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acid) and Its Application to the
Determination of the Methyl Ester
Content of Pectins**

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Enzymatic Determination of Methanol with Alcohol Oxidase, Peroxidase, and the Chromogen 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) and Its Application to the Determination of the Methyl Ester Content of Pectins

A method for the determination of methanol using alcohol oxidase, peroxidase, and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was developed. The procedure, which uses alcohol oxidase, is advantageous over other photometric procedures in that a subsequent reduction step is eliminated. The use of peroxidase and ABTS offers the further advantage of sensitive detection of the formed hydrogen peroxide. The sensitivity of the assay is 0.05–1.0 $\mu\text{g}/(\text{mL}$ of methanol). Moreover, the procedure is very rapid. The methyl ester content in a sample of pectin was determined from the methanol liberated upon its alkaline hydrolysis.

Keywords: *Pectin; methanol; degree of esterification; alcohol oxidase; peroxidase; ABTS*

INTRODUCTION

A method often used to determine methanol for the quantification of pectin methyl ester content (Fishman et al., 1984) is oxidation of methanol to formaldehyde with potassium permanganate, followed by condensation with 2,4-pentanedione and ammonia to yield the colored product 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (Wood and Siddiqui, 1971). The permanganate oxidation method requires the reduction of unreacted permanganate with sodium arsenite. The arsenite reduction step has been reported to be a complex process (Belcher and Nutten, 1960). Wood and Siddiqui reported that trace components of permanganate interfere considerably with the assay. More recently, an improved method was reported employing an enzymatic procedure for oxidizing methanol to formaldehyde (Klavons and Bennett, 1986). This procedure makes use of the fact that in the presence of oxygen alcohol oxidase (alcohol:oxygen oxidoreductase, EC 1.1.3.13; AOD) serves as a rapid and efficient catalyst for the oxidation of lower primary alcohols to the corresponding aldehydes and hydrogen peroxide (Janssen and Ruelius, 1968). Using the alcohol oxidase from *Pichia pastoris*, this method eliminated the reduction step with the toxic sodium arsenite and offered a 2-fold increase in sensitivity (1–20 $\mu\text{g}/\text{mL}$) (Klavons and Bennett, 1986).

Although the specificity of alcohol oxidase and the negligible production of chromophore from the condensation of 2,4-pentanedione with lower primary aldehydes other than formaldehyde provide a direct and specific procedure for the determination of methanol, the sensitivity sometimes is too low, especially if low amounts of pectin are available, e.g. eluates from high-performance gel chromatography. Therefore, we present a modification of the AOD–pentanedione procedure

(Klavons and Bennett, 1986) for the determination of methanol released after alkaline hydrolysis from pectins.

Hydrogen peroxide, the second product of the oxidation of methanol, can be determined with high sensitivity using peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7; POD), e.g. from horseradish. The use of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), ABTS, as donor and a modified assay protocol provide a 20-fold increase in sensitivity over the value from the procedure of Klavons and Bennett (1986).

The POD–ABTS assay was originally introduced to determine the activity of alcohol oxidases (Sahm and Wagner, 1973). An assay using this method can also be used to determine methanol. It can be carried out in less than 4 min, compared to 1 h for the AOD–pentanedione assay and 2 h for the permanganate oxidation method.

EXPERIMENTAL PROCEDURES

Apparatus. A spectrophotometer from Shimadzu Scientific Instruments, Inc., Columbia, MD (UV-2101 PC), was used in its single-beam mode to measure the absorbance of all samples (UVPC Kinetics Software, Version 2.7). Microcuvettes (path length, 10 mm; volume, 200 μL) were used to reduce sample volumes and reagent use.

Reagents. Citrus pectin [85% w/w galacturonic acid content, 10% w/w methoxy content, 5.2% loss on drying (supplier's notes, as supplied) (#P-9135)], alcohol oxidase (alcohol:oxygen oxidoreductase, EC 1.1.3.13) from *P. pastoris* [52 mg of protein/mL, 33 units/(mg of protein) (Biuret) (#A-2404)], peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) type VI-A from horseradish [1380 units/(mg of solid) (#P-6782)], and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (#A-1888) were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals were analytical grade and of the highest purity obtainable.

