

# Optimization of Subcritical Water - Carbon Dioxide Systems for the Extraction and Reactive Processing of Biorenewable Materials

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## ABSTRACT

The coupling of subcritical water with supercritical carbon dioxide offers some unique advantages with respect to processing renewable materials derived from natural sources. Our research group activities are currently devoted to exploiting this “green” platform and applying it for both extraction and reactive conversion processes. Utilizing several predictive approaches, including solubility sphere as well as group contribution – based methods, we have been able to choose starting conditions for processing complex natural product systems using pressurized water, carbon dioxide, and ethanol systems. Recently we have developed several systems for measuring solubility and extraction of naturally occurring flavonoid and sugar moieties from room temperature and pressure to over 600 bar and temperatures exceeding 200°C. These solubility-extraction measurements for the above classes of compounds support extraction studies of flavonoids from waste grape pomace produced by the wine-juice processing industries as well as the generation of sugars from diverse biomass substrates for their conversion to biofuels. Many flavonoids are pH-sensitive solutes for which the subcritical water – CO<sub>2</sub> system can be adjusted to selectively extract them from grape and berry substrates in under 10 minutes processing time. Use of this highly carbonated water (600 bar/120°C) produces a pH of less than 3.0 favoring extraction of red flavanum cation for of anthocyanins found in grape pomace.

Using similar principles, subcritical water – CO<sub>2</sub> systems have been used to treat renewable waste biomass substrates such as corn stover, switchgrass, rice straw, and pine needles – degrading them to oligomeric or monomeric sugar-containing hydrolyzates for further processing to bioethanol. In these studies several reactor systems were used and hydrolysis conditions optimized by incorporating an orthogonal experimental design with respect to temperature (160 – 200°C), pressure (150 - 450 bar), reaction times (30 – 90 min), and particle size (75 – 150 microns) to produce glucose and xylose. Oligomeric sugar content of resultant hydrolyzates were characterized by several chromatographic methods. These experimental results show variable conversion efficiencies depending on the morphology and type of biomass substrate being treated. The subcritical water – CO<sub>2</sub> systems studied seem to hold promise in reducing or eliminating the use of carbohydrase enzyme cocktails for post-hydrolysis treatment of the degraded biomass as well as using excess carbon dioxide in a recycling mode, thereby promoting a “green” and sustainable conversion

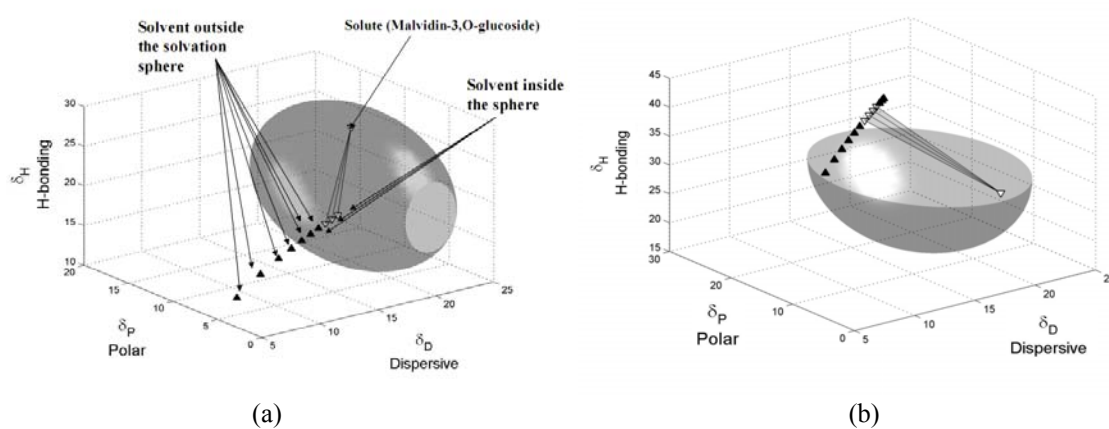
## INTRODUCTION

Our laboratory has for the past three years been developing a program using “green” solvents for extraction and reaction chemistry applied to bio-renewable materials [1]. This program

