Short communication

Determination of Isoflavones Using Liquid Chromatography with Electrochemical Detection

Radka Mikelová,[a] Petr Hodek,[b] Pavel Hanustiak,[c,d] Vojtech Adam,[b] Sona Krizkova,[b] Ladislav Havel,[e] Marie Stiborova,[b] Ales Horna,[f] Miroslava Beklova,[d] Libuse Trnkova,[a] Rene Kizek[c,*]

[a] *Masaryk University, Department of Theoretical and Physical Chemistry, Faculty of Science, Brno, Czech Republic*

[b] *Charles University, Department of Biochemistry, Faculty of Science, Prague, Czech Republic*

[c] *Mendel University of Agriculture and Forestry, Department of Chemistry and Biochemistry, and* [e] *Department of Plant Biology, Faculty of Agronomy, Czech Republic. Tel.: +420(5)45133350, Fax: +420(5)45212044, E-mail: kizek@sci.muni.cz.*

[d] *University of Veterinary and Pharmaceutical Sciences, Department of Veterinary Ecology and Environmental Protection, Brno, Czech Republic*

[f] *Tomas Bata University, Department of Food Engineering and Chemistry, Faculty of Technology, Zlin, Czech Republic*

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Abstract

Among the biologically important roles of isoflavones is also their effect on carcinogenesis. We used flow injection analysis and high performance liquid W with electrochemical detection to simultaneously determine certain isoflavones (biochanin A, formononetin, sissotrin, daidzin, daidzein, glycitin, glycitein and genistein). The most suitable chromatographic conditions were: mobile phase: 0.2 mol L^{-1} acetate buffer (pH 5.0); flow rate 2.0 mL min^{-1}; column and detector temperature: 26 °C; detection potential: 800 mV. Under the optimal conditions, the detection limits were in the range of several ng mL^{-1}. Their simultaneous determination takes 15 min.

Keywords: isoflavone, electrochemical detection, coulometry, estrogen-like compounds, carbon paste electrode

1. Introduction

Nutraceutics are food components having a positive physiological impact on human organism.[1] Certain nutraceutics are beneficial to human health, others increase physical performance or decrease the risk of illnesses. From the chemical point of view, nutraceutics include a huge number of chemically different compounds, and include flavonoids. The interest in biological activity of flavonoids, estrogen-like compounds of plant origin, started only recently.[2,3] Flavonoids comprise a wide-ranging group of plant phenols. Up to now, more than 4,000 flavonoid compounds have been described.[4–7] They are derived from the oxygen-containing heterocyclic compound flavan. These phytoestrogens encompass several classes of compounds, including flavonoids, isoflavonoids, coumestrans (coumestrol), lignans and isoflavones. Isoflavones represent a group of distinct secondary metabolites produced predominantly in leguminous plants. Different isoflavones are derived through oxidation of the three central atoms in the carbon skeleton of 3-phenylbenzpyrone. Their structures differ in the degree of methylation, hydroxylation and glycosylation (Figure 1).

Isoflavones can be considered to be biologically important compounds. Isoflavones (e.g. daidzein) are precursors of phytoalexins and have a role in plant disease resistance, and exhibit antifungal activity (e.g. genistein).[2] The role of these compounds in carcinogenetic processes has been investigated intensively, although it has not been clarified yet.[8–11] It has also been published that the biologically active derivatives of isoflavones could be formed by action of cytochromes.[12–14]

A wide range of analytical techniques have been used for determination of polyphenols and phytoestrogens in food and biological materials.[15–20] Electrochemical techniques and methods are an attractive alternative met-

