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A new method for quantifying iodine in a starch–iodine matrix

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ABSTRACT

A rapid and sensitive method for quantifying iodine in intact starch granules using gas chromatography is described with detection limits as low as 0.2% (w/w) iodine in starch. Sample preparation includes $NaBH_4$ reduction of the various iodine species associated with starch to the colorless soluble iodide ion, followed by its quantitative derivatization to EtI using $Et_3O^+BF_4^-$ in CH_2Cl_2. Identification and quantification of EtI is carried out by extraction and injection of the EtI so generated in CH_2Cl_2 into a gas chromatography–mass spectrometer (GC–MS). Routine quantification of EtI was then performed using GC with a flame ionization detector (GC–FID). Results for different iodine:potassium iodide ratios of the initially bound iodine and for seven different starch matrices showed that in all cases regression coefficients for the standards were high (R^2 >0.96).

1. Introduction

Variations in the iodine-binding characteristics among different polysaccharide materials have long been utilized for diverse applications ranging from differential staining for microscopical examination[1] to qualitative chemical determination of bound iodine relative to the amylose content of starches.[2] Xylan, for example, binds iodine differently than does starch that exhibits different colored complexes.[3] The apparent iodine binding capacities also reflect different amylose contents of various native and modified starches[4–6] especially when compared under similar conditions of moisture content and exposure time to iodine vapor.[6] Iodine appears to form complexes in linear amylose chains and in the longer branches of amylopectin chains in dispersed starch systems where it can form an α-helical complex with the linear chains with polyiodide-rich inclusion complexes. The resulting color formation and wavelength of maximal absorption are dependent on the length of the glucan polymer[6,7] and the various polyiodide species bound.[8] Quantifying the iodine bound within starches thus has potential use in comparative structural studies.

However, practical, reliable, and sensitive iodine quantification methods have been problematic due to a number of complicating factors. Spectrophotometric methods including UV–Vis[9–12], circular dichroism,[10,13] X-ray,[14] and Raman spectroscopy[8,14–16] provide distinctive absorbances at multiple wavelengths due to the presence of different iodine–iodide complexes (I_3^-, I_5^-, I_7^-, I_9^-, etc.) thus making direct spectrophotometric iodine quantification difficult. Rendleman employed a chemical titration of iodine using sodium thiosulfate, but the method suffers from low sensitivity with unreliable results below 5% (w/w) iodine content in starch. Furthermore, the procedure requires several hours for each sample tested.[6] Inductively coupled plasma optical emission spectroscopy shows good quantitative results for iodine,[17] but sample preparation procedures and instrumentation are more involved. Amperometric/potentiometric titration methods are the most commonly employed methods for routine quantification of iodine in starch,[18–21] but these also suffer from low sensitivity, requiring back extrapolation to the zero point of free iodine in solution based on the slope of the response curve.[22,23] Amperometric/potentiometric titrations pose additional drawbacks including prolonged analysis time, as these are diffusion rate-limited with respect to bound iodine entering the bulk solution for titration, as well as being temperature and concentration dependent.[22] Such problems in quantification could also be compounded given the relative instability of amylose–iodine complexes especially at high iodine loads where iodine can be lost from starch samples through volatilization.[24]

Nonetheless, studies of the kinetics of iodine binding to starch and improved quantification of total starch-bound iodine offer potential insights into the structural organization of native and purpose-modified starches.[12,25,26] Our research group is interested in the low-level detection of various iodine chemical species, to less than 1% iodine content for chemical and structural comparisons of various native and modified starches. The existing iodine quantification methods are unsuited for such studies given their poor detection limits (typically only 5%) and varying influence from the bound iodine species. Additionally, low-level iodine detection

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should not be influenced by the type of starch tested, that is, it should quantify similar iodine loads regardless of the starch matrix to which the iodine was initially bound.

