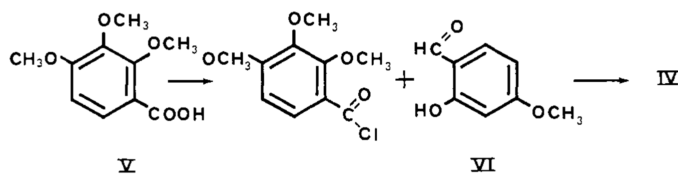
previously reported coumestans (*12, 13*) where this position is unsubstituted. Diehl (*6*) reported a para substituent effect of 0.33 p.p.m. for the methoxyl group in meta- and para-disubstituted benzenes. Thus, the *D* ring is substituted at the 12-position, and the substitution of the *A* ring at the 7- and 8-positions is confirmed. The remaining peaks of the aromatic region can be immediately assigned as shown in Figure 1.

The intermediate aldehyde (IV) must therefore be 4-methoxy-2-(2′,3′,4′-trimethoxybenzoyl)benzaldehyde. This structure was confirmed unequivocally by its synthesis from 2,3,4-trimethoxybenzoic acid and 2-hydroxy-4-methoxybenzaldehyde.



The λ_{max} of sativol in alcohol (Table I) did not undergo a bathochromic shift in the presence of sodium acetate or boric acid–sodium acetate, as would be expected if sativol contained a hydroxyl group at the 7-position (*11*) or an orthodihydroxyl grouping (*10*). Since sativol did not undergo these shifts, the lone methoxyl group must be at the 7-position, and the hydroxyl group must therefore be at the 8-position.

The location of the lone methoxyl group at the 7-position was confirmed by PMR spectroscopy through a comparison of the ether-acetate shift of the aromatic protons of sativol with those observed for several coumestans of known structure. Smith (*14*) suggested an ortho shielding constant (referred to unsubstituted benzene) of 0.21 p.p.m. for the acetate and 0.45 p.p.m. for the methoxyl group from a study of disubstituted benzenes. Thus, substitution of an acetate for a methoxyl group, while both structure and solvent are otherwise constant, should cause a downfield shift of roughly 0.24 p.p.m. Table II shows the shielding of ring protons in acetate and methoxy derivatives of sativol and a series of model compounds. The peak from the 6-proton in sativol shows a small shift which corresponds to the meta shifts of the 5- and 10-protons of coumestrol, its 4′-*O*-methyl ether, 7,11,-12-trihydroxycoumestan, and the 5-proton of trifoliol. This shift shows that the hydroxyl group cannot be at the 7-position and therefore must be at the 8-position. Hence, the methoxyl must be at the 7-position. The shift of the 5-proton resonance as would be expected for para-substitution further confirmed this assignment. The ortho shift of the 13-proton in sativol was entirely consistent with the corresponding shift in 4′-methoxycoumesterol and lucernol and in reasonable agreement with the value from Smith's data (*14*). Very low shifts for H-11 were obtained from sativol, coumestrol, and lucernol, although the corresponding value for trifoliol (where the hydroxyl group is in the 10- rather than the 12-position) appears to be normal. The spin-spin pattern for this proton in sativol is such that no confusion in its assignment is possible, and the shifts in the coumestrol series permit an unambiguous assignment.

Acknowledgment

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CHANGES DURING STORAGE

Effect of Cold Storage on Chlorogenic Acid Content of Potatoes

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THE phenolic compounds of potatoes are involved in the enzymic browning of raw potatoes (*12*) and in the discoloration of cooked potatoes (*8*), and are also associated with injuries and diseases of potatoes (*5, 10*). Because of this, and their importance as metabolic components, phenolic compounds of potatoes have been studied widely.

