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CHANGES IN THE STERYL LIPID CONTENT AND COMPOSITION OF TOMATO FRUIT DURING RIPENING

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Key Word Index—*Lycopersicon esculentum*; Solanaceae, tomato fruit, phytosterols; sterol conjugates, changes with ripening

Abstract—The content and sterol composition of free sterols (FS), steryl esters (SE), acylated steryl glycosides (ASG) and steryl glycosides (SG) were determined in outer pericarp tissue from 'Rutgers' tomato fruit, ranging from mature green to red ripe, and from fruit of three isogenic non-ripening mutants, *rin*, *nor* and *Gr*. The stigmasterol:sitosterol ratio in the four steryl lipids showed a large increase with ripening of Rutgers fruit, but only a small rise in *rin*, *nor* and *Gr* fruit of comparable age. Sitosterol and stigmasterol were the two major sterols in FS, SG and ASG, composing >80% of the total in both 'normal' and mutant tomatoes. In SE, the proportion of stigmasterol was much lower and that of cholesterol and/or methyl sterols substantially higher. The content of FS, SG and particularly SE increased during ripening of Rutgers fruit, while there was little change in the content of ASG. Increases in the content of FS, SG and SE were far less in pericarp of *rin* mutant fruit. Thus, the changes noted in the steryl lipid content and composition of normal tomato fruit appear to be associated with ripening rather than with aging.

INTRODUCTION

Ripening is a highly dynamic and complex process which entails both the synthesis and degradation of cell components [1, 2]. Hence, ripening can be thought of as a phase of reorganization rather than disorganization of fruit tissues [3]. In tomato fruit, the most obvious change with ripening is the transformation of chloroplasts to chromoplasts. The thylakoid membranes, characteristic of chloroplasts, are degraded and supplanted by large, membrane-bounded, crystalline deposits of carotenoids, principally lycopene [4, 5]. The carotenoids synthesized during plastid transformation are products of the mevalonic acid (MVA)-isopentenoid biosynthetic pathway [6]. In 1978, Chow and Jen [7] addressed the question of whether the level of phytosterols, end products of another branch of the MVA-isopentenoid pathway, also increased during fruit ripening in tomato. They determined that there was indeed a substantial increase in the total sterol content of tomato pericarp between the mature green and red ripe stages. It was also noted that the ratio of stigmasterol to sitosterol, the two major phytosterols, increased dramatically with ripening.

In this study, the reported increases in the total steryl lipid content and in the stigmasterol: sitosterol ratio with ripening were confirmed using fruit from a different tomato cultivar (Rutgers). In addition, quantitative analyses of both the content and sterol composition of individual steryl lipids, including free sterols (FS), steryl esters (SE), steryl glycosides (SG) and acylated steryl glycosides (ASG), were performed with fruit representing six stages of ripening covering the span of 29 to 52 days postanthesis. Finally, equivalent analyses were carried out using fruit from three non-ripening tomato mutants (*rin*, *nor* and *Gr*). The fruit of these mutants mature normally, but are essentially nonclimacteric and undergo softening and plastid transformation at a far slower rate than fruit of cv. Rutgers [8]. The results of these comparative biochemical analyses indicated that the changes in steryl lipid content and composition of Rutgers tomato fruit were largely associated with ripening rather than with aging.

RESULTS

Changes in steryl lipid content with ripening

The distribution of total sterol in pericarp of mature green Rutgers tomatoes (29 to 32 days postanthesis) was 60% in ASG, 26% in SG, 11% in FS and 3% in SE. In red ripe fruit (49 to 52 days postanthesis) the distribution was 33% in ASG, 31% in SG, 17% in FS and 19% in SE. As shown in Fig. 1, these changes in the proportions of the four steryl lipids resulted from large increases in the content of FS, SG and especially SE during ripening. On a fresh weight basis, SE, FS and SG increased 12-, 2.6- and 1.9-fold, respectively, while ASG declined 1.1-fold. The increases in SE, FS and SG appear substantially greater when calculated on a dry weight basis, and the level of ASG shows a small rise rather than a decline (data not shown). This is due to the 30% decrease in dry weight of Rutgers pericarp tissue between 29 and 52 days postanthesis.