To enable future research, an improved iodine quantification method has been developed based on the recently reported chemical alkylation and gas chromatographic (GC) procedure for the measurement of various anions reported by D'Ulivo et al.[27] Herein, the authors utilized alkyloxonium tetrafluoroborates to derivatize inorganic anions in an aqueous medium to first create volatile alkylated derivatives of the anions, which are then quantified by headspace GC. D'Ulivo et al. also performed the method specifically for iodine quantification in iodized salt where 1 M triethyloxonium tetrafluoroborate (TEOT) was dissolved in dichloromethane, and a simple solvent extraction of the derivatized ethyl iodide from the organic layer could be performed. The major benefit of this procedure is in allowing for longer storage of the derivatizing agent (since water consumes the derivatizing agent by reacting to ethanol and ethyl ether) and thus greater consistency in sample testing over time. For iodate, these authors first ensured reduction to iodide using sodium borohydride ($NaBH_4$) such that the resultant iodide would be alkylated using TEOT with collection into dichloromethane for subsequent GC analysis. Thus, we have adapted and further developed the procedure by D'Ulivo et al.[27] for the determination of total iodine in both native granular starch and in modified starch. We report a sensitive and reliable chemical procedure for determining total iodine in starch where the total bound iodine content, in the form of polyiodide complexes, is first driven to iodide in solution by reduction with sodium borohydride, alkylated with TEOT, separated and collected from the aqueous milieu into a recoverable dichloromethane collector phase and then quantified as ethyl iodide by sensitive GC–MS and GC–FID instruments.

2. Materials and methods

2.1. Materials

Melojel (Common Corn Starch; CS), Amioca (Waxy Corn Starch; WCS) and Hylon VII (High Amylose Corn; HAC), Perfectamyl D6 (Potato Starch; PS), Eliane 100 (Waxy Potato Starch; WPS), Tapioca Starch (TS), starch samples were all obtained from National Starch and Chemical Company (Bridgewater, NJ, USA). Midsol 50 wheat starch was obtained from MGP Ingredients (Atchinson, KS, USA). Triethyloxonium tetrafluoroborate was purchased from VWR Scientific Co. (Mississauga, ON, Canada), Dichloromethane, 0.1 M iodine–potassium iodide solution (I_2:KI 1.3%:2.6%), sodium borohydride and sodium hydroxide were purchased from Fisher Scientific Co. (Ottawa, Canada). Iodine and potassium iodide crystals (99.99% purity) were purchased from Sigma–Aldrich Chemical Co., (Oakville, ON, Canada).

2.2. Preparation of different I_2:KI solutions

In addition to the 0.1 M standard solution of iodine–potassium iodide (1.3%:2.6% w/v) as purchased from Fisher, three different I_2:KI solutions were prepared, each with a total of 5% (w/v) total iodine The three solutions were a 1:9 (10%:90%), 1:4 (20%:80%), and 1:2 (33%:67%) I_2:KI solutions. Additionally, for testing upper limits of iodine signal linearity, a 2% I_2 (w/v) and 20% KI (w/v) solution was also prepared.

2.3. Preparation of standards

Standards spanning five different amounts of iodine were prepared by pipetting varying amounts of the I_2:KI solutions into 30 mg of starch with addition of Milli-Q water to bring the total iodine solution added to the starch to 400 μL. Control iodine standard samples where no starch was added were also carried through all sample preparation steps. Table 1 shows the various amounts of both the commercial and laboratory-prepared iodine solutions used to prepare the standard curves with the total amounts of iodine (g) and the hypothetical percentage of iodine in the starch if 30 mg of starch is used for the sample preparation. The aqueous samples (400 μL) containing iodine in the form of an I_2:KI mixture, with or without added starch, (typically a 30-mg starch sample was used), were placed into 2-mL polypropylene Eppendorf microcentrifuge tubes. For samples containing both iodine and starch, binding of the iodine to starch was first ensured by vortexing for 10 s and incubating for a minimum of 10 min. Rapid binding of iodine to starch was evident by immediate color formation, which was detectable by eye at added iodine concentrations as low as 0.2% iodine in starch. Samples were then treated with a 400-μL addition of 0.5 M $NaBH_4$ in 0.1 M NaOH to bring the total solution volume to 800 μL. The tubes were capped and mixed by a vortex mixer until all starch had returned to its original white appearance (typically 5 s). The tubes were then uncapped and left to stand open for 15 min in a fume hood to allow excess hydrogen gas to evolve and dissipate. The samples were again capped and let stand for a further 24 h to ensure complete reduction of all iodine species to iodide and to allow the excess hydrogen to dissipate. The sample tubes were then opened briefly to vent any pressure build-up from residual hydrogen gas evolution and then recapped. They were then centrifuged for 10 min at 13,000 rpm to separate the starch from the reaction mixture. A 200-μL subsample of the supernatant obtained from above the centrifuged starch pellet (i.e., if the sample contained starch) was then pipetted into a new 1.5-mL Eppendorf microcentrifuge tube. This subsample was then alkylated with 150 μL of 1 M TEOT in dichloromethane containing 2 μL/mL toluene added as the GC internal reference standard (IS). After a 10-min purge time to allow for the initial derivatization reaction to occur and any gasses to evolve, the sample tubes were capped for a total of 4 h for the derivatization to take place. Finally, a 70 μL subsample of the underlying dichloromethane phase was withdrawn and placed into a 200-μL glass microvial insert and placed into the 8-mm neck of a standard GC vial, ready for injection into the GC instrument. Standards used to quantify iodine in starch were always prepared with added starch (typically 30 mg) as opposed to iodine standards that were prepared without starch in order to achieve most accurate standard curve slope (see Section 3.3 below).

