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Analysis of Resistant Starches in Rat Cecal Contents Using Fourier Transform Infrared Photoacoustic Spectroscopy

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Abstract

Fourier transform infrared photoacoustic spectroscopy (FTIR-PAS) qualitatively and quantitatively measured resistant starch (RS) in rat cecal contents. Fisher 344 rats were fed diets of 55% (w/w, dry basis) starch for 8 weeks. Cecal contents were collected from sacrificed rats. A corn starch control was compared against three RS diets. The RS diets were high-amylose corn starch (HA7), HA7 chemically modified with octenyl succinic anhydride, and stearic-acid-complexed HA7 starch. To calibrate the FTIR-PAS analysis, samples from each diet were analyzed using an enzymatic assay. A partial least-squares cross-validation plot generated from the enzymatic assay and FTIR-PAS spectral results for starch fit the ideal curve with a $R2$ of 0.997. A principal component analysis plot of components 1 and 2 showed that spectra from diets clustered significantly from each other. This study clearly showed that FTIR-PAS can accurately quantify starch content and identify the form of starch in complex matrices.

Keywords

Biochemistry Biophysics and Molecular Biology, Chemistry, Food Science and Human Nutrition, Mechanical Engineering, Resistant starch, Fecal analysis, Fourier transform infrared photoacoustic spectroscopy, Partial least squares, Principal component analysis

Disciplines

Acoustics, Dynamics, and Controls | Food Chemistry | Organic Chemistry

Comments

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Analysis of Resistant Starches in Rat Cecal Contents Using Fourier Transform Infrared Photoacoustic Spectroscopy

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ABSTRACT: Fourier transform infrared photoacoustic spectroscopy (FTIR-PAS) qualitatively and quantitatively measured resistant starch (RS) in rat cecal contents. Fisher 344 rats were fed diets of 55% (w/w, dry basis) starch for 8 weeks. Cecal contents were collected from sacrificed rats. A corn starch control was compared against three RS diets. The RS diets were high-amylose corn starch (HA7), HA7 chemically modified with octenyl succinic anhydride, and stearic-acid-complexed HA7 starch. To calibrate the FTIR-PAS analysis, samples from each diet were analyzed using an enzymatic assay. A partial least-squares cross-validation plot generated from the enzymatic assay and FTIR-PAS spectral results for starch fit the ideal curve with a R^2 of 0.997. A principal component analysis plot of components 1 and 2 showed that spectra from diets clustered significantly from each other. This study clearly showed that FTIR-PAS can accurately quantify starch content and identify the form of starch in complex matrices.

KEYWORDS: *resistant starch, fecal analysis, Fourier transform infrared photoacoustic spectroscopy, partial least squares, principal component analysis*

INTRODUCTION

Foodstuffs contain a varied mixture of complex compounds and materials. One of these compounds, starch, has been characterized and studied for decades. Starch is commonly found in many foods as starch granules, which are a combination of amylose and amylopectin. The ratio of these two compounds varies with source material, but the amount of amylose in the normal starch granule is typically 15−30%, while amylopectin can reach 70 or 100% for waxy starch.[1−3] Amylose can form single helical complexes with many chemicals, such as free fatty acids and iodine, and it can also form double helices.[3] Recently, resistant starches containing high concentrations of amylose (up to 85%, high-amylose corn starch) and those with chemical modifications have increasingly been investigated.[3] These starches have been dubbed resistant starch (RS), because of the fact that they resist degradation and absorption in the small intestine.[4]

The decreased digestibility of RS has garnered attention from researchers who study diabetes.[5] With easily digestible starch, diabetics have difficulty controlling their blood glucose levels, but RS may impart many beneficial effects for diabetics through reduction in blood glucose spikes.[5−8] Another benefit of RS is the potential to control energy intake. Many researchers are attempting to find foods that digest slowly and decrease energy intake, which could help with weight maintenance.[9] RS can also play a role as a prebiotic. Prebiotics encompass many of the dietary fibers, including RS, which are not readily digestible by humans. The undigested RS can be used by microbes within the gut and may release beneficial compounds for the host organism.[10−13]

There are five varieties of RS. Type 1 RS can be found in coarsely ground legumes or whole grain. The cell wall surrounding the type 1 RS makes the starch physically inaccessible to digestion. Type 2 RS is individual C- or B-type crystalline starch granules. Type 2 RS typically is raw banana and potato starch and high-amylose corn starch that retains the crystalline structure. Type 3 RS refers to retrograded amylose.[14] Type 4 RS is chemically modified starch.[10,15] The latest RS is type 5 RS, which is an amylose−lipid complex.[16]

