Supercritical Fluid Extraction of 5-Hydroxymethyl-2-furaldehyde from Raisins

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An extraction method based on supercritical CO_2 has been developed for the analysis of 5-hydroxymethyl-2-furaldehyde in raisins. To optimize extraction variables, a fractional factorial experimental design was applied. Six extraction variables were optimized. The organic modifier used for increasing the extraction fluid solvating power was the most important factor. Methanol as organic modifier produced 10-fold higher recoveries of 5-hydroxymethyl-2-furaldehyde than ethyl acetate. The efficiency of the organic modifier in the static extraction phase was compared with using it in the dynamic extraction phase. Repeatability of the analysis method was evaluated, which resulted in an RSD of <5%. 5-Hydroxymethyl-2-furaldehyde was quantified in raisins, and the concentration was found to be 0.128 mg/g of raisin.

Keywords: *Extraction; 5-hydroxymethyl-2-furaldehyde; raisins; organic modifier*

INTRODUCTION

Food products that contain sugar can undergo Maillard-type reactions when the foods are heated. These reactions produce changes in the flavor, color, and nutritional value of the foods. Furfurals (e.g., furaldehyde derivatives) are the main degradation products of hexoses and pentoses (*1*, *2*). An important flavor compound in this regard is 5-hydroxymethyl-2-furaldehyde (5-HMF), which can be found in several fruits both before and after processing (*3*−*5*). There is considerable interest in developing analytical methods to monitor 5-HMF because its concentration can be used as (1) an index of the heat treatment applied to foods and (2) a marker to measure the extent of deterioration in foods such as tomato paste, honey, and fruit juices (*6*).

The concentration of 5-HMF in various food products covers a broad range. Whereas the values in wine, spirits, and fruit juices have been found to be as high as 200 mg/L, prune juices may have 5-HMF concentrations up to 1000 mg/L (*7*). Caramel solutions, which are added to food products to enhance both color and flavor, are another possible source of 5-HMF because caramels are usually obtained by thermally concentrating fruit juices and extracts. Consequently, the concentration of 5-HMF has been suggested to serve as a criterion for detecting the adulteration of food products with caramel (*8*). In wood-aged drinks, such as wines, vinegars, and brandys, wood cooperage can be an additional source of 5-HMF. For this reason, 5-HMF has been suggested as an aging marker for certain "wood-aged" beverages (*9*−*11*). Furanic aldehydes, which are thought to be produced during the barrel's production, are then extracted by the aged alcoholic beverages inside the barrel (*12*).

From a food-processing point of view, the analysis of 5-HMF is consequently important. Because 5-HMF has been reported to possibly act as an initiator and promoter of colon cancer, its analysis takes on added significance (*13*, *14*). Older analytical methods for 5-HMF involved specific colorimetric reactions, but nowadays most analytical methods are based upon reversed phase HPLC (*11*) and gas chromatography (*15*, *16*). Sample preparation prior to chromatographic analysis is normally via liquid extraction with either methanol or ethyl acetate (*17*).

The analysis of 5-HMF has been reported for a variety of liquid and semisolid matrices. For example, the concentration of 5-HMF in apple juices (*18*, *19*), brandys (*11*), commercial caramels (*20*), and wines (*21*) has been determined by reversed phase HPLC. Similar studies have been performed on various types of milk products (*22*) and formulated infant milk (*23*). The analysis of 5-HMF in honey has also been reported because levels of 5-HMF can reflect an inadequate manufacturing or storing process (*24*).

Supercritical fluid extraction (SFE) has been found to be very effective for the removal of polyphenols from solid matrices (*25*−*27*). Although 5-HMF is not a phenolic compound, its solubility in organic solvents and its chromatographic retention behavior are very similar to those of phenolic compounds (*11*). It mimicks closely the properties of gallic acid, which is the most polar phenolic compound found in grapes and grape-derived products. Consider that the analysis of phenolic compounds and furfural derivatives is similarly carried-out by first extracting the compounds with diethyl ether followed by HPLC-UV analysis (*28*) of the extract.

