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Comparison of Three Colorimetric Reagents in the Determination of Methanol with Alcohol Oxidase. Application to the Assay of Pectin Methylesterase

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Three colorimetric reagents for the determination of formaldehyde, the Nash reagent (ammonia plus acetylacetone), Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole), and *N*-methylbenzothiazolinone-2-hydrazone (MBTH), were compared for the determination of methanol when used in conjunction with alcohol oxidase. The combination of alcohol oxidase plus the commonly used Nash reagent was specific for methanol versus ethanol, but had the lowest sensitivity of the three reagents tested. Substituting Purpald for the Nash reagent increased the sensitivity 3-fold while still maintaining a high (59-fold) selectivity for methanol versus ethanol. Using MBTH increased the sensitivity still further, but with a loss of the selectivity toward methanol. Since MBTH reacted with aldehydes under neutral conditions, it could be included along with the alcohol oxidase to act as an aldehyde trap. This prevented further oxidation reactions by alcohol oxidase and allowed for extended incubations. A procedure for assaying low levels of pectin methylesterase activity that relies on this trapping ability is described. In addition, alcohol oxidase plus Purpald is shown to be a simple and sensitive way to measure the methanol released from plant material following the thermal activation of endogenous pectin methylesterase.

KEYWORDS: Methanol; alcohol oxidase; pectin methylesterase; Purpald

INTRODUCTION

A simple and sensitive method for determination of methanol is important in the study of pectin methylesterase (PME) in plant material. PME-catalyzed demethylation of pectin leads to a significant emission of methanol from leaves (*1*) and accumulation of methanol in ripening fruits (*2*, *3*). A determination of the amount of methanol produced by a particular plant tissue can be used as a way to assess the level of PME activity in that tissue. Quantitative determination of methanol can also be used to assay PME activity in vitro, or as a way to determine the degree of methylation of purified pectins (*4*−*6*).

GC methods have been used to determine the methanol contents of plant materials such as tomatoes and pears (*2*, *3*). These methods can be slow, requiring 30 min or more per sample (*3*), and can involve extensive sample preparation such as distillation (*2*). Other problems, as discussed in ref *1*, include the relatively low sensitivity of flame ionization detectors toward methanol and the fact that the mass of methanol is the same as that of O_2, complicating GC−MS methods. A procedure involving the derivitazation of the methanol with nitrate to form methyl nitrate prior to GC separation has also been described (*4*). This improves sensitivity but also adds an additional step. A simple GC−MS procedure for quantifying both methanol and acetic acid released from pectin has recently been reported (*7*).

An alternative approach is to oxidize the methanol to formaldehyde and then colorimetrically determine the formaldehyde. The oxidation can be done either chemically with permanganate (*5*) or enzymatically with alcohol oxidase AO (*6*). The enzymatic procedure is preferable because it improves the selectivity of the procedure and because it eliminates the use of hazardous chemicals. The formaldehyde produced by the oxidation of methanol can be determined colorimetrically with acetylacetone (pentane-2,4-dione) plus ammonia (*8*) or with the closely related reagent Fluoral-P (4-amino-3-penten-2-one), which yields the same final chromophore (*9*). The sensitivity of these procedures can be increased by using fluorimetry rather than colorimetry to determine the final product (*1*, *10*).

A number of colorimetric methods for the determination of formaldehyde besides the use of acetylacetone or Fluoral-P are known. These vary in their specificity toward formaldehyde, their sensitivity, and their ease of use. The reagent Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) condenses with aldehydes under alkaline conditions to form a cyclic aminal which is then oxidized by the oxygen from ambient air to form a purple tetrazine dye (*11*, *12*). Purpald is more sensitive than acetylacetone and is relatively selective for formaldehyde versus other small aldehydes such as acetylaldehyde (*13*, *14*). Another well-known method for determining aldehydes involves the condensation of an aldehyde with a molecule of *N*-methylbenzothiazolinone-2-hydrazone (MBTH) under neutral conditions. When the medium is acidified and an oxidant such as Fe^{3+} is

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added, this adduct then oxidatively couples with a second MBTH molecule to form a blue formazan dye. The molar absorptivity of this product is about 50000 M^{-1} cm^{-1} (*15*), twice that of Purpald and more than 6 times higher than that reported for acetylacetone (*8*). While highly sensitive and specific for aldehydes, this reaction is not, however, specific for formaldehyde, and other aldehydes such as acetaldehyde react nearly as well as formaldehyde (*14*).

