Developmental and Topophysical Effects on the *n*-Alkanes of Valencia Orange Fruit Epicuticular Wax

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The *n*-alkane fraction was high in the very immature fruit, decreased to minimum values just before fruit color break, and then increased again as fruit reached its maximum size. Odd-numbered *n*-alkanes dominated on fruit from both quadrants but were somewhat more prevalent in the wax from northeast- (NE-) quadrant fruit. In both situations, the quantity of odd-numbered chains was minimum just before fruit color break. A shift in chain length from short to long was observed throughout fruit development, but a slight reversal toward short chains occurred after fruit maturation. Wax of fruit from the NE quadrant had higher amounts of short chains than wax of fruit from the southwest (SW) quadrant until fruit color break, when the situation was reversed. This study supports earlier conclusions that fruit maturation and senescence are accompanied by relative and absolute increases in epicuticular wax *n*-alkanes. This study also suggests that SW-quadrant fruit are physiologically more advanced as they approach maturity than fruit from the NE quadrant.

Fruits, as well as most other aerial plant organs, are covered with waxy substances within the cuticle, as well as on the surface of the cuticle. Epicuticular wax, the outermost layer, has several functions critical for plant development, adaptation, and survival [see reviews by Baker (1982) and El-Otmani (1985)]. Surface wax is composed of alcohols, aldehydes, esters, fatty acids, hydrocarbons, ketones, and related compounds.

Baker et al. (1975) reported that Adamopoulou lemon (*Citrus limon* L. Burm. f.), Willowleaf and Clementine mandarin (*Citrus reticulate* Blanco), and Frost Valencia orange (*Citrus sinensis* L. Osbeck) fruit epicuticular wax contained 23, 36, 42, and 40% hydrocarbons, respectively. Fruit for the study was obtained from Greece, and stage of fruit development for compositional data was not specified. In the epicuticular wax of pineapple and navel oranges (*C. sinensis* L. Osbeck) and Dancy tangerine (*C. reticulata* Blanco), paraffins accounted for 30–44% and 6–12% of the wax of immature and mature fruit, respectively (Freeman et al., 1979). Furthermore, on an absolute basis, paraffins decreased from a high of 24 to below 7 $\mu g/cm^2$ in these cultivars, depending on stage of fruit development (Freeman, 1978). More recently, El-Otmani and Coggins (1985b) reported substantial changes in percentage of straight-chain hydrocarbons during fruit growth, development, maturation and senescence.

n-Alkanes of 20–35 carbon atoms have been found in the epicuticular wax of citrus fruit examined thus far (Nagy et al., 1975; Nordby and Nagy, 1977; El-Otmani and Coggins, 1985b). Linear-chain alkanes were shown to represent at least 98% of the epicuticular wax total alkanes of mature Duncan grapefruit (Nagy et al., 1975). The carbon chains C_{31}, C_{29}, and C_{27} were most abundant, comprising 41, 22, and 11% of the total alkane fraction, respectively.

Fruit development affects the composition and relative abundance of each component of the *n*-alkane fraction of the surface wax of citrus fruit (Nordby and Nagy, 1977b; El-Otmani and Coggins, 1985b). Substantial amounts of C_{20}–C_{27} carbon chains were present in the wax of immature fruit while C_{28}–C_{33} chains predominated in the wax of mature fruit. During fruit senescence, a shift of *n*-alkanes from higher to lower molecular weight components was observed (El-Otmani and Coggins, 1985b).

Because fruit response to biological and physical factors may be influenced by the surface wax layer [see reviews by Baker (1982) and El-Otmani (1985)] and since Valencia orange cultivars (*C. sinensis*) are of considerable value to the California citrus industry, a Valencia orange fruit development/wax *n*-alkane composition study was undertaken. Likewise, because there is evidence of substantial changes in composition during fruit development and since exposure can have significant effects over various aspects of fruit development (Sites and Reitz, 1949; Heinicke, 1966), the potential influence of fruit position within the tree canopy on the composition and relative abundance of *n*-alkane components during fruit development was evaluated.

