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Aroma Biosynthesis in Strawberry: *S*-Adenosylmethionine:Furaneol *O*-Methyltransferase Activity in Ripening Fruits

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Among the most important volatile compounds in the aroma of strawberries are 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Furaneol) and its methoxy derivative (methoxyfuraneol, mesifuran). Three strawberry varieties, Malach, Tamar, and Yael, were assessed for total volatiles, Furaneol, and methoxyfuraneol. The content of these compounds sharply increased during fruit ripening, with maximum values at the ripe stage. An enzymatic activity that transfers a methyl group from *S*-adenosylmethionine (SAM) to Furaneol sharply increases during ripening of strawberry fruits. The in vitro generated methoxyfuraneol was identified by radio-TLC and GC-MS. The partially purified enzyme had a native molecular mass of ~80 kDa, with optimum activity at pH 8.5 and 37 °C. A high apparent K_m of 5 mM was calculated for Furaneol, whereas this enzyme preparation apparently accepted as substrates other *o*-dihydroxyphenol derivatives (such as catechol, caffeic acid, and protocatechuic aldehyde) with much higher affinities (K_m ~ 105, 130, and 20 μM, respectively). A K_m for SAM was found to be ~5 μM, regardless of the acceptor used. Substrates that contained a phenolic group with only one OH group, such as *p*-coumaric and *trans*-ferulic acid, as well as *trans*-anol and coniferyl alcohol, were apparently not accepted by this activity. It is suggested that Furaneol methylation is mediated by an *O*-methyltransferase activity and that this activity increases during fruit ripening.

KEYWORDS: 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (Furaneol); methoxyfuraneol; *O*-methyltransferase; strawberry (*Fragaria* × *ananassa*)

INTRODUCTION

Strawberries are cultivated in nearly all countries of the world and are one of the most popular and important red fruits consumed either fresh or as a conserved or manufactured product (*1*). Strawberry breeding has mainly focused on improving yields, disease resistance, fruit appearance, firmness, size, and storage properties. Selection for flavor has been subjective up to now, depending mainly on breeders' individual tastes and their ability to differentiate aromas, with occasional use of panels to rate final selections. There is a need to better assess and quantify components of flavor to more purposefully improve this trait.

Fruit aroma and taste are the result of a special assortment and relative quantities of a mixture of different metabolites. Whereas sweetness and acidity are caused by sugars and acids, respectively, aroma is composed of unique combinations of volatile molecules. The different proportions of the volatile components and the presence or absence of trace components often determine aroma properties. Strawberry cultivars have been examined to test the characteristic effects of storage and postharvest treatments on their aroma (*2*).

One of the most important flavor compounds in strawberries is 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Furaneol). Furaneol was identified for the first time as a natural aroma component in pineapples (*3*). It has since been detected in several fruits such as strawberry (*4*), raspberry (*5*), mango (*6*), and tomato (*7*). Due to their low odor threshold, and characteristic flavor, Furaneol and its methoxy derivative (methoxyfuraneol and mesifurane) are among the most important aroma compounds in strawberry fruits. Flavor, however, also depends on the presence of small quantities of other volatiles with low threshold values, such as the pleasant-smelling esters γ- and δ-lactones and methylanthranilates or the unpleasant-smelling compounds such as hexanoic acid (*1*, *8*). In addition, the nonvolatile β-D-glucopyranoside (*9*) and the malonylated derivatives of Furaneol

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(*10*) have been detected in strawberry fruits, but their role in conferring aroma to fruits is still unclear.

