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HPLC Separation and Comparison of the Browning Pigments Formed in Grapefruit Juice Stored in Glass and Cans

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Pigments associated with browning in grapefruit juice stored in cans and glass containers were separated by an HPLC procedure employing a ternary solvent gradient with a C_{18} column. The solvent system consisted of water, acetonitrile, and tetrahydrofuran. The types and amounts of pigments are container-dependent. Greater numbers of browning pigments were formed in bottles than in cans, and fewer but more intense browning pigments were formed in cans. Some pigments were unstable and diminished or disappeared at longer storage times, whereas others increased with increasing storage time. Amounts of late-eluting, nonpolar browning pigments increased with increasing storage time. These pigments were a major source of browning in juices stored both in cans and in glass bottles. The number and quantities of browning pigments observed increased dramatically with increasing storage temperature.

Under nonrefrigerated storage conditions, processed grapefruit juice darkens with increasing storage time due to the formation of browning pigments. This nonenzymatic browning has been a visual defect of processed grapefruit juice for many years and is one of the factors determining shelf life. Browning in grapefruit juice is accompanied by an increase in off-flavors, which also limits shelf life.

Browning in citrus juices has been studied by a number of investigators (Joslyn, 1957; Wolfrom et al., 1974; Lee and Nagy, 1988), and the subject has been reviewed recently by Handwerk and Coleman (1988). The general consensus of this review is that citrus browning compounds require the interaction of reducing sugars and/or sugar degradation products with non-sulfur-containing amino acids. The vast majority of citrus browning studies have attempted to investigate the initial reactants associated with the production of browning pigments. By adding specific components to juice or model systems, the compounds that accelerate or inhibit browning have been identified. However, virtually nothing is known about the actual browning compounds formed in citrus juices. In addition, while it is generally known that browning is more of a problem in glass containers than in tin-plated steel cans, the number or types of browning pigments formed in each case are unknown.

Therefore, the purpose of this study was to develop a procedure that would allow characterization of the browning pigments formed in grapefruit juice. Secondary

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goals included examination of container effects (glass vs can), temperature, and time on the number and types of grapefruit juice browning pigments formed.

EXPERIMENTAL SECTION

Samples. Cans (6 oz) and bottles of white, commercial grapefruit juice (from concentrate) (7 oz) were obtained from a Florida citrus processor on the same day and were stored at 10, 20, 30, 40, 50, and 60 °C. Control samples were stored at −10 °C. Cans were tin-plated with enamel-coated lids. For each storage temperature random triplicate samples were analyzed after 0, 3, 6, 9, 12, 15, 18, and 21 weeks of storage.

Sample Preparation. The sample preparation technique was essentially that developed by Klim and Nagy (1988). After shaking, each container was opened and a 50-mL sample taken. Each sample was freeze-dried on a Thermovac Model FD-ULT-6 (Thermovac Industries Corp., Copiague, NY) freeze-drying unit. Freeze-drying consisted of freezing a shell of the juice sample in a 500-mL round-bottom flask and then drying under vacuum (20 Pa) for 16 h. The dried residue was extracted with 30 mL of methanol, filtered through Whatman No. 42 filter paper, and then brought to volume in a 50-mL volumetric flask with methanol. Samples were stored overnight in a freezer at −10 °C, filtered through a 0.45-mm filter (Magna nylon 66 membrane filter), and held at −10 °C until analysis. Browning index, absorbance, was measured at 420 nm. Some of the concentrated samples required dilution with methanol.

Reagents and Chemicals. All solvents were HPLC grade from Fisher Scientific Co. (Fair Lawn, NJ). Laboratory deionized water was further purified with a Milli-Q system (Millipore Corp., Milford, MA).

Equipment. A Perkin-Elmer (Norwalk, CT) Series 4 ternary gradient pump was used to produce the solvent gradient. A Waters Associates (Milford, MA) Model 710B WISP autosampler was used to inject the samples. Column effluent was monitored on a Waters Model 440 UV–vis detector equipped with a 436-nm filter. Chromatographic peaks were integrated on a Shimadzu (Columbia, MD) Model C-R3A recording integrator.