Solutions. Solution A was dissolved ABTS [one tablet of ABTS (=10 mg of ABTS) dissolved in distilled water and diluted to 2 mL (approximately 0.1 mg of ABTS per assay)]. Solution B was dissolved peroxidase [0.65 mg of peroxidase from horseradish dissolved in distilled water and diluted to 1 mL, 20 μL of that solution diluted to 2 mL with distilled water (approximately 0.18 unit per assay)]. Solution C was diluted

alcohol oxidase [30 μL of alcohol oxidase solution from *P. pastoris* diluted to 2 mL with distilled water (approximately 0.5 unit, or 0.3 μL of current lot number per assay)].

Procedure. Solution A (20 μL) and solution B (20 μL) were pipetted into an amber-colored Eppendorf microcentrifuge tube (0.5 mL). Aliquots (100 μL) of methanol standards or unknown (0.1–2.0 μg of methanol/mL) in 0.20 N potassium phosphate buffer (pH 7.5) were added. The tubes were shaken for 2 s with a Vortex mixer. An aliquot (120 μL) of this mixture was pipetted into the microcuvette placed in the light beam of the spectrophotometer. Solution C, diluted alcohol oxidase (20 μL), was added to the cuvette, and the kinetic software was started instantly. The blank, containing 0.2 N phosphate buffer (pH 7.5) instead of the methanol standard solution was treated like the methanol standards, and its absorbance was measured as a function of time. The absorbances were measured at a wavelength of 420 nm against air (without cuvette). The absorbance of the blank was subtracted afterward.

Determination of the Degree of Esterification of Pectin. A stock solution of pectin was prepared at a concentration of 1050 $\mu\text{g}/\text{mL}$ distilled water without purification of the pectin. Pectin methyl esters were hydrolyzed as follows; 5 mL of 1.0 N potassium hydroxide and additional water were added to aliquots of the pectin stock solution to give 10 mL of pectin solutions ranging in concentration from 2.1 to 21 μg of pectin/mL of 0.5 N potassium hydroxide. The solutions were incubated at room temperature for at least 30 min. An aliquot of the pectin hydrolysates (4.0 mL) was neutralized with dilute phosphoric acid to pH 7.5, using a pH meter, and then diluted with distilled water to give 10 mL ranging from 0.84 to 8.4 $\mu\text{g}/\text{mL}$ pectin (hydrolyzed). Aliquots (100 μL) of the hydrolyzed pectin samples were then analyzed for methanol, as above. The demethoxylated pectin solutions were analyzed directly for uronic acid via the *m*-hydroxydiphenyl procedure of Blumenkrantz and Asboe-Hansen (1973), which is more sensitive than the method described by McCready and McComb (1952). Alternatively, the galacturonic acid content was determined according to the method of Birnbaum et al. (1979). After acidification with dilute phosphoric acid to pH 4.0, using a pH meter, the solutions were incubated with a dilute pectinase [poly(1,4- α -galacturonide)glycanohydrolase] solution from *Aspergillus niger* (20 μL , 73 units/mL) for 24 h at room temperature for depolymerization, then adjusted to pH 7.0 with sodium hydroxide (0.1 N), and diluted with distilled water to give 10 mL. Aliquots were subjected to analysis for galacturonic acid.

RESULTS AND DISCUSSION

Solutions of methanol ranging in concentration from 0.1 to 2.0 $\mu\text{g}/\text{mL}$ in 0.2 N potassium phosphate buffer (pH 7.5) were assayed (Figure 1). Pectin solutions, saponified in 0.5 N potassium hydroxide, neutralized to pH 7.5 with phosphoric acid, and then diluted by a factor of 2.5 of their original volume (of 0.5 N KOH), were also assayed (Table 1).

