Determination of 2-(2′-Octenyl)succinic Acid in Lipophilic Modified Starch by Gas Chromatography–Mass Spectrometry/Selected Ion Monitoring

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The covalent bonding of 2-(2′-octenyl)succinic acid (OSA) to starch enables the resulting modified starch to be used as an emulsifier. A gas chromatography (GC)–mass spectrometry (MS) procedure for free and total OSA quantitation in lipophilic modified starch is described. For free OSA, starch was removed from aqueous dispersion by precipitation with methanol and the acidified supernatant (pH<2) was extracted. For total OSA, esterified OSA was hydrolyzed in an alkaline solution and OSA was extracted following acidification (pH <2). OSA after derivatization with ethereal diazomethane was quantified utilizing GC–MS/selected ion monitoring (SIM). The overall OSA recovery from cornstarch was greater than 95%. Free and total OSA levels in five batches of modified starch were 0.15–0.24% and 2.10–2.41% (w/w), respectively. Hydrolytic stability of bound OSA was kinetically investigated. Pseudo-first-order rate constants were calculated from the SIM of free OSA released by hydrolysis. The activation energy for bound OSA hydrolysis was 24.6 kcal/mol.

Keywords: *2-(2′-Octenyl)succinic acid; lipophilic modified starch; gas chromatography–mass spectrometry; selected ion monitoring; activation energy*

INTRODUCTION

Due to polyhydroxyl groups, native starch is hydrophilic and does not possess sufficient affinity for hydrophobic substances such as oil to be an effective emulsifier. However, the lipophilic functionality of starch may be improved through chemical modification. One such modification involves the reaction of starch with 2-(2′-octenyl)succinic anhydride (OSAnh), resulting in the production of 2-(2′-octenyl)succinic acid (OSA) modified starch (Caldwell et al., 1953; Trubiano, 1986). The covalent bonding of OSA to starch through an esterification reaction equips starch with both hydrophilic and lipophilic functionalities due to the addition of carboxyl and octenyl groups, respectively (Figure 1). The presence of dual functional groups makes OSA modified starch useful in food and pharmaceutical applications in which the stabilization of oils exposed to an aqueous environment is desirable without the use of proteins and/or gums for medical or technological reasons. Examples of such systems include salad dressings, beverage emulsions, and encapsulation of fragrances (Marotta et al., 1969; Trubiano, 1986; Light, 1990). Other examples are ready-to-use nutritional products containing protein hydrolysates but no proteins or polypeptides to avoid allergic reactions. The use of OSA modified starch in foods was approved by the U.S. FDA with the stipulation that food starch may be esterified by treatment with 1-octenylsuccinic anhydride, not to exceed 3% (Food and Drug Administration, 1989).

Considering the process used to produce OSA modified starch, it is conceivable that the finished product contains both bound (esterified) and free (unesterified) OSA, although the amount of the latter is likely to be minute. Since ester bonds are inherently subject to hydrolytic cleavage and the bound OSA of lipophilic modified starch is the functional moiety for emulsification, it is important to know the total and bound OSA of lipophilic modified starch to comply with food additive regulations and to assure the sought-after functionality in food applications. The objectives of this study were, therefore, to develop a method utilizing gas chromatography (GC)–mass spectrometry (MS) for the determination of total and free OSA in lipophilic modified starch which enables the calculation of bound OSA by the difference and to assess kinetically the hydrolytic stability of bound OSA using an aqueous solution of modified starch as a simplified model system for a more complex food matrix.

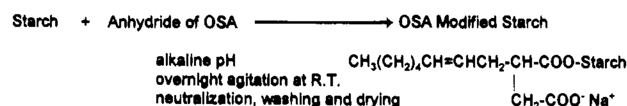

Figure 1. Lipophilic modification of starch producing 2-(2′-octenyl)succinic acid modified starch (Trubiano, 1986).

MATERIALS AND METHODS

Materials. OSAnh was obtained from Milliken Chemical (Inman, SC). Reagents were of either Certified ACS or HPLC grade. Cornstarch, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and pentadecanoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). OSA modified starches were purchased from a commercial supplier.

Preparation of OSA from OSAnh. Five grams of OSAnh was dispersed in 50 mL of 1 N NaOH, and the dispersion was stirred overnight. The pH of the saponified mixture was adjusted to about 5 with 1 N HCl, and the OSA was extracted with three portions of 40 mL of ethyl acetate. Combined extracts were dried over anhydrous Na_2SO_4 and then freed of solvent under vacuum evaporation.

