PRESSURIZED LIQUID EXTRACTION OF PHENOLIC COMPOUNDS FROM FRUIT POMACES

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ABSTRACT

PRESSURIZED LIQUID EXTRACTION OF PHENOLIC COMPOUNDS FROM FRUIT POMACES

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In this study, extraction of phenolic compounds from sour cherry, peach and apple pomaces using high pressure extraction (HPE) and subcritical CO_2 extraction (SCE) was investigated considering total phenolic content (TPC) and antiradical efficiency (AE).

Different combinations of pressure (50, 125, 200 MPa), temperature (20, 40, 60°C), solid/solvent ratio (0.05, 0.15, 0.25 g/ml) and extraction time (10, 25, 40 min) were used for HPE according to the Box-Behnken experimental design. The variables used for SCE were pressure (20, 40, 60 MPa), temperature (40, 50, 60°C), ethanol concentration (14, 17, 20 wt%) and extraction time (10, 25, 40 min).

For HPE, TPC and AE at the optimum conditions were 3.80 mg gae/g sample and 22 mg $DPPH^{\cdot}$/g sample for sour cherry pomace, 0.93 mg gae/g sample and

6.40 mg $DPPH^{\cdot}$/g sample for peach pomace and 2.08 mg gae/g sample and 10.80 mg $DPPH^{\cdot}$/g sample for apple pomace, respectively.

For SCE, TPC and AE at the optimum conditions were 0.60 mg gae/g sample and 2.30 mg $DPPH^{\cdot}$/g sample for sour cherry pomace, 0.26 mg gae/g sample and 1.50 mg $DPPH^{\cdot}$/g sample for peach pomace and 0.47 mg gae/g sample and 3.30 mg $DPPH^{\cdot}$/g sample for apple pomace, respectively.

Efficiency of HPE and SCE methods was compared with solvent extraction (SE). TPC and AE of the extracts obtained by HPE were close to those obtained by SE but the efficiency of SCE was low compared to HPE and SE. SCE was a better extraction method for apple and peach pomaces compared to sour cherry pomace.

Keywords: High pressure extraction, Subcritical carbon dioxide extraction, Sour cherry, Peach, Apple, Pomace, Antioxidant activity, Phenolic compounds

ÖZ

FENOLIK MADDELERİN MEYVE POSALARINDAN BASINÇLI SIVI EKSTRAKSİYONU

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Bu çalışmada, toplam fenolik miktarı (TPC) ve antiradikal verimlilik (AE) göz önünde bulundurularak, yüksek basınç ekstraksiyonu (HPE) ve subkritik CO_2 ekstraksiyonu (SCE) ile vişne, şeftali ve elma posasından fenolik maddelerin elde edilmesi araştırılmıştır.

Basınç (50, 125, 200 MPa), sıcaklık (20, 40, 60°C), katı/çözücü oranı (0.05, 0.15, 0.25 g/ml) ve ekstraksiyon süresi (10, 25, 40 dk) nin fenolik maddelerin yüksek basınç ekstraksiyonu üzerindeki etkisi ve basınç (20, 40, 60 MPa), sıcaklık (40, 50, 60°C), etanol konsantrasyonu (ağırlıkça % 14, 17, 20) ve ekstraksiyon süresi (10, 25, 40 dk) nin fenolik maddelerin subkritik karbon dioksit ekstraksiyonu üzerindeki etkisi araştırılmış.

HPE için, optimum koşullardaki TPC ve AE değerleri, vişne posası için 3.80 galik asit eşdeğeri (gae)/g numune ve 22 mg $DPPH^{\cdot}$/g numune, şeftali posası

için 0.93 mg gae/g numune ve 6.40 mg $DPPH^{\cdot}$/g numune, elma posası için 2.08 mg gae/g numune ve 10.80 mg $DPPH^{\cdot}$/g numunedir.

SCE için, optimum koşullarda TPC ve AE değerleri, vişne posası için 0.60 mg gae/g numune ve 2.30 mg $DPPH^{\cdot}$/g numune, şeftali posası için 0.26 mg gae/g numune ve 1.50 mg $DPPH^{\cdot}$/g numune, elma posası için 0.47 mg gae/g numune ve 3.30 mg $DPPH^{\cdot}$/g numunedir.

HPE ve SCE metodlarının verimliliği, çözücü ekstraksiyonu (SE) ile karşılaştırılmıştır. HPE ile elde edilen ekstraktların TPC ve AE değerleri SE ile elde edilenlerinkine yakındır fakat SCE yönteminin verimliliğinin HPE ve SE yöntemlerininkine göre düşük olduğu görülmüştür. SCE'nin, şeftali ve elma posaları için daha iyi bir ekstraksiyon metodu olduğu görülmüştür.

Anahtar Kelimeler: Yüksek basınç ekstraksiyonu, Subkritik karbon dioksit ekstraksiyonu, Vişne, Şeftali, Elma, Posa, Antioksidan aktivitesi, Fenolik maddeler

To My Daughter, Nur

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INTRODUCTION

1.1 Phenolic compounds

Phenolic compounds are secondary metabolites that are synthesized by plants during normal development and in response to stress conditions as infection, wounding, UV irradiation, herbivores and reactive oxygen species (Beckman, 2000; Dixon and Paiva, 1995; Nicholson and Hammerschmidt, 1992). They are the most stable and powerful type of dietary antioxidants and possess higher *in vitro* antioxidant capacity than other antioxidants like vitamins and carotenoids (Gardner et al., 2000; Lee et al., 2003). In this respect, they protect other compounds or tissues from damage caused by free radicals. Besides their antioxidant property, phenolic compounds exhibit a wide range of physiological properties like anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-Garcia et al., 1997; Manach et al., 2005; Middleton et al., 2000; Puupponen-Pimiä et al., 2001).

1.1.1 Classification of Phenolic Compounds

The general structure of phenolic compounds consists of an aromatic ring with a hydroxyl substituent and a functional residue. These compounds are classified into different groups according to the number of phenol rings that they contain and the structural elements that bind these rings to one another as phenolic acids,

flavonoids, lignans and stilbenes (Figure 1.1). In addition to this diversity, most naturally occuring phenolic compounds are associated with various carbohydrates and organic acids and with one another (Manach et al., 2004; Balasundram et al., 2006).

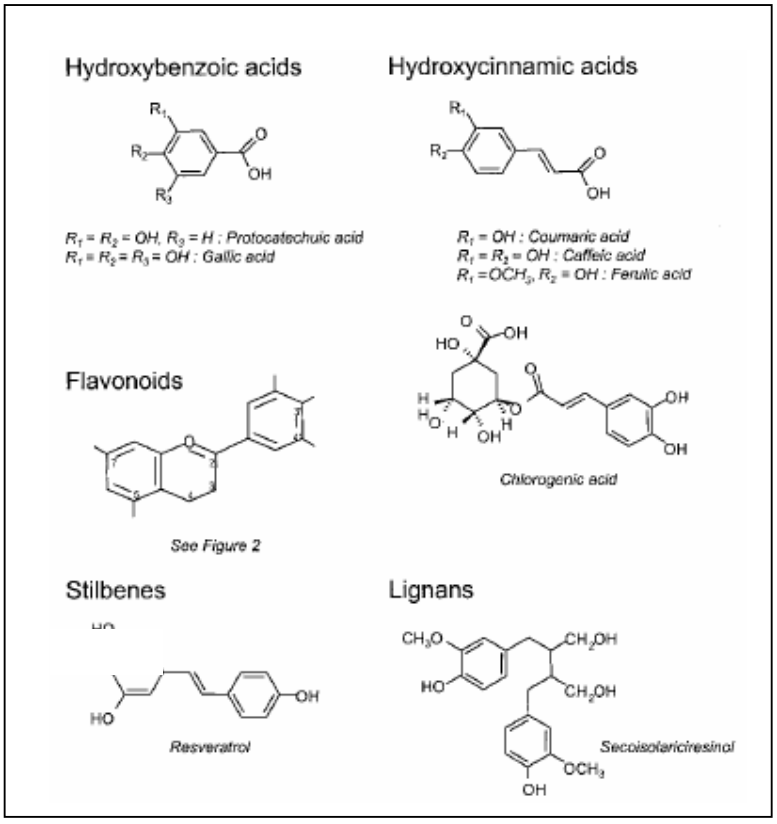

Figure 1.1 Chemical structure of phenolic compounds (Manach et al., 2004)

1.1.1.1. Phenolic acids

Phenolic acids consist of two subgroups; hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids (Balasundram et al., 2006). The hydroxybenzoic acid content of edible plants is generally very low, with the exception of black radish and onions (Manach et al., 2004). Although hydroxybenzoic acids can be detected as free acids in some fruits (e.g. gallic acid in persimmons) or after being released during fruit and vegetable processing, as a general rule they are present as conjugates. Gallic acid may be conjugated as such, or as its dimer, trimer and tetramer (ellagic acid, tergallic acid and gallagic acid respectively). The trimer and tetramer are comparatively rare, but along with gallic and ellagic acids are esterified to glucose in hydrolysable tannins. Gallic acid is also esterified to condensed tannins, their monomers, some derived tannins (particularly in black tea) and quinic acid (theogallin) (Tomás-Barberán and Clifford, 2000).

Hydroxycinnamic acids are more common in edible plants and consist of *p*-coumaric, caffeic, ferulic, and sinapic acids (Clifford, 1999). They are found in both free and esterified form in all parts of fruit, although the highest concentrations are seen in the outer parts of ripe fruit. Concentrations generally decrease during the course of ripening, but total quantities increase as the fruit increases in size (Manach et al., 2004). Ferulic acid is the most abundant phenolic acid found in cereal grains. It is covalently linked to plant cell walls. Caffeic and quinic acid combine to form chlorogenic acid and found at extremely high levels in coffee, also at lower levels in other plant foods (Kroon and Williamson, 1999).

1.1.1.2 Flavonoids

Flavonoids are the largest group of plant phenolic compounds. They can be divided into 6 subclasses according to the type of heterocycle involved: flavonols, flavones, flavanones, isoflavonoids (isoflavones), flavanols and anthocyanidins (Figure 1.2) (Manach et al., 2004; Aharne and O'Brien, 2002).

The main representatives of flavonols are quercetin, kaempferol, isorhamnetin and myricetin. Quercetin is present in various fruits and vegetables but the richest source is onions. Quercetin is present in plants in many different glycosidic forms like quercetin-3-rutinoside, quercetin-3-glucoside, quercetin-4′-glucoside and quercetin-3,4′-diglucoside (Erlund, 2004; Aharne and O'Brien, 2002). Kaempferol is most common among fruits and leafy vegetables. lt is also found in some herbs, legumes and root vegetables. lsorhamnetin occurs in onions and pears. Myricetin is found most often in berries, maize and tea. In fruits, flavonols and their glycosides are found predominantly in the skin.

Flavones are not frequently found in fruits but are found in grains, herbs (parsley, rosemary and thyme), vegetables and their leaves. Common flavones are apigenin and luteolin, which are found in glycosylated form in plants. Nobiletin, sinensetin, and tangeretin are nonglycosidic, highly methoxylated bitter citrus flavones that participate in taste. Flavones can also contribute to plant tissue color, if they occur in high concentrations or are complexed with metal ions (Peterson and Dwyer, 1998).

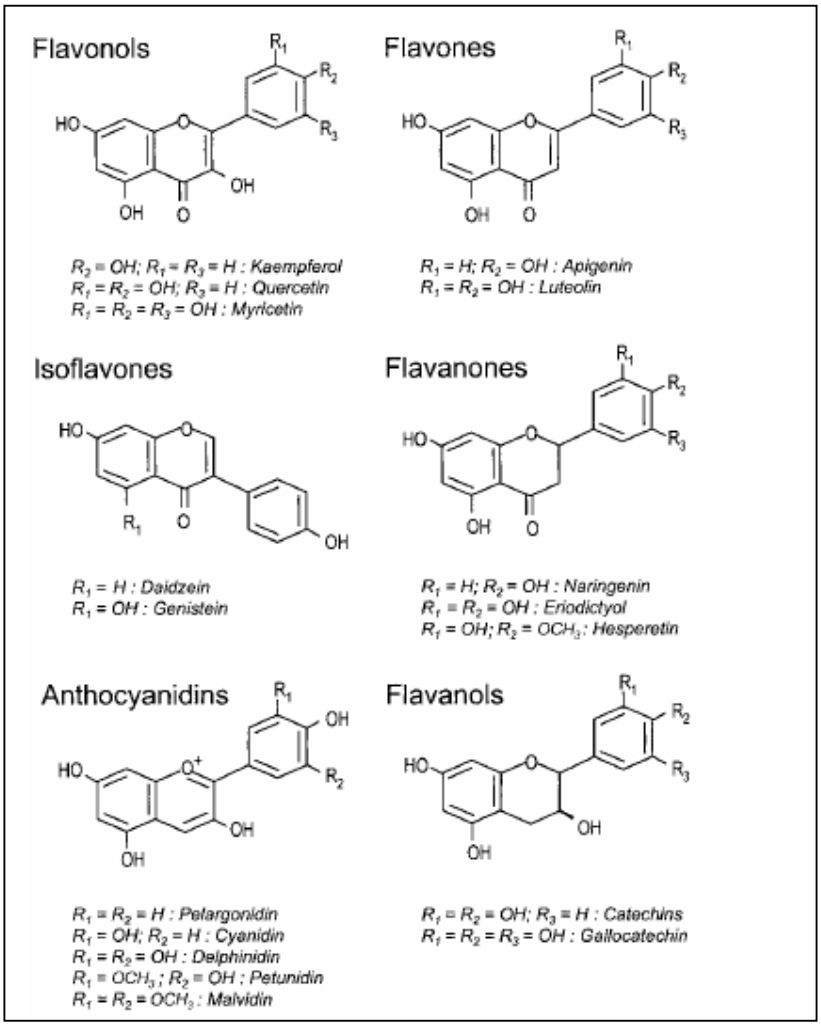

Figure 1.2 Chemical structure of flavonoids (Manach et al., 2004)

The major sources of flavanones are citrus fruits and juices. They are usually found in glycosylated form. Hesperidin (hesperetin-7-rutinoside) and narirutin (naringenin-7-rutinoside) are found in oranges and mandarins where naringin (naringenin-7-neohesperoside) and narirutin are the major flavonoids grapefruits. Low concentrations of naringenin are also found in tomatoes and tomato-based products (Erlund, 2004).

Isoflavonoids are called as estrogenic flavonoids because of their structural similarity to estrogens and estrogenic activity. They are mainly found in the legume family. Soybeans are the major source of daidzein and genistein which are also found in black beans, green split peas, and clover sprouts. Other isoflavonoids, biochanin A, coumesterol, and formononetin, have been found in green beans, chick peas, lima beans, split peas, alfalfa sprouts, clover sprouts, and sunflower seeds (Peterson and Dwyer, 1998).

Flavanols exist in both the monomer form (catechins) and the polymer form (proanthocyanidins). Catechin and epicatechin are the main flavanols in fruits, which occur in combined form as epigallo-catechin gallate and epicatechin gallate, combined with gallic acid in tea or as condensed tannin polymers in fruits, legumes, and grains. Catechins are frequently more abundant in external tissues. In black tea, catechins are oxidized to theaflavins and thearubigens (King and Young, 1999). In contrast to other classes of flavonoids, flavanols are not glycosylated in foods (Van der Sluis, 2005). Proanthocyanidins, which are also known as condensed tannins are dimers, oligomers, and polymers of (+) catechins, flavan-3-ols and (–)-epicatechin. They are determinants of flavor and astringency in teas, wines and fruit juices (Dixon et al., 2005). This astringency changes over the course of maturation and often disappears when the fruit reaches ripeness. The polymerization of tannins probably accounts for the apparent reduction in tannin content that is commonly seen during the ripening of many types of fruit. It is difficult to estimate the proanthocyanidin content of foods because proanthocyanidins have a wide range of structures and molecular weights (Manach et al., 2004).

Anthocyanidins are naturally found in glycosylated form called anthocyanins. They are highly unstable, but anthocyanins are resistant to light, pH, and oxidation conditions. Their degradation is prevented by glycosylation and esterification with various organic acids (citric and malic acids) and phenolic acids. In addition, anthocyanins are stabilized by the formation of complexes

with other flavonoids (Manach et al., 2004). Those phenolic compounds are responsible for the red, blue or violet color of edible fruits, such as plums, apples, eggplant and many berries. The most common anthocyanidins include pelargonidin, cyanidin, delphinidin, and malvidin (Erlund, 2004). The color of anthocyanidins is pH dependent. They are usually red at low pH, becomes colorless (pH 4.5) with sulfur compound and then shifts to blue as the pH increases. Anthocyanins may be complexed with flavones and metal ions such as iron and magnesium in flowers. Anthocyanin content usually increases as the fruit matures (Peterson and Dwyer, 1998).

1.1.1.3 Lignans and Stilbenes

The richest dietary source of lignans is linseed, which contains secoisolariciresinol and low quantities of matairesinol. Generally, other cereals, grains, fruit, and certain vegetables contain traces of these same lignans. Stilbenes are found in only low quantities in the edible plants (Manach et al., 2004). Trihydroxystilbenes *cis*- and *trans*-resveratrol and *cis*- and *trans*-polydatin were determined in low amounts in both red and white wines by Soleas et al. (1997).

1.1.2 Biosynthesis of Phenolic Compunds

Phenolic compounds are secondary metabolites of plants that are synthesized through pentose phosphate, shikimate and phenylpropanoid pathways (Figure 1.3) (Randhir et al., 2004; Ali et al., 2006; Dixon et al., 1995). The oxidative pentose phosphate pathway provides precursor erythrose-4-phosphate for the shikimate pathway. The shikimate pathway converts these sugar phosphates to aromatic amino acids like phenylalanine, which becomes the precursor for the phenylpropanoid pathway (Randhir et al., 2004).

Phenylalanine ammonia-lyase is a crucial enzyme in phenylpropanoid metabolism, which catalyses the deamination of L-phenylalanine to form *trans*-cinnamic acid and a free ammonium ion. It is induced by various biotic (infection by viruses, bacteria, fungi, etc.) and abiotic (low and high temperatures, UV-light, wounding, etc.) stresses, which result in the accumulation of such phenylpropanoids as phenolic acids and flavonoids (Solecka and Kacperska, 2003; Sgarbi et al., 2003; Tomás-Barberán and Espín, 2001). Some of the other key enzymes catalyzing the biosynthesis of phenolic compounds include glucose-6-phosphate dehydrogenase, shikimate dehydrogenase and cinnamyl alcohol dehydrogenase (Ali et al., 2006).

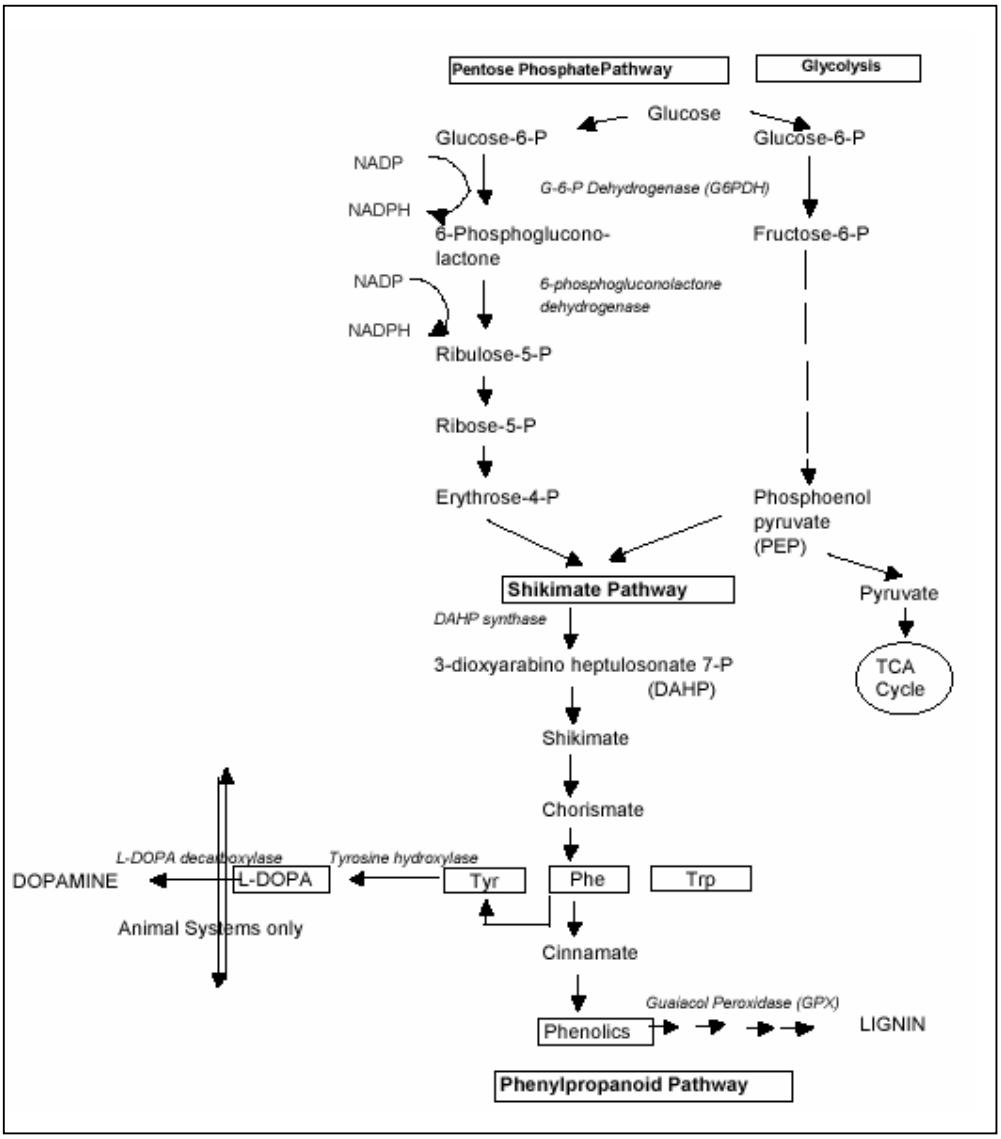

Figure 1.3 Pathway for synthesis of phenolic compounds (Randhir et al., 2004)

1.1.3 Antioxidant Property of Phenolic Compounds

Free radicals such as superoxide ($O_2^{\cdot}$), hydroxyl ($OH^{\cdot}$) and peroxyl ($RO_2^{\cdot}$) are produced as a part of normal metabolic processes or exogenous factors (cigarette smoke, car exhaust fumes and oxidant gases, such as ozone, nitrogen dioxide and sulphur dioxide) and known to attack and damage body cells to get the missing electron they need. Such oxidative damages on lipids, proteins and nucleic acids may induce various chronic diseases, such as coronary heart diseases, atherosclerosis, cancer and aging. Antioxidants are substances that are able to prevent or retard those oxidation reactions and protect compounds or tissues from damage caused by oxygen or free radicals, and hence they have health promoting effect (Wong et al., 2006; Chung et al., 2006; Valentão et al., 2002; Kirkham and Rahman, 2006). Antioxidants also retard lipid oxidative rancidity in food, therefore they are intentional food additives to improve the quality of foods. Synthetic phenolic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly added to foods to inhibit free radical damage to lipids; however, BHA and BHT are suspected as possible carcinogens and there is a growing trend in consumer preference for natural ingredients (Valentão et al., 2002; Duh et al., 1999; Velioğlu et al., 1998; Wang et al., 1998).

A drawback for using natural phenolic antioxidants is that they can affect the sensory or organoleptic properties of foods such as color and taste. As an example, polymerization of phenolic compounds due to autoxidation is responsible for color loss in processed vegetables. Phenolic compounds have other undesirable effects in food systems such as the formation of strong complexes with proteins and enzymes. Therefore, the potential of phenolics to diminish nutrient availability must be characterized when using them as natural antioxidants (Moure et al., 2001).

There are two main types of antioxidant effects, namely, “primary” (chain breaking, free radical scavengers) and “secondary or preventive” (deactivation of metals, inhibition of breakdown of lipid hydroperoxides, regeneration of “primary” antioxidants, singlet oxygen quenching) (Gordon, 1990). In plant extracts and in various foods, antioxidants are present as mixtures and therefore there is currently a great interest in synergistic interaction between antioxidants and between mechanisms of such interaction. Antioxidant synergism may be classified as:

1. Regeneration of the more effective antioxidant in the system by other less effective antioxidants or oxidation retarders.

2. Metal chelation by one antioxidant sparing a chain breaking antioxidant.

3. Interaction of antioxidants with different solubility, i.e. different phase distributions.

4. Interaction of antioxidants with different mechanisms of action, e.g. singlet oxygen quenchers and chain breaking antioxidants, i.e. safeguarding singlet oxygen quenchers from oxidation by free radicals by a chain breaking antioxidant allowing the former to act longer as an efficient quencher protecting the second from oxidation by singlet oxygen (Becker et al., 2004).

When phenolics function as antioxidants, they are univalently oxidized to their respective phenoxyl radicals. Sakihama et al. (2002) suggested that the phenoxyl radicals produced through antioxidative reactions may have the potential to act as prooxidants under certain conditions, for example, they can initiate lipid peroxidation. However, they have shown that phenoxyl radicals usually do not show harmful prooxydant activity under normal conditions, because they are rapidly changed to non-radical products by polimerization reactions and enzymatic (as well as non-enzymatic) reduction of the radicals.

Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables (Parr and Bolwell, 2000; Scalbert and Williamson, 2000; Sun et al., 2002; Cieślik et al., 2006). Different fruits and vegetables were studied as sources of natural antioxidants and various compounds having antioxidant activity were isolated most of which were identified as phenolic compounds. Therefore the beneficial effects derived from those compounds have been attributed to their antioxidant activity (Heim et al., 2002). Mediterranian diet, which is high in fruits, vegetables, fibre, fish and olive oil is indicated to be protective against both cancer and coronary heart disease. This health-promoting property has been attributed mainly to its high phenolic content, besides monounsaturated fatty acid and oleic acids because the major phenolic compounds identified and quantified in olive oil (namely simple phenols, secoiridoids and lignans) have been shown to have potent antioxidant properties (Owen et al., 2000a; Owen et al., 2000b; Owen et al., 2000c).