Effect of Incubation Time. As can be seen in the calibration graph (Figure 1), incubation of methanol with alcohol oxidase, peroxidase, and ABTS to form the green-colored ABTS cation resulted in an instant and fast reaction after addition of AOD which was already finished within 1 min. After this initial rapid linear increase in absorbance, the rate of increase in absorbance decreased but continued at a positive rate proportional to the methanol content. From that point, i.e. 2 min for a methanol concentration of 2.0 mg/L, the absorbance showed a linear positive increase (drift), which did not reach an end point even after several minutes. With increasing concentrations of methanol, the slope of the observed drift increased from 32 (0.1 $\mu\text{g}/\text{mL}$) to 120 mAbs/min (2.0 $\mu\text{g}/\text{mL}$). Since these lines were straight, quantification can be done at any fixed incubation time. Accuracy is a matter of correct timing.

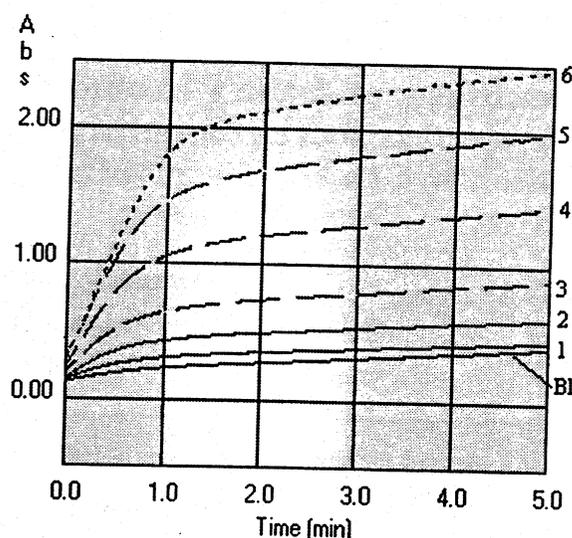


Figure 1. Determination of methanol from one series at various methanol concentrations with the AOD-POD-ABTS assay ($\lambda = 420 \text{ nm}$): Bl = blank, 1 = 0.1 $\mu\text{g}/\text{mL}$, 2 = 0.25 $\mu\text{g}/\text{mL}$, 3 = 0.5 $\mu\text{g}/\text{mL}$, 4 = 1.0 $\mu\text{g}/\text{mL}$, 5 = 1.5 $\mu\text{g}/\text{mL}$, and 6 = 2.0 $\mu\text{g}/\text{mL}$ methanol.

Table 1. Concentration of Methanol and Degree of Methoxylation of Pectin Solutions

pectin ($\mu\text{g}/\text{mL}$)	methanol ($\mu\text{g}/\text{mL}$)	absolute methanol content (% w/w)	relative methanol content (DM %)
0.84	0.084 \pm 0.001 ($n = 5$)	10.02	65.00
1.68	0.167 \pm 0.003 ($n = 5$)	10.06	65.26
4.20	0.420 \pm 0.005 ($n = 5$)	9.99	64.81
8.40	0.843 \pm 0.008 ($n = 5$)	9.97	64.68
			mean: 64.95 \pm 0.25

The absorbance of the blank showed the same behavior as those of the methanol standards. It has been reported that exposure of concentrated solutions of "dried" preparations of purified *P. pastoris* alcohol oxidase to air produced low levels of hydrogen peroxide (apparent autoxidation) in the absence of added substrate (Hopkins and Muller, 1987). Testing alcohol oxidase with chromotropic acid, a reagent specific for formaldehyde, showed that alcohol oxidase contained formaldehyde, reversibly bound to the enzyme. This Schiff adduct is sufficiently stable to survive the purification process but unstable enough to dissociate in aqueous solution at temperatures and pH values where the enzyme is active. It has been found that the color formation resulted from the oxidation of formaldehyde to formic acid and hydrogen peroxide. Titrating the reaction mixture with dithiothreitol, a hydrogen peroxide scavenger, prevented the color formation (Hopkins and Muller, 1987).