2.4. Gas chromatographic analysis

The analyses were performed using a Thermo Trace Ultra GC (Thermo Scientific, Italy). An AS-1 autosampler injected 1 μL of sample into the GC inlet set at 230 °C with a 1:10 split ratio. Carrier gas

Table 1
Standard curve for the preparation of commercial and laboratory-prepared iodine–potassium iodide solutions

	I_2:KI solution amount (μL)	Total iodine (g)	% Iodine in starch[a]
1.3% I_2:2.6% KI commercial solution	2	0.00007	0.2
	10	0.00033	1.1
	50	0.00165	5.5
	100	0.00330	11.0
	150	0.00495	16.5
2% I_2:20% KI laboratory prepared	10	0.00173	5.8
	25	0.00432	14.4
	50	0.00864	28.8
	75	0.01297	43.2
	100	0.01729	57.6

[a] Percentage of iodine in starch (w/w) for 30-mg starch samples.

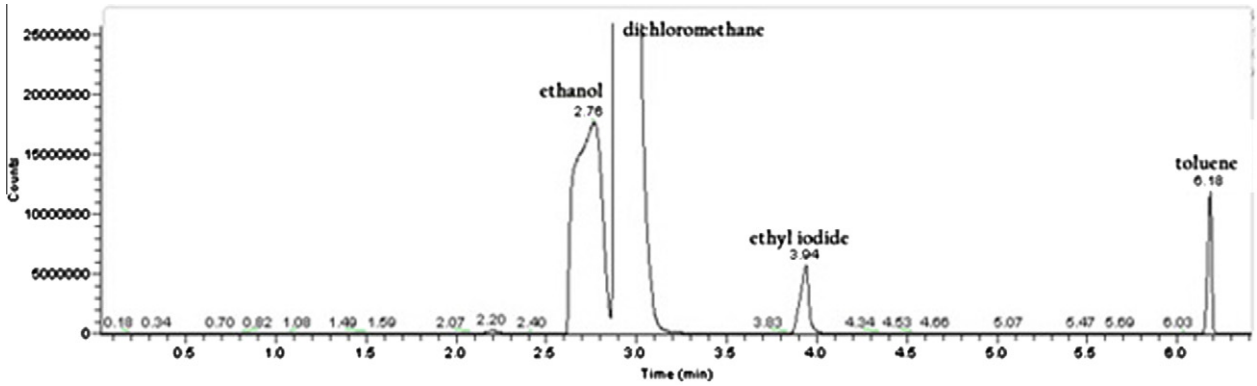

Figure 1. Example GC–FID chromatogram of ethyl iodide analysis with the ethyl iodide peak at 3.94 min and the toluene IS peak at 6. 18 min.

(helium) was set at a constant flow of 1.0 mL/min. A Trace Gold TG-5 column (30 m, 0.25 mm ID, 0.25 μm film thickness, Thermo Scientific, Ottawa, ON, Canada) was employed for the separation of analytes. The temperature program was set at 35 °C for 4 min, ramping to 215 °C over 6 min at 30 °C/min for a total run time of 10 min. Initially, the column was routed to a Thermo mass spectrometer detector to validate peak identities. It was set with an electron-impact energy of 70 eV, with the ionization temperature and the auxiliary MS transfer line temperature set at 250 °C and with vacuum compensation set 'On' for column flow. Peak matches and identities for ethyl iodide (analyte), toluene (IS), and dichloromethane were confirmed by comparison to the mass spectral library database (NIST, 2005). In addition, identities were further verified using Kovats Retention Index numbers obtained from the NIST website (http://webbook.nist.gov/chemistry) for similar column stationary phases (i.e., DB-5/HP-5). Retention times for ethyl iodide and toluene were 3.9 and 6.2 min, respectively. For routine analysis and quantification, the TG-5 column was routed to a flame ionization detector (Thermo Scientific), and the FID was set at 250 °C with hydrogen flow set at 35 mL/min with compressed air flow at 350 mL/min with no makeup gas. Quantification was performed by normalizing the ethyl iodide peak area against the toluene IS peak area and plotting this signal response ratio against known amounts of iodine added.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 16.0 with one-way ANOVA at an alpha level of 0.05 and Duncan's Multiple Range Test for multiple difference comparison. All standard curves were conducted in duplicate at a minimum.

