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THE PROCEDURE OF DETERMINATION DIFFERENT FORM OF PHENOLIC ACID IN RAPESEED FLOURS

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The method for extraction, and quantitative determination of phenolic acids in rapeseed flour was investigated. In examined rapeseed flour (var. Tower), the following phenolic acids were identified: salicylic, p-hydroxybenzoic, p-coumaric, ferulic, sinapic and z-sinapic. Among the identified phenolic acids, sinapic acid was dominated.

INTRODUCTION

Phenolic acids and their esters are common micro-constituents in cereal, oilseed and legume seeds which contribute adverse flavours, colours and odors to food products and, during processing, may cause decreases in protein nutritive value [17]. The development of astringency and discolorations has been associated with the autolytic or enzyme oxidation of polyphenols followed by hydrogen and covalent bounding of the oxidation products to amino, thiol and methylene groups in the proteins [14, 19, 20].

The free phenolic constituents in oilseed flours such as soybean [1], cottonseed and peanut [8], sunflower [14] and rapeseed [7] have been identified and quantitated. Several bound phenolic compounds have also been identified in oilseeds [2, 5, 9, 10]. However, there is little published information on the total composition of free, esterified and bound phenolic compounds in oilseeds and their potential effects on the organoleptic, functional and nutritional properties of the meal products [3].

The objectives of the present investigation were to develop procedures

for the extraction, separation, identification and quantitation of the free, esterified, glycosidic, insoluble-bound and structurally-bound phenolic acids in rapeseed. Rapeseed is particularly suited for an investigation of bound phenolics because of the high level and variety of phenolic acids, esters and glycosides in meal, flours and, more particulary, in protein concentrates and isolates [3, 6, 7, 13]. Although a large proportion of the phenolic compounds and dark pigments are removed during processing, the protein concentrates and isolates exhibit bitter flavours and brown colours when suspended in aqueous slurries or are incorporated into food systems such as ground meats [15, 16, 17]. Therefore, in addition to evaluating the phenolic composition of ether and aqueous extracts, the rapeseed flour residues have been subjected to acidic, alkaline and enzymatic hydrolysis in order to identify and quantitate the insoluble and bound phenolic acids. These bound compounds may be released during heat processing and cooking to adversely influence the organoleptic characteristics of the final product.

MATERIALS AND METHODS

Sample preparation. Analyses were conducted on a seed sample of the low glucosinolate cultivar, Bronowski (*Brassica napus*). The seeds were ground, dehulled by air aspiration and defatted with hexane. After desolventization at room temperature, the meal was pin milled to a particle size of less than 50 μ diameter.

Extraction procedures. Ten gram samples of rapeseed flour were extracted 5 times for 1 hr each with 200 ml of 80% aqueous methanol at room temperature until the supernatants were clear. After centrifugation the combined supernatants were evaporated under vacuum at 40°C to 30-50 ml of aqueous phase. The aqueous suspensions were adjusted to pH 2 (6N HCl), filtered of a cloudy precipitate, and the free phenolic acids extracted by continuous liquid-liquid extraction with diethyl ether for 24 hr [12]. The ether extracts were dehydrated under vacuum at room temperature. The dry residues were dissolved in 5% sodium bicarbonate (pH 8.5) and continuously extracted with ether for 24 hr. The neutral components were isolated by evaporation of the ether extract, while the aqueous phase was adjusted to pH 2 before extracting for acidic components with ether. Both extracts were evaporated and then redissolved in absolute methanol for GLC analyses.

The aqueous phase from the above liquid-liquid extraction and the cloudy precipitate were combined, concentrated under vacuum to 20-30 ml, and the phenolic acid esters hydrolysed with 50 ml of 2N NaOH for 4 hr under N_2 at room temperature. The clear hydrolysate was acidified to pH 2 (6N HCl) and the phenolic acid aglycones extracted by liquid-li-

quid extraction with diethyl ether for 24 h. The ether extracts were dehydrated (Na_2SO_4), filtered and evaporated to dryness. The next procedure was the some as described before for obtaining acid and neutral components.

