POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2003, Vol. 12/53, No 1, pp. 53–57

A RAPID HPLC METHOD FOR DETERMINATION OF MAJOR PHENOLIC ACIDS IN PLANT MATERIAL

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Key words: phenolic acids, HPLC, polymer based reversed phase, canola meal

The present work describes a simplified, and rapid HPLC method for measurement of phenolic acids in plant material. This procedure allows baseline resolution of major phenolic acids commonly found in plants (*p*-hydroxybenzoic, caffeic, syringic, *o*-coumaric, *m*-coumaric, *p*-coumaric, gentisic, ferulic, sinapic, salicylic), it is specific, sensitive, and ensures good reproducibility. The precision and reproducibility obtained using the present approach gave results comparable or better than the more complex, more laborious, and time consuming procedures. The time of HPLC separation of the phenolic acids in the presented procedure was significantly improved by the use of polymer based reverse phase column (PRP-1 column; 4.1 × 150 mm, 5 μm; Hamilton), and careful attention to the pH of the mobile phase, which allowed turnaround times of approximately 15 min and significantly lower solvent use. The present method allows the possibility for processing of a large number of samples rapidly, efficiently, and at a low cost.

INTRODUCTION

Quantitative measurement of phenolic acids have broad application in many disciplines, including food science, plant science, feed industry, and medical research. Several analytical approaches have been described including gas chromatography [Borneman *et al.*, 1990], high performance liquid chromatography [Castanares *et al.*, 1992; Kroon & Williamson, 1996; Scalbert *et al.*, 1985; Ralet *et al.*, 1994; Faulds &Williamson, 1995; Donaghy & McKay, 1995; Donaghy *et al.*, 1998; McKeehen *et al.*, 1999], capillary zone electrophoresis [Donaghy & McKay, 1995; Amarowicz & Kolodziejczyk, 2001]. By far, HPLC is the most popular technique used currently. Commonly used HPLC methods are based on reversed phase, chemically bound octadecyl silica columns, and the separation is carried with considerable usage of solvent in the mobile phase. Typically, elution time required for HPLC separation of major phenolic acids such as *p*-coumaric, ferulic, and sinapic is approximately 40 to 50 min [Kroon & Williamson, 1996; Amarowicz & Weidner, 2001], but some analysis may require as much as 70 to 90 min [Andreasen *et al.*, 1999].

The obvious drawback of the currently used methods is relatively prolonged time of analysis. Here, we present a novel HPLC approach for the measurement of phenolic acids. The method was used to evaluate content of phenolic acids extracted from canola meal and a product of chemical hydrolysis and enzymatic hydrolysis of oat hulls.

MATERIALS AND METHODS

Reagents. All reagents and standards of phenolic acids were purchased from Sigma Chemical Co. (St. Louis, MO). Solvents in this study were of HPLC grade and were obtained from Fisher Scientific Co. (Nepean, ON, Canada).

Plant material. Canola meal and oat hulls were obtained from local canola crushing plant.

Sample preparation. Extraction of phenolic acids from canola meal was carried out as previously described by Amarowicz *et al.* [1995]. Briefly: 20 g of canola meal was extracted with 200 mL of 80% methanol (v/v) for 30 min at 50°C. The slurry was filtered using a Whatman 3 filter, and the extraction of solid residues was repeated twice. The methanol extracts were pooled and methanol was evaporated in a rotary evaporator at 40°C. The remaining residues were acidified with 2 mol/L HCl to pH 2, and separation of phenolic acids into categories of free acids, acids liberated from soluble esters, and acids liberated from soluble glycosides was carried out according to procedure described by Weidner *et al.* [1999].