Methylation of OSA. OSA diluted in methanol was converted to dimethyl ester by the addition of ethereal diazomethane (Figure 2). The derivatization mixture was left at room temperature for about 30 min. Diazomethane was slowly generated (to avoid explosion) and simultaneously trapped in

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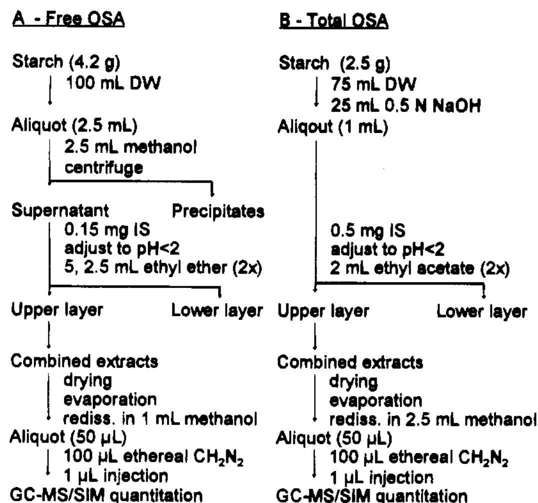

Figure 2. Diagram indicating steps involved in the preparation of (A) free and (B) total OSA from lipophilic modified starch.

diethyl ether according to the recommended procedures of the diazomethane generator supplier (Pierce Chemical Co., Rockford, IL). Briefly, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (ca. 130 mg) and diethyl ether (2 mL) were placed into the inner and outer tubes. The tube assembly was placed into an ice bath. After 0.5 mL of distilled water (DW) was added into the inner tube, it was tightly screw-capped with a Teflon-lined septum followed by slow injection of 5 N NaOH (0.6 mL) for diazomethane generation.

Effect of pH on OSA Extraction. To test the pH effect on OSA extraction efficiency, modified starch (2.5% w/v) was dispersed in aqueous alkaline solution in the same manner as for total OSA extraction (see below). The pH of the 1 mL aliquot was adjusted to different levels (1, 2, 3, 4, and 5) with 1 N HCl. OSA contents from aliquots at different pH values were compared.

Free OSA Extraction. Free OSA was extracted as described in Figure 2A. Modified starch (4.2% w/v) was prepared by stirring (ca. 3–4 h) with intermittent heating not exceeding 80 °C. A 2.5 mL aliquot was transferred into a 15 mL conical centrifuge tube to which 2.5 mL of methanol was added to precipitate starch. After centrifugation at 2500 rpm (Centra-7R, International Equipment Co., Needham Heights, MA) for 10 min, the supernatant was transferred into a test tube. After the addition of pentadecanoic acid (0.15 mg) as the internal standard (IS), the pH of the supernatant was adjusted with 150 μL of 1 N HCl (pH <2). The supernatant was extracted with 5 mL of ethyl ether and then with 2.5 mL of ethyl ether twice. The extracts were combined, dried over anhydrous sodium sulfate, and freed of solvent under a nitrogen stream. The dried extract was redissolved into 1 mL of methanol. A 50 μL aliquot was mixed with 100 μL of ethereal diazomethane for methylation, and 1 μL was injected into a gas chromatograph.

Total OSA Extraction. The extraction procedure for total OSA is shown in Figure 2B. Starch (2.5 g) was dispersed with 75 mL of DW. After 25 mL of 0.5 N NaOH was added, stirring was continued for about 2 h until a homogeneous solution was obtained. A 1 mL aliquot was transferred to a 15 mL conical centrifuge tube, to which 1 mL of 1 N HCl (pH <2) and IS (0.5 mg) were added. Ethyl acetate (2 mL) was added, and the mixture was shaken vigorously followed by centrifugation at 2500 rpm for 20 min. The top layer was transferred into an 1 dram vial. The extraction and centrifugation were repeated once more, and then the combined extracts were dried over anhydrous Na_2SO_4. The extract was freed of solvent under a nitrogen stream. The dried extract was redissolved into 2.5 mL of methanol, 50 μL of which was derivatized by adding 100 μL of ethereal diazomethane. One microliter of the reaction mixture was directly injected into a gas chromatograph.