The residues from the 80% methanol extractions were hydrolysed directly with 100 ml of 2N NaOH under N_2 for 4 h (22°C) or 100 ml of 2N HCl for 30 min in a boiling water bath to release aglycones of the insoluble-bound phenolic compounds. The supernatants were adjusted to pH 2 and extracted with diethyl ether for 24 h. The ether extracts were dehydrated, evaporated to dryness and the residues were egzamined for neutral and acidic components.

Larger samples (50 g) of rapeseed fluor were extracted with 80% methanol to remove soluble phenolic compounds and the residues were hydrolysed with proteolytic enzymes before alkaline or acidic hydrolysis to release additional bound phenolic acid. Pepsin digestion was conducted in 0.1 M glycine buffer at pH 2.1 (2.5 g enzyme in 500 ml buffer) at 40°C for 48 h. After filtration the residue was treated with trypsin (2.5 g) in 500 ml of 0.2 M borate buffer at pH 7.5 for 48 h at 40°C. The supernatants were hydrolysed with 2 N NaOH or 2N HCl as above, and the ether extracts after separation neutral and acidic components, were analyzed by GLC.

GLC techniques — One-two milliliter aliquots of phenolic compounds in ether were combined with known amounts of one or more internal standards and dried under nitrogen at 40°C. Traces of water were removed with benzene and the phenolic compounds silylated with N,O-bis (tri-methylsilyl) acetamide (BSA) by incubation overnight at room temperature. The TMS derivaties of the phenolic compounds were separated on a Pye-Unicam model 104 gas chromatograph equipped with a flame ionization detector and peak area integrator. The fractionation efficiency of a 1.5 m×4 mm glass column packed with 1.5% SE-30 on 80-100 mesh Chromosorb W (HP) [14] was compared with a 2.1 m×4 mm glass column packed with 3% OV-1 [11] on 100-120 mesh Diatomitec Q (Pierce). The flow rate of the carrier gas, helium, was 60 cm^3/min. The injection and detector temperatures were 300°C. Four minutes after injection the oven temperature was raised from 100°C to 260°C at 6°C/min. After identification by co-chromatography with known standards, the TMS derivatives of the phenolic compounds in rapeseed flour extracts and hydrolysates were quantitated from the integrated peak areas and weight ratios of TMS derivatives of authentic phenolic compounds and the internal standards.

RESULTS AND DISCUSSION

Previous investigators have extracted phenolic compounds from oilseeds with aqueous ethanol [1, 3] and methanol [8, 9] under room tem-

perature [9], reflux [14] and boiling [7] conditions. The extraction efficiencies of these procedures were evaluated, and maximum yields of free phenolic acids and esters, with minimal losses due to thermal degradation or polymerization, were obtained with aqueous 80% methanol (80:20 v/v) at room temperature. Five consecutive extractions of 1 h each at a solvent to meal ratio of 20:1 gave essentially complete extraction of free phenolic acids and esters based on analysis of the final extracts. The average yield of methanol-soluble material from rapeseed meal was 22.8% based on four determinations. Kozłowska et al. [7] obtained 25.0 g of methanol-soluble dry matter/100 g of rapeseed meal from a Canadian cultivar.

Crude 80% methanol extracts from rapeseed and other oilseed flours contain a high proportion of nonphenolic constituents such as other organic acids, amino acids, minerals, simple sugars and oligosaccharides and polar lipids [7, 14, 21]. In the present study, the dry matter in the methanol extracts contained 11.7% of crude protein (Kjeldahl N x 6.25) and 15.0% of crude lipids. The methanol-soluble materials was, therefore, fractionated into ether-soluble free phenolic compounds with phenolic acid esters and other compounds being largely isolated in the aqueous phase from the liquid-liquid extraction. The average yield of ether-soluble fraction was 2.6 g/100 g flour while the recovery of dry matter in the aqueous phase was 20.5 g. Before purification of the phenolic acids by liquid-liquid (ether-acidified water) extraction, it was necessary to remove essentially all of the methanol by evaporation to avoid transfer of phenolic acid esters into the ether fraction.