has involved an integrated fluid approach coupling SC-CO<sub>2</sub>, subcritical water, and ethanol for the selective extraction or reaction of residual agricultural products for both food and fuel use. Here we discuss primarily the application of subcritical water above its boiling point coupled with ethanol or SC-CO<sub>2</sub> for obtaining value-added nutraceutical ingredients from waste grape pomace and the conversion of residual biomass to fermentable sugars for conversion to liquid fuels. We have employed a multi-fold approach consisting of theoretical modeling/predictive methods, experimental determination of physicochemical data for subcritical fluid – solute systems, and laboratory-scale optimization studies for the extraction or reaction of agricultural materials primarily using pressurized water as a solvent.

We have previously presented studies based on the thermodynamic correlation of solute solubilities in subcritical water [2] and the use of the Hansen solubility sphere approach [3] to better understand the interaction and solvation between subcritical fluids and molecularly-complex target solutes or reactants. More recently, we have completed experimental studies on the solubility of flavonoids found in grape pomace which we summarize here. We have made extensive use of an accelerated solvent extraction (ASE) module, not as an analytical tool, but to study the extraction of flavonoid moieties from grape pomace and the hot water and enzymatic hydrolysis of several types of biomass. These ASE-based extraction and reaction studies have been supplemented by similar studies using both neat and highly carbonated water to selectively extract the pH-dependent flavylium form of anthocyanins as well as to degrade several types of biomass in a more environmentally-benign manner.

As described in a recent submitted publication [4], the Hansen solubility parameter sphere approach has been applied to understanding and optimizing the extraction conditions between a subcritical fluid solvent and a target solute, which in the case shown below is malvidin-3-O-glucoside, a principal anthocyanin component found in grape pomace. As shown in Figure 1b, the target solute (water) occupies a position inside the solvation sphere and as the temperature is changed for the subcritical fluid ethanol solvent, it initially remains outside the sphere (a case of immiscibility), but over a particular temperature range (100-175°C), enters the sphere resulting in solvation of the target solute. At higher temperatures, the three dimensional solubility parameter coordinates (D, P, H) for subcritical water place the fluid outside the sphere resulting once again in a condition equivalent to immiscibility between the subcritical fluid and the solute.



**Figure 1.** Hansen solubility spheres for the (a) malvidin-3-O-glucoside-ethanol system and (b) malvidin-3-O-glucoside-water system.

For the malvidin-3-O-glucoside-ethanol system (Figure 1a), the Hansen sphere parameters are: center of mass coordinates (D=19.7, P= 8.5, H=19.6) and radius = 7.23 MPa<sup>1/2</sup>. These may be contrasted with the malvidin-3-O-glucoside-water system sphere parameters which has a center of mass (D=17.7, P= 14.3, H=29.9) and radius = 10.35 MPa<sup>1/2</sup>. These sphere parameters show that the solvation of the malvidin-3-O-glucoside in subcritical ethanol is favored over dissolution in subcritical water principally due to the lower polar (P) and hydrogen bonding (H) interaction between the solute and the subcritical fluid. This also reflected in the lower volume of the solvation sphere and radius for the malvidin-3-O-glucoside-ethanol system.

The above modeling has guided us in choosing solvents and optimal conditions for the extraction of a number of natural products from renewable waste streams using subcritical water and/or ethanol, and in degrading diverse biomass substrates using either neat subcritical water with/without dissolved SC-CO<sub>2</sub> [2].

## MATERIALS AND METHODS

### *ASE extractions of grape pomace*

ASE-based extractions of Sunbelt grape pomace were done varying the amount of ethanol in water at 20% increments from 0-100%. A temperature matrix at 20°C intervals was run from 60-140°C. Approximately 0.25 g of freeze-dried grape pomace was placed for all experiments in a 11 mL extraction cell and ~35 mL of solvent was used in the ASE extractions. The ASE program conditions were: pressure = 10.2 MPa, heatup time = 5-7 min, static hold time = 1 min, and purge time = 1.83 min.