OSA Recovery. For free OSA recovery, cornstarch dispersed into DW (4.2% w/v) was spiked with standard OSA at three different levels, i.e., 0.1, 0.3, and 0.5% of starch (w/w). These spiked samples were analyzed for OSA according to the same method as for free OSA. For total OSA recovery, cornstarch (5.25 g) was made into a slurry with 75 mL of DW and 25 mL of 0.5 N NaOH. The slurry was divided into five portions of 19 mL each. Each portion was spiked with different amounts of OSA to represent three different OSA levels at 1, 2, and 3% of starch (w/w). After the addition of IS and dilution to 20 mL with DW, a 1 mL aliquot was subjected to the entire procedure for total OSA quantitation. The percent recoveries were obtained after the OSA content found experimentally was divided by the known amount added.

GC–MS/Selected Ion Monitoring (SIM). A Hewlett-Packard 5890 Series II gas chromatograph equipped with a 5971A mass selective detector was employed. A fused silica capillary column, DB-5 (30 m × 0.25 mm, 0.1 μm, J&W Scientific, Inc., Rancho Cordova, CA), was used. The column oven temperature was programmed from 150 to 190 °C at a 5 °C/min rise. Helium was the carrier gas at 8 psi and the split ratio, 30:1. The energy of the bombarding electrons was 70 eV. Ion source temperature was 190 °C. Data acquisition started from 2.5 min following injection, which was the solvent delay time. For the total ion chromatogram (TIC), mass spectra scanned from mass/charge (m/z) 35 to 400 were recorded using a software package (MS ChemStation G1030A) on a QS/20 Vectra computer (Hewlett-Packard). For OSA quantitation by SIM, the response linearity of OSA relative to IS was studied with four different calibration mixtures representing weight ratios (OSA/IS) of 0.5, 1, 2, and 3. For SIM, quantifier ions were 196.20 and 143.20 for OSA and IS as methyl esters, respectively. OSA concentration was calculated as follows: $\text{OSA}\ \% = (A_{OSA}/A_{IS})(\text{RRF})(\text{IS}/\text{SA})(100)$, where A_{OSA} and A_{IS} are the area responses of OSA and IS, respectively, RRF is the OSA response factor relative to IS, IS is the weight of IS added to starch, and SA is the starch weight.

Hydrolytic Stability of Bound OSA. When an aqueous starch solution (4.2% w/v) was cooled to ambient temperature, its pH (4.7) was adjusted to pH 5.5 and then 0.02% NaN_3 was added. Ten milliliter aliquots of the starch solution were transferred into headspace vials (20 × 75 mm). The vials were sealed with Teflon septa and aluminum caps using a crimper and then stored at 33 ± 1, 43 ± 1, and 53 ± 1 °C. One vial at each temperature was removed at intervals, and free OSA was determined as described above. Prior to methanol addition for starch removal, the pH of the sample solutions was adjusted to pH 4.7 from 5.5. Although the hydrolysis of bound OSA or the formation of free OSA over time (t) would be described by the second-order reaction, $-d[\text{bound OSA}]/dt = d[\text{free OSA}]/dt = k[\text{bound OSA}][H_2O]$, the reaction was analyzed by pseudo-first-order reaction kinetics, since water is present in excess (Schoebel et al., 1969). Accordingly, the rate equation was described by the first-order reaction, $\log[(B_0 - F)/B_0] = (-k/2.303)t$, where B_0 and F are the initial concentration of bound OSA and free OSA concentration at a given time, respectively. The modified starch used in this study had total and free OSA concentrations of 2.7 and 0.2% (w/w), respectively, and thus B_0 estimated by difference was 2.5% (w/w).

Statistical Analysis. All data are mean (M) values and coefficient of variation (CV) from three preparations with a single injection each, unless otherwise stated.