After evaporation of methanol, a cloudy suspension developed in the aqueous evaporate when the pH was adjusted to pH 2 using 6N HCl. This material, on hydrolysis with 2 N NaOH or HCl, gave high yields of sinapic acid. The evaporates were, therefore, filtered and the cloudy material added to the aqueous phase from the liquid-liquid extraction for subsequent alkali and acid hydrolysis.

In addition, anomolous peaks were avoided and yields of phenolic acids increased by thoroughly dehydrating the ether extracts from the liquid-liquid extraction with sodium sulfate before evaporation to dryness under vacuum at room temperature. GLC analysis was further improved by dehydrating the etheric phenolic solutions with benzene before silylation and chromatography.

Chromatography of TMS derivatives of authentic phenolic acids on columns packed with 1.5% SE-30 on Chromosorb W failed to fractionate important seed components such as O-coumaric-genetisic acids and ferulic-isoferulic acids. A longer column of 3% OV-1 on Diatomitic Q W gave essential complete separation of 19 authentic phenolic acids and related compounds including the ferulic acid isomers and several potential internal standards (Fig. 1). On this column, gentisic acid eluted slightly ahead of o-cumaric without interfering with the vanillic acid peak. Similarly,

quinic acid emerged between the p-coumaric and syringic acid peaks. Isoferulic acid eluted sufficiently ahead of E ferulic acid to permit identification and partial quantitation of each peak.

