Examination of Useful Components in Grape Pomace

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Abstract: Grape pomace is the solid waste material（skin, stem, leaf, and seed）that remains after pressing juice from the grapes. At present, it is mainly used as animal feed or for composting. This study has identified some of the useful components of grape pomace with a view to more effective utilization of this material in the future. We quantified the polyphenol concentrations of methanolic extracts of grape pomace from different varieties of grape. For each grape pomace, we used liquid chromatography–mass spectrometry（LC–MS）to detect the presence of anthocyanins and liquid chromatography –tandem mass spectrometry（LC–MS/MS）was used to assign each chromatogram peak. The effects of these components on the differentiation of 3T3-L1 cells were assessed. Polyphenols were found to be effectively retained in the grape skin（campbell, niagara, kerner, and chardonnay）in the grape pomace. Campbell pomace skin, merlot dried pomace leaf, kyoho pomace seed, and chardonnay pomace stem were analyzed, and anthocyanidins with sugars or organic acids were detected in the skin and leaf samples. Delphinidin detected in the skins was observed to inhibit the differentiation of 3T3-L1 cells.

Key words: grape, liquid chromatography-mass spectrometry（LC–MS）, 3T3-L1 cells, differentiation, delphinidin

Introduction

Grapes are cultivated in many regions throughout the world, and most are utilized for winemaking. In recent years, global wine production has stabilized, but consumption in Asian markets continues to gradually increase with population growth and disposable income. Despite continued high production, utilization of grape pomace is not well established. In the grape-producing district of Nagano Prefecture, Japan, about 570 tons of grape pomace is generated annually, only part of which is utilized as animal feed and compost. Most of the pomace is dumped as waste. Therefore, this study aimed to identify the useful components of grape pomace that can be effectively utilized in the future.

Our research group and others have shown that oral administration of grape indicates antioxidant activity of low-density lipoprotein and amelioration of cerebral ischemia/reperfusion-induced neuronal damage.[1,2] Epidemiological studies have also linked moderate wine consumption to a lower incidence of cardiovascular disease as part of the explanation of the so-called French paradox.[3,4] It is known that grapes include many polyphenols（anthocyanins, resveratrol, catechin, tannin, and others）and many of the beneficial health effects of grapes are attributed to these compounds.

Anthocyanins are a group of naturally occurring phenolic compounds that are responsible for the coloration of fruits and vegetables. They have many beneficial effects on human health, including reduced incidence of coronary heart disease, enhancement of visual acuity, maintenance of normal vascular activity, as well as anticarcinogenic, antimutagenic, anti-inflammatory, and antioxidative properties.[5,6] Recent studies in animals also suggest that anthocyanins can reduce body weight and fat mass.[7-9] However, the antiobesity effects of anthocyanins are not fully understood.

Obesity is characterized by the overgrowth of adipocytes, which is caused by an increase in the division and differentiation of pre-adipocytes. Adipocytes also store excess energy in the form of triglycerides and play an

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important role in releasing energy as glycerol and fatty acids.[9]

In this report, we describe the components of pressed grape pomace. We also show that the component of grape pomace inhibit the differentiation of 3T3-L1 cells.

Materials and methods

Plant materials

Pressed grape pomace was obtained from the Nagano Prefecture General Industrial Technology Center. The grape varieties included niagara (*Vitis* (*V.*) *labrusca*), kerner (*V. vinifera*), kyoho (*V. vinifera* × *V. labrusca*), chardonnay (*V. vinifera*), campbell (*V. labrusca*), and merlot (*V. vinifera*). All grapes were cultivated in Nagano Prefecture. The grape samples were separated into skin, stems, leaves, and seeds immediately after wine and juice production.

Sample preparation

Different parts of the grape were separated from the pomace and extracted with 80% ethanol. Respective extractions were performed on kyoho pomace seed; combined pomace skins from niagara, kerner, chardonnay, and campbell varieties; dried pomace leaf from merlot; and pomace stem from chardonnay.

For analysis of anthocyanin in merlot leaves, the grape skin samples were extracted with 12N HCl at 150℃, 30 min. After the sample was freezed, the samples were melted at room temperature (RT). All samples were filtered (0.45 μm), and stored as different aliquots at -20℃ until further analysis.

