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Food Chemistry 90 (2005) 281–286
Food Chemistry
www.elsevier.com/locate/foodchem

In vitro digestibility and intestinal fermentation of grape seed and peel

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Received 23 February 2004; accepted 26 March 2004

Abstract

Grape seed and peel are increasingly being used to obtain functional food ingredients such as natural antioxidants and dietary supplements. The indigestible fraction constitutes the bulk of grape peel and seed (about 80% dry matter). This fraction is neither digested nor absorbed in the small intestine and reaches the colon, where it provides a substrate for fermentative microflora. The objective of this work was to determine the extent of colonic fermentation of grape peel and seed constituents and to evaluate the potential digestibility and bioavailability of their main components (dietary fiber, protein and polyphenols) in the gastrointestinal tract. The extent of fermentation, expressed as per cent disappearance of organic matter (DOM), was similar for the two grape materials (about 32%). The intestinal microflora degraded 95–97% of total polyphenols, 30–32% of dietary fiber and 60–70% of protein in both seed and peel. Total production of short chain fatty acids and molar proportions (acetic:propionic:butyric, 59:27:14) were similar for the two samples. It was estimated that about 25% of grape seed and peel was degraded into the colon, being 50% unavailable in the gastrointestinal tract.

Keywords: Grape seed; Grape peel; Digestibility; Colonic fermentation

1. Introduction

Grape pomace consists of peels, seeds and stems and accounts for as much as 20% of the weight of grape processed into wine and juice. After removal of stems, a wide range of products can be obtained, including ethanol, grape peel oil, tartrate and anthocyanins. Grape seed and peel are increasingly being used to obtain functional food ingredients such as natural antioxidants and dietary supplements (Girard & Mazza, 1998; Shrikhande, 2000).

The composition and physiological properties of grape pomace have been reported by the authors elsewhere (Bravo & Saura-Calixto, 1998; Martin-Carron, García-Alonso, Goñi, & Saura-Calixto, 1997). Indigestible compounds are the main constituents of grape peel and seed accounting for about 80% of sugar-free dry matter. The digestible fraction is made up of oil, protein and minerals (Bravo & Saura-Calixto, 1998).

There are many references in the literature to the composition and antioxidant properties of grape polyphenols (Gonzalez-Paramas et al., 2004; Yilmaz & Toledo, 2004), but there have been very few studies on the digestibility and intestinal degradation of polyphenols and other major grape constituents.

The indigestible fraction (IF) has been defined as the part of plant foods that is not digested or absorbed into the small intestine, but reaches the colon where it provides a substrate for the fermentative microflora (Saura-Calixto, García-Alonso, Goñi, & Bravo, 2000). Colonic microflora may play an important role to metabolize undigestible compounds. Dietary fiber, phenolic compounds, and protein are the main constituents of grape IF.

Short-chain fatty acids (SCFA) are the fermentation end products that most benefit physiological properties

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0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.03.057

in human (Toping & Clifton, 2001). The indigestible and non-fermented fraction is the unavailable matter.

The objective of this work was to determine the extent of colonic fermentation of grape peel and seed constituents and to evaluate the potential digestibility and bioavailability of their main components in the gastrointestinal tract.

A previously validated in vitro model of colonic fermentation, used in dietary fiber studies (Barry et al., 1995; Goñi & Martín-Carrón, 1998) and a recent procedure to determine digestibility and bioavailability of protein (Goñi, Gudiel-Urbano, & Saura-Calixto, 2002) were combined to carry out this study.

2. Materials and methods

2.1. Materials

White grape pomace (*Vitis vinifera* var. Airén) was supplied by Bodegas Los Llanos (Valdepeñas, Spain). Stems were manually removed and pomace was dried in an air-circulating oven at 60 °C for 48 h. Peels and seeds were separated. They were milled to a particle size of less than 0.5 mm in a Cyclone Sample Mill (Tecator, Höganäs, Sweden).