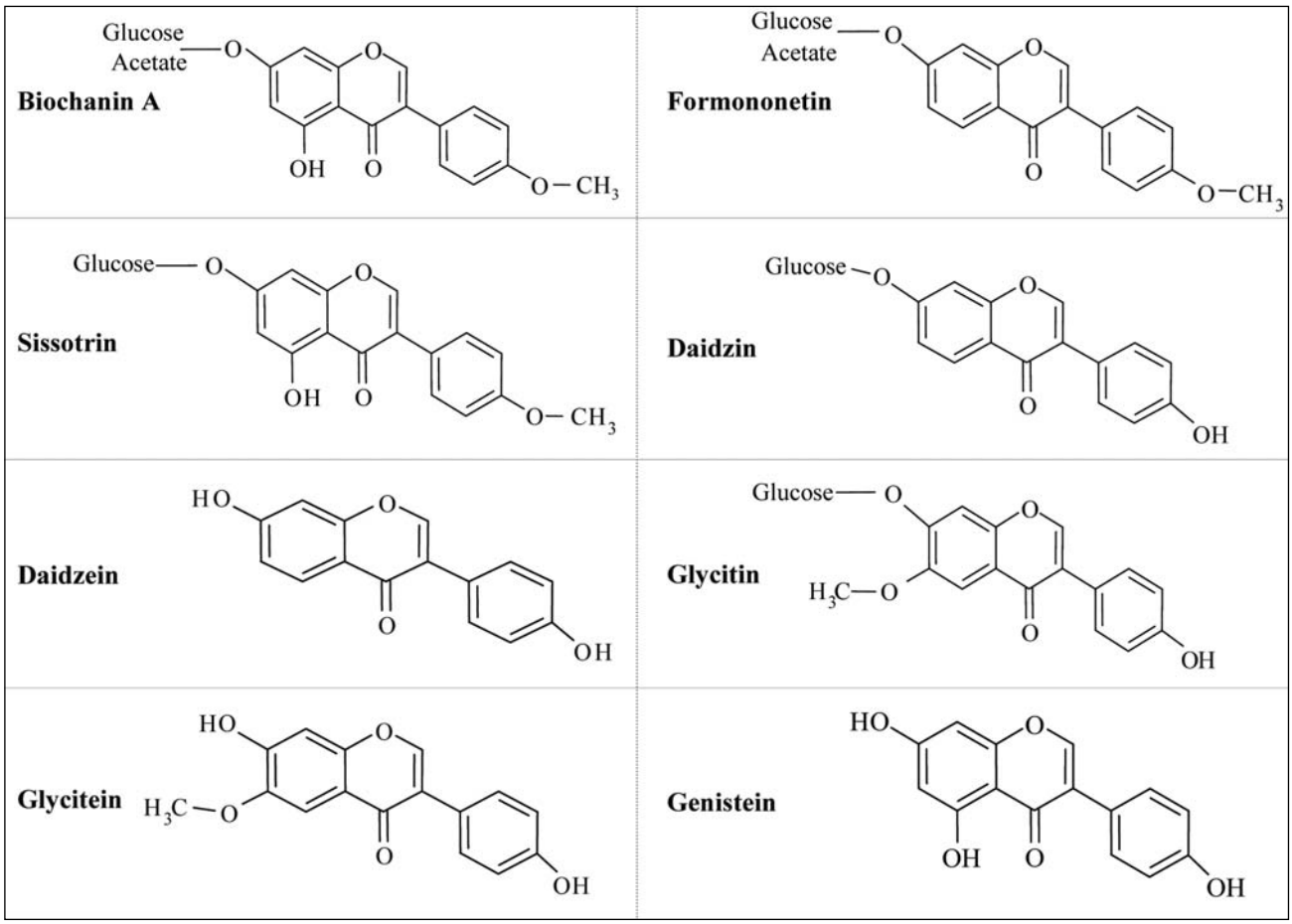

Figure 1. Chemical structures of biochanin A, formononetin, sissotrin, daidzin, daidzein, glycitin, glycitein, and genistein.

hod for detection of electroactive species, because of simplicity, ease of miniaturization, high sensitivity and relatively low cost.[21–29]

Here, we optimized and utilized flow injection analysis and high performance liquid chromatography coupled with electrochemical detection to determine biochanin A, formononetin, sissotrin, daidzin, daidzein, glycitin, glycitein, genistein, simultaneously.

