tested vegetable products did not affect isomerization during the 30-min incubation period. Furthermore, pH 1 solutions showed no change after 15 h of incubation.

It is interesting to note that no isomeric carotenoids were detected in the fresh yellow-orange vegetables, but analysis of all the fresh green vegetables selected indicated that isomers were present. A number of factors such as heat, light, iodine, and acid are well-known catalysts for isomerization (Klaui and Bauernfeind, 1981). Also, chlorophyll has been reported to influence photoisomerization of certain carotenoids through a direct energy-transfer mechanism (Claes, 1961). If chlorophyll can cause carotenoid photoisomerizations, then this reaction may be an important factor during sample workup, thus accounting for the presence of the cis forms found in chlorophyll-containing vegetable extracts. Furthermore, the possible function of carotenoids as accessory pigments in photosynthesis (Krinsky, 1968) may also influence the formation of these isomers in chloroplasts due to their sensitivity to light-induced isomerization. Additional work is needed to determine the factors that influence the formation of cis carotenoid isomers in food products.

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Registry No. *all-trans*-β-Carotene, 7235-40-7; neo-α-carotene B, 83058-23-5; neo-α-carotene W, 86851-59-4; neo-β-carotene B, 6811-73-0; neo-α-carotene U, 29907-61-7; neo-β-carotene U, 13312-52-2; *all-trans*-α-carotene, 432-70-2.

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Identification of Caffeine in Citrus Flowers and Leaves

Ivan Stewart

Caffeine was isolated and identified in extracts from flower buds of several citrus cultivars and from leaves of Valencia oranges (*Citrus sinensis* L. Osbeck). No caffeine was detected in orange juice. Identification was by high-performance liquid chromatography (HPLC), gas chromatography (GC), ultraviolet spectroscopy (UV), and mass spectra (MS).

Our laboratory has been isolating compounds from citrus tissue and assaying them for cytokinin activity using soybean callus growth as a criteria (Miller, 1963). The extraction procedure used not only removed cytokinins from the tissue but various kinds of bases and other compounds. During the separation of a citrus flower extract, one of the fractions was found to induce growth of soybean callus, suggesting the presence of cytokinins. However, upon further fractionation of the extract, the main constituent, as indicated by HPLC, no longer induced callus growth. Obviously, during the purification procedure, the growth-active compound had either been removed or deactivated. The inactive fraction was then subjected to GC–MS analysis and was found to contain virtually pure caffeine.

Caffeine is said to occur in some 60 species of plants (Roberts and Barone, 1983); the more common are from the genera *Caffea*, *Thea*, *Theobroma*, *Cola*, *Paullinia*, *Ilex*, and *Copernecia*. However, this is believed to be the first report of the occurrence of caffeine in citrus. This paper reports on the isolation and identification of caffeine in citrus flowers and leaves, on the amounts of caffeine in these tissues, and a discussion on some of the biological properties of caffeine in plants.

EXPERIMENTAL SECTION

Isolation. Flower buds and open flowers from trees of the Valencia orange (40-kg fresh weight) were collected and extracted in a Waring blender with 80% ethanol and then filtered through a fritted glass funnel. The extract was adjusted to pH 4.0 with HCl, and a portion equivalent to

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10.5 kg of blossoms was passed through a 3000-mL glass fritted funnel containing 2500 g of Dowex 50 H^+-exchange resin. Caffeine and many other compounds were eluted from the resin with 8 L of 6 N NH_4OH in 50% aqueous methanol at 0 °C. The NH_4OH was removed on an evaporator at 50 °C and the water extract concentrated in vacuo. The solution was adjusted to pH 9.0 and extracted three times with butanol. The butanol phase was separated and the alcohol removed by azeotropic distillation with excess water. The resulting aqueous extract was adjusted to pH 3.2 with HCl and passed through a PolyClar AT column. The effluent was concentrated, put on a Sephadex LH 20 column, 2.8 cm × 30 cm, and eluted with 35% aqueous methanol at 1 mL/min. Fifteen-milliliter fractions were collected. Fractions 20–24 contained caffeine. Further purification procedures were carried out with HPLC.

Analysis. Ten grams of flower buds or leaves fresh weight were frozen, extracted with 80% ethanol, and filtered. The extract was adjusted to pH 2.0 with HCl and after 15 min centrifuged to remove the precipitate. The supernatant was adjusted to pH 9.0 with NaOH and extracted three times with butanol as described above. Following azeotropic distillation of the butanol phase with excess water, the resulting aqueous solution was reduced to 10 mL. Aliquots of this solution was taken for HPLC analysis.