Despite its importance as a natural aroma compound, little is known about the biosynthesis and metabolism of Furaneol in plants. Quantification of Furaneol, methoxyfuraneol, and Furaneol glucoside during fruit ripening indicated conversion of Furaneol into these derivatives (*11*, *12*). Recently, a study of the metabolism of Furaneol in detached ripening strawberry fruits demonstrated the incorporation of both *S*-(*methyl*-^{14}C)-adenosyl-L-methionine (SAM) and (^{14}C)Furaneol into methoxyfuraneol (*13*, *14*). This observation indicates the putative role of a methyltransferase enzyme, able to transfer the methyl group from SAM to Furaneol. Methyltransferases are ubiquitous enzymes that catalyze the transfer of a methyl group from SAM to an acceptor substrate, generating *O*-, *N*-, *S*-, and *C*-methyl derivatives and *S*-adenosylhomocysteine (*15*, *16*). SAM-dependent methyltransferases are involved in the O-methylation of many plant natural products such as lignin, flavonoids, phenylpropenes, and alkaloids (*17*, *18*). Some of the known *O*-methyltransferases (OMTs) display strict specificities toward their acceptor substrate and to the position of the methylation of the substrate (*15*). Other OMTs, especially those catalyzing the methylation of catechol (*o*-dihydroxy) moiety substrates, exhibit surprisingly broad substrate specificity: they have been shown to be multifunctional enzymes (*19*) and can also catalyze transformations in two different biosynthetic pathways such as the alkaloid and phenylpropanoid pathways (*19*) or the flavonoid and other phenylpropanoid pathways (*20*).

During the process of fruit maturation Furaneol can be rapidly converted into methoxyfuraneol and its glucoside (*11*, *12*). Zabetakis and Holden (*22*) suggested that the total amount and the ratio of Furaneol and methoxyfuraneol determine the different taste of wild strawberry in contrast to that of cultivated varieties. We hypothesized that methoxyfuraneol is formed due to the action of an OMT activity on Furaneol, utilizing SAM as the methyl group donor (**Figure 1**). This activity has not been reported so far, despite the numerous OMTs known to occur in plants, animals, and microorganisms and the many studies concerning aroma formation in strawberry.

The objective of this study was to prove the existence of a SAM/Furaneol OMT activity in strawberry fruits, to quantify its activity during maturation of the fruits, to assess its presence in several varieties, and to partially characterize some of the biochemical properties of this activity. A better understanding of the enzymes involved in the formation of methoxyfuraneol will assist classical breeding and biotechnological efforts to improve the aroma of strawberries.

MATERIALS AND METHODS

Plant Materials. Fresh (for volatiles) or frozen (for enzymatic analyses) fruits of *Fragaria* × *ananassa* var. Tamar, Yael, and Malach were obtained from the Volcani Institute, Israel. Plants were grown in an unheated greenhouse on raised beds in sandy soil covered with black plastic, 16 plants/m^2. Fertilizer and pest control were applied mimicking commercial growing conditions (*23*). Runner plants were planted at the end of September, flowering began 4−6 weeks later, and fruit harvest was carried out two or three times per week from the end December until mid-May.

Tamar and Yael are sister lines from an Israeli breeding program but differ in that Tamar produces sweet fruits which might be aromatic or not depending on the growing conditions during ripening, whereas Yael produces fruits that are less sweet and not aromatic. Malach, which shares one common parent with Tamar and Yael, produces uniformly sweet, aromatic fruits throughout the growing season.

Extraction of Volatiles. Fresh strawberry fruits (30 g) were homogenized in a food processor (Braun) and extracted vigorously for 2 h with 50 mL of *tert*-butyl methyl ether containing 10 μg of isobutylbenzene as an internal standard. The organic phase was dried on anhydrous Na_2SO_4 and evaporated under nitrogen to 1 mL (*24*).