In addition to producing formaldehyde, the oxidation of methanol by AO also produces 1 equiv of H_2O_2. By including horseradish peroxidase and a suitable chromogenic peroxidase substrate, such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), the amount of H_2O_2 produced by the AO reaction can be quantified. This AO/peroxidase/ABTS system has been used by several workers as a way to quantify methanol (*16*, *17*). However, the presence of antioxidants such as ascorbic acid, which are commonly present in plant extracts, will interfere with this assay by competing for the H_2O_2. In addition, the chromophore, the ABTS radical cation, is unstable, and the absorbance of the product declines with time. For a linear response the absorbance of each sample must be determined at the same specified time interval after the start of the reaction.

AO activity is not specific for methanol. Other short-chain alcohols such as ethanol can also act as substrates, although their rates of oxidation are lower, and the K_m for ethanol is 10-fold higher than that of methanol (*18*). Since alcohols other than methanol can also be oxidized to produce H_2O_2, methods based on H_2O_2 quantification will not be specific for methanol. On the other hand, the colorimetric and fluorimetric procedures for quantifying the aldehyde product with acetylacetone or fluoral-P are relatively specific for formaldehyde versus other aldehydes (*8*, *9*). The combination of AO plus a specific formaldehyde reagent gives a procedure for the specific determination of methanol.

In addition to short-chain alcohols, formaldehyde, the product of methanol oxidation, is also a substrate for AO. It is oxidized to produce formic acid and an additional equivalent of H_2O_2 (*18*, *19*). Fortunately, the rate of formaldehyde oxidation is much lower than that of methanol oxidation such that an incubation containing methanol and AO leads to the rapid accumulation of formaldehyde followed only by its slow disappearance. To obtain a linear response of accumulated formaldehyde versus methanol concentration, it is necessary to incubate the samples for a suitable period of time such that conversion of methanol to formaldehyde is essentially complete but further conversion to formic acid is minimal. The incubation time required for maximal accumulation of formaldehyde depends on the amount and specific activity of the AO used, which can vary with the batch and length of storage of the enzyme. The additional H_2O_2 produced by formaldehyde oxidation also complicates the use of H_2O_2 production as a measure of methanol oxidation.

One important application for methanol determination is the assay of the enzyme PME. PME catalyzes the demethylation of pectin, resulting in the formation of methanol and an equivalent of H^+. The standard method for assaying this enzyme is to quantify H^+ production either titrimetrically (*20*) or spectrophotometrically with a pH indicator dye (*21*). These methods are commonly used because they are simple and allow for a continuous assay. However, in cases where activity is low, and samples contain significant buffering capacity, assay by monitoring pH changes can be very slow and unreliable. This is especially problematic if the number of samples to be assayed is large, as is often the case in studies of enzyme inactivation or protein purification. In these instances a discontinuous PME assay measuring methanol production is a reasonable alternative. The procedure of Klavons and Bennett (*6*), using AO and acetylacetone to measure methanol, has occasionally been used to assay PME (*22*, *23*). We show here that the use of MBTH in conjunction with AO for the determination of methanol is a simple and very sensitive way to assay PME activity.

A second application for the determination of methanol is the quantification of methanol released from intact tissue by the action of PME on the endogenous pectins. It has been known for some time that the heating of vegetables to around 60 °C, in a so-called long-time, low-temperature (LTLT) blanch, activates endogenous PME activity. This leads to the partial demethylation of the pectins in the middle lamella that results in increased calcium cross-bridging within the cell wall matrix and better texture retention through subsequent high-temperature processing (*24*−*26*). Determining the optimal times and temperatures for an LTLT blanch by measuring texture changes is a laborious and time-consuming process. As an alternative, we show here that we can quantify the production of methanol, using AO and Purpald, to follow the time course of pectin demethylation during an LTLT blanch.