Although the knowledge of phenolic compounds of potatoes is increasing, there has been little investigation of the effect of storage on changes in the content of these compounds. Craft *et al.* (*4*) have shown that the total phenolic content in two varieties of potatoes, Russet Rural and Kennebec, does not change significantly during 5 months of storage at 40° and 55° F. or 3 months at 32° F. It increased, however, after 4 to 5 months of storage at 32° F. They suggested that the increase is not due to the storage temperature but is related to injury. The results obtained by Mondy *et al.* (*13*) are not in agreement with those of Craft *et al.* (*4*). The former reported that the total phenolic content of the cortex tissue of potatoes increased 25 to 75% from the time of harvest up to 3 months of storage at 40° F. The

Chlorogenic acid was isolated from two varieties of potatoes by column chromatographic methods, and identified by paper chromatography and by ultraviolet and infrared absorption spectral analyses. Chlorogenic acid was the principal phenolic compound in the inner tissue of tubers and the only compound which increased significantly during the storage of the potatoes at 40° F. No increase in the chlorogenic acid content was observed, however, in potatoes stored at 60° F. It was postulated that the increase in chlorogenic acid during cold storage is due to the accumulation of sugars.

phenol contents of two varieties of potatoes, Pontiac and Ontario, harvested in 1957 reached a maximum level after 4 weeks of storage, but the same varieties harvested in 1956 required 12 weeks to reach the maximum level under the same condition.

Chlorogenic acid is the principal phenolic compound in potatoes (*2*, *7*, *9*). However, the effect of cold storage on changes in the content in potatoes has not been investigated. This investigation was undertaken to find the effect of storage temperature on changes in the chlorogenic acid content in two varieties of potatoes.

Materials and Methods

Unless otherwise stated, two varieties of potatoes, Kennebec and Katahdin, harvested in 1963 were used. They were purchased from the Ohio Potato Growers Association and stored at 40° F. For chlorogenic acid analyses, two 3-pound samples of the potatoes (10 to 12 potatoes in each sample) were taken at random, each representing one replicate of a variety. In addition to the analyses on fresh potatoes, the samples were analyzed after storage for 1, 2, 3, 4, and 8 weeks.

Silicic acid used for column chromatograms was prepared from a commercial preparation, Mallinckrodt 100-mesh as described by Bulen *et al.* (*1*). Chloroform (USP grade) was washed twice with distilled water before use. All other chemicals used were of reagent quality.

Chlorogenic Acid Analysis. Extraction. Plugs were taken from the potatoes by inserting a No. 14 cork borer through the tubes from the stem to the bud end. Both ends of the plugs (5 mm.) were cut off and discarded, providing samples that contained relatively small amounts of subcortical tissue. A 100-gram portion of the plugs was ground for 5 minutes in an AMC blender with 300 ml. of 95% ethanol and the slurry was filtered under reduced pressure through a hardened filter paper (S and S No. 576). The residue was again suspended in 300 ml. of 95% ethanol, blended for 5 minutes, and filtered, then washed with 200 ml. of 95% ethanol.

Isolation. The method used to isolate chlorogenic acid involved partition chromatography on silicic acid columns as described by Bulen *et al.* (*1*), modified by Hanson and Zucker (*7*). Some further changes were made in this method.

Two columns were employed: One (2.8 × 22 cm.) was used for the preliminary separation of phenolic compounds from the ethanol extract of potatoes, and the other (1.2 × 20 cm.) was used for the final separation. Both the preliminary and analytical columns were prepared from silicic acid and $0.5N$ H_2SO_4 by the method described by Hanson and Zucker (*7*).

The solvent system for elution was prepared from cyclohexane, 2-methyl-2-propanol, and chloroform according to the procedure of Hanson and Zucker (*7*): system A, 2-methyl-2-propanol–chloroform (2:3); system B, cyclohexane-chloroform (1:9); and system C, 2-methyl-2-propanol–chloroform (3:7). Each system was saturated with $0.5N$ H_2SO_4.

The ethanol extract was concentrated under reduced pressure with a rotary evaporator at 35° C. to near dryness. The residue was acidified with 0.5 ml. of $0.5N$ H_2SO_4 and mixed thoroughly with 5 grams of silicic acid; the resulting free-flowing powder was transferred to the top of the preliminary column. The column was eluted with 240 ml. of solvent system A. The absorbance of the final eluate at 320 mμ was less than 0.05.