2. Experimental

2.1. Chemicals

Isoflavones (biochanin A, formononetin, sissotrin, daidzin, daidzein, glycitin, glycitein, genistein) were purchased from Karlsroth GmbH (Karlsruhe, Germany). HPLC-grade acetonitrile (>99.9%) was from Merck (Darmstadt, Germany). Acetate buffer, trifluoroacetic acid and other analytical reagents of ACS purity were purchased from Sigma Aldrich (St. Louis, USA) unless noted otherwise. The stock standard solutions of isoflavones (10 mg mL^{-1}) were prepared in methanol (Sigma Aldrich, USA) : water 1:1 v/v and stored in darkness at 4 °C. The working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through a 0.45 μm teflon membrane filters (MetaChem, Torrance, CA, USA) prior to HPLC analysis.

2.2. Instrumentation

The flow injection analysis/high performance liquid chromatography coupled with electrochemical detector (FIA-ED/HPLC-ED) system consisted of two solvent delivery pumps operating in range of 0.001–9.999 mL min^{-1} (Model 582 ESA Inc., Chelmsford, MA), a reaction loop 1 m long and an electrochemical detector (Model 5600A, ESA, USA). Zorbax AAA reversed-phase chromatographic column (150 mm × 4.6 mm, 3.5 μm particle size, Agilent, USA) was used instead of reaction loop to determine the isoflavones simultaneously. The electrochemical detector includes two low volume flow-through analytical cells (Model 6210, ESA, USA). Each analytical cell is consisted of four carbon porous working electrodes, palladium electrodes as reference ones and auxiliary electrodes. The detector and the column were thermostated. The sample (5 μL) was injected manually (Hamilton, USA).

2.3. Statistical Analysis

STATGRAPHICS® (Statistical Graphics Corp®, USA) was used for statistical analyses. Results are expressed as the means ±S.D. unless noted otherwise. Value of $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Flow Injection Analysis

As we already demonstrated, using flow injection analysis coupled to various detectors we can investigate the behaviour of compounds of interest in order to optimise detection.[30–33] Here, we focused on the optimisation of detection of isoflavones in the range of applied potential 300 to 1000 mV. The experiments were carried using acetate buffer pH 5.0 as a mobile phase with flow rate of 0.5 mL min^{-1}; temperature 20 °C. The hydrodynamic voltammograms are shown in Figure 2. The isoflavones of interest gave the highest responses at potentials within the range of 750 to 900 mV. The signals at the same concentration (100 µmol L^{-1}) were similar, except daidzein, where the response was about ten times higher. Based on these results, the potential 800 mV was chosen for the optimisation to follow.

3.2. Influence of the Mobile Phase Composition

The mobile phase composition markedly affects electrochemical determination of the compounds of interest.[34] Two commonly used mobile phases, 0.2 mol L^{-1} acetate buffer and 0.05 mol L^{-1} trifluoroacetic acid (TFA), were tested. We observed an interesting electrochemical response. In the case of acetate buffer, the observed an increase of signal with an increasing pH. If we used TFA, we observed a decrease of the signal with an increasing pH (Figure 3). This behaviour was common to all isoflavones. The highest signals were obtained using 0.2 mol L^{-1} acetate buffer at pH 5 (Figure 3C).