The collected samples which were made up to 50 mL with water were then centrifuged in a refrigerated centrifuge and filtered. The filtered samples were then stored in a refrigerator overnight (at -2°C), and then 5 mL of these solutions were concentrated using a Savant Model 210A Speedvac system for 6-7 hours. The concentrated grape pomace extract was then further diluted with 1 mL of 3% formic acid in water and 0.3 mL of methanol to dissolve the anthocyanins into solution for HPLC analysis as described below.

### *Solubility measurements in subcritical water*

A somewhat similar procedure was followed for the saccharide solubility measurements in subcritical water as described by Montanes et al [5] in these proceedings. A solute saturation cell was made from an empty HPLC column (0.2755 in i.d. x 0.4134 in) and placed in a gas chromatograph oven to provide temperature control. As an example, quercetin (~ 0.9 g) and sand were mixed in 1:2 ratio (by weight) and added to the saturation cell. An Isco Model 260D syringe pump were used to supply water to the saturation cell after passing through a 3-5m length of preheat coil. A mixing tee (HIP15-23AF1) was installed in the oven between outlet of saturation cell to allow additional solvent to be added using another Isco 260D syringe pump. A valve serving as back-pressure regulator (HIP15-11AF1) was placed at the outlet to the system to maintain the water at subcritical conditions. Hence the solute-saturated solution exiting from the cell contacts excess solvent (water) at the mixing tee inside the oven to prevent precipitation of flavonoid (quercetin) upon exiting the oven. The flow rates of the Isco pumps were varied as the experimental temperature of the subcritical water was changed to prevent precipitation of the flavonoid solute in the outlet line, as given in Table 2. After a 10-20 min equilibration period at the desired temperature (higher temperatures required lower equilibration time, higher pump flow rate and hence, lower sampling time interval), 10 fractions were collected every 1-3 minutes. The fractions were diluted appropriately and 0.5

mL of the diluted solution was mixed with methanol and analyzed using HPLC (see below). The flow schematic equipment is very similar to that discussed in these proceedings by Montanes et al [5] except Isco Model 260D pumps were substituted for Isco Model 100D pumps.

*Extraction of grape pomace using carbonated subcritical water with the Spe-ed system*

Approximately 500 mg of pomace was mixed with 18 mL of degassed Milli-Q water and placed inside a 24 mL tubular vessel. This vessel was then placed inside the oven of Applied Separations Spe-ed extraction system for both the neat and carbonated water experiments. All individual experiments were run at 120°C for 7.5, 15, and 30 min extraction times. In the case of the carbonated water extractions, the same experimental matrix parameters were used except that CO<sub>2</sub> was added by using the booster pump of the Spe-ed system. Several levels of carbonation experiments were conducted from 15.0 – 60 MPa at 15 MPa intervals. For safety reasons, it was calculated that 18 mL of water was the maximum solvent volume that could be used in 24 mL vessel. After the above exposure times, the extraction cell was quickly removed from the Spe-ed oven, cooled, and the contents filtered prior to HPLC analysis.

*Biomass hydrolysis using carbonated water in the Spe-ed system*

Hydrolysis of a number of diverse biomass types (corn stover and cobs, switchgrass, several types of rice-derived biomass, pine wood) were treated using a similar experimental setup as described for the carbonated water extractions of grape pomace using the Spe-ed system, except much higher temperatures were used as noted in Table 1 below. The selection of experimental parameters to be investigated were partially determined from previous experiments run in stirred autoclaves and the ASE system, accompanying by enzymatic-aided hydrolysis after exposure of several of the above biomass substrates to much more modest levels of carbonated water [6]. A level 3 – 4 parameter orthogonal experimental design procedure was applied in minimizing the number of required experiments on most of the above listed biomass samples as listed in Table 1. In these cases, 100mg of the biomass was placed in reaction cell along with 20 mL Milli-Q water before running the matrix of nine experimental conditions listed in Table 1. Biomass samples were comminuted and sieved to test the effect of particle size on hydrolysis efficiency using carbonated subcritical water. After the hydrolysis treatment, the water and contents were decanted and dried in an oven overnight at 40°C. Twenty mg of these samples were placed in a tube with 5mL of degassed Milli-Q water and boiled in a 100°C water bath for 30min. Finally the sample was filtered through a 5µl membrane before HPSEC analysis of the hydrolyzate’s molecular weight distribution and/or its sugar content.