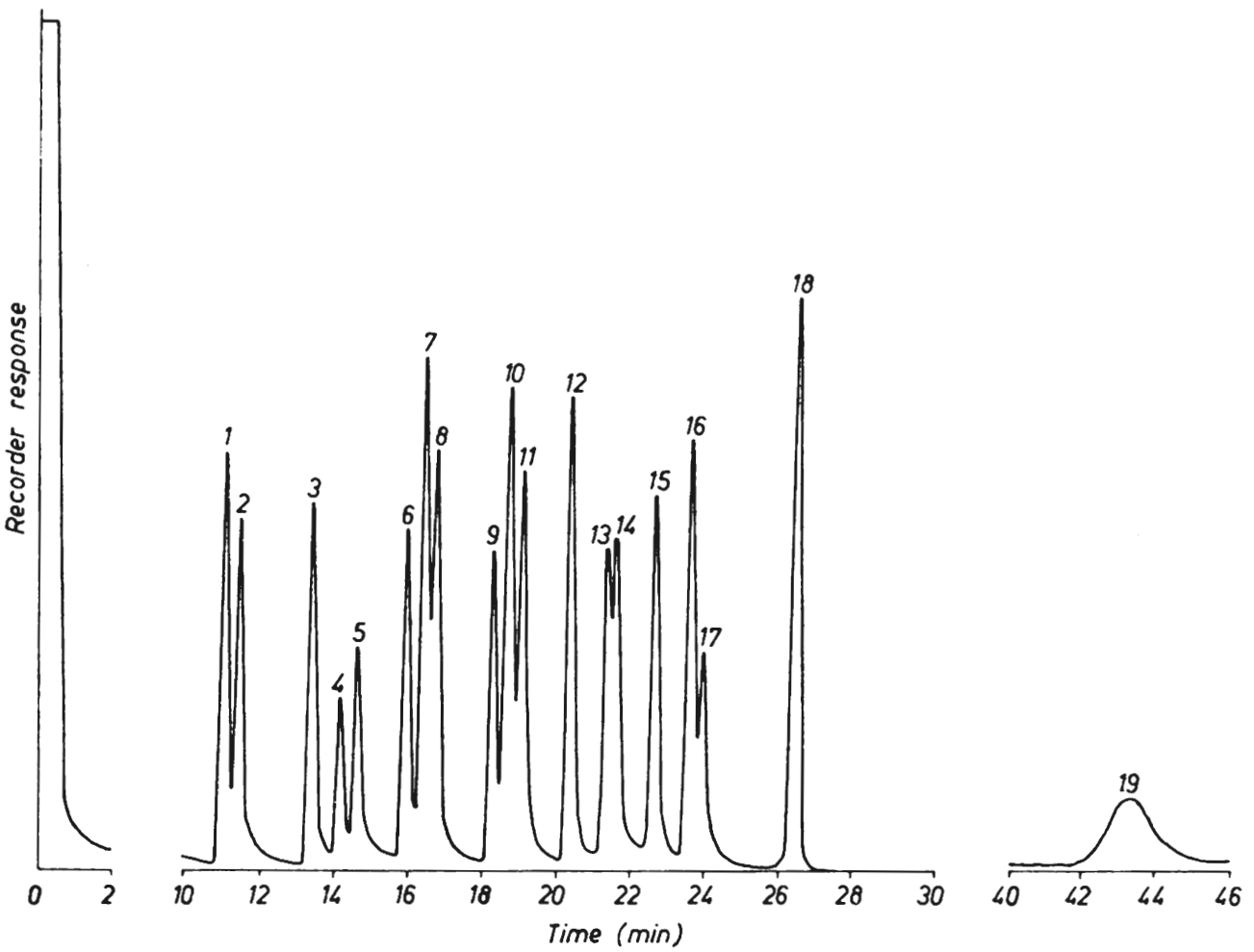

Fig. 1. GLC chromatogram of the TMS derivatives of authentic phenolic acids and related compounds on 2.1 m×4 mm glass columns packed with 3% OV-1 on 100-120 mesh Diatomitec Q; 1 — Salicylic, 2 — E-cinnamic, 3 — p-hydroxybenzoic, 4 — 2,3,6-trichlorobenzoic (IS), 5 — veratric (IS), 6 — vanillic, 7 — gentisic, 8 — o-coumaric, 9 — syringic, 10 — quinic, 11 — p coumaric, 11a — Z-coumaric, 12 — eicosane (IS), 13 — isoferulic, 14 — E-ferulic, 14a — Z-ferulic, 15 — caffeic, 16 — n-docosane (IS), 17 — E-sinapic, 17a — Z-sinapic ester, 18 — n-tetracosane (IS), 19 — chlorogenic acid.

The Z-isomers of p-coumaric, isoferulic, ferulic, sinapic and chlorogenic acids, which were produced by alkali treatment (2H NaOH) or ultrabiolet light, were found to elute approximately 3 min prior to the natural E-isomers on the 3% OV-1 column. These were commonly observed, in lower concentrations than the natural isomer, in the present study because of the hydrolytic procedure employed or exposure of extracts to daylight. Frequently, standards of these phenolic acids contained the Z-isomer as a contaminant.

The internal standards: veratric acid, eicosane and tetracosane, emerged during progressive stages of the temperature program on the GLC to provide accurate quantitation of the phenolic acid series (Fig. 2). N-docosane [11] eluted too closely to the sinapic acid peak to be useful as an

internal standard because of the high proportion of sinapic acid in certain oilseed flours [7, 14]. Tetracosane could also serve as a standards for phenolic acid esters and glycosides. The broad-based double peak obtained for kaempferol [14] was too variable in area for accurate quantitation of chlorogenic acid and other sugar esters.

Salicylic, derulic and sinapic and Z sinapic acids were the principal free phenolic acids in the methanol extracts of rapeseed flour with p-coumaric acid occurring as a minor constituent (Fig. 2). The small peak eluting at 17 min was predicted to be Z-p-coumaric acid since o-coumaric acid (Fig. 1) usually cyclizes into coumarin and is not commonly found in the free form.

