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APPLICATION OF FAST LIQUID CHROMATOGRAPHY FOR ANTIOXIDANTS ANALYSIS

Agnieszka Drożdżyńska[1]✉, Krzysztof Dziedzic[2], Alicja Kośmider[1], Katarzyna Leja[1], Katarzyna Czaczyk[1], Danuta Górecka[2]

[1]Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences
Wojska Polskiego 48, 60-627 Poznań, **Poland**
[2]Department of Food Service and Catering, Poznań University of Life Sciences
Wojska Polskiego 31, 60-624 Poznań, **Poland**

ABSTRACT

Background. An intensive development of the Fast Liquid Chromatography (FLC) has been recently observed. It makes possible to reduce time analysis and improve resolution as well as sensitivity. The aim of this study was to separate the chosen antioxidants optimization using the FLC method.
Material and methods. The three various procedures for antioxidants analysis were compared. Mobile phases containing aqueous solution of formic acid, acetic acid, acetonitrile, and methanol were tested. Limit of detection (LOD), limit of quantification (LOQ), linearity and repeatability of each procedures were determined.
Results. Developed procedure enabled to separate all analytes and allowed to get low LOD levels and good repeatability. This procedure was used for antioxidants analysis in buckwheat and buckwheat products.
Conclusion. Fast Liquid Chromatography allows to reduce time analysis and obtain good validation parameters.

Key words: Fast Liquid Chromatography, antioxidants

INTRODUCTION

Antioxidants are chemical compounds which molecules capable of slowing or preventing the oxidation of other molecules [Sardesai 1995, Halliwell 1990]. There is a growing interest in the use of natural antioxidants, i.e. polyphenolic compounds found in various parts of plants [Kobus-Cisowska et al. 2010, Roman et al. 2009, Zarena and Sankar 2009]. It includes several antioxidants, among other rutin, quercetin, kaempferol and phenolic acids. Recent studies have shown many poliphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C [Rice-Evans et al. 1997]. Polyphenols have been used as a natural medicine for millennia. They can help preserve vascular health and diminish cancer risk [Helliwell et al. 1992, McCarty 2004]. Natural antioxidants detoxify reactive oxygen species and prevent their damage to cellular organelles and macromolecules using different mechanisms [Shahidi 2000]. Phenolic acids (e.g., caffeic, o-coumaric, p-coumaric, ferulic, gallic, sinapic, and p-hydroxybenzoic acid) and flavonoids (catechine, quercetin, kaempferol, and rutin) possess antioxidant activity.

Antioxidant analysis in food is both important and difficult task.

✉agadro@up.poznan.pl

Phenolic compounds can be analysed using chemical (mainly spectrophotometric) or chromatographic methods. It is possible to measure total phenolic compounds concentration by the Folin-Ciocalteau method [Przeciwutleniacze... 2007]. It gives imprecise results because of the reagent's low specificity and does not yield quantitative information about single phenolic compounds. High Performance Liquid Chromatography (HPLC) is a very sensitive and specific method which enables quantitative determination of phenolic compounds. Antioxidants are generally analyzed using C18 column. Acid solutions (formic or acetic) with methanol or acetonitrile are using as eluents. FLC has been fast developing technique recently. It makes possible to reduce time analysis and improve resolution, as well as sensitivity.

The aim of this study was separation of the chosen antioxidants optimisation. The three various procedures were compared. The two from the abovementioned procedures were adopted to FLC technique from HPLC technique. The third procedure is a compilation of the first and the second. Modifications of this procedure were also tested. This procedure was then used for determination of chosen antioxidants in buckwheat and buckwheat products. Buckwheat is one of the source of antioxidants [Górecka et al. 2009].

EXPERIMENTAL

Chemicals and reagents

Phenolic acids: caffeic, o-coumaric, p-coumaric, ferulic, gallic, and p-hydroxybenzoic;

Flavonoids: catechine, quercetin, kaempferol, rutin.

All this reagents were obtained from SIGMA.

Sample. Buckwheat groats were obtained from Podlachian Cereal Industrial Plants (Białystok). Antioxidants were determined in whole buckwheat groats, boiled buckwheat groats, buckwheat bran, and buckwheat wastes.

