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Convenient quantification of methanol in juices by methanol oxidase in combination with basic fuchsin

Min-Chang Wu [a], Chii-Ming Jiang [b], Yen-Yi Ho [a], Szu-Chuan Shen [c], Hung-Min Chang [d,*]

[a] Department of Food Science, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan
[b] Department of Sea Food Science, National Kaohsiung Marine University, Kaohsiung 811, Taiwan
[c] Department of Nutrition, I-Shou University, Kaohsiung County 840, Taiwan
[d] Graduate Institute of Food Science and Technology, National Taiwan University, Taipei 10617, Taiwan

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Abstract

Combination of methanol oxidase (MOX) and basic fuchsin was confirmed effectively in determining methanol content in a model and real systems. The optimal reaction conditions for 20 ppm formaldehyde–0.1% basic fuchsin mixture were determined to be at 35 °C for 2 h in 0.25 N HCl with a maximal absorption wavelength of 560 nm, while those for MOX (0.8 unit MOX/mL)–methanol mixture were at 25 °C for 30 min. Gas chromatography (GC) also confirmed the method developed with an accuracy of >95%. Presence of food additives such as sulfite (100 ppm) interfered greatly (−92%) with the quantification of methanol, while fruit juice components, galacturonic acid, pectin, glucose, did not apparently interfere the quantification results of methanol. Ethanol (>100 ppm) presented competitive inhibition with methanol on MOX. In real samples, fresh fruit juices such as Sunkist, water melon, carrot, carambola, melon, tomato, and papaya were detected to contain 8, 31, 36, 17, 8, 42, and 38 ppm methanol, respectively, with an accuracy of 93–97%, as compared to that determined by a GC, suggesting the feasibility of MOX–basic fuchsin method developed for juice industry.

Keywords: Methanol; Basic fuchsin; Formaldehyde; Methanol oxidase; Gas chromatography

1. Introduction

Pectic substances are polymers composed mainly of (1→4)-α-D-galacturonopyranosyl units, which are found in the middle lamella of plant cells (Keggstra, Talmadge, Bauer, & Albersheim, 1973; van Buren, 1991). In the presence of pectolytic enzymes, high methoxylpectin is converted into low methoxylpectin by releasing methanol and then depolymerized to form pectic acids. Reduction of esterification and depolymerization are crucial to the softening of plant tissues such as fruits and vegetables and are important biological changes during maturation process (Christensen, 1986; Goldberg, 1984; Rothschild, Moyal, & Karsenty, 1974).

Pectolytic enzymes from microorganisms play important roles in the winemaking process due to the facts that they improve the extraction of aroma compounds and color as well as facilitate the clarification and filtration of musts and wines (Brown & Ough, 1981; Fogarty & Kelly, 1983). However, accumulation of methanol during fermentation by commercial pectolytic enzyme preparations is a severe problem, which is an alcohol toxic to humans by producing lactic acidosis and interferes with liver metabolism where it is oxidized. Lactic acidosis is a metabolic disease caused by an increase in blood levels of lactic acid and its symptoms are weakness, vomiting and finally coma and death (Skrzydlewska & Farbiszewski, 1996; Skrzydlewska, Witek, & Farbiszewski, 1998). The human oral lethal dose is 340 mg/kg of body weight.

* Corresponding author. Tel.: +886 233 664 123; fax: +886 223 620 849.
E-mail addresses: changhm@ntu.edu.tw, changhm@ccms.ntu.edu.tw (H.-M. Chang).

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Factors such as fruit species and varieties, some oenological practices, and the yeast strain used are able to influence methanol production. Fruits such as citrus, cherry, apple, and grape contain high pectin, and thus, methanol level in juices and fermented products from those fruits is usually higher than that made from cereal sources. Conventional methanol assays include colorimetric methods using potassium permanganate to convert methanol into formaldehyde that subsequently reacts with basic fuchsin (595 nm) or 2,4-pentanedione (412 nm) to develop color (Puchtler, Meloan, & Brewton, 1975; Wood & Siddiqui, 1970), or using methanol oxidase (MOX) from microbial source (Koultz et al., 1989) to produce formaldehyde to interact with 2,4-pentanedione (Klavons & Bennett, 1986). In spite of costliness, the development of gas chromatography (GC) provides an accurate approach in quantifying limited amounts of volatile compounds in foods.