RESULTS AND DISCUSSION

Preparation of OSA Standard. OSA standard was not commercially available at the time of this study and was thus prepared by the alkaline hydrolysis of its anhydride. The prepared OSA was qualitatively analyzed after methylation by GC–MS to confirm its identity. TIC showed only a single peak, other than the solvent peaks, indicating the purity to be no less than 99% (Figure 3). Its mass spectrum displayed the molecular ion (M) peak (m/z 256, 6%), agreeing with the observation that it is usually possible to observe a weak but noticeable M peak for methyl esters (Pavia et al., 1979). Surprisingly, the base peak (m/z 114) and other major ions such as m/z 196, 146, and 122 were all even-

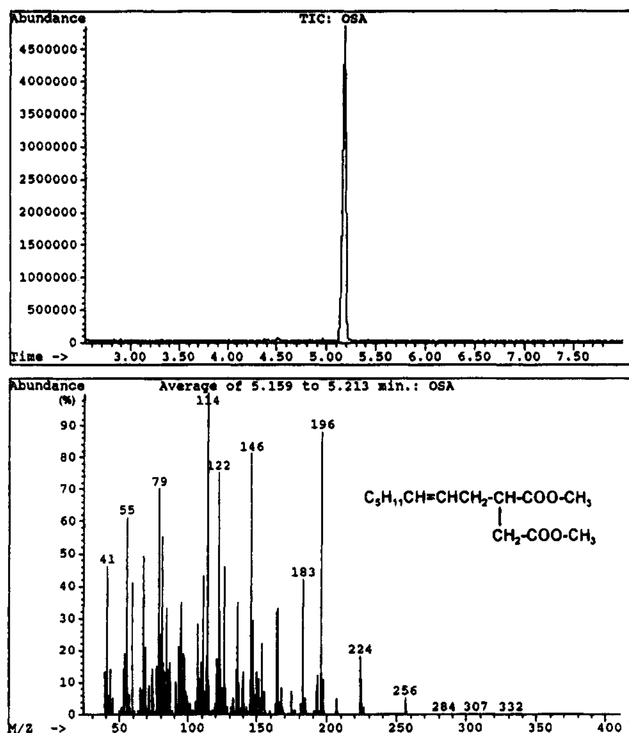

Figure 3. Total ion chromatogram (top) and electron impact mass spectrum (bottom) of 2-(2′-octenyl)succinic acid as dimethyl ester prepared from alkaline hydrolysis of its anhydride and methylation with ethereal diazomethane.

numbered, implying they might have been produced through rearrangement in addition to the usual fragmentation. This makes it almost impossible to figure out how these ions were produced without additional information by other means such as tandem MS−MS. Nevertheless, the observation of M and excellent agreement of the present mass spectrum with that of Giordano et al. (1990) clearly established the identity of the OSA preparation. To confirm that the prepared OSA standard is free from OSAnh, the anhydride diluted in diethyl ether and analyzed by GC−MS without derivatization revealed both major and minor peaks at retention times of 4.94 and 5.03 min, respectively, which were different from that of OSA (5.19 min); the mass spectra of the major and minor peaks were virtually identical with M at *m/z* 210 (45−50%), suggesting that they are cis/trans isomers. Consequently, on the basis of the observation of the OSA peak and the lack of its anhydride peaks in the TIC (Figure 3), the prepared OSA was regarded as pure and thus used without correction for the purity.

SIM Standard Curve. Pentadecanoic acid methyl ester showed a single major peak which was well separated from OSA (retention time, 6.8 min), supporting its candidacy as an IS. Its mass spectrum displayed the well-known diagnostic peak, *m/z* 74, due to McLafferty rearrangement (McLafferty, 1980). Calibration mixtures containing different levels of OSA were run through GC−MS, and the area ratios under the ion peaks (*m/z* 196.2/143.2) were plotted against the corresponding weight ratios (Figure 4). Excellent response linearity was observed over the studied range (weight ratios of 0.5−3.0), yielding the equation $A_{OSA}/A_{IS} = 0.797$ $(W_{OSA}/W_{IS}) + 0.004$ ($r^2 = 0.99$). The RRF of OSA with pentadecanoic acid as IS was 1.25, which is the reciprocal of the curve slope.

Effect of pH on OSA Extraction. The effect of the pH of the starch solution on OSA extraction efficiency was investigated by adjusting the pH of the medium

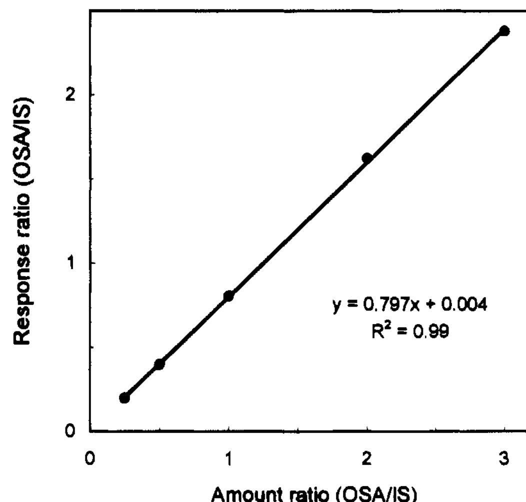

Figure 4. Selected ion monitoring standard curve for 2-(2′-octenyl)succinic acid as dimethyl ester with methyl pentadecanoate as the internal standard.