MATERIALS AND METHODS

Reagents. Alcohol oxidase (from *Pichia pastoris*), Purpald, MBTH, Tris (Trizma grade), acetylacetone, apple pectin (degree of methylesterification approximately 75%), and pectin methylesterase (from citrus) were obtained from Sigma, St. Louis, MO. The preparation and stability of specific reagents and stock solutions were as follows. MBTH was dissolved in water at 3 mg/mL. It is stable for at least a week at 4 °C, after which it develops a brown color. Purpald was dissolved at 5 mg/mL in 0.5 N NaOH. This solution is not stable and must be used within an hour of preparation. The acetylacetone/ammonia solution was prepared the same day it was used by dissolving 41 μL of acetylacetone, 3.08 g of ammonium acetate, and 59 μL of glacial acetic acid in H_2O to a final volume of 20 mL. The acidic iron solution used in the MBTH assay was prepared by dissolving 0.5 g of each ferric ammonium sulfate and sulfamic acid in 100 mL of water. This solution is stable indefinitely at room temperature.

AO/Acetylacetone Procedure. The procedure was essentially as described in ref *6* except the volumes of reagents were reduced. Our standard reaction contained 90 μL of 100 mM phosphate buffer (pH 7.5), 10 μL of AO at 0.01 U/μL (or other concentrations as indicated), 0−100 μL of the solution for which methanol concentration was to be determined, and H_2O to give a final volume of 200 μL. The effect of adding larger or smaller amounts of AO to the assay was also determined (see the Results and Discussion). After addition of the AO solution the sample was incubated in a water bath at 30 °C, and then after 10 min 200 μL of the acetylacetone reagent was added. Samples were transferred to a dryblock at 60 °C and heated for 15 min. After removal from the heat, 0.6 mL of H_2O was added to give a final volume of 1.0 mL. Absorbance at 412 nm was then determined.

AO/Purpald Procedure. The procedure for methanol determination with AO and Purpald is the same as that described for acetylacetone except that formaldehyde was determined with Purpald. After incubation of the samples for 10 min at 30 °C, 200 μL of 5 mg/mL Purpald in 0.5 N NaOH was added, and the samples were vigorously vortexed to ensure oxygenation. After an additional 30 min at 30 °C the samples were removed from the water bath, and 0.6 mL of H_2O was added for a final volume of 1.0 mL. Absorbance at 550 nm was then determined.

AO/MBTH Procedure. Since Fe^{3+} is used as the oxidant to develop color in this procedure, and $FePO_4$ is insoluble, phosphate buffer could not be used in this assay. Either Tris−HCl or MOPS−NaOH was used instead, and both gave essentially the same results. It has been reported (*27*) that Tris binds to aldehydes, which could potentially cause it to interfere with this assay. However, these authors showed that this effect is pH dependent and not significant at pH 7.5. It does not appear to be a problem here since identical results were obtained when Tris was replaced with MOPS−NaOH. We did not choose to use MOPS

routinely, however, since some batches of MOPS−NaOH, particularly those supplied as the free acid rather than the sodium salt, were found to contain substantial amounts of interfering material which gave unacceptably high blanks. Our standard incubation contained 100 μL of 100 mM Tris−HCl (pH 7.5), 10 μL of AO at 0.01 U/μL (or other concentrations as indicated), 40 μL of 3 mg/mL MBTH, and 50 μL of sample or H_2O. After addition of the AO, the samples were incubated for 20 min at 30 °C, and then 200 μL of a solution containing 5 mg/mL each of ferric ammonium sulfate and sulfamic acid was added. After 20 min at room temperature 0.6 mL of H_2O was added to give a final volume of 1.0 mL and absorbance at 620 nm determined.

PME Assay. For the assay of purified citrus PME, incubations contained, in a final volume of 1.0 mL, 20 mM Tris−HCl (pH 7.5), 50 mM NaCl, 0.4 mg/mL apple pectin, 0.6 mg/mL MBTH, and 2.5 U of AO. These components were mixed in a volume of 0.9 mL, and then the assay was started by the addition of 0.1 mL of a solution containing approximately 0.02 U/mL PME activity (determined titrimetrically on a 100-fold more concentrated solution). The mixture was incubated at 30 °C, and then at time points up to 3 h, 0.1 mL aliquots were removed and added to 0.1 mL of a solution containing 5 mg/mL each of ferric ammonium sulfate and sulfamic acid. After 30 min at room temperature 0.8 mL of water was added and absorbance at 620 nm determined.