Antioxidant capacities of fruits, vegetables and other foods have been examined *in vitro* for many years (Kanner et al., 1994; Cao et al., 1996; Velioğlu et al., 1998; Wang et al., 1998; Duh et al., 1999; Kähkönen et al., 1999; Kähkönen et al., 2001; Valentão et al., 2002; Gil et al., 2002; Toor and Savage, 2005; Karadeniz et al., 2005). However, *in vivo* studies on bioavailability and health effects of phenolic compounds are essential in order to elucidate the significance of these compounds in human health because the possible health benefits derived from dietary phenolic compounds depend on their absorption and metabolism (Parr and Bolwell, 2000; Gardner et al., 2000). For that reason, epidemiological studies have been carried out to implicate dietary antioxidant phytochemicals as protecting agents against diseases like cancer and cardiovascular diseases (Hertog et al., 1993; Hertog et al., 1997; Frankel et al., 1993; Knekt et al., 2002; Huxley and Neil, 2003; La Vecchia et al., 2001; Mukhdar and Ahmad, 2000; Di Carlo et al., 1999).

1.1.4 Food Sources of Phenolic Compounds

Although almost all foods of plant origin contain phenolic compounds, the major sources of them in the human diet are fruits, vegetables and beverages (Balasundram et al., 2006). Table 1.1 shows the type of phenolic compounds in different foods. There are many studies in the literature for the determination and characterization of phenolic compounds, however for many plant products, the phenolic composition is still much less known.

The research by Cieślik et al. (2006) in which total phenolic contents of selected fruits (white grapes, pink grapes, plums, pink grapefruits, oranges, kiwi fruit, apples, nectarines and European elder fruit) and vegetables (tomatoes, zucchini, Italian cabbage, carrots, parsley root, celery root, onion, broccoli and Brussels sprouts) available in the Polish market showed that apple and cabbage had the highest content of phenolic compounds.

Leantowicz et al. (2002) who worked on apples, peaches and pears for their influence on lipids and antioxidant capacity also showed that the amount of caffeic, *p*-coumaric and ferulic acids and the total radical-trapping antioxidative potential values are higher in apples and their peels.

Kähkönen et al. (1999) examined the phenolic content and antioxidant activity of extracts obtained from 92 edible and non-edible plant materials including berries, fruits, vegetables, herbs, cereals, tree materials, plant sprouts and seeds by Folin-Ciocalteau method and autoxidation of methyl linoleate, respectively. Berries, especially aronia and crowberry were found to have the highest phenolic content (20 mg gallic acid equivalent/g dry weight) and antioxidant activity. Apple extracts showed strong antioxidant activity although the total phenolic contents were low (12.1 mg gallic acid equivalent/g dry weight). In accordance with this research, Kähkönen et al. (2001) determined the phenolic profiles of 26 berry and 2 apple cultivars by high performance liquid chromatography and found that

phenolic contents among different berry genera varied considerably, where anthocyanins were generally the main phenolic constituent. In apples, hydroxycinnamic acids were the main phenolic subgroup.

Poyrazoğlu et al. (2002) separated and determined the phenolic compounds in pomegranate juice using reversed phase HPLC. They identified minor amounts of phenolic compounds in pomegranates as phenolic acids (gallic, protocatechuic, chlorogenic, caffeic, ferrulic, *o*- and *p*-coumeric acids) and flavonoids (catechin, quercetin and phloridzin). Overall mean concentrations of phenolic compounds were determined as follows: gallic acid 4.55±8.55 mg/L, protocatechuic acid 0.84±0.64 mg/L, catechin 3.72+2.29 mg/L, chlorogenic acid 1.24±1.42mg/L, caffeic acid 0.78±0.79 mg/L, *p*-coumaric acid 0.06±0.07 mg/L, ferulic acid 0.01±0.02mg/L, o-coumaric acid 0.17±0.08 mg/L, phloridzin 0.99±1.47 mg/L, quercetin 2.50±1.96 mg/L.

Kanner et al. (1994) determined the concentrations of phenolic compounds in three grape varieties and two red wines and also the antioxidative powers of wine phenolics. They have reported that the red grape variety and two types of red wines (Cabarnet Sauvignon and Petite Sirah) contain phenolics at concentrations of 920 mg/kg and 1800 and 3200 mg/L, respectively. The inhibition of low density lipoprotein oxidation by wine phenolics was compared with that by α-tocopherol using a system containing low-density lipoproteins oxidized *ex vivo* by Cu^{2+} ions. Their results showed that low-density lipoprotein oxidation was inhibited by lower concentrations of wine phenolics (less than 1 pM) than α-tocopherol, which indicates that wine phenolics are relatively more effective antioxidants.

Table 1.1 Phenolic compounds in foods (Manach et al., 2004)

Phenolic compound	Source
Hydroxybenzoic acids	Blackberry
Protocatechuic acid	Raspberry
Gallic acid	Black currant
p-Hydroxybenzoic acid	Strawberry
Hydroxycinnamic acids	Blueberry
Caffeic acid	Kiwi
Chlorogenic acid	Cherry
Coumaric acid	Plum
Ferulic acid	Aubergine
Sinapic acid	Apple
	Pear
	Chicory
	Artichoke
	Potato
	Corn flour
	Flour: wheat, rice, oat
	Cider
	Coffee
Anthocyanins	Aubergine
Cyanidin	Blackberry
Pelargonidin	Black currant
Peonidin	Blueberry
Delphinidin	Black grape
Malvidin	Cherry
	Rhubarb
	Strawberry
	Red wine
	Plum
	Red cabbage
Flavonols	Yellow onion
Quercetin	Curly kale
Kaempferol	Leek
Myricetin	Cherry tomato
	Broccoli
	Blueberry
	Black currant
	Apricot
	Apple
	Beans, green or white
	Black grape
	Black tea infusion
	Green tea infusion
	Red wine
Flavones	Parsley
Apigenin	Celery
Luteolin	Capsicum pepper
Flavanones	Orange juice
Hesperetin	Grapefruit juice
Naringenin	Lemon juice
Eriodictyol	

Table 1.1 (continued)

Isoflavones	Soy flour
Daidzein	Soybeans, boiled
Genistein	Miso
Glycitein	Tofu
	Tempeh
	Soy milk
Monomeric flavanols	Chocolate
Catechin	Beans
Epicatechin	Apricot
	Cherry
	Grape
	Peach
	Blackberry
	Apple
	Green tea
	Black tea
	Red wine
	Cider

There are wide variations between the phenolic contents of different fruits and vegetables, or even the same type of fruits and vegetables reported by different authors. The phenolic content of bananas, for example, has been found to be 90.4±3.2 mg gallic acid equivalents/100g fresh weight by Sun et al. (2002), whereas that of the same fruit was determined as 11.8±0.4 mg gallic acid equivalents/100g fresh weight by Luximon-Ramma et al. (2003). These differences may be due to the methods of extraction and analysis as well as the variabilities in phenolic content of foods due to intrinsic (genus, species, cultivars) and extrinsic (ripeness at the harvest time, environmental factors, processing and storage) factors (Balasundram et al., 2006; Manach et al., 2004; Pérez-Magariño and González-San José, 2006; Jagetia and Baliga, 2005).

The research by George et al. (2004) shows the variations of phenolic content with respect to genotype. The researchers determined the phenolic content and antioxidant activity of 12 tomato genotypes and found significant differences between lycopene, ascorbic acid and phenolic contents, also the antioxidant

activity among various genotypes. Activity was higher in the hexane fraction containing lycopene than the methanol fraction containing phenolics. Tomato peels, in addition to lycopene, contain significantly high amounts of ascorbic acid and phenolics. Cherry tomato was found to contain the highest content of antioxidants (lycopene, ascorbic acid and phenols) and have highest antioxidant activity.

Mansouri et al. (2005) determined the phenolic content, antioxidant activity and phenolic profile of seven varieties of ripe date palm fruit from Algeria. The total phenolic content was in the range of 2.49±0.01 to 8.36±0.60 mg gallic acid equivalents/100 g fresh fruit and antiradical efficiency (AE) ranged from 0.08±0.00 to 0.22±0.00 µg $DPPH^{\cdot}$/ µg sample. All the varieties were found to contain mainly *p*-coumaric, ferulic and sinapic acids and some cinnamic acid derivatives.

Cevallos-Casals et al. (2006) determined and characterized the total phenolic and anthocyanin contents of fourteen red-fleshed plum and eight peach varieties. Their results showed that the total phenolic content ranged from 298 to 563 mg chlorogenic acid equivalent/100 g for plums and 100 to 449 mg chlorogenic acid equivalent /100 g for peaches.

The degree of ripeness considerably affects the concentrations and proportions of the various phenolic compounds. In general, phenolic acid concentrations decrease during ripening, whereas anthocyanin concentrations increase (Manach et al., 2004). Prior et al. (1998) analyzed different cultivars of highbush, rabbiteye, lowbush and bilberry for total phenolics, total anthocyanins and antioxidant capacity (oxygen radical absorbance capacity, ORAC) and found different antioxidant capacities in various species and cultivars of *Vaccinium*. Increased maturity at harvest increased the ORAC, the anthocyanin and the total phenolic content. Wang and Lin (2000) determined the total antioxidant capacity (ORAC), total phenolic content and total anthocyanin content of different

cultivars of thornless blackberry, red raspberry, black raspberry and strawberry. Blackberries and strawberries had the highest ORAC values during the green stages, whereas red raspberries had the highest ORAC activity at the ripe stage. Total anthocyanin content increased with maturity for all species of fruits.

Environmental factors include soil type, sun exposure, rainfall, culture in greenhouses or fields, biological culture and fruit yield per tree. Exposure to light has a considerable effect on the flavonoid content (Manach et al., 2004). Growth temperature was also found to be effective on the phenolic content and antioxidant activity of strawberries by Wang and Zheng (2001). Iqbal and Bhanger (2006) showed that season and production location have a great impact on the total phenolic content and antioxidant activity of the leaves of moringa, an edible plant grown in Pakistan. Production site was also shown to be effective on the concentration of phenolic compounds in wheat by Gélinas & McKinnon (2006).

The effect of modified atmosphere packaging (MAP) and cooking on the flavonoids and vitamin C content of fresh-cut spinach was evaluated by Gil et al. (1999). The total flavonoid content (approximately 1000 mg/kg fresh weight) remained quite constant during storage in both air and MAP atmospheres, while vitamin C (750 mg/kg fresh weight) was better preserved in MAP-stored spinach. However, a decrease in the total antioxidant activity was observed during storage, particularly important in MAP-stored spinach. The researchers also determined that boiling extracted 50% of total flavonoids and 60% of vitamin C in the cooking water.

Nicoli et al. (1997) evaluated the loss of natural antioxidants in relation to thermal processing and the formation of Maillard reaction products having antioxidant activity in different food systems such as tomato derivatives and coffee. Results showed that, although the concentration of natural antioxidants was significantly reduced as a consequence of the thermal treatments, the overall

antioxidant properties of the food products were maintained or even enhanced by the development of Maillard reaction products.

Storage may also affect the content of phenolic compounds that are easily oxidized. In addition, storage temperature affects the content of phenolics in fruit and vegetables (Manach et al., 2004; Kalt et al., 1999). Ayala-Zavala et al. (2004) investigated the effect of storage temperature on the antioxidant activity and phenolic content of strawberry fruit. They have determined that strawberries stored at 10°C or 5°C showed higher antioxidant capacity, total phenolics, and anthocyanins than those stored at 0°C.

1.2 Phenolic Content of Sour Cherries, Peaches and Apples

Sour cherries contain significant level of anthocyanins with high antioxidant activity. Recent studies have revealed that anthocyanins from sour cherry exhibit *in vitro* antioxidant activities comparable to those from commercial products, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and superior to vitamin E at 2 mM concentration (Wang et al., 1999a). Wang et al. (1999b) identified eight phenolic compounds, 5,7,4'-trihydroxyflavanone, 5,7, 4'-trihydroxyisoflavone, chlorogenic acid, 5,7,3',4'-tetrahydroxyflavonol-3-rhamnoside, 5,7,4'-trihydroxyflavonol-3-rutinoside, 5,7,4'-trihydroxy-3'-methoxyflavonol-3-rutinoside, 5,7,4'-trihydroxyisoflavone-7-glucoside, and 6,7-dimethoxy-5,8,4'-trihydroxyflavone by NMR experiments. The antioxidant assays revealed that 6,7-dimethoxy-5,8,4'-trihydroxyflavone is the most active one, followed by quercetin 3-rhamnoside, genistein, chlorogenic acid, naringenin, and genistin.

Halvorsen et al. (2002) assessed the concentration of total antioxidants in a variety of dietary plants by the FRAP (ferric reducing/antioxidant power) assay and reported that the average total antioxidant concentration in sour cherries

obtained from different sources such as geographical location or manufactures was 5.53 mmol/100g fresh weight, which was one of the highest antioxidant activities among the assessed fruits (berries, pomegranate, grape, orange, plum, pineapple, lemon, date, kiwi, clementine, grapefruit, lime, fig, papaya, apricot, Kaki/sharon, mango, apple, banana, pear, plantain, melon).

Phenolic content and antioxidant activity of peaches are also considerable. Sun et al. (2002) who worked on the total phenolic profiles in common fruits reported the total phenolic content of peaches as 84.6±0.7 mg gallic acid equivalent/100 g fresh weight and the antioxidant activity as (871 mg of vitamin C equiv/100 g). Among the fruits investigated, cranberry had the highest total antioxidant activity (177.0 ± 4.3 μmol of vitamin C equiv/g of fruit), followed by apple, red grape, strawberry, peach, lemon, pear, banana, orange, grapefruit, and pineapple.

Bostock et al. (1999) showed that the major phenolic acids in the epidermis and subtending layers of peach are chlorogenic and caffeic acids. They reported that their concentrations are especially high in peach genotypes with a high level of resistance to the brown rot fungus, *Monilinia fructicola*, and decline with fruit maturation with a corresponding increase in disease susceptibility.

Gil et al. (2002) quantified phenolic compounds of 25 peach, nectarine, and plum cultivars and determined that the total phenolic content of white-flesh peaches ranged between 28 and 111 mg/100g fresh weight and that of yellow-flesh peaches between 21 and 61 mg/100g fresh weight. The same research group identified the phenolic compounds in peach extracts as hydroxycinnamic acid derivatives (neochlorogenic acid and chlorogenic acid), flavan-3-ols (procyanidin B1, catechin, epicatechin) and in the peels, flavonols (cyanidin 3-glucoside, cyanidin 3-rutinoside, quercetin 3-galactoside) (Tomás-Barberán et al., 2001).

The compositions of three types of peach juices were studied by Versari et al. (2002) and it was reported that the main phenolic compounds in peach juice are catechin (20-34 mg/kg), chlorogenic acid (19 mg/kg), caffeic acid (1.3-1.8 mg/kg) and isoquercetin (7.1 mg/kg).

Eight peach genotypes were characterized for their total phenolic and anthocyanin contents by Cevallos-Casals et al. (2006). The main anthocyanin identified in peach was cyanidin 3-glucoside with contribution of cyanidin 3-rutinoside. Apart from anthocyanins, several hydroxycinnamates, flavan 3-ols and flavonols, predominantly chlorogenic acid, neochlorogenic acid, catechin, epicatechin, and quercetin 3-rutinoside were also identified.

Apples were reported to have high amount of phenolic compounds among fruits by many researchers. Sun et al. (2002) found that, among the fruits cranberry, apple, red grape, strawberry, pineapple, banana, peach, lemon, orange, pear, and grapefruit; apple had the highest total phenolic content (296.3±6.4 mg/100 g) after cranberry (527.2 ± 21.5 mg/100 g).

Leantowicz et al. (2002) who compared some bioactive compounds in apples, peaches and pears and their influence on lipids and antioxidant capacity in rats also showed that apples had the highest content of phenolic compounds among the fruits investigated. The content of total phenolic compounds (g/100g) was found as 0.23 ± 0.03; 0.22 ± 0.03 and 0.68 ± 0.1 in peeled fruits and 0.48 ± 0.04, 0.47 ± 0.04 and 1.2 ± 0.12 in peels of peaches, pears and apples, respectively.

The phenolic compounds in apple were identified by Alonso-Salcez et al. (2001) as (+)-catechin, procyanidin B2, (−)-epicatechin, unknown procyanidin, phloretin-2′-xyloglucoside, phloridzin, hyperoside, isoquercitrin, unknown quercetin glycosides+rutin, avicularin, quercitrin, chlorogenic acid and *p*-coumaric acid derivative.

Guyot et al. (1998) identified the main phenolic compounds present in apple as catechins (monomeric flavan-3-ols) and procyanidins (polymeric flavan-3-ols), some dihydrochalcones (phloretin glycosides), some flavonols (quercetin glycosides) and hydroxycinnamic acid derivatives.

Lu and Foo (1997) extracted and identified phenolic compounds from apple pomace. The major phenolics identified were epicatechin, caffeic acid, phloretin-2-glucoside (phloridzin), phloretin-2'-xyloglucoside, 3-hydro-xyphloridzin, quercetin-3-arabinoside (avicularin), quercetin-3-xyloside (reynoutrin), quercetin-3-galactoside (hyperin), quercetin-3-glucoside (isoquercitrin) and quercetin-3-rhamnoside (quercitrin). The total amount of phenolic compounds was determined as 7.24 g/kg dry matter.

Lee et al. (2003) identified and quantified the major phenolic phytochemicals of six apple cultivars as (in terms of mg/100 g of fresh weight of apples): quercetin glycosides, 13.20; procyanidin B(2), 9.35; chlorogenic acid, 9.02; epicatechin, 8.65; phloretin glycosides, 5.59. They also determined their contributions to total antioxidant activity using a 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay and expressed as vitamin C equivalent antioxidant capacity (VCEAC). The estimated contribution of major phenolics and vitamin C to the total antioxidant capacity of 100 g of fresh apples was as follows (in terms of VCEAC): quercetin (40.39) > epicatechin (23.10) > procyanidin B(2) (22.07) > vitamin C (12.80) > phloretin (9.11) > chlorogenic acid (8.75). Their results indicate that flavonoids such as quercetin, epicatechin, and procyanidin B(2) rather than vitamin C contribute significantly to the total antioxidant activity of apples.

1.3 Phenolic Compound Extraction from Industrial By-products

Generally, the researchers that investigated the phenolic content of fruits and vegetables, examined the flesh and peel of the fruits separately. Peels of fruits have been reported to be one of the rich sources of natural antioxidants. Wolfe et al. (2003) investigated the phytochemical content, antioxidant activity, and antiproliferative activity of the peel, flesh and peel+flesh parts of four varieties of apples commonly used in applesauce production in New York State. The total phenolic contents and the antioxidant activities of the extracts obtained from the peels were higher than the flesh. In accordance with these findings, the same researchers (Wolfe and Liu, 2003) developed a value-added ingredient from Rome Beauty apple peels by blanching, drying (oven-dried at 40, 60, or 80°C, air-dried, or freeze-dried) and grinding to a powder form. On a fresh weight basis, the total phenolic and flavonoid contents of these samples were similar to those of the fresh apple peels. The freeze-dried apple peels also had a strong antiproliferative effect.

Leantowicz et al. (2002) searched for the antioxidant capacity of apples, peaches and pears. The authors indicated that the peels have a high content of bioactive compounds and can be used for individual consumption and also for industrial processing. Cevallos-Casals et al. (2006) characterized fourteen red-fleshed plum and eight peach genotypes for their total phenolic and anthocyanin contents. Plums showed a 3- to 4-fold higher phenolic concentration in the skin than in the flesh. Similarly, the anthocyanin concentration in the skin was 3- to 9-fold higher than in the flesh.

Toor and Savage (2005) determined the major antioxidants and antioxidant activity in different fractions (skin, seeds and pulp) of three tomato cultivars (Excell, Tradiro and Flavourine) grown under hydroponic conditions (a method of growing a plant in water containing dilute nutrients) in a commercial greenhouse in New Zealand. They found that the skin fraction of all cultivars had

significantly higher levels of total phenolics, total flavonoids, lycopene, ascorbic acid and antioxidant activity compared to their pulp and seed fractions, and suggested that removal of skin and seeds of tomato during home cooking and processing results in a significant loss of antioxidants.

The high content of phenolics in the peels renders industrial residues as attractive sources for the utilization of these compounds. There are many examples in the literature for the use of fruit and vegetable processing by-products for phenolic compound extraction.

Rødtjer et al. (2006) extracted phenolic compounds from the pomace obtained after production of cherry liquor, which was previously a waste product with no value. Extractions were carried out by shaking (200 rpm) the sample-solvent mixtures at 20°C for 20 min. Different solvents (water, methanol, ethanol, acetone and 2-propanol) and their mixtures with water were examined for their efficiency on extraction. The amount of phenolic compounds obtained by 70% acetone-water mixture, which was the most efficient solvent, was found as 92.8 ± 15.5 mg gallic acid equivalent (gae)/L. The antioxidant capacity of the extracts were determined as 3.47 ± 0.39 mmol Fremy's salt radical quenched per litre extract.

Another research which utilizes grape pomace to obtain phenolic compounds was based on chromotographic properties, stability and antioxidant capacity of anthocyanins in the extracts obtained from different vinification methods: one rose wine vinification (6 h of skin contact time) and three red wine vinifications (4, 8 and 12 days of maceration) (Gómez Plaza et al., 2006). The grape pomaces were extracted for 72 h with sulphited water (1 g/L) at 60°C by stirring. As a result, the aqueous extract obtained from the pomace of a rose wine vinification process gave the highest concentration of anthocyanins and highest color intensity together with an important antioxidant capacity. The chromatic characteristics and stability of the extracts suggested that they could be used for

coloring acidic foods, especially those that are going to be kept at low temperatures for a limited time before consuming. It is also indicated by the authors that they can be used as health supplements and nutraceutical compounds because of their considerable antioxidant capacity.

Su and Silva (2006) evaluated the effects of fermentation type on retention of total anthocyanins, total phenolics and antioxidant activity of blueberry by-products. Total phenolics, total anthocyanins, antioxidant activities (β-carotene bleaching assay and ferric thiocyanate assay) and antiradical activity ($DPPH^{\cdot}$ radical-scavenging assay) of rabbiteye blueberry by-products (juice, wine and vinegar pomaces) were determined. Wine pomace had higher total phenolic content, antioxidant activities and antiradical activity, where vinegar pomace had the lowest. Their results indicate that the antioxidant and antiradical activities of blueberry by-products were not significantly affected by the wine making process. Acetification during vinegar production significantly decreased total phenolic content, anthocyanin content, antioxidant activities and antiradical activity. However, the researchers indicate that vinegar pomace still maintained an important phenolics concentration and antioxidant activity.

Industrial residues from mango processing were utilized by Berardini et al. (2005) to recover pectin and phenolic compounds. Antioxidative capacities were investigated using the $DPPH^{\cdot}$, TEAC (Trolox equivalent antioxidant capacity) and FRAP assays and compared with some referense compounds like gallic acid, mangiferin and quercetin 3-O-glucoside. The antioxidative capacity of the extracts exceeded that of mangiferin and quercetin 3-O-glucoside, thus demonstrating mango peels to be a suitable source of health-beneficial compounds.

Bonilla et al. (1999) extracted phenolic compounds from red grape marc resulting from red winemaking with a mixture of ethyl acetate and water. The extractions were carried out at 20°C for 5, 10, 20 and 30 minutes and the extracts

were further purified by solid phase extraction. The antioxidant activity of the phenolic compounds extracted was determined using the Rancimat method on refined oil and compared with the activities of synthetic food antioxidants. The phenolics of the extract was found to have a lower antioxidant activity than BHA and propyl gallate, but was close to that of BHT.

1.4 Extraction of Phenolic Compounds

Extraction is the first step for the isolation of phenolic compounds. Enzyme activity of the plants, oxygen and light during the extraction affect the efficiency, therefore extreme care must be taken to avoid hydrolysis, oxidation and/or isomerization (Tsao and Deng, 2004).

1.4.1 Solvent Extraction

Classical techniques used to obtain phenolic compounds from plants include Soxhlet extraction and maceration with an appropriate solvent for a certain time.

Soxhlet extraction is a general and well-established technique and is the main reference for evaluating the performance of other solid–liquid extraction (or leaching) methods. In a conventional soxhlet system, plant material is placed in a thimble-holder and filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the holder and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. In the solvent flask, solute is separated from the solvent using distillation. Solute is left in the flask and fresh solvent passes back into the plant solid bed. The operation is repeated until complete extraction is achieved (Wang and Weller, 2006).

During soxhlet extraction, the solvent is usually recovered by evaporation, which represents a major disadvantage when working with toxic solvents. Therefore, the classical method for the extraction of phenolic compounds is maceration. This technique simply involves separation of soluble phenolic compounds by diffusion from a solid matrix using a solvent. The mechanism consists of two stages: initial and diffusion stage. The initial stage is swelling of the solid particles due to sorption of the solvent in the solid phase caused by osmotic forces, capillarity and solvation of the ions in the cells. Therefore, extraction of a certain percentage of phenolics occurs directly in this stage and soluble components dissolve in the liquid phase. In the diffusion stage, phenolics diffuse from the solid phase through the outer layers that surround the particles.

The most widely used solvent for extracting phenolic compounds is methanol and its mixtures with water (Gil et al., 2002; George et al., 2004; Kähkönen et al., 1999; Kanner et al., 1994; Karadeniz et al., 2005; Su and Silva, 2006; Bocco et al., 1998). However, due to the toxicity of methanol, other solvents like ethanol, acetone, ethyl acetate, diethyl ether, acetonitrile, 2-propanol and their mixtures with water can be used although they usually provide lower yields when compared with that of methanol (Perva-Uzunalić et al., 2006; Berardini et al., 2005; Bonilla et al., 1999; Moure et al., 2001; Rødtjer et al., 2006). The presence of glycosylated phenolics tends to render the phenolic compounds more water soluble and combinations of the above solvents with water will increase the efficiency of the extraction. Aglycones which are less polar like isoflavones, flavanones, highly methoxylated flavones and flavonols are more soluble in non-aqueous solvents. Solvent extraction is usually carried out in a few steps in order to increase the efficiency of the extraction (Escribano-Bailon and Santos-Buelga, 2003).

Solvent extraction offers good recovery of phenolic compounds however, has several drawbacks like the use of large amounts of organic solvents, long extraction time, limited solvent choice for health assurance and possible

degradation of target compounds. There are many alternative methods that either eliminate or reduce these drawbacks. These are solid phase extraction, microwave assisted extraction, ultrasound assisted extraction, supercritical fluid extraction and high pressure extraction (Richter et al., 1996).

1.4.2 Solid Phase Extraction

Solid phase extraction is a column chromatography technique in which a solid sorbent material (e.g. alkyl bonded silica) is packed into the extraction column where reversible interactions between the analyte and the stationary phase occur such as non-polar interactions between the C–H links of the analyte and the C–H links of the adsorbent (Van der Waals forces). Then, the analytes are eluted from the system with a small volume of organic solvent. The most common support for non-polar interactions are C_{18} cartridges. Polar interactions include the links created by hydrogen bonding, dipole–dipole forces, etc. These interactions are typical of all the cyano (CN), amino (NH_2), diol or silica (Si) supports. Ion-exchange interactions can also take place when the analytes have negative or positive ionic charges (Ruiz-Gutiérrez and Pérez-Camino, 2000).

