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Obtaining Multiple Coproducts from Red Grape Pomace via Anthocyanin Extraction and Biogas Production

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ABSTRACT: Red grape pomace (RGP), a byproduct of red wine production, is an abundant food processing waste stream in California, rich in both anthocyanins, a class of red-blue pigments, and lignocellulose. Extraction of anthocyanins and biofuel production from RGP have been investigated independently, but no research has examined employing both strategies together for maximal valorization. In this study, anthocyanins were most effectively extracted from RGP at 80 °C. Convection- and vacuum-oven drying of the pomace were found to decrease anthocyanin yield, whereas lyophilization did not significantly affect yield. Fermentable sugars were successfully separated from the crude extract via solid-phase extraction. Ionic liquid pretreatment of RGP was determined to be a nonviable option for application to anaerobic digestion. Extraction reduced biomethane output, but supplementation with the aqueous fraction of the extract mitigated much of this difference, indicating sequential extraction and fractionation of anthocyanins from RGP can minimize the impact on biofuel yields.

KEYWORDS: *red grape pomace, anaerobic digestion, anthocyanins, extraction, pretreatment, ionic liquids*

1. INTRODUCTION

California is a major producer of wine. As of 2016, California accounted for 85% of national wine production, was the fourth largest producer of wine worldwide, and crushed more than four million tons of grapes for wine, about 2.3 million of which were red wine grapes.[1] A byproduct of wine production is pomace: the skins, pulp, and seeds that remain after pressing. Roughly 385 000 tons of grape pomace are produced per year in California alone.[2] Thus far, management strategies for this waste stream have been limited: it has been used as fertilizer or animal feed in the past; however, it is suboptimal for crop yields and animal nutrition, and the quantity of pomace produced outweighs the need of these markets.[3] More recently, grape pomace has been investigated for the retrieval of value-added components, such as phenolics, seed oil, and fiber, as well as for biofuel applications.

Red grape pomace (RGP) is of particular interest because of its rich color. This color comes from anthocyanins, a class of red-, purple-, and blue-pigmented flavonoids that have applications as food colorants. Though much of the anthocyanin content of red wine grapes is imparted into the wine itself, a considerable portion, about ten percent, is retained in the pomace.[4,5] This is significant considering that this is such a readily available, low-cost residue that has limited applications. Many studies have effectively extracted residual anthocyanins from RGP.[5−12] In fact, the current Food and Drug Administration (FDA)-approved method for the production of grape skin extract utilizes grape skins from juice and wine production.[13] The international Food and Agriculture Organization of the United Nations (FAO) also defines grape skin extract as derived from grape skins after pressing.[14] Red grape pomace, therefore, has a solid reputation as a reliable source of colorants for use in food, and because of the recent consumer-driven trend in the food industry to shift from artificial colors to natural ones, natural food dyes are expected to increase in demand.[15]

In addition to anthocyanins, RGP also contains a small portion of nonfermented soluble sugars[16,17] and significant lignocellulose content (roughly half on a dry basis).[16−19] Thus, it represents a potential feedstock for second-generation biofuel technologies such as anaerobic digestion. Several studies have found substantial biomethane yield from the anaerobic digestion of RGP,[18−23] at values comparable to many other lignocellulosic agricultural residues.[24]

However, little research has examined the potential to harvest both of these coproducts from RGP. In this study, extraction of anthocyanins from RGP will be examined using faster, higher-temperature extraction parameters than traditionally explored in the literature. Extracted pomace solids will then be tested as a substrate for anaerobic digestion versus raw pomace to determine if the extraction has any effect on the methane potential of the pomace. A similar sequential process of extraction and fermentation has been explored for purple sweet potato[25] and tomato pomace,[26] with promising results.

It is anticipated that any soluble, fermentable sugars present in the pomace will be extracted along with the anthocyanins. This loss of fermentable sugars in the substrate would likely translate to a loss in methane potential. Therefore, the anthocyanin extract will be subjected to a simple fractionation using solid-phase extraction (SPE) to separate the pigments from soluble sugars. Other studies have successfully fractionated anthocyanin extracts using SPE.[27−29] The aqueous, fermentable-sugar-containing fraction can then be funneled

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back to anaerobic digestion with the expectation of boosting methane output.

Methane output of the pomace may also be boosted by pretreatment technologies. One promising emerging technology is pretreatment with ionic liquids (ILs), salts that are liquid at or near room temperature. The high ionic strength of these liquids is able to disrupt hydrogen bonds and effectively dissolve lignocellulose, making it more accessible for degradation. The digestibility and methane output of many lignocellulosic agricultural residues have been boosted dramatically by IL pretreatment.[30−32] As RGP contains significant lignocellulose content, it seems a reasonable candidate for IL pretreatment. Therefore, this study will also examine whether IL pretreatment can improve the enzymatic digestibility and methane potential of RGP.

2. MATERIALS AND METHODS

2.1. Grape Pomace. Red wine grape pomace (*Vitis vinifera* Barbera), consisting of residual skins and seeds from red wine production, was collected from the UC Davis Vineyards winery in August 2016. The moisture content of the fresh pomace was determined by drying in a vacuum oven for 24 h. Fresh pomace was immediately stored in sealed bags in a −20 °C freezer upon collection. Immediately prior to extraction, frozen pomace was homogenized in a Waring laboratory blender (Stamford, CT) for 90 s on the low setting.