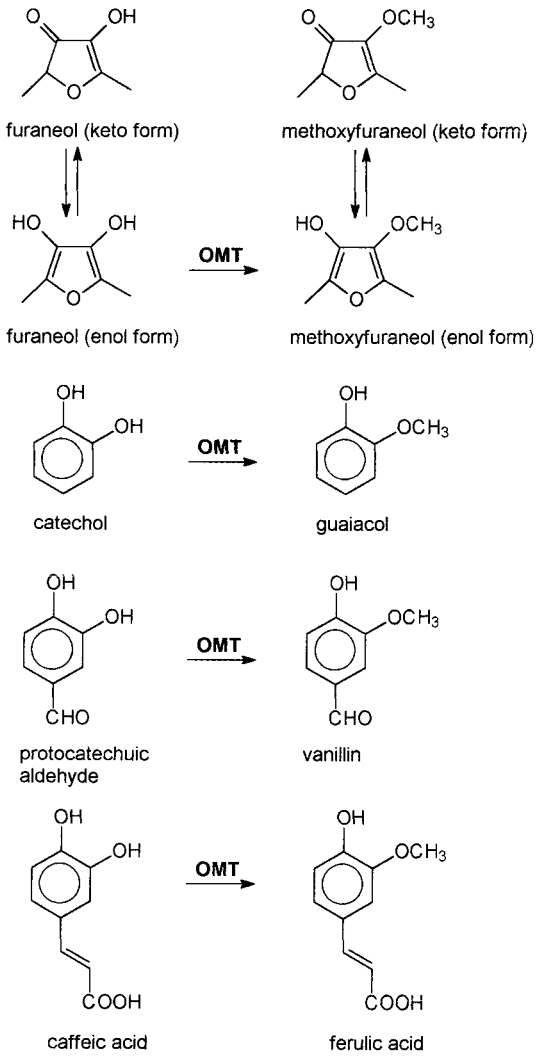

Figure 1. O-methylation of Furaneol to methoxyfuraneol in strawberry fruits by the action of *O*-methyltransferase activity. Other substrates and their methylated products are also shown.

GC-MS Analysis. Volatile compounds were analyzed on an HP-GCD apparatus equipped with an HP-5 (30 m × 0.25 mm) fused-silica capillary column. Helium (1 mL/min) was used as a carrier gas. The injector temperature was 250 °C, set for splitless injection. The oven was set to 50 °C for 1 min, and then the temperature was increased to 200 °C at a rate of 4 °C/min. The detector temperature was 280 °C. Mass range was recorded from *m*/*z* 45 to 450, with electron energy of 70 eV. Identification of the main components was done by comparison of mass spectra and retention time data with those of authentic samples and supplemented with a Wiley GC-MS library. The semiquantitative analyses were determined using isobutylbenzene as an internal standard (*24*, *25*).

Chemicals and Radiochemicals. *S*-Adenosyl-L-methionine was purchased from Sigma Chemical Co. *S*-Adenosyl-L-methyl[^{3}H]-methionine (s.a. = 15 Ci/mmol) and *S*-adenosyl-L-methyl[^{14}C]-methionine (s.a. = 55 mCi/mmol) were purchased from Amersham. Phenolic substrates, including caffeic acid, catechol, protocatechuic aldehyde, Furaneol, and methoxyfuraneol were purchased from Sigma-Aldrich Co.

Preparation of Crude Cell-Free Extracts. Frozen strawberry fruits (10 fruits for each replicate) were cut into ∼1 cm^3 slices, and cell-free extracts were prepared as follows: slices (4−5 g) were weighed and placed in a chilled mortar. The tissues were then ground with a pestle in the presence of ∼0.5 g of polyvinylpolypyrrolidone (PVPP) to

adsorb phenolic materials, and 15 mL of extraction buffer A [100 mM Tris-HCl, pH 8.5, containing 10% glycerol, 5 mM $Na_2S_2O_5$, 10 mM 2-mercaptoethanol, and 1% poly(vinylpyrrolidone)-10 (PVP-10)] was added (*26*). The slurry was mixed at 4 °C for 30 min and then centrifuged at 20000*g* for 10 min at 4 °C. The supernatant (crude extract) was partially purified for the enzymatic assays, see below.