Solid phase extraction offers several advantages like smaller sample and solvent requirements as well as simplicity and ease of handling (Fiorentino et al., 2006; Escribano-Bailon and Santos-Buelga, 2003). However, the method has a drawback that the phenolic compound levels may be under-estimated. For example, the method is not effective for extracting flavanoids located in the solids found as suspension in juices so that an important fraction of phenolic compounds present will be under-estimated (Escribano-Bailon and Santos-Buelga, 2003).

Rostagno et al. (2005) used solid phase extraction for the concentration and clean-up of the extracts obtained by solid-liquid extraction method. The solid

phase extraction method developed by these researchers was indicated to be fast (less than 10 min), extremely accurate and reproducible, achieving high isoflavone recoveries (>98%). The optimized method also concentrates the sample (from 25 ml to 4 ml) allowing measurement of isoflavones at low concentrations and provides cleaner extracts.

Chen et al. (2001) separated, identified and quantitated flavonoids and phenolic acids in cranberry juice using HPLC with UV–VIS photodiode array detection. A solid-phase extraction with a Sep-Pak C_{18} cartridge was used to clean and fractionate free phenolic acids and flavonoids. The described solid phase extraction–HPLC method was indicated to be easy and simultaneous method for determination of flavonoids and other phenolic compounds in cranberry juices and offered to be used in the separation of phenolic antioxidants in other fruit juices.

Palma et al. (2002) coupled PLE in-line with solid-phase extraction to extract phenolic compounds from grapes so that the whole process is carried out under an inert atmosphere. The in-line process reduces the sample handling and therefore the possibility of degradation of the extracted compounds. Using solid phase extraction increased the degree of selectivity in the extraction.

Picinelli et al. (1997) used a C_{18} solid-phase column to extract phenolic compounds of low molecular mass in apple samples. This extraction method was shown to have a greater extraction ability compared with liquid-liquid extraction using the ethyl acetate method.

1.4.3 Microwave Assisted Extraction

Microwave assisted extraction is a method, which uses the energy of microwave radiation to heat solvents quickly and efficiently. A rapid delivery of energy to a total volume of solvent and solid plant matrix, results in subsequent heating of the solvent and solid matrix efficiently and homogeneously. Because water within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of chemicals from the matrix, improving the recovery of nutraceuticals (Wang and Weller, 2006).

The method has many advantages over conventional extraction, which are shorter time, less solvent, higher extraction rate and less polarity limitation for the solvent (Escribano-Bailon and Santos-Buelga, 2003).

There are a few studies in the literature on utilization of microwave energy for the extraction of phenolic compounds from foods. Štěrbová et al. (2004) combined microwave-assisted isolation and solid-phase purification procedures prior to the chromatographic determination of phenolic compounds in plant materials, which provides a useful tool for isolation and purification of bioactive compounds. The highly repeatable results of the microwave assisted extraction procedure and high efficiency of the solid phase extraction clean-up step are indicated to be the main advantages of the proposed analytical procedure.

Pan et al. (2003) presented a microwave-assisted extraction method for the extraction of phenolic compounds from green tea leaves. The extraction yield of phenolic compounds with microwave-assisted extraction for 4 min (after pre-leaching for 90 min at room temperature) were higher than those of extraction at room temperature for 20 h and ultrasonic extraction for 90 min at 20-80°C, respectively. The solvent was ethanol/water (1:1 v/v) for all extractions with the liquid/solid ratio of 20:1 ml/g. Microwave assisted extraction was shown to be

more effective than the conventional extraction methods studied, in terms of extraction time and efficiency.

1.4.4 Ultrasound Assisted Extraction

Ultrasound assisted extraction is often used for the extraction of plant materials using liquid solvents. This extraction process is faster in comparison with solvent extraction, because the surface area in contact between the solid and liquid phases is much greater due to particle disruption taking place (Filgueiras et al., 2000).

Herrera and Luque de Castro (2005) used ultrasound-assisted extraction for the determination of phenolic compounds present in strawberries. In addition, an evaluation of the decomposition of the phenolic compounds when subject to solid–liquid, subcritical water or microwave-assisted extraction and sonication was carried out in order to assess the type of energy that produces a lower degradation of the analytes. As a result, ultrasound-assisted extraction (3 min, solvent: water containing 1.2 M hydrochloric acid) was found to be much faster and producing less analyte degradation than methods as solid–liquid (35-37°C, 15-16 h, solvent: water containing 1.2 M hydrochloric acid), subcritical water (130°C, 10 min, solvent: water) and microwave-assisted extraction (3 min, solvent: water containing 1.2 M hydrochloric acid).

Goli et al. (2005) compared solvent and ultrasound assisted extraction of phenolic compounds from pistachio hull using three different solvents, water, methanol and ethyl acetate. The ultrasound-assisted extraction procedure was carried out by adding 20 ml of solvent to 2.5 g of powdered hulls and sonicating the mixture in an ultrasonic bath for 45 min. As a result, they found no significant difference ($P<0.05$) in the extraction yields between the solvent extraction and ultrasound assisted extraction.

1.4.5 Supercritical Fluid Extraction (SFE)

1.4.5.1 Supercritical Fluids (SCFs)

The temperature above which a gas can not be liquefied regardless of the applied pressure is called the critical temperature (T_c) and the pressure required to liquefy the gas at its critical temperature is called the critical pressure (P_c). The fluid which has a temperature and pressure above the critical values is called a supercritical fluid (SCF) (Mchugh and Krukonis, 1994).

SCFs combine the characteristics of both gases and liquids, which is an advantage for SFE. SCFs are highly compressible in the vicinity of their critical points where large density changes can be caused by relatively small changes in either pressure or temperature. The high density of SCFs results in higher solvent power towards materials that are normally less soluble in the gas or liquid state of the fluid. SCFs also have high diffusion coefficients and low viscosities similar to those of gases, which facilitates diffusion and improves access to phenolic compounds bound to the cell wall (Escribano-Bailon and Santos-Buelga, 2003).

CO_2 is generally the most desirable solvent for SFE of natural products because it is inert, non-toxic, colorless, odorless, clean, inexpensive and readily available. In addition, it has a low critical temperature (31^{o}C), which makes it attractive for the extraction of heat-sensitive compounds (Zhang et al., 1998; Tonthubthimthong et al., 2001). However, since CO_2 is non-polar, it is not a good solvent for polar phenolic compounds. Addition of organic co-solvents like ethanol, methanol, acetone, increases the polarity and the yield of extraction of phenolic compounds. When a co-solvent is added to CO_2, the critical temperature of the resulting mixture is elevated. In cases where the addition of high amounts of the co-solvent to CO_2 is necessary, but elevated temperatures are not preferred, the extraction performed under the critical temperature of the

mixture is called subcritical (or near critical) CO_2 extraction. Table 1.2 shows the change in critical properties of CO_2 with ethanol addition.

Table 1.2 Change in critical properties of CO_2 with ethanol addition (SF-Solver Software, ISCO Inc., Lincoln, NE, USA)

Ethanol concentration (wt %)	T_c (^oC)	P_c (MPa)
0	31.1	7.38
5	42.5	7.32
10	53.7	7.27
15	64.9	7.21
20	76.1	7.15
100	243.3	6.13

1.4.5.2 Principle of SFE

During SFE, raw plant material is loaded into an extraction vessel, which is equipped with temperature controllers and pressure valves at both inlet and outlet to keep desired extraction conditions. The vessel is pressurized with the fluid by a pump. The process occurs in two steps. In the first step, the compounds of interest are solubilized by the supercritical fluid and extracted from the matrix. In the second step, the vent valve of the extractor is opened immediately and the soluble compounds are trapped either in a liquid solvent (usually methanol or ethanol) or on an inert solid matrix. Palma and Taylor (1999a) indicates that the advantage of solid trapping over liquid trapping is the use of less amount of solvent to elute analytes from the solid trap. In case of liquid trap, a reduction in the volume of the solution via evaporation runs the risk of losing extracted volatiles. The authors also indicate that the extraction step may have a great

impact on the trapping step especially when a modified supercritical fluid is used.

The solubility of the compounds to be extracted in the supercritical fluid is probably the most important property that must be determined in order to design a supercritical extraction process. However, solubility data of phenolic compounds are very limited in the literature.

Murga et al. (2002) determined the solubility of some low molecular weight phenolic compounds, 3,4-dihydroxy benzoic acid (protocatechuic acid), methyl 3,4,5-trihydroxybenzoate (gallic acid methyl ester or methyl gallate) and 3,4-dihydroxy benzaldehyde (protocatechualdehyde) in supercritical CO_2 at 10-50 MPa and 313-333 K and found that solubility of the selected phenolic compounds increase with pressure at constant temperature. Their results also show a decrease of solubility with temperature at pressures up to 15 MPa, where temperature has a positive effect at pressures higher than 15 MPa.

Solubility of catechin (Berna et al., 2001a), epicatechin (Cháfer et al., 2002) and resveratrol (Berna et al., 2001b) in supercritical CO_2 + ethanol at 313 K and 8-14 MPa was determined using 5-15 % ethanol as a modifier. The solubility of catechin and epicathechin increased with increasing percentage of ethanol where that of resveratrol showed a maximum at about 7.5 % ethanol. Pressure had a positive effect on solubility of all phenolic compounds of interest.

Cháfer et al. (2004) determined the solubility of quercetin in supercritical CO_2 + ethanol at 313.15 K and 8-12 MPa using 5-30 % ethanol as a modifier and showed that the solubility increased both with pressure and ethanol %.

1.4.5.3 Applications of SFE for Phenolic Compounds

SFE is an alternative extraction method for the food industry due to the advantages such as safety, non-toxicity and easy removal of solvent. Moreover, the absence of light and air during extraction can reduce the risk of degradation reactions. Therefore, SFE has found applications for the extraction of phenolic compounds, which can be oxidized in the presence of light and oxygen.

Goli et al. (2005) compared SFE of phenolic compounds from pistachio hull with solvent extraction and ultrasound-assisted extraction. The extracts were collected in methanol during SFE. Effects of extraction pressure (10.13, 20.26, 35.45 MPa), temperature (35, 45, 55°C) and time (15, 25, 40 min) were investigated. They have also studied the effect of modifier (0, 5, 15 % v/v) by spiking methanol directly on the sample in the extraction vessel prior to extraction. The combination of extraction conditions which gave the best recovery of phenolic compounds (7.8 mg tannic acid equivalents/g dry weight) was 35.45 MPa, 45°C, 15 min and 15% modifier. Although SFE was found to be less efficient than solvent extraction with methanol and water as solvents, it was compatible to solvent and ultrasound assisted extraction carried out with ethyl acetate.

In another research, Grigonis et al. (2005) compared SFE, microwave assisted extraction and Soxhlet extraction for the isolation of antioxidants from sweet grass (*Hierochloë odorata*). The extraction parameters were 40°C, 35 MPa and 2.5-30% (w/w) ethanol as modifier. Although it was found that recovery of compounds continuously increased with increasing modifier content, the authors selected 20% ethanol as optimal composition because increasing the polarity further was found to increase extraction of undesired compounds (the co-extracted "ballast" substances). As a result, the best extraction yield expressed as $(mass_{compound}/mass_{herb})*100$ was obtained with Soxhlet extraction (0.58 %) but yield obtained with SFE was also comparable (0.46 %). The yields obtained with

microwave-assisted extraction and Soxhlet + microwave-assisted extraction were 0.30 % and 0.38 %, respectively.

Luengthanaphol et al. (2004) extracted antioxidants from the seed coat of sweet Thai tamarind using supercritical CO_2 and CO_2 modified with 10% ethanol. The extractions were performed in the range of 35-80°C and 10-30 MPa. The researchers found that both parameters had a negative effect on the amount of cathechins extracted and selected the best conditions as 40°C and 10MPa. As a result of HPLC analysis, only (-)-epicathechin was determined in the extracts. Extraction of (-)-epicathechin with supercrtical CO_2 was very low (22µg/100g seed coat) which increased considerably (13 mg/100g seed coat) with the addition of 10% ethanol as a modifier. The results were compared with solvent extraction carried out using 10 ml of ethanol and ethyl acetate per gram of seed coat at ambient temperature shaking at 200 rpm for 5 h. Accordingly, it was shown that solvent extraction with ethyl acetate (25 mg/100g seed coat) and ethanol (150 mg/100g seed coat) were better than SFE with CO_2 and CO_2 + 10% ethanol.

Palma and Taylor (1999b) used near critical CO_2 to extract phenolic compounds from white grape seeds. CO_2 density, organic modifier, percentage of modifier and extraction temperature were optimized. Gallic acid, catechin and epicatechin were the main phenolic compounds detected in the HPLC chromatogram of each extract. Under optimized conditions (CO_2 density: 0.95 g/ml; solvent: 10 % methanol; 55°C), the recovery was estimated to be 79% with a relative standard deviation equal to 7.3%. In addition, SFE using methanol-modified CO_2 yielded higher catechin and other phenolic compound recoveries from grape seed than liquid–solid extraction. Reproducibility by SFE was found to be lower, but SFE was suggested to be more advantageous since it was fast and the extracts were more protected from degradation processes.

Lin et al. (1999) extracted flavonoids from *Scutellaria baicalensis,* a plant used in Chinese medicine, by SFE. The effect of temperature (40, 50, 60, 70°C), pressure (20, 30, 40 MPa) and two types of modifier (pure methanol and 70% methanol-water; 5, 10, 15 % v/v) were studied. Modifier was directly added onto the sample just before the extraction. Accordingly, the optimal conditions of SFE was found to be 50°C, 20 MPa and 70% methanol-water as modifier with the ratio of 15 % (v/v).

Le Floch et al. (1998) extracted phenolic compounds from olive leaf samples by SFE. The effects of extraction variables such as modifier content (0-20% v/v), pressure (15.5-33.4 MPa, temperature (80-120°C), flow-rate (1-4 ml/min), extraction time (5-140 min), packing materials for analyte trap (ODS and PorapackQ) and rinse solvent (methanol and *n*-hexane) were studied. As a result, 10% methanol, 33.4 MPa, 100°C, 2 ml/min, 140 min was selected to be the best conditions. PorapackQ and methanol were selected as the packing material and rinse solvent, respectively. Feasibility of using ethanol as a modifier instead of methanol was also investigated because the proposed SFE of phenolics could be implemented by the food, cosmetic and pharmaceutical industry which necessitates the modifier to be non-toxic. Ethanol was found to be less effective than methanol: the extraction yield of phenols obtained with 10% ethanol as modifier was 2.0 mg/g dry leaves., the yield obtained with 10% methanol under the same conditions was 3.6 mg /g dry leaves. The optimized method was also compared with ultrasound assisted liquid solvent extraction with solvents methanol, *n*-hexane, diethyl ether and ethyl acetate. The ultrasound assisted extractions were carried out at room temperature using 100 mg sample in four extarction cycles with n-hexane (first extraction for 30 min with 4 ml and the other three extractions for 15 min with 2 ml hexane). SFE at optimum conditions was much more efficient than ultrasound assisted extraction with *n*-hexane, diethyl ether and ethyl acetate but recovered only 45% of the amounts extracted by methanol.

1.4.6 High Pressure Extraction (HPE)

1.4.6.1 Principles of HPE

High pressure extraction is a technique that packs solid samples into an extraction cell and uses an organic solvent at high pressures and temperatures that can be above the boiling point of solvent (up to 200°C) for short periods of time with reduced solvent consumption (Ding & Fann, 2000; Alonso-Salces et al., 2001; Richter et al., 1996). The method has an advantage like performing extractions in an atmosphere protected from light, which prevents degradation of phenolic compounds. Furthermore, the method has advantages offered by high pressure and temperature.

High pressure increases the rate of dissolution. Moreover, the differential pressure between the inner and the exterior of the cell is very large under HPE conditions which facilitates the solvent penetration through the broken membranes into cells or increases the mass transfer rate due to increased permeability (Shouqin et al., 2004). The water in the sample or air may block the entrance into the solutes trapped in pores, so that solvents may not be able to contact these solutes and extract them. The use of elevated pressures (along with elevated temperatures and the reduced solvent surface tensions) will help force the solvent into the pores to contact the solute.

Elevated temperatures improve the efficiency of extraction since the cell walls become more permeable, the solubility and diffusion coefficients of the compounds to be extracted increase and viscosity of the solvent decreases, which facilitates its penetration through the solid matrix. High temperature can also disrupt the strong solute-matrix interactions caused by van der Waals forces, hydrogen bonding, and dipole attractions between the solute molecules and active sites on the matrix because thermal energy can overcome cohesive (solute-solute) and adhesive (solute-matrix) interactions by decreasing the activation

energy required for the desorption process (Escribano-Bailon and Santos-Buelga, 2003; Richter et al., 1996).

1.4.6.2 Applications of HPE for Phenolic Compounds

HPE has gained importance in recent years as an alternative for the other extraction technologies. In the study of Rostagno et al. (2004), isoflavone derivatives from freeze-dried soybeans were extracted by pressurized liquid extraction (PLE) and determined by HPLC. Several extraction solvents (water and 30–80% methanol or ethanol in water), temperatures (60–200°C), pressures (10.13–20.26 MPa), as well as the sample size (0.5–0.05 g) and cycle length (5–10 min) were studied for the optimization of the isoflavone extraction. The optimized extraction conditions were: 0.1 g of sample, 100°C, three (7 min) static extraction cycles and 70% ethanol as extracting solvent. Increasing the pressure from 10.13 to 20.26 MPa did not show any difference in the extraction of isoflavones.

Papagiannopoulos et al. (2002) analysed proanthocyanidins from malt by online coupling of PLE, solid-phase extraction and HPLC. Acetone–water (80:20, v/v) was used as a solvent. A temperature lower than 60°C resulted in inefficient extraction and higher temperatures showed analyte loss due to thermal degradation. However, no thermal degradation during the extraction was observed at 60°C. A pressure setting of 100 or 200 MPa showed no difference. Therefore, the optimized extraction conditions were 60°C, 100 MPa, 25 min of extraction time and 14 ml of extracting volume for 4 grams of sample, which resulted with a recovery of 97 %.

Ju and Howard (2003) used PLE to extract anthocyanins from the freeze-dried skin of a highly pigmented red wine grape with six solvents (0.1% HCl in deionized water (acidified water) (pH 2.3); 0.1% HCl in 60% ethanol (acidified

ethanol) (pH 2.2); 0.1% HCl in 60% methanol (acidified methanol) (pH 2.3); 0.1% HCl in 40:40:20 (methanol/acetone/water; solvent mixture) (pH 1.9); 7% acetic acid in 70% methanol (pH 2.0) and 0.1% trifluoroacetic acid in 70% methanol (pH 2.1) at 50°C, 10.1 MPa, and 3 × 5 min extraction cycles. Temperature (from 20 to 140°C in 20°C increments) effects on anthocyanin recovery by acidified water and acidified 60% methanol were also studied. Acidified methanol extracted the highest levels of total monoglucosides and total anthocyanins, whereas the solvent mixture (40:40:20 methanol/acetone/water with 0.1 % HCl) extracted the highest levels of total phenolics and total acylated anthocyanins. Optimum temperatures for the extraction of total anthocyanins were determined as 80-100°C for acidified water and 60°C for acidified 60% methanol. As a result, the authors indicated that high-temperature PLE using acidified water is effective for isolating anthocyanins from grape skins.

In the study of Palma et al. (2001), the stability of phenolic compounds (*p*-coumaric acid, vanillin, veratric acid, protocatechuic aldehyde, gentisic acid, caffeic acid, syringic aldehyde, catechin and epicatechin) in the extraction conditions with methanol at 10 MPa and different temperatures (40, 50, 100 and 150°C) was tested. The amount of sample was 4 g in the extraction cell volume of 11 ml and the collection vial volume was of 20 ml. After three 10 min cycles, all the assayed phenolic compounds were stable under the extraction conditions with the exception of catechin and epicatechin (recoveries: 87.4% for catechin and 86.0% for epicatechin at 150°C and 94.1% for epicatechin at 100°C. Phenolic compounds kept at the boiling point of methanol at atmospheric pressure (65°C) showed lower recoveries: gentisic acid (85.5%), syringic aldehyde (92.8%), catechin (63.7%) and epicatechin (63.4%).

Alonso-Salces et al. (2001) obtained phenolic compounds from Golden Delicious apple peel and pulp by PLE. The effects of solvent composition (0-100 % methanol in water), temperature (40-100°C), static extraction time (5-15 min) and pressure (6.9-10.3 MPa) on the extraction efficiency were studied.

Accordingly, pure methanol, 40°C, 5 min and 6.9 MPa were determimed to be optimum extraction conditions. As a conclusion, it was indicated that the efficiency of PLE is comparable to conventional techniques to extract phenolic compounds from apple peel and pulp.

1.6 Objectives of the Study

Utilization of industrial by-products for phenolic compound extraction and also extraction of bioactive compounds by using novel technologies have gained importance in recent years. In Turkey, the fruits that are processed in fruit juice industry between years 2000-2005 are apple (65.1%), peach (11.9%), sour cherry (5.9%), orange (5.3%) and apricot (5.0%) (Ekşi, 2006). Apple, peach and sour cherry juice processing by-products can be used for the extraction of phenolic compounds because of their high production capacity as well as their considerable phenolic content.

The objective of this study was to investigate the extraction of phenolic compounds from sour cherry, peach and apple pomace using HPE and subcritical CO_2 extraction. The optimum extraction conditions (pressure, temperature, solid/solvent ratio, extraction time for high pressure extraction and pressure, temperature, ethanol concentration in CO_2, extraction time for subcritical CO_2 extraction) were determined by response surface methodology by considering the total phenolic content and the antioxidant activity of the extracts.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The residue that remains after pressing when fruits are processed for juice, wine or other products is usually called pomace. It consists of pressed skins, pulp residue, seeds and stems and is rich in phenolic compounds (Su and Silva, 2006). Sour cherry (*Prunus cerasus*), peach (*Prunus persica* L.) and apple (*Malus domestica* B.*)* pomaces were obtained from the fruit juice production pilot plant of Ankara University, Department of Food Engineering. The part of the process involved in the production of pomace as a by-product is shown in Figure 2.1. The pomace remaining after pressing was mixed well, placed in 20x25 cm plastic bags and stored at -35ºC.

The pomace was freeze-dried at -5°C and 0.47 kPa (Model FD8, Heto Lab. Equipment, Allerød, Denmark) for 24 hours. Moisture content of the freeze-dried pomaces were determined by placing about 2 g of pomace in previously dried and weighed containers and keeping at 100°C until constant weight. Accordingly, moisture contents of sour cherry, peach and apple pomaces were found as 14.00 ± 0.75 % (n=5), 14.75 ± 0.92 % (n=5) and 13.82 ± 1.07 % (n=5), respectively.

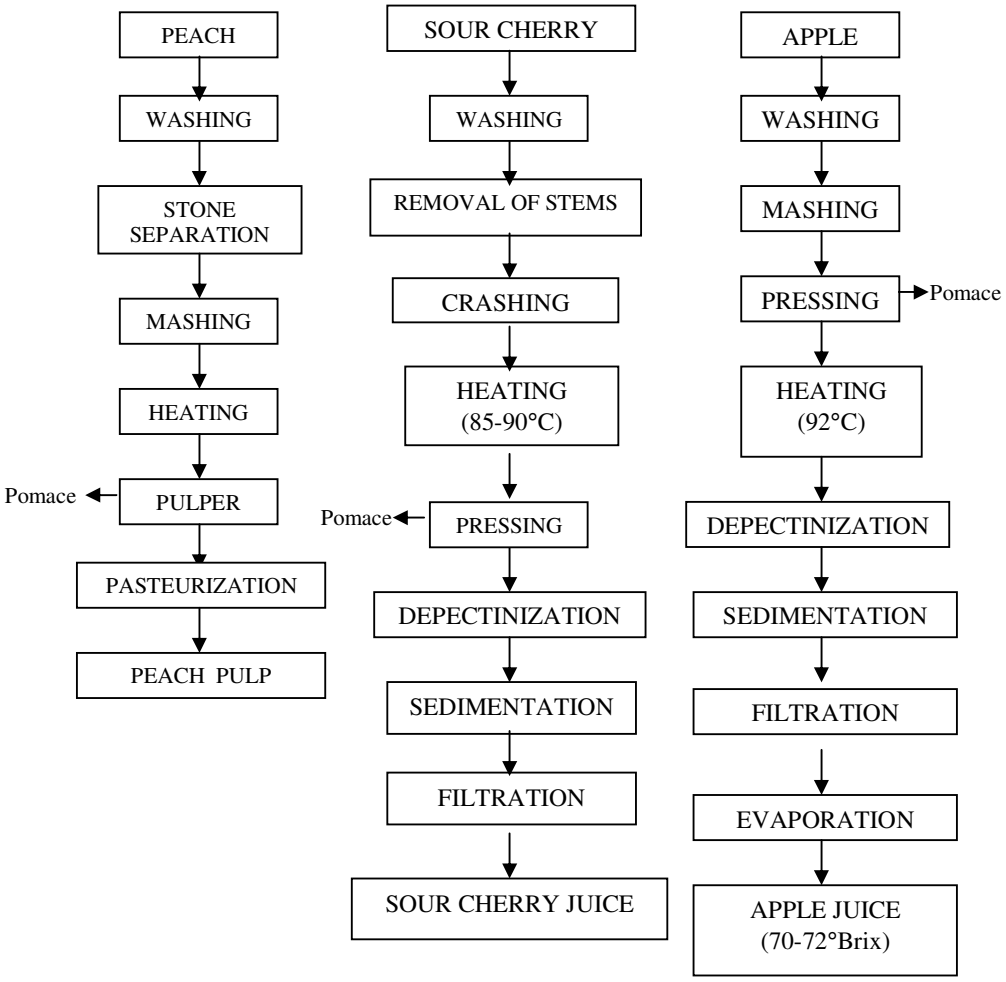

Figure 2.1 Pomace production from sour cherry, peach and apple processing

The dried samples were ground using kitchen-type grinder (Moulinex, France). The ground pomaces were sieved and fractionated according to particle size by certified sieves (Endecotts Ltd, London, England). Sieving was performed by a shaker (Octagon 200, Endecotts Ltd, London, England). The particles that passed through the sieve with an opening size of 0.425 mm and retained on that of 0.850 mm were used. Therefore, the average particle size was 0.638 mm by sieve analysis. The dried and ground samples were also kept at -35°C.