To isolate 5-HMF from raisins, supercritical CO_2 has the advantage in that sugars exhibit low solubility in CO_2 at all experimentally feasible densities. This means that CO_2 extracts will be free of sugars, the primary compounds in raisins. Moreover, SFE affords relatively low extraction temperatures under anaerobic conditions, which should discourage the formation of additional 5-HMF during sample preparation. During the raisin-making process, the fruit is dried under sunlight to reduce the amount of water and to facilitate the reac-

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tions that produce the characteristic flavors. Currently, this loss in weight is the index used for controlling the raisin-making process. Because 5-HMF is one of the more characteristic flavor components in raisins, controlling its formation should afford greater optimization of the raisin-making process, and because raisin extract is used as an additive for some spirituous beverages such as sherry brandy (*29*), extracts are desired with as much furfurals (e.g., 5-HMF) as possible. Therefore, more effective extracts can be prepared if the content of 5-HMF in raisins can be easily monitored. Liquid extracts (thermally produced) many times yield an increase in the amount of 5-HMF in the raisin sample, thus leading to an erroneous value. Furthermore, these extracts contain compounds that coelute with 5-HMF by reversed phase HPLC, thus necessitating some sample cleanup prior to chromatographic analysis (*6*, *30*). Incorporating a solid phase trap during SFE, however, readily affords one the opportunity to fractionate the extract prior to analysis. We therefore wish to describe our work with raisins and pressurized CO_2 with regard to the analysis of 5-HMF.

EXPERIMENTAL PROCEDURES

Samples. Seedless raisins were obtained from a local supermarket. The raisins were sliced rather than ground (which would have been preferred) because 5-HMF is not uniformly distributed throughout the raisin. As 5-HMF is derived from the sugars, it should be more concentrated in the part of the raisin related to the grape pulp and less concentrated in both the raisin skin and center of the raisin where the seeds should be. When raisins are ground in a coffee grinder, a very viscous, nonhomogeneous paste is produced and the temperature increases. Consequently, concern over whether a representative sample could be obtained and whether the 5-HMF analysis would truly reflect the amount in the untreated raisin led us to the "slicing" procedure. For the experimental design phase of the study, sample weights ranged from 55.2 to 150.7 mg.

Apparatus. An Isco-Suprex PrepMaster (Lincoln, NE) equipped with an SSI 222D HPLC pump was used for the supercritical fluid extractions. An HPLC series 1050 from Hewlett-Packard (Little Falls, DE) equipped with an autosampler, a quaternary pump, and a UV−visible multiwavelength detector was used for extract analyses.

Chemicals. The solid phase extraction trap used on the supercritical fluid extractor was packed with silica-based Upchurch-C18 (0.8 g) from Chrom Tech (Apple Valley, MN). Ottawa Sand Standard (20−30 mesh) and HPLC grade solvents, including ethyl acetate, methanol, acetic acid, and water, were obtained from Fisher Scientific (Houston, TX). The 5-HMF and β-resorcylic acid standards were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. Carbon dioxide, with helium headspace, was obtained from Air Products and Chemicals, Inc. (Allentown, PA).

Extraction Protocol. Extractions were conducted in 8 mL stainless steel vessels. Sand was used to support the sample as well as to occupy dead volume in the vessel. For a particular set of conditions, extractions were done in duplicate. An experimental design strategy covering six extraction variables was employed. The high/low values were as follows: density, 0.85/0.95 g/mL; modifier, ethyl acetate/methanol; organic modifier percentage, 20/40%; static extraction time, 20/30 min; dynamic extraction time, 15/30 min; extraction temperature, 35/55 °C. Extraction parameters that were common to each experiment were as follows: 0.3 mL of organic modifier directly added to the sample matrix; liquid CO_2 flow rate = 1 mL/min; solid phase extraction trap temperature = 75 °C, rinse solvent for solid phase trap = methanol; rinse solvent flow rate = 0.2 mL/min. The rinsing time was 10 min.

HPLC Analysis. An Aluna-C_{18} column (150 × 2 mm, 5 μm particle size) from Phenomenex (Torrance, CA) was used. The binary mobile phase consisted of 2% acetic acid in water and 2% acetic acid in methanol. The elution gradient in methanol was as follows (time in minutes) (methanol percentage): (0) (0), (5) (8), (8) (15), (12) (15), (19) (50).

Table 1. SFE Conditions Used in the Experimental Design

expt	density, g/mL	modifier	%	SFE_{sta} (min)	SFE_{dyn} (min)	T_{ex} (°C)	relative recovery[a]
1	0.85	AcOEt	20	20	15	35	0.67
2	0.95	AcOEt	20	20	30	35	0.29
3	0.85	MeOH	20	20	30	55	2.81
4	0.95	MeOH	20	20	15	55	3.05
5	0.85	AcOEt	40	20	30	55	0.30
6	0.95	AcOEt	40	20	15	55	0.36
7	0.85	MeOH	40	20	15	35	3.31
8	0.95	MeOH	40	20	30	35	1.41
9	0.85	AcOEt	20	30	15	55	0.30
10	0.95	AcOEt	20	30	30	55	0.16
11	0.85	MeOH	20	30	30	35	3.85
12	0.95	MeOH	20	30	15	35	6.44
13	0.85	AcOEt	40	30	30	35	0.29
14	0.95	AcOEt	40	30	15	35	0.34
15	0.85	MeOH	40	30	15	55	2.47
16	0.95	MeOH	40	30	30	55	1.82

[a] Relative recovery = (area of 5-HMF)/(area of I.S.)(g of sample).