At 30 days postanthesis, equivalent to the mature green stage of 'normal' tomato development, fruit of the *rin* tomato mutant showed a distribution of total sterol quite close to that of comparable Rutgers fruit, with 59% in ASG, 26% in SG, 11% in FS and 4% in SE. However, in *rin* fruit picked at 60 days postanthesis, more than a week beyond the fully red ripe stage of Rutgers, the sterol

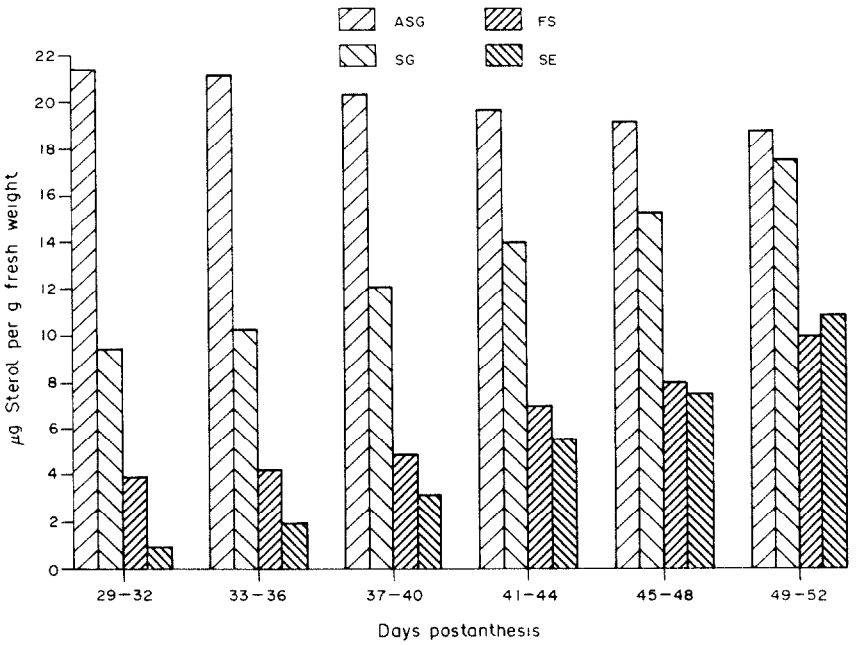

Fig 1 Steryl lipid content of outer pericarp tissue from Rutgers tomato fruit at six stages of ripening ranging from mature green (29 to 32 days postanthesis) to fully red ripe (49 to 52 days postanthesis) Determinations are expressed as μg sterol (recovered from steryl lipid) per gram fresh weight The standard deviation was generally $<10\%$ ($n \geq 5$)

distribution had changed relatively little, with 48% in ASG, 34% in SG, 12% in FS and 6% in SE The results presented in Fig 2 show that between 30 and 60 days postanthesis there was a gradual rise in the content of FS, SE and SG in *rin* pericarp, with little change in the level of ASG On a fresh weight basis, the increases in SE, SG and FS were 1 7-, 1 5- and 1 3-fold, respectively The increase in SG with aging of *rin* fruit most closely approximated the steryl lipid increases during ripening of Rutgers Increases in FS and SE were 2- and 7-fold less, respectively, than those in Rutgers pericarp

Changes in the sterol composition of steryl lipids with ripening

The data in Table 1 show the sterol composition of ASG, SG, FS and SE from pericarp tissue of Rutgers tomatoes at six stages of ripening ranging from mature green to fully red ripe Cholesterol, campesterol, stigmasterol and sitosterol were the four major desmethylsterols in each of the four steryl lipids at all ripening stages Sterols listed as 'other' in Table 1 were determined to be principally mono- and dimethylsterols (see Experimental). These constituted $<5\%$ (by mass) of the total sterols in ASG and SG, and $<10\%$ in FS, but up to 20% in SE depending upon the stage of ripening Among the desmethylsterols, the percentage of cholesterol decreased throughout ripening in SG, FS and particularly SE In contrast, the percentage of campesterol increased to approximately the same extent in all four steryl lipids. The most dramatic change in sterol composition during the ripening of Rutgers tomato fruit was in the ratio of the two C_{29}-phytosterols, stigmasterol and sitosterol The stigmasterol: sitosterol ratio increased sharply in all four steryl lipids between 29 and 52 days postanthesis. As shown in Fig 3, the increase in the stigmasterol sitosterol ratio occurred most rapidly after the 'turning' stage of ripening (*ca* 40 days postanthesis) Also, of the four steryl lipids, the free sterols showed the most pronounced rise (*ca* 5-fold) in the stigmasterol sitosterol ratio