2.2 High Pressure Extraction (HPE)

HPE treatments were performed in a designed and constructed lab-scale unit (capacity: 30 cm^3) (Figure 2.2). The equipment consists of a pressure chamber of cylindrical design, a pressure pump, a hydraulic unit to generate high pressure and a temperature control device. The liquid was heated prior to pressurization to the desired temperature by an electrical heating system surrounding the chamber. Time to reach the desired pressure was approximately 5–10 seconds for the designed system. Pressurization time reported in this study did not include the pressure increase and release times.

Samples were placed inside 3 ml vials, filled with ethanol (≥99.8 %, Riedel, Inc., Steinheim, Germany) and closed. The vials were placed inside the cylindrical vessel of the high hydrostatic pressure equipment and the chamber was closed. Samples were pressurized at constant pressure and temperature for a certain time according to the experimental design (section 2.8) and then immediately removed, cooled and the extract at the top of the vials were transferred to another vial.

2.3 Subcritical CO_2 Extraction (SCE)

Extractions were done by a Supercritical Fluid Extraction System (SFX System 5100, ISCO Inc., Lincoln, NE, USA), which consists of an extractor (SFX 3560) and two syringe pumps (Model 100DX) that enables co-solvent addition (Figure 2.3). 1 gram of sample was placed into 10 ml aluminum sample cartridge. Ethanol absolute (≥99.8 %; Riedel, Inc., Steinheim, Germany) with CO_2 (99.9% ; Bos, İstanbul, Turkey) was used as a solvent for the extractions. The experimental design was given in section 2.8. The solvent flow was downward and the flow rate was kept constant at 2 g/min. The ethanol concentrations and flow rate of (CO_2-ethanol) were calculated in terms of (v/v) and ml/min,

respectively for each extraction condition and adjusted to the calculated values. Solvent containing the extract was passed through a restrictor, the temperature of which was adjusted to 80°C. The extracts were collected in ethanol. After the extraction, all extracts were transferred to closed vials and diluted to a constant volume of 3 ml with ethanol.

Figure 2.2 High hydrostatic pressure equipment

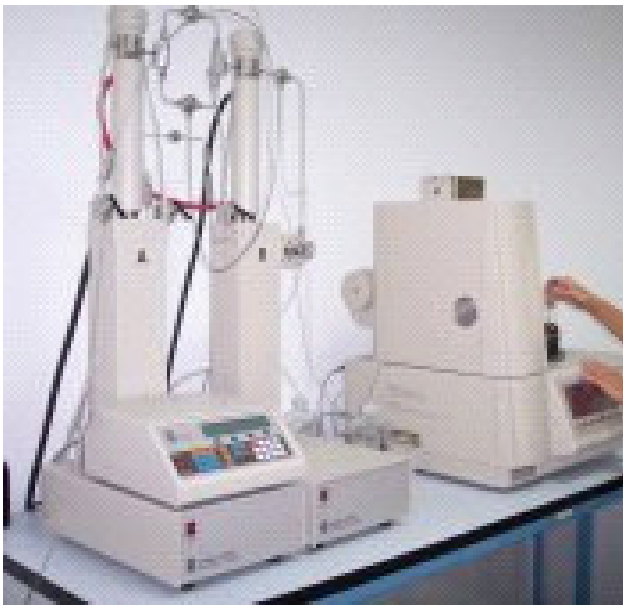

Figure 2.3 SCE equipment

2.4 Solvent Extraction (SE)

SE was used only for comparison. Ethanol (≥99.8 %, Riedel, Inc., Steinheim, Germany) and methanol (≥99.8 %, Riedel, Inc.,Steinheim, Germany) was used for solvent extraction of dried samples. Different mixtures with solid to solvent ratios 0.05, 0.1, 0.2, and 0.3 g/ml were prepared by adding 4 ml solvent on 0.2, 0.4, 0.8, and 1.2 g sample, respectively. The most general methodology for conventional extraction of phenolic compounds involves the use of aqueous methanol for 16–24 h at room temperature (Palma and Taylor, 1999b; Caillet et al., 2007). The mixtures were kept at room temperature in dark for 24 hours. The extracts were transferred into the closed vials.

2.5 Determination of Total Phenolic Content (TPC)

Folin-Ciocalteau method was used for the determination of total phenolic content (Singleton and Rossi, 1965). This method is based on the color change determined at 740 nm caused by reduction of the Folin-Ciocalteau reagent by phenolates produced in the presence of sodium carbonate.

For that purpose, 0.1 ml of extract was placed in a tube and 0.9 ml of distilled water was added. After wortexing, 5 ml of 0.2 N Folin-Ciocalteau's phenol reagent (SIGMA F 9252) was added and the contents were incubated for 5 minutes. Then 4 ml of 75 g/L sodium carbonate (MERCK 106392) solution was placed into the tubes, the tube contents were mixed on a wortex and incubated in dark at room temperature for 2 hours. The absorbance was measured at 740 nm (Pharmacia LKB-Novaspec II model spectrophotometer, UK). TPC was expressed as gallic acid equivalent (gae) using the standard curve prepared at different concentrations of gallic acid for each set of experiment. The equation for the standard curve was

$$A_{740} = 0.0109 \pm 0.0022[\text{gallic acid}] + 0.0622 \pm 0.0096 \quad (R^2 = 0.98\text{-}0.99) \qquad (2.1)$$

where A is absorbance at 740 nm and [gallic acid] is expressed as mg/L.

2.6 Determination of Antioxidant Activity

Free-radical scavenging activity assay was used for the determination of antioxidant activity (Brand-Williams et al., 1995). The assay is based on the color change caused by the reduction of a free radical. The two most widely used free radicals to be used in the assays are 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate ($ABTS^{+\cdot}$) (Gorinstein et al., 2004; Arnao, 2000) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radicals (Brand-Williams et al., 1995; Sánchez-Moreno

et al., 2003; Toit et al., 2001; Kumaran et al., 2006; Matkowski et al, 2006). $DPPH^{\bullet}$ is mostly prefered due to its stability and readiness to dissolve without preparation, where $ABTS^{+\cdot}$ must be prepared by enzymatic or chemical reactions (Arnao, 2000). The presence of phenolic compounds in the reaction medium leads to the disappearance of $DPPH^{\bullet}$, which causes a color change in the reaction medium.

$DPPH^{\bullet}$ (purple) + (PheOH) $\rightarrow$ DPPH-H (yellow) + $PheO^{\bullet}$

Different reaction times were used for the assay by different researchers. The most frequently used times are 30 min (Einbond et al., 2004), 60 min (Toit et al., 2001; Soler-Rivas et al., 2000) or time for the reaction to reach a plateau (Brand-Williams et al., 1995; Sánchez-Moreno, 2003). Because the radical is stable for several hours (Soler-Rivas et al., 2000), the reaction time can be selected as the time for the reaction of the slowest reacting compound to reach a plateau and can be applied for the faster reacting compounds. The most appropriate reaction time was decided by determining the time to reach a plateau for the reaction of gallic acid, which is one of the phenolic compounds with intermediate reaction rate (Sánchez-Moreno et al., 1998). For that purpose, different concentrations of gallic acid was prepared in methanol. 0.1 ml of gallic acid was mixed with 3.9 ml of 0.025 mg/ml $DPPH^{\bullet}$ (SIGMA D 9132) in a tube and absorbance values were measured at different time intervals starting from t=0 until the absorbance values reach to a plateau. As a result, time to reach plateau for the smallest concentration of gallic acid was about 40 min (Figure 2.4), which indicates that a reaction time of 40 min or more will be appropriate for the assay. The experimental incubation times were selected as 60 min during the experiments in order to assure that the plateau is achieved before determination of the absorbances.

For the assay, different amounts of extracts (0-0.2 ml) were placed in tubes and ethanol (≥99.8 %, Riedel, Inc., Steinheim, Germany) in the extracts was evaporated in dark at room temperature. 0.1 ml of methanol (≥99.9 %, Riedel,

Inc., Steinheim, Germany) was added. The tubes were mixed properly to allow the antioxidants dissolve in methanol and 3.9 ml of 0.025 mg/ml $DPPH^{\bullet}$ solution was added. After holding the tubes for 60 minutes in dark at room temperature, the absorbance values were measured at 515 nm (Pharmacia LKB-Novaspec II model spectrophotometer, UK) and were converted to $DPPH^{\bullet}$ concentration using the standard curve prepared for each set of experiment. Methanol was used as blank for the absorbance measurements. The equation for the standard curve was

$$A_{515}=25.023\pm0.2753\ [DPPH^{\bullet}] + 1.113\times10^{-2} \pm 0.0079 \quad (R^2=0.97\text{-}0.99) \qquad (2.2)$$

where A_{515} is the absorbance at 515 nm and $[DPPH^{\bullet}]$ is expressed as g/L

The percentage of remaining $DPPH^{\bullet}$ can be calculated as

$$\%\ DPPH^{\bullet}_{rem} = \left([DPPH^{\bullet}]_t / [DPPH^{\bullet}]_{t=0} \right) \times 100 \qquad (2.3)$$

The percentage of remaining $DPPH^{\bullet}$ against the sample concentration was then plotted to determine EC_{50} (efficient concentration of the sample to decrease the initial $DPPH^{\bullet}$ concentration by 50%). An example for the determination of EC_{50} for gallic acid is presented in Figure 2.5 with trendline. The antioxidant activity was expressed in terms of antiradical efficiency (AE) which is defined as (Mansouri et al., 2005; Atoui et al., 2005; Kallithraka, et al., 2005; Anagnostopoulou et al., 2006),

$$AE = 1/ EC_{50} \qquad (2.4)$$

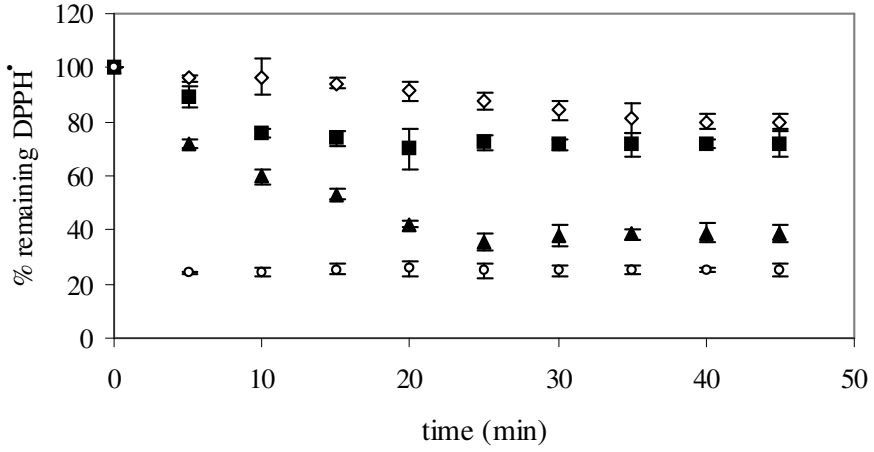

Figure 2.4 DPPH• reduction by gallic acid as a function of time (n=3); ◇ 0.01 g ga/g DPPH·, ■ 0.02 g ga/g DPPH·, ▲0.07 g ga/g DPPH·, ○ 0.1 g ga/g DPPH·

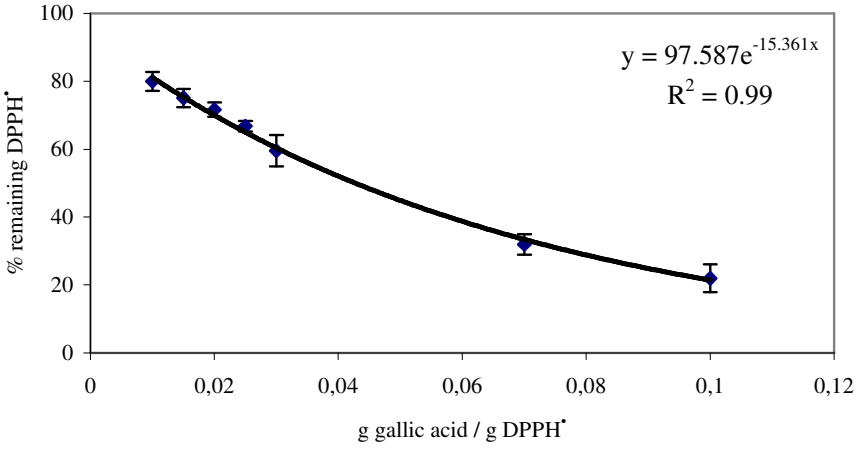

Figure 2.5 The disappearance of DPPH• as a function of gallic acid concentration (n=3)

2.7 Experimental Design and Response Surface Methodology

One of the common experimental designs used in engineering purposes is the Box-Behnken design. Each independent variable is included in the design at 3 levels rather than 5 levels required for a central composite design. Three level Box-Behnken design (Myers and Montgomery, 2002; Thompson, 1982) with four independent variables was used in this study. The independent variables were pressure, temperature, solid/solvent ratio and extraction time for high pressure extraction. Pressure, temperature, ethanol concentration in CO_2 and extraction time were the independent variables of subcritical CO_2 extraction. The uncoded and coded independent variables in the design for high pressure extraction and subcritical CO_2 extraction are given in Table 2.1 and Table 2.2, respectively. The Box-Behnken design is presented in Table 2.3.

Response surface methodology (RSM) is a statistical and mathematical technique useful for analysis and optimization of processes. In a response surface problem, a response variable Y is a function of k number of independent variable codes:

$$Y = f(X_1, X_2, \ldots, X_k) \tag{2.5}$$

The function f is called the response function and is assumed to be a continuous function of the independent variables. The actual form of f in Eqn.2.5 is either unknown or complex, but it is assumed that it can be approximated by a polynomial function of low order. First and second order models are frequently used in RSM studies. Second order models, with the advantage of being easy to fit using multiple regressions are usually preferred:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i \leq j}^{k} \beta_{ij} X_i X_j \tag{2.6}$$

where β_0, β_i and β_{ij} are constant coefficients and X_i are coded independent variables (Myers and Montgomery, 2002).

Table 2.1 Uncoded and coded independent variables for high pressure extraction

		Coded levels		
		-1	0	+1
Code	**Independent variable**		**Variable levels**	
X_1	Pressure (MPa)	50	125	200
X_2	Temperature (°C)	20	40	60
X_3	Solid/solvent ratio (g/ml)	0.05	0.15	0.25
X_4	Extraction time (min)	10	25	40

Table 2.2 Uncoded and coded independent variables for subcritical CO_2 extraction

		Coded levels		
		-1	0	+1
Code	**Independent variable**		**Variable levels**	
X_1	Pressure (MPa)	20	40	60
X_2	Temperature (°C)	40	50	60
X_3	Ethanol concentration (wt %)	14	17	20
X_4	Extraction time (min)	10	25	40

Table 2.3 Box-Behnken experimental design for high pressure and subcritical CO_2 extraction*

Experiment number**	X_1	X_2	X_3	X_4
1	+1	+1	0	0
2	+1	-1	0	0
3	-1	+1	0	0
4	-1	-1	0	0
5	0	0	+1	+1
6	0	0	+1	-1
7	0	0	-1	+1
8	0	0	-1	-1
9	0	0	0	0
10	+1	0	0	+1
11	+1	0	0	-1
12	-1	0	0	+1
13	-1	0	0	-1
14	0	+1	+1	0
15	0	+1	-1	0
16	0	-1	+1	0
17	0	-1	-1	0
18	0	0	0	0
19	+1	0	+1	0
20	+1	0	-1	0
21	-1	0	+1	0
22	-1	0	-1	0
23	0	+1	0	+1
24	0	+1	0	-1
25	0	-1	0	+1
26	0	-1	0	-1
27	0	0	0	0

* Definitions for coded levels (X_1, X_2, X_3 and X_4) are given in Table 2.1 and 2.2

** Experiments were performed in random order

2.8 Statistical Analysis

Experimental data were analyzed by multiple regression to fit the second order models to the dependent variables. The package program MINITAB 13.20 was used to perform regression analysis. The models were used to plot response surfaces using Statistica 5.0 (1995) and contour plots using Surfer 6.01 by keeping two independent variables constant. Contour plots obtained for TPC and AE were superimposed to estimate the optimum extraction conditions.

CHAPTER 3

RESULTS AND DISCUSSION

Recovery of antioxidants from natural sources has gained importance since the replacement of synthetic antioxidants by natural ones. Fresh fruits and vegetables, especially their peels, are the best carriers of antioxidants, therefore industrial residues are valuable sources for the extraction of these compounds.

Due to the long extraction times and relatively large amounts of solvents required for the traditional extraction methods like Soxhlet extraction, there is an increasing demand for new extraction techniques with shortened extraction time, reduced organic solvent consumption and decreased possibility of degradation of the target compounds

The solvents used for the extraction of phenolic compounds in the literature are methanol, ethanol, acetone, ethyl acetate, diethyl ether, acetonitrile, 2-propanol and their mixtures with water (Gil et al., 2002; George et al., 2004; Kähkönen et al., 1999; Kanner et al., 1994; Karadeniz et al., 2005; Su and Silva, 2006; Bocco et al., 1998; Berardini et al., 2005; Bonilla et al., 1999; Moure et al., 2001; Rødtjer et al., 2006). Although methanol was reported to be the most efficient solvent (Peschel et al., 2006; Perva-Uzunalić et al., 2006; Cháfer et al., 2002; Le Floch et al., 1998), ethanol was selected as the solvent in this study due to its low toxicity considering the possible future application of the extracted phenolic compounds in food products.

3.1 High Pressure Extraction (HPE)

Table 3.1 and Table 3.2 show the experimental data and the regression coefficients obtained by fitting experimental data to the second order response models (y) for total phenolic compounds (TPC) and antiradical efficiency (AE) of the extracts obtained by HPE. As a result of the t-tests for $p \leq 0.05$, all independent variables were found to be significant on TPC and AE of the extracts obtained from all samples by HPE. The interaction of temperature with solid/solvent ratio and time for the extraction of TPC and AE from sour cherry pomace was significant ($p \leq 0.05$). Interaction of pressure and temperature was also significant for the AE of the extracts obtained from sour cherry and apple pomaces. Pressure-solid/solvent ratio interaction was found to be significant for the AE of the extracts from peach pomace.

Figure 3.1-3.6 represent response surfaces for the TPC and AE of the extracts obtained from sour cherry, peach and apple pomaces by HPE. The effect of pressure on TPC and AE was positive for all samples. Although the cell walls of the fruits were broken during pressing for juice-making and the sample size was decreased during grinding after freeze drying, the actual packing of the sample in the extraction cell and the possible air bubbles that may be trapped within the solid particles composes a barrier for the diffusion of the solvent through the matrix. Elevated pressures facilitate solvent penetration through the interior of the sample matrix, therefore increases mass transfer rate (Shouqin et al, 2004). The findings in the literature in this respect differ from our results, which may be due to different samples and solvents used or lower pressure ranges selected. Papagiannopoulos et al. (2002) extracted proanthocyanidins from malt (4 g) by online coupling of pressurized liquid extraction, solid-phase extraction and HPLC using acetone–water (80:20, v/v) with a total volume of 14 ml at 60°C for 10 min at 100 and 200 MPa and found that those two pressure setting showed no difference. The findings of Alonso-Salcez et al (2001) also indicate that the effect of pressure at the studied range (6.9-10.3 MPa) was insignificant on the

extraction of phenolic compounds from apple peel and pulp (1 g) at the extraction conditions of 40°C, 5 min extraction time with a total volume of 25 ml of methanol as solvent. Rostagno et al. (2004) evaluated the effect of pressure (10 and 20 MPa) on the extraction of isoflavones from soybeans (0.5 g) at 100°C for 3 × 5 min cycles with 22 ml of 70 % ethanol and showed that increasing the pressure from 10 to 20 MPa did not influence the extraction of tested isoflavones.

In the range studied, the effect of temperature on the TPC extracted and their AE was also found to be positive. This result was expected because high temperature increases the solubility and diffusion coefficients of the compounds to be extracted (Escribano-Bailon and Santos-Buelga, 2003) and decreases the viscosity of the solvent, which facilitates its penetration through the solid matrix (Richter et al., 1996). However, the temperature range to be studied should be selected carefully so that the heat sensitive phenolic compounds should not be inactivated. Therefore, although high pressures were used during extractions so that the solvent can be kept at liquid state at temperatures above its boiling point, the temperature range was selected as 20-60°C in this study to prevent the inactivation of phenolic compounds at elevated temperature. One of the researches showing the negative effect of extremely high temperatures on the extraction of phenolic compounds is that of Bonoli et al (2004) who extracted phenolic compounds (hydroxycinnamic acids, flavan-3-ols, proanthocyanidins and hydrolysable tannins) from barley at 20 MPa and 60, 90, and 120°C with ethanol-water mixture (4:1, v/v) for two 5-min extraction cycles. They showed that increasing temperature has a negative effect on the TPC extracted. They indicated that this may be because of the inactivation of phenolic compounds due to the extremely high extraction temperatures used. Palma et al. (2001) examined the stability of nine phenolic compounds (*p*-coumaric acid, vanillin, veratric acid, protocatechuic aldehyde, gentisic acid, caffeic acid, syringic aldehyde, catechin and epicatechin) in the extraction conditions with the superheated methanol at 10 MPa and different temperatures (40, 50, 100 and 150°C) by using

a model system. After three 10 min cycles, all the assayed phenolic compounds were stable under the extraction conditions with the exception of catechin and epicatechin (recoveries: 87.4% for catechin and 86.0% for epicatechin at 150°C and 94.1% for epicatechin at 100°C). Phenolic compounds kept at the boiling point of methanol at atmospheric pressure in contact with air (65°C) showed lower recoveries: gentisic acid (85.5%), syringic aldehyde (92.8%), catechin (63.7%) and epicatechin (63.4%). Thus, the researchers proved that several phenolic compounds oxidized easily at high temperature (65°C) when they are in contact with the air. When higher temperatures are applied under nitrogen atmosphere, there were no degradations, since the degradation process for phenolic compounds is an oxidative process requiring the presence of oxygen. Ju and Howard (2003) also showed that extreme temperatures have a negative effect on the extraction of anthocyanins. They used pressurized liquid extraction to extract anthocyanins from the freeze-dried skin of a highly pigmented red wine grape with six solvents (acidified water, acidified ethanol, acidified methanol, 40:40:20 methanol/acetone/water, 7% acetic acid in 70% methanol and 0.1% trifluoroacetic acid in 70% methanol) at 50°C, 10.1 MPa, and 3 × 5 min extraction cycles. They studied the effect of temperature (20-140°C) on anthocyanin recovery by acidified water and acidified 60% methanol. Acidified methanol extracted the highest levels of total monoglucosides and total anthocyanins, whereas the solvent mixture (40:40:20 methanol/acetone/water with 0.1 % HCl) extracted the highest levels of total phenolics and total acylated anthocyanins. Optimum temperatures for the extraction of total anthocyanins were determined as 80-100°C for acidified water and 60°C for acidified 60% methanol. They showed that total anthocyanins are degraded at temperatures greater than 100°C. On the other hand, Cacace and Mazza (2003), working on extraction on anthocyanins from milled berries in an agitated vessel found that there was a sharp decrease in the amount of anthocyanins extracted at temperatures higher than 45°C for about 125-275 min, due to the susceptibility of those compounds to high temperatures. In the temperature range studied (6-74°C), the maximum anthocyanin extraction was obtained at 30-35°C using

ethanol as a solvent. Alonso-Salcez et al (2001) examined the effect of temperature on the extraction of phenolic compounds from apple peel and pulp with pure methanol at 6.9 MPa and 10 min extraction time. An increase in temperature from 40 to 60°C resulted with a slight increase in the extraction yield, however increasing the temperature further decreased the yield. The researchers indicated that this may be due to the inactivation of phenolic compounds above 60°C. Papagiannopoulos et al. (2002) showed that a temperature lower than 60°C resulted in inefficient extraction and higher temperatures showed analyte loss due to thermal degradation. No thermal degradation during the extraction was observed at 60°C. Rostagno et al. (2004) evaluated the stability of isoflavones from soybeans at elevated temperatures (60-200°C) at 10 MPa for 3×5 min cycles and their results showed that 100°C is the maximum temperature for extraction of isoflavones since degradation takes place beyond that temperature.

Increasing solid/solvent ratio decreased the TPC and AE for all samples, which may be due to the low concentration gradient at high solid/solvent ratios. Cacace and Mazza (2003), working on mass transfer process during extraction of anthocyanins from milled berries, examined the effect of solid/solvent ratio (0.0135-0.1667 g/ml) on the amount of anthocyanins extracted at 40°C with ethanol and found that anthocyanins extracted was higher at low solid/solvent ratios. Concentration gradient, i.e. the driving force during mass transfer within the solid, was greater when a lower solid-solvent ratio was used. Similar effects were seen by Rostagno et al. (2004), working on extraction of isoflavones from soybeans by accelerated solvent extractor. They found that extraction efficiency of some isoflavones constantly increased with the reduction of the amount of sample from 0.5 to 0.05 g in a total volume of 22 ml in the continuous extraction system.