MATERIALS AND METHODS

Fruit were obtained from a grove of 60-year-old Valencia orange trees on sweet orange rootstock near Visalia, CA. This San Joaquin Valley grove is located on a sandy loam soil with pH 7.0–7.5. Trees have a spacing of 8 m × 9 m and are irrigated by furrows. Rows are oriented north to south.

A block of 180 trees was divided into six replications of 30 trees each from which fruit samples were collected approximately every 4 weeks during 13 months. Sampling started May 22, 1984, approximately 1 month after anthesis, and concluded June 20, 1985, approximately 8 months after fruit color break and approximately 3 months after fruit reached commercial maturity. Twelve fruit samples were collected at each sampling date. This consisted of six replications of fruit from the southwest (SW) quadrant and six replications of fruit from the northeast (NE) quadrant. Initially, each sample consisted of 1000 fruit. As fruit growth occurred, the number of fruit per sample was gradually reduced to 30 in November and was then maintained at this number for the duration of the experiment. Within sampling dates, data were analyzed statistically by a paired comparison test.

Fruit surface area was determined from fruit length and diameter by Turrell's tables (Turrell, 1946), and epicuticular wax extraction was performed by the chloroform dip method, as outlined previously (El-Otmani and Coggins, 1985a). The *n*-alkanes were then isolated from the wax extract by column chromatography with silica gel (28–200 mesh) as the stationary phase and pentane as the moving phase (El-Otmani and Coggins, 1985b).

The composition of the *n*-alkane fraction and relative abundance of each one of its component chains was de-

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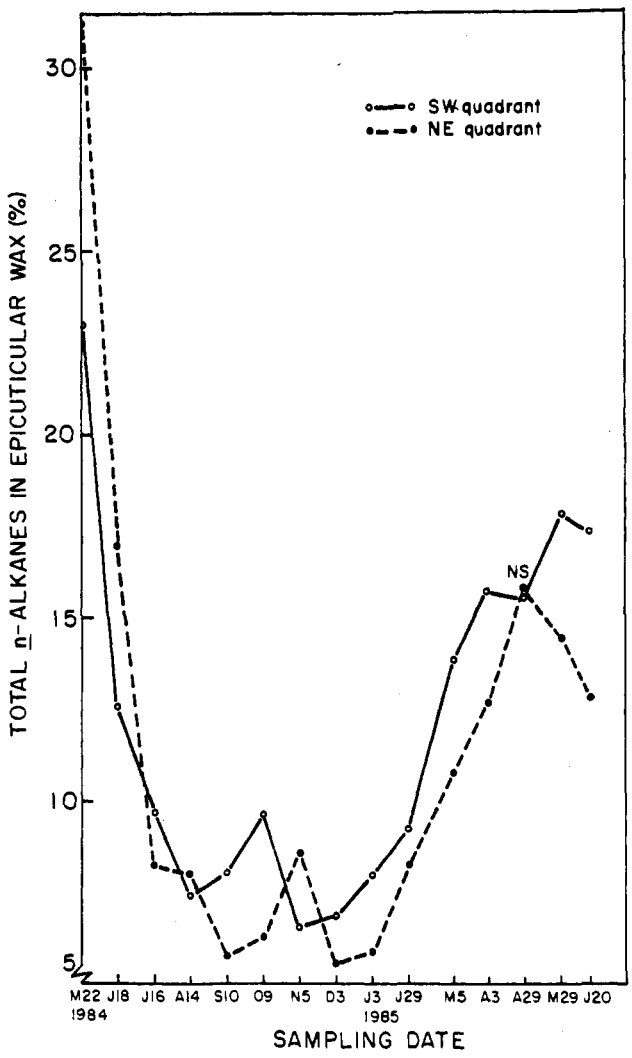

Figure 1. Seasonal variation of, and tree quadrant effect on, the relative abundance of *n*-alkanes in Valencia orange fruit epicuticular wax. Sampling commenced 2 weeks after petal fall, fruit color break occurred in early October, and fruit were legally mature in late March. Within dates, means are significantly different at the 5% level unless the NS designation is present.

termined by gas chromatography as described in an earlier report (El-Otmani and Coggins, 1985b). One analysis was done per sample, and authentic standards of known composition were used to determine retention times of specific *n*-alkanes.

