Supercritical Fluid Extraction of Grape Glycosides

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Supercritical fluid extraction with methanol-modified CO_2 was used to extract glycosides from grapes. An optimization design involving 12 extraction variables was applied to achieve quantitative recoveries. The most important factor was the amount of organic modifier, a consequence of the high degree of glycoside polarity. By the proposed method, the total time of analysis can be decreased relative to that required for more conventional extractions. The full method can also be automated.

Keywords: *Glycosides; supercritical fluid extraction*

INTRODUCTION

Grape aroma compounds are present both as free volatiles and, in much larger concentrations, as nonvolatile sugar-bound glycoside conjugates (Cordonnier and Bayonove, 1974; Williams et al., 1982b). Glycosides are, in part, nonvolatile aroma and flavor precursors. Glycosides have been shown to be hydrolyzed during wine processing (Abbott et al., 1993; Cordonnier and Bayonove, 1974; Sefton, 1998; Williams and Francis, 1996; Williams et al., 1982a; Zoecklein et al., 1997, 1998). I. L. Francis (personal communication, 1998) has also reported a positive correlation between the concentration of total grape glycosides and wine flavor. These studies confirm that grape glycosides have sensory significance and provide justification for their quantification.

Glycosidation is believed to result from glycosyltransferases that catalyze the transfer of carbohydrates from sugar-carrying nucleotides to, for example, monoterpenes. Glycosidically bound grape conjugates include monoterpenes, aliphatic residues, sesquiterpenes, norisoprenoids, and shikimic acid related compounds (Williams et al., 1982a,b). Many nonvolatile aroma precursors are, however, not actually bound analogues of the aroma compounds but molecules that can rearrange or degrade to produce aroma volatiles.

Traditional methods for quantifying glycosides are time-consuming, requiring isolation, hydrolysis, and gas chromatography/mass spectrometry (GC-MS) of individual aglycons (Voirin et al., 1992a). Analytical methods for estimating glycosidically bound secondary metabolites in juice and wine have been developed (Abbott et al., 1993; Voirin et al., 1992b,c; Williams et al., 1995). A common feature of these secondary metabolites is that they are glucosides in which the glucose may or may not be further substituted. Hydrolysis of the glycosides yields an equimolar proportion of the aglycon and D-glucose, referred to as glycosylglucose (GG). The determination of the GG content allows for an estimation of the total pool of glycosides (Abbott et al., 1993; Williams et al., 1995).

In the method of Abbott et al. (1993) and Williams et al. (1995) as modified by Iland et al. (1996), the initial step involves extraction of glycosides from grapes via conventional liquid extraction (C_2H_5OH/H_2O). This is followed by isolation of the glycosides on an octadecyl-silica reversed phase absorbent and the removal of free sugars and free phenolic compounds, which produce interferences. Glycosides are then chemically hydrolyzed and D-glucose quantified by an enzymatic assay.

Due to the possible relationships between total glycosides, glycoside fractions, and grape and wine quality, it would be beneficial to develop an automated system to do the extraction. Supercritical fluid extraction (SFE) with CO_2 could be an alternative method for the isolation of glycosides from grapes. Using this technique and commercially available equipment, the extraction could be fully automated. In other words, after the extracts are collected in an organic solvent or water, the resulting solution could be analyzed via a work station, thus achieving automation.

Glycosides have been previously extracted from natural materials, such as leaves, by SFE (Moore and Taylor, 1996). Due to the high polarity of these compounds, large percentages of organic modifier were needed to achieve quantitative recoveries. Besides the percentage of modifier, other extraction and retention variables were optimized to guarantee good recovery of glycosides from the leafy matrix.

In this study, the development of a new method to extract glycosides from grapes by SFE is presented. An optimization process was performed for 12 different variables. After the extraction step, the method of Williams et al. (1995) as modified by Iland et al. (1996) was applied to the resulting solution for analysis of glycosides.