Chromatographic Conditions. A 3-μm Supelco (Bellefonte, PA) C_{18} column, 4.6 mm (i.d.) × 150 mm, was used with a Brownlee (Santa Clara, CA) Speri-5 C_{18} precolumn, 4.6 mm (i.d.) × 50 mm. A Supelco low dead volume in-line filter was used in front of the precolumn to trap particulates. A ternary solvent system consisting of water, acetonitrile, and 50% aqueous THF was used to develop the solvent gradient. The specific gradient program used is shown:

time	% H_2O	% CH_3CN	% aq THF
0	99	0	1
5	97	0	3
38	89.5	3.5	7
78	70	5	25
98	55	5	40
103	0	90	10
108	0	90	10
111	99	0	1

Flow rate was 1.0 mL/min. All gradient changes were accomplished linearly. The column was equilibrated for at least 15 min before the next sample was injected. Sample injection volume was 15 μL. Solvents were degassed on an aspirator vacuum in an ultrasonic bath. Flow rate was 1.0 mL/min.

Peak Retention Time Precision. The variation in peak retention time was established from successive triplicate injections of the juice stored in glass for 3 weeks at 60 °C. Differences in observed retention times for the major peaks were calculated. To determine long-term precision, the series of juice samples stored in glass at 60 °C for 3–21 weeks were run on an autosampler over a 14-h period. The variation in observed retention time for the peak occurring at 99 min was calculated as a worst case example since it would be affected by errors in earlier gradient steps. Average retention time, standard deviation, and relative standard deviation were calculated for this peak from the seven chromatograms.

In reversed-phase (C_{18}) chromatography, substances eluting at the solvent front are considered to be polar. Those eluting with 90% acetonitrile, 5% THF, and 5% water are very nonpolar, and those in between are of intermediate polarity.

RESULTS AND DISCUSSION

Chromatographic Separation. Resolution of the major browning pigments in grapefruit juice proved to be a formidable task. Since the most severe browning appeared in juices stored for 12 weeks in glass at 60 °C, this sample was used to develop the separation. Juices stored for longer periods of time at this temperature appeared more black than brown and, while analyzed, were not used to develop chromatographic conditions.

Initial separation attempts employing a water–acetonitrile gradient solvent system and a C_{18} column indicated that this sample contained many browning compounds. Browning in citrus juices is usually measured at 420 nm (Meydav et al., 1977). For this study 436 nm was selected to monitor the browning pigments to emphasize those compounds of a yellow-orange hue rather than a yellow or yellow-green hue.

Resolution of the early-eluting peaks was improved through the use of THF rather than acetonitrile as the nonpolar component of the mobile phase. Since some of the browning pigments are not well retained on the C_{18} column, the initial amount of THF used at the start of the gradient was minimal. Use of a 1:1 dilution of THF in the solvent reservoir was necessitated to avoid compositional errors encountered when attempting to pump less than 1% of a solvent. Acetonitrile was used as the other nonpolar solvent modifier to elute the less polar browning pigments. As shown in Figure 2 (60 °C), the first 40 min of the chromatogram contains a large number of small, partially resolved peaks even though the amounts of nonpolar modifiers (THF and acetonitrile) used were very small. It was considered impractical to improve the resolution of the browning pigments in this region of the chromatogram by increasing the gradient time, since total chromatographic run time was already over 2 h. As the proportion of THF increased between 38 and 78 min, several browning pigments of intermediate polarity eluted. These compounds appear to be well separated except for the doublet at 51 min. During the last 10 min of the chromatogram the mobile phase consisted of 90% acetonitrile, 5% THF, and 5% water. The very nonpolar browning pigments eluted under these conditions as a large unresolved peak at 104 min. Attempts to modify the solvent to resolve this peak suggested that it was composed of a large number of compounds of very similar polarity. These compounds are probably the polymeric compounds other workers have predicted.

Retention Time Precision. Since there are no standards for the pigments associated with browning and the solvent gradient employed was complex, it was extremely important to determine the reproducibility of peak retention time. This would assure that peaks occurring at different retention times in different chromatograms were due to different compounds. Triplicate successive injections of the same sample indicated that peak retention times were extremely reproducible with differences ranging from 0.18 min for peaks occurring during the first half of the chromatogram to 0.54 min for peaks occurring during the last half. Long-term reproducibility (14 h) of peak retention times calculated for the peak occurring at 99 min had a relative standard deviation, RSD, of 0.0893%. Since peak retention times were highly reproducible, it was possible to conclude that peaks differing by more than 0.5 min were due to different compounds.