Quantification of Methanol Concentrations. Quantification can be done in three different ways using the kinetic plots (Figure 1); in each of these ways, a graph is drawn in which the absorbance at a distinct time or the absorbance change per time interval (activity) is shown as a function of the methanol concentration: (A) determination of the initial activity ($\Delta\text{Abs}/\text{min}$) from incubation interval $t = 0-0.25 \text{ min}$ (Figure 2), (B) determination of the activity of the drift ($\Delta\text{Abs}/\text{min}$) from incubation interval $t = 2.0-20 \text{ min}$ (Figure 3), and (C) determination of the absorbance (Abs) at $t = 2.0 \text{ min}$ or any other fixed time (Figure 4). All three methods allowed the construction of a calibration curve. While in method A the activity curve is a nonlinear function

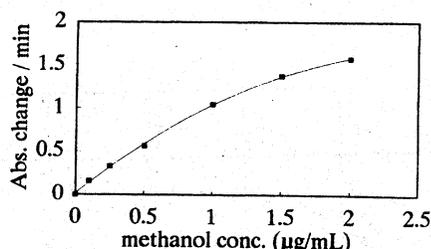


Figure 2. Calibration curve for the AOD-POD-ABTS assay's activity (Δ Abs/min), resulting from the average value of five samples at each methanol concentration, from incubation interval $t = 0-0.25$ min (method A), corrected for the blank activity.

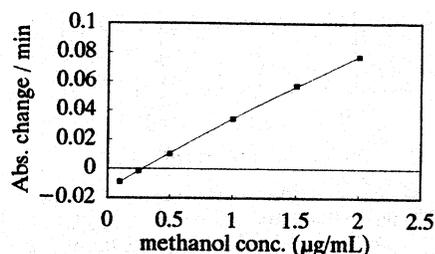


Figure 3. Calibration curve for the AOD-POD-ABTS assay's activity (Δ Abs/min), resulting from the average value of five samples at each methanol concentration, from incubation interval $t = 2.0-10$ min (method B), corrected for the blank activity.

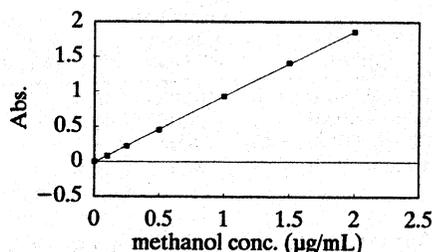


Figure 4. Calibration curve (method C) for methanol standards at $\lambda = 420$ nm using the AOD-POD-ABTS assay, resulting from the average value of five samples at each methanol concentration. Extinctions taken at an incubation time $t = 2.0$ min (see Figure 1).

of the methanol concentration (Figure 2), it is a linear function of the methanol concentration for methods B (Figure 3) and C (Figure 4).

Method B showed an unusual behavior. The drift for the blank is bigger than for 0.1 $\mu\text{g/mL}$ methanol. On the basis of the above-mentioned findings of autoxidation of alcohol oxidase, the phenomenon of higher absorbances with methanol-free blanks than with low concentrations of methanol can be explained on the basis of formaldehyde oxidation by this enzyme. Formaldehyde is one-third as fit a substrate for this enzyme as methanol. Oxidation of formaldehyde in methanol-containing samples could have been suppressed by the methanol. Since the slope of the drift increased with increasing concentrations of methanol, one can assume that subsequent oxidation of formaldehyde, the methanol reaction product, to formic acid and hydrogen peroxide occurred, but at a much slower rate than the oxidation of methanol. This could explain the linear increase in the slope of the drift with increasing methanol concentrations (Figure 3). Since all methanol standards contained the same concentration of alcohol oxidase, quantification of methanol with a calibration curve did not give erroneous methanol concentrations.