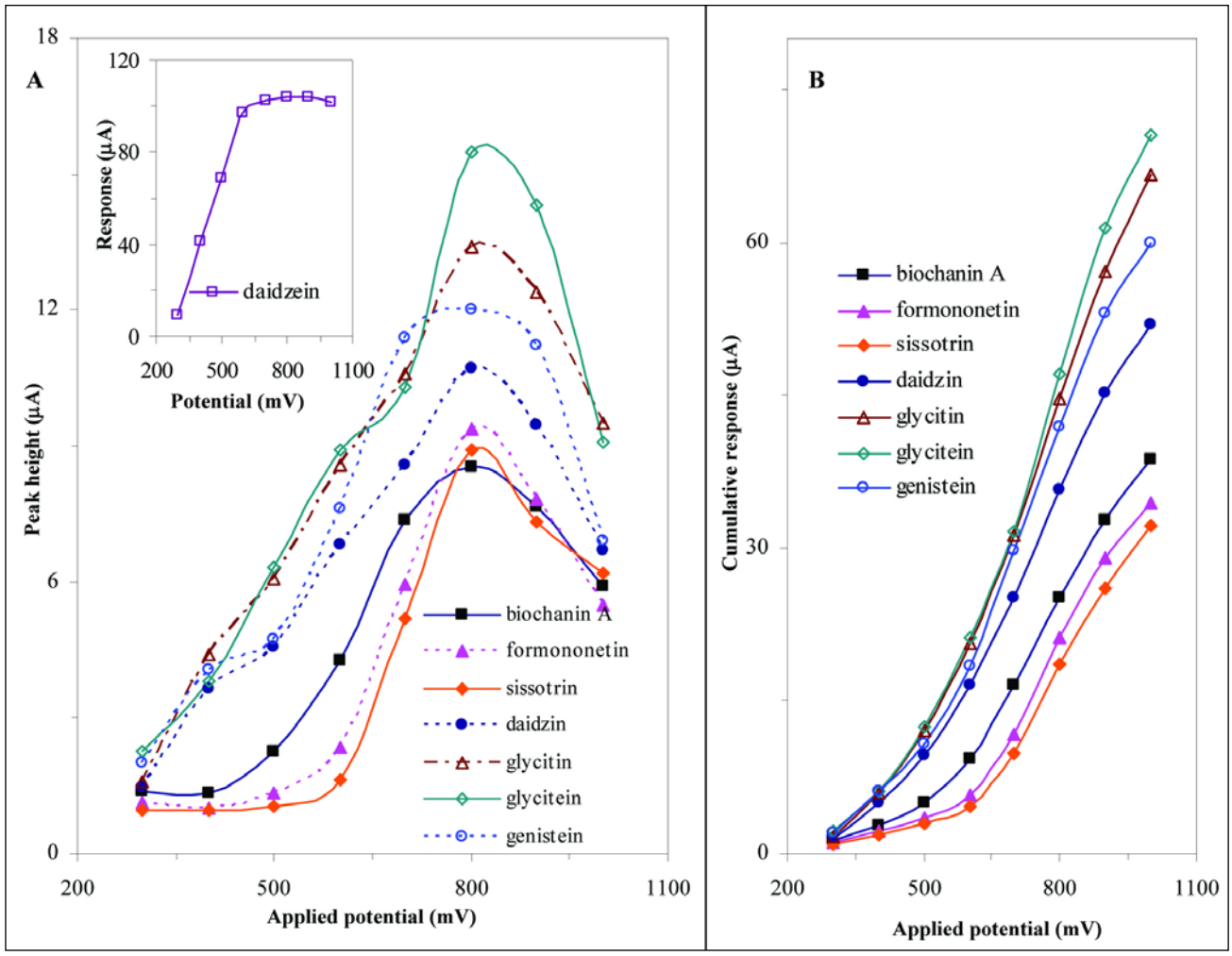

Figure 2. FIA-ED hydrodynamic voltammograms of the isoflavones of interest: peak heights (A) and cumulative responses (B). Inset: hydrodynamic responses for daidzein. Mobile phase: 0.2 mol L^{-1} acetate buffer (pH 5.0); concentration of isoflavones 100 µmol L^{-1}.