Temperature Effects. The effect of temperature on the relative amounts of browning pigments is shown in Figure 1 for juices stored in glass and cans for 6 weeks at

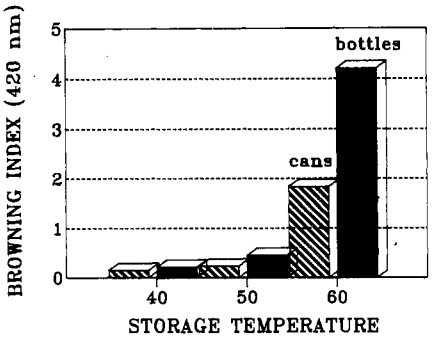

Figure 1. Comparison of total browning measured as a browning index (absorbance, 420 nm) from grapefruit juice stored in bottles and cans for 6 weeks at 40–60 °C.

40, 50, and 60 °C. Similar trends were observed for other storage times. More browning was consistently observed in bottles than in cans. However the amount of browning for either cans or bottles increased slowly and then increased exponentially. This slow initial rate of browning followed by a later rapid acceleration of browning was also noted by Saguy et al. (1978) in grapefruit juice.

With the ability to separate pigments associated with browning, it is now possible to study individual pigments formed under different storage conditions. As shown in Figure 1, the greatest amounts of pigments were formed at the highest storage temperature. However as shown in Figure 2 for bottle stored juice, the greatest *number* of browning pigments are also formed at the highest storage temperature. There were virtually no peaks from the control sample or the sample stored at 30 °C. As storage temperature increased, peaks in the region of 23 and 70 min were observed. Peak heights for some of these peaks increased regularly with increasing storage temperature whereas others decreased. Examples of this behavior are shown in Figure 2 for the peaks labeled a–c. At 40 °C peak a can be scarcely detected whereas peaks b and c are prominent peaks. However at 50 °C peak a has more than doubled in size whereas peaks b and c have diminished to approximately one-third their former size. These trends are continued at 60 °C to the point where peak b can no longer be detected, peak c is barely detected, and peak a is one of the larger peaks in the chromatogram. This behavior suggests that the compound responsible for peak b may be an intermediate product that is rapidly consumed under conditions of more rapid kinetics (higher temperatures).

Another interesting feature is the pronounced increase of the last chromatographic peak(s), which contains the most nonpolar browning pigments. It should be noted that these browning pigments increased most at 60 °C; the increase at 40 and 50 °C was relatively minor. Small peaks in this region were also observed in chromatograms from control juices, suggesting that certain nonbrowning pigments such as carotenoids partially contribute to these peaks.

The effects of storage temperature on the relative amounts of browning pigments formed in grapefruit juice stored in cans differ somewhat from that in bottles. As in the case with bottles, there was virtually no difference in the chromatograms from control juices and those stored at 30 °C in cans. In both instances they were devoid of peaks except at the solvent front and small peaks at the very end. Juices stored at 40 °C exhibited only minor peaks that increased slightly at 50 °C storage. There was no clear trend of specific peaks increasing or decreasing with different storage temperatures as there was with glass-stored juices. However, there was a tremendous

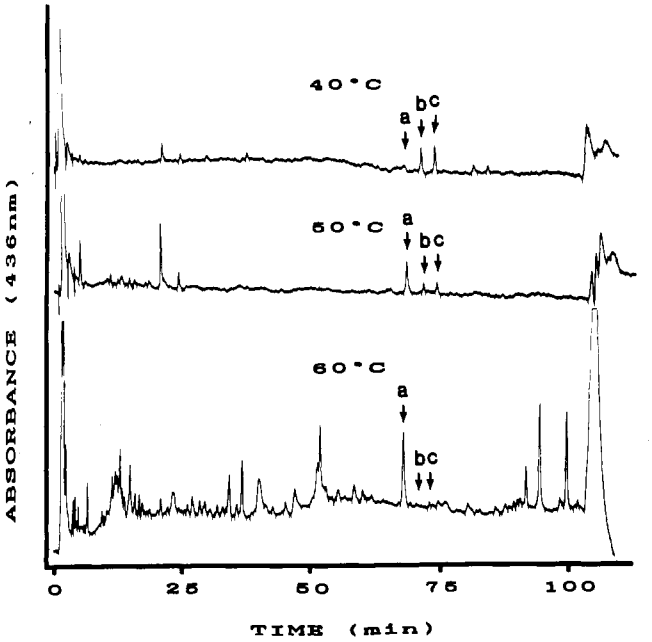

Figure 2. Chromatograms of grapefruit juice browning pigments formed in glass bottles when stored at 40, 50, and 60 °C for 12 weeks. See text for chromatographic details.