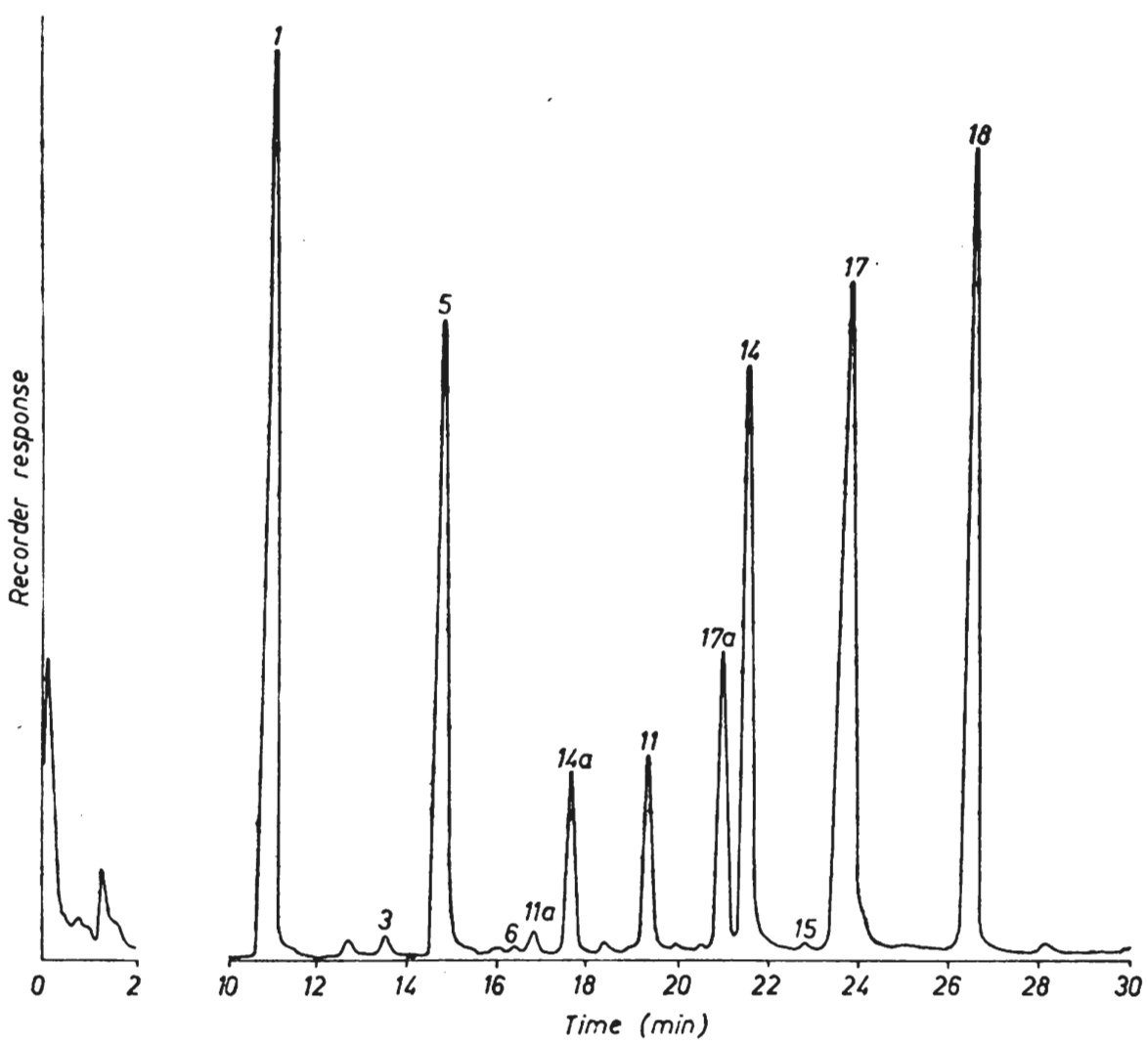

Fig. 2. GLC chromatogram of the TMS derivatives of the free phenolic acids extracted from repeseed flour, explanations — see Fig 1.

