Analysis, isolation, and bioactivities of rapeseed phenolics

Satu Vuorela

ACADEMIC DISSERTATION

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ABSTRACT

The main objective of the work was to investigate the antioxidant and various bioactivity properties of rapeseed phenolics. Rapeseed meal and oil phenolics were analyzed and hydrolyzed, and methods of isolating the phenolics suitable for food applications were evaluated. The bioactivity testing was focused on antioxidant activity.

HPLC analysis of the phenolic extracts showed the main phenolics in rapeseed meal to be sinapine, the choline ester of sinapic acid, and sinapic acid, while those in crude post-expelled rapeseed oil were vinylsyringol and, in smaller amount, sinapine and sinapic acid. Crude post-expelled rapeseed oil had the highest phenolic content of the oils, and the amount of phenolics decreased during processing.

Rapeseed phenolics were hydrolyzed to free sinapic acid with different enzymes and enzyme preparations. Ferulic acid esterase and Ultraflo L enzyme preparation were as effective as sodium hydroxide, hydrolyzing over 90% of sinapine to free sinapic acid. The total phenolic content was unchanged after enzymatic hydrolysis, whereas it was lowered after base hydrolysis.

Rapeseed phenolics were extracted with different systems. With the use of enzymes such as ferulic acid esterase and Ultraflo L, the hydrolysis and extraction could be done simultaneously yielding free sinapic acid as the main phenolic compound and higher total phenolic content than the other isolation methods. Extraction with hot water was also effective.

All rapeseed phenolic extracts showed excellent antioxidant activity toward the oxidation of liposomes and LDL particles. The antioxidant activity was even better than that of sinapic acid, catechin, and α-tocopherol. The extracts were also effective antioxidants of meat lipids. In addition, phenolic extract of crude post-expelled rapeseed oil was an excellent radical scavenger, while the activities of the other extracts were only moderate. Vinylsyringol, the main phenolic compound in crude oil, and sinapic acid, the main phenolic compound in enzyme-assisted extracts, were effective antioxidants toward all oxidation models tested.

Phenolic extract of crude rapeseed oil showed anti-inflammatory properties: it effectively inhibited the formation of prostaglandin E_2 (PGE_2), and it had some effects on nitric oxide (NO). Both are pro-inflammatory mediators. Vinylsyringol effectively inhibited the formation of PGE_2 and NO, while sinapic acid inhibited the formation of NO. Rapeseed meal extract, containing sinapic acid as the main phenolic compound was not effective against these pro-inflammatory mediators. In Caco-2 cell model, enzyme-assisted extract of rapeseed meal enhanced the permeability of ketoprofen and verapamil. Rapeseed oil phenolics had no effect on the permeability of the model drugs. None of the extracts were toxic or mutagenic.

The present results show that rapeseed phenolic extracts contain ingredients that may be valuable when incorporated in health beneficial products such as foods, feeds, and cosmetic and pharmaceutical preparations.

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III **Vuorela S**, Kreander K, Karonen M, Nieminen R, Hämäläinen M, Laitinen L, Galkin A, Salminen J-P, Moilanen E, Pihlaja K, Vuorela H, Vuorela P, and Heinonen M 2005. Preclinical evaluation of rapeseed, raspberry and pine bark phenolics for health related effects. J Agric Food Chem 53: 5922-5931.

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Contribution of the author to papers I-IV

I The author planned the study together with Prof. M Heinonen. Prof. AS Meyer helped in planning the experiments involving enzymes by offering comments and suggestions. The experimental work and the writing of the manuscript were carried out by the author. The study was supervised by Prof. M Heinonen and she participated in the preparation of the manuscript by offering comments and suggestions.

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III The author planned the study together with Prof. M Heinonen in close collaboration with Prof. E Moilanen, Prof. K Pihlaja, Prof. H Vuorela and Ph.D. P Vuorela. The manuscript was written by the author. In addition to the author, experimental work was carried out by M.Sc. K Kreander, M.Sc. M Karonen, Ph.D. JP Salminen, M.Sc. R Nieminen, M.Sc. M Hämäläinen, M.Sc. L Laitinen and M.Sc. A Galkin. All of them also participated, with their comments and suggestions, in the preparation of the manuscript. The study was supervised by Prof. M Heinonen and she contributed to the writing of the manuscript by offering comments and suggestions.

IV The study was planned by the author, M.Sc. H Salminen, Ph.D. R Kivikari and Prof. M Heinonen. The manuscript was written by the author. M. Sc. H Salminen in regard to the part on protein oxidation offered comments and suggestions. The experimental work was carried out by M. Sc. Student M Mäkelä. The study was supervised by Prof. M Heinonen and she also contributed to the writing of the manuscript by offering comments and suggestions.

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1 INTRODUCTION

Phenolic compounds exist widely in plants. They are plant secondary metabolites, and they have an important role as defence compounds. Although the exact contribution of these secondary metabolites is still unclear, phenolic compounds are known to be important in the survival of a plant in its environment (Puupponen-Pimiä et al., 2005). In addition to plants, phenolics exhibit several properties beneficial to humans. Several plant-derived medicines, which can prevent or cure diseases, are rich in phenolic compounds (Scalbert, 1993). In particular, phenolic compounds have been shown to exhibit protection against coronary heart disease and carcinogenesis (Hertog et al., 1995). They can be classified into the following subgroups: phenolic acids, flavonoids, isoflavonoids, lignans, stilbenes, and complex phenolic polymers (Dewick, 2001).

Rapeseed oil is one of the most important edible oils in the world. Its nutritive value is excellent due to the abundant unsaturated fatty acids. Rapeseed meal is the by-product of rapeseed deoiling process and common used for feed. Its amino acid content is ideal and it has a high content of fiber, several minerals, and vitamins (Naczk et al., 1998; Downey et al., 1990). The amount of residual oil depends on the processing method but typically varies between 1 and 10% (Downey et al., 1990; Unger, 1990). Rapeseed contains more phenolic compounds than any other oilseed plant (Nowak et al. 1992). The most significant of these are sinapic acid and its derivatives, most notable sinapine (figure 2). Most of the phenolic compounds remain in the meal when the oil is pressed from the seeds, but are founded in crude rapeseed oil, most abundantly vinylsyringol (figure 5) (Koski et al., 2003).

Rapeseed phenolics are usually extracted from rapeseed with organic solvents such as aqueous methanol, methanol or acetone (Cai and Arntfield, 2001). Because sinapine is not available as a commercial standard, compositional analysis is done by hydrolyzing the extract to release sinapic acid from its esters. Alkaline hydrolysis is the most common procedure for this, but invreasingly enzymatic hydrolysis is being employed as well.

Rapeseed phenolic compounds are potent antioxidants in various environments relevant to food products and cosmetic and pharmaceutical preparations. Nowak et al. (1992) found that rapeseed phenolic compounds, especially sinapic acid, are active in inhibiting the oxidation of emulsions. According to Wanasundara et al. (1996), the antioxidant activity of crude

ethanolic extract in a β-carotene linoleate model system is higher than that of different rapeseed phenolic fractions due to synergism between different phenolics. The most active rapeseed meal phenolic fraction contained several classes of phenolic compounds including phenolic acids, flavones and flavonols. Koski et al. (2003) fractionated crude rapeseed oil and found fraction containing vinylsyringol to be the most effective antioxidant in bulk and emulsified methyl linoleate and lecithin-liposome systems.

The antioxidant activity of rapeseed meal phenolics has not previously been investigated in either liposome or low density lipoprotein (LDL) model systems. Given the cell membrane-resembling properties of liposomes from soybean lecithin (also used as food emulgator, E 322) and the in vitro significance of LDL oxidation as a biomarker for cardiovascular diseases (DiSilvestro, 2001; Halliwell, 1995), these model systems might provide information relevant to the use of rapeseed phenolics in functional foods intended for health benefit. Although extraction with aqueous methanol is a common procedure for isolating rapeseed phenolics, it is not suitable for food applications. Isolation methods based on ethanol, water or enzymatic extraction would be better choices. The phenolic components of rapeseed are of potential value in the development of health-beneficial foods, feeds, and cosmetic and pharmaceutical preparations. An *in vitro* preclinic evaluation of the phenolics is an essential first step before *in vivo* testing or product development.

2 LITERATURE REVIEW

2.1 Rapeseed oil and meal

Rapeseed oil is one of the most widely consumed edible oils in the world, and its production has grown much faster than that of any other edible oil in recent. Earlier, rapeseed oil contained glucosinolates and erucic acid and other undesirable compounds, which limited its use. Canola can be definied as a rapeseed cultivar that contains less than 2% of erucic acid in oil and less than 30 μmol/g of glucosinolates in its defatted meal. Seeds for oil production have to be well matured and contain less than 3% of damaged seeds. Optimal processing is ensured by cleaning, preconditioning, flaking, and cooking of the seeds. (Shahidi, 1990).

Rapeseed oil is usually produced by mechanical pressing of the seeds followed by solvent extraction with hexane. Oil recovery is lower with mechanical pressing alone. Limiting factors for solvent extraction with hexane are its explosiveness, its harmful effects on the environment and the bad image of solvents among consumers. Straight pressing at elevated temperature is becoming more popular as a consequence (Haumann, 1997). The oil obtained by pressing with or without solvent extraction can be described as crude oil since it has not been refined. However, olive oil can be classified as edible without further processing (Johnson, 1998).

Consumers typically prefer an oil with bland aroma and light color as well as good oxidative stability. Crude oil is considered to be edible only after refining process in which undesirable components such as proteins and other solids, phosphatides, various odor and flavour compounds, free fatty acids, pigments, sulfur-containing compounds, trace solvents, and water are removed. There are two different types of refinings: chemical and physical. The chemical refining process includes degumming, neutralizing, bleaching, and deodorizating, whereas in physical refining, free fatty acids and flavors are removed via distillation, where neutralization and deodorization are combined into one operation. Physically refined oil is often called RBD oil (refined, bleached, deodorized) (Johnson, 1998).

Phosphatides are removed from the crude oil in a degumming step. As rapeseed oil is normally rich in phosphatides, degumming is typically performed as a separate operation. In the refining of other oils, it is usually combined with neutralization. In superdegumming, an

organic acid such as citric acid is added to the warm oil, and the mixture is stirred and cooled to precondition the gums (Unger, 1990; Johnson, 1998). Water is added, causing the phosphatides to form liquid crystals, which are easily removed by centrifugation (Johnson, 1998).

Rapeseed meal is the by-product of the rapeseed deoiling process and is commonly used as feed (Downey et al., 1990). Rapeseed meal contains 40% proteins and its amino acid composition has a high nutritive value (Naczk et al., 1998). It is high in fiber, and rich in minerals such as calcium, magnesium, zinc and copper. It also contains a number of vitamins and other bioactive compounds such as tocopherols, several B vitamins and choline, which make the meal nutritionally very valuable. The amount of residual oil in rapeseed meal depends on the processing method and typically varies between 1 and 10% (Downey et al., 1990; Unger, 1990). Rapeseed, especially rapeseed meal, is rich in phenolic compounds. According to Nowak et al. (1992), it contains more phenolic compounds than any other oilseed plant. The most significant of these is phenolic compounds in rapeseed is sinapine, the choline ester of sinapic acid (ca. 80% of the total phenolic compounds) (figure 2) (Kozlowska et al., 1990). Sinapic acid in rapeseed also exists as the glucosidic ester, glucopyranosyl sinapate (Amarowicz and Shahidi, 1994). Only a small part of sinapic acid, less than 16%, is present as the free sinapic acid (figure 2) (Kozlowska et al., 1990). Most of the phenolic compounds remain in the meal when the oil is pressed from the seeds (Koski et al., 2003). Typically the amount of sinapic acid derivatives in rapeseed meal varies between 6390 and 18370 μg/g depending on the variety of oilseed plant and the oil processing method (Kozlowska et al., 1990). Of the phenolic compounds present in the crude rapeseed oil, the most abundant is the newly identified compound, vinylsyringol, which is present in amounts of 245-700μg/g (figure 5) (Koski et al., 2003).

2.2 Phenolic compounds in rapeseed

2.2.1 Biosynthetic origin of plant phenolics

Phenolic compounds can be classified into the following subgroups: phenolic acids, flavonoids, isoflavonoids, lignans, stilbenes, and complex phenolic polymers (Dewick, 2001). In oilseed plants generally, the main phenolic compounds are derivatives of hydroxybenzoic and hydroxycinnamic acids as well as coumarins, flavonoids, and lignins (Kozlowska et al.

1990), while in rapeseed, the main phenolics are hydroxycinnamic acid derivatives. Phenolic acids have carboxylic acid functionality and fall into the subclasses, hydroxycinnamic and hydroxybenzoic acids. The biosynthetic origin of plant phenolics is the aromatic amino acid L-phenylalanine, a three-step sequence referred as general phenylpropanoid metabolism (Robbins, 2003) (figure 1).

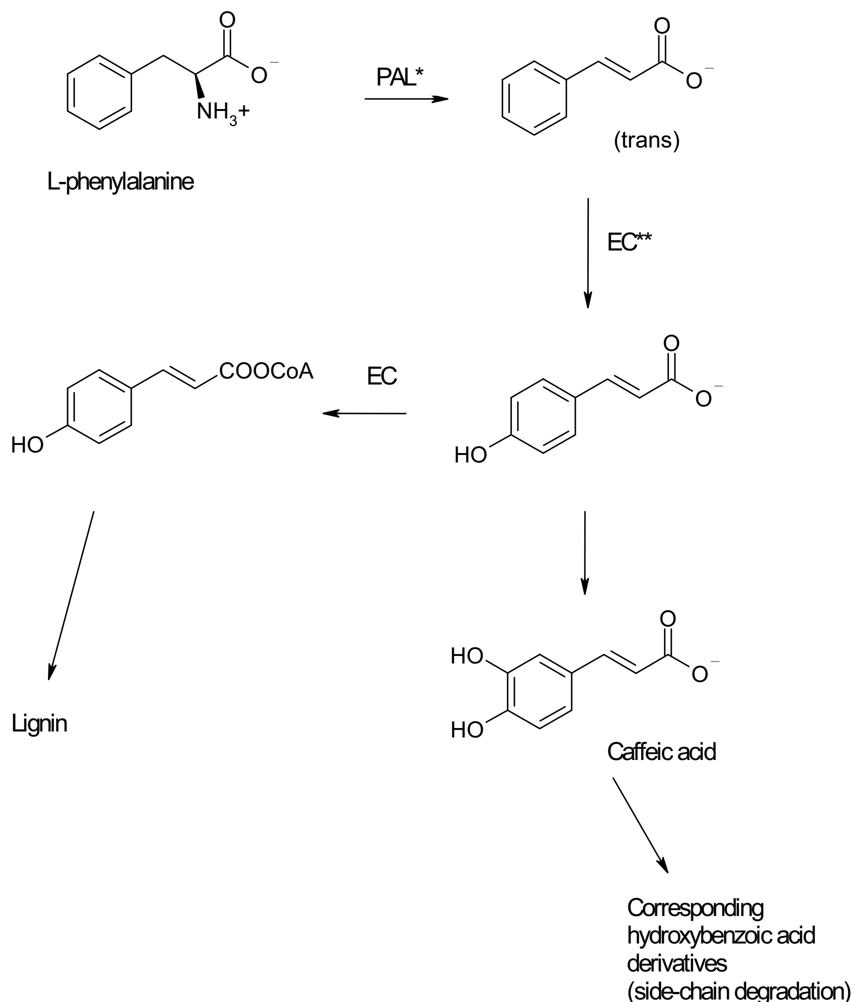


Figure 1. Biosynthetic pathways of hydroxycinnamic and hydroxybenzoic acids from L-phenylalanine (Robbins, 2003). L-phenylalanine ammonia lyase (PAL), enzymatic conversion (EC).