Animal studies are often performed to evaluate RS digestibility from analysis of fecal samples, which are complex materials containing protein, carbohydrate, and lipid. Common quantitative methods for analyzing starch content are starch-hydrolysis enzyme assays. The enzyme assays are useful for starch quantification but have many negative aspects for fecal studies. The enzyme assays cost approximately $3 per sample, take about 20 min per sample, and consume at least 0.2 g of dry sample for analyses in duplicate. Studies with mice or rats tend to produce large numbers of small samples, which may become time-consuming and costly when hundreds of samples need to be analyzed.

Our alternative proposed method of analysis is Fourier transform infrared photoacoustic spectroscopy (FTIR-PAS). Conventional FTIR relies on transmission of IR light through the sample to measure the absorption bands of the compounds of interest. Conventional FTIR does not work well with many food products because of their opaque nature, light scattering properties, and difficulties with sample preparation.[17−19] Alternatively, FTIR-PAS directly measures the IR absorbance spectrum of opaque samples, needs minimal sample preparation, and is fast and nondestructive.[20] FTIR-PAS uses a PAS

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accessory, which has a sample cell with a window to allow a modulated IR beam from the spectrometer to enter and illuminate the sample.[21] The IR light absorbed by the sample heats it. The heat migrates to the gas/sample interface and produces a pressure wave in proportion to the absorbance by the sample. The resultant pressure signal is then picked up by a sensitive microphone, and the signal is converted into a wavenumber versus absorbance intensity spectrum.[21] For further information pertaining to FTIR-PAS theory or explanation of various experimental methods, please see refs 22 and 23.

A handful of studies have successfully analyzed starch and other food-based components using FTIR-PAS. One of the first food analyses used IR-PAS with a near-infrared monochromator to determine the moisture content of protein powders.[24] Later researchers applied FTIR-PAS to analyze protein and carbohydrate but lacked statistical power to quantify the data.[25] It was not until the mid-1990s that FTIR techniques with food materials began to couple spectral results with statistical techniques, such as partial least squares (PLS).[26] PLS uses a small training set of samples analyzed via a non-FTIR standard method to calibrate the FTIR analysis. A multivariate model of the spectral data with the quantitative values can be produced to create a calibration to predict the composition of unknown samples from their spectra. This approach has been confirmed for determining lipid, protein, and carbohydrate concentrations in pea seeds.[27]

The present study went beyond food and single-identity starch analysis by quantifying modified starch in rat cecal contents. Three types of RS were studied along with a control corn starch. The first RS studied was high-amylose corn starch (HA7), a type 2 RS. The second RS was octenyl succinic high-amylose corn starch (OS-HA7), which is a type 4 RS. OS-HA7 is obtained from modifying starch with octenyl succinic anhydride, which forms ester bonds with hydroxyl groups of starch molecules. The third RS was high-amylose corn starch complexed with stearic acid (RS5-HA7), a type 5 RS. RS5-HA7 is based on a physical complex between amylose and stearic acid rather than chemical bonds.

The main goal of this study was to determine if FTIR-PAS and PLS were a practical alternative to the enzymatic assay for starch content. To achieve this, we needed to determine whether FTIR-PAS analysis could produce a linear correlation while accounting for potential interferences from the complex sample matrix and RS modification. Beyond a quantitative fit of the starch, principal component analysis (PCA) was tested to determine if the different diets could be differentiated qualitatively.

MATERIALS AND METHODS

Rat Animal Study. Fischer 344 rats were housed following the procedure by Zhao et al.[28] The animals were on the feeding regimen for 8 weeks before the animals were sacrificed. The trial contained 90 rats total (2 rats died before sacrifice), which were randomly assigned to four diet groups. The four diets consisted of the control (corn starch), HA7, OS-HA7, and RS5-HA7 diets described below. For purposes important to other companion studies based on this same diet trial, the control and RS5-HA7 diet groups each contained 29 rats and were broken down further into four subgroups per diet. The rats were given two injections of either saline or the carcinogen azoxymethane (AOM, Midwest Research Institute, Kansas City, MO), administered following the method by Zhao et al.,[28] and some were fed an antibiotic treatment mixture of vancomycin and imipenem. The treatments resulted in the four subgroups within the control and RS5-HA7 diets that consisted of rats given both AOM and antibiotic, AOM and no antibiotic, saline and antibiotic, and only saline. Both HA7 and OS-HA7 diets contained 15 rats per diet group and were divided into only two subgroups. They were given either AOM and no antibiotic or neither. For purposes of the tests reported here, we have grouped samples only according to diet and not according to AOM or antibiotic treatment. The animal studies were performed in compliance with the guidelines of The Institutional Animal Care and Use Committee of Iowa State University.