However, use of potassium permanganate to quantify methanol suffers some major drawbacks. Oxides converted by this strong oxidizing agent from some ingredients in juice samples such as reducing sugars and carbohydrates usually react with basic fuchsin or 2,4-pentanedione and cause severe interference with absorbance during quantification of methanol (Wood & Siddiqui, 1970). Accordingly, previous distillation of juice samples is required to maximize the quantitative accuracy of methanol, and thus, the entire procedure appears to be lengthy. Use of MOX shows some advantages over potassium permanganate such as specificity, speediness, and most importantly, accuracy. Klavons and Bennett (1986) indicated that MOX–2,4-pentanedione method was effective in quantifying methanol ranging from 0 and 20 ppm in pectin solution with reproducible results during the determination of the degree of esterification (DE) of pectin. However, this method is still suffering some major drawbacks such as evaporation and loss of formaldehyde in the later heating process (58–60 °C, 15 min) during color development (yellowish, 412 nm) of formaldehyde–2,4-pentanedione complexes.

To develop a low cost, convenient, and fast assay for methanol in juices, MOX–basic fuchsin method was tried. First, experimental conditions for formaldehyde–basic fuchsin and MOX–methanol reactions were optimized, respectively, in a respective model system. Then, the possible effects of food additives or ingredients in juices on both reactions were studied. Finally, by comparing the quantitative amount determined by GC, fresh juice samples were tested to evaluate the accuracy of the method developed.

2. Materials and methods

2.1. Materials

Methanol and methanol oxidase (MOX) (from *Pichia pastoris*) (EC 1.1.3.13) are from Sigma (St. Louis, MO, USA), while absolute ethanol (99.5%), potassium pyrosulfite, potassium pyrophosphate, galacturonic acid, glucose, pectin, ascorbic acid, and potassium permanganate are the products of Showa Co., Tokyo, Japan. Isopropanol, *n*-butanol, acetaldehyde, and formaldehyde were purchased from Tedia (Fairfield, OH, USA). Fresh fruits were purchased from a local supermarket.

2.2. Optimization of basic fuchsin–formaldehyde reaction

Mixture of 1 mL of each of methanol solution (20 ppm), 0.1% basic fuchsin (in 1% Na_2SO_3 and 1% H_2SO_4 solution), HCl solution (2, 1, 0.5 or 0.2 N), and 0.01 M phosphate buffer (pH 7.5) were incubated at 35 °C in a water bath for 30 min. Then spectra of the mixtures scanned from 400 to 660 nm were obtained on a Hitachi spectrophotometer (Model U-2000, Tokyo, Japan) to determine the maximal absorption wavelength and optimal acidic solution for color development. To understand the effect of incubation temperature and time on color development, basic fuchsin–formaldehyde mixture were incubated at 25, 35 or 45 °C and then monitored at 560 nm by a Hitachi spectrophotometer for up to 150 min.

2.3. Methanol assays

Mixture of 1 mL methanol solution (20 ppm) and 1 mL MOX solution (0.8 unit/mL in 0.01 M phosphate buffer, pH 7.5) was incubated at 25 °C for 30 min, followed by addition of 1 mL of 1 N HCl and 1 mL of 0.1% basic fuchsin solution and incubation at 35 °C for 2 h. Color of mixture (5 ppm methanol/0.2 unit/mL MOX/0.025% basic fuchsin/0.25 N HCl) developed was monitored at 560 nm by a Hitachi spectrophotometer. Enzyme solution previously incubated in a boiling water bath for 5 min was used as control.

Similar procedures were performed to investigate factors such as enzyme activity (0.1–4.0 units/mL), temperature (25, 35, and 45 °C), and methanol level (1–40 ppm) on MOX–basic fuchsin reaction to optimize the conditions in model systems.

In determining the influence of juice ingredients or additives on MOX–basic fuchsin reaction, similar procedures were performed by previously dissolving 0.1 or 1 mg/mL of potassium pyrosulfite, galacturonic acid, glucose, pectin, isopropanol, *n*-butanol, acetaldehyde or ascorbic acid in 20 ppm methanol solution. To observe the effect of ethanol on the color development, methanol solution containing 0.1 mg/mL–30% (v/v) ethanol was used. De-ionized water (Mili-Q system, Milipore, Osaka, Japan) was used throughout the experiment. Methanol content determined by such colorimetric method was compared with that determined by a gas chromatography (GC) described below for the accuracy (%) (methanol content determined by MOX–basic fuchsin method/methanol content determined by GC × 100%).