The eluate collected from the preliminary column was carefully brought to dryness at a temperature of 30° C. with a rotary evaporator. The residue was dissolved in 0.3 ml. of $0.5N$ H_2SO_4, mixed well into 1 gram of silicic acid, and transferred to the top of the analytical column. The column was eluted by the stepwise method with the mixtures of different proportions of B and C according to the following schedule. At first 50 ml. of B, followed successively by 45 ml. of B and 5 ml. of C, 40 ml. of B and 10 ml. of C, 35 ml. of B and 15 ml. of C, 30 ml. of B and 20 ml. of C, 40 ml. of B and 60 ml. of C, and finally 30 ml. of B and 70 ml. of C. The moisture produced when two solvents were mixed was removed by a dry filter paper. The flow rate was approximately 60 ml. per hour, and 6-ml. fractions were collected. The emergence of materials absorbing at a wavelength of 320 mμ was determined in each fraction using a Beckman DU spectrophotometer.

Identification. The peak corresponding to chlorogenic acid was tentatively determined by descending paper chromatography, comparing R_f values with those obtained using authentic chlorogenic acid. The chromatograms were developed on Whatman No. 1 filter paper at room temperature with the following solvent systems: (1) 1-butanol–acetic acid–H_2O (4:1:5), (2) 1-butanol–acetic acid–H_2O (4:1:2.2), and (3) isoamyl alcohol saturated with $5N$ formic acid. Spots were detected by ultraviolet fluorescence and by spraying the Benedict's solution which was described by Gage *et al.* (*6*) for use with flavonoid-type compounds.

The peak tentatively identified as chlorogenic acid was further examined by ultraviolet absorption spectral analysis with a Bausch and Lomb 505 recording spectrophotometer. For reference purposes, the control spectrum of a known chlorogenic acid was determined in the mixture of B:C::2:3.

For further identification, infrared spectra of isolated and authentic chlorogenic acid were determined with a Beckman IR-5 infrared spectrophotometer. A fraction of approximately 400 ml. of chlorogenic acid collected from the analytical column was brought to near dryness. The residue was dissolved in 150 ml. of water. Any insoluble substances were removed by filtration, and the filtrate was treated with lead acetate according to a modification of the method of Rudkin and Nelson (*14*) as follows. Ten milliliters of lead acetate (10%) were added to the filtrate. After the small amounts of white precipitate, presumably lead sulfate, were removed, the filtrate was adjusted to pH 9.0 with $1N$ NaOH solution. The yellow precipitate formed was collected by centrifugation and suspended in 50 ml. of water. Lead was removed as a PbS precipitate by passing H_2S gas through the suspension, followed by filtration. The resulting clear solution obtained was then freeze-dried. Four milligrams of the dried sample were well mixed with 400 mg. of KBr (infrared grade) in a small mortar. A pellet was made with a Beckman No. 4240 die.

Effect of Sugars on Chlorogenic Acid Synthesis in Potato Slices. The experimental conditions used in this study were similar to those of Zucker and Levy (*16*). Kennebec potatoes stored at 40° F. for 7 months were used. Plugs were prepared as described above, and 35 slices of potatoes (1.2 mm. in diameter and approximately 1 mm. thick) were placed in a Petri dish (9 cm. in diameter) and incubated with exposure to atmospheric oxygen, in 5 ml. of solution containing $10^{-2}M$ of sugar and 25 p.p.m. of neomycin sulfate at 5° or 24° C. Each sample was exposed to a GE No. F8T5W fluorescent lamp during 24 hours of incubation. The light source provided approximately 100 foot-candles at working range. At the end of the incubation period, the slices were washed thoroughly with

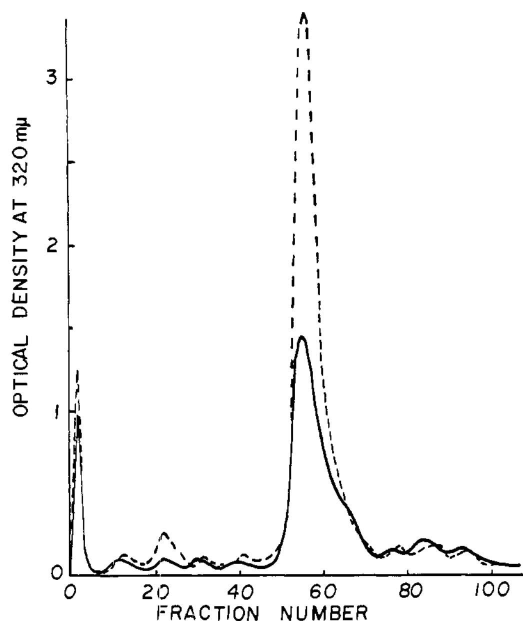

Figure 1. Chromatographic separation of phenolic components from ethanol extract of Katahdin potato