RESULTS AND DISCUSSION

In a related report of this study (El-Otmani, 1985), we found that fruit sampled from the NE quadrant was slightly larger (but not consistently significant) in size than fruit from the SW quadrant. Fruit growth was sigmoidal, which is characteristic of citrus. In the very young fruit, surface wax yield was high in May 1984 (47 $\mu g/cm^2$), decreased to a minimum (17 $\mu g/cm^2$) in June 1984, and then increased significantly with fruit development until the fruit reached legal maturity at which time wax yields started to level off. However, fruit position vs. the amount of epicuticular wax per square centimeter of fruit surface was significant only during the postmaturation stage, with fruit from the SW quadrant accumulating significantly more epicuticular wax than fruit from the NE quadrant (70 and 92 $\mu g/cm^2$ on the SW quadrant vs. 58 and 63 $\mu g/cm^2$ on the NE quadrant fruit for May and June 1985 harvest dates, respectively; El-Otmani, 1985).

The *n*-alkane fraction in the surface wax was high in young fruit, decreased sharply to low values during rapid

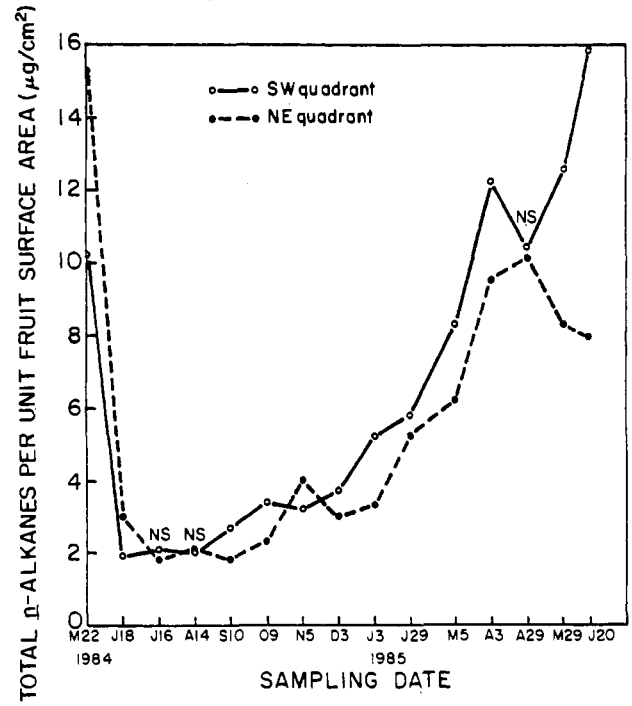

Figure 2. Seasonal variation of, and tree quadrant effect on, yield of epicuticular wax *n*-alkanes per unit surface area of Valencia orange fruit. Within dates, means are significantly different at the 5% level unless the NS designation is present.

fruit growth, and then increased slowly as fruit approached its maximum size (Figure 1). During rapid rates of fruit enlargement (June to November), the rate of accumulation of percent *n*-alkanes was relatively low; however, as fruit growth rates decreased, percent *n*-alkanes accumulated at a rapid rate. These results are somewhat similar to those obtained on navel orange fruits (El-Otmani and Coggins, 1985b).

Young fruit from the SW quadrant contained significantly lower percent wax *n*-alkane content than did fruit from the NE quadrant (Figure 1). During the phase of rapid fruit expansion there were no quadrant differences. However, after fruit approached its maximum size (November to May), significantly higher percent *n*-alkane contents in the epicuticular wax were measured in fruit from the SW quadrant (Figure 1).