In real systems, peeled fruits in pieces were homogenized (cycle blender, 2 min) with one part (1:1) cold (about 5 °C) de-ionized water. After storage at 4 or 30 °C for 10, 60 or 120 min, blended fruits in sealed tested tubes were heated in

a boiling water bath for 15 min to denature enzymes, followed by cooling to room temperature in flowing tap water and centrifugation (4000*g*, 10 min). The supernatant thus obtained was applied for methanol assays by MOX–basic fuchsin method and GC as described below. Dilution of juice supernatant was conducted if needed. Triplicate samples were each analyzed twice.

2.4. Inhibition mode of ethanol on MOX–methanol reaction

The absorbance of MOX–methanol mixture as a function of methanol was investigated under standard MOX–basic fuchsin conditions. Lineweaver–Burk double-reciprocal plots were obtained by plotting 1/A versus 1/[S] (where A is absorbance at 560 nm; and S is the substrate level, 10, 15, 20, and 25 ppm methanol). For the inhibition study, measurements were carried out in the presence or absence of ethanol (100 or 200 ppm) in methanol solution. All parameters are the means of three determinations and are reproducible.

2.5. Gas chromatography

Methanol solution (1–40 ppm) and fruit juices, rested at 30 °C for 120 min, were centrifuged (10,000*g*, 15 min, 4 °C) and then pressed through a 0.45 μm membrane filter for a GC analysis (Varian GC 3800, Varian, California, USA). Experimental conditions: column, CP-Wax (length, 6 m; inner diameter, 0.53 mm); mobile phase, N_2 gas; flow rate, 5.0 mL/min; split ratio, 10/1; injection volume, 20 μL; detector, flame ionization detector (FID); column temperature, 50 °C/12 min, 40 °C/min to 230 °C; injector temperature, 180 °C; detector temperature, 220 °C.

Methanol solution (0–100 ppm) was injected into GC column to construct the standard curve ($R^2 = 0.9986$) for the determination of methanol content in samples and for the identification of methanol peak by comparing the retention time. Triplicate samples were each analyzed twice. Accuracy (%) of methanol content was determined by dividing the determined content of methanol in juice assayed by developed method by that quantified by GC.

3. Results and discussion

3.1. Optimal conditions for basic fuchsin–formaldehyde reaction

Fig. 1 represents the absorption spectra of 0.1% basic fuchsin–20 ppm formaldehyde mixtures added with different HCl solutions. Each mixture was dark purple (data not shown) and the maximal absorption wavelength was observed to be 560 nm, at which the absorbance appeared to be higher (about 0.95) when mixed with 1 N HCl than with other HCl solutions. It suggests that the absorbance of the mixture relies greatly on the acidity (Wood & Siddiqui, 1970). Therefore, 1 N HCl solution was used in the following experiments to prepare the enzyme–methanol solutions.

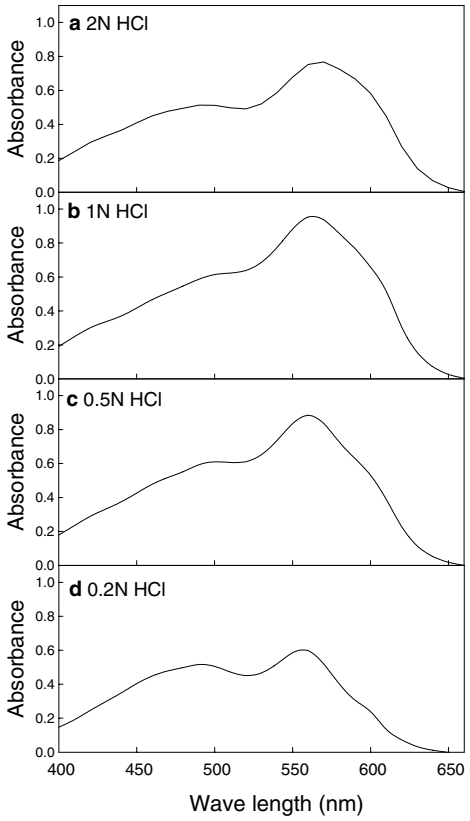

Fig. 1. Absorption spectra of formaldehyde–basic fuchsin solution mixed with various levels of HCl solutions. Mixture of 20 ppm formaldehyde–0.1% basic fuchsin was incubated at 35 °C in a water bath for 2 h before scanning.