EXPERIMENTAL PROCEDURES

Materials. Ottawa Sand standard (20−30 mesh) and HPLC grade solvents, including methanol, ethanol, ethyl acetate, and water, were obtained from Fisher Scientific (Houston, TX). Hydromatrix was obtained from Supelco Inc. (Pittsburgh, PA). A standard solution of *n*-octylglucoside (66.67 mg/mL) was prepared in methanol and stored at −4 °C in dark conditions. Carbon dioxide from Air Products and Chemicals Inc. (Allentown, PA) was used as the extracting fluid. Each extraction for the optimization process was completed in duplicate. The experiments were performed in a pre-established random order. The different extraction conditions that were tested are

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10.1021/jf990957p CCC: $19.00
Published on Web 02/16/2000

Table 1. Experimental Design for Optimization of Extraction Parameters (Selected Experiments)

expt	matrix[a]	density (mg/L)	extrn temp (°C)	CO_2 flow (mL/min)	modifier[b]	% of modifier	SFE time (min)		restrictor temp (°C)	trap temp (°C)	solvent in liq trap[c]	vol of liq trap (mL)	yield[d] (GG/g of sample)
							static	dynamic					
2	1	0.80	40	0.5	−1	20	0	20	50	50	1	8	4
3	−1	0.95	40	0.5	−1	5	15	20	50	50	1	4	21
4	1	0.95	40	0.5	−1	20	15	10	50	30	−1	8	6
5	−1	0.80	60	0.5	−1	20	15	20	100	50	−1	8	48
7	−1	0.95	60	0.5	−1	20	0	10	100	30	1	8	12
10	1	0.80	40	1.5	−1	20	0	20	100	30	1	4	33
11	−1	0.95	40	1.5	−1	5	15	20	100	30	1	8	6
12	1	0.95	40	1.5	−1	20	15	10	100	50	−1	4	19
13	−1	0.80	60	1.5	−1	20	15	20	50	30	−1	4	79
14	1	0.80	60	1.5	−1	5	15	10	50	50	1	8	23
15	−1	0.95	60	1.5	−1	20	0	10	50	50	1	4	24
17	−1	0.80	40	0.5	1	20	15	10	100	50	1	4	20
19	−1	0.95	40	0.5	1	20	0	20	100	30	−1	4	82
20	1	0.95	40	0.5	1	5	0	10	100	50	1	8	16
21	−1	0.80	60	0.5	1	5	0	20	50	30	1	8	15
24	1	0.95	60	0.5	1	20	15	20	50	30	1	4	12
25	−1	0.80	40	1.5	1	20	15	10	50	30	1	8	100
27	−1	0.95	40	1.5	1	20	0	20	50	50	−1	8	80
29	−1	0.80	60	1.5	1	5	0	20	100	50	1	4	16
32	1	0.95	60	1.5	1	20	15	20	100	50	1	8	77

[a] Matrix in the extraction vessel: −1, sand; +1, Hydromatrix. [b] Modifier: −1, EtOH; +1, MeOH. [c] Solvent: −1, EtOH; +1, H_2O. [d] Yield expressed as percentage of recovery relative to experiment 25.

specified in Table 1. Yield is relative to the value obtained in the experiment that gave the highest value for GG (experiment 25). Minitab Release 10Xtra (State College, PA) was used to carry out the data analysis.

The extractions were performed using an ISCO SFX 3560 (Lincoln, NE) configured with cooled pump heads. The samples for the extraction process were placed in 10 mL ISCO Peek extraction vessels.

GC Method. A 50 berry sample of 1995 Cabernet Sauvignon (*Vitis vinifera* L.) grapes was stored at −25 °C prior to analysis of total GG. Frozen berry samples were warmed to 10 °C, macerated, and placed in a Tekmar model 400 laboratory stomacher for 5 min, and the homogenate was uniformly mixed. The fresh juice expelled from the homogenate was analyzed for GG as described by Abbott et al. (1993) and Williams et al. (1995) and involved the isolation of glycosides on a Waters (Milford, MA) C-18 reverse phase Sep-Pak. Glycosides underwent elution and acid hydrolysis followed by passage through an activated C_{18} RP cartridge a second time to reduce the concentration of interfering phenol compounds as suggested by Iland et al. (1996). The glycosides were then eluted, and the liberated glucose was analyzed using hexokinase/glucose-6-phosphate dehydrogenase (Boehringer-Mannheim) measured by 340 nm against standard solution. Results are expressed as micromolar GG in fresh juice. Some of the homogenate was also used for the SFE extractions for comparison.