Chromatography. The HPLC equipment consisted of two Waters pumps with a Model 660 solvent programmer and LDC SpectroMonitor detector. The column (1 cm × 25 cm) was packed with Nucleosil 10 C_{18} packing. The solvents consist of (A) 10% methanol, adjusted to pH 3.2 with H_3PO_4, and (B) methanol. The program was isocratic, using 20% pump B and a flow rate of 3 mL/min. The sample was prepared by adding 10 μL of the plant extract to 40 μL of solvent A and injected. The retention time of the caffeine was 20 min. Analyses were run in duplicate and results calculated on the basis of areas under the peaks. Peak areas were compared with those of known standards.

Gas chromatography separations were made on a Perkin-Elmer Model 3920B instrument using a flame ionization detector. The glass column was packed with 1% OV 17 on Supelcoport. The program started at 150 °C and increased 8°/min to a final temperature of 250 °C. The caffeine retention time was 4 min.

GC–MS samples were run on a Finnigan quadrupole MS with a GC using column packing and conditions as described above. The MS was operated at 55 eV, using both electron impact (EI) and chemical ionization (CI) with isobutane. Other spectra were run on a Kratos Model 25 magnetic sector instrument using both GC and direct probe.

RESULTS AND DISCUSSION

Isolation and Identification. The accidental finding of caffeine in citrus came about as a result of isolating cytokinins. Since the growth hormones occur in very low concentrations, it was necessary to extract large samples of plant tissue in order to obtain sufficient amounts for identification.

Identification of caffeine in citrus tissue was based on several means, including cochromatography of the isolated compound with a known sample using both HPLC and GC. UV spectra (nm) obtained from water solutions showed $\lambda_{max}(H_2O)$ = 271, $\lambda_{min}(H_2O)$ = 243, λ_{max}(0.1 N NaOH) = 270, λ_{min}(0.1 N NaOH) = 245, λ_{max}(0.1 N HCl) = 270, and λ_{min}(0.1 N HCl) = 245 nm. The mass spectra were identical with those of known samples. Using EI, major ions were found at m/e 67, 82, 109, 137, 165, and 194 (M^+). With CI only the mass ion was found at m/e 195 (M + 1). The HPLC, GC, and the total ion current all showed one major peak. Mass spectra were obtained on caffeine extracted from a mixture of Valencia flowers and flower buds collected in 1979 (40 kg) and in 1983 (26 kg) and on leaf samples (25 g) taken in Feb 1984 of young partially expanded and also of old leaves produced the previous year.

Table I. Caffeine Content of Citrus Flowers and Leaves[a]

cultivar	av,[b] μg/g	SD[c]
Dancy tangerine flowers	19	±1.1
Willow leaf mandarin flowers	32	±0.4
Cleopatra mandarin flowers	21	±0.4
Ruby Red grapefruit flowers	29	±0.4
Liane pummelo flowers	24	±0.7
Sour orange flowers	31	±0.2
Lisbon lemon flowers	50	±0.2
Palestine sweet lime flowers	28	±0.4
Valencia orange leaves	6	±0.8

[a] Based on HPLC Analysis. [b] Average of duplicates. [c] Standard deviations.

Table II. Caffeine Content of Valencia Orange Flower Buds Taken at Different Stages of Maturity[a]

bud diam, mm	amt,[b,c] μg/g	bud diam, mm	amt,[b,c] μg/g
4	ND	8	3 ± 0.07
5	ND	open flowers	62 ± 0.28
7	ND		

[a] Based on HPLC analysis. [b] Average of duplicate determinations. [c] ND, not detectable, <25 ng.

Analysis of Plant Extracts. Following the identification of caffeine in citrus tissue, an analytical method was developed for measuring the amounts present. It was found that 10 g of tissue was sufficient to give suitable determinations using HPLC. Also, some of the steps used in the isolation study were not found to be essential for analytical purposes and were deleted.

Caffeine content was compared on the extracts of unopened flower buds of eight citrus cultivars (Table I). The amounts ranged from approximately 19 to 50 μg/g. Highest amounts were found in lemon and the lowest in Dancy tangerine and Cleopatra mandarin. Young leaves not fully expanded, from Valencia orange trees, contained much less, having 6 μg/g. None was observed in extracts from mature leaves.

In another study, caffeine was determined on Valencia orange flower buds at different stages of growth (Table II). Caffeine was not detected in buds 4, 5, or 7 mm in diameter; however, just prior to opening when the buds were elongated to about their maximum, caffeine was detected. The largest amount was found in the fully opened flowers.

In order to determine whether citrus juice contained caffeine, 20 L of Valencia orange juice was extracted and run on HPLC. No caffeine was detected.