Apparently, incubation temperature influenced the color development of 0.1% basic fuchsin–20 ppm formaldehyde mixture (Fig. 2). Absorbance at 560 nm increased rapidly and reached a maximal value (83% of maximal absorption at 35 °C) at about 50 min but dropped fast when the

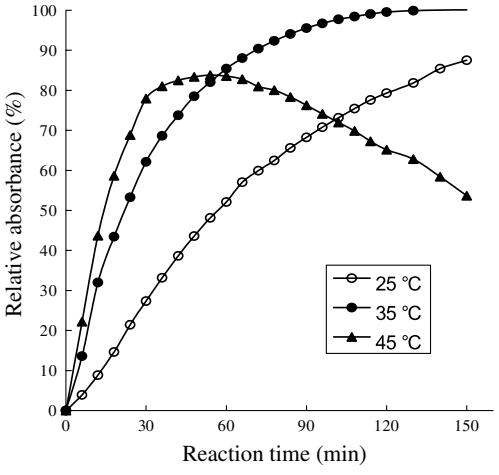

Fig. 2. Effect of reaction temperature on relative absorbance (%) of 20 ppm formaldehyde–0.1% basic fuchsin mixture during incubation for up to 150 min before determination of absorbance at 560 nm. Absorbance of reaction mixture incubated at 35 °C for 120 min was treated as 100%.

mixture was incubated at 45 °C. On the other hand, reaction at 35 °C reached a plateau of absorbance at about 90 min and a maximal value at about 120 min, while that reacted at 25 °C appeared to be incomplete (about 80% of maximal absorption at 35 °C) after incubation for 120 min (Fig. 2). Of note, the color developed at 35 °C for 120 min was stable during the subsequent 1 h-incubation at the same temperature (data not shown), suggesting the convenience and reproducibility of methanol assay by basic fuchsin–formaldehyde reaction at this temperature. Therefore, the optimal reaction conditions for this mixture were determined to be at 35 °C for 120 min.

3.2. Optimization of MOX–methanol reaction

MOX converted methanol into formaldehyde that subsequently reacts with basic fuchsin to develop color. Accordingly, variance in enzyme activity in methanol–basic fuchsin mixtures results in different reaction rates. As shown in Fig. 3, absorbance at 560 nm increased slowly in mixtures prepared with 0.1 or 0.2 unit MOX/mL solution, while reaching maximum at 60 and 30 min in mixtures prepared with 0.4 and 0.8 unit MOX/mL, respectively, during incubation at 25 °C. However, at increased enzyme activity (mixtures prepared with 1.6 and 4 units MOX/mL), sharp changes in absorbance in a very short time were observed and the reaction conditions appeared difficultly to be optimized. In addition, conversion of formaldehyde into formic acid by MOX was confirmed to be responsible for the rapid reduction of absorbance at the increased MOX activity and extended incubation time in preliminary tests (data not shown). Therefore, mixture prepared with 1 mL of 0.8 unit MOX/mL with a reaction time of 30 min was suggested for the following experiments. Similar results were also observed by Klavons and Bennett (1986) that high MOX activity would resulted in sharp reduction in absorbance of formaldehyde–2,4-pentanedione mixture and the optimal enzyme activity and reaction time was suggested to be 0.5–2.0 units/mL and 15–30 min, respectively.

Optimal oxidation temperature of methanol by MOX to formaldehyde was investigated by incubating at 25, 35 and 45 °C. It was apparent that 0.8 unit MOX/mL solution incubated with an equal volume of 10 ppm methanol at 25 °C displayed a highest absorbance at 560 nm, followed by incubation at 30 and 35 °C (Fig. 4). The absorbance of MOX-10 ppm methanol mixture developed at 25 °C was about 30% higher than that at 35 °C, suggesting that obtained formaldehyde was further converted into formic acid and resulted in the reduction of absorbance at increased temperature. Similar trends in the change of absorbance versus temperature were also observed in MOX-20 ppm methanol mixture. Therefore, incubation temperature of 25 °C for 30 min was recommended for MOX–methanol mixture in the following investigations.