2.2. Compositional Analysis. Pomace was analyzed for lipid content via hexane extraction. Raw pomace and methanol-extracted pomace (Section 2.3) were subjected to hexane extraction, using 1.00 g of pomace per 50 mL of hexane (HPLC grade, Sigma-Aldrich, St. Louis, MO). Extraction took place in pressure tubes at 60 °C for 90 min. Extracted solids were filtered and rinsed with hexane using the filtration apparatus described in Section 2.3. Extracts were dried in a centrifugal evaporator and weighed.

Pomace was also analyzed for neutral-detergent-soluble extractives, cellulose, hemicellulose, lignin, and ash contents using a gravimetric method described previously.[33] In brief, 0.5 g of pomace was combined with 100 mL of neutral detergent solution and refluxed to extract soluble compounds. The mixture was filtered and washed through medium porosity crucibles, and solids were dried in a drying oven overnight. Solids were then sequentially processed with acid detergent solution and 72% sulfuric acid solution to determine hemicellulose and cellulose contents, respectively. Solids were then incinerated in a muffle furnace to determine acid-insoluble lignin and ash contents.

2.3. Anthocyanin Extraction. Frozen homogenized pomace was extracted without drying. Approximately 2.253 g of pomace (equivalent of 1.000 g of dry pomace) was combined with a solution of 70% methanol (ACS grade, Fisher Scientific, Hampton, NH) and 30% deionized water in a pressure tube (Ace Glass, Vineland, NJ),[25,34] which was then covered with aluminum foil and placed in a heated oil bath. Extractions were carried out for 60 min[12,35] at various temperatures (20, 50, and 80 °C). For each temperature, extractions were conducted in triplicate. After extraction, samples were cooled to room temperature and filtered using vacuum filtration and filter paper (grade 389, Sartorius, Bohemia, NY).

For subsequent quantification of anthocyanins, the crude extract was syringe filtered and immediately taken through the quantification protocol (Section 2.4). If the extract was not to be quantified, it was dried by centrifugal evaporation (SpeedVac SPD2010, Thermo Scientific, Waltham, MA) at 45 °C under vacuum until dry, approximately 24 h. Dried extracts were weighed, covered with foil, and stored at −20 °C until further analysis. Extracted solids were dried in a vacuum oven at 45 °C and stored in a desiccator until further use.

2.4. Anthocyanin Quantification. Anthocyanins were measured in acidic conditions and quantified on the basis of an analytical standard. Malvidin-3-glucoside (oenin) was chosen as the representative anthocyanin, as it has been shown to be the predominant anthocyanin in red wine grape varieties.[4,7,9,34,36] Oenin chloride standard (Sigma-Aldrich, St. Louis, MO) was suspended in 70% methanol solution and multiple concentrations were prepared via serial dilution. Monomeric anthocyanins were then quantified by adapting the pH differential method,[37] using methanolic instead of aqueous solvents. In addition, as extracts consistently showed no absorbance in the sodium acetate solution of pH 4.5 or at 700 nm, polymerized anthocyanins and haze were determined not to be significant factors impacting the detection of monomeric anthocyanins in filtered extracts; therefore, only the solution corresponding to pH 1.0 was used for calculation of anthocyanin concentrations in this study. A 70% methanol solution containing 0.125 M hydrochloric acid was added to a microplate in a volume of 160 μL per well. Samples were added to the wells in volumes of 40 μL. The total volume in each well was then 200 μL, with a final concentration of HCl being 0.1 M, corresponding to a pH value of approximately 1.0, at which anthocyanins have a maximal red color intensity.[37,38] Microplates were covered with an optically transparent seal (VWR, Radnor, PA) to prevent evaporation and protect plate-reading equipment. The plate was read immediately at 520 nm.[4,5,9,36] Concentrations of monomeric anthocyanins in the extracts were determined using a standard curve. Yield of anthocyanins per unit dry pomace was calculated using the starting dry mass of pomace.

2.5. Comparison of Pomace Drying Methods. To determine whether various methods of drying the grape pomace had a significant effect on the anthocyanin yield, pomace was dried using various methods and analyzed for anthocyanin content in parallel with fresh samples. Pomace was subjected to three methods of drying: a convection oven set at 55 °C for 2.5 h, a vacuum oven set at 45 °C for 5 h, or lyophilization in a centrifugal evaporator (SpeedVac SPD2010, Thermo Scientific, Waltham, MA) for 9 h. The drying times required were determined on the basis of a trial period during which samples were weighed at regular intervals until a constant mass was achieved.

2.6. Anthocyanin Extract Separation. Crude separation of anthocyanin extracts was performed in order to separate polar nutrients such as sugars that could benefit downstream biogas production. A gravity-separation column (Ace Glass, Vineland, NJ) was prepared containing 25.0 g of Amberlite XAD7HP resin (Sigma-Aldrich, St. Louis, MO).[27,28] Frozen dry extracts were resuspended in 2.0 mL of deionized water. The suspended sample was carefully placed atop the column, and polar compounds were eluted with deionized water at a rate of 4 mL/min. Ten vials of 25 mL each were collected, and fractions were analyzed with a reducing sugar assay to determine the amount of water needed to elute all detectable sugars. Aqueous extracts were dried in a centrifugal evaporator and stored at −20 °C until further use. These extracts were later analyzed for reducing sugars using the DNS method described in Section 2.8, and protein content was analyzed using the Bradford method.[39] Biochemical oxygen demands (BODs) of the crude extract and aqueous fraction from the column were also compared (Section 2.9).