3. Results

3.1. Iodine detection by reduction/alkylation/GC

Ethyl iodide was positively identified by comparing the mass spectrum obtained with that for ethyl iodide from the National Institute of Standards and Technology (NIST) Mass Spectral Library 2005 with a greater than 90% probability match based on the mass spectrum of ethyl iodide with characteristic peaks *m/z* 127 and at 156. Once it was confirmed that ethyl iodide was the species detected, methods development continued utilizing GC–FID, which is a simpler operation, less expensive, and more accessible to researchers. Figure 1 provides an example of a GC–FID chromatogram of an ethyl iodide analysis. The first two large peaks at 2.76 and 2.94 min are ethanol/diethyl ether and dichloromethane, respectively. The largest peak, dichloromethane, is the solvent peak, while the ethanol/diethyl ether peak is a result of the reaction of TEOT with water. Thus, a required excess of TEOT can be ensured through ethanol detection of this side reaction. Ethyl iodide and toluene were observed at 3.94 and 6.18 min, respectively. Toluene was selected as an internal standard since it has a relatively similar boiling point to that of ethyl iodide and thus has a similar retention time for analysis. Toluene is also chemically stable allowing it to be added directly to the 1 M TEOT in dichloromethane for better analysis reproducibility. Figure 2 shows the alkylation reaction scheme of the iodide anion with the triethyloxonium ion to form ethyl iodide with diethyl ether as a by-product. Figure 3 shows the relation between the peak retention times of ethanol/diethyl ether (by-products produced from TEOT reaction), dichloromethane, ethyl iodide, and toluene when plotted against their approximate GC retention indices for similar columns, such as DB-5 and HP-5 (95% polydimethylsiloxane (PDMS), 5% phenol) provided from the NIST website, offering another method for peak verification.

3.2. Method optimization for quantifying iodine associated with starch

When adapting the method of D'Ulivo et al.[27] for quantifying iodine in starch, several important modifications were required to ensure reliable, accurate results within a starch matrix. First, the sodium borohydride concentration was adjusted to 0.5 M $NaBH_4$ to ensure complete reduction of all iodine species to iodide,

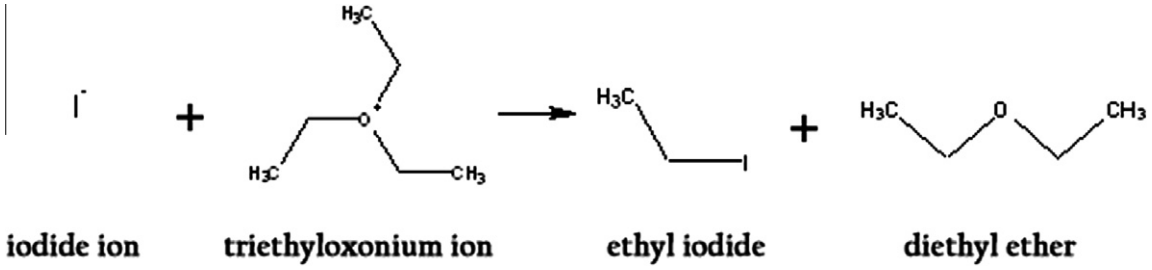

Figure 2. Reaction mechanism of the alkylation of iodide to ethyl iodide.