The seasonal change in the quantity of *n*-alkanes per unit area of fruit surface (Figure 2) followed the pattern found for the relative abundance of this epicuticular wax fraction (Figure 1). This quantity varied from 10.2 $\mu g/cm^2$ for fruit from the SW quadrant to 15.3 $\mu g/cm^2$ for fruit from the NE quadrant in young fruit wax, decreased to a minimum of 2 $\mu g/cm^2$ in both quadrants during rapid fruit growth, and then increased significantly during the period of slow fruit enlargment. Significantly higher values for SW-quadrant fruit were consistently measured during this later period. The sharp decline in total *n*-alkanes per square centimeter of fruit surface seen on the very young Valencia fruit was not observed in Washington navel orange (El-Otmani and Coggins, 1985b). The decline seen in Figure 1 was directly related to a decline in quantity of wax per unit surface area and was further enhanced by a reduction in *n*-alkane content of the wax. Because there was no change in quantity of epicuticular wax on a whole-fruit basis, this decline appears to be due to a low rate of wax synthesis during an early stage of growth. Such differences were not seen on the navel orange, possibly because our first navel orange sample was taken at a more advanced developmental stage. The marked increase in

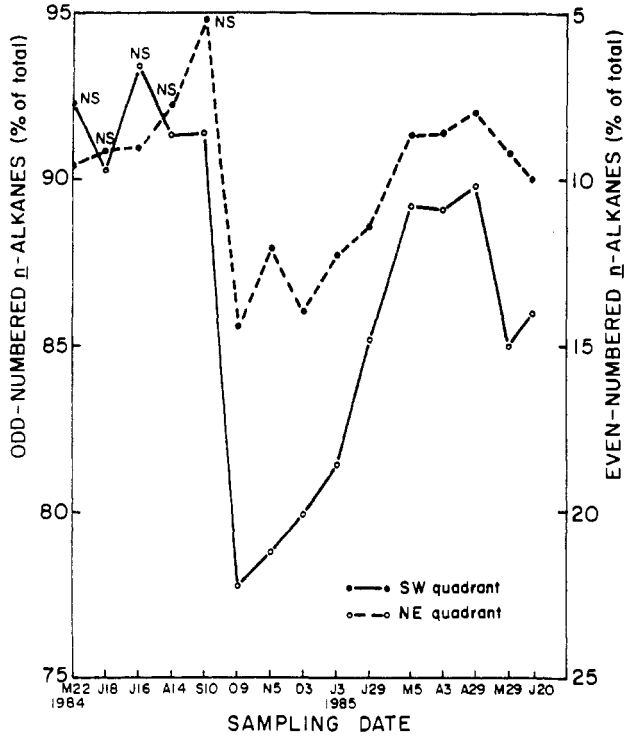

Figure 3. Seasonal variation of, and tree quadrant effect on, the proportion of odd- and even-numbered *n*-alkanes in Valencia orange fruit epicuticular wax. Within dates, means are significantly different at the 5% level unless the NS designation is present.

the *n*-alkane quantity during the period of slow fruit growth was also observed with navels. However, the absolute quantities found on the surface of navel oranges were considerably higher (El-Otmani and Coggins, 1985b).

The *n*-alkane fraction consisted of a mixture of carbon chain lengths, varying from C_{23} to C_{33}. The yield of odd-numbered chains was significantly higher than that of even-numbered chains throughout the season (Figure 3). Odd-numbered chains comprised 90% of *n*-alkanes in the wax of young fruit, declined to a minimum value at fruit color break (October), and then increased thereafter (Figure 3). Fruit position had no effect on the proportions of odd- and even-numbered subfractions prior to fruit color break. However, fruit from the SW quadrant had significantly lower odd-numbered *n*-alkanes following color break (Figure 3). Furthermore, there was a shift in the percentage of total *n*-alkanes from shorter to longer chains during fruit growth and maturation (Figure 4). This was especially apparent for C_{25} vs. C_{31} chains (Figure 5). The most significant shift occurred during early stages of fruit development (Figures 4 and 5). Epicuticular wax of fruit from the NE quadrant, as compared to that of fruit from the SW quadrant, had significantly lower amounts of long *n*-alkanes prior to fruit color break, but this pattern was reversed afterward (Figure 4). Subsequent to fruit maturation, *n*-alkane composition underwent a gradual shift toward shorter chain lengths, regardless of quadrant (Figures 4 and 5).