Anthocyanins were eluted with a 70% methanol solution containing 0.1 M hydrochloric acid at a rate of 4 mL/min until the liquid ran clear (225 mL). The yield of anthocyanins recovered from the column was determined by analyzing both the pre-column and post-column extracts in parallel immediately following elution.

2.7. Ionic Liquid Pretreatment. Raw or extracted pomace (0.5 g) was added to 9.5 mL of 1-ethyl-3-methylimidazolium acetate ($[C_2mim][OAc]$, Sigma-Aldrich, St. Louis, MO) in a glass test tube. Various pretreatment parameters were tested. Pretreated solids were collected as described previously,[33] using vacuum filtration to wash the solids with water and collect the pretreated solids. Solids were dried under vacuum at 45 °C and stored in a desiccator until further use.

The effects of pretreatment temperature and time on enzymatic digestibility, as well as solid recovery from the pretreatment, were examined using a face-centered, 3 × 3 central composite design (CCD) experiment. The pretreatment temperatures were 100, 130, and 160 °C, and the times were 1, 2, and 3 h. The center point (130 °C, 2 h) was repeated five times to gauge variability. Reducing sugar yield after 24 h and percent solid recovery were used as the response

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variables. Data were fitted to a response surface to test the first- and second-order effects of each variable and any interaction effects between them, as previously described:[33]

$$Y(t, T) = \beta_0 + \beta_t t + \beta_T T + \beta_{tT} tT + \beta_{tt} t^2 + \beta_{TT} T^2 \quad (1)$$

where $Y(t, T)$ is the response in terms of reducing sugar yield; t represents the pretreatment time; T represents the pretreatment temperature; β_0 is a constant that describes the intercept; β_t is the main effect of the pretreatment time on the response; β_T is the main effect of the pretreatment temperature on the response; β_{tT} is the interaction effect between the pretreatment time and temperature on the response; β_{tt} is the second-order effect of the pretreatment time on the response; and β_{TT} is the second-order effect of the pretreatment temperature on the response. These parameters were fitted using the standard least squares model-fitting function in JMP Pro (SAS, ver. 12.0.1).

2.8. Enzymatic Digestion and Reducing Sugar Assay. Extracted and pretreated pomace was tested for digestibility using a cellulase cocktail from *Trichoderma reesei* and analyzed for reducing sugars as described previously.[33] In brief, pomace samples were enzymatically digested at 45 °C, and samples were collected at 0, 1, 2, 3, 5, 7, and 24 h. All time points of all samples were tested for reducing sugar content using a dinitrosalicylic acid (DNS) assay using glucose standards, with the results expressed as equivalent masses of glucose. Results were expressed both as per unit dry pomace and, for the treated samples, as per unit dry untreated (raw) pomace by adjusting the values for the yield from each treatment.

Three enzymatic digestion experiments were carried out. The first tested the effect of different pretreatment parameters on digestibility against untreated pomace. The results of this experiment were analyzed according to the methods described in the Section 2.7. In the second, different extraction conditions were tested to determine which, if any, affected digestibility. The effect of extraction temperature was analyzed using a linear regression in JMP Pro (SAS, ver. 12.0.1). In the third experiment, the effect of extraction and pretreatment combined was investigated. The extraction temperature giving the highest anthocyanin yield, 80 °C, was chosen for pretreatment. Extracted samples were pretreated using the conditions demonstrated to most improve digestibility for raw pomace: 160 °C for 3 h. Four treatments were therefore tested in the third digestion: (1) raw pomace, (2) extracted pomace, (3) pretreated pomace, and (4) pomace that was sequentially extracted and pretreated under the same conditions. Yields from different treatments were compared using one-way ANOVA in JMP Pro (SAS, ver. 12.0.1).

2.9. Biochemical Oxygen Demand. Biochemical oxygen demand (BOD) was determined using a HACH BOD protocol (Hach Company, Loveland, CO). Small quantities of dry (1) raw, (2) extracted, (3) pretreated, and (4) extracted and pretreated pomace were milled to a fine powder in a Waring blender using an attachment intended for small samples (MC1 mini container, Waring, Stamford, CT). Pretreated residues (3) and (4) have been demonstrated to have negligible ash residues.[33] For (1) and (2), volatile solids (VS) were determined by combusting the samples in a muffle furnace to constant weight. BOD values were then reported per gram of VS.

BOD was also determined for the crude anthocyanin extract, as well as the aqueous, sugar-containing fraction of extract following anthocyanin separation on the column. BOD was reported in milligrams of O_2 per 1.00 g of extract equivalent.