Determination of total polyphenol content

The total polyphenol content was determined by the Folin–Ciocalteu assay.[10] In brief, the Folin–Ciocalteu phenol reagent (Nacalai Tesque, Kyoto, Japan) was added to the sample and incubated in 1.5% Na_2CO_3 solution for 2 h at 20 ℃. The absorbance was recorded at 750 nm using a DU800 spectrophotometer (Beckman Coulter, Brea, CA, USA). The results are expressed as (+)-catechin (Wako Pure Chemicals, Osaka, Japan) equivalents.

Analysis of specific phenolic compounds

Separation of individual phenolic components in various extracts was performed on an Inertsil ODS C18 column (GL Sciences Inc., Tokyo, Japan) with detection at 520 nm. The mobile phase for chromatographic separation consisted of: solvent A, 0.1% trifluoroacetic acid (TFA) in water (v/v); solvent B, water/acetonitrile/acetic acid/TFA (50.4:48.5:1:0.1). The gradients were 0 min, 70% solvent A, 30% solvent B; 0-30 min, 20% solvent A, 80% solvent B. The flow rate was 0.5 ml/min and the injection volume was 10 μl. The identification of individual phenolics was achieved by comparison with characteristic spectra and their retention times.

Cell culture

3T3-L1 cell line was obtained from the American Type Culture Collection (ATCC, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (D-MEM; high glucose; Sigma, MO, USA) supplemented with 10% fetal calf serum (FCS; Wako, Osaka, Japan), 10,000 units of Penicillin G and 10,000 μg/mL streptomycin sulfate (P/S; Invitrogen, MA, USA) at 37℃ under a 5% CO_2 atmosphere.[11, 12]

Differentiation of 3T3-L1 cells

Pre-adipocytes were induced to differentiate into adipocytes after 48 h of confluence in a 3.5-cm dish (Sigma, MO, USA). The cells were incubated with six types of differentiation media [D-MEM, 0.5 mM isobutylmethylxanthine (IBMX), 0.25 mM dexamethasone, 1 μg/mL insulin, 10% FCS, 1% P/S]. The differentiation pattern was studied by observing the morphological changes and by estimating oil droplet accumulation in the cells. The differentiated adipocytes were taken for oil red O staining after 8 days in culture. [11, 12]

Oil red O staining

The staining procedure was performed as mentioned previously with few modifications.[11, 12] The cells were washed twice with PBS and then were fixed for 2 h with 3.7% formaldehyde. Fixed cells were incubated with oil red O for 15 min at room temperature. After the cells were washed once with water, the stained lipid droplets in the cells were visualized by light microscopy.

Results

Polyphenol concentration

The extracts of campbell, niagara, kerner, and chardonnay pomace skins, kyoho pomace seed, merlot dried pomace leaf, and chardonnay pomace stem all contained recognized polyphenols (Fig. 1). The polyphenol content in dried leaf was considerably higher than in the other pomace materials and this difference was attributed to the much lower water content in the leaf.

Effect on differentiation of 3T3-L1 cells by grape pomace samples

We examined the differentiation of 3T3-L1 cells and observed inhibition of differentiation in cells treated with extracts of niagara, kerner, chardonnay, and campbell pomace skins, and merlot dried pomace leaf (Fig. 2).

Qualitative analysis of anthocyanin

Extracts of campbell pomace skin, merlot dried pomace leaf, kyoho pomace seed, and chardonnay pomace stem