Seasonal changes in quantities ($\mu g/cm^2$) of long vs. short and even- vs. odd-numbered *n*-alkanes are shown in Figures 6 and 7, respectively. Changes in quantity of long- vs. short-chain alkanes during fruit development (Figure 6A,B) were similar to changes in the pattern of their respective percentages (Figure 4).

Beginning 2 months after color break, and for the duration of the study, fruit from the SW quadrant had greater quantities of short-chain *n*-alkanes than fruit from

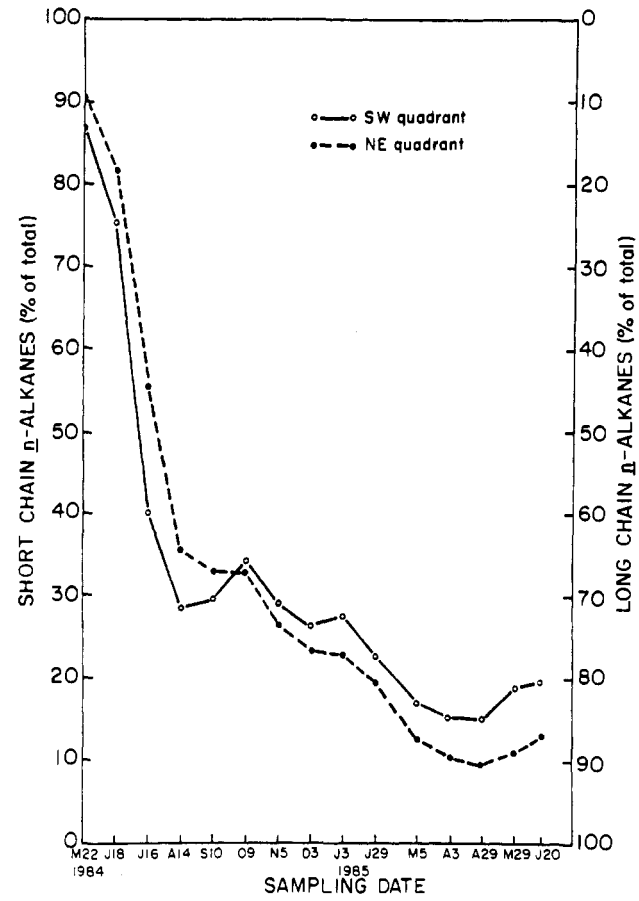

Figure 4. Seasonal variation of, and tree quadrant effect on, the proportion of short- ($\sum C_{23}$–C_{27}) and long- ($\sum C_{28}$–C_{33}) chain *n*-alkanes in Valencia orange fruit epicuticular wax. Within dates, means are significantly different at the 5% level unless the NS designation is present.