—— Fresh potato
– – – Potato stored at 40° F. for 4 weeks

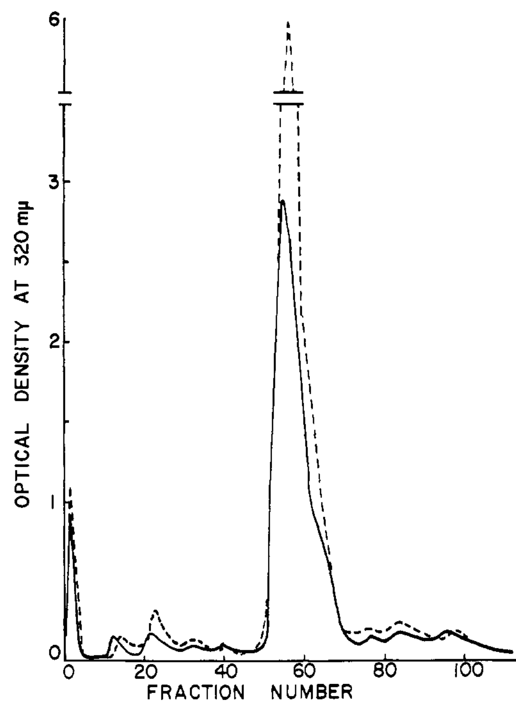

Figure 2. Chromatographic separation of phenolic components from ethanol extract of Kennebec potato

—— Fresh potato
– – – Potato stored at 40° F. for 4 weeks

water and ground with 20 ml. of 95% ethanol in a Virtis 45 homogenizer. The homogenate was filtered and the residue was washed with 30 ml. of 95% ethanol.

The method used for the isolation of chlorogenic acid from the extract and measurement of its concentration was that described previously, except that a small column was used for the preliminary separation of phenolic compounds. The column (0.9 × 15 cm.) was prepared from 1 gram of silicic acid and 0.5 ml. of 0.5*N* H_2SO_4, and was eluted by 50 ml. of solvent system A

Results

The results of the chromatographic separation of phenolic compounds from alcohol extracts of potatoes on the silicic acid columns are shown in Figures 1 and 2. The basic patterns of the elution curves obtained from the Katahdin and Kennebec potatoes were essentially identical. Each had nine peaks and one major peak was located between fractions 50 and 72. Only the major peak increased significantly during storage of the potatoes at 40° F.

Since this study was primarily concerned with chlorogenic acid, the remaining peaks were not identified. The results of the paper chromatographic identification of all the peaks led to the tentative conclusion that the major peak was chlorogenic acid.

Absorption spectra of both the major peak and known chlorogenic acid are shown in Figure 3. The spectra were nearly identical, each having an absorption maximum at 323 mμ in the range of 250 to 360 mμ. Johnson and Schaal (*11*), Cheng and Hanning (*2*), and Uritani and Miyano (*15*) reported a very similar curve for chlorogenic acid.

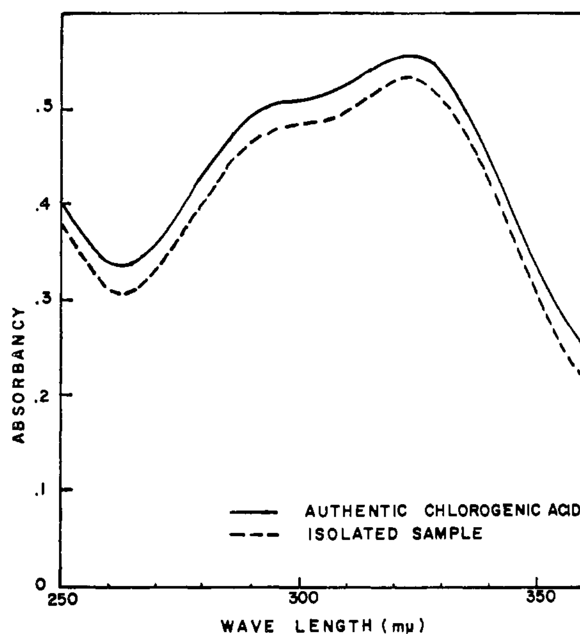

Figure 3. Ultraviolet absorption spectra of authentic and isolated samples of chlorogenic acid