**Table 1.** The orthogonal experimental design parameters applied to corn stover.

Number	P (bar)	T (C)	C (min)	D (micrometer)
#1	150	160	30	75~106
#2	150	170	60	106~125
#3	150	180	90	125~150
#4	300	160	60	125~150
#5	300	170	90	75~106
#6	300	180	30	106~125
#7	450	160	90	106~125
#8	450	170	30	125~150
#9	450	180	60	75~106

### *HPLC analysis*

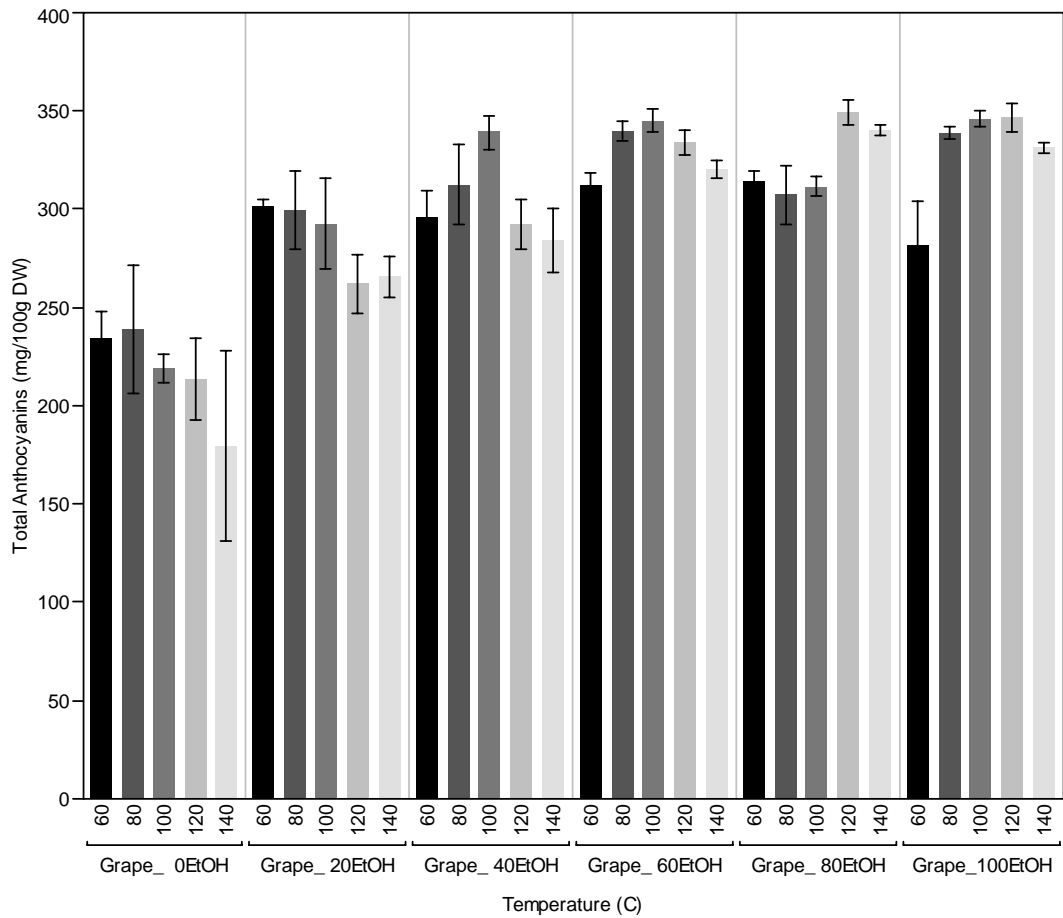
Flavonoid-containing extracts from the neat and carbonated water Spe-ed-based extractions were characterized using a Waters Alliance HPLC system, a Symmetry C 18 column, and UV detection at 510 nm as described by Cho et al. [7]. This same HPLC system and protocol was used for the analysis of the grape pomace extracts from the ASE experiments described above. The analysis of quercetin was analyzed using the HPLC method described by Scheiber et. al.[8]. The method consists of sampling different concentrations of quercetin solution in water through a Phenomenex Aqua C18 column with mobile phase consisting of 2% (v/v) acetic acid in water (eluent A) and (50:50, v/v) 0.5% acetic acid in water and acetonitrile (eluent B) operating in a gradient flow at 1mL/min as follows: 10% B to 55% B (5 mins), 55% B to 100% B (10 mins), 100% B to 10% B (5 min). The injection volume for all samples was 100 $\mu$ L. The samples were monitored at 364 nm using a Waters Model 2998 photodiode array detector.