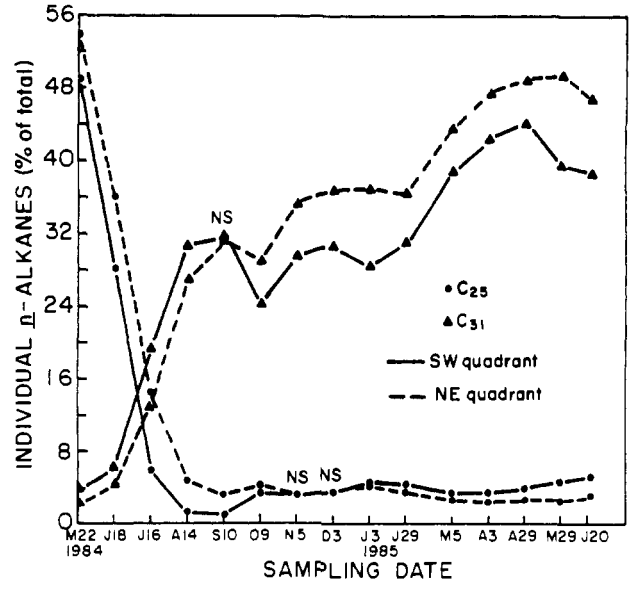

Figure 5. Seasonal variation of, and tree quadrant effect on, the relative abundance of the C_{25} and C_{31} hydrocarbons in Valencia orange fruit epicuticular wax *n*-alkanes. Within dates, means are significantly different at the 5% level unless the NS designation is present.

the NE quadrant (Figure 6A). Such a difference was somewhat less clear for long-chain *n*-alkanes (Figure 6B). The quantity of even- and odd-numbered *n*-alkanes exhibited a sharp decline during the first month of the study and then steadily increased thereafter. During the last 10

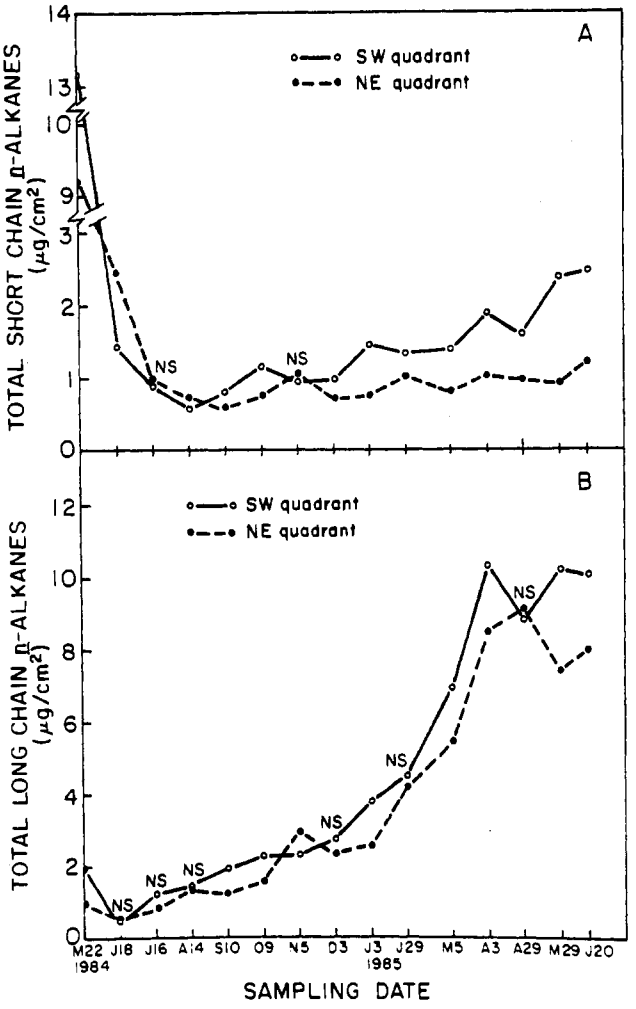

Figure 6. Seasonal variation of, and tree quadrant effect on, the quantity of short- ($\sum C_{23}$–C_{27}) and long- ($\sum C_{28}$–C_{33}) chain *n*-alkanes in Valencia orange fruit epicuticular wax. Within dates, means are significantly different at the 5% level unless the NS designation is present.