The content of phenolic acids in oat hulls was determined after alkaline and enzymatic hydrolyses. To oat hulls (10 mg) 1 mol/L NaOH solution (0.55 mL) was added followed by incubation at 37°C for 24 h. The samples were centrifuged, the supernatants were acidified with 2 mol/L acetic acid to pH 3 and extracted into ethyl acetate five times. Pooled fractions were evaporated to dryness under nitrogen at 40°C. The dry residues were reconstituted in 50% (v/v) methanol, filtered using 0.45 μ membrane filter, and analysed. Enzymatic hydrolysis of phenolic acids esters from oat hulls was carried out according to Yu *et al.* [2002] in 0.1 mol/L MOPS buffer (3-[N-morpholino] propane--sulphonic acid) in a thermostatically controlled shaking incubator at 37°C using *Aspergillus* ferulic acid esterase

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(Finnfeeds International). After enzymatic hydrolysis the sample was prepared for HPLC at the same way like that after alkaline hydrolysis.

HPLC analysis. Phenolic acids were analysed using a Water HPLC system (San Ramon, CA, USA) consisting of a pump and system controller (Model 600), sample processor (715 Ultra WISP) and photo-diode array detector (Model 966). Phenolic acids separation was done by a reversed phase PRP-1 column (4.1 × 150 mm, 5 μm; Hamilton, Reno, NV, USA). The mobile phase comprised of 3.1% methanol (v/v) in 20 mmol/L K_2HPO_4 titrated to pH 9.5 with 1 mol/L KOH. The mobile phase was delivered at a rate of 0.7 mL/min, and samples (10 μL) were introduced into the column using an autosampler. The detection was monitored at 300 nm.

Method validation. The method was used to evaluate content of phenolic acids extracted from canola meal and a product of enzymatic hydrolysis of oat hulls. Phenolic acids in samples were identified and quantified by comparison of retention time and DAD-UV spectra with that of authentic compounds. The procedure was optimised to resolve a mixture of the following phenolic acids: *p*-hydroxybenzoic, caffeic, syringic, *p*-coumaric, gentisic, ferulic, *m*-coumaric, sinapic, salicylic, and *o*-coumaric. Linearity of detector responses was tested for gentisic, *p*-coumaric, ferulic, and sinapic acids. Repeatability of the method was tested by analyzing 10 samples of free phenolic acids and phenolic acids after basic and acid hydrolysis. Samples were prepared from the extract of canola meal. The results were evaluated statistically.

RESULTS AND DISCUSSION

Ten analysed major phenolic acids (*p*-hydroxybenzoic, caffeic, syringic, *o*-coumaric, *m*-coumaric, *p*-coumaric, gentisic, ferulic, sinapic, salicylic), were resolved under the chromatographic conditions described (Figure 1). Chromatographic analysis of the extracted samples of canola meal revealed that phenolic acids found in appreciable concentrations include: ferulic, *p*-coumaric, sinapic, and

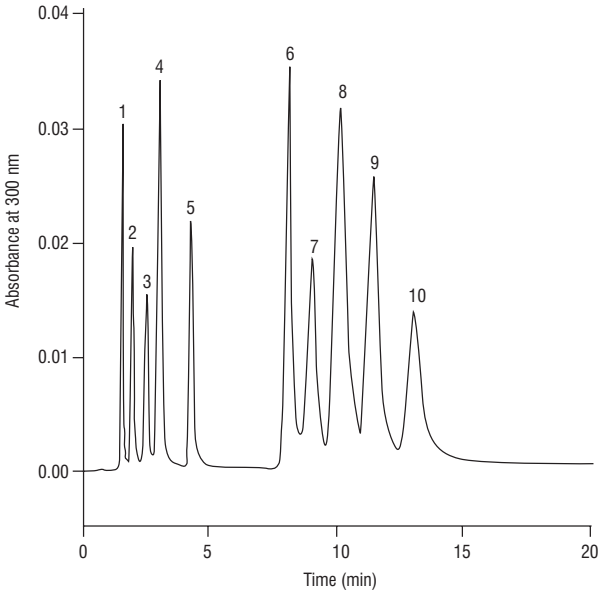

FIGURE 1. HPLC chromatogram of phenolic acids standards. Numbers above each peak correspond to respective phenolic acids: (1) *p*-hydroxybenzoic, (2) caffeic, (3) syringic, (4) *p*-coumaric, (5) gentisic, (6) ferulic, (7) *m*-coumaric, (8) sinapic, (9) salicylic, and (10) *o*-coumaric.

gentisic acids. Figure 2 shows representative chromatograms obtained from analysis of samples extracted using specific method to yield phenolic acids present in the sample classified as free acids, soluble esters, and soluble glycosides. Calibration curves of all analytes routinely yielded correlation coefficients 0.999 or better (Figure 3). Similar results of the calibration of phenolic acids determined by HPLC with diode-array detection was reported by Mattila & Kumpulainen [2002].

