Extraction and Purification of Anthocyanin-rich Fruit Extracts

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Juan Eduardo Cacace

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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"Success is never permanent, failure is never failure, the only thing you can really do is never, never, never give up"

Winston Churchill

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LIST OF SYMBOLS

1. Symbols

a	characteristic dimension of the solid, m,
B	distance of the impeller above the bottom of the vessel, m,
C	concentration of the solute,
C_b	content of a given compound in the frozen berries, mg/g,
C_{eq}	content of a given compound in the final extract, mg/g,
D	diffusivity or diffusion coefficient, $m^2\ s^{-1}$,
D_a	impeller diameter, m,
D_t	vessel diameter, m,
D_0	diffusivity at infinite dilution, $m^2\ s^{-1}$,
D_{AB}	diffusivity of component A in solvent B, $m^2\ s^{-1}$,
D_S	diffusivity or diffusion coefficient of the solute in a solid, $m^2\ s^{-1}$,
DM_B	dry matter weight fractions of the berries,
DM_E	dry matter weight fractions of the extract,
DM_P	dry matter weight fractions of the pomace,
E	extract volume, L,
E_S	weight of total solid in the extract, g,
F	recovery of a given compound, %,
g_c	gravitation coefficient, $m\ s^2$,
H	height of the liquid in the vessel, m,
ΔH_{fus}	molar heat of fusion, $J\ mol^{-1}$,
J	baffles wide, m,
K_L	mass transfer coefficient in the extract; $m\ s^{-1}$,
L	paddle blade length, m,
M	total mass, kg,
M_B	molecular weight of solvent B, $kg\ kmol^{-1}$,
m	partition coefficient,
N	rotational speed of agitator, rps,
n_c	critical stirrer speed, rps,
P	power delivered by agitator, W,
R	universal constant of gases, $J\ (mol\ K)^{-1}$,
S	solid volume, L,
S_{1-6}	dimensionless shape factors,
ΔS_{fus}	molar entropy of fusion, $J\ (mol\ K)^{-1}$
T	absolute temperature, K,

T_m melting point, K,
t extraction time, s,
V extract volume, L,
V_A solute molal volume at normal boiling point, $m^3\ kmol^{-1}$,
W paddle blade wide, m,
W_b weight of frozen berry feed, g,
W_E weight of extract, g,
W_l total loss weight, g,
W_p weight of the wet pomace, g,
W_S initial weight of solvent, g,
x distance,
x_{dm} weight fraction of a given compound in the dry pomace,
x_m weight fraction of the given compound in the wet pomace,
x_0 initial weight fraction of a given compound in the berries,
x_i^{sat} molecular fraction of the solute dissolved in the solvent phase at saturation,
Y dimensionless extract concentration,
y weight fraction of a given compound in the extract at any time t,
y_e weight fraction of a given compound in the extract at equilibrium,
y_0 weight fraction of a given compound in the extract at time zero,
α volume ratio,
ε dielectric constant or permitivity,
ϕ association constant for solvent,
γ_i^{sat} activity coefficient for the solute in the solution,
η dynamic viscosity coefficient, Pa s,
η_0 dynamic viscosity of pure solvent, Pa s,
ρ density of liquid, $kg\ m^{-3}$, $g\ L^{-1}$,
ν kinematic viscosity of the liquid, $m^2\ s^{-1}$,

2. Dimensionless Numbers

Bi Biot number
τ Fick number
N_{Fr} Froude number
N_p Power number
Re Reynolds number
Sc Schmidt number
Sh Sherwood number

Numbers used for dimensionless analysis in empirical plot

$$N_p = \frac{Pg_c}{N^3 D_a^5 \rho} \qquad \text{Re} = \frac{ND_a^2 \rho}{\eta}$$

$$N_{Fr} = \frac{N^2 D_a}{g_c}$$

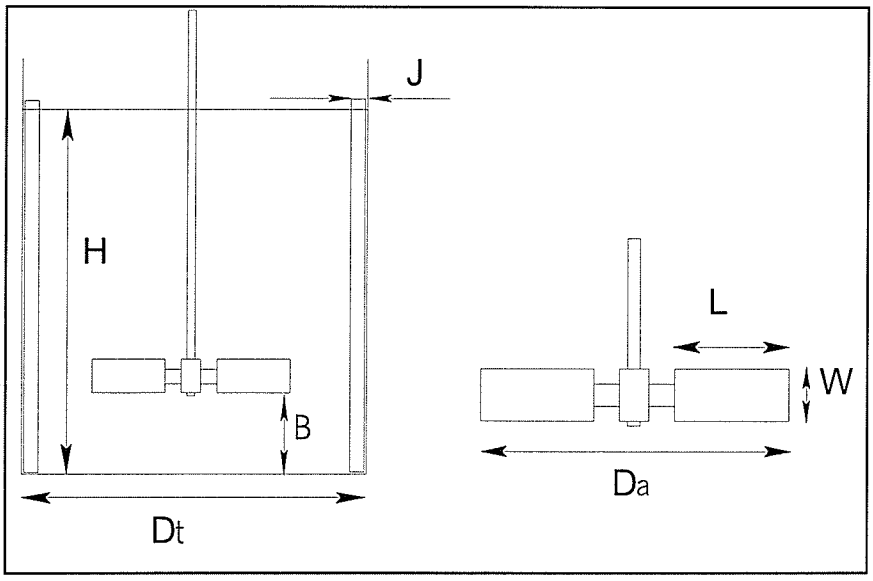
Numbers used in empirical correlations to obtain the mass transfer coefficients

$$Sc = \frac{\eta}{D_S \rho} = \frac{\nu}{D_S} \qquad Sh = \frac{K_L a}{D_S}$$

$$\tau = \frac{D_s t}{a^2} \qquad Bi = \frac{K_L m a}{D_S}$$

3. Dimensionless Shape Factors

$$S_1 = \frac{D_a}{D_t} \quad S_2 = \frac{B}{D_a} \quad S_3 = \frac{L}{D_a} \quad S_4 = \frac{W}{D_a} \quad S_5 = \frac{J}{D_t} \quad S_6 = \frac{H}{D_t}$$



ABSTRACT

Interest in development of efficient methods for extraction and purification of anthocyanins and other phenolic compounds is growing, based on demonstrated health benefits and potential nutraceutical applications. Extraction of phenolic compounds from milled frozen black currants using aqueous ethanol and SO_2 solvents was optimized. Surface response methodology was employed to optimize total phenolic, tartaric ester, flavonol and anthocyanin yields, and antioxidant activity of extracts using the solvent concentration (39, 50, 67, 84, 95 % ethanol or 28, 300, 700, 1100, 1372 ppm SO_2), temperature (6, 20, 40, 60, 74 °C), and solvent to solid ratio (6, 20, 40, 60, 74 mL/g dwb) as independent variables. Four pre-extraction treatments (30, 45 and 60 °C and 45°C using enzymes) and two purification methods (ultrafiltration and adsorption chromatography) were also tested.

Increase of pre-extraction temperature from 30 to 60 °C increased yield and recovery of total phenolics. However, there was no effect of the enzymatic treatment. The type of berry also affected phenolic yields. All purification methods showed anthocyanin recoveries higher than 90% and affected the phenolic profile of extracts. Ultrafiltration showed a higher yield and recovery of total phenolics and anthocyanins which resulted in extracts with higher antiradical activity.

Improved extraction with both solvents was achieved by increased solubility and an enhanced diffusion coefficient of phenolic compounds. Optimum ethanol concentration varied for tartaric esters, flavonols and anthocyanins. Ethanol concentration affected the yield

and the diffusion coefficient of total phenolics with a maximum at approximately 60 % ethanol. Sulfur dioxide concentration also increased total phenolic yield and the diffusion coefficients for anthocyanins and total phenolics. Maximum yield of total phenolics and anthocyanins was obtained at 1,000-1,200 ppm SO_2 concentration.

The solvent to solid ratio had the greatest effect on phenolics extraction with both solvents and a maximum yield was obtained using 19 L/kg of frozen berries. However, the use of a high solvent to solid ratio resulted in more diluted extracts with lower antioxidant activity. Increase of temperature mainly reduced the time to reach equilibrium, by increasing anthocyanins and total phenolics diffusivities. Minimum time for extraction of anthocyanins was obtained using 60-75 % ethanol and 60-70 °C (10 min) and 900 ppm SO_2 and 60-65 °C (28 min). Increasing the temperature beyond 30 - 35 °C resulted in anthocyanin degradation and reduction of yield.

I. INTRODUCTION

Epidemiological evidence indicates that consumption of fruits and vegetables has many health benefits (Block et al., 1992; Ness and Poulens, 1997; Joshipura et al., 1999). These benefits have been attributed in large part to distinct components such as fibre, antioxidant nutrients, plant phenolics and sulfur-containing compounds. Recently the interest in food phenolics has increased greatly, because of their antioxidant capacity and their possible beneficial implications in human health, such as prevention of cancer and cardiovascular diseases; treatment of urinary tract disorders; improvement of visual acuity; anti-inflammatory, antiatherogenic, antibacterial and antiviral activities and prevention of other diseases associated with aging (Rodhes, 1996; Meltzer and Malterud, 1997; Benavente-Garcia et al., 1997; Bravo, 1998; Mazza, 2000; Smith et al., 2000). Plant phenolics constitute a large group of compounds with antioxidant and radical scavenging activities capable of avoiding the oxidation of low density lipoprotein implicated in development of several pathologies. The efficient recovery of phenolic compounds from natural sources for use as nutraceuticals and functional food ingredients has received very little attention.

The extraction of a desirable component from a solid using a liquid phase which combines phase-equilibrium and mass transfer principles constitutes a major step that defines the effectiveness of the whole process. It is generally agreed that there are four factors that affect extraction of phytochemicals: solvent composition, particle size, solvent to solid ratio and temperature. Information on physical and chemical properties of bioactive compounds

and solvents is scarce, their diffusivities in food products are poorly known, and hydrodynamic studies to investigate mass transfer processes and their effects on phenolics extraction rate are needed (Geankoplis, 1978; Schwartzberg.and Chao, 1982; Cortesi et al., 1999; Doulia et al., 2000). Extraction of phenolics to maximize yield has been carried out in the chemistry laboratory but no scalable mixing conditions or pilot plant scale mass balance has been considered (Metivier et al., 1980; García-Viguera et al., 1998; Wettasinghe and Shahidi, 1999). Development of new extraction processes is also required for replacing conventionally employed methods that use non-food grade solvents with more environmentally friendly and safe extraction techniques. The raw material employed may also affect the efficiency of extraction and quality of the extracts.

The wide variety of berries available with different concentrations of phenolic and other bioactive compounds represents a broad spectrum of possibilities for product and process development (Macheix et al., 1990; Mazza and Miniati, 1993; Moyer et al., 2002). The value of a plant raw material depends on its composition and the concentration of desired components. However, characteristics of different plant materials could affect extraction. Availability and bioactive properties of those compounds may also be altered during food processing (Iversen, 1999; Skrede et al., 2000; Smith et al., 2000). Furthermore, many dietary supplements derived from plant sources contain mixtures of phytochemicals that have not been quantified or even identified. Knowledge of characteristic compounds of berries as well as their possible modifications during extraction, purification and concentration is highly desired. Evaluation of bioactivity and optimization of the processes for antioxidant activity of extracts may provide an assessment of the efficacy of products and assure a greater value for the consumer's money.

The objectives of this research were:

1 To optimize extraction of phenolic compounds from black currants, in relation to yield and antioxidant activity.

2 To determine the mass transfer coefficients of anthocyanins during solid-liquid extraction of milled black currants in agitated vessels.

3 To determine the effects of temperature and enzymatic treatments applied before the extraction on yield, antioxidant activity and extract composition.

4 To quantify the purification efficiency of ultrafiltration and adsorption chromatography of anthocyanin-rich berry extracts.

II. LITERATURE REVIEW

A. Phenolic Compounds

1. Occurrence of Phenolic Compounds

It is widely accepted that fruits and vegetables have many health-enhancing properties. The current recommendation of the World Health Organization of eating 5 portions of fruit and vegetables per day is a result of research into the effect of the whole food and food components on biological systems (Williamson, 1996). The distinct components of fruit and vegetables are fibre, antioxidant vitamins and bioactive plant secondary metabolites such as phenolic and sulfur-containing compounds. Traditionally, polyphenols have been considered antinutrients by animal nutritionists, because of the adverse effect of tannins on protein digestibility. However, recently the interest in food phenolics has increased greatly, because of their antioxidant capacity and their possible beneficial implications in human health, such as prevention of cancer, cardiovascular diseases and other pathologies (Rodhes, 1996; Meltzer and Malterud, 1997; Bravo, 1998). A general definition of phenolics is that they include a broad group of substances that possess an aromatic ring with one or more hydroxyl substituents. These compounds are present in all plant tissues and constitute one of the most important class of compounds in higher plants. Distinction between the several thousands of polyphenols that have been described in plants is drawn on the basis of the constitutive carbon atoms and the structure of the basic skeleton. Distribution of the different constituents

is not homogeneous, thus, hydroxycynnamic acids and flavonoids are very widely represented in fruits and vegetables.

Flavonoids occur naturally in fruit, vegetables, nuts, seeds, flowers, and bark. Their properties and occurrence in nature have been thoroughly reviewed (Hrazdina, 1982; Wollenweber, 1982; Peterson and Dwyer, 1998; Harborne and Williams, 2000). The most widely distributed classes of flavonoids are the flavones and flavonols. Other flavonoids are flavanones, isoflavones, flavanols or flavan 3-ols or procyanidins, anthocyanins, chalcones, and aurones. The most important flavanols are catechins, gallocatechins, epigallocatechins and epigallocatechin gallate (Riberau-Gayon, 1972; Hahlbrock, 1981; Harborne and Mabry, 1982). Anthocyanins constitute a large family which is present in most higher plants and is primarily responsible for the color of almost all parts of plants (Mazza and Miniati, 1993; Mazza, 1997).

Chemically, most flavonoids have two aromatic rings enclosing a heterocyclic six-member ring with an oxygen. The benzene ring A is mostly di- or trihydroxylated (resorcinol or phloroglucinol type). The exceptions are the chalcones (isomeric open form) in which one of the hydroxyls is combined in an oxygen heterocycle of six atoms (ring C), and the aurones in which the C ring is a five-member ring (Figure 1). The B ring is mono-, di-, or trihydroxylated. Difference between the A and B rings are due to the fact that the A ring is formed by the condensation of three molecules of acetic acid while the B ring is derived from sugars by the shikimic route (Riberau-Gayon, 1972; Hahlbrock, 1981). Various subgroups of flavonoids are classified according to the substitution pattern of the C ring and to the position of the B ring. Thus the B ring is in the position 2 in flavanones, flavones, flavonol and anthocyanins, and 3 in isoflavonoids (Figure 2).

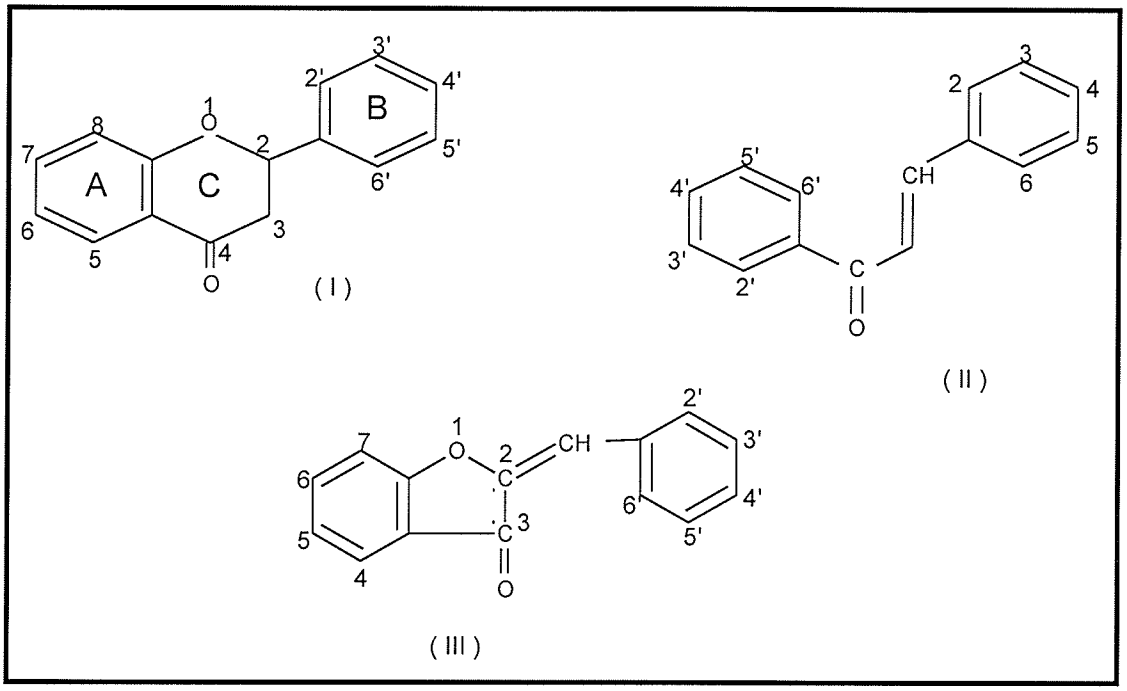


Figure 1. Numbering System in: I) Most Flavonoid Structures; II) Chalcone Structures and III) Aurone Structures (from Riberau-Gayon, 1972)

Chemically flavonols are 3-hydroxyflavones, and this position is usually more or less glycosylated with various sugars which can be further substituted with acyl residues. Thus, substitution of the 3-hydroxyl group of flavonols constitute the main difference between flavonols and flavones. The aglycones of anthocyanins (anthocyanidins) are structurally closely related to each other. There are six major anthocyanidins whose chemical structures are based on the structure of pelargonidin (3,5,7,4'- hydroxyl- substitutions) (Figure 3). Most flavonoids occur as glycoside substituted aglycones.

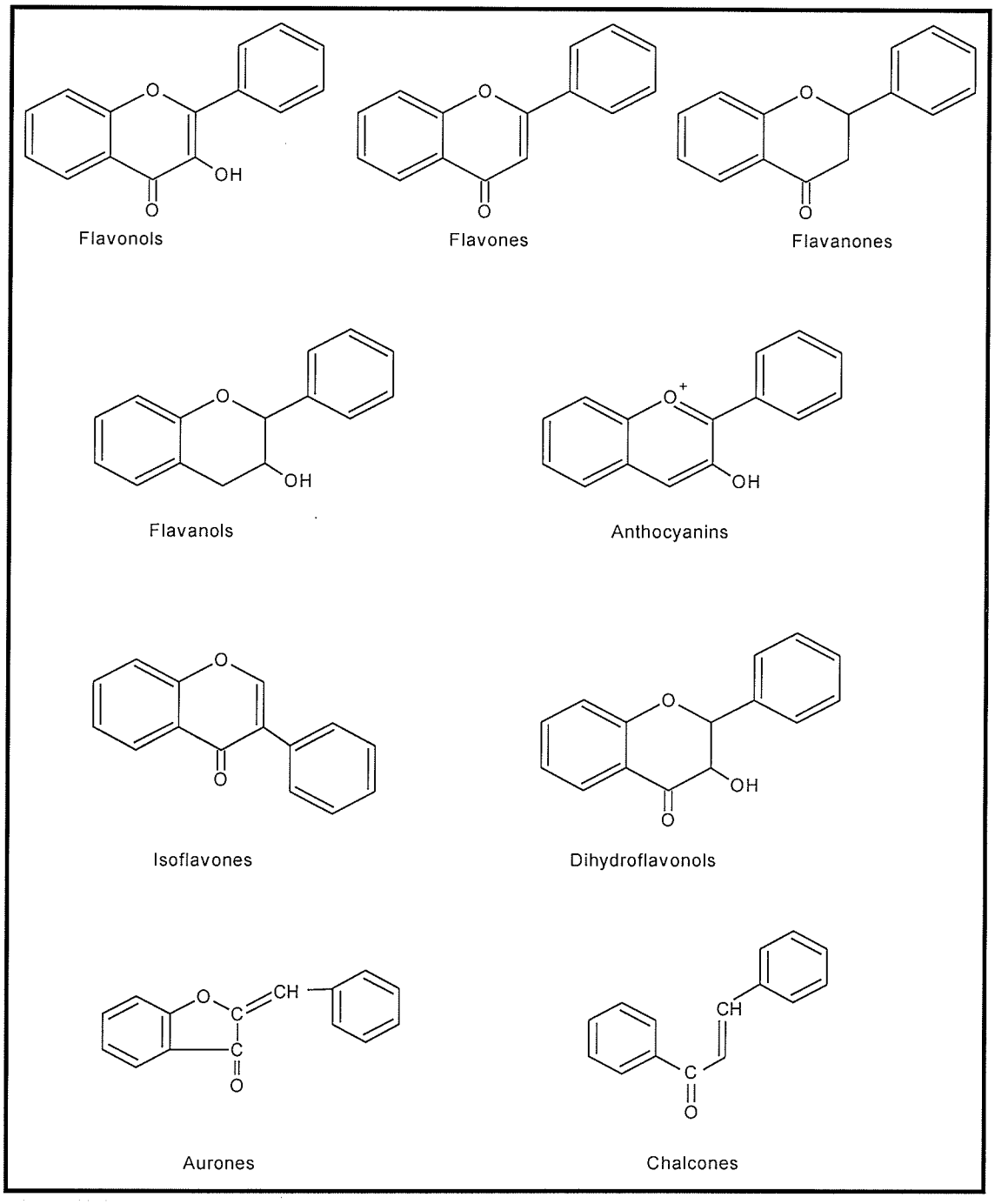


Figure 2. Structures of Major Subgroups of Flavonoids (from Riberau-Gayon, 1972; Harborne et al., 1975; Hahlbrock, 1981)

Anthocyanidin	R_1	R_2
pelargonidin	H	H
cyanidin	OH	H
peonidin	OCH_3	H
delphynidin	OH	OH
malvidin	OCH_3	OCH_3
petunidin	OCH_3	OH

R_3 OH, glycosyl

R_4 OH, glycosyl

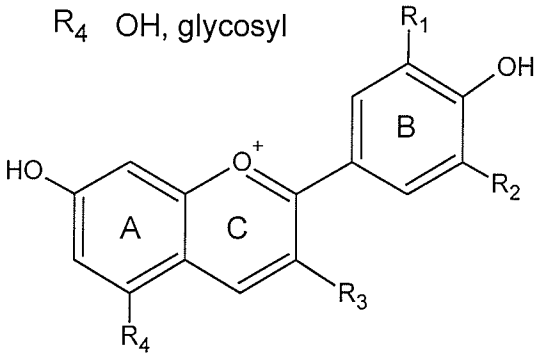


Figure 3. Structure of Major Anthocyanidins (Adopted from Mazza and Miniati, 1993)

Differences in the structure of hydroxybenzoic and hydroxycinnamic acids are due to the hydroxylation and methoxylation pattern of the aromatic rings (Figure 4) (Herrmann,1989; Macheix et al., 1990). Hydroxycinnamic acids are almost exclusively derived from p-coumaric, caffeic, and ferulic acids, while sinapic acid is comparatively rare and o-coumaric and isoferulic acids occur occasionally (Herrmann, 1989). The most common hydroxycinnamic acids are not present in plants in a free state, but two main derivatives have been identified: a) compounds involving an ester bond between the carboxylic function of phenolic acid and one alcoholic group of an organic compound, usually quinic acid or glucose; and b) compounds involving an ester bond with one of the phenolic groups of the molecule. The presence of a double bond in its lateral chain leads to the possible existence of two isomers, cis and trans. Hydroxybenzoic acid compounds are mainly present in the form of glucosides, whereas glucose esters have been found only occasionally. The three most common compounds are derivatives of p-hydroxybenzoic, vanillic and protocatechuic

acids. Syringic acid occurs more rarely. Gallic and ellagic acids are other well known plant acids which participate in the formation of tannins (Macheix et al., 1990).

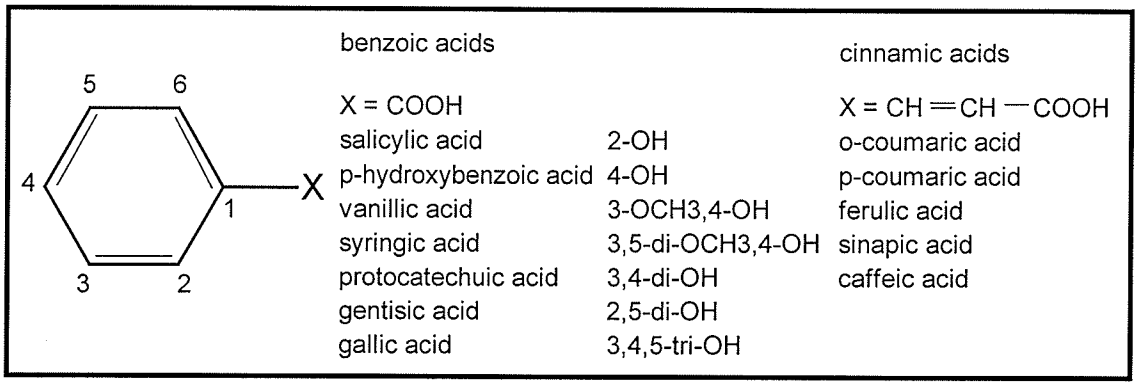


Figure 4. Structure Scheme of Main Hydroxybenzoic and Hydroxycinnamic Acids (from Herrmann, 1989)

2. Phenolic Contents of Berries

Anthocyanin composition of most edible plants has been summarized by Mazza and Miniati (1993) and Mazza (1997). Anthocyanins of six soft fruits are presented in Table 1. Four major and three minor anthocyanins have been found in black currants. The major anthocyanins are cyanidin 3-glucoside, delphinidin 3-glucoside, cyanidin 3-rutinoside and delphinidin 3-rutinoside (Mazza and Miniati, 1993; Renault et al., 1997; da Costa et al., 2000; Degenhardt et al., 2000); pelargonidin 3-rutinoside, cyanidin 3-sophoroside and delphinidin 3-sophoroside are present at low concentration (Mazza and Miniati, 1993). Cyanidin 3-glucoside (57%) and cyanidin 3-rutinoside (30%) are the major anthocyanins in blackberries (Mazza and Miniati, 1993). Anthocyanin (Banaszczyk and Pluta, 1997), flavonol, and phenolic acids (Häkkinen et al.,1999a; Häkkinen et al.,1999b) composition of several berry species (Fukumoto and Mazza, 2000; Moyer et al., 2002), including selected

black currant cultivars (Mikkonen et al., 2001) has been reported.

Table 1. Anthocyanins in Selected Fruits

Plant name	Organ	Anthocyanins [1]
Blackberry	fruit	Cy 3-glucoside, 3-rutinoside, Pg 3-glucoside, 3-sophoroside, 3-glucorutinoside
Currant (black)	fruit	Cy and Dp 3-glucosides, 3-rutinosides, 3-sophorosides, Pg 3-rutinoside
Cherry (sweet)	fruit	Cy and Pn 3-glucoside and 3-rutinoside, Pg 3-rutinoside
Saskatoon	fruit	Cy 3-galactoside, 3-glucoside, and 3-xyloside
Raspberry (red)	fruit	Cy and Pg 3-glucoside, 3-rutinoside, 3-sophoroside, 3-glucorutinoside; Cy 3,5-diglucoside
Blueberry	fruit	Cy, Dp, Pt, Pn and Mv 3-galactoside, 3-glucoside, 3-arabinosides, and their acylated forms

[1] Abbreviations Pg= pelargonidin, Pn = peonidin, Cy = cyanidin, Dp = delphinidin, Pt= petunidin, Mv= malvidin; [2] Adapted from Mazza (1997).

Flavonols in black currants are present primarily in the form of glycosides (Herrmann, 1976). Myricetin and quercetin are the most abundant flavonols, followed by kaempferol in black currant (Mikkonen et al., 2001; Häkkinen et al.,1999a; Häkkinen et al.,1999b) and blackberries (Herrmann, 1976). Percent contribution of each of those flavonols on the total varies with the stage of maturity of berries. Hydroxycinnamic acid derivatives, especially caffeic and p-coumaric acids, occur in high concentration in black

currants and blackberries (Schuster and Herrmann, 1985; Herrmann, 1989; Häkkinen et al.,1999a). Hydroxycinnamic acid derivatives reported in black currant and blackberries include caffeoyl- and p-coumaroyl-quinic acids, caffeoyl-, p-coumaroyl- , feruloyl and galloyl- glucose esters and p-coumaric- and p-hydroybenzoic-O-glucoside esters (Schuster and Herrmann, 1985; Herrmann, 1989). Derivatives of p-hydroxybenzoic, protocatechuic and gallic acids have been found in black currants and blackberries (Schuster and Herrmann, 1985; Herrmann, 1989; Häkkinen et al.,1999a).

B. Extraction of Phenolics from Plant Material

1. Factors Affecting Extraction

The process of extraction of a desired component from a solid using a liquid phase is dependent upon the combination of mass transfer principles with phase-equilibrium relationships. The extraction of phytochemicals from plant material, using solvents, constitutes an important step that defines the yield of the whole process. The mechanism of transport of a particular species may be due to random molecular diffusion or random microscopic fluid motion (turbulent diffusion) as result of a concentration gradient between the two phases or within one phase. Gertenbach (2001) enumerated the factors that affect mass transfer and extraction rate of phytochemicals: solvent composition, particle size, solvent to solid ratio and temperature. Enzymes and sulfur dioxide also affect extraction.

a. Solvent. Solvents are used to dissolve organic solids for a wide variety of processes. The selection of an adequate solvent is essential for many processes especially for solid-liquid extraction. A particularly good choice of solvent helps to improve product quality, allows for a simpler or more robust manufacturing process, or simplifies the technology needed to

achieve environmental, health, and safety goals (Frank et al., 1999). The selection of solvent can change both the amount of phytochemical that extracts into the liquid and the rate at which the phytochemical is extracted (Gertenbach, 2001).

The main required feature of the solvent is the ability to dissolve the solid of interest. The solubility of a pure nonionic solid is modeled (Frank et al., 1999) by the expression

$$\ln x_i^{sat} = \frac{\Delta S_{fus}}{R}\left(1 - \frac{T_m}{T}\right) - \ln \gamma_i^{sat} \qquad for\ T \le T_m \qquad (1)$$

$$\Delta S_{fus} = \frac{\Delta H_{fus}}{T_m} \qquad (2)$$

where x_i^{sat} is the molecular fraction of the solute dissolved in the solvent phase at saturation, ΔH_{fus} molar heat of fusion, J mol^{-1}, γ_i^{sat} is the activity coefficient for the solute in the solution, R is the universal constant of gases, J (mol K)$^{-1}$, T_m is the melting point and T is the absolute temperature (K). Thus, the solubility depends on the properties of the solute (entropy of fusion and melting point) and a property of the mixture such as the activity coefficient. Low melting point and low heat of fusion both favor enhanced solubility. When γ_i^{sat} is equal to 1, the solubility is termed ideal. The temperature dependence of solid solubility is determined not only by the ideal solubility, but also by changes in the activity coefficient with the temperature and composition of the solution (Frank et al., 1999). Compounds having a repulsive interaction with the solvent have an activity coefficient greater than 1 and thus the ideal solubility would be reduced. The solubility of a compound can be enhanced over a limited composition range using a mixed solvent. A solvent based on methanol is 20 % more effective than ethanol and 73 % more effective than water for the extraction of anthocyanins

from wine pomace (Metivier et al., 1980). A 1000 ppm SO_2-water solvent was the most effective for the extraction of anthocyanins from sunflower hulls in comparison with acidified aqueous ethanol and acidified water (Gao and Mazza, 1996). The type of solvent and solvent composition also affect the extraction rate by modifying physical properties of the extraction medium. Organic solvents can be classified on the basis of their structure and dielectric constants as protic, and aprotic polar and non polar. Protic solvents contain relatively mobile protons. Aprotic polar solvents do not have a mobile proton but they have high dielectric constants in comparison with non polar solvents. The dielectric constant is a macroscopic property of the solvent that indicates the ability to reduce the interaction of particles with opposite charges and also determines the solvation characteristics of a solvent (Carey and Sundberg, 1984; Carey, 1987). In terms of structure, dielectric constant depends on both the permanent dipole moment of the molecule and its polarizability or the ease of distortion of the electrons of the molecule. Dielectric constant increases with dipole moment and with polarizability. In a protic solvent with a high dielectric constant, such as water (ε_{H2O} = 78.5) a minimum energy is required to separate a positively charged ion from one with a negative charge; for acetic acid, a protic solvent with a low dielectric constant (ε_{HAc} = 6.2) a greater amount of energy is required to accomplish the process. Methanol and ethanol with dielectric constants of of 33 and 24, respectively are intermediate in their behaviour (Skoog and West, 1982). Non polar aprotic solvents are not effective at stabilizing the development of charge separation because of their small dipole moment and lack of hydrogen atoms capable of forming hydrogen bonds. On the other hand, water is one of the best of all solvents for ions as a result of strong solvating forces for ions combined with a high dielectric constant (Mackay and Mackay, 1981). But water is a very poor solvent for covalent

compounds for which the energy required to separate the solvent molecules and allow the solute molecules to enter between them is a dominant term affecting solubility (Mackay and Mackay, 1981). Then a change in the dielectric constant of the solvent would modify its extraction capacity.

Reduction of dielectric constant of water by modifying pressure and temperature has improved extraction of natural products (Basile et al., 1998; Clifford et al., 1999; Kubatova et al., 2001). Best extraction of anthocyanins from sunflower husks was obtained with distilled water-organic solvent mixtures of intermediate polarity values. An improvement in extraction obtained with alcohols and water-organic solvent mixtures has been related to a decrease in the dielectric constant of the solvent (Pifferi and Vaccari, 1983). The high dielectric constant and the high polarization of O-H bonds with formation of hydrogen bonds of water might be reduced when a substance with a low dielectric constant is incorporated into water to form a mixed solvent. Dielectric constant of pure liquid sulphur dioxide at 20 °C is 14, of pure methanol at 25°C is 32.6 and of pure ethanol at 25 °C is 24.3 (Lide, 1992). These three substances would have potential for reducing the dielectric property of water.

Other properties may also affect the extraction. Density and dynamic viscosity are related to mass transfer coefficients and diffusivity of solute through empirical correlations as it is noted in *Section 2.a and b.*

b. Particle Size. Size reduction is one of the most important preparation steps for extraction or leaching of compounds from plant material. Particle size affects the degree and the rate of extraction. The mass transfer between two phases is proportional to their interfacial area (Kirwan, 1987).Thus, the smaller the size the higher the surface to volume ratio and the higher the extraction. A tinier particle partition allows easier access of the solvent, which

along with a larger surface of contact with the solvent would result in a higher degree of extraction.

Particle size influences the mass transfer process. Thus, higher yields in total phenolic and anthocyanin extraction from sunflower result with a maximum break-up of particles (Pifferi and Vaccari, 1983; Gao and Mazza, 1996) or from a decrease in pomace size of black currant juice press residues (Landbo and Meyer, 2001). Smaller particle size reduces the diffusion step of the solute within the solid and increases the concentration gradient which increases the extraction rate. Since the path of solute to reach the surface is shorter, extraction time is reduced.

Calculations of mass transfer coefficients with empirical correlation for particles in suspension in agitated vessels indicate that there is a range of particle size from approximately 100 to 1000 μm where the mass transfer coefficient is independent of the particle size (Kirwan, 1987). The change of the coefficient falls within a relatively narrow range for a wide range of particle size and agitation conditions especially once the particles are fully suspended (McCabe et al., 1985).

c. Solvent to Solid Ratio. The solvent to solid ratio modifies the rate of extraction by affecting the concentration gradient. An increase in solvent to solid ratio increases the concentration gradient and thus the rate of diffusion of the compounds from the solid to the solvent. Yields are strongly affected by the solvent to powder ratio in the extraction of anthocyanins from sunflower husks (Pifferi and Vaccari, 1983). In the same way, higher water-seed ratios favor the extraction of proteins from flaxseed (Cui et al., 1994). A higher ratio reduces the concentration of solute at the interface increasing the driving force and the extraction rate.

Use of high solvent to solid ratios however, results in dilute solutions. Solute concentration modifies diffusivity, since more dilute solutions have higher diffusivity (Cheryan, 1986). Besides, solute concentration has a close relationship with the activity coefficient of the solute. According to equation (1) the activity coefficient may affect the ideal solubility of the desired compound.

d. Temperature. Temperature is a very important variable that affects most physico-chemical processes. The equilibrium between the solute within the solid and the dissolved molecules in the liquid phase is affected by temperature. Higher temperatures increase the equilibrium constant leading to a larger solute extraction by the solvent. Solubility is affected by the temperature as indicated by equation (1). When temperature is closer to the melting point of the substance, solubility approaches the ideal solubility, which in the case of non-ionic compounds is the maximum value attainable.

Temperature also increases the diffusion coefficient of the solute. The mechanism of action is by increasing the internal energy and the molecular motion within the solid. A higher temperature also reduces the dynamic viscosity which leads to an increase in the diffusivity. Both the direct effect on the energy of the molecules and the reduction of the dynamic viscosity favor the mass transfer process and the extraction of the phytochemical by the solvent.

The use of high temperature can create difficulties with the extraction process, including reduction of volume due to evaporation of the solvent, and changes in concentration. Even more important is the possibility of decomposition of thermally sensitive species. Anthocyanin pigments for instance can be significantly affected by temperature. High temperature treatments reportedly reduce anthocyanins in different processing steps

(Rommel et al, 1990; Iversen, 1999; Skrede et al, 2000) and thermal degradation in sulfurous water solutions follows a first order kinetic (Mok and Hettiarachchy, 1991). The degradation rate of anthocyanins by increasing temperature decreases with increasing SO_2 concentration in the extraction solution. Degradation is also lower at pH 3.0 than at pH 1.0 and 5.0. Anthocyanins from black currant juice completely disappear at 37 °C and pH 2, whereas 60 % remain at 4°C (Mazza and Miniati, 1993).

e. Enzymes. Enzymes can be used as a preparative step for extraction or leaching of compounds from plant material. Enzymes break down membranes and facilitate the access of solvent into the tissue to be extracted. Pectic enzymes sometimes are used in fruit maceration and juice preparation to facilitate pressing or juice extraction and to aid in the separation of flocculent precipitates by sedimentation, filtration or centrifugation. However, some enzymatic processes can degrade polyphenolics susceptible to enzymatic hydrolysis. A loss of up to 47 % anthocyanins has been reported during milling, maceration with enzymes and pressing of blueberries (Skrede et al., 2000).

The use of enzymes reduces the viscosity of fruit juices by depolymerizing the juice pectin (Kilara and Van Buren, 1989). Pectinase is used to improve the extraction of juice from fruits, increasing yield and reducing processing time. However, it has been reported to decrease the yield of phenolic compounds, to cause a loss of anthocyanins and flavan-3-ols in blueberries and sweet cherries (Heinonen et al., 1996). Pectolytic enzymes act on pectic substances by either depolymerizing or deesterifying the substrate. Commercial pectinolytic enzyme preparations alone and in combination with protease significantly increase black currant pomace cell wall break down and enhance yield of phenolics. However, preparations showed no effect or a decrease of the amount of anthocyanins extracted (Landbo and Meyer,

2001).

Degradation of anthocyanins is a recognized adverse side effect of enzyme treatment of fruit. It may be caused by β-glucosidase or β-galactosidase activities in non-specific enzyme preparations. Recently, Matsumoto et al. (2001) reported that β-glucosidase selectively hydrolyses 3-O-β-glucosides of delphinidin and cyanidin, and hesperidinase removes the α-rhamnosyl moieties from delphinidin and cyanidin rutinosides from black currant concentrate. Commercial pectolytic products may also contain other enzymes such as pectin-methylesterases, amylase and hemicellulase deliberately retained or added to improve the clarifying action. However, Skrede et al. (2000) found no glycosidase activity, and there was not anthocyanin degradation that could be associated with the Rapidase® Super BE (Gist-Brocades Int.B.V., Charlotte, NC) depectinization enzymes. Iversen (1999) found that black currant anthocyanin degradation at enzyme doses of 0.0057 % v/v was limited and 97% of the original anthocyanin content in the berries was intact after 2 h of enzyme treatment at 50 °C.

f. Sulfur Dioxide. The use of sulfur dioxide (SO_2) as an antioxidant and antimicrobial is widely accepted as an indispensable aid in wine making. Mild solutions of sulfur dioxide in water can be used to replace ethanol in the extraction of anthocyanins from grape pomace because a considerable increase in recovery of pigments is obtained (Garoglio, 1953). Generally, sulfur dioxide is used to improve the extraction of anthocyanins and to protect the pigments from oxidation and microbial spoilage (Mazza and Miniati, 1993). The exact mechanism for how SO_2 improves extraction is not known, but interactions leading to improved diffusion through the cell walls and increased solubility have been mentioned as possible causes (Gao and Mazza, 1996). The effect of sulphite as modifier of the physical

properties of the solvent has been noted in *Section B.1.a.* Various amount of SO_2 from 200 (Peterson and Jaffe, 1969) to 10,000 mg/L (Yokoyama and Ono, 1981) have been used. Extraction yields of anthocyanins from grapes (Bakker et al., 1998) and from sunflower-hull (Mok and Hettiarachchy, 1991; Gao and Mazza, 1996) were higher when the SO_2 concentration was increased.

In acidic acid solutions the addition of the bisulphite anion to the flavilium cation of cyanidin 3,5-diglucoside results in a colorless adduct which has high stability (Brouillard and El Hage Chahine, 1980). While fruit and must preserved by low levels of SO_2 (200 to 2000 ppm) are bleached by a reversible reaction, very high concentrations (0.8 to 1.5 %) lead to irreversible bleaching (Markakis, 1982). As a general rule, nucleophilic addition to anthocyanins leads to fading either of hydroxyl ion to produce the carbinol base or sulphite ion to produce the adduct SO_2-anthocyanin. The addition of Na_2SO_3 occurs at SO_2 concentration as low as 10^{-3} M (Brouillard and El Hage Chahine, 1980) so that very small amounts of the ion are needed to react with anthocyanins. In the reversible reaction (200 to 2000 ppm SO_2) the SO_2-anthocyanin complex nearly completely dissociates back to the original flavylium salt and free SO_2 upon acidification to pH 1 (Timberlake and Bridle, 1967; Markakis, 1982). Thus, the reaction of nucleophilic addition is not interfering with the quantitative determination of anthocyanins in strong acid media.