Sample preparation. Phenolic compounds were extracted from samples using 80% acetone, at 50°C for 30 minutes. Extraction was carried out in dark-colored flasks using a shaking water bath. Extract was lyophilized and stored at –20°C until used [Amarowicz et al. 2008].

Chromatography

FLC was performed with an Agilent Technologies 1200 series system comprising an autosampler (model G1329B), a pump (model G1312B) and a diode array detector (model G1315C). The FLC system was controlled by ChemStation for LC 3D system. Spectral data from all peaks were accumulated in the range of 190-400 nm. Chromatograms were recorded at 280 nm for gallic acid, p-hydroxybenzoic acid, and catechin, at 320 nm for caffeic, p-coumaric, o-coumaric, sinapic and ferulic acids, and at 360 nm for rutin, quercetin, and kaempferol. Compounds were separated on 50 mm × 4.6 mm, particle 1.8 µm, SB-C18 column (Agilent). This column was thermostated at 25°C. Several mobile phases and gradient profiles were tested to optimize the separation and detection (Table 1).

The basic validation parameters of the three applied procedures were determined and compared**.** Among the validation parameters examined were limit of detection (LOD), limit of quantification (LOQ), repeatability, and linearity.

Limit of detection (LOD). Analyses of measurement series for three standard solution samples containing selected antioxidants (such as caffeic acid, p-coumaric acid, o-coumaric acid, catechin, and rutin) at the three levels of concentration were done. For each level of analytes, six parallel determinations were performed. The standard deviations for each series of measurements were calculated and the linear dependence s = f(c) was determined. The absolute term SD_0 was decoded from the graph. The LOD was determined according to the following dependence [Konieczka and Namieśnik 2007]:

$$LOD = 3 \cdot SD_0$$

Limit of quantification (LOQ). The LOQ was calculated according to the formula: LOQ = 3·LOD [Konieczka and Namieśnik 2007].

Repeatability. Repeatability was determined according to the following dependence:

$$CV = s/\overline{x} \cdot 100\ (\%)$$

CV – coefficient of variation

$\overline{x}$ – mean value of peak area of ten parallel determinations for polyphenols solutions (10 $mg \cdot l^{-1}$)

s – standard deviation.

Table 1. Basis chromatographic parameters

Procedure A		Procedure B		Procedure C	
Mobile phase A: 0.1% formic acid Mobile phase B: Methanol		Mobile phase A: H_2O:acetic acid (98:2) Mobile phase B: H_2O:acetonitrile:acetic acid (78:20:2)		Mobile phase A: H_2O:acetic acid (98:2) Mobile phase B: H_2O:methanol:acetic acid (48:50:2)	
Time, min	% B	Time, min	% B	Time, min	% B
0	20	0	0	0	0
7	50	11	80	22	80
7.4	80	17	90	26	80
11	80	20	95	28	0
12	20	22	100	31	0
		30	100		
Flow rate 0.6 ml·min^{-1}		Flow rate 1.1 ml·min^{-1}		Flow rate 1.1 ml·min^{-1}	

Linearity. Linearity was tested in the range from 0.3 to 300 mg·l^{-1} of selected antioxidants (at nine levels of concentration with the three determinations for each level) by using a graph of measuring instrument calibration. The value of regression coefficient R not lower than 0.999 is considered as a criterion of linearity by CDER (Center for Drug Evaluation and Research) [Reviewer guidance... 1994].