2.2.2 Phenolic compounds in rapeseed meal

Rapeseed meal is rich in phenolic compounds. Indeed, according to Nowak et al. (1992), , rapeseed contains the greatest amount of phenolic compounds of all oilseed plants. The total phenolic acid content of selected oilseed flours is shown in table 1. Rapeseed phenolics include esterified phenolic acids, free phenolic acids, and insoluble-bound phenolic acids (Krygier et al. 1982). The total content of phenolic acids varies between 6400 and 18400 μg/g depending on the variety of the plant and oil processing method. The phenolic content in rapeseed flour (i.e. rapeseed meal without hulls) is lower than in rapeseed meal. In addition, the growing conditions and the degree of maturation affect the phenolic composition. In germination, some sinapine is released as free sinapic acid (Kozlowska et al., 1990).

Table 1. Phenolic acid content of selected oilseed flours (μg/g) (Kozlowska et al., 1990).

Flour	Total phenolic acid content
Soybean	234
Cottonseed	567
Peanut	636
Rapeseed	6399

Esterified phenolic acids

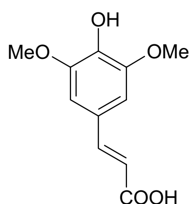
Phenolic acids in rapeseed are present mainly in esterified form. Phenolic esters may constitute as much as 99% of total phenolics in rapeseed flour. The main phenolic ester in rapeseed is sinapine, the choline ester of sinapic acid. Besides choline, sinapic acid can be esterified with another phenolic acid, sugars, or kaempferols (Kozlowska et al., 1990). Amarowicz and Shahidi (1994) isolated glucopyranosyl sinapate, a phenolic glucoside, from rapeseed meal. Other phenolic acids such as p-hydroxybenzoic acid, vanillinic acid, and syringic acid can form ester bonds as well (Kozlowska et al., 1983b). The composition of esterified phenolic acids is genetically controlled but their contents are affected by the cultivation and growing conditions. Rapeseed contains ca. 0.39-1.06% of sinapine depending on the species, growing conditions, and degree of maturation (Kozlowska et al., 1990; Naczk et al., 1998). Sinapine has an important role in rapeseed as storage for sinapic acid and choline in young plants. During seed maturation, some of the sinapine hydrolyzes to free sinapic acid and choline. Sinapic acid is a starting compound in the synthesis of lignins and

flavonoids, while choline is an important metabolic product of the methylation cycle (Kozlowska et al., 1990).

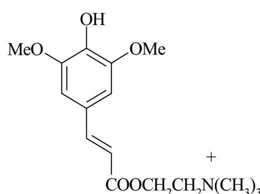
After hydrolysis of the phenolic acid esters, sinapic acid is the predominant phenolic acid. Kozlowska et al. (1983b) released 11 other phenolic acids in addition to sinapic acid from ester linkages, the main ones being ferulic, p-hydroxybenzoic, and syringic acids.

Free phenolic acids

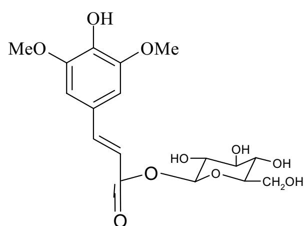
Free phenolic acids constitute 6.5-9.0% of the total phenolics in rapeseed flours and up to 15% in rapeseed meals. Some varieties are exceptional in containing only trace amounts or even no detectable quantity of free phenolic acids. The most significant phenolic compounds in rapeseed are sinapic acid derivatives. Sinapic acid, which belongs to hydroxycinnamic acid group, constitutes 70.2-85.4% of free phenolic acids in defatted rapeseed meals (figure 2). Other phenolic acids besides sinapic acid are ferulic acid, o-coumaric, p-coumaric, caffeic, p-hydroxybenzoic, vanillic, gentisic, protocatechic, syringic, and chlorogenic acids (Shahidi and Naczk, 1992; Kozlowska et al., 1983b; Kozlowska et al., 1983a) (Figure 3). In addition to these, Kozlowska et al. (1983b) found also salicylic and cinnamic acids in rapeseed flour.



Sinapic acid

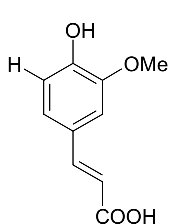


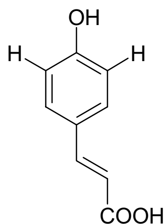
Sinapine (the choline ester of sinapic acid)

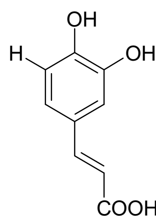


Sinapate (glucopyranosyl sinapate)

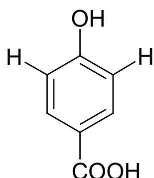
Figure 2. The main phenolics in rapeseed meal.

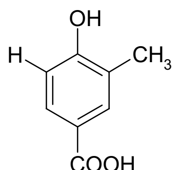


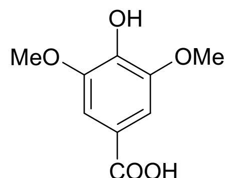




Ferulic acid p-Coumaric acid Caffeic acid







p-Hydroxybenzoic acid Vanillinic acid Syringic acid

Figure 3. Structures of the minor phenolic acids in rapeseed meal.

Insoluble-bound phenolic acids

After extraction of the phenolics from rapeseed, a small amount of phenolics remain in the meal. According to Kozlowska et al. (1983b), these cell-wall bound phenolics are presumably bound to proteins or carbohydrates. Cooking or other processing leads to their release, and they can also be released by alkaline hydrolysis (Kozlowska et al. 1983a). Kozlowska et al. (1983b) found nine phenolic acids in the insoluble-bound phenolic fraction of rapeseed flour, where sinapic acid was the predominant one followed by ferulic, p-coumaric, and o-coumaric acids.

Tannins

Food tannins are polyphenolic compounds, which are widely distributed in plants. They can be classified as condensed or hydrolyzable tannins. Most of the tannins in rapeseed are condensed tannins (figure 4), formed by polymerization of flavan-3-ols or flavan-3,4-diols.

The amount of tannins in rapeseed depends on the variety, the degree of maturation and extraction method, and varies from 0.2 to 3% of defatted rapeseed meal (Naczk et al. 1998).

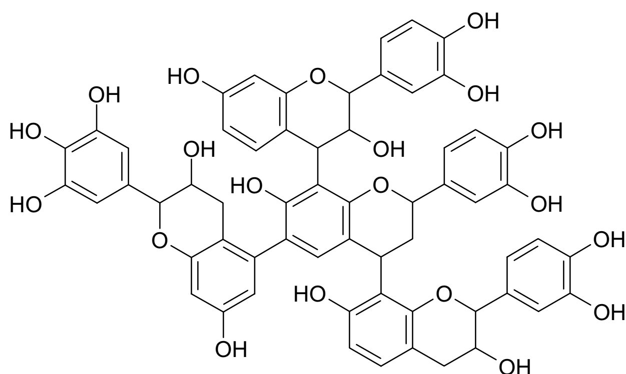


Figure 4. Structure of a condensed tannin.

Some of the tannins in rapeseed exist in insoluble forms. Naczk et al. (2000) measured the content of insoluble-bound tannins in rapeseed hulls and concluded that their insolubility may be due to polymerization as well as to the formation of insoluble complexes with the fiber and protein fractions of the seed.

2.2.3 Phenolic composition in rapeseed oil

In rapeseed oil processing, most of the phenolic compounds especially the polar phenolics remain in the meal. The most abundant nonpolar phenolic compounds in rapeseed are tocopherols. Pekkarinen et al. (1998) analyzed the tocopherol composition of rapeseed oils at different stages of oil processing and found that oil processing did not significantly decrease the amount of α- tocopherol whereas the content of γ-tocopherol decreased slightly (Pekkarinen et al. 1998). Koski et al. (2003) determined the total phenolic content and the contents of tocopherols, sinapic acid and vinylsyringol by HPLC of post-expelled crude rapeseed (RPE) and turnip rapeseed oils (TPE). In addition, turnip rapeseed samples included also superdegummed oil (SDG), a refined, bleached, and deodorized oil (RBD), and an oil refined, bleached and deodorized under modified, milder conditions (MOD). They found the amount of polar phenolicsto be highly dependent on the degree of refinement in rapeseed oil, the total phenolic content in crude post-expelled rapeseed oil was 730 and 1066 µg/g whereas the refined commercial rapeseed oil contained no polar phenolic compounds (table 2).

Table 2. Phenolic composition of crude post-expelled rapeseed oil and different turnip rapeseed oils (μg/g) (Koski et al. 2003).

	RPE	TPE	SDG	MOD	RBD
α-tocopherol	295	243	202	212	154
γ-tocopherol	404	529	520	512	358
Total phenols[a]	1066	730	193	44	16
Sinapic acid	23	13	4	2	0
Vinylsyringol[b]	629	297	48	0	0
Unknown 325 nm	3	19	1	0	0
Unknown 307 nm	5	3	0	0	0

a Caffeic acid equivalents.
b Sinapic acid equivalents.

Koski et al. (2003) identified a new compound, vinylsyringol, in crude post-expelled rapeseed oil (Figure 5), and concluded that it was a decarboxylation product of sinapic acid formed from sinapic acid during oil processing at elevated temperature and pressure. They also concluded that vinylsyringol is the main phenolic compound in crude rapeseed oil. In crude rapeseed oil, the amount of vinylsyringol was 629 μg/g oil quantified as sinapic acid equivalents. Crude rapeseed oil also contained sinapic acid (23 μg/g). Vinylsyringol was subsequently isolated by Kuwahara et al. (2004) under the name canolol.

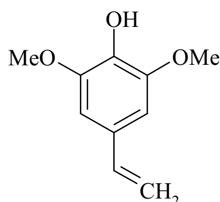


Figure 5. Structure of vinylsyringol.

2.3 Analysis of rapeseed phenolics

The analysis of rapeseed phenolics usually proceeds by the procedure described in figure 6.

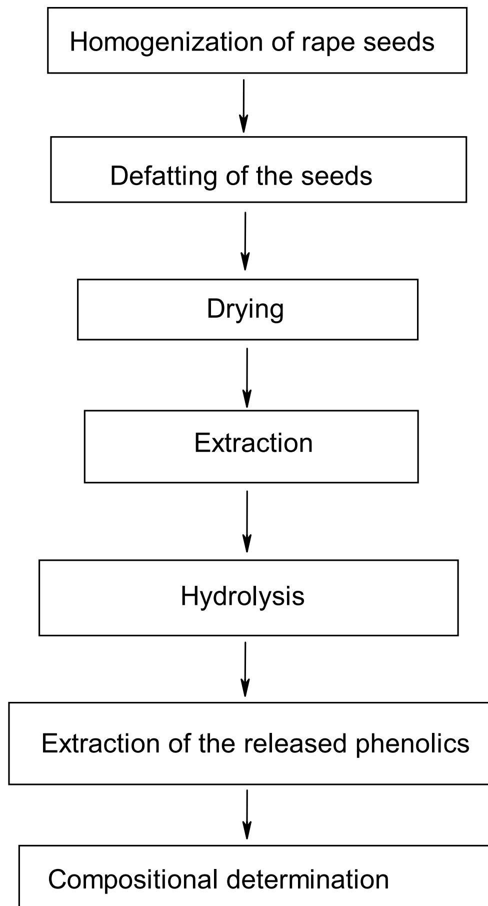


Figure 6. A common procedure for the analysis of rapeseed phenolics.

2.3.1 Pretreatment of the seeds

Since rapeseeds contain 40% of oil, an essential step in isolating the phenolics is to remove the fat from the ground and homogenizated seeds (Shahidi, 1990). as. Extraction with hexane in a Soxhlet apparatus is the most common procedure (Naczk et al., 1992a; Wanasundara et al., 1996). The defatting can also be performed with methanol/ammonium/water (Naczk and Shahidi, 1989) or with carbon tetrachloride (Li and Rassi, 2002). Hulls may be removed after

extraction (Krygier et al., 1982; Kozlowska et al., 1983a; Kozlowska et al., 1983b). However, the flour, i.e. rapeseed meal without rapeseed hulls, contains less phenolics than does the meal with hulls. Finally, the defatted meal is dried with air (Wanasundara et al., 1994; Wanasundara et al., 1996; Xu and Diosady, 1997), in a vacuum oven (Naczk and Shahidi, 1989) or at room temperature (Li and Rassi, 2002).

2.3.2 Extraction of phenolics from rapeseed meal and oil

Defatted rapeseed meal is usually extracted with organic solvents, most commonly aqueous methanol, ethanol, or acetone as described in table 3.

Extraction with aqueous methanol is the most common extraction method. Cai and Arntfield (2001) found refluxing with 100% methanol to be as effective as extraction with 70% methanol at 75°C. According to Naczk et al. (1992a), 70% aqueous methanol is twice as efficient in extracting rapeseed phenolics as is pure methanol. However, the solvent-to-meal ratio was lower, which, according to Cai and Arntfield (2001), may be one reason for this difference. When extracting rapeseed tannins, 70% aqueous acetone is the most common extraction solvent. However, this is not suitable when extracting the insoluble condensed tannins. Naczk et al. (2000) used a combination of methanol, butanol and hydrogen chloride in extracting the insoluble tannins.

Table 3. Methods for the analysis of rapeseed phenolics.

Extracted compounds	Extraction solvent	Hydrolysis yes/no	Method of determination	References
Free, esterified, and insoluble-bound phenolics	MeOH[a]/Ac[b]/ Water (7:7:6)	yes	UV	Pink et al. 1994
Free, esterified, and insoluble-bound phenolic acids	80% MeOH	yes	GC	Zadernowski and Kozlowska 1983
Free, esterified, and insoluble-bound phenolic acids	80% methanol	yes	GC	Kozlowska et al. 1983a
Free, esterified, and insoluble-bound phenolic acids	Hot 80% aqueous methanol	yes	GC	Kozlowska et al. 1983b
Free, esterified, and insoluble-bound phenolics	70% MeOH/70% Ac (1:1)	yes	TLC, GC-MS	Krygier et al. 1982
Free, esterified, and insoluble-bound phenolics	MeOH/Ac/ water (7:7:6)	yes	Folin-Denis	Naczk and Shahidi 1989
Free, esterified, and insoluble-bound phenolics	Several such as 70% MeOH, 70% EtOH 70% Ac, 70% IPA[c], MeOH/Ac/ water (7:7:6)	yes	Folin-Ciocalteu	Thiyam et al. 2004
Phenolic compounds	95% EtOH[d]	no	UV	Wanasundara et al. 1996
Total phenolic acids	60% Ac, pH 3	yes	Folin-Denis	Xu and Diosady 1997
Phenolic acids	70% Ac	yes	TLC, GC, MS	Fenton et al. 1980
Sinapic acid and vinylsyringol from crude rapeseed oil	80% MeOH	no	Total phenolics by Folin-Ciocalteu, sinapic acid and vinylsyringol by HPLC	Koski et al. 2003
Sinapic acid	MeOH/Ac/water (7:7:6)	yes	UV	Pink et al. 1994
Sinapic acid	MeOH/Ac/water (7:7:6)	yes	UV	Naczk et al. 1992a
Sinapine	70% MeOH 75°C 20 min	no	UV-spectrophotometer	Wang et al. 1998
Sinapine	100% methanol	yes	HPLC	Li and Rassi 2002
Sinapate	95% EtOH	no	Semipreparative HPLC	Amarowicz and Shahidi 1994
Sinapate	95% EtOH	no	TLC	Amarowicz et al. 1995
Soluble and insoluble tannins	Soluble tannins: 70% Ac Insoluble tannins: MeOH/BuOH[e]/HCl	no	Proanthocyanidin assay	Naczk et al. 2000
Tannins	70% Ac, N,N-dimethyl formamide or MeOH	no	Folin-Denis	Naczk et al. 1992b

a Methanol (MeOH), b Acetone (Ac), c Isopropanol (IPA), d Ethanol (EtOH), e Butanol (BuOH)

Earlier, it was common procedure to perform several extractions, up to six repetitions, to isolate rapeseed phenolics (Krygier et al. 1982; Zadernowski and Kozlowska, 1983; Naczk et al. 1992a; Pink et al. 1994). Now, procedures with fewer extractions have become common. Cai and Arntfield (2001) and Wang et al. (1998) used just a single extraction. Wang et al. (1998) investigated the effect of number of extractions and the extraction time on the recovery of extracted phenolics varying the number of extractions between 1 and 5 and using extraction times of 10, 30, 60, and 90 min. They found no statistical differences in the amount of extracted phenolics with the number of extractions, or the extraction time. They concluded that a single-extraction with shorter extraction time is suitable for extracting the phenolics from rapeseed meal, especially where large of numbers of samples are to be handled. This facilitates the analysis procedure when analyzing large number of samples. To avoid sinapine degradation, Thies (1991) warned that the extraction should not exceed 20 min. However, Wang et al. (1998) did not observe sinapine degradation.