Timberlake (1980) suggests anthocyanin and sulfur dioxide form a complex and a structure with attachment of SO_2 to the 4 position. The addition in 2 position (Wenzel, 1986) and formation of two adducts (Brouillard and El Hage Chahine, 1980) have also been suggested. However, unfavorable kinetic and thermodynamic conditions determine that a large amount of the 4 adduct in hydroxylated natural anthocyanin never occurs (Brouillard,

1982).

2. Engineering of Extraction

a. Equilibrium and Diffusion Equations. Solid- liquid extraction is the use of a solvent to remove a soluble fraction from an insoluble, permeable solid. The concentration of each compound within the plant tissue develops an equilibrium with the concentration of solute that will dissolve into the solvent. The equilibrium is described by the equation

$$m = \frac{y_e}{x_{dm}} \qquad (3)$$

where $\boldsymbol{m}$ is the partition coefficient, y_e is the weight fraction of a given compound in the extract and x_{dm} is the weight fraction of the given compound in the dry pomace (Schwartzberg and Chao,1982; Gertenbach, 2001). The larger the value of m, the more of a given compound will dissolve in the solvent. The value of m is a function of the characteristic of both the solvent and the compound as well as the temperature. The solvent used for anthocyanin extraction significantly affects the solid-liquid extraction from sunflower hulls (Pifferi and Vaccari, 1983; Gao and Mazza, 1996).

The extraction will also depend on how fast the compound will dissolve and reach the equilibrium concentration in the liquid. Four mass transfer steps are involved but the diffusion of the dissolved solute in the solid is the rate limiting step (Schwartzberg and Chao,1982; Gertenbach, 2001). The rate of diffusion of that step can be described by Fick's second law:

$$\frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta x^2} \qquad (4)$$

where C is the concentration of the solute, t is time, s, D is the diffusion coefficient or diffusivity, $m^2 s^{-1}$, and x is the distance of diffusion, m. The driving force for the extraction is the concentration gradient within the particles. The rate of extraction increases with a larger concentration gradient. The rate of extraction can also be altered by increasing the diffusion coefficient or reducing the particle size. Thus, maximum break-up of sunflower hull resulted in a higher yield of extracted anthocyanins (Gao and Mazza, 1996). An increase in temperature significantly increases diffusivities as it is predicted by the Einstein equation

$$D \propto (T/\eta) \qquad (5)$$

where D is the diffusion coefficient or diffusivity, $m^2 s^{-1}$, T is the absolute temperature (K) and η the dynamic viscosity, Pa s (Loncin and Merson, 1979).

Diffusivity is a very difficult parameter to determine and data in the literature are scarce. Semi-theoretical and empirical equations such as the Wilke-Chang equation (6) have been developed to estimate diffusivities (McCabe et al., 1985; Cheryan, 1986; Kirwan, 1987). Through equation (6) the diffusivity of component A in solvent B (D_{AB}) can be calculated as a function of temperature and dynamic viscosity coefficient:

$$D_{AB} = \frac{(117.3 x 10^{-18})(\phi M_B)^{0.5} T}{\eta V_A^{0.6}} \qquad (6)$$

where D_{AB} is the diffusivity of component A in solvent B, $m^2 s^{-1}$, M_B is the molecular weight of solvent, kg $kmol^{-1}$, T is absolute temperature, K, η is dynamic viscosity of solution, kg $(ms)^{-1}$, V_A is solute molal volume at normal boiling point, m^3 $kmol^{-1}$ and ϕ is an association

constant for solvent.

In fact, this equation represents an empirical modification to the Stokes-Einstein equation for prediction of liquid phase diffusion coefficients to fit available experimental data. It is preferred for estimating infinite-dilution coefficients of low-molecular weight solutes in non polar and non viscous solvents (Kirwan, 1987).

The diffusivity in concentrated solutions is lower than in dilute solution, as expressed by the empirical equation of Gordon (Cheryan, 1986):

$$D = D_0 \left(\frac{\eta_0}{\eta} \right) \left(1 + \frac{d \ln \gamma}{d \ln C} \right) \qquad (7)$$

where $\boldsymbol{D_0}$ is the diffusivity at infinite dilution, $m^2 s^{-1}$, $\boldsymbol{\eta_0}$ and $\boldsymbol{\eta}$ are the viscosities of the pure solvent and the solution, Pa s, $\boldsymbol{\gamma}$ is the activity coefficient of the solute and $\boldsymbol{C}$ is the concentration of the solute. This equation suggests that the diffusivity can be corrected by a thermodynamic activity term (Kirwan, 1987).

In the mass transfer between a fluid and solid particles, in addition to the transfer step in the fluid phase, significant rate processes occur within the particle or at the solid-liquid interface. Diffusion due to random molecular motion dominates in the leaching of foods and the diffusion of the dissolved solute in the solid is usually rate-controlling. The effect of diffusion in the extraction process is described by the mass equivalent to the Biot number:

$$Bi = \frac{K_L \ m \ a}{D_S} \qquad (8)$$

where $\boldsymbol{K_L}$ is the mass transfer coefficient in the extract, m s^{-1}; $\boldsymbol{a}$ is the characteristic dimension of the solid, m; $\boldsymbol{D_S}$ is the diffusivity in the solid, m^2 s^{-1}, and $\boldsymbol{m}$ is the partition coefficient between the solute concentration in the extract and the solute concentration in the

solid. If $Bi > 200$ the error in D_S due to the neglected external resistance will be less than 1% (Schwartzberg and Chao, 1982). A large number of equations have been proposed to represent the process of mass transfer from a sphere shape solid to a fluid. Generally, the correlations assume that the contributions of molecular diffusion and forced convection are additive. Thus, the Frössling equation appears to represent data for a wide range of Schmidt number (Sc) and Reynolds number (Re)

$$Sh = 2 + 0.6\ \mathrm{Re}^{b}\ Sc^{1/3} \qquad (9)$$

$$\text{where } \mathrm{Re} = \frac{ND_a^2\,\rho}{\eta} \text{ and } Sc = \frac{\eta}{D_S\,\rho}, \qquad (10)$$

D_S is the diffusivity in the solid, m^2s^{-1}, N revolutions per unit time, s^{-1}; D_a diameter of the impeller, m; ρ density ($kg\ m^{-3}$) and η dynamic viscosity (Pa s) of the liquid and the exponent b is found to range from 0.5 to 0.62 for $Re > 1{,}000$ (McCabe et al., 1985; Kirwan, 1987).

The same equation can be used for the mass transfer of a suspension of particles in an agitated vessel. Reynolds number can be obtained by using the terminal velocity of the suspended particles and a minimum mass transfer coefficient can be calculated by using the Frössling equation (McCabe et al., 1985). For liquids and Reynolds number of 2,000 to 17,000, the following equation (Geankoplis, 1978) can be used:

$$Sh = 0.347\ \mathrm{Re}^{0.62}\ Sc^{1.3} \qquad (11)$$

b. Mixing of Solids in Agitated Vessel. For the design of an efficient processing vessel, velocity of the fluid circulated is the major factor to be considered. Besides circulation, the turbulence of the moving stream also affects the effectiveness of dispersion and mixing

operations. Circulation and turbulence consume energy and both increase with stirrer speed, although, type and size of impeller influence the relative values of flow rate and power consumption. An important consideration in the design of an agitated vessel is the power required to drive the impeller. To estimate it, empirical correlations using measurements of the tank and impeller, the distance of the impeller from the tank floor and the liquid depth are used. The linear variables can be converted into dimensionless shape factors by dividing each of the factors by the impeller diameter or the tank diameter. The various shape factors depend on the type and arrangement of equipment. The measurements for a typical turbine agitated vessel are :

$$S_1 = \frac{D_a}{D_t} \quad S_6 = \frac{H}{D_t} \quad S_4 = \frac{W}{D_a} \quad S_3 = \frac{L}{D_a} \quad S_5 = \frac{J}{D_t} \qquad S_2 = \frac{B}{D_a} \tag{12}$$

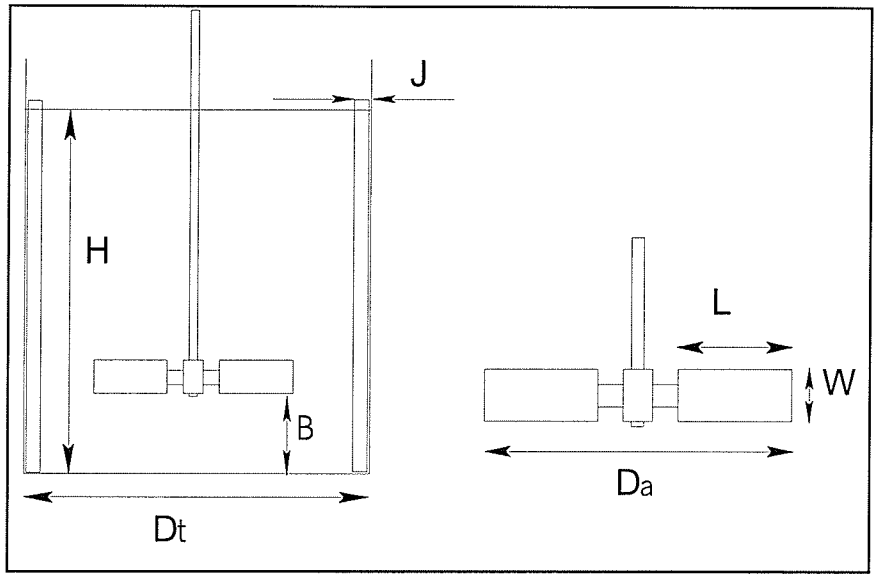


Figure 5. Characteristic Dimensions of the Vessel and Impeller (from McCabe et al., 1985)

i) Design for Uniform Agitation. The variables that enter into the analysis are the shape factors and conditions of the operations, the physical properties of the fluid and the speed of the impeller. Assuming that the fluid is Newtonian and considering dimensionless factors, the power can be equated (McCabe et al., 1985) as the function;

$$\frac{Pg_c}{N^3 D_a^5 \rho} = \Psi\left(\frac{ND_a^2\rho}{\eta}; \frac{N^2 D_a}{g_c}; S_1; S_2; \ldots; S_n\right) \qquad (13)$$

The first dimensionless group $\frac{Pg_c}{N^3 D_a^5 \rho}$ is the power number N_p, the second $\frac{ND_a^2\rho}{\eta}$ is the Reynolds number, N_{Re}, and the third $\frac{N^2 D_a}{g_c}$ is the Froude number, $N_{Fr,}$ $\boldsymbol{P}$ is power delivered by agitator, W; $\boldsymbol{N}$ is rotational speed of agitator, rps; $\boldsymbol{D_a}$ is impeller diameter, m; $\boldsymbol{g_c}$ is gravitation coefficient, m s^2 and $\boldsymbol{\rho}$ density, kg m^{-3} and $\boldsymbol{\eta}$ dynamic viscosity, Pa s of the liquid. Incorporating these substitutions the following equation can be written

$$N_p = \Psi\left(N_{Re}; N_{Fr}; S_1; S_2; \ldots; S_n\right) \qquad (14)$$

Empirical plots of N_p vs. N_{Re} for baffled or unbaffled vessel for different types of impeller and arrangements can be found elsewhere (Charm, 1963; McCabe et al., 1985; Perry et al., 1997). However, at high Reynolds number, when the flow is fully turbulent, the power number is independent of the Reynolds number and the viscosity is not a factor as it is the case in baffled tanks at Reynolds numbers larger than about 10^4 (McCabe et al., 1985).

Then, equation (14) becomes $N_p = K_T = \Psi_T\left(S_1; S_2; \ldots; S_n\right)$ (15)

from which the power of the impeller P is $P = \frac{K_T N^3 D_a^5 \rho}{g_c}$ (16)

where the magnitude of the constant $\boldsymbol{K_T}$ depends on the type of impeller, tank and shape factors. According to this equation, for a given liquid, type and diameter of impeller, and shape factors, the power would depend only on the rotational speed.

The degree of agitation produced by a rotary stirrer has been found to be correlated with mass transfer rates by the equation

$$\frac{K_L \; D_t}{D_S} = 0.16 \left(\frac{N \; D_t^{\,2} \; \rho}{\eta} \right)^{0.62} \left(\frac{\eta}{\rho \; D_S} \right)^{0.5} \qquad (17)$$

for values of $\left(\frac{N \; D_t^{\,2} \; \rho}{\eta} \right)$ higher than 67,000 and where K_L is the mass transfer coefficient, ms^{-1}; N revolutions per unit time; D_t diameter of the vessel, m; ρ density, kg m^{-3} and η dynamic viscosity, Pa s of the liquid (Hixson and Baum, 1941). According to this equation, the mass transfer coefficient depends on the properties of the liquid, diffusivity of the solute and rotational speed. For particles in suspension in an agitated liquid Loncin and Merson (1979) presented a similar equation to (17) that correlates the mass transfer coefficient with the power of the agitator which can be obtained through the empirical plot noted above for different type of impellers, and shape factors. Thus,

$$\frac{K_L \; a}{D_S} = \left(\frac{a^4 \quad P / M}{\nu^3} \right)^{1/6} \left(\frac{\eta}{\rho \; D_S} \right)^{1/3} \qquad (18)$$

where D_S is the diffusivity in the solid, m^2s^{-1}; a is the diameter of the particles, m; and P/M is the power of the agitator per unit of total mass, W kg^{-1}, ν is the kinematic viscosity, $m^2\ s^{-1}$, ρ is the density, kg m^{-3} and η is the dynamic viscosity, Pa s of the liquid.

ii) Design for Rapid Mixing. In the suspension of solids in an agitated vessel, the particles are separated and kept in motion by the fluid flowing between them. There are several ways of defining the suspension of solids in an agitated tank, according to the degree of suspension such as, nearly complete suspension with filleting, complete particle motion, complete off-bottom suspension and uniform suspension. The most widely used technique

for evaluation of suspension of solid particles in agitated liquid is called the "speed for just suspension", N_{js}. According to this theory, with D_a/D_t ratios lower than 0.4 uniformity throughout the rest of the tank is minimal. In D_a/D_t ratios greater than 0.4 the rest of the tank has a very vigorous fluid motion with marked approach to complete uniformity before N_{js} is reached (Perry et al., 1997). McCabe et al. (1985) measured the critical speed for complete suspension which is given by the dimensionless equation

$$n_c D_a^{0.85} = s\nu^{0.1} a^{0.2} \left(g_c \frac{\Delta\rho}{\rho} \right)^{0.45} b^{0.13} \qquad (19)$$

where n_c = critical stirrer speed, ; D_a diameter of the impeller, m; a average particle size, m; ρ density, kg m^{-3} and ν kinematic viscosity, m^2 s^{-1} of the liquid; b = 100 x weight of solid/ weight of liquid; s is a constant depending of the type of impeller and dimensionless shape factors (McCabe et al., 1985). There is a major effect of both shear rate and circulation time on the solid-liquid mass transfer process. The initial increase in power causes more and more solids to be in active contact with the liquid and has a much greater rate of mass transfer than that occurring above the power for suspension (Perry et al., 1997). Then, working at a power level higher than that required for the just suspension speed, the changes in mass transfer due to variations in speed are reduced.

iii) Selected Mixing Conditions

Increase of head velocity of the impeller discharge stream leads to rapid reduction of the concentration differences in the impeller stream, production of large interface area and promotion of mass transfer between phases. At the same power consumption, a higher discharge velocity can be obtained using a smaller diameter impeller at higher rotational

speed. An impeller is considered to have a small diameter when $D_a < D_t/3$ and a large diameter when $D_a > D_t/2$ (Perry et al., 1997). This means a dimensionless factor S_1 lower than 0.33 or larger than 0.5.

For dispersion of particles in liquids and for rapid initial mixing of liquid reactants, a propeller at a distance of $D_t/4$ above the vessel bottom and at a speed of 1150 and 1750 rpm is recommended (Perry et al., 1997). Reynolds numbers in a turbulent regime ($Re>10^4$) assure approximately constant power according to relationship between Reynolds number and power number from experimental plots (McCabe et al., 1985; Perry et al., 1997).

A vessel straight-side-height to diameter ratio (H/D_t) of 0.75 to 1.5 and the location of the impeller at one third of the liquid depth above the vessel bottom is recommended to obtain uniform circulation and mixing (Perry et al., 1997). A clamp- mounted, 15 ° angular off center configuration of the impeller shaft eliminates swirling and vortex formation in a tank with no baffles.

c. Determination of diffusivity. Experimental determination of diffusion coefficients has been achieved using different methods. The diffusion coefficient of phytochemicals can be evaluated from model systems simulating practical extraction conditions. However, the formulation and solution of the appropriate mathematical model have to be properly done for the particular experimental diffusion situation. Mathematical solutions of Fick's second law (equation (4)) have been presented (Carman and Haul, 1954; Crank, 1975; Schwartzberg and Chao, 1982) for the diffusion of a compound during solid-liquid extraction in an agitated vessel.

Solutions of Fick's second law (equation (4)) are used to determine D_S, assuming that D_S is constant; $C = C_b$ for $0 < x < a$ when $t = 0$. The volume ratio α is

$$\alpha = \frac{E\ m}{S} \qquad (20)$$

where E and S are the respective extract and solid volume, L and m is the partition coefficient (equation (3)). In batch extraction tests for determining D_S by stirring particles and solvent together in a vessel the minimum usable E/S ratio is about 2:1 (Schwartzberg and Chao, 1982). Mathematical equations for the determination of diffusivities of phenolics during solid-liquid extraction are included in Appendix I.

C. Purification and Concentration of Phenolic Extracts

1. Purification with Adsorption Column

In adsorption solid/liquid chromatography the stationary phase is a finely divided solid (adsorbent) and the mobile phase is a liquid. The adsorbent has to be chosen to permit interaction with the desired components of the sample. The intermolecular forces primarily responsible for chromatographic adsorption are: van der Waals' forces, electrostatic forces, hydrogen bonds and hydrophobic interactions (Nielsen, 1994).

Adsorption is a concentration dependent process and the adsorption coefficient is not a constant. Some of the factors that affect the adsorption process are the surface area of the adsorbent, preparation of the adsorbent, the nature of both adsorbent and adsorbate, temperature and the adsorbate concentration. Loads exceeding the adsorptive capacity of the stationary phase will result in very poor adsorption because of the saturation of the adsorbent

surface area.

Classical adsorption stationary phases are silica, alumina, charcoal and a few other materials. Cellulose, silica gel, alumina, polyvinylpyrrolidone, molecular sieve and ion exchange and adsorption polymeric resins such as Duolite, Amberlite and Bio-Rex were tested for the purification of commercial grape pigments (Lin and Hilton, 1980). Sixteen solid-phase materials including non polar reversed-phase materials, weak and strong ionic exchange resin, and non-ionic acrylic polymers such as Serdolit and Amberlite were tested on a laboratory scale for the purification of anthocyanins from black chokeberry, var Nero (*Aronia melanocarpa*). Reverse phase silica gel and Amberlite XAD 7 were the two best column materials in terms of negligible loss of color, and anthocyanin quality measured by spectroscopic parameters (Kraemer-Schafhalter et al., 1998). Non-ionic copolymers of styrene-divinylbenzene, Amberlite XAD 2 and Amberlite XAD 4 were not satisfactory because either pigments washed out with the water or it was not possible to get all pigments from the column with black chokeberry (Kraemer-Schafhalter et al., 1998). Grape pigments showed a very strong adsorption on the same Amberlite resins (Lin and Hilton, 1980).

Amberlite XAD-16 is a styrene/divinylbenzene based material that is a non-ionic, hydrophobic, crosslinked polymer with a surface having an aromatic nature (Anonymous, 1996). The benzene ring on the synthetic resin and phenolics enhance organophilic adsorption where two non- polar molecules are held together by van der Waals' forces. Polyphenols are adsorbed by XAD-16 resin by van der Waals' forces between two aromatic rings (Gump and Huang, 1999). Amberlite XAD16 along with six other resins were tested for recovery of anthocyanin. It was the most effective adsorbent-desorbent (Yu, 1998).

In general, acidification of alcoholic eluents as well as the washing water leads to

better anthocyanin recovery. Acidic pH keeps anthocyanins in the more stable flavilium cation form and neutral or slightly acidic solvents result in incomplete elution of anthocyanins (Kraemer-Schafhalter et al., 1998). Use of weak organic acids such as formic, citric or acetic acids is recommended to avoid hydrolysis of acylated anthocyanins (Macheix et al., 1990).

2. Purification with Membrane

The principle of membrane technology uses a membrane as a selective permeable barrier to concentrate, fractionate and/or purify fluids by generating two streams, the permeate and the retentate, with different compositional characteristics. The retentate will be enriched in the retained macromolecules and it will contain some of the permeable solutes also. However, since the retentate shows a much smaller volume than the feed, there have been purification and concentration of the retained species. The permeate, that is recovered on the other side of the barrier, will collect mainly solvent and smaller species passing through the pores of the membrane. The primary factor affecting the separation is the particle size and the driving force of the transport process is the hydraulic pressure applied.

Membrane filtration processes are identified by the pore size of membrane and the solute molecular weight they separate. Although the limits are not sharp, the technologies employed for separating particulate material of very low size are ultrafiltration (UF), nanofiltration and reverse osmosis (RO). Ultrafiltration retains only macromolecules or particles larger than about 0.001 - 0.02 μm (Cheryan, 1986). It is customary to refer to "molecular cut-off" instead of particle size. Thus, UF is the process that works with molecules from 1,000 to about 1,000,000 in molecular weight and nanofiltration from about

200 to 30,000 (Girard and Fukumoto, 2000).

Because a phase change is not involved in the water removal, the application of heat is not required. Furthermore, UF and RO processes can be operated at ambient temperature (Cheryan, 1986). For these reasons, besides the potential savings in energy, membrane technology is a valuable alternative for obtaining high recovery and good quality of heat sensitive products.

Ultrafiltration has been used for concentration of perilla anthocyanins from a 10% citric acid solution (Chung et al., 1986) in the retentate using a membrane of MW cut-off 6000 and for purification of anthocyanins extracted from cranberry pulp wastes in the permeate using membranes of MW cut-off 20,000 and 50,000 (Woo et al., 1980) and for purification of commercial grape pigment extracts in the retentate using a membrane of MW cut-off 10,000 (Lin and Hilton, 1980).

Molecular weight of anthocyanins is around 600. Formation of copigment complexes has allowed researchers recoveries superior to 60% with a membrane with a MW cut-off of 6000 (Chung et al., 1986), about 10 times higher than anthocyanins MW. The nature of the pigment will play a role. A membrane with a MW cut-off of 50,000 caused a significant loss of grape pigments to the permeate but a membrane of 10,000 working at 22 °C with 10% pigment extracts resulted in a loss of less than 10% (Lin and Hilton, 1980). The use of smaller MW cut-off will assure higher recovery of pigments in the retentate, however, flux rate will be reduced. Membranes of 500, 1000 and 5000 MW cut-off yielded permeation rate of less than 1 mL h^{-1} cm^{-2} with grape pigment extracts (Lin and Hilton, 1980).

The concentration of the feed is also a factor. The more concentrated the feed the larger the anthocyanins recovery. An increase of feed concentration from 10 to 15% w/w increased

anthocyanin recovery from perilla extracts from about 70 to 85% (Chung et al., 1986). However, the concentration of the feed affects the flux also. Flux shows an exponential reduction with pigment concentration of the feed. Thus, a reduction from about 3.7 to 0.4 mL h^{-1} cm^{-2} was found when grape extracts increase from 1 to 20 % in the feed (Lin and Hilton, 1980). A similar trend has been reported for UF of perilla extracts at different trans-membrane pressures with the increase of feed concentration (Chung et al., 1986). The type of equipment utilized affects performance. In crossflow filtration, the feed stream moves parallel to the surface and thus reduces the formation of a layer and accumulation of particulate material on the surface of the membrane. In dead-end filtration, the stream moves perpendicular to the membrane and the formed cake affects the filtration flux.

Recovery is also a function of the volume concentration ratio. When UF proceeds for longer time, the concentration of solute in the retentate changes, and thus the recovery. In order to be able to compare UF processes of different samples, the end point of the process must be clearly specified. A volume concentration ratio of 4 was used with perilla anthocyanins and recoveries over 60 % were obtained using a 6,000 MW cut-off membrane (Chung et al., 1986).

The presence of pectins has a negative effect on UF flux. Depectinization reduces the size of the particles and decreases the viscosity resulting in higher flux. This operation is needed to achieve high flux and concentration factors in membrane processes. Other juice components may also have some effects. High molecular weight polysaccharides in sloe juice and wine made filtration more difficult (Girard and Fukumoto, 2000). Other processing steps besides depectinization, such as prefiltration, decantation, vacuum filtration and centrifugation, reduce the particulate matter, improving the flux and concentration factors.

D. Health Effects of Phenolics

1. General Effects

There is currently considerable interest in functional foods and nutraceuticals and this interest is driven by the potential of these products to improve the health of citizens, economic opportunity, help diversification, and contribute to increased sales of high-value products. Epidemiological evidence suggests that consumption of fruits and vegetables has a protective effect against coronary heart disease, stroke and cancer (Block et al., 1992; Ness and Poulens, 1997; Joshipura et al., 1999). Phenolic compounds are responsible for healthy effects such as reduction of coronary heart diseases; treatment of urinary tract disorders; cancer preventive activity; improvement of visual acuity; anti-inflammatory, antiatherogenic, antibacterial and antiviral activities and prevention of other diseases associated with aging (Benavente-Garcia et al., 1997; Mazza, 2000; Smith et al., 2000). Coronary heart diseases have been associated with human low density lipoprotein (LDL) oxidation, the lipid peroxidation chain reaction that has been most widely studied (Frei, 1995; Halliwell et al., 1995). Thus, natural antioxidants have been positively associated with coronary heart diseases such as myocardial infarction, stroke, coronary death, bypass, or angioplasty by epidemiological studies (Machlin, 1995). Phenolic compounds protect other natural antioxidants such as β-carotene and α-tocopherol (Abuja et al., 1996), collaborating in the reduction of disease risk. Most of the beneficial characteristics of phenolic compounds might be related to their antioxidant activity.

2. Antioxidant Activity

Previous studies have examined the antioxidant activity of fruits, vegetables and grain products (Velioglu et al., 1998), fruits (Wang et al., 1996) and berries (Wang and Lin, 2000), as well as the relationship of antioxidant activity with the structure of flavonoids (Shahidi and Wanasundara, 1992; Rice-Evans et al., 1996; Benavente-Garcia et al., 1997; Fukumoto and Mazza, 2000; Burda and Oleszek, 2001). The antioxidant capacity is closely related to three particular structural groups of flavonoids: a) the o-dihydroxy structure in the B ring, b) the 2-3 double bond in conjugation with a 4-oxo function, and c) the presence of both 3-(a) and 5-(b)-hydroxyl groups (Balentine et al., 1997; Benavente-Garcia et al., 1997). Flavonoids have the ability of blocking or scavenging free radicals. This mechanism is mainly based on their structural ability to capture electrons along with a great stability of the flavonoid radical formed which prevents the propagation of the oxygen free radicals. Flavonoids can inhibit lipid peroxidation by scavenging radicals or quenching singlet oxygen. They also may react with peroxy radicals bringing about the termination of radical reactions (Tsuda et al., 1994). Thus, flavonoids may exert their antioxidant action at different points of the oxidation scheme; anti-hydroxyl radical activity, antiperoxidant activity, antioxygen activity, anti-superoxide-oxygen-radical activity, and metal chelating activity (Bombardelli and Morazzoni, 1993).

Flavonoids have proved to be powerful antioxidants (Vinson et al., 1995; Fukumoto and Mazza, 2000). Fruit extracts influence the oxidation of LDL and food lipids. The delay of copper- and peroxyl-induced oxidation of human LDL by elderberry extracts was proportional to the extract concentration (Abuja et al., 1996). Different concentrations of wild blueberry extracts also slowed or suppressed the initiation and the propagation phases

of linolenic acid oxidation (Smith et al., 2000).

Berries, which have a varied content of anthocyanins and other phenolics, have been found to posses a varying antioxidant ability. The antioxidant capacity has been related to anthocyanins and total phenolic content for several berries (Fukumoto and Mazza, 2000) and for several genotypes of ***Vaccinium*** **L.**, ***Rubus*** **L.**, and ***Ribes*** **L.** (Kähkönen et al., 2001; Moyer et al., 2002). Thus, blackberries and black raspberries, with a very high content of anthocyanins have been found to be more active than other berries in the inhibition of LDL oxidation. The percentage of inhibition decreases in the order: blackberries, red raspberries, sweet cherries, blueberries and strawberries (Kalt et al., 1999). Other antioxidant compounds, however, may also play a role. Sweet cherries and blueberries with higher hydroxycinnamate content were more efficient inhibitors of food liposome oxidation than in LDL oxidation (Heinonen et al., 1996).

Antioxidant activity of several anthocyanins and extracts of grapes and berries have been reported. Cyanidin 3-glucoside has been found to be twice as effective as commercially available antioxidants, such as BHA and α-tocopherol (Fukumoto and Mazza, 2000; Mazza, 1997). Anthocyanidins have a different antioxidant effect on human LDL than on lecithin liposome oxidation. Thus, delphinidin and cyanidin, two of the main aglycones of anthocyanins, were the most efficient inhibitors of LDL oxidation. However, in the liposome system malvidin was the best antioxidant species. It has been suggested that the lower pH of the liposome may reduce the ability of hydroxyl groups of delphinidin and cyanidin to ionize and may impair copper chelation. The lower polarity of malvidin may impart a better affinity for the interface and enhance the prevention of oxidation of the lecithin fatty acids (Satué-Gracia, et al., 1997).

Composition and antioxidant capacity change with physiological state and storage of fresh berries (Kalt et al., 1999; Prior et al., 1998). Anthocyanin content continuously increases from green to ripe berries. Fruits show a lower antioxidant activity at the pink stage. This fact relates to the higher concentration of phenolics at the green stage and higher anthocyanins at the ripe stage. Besides, both anthocyanin content and antioxidant activity of raspberries and strawberries increase with storage time and temperature. Blueberry properties are less affected during storage of fresh fruits (Kalt et al., 1999).

III. MATERIALS AND METHODS

A. Materials

1. Berries

Individually quick frozen (IQF) berries obtained from commercial fruit growers were used. Damaged or unripe fruits were discarded.

- Black currants (***Ribes nigrum* L.**) cv. Ben Lomond, from M & G Bros Farm, Abbotsford, BC, 2000 growing season.
- Blackberries (***Rubus fructicosus* L.**) cv. Locknest, from M & G Bros Farm, Abbotsford, BC, 2000 growing season. and
- Saskatoon berries (***Amelanchier alnifolia Nutt.***) cv. Smoky from the Berry Basket, Clairmont, AB

Berries were stored in the dark, at -36 °C, until used.

2. Chemicals

Following chemicals were used: sodium metabisulphite ($Na_2S_2O_5$) from BDH Inc. (Toronto, ON) and quercetin, caffeic acid, chlorogenic acid, β-carotene, 1,1-diphenyl-2-pycril-hydrazyl (DPPH·), butylated hydroxytoluene (BHT), α-tocopherol and catechin hydrate were purchased from Sigma- Aldrich Ltd. (Saint Louis, MO). Cyanidin chloride, kuromanin (cyanidin 3-glucoside) chloride, were purchased from Extrasynthese (Genay, France). Enzymes Rapidase Super BE containing pectinases and hemicellulases derived from

Aspergillus niger, were from DMS Food Specialties Inc. (Charlotte, NC). α-α' azodiisobutyramine dihydrochloride (ADIBA) and linoleic acid were purchased from Fluka Chemika (Sigma- Aldrich Ltd., Saint Louis, MO).

3. Laboratory Equipment

Following equipment were used: absorption column (Pharmacia, model SR 25/100, Amersham Pharmacia Biotech Inc., QB); airfoil axial impeller Lightnin (model A310, Mixing Equipment Co. Inc., Rochester, NY); blender (Waring®, Dynamic Corporation of America, New Hartford, CT); centrifuge Beckman (model Avanti J25 I, rotor JLA 10.5, Beckman Instruments Inc., Mississauga, ON); HPLC (Agilent 1100, Agilent Technologies Inc., Palo Alto, CA); hand refractometer (Atago Co, Japan); HPLC reversed-phase C_{18} column, Zorbax SB 5 μm, 4.6x250 mm (Agilent Technologies Inc., Palo Alto, CA); HPLC guard column Inertsil 5 ODS-2, 5 μm, 4.6x30 mm (Phenomenex, Torrance, CA); micro plate reader (model Spectra max Plus, Molecular Devices Corporation, Sunnyvale, CA); rotary evaporator (model RE51, Yamato Scientific Co. Ltd., Japan), sieve shaker (model Octagon 200, Endecotts Limited, London, England); spectrophotometer (model Beckman DU 640, Beckman Instruments Inc., Fullerton, CA); ultrafiltration thin-channel unit (model Amicon TCF10, Millipore Corporation, Bedford, MA); Wiley mill (model ED-5, Arthur H Thomas Co, Philadelphia, PA).

4. Laboratory Supplies

Following laboratory supplies were used: syringe-filter holders and 0.45 μm PVDF membrane discs, 25 mm diameter (Pall Gelman Sciences, Ann Arbor, MI); Whatman N° 541 filter paper (Fisher Scientific, Nepean, ON); non ionic packing material Amberlite XAD 16

(Rohm & Haas, Philadelphia, PA); 96-flat bottom well EIA microtitration plate (ICN Biomedicals Inc.); ultrafiltration membranes 90 mm disc filters: cellulose acetate 500 MW cut-off membranes (Amicon YC05, Millipore Corporation, Bedford, MA); regenerated cellulose 100,000 and 1,000 MW cut-off membranes (Amicon YM 100 and YM 1, Millipore Corporation, Bedford, MA); 10,000 MW cut-off membranes (Amicon PM 10, Millipore Corporation, Bedford, MA).

B. Methods

1. Sample Preparation

Frozen berries were dipped in liquid nitrogen and milled with a pre cooled Wiley mill (model ED-5, Arthur H. Thomas Co, Philadelphia, PA). Milling conditions were chosen following preliminary trials to obtain a particle size as large as possible. Larger particle size would reduce the diffusion rate, increase extraction time and would help to study the process. The lower the rotational speed, the higher the particle size, because milled particles could more easily get out of the mill (Green, 2001). The retention time was shorter and particle size was larger when using a screen with larger diameter holes and wider gap between the cutting blades. Thus, the rotational speed was selected at 520 rpm, the minimum speed setting of the Wiley mill used. A screen of 6 mm hole diameter was chosen, and blades were set at a distance of 2 mm.

The mill was insulated with styrofoam, put into a cold room at 0°C, and pre-cooled with liquid nitrogen to maintain the particles in a frozen state. During size reduction, berry particles would absorb heat generated in the process and would thaw very easily, because of the large surface to volume ratio. Frozen berries were dipped in liquid nitrogen and some

liquid nitrogen was introduced into the mill with berries. Small containers were used to collect milled berries which were transferred to the storage room at -36°C as soon as the container was filled. Approximately 2 kg of berries were milled each time. During the milling process, berries produced different fractions and particle size depending on what part of the fruit was being milled. For that reason, each batch of milled berries was thoroughly mixed in a 10 L pail to homogenize material to be used for the extractions. Then, berries were transferred to two smaller containers which were flushed with nitrogen gas, closed hermetically and stored at -36°C until used.

Particle size distribution was characterized by sieving 200 g of milled berries at -25 °C with a sieve shaker (model Octagon 200, Endecotts Limited, London, England) and 20 cm diameter US standard sieves N° 5 (4.0 mm), 7 (2.8 mm), 10 (2.0 mm), 16 (1.18 mm), 18 (1.0 mm), and 60 (0.25 mm). Milled berries, shaker and sieves were kept in a refrigerated room at - 25°C for at least 2 h before sieving. The shaker was set at maximum speed (# 9) and run for 20 min. Sieves were weighed before and after sieving and the sample weights remaining in each sieve were recorded. Particle size distribution and average size were obtained by plotting sieve mesh size vs the cumulative passing weight percentage using a log-probability plot as shown by Gertenbach (2001).

2. Extraction Vessel and Mixing Conditions

The rotational speed of the impeller was set at a constant value of 1210 rpm, within the recommended range of 1150 to 1750 rpm (Perry et al., 1997) which was needed to achieve a Reynolds number higher than 10^4 , and a turbulent regime that assured an approximately constant power. In order to eliminate variation from the mixing system

employed, the geometry of the vessel and impeller, diameters ratio, Reynolds number, and position of shaft and impeller were fixed for all the extractions in the study. A fluid foil impeller of 6.35 cm and a vessel of 15.6 cm in diameter were selected, and thus the impeller to vessel diameter ratio was 0.41.

The location of the impeller was at 3.9 cm from the bottom, at the recommended $D_t/4$ distance. A solvent volume of 2.5 L and a height of 11.7 cm were used so that the impeller was also located at one third of the liquid depth above the vessel bottom. Height changed when milled berries were added to the solvent, but all the values were still lower than 23.4 cm, and within the suggested range for the vessel straight-side-height to diameter ratio (H/D_t) of 0.75-1.5 required to obtain uniform circulation and mixing (Perry et al., 1997). A clamp-mounted 15 ° angular off center fluid foil impeller in a tank with no baffles was used to avoid swirling and vortex formation. The distance of the impeller to the wall was 4.1 cm.

3. Extraction Methods

a. Extraction for optimization experiments. Milled frozen samples were dispersed in 2.5 L solvent in an agitated 4 L glass beaker. Aqueous ethanol and water containing sodium metabisulphite ($Na_2S_2O_5$) were used as solvents. The pH of the solvents was adjusted to 3.8 - 4.1 with acetic acid. The selection of this pH was from previous research which showed that extraction of anthocyanins from sunflower-hulls with SO_2 containing water was not affected by pH in a range of 3.5 to 7 (Gao and Mazza, 1996), and that anthocyanins were more thermally stable at pH 3 than at 1 or 5 (Mok and Hettiarachchy, 1991). Sulfur dioxide and ethanol concentrations, and sample weights on a dry weight basis were varied according to the preset experimental design. An airfoil axial impeller (model Lightnin A310, Mixing

Equipment Co. Inc., Rochester, NY) of 6.35 cm diameter at a rotational speed of 1210 rpm was used for mixing and agitation. The beaker was set in a thermostatic water-bath set at the desired temperature. Milled berry sample weights were added to the extractor after the desired temperature was reached.

During extraction, 3 mL samples of liquid were taken every 2-3 min for the first 60 min. After the first hour, samples were taken every 30 min. Extractions were stopped when equilibrium was reached as measured by the lack of change in the 280 and 520 nm absorbance values of the extracts. This procedure establishes a difference with previous extraction research studies which have used a constant time. The technique used here allowed a more thorough extraction and thus evaluation of the maximum capabilities of every tested extraction condition. Besides, the equilibrium time rises as an additional independent variable.

Once the extraction was finished, supernatant was separated from pomace by centrifugation in a refrigerated centrifuge (Centrifuge Beckman, model Avanti J25 I, rotor JLA 10.5, Beckman Instruments Inc., Mississauga, ON). Centrifugation speed was at 8,000-10,000 rpm for 30 min at a temperature range of 10 to 20°C. Weight of the wet pomace, total volume of extract and weight of a 1000 mL volume of extract were recorded. Pomace and extract samples were stored in closed plastic containers at -30 °C until further analyses.

Times to reach equilibrium for anthocyanins and total phenolics were obtained from plots of compound contents vs. extraction time. Curves that best fit experimental points were used to determine equilibrium time. Curves to reach a maximum asymptote and predicted maximum phenolic values were obtained using Sigma Plot® software (SPSS Inc., Chicago, IL). Equilibrium times were times when curves reached the predicted maximum value. The

partition coefficient m was calculated by the equation,

$$m = \frac{y_e}{x_{dm}} \qquad (3)$$

where y_e is weight fraction of a given compound in the extract and x_{dm} is the weight fraction of a given compound in the dry pomace (Schwartzberg and Chao, 1982; Gertenbach, 2001).

Yields in mg/g of frozen berries on a dry weight basis, and recovery F of anthocyanins and total phenolics were evaluated from contents in the final extracts and calculated using the equation

$$F\ (\%) = \frac{C_{eq}}{C_b}\ 100 \qquad (21)$$

where C_{eq} is the content in mg/g of frozen berries on a dwb of a given compound in the final extract and C_b is the content in mg/g in the frozen berries.

b. Extraction of Pre-Treated Material. Extractions used liquid samples from the pre-extraction treatment. The whole volume of sample from pre-extraction treatment was dispersed in 2.5 L solvent volume in an agitated 4 L glass beaker. Water containing sodium metabisulphite at a fixed concentration of 1100 ppm was used as solvent. The pH of the solvent was adjust to 3.8 - 4.1 with acetic acid. Extraction beaker was set in a thermostatic water bath set at 25°C for all extractions.

4. Pre-Extraction Method

Pre-extraction treatments consisted of three temperatures and one enzymatic treatment to induce cell membrane break down. Enzyme Rapidase Super BE, containing pectinases and hemicellulases from *Aspergillus Niger* (Beverage Ingredients, DMS Food Specialties Inc.,

Charlotte, NC) was used. For enzymatic hydrolysis, a temperature of 45°C and incubation time of 60 min were used. Enzyme doses of 1.3 mL/kg dwb were applied. Temperature treatments were 30, 45 and 60°C. An incubation time of 60 min was used in all the pre-treatments. Blackberries, black currants and saskatoon berries were milled as described above in *Section B.1.* Sample weights to maintain an equal solvent to solid ratio of 60 mL/g on a dry weight basis were used. Milled berries were thawed and warmed up with 200 mL of water into a beaker set in a thermostatic bath, to reach pre-treatment temperature before enzymes were added and incubation time began to run. During pre-treatments five samples of 3 mL were taken and analyzed as indicated below.