PME activity in apple juice was determined after the activity was extracted from the particulate fraction with high salt (*28*). A 16000*g* pellet was prepared from a sample of unfiltered Golden Delicious apple juice. This pellet was washed twice with 5.0 mM acetate buffer (pH 4.5) and then resuspended in 0.5 M NaCl and 0.1 M MOPS−NaOH (pH 7.5) and the 16000*g* supernatant collected. This supernatant was assayed for PME activity essentially as described for the purified PME except a 0.2 mL aliquot of the supernatant was used in a final assay volume of 1.0 mL and MOPS−NaOH (pH 7.5) was used as the buffer.

Low-Temperature, Long-Time Blanches. To determine the release of methanol during a low-temperature blanch, thin slices (approximately 1 mm) of pea pods, broccoli stems, carrots, or red bell pepper were prepared. In a 20 mL serum bottle, 18 mL of water was preheated to 60 °C in a water bath, and then 2 g of the sliced vegetable material was added. The bottle was closed with a rubber serum stopper and incubated at 60 °C, with occasional shaking. At various time points 0.1 mL aliquots of the water were removed with a Hamilton syringe, transferred to small sealed vials, and held on ice. To determine methanol content, 50 μL of this blanch water was then assayed with AO and Purpald as described above.

RESULTS AND DISCUSSION

Determination of Methanol. The time course for formaldehyde production from methanol by AO was compared using three colorimetric methods for formaldehyde determination, acetylacetone, Purpald, and MBTH. The time courses for acetylacetone and Purpald (**Figure 1A**,**B**) are very similar and show the optimal time for the incubation is strongly dependent on the amount of enzyme present in the assay. At the lowest AO level used (0.025 U) the oxidation of methanol is not complete even after 60 min. At the next higher AO level, 0.1 U, the amount of formaldehyde formed reaches a peak in 10 min and then declines. This decline is presumably due to the further oxidation of the formaldehyde to formic acid by the AO enzyme. At the highest AO level, 0.4 U, this further oxidation of formaldehyde becomes even more significant and the amount of accumulated formaldehyde declines after only 2 min. As a practical matter, for our standard protocol using Purpald and AO to determine methanol, we used an aliquot of enzyme equal to 0.1 U per assay (based on the activity as reported by the supplier) and an incubation time of 10 min.

The time course for formaldehyde production as monitored by MBTH (**Figure 1C**) is different from that seen with the other two assays in that the amount of formaldehyde does not decline at longer incubations. In this assay, MBTH is included in the incubation along with the methanol and AO. Under these conditions, as the formaldehyde is formed from methanol it reacts with MBTH to form a stable adduct which is blocked from further oxidation to formic acid. This aldehyde trap allows for complete oxidation of the methanol and eliminates the need to determine the exact optimal incubation time for a specific batch of enzyme.

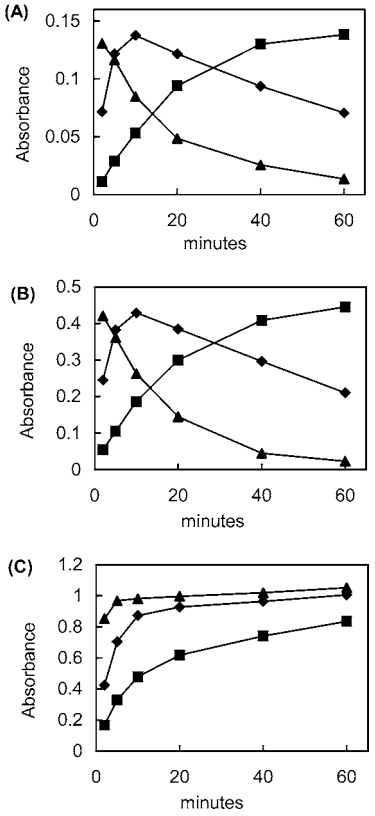

Figure 1. Time course for the formation of a colored product from methanol with AO and acetylacetone (**A**), Purpald (**B**), and MBTH (**C**). In each case three different levels of AO, 0.025 U (■), 0.1 U (◆), and 0.4 U (▲), were included in the assay.