Koski et al. (2003) extracted rapeseed phenolics from crude post-expelled rapeseed oil with 80% aqueous methanol in a separation funnel and concluded that aqueous methanol was the best extraction solvent for extracting rapeseed oil phenolics.

2.3.3 Hydrolysis

After extraction of the phenolics the isolate is usually hydrolyzed with sodium hydroxide to release the esterified phenolics. When the pH is lowered below 2, the released phenolics are available as phenolic acids instead of ionic forms and can be extracted with diethyl ether (Fenton et al., 1980; Kozlowska et al. 1983b; Zadernowski and Kozlowska, 1983; Naczk and Shahidi, 1989) or diethyl ether/ethyl acetate (1:1) (Krygier et al., 1982; Naczk et al., 1992). Hydrolysis of sinapine to sinapic acid is the preferred procedure for analyzing sinapic acid and its derivatives because sinapine is not available as a commercial standard. Moreover, the isolation procedure for sinapine outlined by Clandinin (1961) is very time-consuming.

In place of sodium hydroxide, enzymes have been used in hydrolysis of bound phenolics. Enzymes have been successfully used for hydrolyzing phenolic esters in cereal material, for example in barley spent grain (Bartolomé and Gómez-Cordovés 1999; Faulds et al. 2002). Yu et al. (2002) used ferulic acid esterase and xylanase in hydrolyzing ferulic acid esters in oat. Bartolomé and Gómez-Cordovés (1999) used two commercial enzyme preparations, Ultraflo

L and Viscozyme L, to release ferulic acid from barley spent grain, and found that Ultraflo L hydrolyzed 70% and Viscozyme L 33% of ferulic acid esters. The enzyme activity of Ultraflo L was much higher than that of Viscozyme L, but Viscozyme L had higher specificity in hydrolyzing ferulic acid esters. According to Faulds et al. (2002), Ultraflo L had activity toward the methyl esters of ferulic, caffeic, p-coumaric and sinapic acids. In oat, ferulic acid esterase hydrolyzed only a small part of ferulic acid esters (Yu et al. 2002). The hydrolyzing effect was stronger when ferulic acid esterase and xylanase were added together due to synergistic effects between these enzymes.

2.3.4 Release of insoluble-bound phenolics

Insoluble-bound phenolics are not extractable in the normal common extraction procedureand remain in the meal. Their proportion of the total phenolics is small, but they can be released from the rapeseed matrix by alkaline hydrolysis with sodium hydroxide. After adjustment of the pH under 2 and extraction of the released phenols, they can be measured together with free phenolic acids and the phenolic acids released from their ester bonds (Kozlowska et al. 1983b; Zadernowski and Kozlowska, 1983; Naczk and Shahidi, 1989).

2.3.5 Analysis of sinapine as choline

Li and Rassi (2002) measured the sinapine content in rapeseed meal by first hydrolyzing sinapine to sinapic acid and choline and then oxidizing the choline with choline oxidase (figure 7). The advantages of this method are that sinapic acid and betaine are available as commercial standards, and the amount of sinapine in rapeseed can be calculated without isolation of sinapineas such (Clandinin et al. 961). In addition, calculating of the amount of sinapine through both sinapic acid and betaine (i.e. choline) allows the sinapine content to be measured accurately.

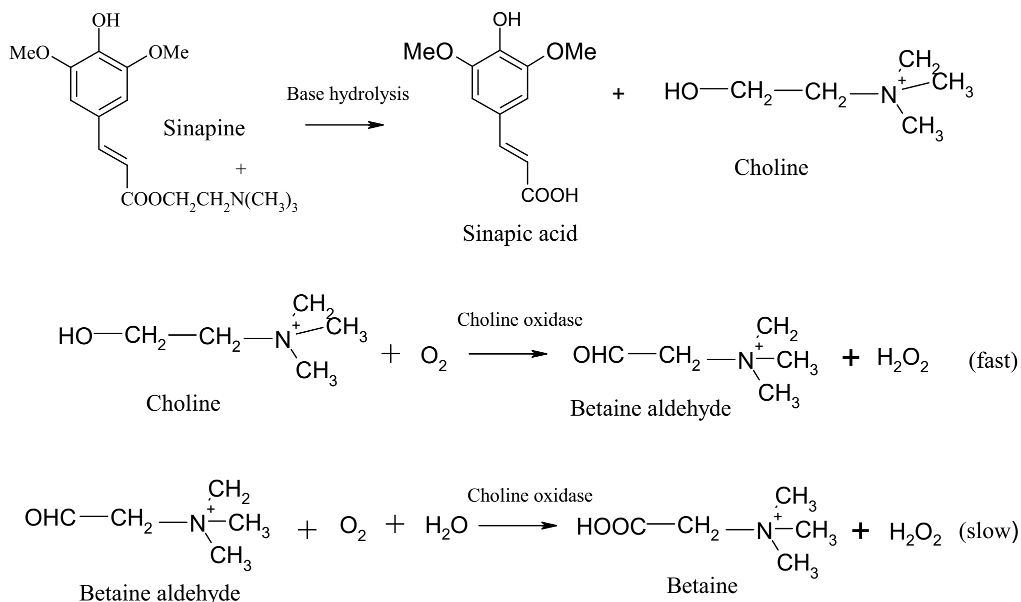


Figure 7. Enzymatic hydrolysis of sinapine to yield choline, which is then oxidized to betaine (Li and Rassi, 2002).

2.3.6 Determination of phenolic compounds

Spectrophotometric methods

The total phenolic content of rapeseed extract is usually determined colorimetrically with a UV-vis –spectrophotometer using Folin-Denis or more commonly Folin-Ciocalteu assays. The main principle of these assays is similar based on the reduction of phosphomolybdic-phosphotungstic acid reagent (Folin reagent) in alkaline solution (Singleton and Rossi, 1965; Schanderl, 1970). During the assay, the methanolic or water-based solutions of rapeseed samples and Folin reagent and sodium carbonate solution are mixed, and after 30 min the absorbance is measured at 725-765 nm (Naczk and Shahidi, 1989; Xu and Diosady, 1997; Naczk et al., 1992a; Koski et al, 2003; Thiyam et al. 2004;). When Matthäus (2002) measured the total phenolics of rapeseed, the samples were in methanol/0.3% HCl (2:3). Sinapic acid is the usual standard in both assays, and the results are expressed as sinapic acid equivalents. Because sinapic acid is practically insoluble in water, which may cause difficulties in

quantification, Koski et al. (2003) preferred caffeic acid in measuring the total phenolic content of differently processed rapeseed oils, noting that caffeic acid as better standard compound produces a more linear dose-response curve than sinapic acid. As sinapic acid is practically insoluble in water, ethanolic/methanolic solution must be used.

Naczk et al. (1992b) applied vanillin assay when determining condensed tannins in rapeseed meal. Vanillin reagent was added to methanolic solutions of condensed tannins, and after 20 min the absorbance was measured at 500 nm with catechin as standard compound. The results were expressed as catechin equivalents. Naczk et al. (2000) used proanthocyanidin assay in measuring the content of insoluble tannins from rapeseed. Rapeseed acetone extracts (70%) were treated with a mixture of methanol, butanol, and HCl, which hydrolyze the condensed tannins to anthocyanidins. The mixture was heated and vortexed, and the absorbance was measured with a UV-Vis –spectrophotometer at 530 nm using cyanidin as standard compound.

Thin layer chromatography

Thin layer chromatography (TLC) has been applied in separating and identifying the phenolic acids in rapeseed meals (Fenton et al., 1980; Krygier et al., 1982). Usually TLC is used to control the purity of samples by gas chromatography. The TLC plates are coated with silica gel IB2-F or G-25 and fluorescent indicator UV-254 (Krygier et al., 1982; Fenton et al., 1980). Krygier et al. (1982a) used benzene-methanol-acetic acid (20:4:1) as a solvent system for the separation of phenolic acids, fatty acids, and other contaminants, while Fenton et al. (1980) used several solvent systems, namely, chloroform-methoxyethanol-formic acid and 88% acetic acid (60:20:8:12), butanol-acetic acid-water (6:1:2) and butanol-acetic acid-water (4:1:5) for separating the polyphenolics from the unhydrolyzed extracts and benzene-diethylether-acetic acid (50:50:0.2) for separating the free phenolic acids. Limitation on the use of TLC in analysis is the difficulties in quantification, as noted by Cai and Arntfield (2001). Krygier et al. (1982) found that before removing the fat residue from the extracts with hexane, the spot of fatty acids was seen on the TLC plate. This spot was well separated from the spots of phenolic compounds, however. This finding indicating that removal of the fat residue from the extracts is not necessary when using TLC.

TLC has also been used to separate individual phenolic compounds such as glucopyranosyl sinapate and other phenolic compounds from rapeseed meal (Amarowicz and Shahidi, 1994; Amarowicz et al., 1995). Quantification of the compounds requires some additional methods, such as purification with Sephadex and semi-preparative HPLC.

Gas chromatography

There are several studies where gas chromatography (GC) has been used for identification and quantification of phenolic compounds in rapeseed meal (Zadernowski and Kozlowska, 1983; Krygier et al., 1982a; Fenton et al., 1980; Kozlowska et al., 1983a; Kozlowska et al., 1983b). In most cases the gas chromatograph has been equipped with a flame ionization detector and a packed glass column with 1.5% SE-30 or 6% or 3% OV-1 on 80-100 mesh Chromosorb W/HP and with nitrogen as a carrier gas (Kozlowska et al., 1983b; Fenton et al., 1980; Krygier et al., 1982a). The chromatographic run has usually been temperature programmed, e.g. from 120 to 300 °C at 4 °C/min, 130-210 °C at 5°C/min or 98-260°C at 6 °C/min (Fenton et al., 1980; Krygier et al., 1982; Kozlowska et al., 1983b). According to Zadernowski and Kozlowska (1983), gas chromatography requires careful removal of the fat residue before analysis, as residues of fatty acids in the samples lead to overestimation of the phenolic compounds as well as generating other errors in quantification and identification. In contrast to this, Krygier et al. (1982) found that free fatty acids do not interfere with the quantification. Before analysis, the samples have to be derivatized to their trimethylsilyl ethers with N, O, -bis (trimethylsilyl) acetamide (BSA). Heptadecanoate (Fenton et al., 1980) or n-tetracosane (Krygier et al., 1982; Kozlowska et al, 1983b; Zadernowski and Kozlowska, 1983) has been used as internal standard in quantification. The isomers of some phenolic compounds (cis- and trans-sinapic and ferulic acids) can be identified in the gas chromatogram.

High performance liquid chromatography

High performance liquid chromatography (HPLC) has recently become a common replacement for gas chromatography. Derivatization is not required and the fat residue does not interfere with the determination. The HPLC system has usually been equipped with a UV detector and a reverse-phase C18 column (Cai and Arntfield, 2001; Li and Rassi, 2002). Li and Rassi (2002) also used a normal-phase silica column for the determination of betaine. Cai

and Arntfield (2001) investigated a rapid HPLC method for the determination of sinapic acid and sinapine in canola (rapeseed) meal using a 10-min isocratic/linear/concave gradient and a 15-min isocratic/linear gradient with a mixture of acetate buffer and methanol as mobile phase. According to Cai and Arntfield (2001), this facilitates the analysis as no purifications or further analyses are required. After extraction, however, the samples had to be purified with CM-Sephadex C-25 resin before HPLC determination. Li and Rassi (2002) used HPLC to determine sinapine in rapeseed. With a C18 column and gradient run, a mixture of ammonium dihydrogen phosphate buffer and methanol was used as the solvents in mobile phase, while with silica column, a mixture of acetonitrile and ammonium chloride was used. Since no commercial standard of sinapine is available, sinapine was first hydrolyzed to free sinapic acid and choline with sodium hydroxide, followed by enzymatic oxidation of choline to betaine. Since betaine and sinapic acid are available as commercial standards, the amount of sinapine can then be quantified via these two fragments, using normal phase chromatography for betaine and reversed phase chromatography for sinapic acid.

Matthäus (2000) used HPLC to analyze the neutral phenolic compounds in rapeseed. After extraction, the phenolics were fractionated by Sephadex LH-20 solid phase extraction method, then isolated with a C_{18}-cartridge and analyzed by analytical and preparative HPLC.

2.4 Sensory and nutritional significance of rapeseed phenolics

2.4.1 Sensory properties

Phenolic compounds may have some unpleasant effects in human nutrition. The sensory properties of phenolics, with their contribution to dark color, bitter taste, and astringency, are not always desirable. The threshold for the objectionable flavors of phenolic acids has been investigated and it has been shown that the threshold is much lower for a combination of phenolic compounds than for individual phenolic acids. Astringency is caused by a precipitation of salivary proteins and manifests as a puckering and drying sensation over the surface of the tongue and the buccal mucosa. The ability of a compound to act as an astringent is linked to its moderate molecular size, from 400 to 3000 daltons, and a number of phenolic groups oriented into 1,2-dihydroxy or 1,2,3-trihydroxy configurations (Shahidi and Naczk, 1992). According to Shahidi and Naczk (1992), at least two such orientations are required to

impart astringency. However, the complex can precipitate protein only when it becomes sufficiently hydrophobic.

In a study, the bitterness and astringency of sinapine and its components, sinapic acid and choline chloride, Ismail et al. (1981) found that sinapine is a precursor of bitterness, while the bitterness of choline chloride is weaker. They also found that sinapine stimulates a minor amount of astringency, while choline chloride is responsible for little if any astringency. The bitterness and astringency of sinapic acid were difficult to measure owing to the sourness. The bitterness of sinapine could be accounted for by the sum of the bitterness of its components.

Sinapine and the degradation products of rapeseed glucosinolates serve as precursors of trimethylamine (TMA), which is responsible for tainting of eggs (Shahidi and Naczk, 1992; Naczk et al. 1998). Normally, TMA is converted to odorless trimethylamine N-oxide by trimethylamine oxidase, but in chickens with a genetic defect, TMA is transferred to egg yolk, where it causes a fishy or crabby taint (Butler et al., 1982; Honkatukia et al., 2005) (figure 8). Honkatukia et al. (2005) recently identified the chicken gene coding for the oxidation of TMA. That microsomal liver enzyme, flavin-containing mono-oxygenase (FMO3) catalyzes the oxidation of TMA to non-odorous trimethylamine N-oxide. Hens with the genetic defect have an inherently low capacity for synthesising TMA oxidase. TMA is produced by microsomal bacteria present in liver and kidneys (Honkatukia et al., 2005; Pearson, 1979). Thus hens, that have this genetic defect may produce tainted eggs if fed with sinapine-containing food, e.g. with rapeseed meal (Honkatukia et al., 2005).