Chlorogenic acid isolated from mixed sample of Kennebec and Katahdin potatoes

The results of the infrared spectral analysis confirmed the above results. The infrared absorption spectra of authentic chlorogenic acid and the sample isolated from the major peak by the lead acetate precipitation method are presented in Figure 4. The isolated and control samples exhibited nearly identical infrared spectra. Slight differences were observed, however, in the range of 10 to 12 microns. In particular, one peak was missing from the isolated sample at the wavelength in the vicinity of 10 microns. The differences might be due to a contamination by an isomer or the partial degradation of chlorogenic

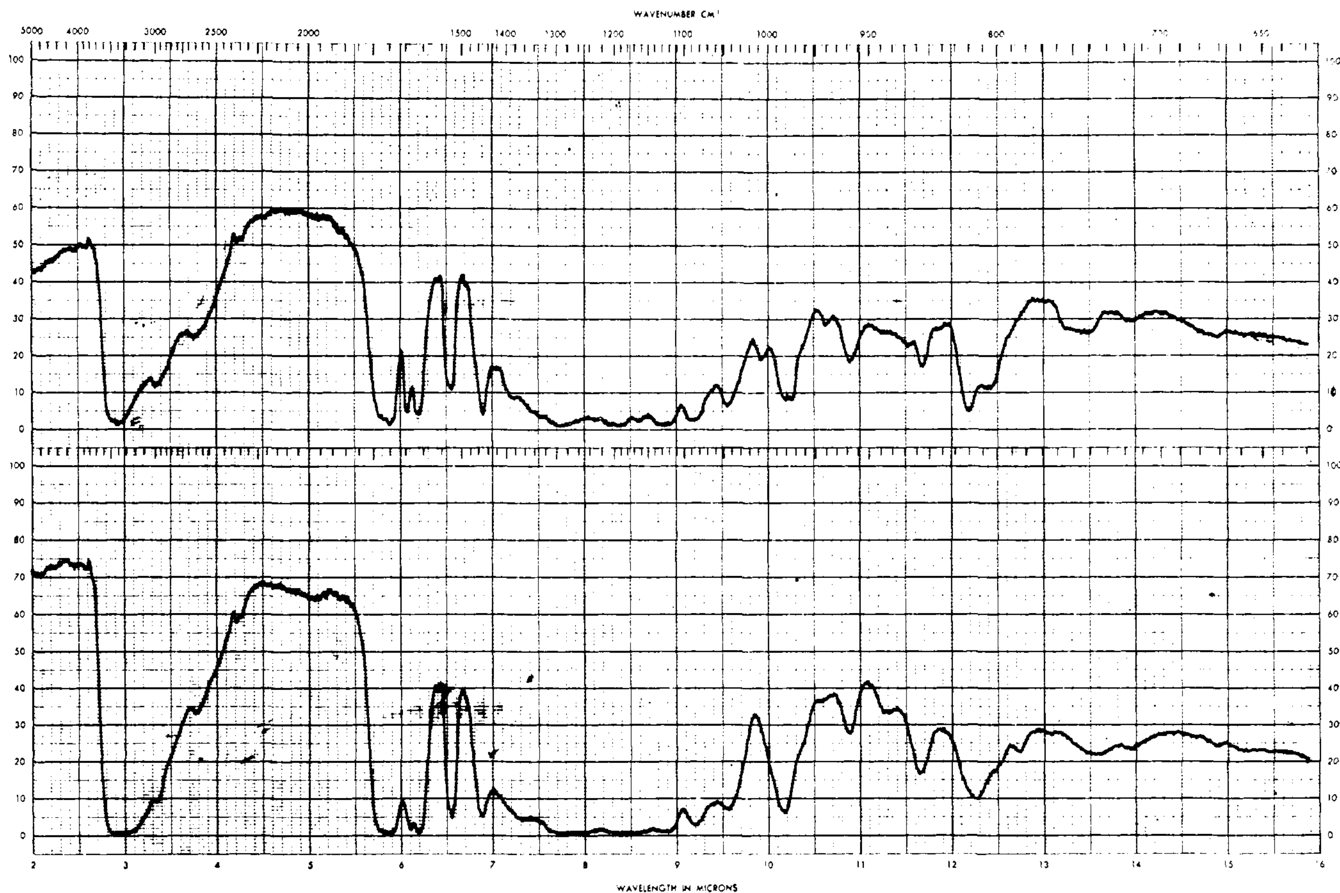

Figure 4. Infrared absorption spectra

Upper. Authentic chlorogenic acid
Lower. Compound isolated from potatoes

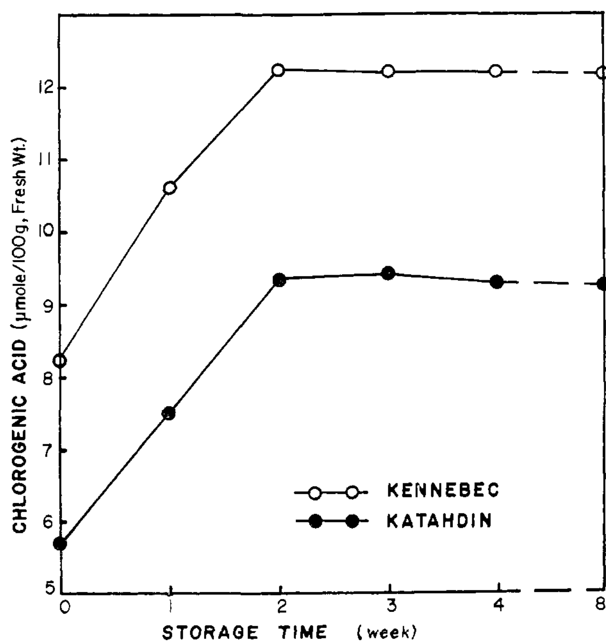

Figure 5. Effect of cold storage (40° F.) on changes in chlorogenic acid content of potatoes

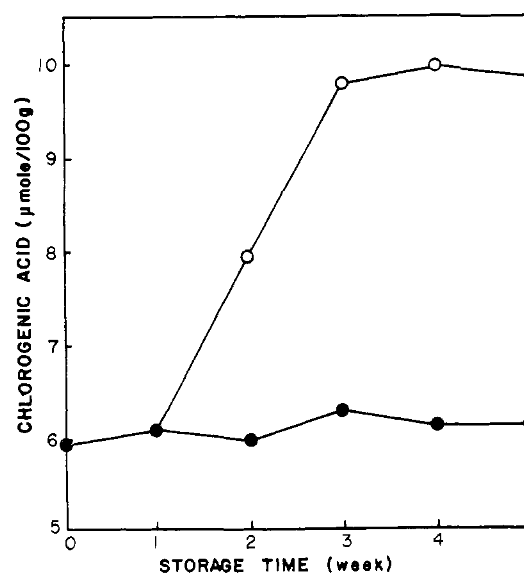

Figure 6. Effect of storage temperature on changes in chlorogenic acid content of potatoes

● Stored at 60° F.
○ Stored at 40° F. after 1 week of storage at 60° F

acid during the described separation.