Quantitative analyses of different forms of phenolic acids in canola meal are shown in Table 1. Among the most abundant phenolic acids found in canola meal was synapic acid, followed by ferulic acid, whereas *p*-coumaric acid and gentisic acid were found in trace amounts. Intra-assay coefficients of variation were below 3.5%. Table 2 shows quan-

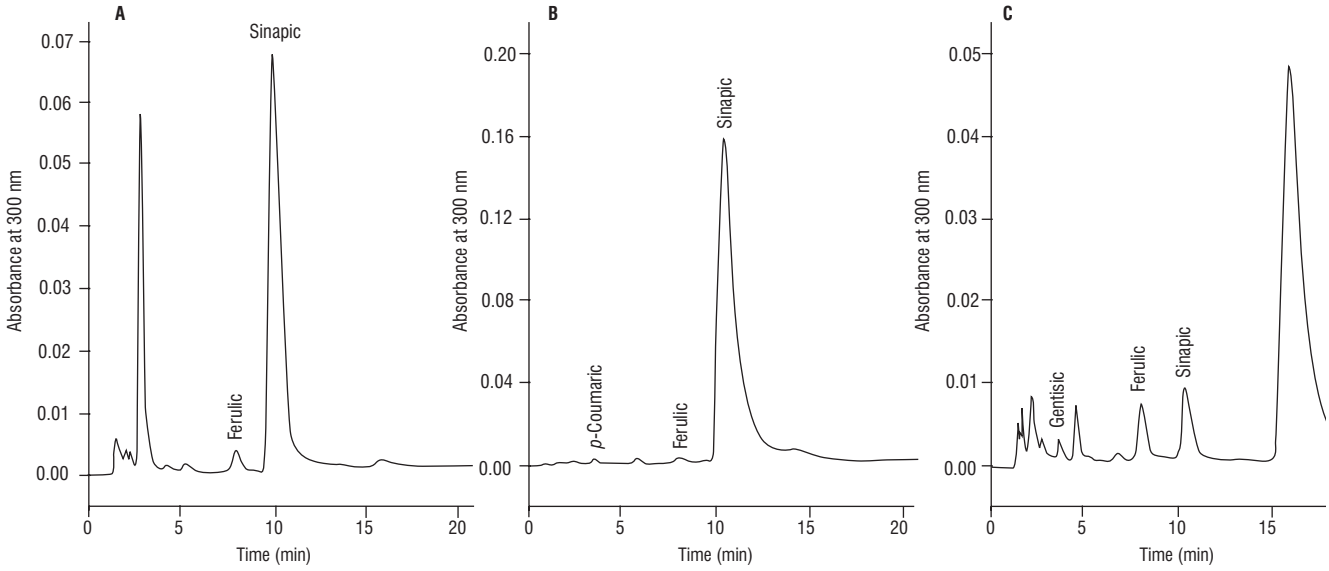

FIGURE 2. HPLC chromatograms of phenolic acids in extracts from canola meal. The chromatograms represent free phenolic acids (A); phenolic acids liberated from soluble esters (B); phenolic acids liberated from soluble glycosides (C).