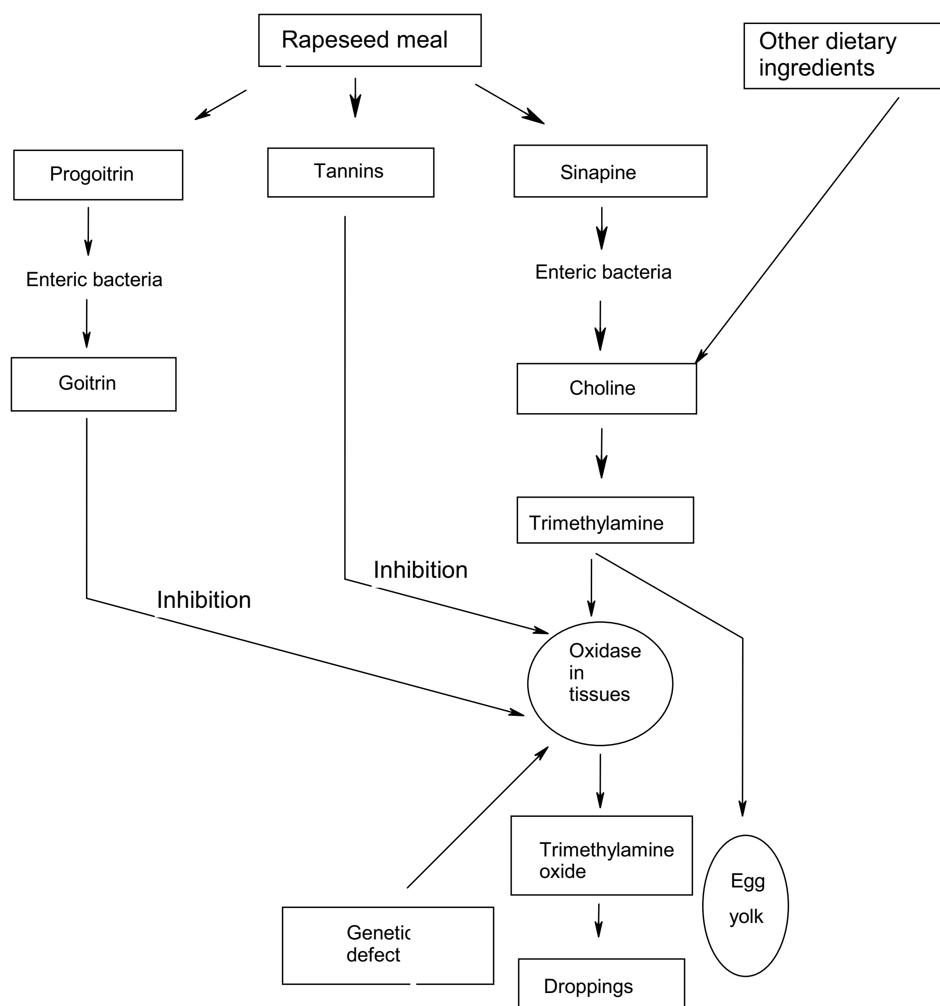


Figure 8. Production of egg taint by rapeseed meal (Butler et al., 1982).

2.4.3 Effects on nutritional availability

Interactions with proteins

Phenolic compounds can form complexes with rapeseed proteins, which may lower their nutritional availability. Phenolic compounds complex with proteins either reversibly by a hydrogen-bonding mechanism or irreversibly by oxidation to quinones, which then combine with reactive groups of protein molecules (Shahidi and Naczk, 1992; Naczk et al. 1998).

There is a correlation between the binding of bovine serum albumin and the pKa of simple phenols, which means that the hydrogen bond is stronger for more acidic phenols. The oxidation products of rapeseed meals, seeds and flours may react with the ε-NH_2 group of lysine and the HC_3S group of methionine to form complexes, which are nutritionally unavailable to monogastric animals. The formation of complexes is favored in a neutral or alkaline pH (Shahidi and Naczk, 1992).

Hydroxycinnamic acid derivatives have been shown to exhibit a somewhat stronger inhibition of pancreatic lipase activity than hydroxybenzoic acid derivatives. The inhibitory effect is influenced by the position of hydroxyl groups and the presence of methoxy groups. Phenolic acids with methoxy groups such as sinapic and syringic acids are the weakest inhibitors of lipase activity (Naczk et al., 1998).

Also, tannins in rapeseed meal can form complexes with meal proteins. The specificity of this interaction depends on the size, conformation, and charge of the protein molecule as well as on the size, length, and flexibility of the tannin molecule. Proteins with a compact globular structure like lysozyme, ribonuclease, and cytochrome C are less prone to complex formation than conformationally open proteins like gelatine and polyproline. Precipitation occurs when the surface of the complex becomes sufficiently hydrophobic. When proteins are present in low concentrations, the precipitation is due to the formation of a hydrophobic monolayer of polyphenols on the protein surface, while in higher concentrations, it is due both to the complexing of polyphenol on the protein surface and to the cross-linking of different protein molecules with polyphenols (Shahidi and Naczk, 1992).

Interaction with minerals

Phenolic compounds are possible inhibitors of iron absorption through the formation of insoluble iron-phenol complexes in the gastrointestinal tract. A relationship has been found between the amount of galloyl groups in foods and the degree of inhibition of iron absorption (Naczk et al. 1998). One possible antioxidant mechanism for phenolic compounds is their formation of complexes with metals (e.g. iron) so that these are unavailable in the gastrointestinal tract and are unable to catalyze the oxidation.

2. 5 Bioactivity properties of rapeseed phenolics

2.5.1 Antioxidant mechanisms

In biological systems, an antioxidant can be defined as any substance that, in low concentration compared with the oxidizable substrate, significantly delays or prevents oxidation of that substrate. The substrate, i.e. the oxidizable compound, is usually a lipid, but can be also a protein, DNA, or carbohydrate. In the case of lipid oxidation, the main mechanism of antioxidants is to act as radical chain-breakers. Another mechanism is to act as a preventive antioxidant oxygen scavenging or blocking the pro-oxidant effects by binding proteins that contain catalytic metal sites (Frankel and Meyer, 2000).

The complexity of antioxidants needs to taken into account in free radical assays.in testing for antioxidant activity. The complexity of a multicomponent oxidative biological material is overlooked compared to oxidation model systems that are models of lipids in their real environment. There is, moreover, no single test to evaluate the antioxidant activity of a compound. The antioxidant activity may vary widely depending on the environment of the lipid substrate. It has been shown that hydrophilic antioxidants are more effective in lipid systems, whereas lipophilic antioxidants work better in emulsions where more water is present (Frankel and Meyer, 2000). In lipophilic environment, hydrophilic antioxidants are oriented to oil-air interface, and give better protection against lipid oxidation than in a more hydrophilic environment, where hydrophilic antioxidants prefer to dilute and thus act poorly against lipid oxidation. Lipophilic antioxidants, in turn, are diluted in lipid environment and are not suitably oriented to the oil-air interface to inhibit the oxidation (Frankel et al., 1994). In testing antioxidants in a radical scavenging test, it should be remembered that this test evaluates only the radical scavenging activity of the compound, and not the other antioxidant mechanisms, such as metal chelation. In addition, the antioxidant action is more complex in real foods and biological systems where several mechanisms become effective (Frankel and Meyer, 2000).

2.5.2 Antioxidant activity of rapeseed phenolics and phenolic acids

Rapeseed phenolics

Some earlier studies have been made on the antioxidant activity of rapeseed phenolics. Several studies of these have shown that phenolic compounds have antioxidant properties. The effect of rapeseed phenolics on radical scavenging has been investigated by Amarowicz et al. (2000) and Matthäus (2002). Amarowicz et al. (2000) fractionated the acetone extract (70%) of rapeseed hulls and found that the radical scavenging activity was highly dependent on the fraction. They found no free phenolic acids in the hulls and concluded that the phenolics exist in the hulls as esters or glucosides.

Wanasundara and Shahidi (1994) found that the antioxidant activity of ethanolic (95%) extract of rapeseed meal toward the oxidation of rapeseed oil was better than that of some widely used synthetic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and the combination of bytylated hydroxyanisole, butylated hydroxytoluene, and monoglyceride citrate (BHA/BHT/MGC). Nowak et al. (1992) found that rapeseed phenolic compounds, especially sinapic acid, were active in inhibiting the oxidation of emulsions. Wanasundara et al. (1996) fractionated a rapeseed ethanolic (95%) extract into five fractions with Sephadex LH-20 and tested the antioxidant activity. When the best fraction against bleaching of β-carotene was found not to contain the greatest amount of phenolics, they concluded that the total phenolic content is not the only thing affecting the antioxidant activity. The antioxidant activity of individual compounds evidently varies according to their chemical properties. In addition, Wanasundara et al. (1996) found that the antioxidant activity of crude ethanolic extract was higher than that of the separate rapeseed phenolic fractions due to the synergism between different phenolics. Related to this, Amarowicz et al. (2000) found no correlation between the total phenolics and the antioxidant activity when testing the radical scavenging activity of different fractions isolated from rapeseed hulls. Thiyam et al. (2004) found rapeseed phenolic extracts to be effective antioxidants in stripped (tocopherol-free) rapeseed oil.

Phenolic compounds present in crude rapeseed oil have also shown antioxidant properties. Koski et al. (2003) fractionated crude rapeseed oil and found vinylsyringol-containing fraction to be the most effective antioxidant in bulk and emulsified methyl linoleate and in

lecithin-liposome systems. Kuwahara et al. (2004) isolated canolol (which is identical with vinylsyringol) from canola oil and found it to have effective antiradical capacity against the endogenous mutagen peroxynitrite.

Phenolic acids

Hydroxycinnamic acid derivatives, e.g. sinapic acid, ferulic acid and caffeic acid, have shown effective radical scavenging activities in several studies (Brand-Williams et al., 1995; Andreasen et al., 2001; Pekkarinen et al., 1999; Chen and Ho, 1997). In radical scavenging, the activity of caffeic acid with its two hydroxyl groups is better than that of ferulic acid with its single hydroxyl group. The two methoxy groups in sinapic acid in addition to one hydroxyl group increase its radical scavenging activity over that of caffeic acid with two hydroxyl groups (Pekkarinen et al., 1999). Kikuzaki et al. (2002) found that the radical scavenging activity was higher in ferulic acid than in its ester derivatives, the same was not true for gallic acid.

Hydroxycinnamic acids have shown potential antioxidant activities in several model systems, including LDL and liposomes. Meyer et al. (1998) isolated hydroxycinnamic acids from fruits, and found that the highest activity toward LDL oxidation (86-97% at a concentration of 5 μM) was obtained with hydroxycinnamic acids with two hydroxyl groups. The activity was closely related to hydroxylation and methylation: the 3-methoxy group in ferulic acid enhanced the antioxidant activity, unlike hydrogen in p-coumaric acid which decreased it. Similarly, Chen and Ho (1997) and Nardini et al. (1995) found that the most active antioxidant of hydroxycinnamic acids was caffeic acid with its two hydroxyl groups. Sinapic acid was not among the tested compounds. Andreasen et al. (2001) found caffeic acid to be more effective than sinapic acid, but sinapic acid was more effective than ferulic acid or p-coumaric acid. In a comparison of Kikuzaki et al. (2002) the antioxidant activities of alkyl gallates and alkyl ferulates in the liposome model system, found that effective antioxidant activity requires the optimum chain length. The higher polarity of a phenoxyl group in alkyl gallates than in alkyl ferulates might require a somewhat longer alkyl chain in the alcohol part. For effective antioxidant activity it is important that the antioxidants locate near the membrane surface. According to Castelli et al. (1999), liposomes are a suitable model for studying membrane structure and properties due to their structural similarity to the lipid

matrix of cell membranes. This similarity allows us to speculate about the *in vivo* bioactivity of the compounds investigated.

2.5.3 Other bioactivity properties of rapeseed and other plant phenolics

Other bioactivity properties of rapeseed phenolics have rarely been considered. In a test of the antimicrobial activity of rapeseed phenolic fractions Nowak et al. (1992) found the fraction of free phenolic acids (FFA) and the sinapic acid (SA) fraction isolated from the ethanolic extract to be highly effective against the growth of gram-negative (*Escherichia coli*, *Enterobacter aerogens*, and *Pseudomonas fluorescens*) and gram-positive (*Bacillus subtilis*, *Bacillus cereus*, *Streptococcus lactis*, and *Streptococcus cremoris*) bacteria. The SA fraction totally inhibited the growth of all tested bacteria on a solid medium and it was effective in liquid culture, where it totally inhibited the growth of *Bacillus cereus* 210, *Streptococcus lactis* 153, and *Pseudomonas fluoresens* 87 and effectively inhibited the growth of other bacterial strains. The FFA fraction was almost as effective.

Kuwahara et al. (2004) tested the antimutagenic properties of canolol (i.e. vinylsyringol) isolated from crude rapeseed oil, and found it to have antimutagenic properties when *Salmonella typhimurium* TA 102 was present. The antimutagenic potency of canolol was higher than that of some flavonoids as well as of α-tocopherol. Canolol also had effects on $ONOO^-$ -induced bactericidal action and it suppressed plasmid DNA (pUC19) strand breakage induced by $ONOO^-$.

The bioactivity properties of some plant extracts have been investigated in a few earlier studies. Pine bark extract was recently shown to have anti-inflammatory activity in inhibiting the production of two pro-inflammatory mediators, nitric oxide and prostaglandin E_2 (Karonen et al., 2004). Cho et al. (2000) reported that pycnogenol, a phenolic extract from maritime pine (*Pinus maritima)* bark, can inhibit the production of proinflammatory cytokine interleukin-1. Pine bark and raspberry extracts have shown antimicrobial activities (Rauha et al. 2000; Puupponen-Pimiä et al., 2001). Rauha et al. (2000) found that raspberry strongly inhibits the growth of *Bacillus subtilis* and *Micrococcus luteus*, while Puupponen-Pimiä et al., 2001; 2005) reported that raspberry phenolics inhibit the growth of gram-negative bacteria such as *Staphylococcus* and *Salmonella* but have no effect on gram-positive lactic acid bacteria. In addition, raspberry phenolics exhibit antiproliferative activities (Liu et al., 2002)

and vasorelaxation properties (Mullen et al., 2002). Laitinen et al. (2004) report that Scots pine bark extract affects the transport of the model drugs verapamil and metoprolol. Tammela et al. (2004) found that the permeabilility of pure flavonoids depends on the degree of hydroxylation and molecular configuration, but, in contrast to other flavonoids, catechin and epicatechin did not penetrate the cell membrane in the Caco-2 colon cell model.

3 OBJECTIVES OF THE STUDY

The main objective of the study was to investigate the bioactivity properties of rapeseed phenolics. The research included analysis and hydrolysis of rapeseed meal and oil phenolics in order to identify the compounds responsible for the bioactivity such as antioxidant, anti-inflammatory, antimicrobial and antimutagenic effects. A further aim was to investigate methods of isolating rapeseed phenolics for application in (functional) foods such as meat products and other biological materials.

The specific aims of the study were:

- to characterize the main phenolics in rapeseed meal and differently processed oils and to explore the efficiency of various enzymes and enzyme preparations for the hydrolysis of sinapic acid esters (**I**)

- to develop an optimal isolation method for rapeseed phenolics not requiring the use of organic solvents and so suitable for food, drug, and cosmetic applications (**II**)

- to investigate the bioactivities of rapeseed phenolics focusing on the antioxidant effects in different environments (**II-IV**), and to study anti-inflammatory, antimicrobial, and antimutagenic (**III**) activities of rapeseed phenolics.

4 MATERIALS AND METHODS

4.1 Rapeseed material

Rapeseed meal (*Brassica rapa*) was the residue of a rapeseed deoiling process, where the oil was expelled from the seeds at elevated temperature by Mildola Ltd, Finland. The rapeseed meal contained 9% fat, which was removed by extraction with petroleum ether (**I**) or was not removed (**II-IV**).

Commercial rapeseed oils were obtained from Finnish manufacturers Mildola Ltd, Finland (oil 1) (**I**) and Raisio (oil 2) (**I**). In addition, three differently processed rapeseed oils, pre-expelled (Pre) (**I**), post-expelled (Post) (**I, III-IV**), and superdegummed (Sdg) (**I**) were obtained from Mildola.