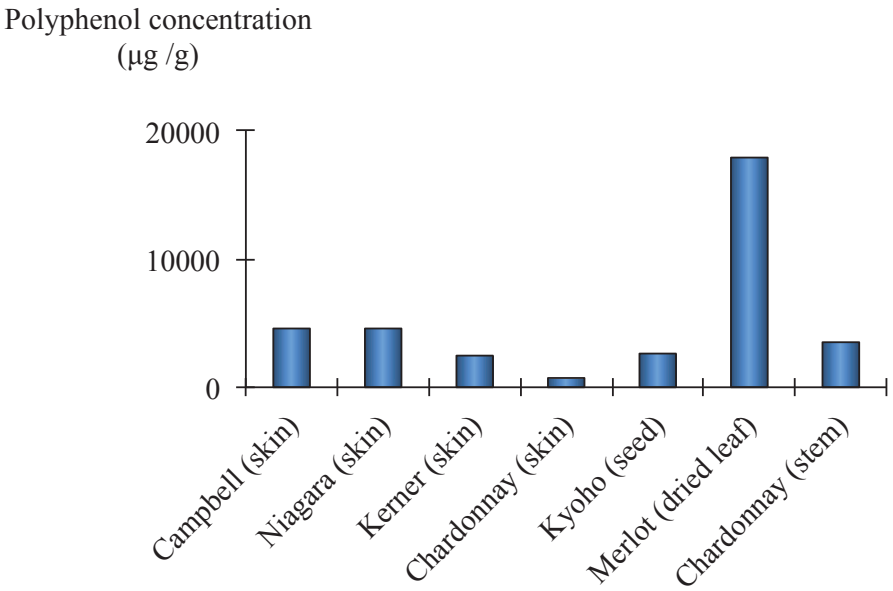


Figure 1. Polyphenol concentration of grape pomace.

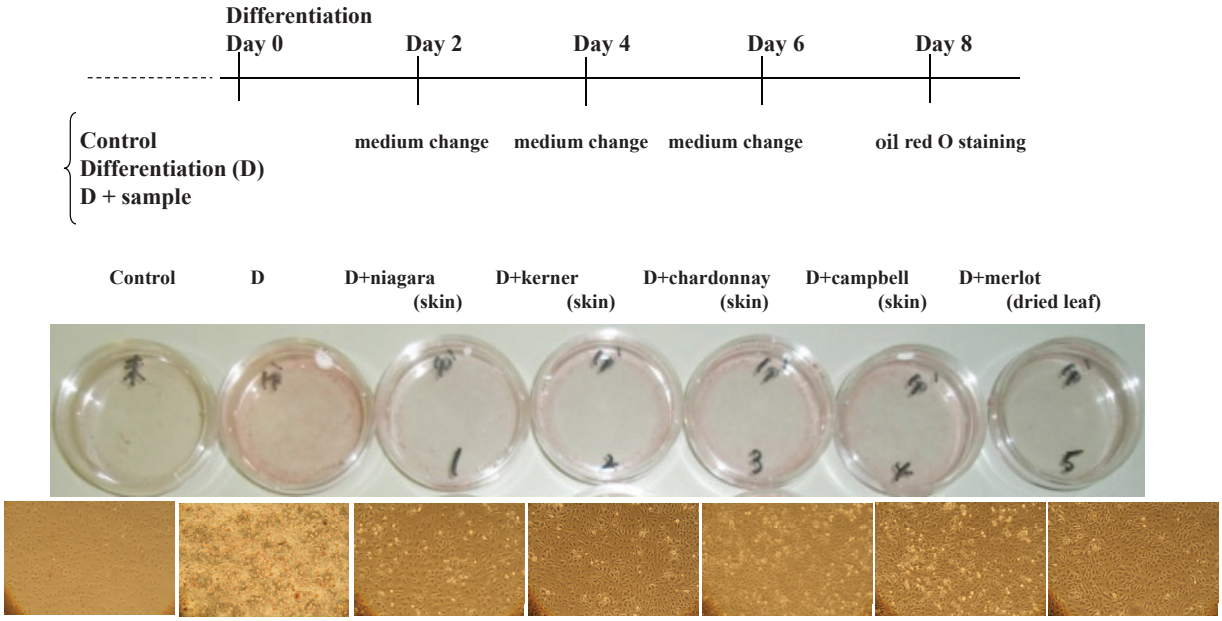


Figure 2. Effect of grape pomace on differentiation of 3T3-L1 cells.

Campbell (skin)