5. Purification Methods

a. Adsorption Column

i) Purification. Extracts from SO_2-containing water extractions of the pre- treatment experiment were used for purification and concentration trials. A 2.5 cm inside diameter column (Pharmacia, model SR 25/100, Amersham Pharmacia Biotech Inc., QB) packed with about 70 g to a 22 cm height with a non-ionic solid phase material (Amberlite XAD 16, Rohm & Haas, Philadelphia, PA) was used. Preparation and conditions were selected from the supplier's information chart (Anonymous, 1991). Measured bed volume of packed column was approximately 40 mL. Samples of 200 mL were fed into the column at a flow of 2.5 - 3.5 mL/min. Adsorbed phenolics were washed to remove sugars and other impurities with 250 to 300 mL of distilled water, acidified to pH 2.9 with acetic acid. Reduction of sugars in the washing water was measured using a hand refractometer (Atago Co, Japan). Then, phenolics were eluded with 80% ethanol, acidified to pH 3 with acetic

acid, using a flow rate of 1.7 to 2.3 mL/min and 450 g (500 mL) of eluate was collected.

ii) Concentration. Ethanol extracts collected from the bottom of the column were concentrated to a final volume of about 100 mL using a rotary evaporator (model RE51, Yamato Scientific Co. Ltd., Japan) operated at 81 kPa vacuum and 30 °C. Samples of extracts after the chromatography column and after concentration were stored at -36°C.

b. Membrane Purification. For ultrafiltration, an internally circulating system was used for efficient concentration and fractionation. Sulfur dioxide-containing water extracts from the pre-treatment experiment were used for purification trials. Samples of 200 mL were fed into a thin-channel ultrafiltration unit (model TCF10, Amicon Division, Millipore Corporation, Bedford, MA) using a peristaltic pump. The unit had an effective filtration area of 40 cm^2 and recirculation rate of 0-500 mL/min. The unit was operated with the speed control setting at 5, and with a nitrogen gas pressure of 276 kPa for an efficient filtration. Ultrafiltration membranes made of cellulose acetate, MW cut-off of 500 (Amicon YC05, (Millipore Corporation, Bedford, MA USA) were selected to collect most of the pigments in the retentate phase. Collected permeate weight was recorded during purification to measure the flow rate. The unit was run until a final permeate volume of around 150 mL (a concentration of approximately 4:1) was collected. Permeate and retentate volumes and weights were recorded. Phenolic concentration and antioxidant activity of retentate and permeate were measured as described below.

6. Chemical Analyses

a. Composition. Phenolics in frozen berries and wet pomace were determined by blending 10 g and 20 g samples for 8 min, with 80 % ethanol using 80 mL solvent. The extract was

filtered through Whatman No. 541 filter paper in a Buchner funnel under vacuum. The solid cake was re-extracted for 8 min in the blender with 60 mL solvent and filtered again. Extracts were combined in a 250 mL volumetric flask and made up to volume with 80% ethanol. Phenolics in milled frozen berries were determined by two separate methods; by extraction with 80% ethanol in a blender (Waring®, Dynamic Corporation of America, New Hartford, CT) and by the equilibrium method of Schwartzberg and Chao (1982). In the equilibrium method, approximately 4 g samples of milled frozen berries were soaked in 100 mL of 60 % ethanol and kept in an incubator at 20 °C for 60 h. Extraction time was selected to assure a complete extraction; thus, extraction time was longer than equilibrium times of 15 to 40 h reported for extraction with ethanol (Metivier et al., 1980). Supernatant was filtered through Whatman No. 541 filter paper in a Buchner funnel under vacuum, collected in a 100 mL volumetric flask and analyzed. Dry matter content was measured by drying 2 to 4 g samples in a vacuum oven at 70 °C for 30 h.

For the determination of phenolic contents, all extracts were filtered through 0.45 μm PVDF 2.5 cm membrane disc held in syringe filter holders (Pall Gelman Sciences, Ann Arbor, MI). Samples were analyzed for total phenolics, tartaric esters, flavonols and anthocyanins using a modified version of the Glories' method (Glories, 1979) as modified by Mazza et al. (1999). Briefly, the method consisted of mixing 0.25 mL of sample with 0.25 mL of 0.1 % HCl in 95% of ethanol and 4.55 mL of 2% HCl. Absorbance was then read at 280, 320, 360, and 520 nm in a spectrophotometer (model Beckman DU640, Beckman Instruments Inc., Fullerton, CA) to measure total phenolics, tartaric esters, flavonol, and anthocyanins, respectively. Standard compounds used included: chlorogenic acid, caffeic acid, quercetin, cyanidin 3-glucoside, and cyanidin chloride.

b. Antioxidant Activity. Antioxidant activity of extracts was evaluated at a uniform concentration range as anti-radical efficiency by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as described by Brand-Williams et al. (1995), and as antioxidant activity by the β-carotene method described by Marco (1968) as modified by Fukumoto and Mazza (2000) for use with a micro-plate reader. Antioxidant activity of extracts was also evaluated keeping existing differences of concentration among extracts by an antioxidant index. Thus, initial and after 90 min absorbances from the β-carotene method were used to calculate antioxidant index as indicated by Wettasinghe and Shahidi (1999).

i) Antiradical Activity by DPPH Method. Briefly, for the modified version of the DPPH method, a 150 mM solution of DPPH was prepared in 80% methanol, and absorbance readings were obtained with a micro plate reader. Dilutions of extracts and standards were prepared to have seven sample solutions with concentrations from about 20 to 500 μM of chlorogenic acid equivalents. A 22 mL sample (or standard) and 200 mL of DPPH solution were added to a well in a flat bottom EIA microtitration plate (ICN Biomedicals Inc.). As soon as the plate was filled, an initial reading of the plate was taken. A micro plate reader, model Spectra max Plus (Molecular Devices Corporation, Sunnyvale, CA) was used for reading at 520 nm. The plate was then covered and left in the dark for 30, 180 and 360 min, when the absorbance was read again. Standards used included butylated hydroxytoluene (BHT), and α-tocopherol. Antiradical activity is defined by the initial slope value in units of μM of DPPH/ μM of antioxidant expressed as chlorogenic acid.

ii) Antioxidant Activity by β-Carotene Method. Briefly, the modified β-carotene method consists of preparing a mixture of 1 mL of β-carotene (2 mg/mL in chloroform), 0.2

mL of linoleic acid and 2 mL of Tween 20. The mixture was vortexed and chloroform was removed using a stream of nitrogen for 1-1.5 h. Air sparged water (20 mL) was then added to the mixture, which was subsequently vortexed to form a clear solution. Standards used included butylated hydroxytoluene (BHT), and α-tocopherol. Dilutions of extracts and standards were prepared to have seven sample solutions with concentrations from about 100 to 1500 μM of chlorogenic acid equivalents. Samples (or standard) of 20 μL and 200 μL of the β-carotene solution were added to a well in a 96-flat bottom well EIA microtitration plate (ICN Biomedicals Inc.). Samples were prepared in triplicate for each concentration used. Azodiisobutyramidine dihydrochloride (ADIBA) (20 μL of 0.3M) was used to initiate the reaction, and the plate was read in a micro plate reader (model Spectra max Plus, Molecular Devices Corporation, Sunnyvale, CA) at 450 nm at 0 min and after 90 min of incubation in the dark. Absorbance at 450 nm after 90 min of incubation was plotted against concentration of sample added. The slope for the initial linear portion of the plot was used to calculate antioxidant activity in units of $Abs_{520}/\mu M$ of antioxidant expressed as chlorogenic acid.

iii) Antioxidant Index Determination. Antioxidant index method is based on spectrophotometric readings at 0 and 90 min from β-carotene method. One dilution of each extract was prepared to have a final value in the plates in a range close to 100 - 200 ppm. All extracts were diluted using the same dilution factor, so that concentration differences among samples were maintained. Procedures for filling the plate and taking readings were the same as for the β-carotene method described above. Antioxidant index was calculated by the ratio of absorbance at 90 min to absorbance at initial time and expressed as a percentage.

c. HPLC Analysis. HPLC analysis was carried out using a liquid chromatograph system (Agilent 1100 Series, Agilent Technologies Inc., Palo Alto, CA) equipped with a photodiode array detector, an auto sampler and a control module. Extracts from treatments 9, 16 and 10, extracted at 6, 40 and 74°C with 67% ethanol and 40 mL/g solvent to solid ratio were analyzed. Extracts from three purification methods and four pre-extraction treatments were also analyzed. Samples of 5 μL were injected into a reversed-phase C_{18} column (Zorbax SB 5 μm, 250 x 4.6 mm, Agilent Technologies Inc., Palo Alto, CA) , preceeded by a guard column (Inertsil 5 ODS-2, 5 μm, 30 x 4.6 mm, Phenomenex, Torrance, CA). A gradient solvent system with solvent A being formic acid -water (5:95v/v) and solvent B being methanol was used. The elution profile had the following proportions (v/v) of solvent B: 0 min, 10%; 0- 30 min, 10-25%; 30- 50 min, 25- 45%, 50- 55 min, 45-100 %, 55- 60, 100% and 60- 65 min, 100-10%, and the solvent flow rate was 1.0 mL/ min. Concentrations were calculated using peak area and standard curves of chlorogenic acid, caffeic acid, quercetin and cyanidin 3-glucoside at 280, 320, 360 and 525 nm, respectively. Identification of peaks was carried out by comparison with retention times and spectra of standard compounds purchased from Extrasynthese (Genay, France) and other compounds purified in our laboratory.

7. Experimental Designs

a. Optimization Designs. Optimization of extraction of phenolics and antioxidant activity was carried out using surface response methodology (Haaland, 1989). Two experiments were carried out. In one experiment extraction of phenolics was optimized using aqueous ethanol

and in a second experiment the extracting solvent was SO_2-containing water. For both experiments the selected experimental design had three factors and five-levels referred to as central composite design. It consisted of 18 runs including four replicates of the center point. Independent variables were solvent concentration, temperature, and solvent to solid ratio (S/S). Ethanol concentrations were 39, 50, 67, 84, and 95 %. Sulfur dioxide concentrations were 28, 300, 700, 1100, and 1372 ppm. Lowest and highest values of solvent to solid ratio and temperature were 6 and 74 (Tables 2 and 3).

Two sets of equipment (beaker, water bath and mixer) were used and two runs were performed each day. In order to avoid bias error, randomized experiments were run in two times the eight combinations of the three equipment factors, beaker, mixer and water bath, plus other two combinations randomly selected.

Data were analyzed using the RSREG, PLOT and REG procedures of SAS ® (1990) (SAS Institute Inc., Cary, NC) and fitted to a second order polynomial equation to optimize the conditions of extraction. Goodness-of-fit test of the model was performed with the REG procedure by backward elimination at the 0.1% level.

The second order equation is:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \beta_{ij} X_i X_j \qquad (22)$$

where Y is the dependent variable, β_0, β_i, β_{ii}, β_{ij} are constant and regression coefficients of the model and X_i are the independent variables in the model. Response surface plots were obtained using predicted values from the fitted model, by keeping the least effective independent variable fixed at a constant value taken from the canonical analysis, while modifying the other two variables.

TABLE 2. Central Composite Experimental Design of Three Variables for the Extraction of Phenolic Compounds with Aqueous Ethanol

Run	Temperature (°C)	Solvent / Solid Ratio (mL/g)	Ethanol Concentration (%)
1	20 (-1) [a]	20 (-1)	50 (-1)
2	20 (-1)	20 (-1)	84 (+1)
3	20 (-1)	60(+1)	50 (-1)
4	20 (-1)	60(+1)	84 (+1)
5	60(+1)	20 (-1)	50 (-1)
6	60(+1)	20 (-1)	84 (+1)
7	60(+1)	60(+1)	50 (-1)
8	60(+1)	60(+1)	84 (+1)
9	6(-1.68)	40 (0)	67 (0)
10	74(+1.68)	40 (0)	67 (0)
11	40 (0)	6(-1.68)	67 (0)
12	40 (0)	74(+1.68)	67 (0)
13	40 (0)	40 (0)	39 (-1.68)
14	40 (0)	40 (0)	95 (+1.68)
15	40 (0)	40 (0)	67 (0)
16	40 (0)	40 (0)	67 (0)
17	40 (0)	40 (0)	67 (0)
18	40 (0)	40 (0)	67 (0)

[a] Numbers in brackets are the coded values of variables in the experimental design

TABLE 3. Central Composite Experimental Design of Three Variables for the Extraction of Phenolic Compounds with SO_2-containing Water Solvent

Run	Temperature (°C)	Solvent / Solid Ratio (mL/g)[a]	SO_2 Concentration (ppm) [b]
1	20 (-1) [c]	20 (-1)	300 (-1)
2	20 (-1)	20 (-1)	1100 (+1)
3	20 (-1)	60(+1)	300 (-1)
4	20 (-1)	60(+1)	1100 (+1)
5	60(+1)	20 (-1)	300 (-1)
6	60(+1)	20 (-1)	1100 (+1)
7	60(+1)	60(+1)	300 (-1)
8	60(+1)	60(+1)	1100 (+1)
9	6(-1.68)	40 (0)	700 (0)
10	74(+1.68)	40 (0)	700 (0)
11	40 (0)	6(-1.68)	700 (0)
12	40 (0)	74(+1.68)	700 (0)
13	40 (0)	40 (0)	28 (-1.68)
14	40 (0)	40 (0)	1372 (+1.68)
15	40 (0)	40 (0)	700 (0)
16	40 (0)	40 (0)	700 (0)
17	40 (0)	40 (0)	700 (0)
18	40 (0)	40 (0)	700 (0)

[a] Expressed in mL of solvent/gram of frozen berries on a dry weight basis (dwb); [b] Calculated as ppm equivalents of sulfur dioxide; [c] Numbers in brackets are the coded values of variables in the experimental design

b. Pre-Extraction Design. Pre-extraction experiments were performed using three berries and three replicates for each extraction. Independent variables were the temperature of the pre-treatment in one experiment, and maceration of berries either with or without enzymes in another. In order to avoid changes in the berry compositions after milling, experiments were run in a sequence of berries, starting with blackcurrant, followed by blackberries and finishing with saskatoon berries. Three berries, three levels of temperature and three replicates were used to measure the effects of temperature, type of berries, and enzyme treatment on the extraction of phenolics.

IV. RESULTS AND DISCUSSION

A. Milling and Composition of Berries

1. Particle Distributions and Sizes

Individual solid particles are characterized by their size, shape and density (McCabe et al., 1985). Using all the product obtained from the milling process, and taking homogeneous samples, these properties should be always the same. However, it is important to know the characteristic particle size of the samples because it affects the extraction (Pifferi and Vaccari, 1983; Gao and Mazza, 1996; Landbo and Meyer, 2001). For equidimensional particles, the specified characteristic dimension is "diameter" (McCabe et al., 1985). In order to evaluate particle size distribution and mean particle size of milled berry samples, cumulative passing weights were plotted against the wire opening size of the sieve meshes.

Preparation of frozen black currant particles yielded almost identical particle size distribution, and an average particle size (P_{50}) of 1.6 mm on three different days berries were milled (Figure 6). Uniformity of milled samples used throughout the experiment can also be evaluated by the standard deviations obtained in the determination of the composition of black currants (Table 4). Particle distributions were narrow as indicated by the shallow slope (0.147) of the linear regression.

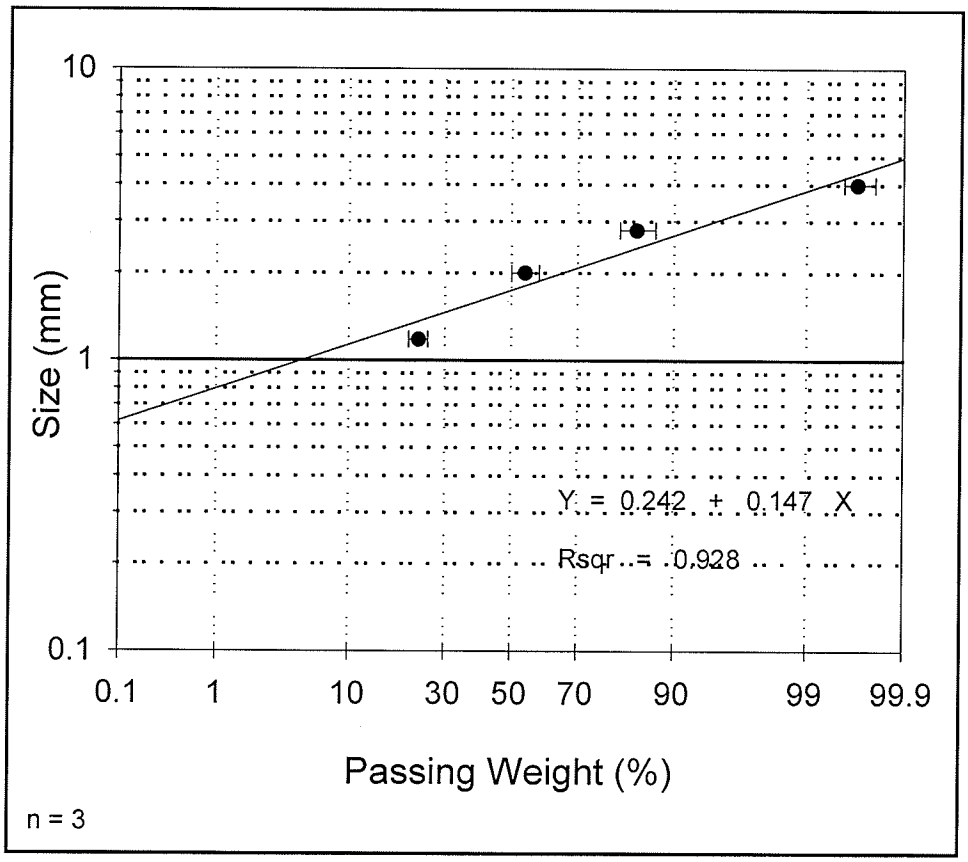


Figure 6. Particle Size Distribution of Frozen Black Currants Milled in a Wiley Mill Set at 520 rpm, with a 6 mm Screen and 2.1 mm Blade Distance

Mean particle size of blackberries (P_{50}), corresponding to a passing weight probability of 50% in the plot of particle size distribution (Figure 7) resulted in 1.7 mm for the milling of frozen blackberries. Distribution in particle sizes was broader than for black currant but still narrow. Slope was 0.202. Blackberries were the easiest berries to mill. Saskatoon berries gave the largest particle size (1.8 mm) and the highest average slope (0.206) (Figure 8).

TABLE 4. Composition of Milled Frozen Black Currants Extracted with Aqueous Ethanol [a]

Sample	Total Phenolics	Tartaric Esters	Flavonols	Anthocyanins		Dry matter (%)
1 [g]	39.6 [b]	2.7 [c]	2.3 [d]	12.5 [e]	14.2 [f]	22.9
2	36.8	2.4	2.0	11.8	13.3	22.1
3	37.2	2.4	2.1	11.8	13.3	22.4
Avg ± SD	37.8 ± 1.5	2.5 ± 0.14	2.1 ± 0.12	12.0 ± 0.40	13.6 ± 0.50	22.5 ± 0.41
1 [h]	36.4	2.4	2.0	12.0	13.1	22.4
2	38.0	2.6	2.3	12.8	14.1	22.6
3	35.5	2.7	2.3	10.2	11.2	23.6
Avg ± SD	36.6 ± 1.3	2.6 ± 0.13	2.2 ± 0.14	11.7 ± 1.3	12.8 ± 1.5	22.9 ± 0.61

[a] Phenolic concentrations in mg /g of frozen berries on a dry weight basis (dwb) expressed as equivalent of: [b] chlorogenic acid; [c] caffeic acid; [d] quercetin; [e] cyanidin chloride; [f] cyanidin 3-glucoside; [e, f] Calculated using standard curves for cyanidin chloride and cyanidin 3-glucoside, respectively; [g] Phenolics determined by equilibrium with 60 % aqueous ethanol with a solvent to solid ratio of 100 mL/g at 20 °C for 60 h; [h] Phenolics determined by two extractions with 80 % aqueous ethanol in a blender for 8 min

Efficiency of the extraction would be affected by the particle size, and the S/S. In preliminary experiments, total phenolic and anthocyanin contents of milled fruit fractions with several particle sizes were different. Use of different fruit fractions would affect results of the evaluation of extract concentration. To overcome this problem, milling conditions were modified to obtain whole milled product with different particle size. However, production of both frozen and freeze-dried milled berries with a significant difference in their average particle size could not be obtained by only modifying milling conditions.

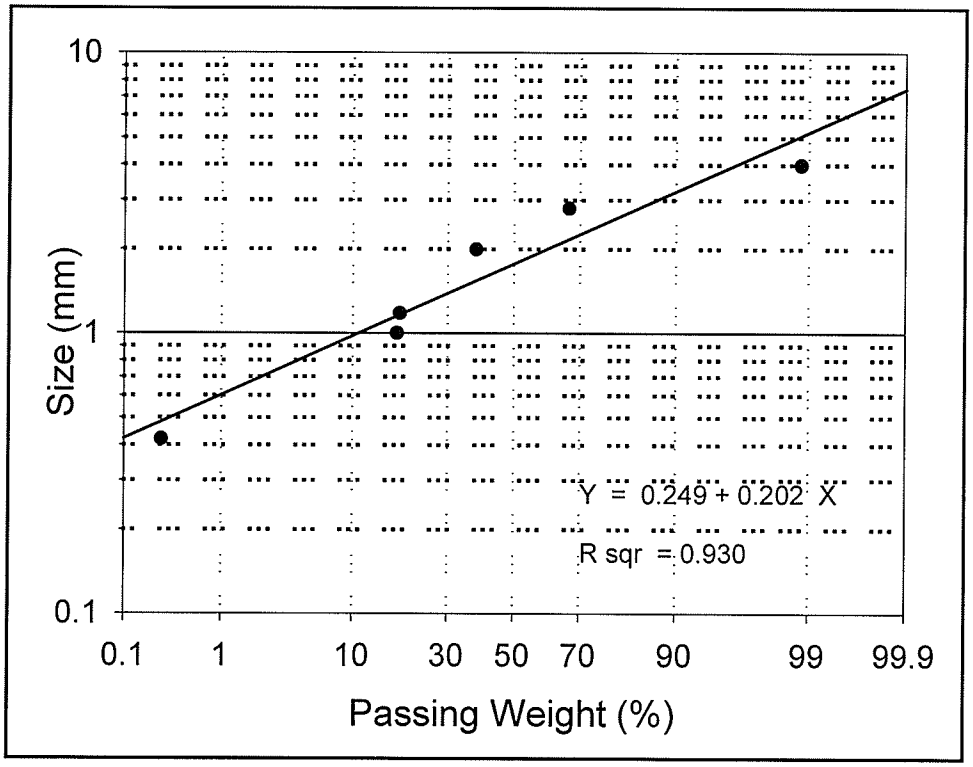


Figure 7. Particle Size Distribution of Frozen Blackberries Milled in a Wiley Mill Set at 520 rpm, with a 6 mm Screen and 2.1 mm Blade Distance

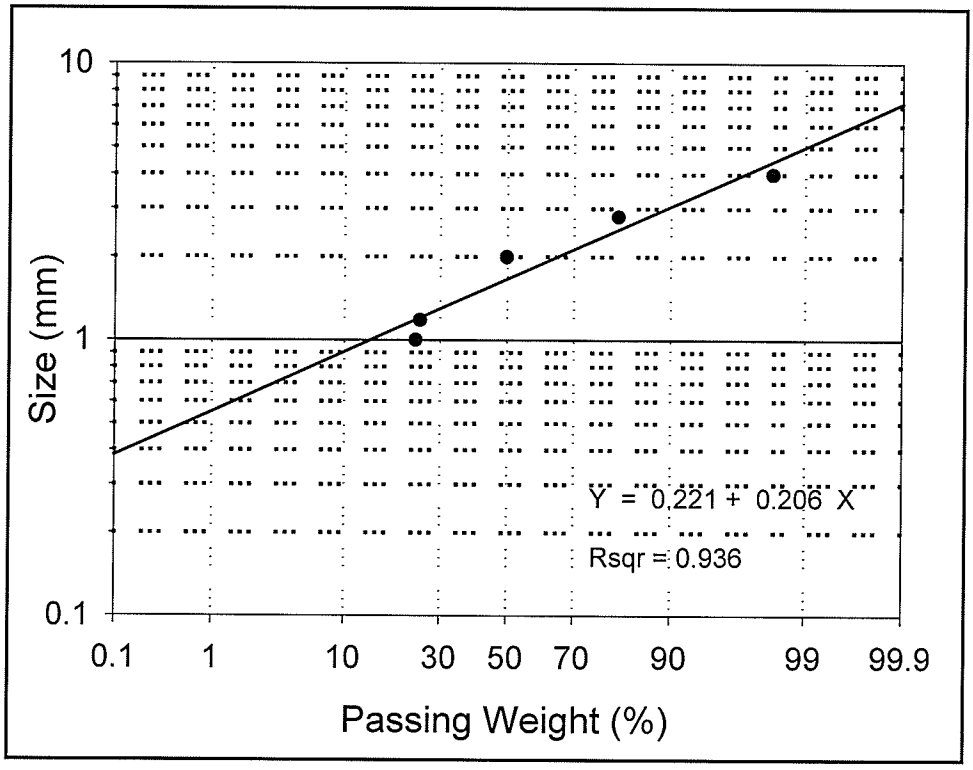


Figure 8. Particle Size Distribution of Frozen Saskatoon Berries Milled in a Wiley Mill Set at 520 rpm, with a 6 mm Screen and 2.1 mm Blade Distance

2. Composition

Composition of phenolics and dry matter content of milled black currants were evaluated for several samples of berries (Table 4). Anthocyanin and flavonol values from the blender method were higher than contents for black currant from Western Europe reported from HPLC determination (Iversen, 1999; Häkkinen et al.1999b; Mikkonen, 2001; Kähkönen et al., 2001). The anthocyanin content was similar to values for cv. Ben Lomond evaluated by a pH differential method (Banaszczyk and Pluta, 1997; Moyer et al., 2002) and was within the range of anthocyanin contents for 40 ***Ribes*** genotypes from Oregon, reported by Moyer et al. (2002). Comparisons of results are difficult since different materials, extraction techniques, methods of analysis and expression of results were used. In addition, cultivar, crop season, growing conditions, degree of ripeness, and storage affect anthocyanin content (Prior et al., 1998; Kalt et al., 1999). Seasonal variations in a six year period for anthocyanin content of Ben Lomond currant grown in Poland ranged from 240 to 420 mg/100 g (Banaszczyk and Pluta, 1997). Values by the equilibrium method with ethanol were higher than values obtained by extraction in a blender for all phenolic compounds evaluated. The long extraction time used might have been the reason for the higher values obtained by the equilibrium method.

In the analysis from the equilibrium method using SO_2-containing water, anthocyanin content of milled black currant (Table 5) was higher than values reported in the literature with organic solvent extraction (Banaszczyk and Pluta, 1997; Fukumoto and Mazza, 2000; Moyer et al., 2002). In this method (Schwartzberg and Chao, 1982), extraction time was longer than equilibrium times reported (Metivier et al., 1980) to assure a complete extraction. Water extraction for long time resulted in high yield of non-phenolic compounds as indicated

by higher total phenolics readings at 280 nm than values from ethanolic extractions. Mok and Hettiarachchy (1991) have reported extraction of non-anthocyanin material with 1,000 ppm SO_2-water from sunflower-hull, and Garcia-Viguera et al. (1998) reported extraneous materials in water extracts of anthocyanins from strawberry. When samples were injected into HPLC to confirm results, values of anthocyanins were close to spectrophotometric values, although total phenolics from HPLC were considerably lower. Proteins absorbing at a wavelength close to 280 nm might be interfering in the spectrophotometric determination of berries composition obtained with the equilibrium method as a result of very long water extraction times.

TABLE 5. Composition of Milled Frozen Black Currants Extracted with SO_2-containing Water Solvent [a]

Sample	Total Phenolics	Tartaric Esters	Flavonols	Anthocyanins		Dry matter (%)
1 [g]	89.4 [b]	2.8 [c]	2.1 [d]	13.6 [e]	15.8 [f]	22.9
2	91.0	2.7	1.8	13.1	14.9	22.1
3	86.3	2.8	1.9	13.2	15.2	22.4
Avg ± SD	88.9 ± 2.4	2.8 ± 0.08	1.9 ± 0.14	13.3 ± 0.31	15.3 ± 0.44	22.5 ± 0.41
1[h]	21.7	2.2	1.6		15.7	
2	19.5	2.0	1.3		14.2	
3	18.7	1.9	1.3		13.5	
Avg ± SD	20.0 ± 1.6	2.0 ± 0.12	1.4 ± 0.17		14.5 ± 1.1	

[a] Phenolic concentrations in mg /g of frozen berries on a dry weight basis (dwb) expressed as equivalents of: [b] chlorogenic acid; [c] caffeic acid; [d] quercetin; [e] cyanidin chloride; [f] cyanidin 3-glucoside; [ef] Calculated using standard curves of cyanidin chloride and cyanidin 3-glucoside, respectively; [g] Phenolics determined by equilibrium with a 1100 ppm SO_2-water solvent at 100 mL/g solvent to solid ratio at 20 °C for 60 h; [h] Calculated from HPLC determination at 280, 320, 360 and 525 nm using standards curves of chlorogenic acid, caffeic acid, quercetin and cyanidin 3-glucoside for quantitation

Blackberries and saskatoon berries were analyzed only by the equilibrium method (Table 6). Anthocyanin content of blackberries was similar to values reported by Moyer et al. (2002) for several ***Rubus*** species. Anthocyanin content of saskatoon berries was similar to reported values (Mazza, 1986; Green and Mazza, 1986). However, total phenolics were approximately 2-3 times higher than values reported by Fukumoto and Mazza (2000) and Moyer et al. (2002). Differences may be attributed to a similar effect to the interference noted with SO_2-containing water in the extraction of black currants, due to absorption of non-phenolic compounds at 280 nm wavelength. The same deviation was also found for total phenolics in saskatoon berries. Differences with reported total phenolics (Fukumoto and Mazza, 2000; Moyer et al., 2002) for black currant and saskatoon berries (4-5 times) were higher than for blackberrries (2-3 times) so the magnitude of the interference could vary with the type of berry.

B. Optimization of Extraction of Phenolics

1. Extraction with Aqueous Ethanol

a. Surface Response for Yields of Phenolics. Models developed by surface response analysis for total phenolics, tartaric esters, flavonols, and anthocyanins, were significant at low (1-5%) level of probabilities and variability could be explained by the models (Table 7). Polynomial second order models (equation 22) were adjusted by backward elimination with the goodness-of-fit test at 0.1% level of REG procedures of SAS ® (1990) (SAS Institute Inc., Cary, NC). Regression coefficients and analysis of variance of the adjusted model for total phenolic and anthocyanin yields, extraction times and diffusion coefficients

are presented in Table 8.

TABLE 6. Composition of Milled Frozen Black Currants, Blackberries and Saskatoon Berries Extracted with SO_2-containing Water Solvent [a]

Berry	Total Phenolics	Tartaric Esters	Flavonols	Anthocyanins		Dry matter (%)
black currant	89.5[b]	2.8 [c]	2.1 [d]	13.6 [e]	15.8 [f]	22.9
	91.0	2.7	1.8	13.1	14.9	22.1
	86.3	2.8	1.9	13.2	15.2	22.4
	88.9 ± 2.4	2.8 ± 0.08	1.9 ± 0.14	13.3 ± 0.31	15.3 ± 0.44	22.5 ± 0.41
blackberries	88.6	2.4	1.7	12.0	13.8	12.4
	86.7	2.4	1.7	12.0	13.6	11.7
	79.6	2.1	1.6	11.7	13.2	12.1
	86.0 ± 4.7	2.3 ± 0.18	1.7 ± 0.09	11.9 ± 0.22	13.5 ± 0.29	12.1 ± 0.38
saskatoon berries	78.1	5.0	3.0	7.7	8.0	23.0
	79.0	4.7	2.8	7.2	7.2	22.8
	86.4	5.3	3.2	8.6	9.1	23.0
	81.2 ± 4.5	5.0 ± 0.31	3.0 ± 0.22	7.8 ± 0.71	8.1 ± 0.95	22.9 ± 0.11

[a] Phenolic compounds in mg /g of frozen berries on a dwb determined by equilibrium with 1100 ppm SO_2-water solvent for 60 h at 20 °C expressed as equivalent of: [b] chlorogenic acid; [c] caffeic acid ; [d] quercetin; [e] cyanidin chloride; [f] cyanidin 3-glucoside; [e,f] Calculated using standard curves of cyanidin chloride and cyanidin 3-glucoside, respectively

Effects of independent variables varied depending on which response variable was analyzed. Increasing the S/S increased the anthocyanin and total phenolic yields and the time to reach constant concentration decreased (Figure 9). An increase of S/S increased the concentration gradient and thus the rate of diffusion of the compounds from the solid to the solvent. In the same way higher water-seed ratios favored the extraction of protein from flaxseed (Cui et al., 1994) and the solvent to powder ratio strongly affected yields in the extraction of anthocyanins from sunflower husks (Pifferi and Vaccari, 1983).

TABLE 7. Surface Response for Yields of Total Phenolics, Tartaric Esters, Flavonols, and Anthocyanins Extracted from Black Currants using Aqueous Ethanol

Run	Ethanol Conc. (%)	Temp. (°C)	S/S Ratio (mL/g)[a]	Total Phenolics	Tartaric Esters	Flavonols	Anthocyanins
1	43	20	20	26.2 [b]	2.4 [c]	1.9 [d]	11.6 [e]
2	72	20	20	30.5	3.2	2.9	12.5
3	47	20	60	32.9	3.1	2.7	14.1
4	80	20	60	34.8	3.8	3.2	13.6
5	43	60	20	29.8	2.7	2.1	9.8
6	72	60	20	31.7	3.4	2.7	12.1
7	47	60	60	33.5	3.3	2.5	12.9
8	80	60	60	32.0	3.4	2.8	13.1
9	62	6	40	35.1	3.3	2.8	14.5
10	62	74	40	35.5	3.7	2.6	8.2
11	43	40	6	26.4	2.6	2.2	11.1
12	64	40	74	37.0	3.8	3.2	14.9
13	36	40	40	32.0	3.1	2.4	12.8
14	88	40	40	30.6	3.1	2.6	12.9
15	62	40	40	34.3	3.4	3.0	13.3
16	62	40	40	33.3	3.1	2.7	13.5
17	62	40	40	32.9	3.0	2.4	13.5
18	62	40	40	33.4	3.2	2.6	12.8
Model				*** [f]	**	**	**
Linear				***	***	***	***
Quadratic				**	NS	NS	NS
Interaction				NS	NS	NS	NS
R^2				0.900	0.809	0.813	0.834
Effects							
Ethanol Conc.				**	NS	**	NS
Temperature				NS	NS	NS	**
S/S Ratio				***	*	NS	*

[a] Solvent to solid ratio in mL/g of frozen berries on a dwb; [b, c, d, e] Phenolics yields in mg /g of frozen berries on a dwb expressed as equivalent of: [b] chlorogenic acid; [c] caffeic acid; [d] quercetin; [e] cyanidin 3-glucoside; [f] *** Significant at 0.01 level, ** significant at 0.05 level,* significant at 0.1 level

TABLE 8. Regression Coefficients and Analysis of Variance of the Second Order Polynomial Model Adjusted by Goodness-of-Fit Test for Total Phenolics and Anthocyanins Yields, Equilibrium Time and Diffusion Coefficient of Black Currant Extractions using Aqueous Ethanol

	Total Phenolics Coefficients			Anthocyanin Coefficients		
Variables[a]	**Yield**	**Time**	**Diffusion Coeff**	**Yield**	**Time**	**Diffusion Coeff**
Intercept	$4.85^{NS\ b}$	154.8^{***}	-11.3^{*}	11.58^{***}	250.6	35.4^{NS}
X_1	$0.64^{***\ c}$					
X_2					-6.66^{***}	-2.65^{*}
X_3	0.19^{***}			0.047^{***}		
X_1^2	-0.005^{***}	0.048^{***}	-0.0028^{NS}		0.034^{***}	
X_2^2	0.001^{**}	0.070^{***}	-0.0073^{*}	-0.00108^{***}	0.090^{***}	0.042^{**}
X_3^2			0.0072^{**}			
X_1*X_2		-0.144^{***}	0.021^{***}	0.00050^{NS}	-0.076^{***}	
X_1*X_3						
X_2*X_3						0.0048^{NS}
$X_1*X_2*X_3$	$-3.5x10^{-5**}$		-0.00017^{*}			
Model	***	***	***	***	***	***
R^2	0.872	0.792	0.708	0.775	0.958	0.550

[a] Polynomial model $Y=\beta_0+\sum_{i=1}^{3}\beta_i X_i+\sum_{i=1}^{3}\beta_{ii}X_i^2+\sum_{i=1}^{3}\beta_{ij}X_iX_j$ adjusted by backward elimination at the level of 0.1 %, where X_1 = ethanol concentration, X_2 = temperature, X_3= solvent to solid ratio; [b]NS non significant ($p>0.1$);[c] *** Significant at 0.01 level, ** significant at 0.05 level,* significant at 0.1 level

Total phenolic extraction was mainly affected by ethanol concentration and the S/S. Surface response analysis (Figure 10) of total phenolics showed a saddle shape and linear and quadratic coefficients of the model were significant. Temperature was not statistically significant for this variable. The critical temperature for the extraction of total phenolics was 60 °C. Total phenolics increased with ethanol concentration up to a maximum at approximately 60 % and then decreased with further increase in ethanol concentration irrespective of the S/S (Figure 10).

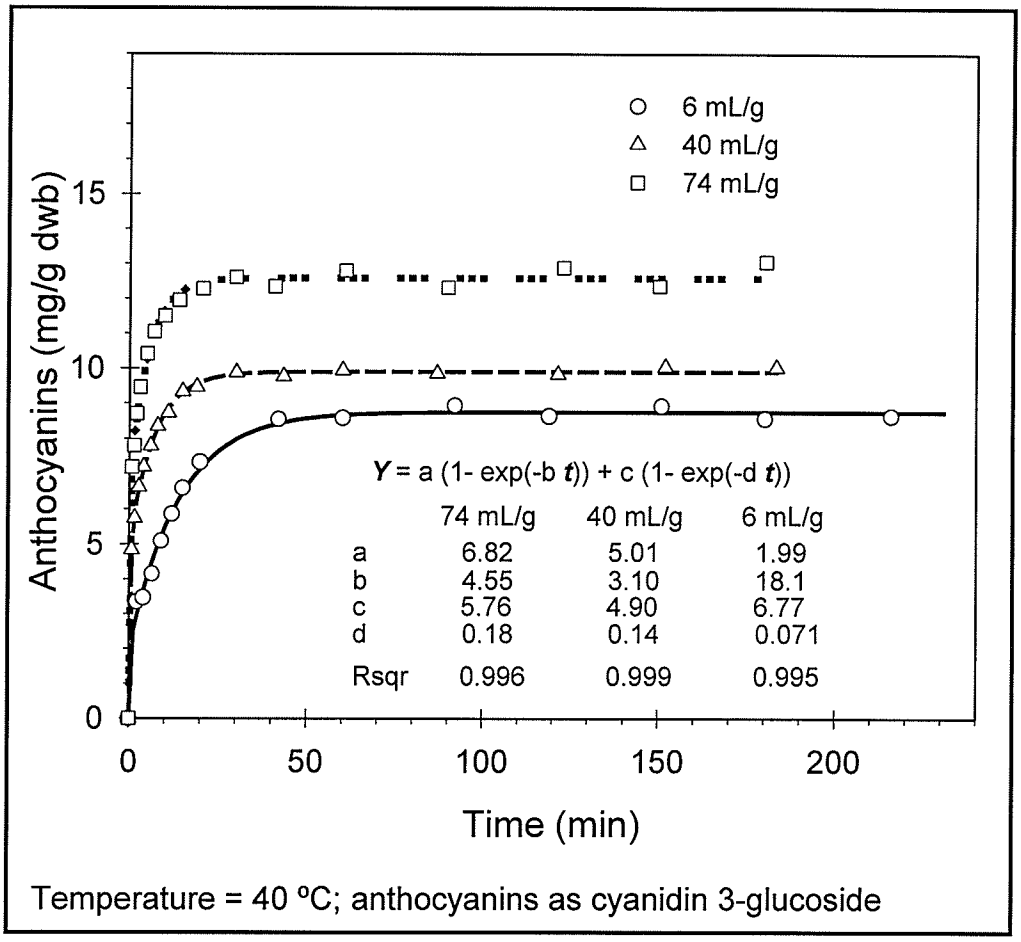


Figure 9. Anthocyanins Expressed as Cyanidin 3-glucoside in mg/g dwb of Frozen Black Currants Extracted with 50% Ethanol at 40°C with Solvent to Solid Ratios of : ○ 6 , △ 40, and □ 74 mL/g dwb of Frozen Berries

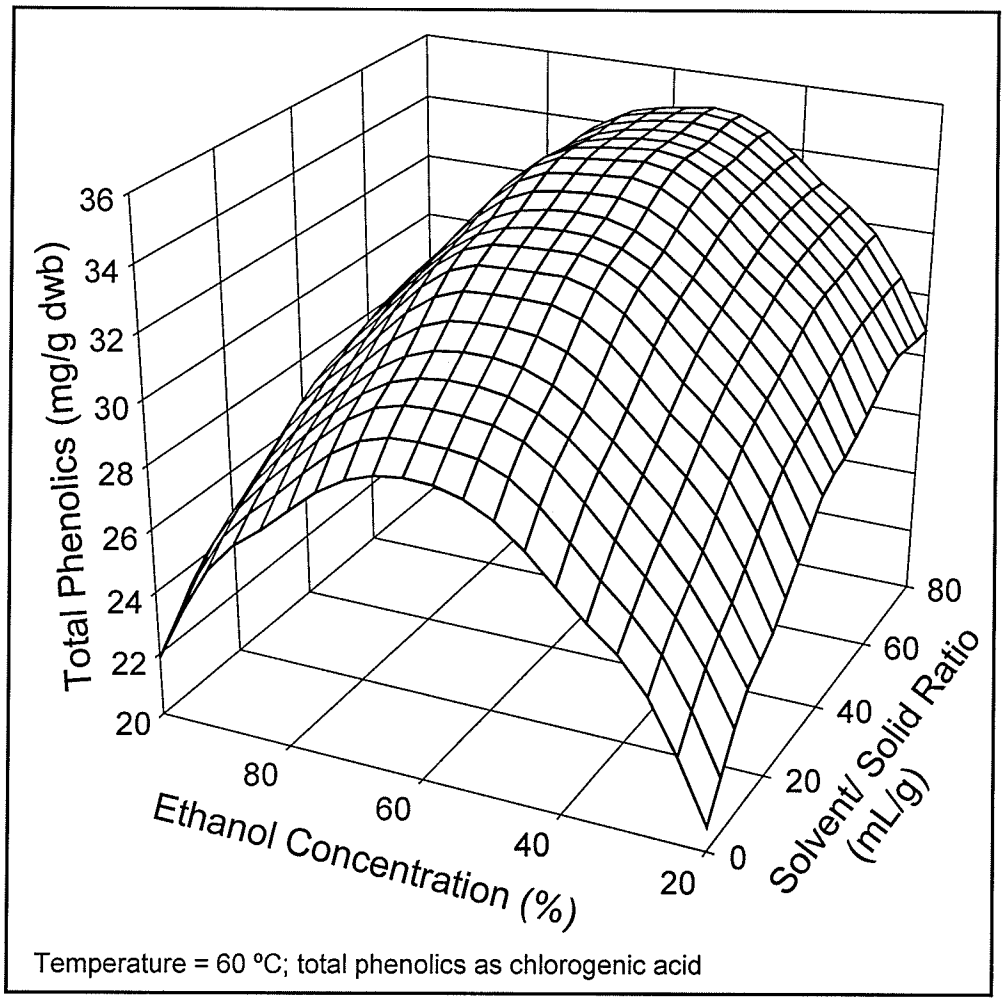


Figure 10. Response Surface for the Effects of Solvent to Solid Ratio and Ethanol Concentration on Total Phenolics Expressed as Chlorogenic Acid Equivalents in mg/g dwb of Frozen Black Currants at a Constant Extraction Temperature of 60 °C

Solubility of a solute can be enhanced using a mixed solvent over a limited compositional range (Frank et al., 1999). Changes in ethanol concentration modify the physical properties of the solvent such as density, dynamic viscosity and dielectric constant. For example, a change from 20 to 60 % ethanol would decrease the dynamic viscosity of the solvent by a factor of 1.12, and an equivalent increase in the diffusion coefficient would be expected. Solubilities of compounds would also be modified by changes in the ethanol concentration and this may influence the extraction of phenolics.