Various levels of methanol (1–40 ppm) were tried to construct the calibration curve (Fig. 5(a)) by the established MOX–basic fuchsin method. Apparently, a linear relationship between absorbance and methanol level (1–20 ppm) was observed and the correlation coefficient (R^2) was 0.9997 (Fig. 5(b)). Subsequently, three methanol solutions (1–20 ppm methanol) were assayed by the established MOX–basic fuchsin method and GC and the results were compared. It was found that the accuracy was between 96% and 98% (data not shown), revealing the reliability and reproducibility of the established method in a model system. Thus, based on the above findings, optimal condi-

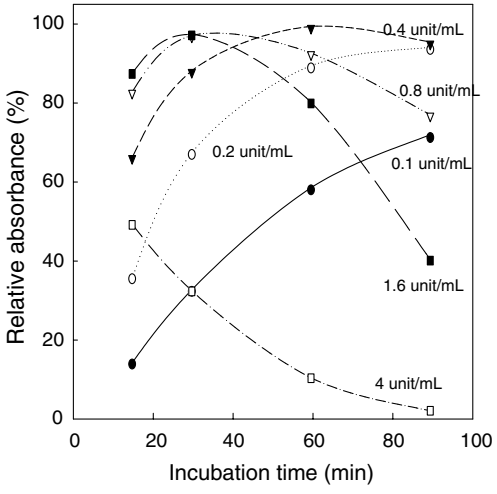

Fig. 3. Effect of methanol oxidase activity on the absorbance of methanol oxidase–methanol mixture during incubation for up to 90 min. Methanol oxidase–20 ppm methanol mixture was incubated at 25 °C in a water bath for 30 min, followed by addition of 1 N HCl and 0.1% basic fuchsin solution and incubation at 35 °C for 2 h before absorbance determination. Methanol oxidase was dissolved in 0.1 M phosphate buffer (pH 7.5) before use. Each value is the average of triplicates.

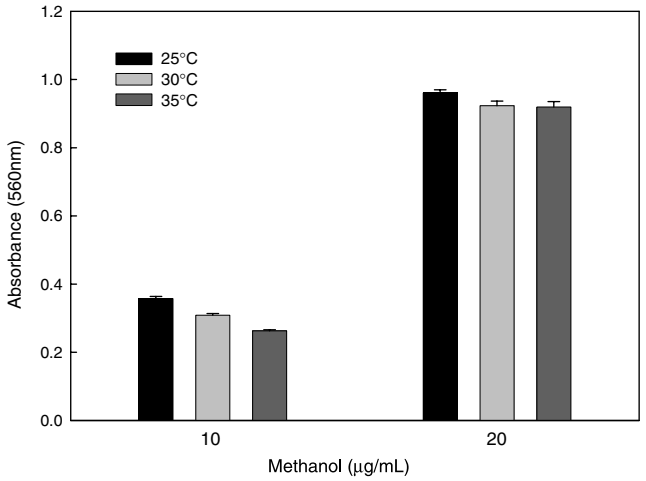

Fig. 4. Effect of reaction temperature on the absorbance of methanol oxidase–methanol solution. Mixture of 0.8 unit/mL methanol oxidase–20 ppm methanol was incubated at various temperatures in a water bath for 30 min before mixing with 1 N HCl and 0.1% acidic basic fuchsin solution to detect the absorbance. Each value is the average of triplicates. Bars in the column refer to SD.

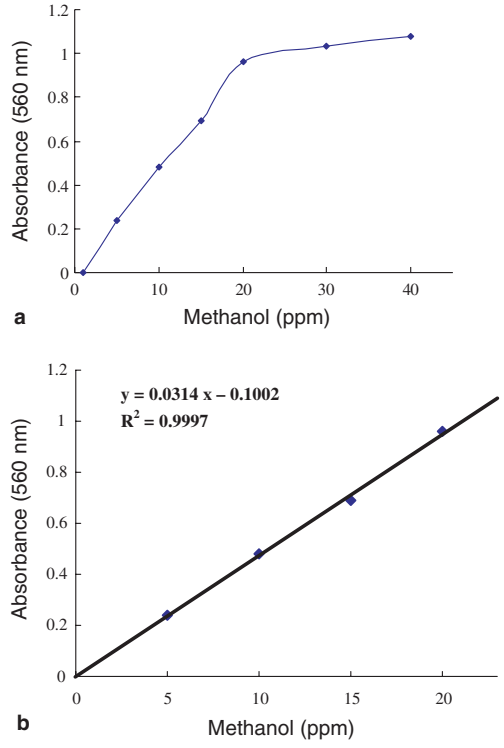

Fig. 5. Changes in absorbance at 560 nm (a) and the calibration curve (b) of methanol determined by methanol oxidase-basic fuchsin method in a model solution. Mixture of 0.8 unit/mL methanol oxidase–20 ppm methanol was incubated at 25 °C in a water bath for 30 min before mixing with 1 N HCl and 0.1% basic fuchsin solution to detect the absorbance.

tions for methanol assay by MOX–basic fuchsin method were decided as follows: mixture of equal volumes of 0.8 unit MOX/mL and 1–20 ppm methanol; incubation temperature and time, 25 °C for 30 min; acid solution, 1 N HCl; dye solution, 0.1% basic fuchsin; color developing temperature and time, 35 °C for 2 h; wavelength, 560 nm.