Partial Purification. All procedures were performed at 0−4 °C, using buffer B (50 mM Tris-HCl, pH 8.5, containing 10% glycerol, 5 mM $Na_2S_2O_5$, 10 mM *2*-mercaptoethanol, and 1 mM EDTA). The crude extract (3 mL) was applied to a Bio-Gel P-6 (Bio-Rad Laboratories, Inc.) column (1 × 10 cm). Column equilibration and protein elution were performed with buffer B. Fractions were tested for enzyme activity, and active fractions were combined. The P-6-active fractions (2 mL) were applied to a HiTrap Q Sepharose column (1 × 5 cm, FPLC, Amersham Pharmacia Biotech), equilibrated with buffer B. Proteins were eluted with a three-step (10 min, 0−30%; 20 min, 30−60%; 10 min, 60−100%, mL/min) linear 0−1 M KCl gradient in buffer B. Fractions were tested for enzyme activity, and active fractions were combined and used for further tests. All assays and enzyme characterizations were done with this partially purified enzyme.

Native Molecular Mass. The molecular mass of the native enzyme was determined by gel permeation chromatography of a 2 mL partially purified enzyme preparation separated through a Superdex 75 Hiload Prep 16/60 (FPLC, Amersham Pharmacia Biotech), using buffer B at a flow rate of 1 mL/min, and compared to molecular masses of known proteins.

Assays of Enzyme Activity. The standard assay mixture consisted of 30 μL of buffer B, 50 μL of enzyme solution (partially purified sequentially by Bio-Gel P6 and HiTrap Q-Sepharose) containing 10−25 μg of protein, 15 mM Furaneol, and 15 μM [^{3}H]SAM, in a total volume of 100 μL. The mixture was incubated at 30 °C for 1−2 h. The reaction was stopped by adding 10 μL of 2 N HCl and mixed. Then 1 mL of ethyl acetate was added to each tube, and the tubes were vigorously vortexed and subsequently centrifuged for 1 min at 20000*g* to separate the phases. The upper ethyl acetate phase layers containing the radioactive labeled enzyme products were transferred to scintillation tubes containing 3 mL of scintillation liquid [4 g/L 2,5-diphenyloxazole (PPO) and 0.05 g/L 2,2′-*p*-phenylenebis(5-phenyloxazole (POPOP) in toluene]. The radioactivity was quantified using a Kontron liquid scintillation counter model BETAmatic.

Effect of pH and Temperature on Enzyme Activity. To determine the optimal pH for activity, enzymatic activity was assayed at 30 °C utilizing buffer B except that the buffer was substituted as follows: 50 mM sodium acetate and 50 mM Tris-HCl for the pH ranges of 5.0−6.0 and 6.0−10.0, respectively. HEPES and MOPS (50 mM) were used for the pH range of 6.5−8.2. To determine the effect of temperature on enzymatic activity, incubations were performed at different temperatures utilizing buffer B (pH 8.5).

Identity of the Biosynthetic Products. *Radio-TLC.* To confirm the identity of the biosynthetic products by radio-TLC, similar incubations were performed except that ^{14}C-labeled SAM (at the same specific activity) was used. In this case the ethyl acetate layer was evaporated to a volume of 20 μL using a gentle stream of N_2 and analyzed by TLC-autoradiography using silica gel 60 F_{254} plates developed with pentane/diethyl ether (5:1). Spots were visualized without any further spraying by fluorescence at 254 nm UV light and compared to authentic standard Furaneol and methoxyfuraneol. Radioactive spots were detected by autoradiography on Kodak X-OMAT paper.

GC-MS. Similar incubations were performed with non-radioactive SAM, and the reaction's products were identified by GC-MS as described above.

Protein Determination. Protein was measured according to the method described by Bradford (*27*), using the Bio-Rad protein reagent (Bio-Rad) and bovine serum albumin (Sigma) as standard.

RESULTS AND DISCUSSION

Determination of SAM/Furaneol *O*-Methyltransferase Activity from Strawberry Fruits. Incubation of strawberry partially purified cell-free extracts with ^{14}C- or ^{3}H-labeled SAM and Furaneol resulted in the accumulation of ethyl acetate soluble radiolabeled product(s). These products were absent when the cell-free extracts were previously boiled for 5 min or without a protein extract. This suggests that a methyltransferase enzymatic activity is involved in the conversion of Furaneol into methoxyfuraneol (**Figure 1**).