RESULTS AND DISCUSSION

Glycosides are highly polar compounds, and supercritical CO_2 is a low polar extracting fluid. Therefore, prior to optimization of the extraction variables, some extractions were performed to determine if it would be possible to extract glycosides quantitatively with supercritical CO_2. A 10 μL aliquot of a standard *n*-octylglucoside (66.67 mg/mL) methanol solution was spiked over sand and allowed to stand for 20 min to evaporate the methanol. The extraction conditions are shown in Table 1. Recovery was strongly affected by the modifier used. When pure CO_2 was used, the recovery of the standard was 16%, whereas when 15% methanol was used as the modifier, the recovery increased to 58.8%. With ethyl acetate, the recovery was 3-fold lower than with methanol. On the basis of these results, we thought it feasible to extract glycosides from grapes by supercritical CO_2 extraction.

SFE methods can be divided into two steps—the extraction of compounds from the matrix and the retention of the extracted compounds in a suitable trap. The trap can be a solid or a liquid. Many references related to the optimization of extraction variables (Maio et al., 1997; Taylor, 1995) are available. The variables related to the extraction step are the matrix where the sample is supported, the fluid density, the flow of the extracting fluid, the extraction temperature, the organic modifier, and the times for the SFE static and dynamic states. Several studies have related the efficiency of liquid traps with respect to retaining different extracted analytes (Burford et al., 1992; Langefeld, 1992; McDaniel et al., 1998). The main variables related to the retention step are CO_2 flow, restrictor temperature, the liquid solvent, solvent volume, and solvent temperature.

There are also variables that relate to both the extraction and the retention steps. The effect of each variable on each step must therefore be analyzed, and the interactions between variables must be studied as well.

Sand and diatomaceous earth (Hydromatrix) to support the sample in the extraction vessel were studied. Sand is an inert matrix, whereas Hydromatrix is more active and can retain a certain concentration of water. The variable matrix was evaluated because grapes have a high water content. Two densities (0.80 and 0.95 g/mL) of the extracting fluids (CO_2) were used. The high densities were used to achieve a high solvating power, which was thought to be advantageous due to the high polarity of grape glycosides. Extraction temperatures of 40 and 60 °C were chosen to avoid analyte degradation.

When SFE is used with a liquid trap, the flow of supercritical fluid is very important. A higher flow allows for a greater recovery within the same extraction time, but a higher flow can also produce analyte losses from the liquid trap. This is due to the fact that each milliliter of supercritical fluid (SF) expands to ∼500 mL of gas at the trap. To reduce extract losses, low flows were studied (i.e., 0.5−1.5 mL/min). For the same SFE dynamic time, 1.5 mL/min should produce a higher extraction recovery than 0.5 mL/min, due to the greater amount of total extracting fluid.

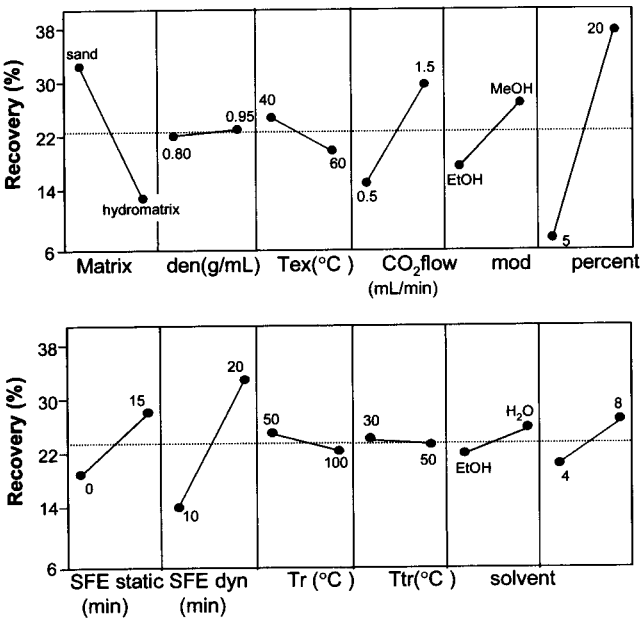

Figure 1. Main effects of extraction/retention variables on the recovery of glycosides.