Sinapic acid was the only phenolic compound released in quantity during the alkaline hydrolysis of the methanol-soluble phenolic acid esters (Fig. 3). Another peak was observed in the position of methylosinapic ester-Z-sinapic acid. Traces of minor components occurred in the positions of salicylic, p-hydroxybenzoic, vanillic, p-coumaric, syringic, caffeic acids and some isomers. The presence of these acids was confirm by paper chromatography method [13]. Sinapine, the choline ester of 3,5-di-

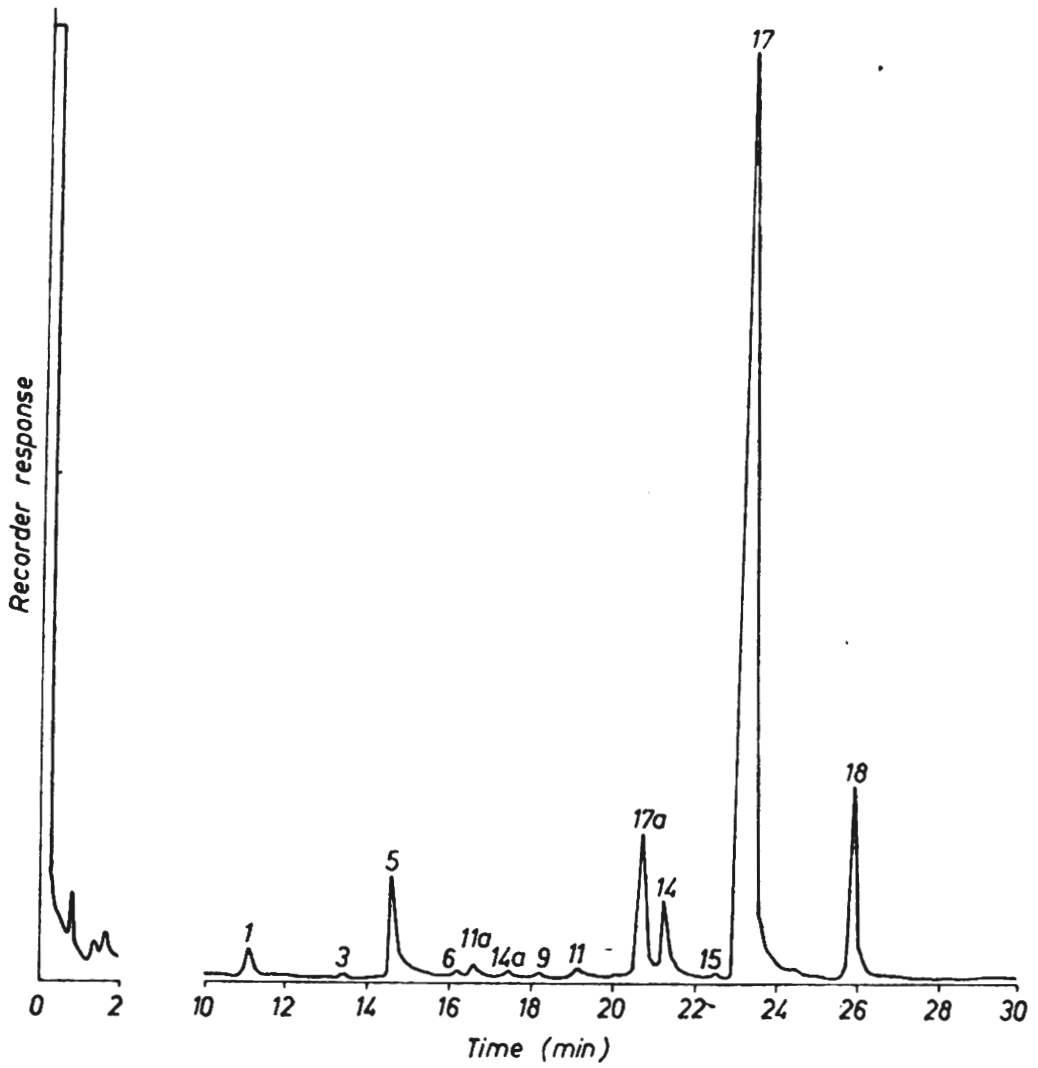

Fig. 3. GCL chromatogram of the TMS derivatives of the soluble phenolic acid esters extracted from rapeseed fluor, explanations — see Fig. 1.