Molecular solids that dissolve as molecules would have a low solubility in water because of the energy required to overcome the attraction between the water molecules. This energy which arises from the attraction between the partial charges of the water dipoles becomes important when a much weaker interaction with covalent molecules is considered (Mackay and Mackay, 1981). For non polar covalent molecules, the energy required to break the configuration of water molecules is dominant, and this could be the situation affecting flavonols which have very low solubility in water. Anthocyanins that would remain in the flavilium cation (AH^+) form at acidic pH might be considered as ionic molecules. Phenolic acids with a carboxillic group and a hydrophobic glycosilated benzene ring may be considered as covalent polar molecules with an intermediate interaction. From other point of view, covalent non-polar compounds that would have a repulsive interaction with a solvent like water would have an activity coefficient higher than 1 and thus the ideal solubility would be reduced (Frank et al., 1999).

Tartaric esters and flavonols were significantly affected by S/S and ethanol concentration, respectively. Both compounds presented a maximum at a particular ethanol concentration. Optimum ethanol concentration changed from 95-100 % at 10 mL/g S/S to 70-75% at 80 mL/g S/S. The main effect on tartaric ester extraction was the S/S which increased extraction in the whole range of ethanol concentration (Figure11). Flavonol yield was increased by increasing S/S at low ethanol concentrations; however, S/S did not affect flavonols at solvent concentrations higher than 65% ethanol (Figure12). A high extraction yield of both tartaric esters and flavonols could be obtained with about 70-75% ethanol concentration and 80 mL/g of S/S. Thus, yields of phenolics were affected by the concentration of ethanol in the solvent, and for the extraction of each group of phenolics studied, there was an optimum ethanol content. The higher the ethanol concentration the lower the dielectric constant and the lower the energy required to break the water arrangement. Thus, covalent non polar flavonols needed higher ethanol concentration (75 - 100 %) to obtain a high yield. For covalent polar phenolic acids the optimum concentration was 70 - 90 % ethanol. Mixture of ionic anthocyanins (flavylium cation, AH^+) and covalent non-polar (quinonoidal base A, pseudobase B and chalcone C) did not require a high ethanol concentration and the maximum yield was at approximately 50 % ethanol.

Anthocyanin extraction was affected significantly by temperature and S/S (Figure 13A and B). At a constant solvent concentration of 85%, there was a quadratic effect of temperature independent of the S/S values, with maximum anthocyanin extraction at around 30 - 35 °C. Increase of temperature would favor extraction by increasing solubility of anthocyanins and increasing the diffusion coefficient.

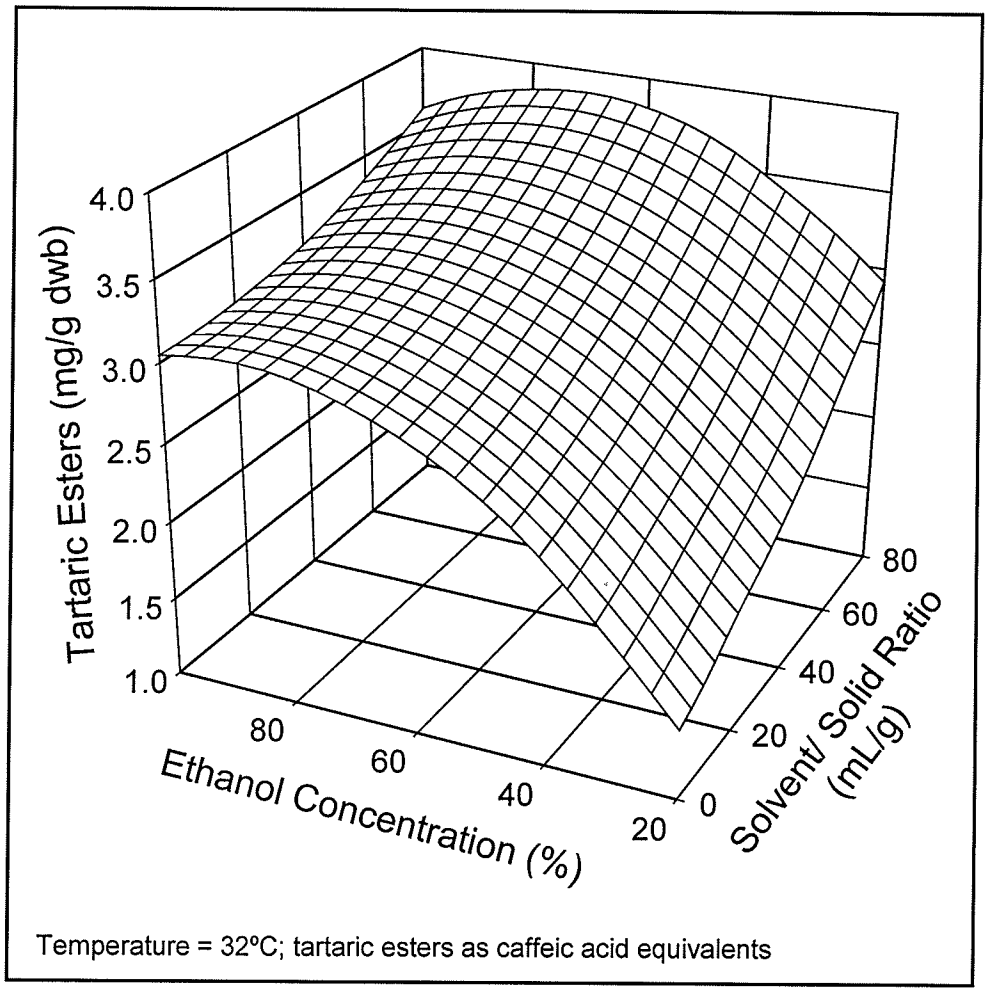


Figure 11. Response Surface for the Effects of Solvent to Solid Ratio and Ethanol Concentration on Tartaric Esters Expressed as Caffeic Acid Equivalents in mg/g dwb of Frozen Black Currants at a Constant Temperature of 32 °C

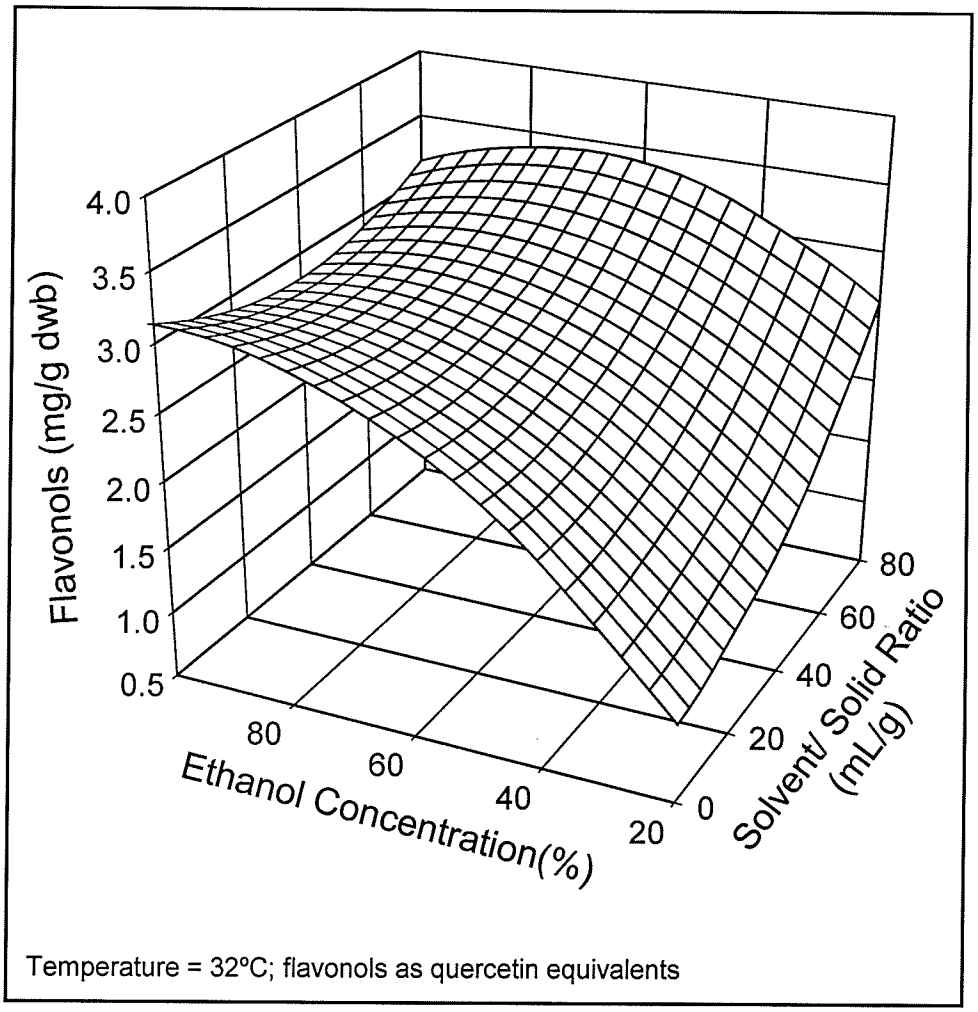


Figure 12. Response Surface for the Effects of Solvent to Solid Ratio and Ethanol Concentration on Flavonols Expressed as Quercetin Equivalents in mg/g dwb of Frozen Black Currants at a Constant Temperature of 32°C

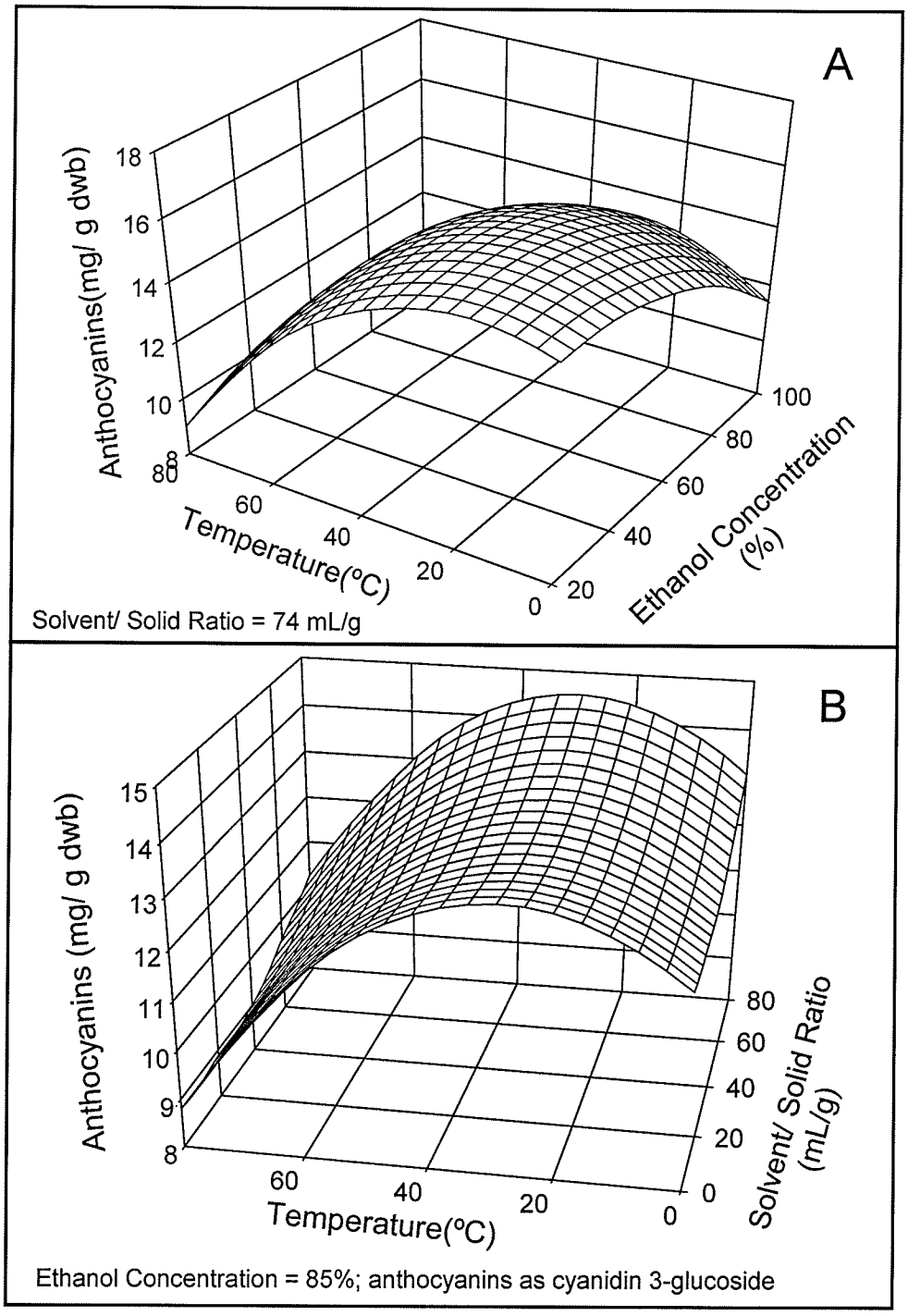


Figure 13. Response Surface for the Effects of Temperature and A) Ethanol Concentration at a Constant Solvent to Solid Ratio of 74 mL/g dwb and B) Solvent to Solid Ratio at a Constant Ethanol Concentration of 85 % on Anthocyanins Expressed as Cyanidin 3-glucoside Equivalents in mg /g dwb of Frozen Black Currants

Ideal solubility of solutes gives an idea of the maximum amount of the compound that could be found in any solvent at a given temperature. Ideal solubilities calculated from $\Delta H_{fus} / R (1/T_m - 1/T)$ (equation 1) using data for selected phenolics measured by differential scanning calorimetry (DSC) (Latorre et al., 2001; Pouzens et al., 2001) at 20 °C indicate that some black currants flavonols have lower solubilities than phenolic acids and these are lower than anthocyanins (Table 9). Besides, phenolic solubilities are affected in a different way by temperature accounting for some of the differences in response to changes in temperature. Thus, increasing temperature from 6 to 74 °C, cyanidin, pelargonidin and peonidin chlorides would increase their solubility by a factor of 4-5, delphinidin chloride by 36, kaempferol and quercetin by 76 and 870, and phenolic acids by 20. Thus, increasing the temperature would favor extraction of phenolic acids and flavonols in comparison with anthocyanins. It must be noted, however, that extracts might not be ideal and that ideal solubility should be modified by the activity coefficient of compounds which depends on the temperature and interaction of solute with solvent. Modification of ideal solubility by the activity coefficient also indicates the change in solubility of a compound due to effect of solvent.

Difference in behavior between total phenolics and anthocyanins can be mainly explained by a higher susceptibility of anthocyanins to high temperature. Increasing the temperature beyond certain values led to a decrease in anthocyanins yield. The lower the ethanol concentration, the lower the critical temperature. Thus, at 20 % ethanol temperatures higher than 25°C decreased anthocyanin extraction. At 95% ethanol concentration however, the critical temperature was around 35°C (Figure 13A). Furthermore, there was a sharp decrease in anthocyanins at temperatures higher than 45 °C.

TABLE 9. Ideal Solubilities of Anthocyanins, Flavonols and Phenolic Acids at 20 °C

Group	Compound	ΔH_{fus} (J/g)	T_m (°C)	Ideal Solubility [a] (10^3 w/w)
anthocyanins [b]	cyanidin chloride	57.5	220	45.5
	delphinidin chloride	125.9	163	3.24
	pelargonidin chloride	62.4	117	141
	peonidin chloride	47.5	272	48
flavonols [c]	kaempferol	179	203	0.307
	quercetin	265	162	0.0217
	myricetin	113	191	4.36
phenolic acids [b]	gallic acid	491	263	0.000176
	HBA	252	220	3.01
	protocatechuic	220	204	4.97
	syringic acid	179.2	211	3.14
	vanillic acid	179.5	213	7.25

[a] Ideal solubility calculated from $\Delta H_{fus} / R (1/T_m - 1/T)$ expressed as weight fraction of the solute dissolved in the solvent; [b] ΔH_{fus} and T_m data from Pouzens et al. (2001); [c] ΔH_{fus} and T_m data from Latorre et al. (2000)

The major factor affecting extraction of anthocyanins was S/S. Anthocyanins increased almost linearly with increase of S/S in the range utilized in the experiment (Figure 13B). The increase of S/S would favor the extraction of anthocyanins by modifying the concentration gradient as noted for total phenolics. The final anthocyanin concentration of the extracts at higher S/S extractions was lower because of the use of smaller berry weights for the same volume of solvent and thus the concentration gradient was higher. Thus, yield of phenolic compounds can be increased with the use of high S/S, but depending on the application of the final product, extracts may require removal of the solvent which would increase the process complexity and cost of product.

b. Surface Response for Antioxidant Activity. For the antioxidant activity parameters the response varied (Table 10); models were not significant, and variabilities explained by the models were low for antiradical activity (R^2= 0.603) and antioxidant activity (R^2= 0.395) measured by the DPPH and β-carotene methods. Antioxidant index model was significant and the variability explained was fairly high (R^2 = 0.888).

Response surface for the antioxidant index had a saddle shape (Figure 14). The main variable affecting the index was the S/S. The higher the S/S, the lower the antioxidant index, irrespective of ethanol concentration. Antioxidant index was measured maintaining extract concentration differences. As a result higher S/S yielded more diluted extracts, which may explain the lower antioxidant indices. Antioxidant index showed a maximum at 60 - 70 % ethanol which may be attributed to the higher extraction obtained with that solvent concentration.

Antioxidant and antiradical activities as measured by DPPH and β-carotene methods did not show differences among treatments. Since the measurement of these activities was done at a fixed concentration, this result indicates that variations found in the compositions of the extracts were not sufficiently large to show differences in antioxidant activities. However, some compositional changes by the effect of the temperature were found. Reduction of antioxidant activity due to decreased anthocyanin content might be compensated by the activity of other compounds whose extractions were increased. Therefore, the antioxidant activity differences determined by the antioxidant index technique is likely due to a difference in concentration between samples rather than to a difference in composition.

TABLE 10. Surface Response for Antiradical Activity, Antioxidant Activity, and Antioxidant Index of Black Currant Extracts with Aqueous Ethanol

Run	Ethanol Conc. (%)	Temp. (°C)	S/S Ratio (mL/g)	Antiradical activity	Antioxidant activity	Antioxidant index (%)
1	43	20	20	-3.8 [a]	776 [b]	74.4
2	72	20	20	-3.8	630	75.7
3	47	20	60	-4.1	675	50.3
4	80	20	60	-3.1	845	53.4
5	43	60	20	-3.5	821	79.9
6	72	60	20	-3.6	671	70.1
7	47	60	60	-3.8	908	51.6
8	80	60	60	-3.7	672	53.4
9	62	6	40	-3.9	525	55.2
10	62	74	40	-4.8	635	73.8
11	43	40	6	-4.8	755	82.2
12	64	40	74	-3.4	649	52
13	36	40	40	-3.9	639	46.3
14	88	40	40	-3.3	692	58.6
15	62	40	40	-3.5	757	65
16	62	40	40	-3.4	737	62.3
17	62	40	40	-3.5	752	67.6
18	62	40	40	-3.4	614	66.5
Model				NS	NS	*** [c]
Linear				NS	NS	***
Quadratic				NS	NS	NS
Interaction				NS	NS	NS
R^2				0.603	0.395	0.888
Effects						
Ethanol Conc.				NS	NS	NS
Temperature				NS	NS	NS
S/S Ratio				NS	NS	***

[a] Slope coefficient calculated by linear regression in μMDPPH / μM of antioxidant, expressed in total phenolics as chlorogenic acid, [b] Slope coefficient calculated by linear regression in 10^{+6} absorbance units/ μM of antioxidant, expressed in total phenolics as chlorogenic acid, [c] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level

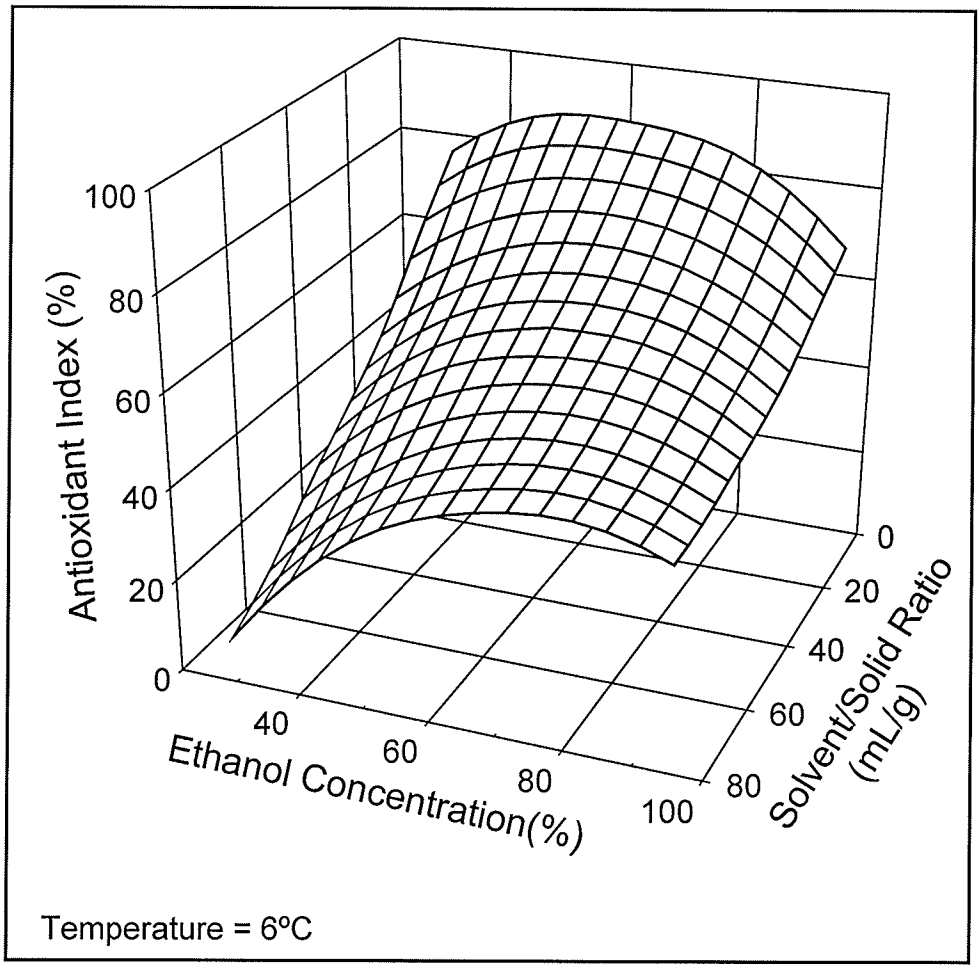


Figure 14. Response Surface for the Effects of Solvent to Solid Ratio and Ethanol Concentration on Antioxidant Index of Extracts at a Constant Temperature of 6 °C

c. Anthocyanins and Temperature Effects. As previously noted, extraction temperature had a statistically significant effect on anthocyanins, and no significant effect on total phenolics. To further investigate these effects HPLC analysis of 6, 40 and 74°C extracts (67% ethanol and 40 mL/g S/S) were performed. The results of the analyses confirmed an increase of anthocyanins to a maximum and then a decrease with further temperature increase. It was also observed that tartaric esters and total phenolics increased with temperature, and anthocyanins decreased from about 70 % to 54 % of total phenolics (Table 11).

HPLC chromatograms (Figure 15) show that black currants contain four major (peaks # 11, 12, 13, 14) and at least four minor (peaks # 15, 16, 18 and 19) anthocyanins. Major anthocyanins were confirmed as delphinidin3-rutinoside (peak #12), cyanidin 3- rutinoside (peak #14), delphinidin 3-glucoside (peak #11) and cyanidin 3-glucoside (peak #13) (Mazza and Miniati, 1993; Renault et al., 1997; da Costa et al., 2000; Degenhardt et al., 2000). Peak # 15 (35.9 min) showed an absorption maximum ($\lambda_{max\ vis}$ = 528) indicative of a delphinidin derivative (Figure 16A and Table 12), E_{440}/E_{vis} ratio = 29 indicative of a 3- glycoside substitution, a peak at 310 nm indicative of acylation by hydroxylated aromatic organic acid, and an acyl to visible absorbance ratio (E_{acyl}/ E_{vis}) of 46 indicative of a molar ratio of the cinnamic acid to anthocyanin of 1:1 (Markham, 1982; Hong and Wrolstad, 1990). Its retention time was similar to that of acetylated anthocyanins, which suggests that this peak is a caffeoyl derivative of delphinidin (Baldi et al., 1995). Peak #16 (Figure 16 B) had an absorption maximum ($\lambda_{max\ vis}$ = 520) similar to cyanidin; it did not have absorption peak at 310 indicative of no cinnamic acid acyl function, and a very high E_{440}/E_{vis} ratio of 52. This peak is likely a 3 - glycoside acetylated derivative of cyanidin. Compounds with retention

TABLE 11. Total Phenolics, Tartaric Esters and Anthocyanins Measured by HPLC-DAD at 280, 320, and 525 nm, respectively from Black Currant Extracts Prepared Using Aqueous Ethanol at 6°C and 74°C [a]

Peak #	Retention Time (min)	Total Phenolics (mg/L) [b] at 280 nm		Tartaric Esters (mg/L) [c] at 320 nm		Anthocyanins (mg/L) [d] at 525 nm	
		6 °C	74 °C	6 °C	74 °C	6 °C	74 °C
1 [e]	6.09	0 [f]	44.3	0	7.8	0	0
2	7.91	0	6.9	2.7	3.4	0	0
3	9.20	0	8.0	2.5	3.9	0	0
4	11.27	15.9	16.6	5	8.1	0	0
5	11.99	0	6.8	2.1	6.3	0	0
6	14.03	5.8	7.2	2.8	5.1	0	0
8	17.03	0	15.5	0	2.2	0	0
10	20.48	10	17.6	2.4	4.2	0	0
11	23.34	41.9	32.1	0	0	33.2	23.4
12	26.52	187.7	150.6	6.4	5.4	154.6	119.9
13	28.32	21.3	19.2	0	0	14.9	13.6
14	32.04	165.4	154.1	6.9	6.6	119	111.8
17	42.71	7.5	19.1	4.8	8.7	0	0
18	52.25	17.4	15.6	4.3	3.7	8.3	7.4
Total		472.9	513.7	39.9	65.4	330.0	276.1
Change (%)[g]			8.6		63.9		-16.3

[a] Phenolics extracted with 67% ethanol and a solvent to solid ratio of 40 mL/g in mg/L expressed as equivalent of: [b] chlorogenic acid; [c] caffeic acid; [d] cyanidin 3-glucoside; [e] Only peaks affected by the change of temperature are presented in the table; [f] Zero value means there was no peak or the concentration was negligible; [g] Expressed as percentage of total at 6°C

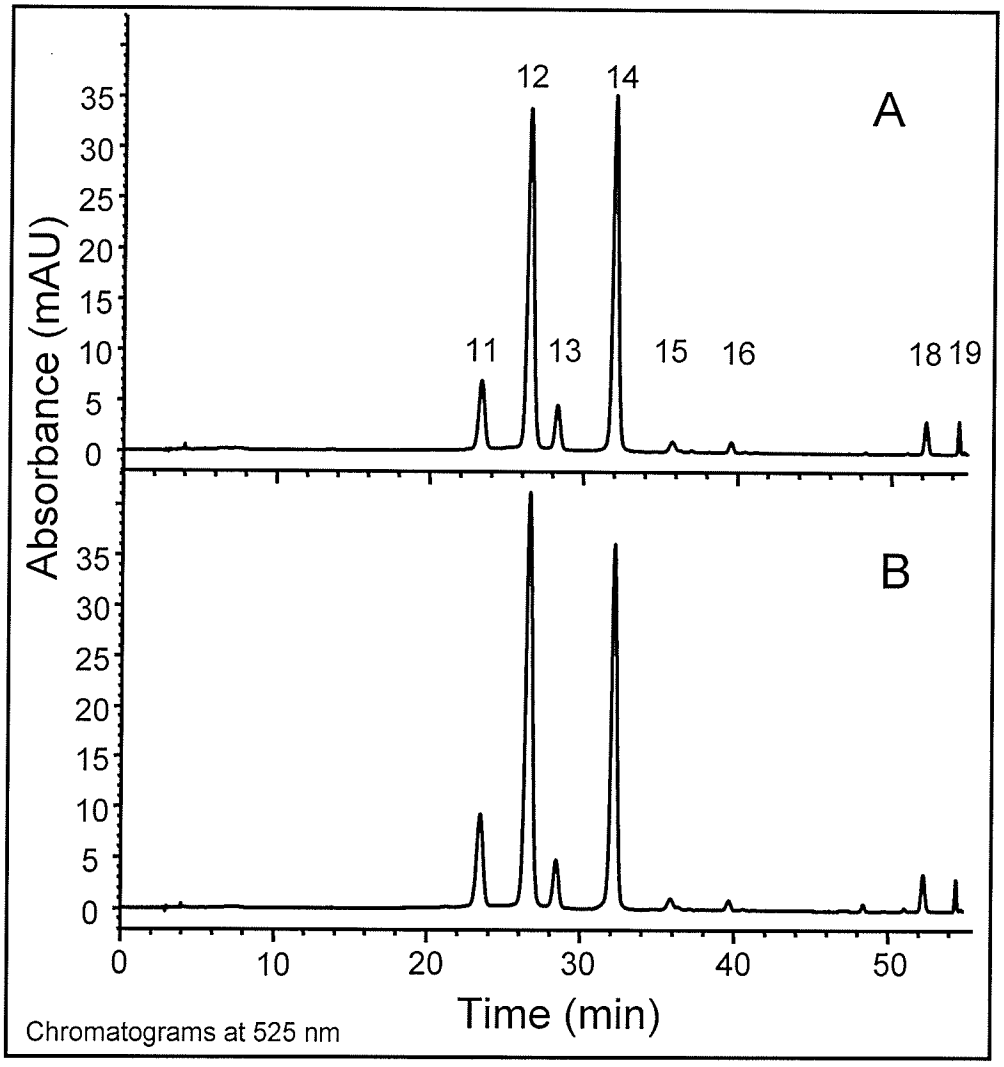


Figure 15. HPLC Chromatograms at 525 nm of Black Currant Extracts prepared using a Solvent to Solid Ratio of 40 mL/g dwb and an Ethanol Concentration of 67% at A) 74°C and B) 6°C

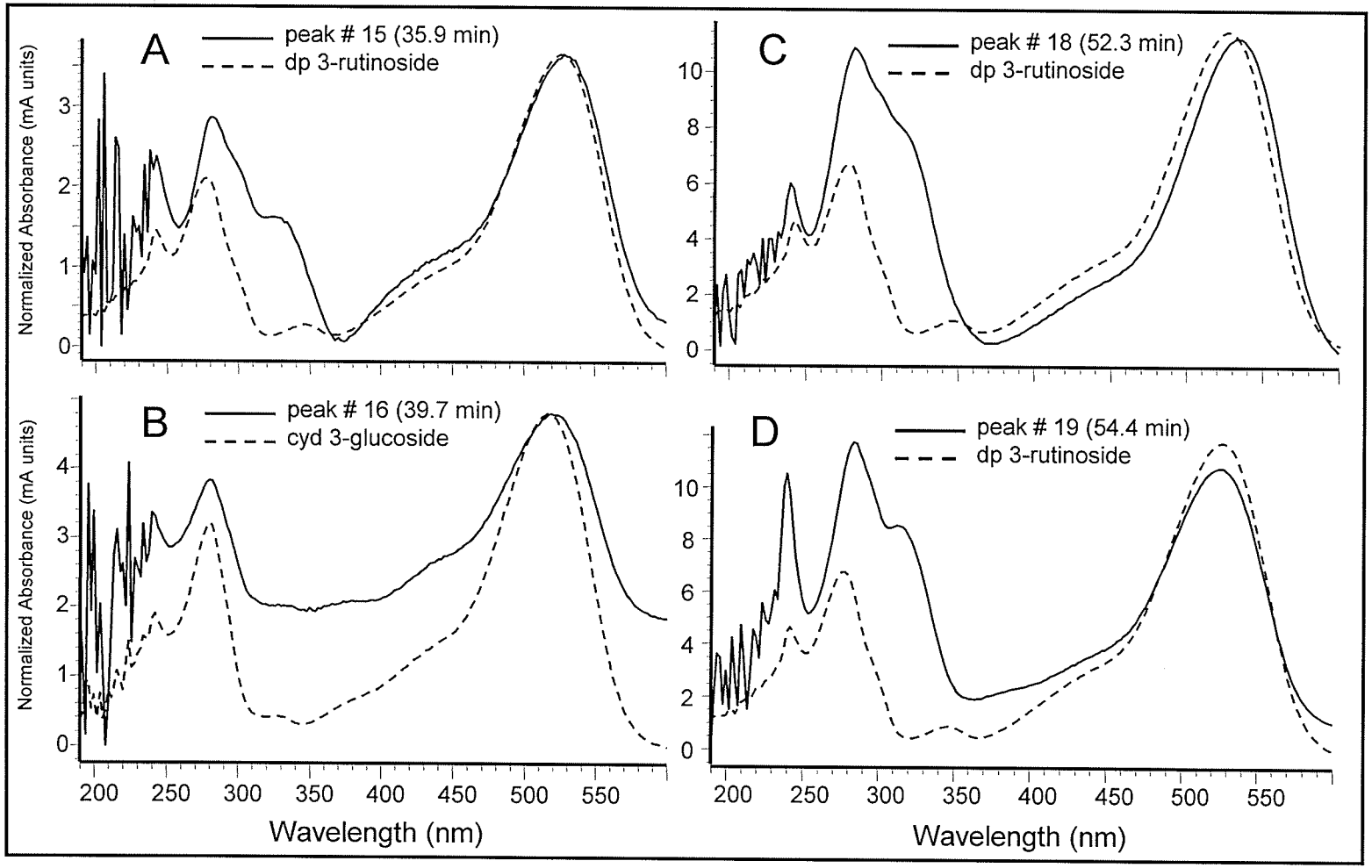


Figure 16. UV-VIS Spectra of Peaks from HPLC Analysis of Black Currant Extracts for: solid line, Peaks with Retention Times of A) 35.9 min (#15), B) 39.7 min (#16), C) 52.3 min (#18) and D) 54.4 min (#19); dashed line, Peaks of A), C), D) delphinidin 3- rutinoside and B) cyanidin 3-glucoside

time of 52.3 (peak #18) and 54.4 min (peak #19) (Figure 16 C and D) had visible absorption maxima around 530 indicative of delphinidin derivatives, absorption maxima at 310, indicating cinnamic acyl function, and higher absorbance in the range of 270- 330 nm than 3-O-glucoside standards. An HPLC peak from grapes, identified as 3-(6-O-p- coumaroyl) glucoside (Baldi et al., 1995) with spectral characteristics similar to peak # 18 has been reported. Peak # 19 showed an acyl to visible absorption ratio around 80 that suggests an acid to anthocyanin molar ratio of 2 (Hong and Wrolstad, 1990). Thus, peaks #18 and #19 are probably p-coumaroyl 3-O-glycoside and caffeoyl 3-O-glycoside of delphinidin, respectively.

TABLE 12. Spectral Data for Anthocyanin Peaks from HPLC Analysis of Black Currant Ethanolic Extracts

Peak # [a]	Ret. Time (min)	λ_{max} nm	E_{UV}/E_{vis} (%)	E_{440}/E_{vis} (%)	E_{acyl}/E_{vis} (%)
11	23.5	278, 528	52.7	28	
12	26.6	278, 528	52.7	27	
13	28.5	280, 518	54.1	31	
14	32.1	280, 514	54.5	30	
15	35.9	282.528	53	29	46
16	39.7	280, 520	53.8	52	
18	52.3	282, 535	52.7	28	70
19	54.4	285, 528	54	33	79

[a] Peak # reference as in Figure 15

The results of the HPLC analyses showed that reduction of anthocyanin concentration at higher temperature was mainly due to reduction of delphinidin 3-glucoside (30%) (peak #11) and delphinidin3-rutinoside (22%) (peak #12) concentrations and lesser to decrease of

cyanidin 3-glucoside (peak #13) and cyanidin 3- rutinoside (peak #14) (Figure 15 and Table 11). Other compounds (peak #18) also showed a minor reduction in their concentration due to high temperatures. In spite of a reduction in anthocyanins, chromatograms at 280 and 320 nm of samples extracted at 74°C showed a higher number of peaks and increased peak areas in comparison with chromatograms of sample extracted at 6°C (Figure 17). This indicates that increased number and higher amounts of tartaric esters, or cinnamic acid derivatives were extracted at the higher temperature. The largest increase was due mainly to the increase of three compounds with elution times at 6.09 min (peak #1), 17.03 min (peak #8) and 42.7 min (peak #17) (Figure 17 and Table 11). Along with those peaks, the concentration of other compounds (peaks # 2, 3, 5 and 10) increased at 74 °C. Compounds #1 and #8 which were practically absent in the 6°C extracts may have been products of degradation of other compounds by the high temperature or products of a more exhaustive extraction. Identification of those three compounds was not pursued at this time; however, their retention times did not match those of 3-hydroxybenzoic acid, p-coumaric acid, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, catechin, or epicatechin; although the spectral characteristic of compounds #1 and #8 were similar to those of p-coumaric acid. Glucosylated derivatives of p-coumaric, caffeic and ferulic acids and 3-hydroxybenzoic acid have been reported in black currant (Herrmann, 1989; Häkkinen et al., 1998). Peak #18, believed to be a coumaroyl derivative of delphinidin, decreased by about 16% when the extraction temperature was increased from 6 to 74°C. It is likely that this reduction of peak # 18 was due to the deacylation of the acylated delphinidin which released cinnamic acid derivatives that were detected as peaks #1 and #8.