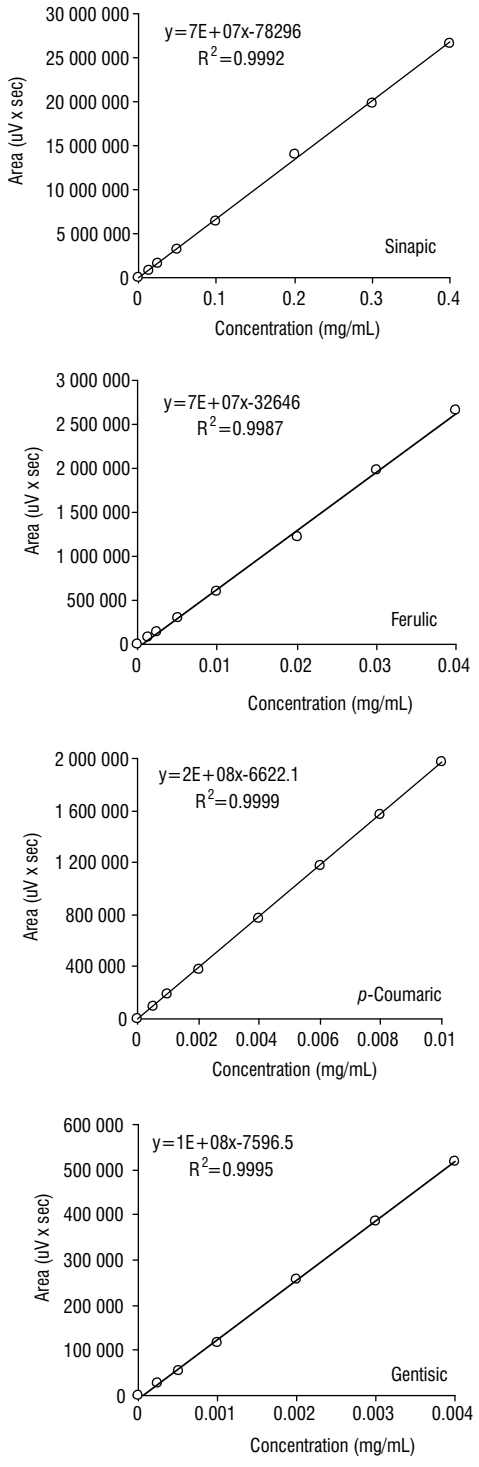

FIGURE 3. Calibration curves for sinapic, ferulic, p-coumaric, and gentisic acids. The detection was monitored at 300 nm.

titative analysis of products from chemical and enzymatic hydrolysis of oat hulls. Of note, enzymatic hydrolysis released only 1.9% and 6.9% respectively of total alkali--extractable *p*-coumaric and ferulic acids.

Among the most desirable attributes sought after in preparations of analytical procedures are rapid turnover and minimal usage of chemicals. In this context, the present method offers considerable improvement over the previously used procedure. Notably, in comparison to previously described methods, the present approach requires only

TABLE 1. Quantitative analysis of phenolic acids in canola meal (mg/100 g on dry weight).

Phenolic acid	Free phenolic acids	Phenolic acids liberated from soluble esters	Phenolic acids liberated from soluble glycosides
p-Coumaric	ND	0.72±0.02 CV 2.77	ND
Gentisic	ND	ND	0.21±0.003 CV=1.43
Ferulic	3.49 ±0.09 CV=3.49	5.88±0.15 CV=2.55	2.73±0.09 CV=3.30
Sinapic	66.9±3.09 CV=3.09	706.3±15.0 CV=2.55	3.93±0.11 CV=3.30

Values are means ± standard deviation of 10 separate analyses; ND – not detectable, CV – coefficient of variation.

TABLE 2. Phenolic acids (μg/ 100 mg on dry weight) released from oat hulls sample by alcaline hydrolysis or upon enzymatic hydrolysis with ferulic acid esterase.

Phenolic acid	Alkaline hydrolysis	Enzymatic hydrolysis*
p-Coumaric	521	10
Ferulic	383	26

*Based on 3276.9 U of ferulic acid esterase per assay.

a third of the analytical time and reduces to requirement of organic modifier content in mobile phase to approximately 3%.