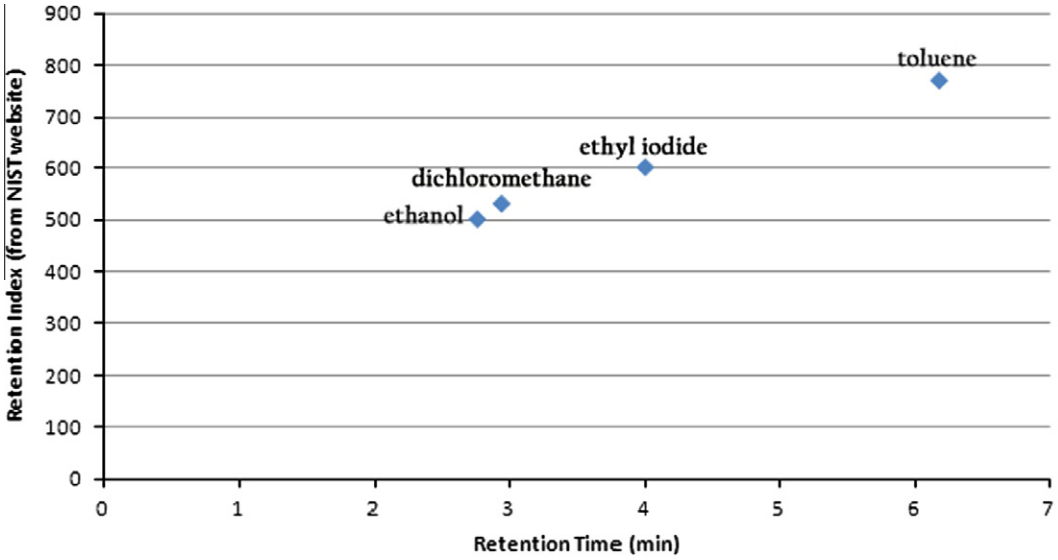

Figure 3. Retention time versus retention index of ethanol + diethyl ether, dichloromethane, ethyl iodide and toluene.

especially since the expected iodine quantities in starch would be much higher than that used by D'Ulivo et al. in the analysis of iodine in iodized salt.[27] Additionally, the sodium hydroxide solvent for the sodium borohydride was lowered from 1 M to 0.1 M, that is, sufficiently alkaline to retain $NaBH_4$ activity, but not too alkaline so as not to cause gel formation of starch in the samples. Gel formation of starch was found to interfere with the derivatization reaction and the extraction of ethyl iodide. The amount of added $NaBH_4$ was increased to 400 μL, and the amount of 1 M TEOT in dichloromethane was increased to 150 μL to ensure excess reducing and derivatizing agents, respectively, since the expected iodine concentrations in the starch samples were much higher.

3.3. Ethyl iodide standard curves

The main objective in this phase is to determine if the total amount of added iodine can be recovered and quantified using the adopted procedure. This was first accomplished by developing standard curves using only iodine–potassium iodide solutions without starch in the matrix. Figure 4 shows the slope of the equation for the standard curve developed using only two different iodine–potassium iodide solutions; one with a 1:2 ratio of I_2:KI (1.3% I_2:2.6% KI) and one with a 1:10 ratio of I_2:KI (2% I_2:20% KI). Even with two different solutions, the signal response is very linear with a 0.9941 regression coefficient. To validate the adapted method to quantify iodine in the presence of starch, standard curves of I_2:KI solutions without starch, as well as with different types of starch in the matrix, were used to determine if differences existed in the response. Initially, samples were prepared without a centrifugation step to remove the starch after reduction, by adding the 1 M TEOT in dichloromethane directly to the aqueous starch suspension. The starch would partition in the top water phase, allowing sampling of the bottom dichloromethane layer that contained the newly-derivatized ethyl iodide; however, results for the various starches showed dramatically different standard curve slopes and with greater standard deviations. Table 2 shows the slopes of the equations for the various standard curves developed using different starches in the sample matrix, both with and without adding the centrifugation step after all colored iodine species had been reduced with sodium borohydride. The standard curve slope was