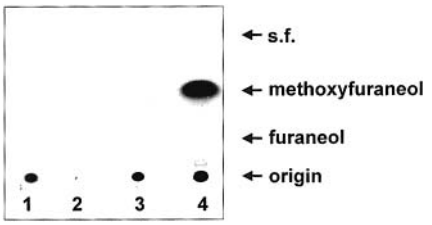

Figure 2. Radio-TLC determination of enzymatically formed methoxyfuraneol by strawberry cell-free extracts: lane 1, enzyme + [^{14}C]SAM; lane 2, enzyme + Furaneol; lane 3, [^{14}C]SAM + Furaneol; lane 4, complete assay (enzyme, + Furaneol + [^{14}C]SAM). Partially purified (after P6 and Hi-Trap Q Sepharose chromatography) cell-free extracts from strawberry fruits were incubated with the corresponding additions. The products of the reactions were analyzed as described under Materials and Methods. S.f. indicates the solvent front.

Table 1. Substrate Specificity of OMT Activity from Strawberry Fruits[a]

substrate	concn (mM)	product identified[b]	relative activity[c]
caffeic acid	1	ferulic acid	100
catechol	1	guaiacol	131
protocatechuic aldehyde	1	vanillin	140
Furaneol	10	methoxyfuraneol	27

[a] Assays were made and analyzed as described under Materials and Methods. *p*-Coumaric acid, cinnamic acid, ferulic acid, vanillic acid, *trans*-anol, chavicol, coniferyl alcohol, phenol, hydroquinone, and *o*-cresol were not methylated in detectable levels by the enzyme preparation. [b] As determined by GC-MS, by comparison of retention times and mass spectra with authentic standards. [c] Percent rate of caffeic acid methylation.

To confirm the identity of the radiolabeled compounds formed, the ethyl acetate extracts were evaporated and analyzed by TLC-autoradiography and GC-MS. Only one radioactive substance originating in Furaneol and [^{14}C]SAM fed extracts was detected, and its R_f coincided with that of authentic methoxyfuraneol (**Figure 2**). These results indicate that methyltransferase activity, present in strawberry fruits, is able to O-methylate Furaneol and release methoxyfuraneol. Further confirmation of the product was performed utilizing GC-MS (**Table 1**).

Extraction and Properties of the SAM/Furaneol *O*-Methyltransferase Activity from Strawberry Fruits. In crude cell-free extracts prepared from ripe strawberry fruits, we noticed the formation of unidentified radioactive, ethyl acetate soluble products, even without added Furaneol (up to 10−30% of the levels obtained in complete assays with Furaneol), indicating the presence of endogenous substrates. These interfering substances were effectively removed after partial purification using adsorption to vinylpyrrolidone polymers, gel filtration, and ion exchange chromatography. The inclusion of PVP and PVPP during the extraction was also crucial for stabilizing the enzymatic activity, which could be kept (after partial purification) for >3 months at −20 °C without an apparent loss of activity.

The activity levels linearly increased with incubation time for up to 2 h and were linearly dependent on protein concentration up to 25 μg of protein. Activity increased linearly with increasing assay temperature up to an optimal 37 °C (**Figure**

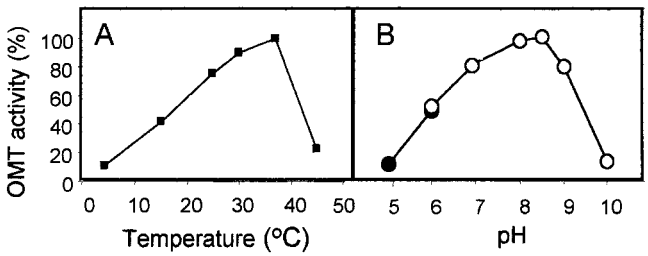

Figure 3. Effect of assay temperature (A) and pH (B) on OMT activity. The partially purified enzyme was incubated with SAM and Furaneol as described under Materials and Methods. A temperature of 37 °C and a pH 8.5 were optimal for activity.