2.10. Anaerobic Digestion. Anaerobic digestion was conducted in batches. Pomace (2.50 g) was added to 250 mL bottles with 250 mL of anaerobic digester sludge to minimize headspace. Sludge was obtained from a local, active anaerobic digestion facility (Clean World, Sacramento, CA) that utilizes food waste. Four replicates of each of the following treatments were run in parallel: (1) raw pomace, (2) extracted pomace, (3) extracted pomace supplemented with the aqueous fraction of the extract from the column, and (4) a sludge-only control to correct for background biogas production.

Reactors were purged with nitrogen (Airgas, Lawrenceville, GA) and then incubated at 55 °C for 30 days or until biogas production became negligible. Reactors were connected via airtight tubing connections to a syringe (Monoject, Fisher Scientific, Hampton, NH) for biogas collection. One-way stopcocks (Qosina, Ronkonkoma, NY) were strategically placed to allow for syringe replacement without oxygen contamination. Syringes were removed at regular intervals as needed on the basis of syringe capacity, or roughly as follows: twice daily for the first 12 days, once daily for the next 8 days, and once every 2 days for the remaining 10 days. Methane and CO_2 in the collected syringes were analyzed using Fourier-transform infrared spectroscopy (FT-IR, IRTracer-100, Shimadzu, Columbia, MD). A 23-gauge beveled needle (Fisher Scientific, Hampton, NH) was connected to the syringe; then, a set volume of biogas was injected into the IR gas cell equipped with sodium chloride windows (Pike Technologies, Madison, WI).

A gas mixture containing known quantities of methane and CO_2 (Airgas, Lawrenceville, GA) was utilized as a standard. Gas concentrations were determined in sample spectra utilizing a calibration curve generated from the standard spectra. Methane and CO_2 accumulation values were adjusted and reported per gram of VS. Biogas quality was determined as the percent of methane out of the total amount of methane and CO_2 produced. Different treatments were compared using both one-way ANOVA and two-way ANOVA with Tukey's post hoc analysis in JMP Pro at $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1. Compositional Analysis. The moisture content of the pomace was determined to be 55.61% (wet basis). The hexane-soluble lipid content of raw pomace was found to be 9.281 ± 0.72%, which is comparable to literature values.[8,40] The lipid content of the methanol-extracted pomace was determined to be 10.25 ± 0.25% and to be 8.590% when adjusted for solid recovery from the extraction. This difference was deemed statistically nonsignificant ($P = 0.185$). Therefore, the lipid content of the grapeseed was preserved in the pomace following anthocyanin extraction, a potential benefit for downstream bioconversion to fuel.

The results of the gravimetric lignocellulosic compositional analysis revealed a neutral detergent extractives (NDE) content (which can include pectin, soluble carbohydrates, anthocyanins, and other polyphenolics) of 31.11%, a hemicellulose content of 11.20%, a cellulose content of 10.49%, and an acid-insoluble lignin (AIL) content of 47.20%. The NDE content aligns well with other compositional analyses,[18,19,40,41] though some have found even higher extractives content.[17,42] The cellulose and hemicellulose contents of just over 10% each are comparable to some reported values[16] but lower than others.[18,19] Many studies report elevated AIL in fermented pomaces like RGP, and though the value reported here is higher than some reported values,[16,18,40] it is comparable to others.[17,42] It has been established that RGP has lower cellulose and hemicellulose contents and an elevated acid-insoluble lignin content than those of a white grape pomace that has not undergone fermentation, which has been postulated to be due to partial fermentation of the polysaccharides. However, due to the nature of gravimetric analysis, recalcitrant protein and polymerized phenolics such as condensed tannins can lead to artificial elevation of calculated AIL.[17,40,42] Because of the brown color of the pomace following extraction, this mechanism is theorized to be a critical factor behind the high elevation of AIL in this study. The ash content of raw pomace was determined to be 7.047% (92.95% volatile solids), comparable to many literature values;[16,17,19,40,42] the ash content of extracted pomace was determined to be 3.346% (96.65% VS), a statistically significant reduction in ash ($P = 0.0007$).

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3.2. Anthocyanin Extraction. Average anthocyanin yields at 20, 50, and 80 °C were 1399 ± 22.89, 1512 ± 15.38, and 1861 ± 84.78 μg per gram of dry pomace, respectively. These values align closely with some reported values;[6,11,17] however, the range of reported values for anthocyanin content of RGP is wide, spanning up to an order of magnitude. Much of this difference can be attributed to variety,[5,17,43] but duration of maceration can also be a significant factor.[43] In addition, many studies do not report anthocyanin contents of RGP per unit dry mass; they are often reported per gram of extract,[34] percent of extract,[35] per unit wet mass,[4] or per gram of separated skins,[12] which are not representative of pomace as a whole. As such, in this study, choosing a variety or fermentation process designed to maximize the anthocyanin content of the pomace was not of paramount importance, but rather the comparison of different extraction temperatures and the efficacy of the extraction process within the larger processing pipeline.

Linear regression indicated that higher temperature yielded significantly more anthocyanins (P = 0.0002, Figure 1).