The sterol composition of steryl lipids from fruit of the three non-ripening mutant tomatoes *rin*, *nor* and *Gr*, picked at either 30 or 60 days postanthesis, was also determined and Fig 4 shows the stigmasterol sitosterol ratios There were small increases in the stigmasterol sitosterol ratios of the four steryl lipids with aging of mutant tomato fruit, but far less than those observed with ripening of normal fruit (Fig 3) For example, the increase in the stigmasterol sitosterol ratio in the FS fraction from the non-ripening mutant tomatoes averaged 1 4-fold between 30 and 60 days postanthesis, compared to the 5-fold increase in the FS from Rutgers pericarp between 29 and 52 days postanthesis

DISCUSSION

Changes in the distribution and metabolism of steryl lipids have been shown to occur during the growth and development of a variety of plants and plant organs [9–11] These observations raise the question of the physiological role of sterols and sterol conjugates in plant tissues and organelles The pericarp of tomato fruit, and particularly the plastids therein, undergo drastic reorganization during ripening [1, 4, 12] In this and in one previous study [7], it was found that a large increase in the content of steryl lipids, as well as a dramatic change in their sterol composition, occur with ripening in tomato It was also shown in the present study, through comparison with fruit of non-ripening mutants, that these steryl lipid changes are 'ripening specific', i e they are much more

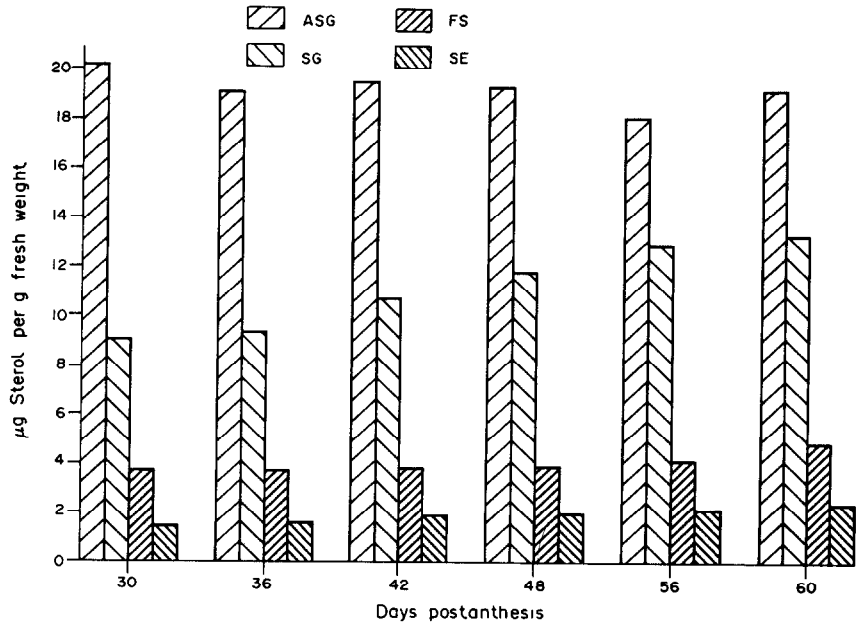

Fig 2 Steryl lipid content of outer pericarp tissue from fruit of the non-ripening tomato mutant *rin* at six ages ranging from 30 to 60 days postanthesis At 30 days postanthesis the size and seed development of *rin* fruit was equivalent to that of mature green Rutgers fruit Determinations are expressed as μg sterol (recovered from steryl lipid) per gram fresh weight The standard deviation was generally $<10\%$ ($n=3$)

Table 1 Sterol composition of steryl lipids from pericarp of Rutgers tomato fruit at six stages of ripening (values expressed as mean weight per cent of total steryl lipid sterols ±s d of 5 to 10 determinations)