It is reasonable to conclude from the above results coupled with the results obtained by Hanson and Zucker (*7*) that the major peak is chlorogenic acid. On the basis of data obtained in this study on the extinction coefficient of chlorogenic acid (16,800 at 320 mμ) and the results of recovery tests (85%), the chlorogenic acid contents in the Katahdin and Kennebec potatoes were estimated to be 6.5 and 9.5 μmoles per 100 grams on a fresh weight basis, respectively.

The results of the detailed study of the storage effect on changes in the chlorogenic acid content are shown in Figure 5. In both the Katahdin and Kennebec

Table I. Effect of Sugars on the Synthesis of Chlorogenic Acid in Potato Tuber Slices

Substrates	Incubation Temp., ° C.	Chlorogenic Acid, μmoles/35 Slices
Control	5	0.28
	24	0.43
Glucose	5	0.61
	24	2.54
Fructose	5	0.59
	24	1.29
Sucrose	5	0.71
	24	2.05

Incubation period. 24 hours.
Chlorogenic acid content of zero time control. 0.19 μmole per 35 slices.

varieties, its content increased sharply and steadily during the early period of storage and reached a maximum level after 2 weeks of storage. After reaching a maximum level, the chlorogenic acid content remained constant at least until 8 weeks. At the maximum level, the increases in Katahdin and Kennebec were 64 and 50%, respectively.