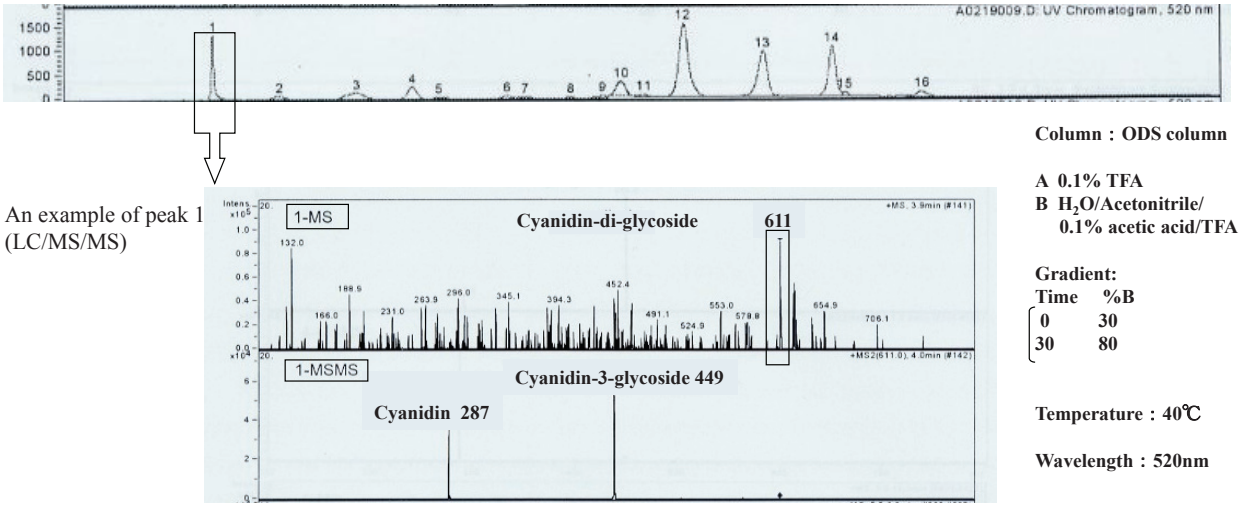


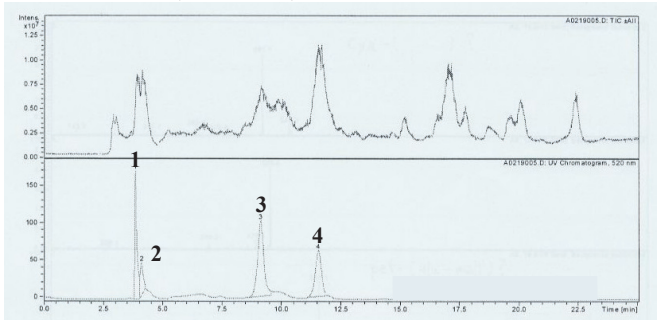
Figure 3. Qualitative analysis of anthocyanin in campbell skin.

Table 1. The chromatogram of the campbell pomace skin sample showed 16 peaks.
Campbell (skin)

Peak NO.	Anthocyanin	Area	Peak NO.	Anthocyanin	Area
1	cya-di-glu	7569	10	del-di-glu-cou	6530
2	cya-di glu	2081	11	cya-glu-ace	1295
3	peo-di-glu	4811	12	cya-di-glu-cou	28307
4	cya-glu	4333	13-1	peo-di-glu-cou	17530
5	pet-glu	852	13-2	del-glu-cou	
6	peo-glu	897	14	cya-glu-cou	14219
7	mal-glu	647	15	pet-glu-cou	1008
8	del-glu-ace	308	16	peo-glu-cou	98
9	unknown			Total	21498

cya: cyanidin, glu: glucose, peo: peonidin, pet: petunidin, mal: malvidin, del: delphinidin, ace: acetic acid, cou: coumaric acid.

Merlot (dried leaf)



LC/MS/MS

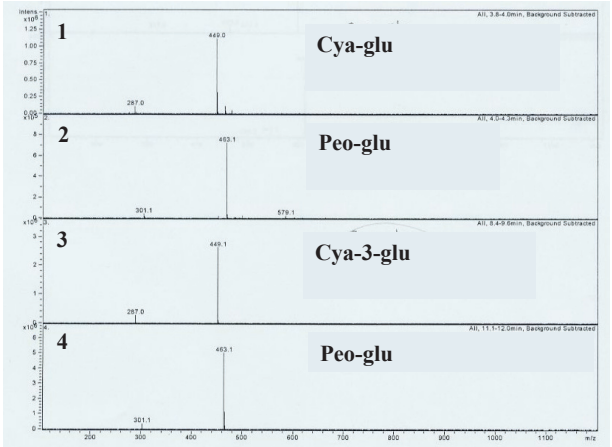


Figure 4. Qualitative analysis of anthocyanin in merlot dried leaf.

were analyzed for anthocyanins, and anthocyanidins with sugars and organic acids were detected. After acid decomposition, analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS) detected many anthocyanidins and associated sugars.