2.2. Experimental procedure

A scheme of the experimental design is shown in Fig. 1. Samples were treated with protease (Sigma, P-3910), (buffer pH 6, 60 °C) followed by centrifugation (15 min, 3000*g*) to isolate the indigestible compounds such as dietary fiber, protein and polyphenols (Residue 1). Since these are starch-free samples, no treatment with heat-stable α-amylase and amyloglucosidase was needed. The digestible constituents were discarded in the supernatant.

Residue 1 was fermented under anaerobic conditions, following the procedure described above. Residue 2 contains non-fermented indigestible compounds (dietary fiber, protein and polyphenols) along with bacterial

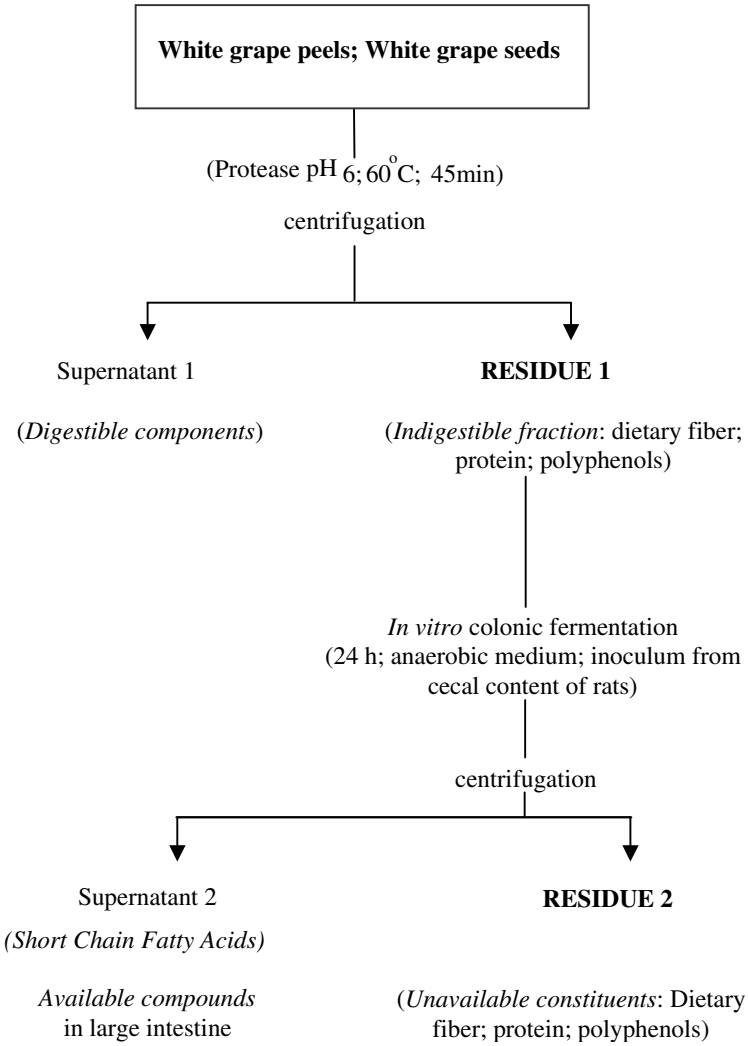

Fig. 1. Scheme of the method for determination the digestibility and fermentability of grape constituents.

mass. The supernatant 2 contains fermentation end products, mainly SCFA.

2.2.1. Total dietary fiber

The AOAC enzymatic–gravimetric method for dietary fiber determination in original grape materials, non-digested residues and non-fermented residues was followed (Prosky, Asp, Schweizer, Vries, & Furda, 1992). Samples were treated with heat stable amylase (Sigma, A-3306), protease (Sigma, P-3910) and amyloglucosidase (Sigma, A-9913). Soluble dietary fiber was precipitated by addition of ethanol. Residue was separated by centrifugation (15 min, 3000*g*) and successively washed with 95% ethanol and acetone, dried and weighed.