difference between the browning observed at 40 and 50 °C and that observed at 60 °C. Juices stored at 60 °C yielded chromatograms that were characterized by a single large peak at 63 min and two moderately tall peaks after it. One of these later peaks was the broad peak that occurs at approximately 105 min. In general this peak increased with increasing storage temperature, albeit not as markedly as with glass-packed juice.

Since both the amounts and numbers of compounds formed at 60 °C are considerably different from that observed at lower temperatures, there is a natural question as to whether this represents an expected 2-fold increase in the rate of browning (compared to juices stored at 50 °C) or whether this storage temperature is so high that completely different reactions are occurring. The answer to this question is container dependent. Juice stored in bottles at 60 °C continue trends observed at lower storage temperatures (see Figure 2). However, visual observations suggest that juices should not be stored in cans at 60 °C for more than 12 weeks. Excessively dark juices and extensive can corrosion indicated that additional reactions of an indeterminate nature were occurring.

Can vs Glass Containers. As shown in Figure 3, a greater number of browning pigments were formed in glass than in cans. Grapefruit juices stored in cans rather than glass exhibited fewer but larger peaks. Perhaps one of the most surprising observations was the general lack of common browning compounds. The types of browning pigments observed appear to be highly container dependent. Chromatograms from juices stored in cans for 9 weeks exhibited a major peak in the region of 63 min and a few minor peaks eluting before and after it. This is in contrast to the vast number of smaller peaks observed for a similar sample stored in glass. Few if any of the browning pigments observed in chromatograms from juice stored in glass containers were also observed in juices stored in cans.

Another feature worth noting is the difference in the last peak of the chromatogram. Both examples shown in Figure 3 contain large amounts of nonpolar browning pigments eluting after 100 min, but juice stored in bottles contained substantially more of these pigments than juice stored in cans. As discussed earlier, this late-eluting peak

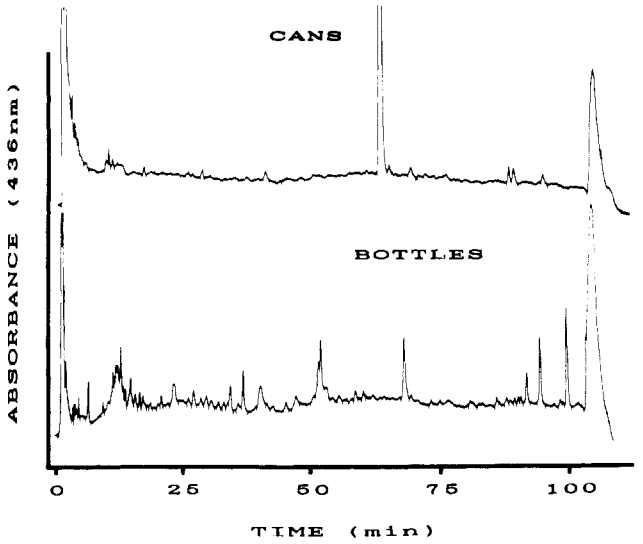

Figure 3. Chromatograms of browning pigments formed in grapefruit juice stored in glass and cans at 60 °C for 9 weeks. See text for chromatographic conditions.

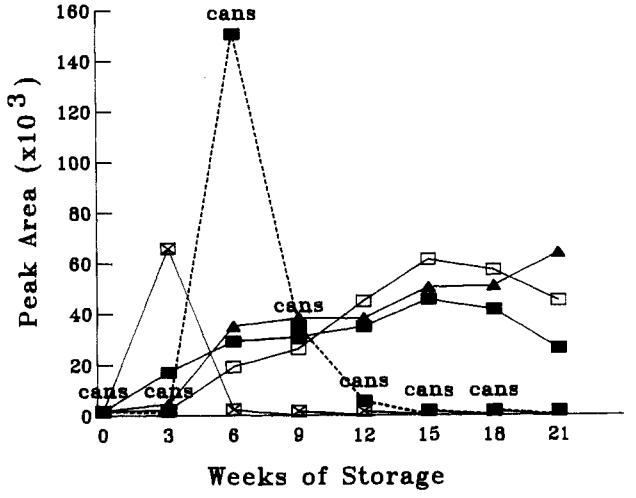

Figure 4. Changes in selected browning pigment peaks formed in grapefruit juice stored in glass and cans at 60 °C from 0 to 21 weeks: ■, peak at 63 min (cans) (peak area/10); ⊠, peak at 27 min (glass); ■, peak at 67 min (glass); □, peak at 94 min (glass); ▲, peak at 99 min (glass).