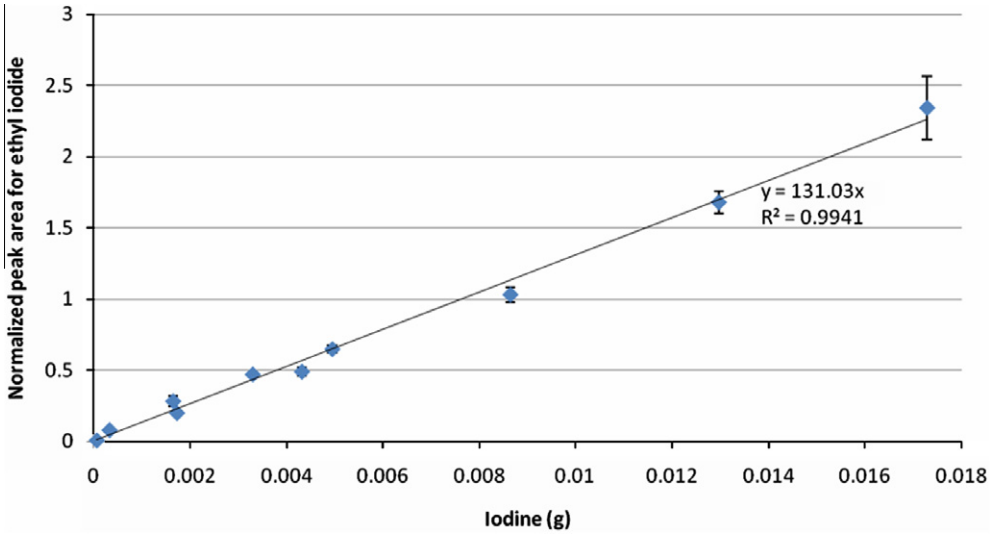

Figure 4. Range and linearity of iodine detection/quantification (response curve for standards).

Table 2
Response curve slopes for iodine standards spanning a range of 0.2% (w/w) to 16.5% (w/w) iodine in starch as determined for seven different starch matrices with and without a post-reduction centrifugation starch-removal step in the sample preparation

	Removal of starch (centrifugation)			No starch removal		
	Average slope	Std dev	Significance	Average slope	Std dev	Significance
Corn	102.4	8.0	a,b*	119.3	19.7	a,b,c
High amylose corn	102.9	10.8	a	131.8	9.2	b,c
Waxy corn	103.1	4.7	a,b	126.1	0.7	a,b
Potato	104.9	2.8	a,b	143.0	4.5	c
Waxy potato	104.2	0.3	a,b	147.6	18.4	c
Tapioca	102.9	8.7	a,b	144.7	31.8	c
Wheat	101.2	8.2	a	138.1	27.2	c
Overall average	**103.1**	**5.5**		**136.2**	**18.8**	

* Slope values with the same letter are not significantly different ($p = 0.05$) for that type of starch whether centrifuged or not.

generated by graphing the peak area of ethyl iodide (normalized against toluene IS) on the *y*-axis and the weight (g) of total iodine (I_2 + iodine from KI) on the *x*-axis, such that a decrease in the slope means a loss of signal sensitivity. Since the same known amounts of iodine were added in with each different type of starch, the slopes should all be identical to one another if there are no interaction effects. However, there is much greater variation seen in the slopes of the standard curves when the starch is left in. This is possibly due to the different types of starch α-helices binding different amounts of dichloromethane and thereby concentrating the ethyl iodide in the remaining dichloromethane layer. It is clear that with the centrifugation step employed, the slopes for the various starches are more consistent with each other and with overall lower standard deviations. When averaging all standard curve slopes for the different types of starches without the centrifugation step employed, the mean slope was 136.2 ± 18.8, while the mean slope when the centrifugation was employed was 103.1 ± 5.5. This constitutes a drop in signal sensitivity of approximately 30%; however, the standard deviation is greatly reduced, indicating better slope reproducibility. Based on one-way ANOVA, there was no significant difference seen in the standard curve slope among any of the starch types (corn, high amylose corn, waxy corn, potato, waxy potato, tapioca and wheat) when the centrifugation step was employed, indicating that any starch may be used to generate a standard curve to allow reliable quantification of unknown starch samples with bound iodine. Table 2 shows the average standard curve slopes, including standard deviations for all different starch types without and with the centrifugation step employed with the significance among starch samples denoted by letters. There was a significant difference observed between all centrifuged and all non-centrifuged sample slopes at an alpha level of 0.05; however, individual starch samples showed different significance in slopes when compared using Duncan's test, whether or not the centrifugation step was employed (see Table 2). It was found in all cases that the regression coefficients for each standard curve were high (R^2 >0.96) with the average R^2 of 0.9927 ± 0.0099, indicating that the slope responses were linear. With pure I_2:KI solutions (without added starch) the standard curve slope averaged 125.5 ± 16.3, which was slightly higher than the slopes for the centrifuged samples, indicating a small loss of sensitivity for samples with starch added.

3.4. Iodide to ethyl iodide derivatization reaction kinetics

Derivatization reaction kinetics for iodide in the 1 M TEOT in dichloromethane were investigated given the higher expected initial iodine concentrations and the reagent modifications that were made in comparison to the previous method of D'Ulivo et al. for iodized salt.[27] These tests were performed using wheat starch loaded with an intermediate iodine concentration of 11.0% (w/w) total iodine employing the 0.1 M I_2:KI (1.3%:2.6%). Samples were obtained at various derivatization times from 0 to 48 h (Fig. 5). These results showed that within 2–4 h the derivatization reaction had gone to completion and thus, a 4-h derivatization time was adopted.