RESULTS AND DISCUSSION

For the samples in our experimental design, an internal standard was added to the extract before the chromatographic analysis. In that case, relative recovery to the internal standard was used as the target variable. To quantify the amount of 5-HMF, some extracts were diluted to 10 mL, before the chromatographic analysis. For all of the extracts, 20 μL was injected in the chromatographic system.

A fractional factorial experimental design was performed to optimize the extraction variables. The working variables were density of the supercritical fluid, organic modifier (mod), percentage of organic modifier, time for static extraction (SFE_{sta}), time for dynamic extraction (SFE_{dyn}), and extraction temperature (T_{ex}). There were more variables involved in the extraction process; however, these were established to be less critical on the basis of our previous work done with polyphenolic compounds and grape seeds (*25*, *26*).

A fractional experimental design was utilized to reduce the number of experiments. Normally, 64 experiments would be needed to evaluate the six variables in two levels, whereas a fractional design allows for doing 16 experiments instead of 64. Statistical treatment of the extracts affords enough information to ascertain the best extraction conditions. This kind of experimental design has produced good results in previous work with both real and spiked samples (*25*, *26*). A Minitab program (State College, PA) has been used for the experimental design and for the treatment of the resulting information. The actual experimental design is shown in Table 1. The values for the extracting variables were established on the basis of our previous work with phenolics.

Raisins were sliced to increase surface area. This strategy was very successful because recovery was generally 5-fold higher than when using whole raisins. Approximately 50 raisins were cut (not ground) into ~0.1 g portions to create a sample pool. All of the pieces from different raisins were mixed together, so that experiments were not done over different parts of the same raisin but over different parts of different raisins.

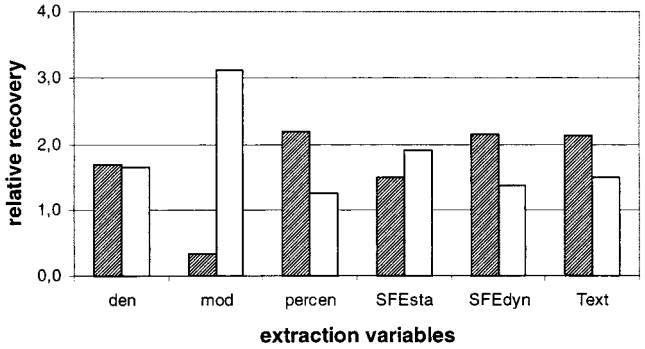

Figure 1. Main effects of variables studied concerning the relative recovery of 5-HMF from raisins: (slashed bars) lowest level; (white bars) highest level.

The weight of a single raisin was ~0.5 g. Each raisin was cut into approximately four pieces of 0.1 g/each. Because slightly different weights of sample were taken and different volumes of extract were obtained due to the different extraction conditions assayed, an internal standard (e.g., β-resorcylic acid) was employed to compare recoveries. The target value we have used in the experimental design is the ratio of the peak area of 5-HMF and the product of the peak area of the internal standard and weight of the sample. All extractions were performed in duplicate.

The amount of sample taken for each extraction (~0.1 g) was relatively high; consequently, no quantitative extractions were realized because of the limited extracting fluid used. Because no experiments yielded 100% recovery of 5-HMF, it was, therefore, possible to compare recovery for all of the assayed conditions. The incomplete recovery was determined by re-extraction of the raffeinate and analysis for 5-HMF in the second extracts for all of the experiments in the experimental design.

The relative recoveries obtained from the experimental design approach are shown in Figure 1. The most important factor appears to be the nature of the organic modifier. Extractions developed using ethyl acetate (EtOAc) showed a 10-fold lower recovery than extractions done using methanol (MeOH) as organic modifier (e.g., ratio 0.3% versus 3.1%). Other variables either showed lower or no effect on the recoveries. The same average recovery was recorded for both 0.85 and 0.95 g/mL CO_2. For the other variables, the average recoveries were ~30% higher using (a) 20% organic modifier instead of 40%, (b) 30 min of static extraction time instead of 20 min, (c) 15 min of dynamic extraction time instead of 30 min, and (d) 35 °C extraction temperature instead of 55 °C.