is not as pronounced at lower storage temperatures for either cans or bottles.

Storage Time. In general, browning increased with increased storage time. For example, total browning (browning index) for juices stored at 50 °C in glass for 0, 3, 6, 9, 12, 15, and 18 weeks were 0.108, 0.300, 0.451, 0.553, 0.724, 0.821, and 0.927 AU, respectively. The increase in total browning was due to a combination of an increase in concentrations of individual browning pigments and formation of a greater number of browning pigments. However, some of the browning pigments were unstable. Shown in Figure 4 are changes in peak area for grapefruit juice browning pigments from cans and glass stored at 60 °C for various lengths of time. Some browning pigments such as the peak observed at 27 min from glass containers and also the major peak at 63 min from cans formed rapidly and then diminished just as rapidly. It should be noted that the peak area for the peak at 63 min from cans is actually 10 times greater. It was drawn at one-tenth scale to allow comparison of the trends of the smaller peaks from glass containers on the same graph. Other peaks from glass-stored juice, as for example the peak at 67 min, increase regularly with storage time, reaching a maximum

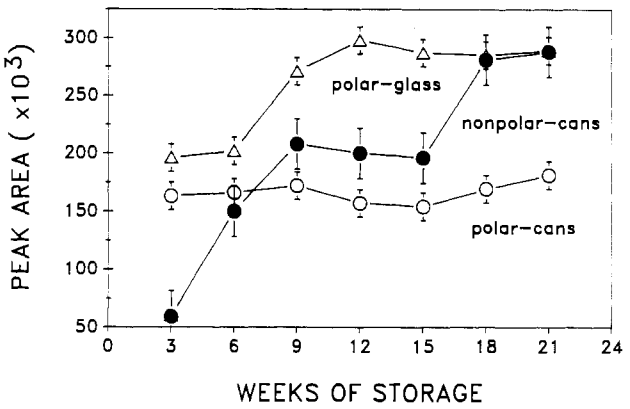

Figure 5. Grapefruit juice polar and nonpolar browning pigment peak area changes. Juices were stored in cans and glass bottles at 50 °C. Polar compounds are those compounds eluting during the first 2 min and nonpolar pigments are those pigments eluting after 103 min.

at 15 weeks of storage. After 15 weeks the compound responsible for this peak and the peak at 94 min decrease steadily upon further storage. The instability of these peaks suggests that they are intermediate pigmented compounds that are subsequently converted to less polar pigmented material since the last broad peak also increased as did total browning.

Time vs Temperature Effects. In general, the most browning pigments were observed for those juices stored at the highest temperature (60 °C) and the longest time (21 weeks).

Storage temperature appeared to have a greater effect than storage time. Juices stored at 30 °C in both glass and cans exhibited little browning regardless of storage time. Juices stored at 60 °C exhibited a number of browning pigment peaks after only 3 weeks of storage. The relative number and intensity of these peaks increased up to 6 weeks of storage and then remained essentially unchanged from 6 to 21 weeks except for the last peak in the chromatogram. This peak contained a host of nonresolved highly nonpolar browning pigments whose peak areas increased with increasing storage time. For similar samples stored in cans at 60 °C the numbers and amounts of intermediate polarity browning pigments increased in the same proportion as the very nonpolar pigments.

Polar vs Nonpolar Browning Pigments. Shown in Figure 5 are the changes in peak area for both the very polar and nonpolar pigments formed in grapefruit juice stored in glass and tin cans stored at 50 °C. It is recognized that the peak areas from the nonresolved compounds at the solvent front of the chromatogram are due to more than browning pigments. However, contributions such as changes in refractive index should be constant as the injection volume was constant. Therefore, any changes in these peaks should be due to very polar browning pigments. As shown in Figure 5 the amount of very polar browning pigments formed in cans is essentially unchanged with increasing storage time. Polar pigments in glass-stored juice increase slightly with increasing storage time and then plateau after about 12 weeks of storage.