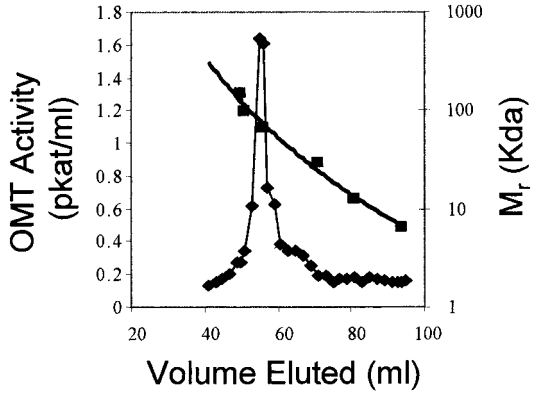

Figure 4. Determination of native molecular mass (MW) of Furaneol *O*-methyltransferase activity from strawberry fruits. The partially purified enzyme was further separated on a Superdex 75 Hi Load 16/60 column (Pharmacia FPLC) using a buffer solution of 50 mM Tris-HCl (pH 8.5) containing 10% glycerol, 5 mM $Na_2S_2O_5$, 10 mM 2-mercaptoethanol, and 1 mM EDTA (flow rate = 1 mL/min). Fractions were assayed for OMT activity as indicated under Materials and Methods. A molecular weight of 80 ± 2 was estimated by comparison to protein standards [alcohol dehydrogenase (MW ~ 150 kDa), phosphorylase *b* (MW ~ 97 kDa), bovine serum albumin (MW ~ 66 kDa), carbonic anhydridrase (MW ~ 29 kDa), cytochrome *c* (MW ~ 12.4 kDa), and aprotinin (MW ~ 6.5 kDa), all from Sigma].

3A). Activity was irreversibly lost at incubation temperatures >45 °C, probably due to protein denaturation. The optimum pH was found to be ~8.5 (**Figure 3B**). The preparation was virtually inactive at pH values <6 or >10.

Some methyltransferases require the presence of metal cofactors for activity. To test whether the Furaneol−*O*-methyltransferase from strawberry requires any metal cofactor for activity, 1 or 10 mM of either $CaCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $ZnSO_4$, or $FeSO_4$ was added to the assays. All of the additions caused only a minor diminution of the activity as compared to the untreated controls. This indicated that the Furaneol-sustained OMT activity from strawberry apparently does not require a metal cofactor to be active.

A native molecular mass of ~80 kDa was determined for the strawberry OMT by gel permeation chromatography (**Figure 4**). This is consistent with the reported molecular mass of other plant OMTs (*16*, *28*), many of which consist of homodimers, with ~40 kDa subunits (*18*).

The OMT activity had an apparent K_m for Furaneol of ~5 mM utilizing both Lineweaver−Burk and Eadie−Hofstee equations. This is **not** within the range of K_m values obtained for substrates of OMTs from other sources, which normally display K_m values lower by orders of magnitude (*16*). Conversely, keeping a constant 15 mM of the acceptor substrate Furaneol, a K_m for SAM was found to be ~5 μM.

Substrate Specificity of the *O*-Methyltransferase Activity. We have provided evidence that, in strawberry, Furaneol can be enzymatically O-methylated, to generate methylfuraneol by SAM-dependent OMT activities. Nevertheless, we found that the K_m for this activity (5 mM) is relatively high in comparison with usual K_m values of acceptor substrates of other OMTs (K_m = 5−500 μM) (*16*, *26*). It was therefore of interest to study the substrate specificity of this Furaneol OMT activity.