Table 2. Reproducibility of Absorbance Rates and Absorbances

methanol ($\mu\text{g/mL}$)	Δ mAbs/min	Δ mAbs/min	mAbs
	($t = 0-0.1$ min) method A	($t = 2.0-3.0$ min) method B	($t = 2.0$ min) method C
0.0	159 \pm 1.72 ^a	41.6 \pm 0.84 ^a	0 \pm 2.24 ^a
0.1	319 \pm 1.75 ^a	32.7 \pm 0.81 ^a	77 \pm 2.46 ^a
0.2	485 \pm 1.75 ^a	40.1 \pm 0.81 ^a	223 \pm 3.87 ^a
0.5	721 \pm 1.87 ^a	51.9 \pm 1.24 ^a	454 \pm 3.44 ^a
1.0	1199 \pm 3.87 ^a	75.9 \pm 1.62 ^a	932 \pm 3.61 ^a
1.5	1530 \pm 5.70 ^a	98.9 \pm 1.91 ^a	1411 \pm 5.95 ^a
2.0	1735 \pm 7.32 ^a	119.0 \pm 1.81 ^a	1855 \pm 9.40 ^a

^a Standard deviation (σ), resulting from the average value of five samples at each methanol concentration (methods A and B, values not corrected for the blank).

Polynomial fitting calculations gave calibration curves with the formula $y = -0.003x^2 + 0.051x - 0.014$ for method B, where $y =$ absorbance change and $x =$ methanol concentration, and $y = -0.008x^2 + 0.952x - 0.012$ for method C, where $y =$ absorbance and $x =$ methanol concentration. This validates the subjective impression that the responses are linear.

On the basis of these facts, method C was judged to be the most appropriate means of calculating methanol concentrations (Figure 4). Using this calibration curve, the methanol concentrations for any unknown sample could be found easily. Nevertheless, methods A and B can be very valuable for the examination of interference. Table 2 shows the precision of five series at each methanol concentration at 1 day with all three methods. The precision of the assay from series to series from day to day exhibited a variation coefficient of 2.2% for method C.

Determination of the Degree of Esterification of Pectin. Aliquots of pectin ranging in concentration from 0.84 to 8.4 $\mu\text{g/mL}$ (final concentration) were analyzed for methanol. The increase in absorbance was a linear function of the pectin concentration (not shown). A spectral overlapping of the deesterified pectin solutions with the dye formed in the indicator reaction could be observed. The slope of the methanol calibration curve with the pectin hydrolysates was steeper than with the methanol standards. The reason for this behavior could be caused by colored degradation products during alkaline demethoxylation. Therefore, standard addition with methanol was employed for each pectin sample. We also recommend the absorbance measurement of pectin hydrolysates against water at 420 nm. The pectin used was found to have a galacturonate content of 85.0% employing the method of Blumenkrantz and Asboe-Hansen (1973). This is in accordance with the data given by the supplier. The method of Birnbaum et al. (1979) resulted in distinct lower values probably because of incomplete depolymerization by the employed endopectinase (data not shown). The absolute methanol content (% w/w) and the relative methanol content (DM) are given in Table 1. They were in accordance with the values determined by the supplier of the pectin.

Effect of pH. A pH of 7.5 the optimum pH of alcohol oxidase, was found to give maximum activity for the assay, although peroxidase has an optimum pH of 5.0 (Figure 5C).

Effect of Light. Mixtures of the two enzymes (AOD and POD) and ABTS in phosphate buffer at pH's from 6.0 to 8.0 showed a visible change in color in the sunlight (Figure 5B), whereas light-protected solutions did not change (Figure 5A). Determination of the

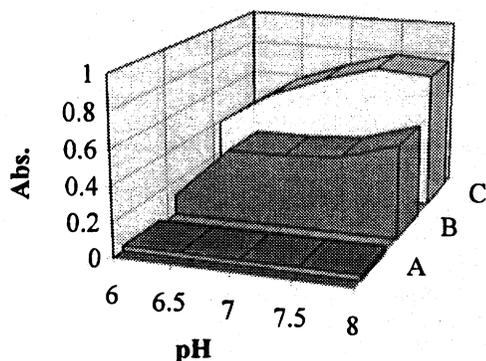


Figure 5. Incubation of (A) a blank, protected from light, (B) a blank, exposed to sunlight for 60 min, and (C) a methanol standard (0.5 $\mu\text{g/mL}$), protected from light, with ABTS, AOD, and POD under the conditions described, resulting from the average absorbance of five samples at each pH value.