Biological Properties of Caffeine in Plants. Caffeine is known to have interesting physiological properties in both animals and plants. Rabechault and Cas (1973) found that $<10^{-4}$ M caffeine in culture media stimulated growth of coffee embryos, while higher concentrations inhibited growth. In my studies, crude extracts of citrus flowers containing endogenous caffeine stimulated growth of soybean callus when added to the media. In order to observe the effet of caffeine on soybean growth, a study was made where increasing rates of caffeine ranging from 10^{-7} to 10^{-2} M with and without the addition of kinetin were added to growth media. There was no growth in any of the minus kinetin cultures due to the absence of a cy-

tokinin. Caffeine concentrations of 10^{-5} M and less with kinetin in the media did not affect the yield of soybean callus over that growing on media containing no caffeine. However, when 10^{-4} M of caffeine was used, yields were reduced and the 10^{-2} M rate completely inhibited growth. Thus, the increased callus growth of the soybean bioassay from citrus extracts in my original observations was not due to the caffeine.

In recent studies, Nathanson (1984) pointed out that caffeine may be a naturally occurring pesticide. He found that relatively low concentrations of methylxanthines were potent synergists when combined with certain other compounds. One of these synergistic compounds was octopamine, which we have reported previously to occur in citrus and other plants (Stewart and Wheaton, 1964; Wheaton and Stewart, 1970). It is tempting to speculate that caffeine might function as a deterrent to herbivores in reproductive buds and flowers.

In plants, caffeine sprays have been reported to increase flowering of potatoes and olives whereas xanthine was shown to increase flowering of grapefruit seedlings (Kessler and Bak, 1959). Other researchers have investigated some of the more basic properties of caffeine and related xanthine derivatives with cell division in plants. These compounds were found to prevent cytokinesis and induce formation of binucleate cells in plants (Kihlman, 1955; Kihlman and Levan, 1949). Binucleate cells induced with caffeine may be held in the G_1 stage for extended periods (Davidson, 1983). This stage is prior to the S phases where DNA synthesis occurs. Davidson (1983) has suggested that caffeine may be chemically similar to the naturally occurring cell regulator that controls mitosis and/or differentiation. If this hypothesis can be supported, it may be that caffeine or similar methylxanthines are synthesized in many plants or at least in juvenile tissues of woody plants and that these compounds may function as growth regulators.

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Use of a Standard-Addition Bromide-Selective Electrode Technique To Determine Bromide and Trace Its Migration in Peaches

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A bromide-selective electrode (BrSE), coupled with a standard-addition procedure, was successful in detecting known levels of bromide ion in peach (*Prunus persica* (L.) Batsch) extract. Bromide, added as 0, 1, 5, 10, 25, 50, or 100 mg/L of deionized H_2O (bromide standard solution) to ground peaches, was detected with a mean recovery of 103 ± 2%. BrSE analysis yielded similar results as X-ray fluorescence and thiosulfate titration when bromide content was measured in MeBr-fumigated peaches or in peach puree fortified with NaBr. The concentrations of bromide in the peel and flesh of peaches that had been fumigated with MeBr at 32 g/m^3 for 3.5 h averaged 11 and 4 mg/kg, respectively, both 1 and 7 days after treatment. A standard-addition BrSE method can be useful for determining residues when fruit has to be fumigated for quarantine purposes.

Methyl bromide (MeBr), when absorbed in many plant tissues, decomposes to yield a methylated protein product and bromide ions (Winteringham et al., 1955). Bromide may be measured by thiosulfate titration (Shrader, 1942), X-ray fluorescence (Getzendaner, 1961), and ion-selective electrode potentiometry (Pflaum et al., 1962). Potentiometric analyses of bromide have usually been direct estimations of bromide concentrations utilizing a calibration curve of electrical potential vs. bromide content.

Some researchers have used BrSE to analyze vegetables and fruit grown in soil fortified with KBr or fumigated with MeBr for bromide (Abdalla and Lear, 1975; Basile and Lamberti, 1981). Others have analyzed MeBr-fumigated fruits and cereals with a BrSE (Banks et al., 1976; Gnanasunderam and Triggs, 1983). In some studies, ashing and drying of the commodity preceded extracting of the bromide. Only Banks et al. reported recovery data or comparisons with established analyses of bromide determination on fruits and vegetables with residues below 3000 mg/kg of bromide. Their bromide recoveries ranged from 94 to 118% for wheat fortified with KBr at 25–75 mg/kg and from 95 to 112% for maize, copra, sorghum, and soybeans fortified with KBr at 50 mg/kg. Their results with BrSE were similar to those with neutron activation analysis, Volhard titration after exchange chromatography, and X-ray fluorescence for a concentration range of 20–53 mg/kg.

Each of the above studies was conducted utilizing a calibration curve to estimate bromide concentration. An alternative to the use of a calibration curve is the use of

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