RESULT AND DISCUSSION

HPLC methods are more specific than other methods (for example spectrophotometric methods) [Przeciwutleniacze... 2007]. By this reason, HPLC technique has been commonly used to antioxidants determination [Olszewska 2007, Vallejo et al. 2004, Amarowicz et al. 2008]. Recently, "fast chromatography" has been developing. This technique allows to shorten time analysis and save reagents. There are some literature data concerning using "fast chromatography" for antioxidants determination. In this paper, the three FLC procedures (various composition of eluents and chromatographic parameters) and StableBond-C18 column were tested for their ability to perform antioxidants separation. Figure 1 shows chromatograms obtained from a reversed-phase C18 column after injection of the selected antioxidants solutions (such as caffeic acid, o-coumaric acid, p-coumaric acid, ferulic acid, gallic acid, p-hydroxybenzoic acid, sinapic acid, catechine, quercetin, kaempferol, and rutin). In the A procedure the mobile phase was prepared according to Vallejo et al. method [2004]. The eluent contained 0.1% aqueous solution of formic acid (the A component) and methanol (the B component) [Vallejo et al. 2004]. Gradient profile was adopted to FLC technique using Agilent Method Translator with modification. This procedure allowed to reduce analysis time almost four times (according to literature date) [Vallejo et al. 2004]. In this case baseline separation was not achieved (Fig. 1 A). Co-elution of sinapic and ferulic acids was observed. This procedure allowed to get low LOD levels and low CV values (CV < 3% for catechin and CV < 1% for other analytes; Table 2).

The second examined chromatographic parameters were based on Amarowicz et al. [2008]. Mobile phase, which was used in these analysis, consisted of 2% aqueous solution of acetic acid (the A component) and aqueous solution of acetonitrile and acetic acids (the B component). Gradient profile was adopted to FLC technique using Agilent Method Translator with modification. This procedure allowed to reduce analysis time more than three times (according to literature date) [Amarowicz et al. 2008]. It enabled to separate all analytes. It also allowed to obtain good validation parameters. However, there were no significant differences

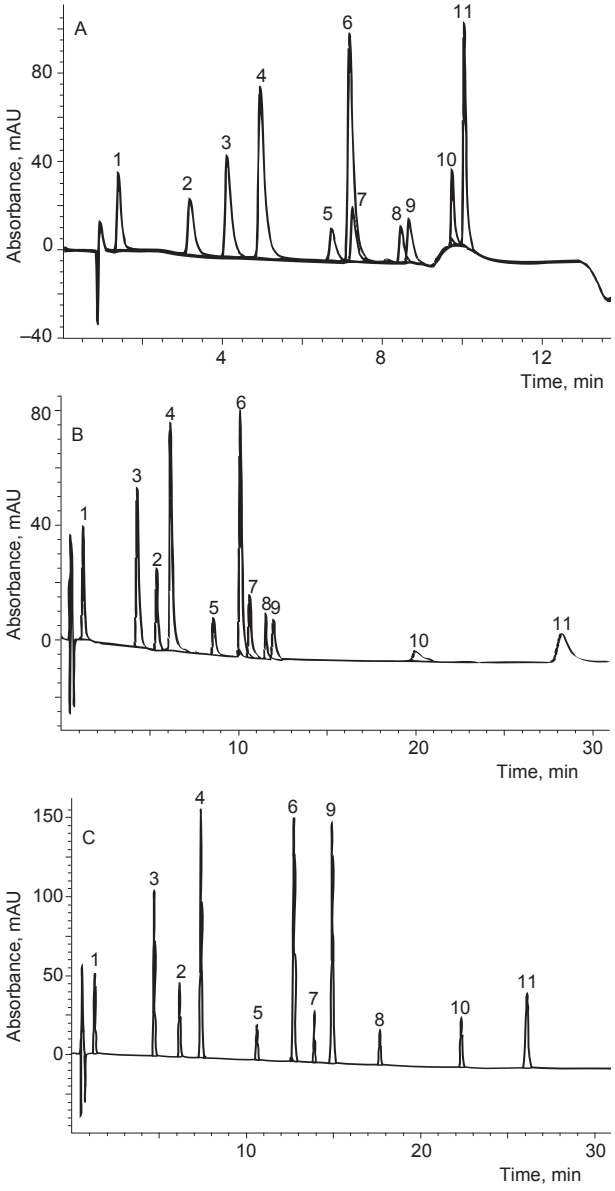

Fig. 1. Chromatograms of chosen antioxidants on SB-C18 column obtained with three procedures: A, B and C. Operating conditions – see Table 1. Peaks identification: 1 – gallic acid, 2 – catechin, 3 – p-hydroxybenzoic acid, 4 – caffeic acid, 5 – p-coumaric acid, 6 – ferulic acid, 7 – sinapic acid, 8 – rutin, 9 – o-coumaric acid 10 – quercetin, 11 – kaempferol

in retention times of ferulic acid, sinapic acid, rutin, and o-coumaric acid (Fig. 1 B). Thus, baseline separation of these compounds was not possible when they occurred at a high concentration.