Heat treatment of formaldehyde–2,4-pentanedione mixture at 58–60 °C is required for color development (Wood & Siddiqui, 1970), which results largely in the variation of methanol content due to the vaporization-induced loss of formaldehyde. Besides, the color developed is yellowish (412 nm) and is liable to be interfered by acetaldehyde–2,4-pentanedione reaction mixture (388 nm) when potassium permanganate acts as an oxidizing agent in the presence of ethanol (Wood & Siddiqui, 1970).

3.3. Influence of additives on absorption

Tables 1 and 2 represent the effects of food additives on formaldehyde–basic fuchsin reaction and MOX–methanol reaction, respectively. By comparing the results in Tables 1 and 2 in model systems, potassium pyrosulfite, a common reductant in wines, reduced the absorbance by mainly interfered with the formaldehyde–basic fuchsin reaction, and thus, reduced severely the absorbance by 92% (−91.8%) at a level of 0.1 mg/mL in 0.1% basic fuchsin–20 ppm methanol mixture (Table 1). Accordingly, wines contain sulfite

Table 1
Effect of antioxidants, reducing sugars, carbohydrates, and alcohols, and acetaldehyde on formaldehyde–basic fuchsin reaction

Substance	Concentration (mg/mL)	A 560 nm	Change of absorbance (%)[a]
Potassium pyrosulfite	0.1	−0.888 ± 0.001	−91.8
	1	−0.901 ± 0.003	−93.2
Galacturonic acid	0.1	−0.024 ± 0.003	−2.5
	1	−0.025 ± 0.003	−2.6
Glucose	0.1	−0.013 ± 0.003	−1.3
	1	−0.019 ± 0.002	−2.0
Pectin	0.1	−0.025 ± 0.003	−2.6
	1	−0.027 ± 0.004	−2.8
Ethanol	0.1	0.003 ± 0.003	0.3
	1	0.002 ± 0.010	0.2
	50	−0.009 ± 0.009	−1.0
	120	0.019 ± 0.005	2.0
	300	0.072 ± 0.008	7.5
Isopropanol	0.1	−0.028 ± 0.001	−2.9
	1	−0.026 ± 0.001	−2.7
n-butanol	0.1	−0.035 ± 0.009	−3.6
	1	−0.029 ± 0.004	−3.0
Acetaldehyde	0.1	0.013 ± 0.001	1.3
	1	0.055 ± 0.007	5.7
Ascorbic acid	1	−0.022 ± 0.011	−2.3

Mixture of 20 ppm formaldehyde–0.1% basic fuchsin solution was incubated at 35 °C for 2 h before absorbance determination.
Each value is the average of triplicates.
[a] −[1 − (Absorbance of sample/absorbance of control)] × 100%, negative values represent reduction of absorbance.

Table 2
Effect of antioxidants, reducing sugars, carbohydrates, and alcohols, and acetaldehyde on MOX–methanol reaction

Substance	Concentration (mg/mL)	A 560 nm	Change of absorbance (%)[a]
Potassium pyrosulfite	0.1	−0.814 ± 0.008	−92.7
	1	−0.832 ± 0.002	−95.2
Galacturonic acid	0.1	0.001 ± 0.005	0.2
	1	0.015 ± 0.009	1.7
Glucose	0.1	0.001 ± 0.003	0.1
	1	0.003 ± 0.003	0.4
Sucrose	1	−0.029 ± 0.005	−4.6
Pectin	0.1	−0.002 ± 0.010	−0.3
	1	−0.019 ± 0.009	−3.0
Ethanol	0.1	−0.125 ± 0.005	−17.3
	1	−0.562 ± 0.007	−64.4
	50	−0.849 ± 0.002	−97.4
	120	−0.840 ± 0.008	−96.3
	300	−0.837 ± 0.007	−95.9
Isopropanol	0.1	−0.005 ± 0.003	−0.5
	1	0.008 ± 0.006	0.9
n-butanol	0.1	−0.022 ± 0.002	−2.5
	1	−0.179 ± 0.004	−20.5
Acetaldehyde	0.1	0.009 ± 0.018	1.4
	1	0.149 ± 0.027	23.7
Ascorbic acid	1	−0.126 ± 0.018	−20.0