All three colorimetric procedures gave linear responses with methanol. Typical standard curves are given in **Figure 2A**. The relative sensitivities of the three reagents were calculated from the slopes of these lines (**Table 1**). The apparent molar absorptivity at 412 nm obtained for methanol in the AO plus acetylacetone procedure was 7100 $M^{-1}cm^{-1}$. This is similar to the value of 6100 M^{-1} cm^{-1} which we calculate from previously reported data using this same procedure (*6*). The sensitivities of the Purpald and MBTH methods toward methanol were 3-fold and 7-fold higher. For the standard curves reported in **Table 1** the apparent molar absorptivities for Purpald at 550 nm and MBTH at 620 nm were 22600 and 48300 M^{-1} cm^{-1}, respectively. These two procedures also showed good reproducibility. Multiple standard curves generated on different days with these two reagents yielded apparent molar absorptivities of 49700 ± 2900 M^{-1} cm^{-1} ($n = 8$) for MBTH and 20800 ± 1100 M^{-1} cm^{-1} ($n = 8$) for Purpald.

The apparent molar absorptivity for the MBTH reaction is in close agreement with the reported value of 50000 M^{-1} cm^{-1} for the reaction of formaldehyde with MBTH (*15*). This indicates that conversion of methanol to formaldehyde by AO was complete in this assay. Reported molar absorptivities for the reaction of Purpald with formaldehyde are 28200 M^{-1} cm^{-1} (*13*) and 26300 M^{-1} cm^{-1} (*11*). These values are slightly higher than what we found with methanol, which indicates that

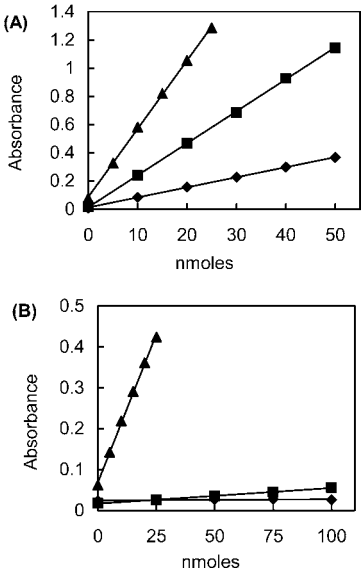

Figure 2. Standard curves for methanol (**A**) and ethanol (**B**) using AO and MBTH (▲), Purpald (■), and acetylacetone (◆). The final volume in all three assays was 1.0 mL.

Table 1. Relative Sensitivities of Acetylacetone, Purpald, and MBTH toward Methanol and Ethanol after Oxidation with AO[a]

	apparent molar absorptivity (M^{-1} cm^{-1} × 10^3)		selectivity of
reagent	methanol	ethanol	methanol/ethanol
acetylacetone	7.1	<0.03	>237
Purpald	22.6	0.38	59
MBTH	48.3	14.5	3.3

[a] Values were calculated from the slopes of the lines in **Figure 2**.

conversion of methanol to formaldehyde by AO was not complete in the Purpald assay. This discrepancy is consistent with our interpretation of the data in **Figure 1**. Complete conversion of the methanol to formaldehyde is not possible in the Purpald assay since some of the formaldehyde is further oxidized to formic acid before the oxidation of the methanol is complete. In the MBTH assay complete conversion can occur since the formaldehyde reacts immediately with MBTH, which prevents the further oxidation. The Purpald assay is nonetheless a useful method for methanol determination, even if the conversion of methanol to formaldehyde is not complete, since a linear standard curve was still obtained (**Figure 2**).

When tested with ethanol, only the MBTH procedure gave a significant response (**Figure 2B**). The response of the MBTH procedure toward ethanol was, however, only about a third of that seen with methanol. This lower reactivity toward ethanol is most likely the result of both the specificity of the AO enzyme and the slightly lower reactivity of MBTH toward acetaldehyde versus formaldehyde (*14*). The Purpald and acetylacetone methods showed a much higher selectivity toward methanol versus ethanol (**Table 1**). The combination of AO and Purpald was 60-fold more reactive toward methanol than ethanol; the selectivity of acetylacetone toward methanol was even higher. This is in agreement with previous reports (*5*, *13*, *14*) that both Purpald and acetylacetone are relatively specific for formaldehyde.