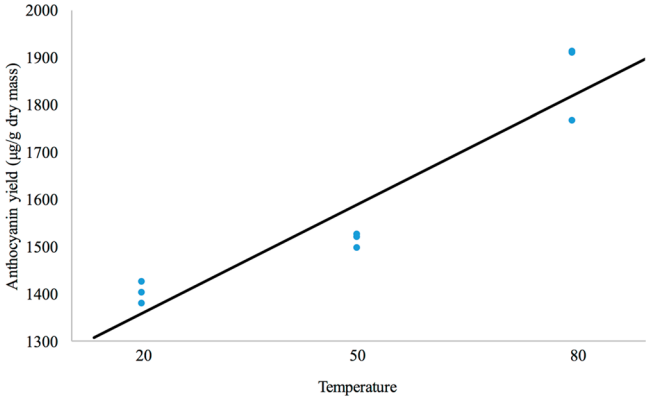

Figure 1. Linear regression of anthocyanin yield vs temperature.

Despite an abundance of studies extracting anthocyanins from RGP,[5,9,12,17,34,44] few have used temperatures elevated above room temperature,[29,35] and of those that have compared room temperature with higher temperatures, one found anthocyanin yield to actually decrease with increased temperature.[6] However, long extraction times of several hours were used in that study, which may have contributed to anthocyanin loss. A study on the extraction of anthocyanins from purple sweet potato found that a rapid, 1 h extraction at 80 °C yielded more anthocyanins than a 20 or 50 °C extraction.[25] In the same vein, in addition to the increased solubility and mass diffusivity of anthocyanins with increased temperature, the beneficial effect found in this study is likely also due to the rapid nature of the extraction process. Monomeric anthocyanins are more susceptible to oxidation and polymerization at higher temperatures. A fast, higher-temperature extraction seems to reap the benefit of the higher temperature while avoiding the deleterious effect it can have on anthocyanin stability. The highest and most effective extraction temperature, yielding the most anthocyanins (80 °C), was therefore chosen for further analysis.

Solid recoveries for the different extraction temperatures are shown in Figure 2. Linear regression confirmed that temperature was negatively correlated with solid recovery (P = 0.0028).

The three different extraction conditions for pomace were also compared for enzymatic digestibility. Linear regression found no statistical differences among the temperatures in terms of reducing sugar yield (data not shown).

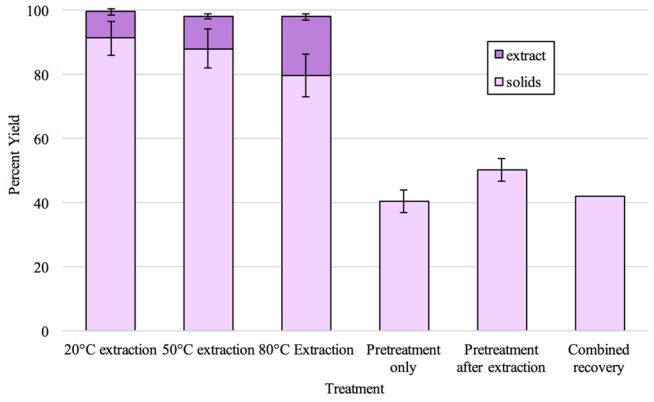

Figure 2. Solid and extract recovery following different treatments. The pretreatment parameters were 160 °C for 3 h, as selected by the response surface. The extraction temperature chosen for subsequent pretreatment was 80 °C, as determined by linear regression. "Combined recovery" indicates the cumulative recovery from both the extraction and subsequent pretreatment.

3.3. Comparison of Different Drying Methods. Average anthocyanin yields for convection-oven-, vacuum-oven-, and lyophilization-dried pomace were 1568, 1624, and 1784 μg per gram of dry pomace, respectively. ANOVA was performed on these samples versus undried pomace; these results are shown in Figure 3. Significant differences were

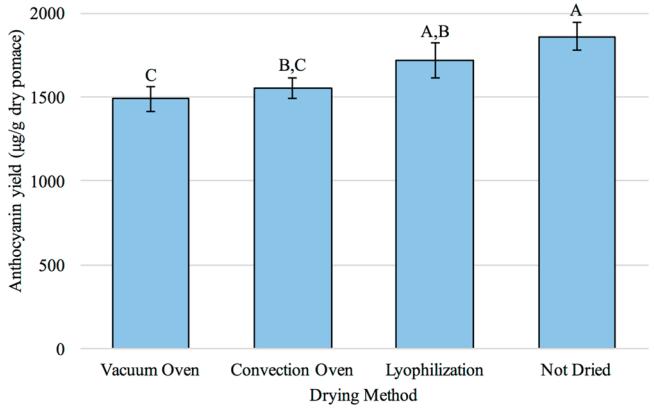

Figure 3. Anthocyanin yields of different drying methods in micrograms per gram of dry material. All samples were extracted at 80 °C for 60 min. Values that do not share a letter are significantly different.

observed between several pairings. The only drying method that did not significantly reduce anthocyanin yield was lyophilization. Other studies that have extracted anthocyanins with and without drying have demonstrated that drying can significantly impact anthocyanin yield.[45,46] Extended exposure to both heat and oxygen can cause anthocyanin degradation, a subject that has been reviewed at length.[47] The least destructive effect of lyophilization may be explained by the nature of the process: near-freezing temperatures are maintained as a result of constant evaporation, and the vacuum allows for low-oxygen conditions. The vacuum oven, also with low-oxygen conditions but with increased heat and with the longest duration of drying, resulted in more anthocyanin loss. The convection oven, though it was the

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fastest method, resulted in the biggest loss. However, there is no protection from oxygen or heat using this method.