A high performance size exclusion chromatography (HPSEC) using a Waters Model 2414 refractive index detector (Milford, MA, USA) coupled with Shodex OH Pak SB-802 and SB-804 HQ columns (Shodex - Kawasaki, Japan) was used to measure both the degradation profiles of the biomass substrates as well as sugar (glucose and xylose) content as well as their solubility in subcritical water [5] . The mobile phase was 0.003 mol/L  $\text{NaN}_3$  and 0.1 mol/L  $\text{NaNO}_3$ . All samples were filtered before injection into the HPSEC system. A pullulan molecular weight (MW) standard set (MWs = 342-710,000) from Sigma-Aldrich (St. Louis, MO) was used for MW calibration of the elution volume axis.

## **RESULTS AND DISCUSSION**

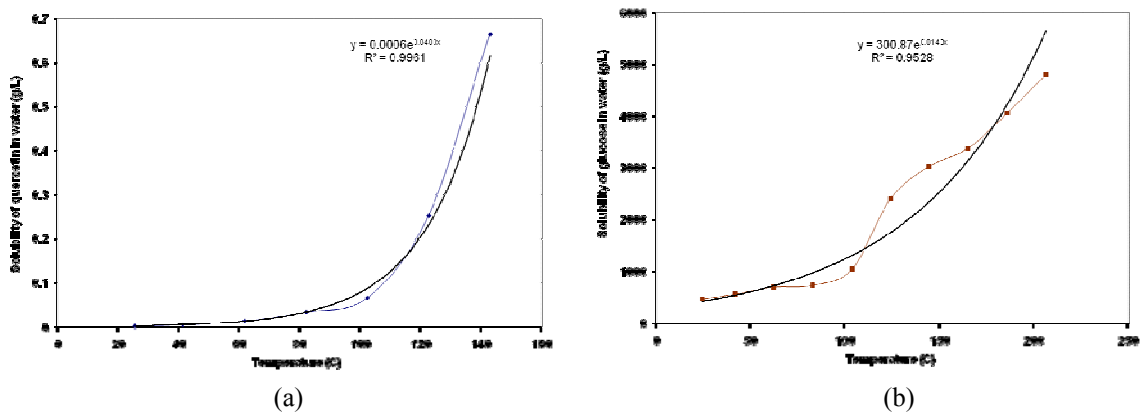
Extraction of anthocyanins from grape pomace can be conveniently studied using the above described ASE procedure. An ASE system has been previously used in studying and optimizing the temperature and pressure conditions for extraction of flavonoid compounds from grape pomace using mixtures of water and ethanol and dilute acid as solvents [9]. In this study, it was found that a brief extraction time of only 1 minute in the ASE extraction cell at a pressure of 10.2 MPa, using 60-100% levels of ethanol at temperatures between 100-120 $^{\circ}$ C, was optimal for the extraction of anthocyanins from freeze-dried grape pomace. Further statistical analysis of the results shown in Figure 2 indicated an interplay between the temperature of the extraction and the composition on the solvent. For example, the maximum value for total anthocyanin extraction would appear to occur at 80% ethanol and 120 $^{\circ}$ C, but similar results can also be achieved using 60% ethanol at 100 $^{\circ}$ C or 40% ethanol at 100 $^{\circ}$ C from inspection of Figure 2 .