2.2.2. Protein

Grape original materials and fractions isolated following the scheme in Fig. 1 were analyzed for total nitrogen by using an automated nitrogen analyzer (LEKO, FP-2000, LEKO Corporation, MI, USA).

Samples were combusted in the pure oxygen environment of the furnace. After passing through a thermoelectric cooler to condense water, an aliquot from the combustion gases was taken. Gases were bubbled and all nitrogen-containing materials reduced to nitrogen and detected by a thermal-conductivity cell. An air blank was carried out and the instrument calibrated with EDTA. Protein was calculated as nitrogen $\times$ 6.25.

2.2.3. Total polyphenols

Total extractable polyphenols (EPP) were extracted from samples by shaking at room temperature with methanol-water (50:50 v/v, 50 ml/g sample, 60 min, room temperature; constant shaking) and acetone–water (70:30 v/v, 50 ml/g sample, 60 min, room temperature; constant shaking). After centrifugation (15 min, 3000*g*) supernatants were combined and made up to 100 ml. Total polyphenols were quantified as gallic acid equivalents using the Folin–Ciocalteau reagent (Montreau, 1972).

2.2.4. In vitro colonic fermentation

Preparation of inoculum: Cecal content of adult rats and sterilized anaerobic medium (100 $g\,l^{-1}$) were mixed for 10 min in a Stomacher 80 Lab Blender (Seward Medical, London, UK) and filtered (1 mm mesh).

2.2.4.1. Procedure. Substrates were hydrated in fermentation medium (8 ml/100 mg of substrate dry matter) at 4 °C for 16 h. The medium contained trypticase, micro and macromineral solutions and resazurin as anaerobic redox indicator in proportions described elsewhere (Goñi & Martín-Carrón, 1998). Vials were sealed with rubber caps (Ormacisa, 407-0-13, Madrid, Spain) and substrates were left overnight at 4 °C. 2 ml of inoculum was added into each vial and the headspace rinsed with carbon dioxide. Vials were placed in a shaking water bath at 37 °C for 24 h. Lactulose (Sigma, L-7877) was included in the experiment as a completely fermentable substrate. Substrates and blanks (inoculum without substrate) were fermented in triplicate for 24 h.

The pH was measured in each sample and the fermentation process was stopped by adding 1 M sodium hydroxide. Samples were centrifuged and the supernatant was taken in duplicate for SCFA determination. Residues were stored at −20 °C until use.

2.2.4.2. SCFA analysis. The method of Spiller et al. (1980) was followed with some modifications. A 400 μl aliquot of supernatant from the fermentation samples with 100 μl internal standard (50 $mmol\,ml^{-1}$ 4-methyl valeric acid) (Sigma, 27,782-7) and 10 μl of formic acid, (Merck, 264.0100, Farma-Química SA, Madrid, Spain) were made up to 1 ml with water and centrifuged (4 °C, 6000*g*, 15 min). 2 μl of supernatant were injected into a 5890 Hewlett–Packard gas chromatograph equipped with a flame ionisation detector and a capillary column (HP-FFAP, 10 m $\times$ 0.53 mm i.d., Hewlett–Packard 19095F-121, Delta Científica SL, Madrid, Spain). Nitrogen was the carrier gas at a pressure of 17 kPa. Injector and detector temperatures were 200 and 220 °C, respectively. The column temperature program was: initial temperature 100 °C for 2 min, rate 10 °C/min and final temperature 120 °C for 2 min. SCFA were identified and quantified by comparison with known fatty acid standards. SCFA produced were expressed as μmol/mg of dry substrate.

The percentage of fermentability of samples was calculated by considering total SCFA produced from lactulose as 100% fermentability.

2.2.4.3. Determination of dry matter disappearance. The method followed to quantify the non-fermented residue was adapted from Guillon, Renard, Hospers, Thibault, and Barry (1995). Fermentation residues were suspended in 50 ml of saline solution, stirred in a Stomacher 80 for 3 min. They were washed twice and were filtered through Dacron cloth (mesh size 150 mm) to remove bacterial mass. Finally, they were washed with solvent exchange (95% ethanol and acetone) and dried at 60 °C overnight.