Table 3.1 Experimental results for HPE

	Sour cherry pomace		Peach pomace		Apple pomace	
Exp. no	TPC[a]	AE[b]	TPC[a]	AE[b]	TPC[a]	AE[b]
1	3.26	17.31	0.89	7.00	1.84	9.54
2	2.28	12.58	0.53	4.37	1.15	7.91
3	2.66	14.67	0.70	5.21	1.86	8.99
4	2.22	13.77	0.19	1.38	0.80	6.58
5	3.01	10.10	0.77	4.69	1.53	8.24
6	2.37	9.36	0.59	3.59	1.37	6.63
7	2.82	19.23	0.97	6.06	1.84	8.21
8	2.62	15.87	0.66	4.96	1.79	7.65
9	2.70	14.67	0.67	4.59	1.42	9.33
10	2.90	16.25	0.77	6.25	1.64	8.03
11	2.73	13.81	0.48	3.91	1.46	6.64
12	2.44	14.80	0.56	4.03	1.57	7.11
13	2.36	11.91	0.38	3.77	1.31	6.12
14	2.71	12.13	0.66	4.19	1.75	7.58
15	3.73	21.03	0.88	7.40	1.95	10.45
16	2.11	9.80	0.41	2.64	1.05	7.53
17	1.84	15.82	0.54	4.36	1.25	8.39
18	2.79	13.70	0.58	4.18	1.39	9.51
19	2.31	11.44	0.67	3.94	1.51	5.94
20	2.99	20.52	0.78	8.55	2.12	9.29
21	2.25	11.83	0.40	2.86	1.36	5.75
22	2.38	19.08	0.52	5.01	1.76	8.22
23	3.32	16.43	0.97	6.50	1.65	10.36
24	3.38	15.33	0.52	4.71	1.53	8.54
25	2.55	15.03	0.38	3.55	1.17	8.01
26	1.79	9.84	0.21	2.31	1.04	7.93
27	2.74	14.63	0.61	4.79	1.52	10.09

[a] Total Phenolic Content (mg gae/g sample)

[b] Antiradical Efficiency (mg $DPPH^{\cdot}$/g sample)

Table 3.2 Second order response model constants and regression analysis for TPC and AE of the extracts obtained by HPE[a]

	Sour cherry pomace		Peach pomace		Apple pomace	
	TPC[b]	AE[c]	TPC[b]	AE[c]	TPC[b]	AE[c]
a_0	2.7433**	14.3333**	0.6200**	4.5200**	1.4433**	9.6433**
a_1	0.1800**	0.4875*	0.1142**	0.9800**	0.0883*	0.3817*
a_2	0.5225**	1.6717**	0.1967**	1.3667**	0.3433**	0.7592**
a_3	-0.1350**	-3.9075**	-0.0708**	-1.2025**	-0.1783**	-0.8783**
a_4	0.1492**	1.3100**	0.1317**	0.6525**	0.0750*	0.5375**
a_{11}	-0.1504	0.5821	-0.0525	0.1400	0.0633	-1.4583**
a_{22}	-0.0167	0.0533	-0.0513	-0.1925	-0.1042	0.0029
a_{33}	-0.1054	0.3621	0.0700	0.3863	0.1758**	-0.9883**
a_{44}	0.0383	-0.6692*	-0.0038	-0.1038	0.0033	-1.0396**
a_{12}	0.1350	0.9575*	-0.0375	-0.3000	-0.0925	-0.1950*
a_{13}	-0.1375	-0.4575	0.0025	-0.6150*	-0.0525	-0.2200
a_{14}	0.0225	-0.1125	0.0275	0.5200	-0.0200	0.1000
a_{23}	-0.3225**	-0.7200*	-0.0225	-0.3725	-0.0000	-0.5025
a_{24}	-0.2050*	-1.0225**	0.0700	0.1375	-0.0025	0.4350
a_{34}	0.1100	-0.6550	-0.0325	0.0000	0.0275	0.2625
R^2	0.941	0.979	0.926	0.943	0.939	0.908
F	13.58	40.92	10.70	14.21	13.13	8.49
Sig F	0.000	0.000	0.000	0.000	0.000	0.000
Std. Error	0.1645	0.6736	0.0818	0.5566	0.1130	0.5881

[a] $y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4$

[b] Total Phenolic Content (mg gae/g sample)

[c] Antiradical Efficiency (mg $DPPH^{\cdot}$/g sample)

* significant at $p \leq 0.05$

** significant at $p \leq 0.01$

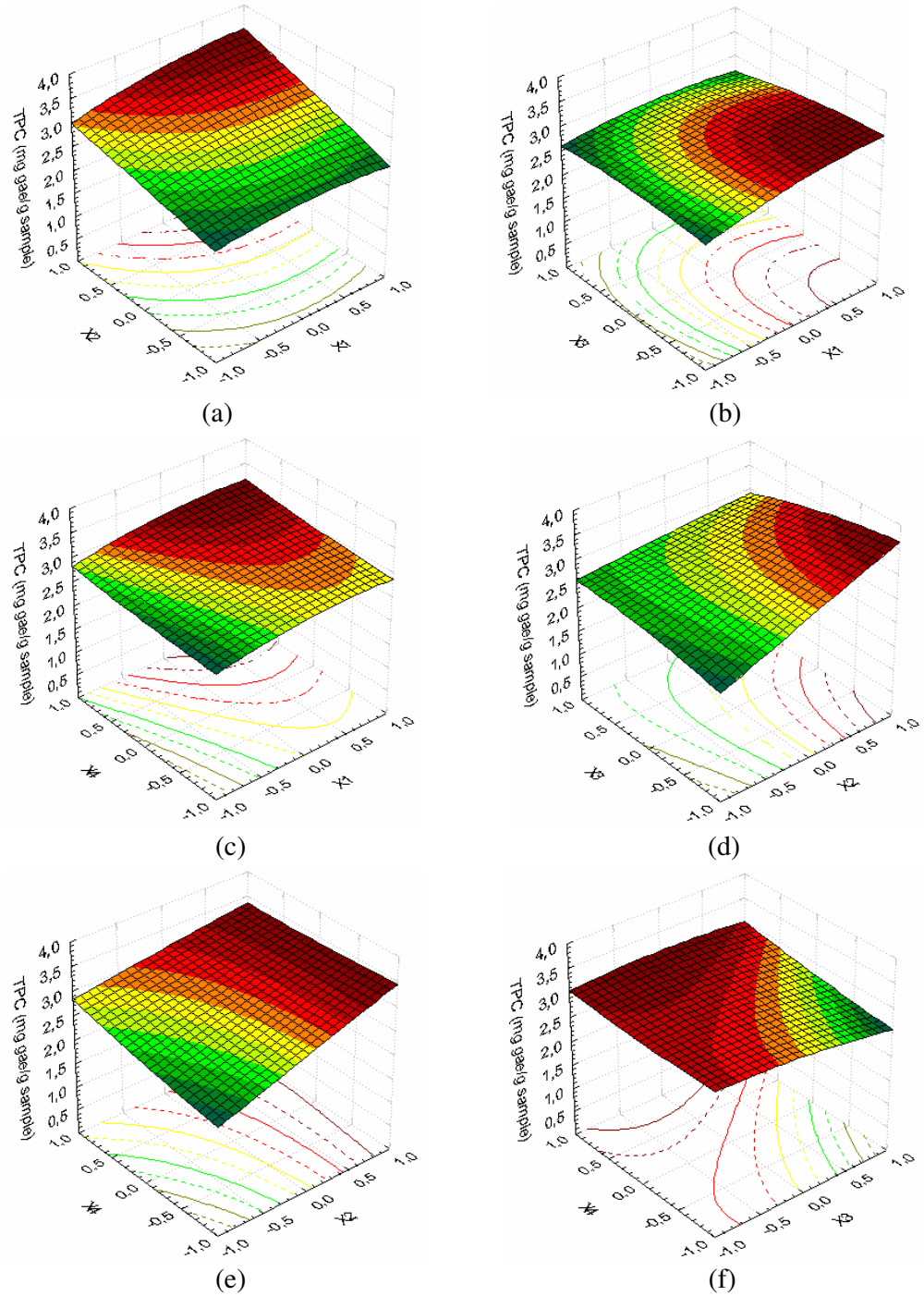

Figure 3.1 Response surfaces of TPC in the extracts obtained by HPE from sour cherry pomace (a) $X_3=X_4=0$, (b) $X_2=X_4=0$, (c) $X_2=X_3=0$, (d) $X_1=X_4=0$, (e) $X_1=X_3=0$, (f) $X_1=X_2=0$

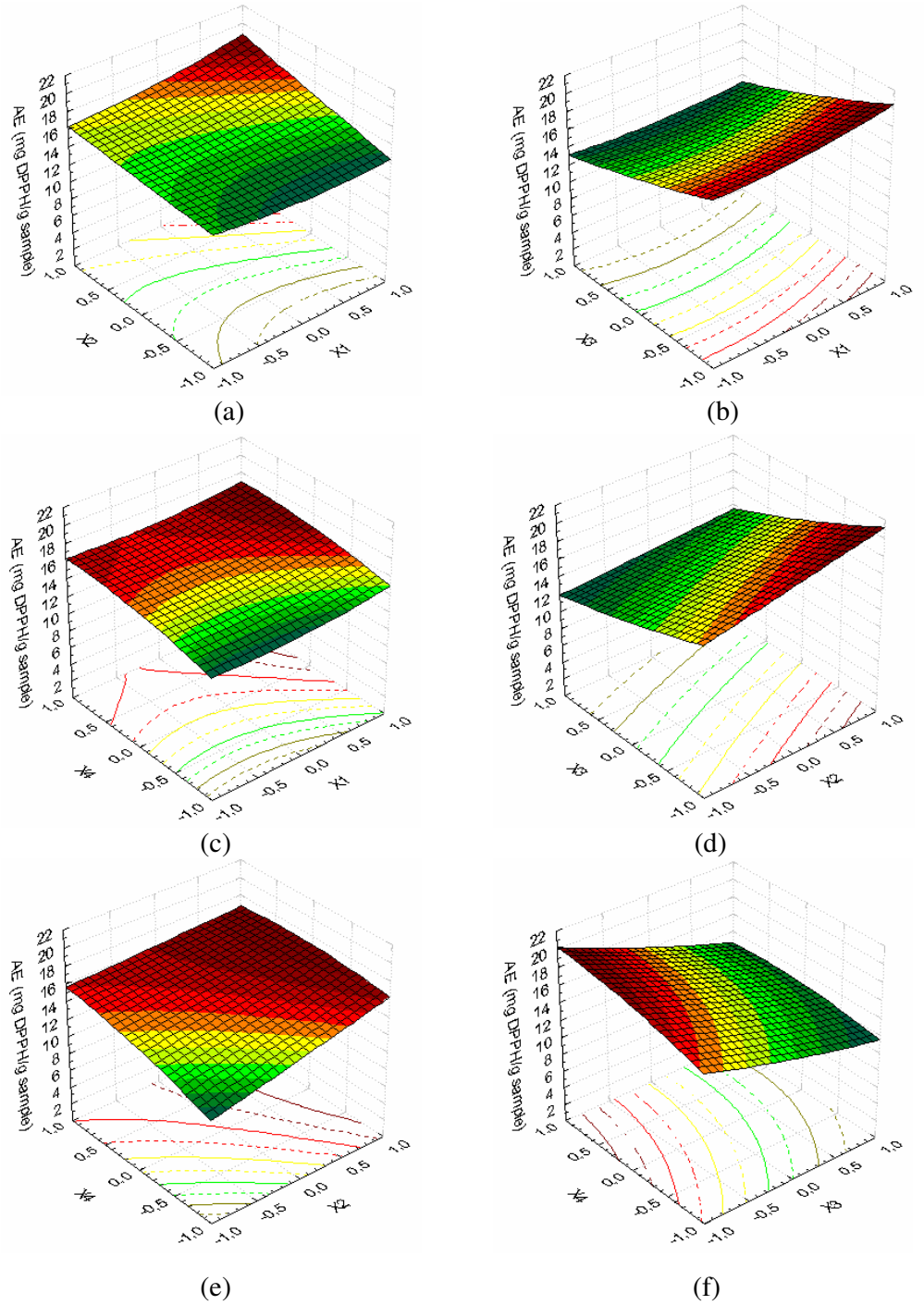

Figure 3.2 Response surfaces of AE in the extracts obtained by HPE from sour cherry pomace (a) $X_3=X_4=0$, (b) $X_2=X_4=0$, (c) $X_2=X_3=0$, (d) $X_1=X_4=0$, (e) $X_1=X_3=0$, (f) $X_1=X_2=0$

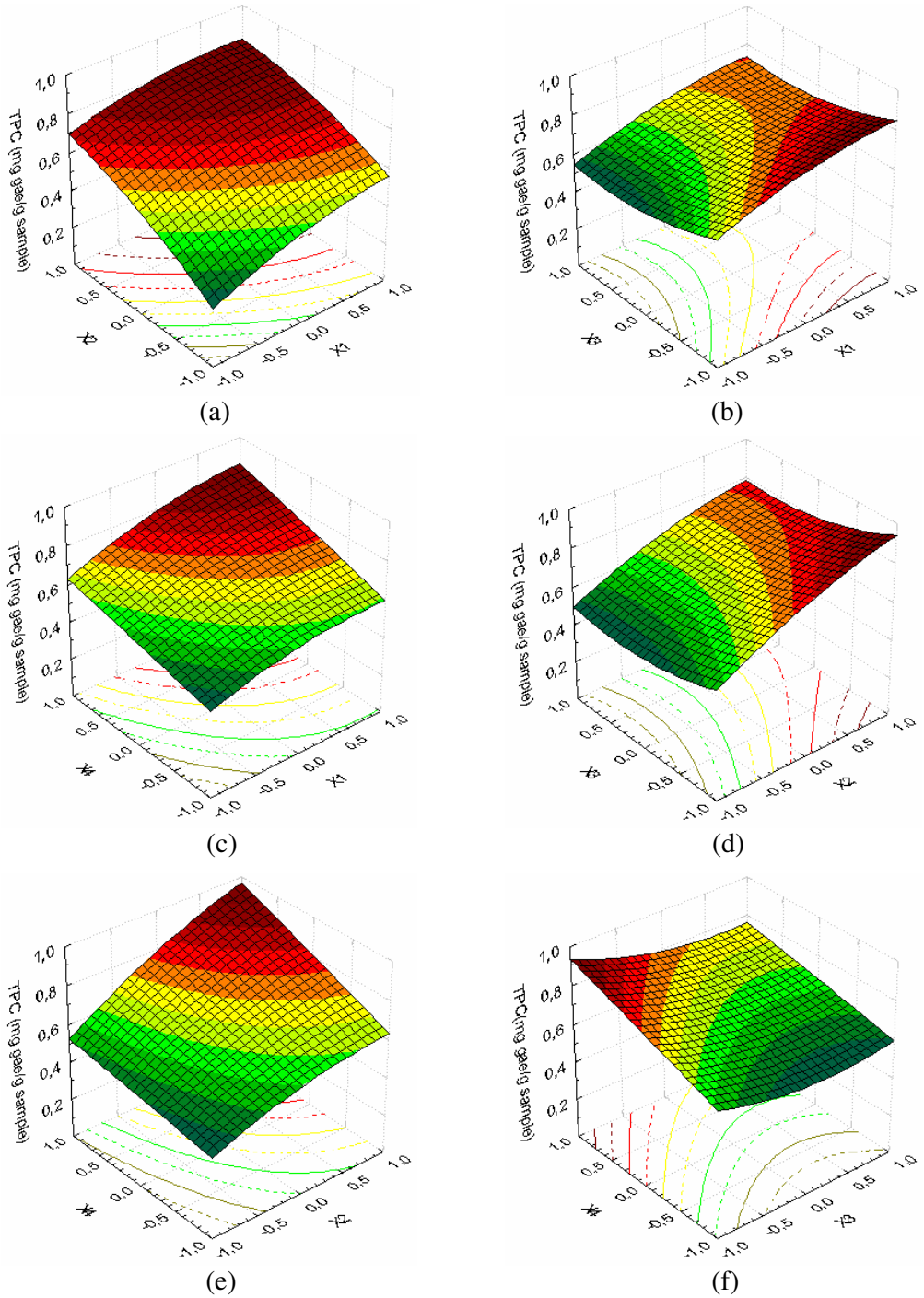

Figure 3.3 Response surfaces of TPC in the extracts obtained by HPE from peach pomace (a) $X_3=X_4=0$, (b) $X_2=X_4=0$, (c) $X_2=X_3=0$, (d) $X_1=X_4=0$, (e) $X_1=X_3=0$, (f) $X_1=X_2=0$

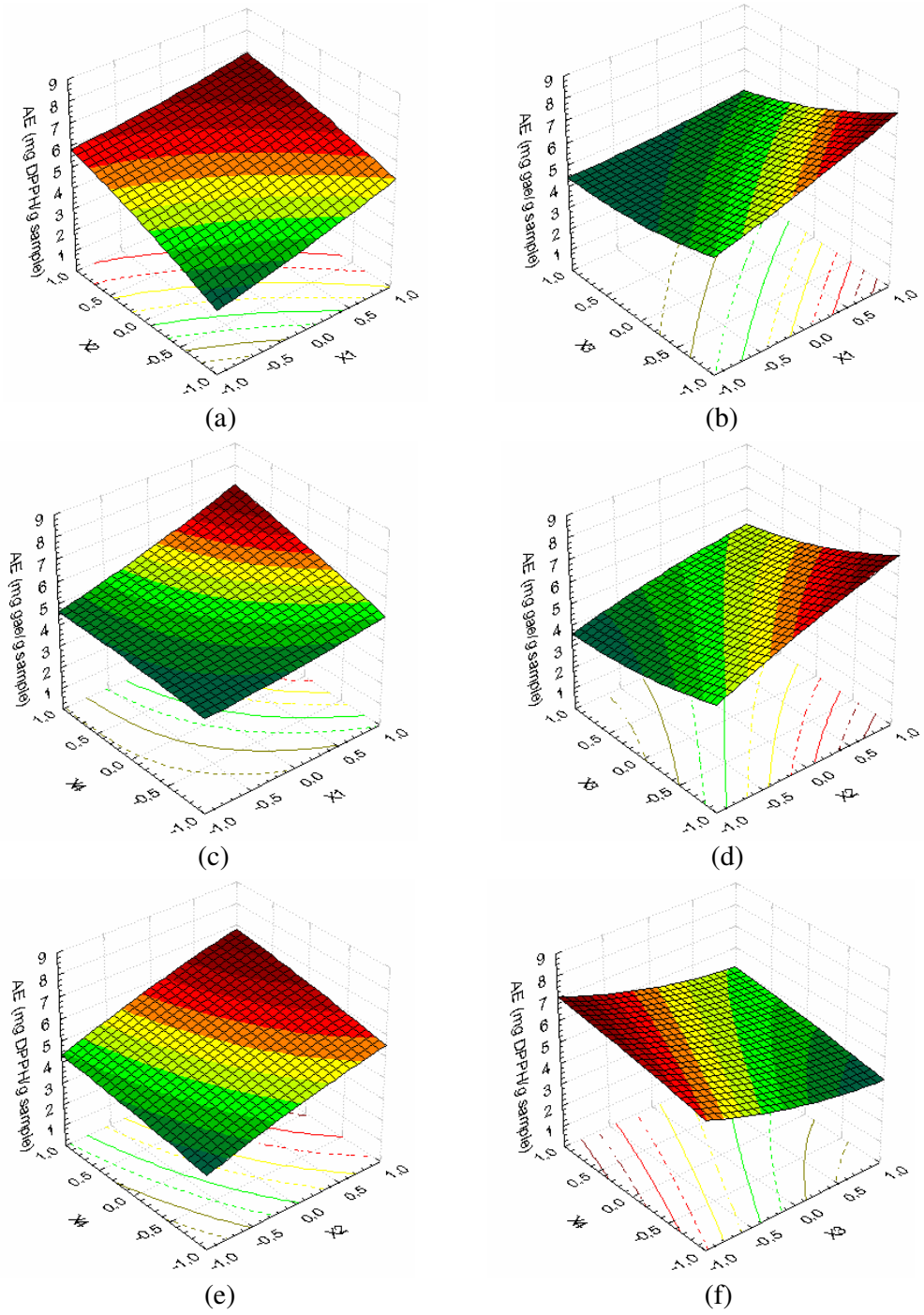

Figure 3.4 Response surfaces of AE in the extracts obtained by HPE from peach pomace (a) X_3=X_4=0, (b) X_2=X_4=0, (c) X_2=X_3=0, (d) X_1=X_4=0, (e) X_1=X_3=0, (f) X_1=X_2=0

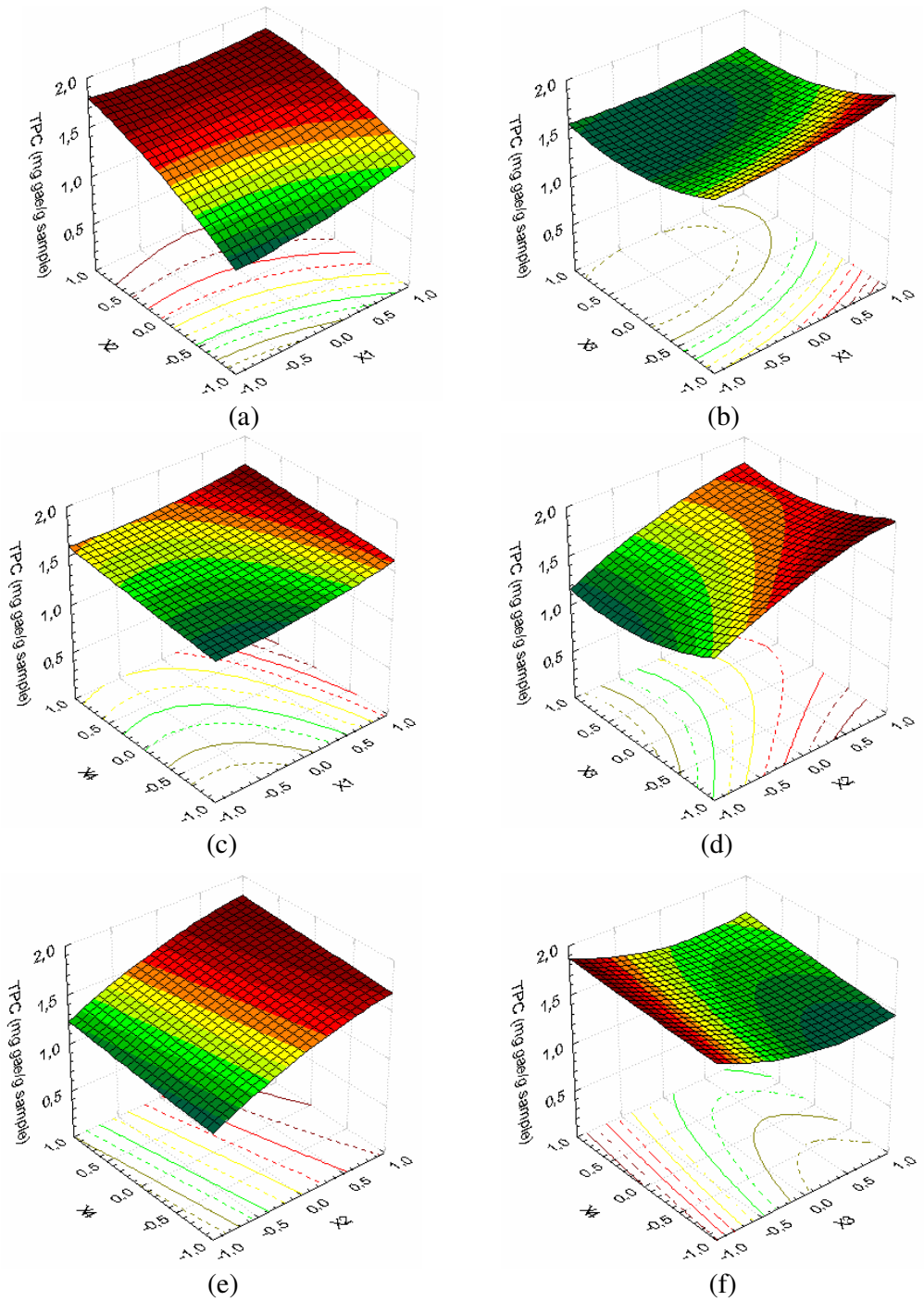

Figure 3.5 Response surfaces of TPC in the extracts obtained by HPE from apple pomace (a) X_3=X_4=0, (b) X_2=X_4=0, (c) X_2=X_3=0, (d) X_1=X_4=0, (e) X_1=X_3=0, (f) X_1=X_2=0

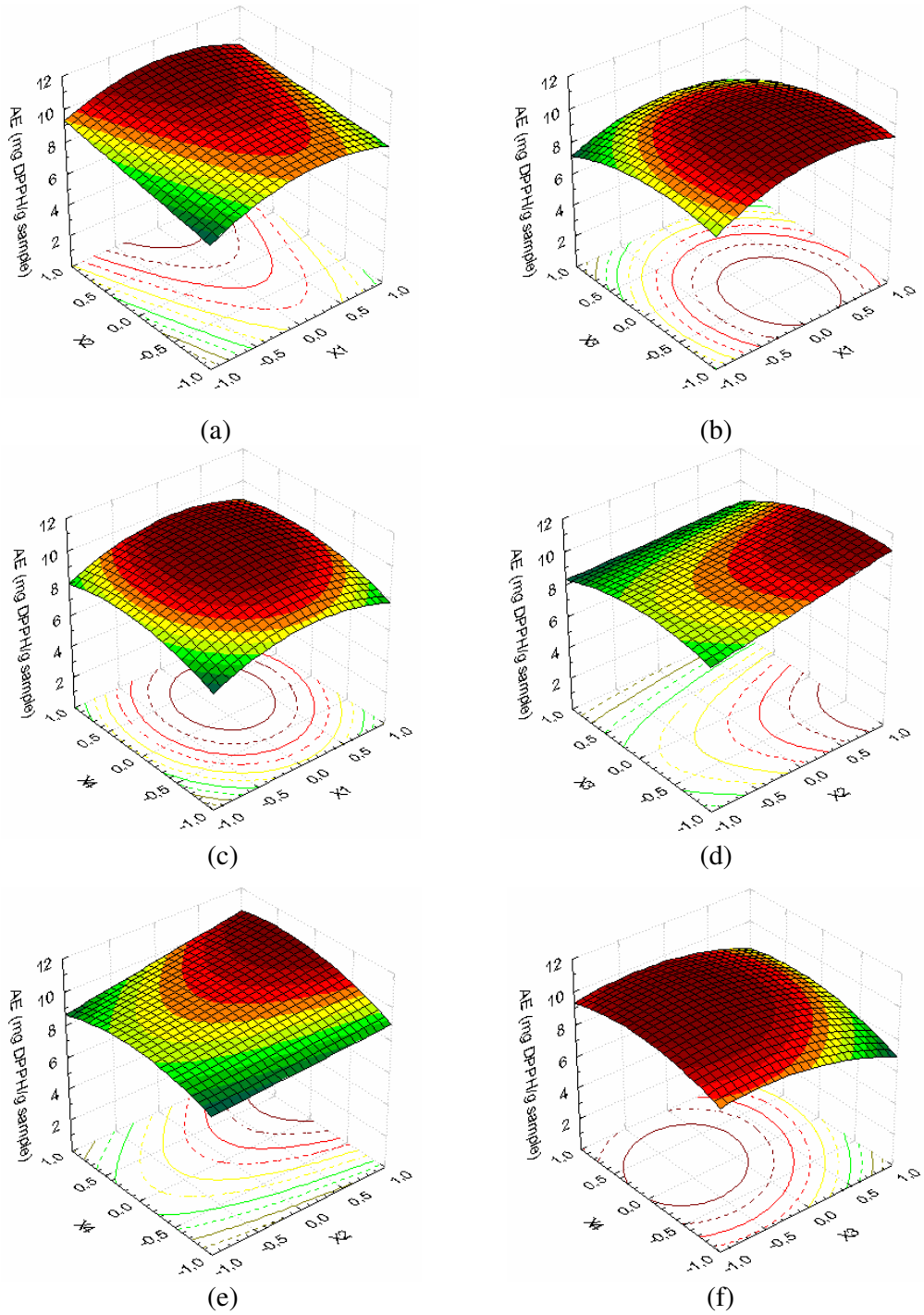

Figure 3.6 Response surfaces of AE in the extracts obtained by HPE from apple pomace (a) $X_3=X_4=0$, (b) $X_2=X_4=0$, (c) $X_2=X_3=0$, (d) $X_1=X_4=0$, (e) $X_1=X_3=0$, (f) $X_1=X_2=0$

Extraction time had a positive effect on both responses. Rostagno et al. (2004) examined the effect of extraction time on the extraction of isoflavones from soybeans. The length of the static extraction cycle was three cycles of 5, 7 and 10 min with the extraction conditions of 100°C, 100 atm and 0.1 g of sample using 70% ethanol as solvent). Extraction efficiency of all tested isoflavones increased with the extension of the static extraction cycle length from 5 to 7 min and no clear difference between 7 and 10 min was observed. Alonso-Salcez et al. (2001) found that extraction times of 5, 10, 15 min did not have any effect on the extraction yield of phenolics from apple peel and pulp at 6.9 MPa and 40°C using pure methanol as a solvent.