Starch Diets Fed to Rats. Four starch varieties were used for the feeding study: control (corn starch, Cargill Gel 03420; Cargill, Inc., Minneapolis, MN), HA7 (AmyloGel 03003; Cargill, Inc.), OS-HA7 (processed HA7 bound to octenyl succinate in the Department of Food Science and Human Nutrition, Iowa State University), and RS5-HA7 (processed using HA7 and stearic acid in the Department of Food Science and Human Nutrition, Iowa State University).[16,29] The starches were cooked before being added to the diets following the procedure by Zhao et al.[28] The cooked starch was then added to a diet formulated on the basis of the standard diet recommended by the American Society for Nutritional Sciences for mature rats (AIN-93M).[30] Starch diets were prepared every other day and served fresh to the rats.

Rat Cecal Samples. This study collected only the rat ceca and placed the contents into Corning 15 mL centrifuge tubes (Tewksbury, MA) on dry ice before storage at −80 °C. Because of two other companion studies obtaining samples prior to this experiment, much of the cecal contents from the samples was exhausted. Adequate material from only 28 samples, seven from each of the four feeding groups, could be randomly obtained. The wet cecal samples were placed in aluminum weighing pans and dried in an oven at 105 °C for 3 h. After drying, the cecal material formed dry wafers, which were ground using mortar and pestle. The ground cecal material was then placed in 1.7 mL microcentrifuge tubes purchased from Marsh Bio Products (Rochester, NY) and stored sealed at room temperature prior to analysis.

Enzymatic Assay for Starch Content. The starch content of the cecal materials was measured using Total Starch Assay Kit (Megazyme International Ireland, Ltd., Co., Wicklow, Ireland) following American Association of Cereal Chemists (AACC) Method 76-13.[31]

FTIR-PAS. The FTIR-PAS analysis was performed using a MTEC Photoacoustics PAC300 detector mounted in a Digilab FTS 7000 FTIR spectrometer. The sample detector has a 1 cm interior diameter and a window at the top for the infrared beam to enter the chamber and illuminate the sample. The dried and ground cecal material was placed in a disposable aluminum cup, which was fully illuminated by the infrared beam. Immediately before analysis, the detector was purged with helium gas to remove atmospheric water vapor and carbon dioxide, which have strong mid-IR absorptions. Also, a desiccant, magnesium perchlorate, was added beneath the sample to remove any moisture that might evolve from the sample during analysis. Spectra were taken at 8 cm^{-1} resolution and a 2.5 kHz scan speed, with the co-addition of 256 scans.

PLS and PCA. The spectra were correlated with starch levels determined by the enzymatic assay via PLS using commercial software (Thermo Galactic GRAMS/AI PLSplus IQ, Version 5.1).[32−34] PLS uses a training set of spectra from samples whose relevant properties are known and span the range of interest. In the present case, the enzymatic assay provided the known property values. PLS modeling determines a small set of basis-vector spectra, called factors, by which it can describe all of the training set spectra. Each training set spectrum is then just a weighted sum of the factors. The factors with the smallest weightings consist mostly of noise and are dropped from the model. PLS then performs a multiple linear regression correlating the factor weightings with the known values of the property being predicted. Once the PLS model is built, the correlated property can be determined for unknown samples directly from the model, as long as the properties of the unknowns fall within the range of those covered by the original training set.

Because the starch level was determined for only 28 samples (seven per diet), the sample set was not split into separate training and

validation sets. Instead, all of the samples were used in creating the PLS model, and a single-elimination cross-validation was used to measure model quality. In such a cross-validation, one member of the training set is removed and a model is built from the remaining members. The removed spectrum is then analyzed as an unknown. The removed spectrum is returned to the training set, then a different one is removed, and the process is repeated. This is performed until all training set members have been removed and analyzed as unknowns. Plots comparing the known values and the predicted values from the cross-validations are included in the Results and Discussion. The standard error of cross-validation (SECV) is a measure of model quality. It is the root-mean-square difference between the values of the predicted property determined during the cross-validation and their known values.