4.2 Reagents and enzymes

Liposomes in the liposome model system were prepared from soybean lecithin (containing 40% phosphatidyl choline). According to HPLC analysis of phosphatidyl choline, it contained α-, γ-, and δ-tocopherols (19, 117, and 59 μg/g, respectively). The fatty acid composition of phosphatidyl choline was similar to that of soybean oil. The oxidation was catalyzed by copper acetate. Human low density lipoprotein (LDL) was used in the LDL model system (Sigma Chemical Co., St. Louis, MO). The LDL solution was diluted in phosphate buffer (pH 7.4). The oxidation was catalyzed by copper sulfate.

Ultraflo L (source: *Humicola insolens*), Viscozyme L (source: *Aspergillus aculeatus*), and Celluclast (source: *Trichoderma reesei)* are multicomponent cellulytic and xylanolytic enzyme preparations that were obtained from Novozymes A/S (Bagsvaerd, Denmark). Ferulic acid esterase (FAE) was a *Humicola insolens* monocomponent enzyme also provided by Novozymes A/S. β-Glucosidase was obtained from Fluka, BioChemika (source: almonds, activity: 6.2 U/mg), and esterase from Sigma (source: porcine liver, activity: 250 U/mg protein).

4.3 Extraction and analysis of rapeseed phenolics

4.3.1 Extraction of rapeseed phenolics from rapeseed meal (I-IV)

Rapeseed phenolics were extracted from meal with 70% aqueous methanol (**I-IV**), 70% aqueous ethanol (**II**, **IV**), and hot water (**II, IV**), and enzyme-assisted extractions were done with ferulic acid esterase (**II**) or Ultraflo L enzyme preparation (**III-IV**).

*Extraction with organic solvents and hot water (***I-IV***)*

Solvent and hot water extraction of rapeseed phenolics was performed according to the method outlined by Cai and Arntfield (2001). Rapeseed meal (0.2 g in studies **I-III**, 0.8 g in study **IV**) and 20 ml 70% aqueous methanol (**I-II**), 70% aqueous ethanol (**II** and **IV**), or hot water (**II** and **IV**) were put in a centrifuge tube, which was then shaken in a water bath (75°C) for 20 min (**II-III**) or 1 h (**IV**). After centrifugation (3500 rpm, 15 min), the clear phenolic extract was collected.

Enzyme-assisted extraction **(II-IV)**

Rapeseed meal (0.2 g in studies **II-III**, 0.8 g in study **IV**) and 20 ml of 0.02 M ammonium diphosphate buffer solution (pH 5.5) containing 0.1% ferulic acid esterase (**II**) or Ultraflo L enzyme preparation (**III-IV**) (calculated against total phenolic content in rapeseed meal) were put in a shaking water bath (37°C) for 2 h. The enzymatic reaction was stopped by boiling the mixture for 10 min. After centrifugation (3500 rpm, 20 min), the phenolic extract was collected. The ferulic acid esterase and Ultraflo L enzyme preparation were confirmed by HPLC not to contain phenolic compounds.

Extraction with supercritical carbon dioxide **(II)**

Supercritical carbon dioxide extraction was performed at 50°C. The pressure was 460 bar (CO_2 1) or 300 bar ($C0_2$ 2), and the carbon dioxide flow was 0.4 mL/min. The extraction was performed by Aromtech Ltd, Finland.

4.3.2 Extraction of the phenolic compounds from rapeseed oils (III-IV)

Rapeseed oil phenolics were extracted according to the method outlined by Koski et al. (2003). Rapeseed oil was dissolved in heptane and extracted with 80% methanol. The methanol phases were combined and washed with heptane. The methanol phase was collected and filtered, and the eluent was evaporated to dryness and dissolved in methanol.

4.3.3 Hydrolysis of sinapic acid esters (I)

Hydrolysis was applied to hydrolyze sinapine in rapeseed meal and oil methanolic extracts and obtain free sinapic acid. The hydrolysis was performed with NaOH and with various enzymes or enzyme preparations.

NaOH-hydrolysis

NaOH hydrolysis was performed according to the method outlined by Naczk et al. (1992a) under the hydrolysis conditions shown in table 4. After four hours, the hydrolysate was acidified to pH 2 with 1 M HCl. The released phenols were then extracted with ethyl acetate/diethyl ether (1:1) and the combined upper phases were evaporated to dryness and dissolved in methanol

Enzymatic hydrolysis

Enzymatic hydrolysis of meal was performed with six different enzymes or enzyme preparations: Ultraflo L, ferulic acid esterase, Viscozyme L, Celluclast, esterase, and β-Glucosidase (table 4). Acetic acid was added to stop the enzymatic reaction. After the enzyme treatment the released phenolics were extracted three times with diethyl ether/ethyl acetate (1:1). The upper phases were collected, evaporated to dryness, and dissolved in methanol.

Table 4. Conditions of hydrolysis of sinapic acid esters (**I**).

	Solution	Enzyme concentrations	Conditions
NaOH	70% methanol	2 and 4 M	room temperature 2 and 4 h
Ultraflo L	0.02 M ammonium dihydrogen phosphate (pH 5.5)	0.1 and 0.05% of total phenolics	37°C 0.5, 1 and 4 h
Ferulic acid esterase	0.02 M ammonium dihydrogen phosphate (pH 5.5)	0.1 and 0.05% of total phenolics	37°C 0.5, 1 and 4 h
Viscozyme L	0.02 M ammonium dihydrogen phosphate (pH 5.5)	0.1 and 0.05% of total phenolics	37°C 0.5, 1 and 4 h
Celluclast	0.02 M ammonium dihydrogen phosphate (pH 5.5)	0.1 and 0.05% of total phenolics	50°C 0.5, 1 and 4 h
Esterase	3.2 M ammonium sulfate buffer (pH 8.0)	10 and 20 mU/mL, 10 and 20 u/mL, 75 U/mL	37°C 4 and 16 h
β-Glucosidase	Dimethyl sulfoxide	20 and 40 mg/mL	37°C 15 h

Enzymatic hydrolysis of rapeseed oil was applied to test the effect of enzymes on the release of free phenolics from sinapic acid esters. The enzymatic hydrolysis was performed on the phenolic extracts of pre-expelled (Pre) and post-expelled (Post) oils with ferulic acid esterase (0.1%, 4 h), which had given best sinapic acid yield in the hydrolysis of rapeseed meal phenolics.

4.3.4 Determination of total phenolic content (I-IV)

The total phenolic content of the phenolic extracts of rapeseed meal and oils was determined according to Folin-Ciocalteu procedure with some modifications. Since sinapic acid is insoluble in water, all solutions were prepared in methanol/water (1:2). The phenolic extract (0.2 mL) was evaporated to dryness, and 0.2 mL of methanol/water (1:2), 1 mL Folin-Ciocalteu reagent (1:10), and 0.8 mL sodium dicarbonate solution (7.5%) were added. After 30 min the total phenolic content was measured at 765 nm with a Perkin-Elmer λ15 UV-Vis spectrophotometer, Norwalk, CT (Singleton and Rossi, 1965). Sinapic acid was used as standard compound.

4.3.5 HPLC (I-IV)

Rapeseed meal and oil phenolics were analyzed by HPLC according to the method outlined by Koski et al. (2003) using a Model 1090 II (Hewlett-Packard) diode array detector. The column was Nova-pak C18 (150 x 3.9 mm, 4 μm, Waters, Millipore, Mass.) equipped with a C18 guard column. The solvent system was as follows: solvent A: 0.02 M ammonium dihydrogen phosphate buffer (pH 2.15)/methanol (75:25) and solvent B: methanol. The gradient system was: 5% B (0-15 min), 5-35% B (15-20 min), 35-100% B (35-45 min), 100-5% B (45-50 min), 5% B (50-52 min), and an 18-min post run period with 5% solvent B. For detection, wavelengths 325 nm (for sinapic acid and sinapine) and 275 nm (for vinylsyringol) were recorded. The flow rate was 1 mL/min and the temperature 27°C. The phenolic extracts of rapeseed meal and oils for HPLC analysis were first dried under nitrogen and then diluted with methanol / ammonium dihydrogen phosphate buffer (0.02 M, pH 2.15) (1:2). The injection volume was 100 μl. The results were expressed as sinapic acid equivalents, μg/g SAE. The calibration curve of sinapic acid was calculated at 325 nm. Sinapine was isolated from rapeseed meal according to the method outlined by Clandinin (1961).

4.4 DPPH radical scavenging test (II-III)

Rapeseed meal and oil extracts, except the supercritical extract, were tested in the DPPH free radical scavenging test, performed by the method outlined by Kähkönen et al. (2003). Methanolic 0.1 mM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) solution (2.950 mL) was mixed with 0.050 mL of the phenolic extract. The concentrations of the phenolic extracts were 0.5, 1.0 (**II-III**), and 1.5 (**II**) mg/mL according to the content of total phenolics. The absorption (517 nm) was monitored every 30 s for 5 min using a Perkin-Elmer λ15 UV-Vis spectrophotometer (Norwalk, CT). The results were expressed as the percentage of radicals scavenged after four min reaction time. The percentage of radical scavenging activity was calculated as

$$\text{Radical scavenging activity (\%)} = 100 \times (A1-A2) / A1$$

A1= Initial absorbance immediately after adding the antioxidant (t=0)
A2= Absorbance after 4 min reaction time

The DPPH● free radical scavenging test was performed in triplicate. Pyrogallol (0.5 mg/mL) was a control sample in each measurement and its radical scavenging activity was between 90 and 95%.

4.5 Oxidation model systems

4.5.1 LDL model system (II)

Rapeseed meal extracts, except the extract obtained with the Ultraflo L enzyme preparation, were tested in the LDL model system. This test was performed by the method outlined by Kähkönen and Heinonen (2003) using human LDL (Sigma) solution. Copper sulfate was used as a catalyst for the oxidation. The methanolic or ethanolic extracts were dried and then the LDL solution and phosphate buffer were added to the headspace vials. The oxidation tests were performed in triplicate. After 2 hours of incubation at 37°C in a shaking water bath, the formation of hexanal due to lipid oxidation was measured by static headspace gas chromatography with an automatic sampler (Perkin Elmer HS 40XL). Vials were thermostated for 13 min at 60°C. The gas chromatograph (Perkin Elmer AutoSystem XL) was equipped with a capillary column (Nordibond NB-54, 25 m, 0.32 mm) and a flame ionization detector. Oven temperature was held constant at 80°C. Identification was based on comparing the retention times of peaks with the peak of a commercially available hexanal standard. The control sample contained no added antioxidants. Sinapic acid was used as a reference. The inhibiting effect of rapeseed phenolics against the formation of hexanal was calculated as

$$(A_0-A_1) / A_0 * 100$$

where A_0 is the area of hexanal in the control sample and A_1 the area of hexanal in the tested sample. The inhibitions were expressed as percentages.

4.5.2 Liposome model system (II-III)

Rapeseed meal and oil extracts, except the supercritical extract, were tested in the liposome model system according to the method outlined by Huang and Frankel (1997). The liposomes were prepared from soybean lecithin, which contained 40% phosphatidyl choline, and the concentration of phosphatidyl choline in samples was 0.8 mg/mL. Cupric acetate was used as

a catalyst. The methanolic or ethanolic extracts of rapeseed were put in Erlenmeyer flasks and dried under nitrogen; the other extracts were not dried. After that, the liposome solution and water were added. The samples were put into a shaking water bath (100 rpm, 37°C) for 3 days. The oxidations were performed in triplicate. Sinapic acid was used as a reference and inhibited the formation of conjugated dienes and hexanal by 90-95%. The inhibition against liposome oxidation was calculated at day 3 by measuring the formation of oxidation products.

Measurement of conjugated diene hydroperoxides

A liposome sample (0.1 mL) was dissolved in methanol (5 ml). The formation of conjugate dienes was measured at 234 nm with a Perkin-Elmer λ15 UV-Vis spectrophotometer. The inhibition against formation of conjugated dienes was calculated as

$$(A_0-A_1)/A_0*100$$

where A_0 is the absorbance of a control sample and A_1 is the absorbance of a tested sample. The inhibitions were expressed as percentages.

Measurement of hexanal formation

A liposome sample (0.5 mL) was put into a headspace vial and the formation of hexanal was measured by headspace gas chromatography as in the LDL oxidation test, except that the vials were thermostated for 18 min at 60°C and the oven temperature was 80°C. The control sample contained no added antioxidants. The inhibition against formation of hexanal was calculated as in the LDL oxidation model system.

4.5.3 Meat model system (IV)

Preparation of meat model system

The meat model (**IV**) was prepared according to the method of Kivikari et al. (2005) using red meat from *longissimus dorsi* muscle of pork and fat from pork back (2:1). The rapeseed phenolic extracts and dry rapeseed meal were added to the minced meat with salt and phosphate after which the meat was cooked for 10 min in plastic bags. The bags were opened and put into decanter flasks where the samples were oxidized for 9 days at 5°C under light. The oxidation tests were performed in triplicate. The control sample contained no antioxidants. Sinapic acid was used as a reference; the inhibition against formation of hexanal was 89%. Oxidation was followed by measuring the lipid oxidation on the starting day, the sixth day, and the ninth day.

Measurement of hexanal formation

A sample (0.5 g) was put in a headspace vial, and the formation of hexanal was measured by headspace gas chromatography as in the liposome oxidation model.

Aqueous ethanolic (70%) and enzyme-assisted extracts of rapeseed meal, dry rapeseed meal, and phenolic extract of crude post-expelled rapeseed oil were tested in cooked pork meat patties (**IV**). The extracts and materials were tested with different amounts added to achieve at least 80% inhibition against hexanal formation in cooked pork meat. The levels of plant materials exhibiting an inhibitory effect of more than 80% toward lipid oxidation after nine days of oxidation were selected for further analysis. Rapeseed meal was tested at four different addition levels (141-424 mg/100 g meat, which contained 0.67-2.0 mg of phenolic compounds). Rapeseed phenolic extracts were tested at levels of 15 and 29 mL(the amounts of phenolic compounds were 2.9 and 5.6 mg in rapeseed ethanolic extract and in extracts prepated by the use of enzymes, the amounts were 3.5 and 6.9 mg). Similarly, phenolic extract of crude rapeseed oil was tested at levels of 2 and 5 mL (the amounts of phenolic compounds were 0.9 and 2.2 mL), respectively. Sinapic acid was tested at levels of 7-24 mg/100 g meat.

4.6. Other bioactivity testing (III)

Rapeseed meal phenolic extract obtained with the use of Ultraflo L enzyme preparation and rapeseed oil phenolic extract obtained from crude post-expelled rapeseed oil were tested further in other bioactivity tests.

4.6.1 Anti-inflammatory properties

The anti-inflammatory properties of the extracts and standards were tested by measuring their effects on the pro-inflammatory mediators nitric oxide (NO) and prostaglandin E_2 (PGE_2) in activated macrophages (J774 murine macrophages). Measurement of nitrite accumulation in the culture medium was used to determine NO production. The culture medium was collected after 24 h incubation, and the nitrite concentration was measured by Griess reaction (Green et al., 1982). For determining prostaglandin E_2, the culture medium was collected after 24 h incubation, and PGE_2 concentrations were determined by radioimmunoassay.

XTT test

Cell viability was tested using Cell Proliferation Kit II (Boehringer Mannheim, Indianapolis, IN). The cells were incubated with the tested compounds for 20 h before the addition of sodium 3'- [1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) (final concentration 0.3 mg/mL) and N-methyldibenzopyrazine methyl sulfate (1.25 mM). The cells were then further incubated for a further 4 h, and the amount of formazan accumulated in the growth medium was assessed spectrophotometrically. Triton-X –treated cells were used as a positive control. The conditions were considered toxic if the cells' ability to metabolize XTT to formazan was lowered by more than 20% in comparison with that of the untreated cell culture.