There was a positive significant ($p<0.01$) correlation between TPC and AE in the extracts. Correlation between TPC and AE was high in case of peach pomace ($r=0.86$) while in sour cherry ($r = 0.56$) and apple ($r = 0.50$) pomaces, the correlations were lower. This might be due to the contribution of non-phenolic antioxidative compounds to the assays or due to the synergistic effect among the free phenolic antioxidants. The related explanations are as follows:

One reason can be the contribution of ascorbic acid and carotenoids to antioxidant activity and TPC assay. Gil at al. (2002) and Cevallos-Casals et al. (2006) showed that phenolics in peaches were the only compounds that correlated with antioxidant capacity when compared with vitamin C and carotenoids. Ascorbic acid had only a minor contribution to the antioxidants in fruits with the exception of citrus fruits (app.73 %) and strawberry (app. 40 %) (Vinson et al., 2001). The same research group reported that ascorbic acid was destroyed in the total phenol extracts under heat. The material used in this study for extraction was the pomace obtained after heat treatment. Ascorbic acid is also slightly soluble in ethanol (FAO, ascorbic acid), therefore the interference from ascorbic acid is negligible. In addition, a statistically significant decrease in ascorbic acid content was reported in freeze dried marionberry and strawberry in the study of Asami et al. (2003) related to extraction of phenolic compounds by acetone, water and acetic acid (70:29:0.5 v/v).

Therefore the total phenol concentration could be determined directly from Folin assay that is widely used in the literature without ascorbic acid correction. George et al. (2005) showed that ascorbic acid exhibited a lower response than gallic acid (app. 80 % of the gallic acid absorbance at the same concentration). By contrast, carotenoids apperared to exhibit a higher response value (app. 2 -3 fold higher than that of gallic acid) that overestimation of the phenolic content if the extract is rich in carotenoids. However carotenoids are poorly extractable or soluble in polar solvents therefore contribution in calorimetric assay is negligible.

Phenolics in fruits are in both soluble and bound forms. Bound ones are mainly in the form of β-glycosides. In the literature there is a large variation in free phenol content. Vinson et al. (2001) reported that 51.9, 53.0 and 65.2 % of total phenols were the bound ones for apple, cherry and peach, respectively, with lower contribution to Folin assay. Sun et al. (2002) showed that phenolics in fruits were mainly in soluble free form (91.8 % for apple, 77.2 % for peach, 92.3 % for strawberry and 96.2 % for cranberry). The same authors reported that ascorbic acid only contributed 0.4 % and 0.76 % of the total antioxidant activity in apples and peach, respectively. This number is 0 and 3 % for cranberry (47 mg ascorbic acid/100 g fruit) and strawberry (257 mg /100g fruit) for which ascorbic acid content is higher than sour cherry (42 mg/g fruit). Another reason for lower correlation can be the synergism among the free phenolic antioxidants in the extracts (Vinson et al., 2001). The similar explanation was given for apple peel, flesh and whole apple extracts with 80% methanol (Pearson et al., 1999)

Being rapid, simple and independent of sample polarity, DPPH method is widely used for the estimation of antioxidant activity. But, in alcohols, the hydrogen abstraction from some phenols by DPPH was found enhanced due to partial ionization of the phenols and a very fast electron transfer from the phenolate anion to DPPH, in fact changing the antioxidative mechanism from hydrogen atom donation to electron transfer as explained in the article of Becker et al. (2004).

Superimposition of contour plots is frequently used in response surface analysis to determine the optimum regions when focus of interest is more than one variable. Therefore, contour plots for TPC and AE were superimposed in order to find the region giving the maximum value for both variables. The superimposed contour plots giving the optimum regions are given in Figures 3.7-3.9.

Table 3.3 and Table 3.4 show the optimum extraction regions for coded and uncoded factor levels, respectively. The optimum pressure ranges and temperature values are similar for all samples, which are close or equal to the upper value of the design. Those results support the positive effect of these two independent variables on both responses. The optimum ranges for solid/solvent ratio is similar for sour cherry and apple pomaces, but higher for peach pomace. The optimum extraction times are also close to each other for all samples, where the shortest time belongs to the sour cherry pomace.

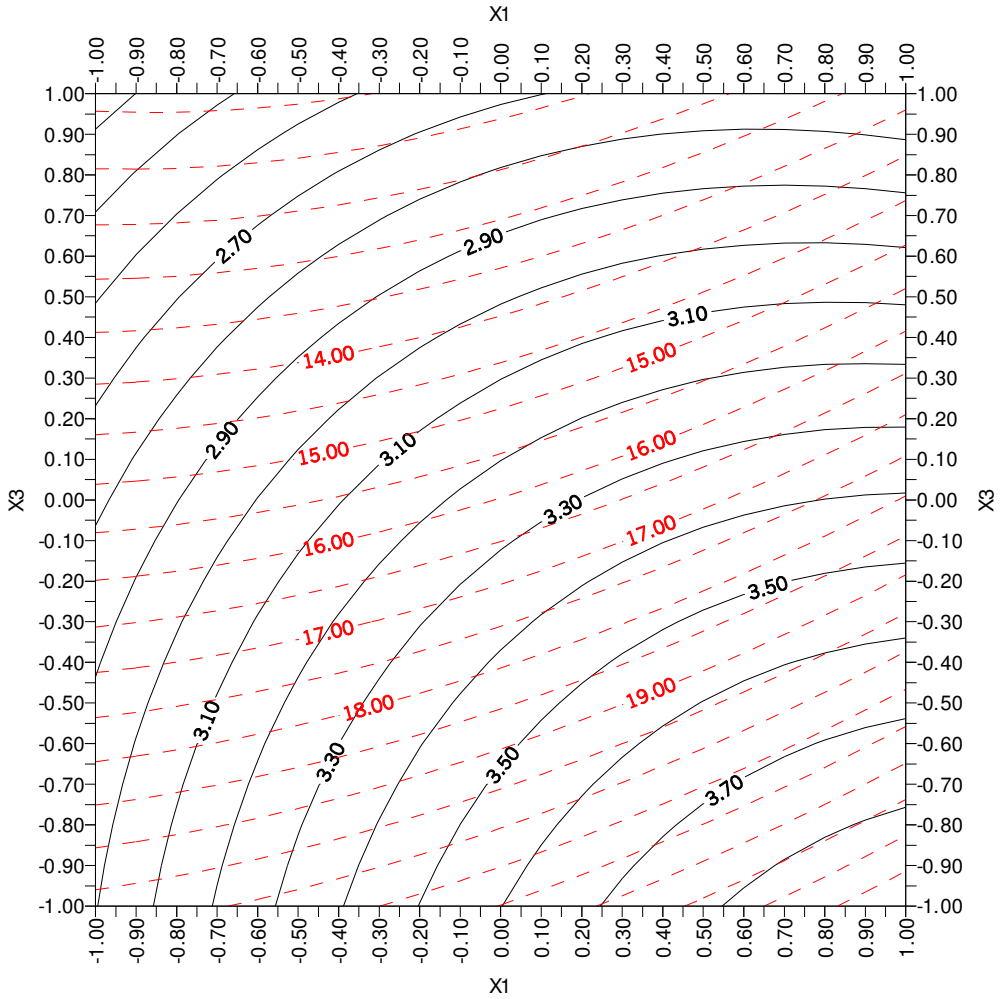

Figure 3.7 Superimposed contour plots for HPE of phenolic compounds from sour cherry pomace (X_2=1.0, X_4=0); —— TPC (mg gae/g sample); ------ AE (mg DPPH˙/g sample)

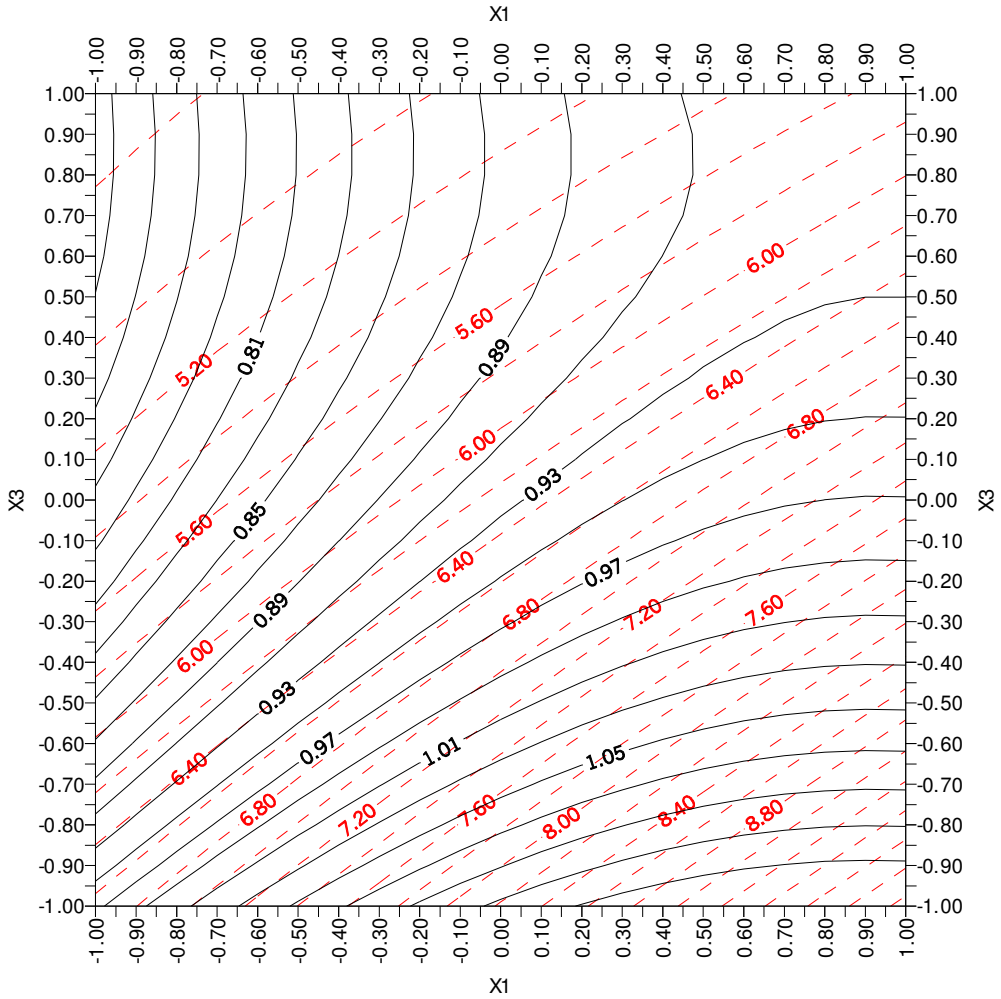

Figure 3.8 Superimposed contour plots for HPE of phenolic compounds from peach pomace (X_2=1.0, X_4=0.8); ——— TPC (mg gae/g sample); ------ AE (mg DPPH˙/g sample)

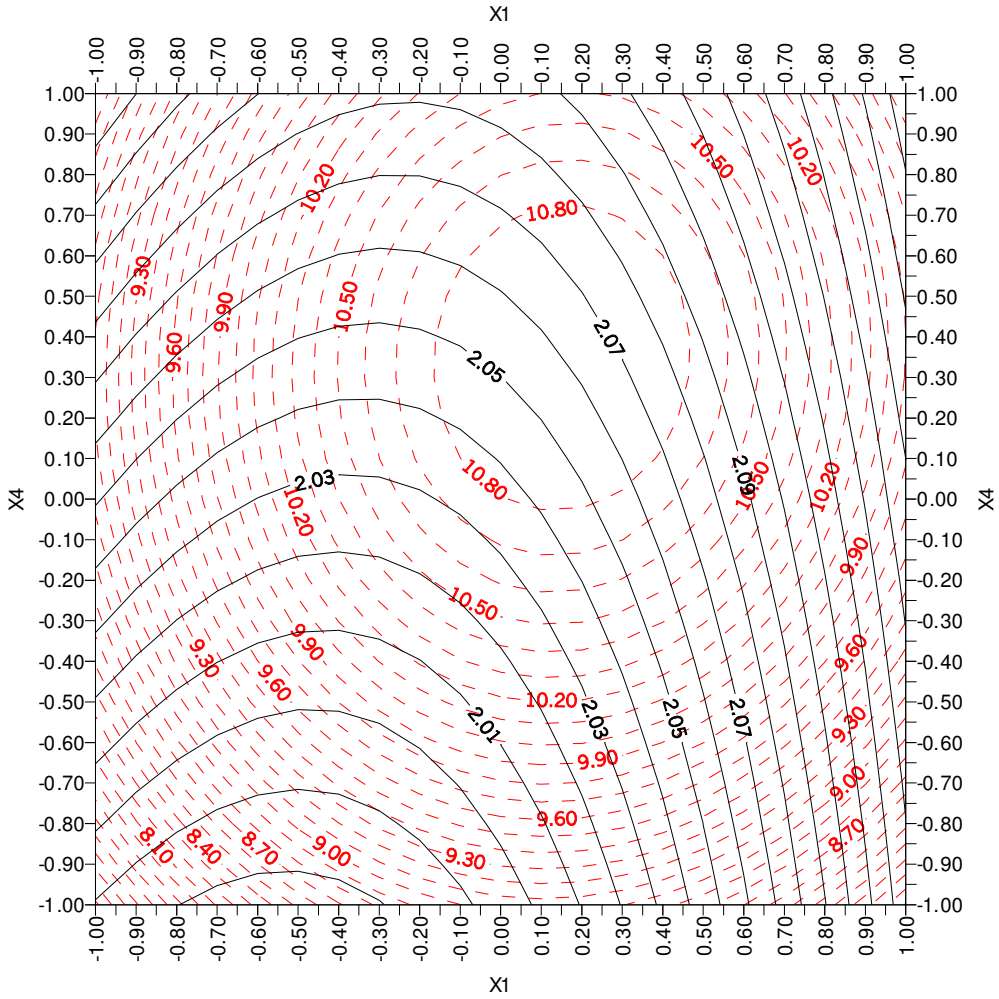

Figure 3.9 Superimposed contour plots for HPE of phenolic compounds from apple pomace (X_2=1.0, X_3=-1.0); ——TPC (mg gae/g sample); ------ AE (mg DPPH˙/g sample)

Table 3.3 Optimum conditions for HPE of TPC and their AE at coded factor levels

	Sour cherry pomace	Peach pomace	Apple pomace
X_1	0.68 – 0.90	-0.65 - 0.9	0.21-0.45
X_2	1	1	1
X_3	-0.9 – -0.78	-0.55 – 0.5	-1
X_4	0	0.8	0.25 – 0.72

Table 3.4 Optimum conditions for HPE of TPC and their AE at uncoded factor levels

	Sour cherry pomace	Peach pomace	Apple pomace
Pressure (MPa)	176-193	76-193	141-159
Temperature (°C)	60	60	60
Solid/solvent ratio (g/ml)	0.06-0.07	0.095-0.20	0.05
Time (min)	25	37	29-36

Table 3.5 shows the values of response variables at the optimum extraction conditions. Sour cherry pomace was found to have the highest phenolic content and AE, followed by apple and peach pomace. Sun et al. (2002) who worked on the total phenolic profiles in common fruits reported the total phenolic content apples as 2.963±0.064 mg gae/g fresh weight and peaches as 0.846±0.007 mg gae/g fresh weight. Kim et al. (2005) extracted phenolic compounds from sweet and sour cherry and found that the amount of phenolic compounds in different sour cherry varieties range between 1.617-3.124 mg gae/g fresh weight. Although the findings seem to be close to our results, a major difference comes from the type of sample used for

the extractions which was wet fresh fruit in their research, where it was dry pomace in our work. If their results are converted to dry weight basis using the moisture content of apple, cherries and peach (84, 81 and 88 %, respectively) (Bastin and Henken, 1997), the findings of Sun et al (2002) will be 18.52 mg gae/g dry weight for apple and 7.05 mg gae/g dry weight for peach. Similarly, TPC for sour cherries is 8.51-16.44 mg gae/g dry weight according to the findings of Kim et al. (2005). Those results are much higher than our findings. This difference majorly comes from the losses during fruit juice production, especially the heating process (Figure 3.1). Çalımlı (2003) who extracted anthocyanins from sour cherry pomace by solvent extraction and supercritical fluid extraction also found that the amount of anthocyanins extracted by solvent extraction with methanol was much lower than those given in the literature for fresh sour cherries (18 μg anthocyanins/g dry pomace vs 350-820 μg anthocyanins/g fresh fruit). They indicated that this difference may be due to the losses during fruit juice production, pomace receiving, drying, grinding, sieving and keeping in the freezer.

Table 3.5 Values of response variables at the optimum conditions of HPE

	Sour cherry pomace	Peach pomace	Apple pomace
TPC (mg gae/g sample)	3.80	0.93	2.08
AE (mg $DPPH^{\cdot}$/g sample)	22.00	6.40	10.80
AE/TPC (mg $DPPH^{\cdot}$/ mg gae)	5.79	6.88	5.19

3.2 Subcritical CO_2 Extraction (SCE)

Preliminary experiments were performed with sour cherry pomace in order to decide about the levels of the design variables. The ethanol concentration range was decided by performing extractions at 60 MPa, 60°C, using 2 g/min solvent flow rate for 30 minutes with 0-20 wt% ethanol in CO_2 (Fig 3.10). At 60°C by 0-10 wt% ethanol addition, supercritical CO_2 extraction was performed (Table 1.2) where TPC and AE of the extracts were low, which increased about four fold at higher ethanol concentrations where the extraction was subcritical. Therefore, it was decided that subcritical CO_2 was advantageous than supercritical CO_2 extraction.

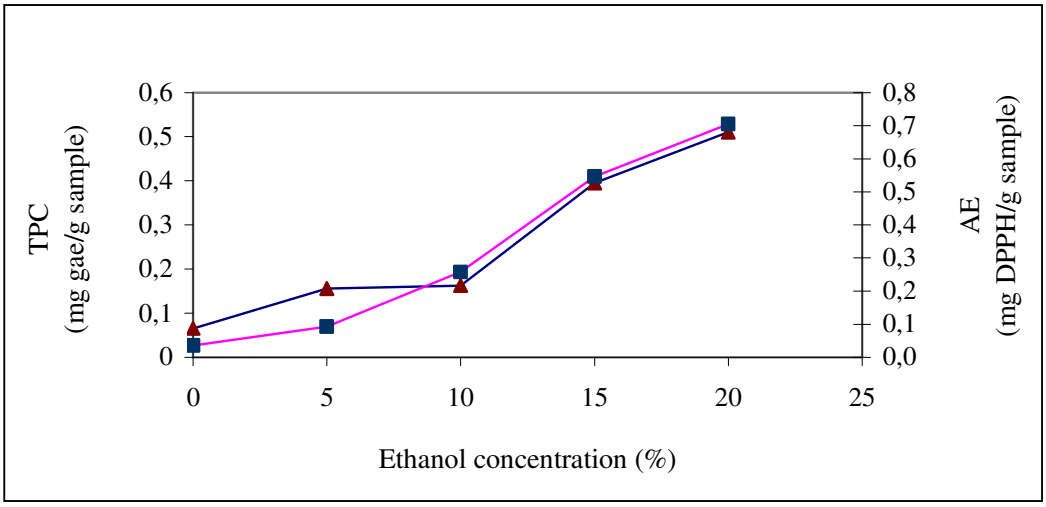

Figure 3.10 Effect of ethanol concentration on the TPC and AE of phenolic compounds extracted from sour cherry pomace at 60 MPa, 60°C and 30 min of extraction time ■: AE, ▲: TPC

The extraction time was decided by performing extractions at 60 MPa, 60°C, using 2 g/min solvent flow rate for about 120 minutes. The extracts were collected in separate tubes in every 15 minutes. The extraction curve (Fig 3.11) shows that in the first period of extraction the mass transfer was majorly controlled by the solubility of phenolic compounds in CO_2-ethanol mixture, and by the diffusion of phenolic compounds in sour cherry pomace particles in the second period of extraction. The maximum extraction time was selected to be 40 minutes to assure that TPC of the extracts increase significantly with extraction time at all extraction conditions.

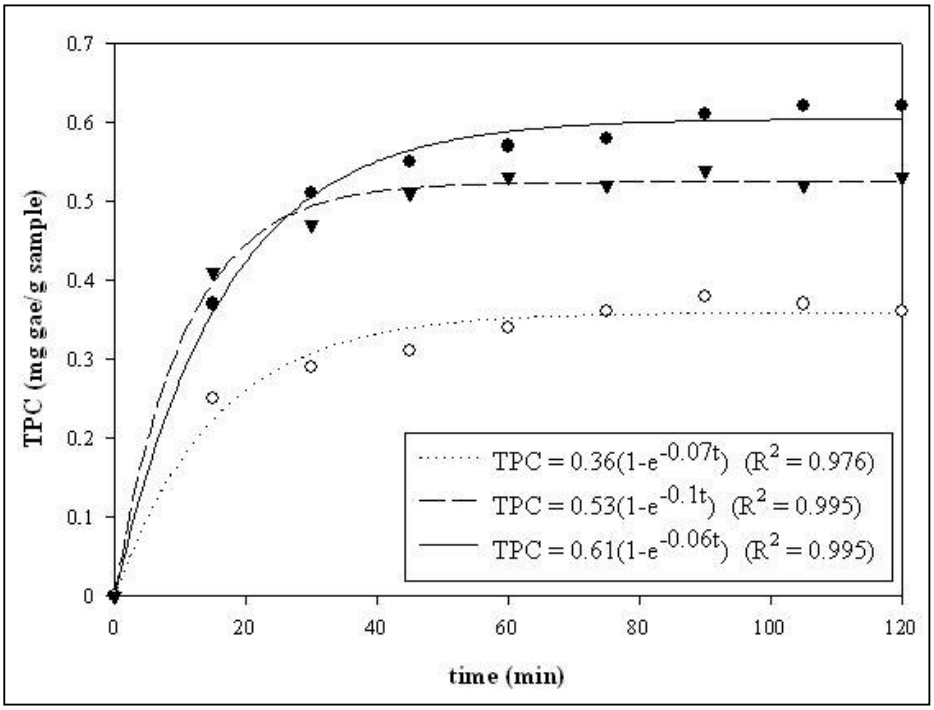

Figure 3.11 Change in TPC of the extracts obtained at different extraction times at 60 MPa, 60°C and 20 % (w/w) ethanol concentration; •: sour cherry pomace, ▼ peach pomace, ○ apple pomace

Table 3.6 and Table 3.7 show the experimental data and the regression coefficients obtained by fitting experimental data to the second order response models for TPC and AE of the extracts obtained by SCE. t-tests for $p \leq 0.05$ show that all independent variables are significant for the extraction of TPC from all fruits by SCE and also for their AE. Temperature seems to interact with ethanol concentration and time for TPC from sour cherry pomace and peach, where the interaction of temperature is only with time for AE from the same fruits. Interaction of temperature and pressure with time are significant for AE from apple pomace, where only temperature interacts with time for TPC from apple. For sour cherry pomace, ethanol concentration and time interaction was also found to be significant for TPC ($p \leq 0.05$).

Figure 3.12-3.17 represent response surfaces for the TPC and AE of the extracts obtained from sour cherry, peach and apple pomace by SCE. The effect of pressure on TPC and AE was positive for all samples. This is mainly due to the increase in the density of CO_2, i.e. increase in the solvating power with increasing pressure (Escribano-Bailon and Santos-Buelga, 2003; Hamburger et al., 2004). The effect of pressure was more significant at high ethanol concentration. The same result was obtained by Cháfer et al. (2002) who work on solubility of epicatechin in supercritical CO_2 + ethanol at 40°C and 8-14 MPa. As the concentration of ethanol increased from 5 to 25%, a sharper increase in solubility was observed with an increase in pressure. Murga et al. (2002) determined the effect of pressure (10-50 MPa) on the solubility of some natural, low molecular weight phenolic compounds in supercritical CO_2 at 40-60°C and found that solubility increases with increasing pressure. Other researches on the solubility of catechin (Berna et al, 2001a), epicatechin (Cháfer et al, 2002), quercetin (Cháfer et al, 2004) and resveratrol (Berna et al, 2001b) in supercritical CO_2 + ethanol at 40°C and 8-14 MPa show that pressure has a positive effect on solubility of the phenolic compounds of interest. Le Floch et al. (1998) studied the effect of extraction pressure (15.5 - 33.4 MPa) on the amount of phenolic compounds extracted from olive leaves using CO_2 modified with 10% methanol for 20 min at a constant temperature of 100°C and found that

the amounts extracted increased linearly with increasing pressure, i.e. CO_2 density. The only finding in the literature showing the negative effect of pressure on the extraction of phenolic compounds belongs to Lin et al. (1999) who extracted flavonoids from *Scutellaria baicalensis,* a plant used in Chinese medicine, by SFE at 40-70°C, pressure 20-40 MPa adding two types of modifier (pure methanol and 70% methanol-water; 5-15 % v/v) directly on the sample before the extraction.