prior to ethyl acetate extraction. As the pH was decreased from 5 to 1, higher amounts of OSA were extracted. The highest extraction was obtained at pH 1 (taken as 100%) followed by that at the pH 2 (98.5%) and that at pH 3 (96.1%). While the CV of the extraction below pH 3 was smaller than 2%, a large CV (7.2%) with a drastic decrease in OSA extraction (down to 70.6%) resulted at pH 4. At pH 5 only 26.3% OSA was extracted with a CV of 44.3%. Considering that the pK_{a1} and pK_{a2} of succinic acid are 4.21 and 5.72, respectively (Dawson et al.,1986), the poor extraction of OSA into organic solvent from a sample solution at pH 4 or above, therefore, seems to be reasonable. Consequently, the pH of a sample solution was adjusted to below 2 before extraction throughout the present study.

Free OSA Quantitation. Initially, we directly extracted starch powders with ethyl acetate for free OSA, ending up with a low level of free OSA which was about $^1/_{10}$ of values the supplier provided (analytical method not disclosed). Next, we examined prolonged leaching of free OSA from sample powder with methanol by repetitive extraction overnight using the Soxhlet apparatus. However, the Soxhlet extraction method was not pursued further because more than a 3-fold increase in free OSA content resulted, suggesting the methanolysis of bound OSA as the extraction was extended from 17 to 42 h. At this point, we surmised that a portion of free OSA might be physically entangled by or within modified starch particles. If so, solubilization or dispersion of starch particles may render free OSA more readily extractable. Prior to extraction, modified starch was therefore dispersed in DW as described under Materials and Methods. An aliquot (2.5 mL) of the starch solution (4.2% w/v) was transferred into tubes. When free OSA was extracted without starch removal, the free OSA levels were unrealistically high (0.89−2.77%). A review of peak area response data revealed that the higher OSA levels were the result of the relatively poor recovery of IS rather than higher OSA extraction. When the extraction was carried out following starch removal as described in Figure 2A, the resulting free OSA content not only approximated the values of the supplier but also showed good reproducibility. The repeated analyses of a starch solution showed a CV of 2.9%, and the analyses for repeated preparations of starch solutions had a CV of about 3.5%. This procedure also obviated concern about the potential

Table 1. Free and Total OSA Contents (Percent) in Modified Starch

starch batch	free OSA M	free OSA CV%	total OSA M	total OSA CV%
1	0.18	4.9	2.10	3.5
2	0.23	2.3	2.17	2.9
3	0.24	2.8	2.26	4.1
4	0.15	4.7	2.20	1.9
5	0.19	4.1	2.41	2.7
overall	0.20	18.7	2.23	5.3

contribution of bound OSA to free OSA, if hydrolysis takes place due to pH adjustment prior to extraction. The free OSA level of five modified starch batches, determined as described in Figure 2A, was 0.15–0.24% (Table 1).

Total OSA Quantitation. Total OSA levels in OSA modified starch samples were determined after alkaline hydrolysis of the ester bond of bound OSA and thus represented the sum of free and bound OSA. The OSA content of five starch batches determined as described in Figure 2B was 2.10–2.41% (Table 1), showing that OSA levels were in good compliance with the FDA regulation (below 3%). A small aliquot, equivalent to about 50 mg of starch, was used for quantitation, although 5 g of starch was dispersed to better represent the powder samples. This microscale analysis not only allowed less generation of chemical waste and higher sample throughput but also overcame limited supply of ethereal diazomethane available from single-batch production. It is, therefore, important to make sure that starch samples are dispersed thoroughly and free of any observable lumps, so that a 1 mL aliquot can be representative of the entire sample dispersion. Similar to free OSA determination, the precision of the total OSA determination procedure was regarded to be satisfactory as represented by narrow CV, i.e., 1.2% for the repeated analyses of a starch solution and 3.2% for the repeated preparations of starch solutions.