Application to the Assay of PME Activity. PME activity produces methanol and thus can be assayed by quantifying the rate of methanol production. Of the three methods described here for the determination of methanol using AO, the MBTH method is the most suitable for the assay of PME. By including both AO and MBTH in the PME incubation, the methanol formed by the PME activity will be oxidized to formaldehyde. This formaldehyde then reacts with the MBTH to form a stable MBTH−aldehyde adduct. Adding acid and Fe^{3+} terminates the enzyme reaction and causes the accumulated MBTH−aldehyde adduct to react with a second molecule of MBTH to form the highly colored formazan dye. This product can then be quantified spectrophotometrically. The acetylacetone and Purpald methods are less suited for use in a PME assay because in these methods there is no trapping mechanism to prevent the AO from further oxidizing the formaldehyde to formic acid. To use either of these methods, a more complicated two-step procedure would be needed in which methanol is allowed to accumulate during a PME incubation, and then, once the reaction is terminated by boiling or the addition of acid, an aliquot of the incubation medium is assayed for methanol.

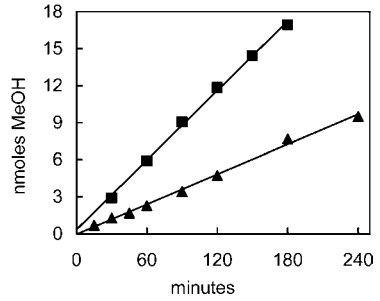

Figure 3. Time course for the formation of methanol by PME using either purified citrus PME (■) or an extract prepared from apple juice (▲). Methanol was determined using AO and MBTH.

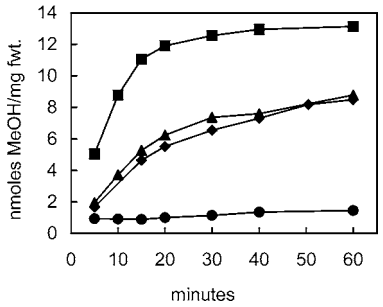

Figure 4. Time course for the release of methanol from pea pods (■), broccoli (▲), carrots (◆), and red bell peppers (●) when heated to 60 °C.

The suitability of the AO/MBTH method for assaying PME activity was initially examined using purified citrus PME. The time course for methanol production by PME was linear for up to 3 h (**Figure 3**). Activity was also linear with the amount of added PME enzyme (data not shown). We also tested this assay with an extract prepared from apple juice that contained no detectable PME activity when assayed titrimetrically. When assayed for methanol production over 4 h, a linear time course was again obtained and a rate of 0.002 μmol mL^{-1} min^{-1} calculated. This is an activity level roughly 50-fold lower than what we can accurately measure titrimetrically using a pH electrode. The sensitivity of the MBTH procedure could be increased further by not diluting the samples with water to the final volume of 1.0 mL, but instead reading the absorbance in a microcuvette or on a microplate.

Release of Methanol in an LTLT Blanch. To determine the release of methanol in an LTLT blanch, the AO/Purpald procedure was used since it has a much higher specificity toward methanol versus other alcohols than the AO/MBTH procedure.

This eliminates any interference from ethanol, which could possibly be present in, or produced by, the plant material and released during the incubation. For the determination, thin slices of four different vegetables were immersed in 60 °C water, and then, at various time points, aliquots of the water were removed and analyzed for methanol content. With carrots, pea pods, and broccoli, the methanol concentration in the water rose rapidly over the first 20 min of heating (**Figure 4**). This is the same duration of heating at this temperature that both we (Li and Barrett, manuscript in preparation) and others (*24*−*26*) have observed to induce firming of vegetables. By contrast, there was essentially no release of methanol from red bell peppers. This is consistent with the finding that red bell peppers contain very low levels of PME activity (*29*) and our own observation that an LTLT blanch does not induce firming in this vegetable (Li and Barrett, manuscript in preparation).

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); AO, alcohol oxidase; LTLT, long time, low temperature; MBTH, *N*-methylbenzothiazolinone-2-hydrazone; PME, pectin methylesterase.

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