methoxy-4-hydroxycinnamic acid, is known to be the principal sinapic acid ester, in rapeseed. Fenwick and Hoggan [4] have reported that sinapine represents 0.6 to 1.3% of a series of European and Canadian rapeseed meals. The level of sinapic acid found in Bronowski (477 mg% was somewhat higher similar (Table 1).

Sinapic acid was also the principal aglycone in the insoluble-bound fractions of the rapeseed flour (Fig. 4). The identity of other compounds in the hydrolysates is currently underway. The total bound phenolic acid fraction appeared to be less than 5.3 mg% of the flour (Table).

CONCLUSIONS

1. The described method permit to make separation, identification, and quantitative evaluation of free esterified, and bound phenolic acids with insoluble in alcohol part of rapeseed flour.

2. Rapeseed flour revealed the presence of 50.0 mg% free phenolic acids, 622.1 mg% phenolic acid esters and 5.32 mg% of insoluble-bound phenolic acids.

Table 1. Phenolic acids in rapeseed flour

	Phenolic acid or aglycones in mg % dry wt.												
	Salicylic	Cinnamic	p-hydroxy-benzoic	Veratric	Vanillic	Cumaric	Protocate-chuic	- m - Cumaric	Syringic	- p - Cumaric	Sinapic (Like)	Ferulic	Sinapic
Free phenolic acids	1.04	2.90	1.10	Tr	Tr	0.90	Tr	—	1.00	1.67	5.43	3.53	32.50
Phenolic acid ester	4.08	Tr	10.90	11.05	4.84	Tr	7.23	—	9.09	Tr	66.00	31.42	477.50
Insoluble bound	0.31	—	—	0.07	0.08	0.33	0.20	0.18	0.17	0.73	0.63	0.91	1.66