The disappearance of each indigestible compound was calculated as the difference between initial amount of these compounds in the substrate for fermentation and the non-fermented residue. It was expressed as a percentage.

3. Results and discussion

Plant foods contain a significant indigestible fraction that is neither digested nor absorbed in the small intestine

but passes into the colon as a substrate for fermentative microflora. As such, it comprises not only DF but also other compounds such as a protein fraction, polyphenols and other associated compounds (Saura-Calixto et al., 2000). The non-digested and non-fermented fraction are unavailable matter excreted in feces.

Grape seed and peel are obtained from pomace of wine production. White seed and peel were chosen because the total polyphenol content in white grape pomace was higher than in red grape pomace, which is related to the fact that in the preparation of white wine, the pomace is removed before alcoholic fermentation (Larrauri, Ruperez, & Saura-Calixto, 1996). The indigestible fraction (IF) is a major component of grape pomace (about 80% of dry matter). The detailed composition of the IF in grape peel and seed was previously reported (Bravo & Saura-Calixto, 1998).

The IF and the unavailable matter of the samples were isolated following the experimental procedure indicated in Fig. 1. The composition of indigestible residues of grape peel and seed (residue 1 in Fig. 1) is indicated in Table 1. DF was the main constituent of grape samples (72–79%) remaining in the residue after enzymatic treatments. The protein concentration in the indigestible residues was high (14%), indicating that about 88% of the protein content in grapes was not digested by the digestive enzymes and may reach the colon along with DF constituents. Such an exceptionally high amount of indigestible protein was also found in other vegetables rich in polyphenolic compounds, such as cacao (Bartolome, Jimenez-Ramsey, & Butler, 1995) and carob pods (Bravo, Mañas, & Saura-Calixto, 1993). The formation of protein–polyphenol complexes and the enzymatic inhibition of polyphenolic compounds prevent the removal of protein (Butler, 1992).

As regards polyphenols, only part of the EPP in the original matter was solubilized in intestinal fluids and became potentially available in the small intestine but a significant part remained in the IF (3.1%), subject to bacterial fermentation in the colon. Indigestible residues were partially degraded in the large intestine by colonic microflora and are therefore not an inert part of the grape materials. To the contrary, indigestible food components are essential to feed intestinal bacteria and maintain health in the intestinal tract. The amount and composition of the non-fermented substrates (residue 2 in Fig. 1) are shown in Table 2. The extent of fermentation, expressed as per cent disappearance of organic matter (DOM), was similar for the two grape samples (about 32%).

Table 2
Disappearance of organic matter (DOM)[a] and major constituents of non-fermented residues in white grape peel and seed[b]

	Grape peel	Grape seed
DMO[a]	31.5 ± 7.85	31.5 ± 8.07
Dietary fiber	74.8 ± 0.41	79.9 ± 0.35
Protein	8.13 ± 0.30	6.00 ± 0.21
Extractable polyphenols	0.19 ± 0.09	0.17 ± 0.02

[a] [(mg of substrate−mg of residue non-fermented) × mg of substrate^{-1}] × 100.
[b] Mean values ± standard deviation, $n = 3$; % on dry basis.

Inhibitory effects of polyphenols on fermentation patterns have been described (Ahn et al., 1993). However, previous research by the authors found that when adult rats ingested these grape by-products and the fermentative capacity of the cecum bacteria was measured after 6 weeks of dietary treatment, that fermentative capacity was not inhibited (Martín-Carrón & Goñi, 1998).

Intestinal bacteria showed a high capacity to degrade EPP, with up to 95% of EPP in indigestible residues being degraded during fermentation (Tables 2 and 3). Déprez et al. (2000), also reported that major phenolic constituents of grape pomace (polymeric proanthocyanidins) were degraded by human colonic microflora. EPP in intestinal ecosystem were equally available to bacteria and to the host. These results are in agreement with values reported by other authors, who found low EPP content in faeces of animals (Bravo, Saura-Calixto, & Goñi, 1992) and humans (Garsetti, Pellegrini, Baggio, & Brighenti, 2000) fed with rich polyphenol materials.