MOX (0.8 unit/mL)–20 ppm methanol reaction mixture was incubated at 25 °C for 30 min, followed by mixing with 1 N HCl and 0.1% basic fuchsin solution and incubation at 35 °C for 2 h before absorbance determination.
Each value is the average of triplicates.
[a] −[1 − (Absorbance of sample/absorbance of control)] × 100%, negative values represent reduction of absorbance.

higher than 100 ppm would possibly lead to severe down-evaluation of methanol content determined by this method. Therefore, the reliability of MOX–basic fuchsin method is poor when used in wines. The reducing sugar at levels of 0.1 and 1 mg/mL only caused insignificant interfering effects on the quantification of methanol (Tables 1 and 2), while 0.1 mg/mL sucrose caused reduction of absorbance by 4.6% (−4.6%) (Table 2) in MOX–methanol reaction. It could be due to the hydrolysis of sucrose to form double moles of monosaccharides. Moreover, at levels between 0.1 and 50 mg/mL, ethanol increased absorbance of formaldehyde–basic fuchsin mixture by 0.2–1% (Table 1), whereas it reduced remarkably the absorbance of MOX–methanol mixture by about 17–97% (−17.28 ∼ −97.36%) (Table 2), revealing the strong inhibition of ethanol on MOX–methanol reaction and the poor reliability of MOX–methanol method on determining methanol content in the presence of ethanol or ethanol containing beverages. Similarly, by inhibiting the MOX–methanol reaction, *n*-butanol at a level of 1 mg/mL interfered severely with MOX–methanol reaction and reduced the absorbance by about 20% of (−20.5%) (Table 2). On the other hand, acetaldehyde, similar to formaldehyde in structure, reacted with basic fuchsin and enhanced the absorbance by about 5.7 (Table 1) and 24% (Table 2) at a level of 1 mg/mL, suggesting the up-evaluation of methanol in the presence of acetaldehyde. Presence of acetaldehyde in alcoholic beverages due to the conversion from ethanol by the conventional potassium permanganate–basic fuchsin method was also a severe problem during the determination of methanol (Puchtler et al., 1975). Ascorbic acid, a natural antioxidant in fruits, at an added level of 1 mg/mL, also presented apparent interfering effect (about 20% reduction) on MOX–methanol reaction in a model system (Table 2). Lemon, Sunkist, pineapple, and spinach contain about 0.4, 0.38, 0.05, and 0.13 mg/mL ascorbic acid, respectively (Liao, Jiang, Hwang, & Chang, 2001), and therefore, the reduction of absorbance results from the presence of ascorbic acid during the methanol assay by MOX–methanol method developed is expected. Conclusively, any food component or additive that interferes with the MOX–methanol reaction or reacts with basic fuchsin would lead to changes in absorbance. However, the developed method is available for preliminary determination of methanol in fresh fruit juices.

Inhibition mode of ethanol on MOX–methanol reaction was investigated by the Lineweaver–Burk double-reciprocal plots (Fig. 6). Absorbance at 560 nm decreased with the increasing level of ethanol (0, 100 and 200 ppm) in methanol solution. Apparently, ethanol displayed competitive inhibition on MOX–methanol reaction. Thus, presence of other alcohols in juices or beverages would lead to down-evaluation of methanol by MOX–methanol method developed.

3.4. Real systems

Fresh juices were incubated at 4 or 30 °C for 10, 60 or 120 min, and then the methanol content was determined by MOX–basic fuchsin method developed. Interestingly, methanol level of Sunkist juice maintained at about 8 ppm, regardless of the difference in resting times and temperatures, suggesting the fast release of methanol from citrus pectin by PE. However, methanol level in water melon juice was relatively low (24 ppm) at 4 °C for 10 min but it increased to 31 ppm after resting for extended period of time. Similar results were also observed in carrot juice. Among the samples tested, tomato juices contained highest level of methanol (about 41–42 ppm). The fast and high release of methanol at 4 °C for only 10 min revealed the strong PE activity in tomato juice.