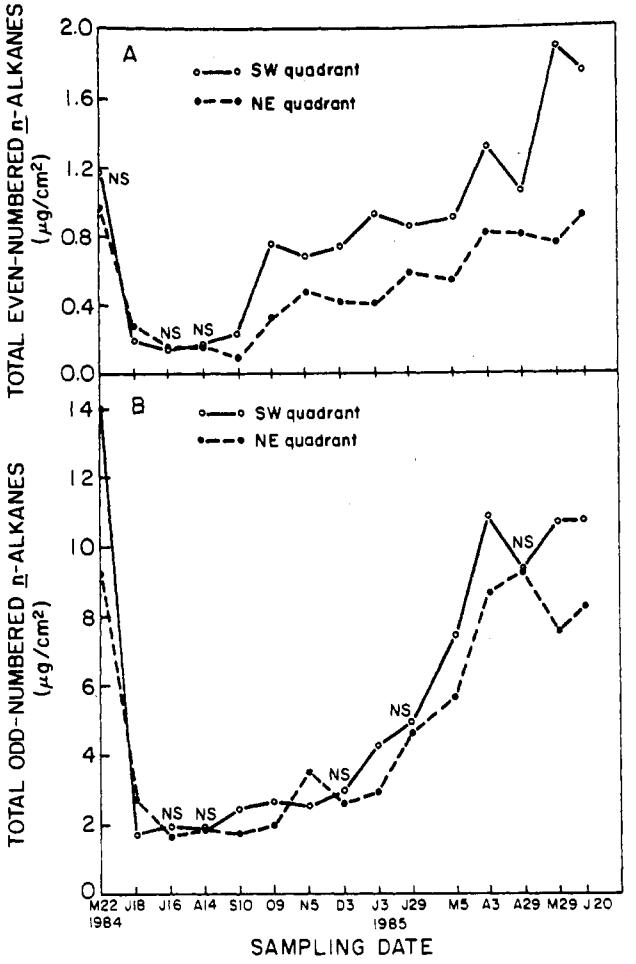

Figure 7. Seasonal variation of, and tree quadrant effect on, the quantity of even- and odd-numbered *n*-alkanes in Valencia orange fruit epicuticular wax. Within dates, means are significantly different at the 5% level unless the NS designation is present.

months of the study, the quantity of even-numbered *n*-alkanes was higher on SW quadrant fruit (Figure 7A). During this same period of time, there were no major influences of fruit exposure on quantity of odd-numbered *n*-alkanes (Figure 7B).

This study revealed differences and similarities with respect to *n*-alkane changes during development of the Valencia vs. the Washington navel orange (El-Otmani and Coggins, 1985b). The presence of C_{23}–C_{33} chains, the dominance of odd-numbered chains, and the shift from low to high molecular weight chains during rapid stages of fruit growth were common to both cultivars. The sharp reduction in *n*-alkanes per unit surface area early in the life of the fruit (Figure 2) and the significant increase in percentage of *n*-alkanes during the stage of slow fruit enlargement and maturation (Figure 1) were not observed in navel orange wax. Moreover, in navels, the shift of *n*-alkanes from short to long chains took place during rapid fruit expansion and reached a limit 4–6 weeks after fruit color break, and then a return toward shorter chain length occurred. In contrast, Valencia orange was characterized by a slow and protracted shift from short to long chains, followed by a slight shift toward short chains beginning 12–13 months after anthesis (Figure 4). We speculate that the faster decline and the early rise in short-chain *n*-alkanes seen in the Washington navel orange vs. the slower and less abrupt changes seen in the Valencia orange are due to vastly different rates of maturation of these two cultivars. The navel orange attains maturity approximately 7 months after anthesis, whereas the Valencia orange requires approximately 11 months to mature. Thus, we suggest that the earlier and faster alkane changes seen for the Washington navel orange are related to earlier maturation and senescence. Furthermore, we followed the Washington navel orange for 4 months subsequent to maturation whereas we followed the Valencia orange for only 2.5 months beyond maturation. It is possible that further shifts toward short chains could have been seen had we followed Valencia orange for several more months.