3.5. Effect of speciation of initial iodine on detection

Another important aspect explored was the influence of the initial iodine species bound to starch since previous results have shown that the starting ratios of I_2 to KI can affect the bound polyiodide species; for example, I_3^- or I_5^- is observed from different UV–vis and Raman spectroscopic absorbance techniques.[8,11,15] Figure 6 demonstrates that the reduction and derivatization procedures employed provided similar detection for total iodine when comparing three samples with different I_2:KI species ratios.

3.6. Effect of amount of starch in matrix

Most of the method development utilized a 30-mg starch sample, but the influence of higher or lower starch loads was investigated to establish potential influence and useful limits for analyses of varying starch quantities. Recoveries of standard iodine quantities added to either corn, waxy corn, wheat and using 20, 30 or 40 mg of the starches were compared. There were no significant differences observed in the standard curve slopes for these samples (data not shown). Thus, the method can be extended to larger sample amounts if higher sensitivity is desired or to smaller sample amounts if samples are limited.

4. Conclusion

A simple, rapid and sensitive method for quantifying iodine in starch–iodine medium has been developed. Compared to previously employed methods for quantifying iodine such as titration, this new method will allow quantification of lower than 5% iodine (w/w) in starch with a linear range of quantification from 0.2% up to the maximum binding capacity of pure amylose (~35%). Furthermore, the method is indiscriminate as to the form of iodine species as it quantifies bound iodine as iodide pulled into solution and then derivatized to ethyl iodide. Finally, the method will allow rapid sample throughput of many different starch samples with the potential to analyze over 50 samples per day. This will allow for many applications for quickly screening maximum iodine-binding capacities in different intact starches or modified starches of different botanical origins and correlating these to spectrophotometric absorbance data. An added feature is in measuring the iodine binding based on exposure to iodine vapor at different equilibrated water activities, which have been shown to increase with increasing water activities due to increased molecular mobility.[12,25,26] In addition, the method will allow comparison of