		Stage of ripening* (days postanthesis)					
Steryl lipid	Sterol moiety	MG (29–32)	BK (33–36)	TN (37–40)	PK (41–44)	OR (45–48)	RR (49–52)
Acylated steryl glycoside	Cholesterol	4 4±0 3	4 3±0 1	4 4±0 4	3 9±0 1	4 3±0.3	3 7±0 3
	Campesterol	6 7±0 4	7 7±0 5	7 7±0 2	8 4±0.4	10.2±0 5	10.6±0.8
	Stigmasterol	14 1±0 5	16 3±0 9	17 4±1 0	22.8±0 4	26.1±0 5	36.0±1 4
	Sitosterol	71.4±0.4	69 1±0 6	67 9±1.1	61 3±0 8	57 3±0 8	45 9±1.2
	Other	3 4±0.4	2 6±0.3	2 5±0 3	3 5±0 4	2 1±0.4	3.8±0.7
Steryl glycoside	Cholesterol	6 2±0 6	5.6±0 5	5 3±0 4	4.8±0 3	4 5±0.4	3.9±0.3
	Campesterol	7 1±0 6	7 9±0 4	7 8±0 1	8 9±0 5	10.1±0.3	10.8±0.7
	Stigmasterol	15 2±0 6	16 7±0 7	19.0±0.5	26.6±0.4	29 5±0 9	39.9±0.9
	Sitosterol	70 1±1 1	68 5±1 1	67 0±1.0	58 8±0.4	54.8±1 0	43 8±0.8
	Other	1 3±0 2	1 3±0 2	1 0±0 1	1 0±0 2	1 1±0 1	1 7±0 7
Free sterol	Cholesterol	8.1±0 4	7 0±0.4	6 2±0 5	5 9±0 3	4 5±0.5	3.6±0.4
	Campesterol	7 7±0 2	8 7±0 4	9 1±0 6	10 3±0 3	10.4±0.4	11.0±0.2
	Stigmasterol	23 0±1 3	26 0±0.7	30 7±0 7	42 6±0.9	49.3±1.1	57.7±0.4
	Sitosterol	53 7±2 0	50 9±1 6	45 5±1 1	32.8±0 9	28.6±1.7	22.8±0.5
	Other	7 7±0 6	7 4±0 7	8 6±0.9	8 3±0 6	7 1±0 5	4.9±0 4
Steryl ester	Cholesterol	20 2±1 6	15 9±2 4	6 3±0 6	5 1±0 5	4 3±0 5	3.2±0.2
	Campesterol	6 4±1 3	8 0±0.8	7.7±0 3	10 0±0 4	10 9±0.8	11.1±0.6
	Stigmasterol	3 1±0 7	4 5±1.1	5.3±0 4	8 2±0 2	12.8±0.7	17.9±1.8
	Sitosterol	50 8±2 1	60 5±1.2	62.9±1 5	59 0±0.8	57.9±1.0	54.8±1.7
	Other	19 5±1 1	11 1±1 7	17 9±0 7	17.7±0.7	14.1±1.0	12.9±0.6

*Abbreviations MG = mature green, BK = breaker, TN = turning, PK = pink, OR = orange, RR = red-ripe.

pronounced with ripening than merely with aging of the fruit.

The proportions of the four steryl lipids determined for the pericarp of mature green tomato fruit were quite similar to those reported for leaves of fully grown tomato plants by Dupéron *et al.* [13]. Most notably, glycosylated sterols (ASG + SG) constituted $>80\%$ of the total sterols in both organs. This percentage is quite high in com-

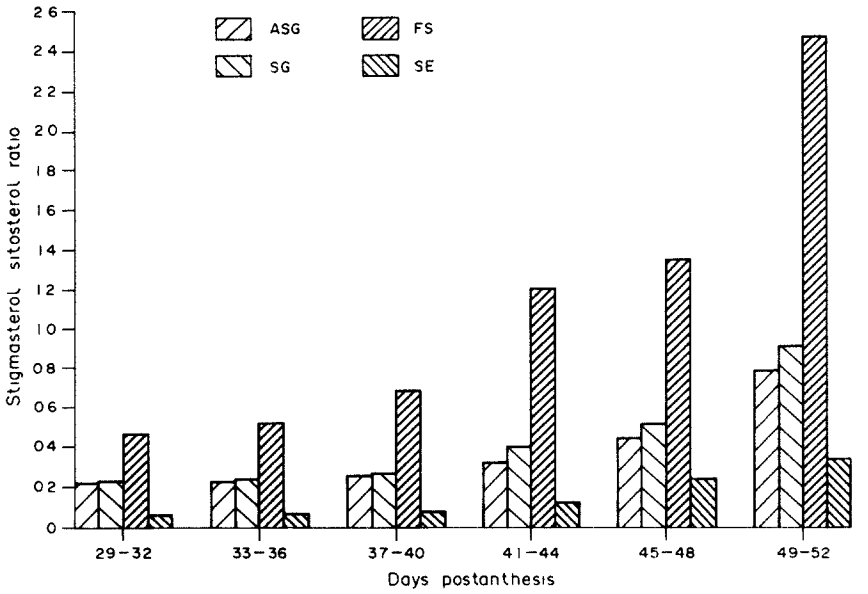

Fig. 3 Ratio of stigmasterol to sitosterol in steryl lipids from outer pericarp tissue of Rutgers tomato fruit at six stages of ripening ranging from mature green (29 to 32 days postanthesis) to fully red ripe (49 to 52 days postanthesis) The standard deviation was generally $<10\%$ ($n \geq 5$)