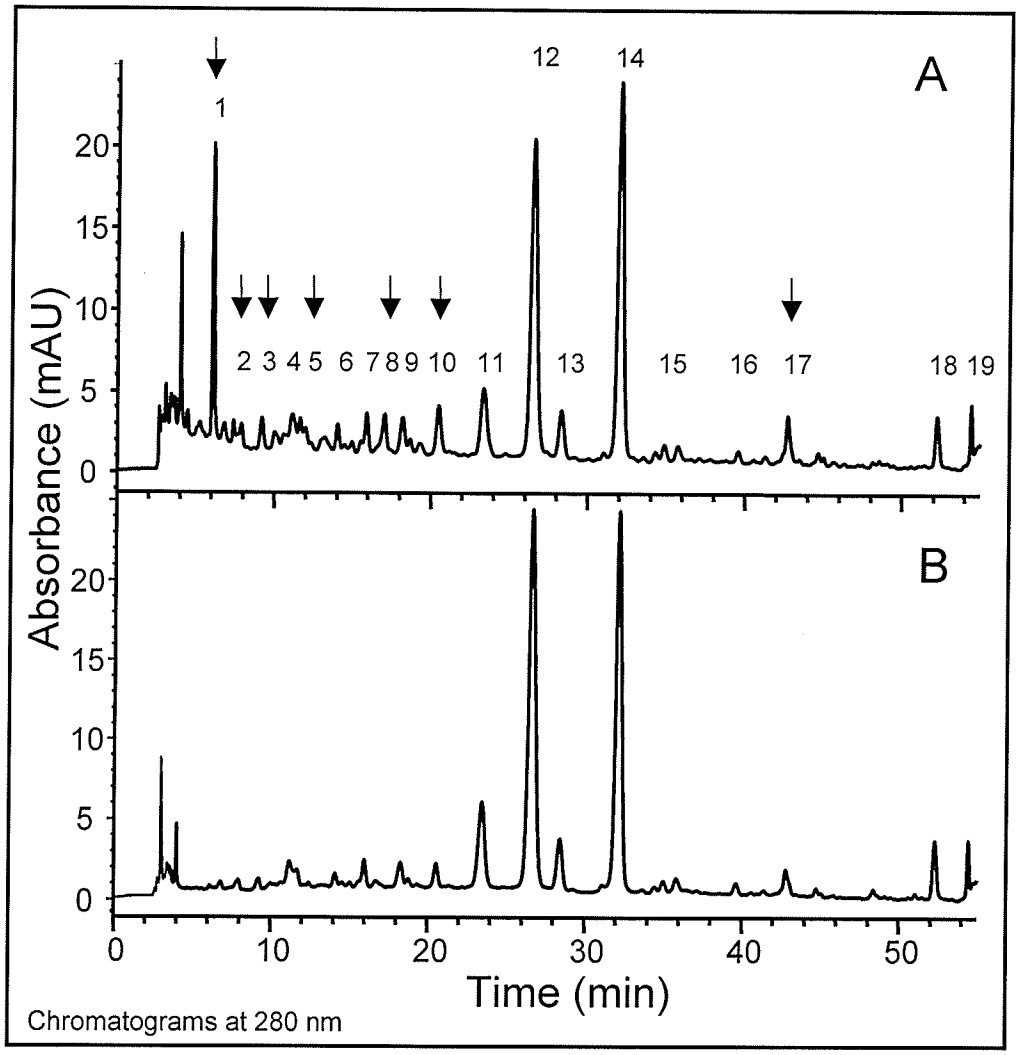


Figure 17. HPLC Chromatograms at 280 nm of Black Currant Extracts prepared using a Solvent to Solid Ratio of 40 mL/g dwb and an Ethanol Concentration of 67% at A) 74°C and B) 6°C

d. Surface Response for Equilibrium Factors. Models developed by surface response analysis for partition coefficient and equilibrium time were significant at low (1-5%) levels of probabilities. Partition coefficients and equilibrium times for anthocyanin and total phenolic extraction were significantly affected by the S/S and by ethanol concentration and temperature, respectively (Tables 13 and 14). Equilibrium times measured from concentration vs time curves similar to those presented in Figure 9, were not affected by S/S, and they were independent of the final extract concentration. Theoretically, equilibrium between a given solute in the solid and in the solvent is a function of the properties of both solute and solvent and temperature. Equilibrium time was affected by these two variables so that it was the variable that best represented equilibrium. Extractions were run until equilibrium was reached. Lower temperature or, lower S/S might extract similar amounts of anthocyanin than more rigorous conditions because they used longer times. Thus, yield of compounds would not show an effect as large as equilibrium time.

Response surface for equilibrium time for anthocyanin (Figures 18) and total phenolic (not shown) extraction showed a minimum shape under the conditions of the experiment. Extraction time increased when the temperature decreased and the raise was more noticeable at higher ethanol concentration. Reduction of equilibrium time at high temperature is likely related to an increase in diffusivity. Maximum diffusivity should be obtained at the minimum extraction time. Thus, measured diffusivities of anthocyanins at 95 % ethanol concentration and at 6 °C were lower and as a consequence the time required for extraction was longer. A minimum extraction time of 10 min for anthocyanins was obtained with 55°C at low ethanol concentration as well as with 70°C for solvent concentration higher than 75% (Figure 19).

TABLE 13. Surface Response for Partition Coefficient, Equilibrium Time, and Diffusion Coefficient of Anthocyanins from Black Currant Extractions using Aqueous Ethanol

Run	Ethanol Conc. (%)	Temp. (°C)	S/S Ratio (mL/g)	Partition Coefficient	Equilibrium Time (min)	Diffusion Coefficient (10^{11} m^2s^{-1})
1	43	20	20	0.31	133	1.8
2	72	20	20	0.46	215	4.0
3	47	20	60	0.14	150	9.8
4	80	20	60	0.22	260	6.9
5	43	60	20	0.2	36	1.2
6	72	60	20	0.33	45	15.9
7	47	60	60	0.11	29	22.8
8	80	60	60	0.10	38	5.0
9	62	6	40	0.27	328	4.2
10	62	74	40	0.17	26	133.1
11	43	40	6	0.64	108	2.8
12	64	40	74	0.13	44	26.0
13	36	40	40	0.15	62	10.1
14	88	40	40	0.24	113	5.6
15	62	40	40	0.15	82	5.9
16	62	40	40	0.23	58	13.7
17	62	40	40	0.24	85	19.4
18	62	40	40	0.18	61	10.3
Model				*** [a]	***	NS
Linear				***	***	NS
Quadratic				**	***	NS
Interaction				NS	*	NS
R^2				0.888	0.972	0.595
Effects						
Ethanol Conc.				NS	**	NS
Temperature				NS	***	NS
S/S Ratio				***	NS	NS

[a] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level

TABLE 14. Surface Response for Partition Coefficient, Equilibrium Time, and Diffusion Coefficient of Total Phenolics from Black Currant Extractions using Aqueous Ethanol

Run	Ethanol Conc. (%)	Temp. (°C)	S/S Ratio (mL/g)	Partition Coefficient	Equilibrium Time (min)	Diffusion Coefficient (10^{11} m^2s^{-1})
1	43	20	20	0.23	130	1.0
2	72	20	20	0.40	160	2.5
3	47	20	60	0.15	147	6.1
4	80	20	60	0.28	270	7.8
5	43	60	20	0.29	100	2.2
6	72	60	20	0.58	50	34.3
7	47	60	60	0.11	104	10.0
8	80	60	60	0.19	91	24.2
9	62	6	40	0.17	350	2.0
10	62	74	40	0.38	24	10.2
11	43	40	6	0.86	119	5.4
12	64	40	74	0.16	91	34.6
13	36	40	40	0.15	188	6.0
14	88	40	40	0.22	119	6.3
15	62	40	40	0.18	87	13.1
16	62	40	40	0.27	63	10.9
17	62	40	40	0.23	63	12.9
18	62	40	40	0.16	70	4.2
Model				** [a]	***	*
Linear				***	***	**
Quadratic				*	**	*
Interaction				NS	NS	NS
R^2				0.822	0.875	0.786
Effects						
Ethanol Conc.				NS	*	*
Temperature				NS	***	*
S/S Ratio				***	NS	NS

[a] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level

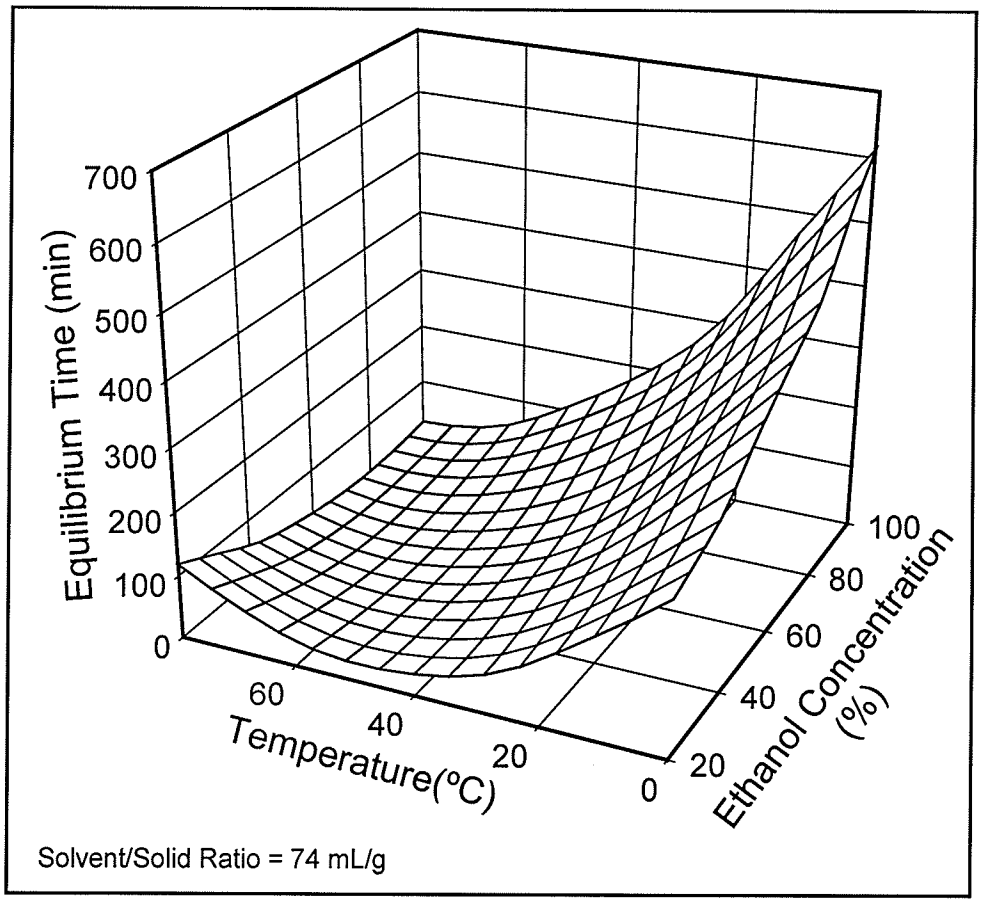


Figure 18. Response Surface for the Effects of Temperature and Ethanol Concentration on Equilibrium Time for the Extraction of Anthocyanins at a Constant Solvent to Solid Ratio of 74 mL/ g dwb of Frozen Black Currants

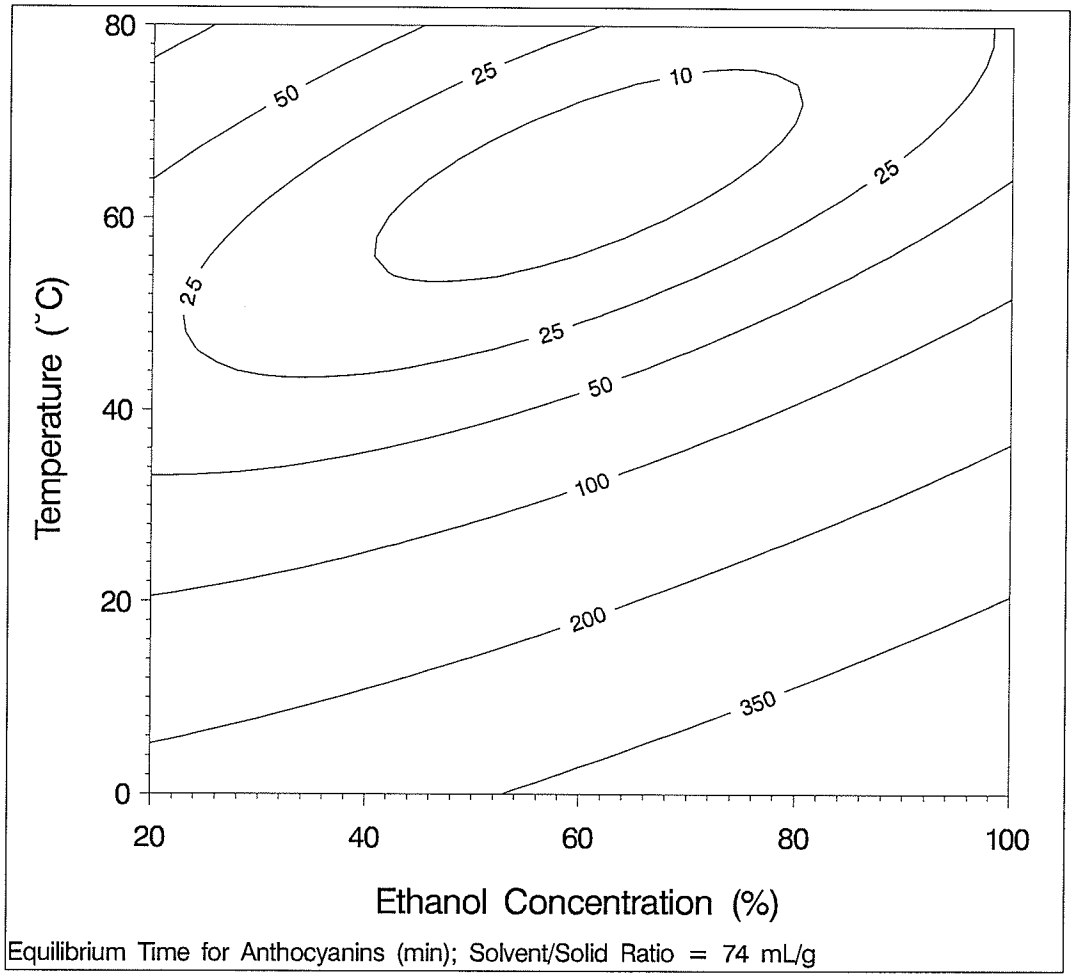


Figure 19. Contour Plot for the Effects of Temperature and Ethanol Concentration on Equilibrium Time for Anthocyanin Extraction at a Constant Solvent to Solid Ratio of 74 mL/g dwb of Frozen Black Currants

However, because of anthocyanin degradation at high temperature, conditions to reach the minimum extraction time could not be applied. Thus, the minimum time for anthocyanin extraction without resulting in degradation would be 50 to 100 min. Minimum extraction time for total phenolics which was restricted to narrower conditions than anthocyanin time was obtained around 65% ethanol and 60 °C (Figure 20).

The selected settings of mixer and agitation speed allowed rapid mixing and suspension of particles in the solvent even at the lowest S/S (6 mL/g). Equilibrium time for the extraction of anthocyanins varied from 26 to 330 min. Slightly shorter times, from 15 min to 120 min, were required for ethanol extraction of anthocyanins from very refined sunflower hulls (20 - 40 mesh) (Gao and Mazza, 1996). These conditions allowed a rapid extraction process in comparison with the 15 to 40 h for unagitated extraction of anthocyanins from grape pomace at an S/S ratio of 22 mL/g reported by Metivier et al. (1980). Conditions of mixing and extraction were very efficient as indicated by the short extraction times required and by the recovery of total phenolics (69 to 98%) and anthocyanins (60 to 100%) obtained.

e. Diffusion Coefficients of Phenolics. The surface response model for anthocyanin diffusion coefficient was not significant and showed no effect from any of the variables. The model for diffusivity of total phenolics was significant and was affected by the ethanol concentration and temperature. There was no significant effect of extract concentrations on the diffusivity of total phenolics in the concentration range used in the experiment (Tables 13 and 14). Fick's number (τ) is

$$\tau = \frac{D_s\, t}{a^2} \qquad (23)$$

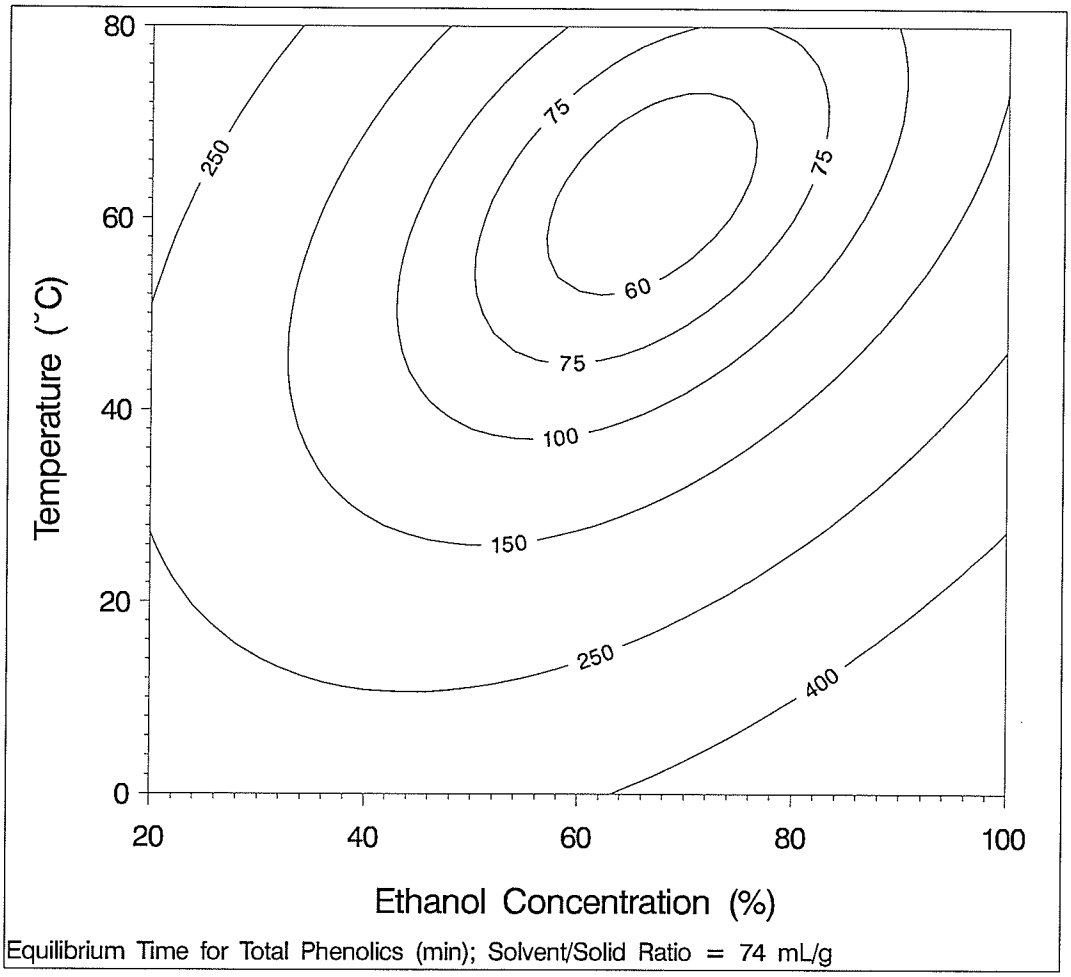


Figure 20. Contour Plot for the Effects of Temperature and Ethanol Concentration on Equilibrium Time for Total Phenolic Extraction at a Constant Solvent to Solid Ratio of 74 mL/g dwb of Frozen Black Currants

Thus, diffusion coefficient D_s is directly proportional to the slope of straight line from plots of Fick's numbers (τ) versus time (t) (Figure 21 and 22 and Appendix 1). Both anthocyanin and total phenolic diffusion coefficients increased with ethanol concentration from 39 % to 67 % ethanol and then decreased with further increase of concentration from 67 to 95 % ethanol. Increase of diffusivities with increase of ethanol percentage in the solvent composition would be an effect of the solvent on the solvation of the molecules. Higher ethanol composition would reduce the dielectric constant of the solvent, thus reducing the solvation of particles and increasing their diffusion. This is in agreement with enhanced total phenolic extraction at 60 % ethanol concentration noted in *Section B.1.a.*. Total phenolic diffusion coefficient increased with temperature from 6 to 40 °C and decreased with a further increase to 74°C. Anthocyanin diffusivity however, increased in the entire range from 6 to 74 °C. At 74 °C a large volume of solvent evaporated and that changed the solvent composition. Evaporation of a binary mixture results in a liquid phase richer in the less volatile compound. In the situation of an ethanol-water mixture, with an initial composition of 40 % ethanol, evaporation would lead to a liquid with a higher weight fraction of water. The reduction of ethanol concentration may have affected the solvent-solute interaction, thus reducing the diffusivity of solutes. This change would more likely affect non polar covalent compounds such as flavonols and tartaric esters than partially ionic compounds like anthocyanins because the energy of breaking apart the solvent is more important for the first type of compounds (Mackay and Mackay, 1981), and thus there would be a greater effect in their solvation.

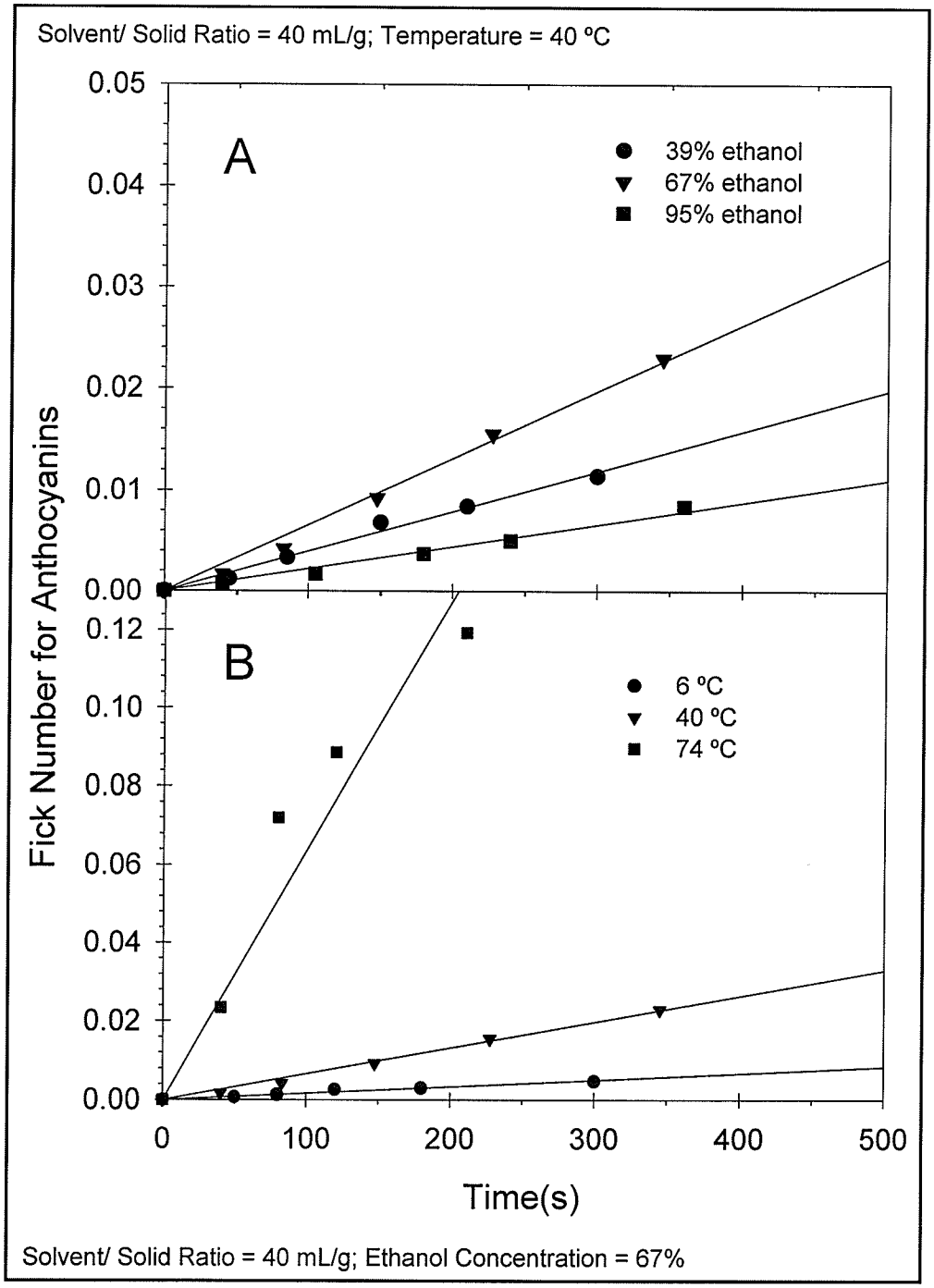


Figure 21. Anthocyanin Fick Number for the effects of A) Ethanol Concentration at Constant Temperature of 40 °C and B) Temperature at Constant 67 % Ethanol Concentration for Black Currant Extraction at a Solvent to Solid Ratio of 40 mL/g dwb of Frozen Berries

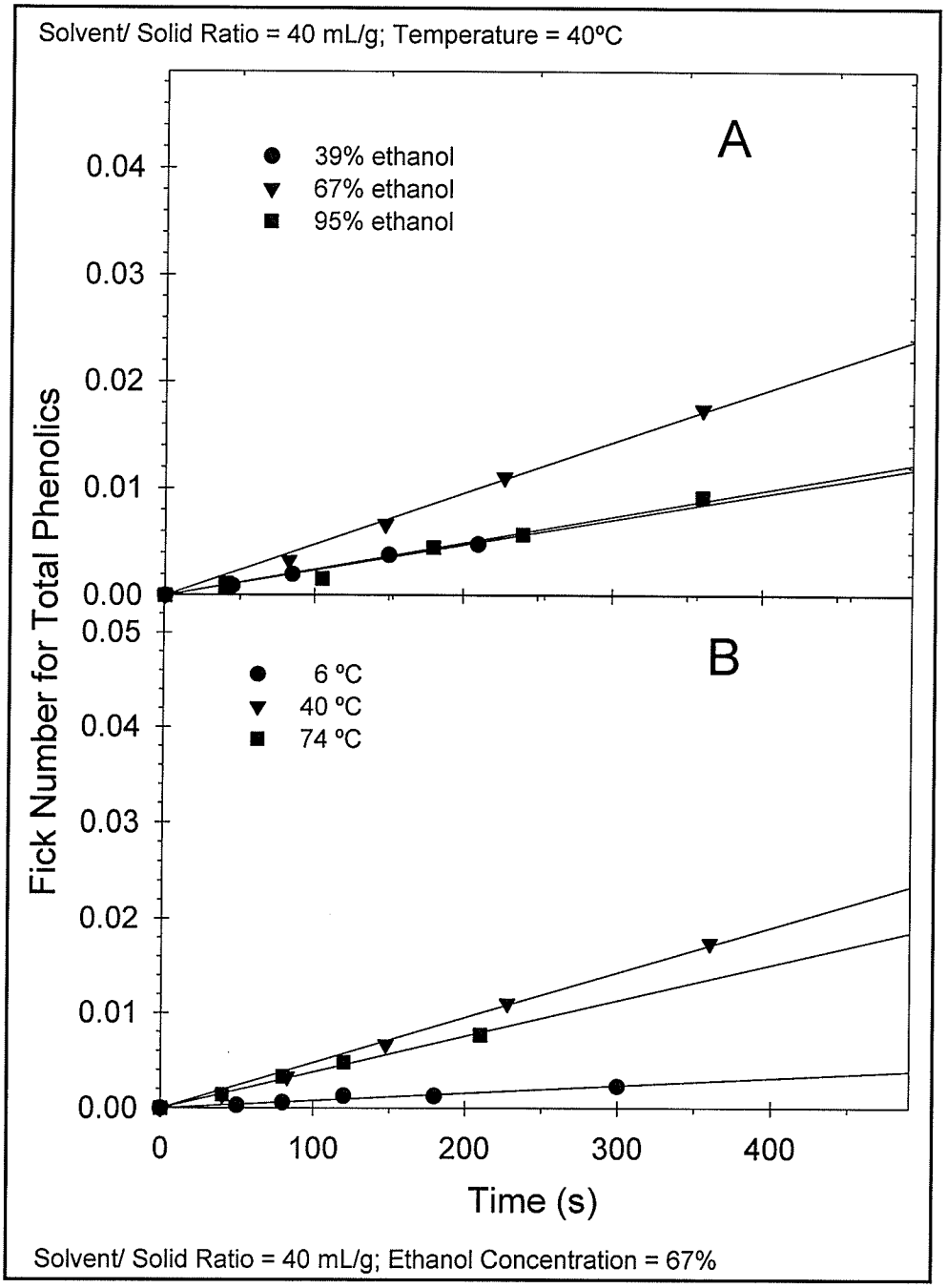


Figure 22. Total Phenolic Fick Number for the effects of A) Ethanol Concentration at Constant Temperature of 40 °C and B) Temperature at Constant 67 % Ethanol Concentration for Black Currant Extraction at a Solvent to Solid Ratio of 40 mL/g dwb of Frozen Berries

Mass transfer coefficients were calculated from equation (18) (Loncin and Merson, 1979) using an empirical correlation of power number and Reynolds number (Charm, 1963) to obtain the power delivered by the agitator. For an axial turbine with four blades at 45° angle and at the Reynolds number of the center point extraction (90,000), the power of the agitator was 4.0 W. Maximum power stamped on the motor of the agitator is 49.7 W however, this would be the power at maximum speed. Besides, the power uptake by the motor depends on the resistance by the fluid-impeller system. A low viscosity solvent such as the aqueous ethanol used did not provide a high resistance for the impeller. Voltage and current of the used agitator-vessel system working with water at 1210 rpm were measured and the power consumption calculated was 3.84 W. Thus, mass transfer coefficients for anthocyanins and total phenolics from extractions using 40 mL/g of 67% ethanol at 40°C, calculated with a power of 4.0 W, were 2.36 10^{-5} and 2.09 10^{-5} ms^{-1}.

f. Mass Balance of Extraction. Extraction was achieved by suspending milled frozen particles of berries with a solvent in an agitated container. A scheme of the extractor displays the mass flow of the components used for extraction of phenolics (Figure 23).

Composition data of berries (Table 4), extract weight fractions (y_e), pomace weight fractions (x_m) and measured extract volume, density of extracts and pomace weights were used to calculate extract yields of phenolics and pomace losses (Table 15, Appendix II).

Extract yields of phenolics were calculated from

$$yield(\%) = \frac{V(L).\rho(g/L).y_e.100}{W_b(g).C_b} \qquad (24)$$

where V is the volume, ρ is the density, and y_e is the weight fraction of the extract for the

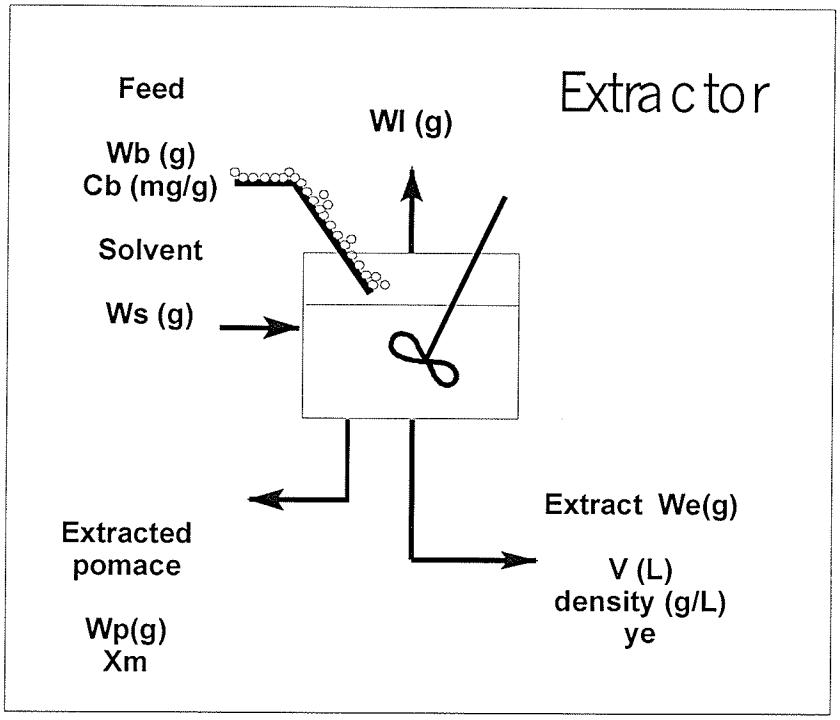


Figure 23. Mass Flow Scheme for the Extraction of Phenolic Compounds from Milled Frozen Berries in an Agitated Container

phenolic whose yield is being calculated, W_b is the weight of the feed of berries and C_b is the content of the phenolic in the berries.

TABLE 15. Total Phenolic and Anthocyanin Extract Mass Yields and Pomace Mass Loss, Total Mass Loss, Dry Matter Content of Extract and Total Solids Extracted from Black Currants using Aqueous Ethanol

Run	Total Phenolics [a]		Anthocyanins [b]		Total Loss (g) [e]	Total Loss (%) [f]	Extract DM (%)	Extract Solid (g) [g]	Extract Solid (%)[h]
	extract yield [c]	pomace loss [d]	extract yield	pomace loss					
1	72	18	89	14	308	10.8	3.35	72.8	13.7
2	84	22	96	17	240	8.9	3.09	62.3	11.8
3	91	7.3	108	4.4	210	8.5	1.22	26.5	15.0
4	96	6.0	104	2.8	180	7.8	1.26	25.6	14.2
5	82	19	75	15	650	22.8	3.87	73.2	13.8
6	88	14	93	13	409	15.2	3.2	63.6	12.0
7	93	9.9	99	2.8	405	16.3	1.35	26.7	15.0
8	98	8.8	101	4.7	331	14.5	1.29	23.9	13.5
9	97	9.3	112	4.7	130	5.2	1.72	38.5	14.4
10	94	15	60	8.6	986	39.7	1.93	25.9	9.7
11	73	15	86	15	495	12.1	7.91	236	13.2
12	106	8.0	110	5.0	284	12.1	0.99	19.6	13.6
13	88	14	98	7.9	257	9.8	1.67	36.8	13.8
14	84	9.2	99	6.2	399	17.2	2.07	37.5	14.1
15	100	10	102	7.2	274	11.1	1.81	37.7	14.3
16	92	10	104	7.0	334	13.5	1.9	37.7	14.2
17	91	6.5	104	3.6	277	11.1	1.87	38.9	14.7
18	90	17	96	4.1	315	12.7	1.56	31.1	11.8

[a b] Phenolics expressed in weight percent of the feed weight as equivalents of: [a] chlorogenic acid; [b] cyanidin 3-glucoside; [c] Phenolic weight of the extract in percent of the feed weight; [d] Phenolics weight lost in the pomace in percent of the feed weight; [e,f] Total mass loss expressed as [e] weight in grams or [f] percentage of the total initial weight; [g,h] Total solids expressed as [g] weight in grams or [h] percentage of the initial berry weight

Phenolic loss in the pomace was calculated from

$$pomace\ loss(\%) = \frac{W_p(g).x_m}{W_b(g).C_b} \qquad (25)$$

where W_p is the weight of the wet pomace and x_m is the weight fraction of a given phenolic in the wet pomace. Dry matter of the extracts were calculated from a mass balance of solids

$$W_b.DM_B = W_p.DM_P + W_E.DM_E \qquad (26)$$

where DM_B, DM_P, and DM_E are dry matter weight fractions of the berry, pomace and extract and W_E is extract weight calculated with extract volume and density of extract. Total loss which was mainly due to evaporation of solvent and secondly to degradation and loss of volatile compounds was calculated from a total mass balance

$$W_b + W_S = W_p + W_E + W_l \qquad (27)$$

W_S is the initial weight of solvent loaded into the extractor. Total loss was expressed as weight W_l and as percentage of the initial mass (Table 15). Because aqueous ethanol is a very volatile solvent, its evaporation represented a large part of the loss and thus there was a significant effect of temperature on the total loss. Treatment at the highest and lowest temperatures had the largest and smallest total loss, respectively. Besides increase of extraction temperature from 6 to 74 °C has not reduced the loss of total phenolics and anthocyanins in the pomace. Furthermore the 74°C extraction had a very low anthocyanin yield indicating degradation of anthocyanins which added to the solvent evaporation contributed to increase the total loss.

Total solids in the extract, E_S were calculated using the dry matter of extracts and the weight of extract

$$E_S = W_E(g).DM_E \qquad (28)$$

The highest dry mater content and total solid in the extracts (7.9 % and 236 g) were obtained with the extraction that used a S/S of 6 mL/g (Table 15). This was a consequence of the very low S/S leading to a high concentration of phenolics in the extract. However, extraction under these conditions was not efficient because phenolics yields obtained were relatively low (Tables 7 and 15). On the other hand, extraction with the highest S/S led to an effective process with very high yields but with the lowest extract dry matter. Thus, a high input of additional energy would be needed to concentrate and dry this extract if the intended final product were a dry powder. Effect of the S/S on the total solid extracted can be better evaluated with the solid content expressed as a percentage of the initial berries (SE). Maximum SE was obtained from extractions using 74 mL/g of 67 % ethanol at 20 to 30 °C. Solids percentage increased with the increase of the S/S and remained constant with ethanol concentration at S/S higher than 40 mL/g. At lower S/S however, SE slightly decreased when the ethanol concentration was increased. The diffusion might be the controlling step of the extraction. Because of the lower gradient at low S/S, the diffusion rate could have been not sufficiently high to remove compounds from the solid. However, SE includes not only phenolics but also any other soluble compounds that would be extracted by the solvent.

The highest anthocyanin yield was obtained with the 6°C treatment which used intermediate S/S and ethanol concentration. However, this extraction had 1.72 % solid content (Table 15) and because of the low temperature employed, the extraction process was

slow and required the longest time (328 min) to reach equilibrium. Optimum technical conditions for maximum yield of bioactive phenolic compounds in the extraction with aqueous ethanol solvents would be 60 % ethanol, 74 mL/g and 30- 35 °C. However, optimization needs to consider other aspects involved in the process such as product specifications, final use of the product, spent energy, costs of energy, cost of raw material and price of final product. Information reported in this research provides data for a better selection of process conditions for the extraction of phenolics to meet the requirements of most diverse processing objectives.

2. Extraction with SO_2-containing Water

a. Surface Response for Phenolic Yields. The model developed by surface response analysis for total phenolics was significant at a low levels of probabilities ($p<0.01$) and variability could be very well explained by the model (Table 16). Surface response models for anthocyanins, tartaric esters, and flavonols were not statistically significant ($p>0.1$). Polynomial second order models (equation 22) were adjusted by backward elimination with the goodness-of-fit test at 0.1% level of REG procedures of SAS ® (1990) (SAS Institute Inc., Cary, NC). Regression coefficients and analysis of variance of the adjusted model for total phenolic and anthocyanin yields, extraction times and diffusion coefficients are presented in Table 17.

TABLE 16. Surface Response for Yields of Total Phenolics, Tartaric Esters, Flavonols, and Anthocyanins Extracted from Black Currants using SO_2-containing Water Solvent [a]

Run	SO_2 Conc. (ppm)	Temp. (°C)	S/S Ratio (mL/g)	Total Phenolics	Tartaric Esters	Flavonols	Anthocyanins
1	258	20	20	25.4 [b]	2.1 [c]	1.9 [d]	12.2 [e]
2	947	20	20	32.1	2.2	1.9	13.4
3	285	20	60	33.3	2.3	2.0	12.7
4	1044	20	60	55.6	2.6	2.1	13.4
5	258	60	20	23.5	2.1	1.8	11.4
6	946	60	20	35.6	2.6	2.2	13.3
7	285	60	60	26.6	2.0	1.8	13.1
8	1043	60	60	39.2	2.4	2.0	13.7
9	647	6	40	36.4	2.0	1.7	13.5
10	647	74	40	23.4	2.1	1.7	9.7
11	455	40	6	23.7	2.0	1.8	11.6
12	671	40	74	41.3	2.1	1.7	13.3
13	26	40	40	24.3	2.2	1.9	11.7
14	1269	40	40	40.6	2.2	1.7	12.5
15	647	40	40	36.9	2.5	2.1	13.1
16	647	40	40	31.4	2.0	1.7	12.0
17	647	40	40	32.5	2.2	2.0	12.8
18	647	40	40	31.0	2.2	1.9	13.7
Model				***[f]	NS	NS	NS
Linear				***	NS	NS	*
Quadratic				NS	NS	NS	NS
Interaction				*	NS	NS	NS
R^2				0.928	0.461	0.275	0.630
Effects							
SO_2 Conc.				***	NS	NS	NS
Temperature				**	NS	NS	NS
S/S Ratio				***	NS	NS	NS

[a] Phenolics yields in mg /g of frozen berries on a dwb expressed as equivalents of: [b] chlorogenic acid; [c] caffeic acid ; [d] quercetin; [e] cyanidin 3-glucoside; [f] *** Significant at 0.01 level, ** significant at 0.05 level,* significant at 0.1 level

TABLE 17. Regression Coefficients and Analysis of Variance of the Second Order Polynomial Model Adjusted by Goodness-of-Fit Test for Total Phenolic and Anthocyanin Yield, Equilibrium Time and Diffusion Coefficient of Black Currant Extractions using SO_2-containing Water Solvent

	Total Phenolics Coefficients			Anthocyanin Coefficients		
Variables[a]	**Yield**	**Time**	**Diffusion Coeff**	**Yield**	**Time**	**Diffusion Coeff**
Intercept	25.41^{***}	241.5^{***}	-21.4^{NS}	12.2^{***}	338.0^{***}	20.3^{*}
X_1	0.014^{*} [c]	-0.16^{***}		0.0012^{*}	-0.29^{***}	
X_2		-4.06^{***}			-6.30^{***}	
X_3		-1.20^{**}			-2.85^{**}	-1.23^{**}
X_1^2		$5.1\times10^{-5*}$	$9.1\times10^{-6\,NS}$		$4.5\times10^{-5***}$	
X_2^2	-0.0015^{*}	0.032^{***}		-0.00054^{***}	0.025^{**}	
X_3^2						0.016^{**}
X_1*X_2	0.0004^{**}		0.0037^{***}		0.0040^{**}	0.00088^{***}
X_1*X_3	0.0008^{***}	-0.0012^{*}			0.0031^{**}	
X_2*X_3				0.00047^{*}	0.044^{*}	
$X_1*X_2*X_3$	$-1.2\times10^{-5**}$				$-5.5\times10^{-5\,*}$	
Model	***	***	***	**	***	***
R^2	0.945	0.919	0.756	0.508	0.946	0.763

[a] Polynomial model $Y=\beta_0+\sum_{i=1}^{3}\beta_i X_i+\sum_{i=1}^{3}\beta_{ii}X_i^2+\sum_{i=1}^{3}\beta_{ij}X_iX_j$ adjusted by backward elimination al the level of 0.1 % with the goodness-of-fit test, where X_1 = SO_2 concentration, X_2 = temperature, X_3= solvent to solid ratio; [b] NS non significant ($p>0.1$);[c] *** Significant at 0.01 level, ** significant at 0.05 level,* significant at 0.1 level

Increasing the S/S, anthocyanins and total phenolics increased and the time to reach constant concentration decreased (Figure 24). This effect is more noticeable at low (300 ppm) SO_2 concentration than at high (1,100 ppm) SO_2 concentration. An increase in extraction temperature from 20 to 60 °C did not result in a higher anthocyanin extraction, but did reduce the time to reach equilibrium. Temperature can affect the extraction of a given compound by modifying the diffusion coefficient or its solubility in the solvent. Increasing the

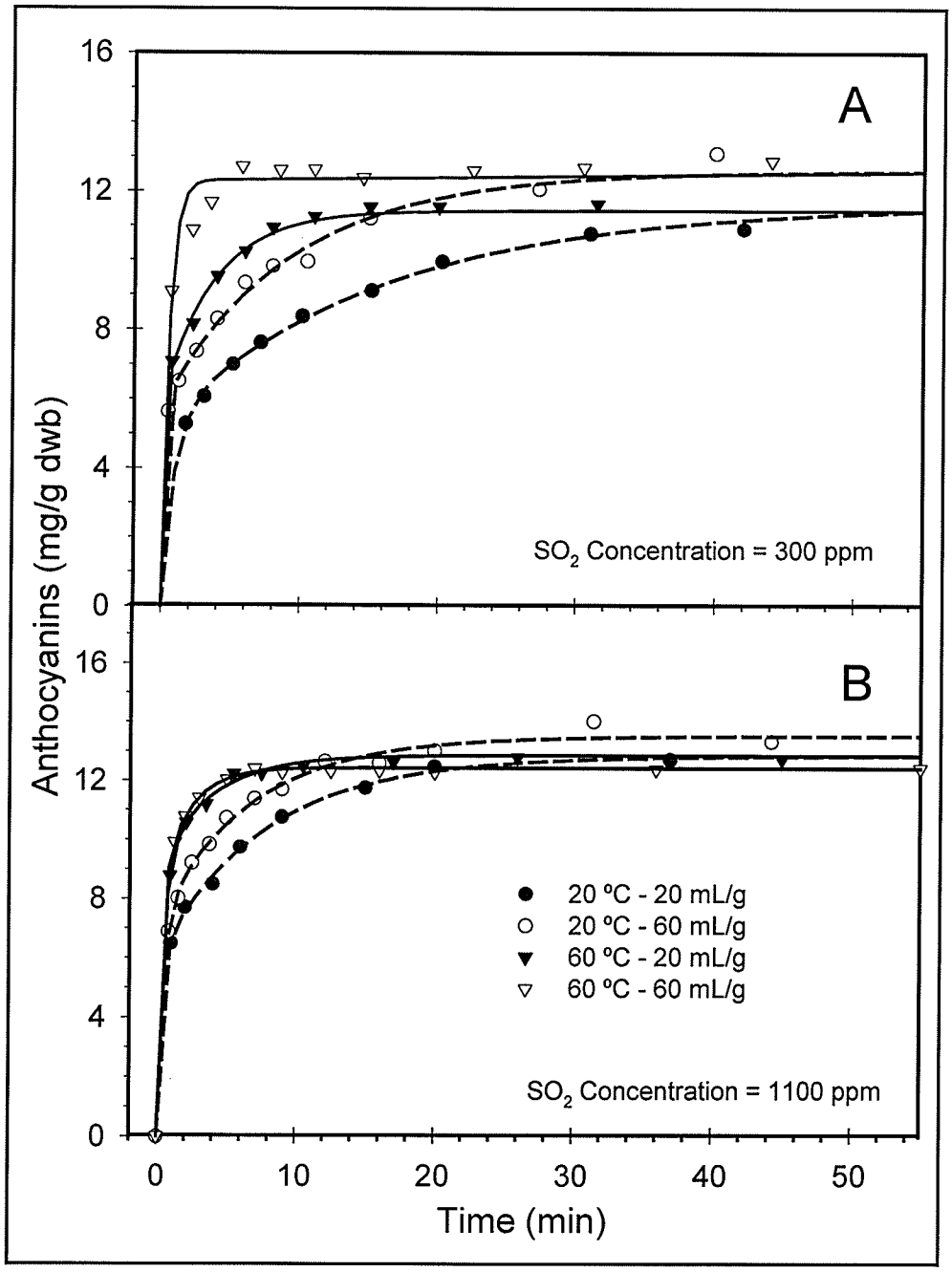


Figure 24. Anthocyanins Expressed as Cyanidin 3-glucoside Equivalents in mg/g dwb of Frozen Black Currants Extracted with A) 300 ppm SO_2-containing Water and B) 1100 ppm SO_2-containing Water at the Following Temperatures and Solvent to Solid Ratios: ● 20°C and 20 mL/g: ○ 20°C and 60 mL/g; ▾ 60°C and 20 mL/g, and ▽ 60°C and 60 mL/g

temperature would increase the diffusion coefficient and thus the rate of diffusion leading to a reduction in the extraction time. At 1,100 ppm SO_2 concentration, a high temperature of extraction (60 °C) in combination with a high S/S led to lower anthocyanin extraction than the same high S/S with low temperature. This effect can be a consequence of degradation of anthocyanins produced by the high temperature.