Since the detailed postharvest history of the two varieties of potatoes was unknown, a quantity of freshly harvested, fully mature potatoes of unknown variety was purchased from a local farmer, to confirm the results described above, starting with fresh material. The potatoes were stored at 60° F. immediately after being taken from the ground. One week later, one half of the potatoes was transferred to a 40° F. storage room, and kept at this temperature for the remainder of the study period. As shown in Figure 6, the chlorogenic acid content of the potatoes stored at 60° F. remained fairly constant throughout 5 weeks of storage. On the other hand, an increase was observed in the potatoes stored at 40° F., with the pattern similar to those observed with the Katahdin and Kennebec varieties.

Fructose and sucrose were as effective substrates as glucose for the synthesis of chlorogenic acid (Table I). When the potato slices were incubated in air in the presence of $10^{-2}M$ glucose, fructose, or sucrose for 24 hours at 24° C., chlorogenic acid increased in concentration to levels as great as 13.4, 6.4, or 10.8 times the original amount. When the incubation was carried out at 5° C., the chlorogenic acid produced in the presence of each of these sugars was approximately 3 times that found in the zero control.

Discussion

The efficiency of the silicic acid columns used for the isolation of chlorogenic acid was determined by the addition of standard amounts of chlorogenic acid to extracts of potatoes before concentrating the extracts. The recovery of the chlorogenic acid from the preliminary column was 99%. The recovery from the analytical column was, however, 85%. As suggested by Hanson and Zucker (*7*), the loss was probably due to the oxidation and polymerization involved during the concentration of the acidic eluate obtained from the analytical column. Although the loss was considerable, the entire analytical steps were reproducible (variations between columns were less than 5%).

The elution patterns obtained upon chromatography of the potato extracts (Figures 1 and 2) were similar to those of Hanson and Zucker (*7*). The latter, however, found a few peaks in addition to those obtained in this study. The failure to isolate these minor peaks could be due to the differences in elution techniques used, or different parts of the potato may have been used in the two studies. It has been reported that the cortex tissue contains higher concentrations and a greater variety of phenolic compounds than the inner tissue (*2–4, 9*).

Chlorogenic acid was found to be the principal phenolic compound in both the Katahdin and Kennebec potatoes. This supports the earlier investigations (*2–4, 7, 9*). Chlorogenic acid accounted for 70% of the total phenolic content in the inner tissue of both the Kennebec and Katahdin potatoes. This value is higher than that of the cortex tissue (*7*), in which chlorogenic acid accounted for 60% of the total phenolic content. This could be one of the reasons why Cheng and Hanning (*2*) were unable to detect phenolic compounds other than chlorogenic acid from the inner tissue of potatoes.

The chlorogenic acid concentrations obtained in this study agreed well with the results of Craft *et al.* (*4*), whose estimates, based on direct paper chromatographic examination of tuber extracts, were 8.5 μmoles or less per 100 grams of pith tissues

The important finding, shown in Figures 1 and 2, was that among the compounds isolated, only chlorogenic acid increased significantly during the cold storage of the potatoes. The increases, 50% in Kennebec and 64% in Katahdin, are in agreement with the results of Mondy *et al.* (*13*). Although their increases were observed to occur in the cortex tissue, it is reasonable to suggest that the increase in the total phenolic content may be due to the increase in chlorogenic acid. These findings, however, disagree with the results obtained by Craft *et al.* (*4*), who reported that the total phenolic content remained constant during storage at 32°, 40°, and 55° F.

Upon storage of potatoes at 60° F., the chlorogenic acid content remained fairly constant. The question arises concerning the reason why cold storage resulted in the accumulation of chlorogenic acid in the tuber, while high temperature storage yielded no increase. During the study on chlorogenic acid synthesis, Zucker and Levy (*16*) found that glucose was one of the substances which stimulated chlorogenic acid synthesis in potato slices. Fructose and sucrose were also very effective substrates for the chlorogenic acid synthesis.

From the above results, coupled with the well known fact that glucose, fructose, and sucrose accumulate in potatoes during cold storage, it is postulated that the increase in chlorogenic acid during the cold storage is due to the accumulation of sugars.

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