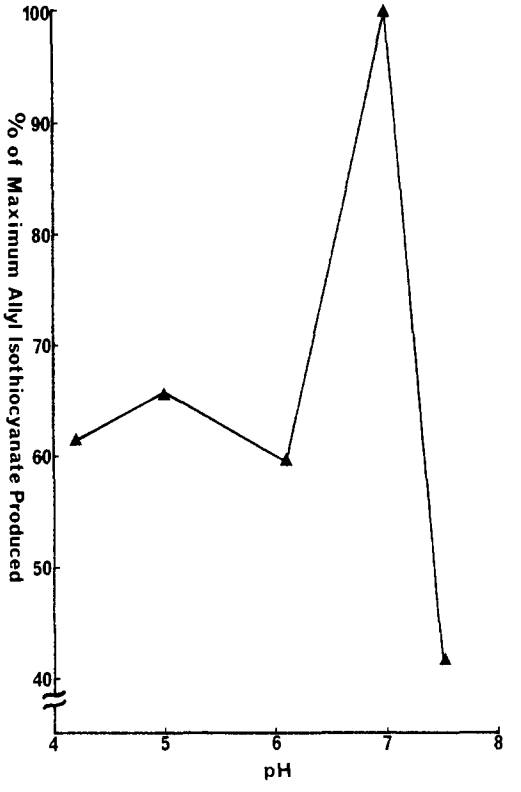

Figure 2. pH–activity plot for cabbage leaf cell-free extract.

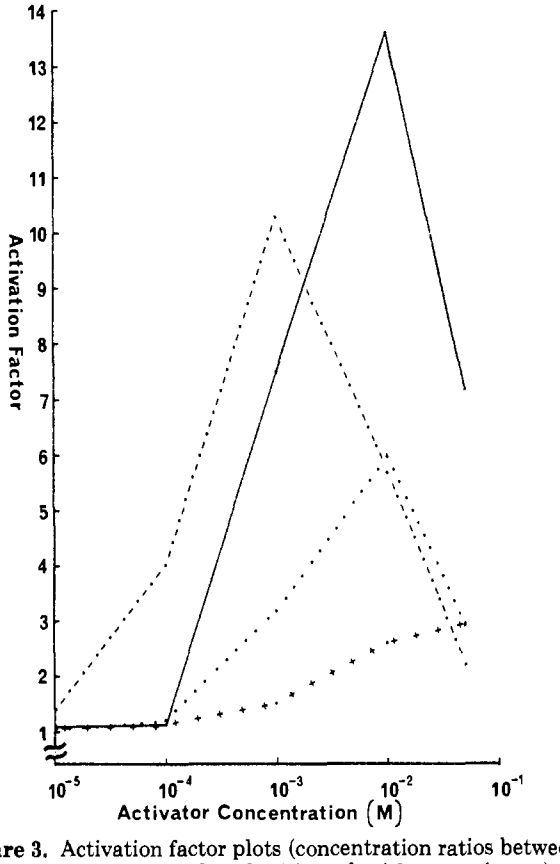

Figure 3. Activation factor plots (concentration ratios between allyl isothiocyanate produced with and without activator) of cabbage leaf cell-free extract using ascorbate derivatives: (—) *l*-ascorbic acid, pH 7.0; (----) *l*-ascorbic acid, pH 4.45; (····) *d*-ascorbic acid, pH 4.45; (·++++·) ascorbigen, pH 4.45.

isothiocyanate decreases and the concentration of allyl cyanide increases (Ettlinger et al., 1961). The protonated intermediate apparently loses sulfur to a greater extent

than the nonprotonated species. Our results are consistent with this observation. With *l*-ascorbate activation at pH 7.0, allyl cyanide was measurable in only those vials containing 10^{-2} M and 5×10^{-2} M *l*-ascorbic acid and represented 1.1 and 5.2% of the allyl isothiocyanate peak, respectively. At pH 4.45, the measurable quantities were in the vials containing 10^{-4} M, 10^{-3} M, 10^{-2} M, and 5×10^{-2} M *l*-ascorbic acid and now represented 11.0, 11.6, 12.8, and 16.4% of the allyl isothiocyanate peak. The absolute amounts of allyl cyanide exactly duplicated the trends seen with allyl isothiocyanate, i.e., the most amount at 10^{-2} M *l*-ascorbate at pH 7.0 and 10^{-3} M *l*-ascorbate at pH 4.45. However, its relative contribution as compared to allyl isothiocyanate increased throughout the experiment. As will be discussed later, the amount of allyl cyanide in cole slaw at pH 4.45 was 10–12% of the allyl isothiocyanate concentration.