Previously described HPLC methods used chemically bound octadecyl silica columns. Because silica based columns are very sensitive to alkaline conditions, elution is carried out in acidic conditions. This requires considerable usage of solvent in the mobile phase and prolonged elution time. Typically, elution time required for HPLC separation of major phenolic acids such as *p*-coumaric, ferulic, and sinapic was approximately 40 to 50 min [Kroon & Williamson, 1996; Amarowicz & Weidner, 2001], but using a LUNA C18 column (Phenomenex) may be as high as 70 min [Amarowicz *et al.*, 2002]. Gradient elution employed by Mattila & Kumpulainen [2002] for ODS-3 column reduced time of determination of phenolic acids but ferulic and sinapic acids with retention time of 27 min were not separated.

In the present procedure we used polymer based reverse phase column, which permits considerable flexibility with respect to pH conditions of the mobile phase. Considering strongly hydrophobic characteristics of the column, a careful attention to the pH of the mobile phase allowed us to lower the requirement for organic modifier to a very minimal level of 3%. Taken together this procedure allowed turnaround times of approximately 15 min and at the same time ensured good resolution of compounds.

Notably, under the described chromatographic conditions, this approach allowed good resolution of mixture of 10 major phenolic acids commonly encountered in plant material, including those that are most abundant. This shows that the procedure ensures good specificity. As evidenced by high correlation coefficients for calibration curves of all analytes, and excellent reproducibility (very low coefficients of variation), this method also ensures a high degree of precision.

With such a considerably shortened time of analysis and very small amount of organic modifier required in the mobile phase, the present method offers considerable advantages, particularly in laboratories that process large number of samples.

We have applied this method in routine measurement of phenolic acids content is canola meal and to measure the product of enzymatic hydrolysis of oat hulls. In both cases, this procedure allowed very good resolution of all phenolic acids of interest in these samples. It is noteworthy, that even at a very high sensitivity (see Figure 2c), there was very little sample matrix interference, which allowed quantitative measurement of very small amounts of phenolic acids in the native sample. As evidenced by the data, the present procedure offers a wide range of application in phenolic acid analysis commonly used in various disciplines. The specificity, precision, and reproducibility obtained using the present approach gave results comparable or better to more complex, more laborious and time consuming procedures.

There is no single method that would be applicable or practical to fulfil all analytical requirements of phenolic acids. The present method was developed for specific experimental objectives. The advantages of our procedure have been discussed and contrasted with methods currently used for similar applications. However, it is noteworthy that within the context of our work we also introduced a novel conceptual approach that allows practically unlimited manipulation of mobile phase with respect to pH as well as the content of organic modifier. These two factors permit considerable flexibility of methodological approaches that can be further developed for more specific applications of phenolic acids analysis.

CONCLUSIONS

The above-discussed results prove that the proposed method can be used in analysis of major phenolic acids extracted from oil seeds and cereals grains. The present method allows the possibility for processing of a large number of samples rapidly, efficiently, and at a low cost.

ACKNOWLEDGEMENTS

Financial assistance for this work was provided by Saskatchewan Agriculture Development Fund.

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Received December 2002 and accepted January 2003.

SZYBKA METODA HPLC OZNACZANIA GŁÓWNYCH FENOLOKWASÓW W MATERIALE ROŚLINNYM

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W pracy opisano prostą i szybka metodę HPLC do oznaczania fenolokwasów w materiale roślinnym. Pozwala ona całkowicie rozdzielić i oznaczyć ilościowo główne fenolokwasy występujące w materiale roślinnym: *p*-hydroksybenzoesowy, kawowy, syryngowy, *o*-kumarowy, *m*-kumarowy, *p*-kumarowy, gentyzowy, ferulowy, sinapowy, salicylowy. Metoda charakteryzuje się dużą specyficznością, czułością i odtwarzalnością. W metodzie zastosowano kolumnę z odwróconą fazą (kolumna PRP-1; 4,1 × 150 mm, 5 μm; Hamilton). Jako fazę ruchomą stosowano układ 3,1% metanolu (v/v) w 20 mmol/L K_2HPO_4, doprowadzony do pH 9,5. Przeciętny czas analizy fenolokwasów w porównaniu z innymi metodami został znacząco skrócony do 15 min.