Total phenolic extraction was affected by the three variables studied, but it was mostly affected by the SO_2 concentration and the S/S (Table 16). Solvent to solid ratio significantly increased extraction of total phenolics. Total phenolics increased linearly with S/S; however, the slope was steeper at low temperature, increasing from 25 to 78 mg chlorogenic acid equivalents per gram of frozen berries on a dry weight basis (Figure 25A). The slower increase of total phenolics at high temperature might be due to susceptibility of some phenolics to thermal degradation. At high temperature, the amount of compounds available for extraction could be removed at the diffusion rate obtained with 20 mL/g S/S (low value). Then, an increase in S/S did not show a high increase of yield. A lower effect of temperature on extraction of total phenolics compared to anthocyanins from lowbush blueberry has been reported by Kalt et al. (2000). Solvent to solid ratio also increased total phenolics at high SO_2 concentration but values were essentially constant at very low SO_2 concentrations (Figure 25B). The limiting step of extraction at low SO_2 concentration would be the reduction of the solubility. At high SO_2 concentration, solubility would have increased and then, an increase in S/S resulted in a higher extraction of total phenolics.

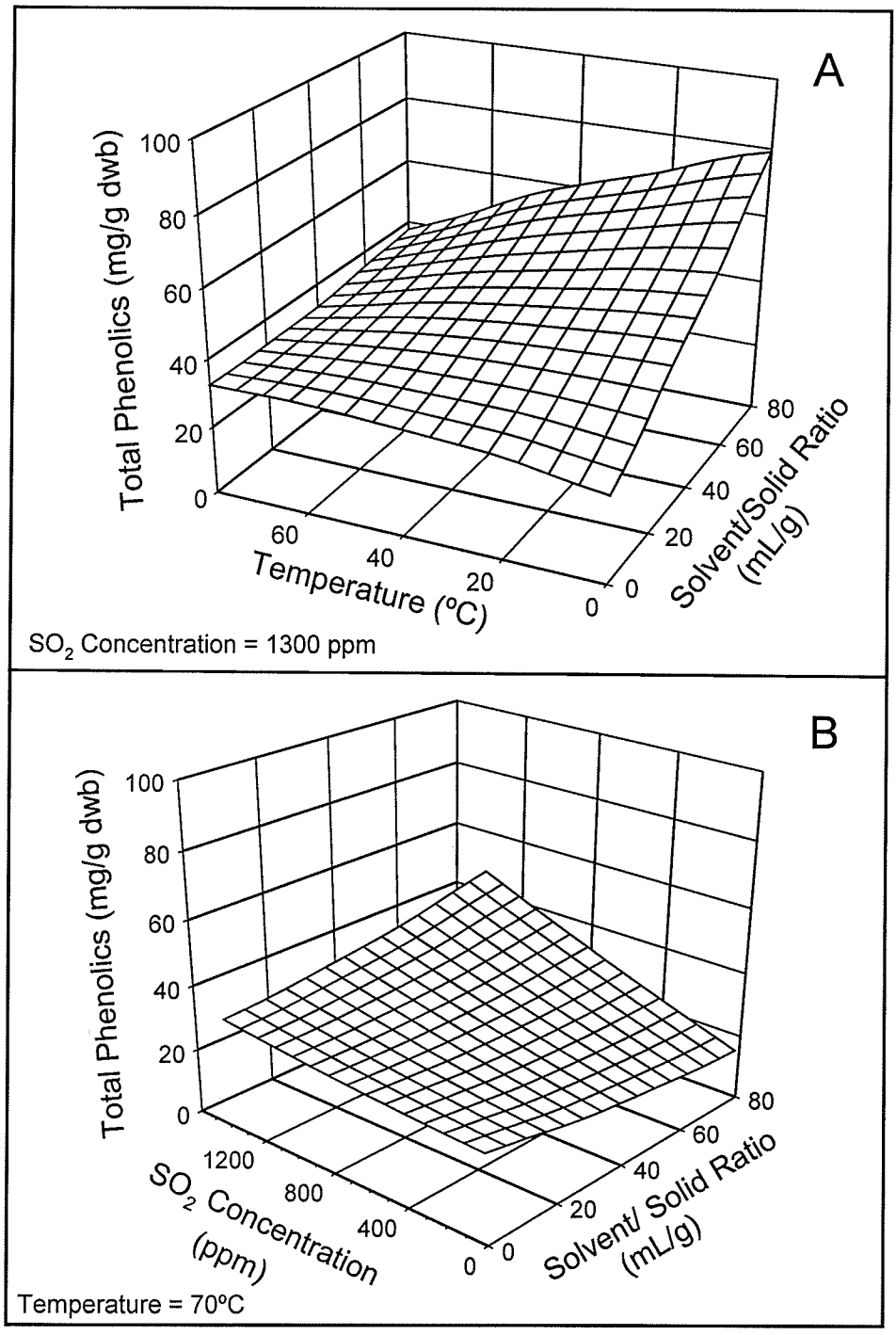


Figure 25. Response Surface for the Effects of A) Solvent to Solid Ratio and Temperature at a Constant SO_2 Concentration of 1300 ppm and B) Solvent to Solid Ratio and SO_2 Concentration at a Constant Temperature of 70°C on Total Phenolics Expressed as Chlorogenic Acid Equivalents in mg/g dwb of Frozen Berries

Total phenolics increased with SO_2 concentration in the whole range of S/S but the increase was higher at higher S/S (Figure 25B). Anthocyanin extraction also increased by increasing SO_2 concentration but the effect was not statistically significant. The exact mechanism for how SO_2 improves extraction is not known, but interactions leading to improved diffusion through the cell walls and increased solubility have been mentioned as possible causes (Gao and Mazza, 1996). Higher anthocyanin yields from grapes (Bakker et al., 1998) and from sunflower-hull (Mok and Hettiarachchy, 1991; Gao and Mazza, 1996) have been reported from extractions with higher SO_2 concentration.

Extraction of anthocyanins and total phenolics from black currant berries with 100% water at room temperature have resulted in yields (Kähkönen et al., 2001) similar to those achieved with our lowest SO_2 treatment (28 ppm). Solvent composition changes physical properties such as density and dynamic viscosity that may affect diffusion and the rate of extraction. It also influences the activity coefficient and thus the solubility of a given compound in the solvent. Another physical property of the solvent that would be modified with changes of solvent composition is the electric permitivity or dielectric constant. This property measures the ability to reduce the interaction of particles with opposite charges and also determine solvation characteristics of a solvent (Carey and Sundberg, 1984; Carey, 1987). Reduction of dielectric constant of a protic solvent (contains relatively mobile protons), such as water ($\varepsilon_{H2O} = 78.5$) into the range of intermediate behavior solvents such as methanol ($\varepsilon_{MeOH} = 32.6$) or ethanol ($\varepsilon_{EtOH} = 24.3$) by modifying pressure and temperature has improved extraction of natural products (Basile et al., 1998; Clifford et al., 1999; Kubatova et al., 2001). The high dielectric constant of water may have been reduced when SO_2 concentration increased. Dielectric constant of pure liquid SO_2 at 20°C is 14 (Lide,

1992). Lower dielectric constant reduces the energy required to separate the solvent molecules and allow the solute molecules to enter between them (Mackay and Mackay, 1981). Best extractions were obtained with distilled water-organic solvent mixtures of intermediate polarity values. The improvement of extraction of anthocyanins from sunflower husks with alcohols and water-organic solvents mixtures has been attributed to a decrease in the dielectric constant of the solvent (Pifferi and Vaccari, 1983). From these research results it is suggested that one of the mechanisms of SO_2 to improve the extraction is by increasing the solubility of phenolic molecules.

The effect of the temperature on the extraction of total phenolics is not clear. Total phenolics had a possibly significant ($p<0.1$) interaction effect, and the temperature effect was not significant for anthocyanins. At low SO_2 concentration (28 to 300 ppm) a change in color from pale pink to reddish pink and red was noticed as extraction proceeded. Nucleophilic addition of HSO_3^- ion to anthocyanins is responsible for fading of pigment extracts (Brouillard and El Hage Chahine, 1980). The noted color shift may indicate that the reaction of HSO_3^- addition was limited by the relatively low SO_2 concentration in the extracts. Thus, once the SO_2 available was consumed, further extraction of anthocyanins resulted in an increase of red color because the pigment remained in the flavilium cation or quinonoidal base forms. A similar effect was observed at intermediate SO_2 concentration (700 ppm) and 74 °C. High temperatures may have reduced the SO_2 availability. Solubility of SO_2 in water decreases when temperature is increased (Schroeter, 1966). As a result, a limited HSO_3^- addition reaction may have occurred even at 700 ppm SO_2 by an effect of high temperature. This reduction in SO_2 concentration may have been responsible for reduced phenolics yield at high temperature resulting in a confounding temperature effect and a significant interaction

coefficient. As a result, temperatures as high as 74 °C are not recommended for extraction with SO_2 containing solvents because the beneficial effect of SO_2 on extraction may be negated by the high temperature.

Extraction of anthocyanins was affected by S/S and temperature (Figure 26), although the effects were not statistically significant. At a constant SO_2 concentration of 1,100 ppm, anthocyanin content increased with temperature up to a maximum at approximately 40 °C and then decreased with further increases in temperature, independent of the S/S values. Increase of temperature would be favoring extraction by increasing both solubility of anthocyanins and diffusion coefficient, although, a major effect was to speed up extraction by increasing the diffusion coefficient. Reduction of anthocyanin extraction by increasing temperature beyond 35- 40 °C can be attributed to anthocyanin degradation at high temperatures. This is in full agreement with Pifferi and Vaccari (1983). Both degradation of anthocyanin (Markakis, 1982; Mok and Hettiarachchy, 1991) and increased permeability of membranes (Spanos et al., 1990) have been attributed to high temperatures, and together these factors would result in confounded effects on anthocyanins extraction. However, the results presented in Figure 26 appear to indicate that degradation had a greater effect than permeability. An increase in extracted anthocyanins by about 15 times over the initial values was reported during water extraction of anthocyanins at 60 °C from lowbush blueberry (Kalt et al., 2000). The results of this study showed that the amounts of anthocyanins extracted at 60°C were lower than values obtained at 25 °C in the whole range of S/S. Anthocyanins were also affected by S/S. There was an almost linear increase in anthocyanins with the increase of S/S in the range used in the experiment (Figure 26). Increase of S/S would favor the extraction of anthocyanins by modifying the concentration gradient and thus increasing the diffusion rate.

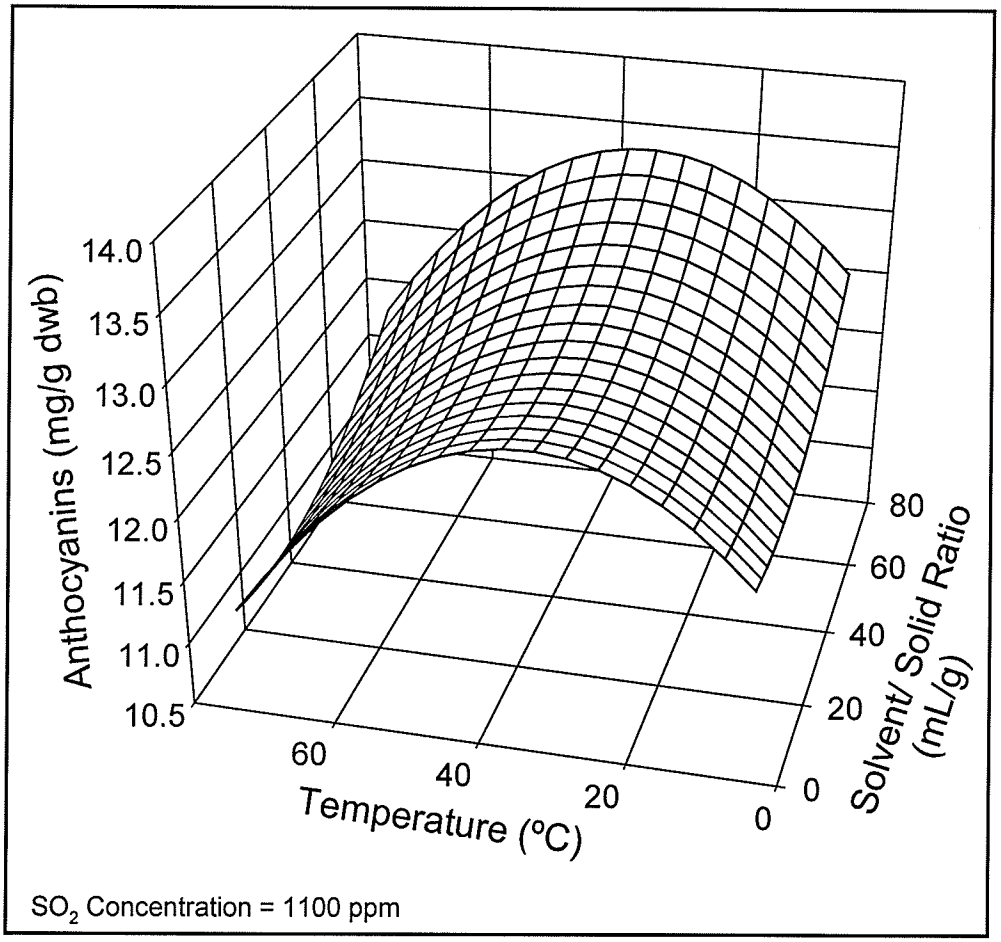


Figure 26. Response Surface for the Effects of Temperature and Solvent to Solid Ratio on Anthocyanins Expressed as Cyanidin 3-glucoside Equivalents in mg/g dwb of Frozen Black Currants at a Constant SO_2 Concentration of 1100 ppm

b. Surface Response for Antioxidant Activity. Surface response varied for the antioxidant activity parameters (Table 18). The model was not significant and variability explained was low for antioxidant activity measured by the β-carotene method, but models were significant and the variability explained was fairly high for the antiradical activity measured by the DPPH method and for the antioxidant index.

Surface response plots for antioxidant index had a saddle shape (Figure 27). The index was affected by all three experimental variables evaluated. The main variable affecting the index however, was S/S. The higher the S/S, the lower the antioxidant index and the effect was higher at low temperatures. Higher S/S yielded more dilute extracts which may explain the lower antioxidant indices. Antioxidant activity as measured with the β-carotene method did not show differences among treatments. However, temperature and S/S affected antiradical activity measured by the DPPH method (Table 18). The highest antiradical activity was obtained from extractions using a combination of high S/S and temperature. Antiradical activity ranged from -3.5 to -6.1 μM DPPH/μM of antioxidant expressed as chlorogenic acid. Antioxidant activity of extracts compared favorably with the activity of well known antioxidants used as reference such as α-tocopherol (-1.71 μM DPPH/μM) and BHT (-2.75 μM DPPH/μM). Differences in antiradical activity are related to variations in extract compositions. Some compositional changes were observed in samples extracted at varying temperatures, and to further investigate this effect, samples from extractions at 6°C, 40 °C and 74 °C were analyzed by HPLC.

TABLE 18. Surface Response for Antiradical Activity, Antioxidant Activity, and Antioxidant Index of Extracts with SO_2-containing Water Solvent

Run	SO_2 Conc. (ppm)	Temp. (°C)	S/S Ratio (mL/g)	Antiradical activity	Antioxidant activity	Antioxidant index (%)
1	258	20	20	-4.02 [a]	892 [b]	85.8
2	947	20	20	-3.75	694	83.0
3	285	20	60	-3.53	711	58.5
4	1044	20	60	-4.30	745	42.8
5	258	60	20	-4.14	937	87.9
6	946	60	20	-4.16	747	87.0
7	285	60	60	-4.99	1331	70.1
8	1043	60	60	-6.14	501	70.4
9	647	6	40	-4.43	1189	67.6
10	647	74	40	-5.36	569	80.5
11	455	40	6	-5.21	1142	91.8
12	671	40	74	-5.56	263	51.6
13	26	40	40	-3.59	822	74.2
14	1269	40	40	-5.50	416	63.8
15	647	40	40	-4.64	656	70.8
16	647	40	40	-4.75	555	72.1
17	647	40	40	-4.18	369	75.9
18	647	40	40	-4.36	348	70.3
Model				*	NS	***
Linear				**	NS	***
Quadratic				NS	NS	NS
Interaction				NS	NS	***
R^2				0.772	0.627	0.977
Effects						
SO_2 Conc.				NS	NS	**
Temperature				*	NS	***
S/S Ratio				*	NS	***

[a] Slope coefficient calculated by linear regression in μMDPPH / μM of antioxidant, expressed in total phenolics as chlorogenic acid, [b] Slope coefficient calculated by linear regression in 10^{+6} absorbance units/ μM of antioxidant, expressed in total phenolics as chlorogenic acid, [c] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level

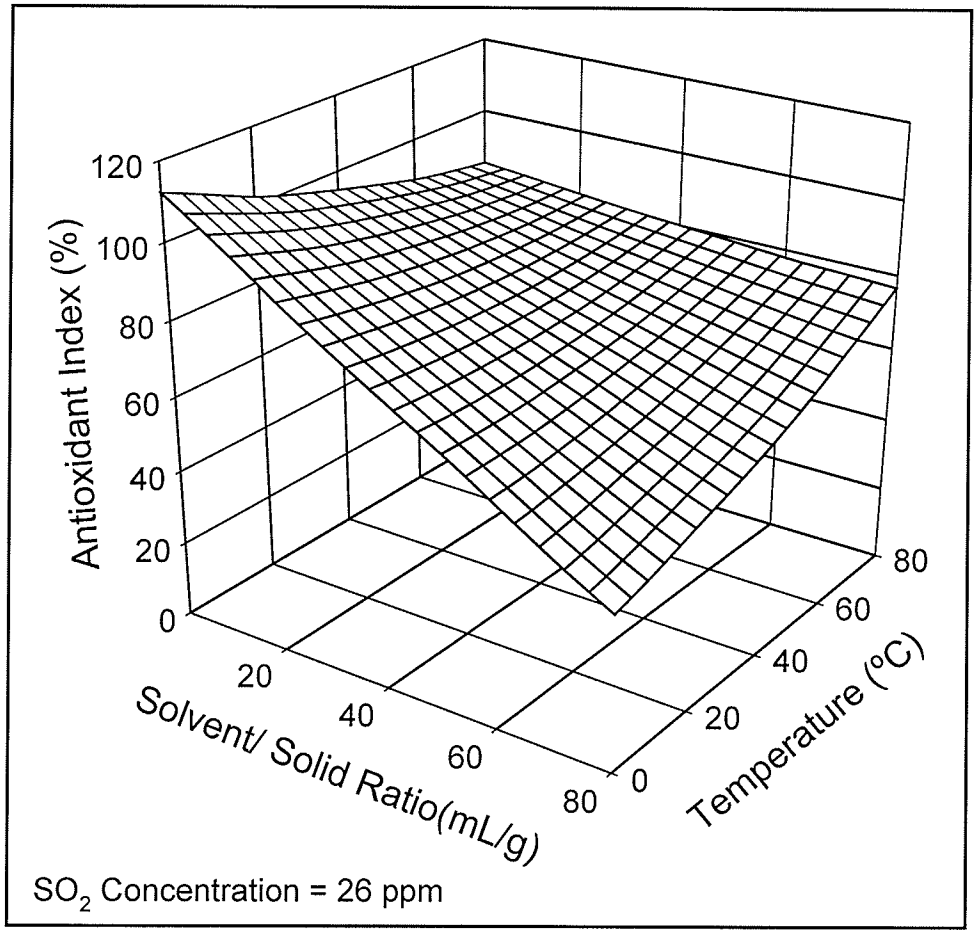


Figure 27. Response Surface for the Effects of Solvent to Solid Ratio and Temperature on Antioxidant Index of Extracts at a Constant SO_2 Concentration of 26 ppm

c. HPLC Results and Temperature Effects. Phenolic determination by HPLC showed a decrease in anthocyanins with an increase in temperature (Figure 28). As seen in Figure 26, anthocyanins extraction decreased with the increase in extraction temperature beyond 35-40°C. From the HPLC determination, a decrease in anthocyanins of approximately 53% was recorded when samples extracted at 74°C were compared to samples extracted at 6°C. Contents of all four major anthocyanins (peaks # 2, 3, 4 and 5) found in black currant decreased by the same percentage when the temperature was increased from 6 to 74°C. Minor anthocyanins (peaks # 6, 7, 10 and 11) were present in negligible amounts in samples extracted at 74 °C.

In addition to the reduction in anthocyanins, HPLC chromatograms at 280 and 320 nm of extracts processed at 74°C showed a decrease of about 48 and 21 % of total phenolics and tartaric esters, respectively, as compared to values at 6°C (Figure 29). A lower number of peaks and reduced peak areas were observed in samples extracted at 74°C than in samples extracted at 6°C. An increase of phenolic acids and tartaric ester extraction by effect of the temperature has been found in the extraction of phenolic compounds using aqueous ethanol from black currants (*Section B.1.c.* and Figure 17). Thus, at high temperature the extraction of those compounds with ethanol was higher than extraction with SO_2-containing water. The noted effect of high temperature on SO_2 concentration might be responsible for a smaller extraction of tartaric esters and phenolic acids with sulfurous water.

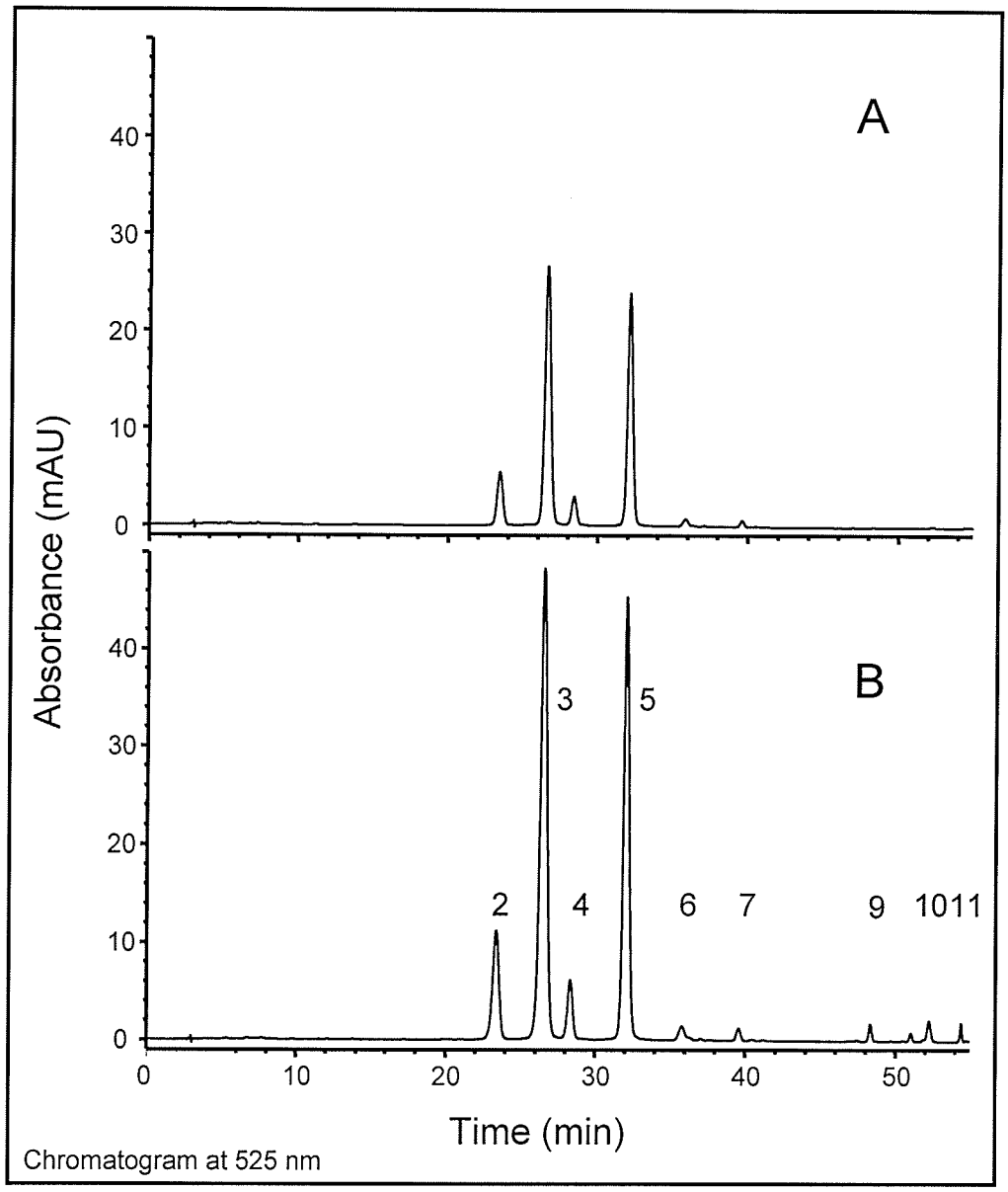


Figure 28. HPLC Chromatograms at 525 nm of Black Currant Extracts prepared using a Solvent to Solid Ratio of 40 mL/g dwb of Frozen Berries and a SO_2 Concentration of 700 ppm at A) 74°C and B) 6°C

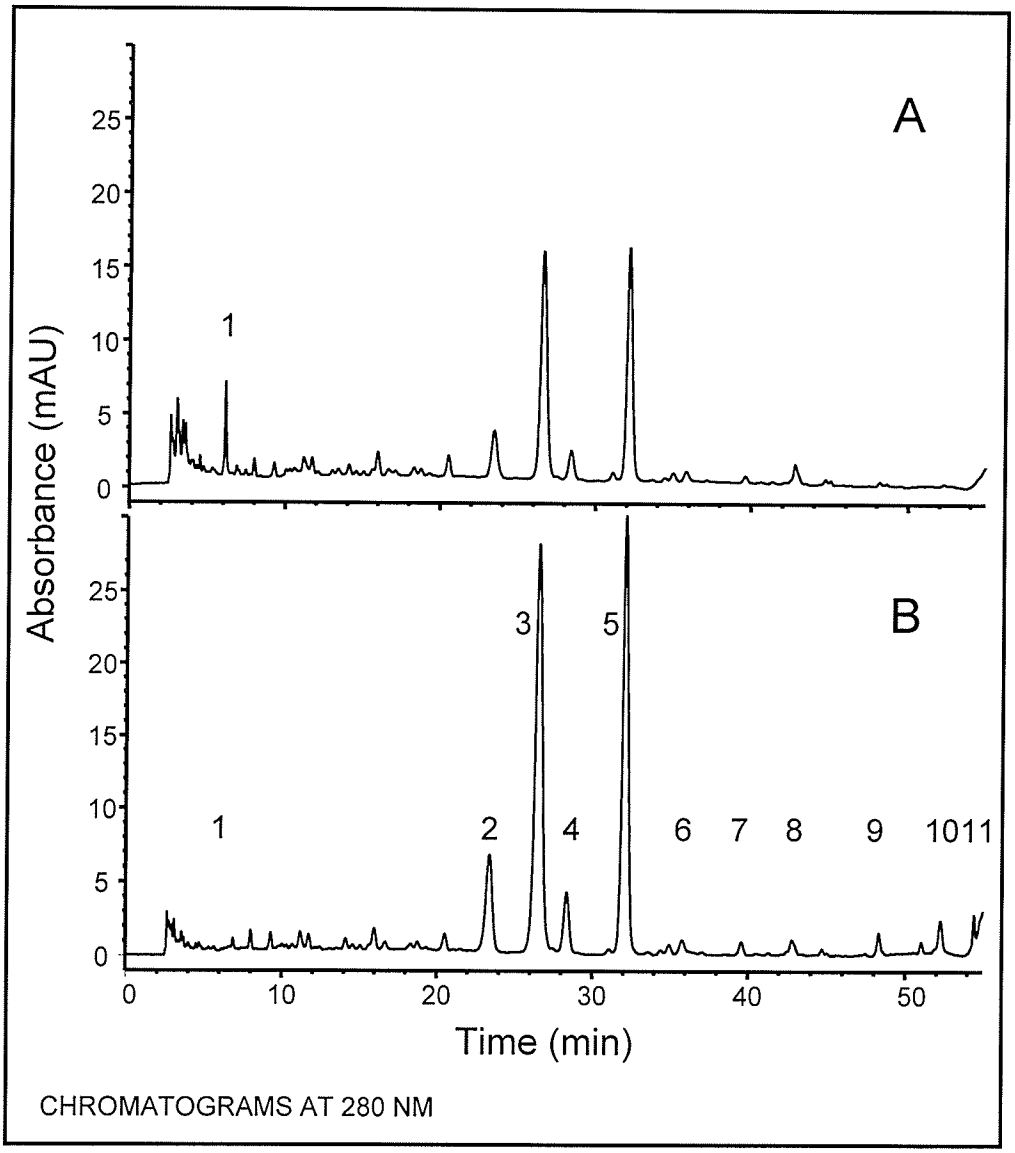


Figure 29. HPLC Chromatograms at 280 nm of Black Currant Extracts prepared using a Solvent to Solid Ratio of 40 mL/g dwb of Frozen Berries and a SO_2 Concentration of 700 ppm at A) 74°C and B) 6°C

d. Surface Response for Equilibrium Factors. Surface response models for partition coefficient and equilibrium times of anthocyanins (Table 19) and total phenolics (Table 20) were statistically significant. The major effect on the constants was due to changes in S/S. Equilibrium times for anthocyanins and total phenolics were mainly affected by SO_2 concentration and temperature. The same variables affected equilibrium times in the extraction of phenolics with aqueous ethanol solvents.

Response surfaces for the equilibrium time of anthocyanins and total phenolics were similar, and both models showed a large increase in the extraction time when temperature was reduced (Figure 30). Increase of extraction time is related to a lower diffusion rate by the effect of a lower temperature. Increase in time was higher at lower SO_2 concentration. At higher SO_2 concentrations the favorable effect on diffusion by the SO_2, might have compensated for the reduction of extraction rate caused by the lower temperature preventing a higher increase in extraction time. This effect is more evident in the anthocyanins response (Figure 30 A) than on total phenolics (Figure 30 B). Minimum extraction times were obtained at a SO_2 concentration of about 900 ppm and temperature of 65°C for anthocyanins and at S/S of 10 mL/g and about 900 ppm and 60°C for total phenolics at a S/S of 45 mL/g (Figures 31 and 32). Extraction of anthocyanins at a temperature equal or below 35 °C would allow a minimum time from 40 to 100 min depending on the SO_2 concentration in the range of 200 to 1300 ppm.

TABLE 19. Surface Response for Partition Coefficient, Equilibrium Time, and Diffusion Coefficient of Anthocyanins Extracted using SO_2-containing Water Solvent

Run	SO_2 Conc. (ppm)	Temp. (°C)	S/S Ratio (mL/g)	Partition Coefficient	Equilibrium Time (min)	Diffusion Coefficient (10^{11} m^2s^{-1})
1	258	20	20	0.25	130	6.7
2	947	20	20	0.37	55	20.8
3	285	20	60	0.09	70	8.1
4	1044	20	60	0.12	51	26
5	258	60	20	0.21	32	14.7
6	946	60	20	0.38	38	67.9
7	285	60	60	0.08	22	12.1
8	1043	60	60	0.12	22	70.4
9	647	6	40	0.14	136	4.6
10	647	74	40	0.11	20	16.4
11	455	40	6	1.17	80	22.4
12	671	40	74	0.1	29	42.3
13	26	40	40	0.08	92	0.07
14	1269	40	40	0.13	40	27.8
15	647	40	40	0.13	42	14.6
16	647	40	40	0.13	37	19.3
17	647	40	40	0.16	46	31.9
18	647	40	40	0.17	40	31.5
Model				** [a]	***	**
Linear				**	***	***
Quadratic				**	NS	NS
Interaction				NS	NS	NS
R^2				0.813	0.921	0.817
Effects						
SO_2 Conc.				NS	**	**
Temperature				NS	***	*
S/S Ratio				***	NS	NS

[a] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level

TABLE 20. Surface Response for Partition Coefficient, Equilibrium Time, and Diffusion Coefficient of Total Phenolics Extracted using SO_2-containing Water Solvent

Run	SO_2 Conc. (ppm)	Temp. (°C)	S/S Ratio (mL/g)	Partition Coefficient	Equilibrium Time (min)	Diffusion Coefficient (10^{11} m^2s^{-1})
1	258	20	20	0.13	128	10.8
2	947	20	20	0.18	52	38.4
3	285	20	60	0.06	68	15.8
4	1044	20	60	0.11	48	67.4
5	258	60	20	0.12	45	44
6	946	60	20	0.3	15	198.1
7	285	60	60	0.05	18	90.9
8	1043	60	60	0.31	10	258.3
9	647	6	40	0.09	135	6.7
10	647	74	40	0.2	20	64.2
11	455	40	6	0.47	62	28.4
12	671	40	74	0.12	29	70.5
13	26	40	40	0.04	88	4.1
14	1269	40	40	0.3	32	155.8
15	647	40	40	0.09	40	30.6
16	647	40	40	0.16	33	61.3
17	647	40	40	0.07	22	51.9
18	647	40	40	0.11	40	58.4
Model				** [a]	***	***
Linear				***	***	***
Quadratic				*	**	NS
Interaction				NS	NS	NS
R^2				0.841	0.944	0.867
Effects						
SO_2 Conc.				**	***	***
Temperature				NS	***	**
S/S Ratio				**	*	NS

[a] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level

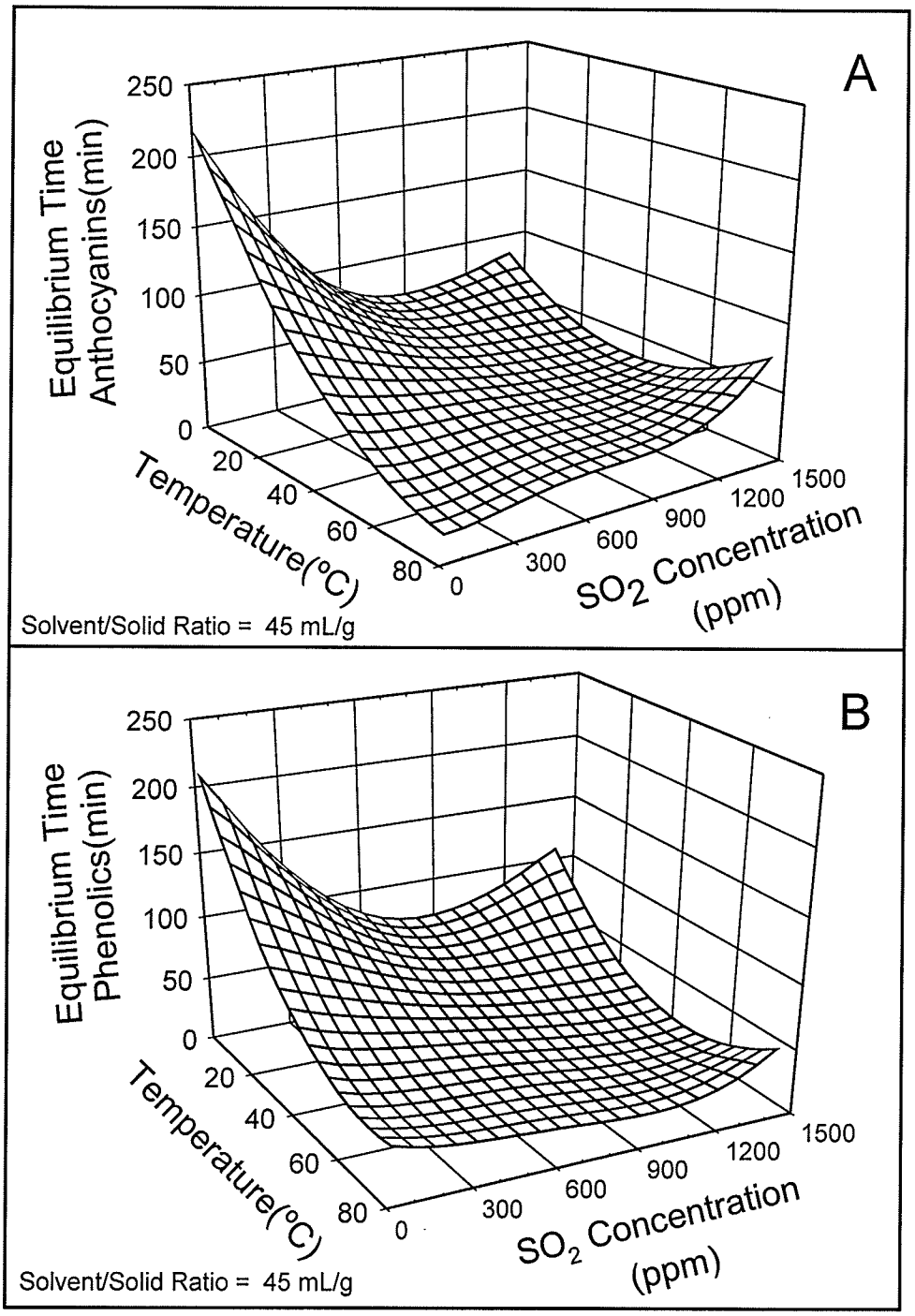


Figure 30. Response Surface for the Effects of Temperature and SO_2 Concentration at a Constant Solvent to Solid Ratio of 45 mL/ g dwb of Frozen Black Currants on A) Equilibrium Time for the Extraction of Anthocyanins and B) Equilibrium Time for the Extraction of Total Phenolics