absorbances of the samples showed that at pH 6.5–8.0 the samples were most affected by light, whereas at a pH of 6.0, only about 25% of that absorbance was observed (Figure 5B). Because of the tendency for higher blank values with increasing pH, we suggest that the enzyme alcohol oxidase is implicated in this unexpected behavior; however, there is no proof of this yet. The enzyme or its cofactor, flavin, could act as a photosensitizer and, perhaps, be responsible for the production of singlet oxygen which itself oxidizes ABTS. Reagents therefore were kept separately under exclusion from light and mixed shortly before the assay was performed. Light impermeable tubes were used. AOD was added to the mixture of POD, ABTS, and sample, which had been pipetted into the cuvette shortly before, to start the reaction. It is not clear yet if the release of formaldehyde from alcohol oxidase and, subsequently, the observed linear drift in absorbance of the blank has been accelerated by the continuous exposure to the light beam in the spectrophotometer. Further examinations have to be done in this field, especially the determination of the spectral changes of the enzyme–substrate complex during the reaction.

Effect of Possible Interfering and Inhibiting Substances. The effect of possible interfering substances with alcohol oxidase, particularly that of primary alcohols such as ethanol, 1-propanol, and 2-propanol, is well-known (Janssen and Ruelius, 1968; Sahm and Wagner, 1973). Error may arise due to adsorbed ethanol or 2-propanol present in many commercial alcohol-precipitated pectin preparations. Due to the high sensitivity of the assay toward such alcohols, purification of most of the commercial pectins is recommended to remove alcohol contaminants. This purification can be achieved by using well-accepted procedures, i.e. multiple humidification and drying, dialyzing against water, or reprecipitation with acetone. Precipitation with Cu^{2+} ions is not recommended as they are known to inhibit alcohol oxidase (Sahm and Wagner, 1973). While peroxidase is specific for the hydrogen acceptor hydrogen peroxide, it is not specific for the hydrogen donor. A large number of phenols, aminophenols, diamines, indophenols, leuco-dyes, ascorbate, and several amino acids are able to serve as alternative electron donors for hydrogen peroxide (Paul, 1963). Other reasons may be the breakdown of hydrogen peroxide by radical species or the reductive reversal of dye color. To suppress reactions with minor concentrations of competitive hydrogen donors and to inhibit disproportionation of the ABTS radical cation, a 100-fold molar excess

of ABTS over the concentration of the analyte hydrogen peroxide has been used as recommended (Werner et al., 1970). Several papers and patent applications have dealt with the minimization or elimination of other interfering substances [summarized in Michal et al. (1983)] which were not investigated in our department as they were absent in the used pectin.

Effect of Temperature. Like all enzymatic reactions, the AOD–POD-catalyzed reaction is sensitive to temperature changes, and therefore, the greatest possible constancy of temperature should be applied. All reactions have been done at room temperature without a thermally controlled environment for the spectrophotometer. As the procedure is very rapid, the setup of a calibration curve and the multiple measurement of a sample and a blank can be done in 0.5 h. Long term temperature drift was controlled by single-standard recalibration. Investigations on the determination of pectinesterase activity in juices are currently being carried out in our department employing the AOD–POD–ABTS assay to evaluate its reproducibility under optimized conditions from series to series and from day to day.

Reproducibility and Stability of the Enzymes and the Chromogen. Loss of stability of the commercial enzyme preparation stored under conditions advised by the supplier was negligible for months. In the solid state, ABTS is stable at room temperature for at least 24 months. Although stable for at least 3 days, working solutions of both enzymes and ABTS were prepared daily. Batch to batch variations were controlled by standard calibrations.