4.6.2 Mutagenicity

The mutagenic effects were determined according to Flamand et al. (2001) using *Salmonella typhimurium* strains TA98 and TA100 (Wenometrix Inc., San Diego, CA) grown in nutrient broth (Beckton Dickinson, le Pont de Claix, France) at 37°C for 24 h. The test was performed with buffer (B) or in the presence of 10% rat liver enzyme S9-mix (S9) as a metabolic

activator. When B was present, 2-nitrofluorone (2N) 0.1 μg/well was used as a positive control for TA98, and sodium azide (Na) 0.05μg/well for TA100. When S9 was present, 2-aminoanthracene (2A) was used as a positive control, for TA98 strain 0.5 μg/well and for TA100 0.75 μg/well. The final dilutions in wells were 1/27 of the original extract concentrations. The results were expressed as positive (+) or negative (-) effects relative to the control sample.

The antimutagenic properties were determined according to Yen et al. (2001) using TA98 and TA100 strains without (B) and with (S9) metabolic activation. Vinylsyringol or the extracts together with positive controls were added to wells and incubated for 48 h.

4.6.3 Antimicrobial properties

The antimicrobial activity of rapeseed extracts was tested using the following bacterial strains colonizing in the colon: *Escherichia coli* (FOMK), *Salmonella typhimurium* (TA100), *Klebsiella oxytoca* (FOMK), *Proteus mirabilis* (FOMK), *Lactobacillus acidophilus* (ATCC 4356), and *L. crispatus* (A269-21). Strains were grown in nutrient broth (Beckton Dickinson, le Pont de Claix, France) at 37°C before the test. The antimicrobial assay was performed according to Skyttä and Mattila-Sandholm (1991) in 96-well plates at concentrations of 4-100 μg/mL. The samples were incubated for 24 h and the test was performed in triplicate.

4.6.4 Drug permeability

In drug permeability test, the aim was to see if rapeseed affects the absorption of the widely used highly permeable drugs verapamil, metoprolol, paracetamol, and ketoprofen when co-administered. The test was performed according to the method outlined by Laitinen et al. (2004). The permeabilities of verapamil, metoprolol, paracetamol, and ketoprofen across Caco-2 cell monolayers were studied in an apical-to-basolateral direction at pH 7.4.

MTT test

This colorimetric assay was used to determine cell viability (mitochondrial activity) by measuring the extent of formazan formation after solubilization of the Caco-2 cells. Briefly, cells were seeded on to 96-well plates (Costar Corp., Cambridge, MA) at a density of 5.0 x 10^4 cells/well and incubated for 20-24 h. The cells were exposed for 60 min to solutions of the drugs, extracts or drugs and extracts, at 37°C. Subsequently, the medium was aspirated, MTT solution (5 mg/mL) was added, and the cells were further incubated for 1.5 h. Formazan crystals were then dissolved in a solution of 10% SDS and 0.01 M HCl in isobutanol. The color that developed was measured at 590 nm. Results (n = 8) were expressed as percentages of the control value (cells treated with NBSS only), and reduction by over 20% was evaluated as toxic conditions.

4.7 Statistical analysis

Statistical analysis was performed using Statgraphics (STCC Inc., Rockville, ML, USA) one-way ANOVA.

5 RESULTS

5.1 Analysis and hydrolysis of rapeseed meal phenolics (I-IV)

A primary aim of the study was to characterize the main phenolics in rapeseed meal and differently processed oils and to explore the efficiency of different enzymes and enzyme preparations for hydrolysis of sinapic acid esters.

When rapeseed meal (fat residue removed) was extracted with 70% methanol, sinapine (5048 μg/g) and sinapic acid (454 μg/g) were the main phenolics according to HPLC analysis (figure 9a). The total phenolic content was 11183 μg/g measured with Folin-Ciocalteu assay (**I**; table 1).

The aqueous methanolic extract of rapeseed meal was hydrolyzed with sodium hydroxide or with different enzymes and enzyme preparations to release sinapic acid from its esters. With sodium hydroxide, all sinapic acid esters were hydrolyzed to free sinapic acid. Ferulic acid esterase and Ultraflo L were also effective in hydrolyzing rapeseed phenolics in the extract: over 90% of rapeseed sinapine was hydrolysed to free sinapic acid (figure 9b). Ferulic acid esterase hydrolyzed 97% and Ultraflo L 90% of sinapic acid derivatives to free sinapic acid. Viscozyme L and Celluclast were not as effective: Celluclast hydrolyzed 42 %, esterase 20%, and Viscozyme L only 11 % of sinapic acid derivatives. β-Glucosidase did not work at all on these conditions. (**I**; table 1).

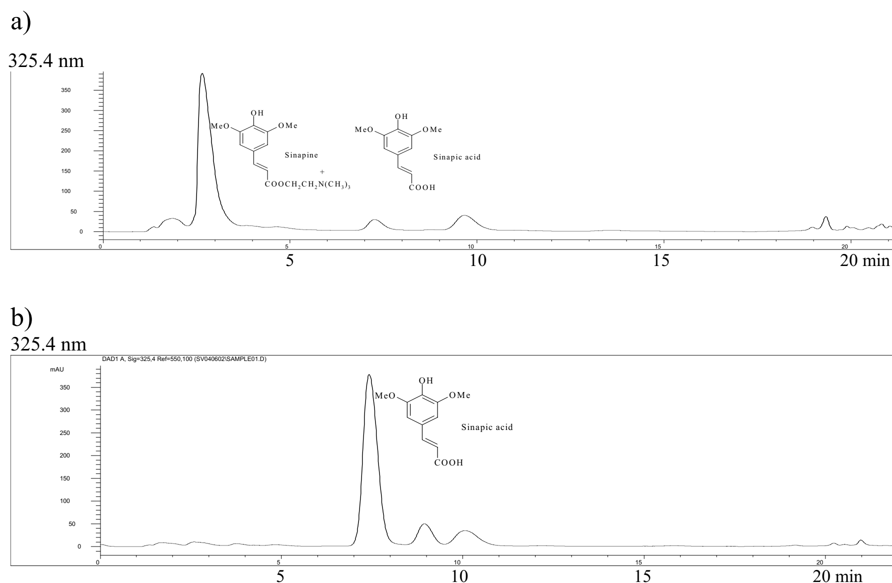


Figure 9. HPLC chromatograms of rapeseed meal phenolic extract a) before and b) after enzymatic hydrolysis (ferulic acid esterase) with UV-detection at 325.4 nm.

The total phenolic content measured by the Folin-Ciocalteu method remained unchanged after enzyme treatments effective in hydrolyzing sinapic acid derivatives to sinapic acid. Ultraflo L and ferulic acid esterase hydrolyzed over 100% of sinapic acid derivatives and the total phenolic content remained unchanged or was slightly increased. Esterase and Viscozyme L hydrolyzed less than 20% of sinapic acid derivatives and the total phenolic content decreased sharpy. Statistically, there was a strong correlation between the total phenolic content and the sinapic acid concentration ($r^2 = 0.9288$) in enzymatic hydrolysis. (**I**; table 1). Moreover, the hydrolysis did not destroy the phenolics when the enzyme was effective. Base hydrolysis destroyed 20% of the phenolics according to Folin-Ciocalteu analysis of total phenolics.

5.2 Analysis and hydrolysis of differently processed rapeseed oils (I)

Rapeseed oil phenolics were extracted with 80% methanol. Vinylsyringol was the predominant phenolic compound in crude post-expelled rapeseed oil, while sinapine (19 µg/g) and sinapic acid (16 µg/g) were present in small amounts. The total phenolic content was 439 µg/g. Pre-expelled crude oil contained the same phenolics but in smaller amounts (under 5 µg/g), and the total phenolic content was only 25 µg/g. In superdegummed oil, there was still some vinylsyringol and sinapic acid left but no sinapine. The total phenolic content was

some vinylsyringol and sinapic acid left but no sinapine. The total phenolic content was highest in post-expelled oil and decreased in processing. However, the pre-expelled crude oil contained only a small amount of phenolics. In commercial rapeseed oils, only the oil produced by pressing at elevated temperature (Oil 2) contained some phenolics; the oil produced by solvent extraction (Oil 1) contained no phenolic compounds measured by Folin-Ciocalteu assay (figure 10).

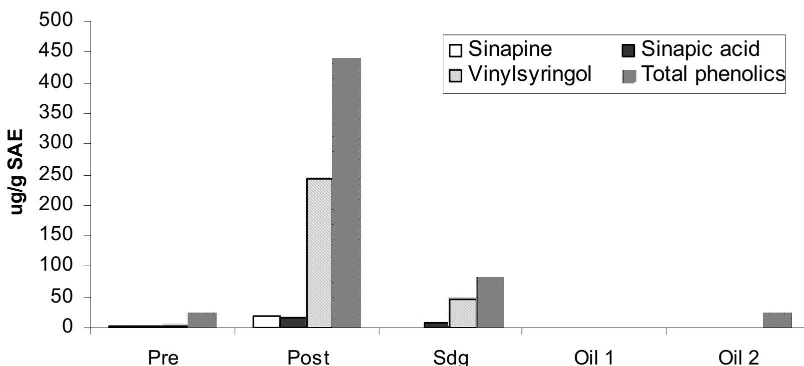


Figure 10. Phenolic composition in differently processed oils (μg/g sinapic acid equivalents, SAE). Pre-expelled (Pre), post-expelled (Post), and superdegummed (Sdg) rapeseed oils as well as commercial rapeseed oils (Oil 1 and oil 2).

Pre-expelled (Pre) and post-expelled (Post) rapeseed oils were hydrolyzed with sodium hydroxide and ferulic acid esterase to release sinapic acid from its esters. The best method for rapeseed oils was enzymatic hydrolysis. After hydrolysis, there was no sinapine left, while the amount of sinapic acid was increased (**I**; table 2). The hydrolysis was most effectively applied to the post-expelled crude rapeseed oil, which had high sinapine content. However, the total phenolic content decreased which indicates some loss of the phenolics during hydrolysis process. The loss was higher after base hydrolysis.

5.3 Effect of isolation method on the main phenolics in rapeseed meal phenolic extract (II-III)

Rapeseed meal phenolics were extracted with aqueous methanol (70%), aqueous ethanol (70%), hot water, or supercritical CO_2, and enzymatically with ferulic acid esterase (**II**) or Ultraflo L enzyme preparation (**III**). The total phenolic content in rapeseed meal phenolic isolates measured by Folin-Ciocalteu assay varied between 5310 and 6937 µg sinapic acid equivalents/g with the highest amount found in enzyme assisted isolate and the lowest in aqueous ethanolic isolate. According to HPLC analysis, the main phenolics in rapeseed meal were sinapine and sinapic acid. The rapeseed meal phenolic isolates contained 17-3070 µg/g of sinapine and 120-1700 µg/g of sinapic acid depending on the method of isolation (Figure 11). The phenolic profile of rapeseed obtained with ferulic acid esterase was different from that obtained with the other methods: the main phenolic compound was sinapic acid and the content of sinapine was about tenfold lower. In the enzymatic hydrolysis, nearly all sinapine was hydrolyzed to free sinapic acid. In enzymatic hydrolysis, rapeseed meal phenolics were extracted with ammonium diphosphate buffer with the addition of ferulic acid esterase (**II**) or Ultraflo L enzyme preparation (**III**). It was found that the hydrolysis and extraction could be done simultaneously. HPLC analysis showed that, during two hours of extraction, nearly all sinapine was hydrolyzed to free sinapic acid. However, the total amount of sinapine and sinapic acid (1870 µg/g) isolated with ferulic acid esterase was less than the amount obtained with solvents (2260-3240 µg/g) or hot water (2020 µg/g).

The use of supercritical CO_2 for isolation of rapeseed meal phenolics was not successful. The amount of phenolics isolated with supercritical CO_2 was low; total phenolic content was just 459 and 424 µg/g. In the two supercritical CO_2 extracts obtained with two sets of extraction conditions, the main phenolic compound in supercritical CO_2 extract was vinylsyringol with the contents 250 and 250 µg/g, and the contents of sinapic acid were 16 and 10 µg/g (**II**; table 1).

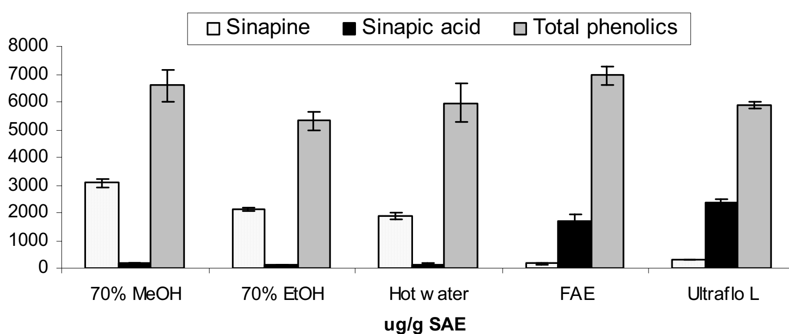


Figure 11. Phenolic composition (sinapic acid equivalents, SAE) of rapeseed meal phenolic extracts obtained by different isolation methods. Extractions were performed with aqueous methanol (70% MeOH), aqueous ethanol (70% EtOH), and hot water, enzyme-assisted with ferulic acid esterase (FAE), and enzyme-assisted with Ultraflo L enzyme preparation (Ultraflo L).

5.4 Antioxidant activity of rapeseed phenolic extracts (II-IV)

5.4.1 Aqueous methanolic, aqueous ethanolic, and water extracts and dry rapeseed meal (II, IV)

The main phenolic compound in these extracts, as well as in dry rapeseed meal was sinapine, the choline ester of sinapic acid. Rapeseed meal methanolic extract, ethanolic extract, and the extract obtained with hot water showed moderate radical scavenging activity in DPPH radical scavenging test. The radical scavenging activity was highly dependent on concentration (**II**; figure 1). At a concentration of 1.5 mg/mL, the highest concentration tested, calculated as total phenolics, all extracts scavenged less than 70% of free radicals. The methanolic extract was the best radical scavenger followed by the ethanolic extract and water extract. All of these extracts showed excellent antioxidant activity in the liposome and LDL model systems. The antioxidant activity was over 95% even at a concentration of 1.4 μg/mL. There was no difference between the extracts. Ethanolic extract, at an amount of 5.6 mg of total phenolics per 100 g meat in a sample, inhibited 94% of hexanal formation in cooked pork meat (Figure 12). As well, dry rapeseed meal, i.e. rapeseed meal without extraction, was tested in a meat model system. At an amount of 1.3 mg /100 g meat total phenolics, it inhibited the formation of hexanal by 80% (**IV**; table 2).

5.4.2 Enzyme-assisted extracts of rapeseed meal (II-IV)

The main phenolic compound in the extracts prepared with enzymes was sinapic acid due to hydrolysis of sinapine. Rapeseed meal extracts obtained with the use of ferulic acid esterase (**II**) and with Ultraflo L, where β-glucanase is the main activity and ferulic acid esterase the side activity (**III**), showed moderate radical scavenging activity in DPPH radical scavenging test at a concentration of 1.0 mg/mL. The extract obtained with the use of ferulic acid esterase scavenged ca. 80% of free radicals at a concentration of 1.5 mg/mL and was the most effective radical scavenger of the tested extracts (**II**; figure 2). In the liposome model system, both extracts showed excellent antioxidant activity at a concentration of 8.4 µg/mL (over 95%) (**II-III**) The extract obtained by the use of ferulic acid esterase showed also excellent antioxidant activity against oxidation of LDL particles at a concentration of 4.2 µg/mL (over 95%), where it was quite effective (60%) even at the lowest concentration tested (0.83 µg/mL) (**II**) The extract obtained by the use of Ultraflo L showed also excellent antioxidant activity (over 90%) against hexanal formation in cooked pork meat patties at an amount of 7 mg phenolics per 100 g of meat (**IV**) (figure 12).