Differences observed in the *n*-alkane composition with respect to fruit position on the tree suggests a possible effect of light and of temperature on the physiology or biochemistry of wax formation and deposition. This suggestion is presented because the SW quadrant receives higher amounts of direct sunlight and is typically warmer than the NE quadrant, and that light (Macey, 1970; Giese, 1975; Avato et al., 1980) and temperature (Giese, 1975) are reported to influence synthesis of wax components. Such environmental factors have been shown to influence other fruit characteristics. For example, Valencia orange rind color and juice percentages of soluble solids were shown to be closely related to the amount of light to which the fruit and leaves had been exposed. Shaded fruit had lower soluble solids and higher percentage of fruit with green color at maturity than the most exposed fruit (Sites and Reitz, 1949). In apples, fruit color development was directly related to exposure to light, with best color in fruit

exposed to more than 70% full sunlight (FS) (Heinicke, 1966). Also, apples exposed to 90–100% FS had the highest content of soluble solids. In navel oranges, it was shown that fruit composition was associated with the available heat units (Jones et al., 1962) and that fruit from widely spaced trees colored faster and matured earlier than fruit from trees closely planted (Boswell et al., 1982). Higher minimum and maximum temperatures and better light penetration prevail in orchards with widely spaced trees.

When gibberellic acid (GA_3) is applied to navel oranges, it delays rind senescence and moderates compositional changes of epicuticular wax *n*-alkanes (El-Otmani and Coggins, 1985b). In particular, GA_3 moderates the shift from short to long chains during development and reduces the subsequent shift toward short chains during postmaturation and senescence. Also, when expressed as a percentage, wax composition of the GA_3-treated fruit had significantly lower *n*-alkane percentages than wax of untreated fruit. In addition, on an absolute basis ($\mu g/cm^2$), GA_3-treated fruit accumulated significantly less *n*-alkanes than did control fruit. Therefore, because SW-quadrant Valencia orange epicuticular wax contained a higher percentage of total *n*-alkanes (Figure 1) and of short-chain components (Figure 4) and a higher quantity ($\mu g/cm^2$) of *n*-alkanes (Figures 2, 6, and 7) from fruit color break onward, and because most of these differences appeared to increase over time, we suggest that SW quadrant fruit were physiologically more advanced as they approached maturity than fruit from the NE quadrant.

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High-Performance Liquid Chromatographic Analysis of Anticarcinogenic Indoles in *Brassica oleracea*

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Vegetables of the *Brassica* genus contain an (indolylmethyl)glucosinolate, glucobrassicin (GB). The autolysis products of this glucosinolate include indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (I33′), all of which inhibit chemically induced neoplasia in rodents. Analytical methodology, utilizing RP-HPLC, was developed to quantify this potentially important series of indolylic metabolites in foods and to describe the thioglucosidase-mediated autolytic process. Our results demonstrate that I3C is the major GB metabolite generated when plant material is disrupted, although it is not stable in the autolytic milieu (84% conversion to other products in 24 h). Additionally, these results indicate that common cooking practices employed on cruciferous vegetables do not inactivate the thioglucosidase to a significant extent, and thus autolytic products are likely to occur in cooked as well as raw vegetables.

The National Research Council, Committee on Diet, Nutrition and Cancer, has recently recommended increased consumption of cruciferous vegetables as a measure to decrease human cancer incidence (National Research Council, 1982). This recommendation is based on epidemiological evidence (Graham, 1983) and results from animal experiments (Stoewsand et al., 1978; Wattenberg, 1983) that suggest that these vegetables possess cancer-modulating properties. The inhibitory effects may result from exposure to a number of nutritive and nonnutritive constituents known to inhibit chemically induced carcinogenesis in experimental animals [see Wattenberg (1983)].

Vegetables of the *Brassica* genus (family Cruciferae) contain an (indolylmethyl)glucosinolate, commonly known as glucobrassicin (GB). Levels of this thioglucoside are reported to be as high as 1100 $\mu g/g$ in some cultivars (Fenwick et al., 1983). Upon disruption of the plant material a thioglucosidase-mediated autolytic process ensues, yielding a series of 3-substituted indoles; among these are indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (I33′) (Virtanen, 1965; Figure 1). Rodents exposed to these indoles, via intubation or diet,

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