The activation observed with the *d*-ascorbic acid parallels the activation seen with the *l* isomer, but the activation factor (6.0) is lower (Figure 3). This decrease in activity has been observed with other Crucifer myrosinases (Ettlinger et. al., 1961) and might be explained by proposing that the arrangement of the hydroxyl group affects the binding of the ascorbate to the enzyme surface.

Ascorbigen has not previously been tested with regard to myrosinase activation. The activation factor of 2.9 is much lower than observed with the other derivatives, and, in the concentration range tested, its activity does not parallel ascorbate. The amount of residual *l*-ascorbic acid present cannot account for activation at even the highest concentration of ascorbigen; the titratable vitamin C in the 5×10^{-2} M ascorbigen amounts to only 6.5×10^{-6} M ascorbate, which is far below the activating range. The decomposition of ascorbigen to ascorbic acid and other products during the course of the experiment is doubtful since the compound is prepared in best yields from ascorbic acid and 3-indolecarbinol at pH 4.0 (Piironen and Virtanen, 1962). The fact that ascorbigen still retains vitamin C activity in guinea pigs may or may not be related, but it does seem to add support to the idea that the compound is capable of cofactor activity (Virtanen and Kiesvaara, 1963).

The oxidation of ascorbic acid by ferric ion had a significant effect upon activity. The addition of ferric ion decreased the formation of allyl isothiocyanate slightly by increased conversion to allyl cyanide, but mainly by oxidizing the ascorbic acid. In both the ascorbate and nonascorbate containing vials, the addition of ferric ion increased the amount of allyl cyanide by 3 to 5%. However, the addition of ferric ion to the ascorbate containing vials decreased allyl isothiocyanate concentration by more than three times as compared to nonascorbate-containing controls. These observations have previously been documented with other Crucifer myrosinases (Ettlinger et al., 1961; Tapper and Reay, 1973).

As shown by this study, the concentration of allyl isothiocyanate is significantly higher, by an average of six times, in the food product than in the dry slaw mix (Figure 4). When processed into cole slaw, the allyl isothiocyanate concentration was approximately 17 ppm as opposed to 3 ppm for the shredded plant material. Allyl cyanide was present in cole slaw in concentrations of about one-tenth of the allyl isothiocyanate. This difference very closely approximates the values observed with *l*-ascorbic acid activated cell-free cabbage extracts at the same pH.

In the dry slaw, allyl isothiocyanate and allyl cyanide concentrations are not significantly different. The similarity in the concentrations of these two compounds has

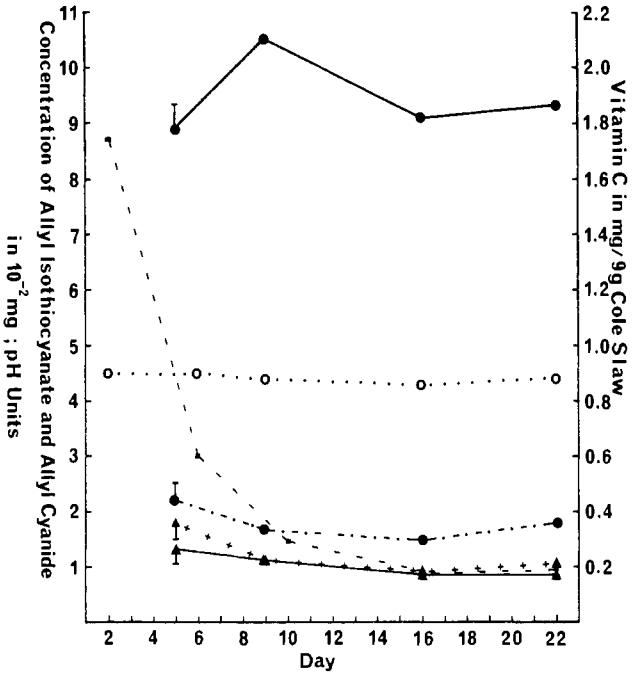

Figure 4. Food product analysis: allyl isothiocyanate: (·——·) cole slaw, (·····) dry slaw; allyl cyanide: (▲——▲) cole slaw, (▲+++▲) dry slaw; (O···O) pH of cole slaw; (■- - -■) vitamin C in cole slaw.

been observed when studying the cooking volatiles from cabbage (Macleod and Macleod, 1968), but these results are difficult to compare to fresh cabbage since the addition of heat quite likely alters the composition. Recently, the autolysis of fresh cabbage at natural pH (5.6–6.3) revealed a greater concentration of nitriles instead of the related isothiocyanates; however, the hydrolysis products of sinigrin were not specifically studied (Daxenbichler et al., 1977). The importance of the nitriles in foods with regard to taste or toxicity is not completely known, but about 10 mg of a nitrile, benzyl cyanide, is consumed in 30 g of garden cress with no known effect on man (Virtanen, 1965).