The model with the lowest prediction residual error sum of squares (PRESS) value was selected as the most accurate model. PRESS is given by

$$\mathrm{PRESS} = \sum_{i=1}^{N} (k_i - p_i)^2$$

where k_i and p_i are the known and predicted values for the ith sample, respectively, and there are N samples in the training set. In that most accurate model, the 4000−397 cm^{-1} range of the spectra was used and the spectra were preprocessed using multiplicative scatter correction (MSC)[35] and by conversion to first derivatives (19-point Savitsky−Golay). The resulting model had 10 factors.

Classification of the spectra according to diet was performed using PCA.[36,37] The same 4000−397 cm^{-1} range and the same first derivative and MSC preprocessing were applied to the data as in the PLS modeling. This was sufficient to cleanly separate the samples into clusters according to diet.

RESULTS AND DISCUSSION

Enzymatic Assay for Starch Content. Starch contents of the cecal material from the rats fed different diets are shown in Table 1. The cecal content from the rats fed the OS-HA7 diet had the highest starch content, ranging from 47 to 50.1%, whereas that from the rats fed the control diet with normal corn starch had the lowest starch content, ranging from 0.3 to 1.1%. There was no significant difference among the food disappearance (used to estimate intake but includes loses) of the rats fed the different diets (data not shown). These results suggest that OS-HA7 has the highest resistance to *in vivo* digestion, followed by RS5-HA7, HA7, and normal corn starch.

FTIR-PAS. The FTIR-PAS data were measured from 4000 to 397 cm^{-1}. Spectra from all four diets are shown in Figure 1. All samples show many bands in common, but in the fingerprint region (1800−397 cm^{-1}), there are visible differences among the cecal samples from different diets. A study by Irudayaraj and Yang using FTIR-PAS identified bands in pure starch and protein spectra.[38] However, because of the complexity of the cecal samples, the present spectra have substantial peak overlap; therefore, manual interpretation is not sufficient. The use of chemometric software can analyze the data and draw out the quantitative and qualitative data needed.

PLS was successfully used for starch to model the relation between the enzymatic assay results and the FTIR-PAS spectra of the rat cecal contents. Figure 2 shows the cross-validation for the best fitting model. The plot correlates the known starch content (dry basis) with the starch content predicted by the PLS model. The diagonal line is the ideal (i.e., predicted = known). The SECV is 1.055 wt %, and R^2 is 0.997. The SECV is only 2% of the starch content range in the sample set (0.3−50.1 wt %); therefore, the predictions are of good quality. The high quality of the predictions from the training set would allow for unknown samples to be quantitatively analyzed for starch content using the chemometric model developed. Also, because the model was able to accurately fit every modified starch, the model should be useful for any of the four starch diets used.

Besides the quantitative starch information, qualitative information to identify which starch was measured is very

Table 1. Summary of Enzymatic Assay Analysis of *in Vivo* Starch (Dry Basis) in Cecal Contents by Rat

diet	rat number	average starch (wt %)	standard deviation
control	87	0.3	0.2
	23	0.6	0.1
	27	0.8	0.0
	49	0.7	0.0
	84	0.8	0.0
	81	1.1	0.3
	70	1.0	0.1
	average	0.7	
HA7	33	12.7	0.4
	40	19.4	0.5
	15	21.2	0.5
	9	21.3	0.5
	18	18.1	0.1
	22	20.5	0.1
	28	14.8	0.6
	average	18.3	
OS-HA7	14	47.9	0.1
	16	47.9	0.1
	10	49.8	0.3
	44	47.1	0.4
	6	50.1	0.4
	50	50.0	0.1
	52	49.1	0.7
	average	48.8	
RS5-HA7	12	24.0	0.1
	1	19.2	0.3
	20	17.1	0.6
	29	13.3	0.5
	43	28.1	0.2
	57	30.5	0.7
	64	21.5	0.4
	average	21.9	

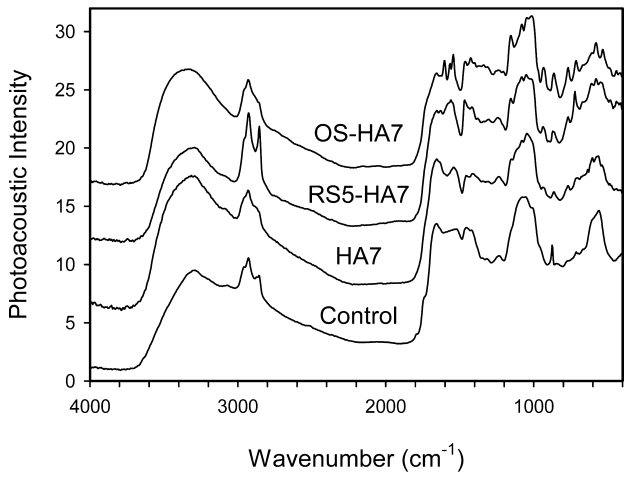

Figure 1. FTIR-PAS spectra collected from rat cecal contents. The spectra are from single representative rats from each of the diet groups. Spectra are scaled and displaced vertically.