5.4.3 Methanolic extract of crude rapeseed oil (III-IV) and supercritical extract of rapeseed meal (II)

The predominant phenolic compound in the methanolic extract of crude rapeseed oil and the supercritical extract of rapeseed meal extract vinylsyringol, a decarboxylation product of sinapic acid. In DPPH free radical test, the phenolic extract of crude post-expelled rapeseed oil was excellent radical scavenger; it scavenged over 95% of free radicals at a concentration of 1.0 mg/mL. The activity was highly dependent on concentration: the activity was under 50% at a concentration of 0.5 mg/mL. Crude rapeseed oil phenolics also showed excellent antioxidant activity in the liposome model system; the inhibition was 98% at both concentrations tested (4.2 and 8.4 µg/mL calculated as total phenolics) (**III**). Crude oil phenolics were effective antioxidants in the meat model system; 2.2 mg of the crude oil phenolics per 100 g of meat inhibited 84% of hexanal formation (**IV**) (figure 12). Phenolic extracts obtained by supercritical CO_2 showed excellent antioxidant activity toward oxidation of LDL particles; at a concentration of 4.2 µg/mL, the inhibitions were 89 and 97%, respectively (**II;** table 3).

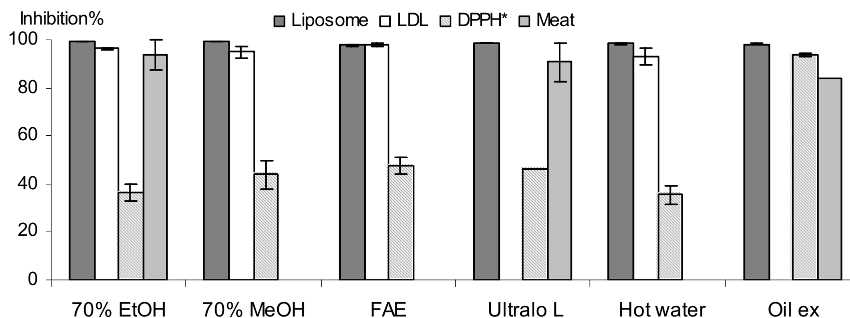


Figure 12. Antioxidant activities of rapeseed phenolic extracts toward oxidation of liposomes, LDL particles, and meat, together with their radical scavenging activity*. Extracts as described in figures 10 and 11. Concentrations: liposome: 8.4 μg/mL, LDL: 4.2 μg/mL, DPPH: 1.0 mg/mL; Meat: EtOH: 5.6 mg/100 g, Ultraflo L: 6.9 mg/100 g, oil ex: 2.2 mg/100 g.

5.5 Other bioactivities of rapeseed phenolics (III)

5.5.1 Anti-inflammatory properties

At a dilution of 1:500 of the original extract, crude rapeseed oil phenolics inhibited the formation of PGE_2 by 54% and the formation of NO production by 17% (table 5). These results indicate that crude rapeseed oil extract with vinylsyringol as the main phenolic compound has anti-inflammatory properties. At a concentration of 100 μM, sinapic acid and vinylsyringol, the tested standards, inhibited the formation of NO by 5.2 and 43.8% and the formation of PGE_2 by 58.2 and 96.7%, respectively. Rapeseed meal extract obtained by enzyme-assisted extraction showed no anti-inflammatory properties. These reults indicate that vinylsyringol was responsible for the anti-inflammatory effect of crude rapeseed oil while sinapic acid in the rapeseed meal had no effect. The extracts were not toxic in XTT test.

5.5.2 Antimutagenicity

The effects of rapeseed meal extract obtained by enzymatic treatment and the phenolic extract of crude rapeseed oil on antimutagenicity were tested in the modified Ames test. Both extracts were found to be antimutagenic without S9 mix; i.e., without metabolic activation both extracts showed antimutagenic properties to prokaryotic cells. With S9 mix (with metabolic

activation), the extracts showed no antimutagenic properties, which indicates they had no antimutagenic properties against eukaryotic cells (table 5) (**III**).

5.5.2 Antimicrobial activity

In test of the effect of rapeseed phenolics on the growth of bacterial strains rapeseed phenolics had only a slight effect on *Escherichia coli* (FOMK), *Salmonella typhimurium* (TA100), *Klebsiella oxytoca* (FOMK), *Proteus mirabilis* (FOMK), *Lactobacillus acidophilus* (ATCC 4356), and *L. crispatus* (A269-21) and thus exhibited no antimicrobial activity (table 5).

5.5.3 Cell permeability

In cell permeability test, the effect of rapeseed meal extract obtained with enzymatic treatment as well as the phenolic extract of crude rapeseed oil on verapamil, ketoprofen, metoprolol, and paracetamol permeability was investigated. The crude rapeseed oil phenolic extract had no significant effects on the permeability of the model drugs. Rapeseed meal phenolics enhanced the permeability of verapamil and ketoprofen indicating that they may have an impact on drugs and other components being actively transported across the cell membrane (table 5). The extracts did not show toxicity in MTT test.

Table 5. In vitro testing of rapeseed phenolics.

Bioactivity	Rapeseed meal ex[a]	Rapeseed oil ex[b]
Anti-inflammatory	12% (NO) negat. (PGE_2)	17% (NO) 54% (PGE_2)
Antimicrobial	No effect	No effect
Antimutagenic	Prokaryotic cells	Prokaryotic cells
Cell permeability	Verapamil ↑ Ketoprofen ↑	No effect
Toxicity	No	No

a Enzyme-assisted extract of rapeseed meal prepared with the use of Ultraflo L (Rapeseed meal ex), b Aqueous methanolic extract of crude post-expelled rapeseed oil (Rapeseed oil ex).

6 DISCUSSION

6.1 Analysis and hydrolysis of rapeseed phenolics (I)

A primary aim of the study was to characterize the main phenolics in rapeseed meal and differently processed oils and to explore the efficiency of various enzymes and enzyme preparations for hydrolysis of sinapic acid esters. Phenolics were extracted from rapeseed meal and oils with aqueous methanol (70% for meal and 80% for oils). The main phenolic compounds in rapeseed meal were sinapine (the choline ester of sinapic acid) and sinapic acid. Sinapine was the predominant phenolic compound in rapeseed meal, while the amount of sinapic acid was one-tenth that of sinapine. The results are in accordance with previous results (Naczk et al., 1998; Cai and Arntfield, 2001), although, Cai and Arntfield (2001) measured higher sinapine content in rapeseed flour. According to Kozlowska et al. (1990), rapeseed flour (meal without seed hulls) contains more esterified phenolics than rapeseed meal (with seed hulls). The total phenolic content in the study of Cai and Arntfiled (2001) was also higher than the present results. The higher value may be due to a different species, growing conditions, or the processing method. Typically the amount of sinapic acid derivatives in rapeseed meal varies between 6390 and 18370 μg/g depending on the plant variety and oil processing method (Kozlowska et al., 1990).

The main phenolic compound in crude post-expelled rapeseed oil was vinylsyringol, a decarboxylation product of sinapic acid, followed by sinapine and sinapic acid. The amount of phenolics decreased during processing, in agreement with the results of Koski et al. (2003). Kuwahara et al. (2004) found canolol (vinylsyringol) in crude rapeseed oil. The pre-expelled rapeseed oil contained only a small amount of phenolics indicating that higher temperature (<100°C) and pressure are necessary for release of the phenolics into rapeseed oil.

Hydrolysis was performed with sodium hydroxide and various enzymes and enzyme preparations. Sodium hydroxide (4 M) hydrolyzed all sinapic acid esters to sinapic acid. In enzymatic hydrolysis, ferulic acid esterase and Ultraflo L were highly effective in hydrolyzing rapeseed phenolics in the extract: over 90% of sinapine was hydrolyzed to free sinapic acid. Viscozyme L and Celluclast were less effective: Viscozyme L hydrolyzed only 11% and Celluclast 42% of sinapic acid derivatives. There are no previous studies on the enzymatic hydrolysis of rapeseed phenolics. Bartolomé and Gómez-Cordovés (1999) found that Ultraflo L hydrolyzed 70 % and Viscozyme L 33 % of ferulic acid esters to free ferulic acid in barley

spent grain. They found that the enzyme activity of Ultraflo L was much higher than that of Viscozyme L. Therefore the hydrolyzing effect of Viscozyme L could not be as effective as in Ultraflo L, because the enzyme concentration was too low. Since Ultraflo L and Viscozyme L both have β-glucanase activity, their effects in hydrolyzing sinapic acid esters would be expected to be about the same. According to Faulds et al. (2002), Ultraflo L has activity toward methyl esters of ferulic, caffeic, p-coumaric, and sinapic acids. In oat, ferulic acid esterase was reported to hydrolyze only a small part of ferulic acid esters (Yu et al. 2002). The hydrolyzing effect was stronger when both ferulic acid esterase and xylanase were added owing to the synergistic effect of these enzymes. In the present study, esterase was poorly effective in hydrolyzing sinapic acid esters; only 20% of sinapic acid esters hydrolyzed to free sinapic acid. β-Glucosidase did not work at all under these conditions, possibly due to the strong ester bonds in phenolic compounds that contain glucose.

The phenolic content remained unchanged when enzymes effective in hydrolyzing sinapic acid derivatives as free sinapic acid were employed. Ultraflo L and ferulic acid esterase hydrolyzed almost 100% of sinapic acid derivatives and the total phenolic content remained the same or increased a little. Esterase and Viscozyme L hydrolyzed less than 20% of sinapic acid derivatives and the total phenolic content highly decreased. There was a strong correlation between the total phenolic content and the sinapic acid concentration in enzymatic hydrolysis.

Pre-expelled and post-expelled rapeseed oils were hydrolyzed with sodium hydroxide and ferulic acid esterase to release sinapic acid from its esters. There was no sinapine left in post-expelled rapeseed oil phenolic extract after either base or enzymatic hydrolysis and the amount of sinapic acid was increased, the sinapic acid content was not increased significantly in the pre-expelled oil extract. Thus, the hydrolysis worked only in post-expelled crude rapeseed oil with its high sinapine content. Total phenolic content decreased in both extracts, during the hydrolysis process. The loss was higher after base hydrolysis. Thus, enzymatic hydrolysis was better hydrolysis method than base hydrolysis. This was first time that enzymes were used in the hydrolysis of rapeseed oil phenolics.

In conclusion, the main phenolic compounds in rapeseed meal are sinapine and sinapic acid, while in crude rapeseed oil, vinylsyringol is the predominant phenolic compound followed by sinapine and sinapic acid. Hydrolysis with ferulic acid esterase and Ultraflo L enzyme

preparation was highly effective in rapeseed meal; nearly 90% of sinapine was hydrolyzed to free sinapic acid, with no statistical difference between enzymatic and base hydrolysis. In the case of crude post-expelled rapeseed oil, enzymatic hydrolysis was more effective than base hydrolysis. Enzymes can thus be applied to the hydrolysis of rapeseed phenolic esters.

6.2 Effect of isolation method on the composition of rapeseed meal phenolics (II-III)

A further aim of the study was to develop an optimal isolation method for rapeseed phenolics suitable for food, drug and cosmetic applications. The method should not require the use of organic solvents. The isolation methods selected for study included extraction with aqueous ethanol, hot water, enzymes, and supercritical CO_2. In study **I**, ferulic acid esterase was shown to be effective in hydrolyzing sinapic acid esters in both rapeseed meal and crude rapeseed oil. In studies **II** and **III**, enzymatic extraction with ferulic acid esterase or with Ultralflo L was also shown to be an effective procedure for extraction of rapeseed meal phenolics. Simultaneous release of sinapic acid may improve the antioxidant activity as sinapic acid is a known antioxidant (Pekkarinen et al., 1999). Isolation of rapeseed meal phenolics by enzymatic extraction facilitates the analysis, because extraction and hydrolysis can be performed in a single step. Enzymatic isolation resulted in a higher amount of phenolics measured by Folin-Ciocalteu method than the other methods of isolation, although the amount was not significantly different from that obtained in isolation with methanol. However, there was a 2-fold difference between the total phenolic content measured with Folin-Ciocalteu method and the total amount of sinapine and sinapic acid measured by HPLC due to methodological differences. Folin-Ciocalteu procedure is highly sensitive to all reducing hydroxyl groups such as those present not only in phenolic compounds but also in some sugars and proteins (Singleton and Rossi, 1965). Thus, the sum of HPLC identified phenolic compounds such as sinapine, sinapic acid and vinylsyringol (in the case of supercritical CO_2) provide better grounds for comparing the effect of different isolation methods.

Measured by HPLC, isolation of rapeseed phenolics was statistically the most efficient with 70% methanol followed by use of ethanol, hot water or ferulic acid esterase. There was no significant difference between the isolations with 70% ethanol and hot water.

Isolation of rapeseed meal phenolics with supercritical CO_2 yielded a much lower amount of phenolics, and is not a method of choice for the isolation of rapeseed phenolics. The phenolic profile obtained with supercritical CO_2 was similar to that in post-expelled crude rapeseed oil, where the content of vinylsyringol formed during processing was 245-700 μg/g (Koski et al., 2003). Supercritical CO_2 extraction is best applied to the isolation of non-polar compounds with the more significant rapeseed meal phenolics remaining in the solid material (Ribeiro et al., 2001).

In conclusion, rapeseed meal phenolics can be extracted with hot water or with the assistance of enzymes. Enzyme-assisted extraction yielded the highest amount of phenolics, while the phenolic content in water extract was nearly as high as that in methanolic extract. The phenolic profile of enzyme-assisted extracts differed from the solvent and water extracts since the main phenolic compound was sinapic acid due to the hydrolysis of sinapine during extraction.

6.3 Antioxidant activity of rapeseed phenolic extracts (II-IV)

Rapeseed phenolics were studied for their antioxidant effects (**II-IV**) and for their anti-inflammatory, antimicrobial, and antimutagenic (**III**) activities in different environments.

6.3.1 Rapeseed meal phenolic extracts obtained with organic solvents, hot water, and dry rapeseed meal (II, IV)

In DPPH radical scavenging test, the radical scavenging activity of rapeseed methanolic and ethanolic extracts and the extract obtained with hot water was moderate. However, the effect was dependent on concentration, 1.5 mg/mL being the highest concentration tested. Aqueous methanolic extract was the most effective extract, followed by ethanolic and water extracts. Amarowicz et al. (2000) tested the radical scavenging activity of phenolic compounds isolated from rapeseed hulls and found that the radical scavenging activity of phenolic compounds was 6-81% (1 mg/mL) depending on the fraction. None of these fractions contained free sinapic acid.

All rapeseed phenolic extracts with sinapine as the main phenolic compound showed excellent antioxidant activity toward liposome oxidation at all concentrations tested. Even at

the lowest concentration, the activity was over 95%. These results indicate that the presence of sinapine as main phenolic compound may in part explain the antioxidant activity of these extracts. In a comparison of Kikuzaki et al. (2002) the antioxidant activities of alkyl gallates and alkyl ferulates in the liposome model system concluded that the effective antioxidant activity requires the optimal chain length. According to them, the higher polarity of a phenoxyl group in alkyl gallates than in alkyl ferulates might require a somewhat longer alkyl chain in the alcohol part. For effective antioxidant activity it is important that the antioxidants are located near the membrane surface. Sinapine was the main phenolic compound in the present extracts, and it showed excellent antioxidant activitiy toward liposome oxidation. Sinapine is a more polar compound than sinapic acid due to the positive charge in the choline part. From this it can be assumed that this polar charged molecule, sinapine, easily moves to the lipid bilayer due to lower solubility to phosphatidyl choline in liposomes. This finding is not in accordance with the polar paradox, which suggests that other factors may be affecting the antioxidant activity e.g., charge and pH of the compound. A dissociated phenolic acid may act differently from a non-dissociated acid in the liposome system.