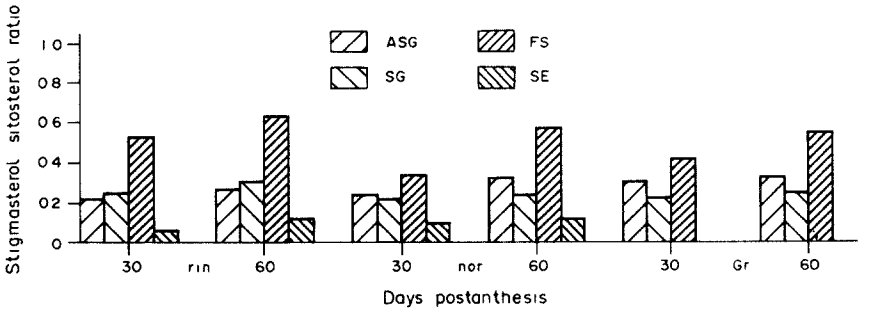

Fig 4 Ratio of stigmasterol to sitosterol in steryl lipids from outer pericarp tissue of fruit from the three non-ripening tomato mutants *rin*, *nor* and *GR* at 30 and 60 days postanthesis At 30 days postanthesis the size and seed development of mutant fruit was equivalent to that of mature green Rutgers fruit The standard deviation was generally $<10\%$ ($n=3$)

parison to that reported for many other species and appears to be a peculiarity of a few closely related members of the genera *Solanum* and *Lycopersicon* [13]. It is currently thought that both ASG and SG serve as membrane structural lipids [11, 14, 15], and the unusually high proportion of these sterol conjugates in several solanaceous plants has led to speculation that they have a specialized role in governing the physical properties of cell membranes in these species [13]

The changes observed in the proportions of the steryl lipids during ripening of tomato fruit were similar to those found to occur in senescing leaves of tomato plants [13] Specifically, the percentage of total sterols represented by SE and FS rose while ASG declined. However, the total sterol content of tomato fruit increased dramatically with ripening, in contrast with senescing leaves, in which the total sterol content remained unchanged or decreased slightly. Hence, changes in the proportions of ASG, SG, FS and SE in ripening fruit probably result from increased steryl lipid synthesis, while the changes in senescing leaves apparently arise via interconversion of steryl lipids

The 12-fold increase in the SE content of tomato fruit pericarp was the greatest change in the steryl lipids during ripening A rise in the level of SE has been observed with aging or senescence in a number of plant tissues [9, 13, 16], and it has been suggested that they function in the removal of FS and SG from senescing membranes for transport elsewhere [13, 17] This role for SE has been disputed [9, 11] and seems unlikely in tomato fruit, as the levels of FS and SG also increased substantially during ripening While there is some evidence that SE synthesis occurs in plant mitochondria, this is far from certain [11, 18] Ripening pericarp of tomato appears to be an ideal tissue for further study of steryl ester synthesis and function

The sterol composition of SE, ASG, SG and FS changed dramatically during ripening of tomato fruit. Most notable was the large increase in the ratio of

stigmasterol:sitosterol, particularly in the free sterol fraction. Similar, but less pronounced shifts in the stigmasterol: sitosterol ratio have been reported to occur with maturation or aging of leaves, hypocotyls and whole plants [9, 10, 19]. There is also evidence that light, perhaps through phytochrome, can influence the ratio of stigmasterol to sitosterol in plant tissues, including etiolated shoots of barley and pericarp of ripening tomato fruit [7, 9, 20]. Thus, a shift in the proportions of the two predominant phytosterols is often associated with the later stages of plant growth and development, and this compositional change can be under photoperiodic control.

The changes in steryl lipid composition in ripening tomato fruit and other plant tissues have no known physiological significance, but are likely to affect membrane fluidity. The increasing steryl lipid.phospholipid ratio during ripening of tomato fruit should also influence the physical properties of cell membranes, the expected effects including increased ordering and condensation of the bilayer due to sterol-phospholipid interactions, with resultant decreases in membrane fluidity and permeability [21, 22]. A recent study by Legge *et al.* [23] has in fact indicated that the plasma membrane of cells in tomato pericarp undergoes a large decrease in fluidity during ripening, although an earlier study of potassium flux in tomato found no change in the permeability of the plasma membrane or tonoplast to K^+ with ripening [3]. Possibly, effects on the physical properties of cell membranes produced by alteration of their steryl lipid content and composition may serve to modulate the activities of membrane-bound enzymes which are involved in the processes of ripening and senescence [15, 23].