Recovery of Added OSA. Recoveries of OSA spiked at levels of 0.1, 0.3, and 0.5% to cornstarch which was subjected to free OSA quantitation procedure were 94.6, 96.3, and 94.9%, respectively, with the overall recovery of 95.3%. This high recovery implied that the method recovers most of the free OSA and, therefore, the resulting free OSA value would be accurate after correction for recovery percent. Also, the precision of the method was reaffirmed as indicated by the narrow standard deviation observed (overall CV of 0.95%). To determine percent recovery of total OSA, cornstarch free of OSA was similarly spiked at higher OSA levels of 1, 2, and 3%. Recoveries were 99.0, 95.2, and 101.4%, respectively, the overall recovery being 98.5% (CV, 3.1%). At higher concentration ranges, i.e., 2 and 3%, CV was even narrower, less than 1%. This excellent recovery with narrow CV suggests that the present method, extraction of OSA released from starch and its subsequent GC–MS quantitation, should provide precise and accurate information about the total OSA content of OSA modified starch.

Hydrolytic Stability of Bound OSA. The knowledge of nutrient stability and/or chemical stability of ingredients in food products is important for shelf life as well as consumer acceptance. As ester bonds are inherently subject to hydrolytic cleavage, the emulsification effected by lipophilic modified starch may deteriorate. The effects of storage time and temperature on the hydrolytic loss of bound OSA were studied by monitoring free OSA level in starch solutions. The semilog plot of residual bound OSA (percent) at each temperature vs storage time is shown in Figure 5. The

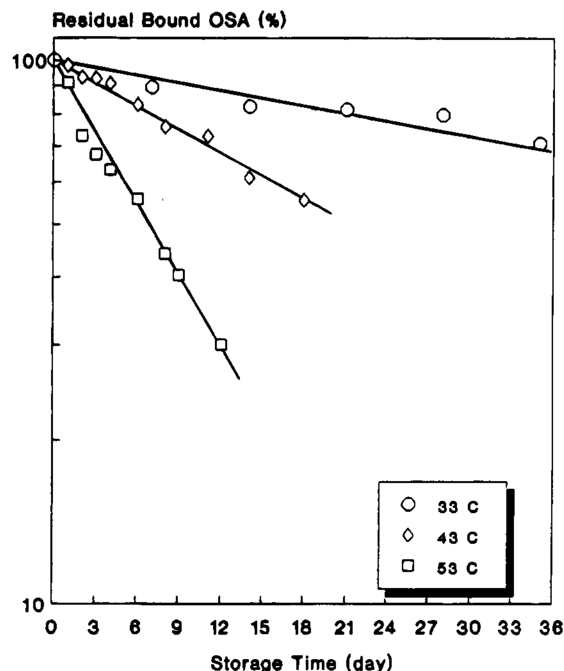

Figure 5. Hydrolysis curves of bound OSA at three different temperatures. The aqueous dispersion of starch (4.2% w/v) had an initial concentration of bound OSA of 2.50% w/w.

Table 2. First-Order Rate Constants of Bound OSA Hydrolysis

temp, °C	y-intercept	rate, ×100/day	r^2	r^{2a}
33	96.5	0.85	0.92	0.91
43	99.9	3.02	0.97	0.96
53	96.6	10.15	0.98	0.93

[a] For zero-order kinetics.

plot yielded in all cases a straight line fit with r^2 being not lower than 0.92 (Table 2), indicating a first-order reaction. From the slopes of the regression lines, first-order rate constants were obtained. When the zero-order reaction was assumed, the regression analysis of the plot, $(B_0 - B)$ vs t, showed that the goodness-of-fit at 53 °C was not as strong as that for the first-order plot. However, the r^2 values at 33 and 43 °C for the zero-order kinetics were slightly lower than those for the first-order reaction (Table 2). This is partly because the extents of hydrolysis at 33 and 43 °C were not sufficient due to slow reaction to differentiate more clearly zero- and first-order kinetics, although the experiments were carried out far longer than that at 53 °C. Labuza and Riboh (1982) pointed out that most quality-related reactions are either zero- or first-order and statistical differences between the two types may be not significant. Since many hydrolysis reactions are known to follow first-order kinetics (Schoebel et al., 1969) and higher r^2 values were observed for the first-order than for the zero-order kinetics in the present hydrolysis reaction, the hydrolytic breakdown of bound OSA to free OSA was considered to be better described by the first-order kinetics as shown in Figure 5.