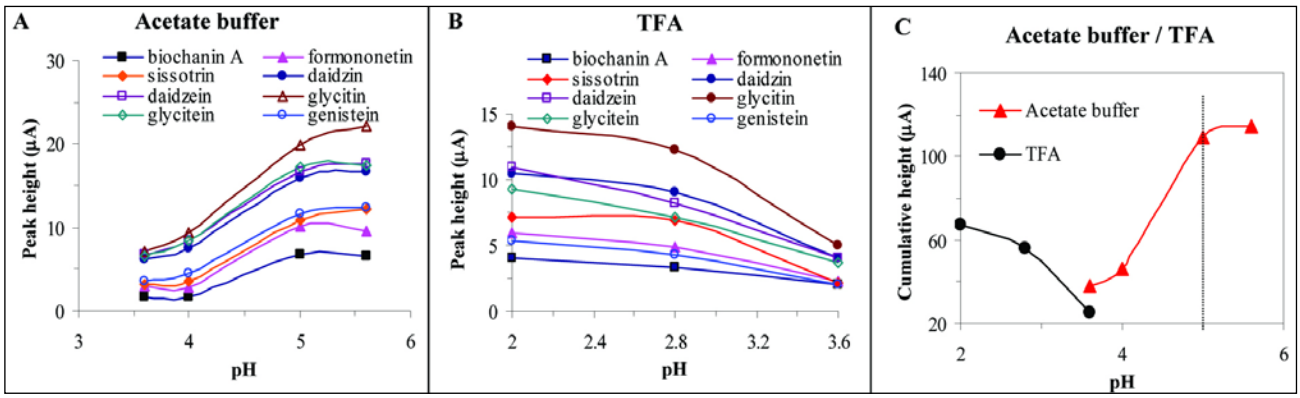

Figure 3. FIA-ED dependence of peak heights on pH of the mobile phase: 0.2 mol L^{-1} acetate buffer (A), 0.05 mol L^{-1} trifluoroacetic acid (B), influences on cumulative peak heights (sum of peak heights of the individual isoflavone at the respective pH) of the studied isoflavones (C). Potentials of all electrodes: 800 mV (peak height and area was determined from peaks at the first detector electrode). Other details see Figure 3.

3.3. Influence of the Flow Rate and Temperature

Flow rate within the range of 1–3 mL min^{-1} was investigated. At higher flow rates, signals of the isoflavones increased (Figure 4A). The optimal flow of mobile phase was 2 mL min^{-1} at 20 °C. The influence of detector temperature is shown in Figure 4B. The current response of isoflavones increased with temperature up to 30 °C, then we observed a slight decrease of the signal of 10–15%. On the other hand, a higher standard deviation (>5%) was observed at 30 °C. Therefore, we chose the detector temperature of 26 °C as the most suitable, where the relative standard deviation was ~3.5% (n = 5).

3.4. Calibration Curves

At the optimized conditions, the relationship between signal intensity and isoflavone concentration was determined. The calibration curves were linear within the concentration range of 50–1000 ng mL^{-1} (R^2 = 0.99), see Table 1. The relative standard deviation varied from 2.4–4.1%.