Nonpolar pigments were inadequately quantified because it was difficult to control integration of the very broad and ill-defined peaks that eluted in the chromatogram. Small peaks in this region were also observed in the control juice and were probably due to carotenoids (Gross, 1977). The total carotenoid content of Marsh white grapefruit pulp is very small, i.e., about 0.24 μg/g fresh weight (Romjaro et al., 1979). The contribution from these cartenoids should remain constant or diminish under the

accelerated storage conditions used in this study. Therefore, any increase in these late-eluting peaks must be due to highly nonpolar browning pigments. These peaks appeared to be quite large in glass-stored juice in comparison to juice stored in tin-plated cans. (The trend for the nonpolar pigments in glass-stored juice could not be accurately quantified and, therefore, is not shown in Figure 5.) The trend for nonpolar pigment peaks from cans is shown in Figure 5. There is a dramatic increase in these pigments with increasing storage time. These late-eluting, nonpolar pigments appear to be a major contributor to the browning observed in canned grapefruit juice. It can be seen from Figures 2 and 3 that the late-eluting, nonpolar pigments in glass-stored grapefruit juice also constitute an important component of the total browning observed.

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Effect of γ-Irradiation on Thiol Compounds in Grapefruit

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The effect of ^{60}Co γ-irradiation on thiol compounds in grapefruit was investigated. Thiols were separated by HPLC and measured with a fluorescence detector. Reduced glutathione (GSH), cysteine (CySH), cysteinylglycine (CySGly), and a number of unknown peaks were observed in unirradiated grapefruit. GSH was the main thiol at an average concentration of 143.3 μM. GSH content exponentially decreased with increased radiation doses, and after 100 krad only 80% of the original remained. The *G* value based on the result of 100 krad was 0.29. Authentic GSH in water or citrate buffer (pH 3) was converted mainly to its oxidized form (GSSG) with γ-irradiation. GSSG in irradiated grapefruits showed no equivalent increase, however.

Recent animal feeding studies have explicitly proved the safety of irradiated foodstuffs (International Food Irradiation Project, Report R51, 1979). On the basis of these results, all foods irradiated below 10 kGy (1 Mrad) were recognized as suitable for human consumption by a Joint Expert Committee of the International Atomic Energy Agency (IAEA), the Food and Agriculture Organization (FAO), and the World Health Organization (WHO). This decision and progress in irradiation technique development soon led to the appearance of irradiated foodstuffs on the international market. An identification method for irradiated foods thus became desirable to distinguish them from unirradiated foods.

Various components (proteins, starch, vitamins, carbohydrates, lipids, etc.) (Elias and Cohen, 1983; Stockhausen and Bögl, 1978; Simic, 1978; Schubert et al., 1973; Drijver et al., 1986; Moshonas and Shaw, 1982, 1984) were analyzed in food before and after irradiation. The use of measurements of such components for identification however was limited because changes could also occur in these compounds as a result of heating or storage conditions.

As a part of our research on the identification of irradiated foodstuffs, the differences between the components in grapefruit before and after irradiation have been investigated. Among various components in grapefruit, thiols seem to be one of the most sensitive group of compounds, as suggested by radiation biochemical studies (Meyers and Bide, 1966; Sutherland and Pihl, 1968). The determinations of thiol compounds are carried out generally by the following methods: electrochemical detection (Saetre and Rabenstein, 1978; Kreuzig and Frank, 1981; Bergstrom et al., 1981; Shimada et al., 1982), UV–vis labeling (Kuwata et al., 1982; Chang et al., 1983; Nishiyama and Kuninori, 1984; Moroney et al., 1984), and fluorescence labeling (Kanaoka, 1980; Lankmayr et al., 1981; Newton et al., 1981; Werkhoven-Goewie et al., 1981). The most appropriate method of estimating the individual thiol seems to be fluorometry due to its selectivity and sensitivity.

The derivatizing agent ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) (Imai et al., 1983; Toyo'oka and Imai, 1984) was used in this study. It is highly soluble in water, is selective with respect to thiol-containing compounds, and has well-defined fluorescence characteristics (excitation at 380 nm, emission at 510 nm) (Figure 1); its

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