3.4. Anthocyanin Extract Separation. The first four of the ten water fractions from the separation column contained reducing sugars. The total quantity amounted to 11.38 mg. A follow-up experiment involving the collection of five vials of 25 mL each confirmed that only the first 100 mL contained any sugars, with the total amounting to 12.02 mg. No Bradford protein was detected in any vials for either experiment. Anthocyanin recovery from the column was determined to be 90.46%, which corroborates other studies that examined anthocyanin separation from sugars using similar or identical resins.[27−29] At the same time, the fermentable contents of the extract, likely in the form of sugars and other small carbohydrates, were maintained in the aqueous fraction. BOD values of the pre-column crude extract (27.9 mg of O_2) and the post-column aqueous fraction (24.9 mg of O_2) were determined to be statistically indistinguishable (P = 0.239). These data indicate that this simple, preliminary separation with the XAD7 resin was an effective way of recovering fermentable components from the extract that would otherwise be lost as byproducts during downstream purification. The resulting aqueous fraction of the extract may be able to boost the biogas potential of the pomace while still offering an anthocyanin stream as a value-added product.

3.5. Ionic Liquid Pretreatment. On the basis of the raw data from the enzymatic digestion of RGP pretreated under different conditions, there was a positive, first-order effect of both temperature (P = 0.0002) and time (P = 0.0279) of pretreatment on the reducing sugar yield of the pomace (Table 1 and Figure 4A). When solid recovery following pretreatment was factored into the reducing sugar yield, the statistical significance of the variables changed: only a positive, second-order effect of temperature was observed (P = 0.0238, Table 2 and Figure 4B). On the basis of these results, ionic liquid pretreatment of the pomace appeared to be a superbly effective option, improving digestibility roughly six times over that of the untreated pomace. Even taking into account the poor solid recovery from the pretreatment (roughly 40%), pretreated pomace still outperformed the control by more than 100%. Subsequently, the best performing pretreatment parameters within the design space on a per gram basis (i.e., 160 °C for 3 h) were selected for further analysis.

Table 1. Parameter Estimates for Reducing Sugar Yield Following Ionic Liquid Pretreatment

Parameter	Estimate[a]	Std error	P-value[b]
β_0	134.9	5.083	**<0.0001**
B_T	34.1	4.997	**0.0002**
B_t	13.82	4.997	**0.0279**
β_{tT}	4.5	6.121	0.4861
B_{TT}	14.17	7.366	0.0958
B_{tt}	−10.53	7.366	0.196

[a]Parameter estimates are based on the response surface model in units of milligrams of reducing sugars recovered per gram of dry pomace and with coded values for the independent variables. [b]Bold values indicate P-values that are below the 0.05 threshold for statistical significance.

3.6. Analysis of Combined Treatments. The combination of extraction and pretreatment was also tested in an enzymatic digestion assay. Pomace extracted at the selected temperature of 80 °C was compared with raw pomace, pretreated pomace, and sequentially extracted and pretreated pomace; these results are shown in Figure 4C. In Figure 4D, the solid recoveries from different treatments were taken into account in the comparisons. The corresponding solid recoveries from these different treatments are displayed in Figure 2. Even when extraction was performed first, pretreatment still effectively improved the digestibility of the pomace: more than 4 times on a per gram basis and more than double when solid recovery was taken into account. These data indicated pretreatment could be a viable option for improving the digestibility of the spent pomace solids following extraction.

The results of the BOD assay of the homogenized pomace samples for the different treatments (Figure 5) contrasted sharply with those from the enzymatic digestion assay. ANOVA analysis found significant differences between groups (P = 0.000 169); post-hoc analysis found that specifically, all treatments yielded a lower BOD than raw pomace. It is conventional to utilize liquid residues for BOD assays, and using solids relies on the assumption that the biomass is highly homogeneous. On the basis of the relatively high standard deviations within treatments, it may be inferred that the homogeneity required to discern differences between treatments was not achieved because of the very small sample size (∼7.5 mg) required to stay within the range of detectable changes in oxygen concentration. However, BOD has been demonstrated in previous literature to provide a valuable prediction for biomass performance during anaerobic digestion.[48] These data suggested that pretreated pomace would likely not perform as well during anaerobic digestion as during enzymatic digestion. This discrepancy is something that has been observed during a prior study using tomato pomace.[33] The high content of extractives and lipids present in tomato pomace was postulated to generate microbial inhibitors during the high temperature of pretreatment. As RGP is also higher in non-lignocellulosic matter compared with traditional bioenergy crops, a similar process may have occurred here. Therefore, pretreated material was not included in the anaerobic digestion study. As this is the second investigation in which ionic liquid pretreatment was found to have a deleterious effect on the microbial degradation processes despite the initial promise from enzymatic digestion trials, caution should be advised when pursuing IL pretreatment for biofuel applications of fruit and vegetable waste residues such as tomato and grape pomace.