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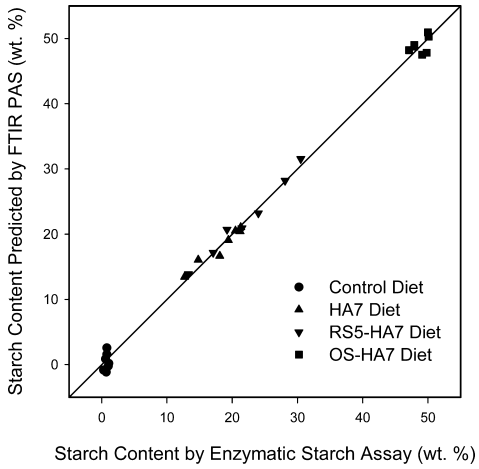

Figure 2. Plot of cross-validation for starch content measured by the enzymatic starch assay from each cecal sample (dry basis) from all four diets versus the predicted values by FTIR-PAS. The R^2 of the data to the ideal best fit line was 0.997.

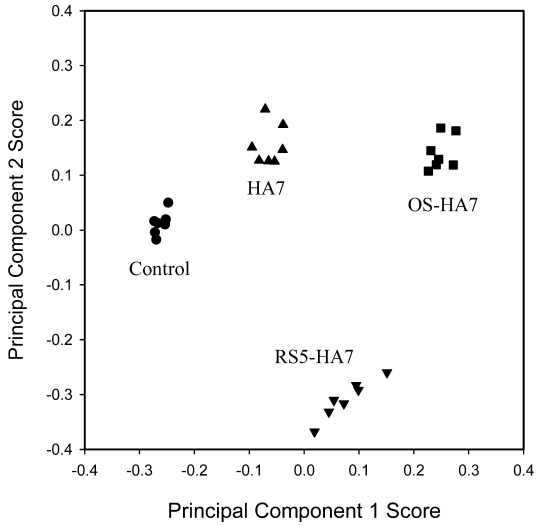

Figure 3. Scores for the first two principal components in the PCA modeling of the spectra of 28 dried cecal samples separate the samples according to the RS diets of the rats.

useful. The spectral data were analyzed by PCA to aid in sample identification. The first two principal components from the PCA of the spectra cleanly separated the samples according to diet, as shown in Figure 3. These two components account for 83.5% of the variance in the data. The PCA analysis gives a simple and clearly visible means to match the cecal samples to the corresponding starch diets.

Despite the similarity of the measured spectra for the different cecal materials, chemometric analysis produced a successful model of the data. The FTIR-PAS data coupled with the enzyme starch assay results clearly were able to produce a cross-validation plot that gave high-quality quantitative results. The first two principal component scores were also able to show clustering that would allow for qualitative identification of starch in future unknown cecal samples. No clustering among the antibiotic or AOM subgroup treatments was observed using the PCA components. This finding should give credence to the robustness of FTIR-PAS to see through minor effects even within complex matrix materials.

This study was proof of concept for FTIR-PAS analysis of starch to replace future high-volume enzymatic assay analysis. Future work will incorporate timed fecal collections and FTIR-PAS starch analysis and metabolic analysis. This analysis would be used to track how the chemistry of the gut microbiome changes as the animal adapts over time to a RS diet.

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Notes

The authors declare the following competing financial interest(s): John McClelland has a financial interest in MTEC Photoacoustics, Inc., the manufacturer of the photoacoustic detector used in this study.

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ABBREVIATIONS USED

RS, resistant starch; FTIR-PAS, Fourier transform infrared photoacoustic spectroscopy; PLS, partial least squares; HA7, high-amylose corn starch; OS-HA7, octenyl succinic high-amylose corn starch; RS5-HA7, high-amylose corn starch complexed with stearic acid; PCA, principal component analysis; PRESS, prediction residual error sum of squares; MSC, multiplicative scatter correction; SECV, standard error of cross-validation

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