Determination of Cysteine and Glutathione in Fruit by High-Performance Liquid Chromatography

A new method is described for the determination of cysteine and glutathione in fruit by high-performance liquid chromatography (LC). The procedure involves acidifying, centrifuging, and filtering the fruit juice, followed by a liquid chromatographic separation on Zipax SCX cation-exchange resin and detection with a mercury-based electrochemical detector. The LC analysis time is approximately 6 min, with detection limits of ca. 2×10^{-6} M glutathione and 3×10^{-6} M cysteine. The procedure is general and can be extended to the determination of cysteine and other nonprotein thiols in plant protein hydrolysates and other food products.

The main nonprotein thiols in plants are cysteine and glutathione. Exceptions include the mung bean, which contains homoglutathione (Carnegie, 1963). Nonprotein thiols are thought to be involved in various aspects of plant physiology, including such processes as protoplasmic streaming (Kamiya, 1960), electron transport and phosphorylation during photosynthesis (Jocelyn, 1972), cell division (Stonier and Jang, 1973), and keeping ascorbic acid in its reduced form (Zuman, 1951). They also are thought to be involved in frost hardiness (Levitt et al., 1961, 1962; Kohn and Levitt, 1966), and it has been demonstrated that thiol compounds and thiol reagents can regulate the ripening of Bartlett pears (Frenkel, 1976). Knowledge about these processes at the molecular level, which at present is limited, requires selective and sensitive methods of analysis. There are few reports in the literature on the determination of nonprotein thiols in fruits and vegetables.

In this paper, we describe a high-performance liquid chromatography (HPLC) method for the simultaneous determination of cysteine and glutathione in fruits. The procedure involves separation on Zipax SCX cation-exchange resin followed by detection with a sensitive mercury-based electrochemical detector (Rabenstein and Saetre, 1977). The approach described is generally applicable, and it should be easily extendable to the determination of cysteine and other thiol compounds in plant protein hydrolysates and other food products.

EXPERIMENTAL SECTION

Chemicals. Cysteine (Nutritional Biochemicals Corporation) and glutathione (Sigma) were used as received. Their purities were determined to be 95.0 ± 0.5 and 97.3 ± 0.5%, respectively, by titration procedures involving coulometrically generated Br_2 for the cysteine assay and I_2 for the glutathione assay. The assay values were used in the preparation of standard solutions. Doubly distilled water was used throughout.

Apparatus. The LC system was constructed from a Milton Roy minipump; tubing, connectors, valves, and columns from the Cheminert Division of Laboratory Data Control, and a mercury-based electrochemical detector (Rabenstein and Saetre, 1977). The LC system has been described in detail (Rabenstein and Saetre, 1977; Saetre and Rabenstein, 1978). The electrochemical detector was operated at +0.1 V vs. the saturated calomel electrode (SCE). Separation of cysteine and glutathione was performed on a 50 × 0.2 cm column dry-packed with Zipax SCX strong cation-exchange resin. Aliquots of 10 μL were injected onto the column with a syringe. A 0.5% H_3PO_4 solution deaerated with O_2-free nitrogen was used as eluent at a flow rate of 0.5 mL/min.

Procedure. One-half milliliter of fruit juice was added to a 10 × 75 mm test tube containing 0.5 mL of 2% H_3PO_4. The contents were mixed well and centrifuged for 5 min at 2500 rpm. The supernatant was withdrawn with a 1-mL tuberculin syringe and forced through a 0.45-μM pore diameter filter, which was held in a filter holder (Millipore) fitted to the syringe. The filtrate was collected in a 10 × 75 mm test tube. All fruits were obtained from a commercial distributor. Juice was obtained from the fruits by making a small cut in the skin and squeezing.

Standard mixtures of glutathione and cysteine were prepared in 1% H_3PO_4. Calibration curves were prepared in terms of the height of the chromatographic peak (Figure 1) vs. thiol concentration.

Table I. Concentrations of Cysteine and Glutathione in the Juice of Various Fruits[a]

Sample	[Glutathione], M, × 10^5	[Cysteine], M, × 10^5
Tomato 1	16.5	17.1
Tomato 2	6.6	10.6
Tomato 3	13.6	9.2
Tomato juice (Libby's)	2.2	2.1
Orange 1	11.6	0.9
Orange 2	7.6	0.8
Green grapes	18.5	0.9
Lemon	1.7	<0.3
Kiwi fruit	22.5	1.7
Cantaloupe	6.9	0.4

[a] The concentrations given are the result of replicate analyses with average relative standard deviations of ±2.0%.

RESULTS

Figure 1 shows a typical chromatogram obtained for juice from a ripe tomato. The unretained peak at about 1.5 min after sample injection is due to the double-layer capacitance effect (Rabenstein and Saetre, 1977) combined with detector response to chloride in the sample. The peaks at ca. 3 and 4.5 min are due to glutathione and cysteine, respectively. The total elution time is about 6 min. The limits of detection for the described conditions are ca. 2×10^{-6} M for glutathione and ca. 3×10^{-6} M for cysteine. The levels of glutathione and cysteine in the various fruits investigated are listed in Table I.