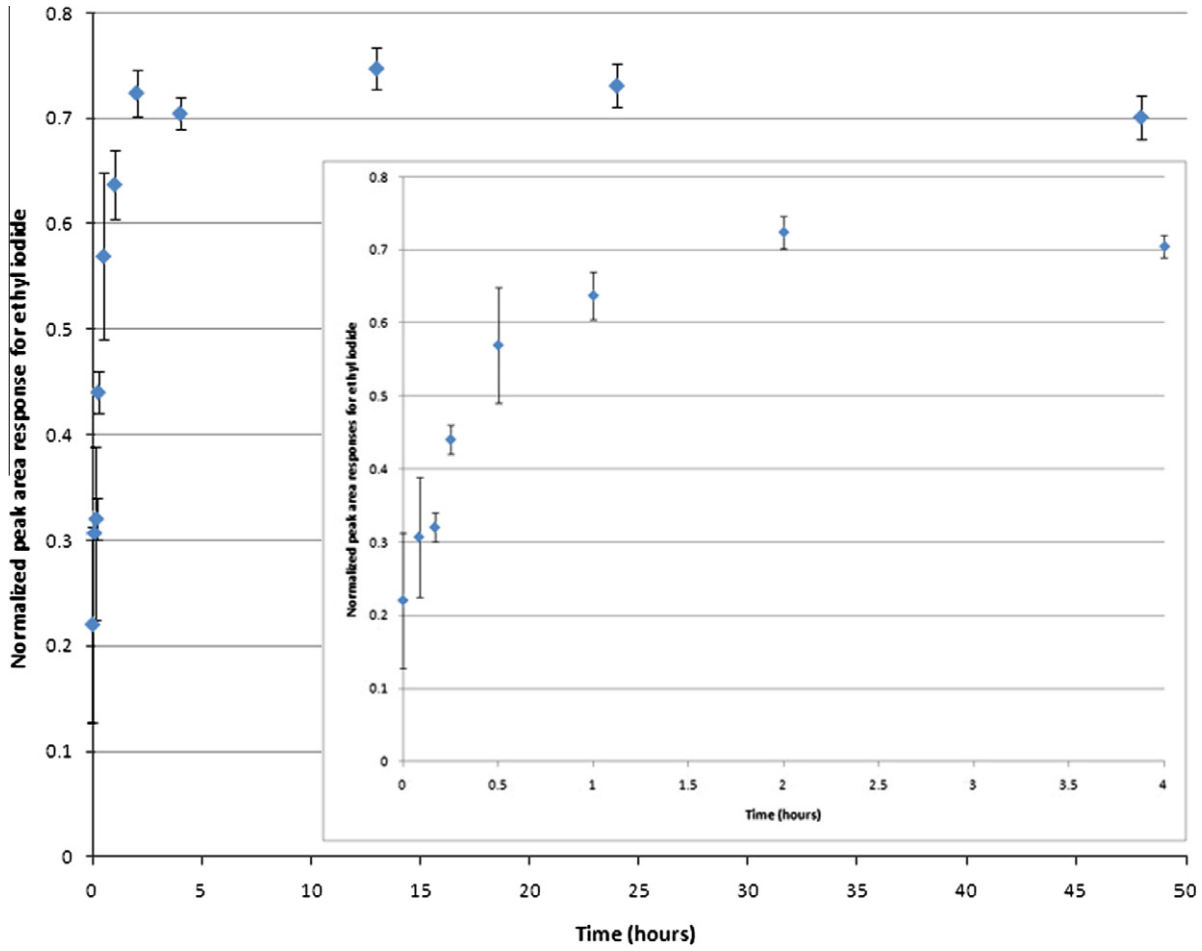

Figure 5. Kinetics of the alkylation of iodide to ethyl iodide. Inset includes kinetics of the derivatization for the first 4 h.

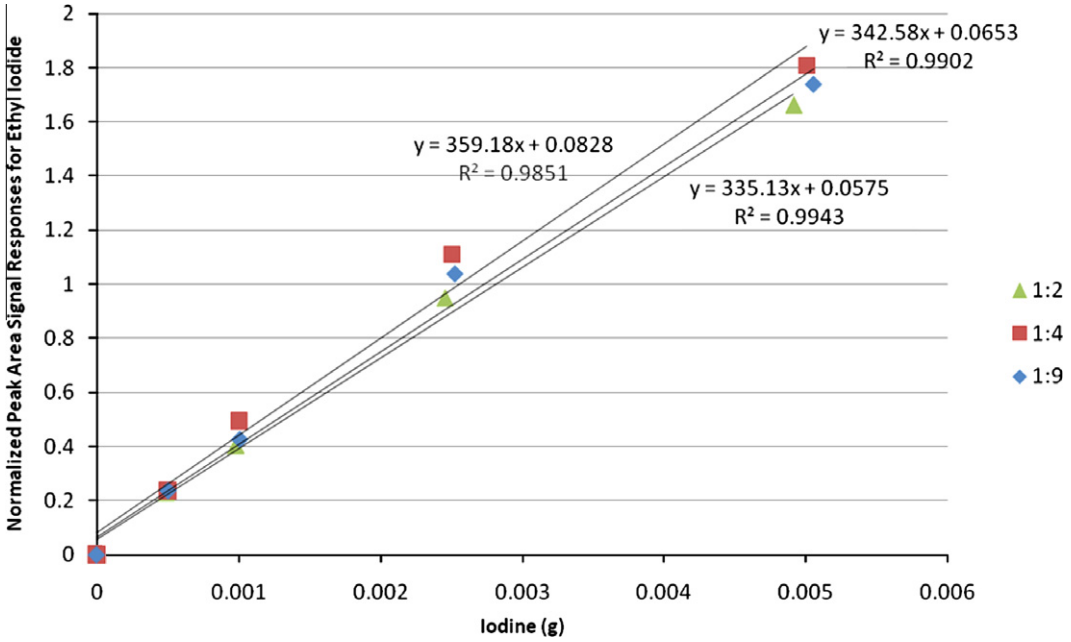

Figure 6. Effect of different ratios of initial iodine to iodide species on their detection as ethyl iodide.

previous researchers' results performed by titrations with the potential for new conclusions to be drawn about starch granular architecture. Furthermore, the kinetics of the binding of iodine from iodine vapor can be explored, possibly offering an indication

of how quickly accessible the α-helices of glucose chains are to include polyiodide complexes via transfer by air, possibly offering information on the relative spacing between chains, their inherent crystallinity, as well as the overall degree of branching.

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