To test for the substrate specificity of this activity, we chose several compounds, in concentrations of 1 and 10 mM (**Table 1**). Interestingly, only compounds containing *o*-dihydroxyphenols (catechol, caffeic acid, and protocatechuic aldehyde; **Figure 1**) or similar *o*-dihydroxy compounds (enolic tautomer of Furaneol, Figure 1; and dithiotreitol, not shown) were accepted by this enzyme activity, resulting in the transfer of a methyl group from SAM to create methylhydroxy compounds. The enzyme preparation had a much lower K_m toward catechol (~105 μM), protocatechuic aldehyde (~20 μM), and caffeic acid (~130 μM) than toward Furaneol (~5 mM). Substrates that contained only one OH group, such as *p*-coumaric or ferulic acid, *trans*-anol, and coniferyl alcohol, were not efficiently methylated by this activity. A K_m for SAM was found to be ~5 μM, regardless of the acceptor used (Furaneol, caffeic acid, protocatechuic aldehyde, or catechol). The resulting methylated products (guaiacol for catechol; vanillin for protocatechuic aldehyde; ferulic acid for caffeic acid) were identified by GC-MS (**Table 1**). In all cases, the activity toward these substrates was found in the same fractions collected along the purification process as the activity toward Furaneol.

In the case of substituted *o*-diphenols (caffeic acid and protocatechuic aldehyde), the O-methylation was observed only on the hydroxy group at the meta position to the substituent, similary to other caffeic acid OMTs from other plants (*16*, *29*).

Furaneol, Methoxyfuraneol, and Methyltransferase Activity in Different Varieties and Maturation Stages. Three strawberry varieties, Malach, Tamar, and Yael, differing in their aroma, were assessed for total volatiles, Furaneol, methoxyfuraneol, and methyltransferase activity during ripening. The unripe green stage was devoid of Furaneol and methoxyfuraneol, and, accordingly, no Furaneol-sustained OMT activity was detected. In all three varieties, the content of both Furaneol and methoxyfuraneol sharply increased during fruit ripening, with maximum values at the ripe stage (**Table 2**). This is in agreement with the results reported by Sanz et al. (*11*), who studied seven strawberry varieties and found that in most cases the content of these compounds sharply increased during fruit ripening, with maximum values at the overripe stage. The Furaneol OMT activity measured sharply increased in all varieties during ripening. Malach is an aromatic variety and accumulates relatively high levels of total volatiles, including both Furaneol and methoxyfuraneol (**Table 2**). This variety also contains the highest values of Furaneol-sustained OMT at the ripe stage (**Figure 5C**). Cell-free extracts from all varieties also displayed significant OMT activity when catechol and caffeic acid were used as substrates, much higher than when Furaneol was used as substrate (Figure 5). Tamar and Yael varieties are much less aromatic, and this is reflected in the lower levels of total volatiles (<$^1/_{10}$ of the levels found in Malach; **Table 2**). Still, cell-free extracts from ripe fruits of these two varieties displayed significant OMT activities toward Furaneol, catechol, and caffeic acid, close to the levels found in Malach (**Figure 5**). Thus, the various amounts of methoxyfuraneol detected in

Table 2. Furaneol, Methoxyfuraneol, and Total Volatile Content in Three Strawberry Varieties at Different Ripening Stages

	Tamar			Yael			Malach		
compound	green	pink	red	green	pink	red	green	pink	red
Furaneol[a]	0.00	0.00	0.73 ± 0.16	0.00	0.09 ± 0.02	0.28 ± 0.05	0.00	0.36 ± 0.12	11.28 ± 3.87
methoxyfuraneol[a]	0.00	0.00	0.77 ± 0.06	0.00	0.00	0.08 ± 0.02	0.00	0.08 ± 0.02	9.77 ± 1.79
total volatiles[a−c]	0.92 ± 0.11	1.42 ± 0.10	3.45 ± 0.39	1.11 ± 0.19	1.76 ± 0.05	2.64 ± 0.4	1.95 ± 0.34	4.50 ± 0.92	29.79 ± 6.01

[a] Volatile compounds from freshly cut strawberry fruits were extracted and analyzed by GC-MS as described under Materials and Methods. [b] μg/g of FW. Means ± SE, $n = 3$. [c] Mainly aliphatic alcohols and esters.