The organic modifiers tested were methanol and ethanol. Methanol was chosen on the basis of our initial extraction results, and ethanol was chosen because it is the classic extracting solvent for glycoside extractions. The modifier percentages assayed were 5 and 20%. The times for SFE static and dynamic steps were chosen with the hope of achieving an extraction method that was shorter than the classic extraction method. Restrictor temperatures of 50 and 100 °C were chosen to guarantee that no extract degradation or plugged restrictor occurred.

Ethanol and water were assayed as liquid traps because mixtures of ethanol and water are the usual extracting solvents in classic extractions of glycosides. Water was also assayed in an effort to avoid the concentration steps, which are usually necessary when an organic solvent is used as the liquid trap.

For liquid trap temperatures, temperatures of 30 and 50 °C were assayed. The solvating power of the liquid increases with increased temperature. Therefore, greater recovery should result from higher temperatures. However, lower temperatures result in smaller losses of trap solvent and analytes due to evaporation. Lower temperatures also mean higher viscosities and, thus, lower speed of ascending bubbles of CO_2 through the liquid trap, that is, easier mass transfers and greater recoveries. The volumes used in the liquid trap were chosen to determine the effect of two different factors. A higher volume leads to a higher recovery due to better mass transfer. However, a longer concentration step is necessary when a higher volume is used and, therefore, degradation becomes more probable.

A fractional factorial experimental design was chosen for optimization given 12 variables in two different levels. The total number of extractions was 32 from 4096 possible experiments. The extraction conditions are shown in Table 1. Figure 1 shows the effects of 12 variables used in the experimental design over the GG values. All of the recovery data refer to the extraction with the highest value of GG (extraction 25). The greatest effect was shown by the amount of organic modifier. If 20% was used instead of 5%, the recovery

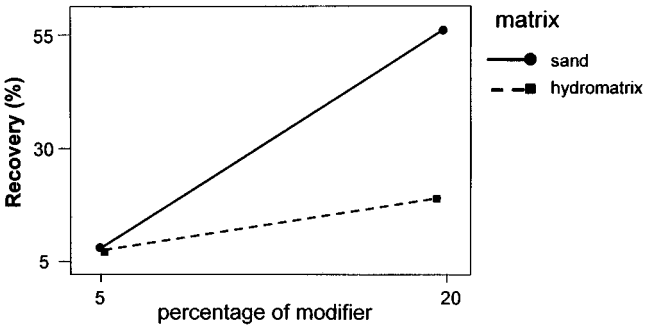

Figure 2. Interaction effects between the matrix and the percentage of modifier on the extraction of glycosides.

was 5-fold higher (37 versus 7%). These values are the average of 16 of the 32 extractions performed with 20% and the remaining 16 extractions performed with 5% modifier. This result is similar to the initial results, and it is due to the high polarity of the analytes. Because of the large effect on the recovery when the percentage of modifier was increased, even higher percentages of modifier were evaluated in later extractions (after the optimization process).

Sand was a more inert matrix for sample support than was Hydromatrix. The average recovery was 32% when sand was used versus 12% when Hydromatrix was used. Any interaction between "percentage of modifier" and "matrix" is shown in Figure 2. This graph shows the average recovery of eight extractions with 20% modifier and sand, eight extractions with 20% modifier and Hydromatrix, eight extractions with 5% modifier and sand, and eight extractions with 5% modifier and Hydromatrix. From Figure 2, it is possible to determine if there is interaction between the two variables. A similar slope for both lines signifies no interaction, whereas a different slope indicates that there are interactions. When 5% modifier was used, the recovery was 6% for both matrices (sand and Hydromatrix). However, with a higher percentage modifier, the recovery was much greater with sand (solid line = 6−55%) than with Hydromatrix (dashed line = 6−13%). Therefore, these variables are not independent. The effects of one on the recovery are modulated by the value of the other one. The reason for this interaction could be due to the fact that Hydromatrix can retain more modifiers than sand. If there is too much modifier in the vessel, the analytes can be transferred from the sample to the modifier instead of to the supercritical fluid, so the recovery is lower. When the percentages of modifier are low, there are few differences in the recoveries, but if the percentage of modifier is high, the differences are greater.