Accuracy. Klavons and Bennett (1986) found a distinct decrease in the final absorbance after prolonged incubation with AOD and interpreted it as an impurity of the AOD preparation which further oxidized formaldehyde to formic acid. As stated above, “formaldehyde oxidase” activity is not an impurity of this AOD preparation but a general property of alcohol oxidases. The K_m value of the enzyme for formaldehyde is usually 3–10 times higher than that for methanol (Harder and Veenhuis, 1989). Our investigations did not show such behavior because hydrogen peroxide was detected instead of formaldehyde. This led to the mentioned increase in the positive drift because of the equimolar production of hydrogen peroxide during the oxidation of formaldehyde. Detection of hydrogen peroxide is, therefore, assumed to give more accurate results than detection of formaldehyde due to its subsequent “uncontrolled” oxidation to formic acid. Klavons and Bennett (1986) measured samples against a blank containing phosphate buffer instead of methanol, a common procedure in quantitative analysis. As investigations in our laboratory have shown, the absorbance of the methanol-free blank assayed with this procedure led to marginally higher extinctions than with low-level methanol samples at the lower detection limit because of the release of formaldehyde, reversibly bound to alcohol oxidase. It is therefore recommended that one use sample sizes sufficient to generate absorbances above the very low background value due to enzyme-bound formaldehyde; determination of the degree of esterification of pectins showed values from 43.2 to 48.2% with increasing pectin concentration (Klavons and Bennett, 1986).

Comparison with Other Methodology. Methanol is routinely determined by methods such as high-performance liquid chromatography (HPLC), gas chro-

matography (GC), headspace gas chromatography (HS-GC) (Gessner, 1970), and spectrophotometry. Another method utilizing alcohol oxidase, immobilized onto an oxygen electrode, is also available (Guibault et al., 1983). The HPLC procedure (Galensa et al., 1988) is very sensitive (1.2 ng per 20 μ L injection, 60 μ g/L), but it requires a postcolumn reactor and electrochemical detection of hydrogen peroxide. Several GC methods for analyzing lower alcohols have been published in the past. Many of the earlier ones were not used for concentrations lower than 10–20 mg/L (Dyer, 1971). Today, methanol can be determined in concentrations of about 1 mg/L with GC (Berger et al., 1974); however, the sensitivity of the detector can be affected by the water in the sample, and low concentrations (<10 mg/L) in aqueous samples appeared to give irregular response (Krop, 1974). Samples can be distilled or pretreated before injection, but these procedures can be very time-consuming. With HS-GC and derivatization of alcohols to nitrite esters, 1 mg/L can be determined quantitatively (Gessner, 1970), a sensitivity similar to that for photometric methods used so far.

The spectrophotometric method presented here offers a sensitivity of 50–1000 μ g of methanol per liter and, therefore, can be seen as a breakthrough beyond the 1 mg/L concentration limit of conventional analytical methods. The sensitivity of the AOD–POD–ABTS assay for methanol determination diminishes the importance of the selectivity of the AOD–pentanedione procedure toward methanol. This latter assay is now only necessary if more than one lower alcohol is present in the sample. The procedure is also very rapid; with an analysis time of less than 4 min, it is also one of the fastest assays reported to date.