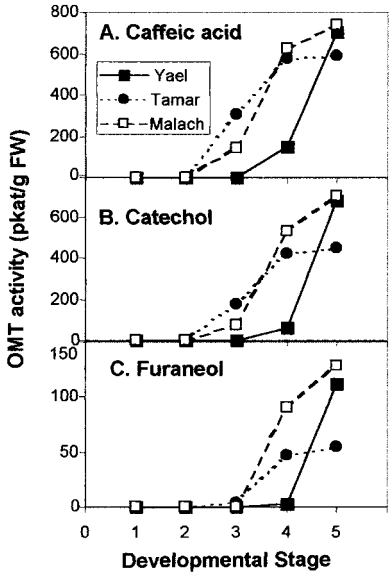

Figure 5. Changes in *O*-methyltransferase activity in three strawberry varieties during maturation: (□) Malach; (●) Tamar; (■) Yael. Partially purified cell-free extracts were incubated with [^{3}H]SAM with either 1 mM caffeic acid (A), 1 mM catechol (B), or 15 mM Furaneol (C). Formation of the products was monitored as described under Materials and Methods. Developmental (ripening stages): 1, green; 2, white; 3, pink; 4, red; 5, red overripe. Each value is the average of three replicates, each prepared by pooling 10 individual fruits.

these strawberry cultivars might be dependent on the availability of Furaneol, rather than on the OMT activity levels. Breeding programs aimed at improving strawberry aroma should therefore focus on increasing Furaneol biosynthesis and/or on preventing its degradation, and less on its methylating enzyme. Furaneol has better organoleptic properties and a lower odor threshold than methoxyfuraneol but is less stable than methoxyfuraneol, which is especially important for the aroma of processed strawberry products. High Furaneol/methyltransferase activity in fruits may contribute to higher levels of total Furaneol derivatives, due to reduced degradation of methoxyfuraneol.

It is somewhat surprising that developing strawberry fruits display such high OMT activity levels toward caffeic acid, protocatechuic aldehyde, and catechol, [forming ferulic acid, vanillin, and guaiacol, respectively (**Table 1**)]. Although vanillin has been found in wild and some cultivated strawberries (*30*) and probably contributes to their aroma, we found only traces of this compound in the cultivars we analyzed (data not shown). Caffeic and ferulic acids are ubiquitous precursors of lignin, but catechol and guaiacol have not been reported in strawberry to the best of our knowlegde. Caffeic and ferulic acids might be involved in lignin formation of the developing seeds and in vascular tissues. Caffeic acid is not usually an intermediate in anthocyanin pigmentation biosynthesis, a process that takes place concomitantly with ripening and methoxyfuraneol formation in strawberry. Ripening-related gene sequences that code for proteins involved in key metabolic events including anthocyanin biosynthesis were isolated from strawberry (*31*) and were not found to be active in green fruits. Cyanidin, an anthocyanin precursor in strawberry, also contains an *o*-dihydroxyphenol structure typical of that of caffeic acid and might be recognized by the OMT activity. Peonidin glucoside (the *O*-methyl derivative of cyanidin glucoside) has been found in strawberry cell suspensions (*32*).

It is presently unknown whether one enzyme with a relatively low substrate specificity is able to catalyze the transfer of a methyl group to each of the several substrates, or if in a mixture of more than one enzyme (unseparable under our conditions), each is capable to O-methylate a different substrate. The availability of cDNAs coding for the respective enzymes and their functional expression will be valuable in attempts to answer this question. Many enzymes of secondary metabolism are known to be able to recognize more than one substrate, although they often have different catalytic rates toward them (*33*−*35*). It has been postulated that this phenomenon is probably due to gene evolution of ancestor genes, involved in primary metabolism, such as the caffeic acid methyltransferase involved in lignification (*17*). For example, the gene from *Carkia breweri* that encodes an isoeugenol *O*-methyltransferase (IEMT) has been suggested to have arisen from a caffeic acid *O*-methyltransferase gene (*17*, *33*). Multiple OMTs displaying small but defined substrate discrimination can be found within the same plant and even within the same tissues (*36*, *37*). Therefore, it could be that Furaneol methylation occurs as a result of and in parallel with other reactions involving the methylation of caffeic acid, catechol, anthocyanidin, or other as yet unidentified *o*-diphenols that increase during fruit ripening.

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