To determine the best value of density to use, interaction between variables was studied. The interaction effects between density and (a) percentage of modifier, (b) time of static extraction, and (c) time of dynamic extraction are shown in Figure 2. Our initial consideration of the data had suggested that the best values for these variables were 20% modifier, 30 min static time, and 15 min dynamic time; the best value for CO_2 density in all three cases was determined to be 0.95 g/mL CO_2. Therefore, the extraction parameters that provided a "best" recovery are 0.95 g/mL CO_2, 20% methanol-modified fluid, 30 min of static time, 15 min of dynamic time, and 35 °C extraction temperature.

The resulting optimized extraction conditions for raisins are very similar to those obtained for phenolic

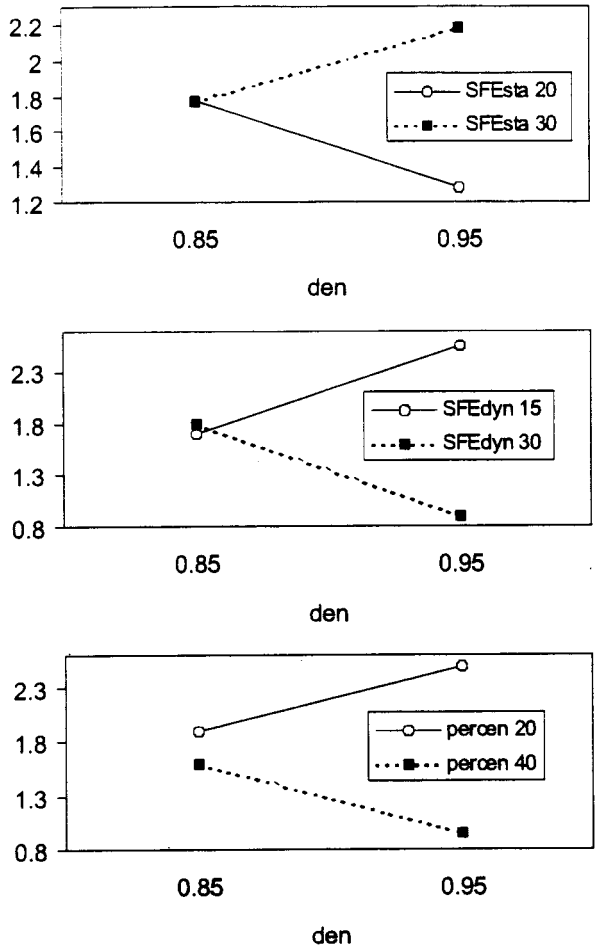

Figure 2. Effects of interaction between static extraction time, dynamic extraction time, and percentage of modifier with density of the extracting fluid on the recovery of 5-HMF from raisins.

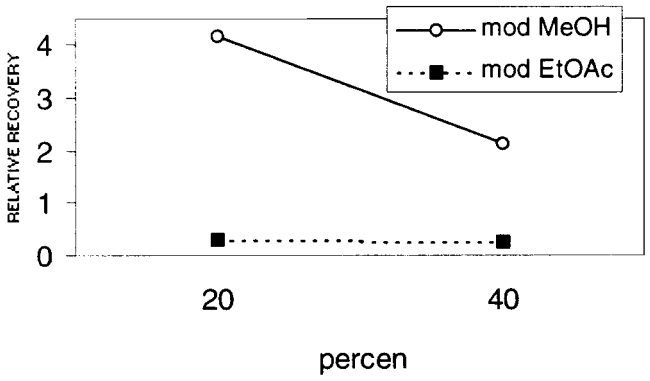

Figure 3. Interactions between percentage and kind of modifier on the recovery of 5-HMF from raisins.

compounds from grape seeds (*26*). The main differences are the organic modifier and its percentage. For phenolics, EtOAc (40%) gave better results, whereas for 5-HMF, MeOH (20%) gave better results. As Figure 3 shows, we studied both modifiers at the 20 and 40% levels. At 40%, the MeOH modifier yielded a higher recovery than EtOAc. The same was true at 20% modifier, but the difference in recoveries for the two modifiers was surprisingly greater at the lower modifier concentration. Therefore, MeOH has a greater effect on changing the solvating power of the CO_2 than EtOAc when 20% of modifier was used. It is well-known that for modifier levels >5% and subambient solid phase trap

Table 2. Influence of the Percentage of MeOH Modifier on Recovery of 5-HMF[a]

expt	%	recovery[b]
a	15	91
b	20	100
c	25	80

[a] Common conditions: density = 0.95 g/mL; static modifier = 0.3 mL of MeOH; modifier = MeOH; SFE_{sta} = 30 min; SFE_{dyn} = 15 min; T_{ex} = 35 C. [b] Recovery relative to optimized method b.