Another important pair of variables is the flow of the supercritical fluid and the dynamic extraction time. These two variables determine the total amount of extracting fluid used for the extraction. As explained previously, higher amounts of extracting fluid should produce higher recoveries if the effect of bubble size and loss of volatile compounds are not important. The results in this study showed that the more important variable is the total amount of CO_2. From Figure 3, it can be seen that there is no interaction between the flow of CO_2 and the dynamic extraction time. Increasing either variable caused the recoveries of the extractions to increase. Both lines are parallel, indicating that they are independent factors.

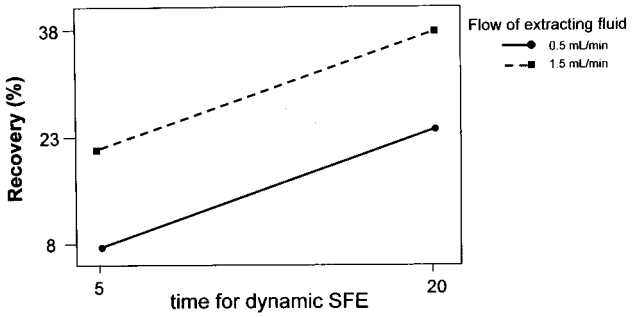

Figure 3. Interaction effects between the flow of the extracting fluid and the time for dynamic SFE on the extraction of glycosides.

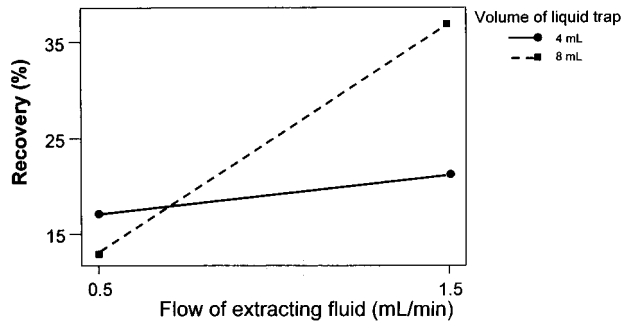

Figure 4. Interaction effects between the volume of liquid trap and the flow of extracting fluid on the extraction of glycosides.

Other variables of less importance to the extraction/retention process were the extraction temperature, the static time, the solvent used in the liquid trap and its volume, and the CO_2 density (Figure 1). Even though the volume of the liquid trap showed little effect on average recoveries, it had a high degree of interaction with the flow of extracting fluid (Figure 4). When the flow was low (0.5 mL/min), the recoveries were similar (17 versus 14%) for any volume in the liquid trap (4 or 8 mL). However, when the flow was high (1.5 mL/min), there was a large difference between recoveries (22% for 4 mL versus 36% for 8 mL of liquid trap). Therefore, it was necessary to take into account the volume of the liquid trap if the optimal flow was 1.5 mL/min. Restrictor temperature and collection temperature did not show an effect on the recovery of glycosides (Figure 1).

Within the ranges studied, the best conditions for grape glycoside extraction were deemed to be as follows: matrix, sand; density of the extracting fluid, 0.95 g/mL; extraction temperature, 40 °C; flow of the extracting fluid, 1.5 mL/min; organic modifier, 20% of methanol; static SFE, 15 min; dynamic SFE, 20 min; restrictor temperature, 50 °C; liquid trap, H_2O; volume and temperature of the liquid trap: 8 mL and 30 °C.

To determine the recovery of glycosides extracted using these conditions, a standard solution of a glycoside (*n*-octylglucoside) was used at a concentration similar to the total value of glycosides in grapes (1.33 mg/mL). As can be seen in Table 4, the recovery of the glycoside standard from sand using the best extraction conditions suggested by the optimization design study was 42.1% (experiment a). Some changes were made to try to increase the recovery. The most important variable was the amount of organic modifier. Therefore, 1 mL of organic modifier was used in the static SFE step (experiment b). With this method, the recovery was increased (57.5%).