Temperature also had a positive effect on TPC and AE for all samples. In most of the findings in the literature, the effect of temperature was reported to be negative on the extraction of phenolic compounds at very low pressures (10-15 MPa) up to a certain value, beyond which the effect of temperature becomes positive. The pressure value at which the effect of temperature on the solubility changes is called the cross-over pressure (Özkal et al., 2006). Roy et al. (1996) has explained that this cross-over property arises from the solubility being controlled by a balance between the solvent density and the change in the solute vapour pressure with changes in the pressure and temperature. Murga et al. (2002) determined the effect of temperature (313-323°C) on the solubility of some natural, low molecular weight phenolic compounds (3,4-dihydroxy benzoic acid, methyl 3,4,5-trihydroxybenzoate and 3,4-dihydroxy benzaldehyde) in supercritical CO_2 at 10-50 MPa. They found that temperature had a negative effect on the solubility up to 15 MPa. After this cross-over pressure, solubility increased with increasing temperature. The cross-over effect of pressure was not seen in our results because the extraction pressures were beyond the cross-over values. Le Floch et al. (1998) found that increasing temperature (80-120°C) has a positive effect on both the rate and efficiency of the phenolic compounds from olive leaves using CO_2 modified with 10% methanol at 33.4 MPa for 20 min. Palma and Taylor (1999b) observed an increase in the recovery of phenolic compounds from grape seeds with near critical CO_2 with an increase in temperature from 35 to 55°C with 10% methanol and CO_2 density of 0.95 g/ml.

Table 3.6 Experimental results for SCE

	Sour cherry pomace		Peach pomace		Apple pomace	
Exp. no	TPC[a]	AE[b]	TPC[a]	AE[b]	TPC[a]	AE[b]
1	0.300	1.038	0.150	0.792	0.238	1.999
2	0.324	1.149	0.160	0.760	0.281	1.362
3	0.264	0.932	0.130	0.629	0.168	1.298
4	0.179	0.607	0.090	0.405	0.143	0.879
5	0.542	1.863	0.241	1.232	0.383	2.573
6	0.298	1.010	0.187	0.773	0.297	1.477
7	0.258	0.923	0.150	0.613	0.238	0.435
8	0.188	0.439	0.092	0.222	0.146	0.582
9	0.353	1.103	0.215	0.913	0.340	1.981
10	0.396	1.613	0.214	1.275	0.356	2.767
11	0.139	0.502	0.143	0.334	0.222	0.725
12	0.282	1.056	0.140	0.604	0.222	1.121
13	0.099	0.363	0.077	0.346	0.122	0.751
14	0.419	1.604	0.210	1.079	0.365	1.783
15	0.256	0.909	0.130	0.666	0.206	1.245
16	0.495	1.579	0.238	1.053	0.326	0.485
17	0.170	0.551	0.071	0.368	0.097	0.499
18	0.349	1.220	0.227	0.935	0.355	2.029
19	0.503	2.000	0.250	1.335	0.427	2.193
20	0.228	0.860	0.145	0.573	0.246	1.043
21	0.283	1.071	0.184	0.718	0.265	1.249
22	0.126	0.405	0.092	0.276	0.146	0.586
23	0.446	1.724	0.225	1.169	0.502	2.246
24	0.150	0.541	0.075	0.362	0.194	0.781
25	0.261	0.826	0.138	0.751	0.235	0.950
26	0.157	0.486	0.117	0.321	0.160	0.889
27	0.353	1.056	0.198	0.904	0.333	1.962

[a] Total Phenolic Content (mg gae/g sample)

[b] Antiradical Efficiency (mg $DPPH^{\cdot}$/g sample)

Table 3.7 Second order response model constants and regression analysis for TPC and AE of the extracts obtained by SCE[a]

	Sour cherry pomace		Peach pomace		Apple pomace	
	TPC[b]	AE[c]	TPC[b]	TPC[b]	AE[c]	TPC[b]
a_0	0.3517**	1.1263**	0.2133**	0.9173**	0.3427**	1.9907**
a_1	0.0547**	0.2273**	0.0291**	0.1742**	0.0587**	0.3504**
a_2	0.0207*	0.1292**	0.0088*	0.0866**	0.0359*	0.3573**
a_3	0.1095**	0.4200**	0.0525**	0.2893**	0.0820**	0.4475**
a_4	0.0962**	0.3887**	0.0347**	0.2738**	0.0662**	0.4072**
a_{11}	-0.0673**	-0.1158	-0.0368**	-0.1468**	-0.0662**	-0.2453
a_{22}	-0.0300	-0.0720	-0.0419**	-0.1083**	-0.0563*	-0.4399**
a_{33}	0.0131	0.0865	-0.0100	-0.0392	-0.0280	-0.4732**
a_{44}	-0.0556**	-0.1470*	-0.0338**	-0.1524**	-0.0361	-0.3298*
a_{12}	-0.0272	-0.1090	-0.0125	-0.0480	-0.0170	0.0545
a_{13}	0.0295	0.1185	0.0032	0.0800*	0.0155	0.1217
a_{14}	0.0185	0.1045	0.0020	0.1707**	0.0085	0.4180*
a_{23}	-0.0405*	-0.0832	-0.0217**	-0.0680	-0.0175	0.1380
a_{24}	0.0480**	0.2108**	0.0322**	0.0942*	0.0582*	0.3510*
a_{34}	0.0435*	0.0922	-0.0010	0.0170	-0.0015	0.3108
R^2	0.965	0.961	0.978	0.980	0.900	0.891
F	23.61	21.36	38.24	42.14	7.72	7.04
Sig F	0.000	0.000	0.000	0.000	0.001	0.001
Std. Error	0.03349	0.1348	0.01224	0.06844	0.04692	0.3321

[a] $y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4$

[b] Total Phenolic Content (mg gae/g sample)

[c] Antiradical Efficiency (mg $DPPH^{\cdot}$/g sample)

* significant at $p \leq 0.05$

** significant at $p \leq 0.01$

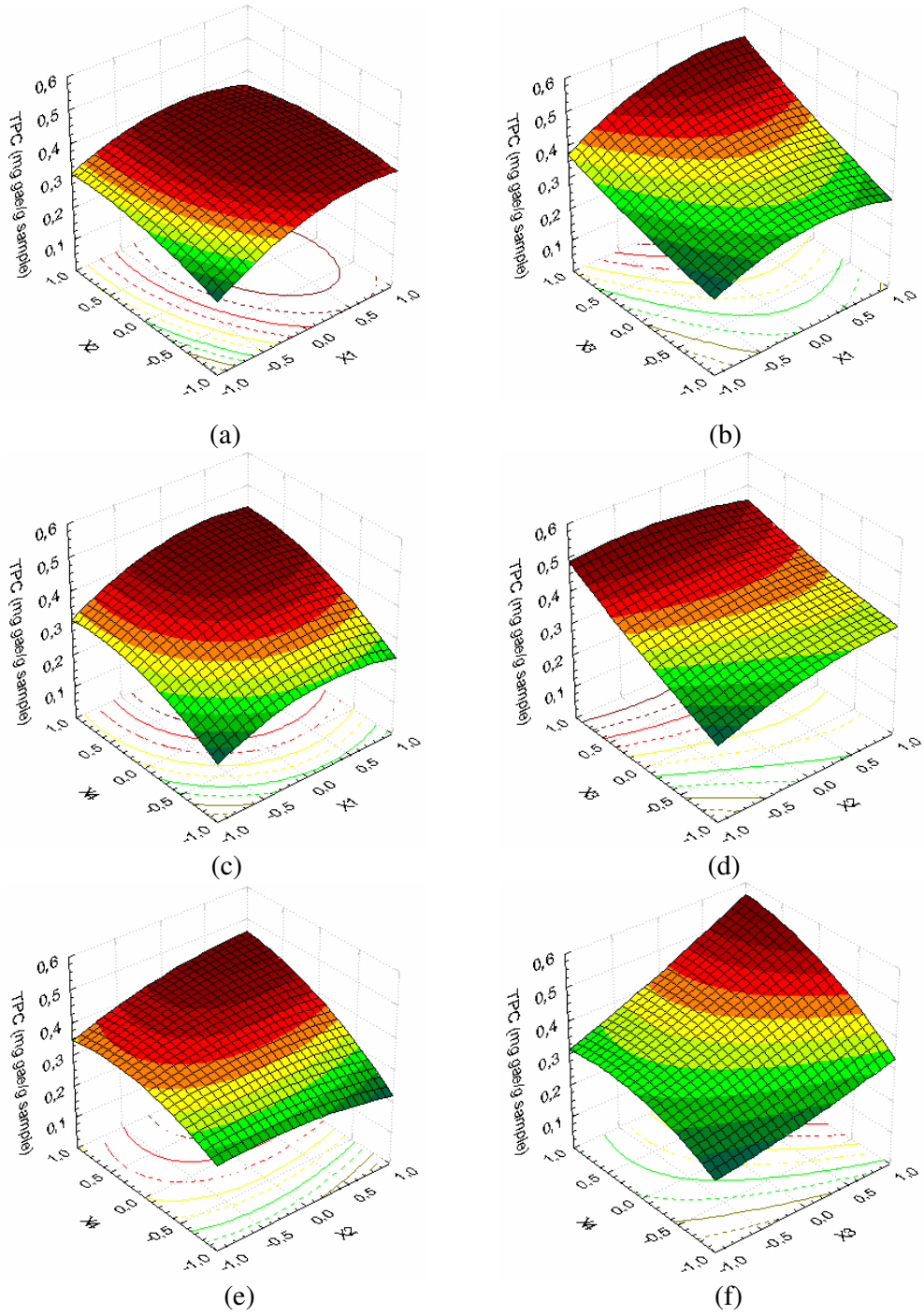

Figure 3.12 Response surfaces of TPC in the extracts obtained by SCE from sour cherry pomace (a) $X_3=X_4=0$, (b) $X_2=X_4=0$, (c) $X_2=X_3=0$, (d) $X_1=X_4=0$, (e) $X_1=X_3=0$, (f) $X_1=X_2=0$

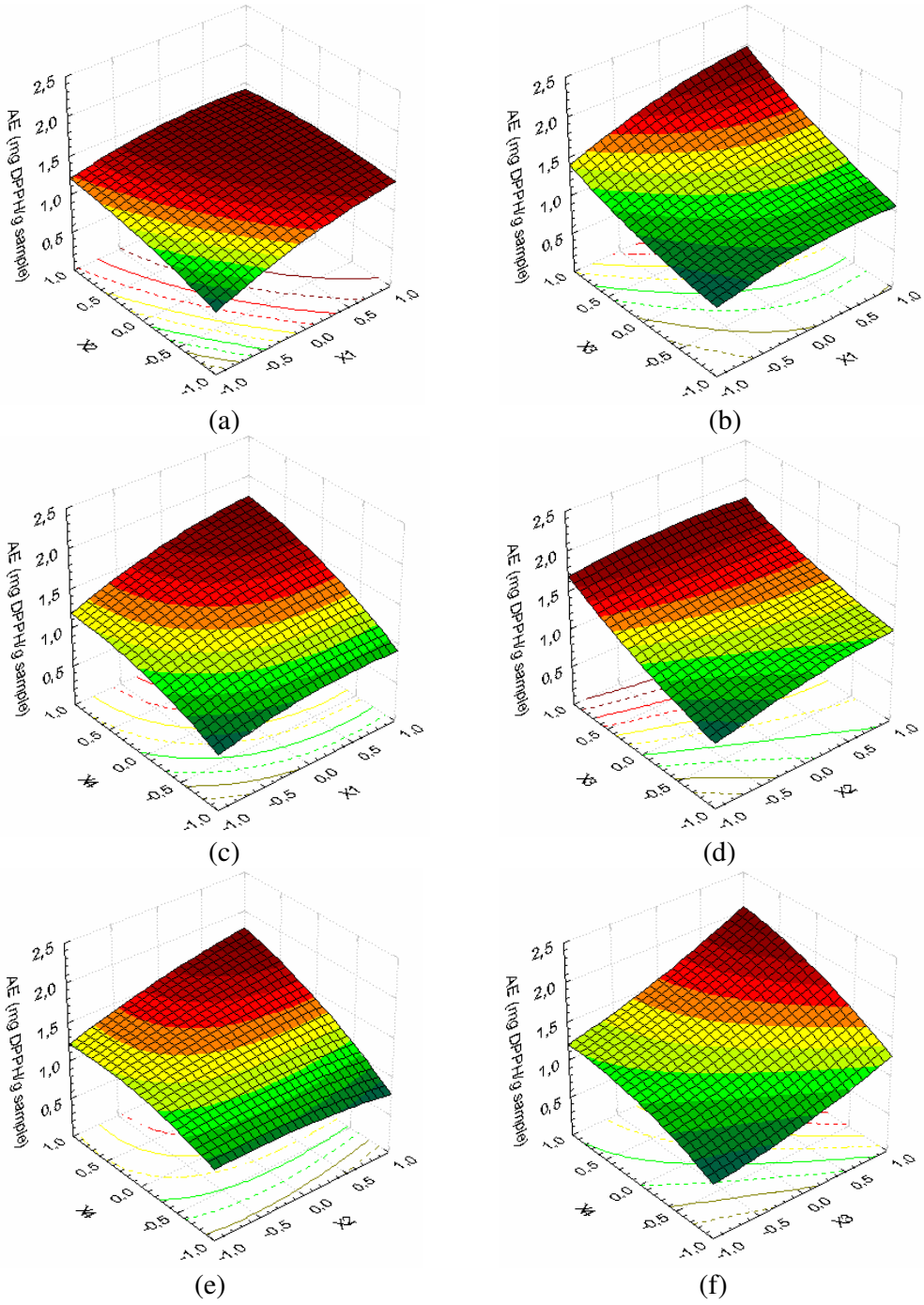

Figure 3.13 Response surfaces of AE in the extracts obtained by SCE from sour cherry pomace (a) $X_3=X_4=0$, (b) $X_2=X_4=0$, (c) $X_2=X_3=0$, (d) $X_1=X_4=0$, (e) $X_1=X_3=0$, (f) $X_1=X_2=0$

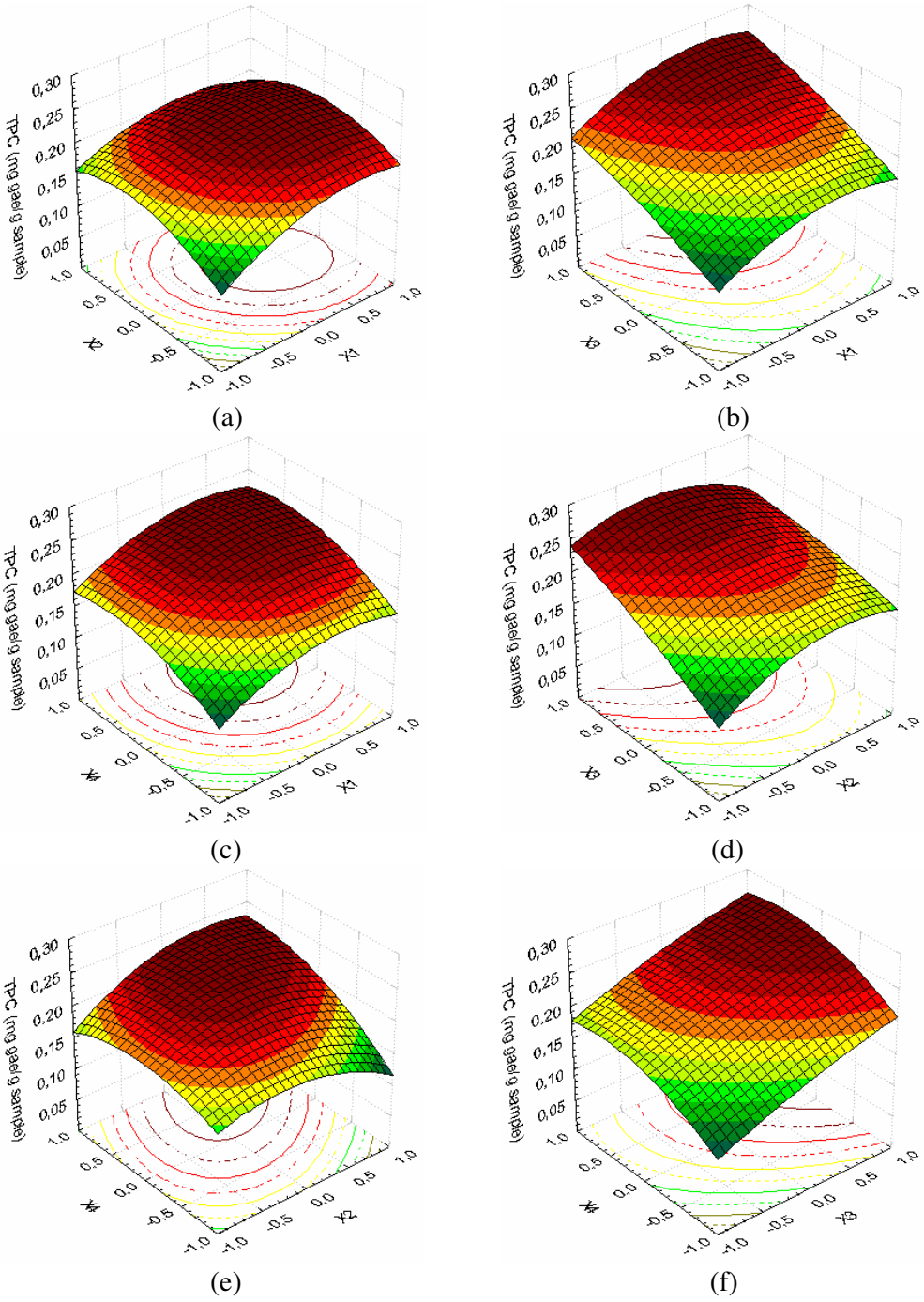

Figure 3.14 Response surfaces of TPC in the extracts obtained by SCE from peach pomace (a) X_3=X_4=0, (b) X_2=X_4=0, (c) X_2=X_3=0, (d) X_1=X_4=0, (e) X_1=X_3=0, (f) X_1=X_2=0

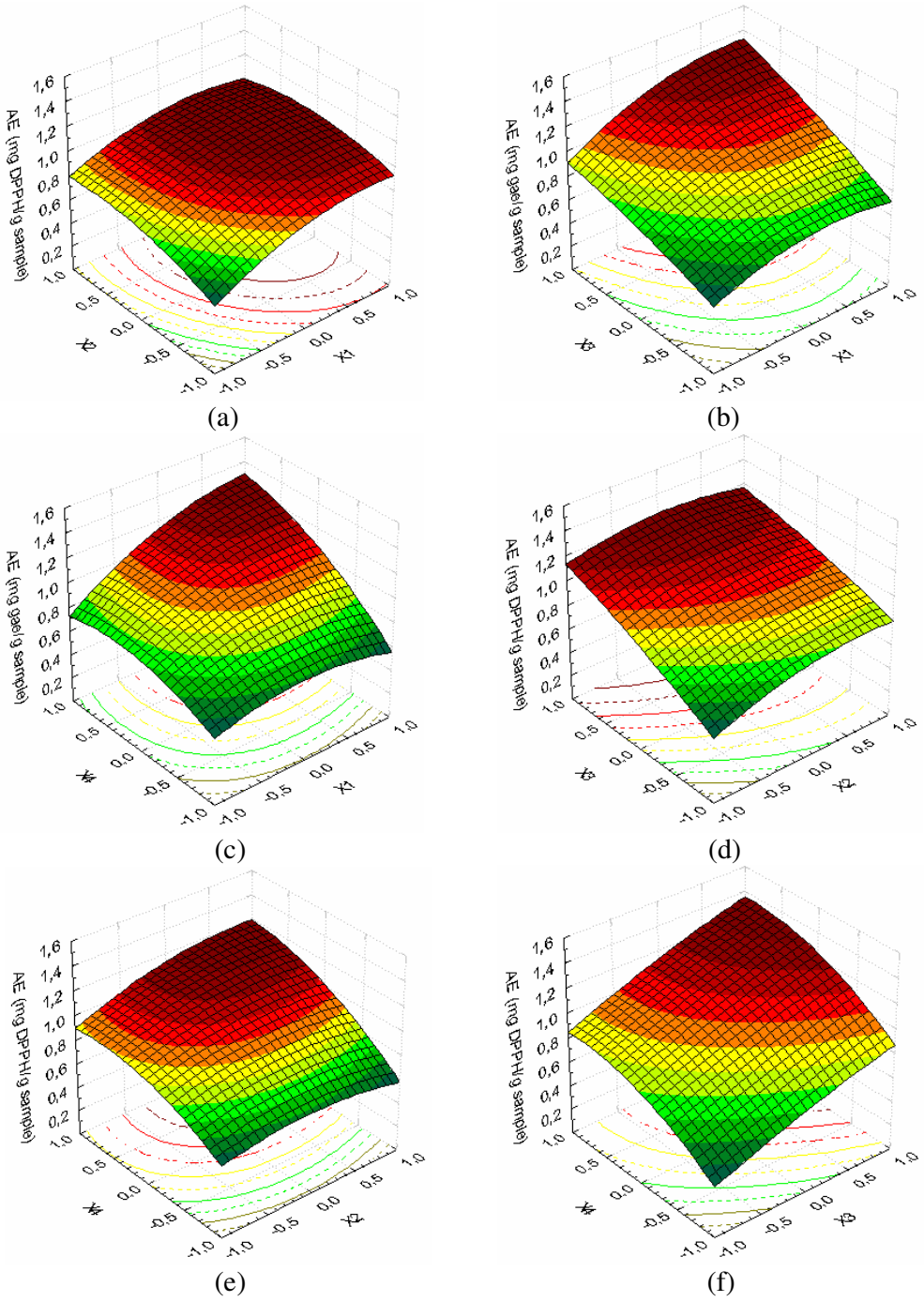

Figure 3.15 Response surfaces of AE in the extracts obtained by SCE from peach pomace (a) X_3=X_4=0, (b) X_2=X_4=0, (c) X_2=X_3=0, (d) X_1=X_4=0, (e) X_1=X_3=0, (f) X_1=X_2=0

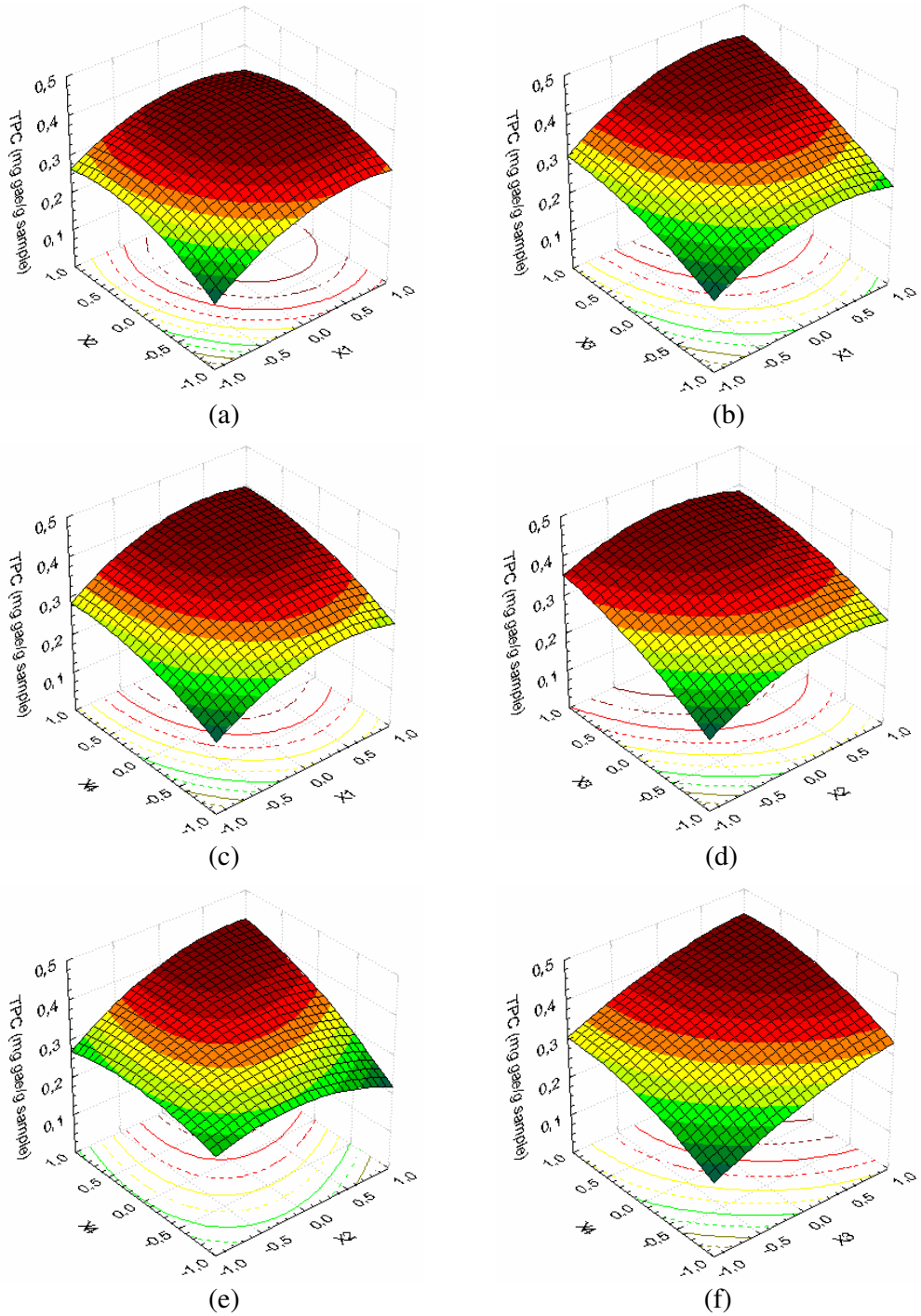

Figure 3.16 Response surfaces of TPC in the extracts obtained by SCE from apple pomace (a) $X_3=X_4=0$, (b) $X_2=X_4=0$, (c) $X_2=X_3=0$, (d) $X_1=X_4=0$, (e) $X_1=X_3=0$, (f) $X_1=X_2=0$