The aqueous fraction of the extract following crude separation was shown to have appreciable BOD. Though extracted pomace yielded a lower BOD than raw pomace, adding its BOD value to that of the aqueous extract would make up much of the difference, bringing the hypothetical total to 74.5 mg of O_2. Therefore, in addition to testing both untreated and extracted pomace in an anaerobic digestion trial (Section 3.7), a third treatment group containing both extracted pomace and its corresponding volume of aqueous extract was tested to determine if this supplementation would boost biogas production as predicted by the BOD. Eleutheria and colleagues found evidence to support such a hypothesis; after an aqueous extraction of white grape pomace, extracts were diverted to anaerobic digestion and found to be highly digestible.[49]

3.7. Anaerobic Digestion. The average methane yields of the raw pomace, extracted pomace, and extracted pomace with

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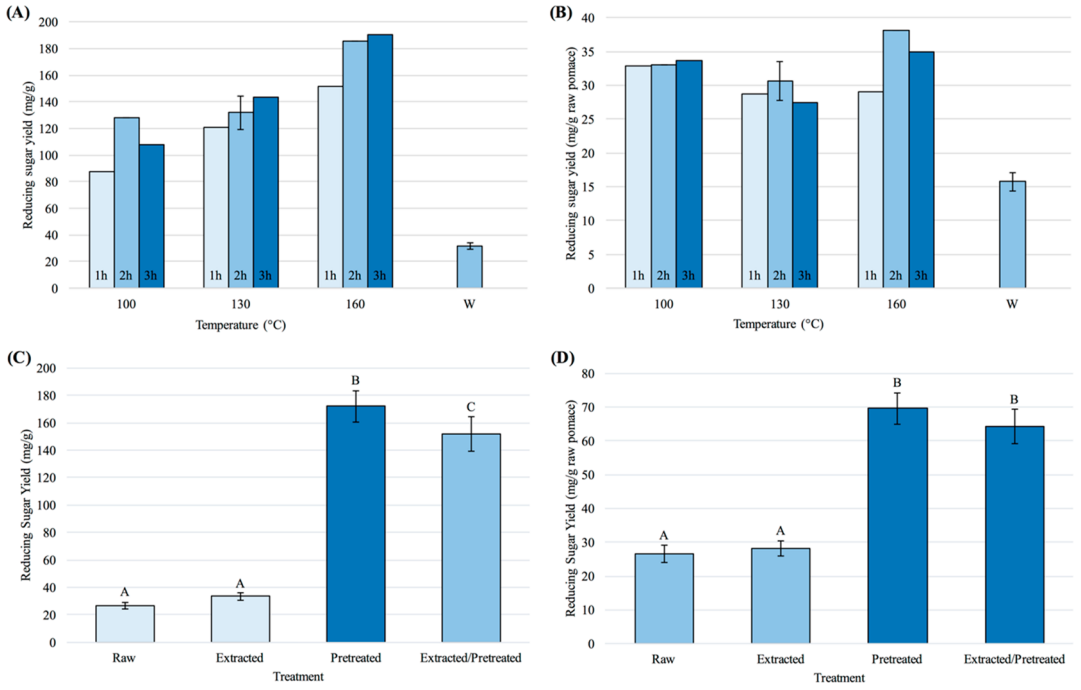

Figure 4. Reducing sugar yield from the enzymatic digestion of pomace following different treatments: (A) after pretreatment under different conditions, (B) after pretreatment under different conditions and adjusted for solid recovery, (C) after selected extraction and pretreatment conditions, and (D) after selected extraction and pretreatment conditions and adjusted for solid recovery. W represents the washed pomace control. Values that do not share a letter are significantly different. All values are reported on a dry mass basis.

Table 2. Parameter Estimates for Reducing Sugar Yield Following Ionic Liquid Pretreatment, Adjusted for the Recovery of Solids

Parameter	Estimate[a]	Std error	*P*-value[b]
β_0	30.69	1.147	**<0.0001**
B_T	0.43	1.128	0.7144
B_t	0.8783	1.128	0.4617
β_{tT}	1.273	1.382	0.3877
B_{TT}	4.781	1.663	**0.0238**
B_{tt}	−2.734	1.663	0.1441

[a]Parameter estimates are based on the response surface model in units of milligrams of reducing sugars recovered per gram of dry pomace and with coded values for the independent variables. [b]Bold values indicate *P*-values that are below the 0.05 threshold for statistical significance.

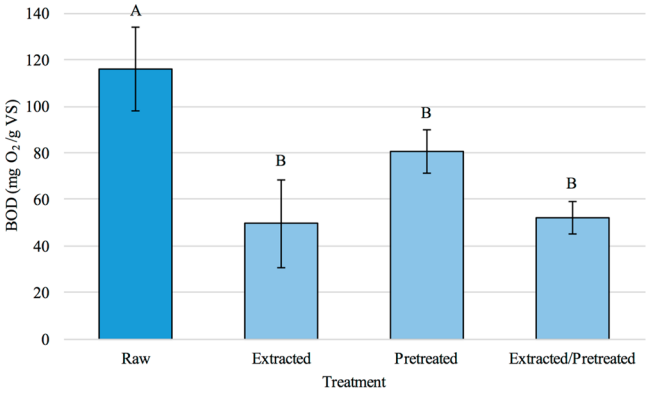

Figure 5. BOD results for pomace undergoing different treatments. Values are reported in milligrams of O_2 per gram of dry material. Values that do not share a letter are significantly different.