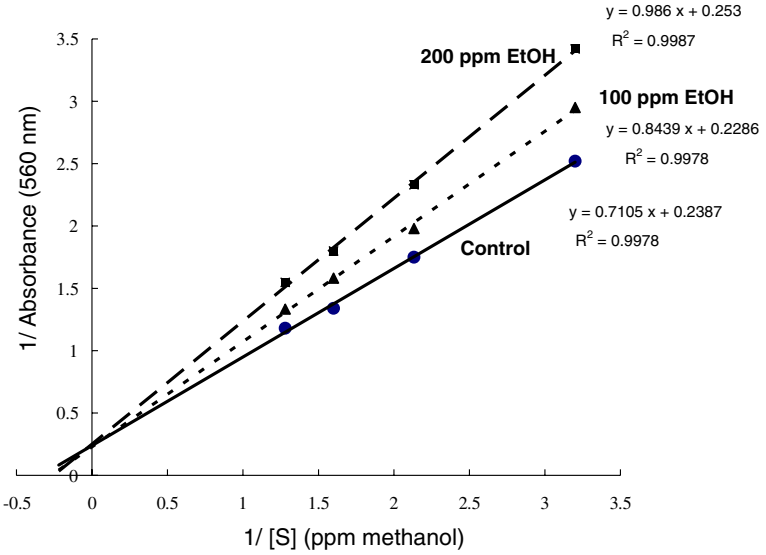

Fig. 6. Lineweaver–Burk double-reciprocal plots for methanol oxidase in the absence or presence (100 and 200 ppm) of ethanol. MOX (0.8 unit/mL)–methanol (15, 20, 25 or 30 ppm) reaction mixture was incubated at 25 °C for 30 min, followed by mixing with 1 N HCl and 0.1% basic fuchsin solution and incubation at 35 °C for 2 h before absorbance (560 nm) determination. Each value is the average of triplicates. Currant rosehip nectars.

Table 3
Contents (ppm)[a] of methanol in fruit juices rested at 4 or 30 °C for 10, 60 or 120 min before determination by MOX–basic fuchsin method

Resting time (min)	4 °C			30 °C			Accuracy (%)[b]
	10	60	120	10	60	120	
Sunkist	7.82 ± 0.02	8.04 ± 0.31	8.11 ± 0.11	8.05 ± 0.02	8.12 ± 0.18	8.21 ± 0.08	93.0 ± 0.2
Water melon	24.12 ± 0.13	31.54 ± 0.33	31.25 ± 0.76	30.23 ± 0.28	31.54 ± 0.30	31.54 ± 0.16	95.3 ± 0.1
Carrot	21.24 ± 0.43	36.36 ± 0.33	36.57 ± 0.13	35.23 ± 0.37	36.21 ± 0.72	36.36 ± 0.74	94.4 ± 0.2
Carambola	15.25 ± 0.33	17.47 ± 0.22	17.36 ± 0.29	16.21 ± 0.10	17.11 ± 0.48	17.23 ± 0.40	96.7 ± 0.1
Melon	7.23 ± 0.02	7.54 ± 0.07	8.24 ± 0.06	7.12 ± 0.05	7.96 ± 0.06	8.02 ± 0.10	94.5 ± 0.2
Tomato	41.41 ± 0.19	42.24 ± 0.34	42.13 ± 0.76	41.25 ± 0.21	42.25 ± 0.23	42.56 ± 0.19	97.3 ± 0.1
Papaya	36.35 ± 0.82	37.23 ± 0.30	38.31 ± 0.46	38.17 ± 1.08	38.23 ± 0.39	38.25 ± 0.51	96.6 ± 0.2

Peeled fruit was homogenized with one part cold de-ionized water, followed by incubation at required temperature for desired period of time and heating before centrifugation to obtain supernatant for analysis.
MOX (0.8 unit/mL)–juice mixture was incubated at 25 °C for 30 min, followed by mixing with 1 N HCl and 0.1% basic fuchsin solution and incubation at 35 °C for 2 h before absorbance determination.
[a] Each value is the average of triplicates.
[b] (Methanol content determined by MOX–basic fuchsin method/methanol content determined by GC) × 100%.

For accuracy determination, methanol content in juice incubated at 30 °C for 120 min was determined by MOX–methanol method and compared with that determined by a GC. Of note, the accuracy was between 93% and 97% (Table 3), revealing the feasibility of the method developed in the present study, which was dependent on the level of reducing sugar, carbohydrates, and ascorbic acid in fruit juices (Tables 1 and 2).

4. Conclusions

Conditions for MOX–methanol reaction were optimized for the fast, accurate, and most importantly, costless quantification of methanol in juices with the aid of basic fuchsin. The specificity of enzyme facilitates the determination of methanol. However, presence of ethanol in methanol sample strongly inhibited the MOX–methanol reaction, and thus, the method developed in the present study is limited to the application in alcoholic beverages. Besides, down-evaluation of methanol may occur as a result of the co-existence of reducing agents such as glucose and ascorbic acid.

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