The chromatogram of the campbell pomace skin sample showed 16 peaks (Fig. 3). The molecular weight and retention time of each peak were used to identify each component. Table 1 shows an example of the analytical data used for the identification. On the basis of the molecular weight, peak 1 was considered to be cyanidin with two glucosides. Further LC–MS/MS analysis also detected cyanidin (molecular weight 287) and cyanidin-glycoside (molecular weight 449). Using standard samples of cyanidin and cyanidin-glycoside, each peak was confirmed by molecular weight and retention time. Peak 1 was assigned as cyanidin-di-glucoside. By using the same method, all 16 peaks were assigned as anthocyanins.

Analysis of merlot dried pomace leaf anthocyanin showed four peaks in the chromatogram (Fig. 4). These were assigned as cyanidin glucoside (Cya-glu) and its isomer, and peonidin glucoside (Peo-glu) and its isomer. However, analysis of kyoho pomace seed (Fig. 5) and chardonnay pomace stem (Fig. 6) did not detect anthocyanins.

Effect on differentiation of 3T3-L1 cells by anthocyanins

In addition to the action of crude pomace extracts, delphinidin and cyanidin-3-glycoside were also found to inhibit differentiation of 3T3-L1 cells. A delphinidin concentration of 1 μM was found to affect the differentiation of 3T3-L1 cells (Fig. 7).

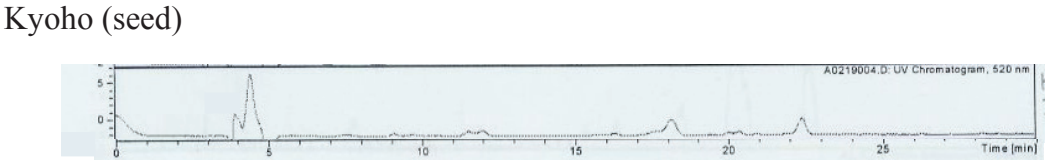


Figure 5. Qualitative analysis of anthocyanin in kyoho seed.

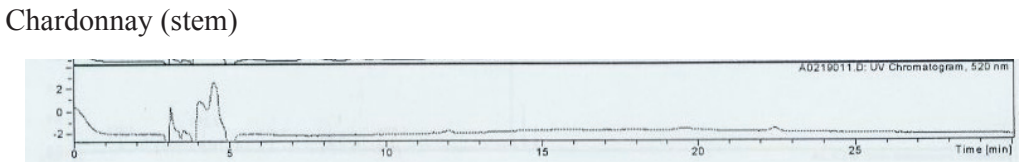


Figure 6. Qualitative analysis of anthocyanin in cardonnay stem.

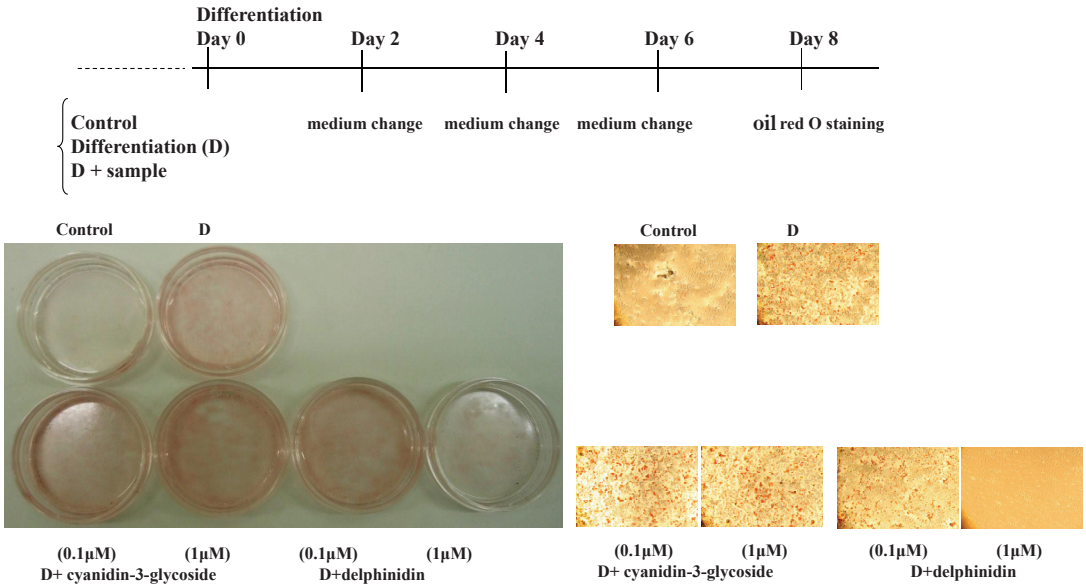


Figure 7. Effect of cyanidin-3-glycoside and delphinidin on differentiation of 3T3-L1 cells.