All extracts with sinapine as the main phenolic compound showed excellent antioxidant activity toward oxidation of LDL particles at all concentrations except the lowest. This indicates that sinapine is the major contributor to the antioxidant activity. With 5.6 mg of phenolics per 100 g of meat, the ethanolic extract also showed excellent inhibition of meat lipid oxidation. Dry rapeseed meal with only 1.3 mg of phenolics provided the sufficient antioxidant activity (80%); the amount of meal in the meat was optimal for good antioxidant activity. However, the higher amount of meal was not reasonable because of the possible unfavourable sensory effects. Rapeseed meal may contain some tocopherols, which would explain some of the antioxidant activity in rapeseed meal. There were no tocopherols in the extracts, however, because the extraction with polar solvents. In the extracts, Likewise, proteins in rapeseed meal may act as antioxidants and thus give more pronounced protection against oxidation in meat. Kivikari et al. (2005) found rapeseed meal effective antioxidant against meat lipid oxidation. At an amount of 100 mg of rapeseed meal per 100 g of meat inhibited 58% of hexanal formation in pork meat. The inhibition increased with an increased amount of meal in meat, an amount of 1.17 g meal per 100 g of meat inhibited the hexanal formation completely. Camelinaseed meal was almost as effective, with 98% inhibition at the same amount of meal in meat. Several other plants or their phenolic extracts such as rosemary (Karpinska et al., 2000; Mc Carthy et al., 2001), potato peel (Kanatt et al., 2005), tea

catechins (He and Shahidi, 1997; Mc Carthy et al., 2001; Tang et al., 2001; Tang et al., 2002; Rababah et al., 2004), sage (Mc Carthy et al., 2001; El-Alim et al., 1999), cloudberry, beetroot, and willow herb (Rey et al., 2005) have shown an ability to act as lipid antioxidants in different meat model systems. In addition to this, Rababah et al. (2004) found that tea catechins inhibit protein oxidation in meat.

In conclusion, rapeseed phenolic extracts with sinapine as the main phenolic compound showed excellent antioxidant activities toward oxidation of liposomes, LDL particles, and cooked pork meat patties. Their radical scavenging was moderate, but was dependent on concentration.

6.3.2 Enzyme-assisted extracts of rapeseed meal (II-IV)

Rapeseed meal phenolics isolated with ferulic acid esterase (**II**) or Ultraflo L (**III**) with sinapic acid as the main phenolic compound showed moderate radical scavenging activity. However, the activities were slightly higher than those of extracts with sinapine as the main phenolic compound, which indicates the better effect of sinapic acid compared to sinapine as radical scavenger. Kikuzaki et al. (2002) similarly found the radical scavenging activity of phenolic acids to be higher than that of their ester derivatives. This finding is in accordance with the results of the present study. Pekkarinen et al (1999) investigated the radical scavenging activity of some hydroxycinnamic and benzoic acids. They found that the activity was higher in hydroxycinnamic acids with two hydroxyl groups (caffeic acid) due to the electron-donating ability. The second methoxy group in sinapic acid in addition to the one hydroxyl group increased the radical scavenging activity more than the second hydrogen in caffeic acid.

Enzyme-assisted extracts showed excellent antioxidant activities toward oxidation of liposomes at the highest concentration tested. However, the effect was slightly less than for the other extracts. This indicates that extracts with sinapic acid as the main phenolic compound are less effective at the lower concentrations than the extracts with sinapine as the main phenolic compound. Although sinapic acid as pure standard was very effective against the oxidation of liposomes, the higher sinapic acid content in the enzymatically obtained phenolic extracts did not result in superior antioxidant activity toward the oxidation of liposomes.

Rapeseed phenolic extract obtained with the use of ferulic acid esterase was highly effective in inhibiting LDL from oxidation. At higher concentrations there was no difference in the antioxidant activity toward LDL oxidation between the rapeseed meal phenolics isolated by other methods. However, at the lowest concentration, extraction with ferulic acid esterase exhibited significantly better antioxidant activity compared to the other extracts, which indicates the effect of sinapic acid against oxidation of LDL particles being more effective that of sinapine. The effect of hydroxycinnamic acids in inhibiting LDL oxidation has been investigated earlier. Meyer et al (1998) isolated hydroxycinnamic acids from fruits, and found that the highest activity (86-97% at a concentration of 5 μM) was due to hydroxycinnamic acids with two hydroxyl groups. The activity was highly related to hydroxylation and methylation. The 3-methoxy group in ferulic acid enhanced the antioxidant activity compared to the decreased antioxidant activity due to hydrogen in p-coumaric acid. Chen and Ho (1997) and Nardini et al. (1995) found too that the most active antioxidant among the hydroxycinnamic acids is caffeic acid with two hydroxyl groups. Sinapic acid was not among the tested compounds. Andreasen et al. (2001) found caffeic acid with two hydroxyl groups to be more effective than sinapic acid, but sinapic acid was more effective than ferulic acid or p-coumaric acid. Thus, the amount of free sinapic acid in the enzymatic isolate of rapeseed phenolics may in part explain the antioxidant activity at the lowest concentration.

Rapeseed extract obtained with Ultraflo L enzyme preparation showed excellent antioxidant activity toward oxidation of cooked meat with 6.9 mg of phenolics. This effect did not differ from rapeseed ethanolic extract where sinapine is the main phenolic compound.

In summary, enzyme-assisted extracts of rapeseed meal with sinapic acid as the main phenolic compound showed excellent antioxidant activity toward the oxidation of liposomes, LDL particles and, cooked pork meat patties. Their radical scavenging activity was dependent on concentration and was better than that of extracts containing sinapine as the main phenolic compound.

6.3.3 Aqueous methanolic extract of crude rapeseed oil and supercritical extract of rapeseed meal (II-IV)

The radical scavenging activity of crude rapeseed oil phenolics was excellent as it scavenged 95% of DPPH radicals at a concentration of 1.0 mg/mL. The main phenolic compound in

crude rapeseed oil is vinylsyringol, a decarboxylation product of sinapic acid. It can be concluded that vinylsyringol, the main phenolic compound in post-expelled crude rapeseed oil explains the excellent radical scavenging activity as its content was 10-fold compared to the other main phenolics, sinapine and sinapic acid. Also Kuwahara et al. (2004) who isolated canolol (which is identical with vinylsyringol) from canola oil, found that canolol had effective antiradical capacity against the endogenous mutagen peroxynitrite. In the study, the radical activity of sinapic acid was over 90% at a concentration of 1.0 mg/mL. The radical scavenging activity of sinapic acid was higher than that of vinylsyringol, but their activities were lower than that of rapeseed oil phenolics suggesting that there may be synergistic effects between different rapeseed phenolics. In crude rapeseed oil, the main phenolic compound is vinylsyringol followed by sinapic acid and sinapine in small quantities.

Crude rapeseed oil phenolics showed excellent antioxidant activity toward liposome oxidation mainly due to vinylsyringol as the main phenolic compound. Koski et al. (2003) reported that the crude rapeseed oil fraction containing vinylsyringol was the most effective antioxidant in inhibiting the oxidation of lecithin-liposomes. The supercritical extract of rapeseed meal showed excellent antioxidant activity toward oxidation of LDL particles. However, there may be some tocopherols present in the extract, which may have an impact on the activity. According to Ribeiro et al. (2001), supercritical extraction is best applied to the isolation of non-polar compounds, which may be more favorable to tocopherols than to polar phenolics.

Just 2.2 mg of phenolics of crude rapeseed oil phenolics had also excellent inhibition against oxidation of cooked meat lipids. As the rapeseed meal extracts with 3-fold more phenolics showed as pronounced effect, it can be concluded that rapeseed oil extract with vinylsyringol as the main phenolic compound had the most potent phenolic composition.

In summary, phenolic extract of crude rapeseed oil with vinylsyringol as the main phenolic compound showed excellent radical scavenging activity, the activity was significantly better than that of rapeseed meal extracts. Crude rapeseed oil also showed excellent antioxidant activity against oxidation of liposomes and cooked pork meat patties as well. Supercritical CO_2 extract with vinylsyringol as the main phenolic compound inhibited the oxidation of LDL particles effectively. However, rapeseed oil phenolics were effectively extracted with aqueous methanol, which is not suitable for food applications. The extraction of rapeseed oil phenolics needs to be developed further.

6.4 Other bioactivities of rapeseed phenolics (III)

Rapeseed, as well as raspberry and pine bark are promising bioactive sources of plant phenolics selected among ca. 100 previously screened plant materials for *in vitro* preclinical evaluation of health related effects (Kähkönen et al., 1999).

Crude rapeseed oil phenolics inhibited the formation of NO and PGE_2, whereas rapeseed meal phenolics extracted with ferulic acid esterase showed no anti-inflammatory properties. From this it can be concluded that the anti-inflammatory properties of rapeseed oil phenolics are due to vinylsyringol, which effectively inhibited the formation of NO and PGE_2. The anti-inflammatory effects of sinapic acid are weaker, which partly explains the lack of effect with rapeseed meal phenolics consisting mainly sinapic acid (64%). Compared with the anti-inflammatory effects of other phenolic rich plant materials such as pine bark (Karonen et al., 2004), the effect of rapeseed phenolics was moderate. Phenolic fractions of pine bark, with ferulic acid as well as lignans pinoresinol and matairesinol as main phenolics, show effective anti-inflammatory properties (Karonen et al., 2004).

The effects of rapeseed meal extract obtained with enzymatic treatment as well as the phenolic extract of crude rapeseed oil on the antimutagenicity was tested and it was shown that without metabolic activation all tested samples showed antimutagenic properties to prokaryotic cells. However, crude rapeseed oil extract and rapeseed meal extract obtained with enzymatic treatment had no antimutagenic properties in the modified Ames test with metabolic activation, which indicates they had no antimutagenic properties against eukaryotic cells. This finding is in accordance with Kuwahara et al. (2004), who concluded that canolol is an antimutagenic compound without S9 mix. The antimutagenic potency of canolol is reported to be higher than that of some flavonoids as well as of α-tocopherol.

Test of the effect of rapeseed phenolics on the growth of *Escherichia coli* (FOMK), *Salmonella typhimurium* (TA100), *Klebsiella oxytoca* (FOMK), *Proteus mirabilis* (FOMK), *Lactobacillus acidophilus* (ATCC 4356), and *L. crispatus* (A269-21) showed there to be no no antimicrobial properties as they showed only a slight impact of the tested bacteria. In an earlier study, Nowak et al. (1992) tested the antimicrobial activity of different rapeseed phenolic fractions. They found rapeseed phenolic fractions, fraction of free phenolic acids (FFA) and sinapic acid (SA) isolated from the ethanolic extract being very effective against

the growth of several gram-negative (*Escherichia coli*, *Enterobacter aerogens,* and *Pseudomonas fluorescens*) and gram-positive (*Bacillus subtilis*, *Bacillus cereus*, *Streptococcus lactis,* and *Streptococcus cremoris*) bacteria. The sinapic acid (SA) fraction was the most effective fraction as it inhibited completely the growth of all tested bacteria on solid foundation and was also effective in liquid culture when it totally inhibited the growth of *Bacillus cereus* 210, *Streptococcus lactis* 153, and *Pseudomonas fluoresens* 87 and inhibited the growth of the other bacterial strains by 97.7-99.1%. The FFA fraction was almost as effective on solid foundation, when it totally inhibited the growth of *Pseudomonas fluorescens* 87. In liquid culture, FFA inhibited the growth of the tested bacteria by 70-96.5%. The fraction containing sinapine had no antimicrobial activity against the tested bacteria. Nowak et al. (1992) used other bacteria in their study, which may explain their different result. Rauha et al. (2000) report that raspberry strongly inhibits the growth of *Bacillus subtilis* and *Micrococcus luteus* and raspberry phenolics were mainly reported to inhibit the growth of gram-negative bacteria such as *Staphylococcus* and *Salmonella*, but have no effect on gram-positive lactic acid bacteria (Puupponen-Pimiä et al., 2001; Puupponen-Pimiä et al., 2005). The main phenolics in raspberry are ellagitannins, which indicates their effects as potential antimicrobial compounds. Rauha et al. (2000) found only slight effects of phenolic acids, caffeic acid and gallic acid on the growth of tested bacteria and fungi, which is in accordance with the present results for rapeseed where the main phenolics are phenolic acids and their derivatives.

In cell permeability test, the effect of rapeseed meal extract obtained by enzymatic treatment and the phenolic extract of crude rapeseed oil on verapamil, ketoprofen, metoprolol, and paracetamol permeability was investigated. Crude rapeseed oil phenolic extract had no significant effects on the permeability of the model drugs. Rapeseed meal phenolics enhanced the permeability of verapamil and ketoprofen indicating that they may have an impact on drugs and other components being actively transported across the cell membrane. Tammela et al. (2004) found the permeabilility of pure flavonoids to depend on the degree of hydroxylation and molecular configuration, but, in contrast to other flavonoids, catechin and epicatechin did not penetrate the cell membrane in the Caco-2 cell model. The extracts were not toxic to Caco-2 cells or macrophages and showed no mutagenic properties.

The results show that phenolics from crude rapeseed oil had anti-inflammatory properties when they inhibited the formation of NO and PGE_2. Rapeseed meal phenolics had no anti-

inflammatory properties, which indicates that vinylsyringol is the compound responsible for the anti-inflammatory effect. Rapeseed meal phenolics enhanced the permeability of verapamil and ketoprofen, while the oil phenolics had no effect. Both extracts had antimutagenic properties against prokaryotic cells. The extracts were neither toxic nor mutagenic.

7 CONCLUSIONS

The main phenolics in rapeseed meal are sinapine, the choline ester of sinapic acid, and sinapic acid, while in crude post-expelled rapeseed oil, vinylsyringol was the predominaint phenolic compound followed by sinapine and sinapic acid in smaller amounts. The amount of phenolics decreases during processing. However, pre-expelled crude oil contains only a small amount of phenolics due to the lower temperature and pressure used in processing.

With the use of ferulic acid esterase or Ultraflo L enzyme preparation, rapeseed phenolics can be successfully hydrolyzed to sinapic acid. These enzymes were as effective as sodium hydroxide as they hydrolyzed over 90% of sinapine to sinapic acid. The total phenolic content was the same after as before enzymatic hydrolysis, but it was lowered by 20% of the original after base hydrolysis. Thus enzymes can be applied to the hydrolysis of phenolic esters instead of sodium hydroxide.

Rapeseed meal phenolics can be isolated by various procedures without the use of organic solvents. With the use of enzymes such as ferulic acid esterase and Ultraflo L hydrolysis and extraction can be done simultaneously. Free sinapic acid is obtained as the main phenolic compound and the amount of phenolic compounds is greater than that obtained by other extraction methods.

All of the rapeseed phenolic extracts showed excellent antioxidant activity toward oxidation of liposomes and LDL particles. The antioxidant activity was better than that of sinapic acid, catechin, or α-tocopherol. The extracts were also effective antioxidants of meat lipids. In addition, the phenolic extract of crude post-expelled rapeseed oil was an excellent radical scavenger. The activities of the other extracts were only moderate. Vinylsyringol, the main phenolic compound in crude oil, and sinapic acid, the main phenolic compound in extracts prepared by enzymatic treatment were effective antioxidants in all oxidation models tested.

The phenolic extract of crude rapeseed oil showed anti-inflammatory properties, effectively inhibiting the formation of PGE_2 and having some effect on NO. Both are pro-inflammatory mediators. Vinylsyringol effectively inhibited the formation of NO and PGE_2, while sinapic acid inhibited the formation of NO. Rapeseed meal extract, which contains sinapic acid as the main phenolic compound had no effects against these pro-inflammatory mediators.

According to the Caco-2 cell model, the enzyme-prepared extract of rapeseed meal enhances the permeability of ketoprofen and verapamil, which are actively transported across the cell membrane. Rapeseed oil phenolics had no effect on the permeability of the model drugs. The extracts were not toxic to Caco-2 cells.

As demonstrated in this work, rapeseed phenolic extracts contain ingredients which can be used in developing health beneficial products such as foods, feeds, and cosmetic and pharmaceutical preparations.

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