Only a portion of the solubility solute data in subcritical water is presented here as determined using the above described system, but as noted previously, sugar solubility data as a function of temperature is presented in these proceedings by Montanes et al. [5] using the same system. In Figure 3 below is presented the solubility data for quercetin, a biologically important flavonoid, up to 140 $^{\circ}$ C. Similarly, glucose's solubility in subcritical water has been determined up to 200 $^{\circ}$ C using a nearly identical experimental system. It is interesting to contrast the magnitudes of the experimentally-determined solubilities in terms of their g/L in pressurized water: (0.0021 - 0.67 g/L from 25-140 $^{\circ}$ C for quercetin and 465 – 4,800 g/L from 25 – 200 $^{\circ}$ C for glucose). This is over a  $10^4$ -fold difference in the observed solubility between



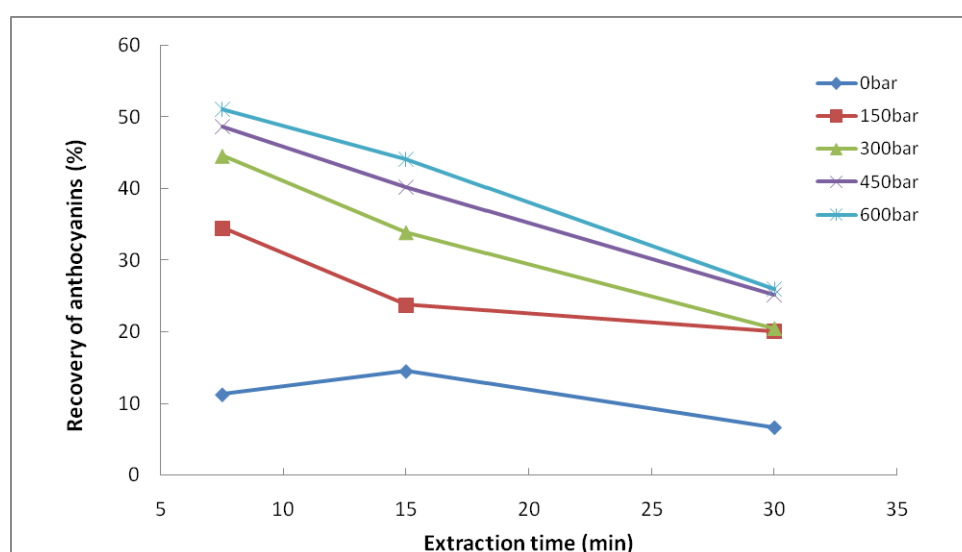
**Figure 2.** Total anthocyanins recovered from grape pomace as a function of ethanol concentration as determined using the ASE system.

these members of two different solute classes, which illustrates the potential of this measurement technique. A trend line fit has also been superimposed on the experimental data given in Figures 3a and b, and both show a good  $R^2$  correlation (0.9961 and 0.9528) approximating an exponential dependence of g/L solubility on water temperature ( $^{\circ}\text{C}$ ).