Indigestible protein was considerably degraded by colonic bacteria (60–70%). They may induce changes in bacteria proliferation and in the formation of fermentation end products (Morita, Kasaoka, Hase, & Kiriyama, 1999). Further studies are needed to determine possible detrimental effects on health of colonic fermentation of protein.

DF was fermented by 30–32%. This degree of fermentability is typical of insoluble DF such as cellulose and lignin-rich materials (Bourquin, Titgemeyer, Garleb, & Fahey, 1992).

Indigestible components of the foods may be fermented by bacteria in the large intestine to produce

Table 1
Major indigestible compounds in white grape peel and seed[a]

	Grape peel	Grape seed
Dietary fiber	77.2 ± 1.43	78.9 ± 2.01
Protein	13.9 ± 0.31	13.6 ± 0.21
Extractable polyphenols	3.10 ± 0.91	3.61 ± 0.21

[a] Mean values ± standard deviation, $n = 4$; % on dry indigestible fraction.

Table 3
Fermentability of grape indigestible components[a]

	Grape peel	Grape seed
Dietary fiber	33.7 ± 0.61	30.6 ± 0.51
Protein	60.0 ± 0.30	69.8 ± 0.22
Extractable polyphenols	95.8 ± 0.9	96.81 ± 0.21

[a] Percentage of disappearance of indigestible compounds; Mean values ± standard deviation, $n = 3$; % on dry basis.

Table 4
Fermentability[a] and production of short-chain fatty acids (SCFA) (μmol/mg dry matter) of white grape peel and seed[b]

	Grape peel	Grape seed
Fermentability	33.2 ± 0.51	29.9 ± 1.11
Total SCFA	6.96 ± 0.11	6.27 ± 0.22
Acetic acid	4.09 ± 0.09	3.63 ± 0.15
Propionic acid	1.85 ± 0.03	1.70 ± 0.04
Butyric acid	1.02 ± 0.04	0.94 ± 0.08

[a] Fermentability: [(SCFA from substrate) × (SCFA from lactulose^{-1}) × 100.
[b] Mean values ± standard deviation, $n = 3$; % on dry basis.

short-chain fatty acids (SCFAs), predominantly acetic, propionic and butyric, which are metabolized by the colonic epithelium (butyrate), muscle (acetate) and liver (propionate) (Cummings & Macfarlane, 1997). There are numerous studies pertaining to the complex physiological effects of dietary fiber, and it is becoming clear that some of the effects are mediated by SCFAs. The architecture of the cell wall and the linkages between indigestible compounds may change the fermentability and turn the metabolism products into specific SCFAs (Titgemeyer, Bourquin, Fahey, & Garleb, 1991). Not only the total amount, but also the patterns of individual SCFAs, vary depending on the chemical composition.

Total SCFAs, percentage of fermentability (30–33%) and molar proportions acetic:propionic:butyric (59:27:14) were similar for the two grape samples (Table 4). Acetic acid was the most abundant product in the medium, but production of propionic and butyric acids was higher than in other dietary fibers such as pectin (79:13:7) or cellulose (73:21:4) (Berggren, Björck, & Nyman, 1993).

To summarize, grape seed and peel contain three different fractions: (I) Digestible components, (available in small intestine); (II) Indigestible compounds degraded by intestinal microflora, (available in large intestine); (III) Indigestible and non-fermented fraction (unavailable). Protein, dietary fiber and polyphenolic compounds of grape seed and peel were not equally utilized by intestinal bacteria during in vitro fermentation in terms of the extent of degradation.

Acknowledgements

The authors thank the support of the Spanish Comisión Interministerial de Ciencia y Tecnología (Project AGL2002-04104-C04-01) and CYTED (Project XI.18/CNPq).

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