The relationship between the storage temperature in terms of the absolute temperature (T) and the measured rate constant (k) was investigated using the Arrhenius plot (Figure 6), in which the semilog plot of log k vs $1/T$ yields a straight line with the slope equal to $-E_a/2.303R$ (Labuza, 1972). The slope of the regression line in Figure 6 is −5.38 (K) and, therefore, the activation energy (E_a) was estimated to be 24.6 kcal/mol, which was larger than the E_a for hydrolysis reactions in general, 15 kcal/mol (Saguy and Karel, 1980). However, it was smaller than the E_a for the acid-catalyzed

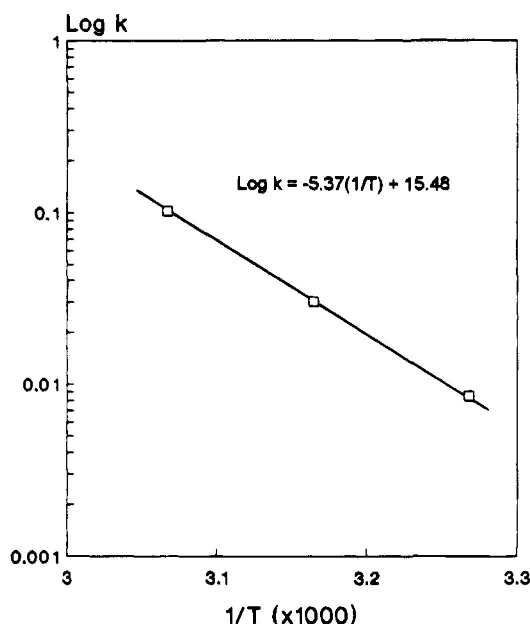

Figure 6. Arrhenius plot of the bound OSA hydrolysis reaction over the temperature range 33–53 °C. Regression analysis of data shows that the E_a was 24.6 kcal/mol.

hydrolysis of sucrose α-1,2-glucosidic linkage, 24.7–30.0 kcal/mol (Heidt and Purves, 1938; Lund et al., 1969; Zhong et al., 1984), and lactose α-1,4-glucosidic linkage, 29.5–30.1 kcal/mol, calculated from the data of Coughlin and Nikerson (1975), or for the hydrolysis of β-1,6-glucosidic linkage using melibiose, 42.4 kcal/mol (Heidt and Purves, 1938). Since the ester bond is generally less stable in aqueous medium, it is not surprising that the E_a requirement for bound OSA hydrolysis is lower than that for even the acid-catalyzed saccharide hydrolysis. Nevertheless, it appeared to be more stable than ascorbic acid and aspartame (methyl ester of dipeptides); the E_a for vitamin C degradation was 18.2 kcal/mol during the storage of canned single-strength grapefruit juice (Smoot and Nagy, 1980) and the E_a for aspartame degradation, when calculated from the data of Fellows et al. (1991), was about 18 kcal/mol in fruit preparations used in yogurt.

A GC–MS method was developed for the determination of free and total OSA in lipophilic modified starch. For free OSA determination, starch was removed from its aqueous dispersion by the addition of methanol and the resulting supernatant was extracted. For total OSA determination, starch was dispersed in alkaline solution and bound OSA was hydrolyzed prior to extraction. The extracted OSA was quantified as the dimethyl ester by GC–MS/SIM using m/z 196.2 and 143.2 for OSA and IS, respectively. The analysis of five batches of lipophilic modified starch showed that most OSA was in the esterified form as represented by low levels of free OSA (0.15–0.24% w/w), and the total OSA level (2.10–2.41% w/w) did not exceed the regulatory limit, 3%. The hydrolysis of bound OSA of modified starch dispersion was studied kinetically as a simple model for liquid foods and the reaction could be described as a pseudo-first-order reaction. The activation energy for bound OSA hydrolysis was about 24.6 kcal/mol, which appeared to be lower than that of acid-catalyzed saccharide hydrolysis (24.7–42.4 kcal/mol) but higher than that of vitamin C and aspartame degradation (ca. 18 kcal/mol). Since the present method utilized GC with mass spectrometric detection and pentadecanoic acid (used as IS) occurs at usually negligible levels in oils and fats, this method should perform with good specificity in determining OSA in samples having more complicated matrices than the present modified starch solution.

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