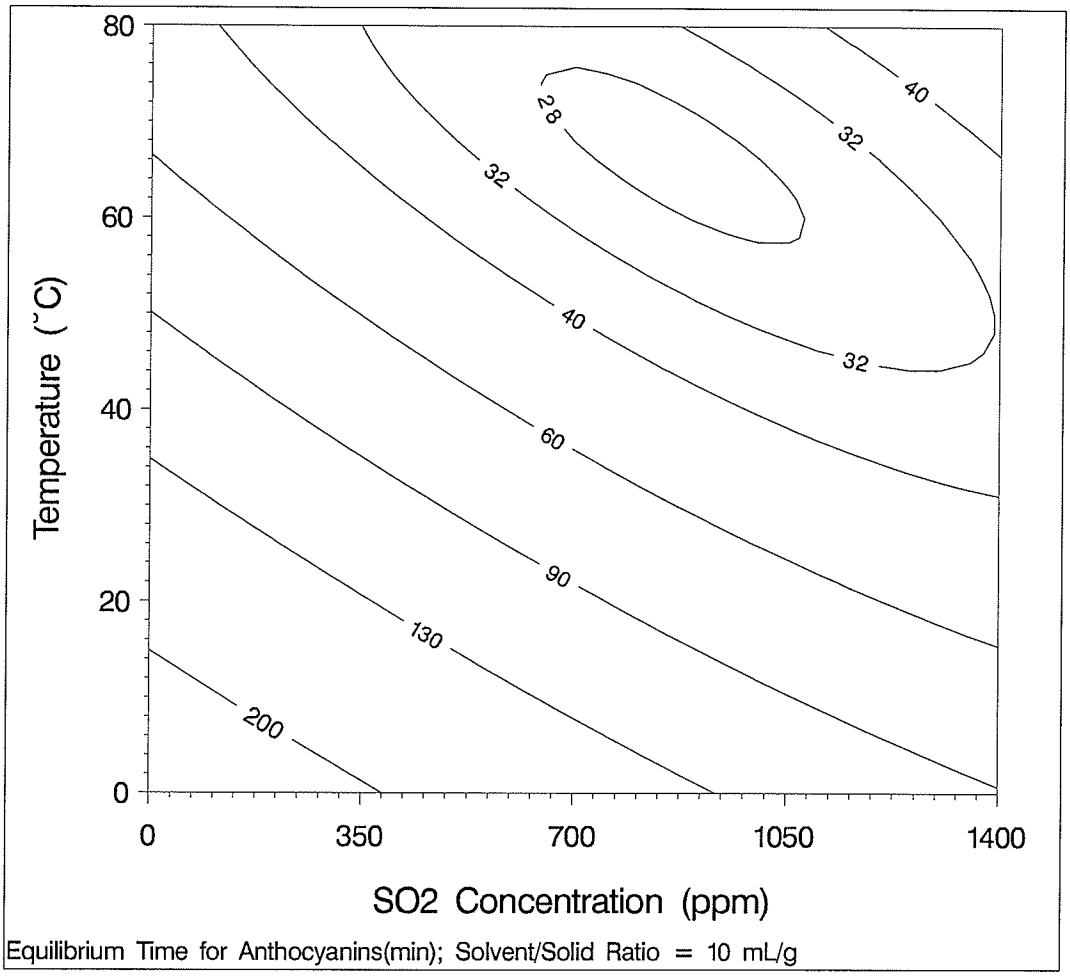


Figure 31. Contour Plot for the Effects of Temperature and SO_2 Concentration on Equilibrium Time for Anthocyanin Extraction at a Constant Solvent to Solid Ratio of 10 mL/g dwb of Frozen Black Currants

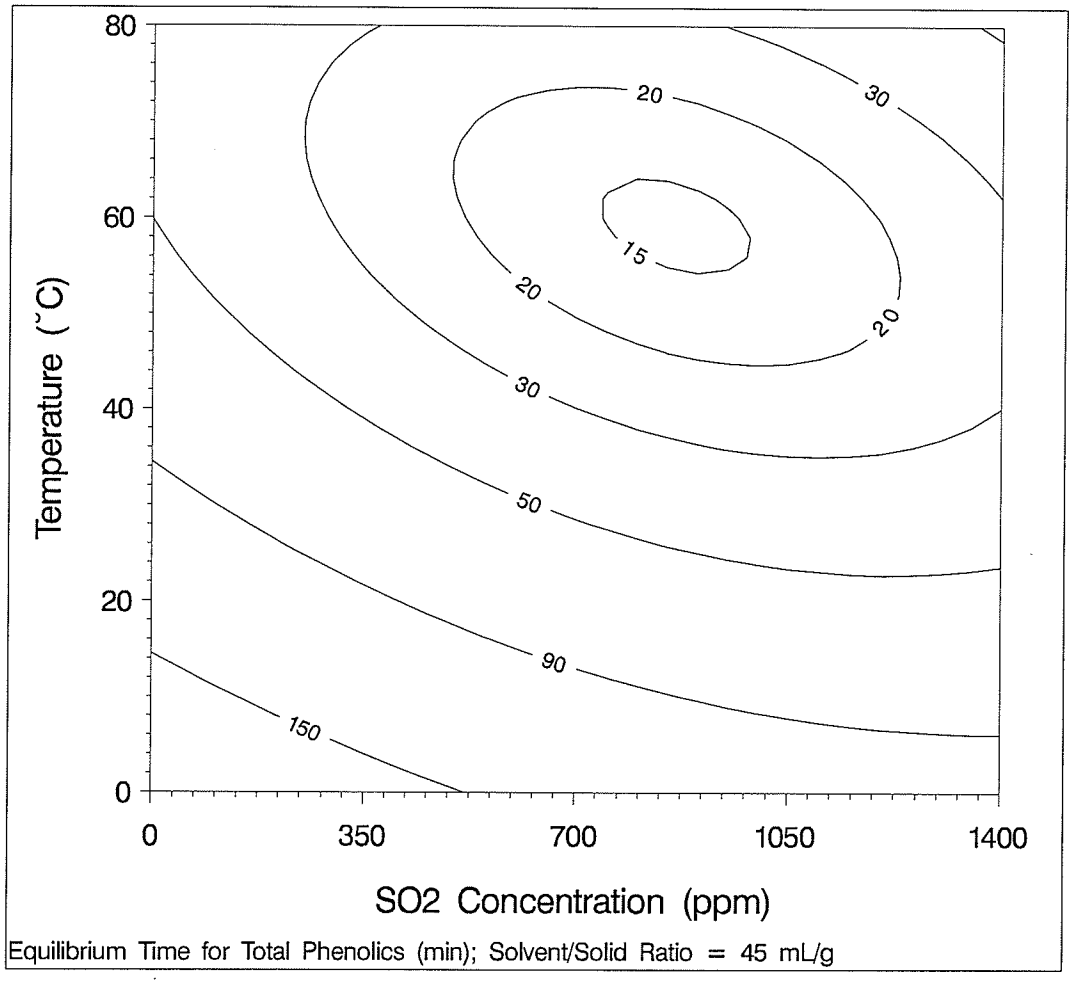


Figure 32. Contour Plot for the Effects of Temperature and SO_2 Concentration on Equilibrium Time for Total Phenolic Extraction at a Constant Solvent to Solid Ratio of 45 mL/g dwb of Frozen Black Currants

Extraction with SO_2-containing water resulted in very short extraction times to obtain a high anthocyanin recovery which varied from approximately 60 to 90%. However, the recovery for total phenolics was considerably lower, and ranged from 26 to 62%. This may have been caused by high absorbance readings at 280 nm attributable to interference of non phenolic compounds in the determination of total phenolics of the milled berries, as mentioned previously. Equilibrium times for anthocyanins varied from 20 to 136 min and for total phenolics from 10 to 135 min. Under similar extraction conditions, equilibrium times for extraction with aqueous ethanol were considerably longer (26 to 328 min). Shorter times for extraction of pigments with water than extraction times with alcohols was also reported by Pifferi and Vaccari (1983). Times from 10 min to about 60 min were required for reaching a complete extraction of anthocyanins from very refined sunflower hulls (0.42- 0.85 mm) (Gao and Mazza, 1996). Very short equilibrium times indicate a very high extraction rate; however, it should be noted that effects of SO_2 concentration or temperature dictate careful selection of extraction time to maximize yields.

e. Diffusion Coefficients of Phenolics. Surface response models for diffusion coefficients of anthocyanins and total phenolics were significant with a high explanation of the variability (R^2= 0.817 and 0.867). Diffusivities of total phenolics and anthocyanins were affected by both SO_2 concentration and temperature. There was no effect of S/S which represents no concentration effect (Tables 19 and 20). Diffusivity in concentrated solutions is lower than in dilute solutions as expressed by the empirical equation of Gordon (equation 7) (Cheryan, 1986). However in this research, the diverse concentrations of extracts that resulted from different S/S used did not affect diffusion coefficients.

Diffusion coefficients for anthocyanins and total phenolics increased with increasing SO_2 concentration (Figures 33 and 34). Both anthocyanin and total phenolic diffusivities using 28 ppm SO_2 water were extremely low. Diffusion coefficients depend on both solute and solvent. An increase of SO_2 concentration would have reduced the dielectric constant of the solvent and thus reduced solvation of molecules. Then the increase of SO_2 concentration would lead to an increase in the diffusion of the molecules by reduction of the interaction with the solvent. In *Section B.2.a.* it was noted that a major effect of SO_2 for enhanced extraction of phenolics was the increase of solubility of the solute. Sulfur dioxide also increased diffusivity of phenolics through the solid. Thus, it is believed that the mechanism responsible for improved extraction of phenolic compounds by SO_2-containing water is caused by two combined factors, increased solubility and enhanced diffusion coefficient of the molecules through the solid.

Total phenolics diffusivity increased when extraction temperature was increased from 6 to 40 and 74 °C. However, anthocyanins diffusivity at 74 °C was lower than at 40 °C. Reduction of SO_2 concentration by effect of the temperature might have impaired the beneficial effect of this compound on the diffusivity of anthocyanins. Diffusivities of urea, glycerin and sucrose in water increase with increase of temperature from 5 to 10, 15 and 20 °C (Schwartzberg and Chao, 1982). Diffusion coefficient of sucrose in 2.87 mm coffee grounds and water increases markedly from 0.91 to 1.85 10^{-10} $m^2 \cdot s^{-1}$ when the extraction temperature is increased in the range from 25 to 50 °C (Schwartzberg et al., 1982). The increase in diffusivity with the raise of temperature may be caused by an increase of the internal energy of the molecules and thus their mobility, and a reduction of the dynamic viscosity coefficient. Effect of temperature was higher on total phenolics diffusivity than on

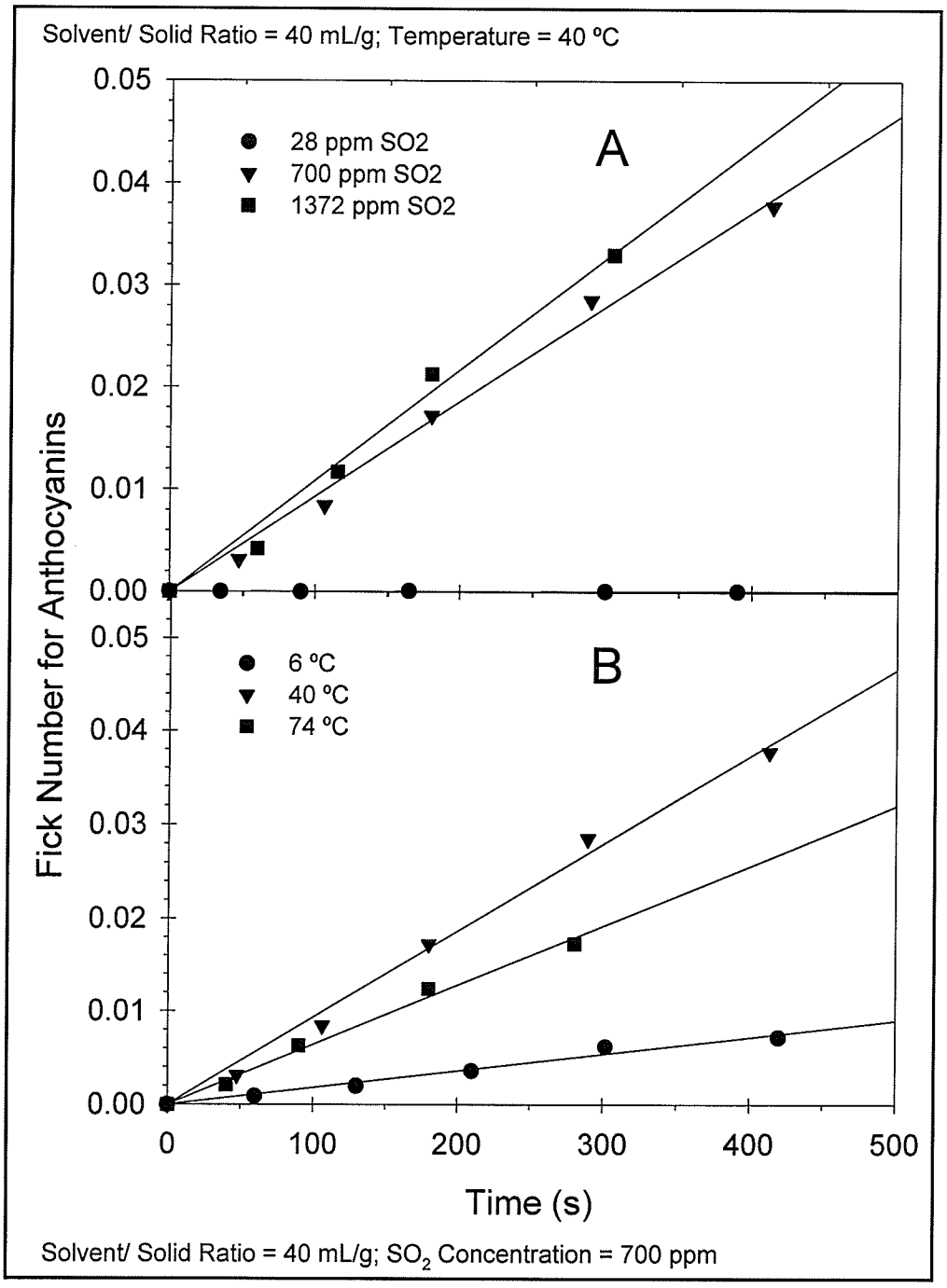


Figure 33. Anthocyanin Fick Number for the effects of A) SO_2 Concentration at Constant Temperature of 40 °C and B) Temperature at Constant 700 % SO_2 Concentration for Black Currant Extraction at a Solvent to Solid Ratio of 40 mL/g dwb of Frozen Berries

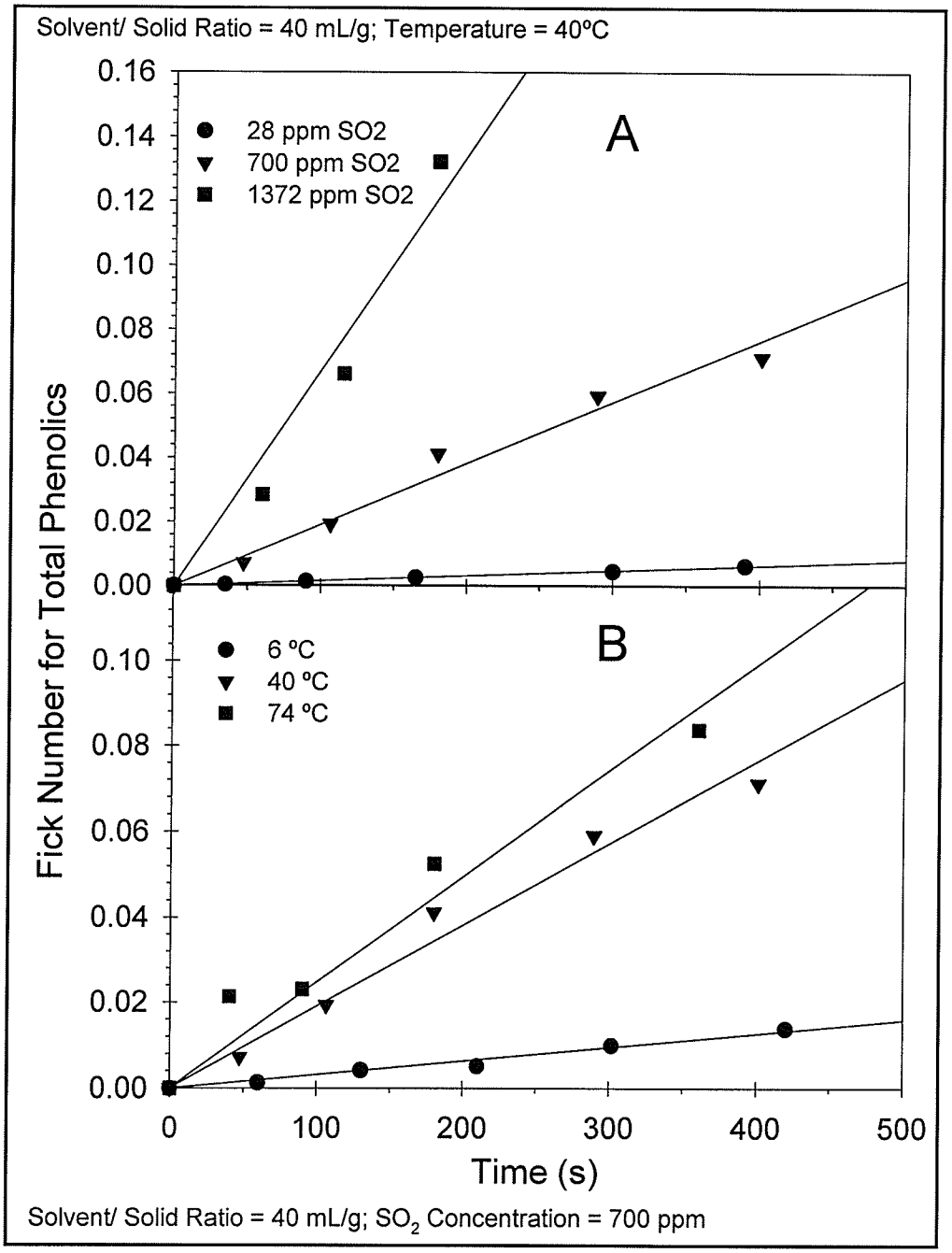


Figure 34. Total Phenolic Fick Number for the effects of A) SO_2 Concentration at Constant Temperature of 40 °C and B) Temperature at Constant 700 % SO_2 Concentration for Black Currant Extraction at a Solvent to Solid Ratio of 40 mL/g dwb of Frozen Berries

anthocyanin diffusivity. Most anthocyanins diffusivities ranged from 4 to 70 10^{-11} $m^2 \cdot s^{-1}$ and total phenolics diffusivities varied from 4 to 260 10^{-11} $m^2 \cdot s^{-1}$.

The power delivered by the agitator was obtained from empirical plots that correlate the power number and Reynolds number (Charm, 1963) using the Reynolds number of the extractions 15 to 18 (center point), type of impeller and characteristic shape factors of the experiment. Mass transfer coefficients were calculated from equation (18) (Loncin and Merson, 1979). For an axial turbine with 4 blades at 45° angle and Reynolds number of the center point extraction (120,000), the power of the agitator was 4.3 W. The power uptake by the motor depends on the resistance by the fluid-impeller system. A low viscosity solvent such as the used SO_2-containing water provided very low resistance to the impeller. The power consumption calculated by measuring the voltage and current used by the agitator-vessel system operated with water at 1210 rpm was 3.84 W. Thus, mass transfer coefficients for anthocyanins and total phenolics from the extraction using 40 mL/g of 700 ppm SO_2 at 40°C, calculated with a power of 4.3 W, were 3.85 10^{-5} and 6.27 10^{-5} ms^{-1}.

f. Mass Balance of Extraction. A scheme of the extractor and mass flow diagram of the components involved in the extraction were displayed above (Figure 23). Phenolics weight fraction of extracts (y_e) and phenolics weight fraction of black currant pomace (x_m) were measured by spectrophotometric analysis. Weight of berry feeds, volume and density of extract and dry matter content and weight of wet pomace were also measured. Extract yields and pomace loss for total phenolics and anthocyanins were calculated using equations 24 and 25, respectively (Table 21).

Total phenolic yields were very low (ranged from 27 to 79 %), because they were calculated using overestimated total phenolic content of berries measured by the equilibrium

method with SO_2-containing water. Anthocyanin yields however, were high with values around 85-90 % (ranged from 66 to 94 %). This indicates good efficiency of the extraction condition for the production of anthocyanin-rich extracts.

TABLE 21. Total Phenolic and Anthocyanin Extract Mass Yields and Pomace Mass Loss, Total Mass Loss, Dry Matter Content of Extract and Total Solids Extracted from Black Currants using SO_2-containing Water Solvent

Run	Total Phenolics [a]		Anthocyanins [b]		Total Loss (g)[e]	Total Loss (%)[f]	Extract DM (%)	Extract Solid (g) [g]	Extract Solid (%) [h]
	extract yield [c]	pomace loss [d]	extract yield	pomace loss					
1	30	7.7	83	10.8	153	5.0	2.9	73.5	13.8
2	40	6.3	91	7.2	271	9.0	3.14	79.4	15.1
3	42	6.4	86	4.4	116	4.3	1.09	26.3	14.9
4	79	7.4	92	3.5	106	4.0	1.06	26	14.7
5	30	7.1	78	10.4	269	8.9	3.17	77.9	14.7
6	43	6.3	91	8.8	228	7.5	3.14	78.8	14.8
7	36	7.0	89	6.3	229	8.6	1.16	26.5	14.9
8	52	3.4	94	3.9	384	14.3	1.35	29.2	16.4
9	53	8.8	92	7.1	122	4.4	1.56	38.5	14.4
10	27	2.7	66	5.1	232	8.4	1.84	43.9	16.5
11	29	9.7	79	17.9	197	4.6	7.0	222.1	12.6
12	53	3.3	91	5.2	132	5.0	0.9	21.2	14.7
13	29	8.4	79	11.6	196	7.1	1.67	39.5	14.8
14	50	7.9	86	8.5	152	5.5	1.57	36.4	13.7
15	47	9.3	89	7.9	158	5.7	1.62	38.5	14.4
16	39	6.1	82	8.1	133	4.8	1.6	38.2	14.4
17	43	9.8	87	7.0	156	5.6	1.6	37.8	14.2
18	43	7.5	93	6.8	188	6.8	1.47	34.9	13.2

[a b] Phenolics expressed in weight percent of the feed weight as equivalents of: [a] chlorogenic acid; [b] cyanidin 3-glucoside; [c] Phenolic weight of the extract in percent of the feed weight; [d] Phenolics weight lost in the pomace in percent of the feed weight; [e,f] Total mass loss expressed as [e] weight in grams or [f] percentage of the total initial weight; [g,h] Total solids expressed as [g] weight in grams or [h] percentage of the initial berry weight

Total loss was calculated from a total mass balance (equation 27) and expressed as weight, W_l and as percentage of the initial mass (Table 21). Dry matter contents of the extracts were calculated from a solids mass balance (equation 26) and the total solids in the extract were calculated from equation 28, using the volume and density of extracts. Production of total solids varied from values as low as 22g to a very high solid weight of 222g with the conditions employed for the extractions. Total solids extracted with SO_2-containing water were higher than solids obtained with aqueous ethanol. This might be due to extraction of non-phenolic water soluble compounds. Higher purity of pigments extracted with alcohols and reduced purity when pigments were extracted with water have been reported (Pifferi and Vaccari, 1983). Extract dry matter content and total solids were highly correlated with the S/S (-0.824) and berry feed weight (0.982). Apart from the effect that S/S and berry feed may have had on the extraction, S/S yielded extracts with different concentrations which determined the solid content of the extract because more concentrated extracts contain higher solids. Total solid expressed as percentage of the feed (SE) gives a more accurate indication of the effect of S/S on the extraction of solids. The percentage of solids, SE increased with the S/S and the slope of increase was steeper at high temperature. A reduced solubility at low temperature may have been a limiting factor.

Optimization of extraction from a technical point of view means the selection of the best conditions for reaching a goal. For the production of dry solid, it would be convenient to maximize the total solid content. Thus, extraction with a S/S of 6 mL/g would be recommended, provided it gave the highest dry matter content and total solid of extract (Table 21). However, if a maximum recovery of phenolics is pursued this extraction would not be recommended because it had a high pomace loss of total phenolics and anthocyanins

indicating the extraction was deficient. Anthocyanin yields higher than 90 % were obtained with several treatments. Sulfur dioxide concentration improved the extraction of total phenolics. However, high temperature treatments might have impaired the beneficial effect of low to mild SO_2 concentration by elimination of this compound. The extraction at 20 °C using 60 mL/g of 1100 ppm SO_2 water produced high yields and the lowest total loss.

C. Pre-Extraction Treatments

1. Effects of Temperature and Type of Berries

Pre-extraction treatments are intended to improve extraction of desired bioactives by facilitating the access of extraction solvent to the tissue. The use of high temperature as a preparative step for the extraction may reduce the viscosity of fruit juices and favor the break down of membranes. Thus, the use of high temperature (50 °C versus 20 °C) had a positive effect on the cell wall break down of black currant pomace (Landbo and Meyer, 2001). Increasing temperature of the pre-extraction step increased yield and recovery of total phenolics. A 60 °C temperature was significantly better than 30°C (Table 22). Hydroxycinnamic acids form part of the structure of various polymers of the cell wall as precursors of lignin monomers, or esterified with lignin or with cell-wall polysaccharides (Macheix et al., 1990; Meyer et al., 1998). It may be possible that a 60 °C temperature had caused cell-wall to break down resulting in a higher hydroxycinnamic acid derivative extraction. Pre-extraction temperature did not affect significantly the antioxidant activity of extracts.

TABLE 22. Effect of Temperature Pre-Extraction Treatment on Total Phenolic, Tartaric Ester, and Flavonol Yield, Recovery and Antiradical Activity of Black Currant, Blackberry and Saskatoon Berry Extracts using 60 mL/g of 1100 ppm SO_2 water at 25 °C

Pre-Extraction Temp. (°C)	Yield [a]			Recovery [e]			Antirad Activity
	Total Phen	Tart Esters	Flav	Total Phen	Tart Esters	Flav	
30	53.9 [b] b[g]	2.80 [c] a	2.03[d] a	63 b	82 a	92	- 3.91[f] a
45	55.9 ab	2.84 a	2.06 a	66 ab	83 a	94	- 3.88 a
60	57.4 a	2.95 a	2.16 a	68 a	86 a	99	- 4.06 a
R^2	0.63	0.99	0.83	0.55	0.84	NS	0.58
Effects	** [h]	NS [i]	NS	**	NS		NS

[a] Phenolics yields in mg /g of frozen berries on a dwb expressed as equivalents of: [b] total phenolics as chlorogenic acid; [c] tartaric esters as caffeic acid ; [d] flavonols as quercetin; [e] Phenolics recoveries in percentage of phenolics content of samples; [f] Antiradical activity as slope coefficient calculated by linear regression in μMDPPH / μM of antioxidant, expressed in total phenolics as chlorogenic acid, [g] Means (n=9) with the same letter do not differ significantly (p>0.05); [h] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level; [i] NS Treatment effect or model is non significant (p> 0.1)

Total phenolic yield was higher for blackberries and black currant than for saskatoon; however, total phenolic recovery from blackberries was higher than from black currants (Table 23). Tartaric ester and flavonol yields were different for all three berries studied, and yield from saskatoon was the highest followed by yield from black currants. Antioxidant activity of saskatoon berry extracts was also significantly higher than antioxidant activity for the other two berries. Effects of berries on tartaric esters, flavonols and antioxidant activity may be attributed to the higher content of tartaric esters and flavonols in saskatoon berries (Table 6) according with reported composition of berries (Fukumoto and Mazza, 2000; Wang and Mazza, 2002).

TABLE 23. Effect of the Type of Berry on Total Phenolic, Tartaric Ester, and Flavonol Yield, Recovery, and Antiradical Activity of Blackberry, Black Currant and Saskatoon Berry Extracts using 60 mL/g of 1100 ppm SO_2 water at 25 °C

Berry	Yield [a]			Recovery [e]			Antirad Activity
	Total Phen	Tart Esters	Flav	Total Phen	Tart Esters	Flav	
blackberry	58.0[b] a[g]	1.72[c] c	1.53[d] c	68 a	75 b	92	-3.71[f] a
black currant	57.0 a	2.46 b	1.99 b	64 b	89 a	103	-3.74 a
saskatoon	53.3 b	4.48 a	2.80 a	66 ab	89 a	93	-4.46 b
R^2	0.61	0.99	0.83	0.55	0.84	NS [i]	0.58
Effects	*** [h]	***	***	**	***		***

[a] Phenolics yields in mg /g of frozen berries on a dwb expressed as equivalents of: [b] total phenolics as chlorogenic acid; [c] tartaric esters as caffeic acid ; [d] flavonols as quercetin; [e] Phenolics recoveries in percentage of phenolics content of samples; [f] Antiradical activity as slope coefficient calculated by linear regression in µMDPPH / µM of antioxidant, expressed in total phenolics as chlorogenic acid, [g] Means (n=9) with the same letter do not differ significantly (p>0.05); [h] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level; [i] NS Treatment effect or model is non significant (p> 0.1)

There was a significant interaction between temperature and the type of berries on anthocyanin yield and recovery (data not shown). Thus, temperatures of 30 °C for saskatoon berries, 30 and 45°C for blackberries and 60 °C for black currant led to highest yield and recovery (Table 24). At all temperatures, blackberries and black currants had similar anthocyanin yields which were higher than yields from saskatoon. Most probably the effect of berries on total phenolic and anthocyanin yields is related to differences in content of these compounds in the berries. Thus, black currant and blackberries had higher anthocyanin content than saskatoon berries (Table 6). At 30, 45 and 60 °C pre-extraction temperatures, anthocyanin recoveries from blackberries were higher than those from black currants which, in turn, were higher than recoveries from saskatoon. There was an important reduction of

recovery from saskatoon using pre-extraction temperatures higher than 30 °C (Figure 35). It seems that saskatoon berries are more susceptible than the other two berries to high temperature. It is possible that cell wall material released with high temperature pretreatment decreased the extraction of anthocyanins by increasing extraction medium viscosity.

TABLE 24. Interaction Effect of the Type of Berry and Temperature of Pre-Extraction Treatment on Yield and Recovery of Anthocyanins from Black Currant, Blackberry, and Saskatoon Berry Extractions using 60 mL/g of 1100 ppm SO_2 water at 25 °C

Berry	Temp. °C	Yield [a]	Recovery [b]	Temp. °C	Berry	Yield	Recovery
blackberry	30	12.5 a[c]	92 a	30	Bb [d]	12.5 a	92 a
	45	12.5 a	93 a		Bc	11.8 a	77 b
	60	12.3 a	91 b		S	5.90 b	73 c
black currant	30	11.8 a	77 b	45	Bb	12.5 a	93 a
	45	11.8 a	77 b		Bc	11.8 a	77 b
	60	12.1 a	79 a		S	4.66 b	57 c
saskatoon	30	5.90 a	73 a	60	Bb	12.3 a	91 a
	45	4.65 b	57 c		Bc	12.1 a	79 b
	60	4.78 b	59 b		S	4.78 b	59 c

[a] Anthocyanin yields in mg /g of frozen berries on a dwb expressed as equivalents of:cyanidin 3-glucoside; [b] Anthocyanin recoveries in percentage of anthocyanin content of samples; [b] Means (n=3) with the same letter do not differ significantly ($p>0.05$); Bb blackberries, Bc black Currants, S Saskatoon berries

Noted similarities of black currant and saskatoon and differences with blackberries on recoveries may be consequence of structural characteristics of the berries. Anthocyanins of black currant and saskatoon are in the peel. The skin of black currants contains at least 2% of pigments on a fresh mass basis (Mazza and Miniati, 1993; Landbo and Meyer, 2001). However, blackberry fruits are formed with many small closely packed druplets (Bailey, 1942) and more uniformly distributed pigments.

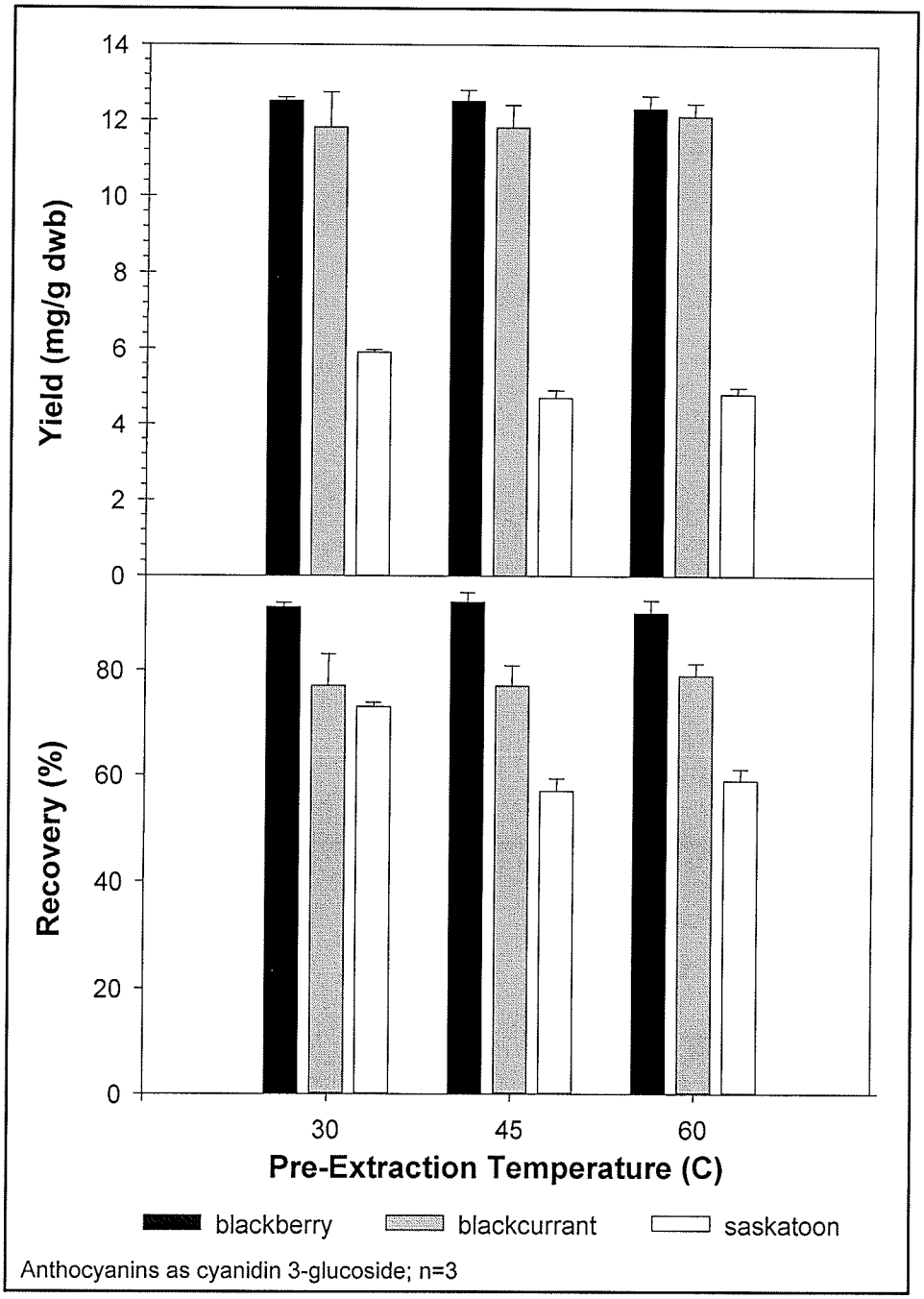


Figure 35. Anthocyanin Yields in mg/g dwb of Frozen Berries as Cyanidin 3-glucoside Equivalents and Recoveries in Percentage of Sample Content for the Effect of Pre-Extraction Temperature in Black Currants, Blackberries and Saskatoon Berries

2. Effect of Enzymes

Enzymatic treatment of berries before the extraction with SO_2-containing water did not significantly improve extraction of phenolics compounds (Table 25). None of the variables studied, phenolic yields, recoveries, or antiradical activities of extracts was significantly affected by the use of enzymes in the pre-extraction step. However, all the variables increased with use of enzymatic pre-extraction treatment.

TABLE 25. Effect of Enzymatic Pre-Extraction Treatment on Yield and Recovery of Total Phenolics, Tartaric Esters, Flavonols and Anthocyanins and Antioxidant Activity of Blackberry, Black Currant and Saskatoon Berry Extractions using 60 mL/g of 1100 ppm SO_2 water at 25 °C

Pre-Extraction Treatment	Yield [a]				Recovery [f]				Antirad Activity
	Total Phen	Tart Ester	Flav	ACY	Total Phen	Tart Esters	Flav	ACY	
no enzymes	55.9[b]a[h]	2.84[c]a	2.06[d] a	9.67[e] a	66 a	83 a	94	76 a	-3.88 [g] a
enzymes	57.3 a	2.95 a	2.17 a	9.80 a	67 a	86 a	100	77 a	-4.03 a
R^2	0.63	0.99	0.83	0.99	0.55	0.84	NS	0.97	0.58
Effects	NS [i]	NS [i]	NS	NS	NS	NS		NS	NS

[a] Phenolics yields in mg /g of frozen berries on a dwb expressed as equivalents of: [b] total phenolics as chlorogenic acid; [c] tartaric esters as caffeic acid ; [d] flavonols as quercetin; [e] ACY anthocyanins as cyanidin 3-glucoside; [f] Recoveries calculated as percentage of phenolics content of samples; [g] Antiradical activity as slope coefficient calculated by linear regression in μMDPPH / μM of antioxidant, expressed in total phenolics as chlorogenic acid, [h] Means with the same letter do not differ significantly ($p>0.05$); [i] NS Treatment effect or model is non significant ($p> 0.1$)

Four out of five pectinase enzymes at a doses from 0 to 10 % enzyme to substrate ratio enhanced the amount of total phenolics extracted, but two enzyme treatments decreased and other two had no effect on anthocyanins extracted from black currant pomace (Landbo and Meyer, 2001) and only one of two enzyme treatments had a positive effect on total phenolics extraction from grape pomace (Meyer et al., 1998). Degradation of anthocyanins

is a recognized adverse side effect of enzyme treatment of fruit. It may be caused by β-glucosidase or β-galactosidase activity in non-specific enzyme preparations. Rapidase® Super BE had neither a positive nor a negative effect on anthocyanins extraction. This is in agreement with reports of no glycosidase activity and no anthocyanin degradation associated with the same enzyme (Skrede et al., 2000).

Low enzymatic activity may have been affected by the formation of cell wall material resistant to chemical and biological degradation and the shielding of cell wall substrates to the accessibility of hydrolytic enzymes by lignin and low molecular weight phenolic compounds (Meyer et al., 1998). Besides, enzymes are useful for recovering compounds remaining in the fruit pomace after juice production or from the wine industry. However, the relative improvement achieved in the solvent extraction of berries may not be sufficiently large to give a significant increase in comparison with the amount of phenolics recovered from unpressed fruits. In plant cells, phenolic compounds accumulate in the vacuoles or in the cell wall. Soluble phenolic compounds present in the vacuoles of the cells may be extracted easily without additional help. The amount of phenolic compounds retained in the cell walls that might be savaged by the effect of the enzymatic pre-extraction step would be comparatively small.

D. Purification of Extracts

1. Effect of Methods

Extracts from the purification method using an adsorption column were evaluated before and after concentration in a rotary evaporator. In this way, the purification by adsorption was evaluated as two methods. One method whose final product was the extract

collected at the end of the column referred to as adsorption purification (ADS), the second method referred as adsorption/concentration(ADS/CONC), obtained as final product from the concentrated extract, this means the extract obtained from the rotary evaporator. The third purification method was ultrafiltration which purified and concentrated the extracts in one operation without using heat or thermal treatment.

The effect of purification methods on phenolic yields and recoveries are presented in Table 26. Ultrafiltration had a higher yield and recovery of total phenolics and anthocyanins and a lower recovery and yield of tartaric esters than adsorption methods. The UF process lost some compounds through the membrane into the permeate. Phenolic acids present in black currant are mainly hydroxycinnamic acid derivatives and secondly derivatives of p-hydroxybenzoic, protocatechuic and gallic acids (Schuster and Herrmann, 1985; Herrmann, 1989; Häkkinen et al.,1999a). As an example the molecular weight of p-coumaric acid ($C_9H_8O_3$) is 166.15 (Windholz, 1976). Compounds measured as tartaric esters have smaller molecular weight than flavonols (300) or anthocyanins (450- 700). Some phenolic acids are also measured as total phenolics at 280 nm; however, the major fraction at 280 nm is still anthocyanins. The membrane used (Amicon YC05, Millipore Corporation, Bedford, MA) had 500 MW cut off and thus it is likely that some of the smaller molecular weight phenolic acids passed through the membrane. Thus, lower yields and recoveries of tartaric esters were obtained using UF.

TABLE 26. Effect of Purification Methods on Yield and Recovery of Total Phenolics, Tartaric Esters, Flavonols and Anthocyanins and Antioxidant Activity of Black Currant Extracts using 60 mL/g of 1100 ppm SO_2 water at 25 °C

Method	Yield [a]				Recovery [f]				
	Total Phen	Tart Ester	Flav	ACY	Total Phen	Tart Esters	Flav	ACY	Antirad Activity
UF [k]	29.6[b] a[h]	1.52[c] b	1.47[d] b	11.8[e] a	57 a	69 c	86 b	94 a	-3.63[g] b
ADS	27.1 b	1.93 a	1.51 b	11.4 b	57 a	86 b	85 b	90 b	-2.22 a
ADS/CONC	25.5 b	2.03 a	1.69 a	11.3 b	54 a	91 a	95 a	90 b	-2.55 a
R^2	0.76	0.92	0.80	0.74	0.83	0.93	0.81	0.74	0.91
Effects	*** [i]	***	***	**	NS [j]	***	***	***	***

[a] Phenolics yields in mg /g of frozen berries on a dwb expressed as equivalents of: [b] total phenolics as chlorogenic acid; [c] tartaric esters as caffeic acid ; [d] flavonols as quercetin; [e] ACY anthocyanins as cyanidin 3-glucoside; [f] Recoveries calculated as percentage of phenolics content of samples; [g] Antiradical activity as slope coefficient calculated by linear regression in μMDPPH / μM of antioxidant, expressed in total phenolics as chlorogenic acid, [h] Means with the same letter do not differ significantly ($p>0.05$); [i] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level; [j] NS Treatment effect is non significant ($p> 0.05$); [k] UF Ultrafiltration, ADS adsorption column, ADS/ CONC Adsorption followed by concentration

Anthocyanins form intermolecular association with other flavonoids, polyphenols, organic acids, other compounds and with other anthocyanins (Mazza and Miniati, 1993). Flavonols are one of the most efficient copigments so that they would also have less chance to pass through the membrane. The polymeric nature of the pigments may be responsible for a very high recovery with membrane cut-off close to the molecular weight of anthocyanins and flavonols. Recoveries of flavonols and anthocyanins were 86 and 94 % respectively, and this shows that a very high proportion of both classes of flavonoids present in the sample were recovered after purification (Table 26). Loss of pigment from commercial grape extracts to the permeate were less than 10 % using a membrane with 10,000 cut-off and feed concentration higher than 10% (Lin and Hilton, 1980). Preliminary experiments with 10,000

and 1,000 cut-off membranes (Amicon PM 10 and YM 1, Millipore Corporation, Bedford, MA) and a feed of 0.02 % anthocyanin concentration, gave anthocyanin recoveries approximately of 51 and 71 %, respectively. Copigmentation did not have any effect on retention of anthocyanins at the very low concentration range of the extracts used so membranes with a lower cut-off had to be used.