Table 2. Initial SFE Conditions Applied to *n*-Octylglucoside Spiked on Sand[a]

extraction	organic modifier (percentage)	recovery[b]
1	methanol (15%)	58.8
2	ethyl acetate (15%)	19.0
3	no modifier	16.0

[a] Extraction conditions: CO_2 density, 0.95 g/mL; SFE static time, 15 min; SFE dynamic time, 15 min; extraction temperature, 50 °C; liquid trap, H_2O (35 °C); restrictor temperature, 85 °C. [b] Average of two extractions.

Table 3. Extraction Conditions for Postoptimization Experiments[a]

expt	SFE dynamic modifier (%)	SFE static modifier (mL)	SFE dynamic % modifier	recovery
a	20	0	0	41.2
b	20	1	0	57.5
c	30	1	0	13.5
d	40	1	0	6.0
e	20	1	10	64.2
f	30	1	10	30.1
g	40	1	10	19.3
h	20	2	10	94.1

[a] Matrix: sand; density, 0.95 g/mL; extraction temperature, 40 °C; SF flow, 1.5 mL/min; organic modifier, MeOH; SFE static time, 15 min; SFE dynamic time, 20 min; restrictor temperature, 50 °C; liquid trap temperature, 30 °C; liquid trap solvent, H_2O; liquid trap volume, 8 mL.

Table 4. GG for Six Replicate Analyses Using the Best Overall Extraction Conditions[a]

wt of sample (g)	GG (mM)	GG (mM)/ g of sample
0.4456	20.15	45.21
0.5149	22.60	43.90
0.5357	27.52	51.37
0.5000	22.69	45.38
0.5476	23.83	43.52
0.5092	26.29	51.63
av		46.84
RSD (%)		7.2

[a] For extraction conditions see Table 3, expt h.

Additional trials were performed with the modifier added in the static mode and increased percentages of modifier in the dynamic mode [30% (experiment c) and 40% (experiment d)]. The results were worse than the extraction developed with 20% modifier. The reason for the decreased recovery when the amount of modifier is increased could be that the more polar analytes are transferred to the CO_2 insoluble solvent instead of the extracting fluid, which is less polar. Therefore, a higher amount of modifier means lower recoveries. In a previous work with polar compounds (phenolic acids and aldehydes) a similar problem was found (Palma and Taylor, 1999). An additional dynamic extraction step using 100% CO_2 proved to be a good solution. Three more experiments were done using the same conditions but with a 10 min second dynamic extraction step using 100% CO_2 (experiments e−g in Table 3). The recoveries were higher than in previous experiments and the differences in recovery more notable in the cases of trials with higher percentages of modifier.

These results point out that it is necessary to increase the time for drying the extraction vessel using 100% CO_2. However, with this method the total time for the extraction could be as long as the classic extraction method. Therefore, the amount of modifier in the static

mode was increased as a way of increasing the recovery without increasing the extraction time. The result is shown in Table 4 (experiment h). Experiment h was done using the same conditions as in the other experiments except for using 2 mL of methanol in the static step. As a result, the recovery increased to 94.1%, which can be considered quantitative.

The extraction conditions for experiment h were then applied to real samples. The weight of real sample used was 0.4456−0.5476 g, and the results for the analysis of GG per gram are shown in Table 4. Six extractions were done to measure the reproducibility and to compare the extraction recovery with that of the classic extraction method. The relative standard deviation (7.2%) was similar to those obtained by Williams et al. (1995). The average GG concentration obtained using the method of Williams et al. over the same samples was 45.95 mmol/g of sample, so our recovery relative to that of the classic method was 102%. Therefore, the analytical method developed to determine glycosides using SFE has the same reproducibility and the same recovery as the more conventional method. There are two advantages to using SFE. The first is that the extraction time is 25% shorter, and the second is that, by SFE, the method could be fully automated.

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Received for review August 24, 1999. Revised manuscript received December 14, 1999. Accepted December 21, 1999.

JF990957P