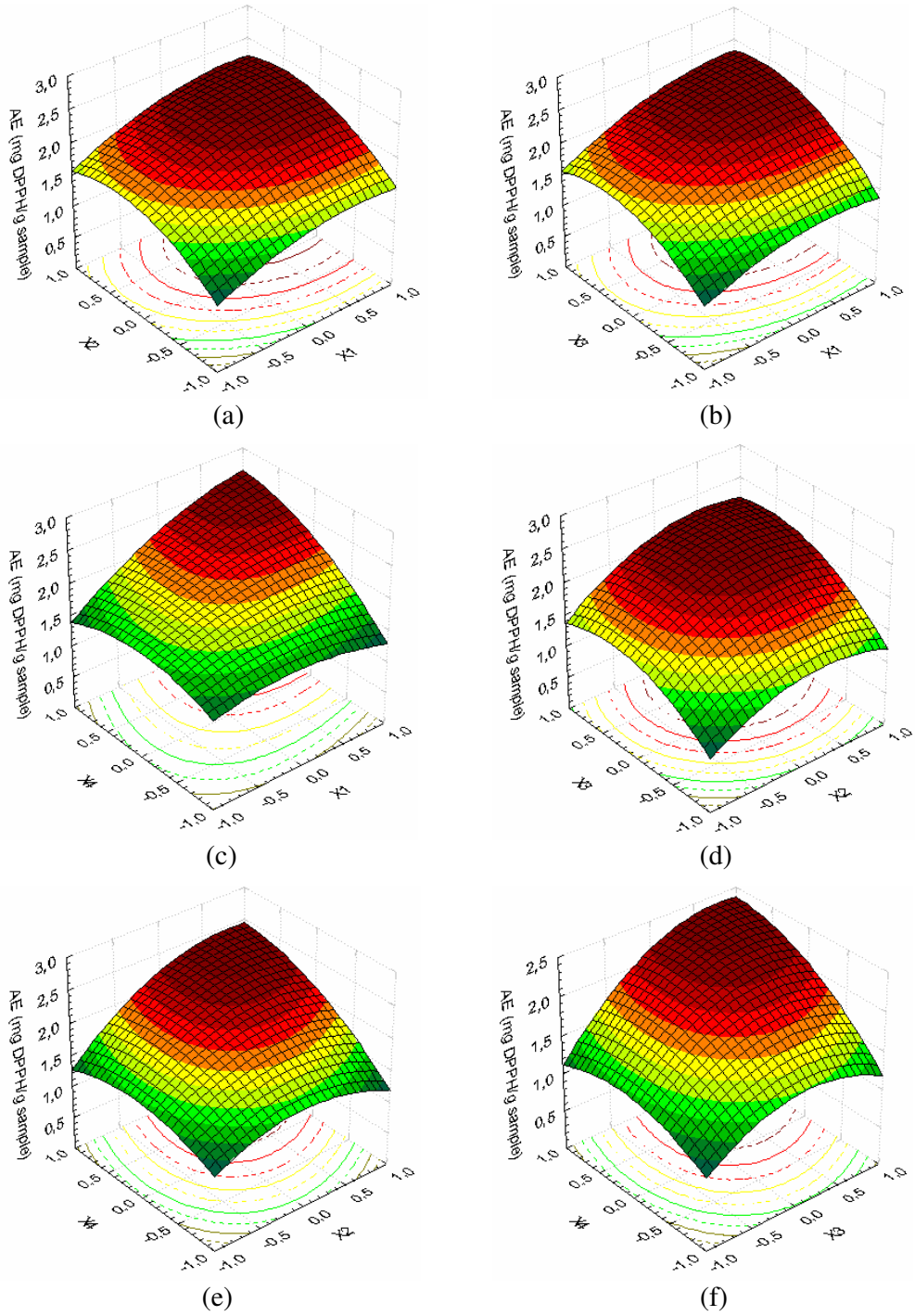

Figure 3.17 Response surfaces of AE in the extracts obtained by SCE from apple pomace (a) X_3=X_4=0, (b) X_2=X_4=0, (c) X_2=X_3=0, (d) X_1=X_4=0, (e) X_1=X_3=0, (f) X_1=X_2=0

The most effective variable was ethanol concentration among four variables. It affected both responses positively as expected, because CO_2 itself is not polar enough to extract phenolic compounds. The polarity of CO_2 increases as the ethanol concentration is increased, which results with more hydrogen bonding and dipole-dipole interactions, i.e. increased solubility of phenolic compounds. Effect of ethanol concentration on solubility of phenolic compounds depend on their polarity. Solubility of catechin, epicatechin, quercetin and resveratrol as a function of ethanol concentration was studied by Berna et al., 2001a, Cháfer et al., 2002; Cháfer et al., 2004 and Berna et al., 2001b at 40°C and 9 MPa. All phenolic compounds showed different solubility behaviours with respect to ethanol concentration. Solubility of quercetin was not effected much by the ethanol concentration, where solubility of catechin and epicatechin increased as more ethanol was added into the CO_2. Epicatechin, which is the isomer of catechin was more soluble in CO_2+ethanol than catechin because of its higher polarity. The behaviour of resveratrol was much more different. Its solubility increased up to an ethanol concentration of 7.5 %, and decreased beyond that value. Le Floch et al. (1998) used 0-20% methanol as a modifier for the extraction of phenolic compounds from olive leaves. The effect of the modifier content on the extraction yield was examined at 100°C and 34.4 MPa and it was found that a 10% methanol–CO_2 mixture provided the highest recovery of phenols while a modifier content of 20% produced undesirable methanol condensation on the analyte trap (ODS or PorapackQ trap) because they used a solid trap for collecting the analytes. The feasibility of replacing methanol with ethanol as modifier was also investigated because the proposed SFE of phenols could be implemented by the food, cosmetic and pharmaceutical industry, which necessitates the use of a non-toxic modifier. As a result, ethanol was found to be useful as a modifier, but less effective than methanol.

The TPC and AE of the extracts obtained at longer extraction times were higher. This was also expected since the maximum extraction time was selected to be 40 minutes according to the preliminary experiments to assure that TPC of the extracts increase significantly with extraction time at all extraction conditions. In fact, the

extraction curves for sour cherry, peach and apple pomace at 60 MPa, 60°C and 20 % (wt/wt) ethanol concentration, show that the amount of phenolic compounds extracted after approximately 45 min was not significant (Figure 3.11). Moreover, Le Floch et al. (1998) found that exhaustive extractions of phenolic compounds from olive leaves were obtained after 140 min at 33.4 MPa, 100°C, using 10% methanol.

Although, there was the possibility of extraction of other antioxidative compounds such as carotenoids, there was a positive significant relationship ($p<0.01$) between TPC and AE in the extracts with high correlations for SCE. The correlation values (r) were 0.97, 0.92 and 0.78 for sour cherry, peach and apple pomaces, respectively. This result might be due to the low concentration of carotenoids in the selected fruits (USDA-NCC Carotenoid Database for U. S. Foods).

The superimposed contour plots giving the optimum regions are given in Figures 3.18-3.20. Table 3.8 and Table 3.9 show the optimum extraction conditions for coded and uncoded factor levels, respectively. The optimum conditions of the independent variables for all samples were similar and close or equal to the upper values of the design. This result was expected since, according to the statistical analysis, all independent variables had a positive effect on both responses. Table 3.10 shows the values of response variables at the optimum extraction conditions. As in HPE, sour cherry pomace was found to have the highest TPC and AE in the extracts obtained by SCE. Although the TPC of the extracts obtained by SCE from apple pomace was a little lower than that of sour cherry, their AE was higher, which may indicate that less but more active phenolic compounds were extracted from apple by SCE. This is consistent with the findings of Kähkönen et al. (1999) who reported that apple extracts showed strong antioxidant activity although the total phenolic contents were low compared with different type of fruits.

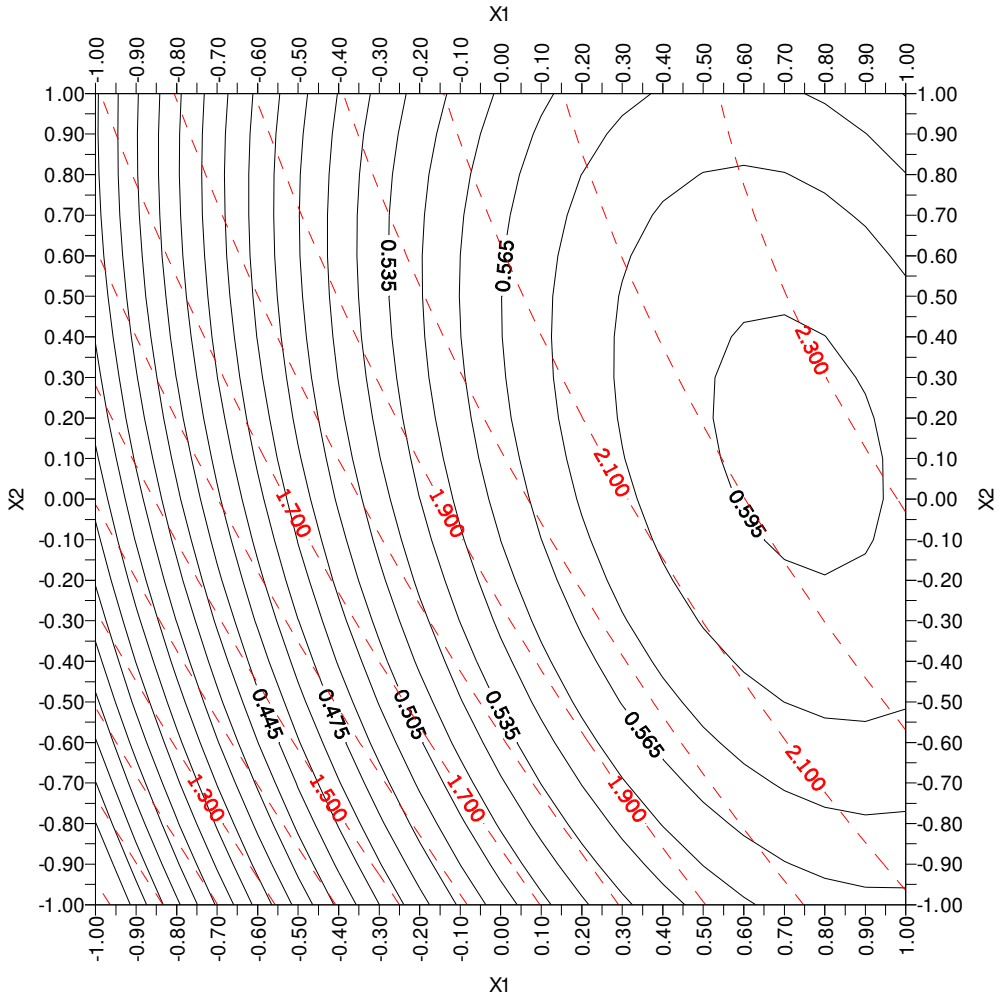

Figure 3.18 Superimposed contour plots for SCE of phenolic compounds from sour cherry pomace (X_3=1.0, X_4=1.0); —— TPC (mg gae/g sample); ------ AE (mg DPPH˙/g sample)

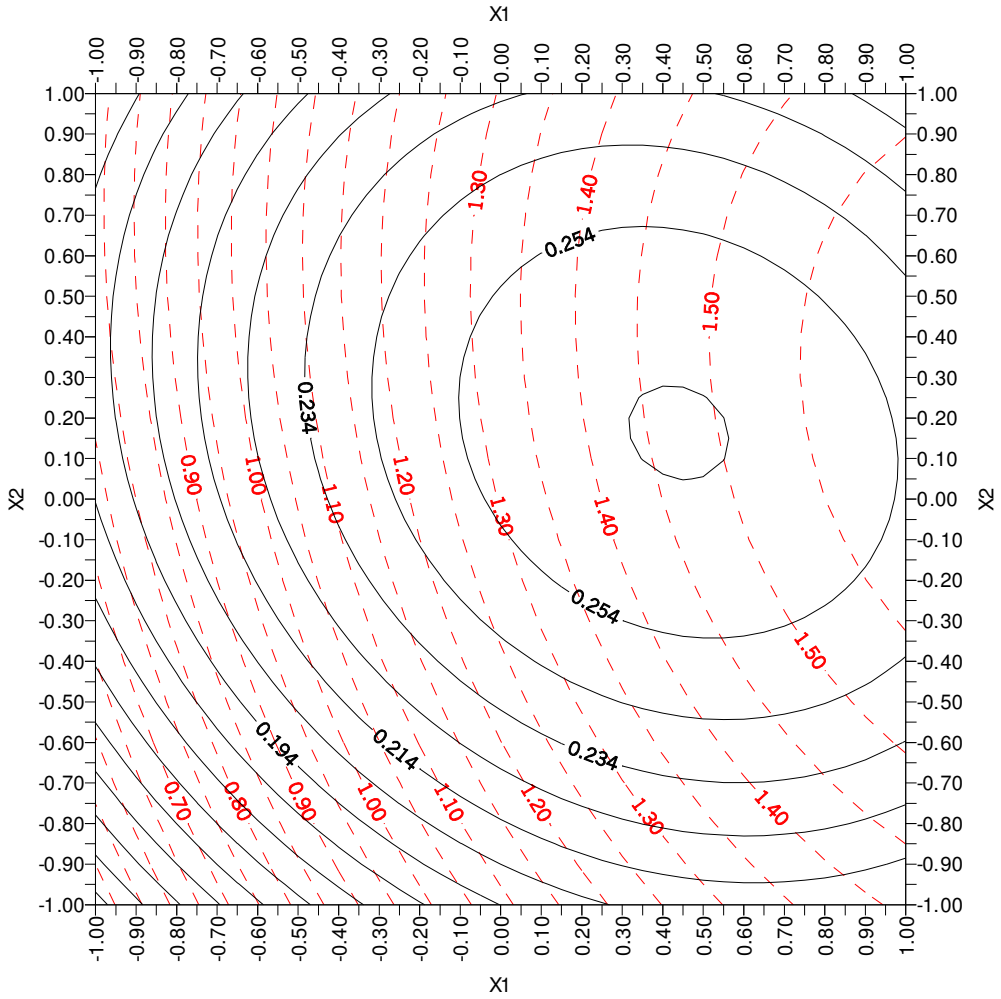

Figure 3.19 Superimposed contour plots for SCE of phenolic compounds from peach pomace (X_3=1.0, X_4=1.0);—— TPC (mg gae/g sample); ------ AE (mg DPPH˙/g sample)

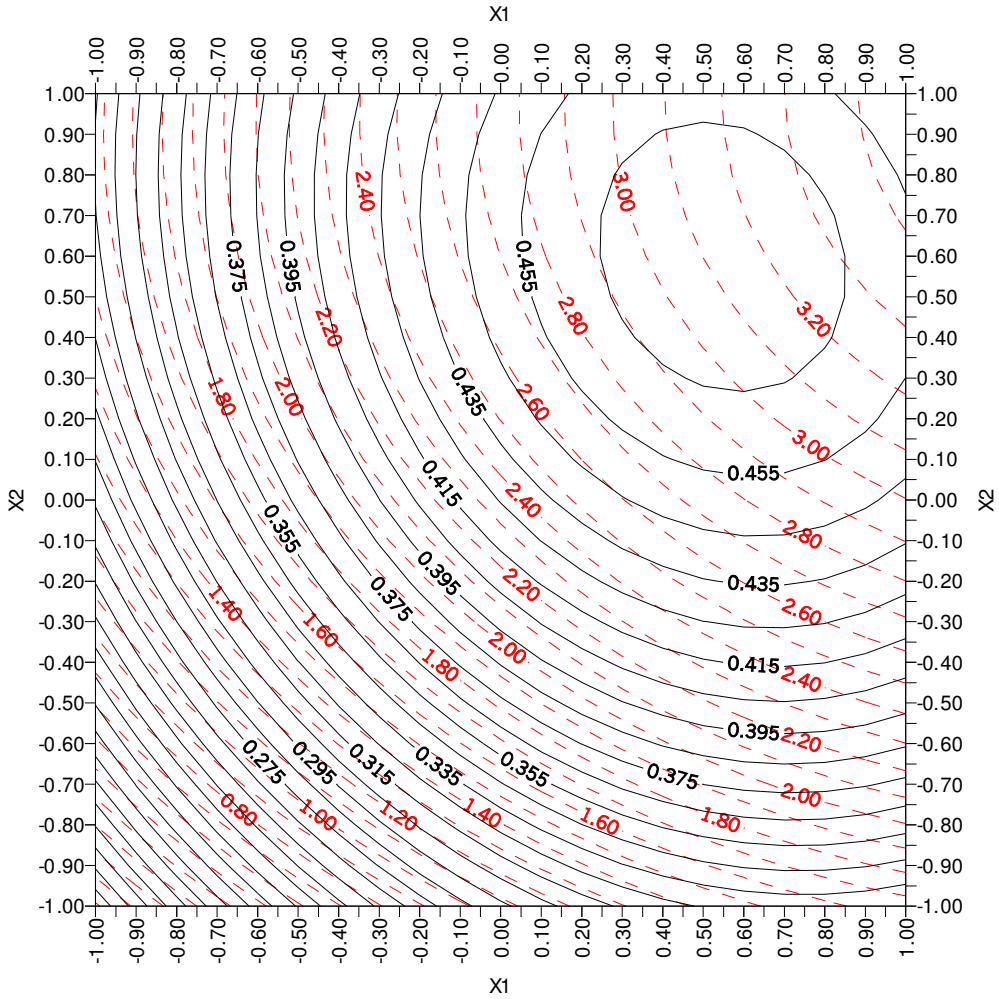

Figure 3.20 Superimposed contour plots for SCE of phenolic compounds from apple pomace (X_3=1.0, X_4=1.0); —— TPC (mg gae/g sample); ------ AE (mg DPPH˙/g sample)

Table 3.8 Optimum conditions for SCE of TPC and their AE at coded factor levels

	Sour cherry pomace	Peach pomace	Apple pomace
X_1	0.74-0.95	0.53-0.55	0.73-0.85
X_2	0.06-0.44	0.09-0.23	0.57-0.84
X_3	1	1	1
X_4	1	1	1

Table 3.9 Optimum conditions for SCE of TPC and their AE at uncoded factor levels

	Sour cherry pomace	Peach pomace	Apple pomace
Pressure (MPa)	54.8-59	50.6-51	54.6-57
Temperature (°C)	50.6-54.4	50.9-52.3	55.7-58.4
Ethanol concentration (wt %)	20	20	20
Time (min)	40	40	40

Table 3.10 Values of response variables at the optimum conditions of SCE

	Sour cherry pomace	Peach pomace	Apple pomace
TPC (mg gae/g sample)	0.60	0.26	0.47
AE (mg $DPPH^{\cdot}$/g sample)	2.30	1.50	3.30
AE/TPC (mg $DPPH^{\cdot}$/ mg gae)	3.83	5.77	7.02

3.3 Comparison of HPE, SCE and SE

HPE and SCE methods were compared by considering solvent extraction (SE). Both ethanol and methanol was used as solvents for SE. Different mixtures with solid/solvent ratios 0.05, 0.1, 0.2, and 0.3 g/ml were prepared which was in parallel with the solid/solvent ratios used in HPE (0.05-0.25 g/ml).

Tables 3.11, 3.12 and 3.13 show the TPC and AE of the extracts obtained by SE at different solid/solvent ratios using methanol and ethanol as solvent and at the optimum conditions of HPE and SCE using sour cherry, peach and apple pomaces. Although both solvents are able to produce hydrogen-bonding and dipole-dipole interactions with phenolics, methanol was a better solvent for the extraction of phenolic compounds from all samples by SE, which was in parallel with the findings in the literature (Peschel et al., 2006; Perva-Uzunalić et al., 2006; Cháfer et al., 2002; Le Floch et al., 1998). However, considering the possible future application of the extracted phenolic compounds in food products, ethanol was selected as a solvent due to its lower toxicity. A positive significant ($p<0.01$) correlation between TPC and AE was found for SE with methanol and ethanol for all pomaces. The correlations (r values) are 0.99, 0.94 and 0.99 for SE extraction with methanol for sour cherry, peach and apple pomaces, respectively. For ethanol, the correlations are 0.89, 0.85 and 0.99 for sour cherry, peach and apple pomaces, respectively.

HPE of phenolic compounds with ethanol at optimum conditions yielded much higher TPC and AE close to those obtained by SE using methanol with a solid/solvent ratio of 0.05 g/ml. Using ethanol as a solvent, high pressure and temperature combination enhanced extraction as compared to SE at the given conditions, as also indicated in the literature (Shouqin et al, 2004; Escribano-Bailon and Santos-Buelga, 2003; Richter et al., 1996).

Table 3.11 TPC and AE of the extracts obtained by SE (n=2) and at the optimum conditions of HPE and SCE from sour cherry pomace

Extraction method	Solid/solvent ratio (g/ml)	TPC (mg gae/g sample)	AE (mg DPPH˙/g sample)	AE/TPC (mg DPPH˙/mg gae)
SE with methanol	0.05	3.52	25.6	7.27
	0.1	3.21	12.5	3.89
	0.2	3.01	4.60	1.53
	0.3	2.96	2.97	1.00
SE with ethanol	0.05	2.92	24.8	8.49
	0.1	2.62	12.1	4.62
	0.2	2.50	4.50	1.80
	0.3	2.06	2.87	1.39
SCE		0.60	2.30	3.83
HPE	0.06-0.07	3.80	22.0	5.79

Table 3.12 TPC and AE of the extracts obtained by SE (n=2) and at the optimum conditions of HPE and SCE from peach pomace

Extraction method	Solid/solvent ratio (g/ml)	TPC (mg gae/g sample)	AE (mg DPPH˙/g sample)	AE/TPC (mg DPPH˙/mg gae)
SE with methanol	0.05	1.07	7.12	6.65
	0.1	0.91	6.80	7.47
	0.2	0.85	6.12	7.20
	0.3	0.79	6.03	7.63
SE with ethanol	0.05	0.81	6.21	7.67
	0.1	0.72	6.13	8.51
	0.2	0.67	5.99	8.94
	0.3	0.58	4.12	7.10
SCE		0.26	1.50	5.77
HPE	0.095-0.19	0.93	6.40	6.88

Table 3.13 TPC and AE of the extracts obtained by SE (n=2) and at the optimum conditions of HPE and SCE from apple pomace

Extraction method	Solid/solvent ratio (g/ml)	TPC (mg gae/g sample)	AE mg DPPH˙/g sample)	AE/TPC (mg DPPH˙/mg gae)
SE with methanol	0.05	2.22	12.1	5.45
	0.1	2.10	10.9	5.19
	0.2	1.98	9.7	4.90
	0.3	1.88	8.6	4.57
SE with ethanol	0.05	1.71	9.3	5.44
	0.1	1.62	8.6	5.31
	0.2	1.56	7.7	4.94
	0.3	1.40	6.1	4.36
SCE		0.47	3.30	7.02
HPE	0.05	2.08	10.8	5.19

Compared to SE and HPE methods, SCE was not so efficient in the extraction of phenolic compounds from the pomaces in spite of the increased polarity of CO_2 with the addition of 20 % (w/w) ethanol. However, SCE can be a suitable extraction method for apple and peach pomaces compared to sour cherry pomace. Çalımlı (2003) also determined that SFE of anthocyanins from sour cherry pomace was inefficient as compared to SE: the amount of anthocyanins extracted by shaking the dry pomace-ethanol mixture at 150 rpm in a water bath at 40°C for 30 min was 18 µg/g dry pomace, where that obtained by SFE at 40 MPa and 50°C without any modifier was 0.893 µg/g dry pomace, which was 5 % of that obtained by SE. Another research on comparison of SFE with SE belongs to Le Floch et al. (1998), who compared extraction of phenolic compounds by SFE with ultrasound-assisted extraction with different solvents at room temperature for 45 minutes. SFE at 33.4 MPa, 100°C for 140 min with CO_2 modified with 10 % methanol was much more efficient than ultrasound-assisted extractions with *n*-hexane, diethyl ether or ethyl

acetate, however recoveries obtained by SFE was 45 % of those obtained by ultrasound-assisted extraction with methanol.

The TPC of the extracts from sour cherry pomace was the highest for all extraction methods, followed by that of apple and peach pomace. The same trend was observed for AE values obtained by HPC and SE. For SCE, the AE of apple pomace extract was higher than that of sour cherry pomace, although its TPC was lower. This result may be due to the selectivity of SCE according to the polarity of phenolic compounds. Accordingly, less but more active phenolic compounds may have been extracted from apple pomace by supercritical CO_2-ethanol mixture. Another reason for low AE of the extracts obtained by HPE and SCE from sour cherry pomace may be the loss of antioxidant activity because of isomerization caused by heat. In fact, Palma et al. (2001) indicates that, of the two isomeric forms of resveratrol, only the *trans* configuration shows biological activities.

The AE/TPC values obtained by SE using ethanol were higher than those for HPE and especially for SCE, which indicates that phenolic compounds extracted by these two methods are less active. This may be due to the inactivation of some heat-sensitive phenolic compounds at 50-60°C. Although most of the phenolic compounds were shown to be stable up to 60°C (Palma et al., 2001; Ju and Howard, 2003; Alonso-Salcez et al., 2001; Papagiannopoulos et al., 2002; Rostagno et al., 2004), some was more sensitive to heat (Cacace and Mazza, 2003).

CHAPTER 4

CONCLUSIONS

In this study, modeling of extraction of phenolic compounds from sour cherry, peach and apple pomaces using high pressure extraction (HPE) and subcritical CO_2 extraction (SCE) was presented in terms of total phenolic content (TPC) and antiradical efficiency (AE). Efficiency of the two methods was compared by considering solvent extraction (SE).

In general, the optimum temperature for HPE for all samples was determined to be 60°C. The optimum values of pressure, solid-solvent ratio and time were between 76-193 MPa, 0.05-0.02 g/ml and 25-37 min. For SCE, the optimum ethanol concentration and extraction time was the same for all samples, which were 20 % (w/w) and 40 min. The optimum values for pressure and temperature were also close to each other, which were between 50.6-59 MPa and 50.6-58.4°C.

Among the three samples, the highest TPC was obtained from sour cherry pomace by all extraction methods, followed by apple and peach pomaces. However, AE of apple pomace extract was the highest for SCE, which may be attributed to the selectivity of SCE according to the polarity of phenolic compounds.

The results indicate that, by using elevated temperatures and pressures, extraction of phenolic compounds from fruit pomaces can be enhanced. HPE with ethanol gives recoveries higher than those obtained by SE with the same solvent. The results are even comparable with those obtained by SE with methanol. In the light of these findings, HPE can be a useful alternative for SE in terms of high efficiency, reduced

extraction time and less amount of solvent. In comparison to ethanol extracts, different solvents can be tested for HPE.

SCE was not so efficient as HPE for the extraction of phenolic compounds from all samples, which is due to the low polarity of CO_2. Addition of ethanol increased the extraction efficiency, however it was not enough to be comparable with HPE and SE. SCE was a better extraction method for apple and peach pomaces compared to sour cherry pomace.

Overall results indicated that fruit pomaces can be used as a source of phenolic compounds. The extracts with antioxidant activity may be potential food ingredients for protecting food and consumers.

As a recommendation, further investigations of antioxidant interactions in relation to food stability can be carried out. Another study can be characterization of the phenolic compounds extracted. The effects of other solvents on the extraction of phenolic compounds can also be investigated. In the further stages, the bioavailability of the extracted phenolic compounds and their health effects can be searched.

CHAPTER 5

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