Discussion

To examine the potential use of grape pomace, we first investigated the residual chemical components of pomace. The retention of polyphenol in niagara, kerner, chardonnay, and campbell grape skins in the grape pomace was relatively high (Fig. 1)—about 50–80% of the levels found in normal unprocessed grape skin (data not shown). Polyphenol was also detected in kyoho pomace seed, merlot dried pomace leaf, and chardonnay pomace stem.

Grapes, berries, and some tropical fruits typically contain high levels of anthocyanins. Anthocyanins are colored water-soluble pigments that belong to the phenolic group. Structurally, anthocyanins are the glycosylated forms of anthocyanidins. The most abundant of the naturally occurring anthocyanins in grapes are the glycosides of cyanidin, followed by those of malvidin, peonidin, and delphinidin.[12] Grape pomace samples were subjected to qualitative analysis for anthocyanins. Grape pomace skin was found to include 16 anthocyanidins associated with sugars or organic acids (Table 1). Grape leaf is known to include anthocyanin, and our analysis of merlot dried leaf identified Cya-glu and Peo-glu (Fig. 4). While grape leaf utilization is unknown at present, there may be some potential for extraction of anthocyanins.

Delphinidin is one of the most abundant anthocyanidins in nature and is commonly found in pigmented fruits and vegetables. Among anthocyanidins, delphinidin shows high radical scavenging activity because it contains a large number of hydroxyl groups that enhance reactivity.[13] We investigated the antiadipogenic effect of delphinidin on 3T3-L1 pre-adipocyte differentiation. We found that delphinidin (1 μM; plasma level) effectively suppressed lipid accumulation (Fig. 7). Although the mechanistic detail of the effect is not known, including the target genes, delphinidin may be a promising candidate for the prevention of metabolic diseases, including obesity.

Depending on eating habits and diet, the daily intake of anthocyans (anthocyanins and anthocyanidins) in humans is estimated to be a few hundred milligrams per day. They are absorbed in the stomach and by intestinal cells and are rapidly detected in plasma in vivo, suggesting that they are bioavailable to exert their biological effects. Studies of the pharmacokinetics of these compounds after their consumption as single agents, anthocyan mixtures, or berry extracts suggest that anthocyans reach levels of 10^{-8} to 10^{-7} M in human blood. [14, 15] Lamy S. et al.[15] reported that low concentration (2 μM) of delphinidin inhibits vascular endothelial growth factor (VEGF)-dependent tyrosine phosphorylation of VEGFR-2, and that the inclusion of berries in the diet may have chemopreventive effects through the inhibition of angiogenesis.

Resveratrol (3,4,5-trihydroxystilbene) [16], a natural bioactive phytochemical mainly found in grapes and berries, has been previously identified as a potent antioxidant and anti-inflammation agent. Resveratrol is known to possess broad bioactivities that are effective in prevention of cardiovascular disease, cancer progression, neurodegenerative disorders, and metabolic disorders. However, the detailed mechanism of resveratrol activity needs to be further elucidated in terms of the composition of various cellular activities and its effects against obesity. In this investigation, chardonnay skins held a resveratrol concentration of 3.8 mg/100 g (ethyl acetate fraction) (data not shown). To clarify the anti-obesity effects of resveratrol, we evaluated its effects on the differentiation of 3T3-L1 cells. But inhibition of differentiation was not recognized in the concentration range of 0–1 μM (plasma level; data not shown).

Grape pomace and its components, including delphinidin and cyanidin-3-glycoside were found to inhibit the differentiation of 3T3-L1 cells. Delphinidin showed effective inhibition at a plasma level of 1 μM.

Conclusions

Polyphenols are effectively retained in grape pomace. Skin and leaf pomace samples have the ability of inhibit the differentiation of 3T3-L1 cells. Delphinidin was detected in campbell skin samples and was also observed to inhibit the differentiation of 3T3-L1 cells at a plasma concentration of 1 μM. A good variety of anthocyanins were detected in pressed grape pomace (skin and leaf).

Acknowledgments

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