The concentration of allyl cyanide in cole slaw is not significantly different from that in dry slaw. The activation observed for allyl isothiocyanate formation did not change the allyl cyanide concentration.

Although not represented on the graph, the analysis of freshly cut cabbage reveals no detectable amounts of either allyl isothiocyanate or allyl cyanide. Future experiments will be needed to determine how rapidly the rise of allyl isothiocyanate and allyl cyanide occurs in both cole slaw and cabbage.

Also not graphed was the observation that the cole slaw dressing contains no detectable quantities of either compound. Although both horseradish and mustard contain allyl isothiocyanate, their small concentrations in the dressing makes their detection impossible using these procedures. The effect of the extremely diluted myrosinase from these ingredients was anticipated to be negligible.

For the cole slaw samples, the relative standard deviation for allyl isothiocyanate determinations was ±4.4% and for allyl cyanide, ±11.3%. In dry slaw, the relative standard deviation for allyl isothiocyanate was ±16.4% and for allyl cyanide, ±13.5%. The poorer precision for the components in lower concentration was most likely the result of less consistent recorder response when using maximum attenuation.

It seems reasonable to suggest that ascorbic acid is at least partly responsible for the increased quantities of allyl isothiocyanate in cole slaw. If one assumes that cole slaw

is 80–90% water, the concentration of vitamin C on day 2 of the experiment would range from 1.2×10^{-3} to 1.4×10^{-3} M. As shown in the cell-free experiments, this is definitely within the range of maximum activation at pH 4.45.

The results of this study indicate that food processing may have a significant effect upon the enzymatic release of natural flavorants and toxicants.

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Volatile Flavor Components of Licorice

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Sixty-three compounds never found before in heated licorice essential oil have been identified by use of GLC, GLC–MS coupling, and IR spectrometry. In addition, a few preliminary results on some components identified in unheated licorice juice are reported. Many heated licorice compounds are furan derivatives; this fact may be due to pyrolysis and condensation reactions which may occur during heating between sugars of which licorice roots are very rich. The most abundant components are: acetol, propionic acid, 2-acetylpyrrole, 2-acetylfuran, and furfuryl alcohol. None of the identified compounds is alone responsible for licorice flavor, while on the other hand, total extract shows a typical licorice aroma, indicating that this may be due to an integrated response to the proper mixture of the proper volatiles, rather than to the odor of one or two components.

Continuing our past studies on volatile drug components (Frattini et al., 1976), we have obtained some results in a few preliminary experiments carried out on samples of unheated licorice (Frattini et al., 1976). We now report our data on heated licorice flavoring components compared with those of unheated licorice.

Licorice (*Glycyrrhiza glabra* L.) is a very well-known plant since antiquity, known in ancient Egypt and in India as well as in Greek–Roman medicine. Since early times it has been employed as an emollient and expectorant; it is still under discussion whether it has therapeutic value for the treatment of gastric ulcer or not. Generally, licorice is employed in confectionery and beverages industries; the commercial product comes from the roots heated at 130–150 °C: juice is collected and solidified in black pats with typical flavor and taste.

Glycyrrhiza glabra L. var. *typica* grows in Italy, while the variety *glandulifera*, also named Russian licorice, grows in Russia, Turkey, and Asia Minor.

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Owing to its great pharmacological and commercial interest, components of licorice have been widely studied; we know many of its active principles and, particularly, substances which give it taste: glycyrrhetic acid, liquiritigenin, liquiritin, and glycyrrhizin (the latter is responsible for its sweet taste, with a sweetening power 50 times greater than sugar). Very little, however, is known of its volatile flavor components, although its essence oils have been mentioned (Haensel, 1899; Isaev, 1934).

In our analyses we use GLC, GLC–MS coupling, and infrared spectrometry. Each approach is discussed below. Sixty-three compounds (Table I), never found before in heated licorice, have been identified by comparison of their retention times and mass and/or infrared spectra with those of authentic samples or on the basis of literature spectral data.

EXPERIMENTAL SECTION

Licorice pats (8 kg) (manufactured in Calabria by handicraftsmen or obtained from foreign sources) were crushed and suspended in water (6 L). This slurry was extracted in Soxhlet with 6 L of dichloromethane for 1 week. The extract, concentrated to 250 mL, was again water-suspended and steam distilled in a two-phase continuous distiller (1 L of water, 0.3 L of dichloromethane). Distillation was controlled at regular intervals; the qualitative composition of the extract was constant