The stability of cysteine and glutathione to oxidation in some of the juices was also studied. The juices were left exposed to the air at room temperature for varying periods of time before acidification with H_3PO_4. Typical results are presented in Table II. In the juice from tomatoes, glutathione appears to be oxidized faster than cysteine, which is surprising in view of the report that glutathione is the more stable toward oxidation (Jocelyn, 1958). In general, it is to be expected that the stability of the thiols will depend on the pH of the juice, and previous studies have shown that, once the sample is acidified, both cysteine and glutathione are stable on the time scale of these determinations (Rabenstein and Saetre, 1978). Recovery studies indicate negligible loss of cysteine and glutathione

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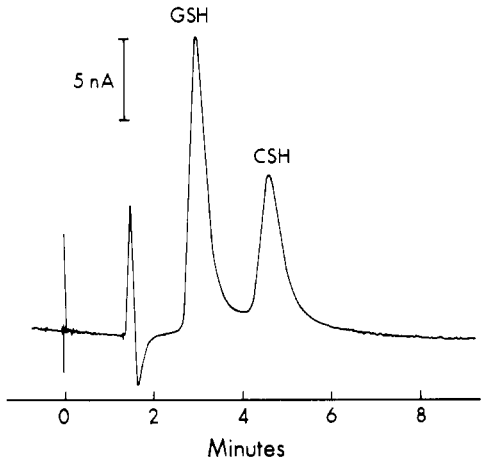

Figure 1. Representative chromatogram obtained from juice from a ripe tomato. Peak heights correspond to 6.8×10^{-5} M glutathione (GSH) and 4.6×10^{-5} M cysteine (CSH).

Table II. Stability of Cysteine and Glutathione in Fruit Juices to Oxidation

	Tomato juice		Orange juice	
Time,[a] h	[Glutathione], M, $\times 10^5$	[Cysteine], M, $\times 10^5$	[Glutathione], M, $\times 10^5$	[Cysteine], M, $\times 10^5$
0	16.5	17.1	11.6	0.9
0.5	16.9	16.3	11.8	0.9
1	14.2	16.4	11.4	0.9
12	11.9	18.0		

[a] The length of time the fruit juice was exposed to air before acidification.

from the acidified samples on the ion-exchange column. No thiols other than cysteine and glutathione were detected in the fruit juices.

DISCUSSION

The LC method described in this paper is fast and sensitive, and with this method both the cysteine and the glutathione levels can be determined simultaneously. The speed and sensitivity are due largely to the characteristics of the mercury-based electrochemical detector. In this determination, the detector is operated at +0.1 V vs. the SCE, and signals are obtained only as those compounds which are electroactive at this potential are eluted through the detector. Since the thiol compounds are the only components of fruit which are electroactive at this potential, the only requirement of the chromatographic step is that it separate cysteine from glutathione. To test for potential interference from ascorbic acid, a 1.0×10^{-3} M solution of this vitamin was injected onto the column. This gave no response apart from the unretained peak at about 1.5 min.

The present study has focussed on the development of a method for the determination of the reduced forms of cysteine and glutathione. With a preelectrolysis of the sample at a mercury pool electrode (Saetre and Rabenstein, 1978), it should be possible to determine the totals of the cysteine and of the glutathione in the reduced and the nonprotein disulfide forms by this method.

Other methods which have been used for the determination of the nonprotein thiol content of fruits and other plant materials include an argentimetric–amperometric titration (Kolthoff and Harris, 1946; Weissman et al., 1950; Levitt et al., 1961), polarography (Zuman, 1951, 1952), colorimetry based on reaction with Ellman's reagent (Ellman, 1959), a *p*-chloromercuribenzoate titration (Jansen and Jang, 1952), and filter paper chromatography (Miller and Rockland, 1952). In comparison to the LC method, the titration, colorimetric, and polarographic methods provide only the total nonprotein thiol content. The filter paper chromatography method is selective but considerably more involved. Jansen and Jang (1952) achieved selectivity by an elaborate procedure which required some 60 L of juice.

It is of interest that, of the various fruits investigated, only the tomatoes contained a significant amount of cysteine. This disagrees with the results of Zuman (1952) who concluded from polarographic measurements that his tomato samples contained only glutathione. It may be that the different kinds of tomatoes studied contain different relative amounts of cysteine and glutathione. The two oranges tested contained less glutathione and cysteine than found by Miller and Rockland (1952). Again, this may be due to different varieties or to the oxidation of thiols during storage of the oranges. The highest glutathione concentrations were found in kiwi fruit and green grapes, whereas the lowest level appeared in the lemon.

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