Table 3. Influence of the Amount of Organic Modifier on the Recovery of 5-HMF

method	static modifier (mL)	static time (min)	extraction fluid (g)	% of modifier	recovery[a]
1	0.3	30	15	20	96.2
2	0	30	15	20	77.8
3	0	20	15	30	85.6
4	0	20	15	40	93.7
5	0	5	15	40	78.1

[a] Recovery calculated relative to the amount of 5-HMF spiked in the extraction vessel.

temperature, modifier can condense on the solid trap, thus causing the possible elution of extracted compounds from the trap. MeOH has a higher solvating power for rinsing the C_{18} solid phase trap than EtOAc, so higher percentages of MeOH can result in greater losses during the dynamic extraction step. For 5-HMF analysis in real samples it is very interesting to be able to use the solid trap as a cleaning step after the SFE and prior to HPLC analysis, so it is necessary to guarantee no losses of analyte from the solid trap during the dynamic extraction. Fractionation of the extract in the solid trap has given very useful results in a previous work (*31*).

Due to the dependence of modifier percentage on 5-HMF recovery, several other percentages were examined to guarantee that the optimal modifier amount was being used. As 20% gave the better results, both 15 and 25% modifier levels were assayed using the values previously optimized for the other extraction variables. Table 2 shows the recovery results relative to the method using 20% MeOH. It is demonstrated that lower recoveries were obtained at both 15% (e.g., poorer solvating fluid) and 25% (e.g., trapping losses) modifier.

In all raisin extracts 0.3 mL of organic modifier was added to the sample matrix prior to initiation of the extraction. To evaluate the influence of this practice, additional experiments were performed without this matrix modifier. Because we wish to evaluate the solubility of 5-HMF in the extracting fluid, extractions were done on a spiked sand matrix. Table 3 shows the actual extraction conditions and the resulting recoveries compared to the amount of 5-HMF spiked onto the sand. Two sets of extraction conditions produced recoveries >90%. The first method (1) was the previously optimized with 0.3 mL of matrix modifier. The second method (4) used 40% modifier in the fluid phase and no matrix modifier. If the static modifier was not used under the same fluid conditions, the recovery was 18.4% lower (1 versus 2). Increasing the modifier from 20 to 30% in the dynamic step increased recovery only 8% (3 versus 4). If the static extraction time was reduced slightly and the percent organic modifier increased, recoveries were comparable (2 versus 3). Static time was, however, critical because a dramatic reduction from 20 min caused the recovery to be greatly diminished (4 versus 5).

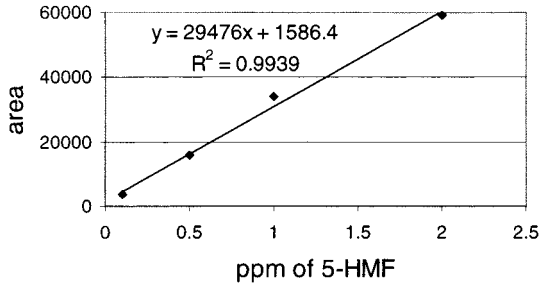

Figure 4. Calibration results for 5-HMF. Limit of detection: 0.192 ppm. Limit of quantification: 0.645 ppm.

The repeatability of the developed method was of interest to us. In this regard, five extractions of raisins were performed under the optimized extraction conditions. The ratio of the 5-HMF chromatographic peak area and the product of the internal standard peak area and sample weight were used to express repeatability. The obtained RSD was 5.0%.

Last, the amount of 5-HMF was determined in raisins using SFE as the method of sample extraction. Extracts were diluted to 10 mL with MeOH. Prior to analysis, a calibration curve over a suitable concentration range was constructed. Four levels of concentration were employed in duplicate (Figure 4). A limit of detection (0.192 ppm) and limit of quantification (0.645 ppm) were determined using the Alamin program (University of Granada, Spain) following a published method (*32*). The signals produced by the diluted extracts were always above the limit of quantification. Taking into account the weight of the raisin sample used in each extraction, the average amount of 5-HMF was 0.128 mg/g of raisin.

In conclusion, the developed SFE method appears to be viable for the determination of the content of 5-HMF in raisins. The method is reliable and fast. Because the extractions conditions are "soft" and nonpolluting, the methodology should be extendable to other fruits and wood products.

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