LITERATURE CITED

- Belcher, R.; Nutten, A. G. *Quantitative Inorganic Analysis*; Butterworth: London, 1960; pp 221, 258.
- Berger, G.; Agnel, J. P.; Saint-Lébe, L. Quelques Composés Volatils Formés au Cours de l'Irradiation Gamma de l'Amidon de Mais (Volatile compounds formed during gamma irradiation of corn starch). *Stärke* **1974**, *26*, 185–189.
- Birnbaum, A.; Dörreich, D.; Ly, T. B.; Sabir, D. M.; Wadud, S.; Gierschner, K. Beitrag zur Enzymatischen Bestimmung der Galakturonsäure (GA) (The enzymic determination of galacturonic acid (GA)). *Lebensm.-Wiss. Technol.* **1979**, *12*, 231–233.
- Blumenkrantz, N.; Asboe-Hansen, G. New Method for Quantitative Determination of Uronic Acid. *Anal. Biochem.* **1973**, *54*, 484–489.
- Dyer, R. H. Comparison of GLC and Colorimetric Methods for Determination of Methanol in Alcoholic Beverages. *J.-Assoc. Off. Agric. Chem.* **1971**, *54*, 785–786.
- Fishman, M. L.; Pfeffer, P. E.; Barford, R. A.; Doner, L. W. Studies of Pectin Solution Properties by High-Performance Size Exclusion Chromatography. *J. Agric. Food Chem.* **1984**, *32*, 372–378.
- Galensa, R.; Müller, G.; Schirmer, A.; Hippe, H.; Stadler, H. Bestimmung von Lebensmittelinhaltsstoffen durch HPLC-Enzymreaktor-Kopplung (Determination of food constituents by HPLC-enzyme reactor coupling). *Lebensmittelchem. Gerichtl. Chem.* **1988**, *42*, 84–85.
- Gessner, P. K. Method for the Assay of Ethanol and Other Aliphatic Alcohols Applicable to Tissue Homogenates and Possessing a Sensitivity of 1 μ g/ml. *Anal. Biochem.* **1970**, *38*, 499–505.
- Guibault, G. G.; Danielsson, B.; Mandenius, C. F.; Mosbach, K. Enzyme Electrode and Termistor Probes for Determination of Alcohols with Alcohol Oxidase. *Anal. Chem.* **1983**, *55*, 1582–1585.
- Harder, W.; Veenhuis, M. Metabolism of one-carbon compounds. In *The Yeasts*; Rose, A. H., Harrison, J. S., Eds.; Academic Press: London, 1989; Vol. 3, Chapter 8.
- Hopkins, T. R.; Muller, F. Biochemistry of Alcohol Oxidase. In *Proceedings of the 5th International Symposium on Microbial Growth on C1 Compounds*; van Verseveld, H. W., Duine, J. A., Eds.; Kluwer Academic Publishers: Dordrecht, 1987; pp 150–157.
- Janssen, F. W.; Ruelius, H. W. Alcohol Oxidase, a Flavoprotein from Several Basidiomycetes Species. *Biochim. Biophys. Acta* **1968**, *151*, 330–342.
- Klavons, J. A.; Bennett, R. D. Determination of Methanol Using Alcohol Oxidase and Its Application to Methyl Ester Content of Pectins. *J. Agric. Food Chem.* **1986**, *34*, 597–599.
- Krop, J. J. P. The Mechanism of Cloud Loss Phenomena in Orange-Juice. *Versl. Landbouwk. Onderz.* **1974**, No. 830.
- McCready, R. M.; McComb, E. A. Extraction and Determination of Total Pectic Materials in Fruits. *Anal. Chem.* **1952**, *24*, 1986–1988.
- Michal, G.; Möllering, H.; Siedel, J. Chemical Design of Indicator Reactions for the Visible Range. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Verlag Chemie: Weinheim, 1983; Vol. 1, pp 197–232.
- Paul, K. G. Peroxidases. In *The Enzymes*; Boyer, P. D., Lardy, H., Myrbäck, K., Eds.; Academic Press: New York, 1963; Chapter 7.
- Sahm, H.; Wagner, F. Microbial Assimilation of Methanol. The Ethanol- and Methanol-Oxidizing Enzymes of the Yeast *Candida Boidinii*. *Eur. J. Biochem.* **1973**, *36*, 250–256.
- Werner, W.; Rey, H.-G.; Wielinger, H. Über die Eigenschaften eines neuen Chromogens für die Blutzuckerbestimmung nach der GOD-POD-Methode (Screening of chromogens for the determination of glucose in blood according to the GOD/POD method). *Z. Anal. Chem.* **1970**, *252*, 222–224.
- Wood, P. J.; Siddiqui, I. R. Determination of Methanol and its Application to Measurement of Pectin Ester Content and Pectin Methyl Esterase Activity. *Anal. Biochem.* **1971**, *39*, 418–428.

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