aqueous supplement (E+S), adjusted per gram of volatile solids (VS), were 213.6 ± 10.15, 190.5 ± 18.27, and 205.9 ± 18.39 mL, respectively. These values are comparable to those from other studies of anaerobic digestion of RGP, in the range of 110−360 mL per gram of VS.[18−22,50] Methane production over time for each treatment is depicted in Figure 6. One-way

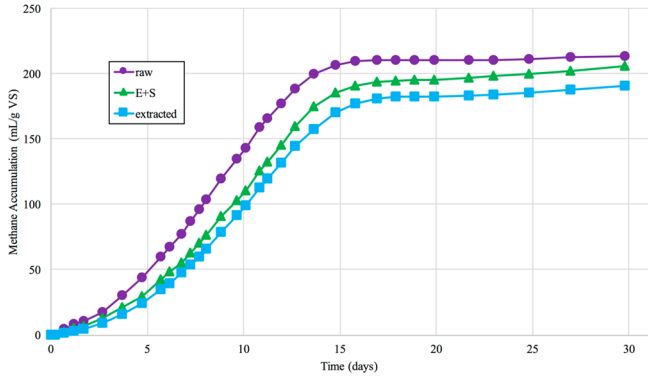

Figure 6. Methane accumulation for different treatments during anaerobic digestion. Values at each time point are averages of four replicates normalized to blank reactors. E+S indicates extracted pomace supplemented with its corresponding value of aqueous extract. All values are adjusted and reported per gram of VS.

ANOVA analysis of the terminal methane could not discern any significant differences (P = 0.17). However, two-way ANOVA with Tukey's post-hoc analysis, taking into account time in addition to treatment, indicated a highly significant effect of treatment on methane production (P < 0.0001). Two-way analysis of only steady-state values also yielded a significant effect of treatment (P < 0.0001). The biogas qualities for the raw, extracted, and E+S pomace were 57.4 ±

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0.924, 58.0 ± 1.22, and 58.9 ± 1.47%, a difference determined to be nonsignificant (P = 0.26). These values are comparable to the value obtained by Gersl et al. (62%),[22] though other studies report lower quality values of 31−41%.[19,51]

BOD results predicted a decrease in methane production following extraction and that the aqueous supplement would boost that production, and these results largely support these predictions. Because the repetition of comparisons in the two-way ANOVA was able to discern differences between treatments, this suggests a true difference in methane yields exists. There was a relatively high coefficient of variation (CV %) of the methane yields for both the extracted pomace and extracted pomace with aqueous supplement (9.6 and 8.9%, respectively). The relatively high variability between replicates may have prevented one-way ANOVA analysis from discerning significant differences. Replications for each treatment were limited to four in this study because of space, but prior to application of such a technology, more replicates could be run to produce more statistical power to increase the confidence in the ability to discern differences in final methane yield.

Though a significant difference in performance was observed between raw and extracted pomace, these differences were relatively small in quantity. The crude decrease in methane yield found in this study was only about 11%, with the aqueous supplement bringing that difference to only 3%. This suggests that, provided an appropriate protocol is used, extraction of anthocyanins can have a minimal effect on biofuel yields. Pellera and colleagues found an increase in biomethane yield from RGP after moderately high temperature (75−100 °C) pretreatment in water.[23] Thus, the elevated extraction temperature of 80 °C could have served as a de facto pretreatment for the pomace that was able to cancel out some of the effect of soluble sugar loss on methane production. Regardless of the mechanism, these results demonstrate an opportunity to create a valuable coproduct stream of anthocyanins without sacrificing the ability to repurpose this spent waste stream for biofuel production, which could help reduce waste and offset fossil fuel consumption by the wine industry. Fractionating the extract as a preliminary step in purifying anthocyanins enables fermentable nutrients to be harvested rather than lost as impurities during anthocyanin refinement, further minimizing the impact on methane production. However, this extra step also may not be necessary to achieve a successful sequential process to obtain both anthocyanins and biomethane.

With an ever-increasing need to improve waste management strategies and shift toward renewable energy, innovations to combine these goals are particularly attractive. Red grape pomace has been evaluated for both coproduct extraction and biofuel applications. However, there is a dearth of investigation into combining these two avenues for valorization. In this study, a multi-coproduct pipeline was developed for RGP to target both anthocyanins and biomethane. Anthocyanins were most effectively extracted from RGP at an elevated temperature of 80 °C. Water-soluble labile nutrients were successfully fractionated from the crude anthocyanin extract using SPE. Extraction reduced methane yield during anaerobic digestion; however, the aqueous extract significantly boosted the methane yield of extracted pomace. Sequential extraction of anthocyanins and the anaerobic digestion of RGP therefore creates a valuable coproduct stream while minimizing downstream effects on biomethane production. These promising results indicate that, as research in this area continues, similar processes may be applied to other food and agricultural waste streams or other biofuel production technologies.

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Notes

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ABBREVIATIONS USED

AIL,acid-insoluble lignin; BOD,biochemical oxygen demand; CCD,central composite design; DNS,dinitrosalicylic acid; IL,ionic liquid; NDE,neutral detergent extractives; RGP,red grape pomace; SPE,solid-phase extraction; VS,volatile solids

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