The use of a membrane with lower MW cut-off did however, reduce the flux rate of permeate. Preliminary experiments gave a flux rate of 4.8 to 1.0 and 0.7 mL cm^{-2} h^{-1} for membranes with 10,000, 1,000 and 500 MW cut-off, respectively (data not shown). Permeation rates lower than 1.0 mL cm^{-2} h^{-1} were reported for membranes with small (500 to 5,000) molecular weight cut-off (Lin and Hilton, 1980). An UF procedure tried on the preliminary experiments that used 100,000 and 500 MW cut-off membranes (Amicon YM 100 and YC05, Millipore Corporation, Bedford, MA) in two successive steps yielded an anthocyanin recovery equivalent to that of the 500 MW cut-off membrane used alone (data not shown). The flux for the process combining membranes was approximately 1.0 mL cm^{-2} h^{-1}, similar to the flux obtained with a 1,000 MW cut-off membrane in a single step. Removal of large molecular weight compounds such as proteins or pectin material with the retentate in the first UF step and retention of most phenolic compounds in the second step improved UF flux and assured a high anthocyanin recovery. However, because of the increased time and cost of operation needed to run this dual membrane, the process may not be viable.

Purification using the adsorption column retained more phenolic acids than UF where some of those compounds passed through the membrane. Tartaric ester recovery of adsorption was close to 90% in comparison with UF recovery of 69% (Table 26). On the other hand, yield and recovery of anthocyanins were lower than UF, but the values were still

high (11.3 mg/g and 90 %). The intermolecular forces primarily responsible for chromatographic adsorption are: van der Waals' forces, electrostatic forces, hydrogen bonds and hydrophobic interactions (Nielsen, 1994). Amberlite® XAD-16 is a nonionic, hydrophobic polymer which adsorbs polyphenols by van der Waal's forces between two aromatic rings (Gump and Huang, 1999). Hydroxycinnamic and hydroxybenzoic acids and their derivatives have a polar carboxylic group capable of forming hydrogens bonds, and they also have a hydrophobic aromatic ring that can attach to the resin by hydrophobic van der Waals' interactions.

Apart from the desired adsorption to the column for purification, anthocyanins may be adsorbed too strongly by the resin, so that pigments might have been retained even after elution with the acidified ethanol, thus reducing the recovery. Incomplete elution of grape pigment from columns with styrene/divinylbenzene and phenolic based resins has been reported by Lin and Hilton (1980). Nevertheless, anthocyanin recovery for adsorption (ADS) and adsorption concentration (ADS/CONC) purification techniques in this research were very high (90 %). In agreement with this result recoveries of anthocyanins from Amberlite® XAD-16 greater than 90 % were found when the concentration of acetic acid in the ethanol elution solvent was lower than 5% (Yu, 1998).

Compositional changes of extracts obtained by application of the three purification procedures resulted in different antiradical activity measured by the DPPH method. Thus, the UF technique led to extracts with higher antiradical activity (Table 26). This effect may be attributed to richer anthocyanin extracts obtained by UF. Antiradical activity of UF purified extracts (-3.65 μM DPPH/μM) was similar to unpurified samples; thus the UF purification method did not reduce antioxidant properties of the extracts. Furthermore, antiradical activity

of UF purified phenolic-rich berry extracts were higher than the activities of commercial antioxidants such as α-tocopherol (-1.15 μM DPPH/μM) and BHT (-2.61 μM DPPH/μM) used as reference.

Varied antiradical activity is related to differences in composition of extracts. As noted previously, UF had significantly higher antiradical activity than extracts from adsorption purification. In order to investigate compositional differences that might have caused a higher antiradical activity, total phenolic, tartaric ester, flavonol and anthocyanin concentrations of extracts from the three purification methods were measured by HPLC. The change in the phenolic profile of purified extracts was confirmed by HPLC analysis. In order to describe the composition profile of extracts, percentage of each of the four groups of compounds were calculated in relation to the total amount of phenolics obtained by adding measured HPLC total phenolics, flavonols, tartaric esters and anthocyanins. Percentage of each fraction of the extracts from UF, adsorption and adsorption-concentration were calculated and plotted (Figure 36). In agreement with spectrophotometric measurements, tartaric esters were higher in extracts purified by any of the adsorption methods and anthocyanins were higher in extracts from UF than in those from adsorption. Thus, the purification method changed the phenolic profile of the purified extracts.

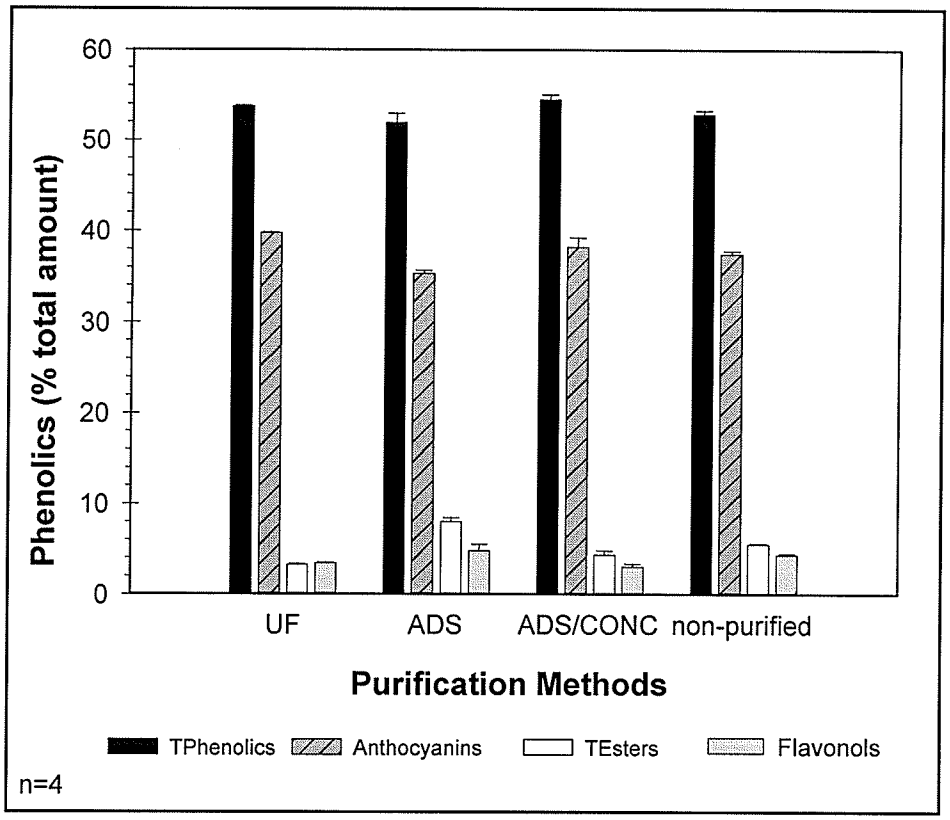


Figure 36. Total Phenolic, Tartaric Ester, Flavonol and Anthocyanin Contents as Weight Percentages of the Total Amount of Phenolic Compounds in Ultrafiltration (UF), Adsorption Column (ADS), Adsorption Column Followed by Concentration (ADS/CONC) and Non Purified Black Currant Extracts

2. Effect of Pre-Extraction Treatments

Pre-extraction treatments are preparative steps to facilitate removal of desired compounds from the tissue. Treatment of black currants using high pre-extraction temperature affected the purification yield and recovery of phenolics (Table 27). Higher recoveries were obtained using pre-extraction treatment at 30 °C than at 60 °C for all of the four classes of phenolics measured. Intermediate treatment at 45 °C was equal to that at 30 °C for flavonols and anthocyanins and equal to that at 60 °C for tartaric esters and total phenolics.

TABLE 27. Effect of Pre-Extraction Temperature on Yield and Recovery of Total Phenolics, Tartaric Esters, Flavonols and Anthocyanins and Antioxidant Activity of Purified Black Currant Extracts using 60 mL/g of 1100 ppm SO_2 water at 25 °C

Pre-Extraction Treatment	Yield [a]				Recovery [f]				Antirad Activity
	Total Phen	Tart. Ester	Flav	ACY	Total Phen	Tart Esters	Flav	ACY	
30	27.8[b]a[h]	1.79[c] a	1.53[d] a	11.7[e] a	62 a	85 a	91 a	92 a	- 2.97[g] a
45	25.6 b	1.74 a	1.51 a	11.0 b	51 b	79 b	91 a	93 a	- 3.17 a
60	28.5 a	1.87 a	1.58 a	11.6 a	55 b	79 b	84 b	89 b	- 2.88 a
R^2	0.76	0.92	0.80	0.74	0.83	0.93	0.81	0.74	0.96
Effects	* [i]	NS [j]	NS	***	***	**	**	**	NS

[a] Phenolics yields in mg /g of frozen berries on a dwb expressed as equivalents of: [b] total phenolics as chlorogenic acid; [c] tartaric esters as caffeic acid ; [d] flavonols as quercetin; [e] ACY anthocyanins as cyanidin 3-glucoside; [f] Recoveries calculated as percentage of phenolics content of samples; [g] Antiradical activity as slope coefficient calculated by linear regression in μMDPPH / μM of antioxidant, expressed in total phenolics as chlorogenic acid, [h] Means (n=3) with the same letter do not differ significantly ($p>0.05$); [i] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level; [j] NS Treatment effect or model is non significant ($p> 0.1$)

The use of enzymes as a pre-treatment affected phenolics purification yields and recoveries. Higher yields of tartaric esters, flavonols and anthocyanins were obtained when a pre-extraction treatment with enzymes had been used (Table 28). Pectolytic enzymes significantly increase black currant pomace cell wall breakdown by acting on pectic substances by either depolymerizing or deesterifying the substrate (Landbo and Meyer, 2001). This action may facilitate removal of substances interfering in the process of adsorption-desorption or diffusion through the ultrafiltration membrane resulting in a more efficient purification processes. Thus, lignin is composed of hydrophobic polymers derived from p-coumaric, coniferyl, and sinapyl alcohols. The hydrophobicity of lignin matrix strengthens the hydrogen bonds between adjacent polysaccharides (Meyer et al., 1998) and may interfere in the adsorption of phenolics. Besides, the use of enzymes reduces the viscosity of fruit juices by depolymerizing the juice pectin (Kilara and Van Buren, 1989). This reduction of viscosity increases the diffusivity of phenolics and may improve purification yield. Compositional changes due to either enzyme pre-extraction treatment or to temperature pre-extraction treatment did not affect the antiradical activity of purified extracts (Table 27 and 28).

TABLE 28. Effect of Enzymatic Pre-Extraction Treatment on Yield and Recovery of Total Phenolics, Tartaric Esters, Flavonols and Anthocyanins and Antioxidant Activity of Purified Black Currant Extracts using 60 mL/g of 1100 ppm SO_2 water at 25 °C

Pre-Extraction Treatment	Yield [a]				Recovery [f]				
	Total Phen [b]	Tart Ester [c]	Flav [d]	ACY [e]	Total Phen	Tart Esters	Flav	ACY	Antirad Activity [g]
no enzymes	25.6 a [h]	1.74 b	1.51 b	11.0 b	51 b	79 b	91 a	93 a	-3.17 a
enzymes	27.7 a	1.90 a	1.61 a	11.8 a	56 a	85 a	90 a	91 a	-3.16 a
R^2	0.76	0.92	0.80	0.74	0.83	0.93	0.81	0.74	0.91
Effects	NS [j]	** [i]	**	***	**	**	NS	NS	NS

[a] Phenolics yields in mg /g of frozen berries on a dwb expressed as equivalents of: [b] total phenolics as chlorogenic acid; [c] tartaric esters as caffeic acid ; [d] flavonols as quercetin; [e] ACY anthocyanins as cyanidin 3-glucoside; [f] Recoveries calculated as percentage of phenolics content of samples; [g] Antiradical activity as slope coefficient calculated by linear regression in μMDPPH / μM of antioxidant, expressed in total phenolics as chlorogenic acid, [h] Means (n=3) with the same letter do not differ significantly ($p>0.05$); [i] *** Significant at 0.01 level, ** significant at 0.05 level, * significant at 0.1 level; [j] NS model is non significant ($p> 0.1$)

V. SUMMARY AND CONCLUSIONS

Extraction of phenolic compounds from milled frozen black currants using aqueous ethanol and SO_2 solvents was optimized. Surface response methodology was used to optimize total phenolic, anthocyanin, tartaric ester, and flavonol yields, and antioxidant activity of extracts obtained by modifying the solvent concentration, temperature, and solvent to solid ratio. Four pre-extraction treatments and two purification methods were also tested. The following conclusions can be drawn:

1. Milling of frozen berries with a Wiley mill resulted in a narrow distribution and average particle sizes of 1.6 mm, 1.7 mm and 1.8 mm for black currants, blackberries and saskatoon berries, respectively.

2. Solvent type and concentration affected extraction of phenolics from berries and both aqueous ethanol and sulfurous water were effective solvents. Improved extraction with both solvents was achieved by increased solubility and an enhanced diffusion coefficient of phenolic compounds.

3. Optimal ethanol concentration varied for tartaric esters, flavonols and anthocyanins. Ethanol concentration affected the diffusion coefficient and yield of total phenolics which were maximized at 60 % ethanol. SO_2 concentration increased yield and diffusion coefficients in the whole range studied and a maximum yield was obtained at 1,000-1,200 ppm SO_2.

4. Higher solvent to solid ratio increased the diffusion rate of phenolics by increasing the concentration gradient and diffusion coefficients with both ethanol and sulfurous water solvents. Higher solvent to solid ratio led to higher anthocyanin and total phenolic yields with a maximum at 19 L/kg of frozen berries.

5. Temperature reduced the time to reach equilibrium by increasing diffusivities of anthocyanins and total phenolics. Minimum times for anthocyanin extractions were obtained with 60-75 % ethanol and 60-70 °C (10 min) and with 900 ppm SO_2 and 60- 65°C (28 min). Temperatures higher than 30-35°C resulted in degradation of anthocyanins and reduction of yields.

6. Variation in composition of ethanolic extracts was not sufficiently large to affect antiradical activity; however, in SO_2-water extracts, the highest antiradical activity was obtained using a combination of high solvent to solid ratio and temperature. In both ethanol and SO_2-water extractions, the use of high solvent to solid ratio resulted in more diluted extracts which had lower antioxidant indices.

7. Increase of pre-extraction temperature from 30 to 60 °C increased yield and recovery of total phenolics, but there was no effect of enzymatic treatment.

8. Purification methods affected the phenolic profile of extracts. Purification of extracts using ultrafiltration led to a higher yield and recovery of total phenolics and anthocyanins which result in extracts with higher antiradical activity. All purification methods showed anthocyanin recoveries higher than 90%.

In conclusion, solvent type and concentration influenced the extraction of phenolics from berries. The solvent to solid ratio and temperature also affected the extraction and antioxidant activity of extracts. Extraction conditions must be selected carefully in order to

optimize the process. Information reported in this research provides data for a better selection of process conditions for the extraction of phenolics to meet the requirements of most processing objectives. Solid-liquid extraction in an agitated vessel was an efficient method of processing with high yield and short time of extraction that can be successfully scaled up. Sulfurous water was as efficient as ethanol and thus, it may be used as an alternative, less expensive solvent.

Future research could be extended to studies of other solvents such as acetone and processing methods such as supercritical CO_2 and superheated water extractions.

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APPENDIX I

Experimental Determination of Diffusivity

1. Diffusion for Solid-Liquid Extraction in Agitated Vessels

Diffusion due to random molecular motion dominates in the leaching of foods and the diffusion in the solid is usually rate-controlling. The rate of diffusion of that step can be described by Fick's second law:

$$\frac{\delta C}{\delta t} = D \frac{\delta^2 C}{\delta x^2} \qquad (1)$$

where C is the concentration of the solute, t is time, s, D is the diffusion coefficient or diffusivity, $m^2 s^{-1}$, and x is the distance of diffusion, m. The driving force for the extraction is the concentration gradient within the particles.

Various solutions of the Fick's second law have been presented (Carman and Haul, 1954; Crank, 1975; Schwartzberg and Chao, 1982) for the diffusion of compound during solid-liquid extraction. Solutions of Fick's second law are used to determine D_S assuming that D_S is constant; $C = C_b$ for $0 < x < a$ when $t = 0$; and negligible external resistance $x_{dm} = y_e / m$ at $x = a$ for $t > 0$. The most commonly used solution of the Fick's second law is a series solution (Carman and Haul 1954; Crank, 1975; Schwartzberg and Chao, 1982) which are expressed in general form by the following equation

$$Y = \sum_{n=1}^{\infty} C_n \exp\left[-q_n^2 . \tau\right] \qquad (2)$$

where $\tau = \frac{D_s\, t}{a^2}$ is the Fick's number and (3)

Y is the dimensionless extract concentration, $\mathbf{q_n}$ are a function of the boundary conditions and $\mathbf{C_n}$ are a function of the boundary conditions and the initial conditions. $\mathbf{C_n}$ and $\mathbf{q_n}$ are presented by Carman and Haul (1954) and Crank (1975) for values of $\boldsymbol{\alpha}$ corresponding to several values of final fractional uptake. Equations for eigenvalues and series coefficients can be obtained from Crank (1975), Schwartzberg and Chao (1982) and Schwartzberg (1987). Equation (2) is valid for all values of the Fick's number ($\boldsymbol{\tau}$), but for small values of $\boldsymbol{\tau}$, more terms of the series have to be considered to have satisfactory accuracy. For a large number of cases in which τ is large enough, only the first term of the series is needed and equation can be used to calculate the diffusivity. Values of Fick's number ($\boldsymbol{\tau}$), α and y ranges in which various solutions of the differential Fick equation yield less than 1% error are reported by Schwartzberg and Chao (1982).

When τ is small the number of terms to be considered in the equation makes the solution too complicated and it is preferable to use an alternative solution applicable to large values of Y, i.e. for small times (Carman and Haul, 1954; Schwartzberg and Chao, 1982)

$$(1 - Y) = \left(1 + \alpha\right)\left[F\phi - G\phi^2 + H\phi^3\right] \qquad (4)$$

where $\phi = \frac{\sqrt{\tau}}{\alpha}$ and coefficients depend on geometries; for a sphere, short contact times, and finite values of $\boldsymbol{\alpha}$,

$$F = \frac{6}{\sqrt{\pi}} \; ; \; G = -3(3+\alpha) \; ; \text{ and } \; H = \frac{12(3+2\alpha)}{\sqrt{\pi}} \qquad (5)$$

For infinite values of α and moderately short times, the equation for a sphere shape (Schwartzberg and Chao, 1982) is

$$1 - y = 6\sqrt{\frac{\tau}{\pi}} - 3\tau \qquad (6)$$

2. Calculations. The dimensionless extract concentration Y of anthocyanins was calculated from the equation (Schwartzberg and Chao, 1982; Schwartzberg, 1987)

$$Y = \frac{(y - y_e)}{(y_0 - y_e)} \qquad (7)$$

where y_e is the measured weight fraction of a given compound in the extract at equilibrium and y_0 was calculated by extrapolating to time zero a plot of the measured concentration y vs $\sqrt{t}$, since y varies linearly with $\sqrt{t}$ at very small t (Schwartzberg and Chao, 1982; Schwartzberg et al., 1982).

Partition coefficients m for a solvent and for each extraction were calculated from the equation

$$m = \frac{y_e}{x_{dm}} \qquad (8)$$

where y_e is the weight fraction of a given compound in the extract at equilibrium and x_{dm} is the weight fraction of a given compound in the dry pomace at equilibrium. The weight fraction of the compound in the dry pomace was calculated from the mass balance equation

(Gertenbach, 2001),

$$x_{dm} = \frac{x_m - (1 - DM_p).y_e}{DM_p} \qquad (9)$$

where x_m is the weight fraction of a given compound in the wet pomace, x_{dm} is the weight fraction of a given compound in the dry pomace and y_e is the weight fraction of a given compound in the liquid extract. The factor DM_p is the weight fraction of dry matter in the wet pomace. Thus, measuring y_e, x_m, and DM_p the weight fraction of a given compound in the dry pomace and equilibrium constant can be obtained.

Values of volume ratio α were determined by a solute material balance:

$$\alpha = \frac{m x_0 - y_e}{y_e - y_0} \qquad (10)$$

where x_0 is the initial weight fraction of a given compound in the solid, that means the compound concentration of the milled frozen berries measured by the equilibrium method. Three different calculation procedures can be applied according to various situations of the extraction. Two of them apply to short times of extraction, one for finite values of the volume ratio α and the second for larger values of α. The third possibility that can be applied to long extraction times is presented here but has not been used for the calculations of the results presented in this thesis.

a. Case for very short extraction times. When τ is small (τ <0.0189, α =1 to 4) equation (4) (Carman and Haul, 1954; Schwartzberg and Chao, 1982) can be used to calculate the Fick's numbers with less than 1% error. Fick's numbers τ calculated with equations (4) and (5) using dimensionless concentrations Y were plotted against time (Figure AI.1).

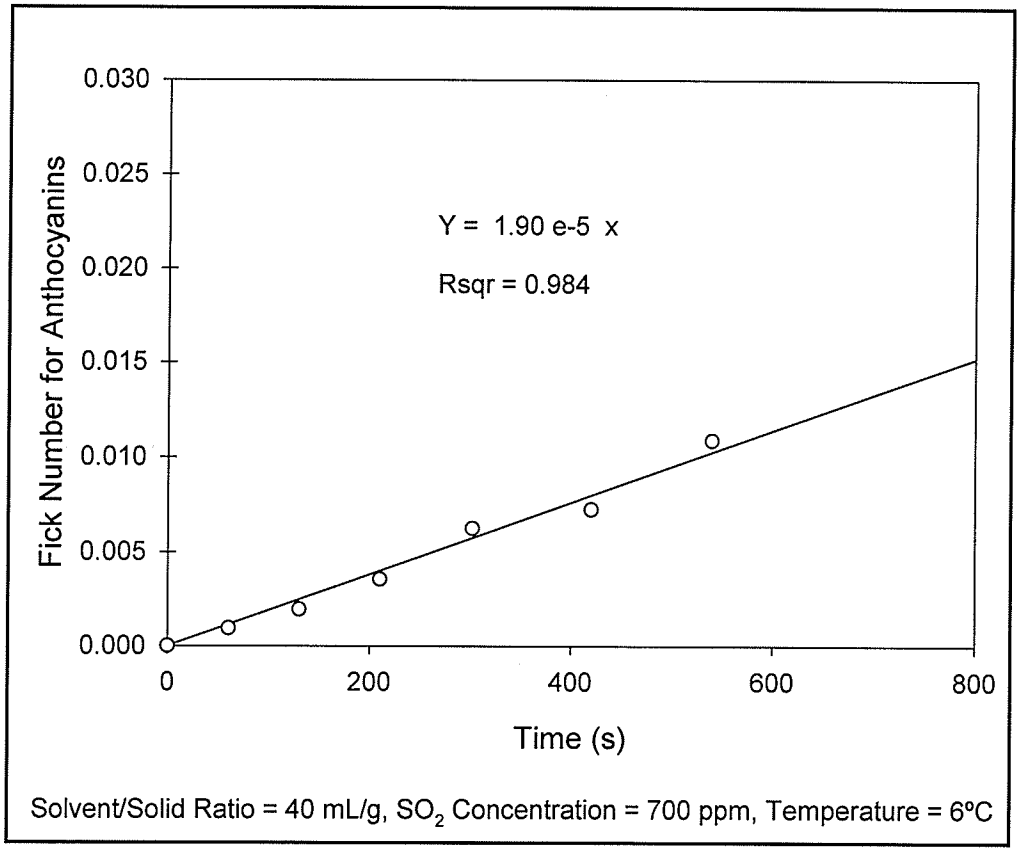

Figure AI.1. Fick Number for the Extraction of Anthocyanins with 40 mL/g dwb Frozen Berries of 700 ppm SO_2 at 6 °C

Slope of the regression straight line is equal to $\frac{D_S}{a^2}$ where D_S is the diffusivity of anthocyanins in the solid and a is the diameter of the particles, assuming particles have spherical shape.

Then diffusivity was calculated from

$$D_S = slope\ a^2 \qquad (11)$$

The range of validity of solution increases when α increases, attaining maximum accuracy at values of α between 2 and 4, but the measurable change of **Y** diminishes (Schwartzberg and Chao, 1982).

b. Case for moderately short extraction times. For large values of α and moderately short times the foregoing procedure can not be applied, so equation (6) can be used instead of (4) to calculate the Fick's number. Then calculation procedure follows as for very short extraction times, and diffusivity is calculated using the slope of plots of τ vs. time and equation (11).

c. Case for long extraction times. When τ is large ($\tau > 0.1$) diffusion coefficients can be calculated from the plot of the dimensionless anthocyanin concentration Y vs. t. When τ is large enough only the first term of the series solution (equation 2) of the Fick's second law is needed. Then, equation is

$$Y = C_1 \exp\left[-q_1^2.\tau\right] \qquad (12)$$

equations of C_1 and q_1 are a function of the volume ratio, and the shape of the solid particles.

Dimensionless concentrations were plotted against extraction time (Figure AI.2). Slope of regression straight line would be equal to $-q_1^2 . D_s/a^2$. Slopes of the linear regression lines were used for calculation of the diffusivity from the following equation

$$D_S = -slope\left(\frac{a}{q_1}\right)^2 \qquad (13)$$

where a is the diameter of spherical particles and q_1 is the first root of the series equation (2).

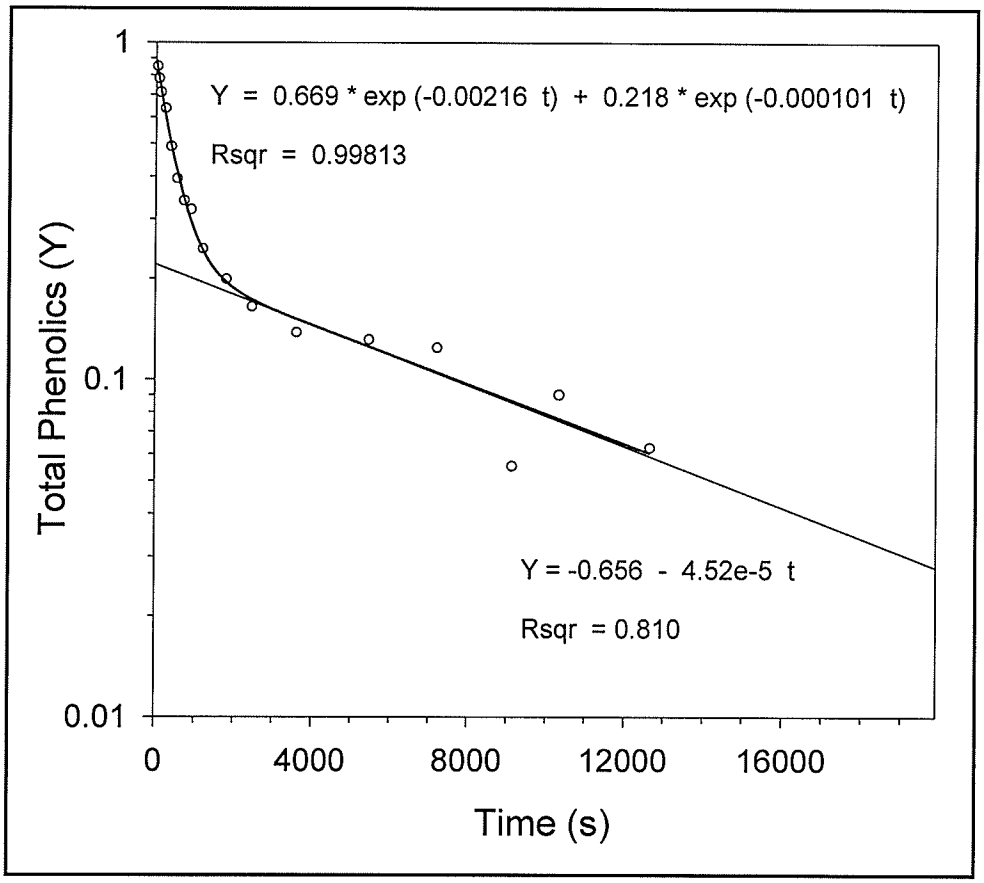

Figure AI.2. Dimensionless Concentration for the Extraction of Total Phenolics with 40 mL/g dwb of Frozen Berries of 67% ethanol at 40°C

APPENDIX II

Mass Balance Calculations

1. Equations

Extract yields of phenolics were calculated from

$$yield\,(\%) = \frac{V(L).\rho(g/L).y_e.100}{W_b(g).C_b} \qquad (1)$$

where V is the volume, ρ is the density, and y_e is the weight fraction of the extract for the phenolic for which yield is being calculated, W_b is the weight of berry feed and C_b is the content of the phenolic in the berries. Phenolic loss in the pomace was calculated from

$$pomace\,loss\,(\%) = \frac{W_p(g).x_m}{W_b(g).C_b} \qquad (2)$$

where W_p is the weight of the wet pomace and x_m is the weight fraction of a given phenolic in the wet pomace. Dry matter of the extract was calculated from a mass balance of solids

$$W_b.DM_B = W_p.DM_P + W_E.DM_E \qquad (3)$$

where DM_B, DM_P, and DM_E are dry matter weight fractions of the berry, pomace and extract and W_E is extract weight calculated with extract volume and density of extract. Total loss, W_l was calculated from a total mass balance

$$W_b + W_S = W_p + W_E + W_l \qquad (4)$$

W_S is the initial weight of solvent loaded into the extractor. Total loss W_l was expressed as

weight and as percentage of the initial mass.

Total solids in the extracts, E_S were calculated using the dry matter fraction of extract and the weight of extract

$$E_S = W_E(g).DM_E \qquad (5)$$

2. Data from Aqueous Ethanol Extraction

Composition data of berries (Table 4), extract weight fractions (y_e), pomace weight fractions (x_m) (Table AII.1) and measured extract volume, density of extracts and pomace weights (Table AII.2) were used to calculate phenolics extract yields and pomace losses.

Thus, anthocyanin yield in the first run was,

$$yield\,(\%) = \frac{V(L).\rho(g/L).y_e.100}{W_b(g).C_b}$$

$$yield\,(\%) = \frac{2.27(L).958(g/L).66.4.10^{-5}.100}{531(g).\ 0.225\ .\ 13.6(mg/g_{dwb})\ .10^{-3}}$$

$$yield_{anthoc} = 89\%$$

Pomace loss of anthocyanins in the first run was

$$pomace\,loss\,(\%) = \frac{W_p(g).x_m}{W_b(g).C_b}$$

$$pomace\,loss\,(\%) = \frac{371(g).\ 62.2.10^{-5}\ .\ 100}{531(g).\ 0.225\ .\ 13.6(mg/g_{dwb}).10^{-3}}$$

TABLE AII.1. Phenolic Weight Fractions of Extracts and Wet Pomace from Black Currant Extractions using Aqueous Ethanol

Run	Total Phenolics[a]		Tartaric Esters[b]		Flavonols[c]		Anthocyanins[d]	
	extract[e]	pomace[f]	extract	pomace	extract	pomace	extract	pomace
1	150	219	13.6	16.4	10.8	14.4	66.4	62.2
2	189	228	19.7	18.2	18.1	14.8	77.1	64.7
3	63	116	5.8	11.2	5.2	9.9	27.0	24.9
4	73	107	7.9	9.0	6.7	0.9	28.4	17.9
5	196	270	18.1	45.0	13.7	43.9	64.5	78.9
6	200	211	21.2	33.5	16.9	29.5	76.1	71.5
7	71	149	7.0	12.1	5.3	2.9	27.5	15.0
8	79	138	7.6	14.8	6.2	13.2	29.3	26.4
9	98	191	9.2	15.1	7.9	11.9	40.6	35.0
10	160	219	16.8	38.9	11.5	36.8	36.8	45.5
11	372	364	36.1	37.6	31.0	32.5	157	126
12	66	124	6.3	15.6	5.3	15.9	24.5	27.8
13	91	188	8.7	23.5	6.9	20.3	36.2	38.8
14	105	181	10.7	14.5	9.1	12.4	44.4	43.5
15	108	182	10.3	21.6	8.8	23.6	39.8	45.6
16	104	144	9.7	11.1	8.4	10.0	42.3	35.6
17	98	120	8.9	10.3	7.1	2.8	40.2	24.3
18	101	213	9.7	16.8	8.0	3.6	38.9	18.7

Phenolics expressed in weight fractions as equivalents of: [a] chlorogenic acid; [b] caffeic acid; [c] quercetin; [d] cyanidin 3-glucoside; [e] Extract weight fractions (y_e) in 10^5 g of equivalent/ g of liquid extract; [f] Pomace weight fractions (x_m) in 10^5 g of equivalent/ g of wet pomace

TABLE AII.2. Extract Volume and Density, Pomace Weight and Dry Matter Content and Berry Feeds from Black Currant Extractions using Aqueous Ethanol

Run	Berry Feed (g)	Extract Volume (L)	Density (g/L)	Pomace Weight (g)	Pomace DM (%)
1	531	2.27	958	371	12.8
2	530	2.27	889	430	13.5
3	177	2.31	941	95	14.3
4	180	2.35	861	87	17.7
5	530	1.95	969	312	15.0
6	530	2.24	886	291	19.5
7	178	2.1	942	101	13.7
8	177	2.17	856	95	16.9
9	267	2.46	913	110	20.0
10	268	1.43	940	155	22.5
11	1785	3.05	977	633	26.8
12	144	2.21	899	79	16.5
13	266	2.29	963	165	14.2
14	265	2.15	842	115	19.8
15	264	2.28	912	128	17.4
16	265	2.18	912	159	14.0
17	265	2.28	914	121	17.5
18	264	2.19	909	175	16.4

$$pomace\,loss_{anthoc} = 14\%$$

The total loss of the first run was

$$W_b + W_S = W_p + W_E + W_l$$

$$W_l\,(g) = W_b + W_S - W_p - W_E$$

$$W_l\,(g) = 531\,g + 2.5\,L\,.\,929\,g/L - 371\,g - 2.27\,L\,.\,958\,g/L$$

$$W_l = 308\ g$$

and the dry matter of the extract was

$$W_b\,.\,DM_B = W_p\,.\,DM_P + W_E\,.\,DM_E$$

$$DM_E = \frac{W_b\,.\,DM_B - W_p\,.\,DM_P}{W_E}$$

$$DM_E\,(\%) = \frac{(531\,g\,.\,0.225 - 371\,g\,.\,0.128).100}{2.27\,L\,.\,958\,g/L}$$

$$DM_E = 3.3\ \%$$

The total solids in the extract were

$$E_S = W_E\,(g).DM_E$$

$$E_S = 2.27\,g\,.\,958\ g/L\,.\,0.033$$

$$E_S = 72.8\ g$$

Results of mass balance calculations for all the extractions of black currants using aqueous ethanol are presented in Table 15.

3. Data from SO_2-containing Water Extraction

Composition data of berries (Table 5), extract weight fractions (y_e), pomace weight fractions (x_m) (Table AII.3) and measured extract volume, density of extracts and pomace weights (Table AII.4) were used to calculate phenolics extract yields and pomace losses.

Results of the mass balance calculations for all the extractions of black currants using sulfurous water are presented in Table 21.

TABLE AII.3. Phenolic Weight Fractions of Extracts and Wet Pomace from Black Currant Extractions using SO_2-containing Water Solvent

Run	Total Phenolics[a]		Tartaric Esters[b]		Flavonols[c]		Anthocyanins[d]	
	extract[e]	pomace[f]	extract	pomace	extract	pomace	extract	pomace
1	126	238	10.9	22.2	9.4	19.9	60.2	57.6
2	168	291	11.8	23.0	9.79	19.8	65.6	57.1
3	61.3	165	4.19	14.2	3.35	11.0	21.7	19.7
4	114	217	4.99	14.6	3.59	12.8	22.8	17.9
5	128	247	11.6	24.7	10.1	20.8	58.0	62.5
6	181	230	13.6	20.0	11.5	18.6	66.4	54.7
7	56.2	144	4.38	13.7	3.77	11.3	24.0	22.4
8	85.1	101	5.52	5.52	4.55	6.0	26.4	19.9
9	115	256	6.6	19.8	5.41	18.7	34.3	35.9
10	60.4	102	5.8	10.6	4.73	10.8	25.3	32.8
11	320	384	28.9	35.4	24.9	27.9	152	121
12	64.7	61	3.52	6.5	2.87	8.3	19.1	16.7
13	64.3	218	5.89	17.4	5.27	15.2	30.6	51.7
14	115	140	6.08	7.16	4.93	5.1	33.9	26.0
15	106	207	6.85	13.3	5.63	12.1	34.6	30.1
16	86.2	130	5.23	7.05	4.55	8.1	31.6	29.7
17	97.6	213	6.54	15.0	5.26	13.7	33.9	26.0
18	95.9	200	6.74	12.5	5.73	10.7	35.9	31.0

Phenolics expressed in weight fractions as equivalents of: [a] chlorogenic acid; [b] caffeic acid; [c] quercetin; [d] cyanidin 3-glucoside; [e] Extract weight fractions (y_e) in 10^5 g of equivalent/ g of liquid extract; [f] Pomace weight fractions (x_m) in 10^5 g of equivalent/ g of wet pomace

TABLE AII.4. Extract Volume and Density, Pomace Weight and Dry Matter Content and Berry Feeds from Black Currant Extractions using SO_2-containing Water Solvent

Run	Berry Feed (g)	Extract Volume (L)	Density (g/L)	Pomace Weight (g)	Pomace DM (%)
1	531	2.51	1012	344	13.7
2	527	2.5	1011	229	17.5
3	176	2.41	1006	137	10.0
4	177	2.44	1006	121	11.7
5	530	2.43	1013	303	14.0
6	533	2.48	1012	294	14.3
7	177	2.27	1002	173	7.93
8	178	2.16	1007	120	9.28
9	266	2.45	1005	183	12.0
10	266	2.38	1007	142	11.5
11	1766	3.09	1026	897	19.9
12	144	2.35	1003	155	7.35
13	266	2.35	1008	206	10.1
14	266	2.29	1011	300	8.01
15	267	2.35	1008	240	9.17
16	266	2.37	1008	250	8.86
17	266	2.35	1008	246	9.16
18	265	2.36	1007	200	12.6

APPENDIX III

Flow Diagram of Processes

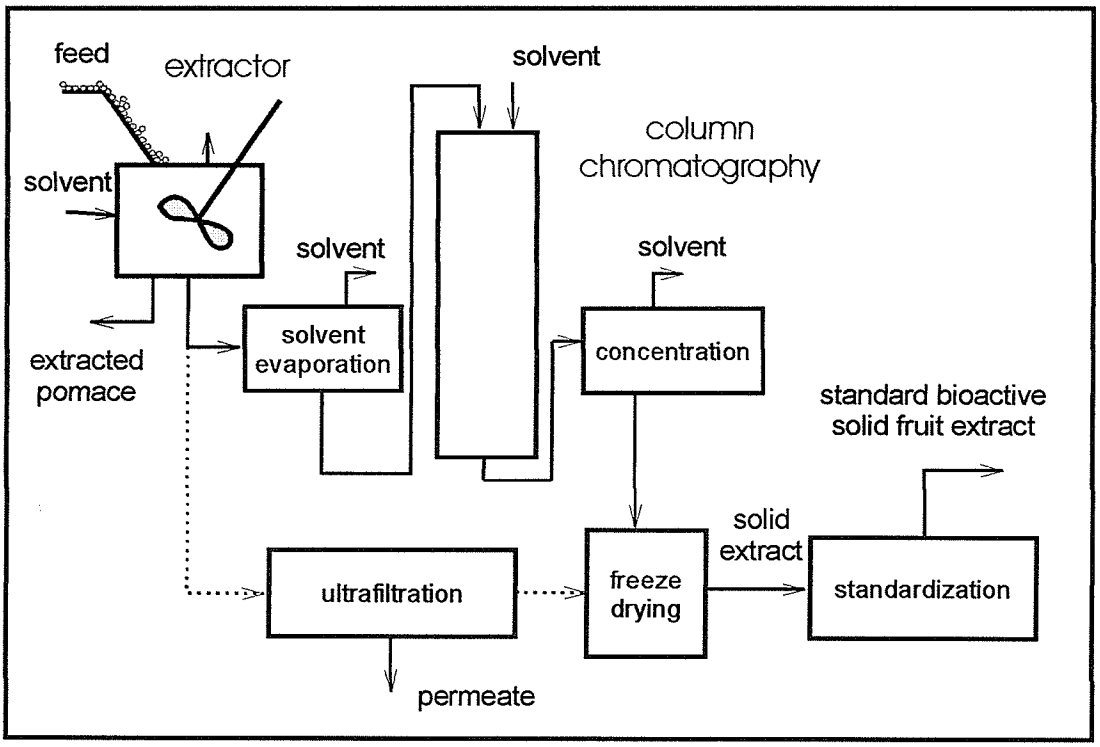

Figure AIII.1. Scheme of Operations for the Extraction, and Purification of Bioactive-rich Extracts