The world-wide shortage of acetonitrile was hitting the HPLC market in the past. By that reason substitutes for acetonitrile in the mobile phase have been searched. The most frequently used reverse-phase solvents are water, acetonitrile, methanol, and tetrahydrofuran (THF). Chemical and physical properties of the solvents and their effects on the chromatographic process when replacing one solvent with another should be considered. Selecting new solvent is mainly based on UV cutoff. Tetrahydrofuran has the highest UV cutoff equals 212 nm. Moreover, storage of THF leads to its degradation and forming unstable and chemically reactive peroxides. Methanol has UV cutoff 205 nm (acetonitrile < 195 nm). Achieving a background absorbance contribution from methanol of less than 0.05 AU requires working at $\lambda > 235$ nm [Sadek 2002].

Among the examined antioxidants p-hydroxybenzoic acid had the lowest maximum absorption at 255 nm, thus methanol could be used as an alternative for acetonitrile successfully. Switching solvents affects retention time, as well as selectivity. Selectivity differences are based on the different solvation properties of methanol and acetonitrile. The type of solvent may also influence column efficiency and peak symmetry [Campbell 2008].

The new chromatographic parameters were proposed. An expensive acetonitrile was replaced with cheaper and commonly available methanol. Acetic acid was used to acidize mobile phase. According to the eluotropic strength nomogram, 20% acetonitrile corresponds to 28% methanol [Meyer 2004]. Analysis were performed using mobile phase consisting of 2% aqueous solution of acetic acid (the A component) and aqueous solution of 25% methanol and 2% acetic acid (the B component). The gradient profile was the same as the gradient profile of the method described by Amarowicz et al. [2008]. It made possible to separate almost all analytes. However, kaempferol and quercetin were bound so much to the column that they did not elute until 40 minutes (data not present). Thus, the concentration of methanol in mobile phase should be increased. The proposed chromatographic parameters (the C procedure) included the B component consisting of 50% methanol and 2% acetic acid were tested. The gradient profile was changed to allow separation of all compounds. It enabled both baseline separation of all analytes and elution of kaempferol and quercetin

Table 2. Limits of detection, limits of quantification, precision and linearity of selected antioxidants by the three procedures

Substance	Procedure A				Procedure B				Procedure C			
	LOD mg·l^{-1}	LOQ mg·l^{-1}	CV %	linearity mg·l^{-1}	LOD mg·l^{-1}	LOQ mg·l^{-1}	CV %	linearity mg·l^{-1}	LOD mg·l^{-1}	LOQ mg·l^{-1}	CV %	linearity mg·l^{-1}
Caffeic acid	0.065	0.195	0.162	0.3-305*	0.054	0.161	0.291	0.3-305*	0.040	0.121	0.647	0.3-305*
o-coumaric acid	0.047	0.140	0.229	0.3-302*	0.059	0.178	0.383	0.3-302*	0.046	0.137	0.511	0.3-302*
p-coumaric acid	0.007	0.021	0.775	0.3-100	0.015	0.045	0.208	0.3-300*	0.011	0.032	0.522	0.3-300*
Catechin	0.298	0.895	2.665	0.3-319*	0.298	0.893	0.986	0.3-319*	0.201	0.603	0.913	0.3-319*
Rutin	0.054	0.162	0.619	0.3-301*	0.036	0.107	0.345	0.3-301*	0.058	0.175	0.850	0.3-301*

*Linear detector response was achieved in a whole analysed concentration range.