EXPERIMENTAL

Plant material. Tomato plants (*Lycopersicon esculentum* Mill cv 'Rutgers' and the isogenic non-ripening mutants *rin, nor* and *Gr*) were grown in a greenhouse without supplemental lighting. Flowers were tagged at anthesis and hand-pollinated Only two fruit were allowed to develop from each inflorescence to ensure uniformity. Fruit of uniform size (148 ± 21 g fr wt) were hand-harvested between the mature green and red ripe stages of ripening (29 to 52 days postanthesis for cv Rutgers), or in the case of the non-ripening mutants, between 30 and 60 days postanthesis. Fruit were quartered following excision of the stem scar region and the locular tissue was removed Outer pericarp sections (including the epidermis) from two or three fruit were quickly blotted and immersed in liquid nitrogen The randomized, frozen pericarp quarters were fractured and stored at −20° prior to lipid extraction At least three separate tissue samples were analysed for each stage of ripening or age postanthesis.

Lipid extraction and fractionation Frozen pericarp tissue (between 30 and 40 g fr wt per sample) was diced, immersed in 40 ml of hot *i*-PrOH and boiled gently for 5 min After cooling on ice, the propanolic tissue suspension was homogenized with three 15 sec bursts (at setting 4) of a Polytron tissue homogenizer. The homogenate was filtered through a sintered glass funnel The filtrate was dried down to an aq residue at 50° under N_2 and resuspended in three volumes of $CHCl_3$–MeOH (2 1). The propanol-extracted tissue was resuspended in 40 ml of $CHCl_3$–MeOH, (2·1) in a 250 ml flask, which was flushed with N_2, sealed and agitated on a rotary shaker for 1 hr. Following filtration and further rinses of the tissue with $CHCl_3$–MeOH (2·1) the combined lipid extracts were washed [24], and the $CHCl_3$ phase containing total lipids was evaporated under N_2. The lipids were dissolved in 2 ml of $CHCl_3$ prior to CC The extracted tissue was scraped into a small aluminium weighing dish and dried overnight in an oven at 60° prior to dry wt determination.

The neutral lipid fraction containing FS and SE, and the glycolipid fraction containing SG and ASG, were recovered in sequence from a silicic acid column (100–200 mesh) by elution with $CHCl_3$ (FS + SE), then Me_2CO (ASG + SG) [25] The neutral lipid fraction was dried under N_2 and dissolved in hexane–Et_2O (10 1), followed by separation of SE and FS by passage through a silica gel Sep-Pak (Waters) SE were eluted first with hexane–Et_2O (10 1) then FS were eluted with hexane–Et_2O (2 1) ASG and SG were separated by elution from a second silicic acid column with $CHCl_3$–Me_2CO (2 1) and Me_2CO, respectively

Sterol isolation and analysis. SE were cleaved by saponification (1 M KOH in 80% EtOH), while SG and ASG were acid hydrolysed [26]. The sterols freed by hydrolysis were recovered by hexane extraction Following saponification of the SE fraction from orange and red ripe 'Rutgers' fruit, a Sep-Pak separation was performed as described above to isolate the sterols from the bulk of the lycopene Sterols derived from the four steryl lipid fractions were precipitated with digitonin and recovered as described elsewhere [27]. Prior to digitonin precipitation, 10 μg of lathosterol (cholest-7-en-3β-ol) were added to each fraction as internal standard Quantitative analysis of underivatized sterols was performed by GLC on 1 m × 2 mm glass coil columns packed with either SP 2100 or SP 2250 (Supelco) Column oven, injector and detector (FID) temperatures were 240° (isothermal), 300°, and 350°, respectively Carrier gas (N_2) flow rate was 30 ml/min [27]. Retention times were compared with authentic cholesterol, campesterol, stigmasterol and sitosterol standards. Unidentified sterols constituted from *ca* 1 to 20% of the total in the different steryl lipid fractions, the percentage varying with the stage of ripening Based on their separation from the desmethylsetrols on a neutral alumina column and on the relative retention times of the free sterols vs their acetates, most of these unknowns appear to be methylsterols [28]

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