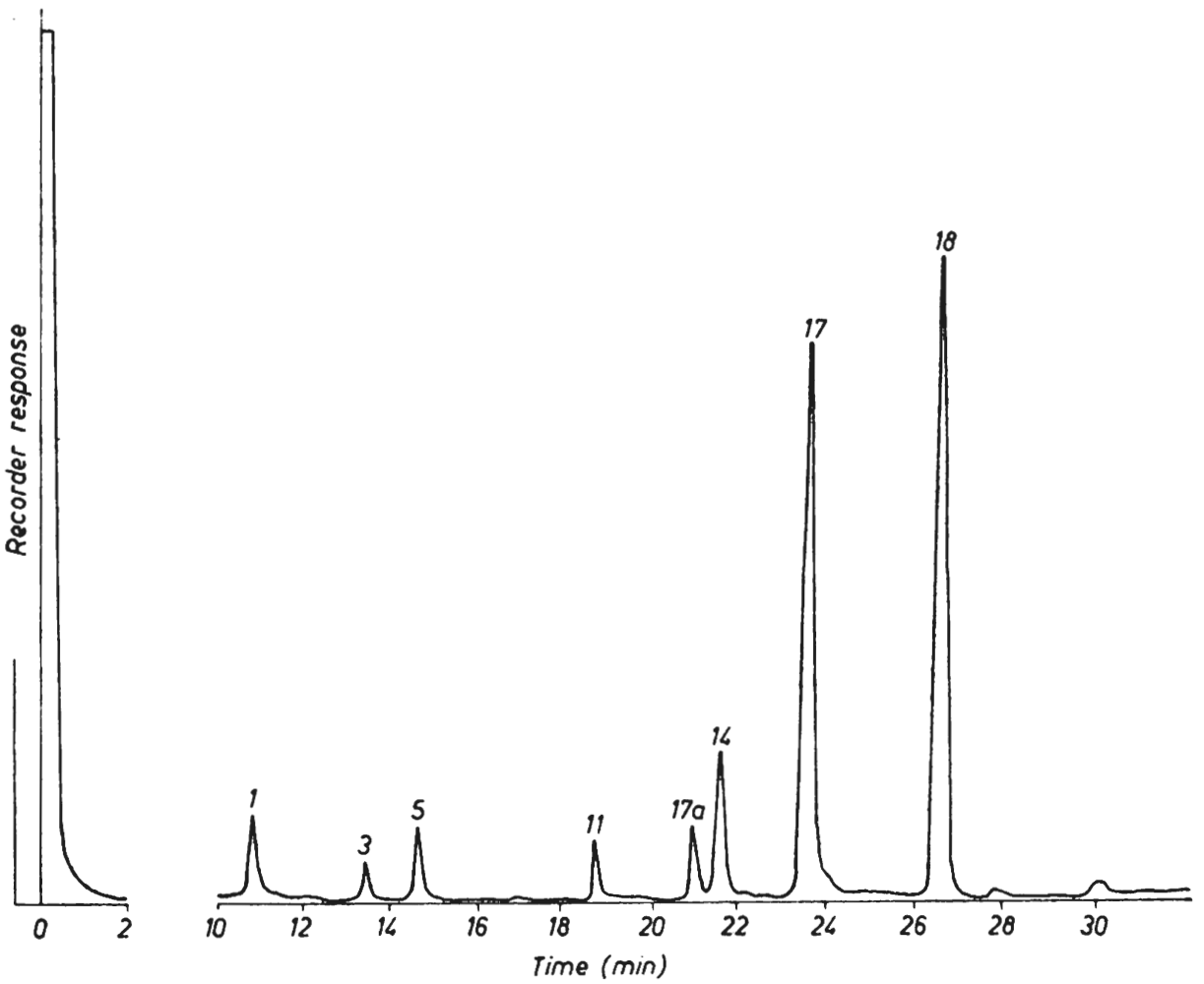

Fig. 4. GLC chromatogram of the TMS derivatives of the insoluble bound phenolic compounds in rapeseed flour after acid hydrolysis of the methanol-extracted residues, explanations — see Fig. 1.

3. The principal phenolic compounds in rapeseed was sinapic acid. Sinapic acid appeared as a doublet in the form of chromatograms in the rapeseed, suggesting the presence of Z and E isomers.

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OZNACZANIE RÓŻNYCH FORM KWASÓW FENOLOWYCH W MĄCE RZEPAKOWEJ

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Streszczenie

W pracy opisano sposób wyodrębniania, identyfikacji oraz ilościowego oznaczania wolnych, zestryfikowanych oraz związanych w formy nierozpuszczalne kwasów fenolowych występujących w mące rzepakowej otrzymanej z nasion rzepaku kanadyjskiego odmiany Tower.

Stosując opracowaną metodę opartą na identyfikacji oraz ilościowym oznaczeniu kwasów fenolowych metodą chromatografii gazowej ustalono, że najwięcej kwasów fenolowych w mące występowało w postaci estrów (2833 mg/100 g) (tab.), znacznie mniej jako wolne (303 mg/100 g) (tab.) i najmniej związanych w formy nierozpuszczalne, z których je uwalniano stosując hydrolizę zasadową (176 mg/100 g) (tab.).

Wśród zidentyfikowanych związków dominował kwas sinapinowy, który występował prawdopodobnie w postaci izomerów Z i E (rys. 1). Stanowił on podstawowy składnik frakcji kwasów fenolowych występujących w postaci estrów oraz związanych w formy nierozpuszczalne.