Table 3. Contents of some selected antioxidants in whole buckwheat groats, boiled whole buckwheat groats, buckwheat bran and buckwheat waste

Products	Compound, mg·kg^{-1} d.m.				
	rutin	p-coumaric acid	p-hydroxybenzoic acid	caffeic acid	gallic acid
WBG	70.18^{c}	7.31^{a}	22.13^{a}	nd^{b}	3.84^{b}
BWBG	71.02^{c}	0.91^{b}	nd^{c}	nd^{b}	4.08^{b}
BB	619.06^{b}	5.89^{a}	13.33^{b}	nd^{b}	6.11^{a}
BW	224.78^{a}	nd^{b}	nd^{c}	95.08^{a}	4.76^{b}

WBG – whole buckwheat groats, BWBG – boiled whole buckwheat groats, BB – buckwheat bran, BW – buckwheat waste.
The numerical values denoted by different letters in line differ statistically significantly at $p < 0.05$.
nd – not detected.

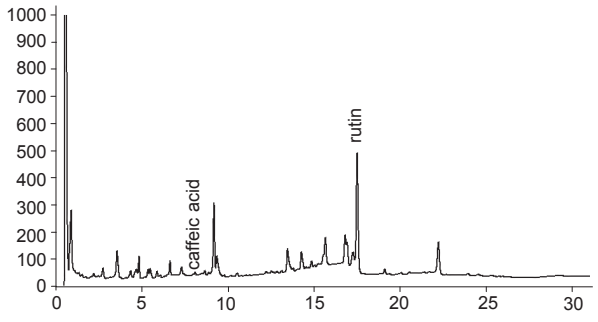

Fig. 2. Chromatogram of chosen antioxidants in buckwheat waste

from the column in less than 30 minutes (Fig. 1 C). This procedure allowed to get low the LOD levels and low the CV values (CV < 1% for all analytes; Table 2). This eluent and chromatographic parameters (the C procedure) were then used for antioxidant analysis in extracts from buckwheat and products which occurred during its processing. The content of phenolic compounds in the samples extract is reported in Table 3.

Rutin was the dominant phenolic compound in all examined samples. The largest amount of rutin was found in buckwheat bran. Caffeic acid occurred only in buckwheat waste. Boiling buckwheat groat influenced on amount of p-coumaric acid, p-hydroxybenzoic acid, and gallic acid. The chosen procedure allowed good resolution of antioxidants in the analysed materials (Fig. 2).

CONCLUSION

The FLC technique used in this investigation enabled significantly faster analysis in comparison to conventional liquid chromatography. All three tested chromatographic parameters can be used for antioxidants analysis. These procedures were characterized by low limits of detection and good precision. The C procedure allowed to get the best chromatographic resolution of all chosen antioxidants and was used for antioxidants determination in buckwheat and products which occurred during its processing.

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WYKORZYSTANIE TECHNIKI SZYBKIEJ CHROMATOGRAFII CIECZOWEJ DO ANALIZY ANTYOKSYDANTÓW

STRESZCZENIE

Cel. Szybka chromatografia cieczowa (FLC – Fast Liquid Chromatography) jest szeroko wykorzystywaną techniką. Pozwala ona na skrócenie czasu analizy i umożliwia osiągnięcie większej czułości i rozdzielczości w porównaniu z „klasyczną" chromatografią cieczową. Celem pracy była optymalizacja rozdziału chromatograficznego wybranych antyoksydantów z wykorzystaniem techniki FLC.

Materiał i metody. Przetestowano różne procedury oznaczania antyoksydantów. Wykorzystano wodne roztwory kwasu mrówkowego, kwasu octowego, metanolu oraz acetonitrylu jako fazy ruchome i sprawdzono ich wpływ na efektywność rozdziału badanych antyoksydantów. Wyznaczono granice wykrywalności, oznaczalności, zakres liniowości oraz precyzję.
Wyniki. Opracowana procedura umożliwiła całkowity rozdział badanych związków polifenolowych, uzyskanie niskich granic wykrywalności i oznaczalności oraz dobrej precyzji. Procedurę wykorzystano do analizy wybranych antyoksydantów w gryce i jej produktach.
Podsumowanie. Technika szybkiej chromatografii cieczowej cechuje się dobrymi parametrami walidacyjnymi i pozwala na znaczne skrócenie czasu analizy (w porównaniu z tradycyjną chromatografią cieczową).

Słowa kluczowe: szybka chromatografia cieczowa, antyoksydanty

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