**Figure 3.** Comparison of solubilities of quercetin and glucose as a function temperature in subcritical water.

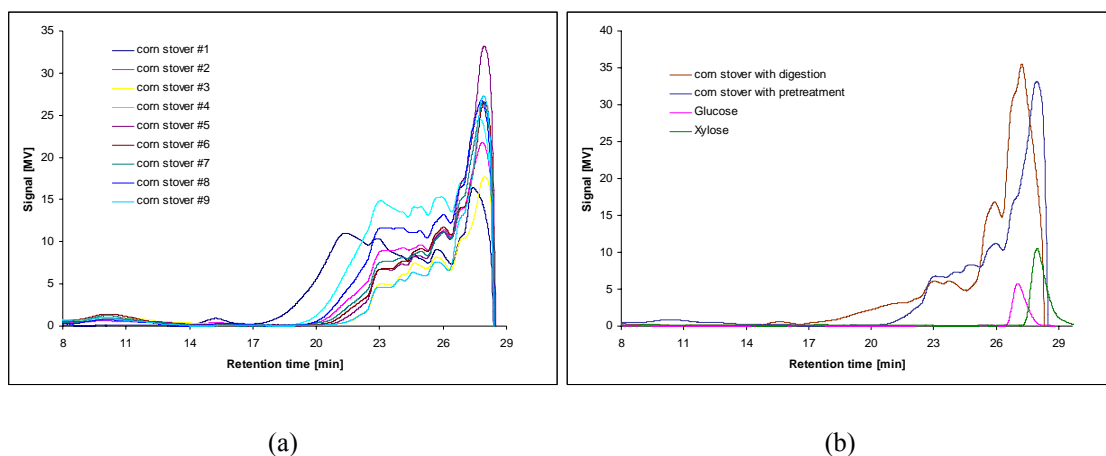
The use of carbon dioxide with pressurized water has been advocated by other researchers for the pretreatment of biomass substrates [10,11] and has been reviewed by Rayner [12] in the context of reaction chemistry, but few researchers if any have employed the principle directly for the extraction of a desired pH-dependent form of a target solute. Figure 4 below shows the effect of dissolving carbon dioxide as an acidifying agent in stabilizing the flavylium ion form of anthocyanin moieties extracted from grape pomace - the form responsible for the red color of wine. The maximum recovery of the anthocyanins from the pomace as determined by HPLC is over 50% for 60 MPa dissolution of SC-CO<sub>2</sub> at 7.5 min at 120°C, the yield decreasing as the CO<sub>2</sub> pressure is dropped and the extraction time extended (each experimental point is the average recovery from triplicate extractions). This maybe contrasted with the yield using neat water which is in the range of 10-15% recovery depending on the extraction time. Aqueous extracts recovered from the batch extractor vessel were red-purple in color, a resultant of the acidic pH of the highly carbonated solution [ 10,13].



**Figure 4.** The recovery of anthocyanins as a function for different extraction times and CO<sub>2</sub> pressures at 120°C.

The above principle has also been applied for the subcritical water treatment of biomass substrates as noted in the experimental section using pressures from 15.0 - 45.0 MPa at temperatures of 160 – 200°C, and exposure times of 30 – 90 min. In the orthogonal design in Table 1, it was frequently found that conditions listed for experimental run #5 were optimal for degrading the carbohydrate-laden biomass as judged by the change in the HPSEC elution profiles as well as producing the most total monomeric sugars (e.g. - for corn stover – 30.0 MPa, 170°C, 90 minutes, and a particle size range of 75 – 106 mm). However it was also found that the variable recalcitrance of different biomass types could require slightly different conditions than those found for the corn stover and/or produce different amounts of glucose/xylose. For example, when degrading a corn cob substrate, the orthogonal design statistics showed that the experimental parameters for obtaining the maximum yield of xylose were of the order of pressure>temperature>particle size>reaction time, while for maximizing glucose yield they were particle size>time>temperature>pressure. Figure 5a below shows the overlaid HPSEC elution profiles for all nine experimental runs listed in Table 1. The profile corresponding for the optimal carbonated subcritical water hydrolytic pretreatment of corn stover (#5) is shown in Figure 5b along with the carbohydrase (DePol 692L) digestion of this hydrolyzate and the elution positions of glucose and xylose. Clearly the described

pretreatment has facilitated further enzymatic-based hydrolysis of this particular biomass substrate to monomeric sugars and reduced the amount of required enzyme relative to other pretreatment/degradation methods.



**Figure 5.** HPSEC elution profiles for corn stover pretreated under conditions specified in Table 1 and the hydrolyzate produced under reaction condition #5 with and without enzymatic post treatment.

## ACKNOWLEDGMENTS

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