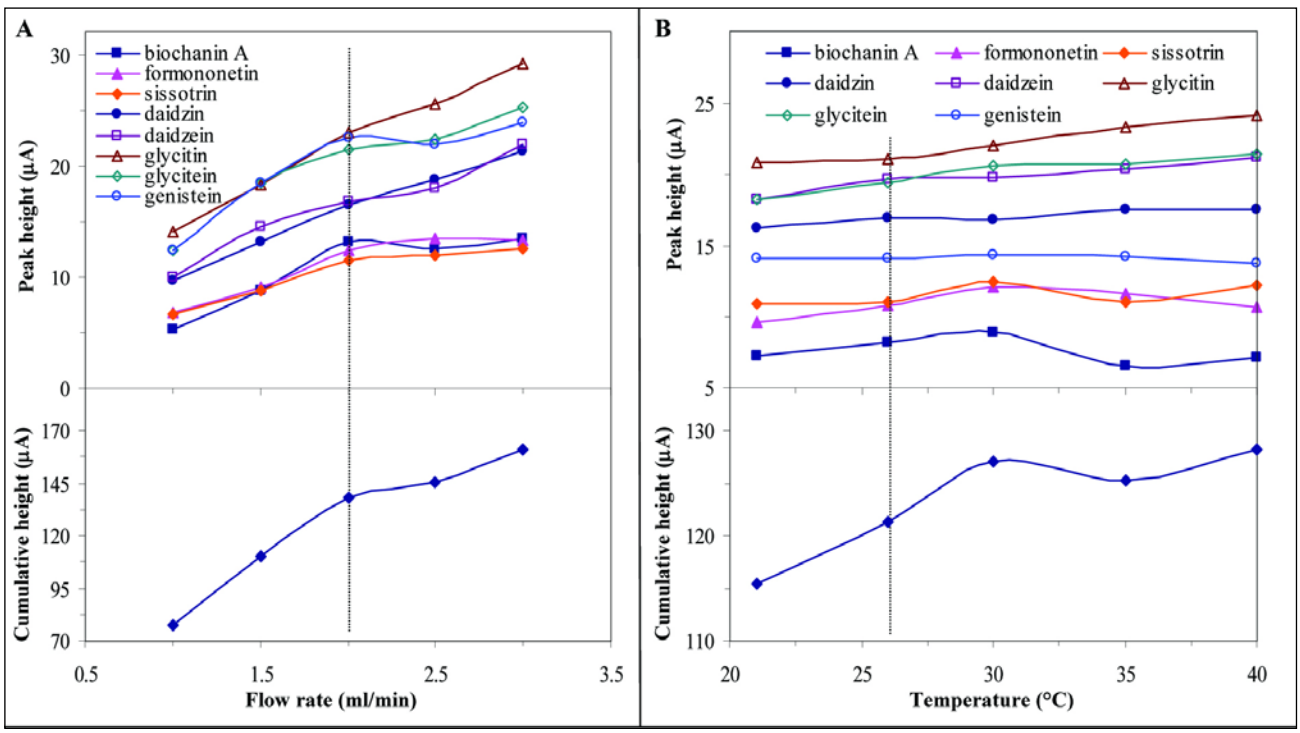

Figure 4. Dependence of peak heights and cumulative peak heights of isoflavones of interest at different flow rates (A) and temperature (B). For other details see Figure 3.

Table 1. Method parameters for determination of isoflavones (n = 5). Applied potential: 800 mV.

Isoflavones	Regression equation	$R^{2,a}$	LOD^b (ng mL^{-1})	LOD^b (nmol L^{-1})	LOD^c (fmol)	LOQ^d (ng mL^{-1})	LOQ^d (nmol L^{-1})	R.S.D.[e] (%)
Biochanin A	y = 13.40x + 1.09	0.9926	0.40	1.4	7.0	1.3	4.7	4.1
Formononetin	y = 4.99x + 1.33	0.9946	0.60	2.2	11	2.0	7.5	3.2
Sissotrin	y = 4.27x + 1.02	0.9909	0.74	2.6	13	2.5	8.7	3.6
Daidzin	y = 15.91x + 0.63	0.9971	0.46	1.1	5.6	1.5	3.7	2.4
Daidzein	y = 11.91x + 1.74	0.9946	0.42	1.7	8.3	1.4	5.5	3.1
Glycitin	y = 16.30x + 1.81	0.9943	0.30	0.6	3.4	1.0	2.2	2.6
Glycitein	y = 19.36x + 0.73	0.9915	0.40	1.4	7.0	1.3	4.7	3.9
Genistein	y = 15.90x + 0.63	0.9917	0.41	1.5	7.7	1.4	5.1	3.2

a … Regression coefficients,
b … Limits of detection (3 S/N),
c … Limits of detection per injection (5 μL),
d … Limits of quantification (10 S/N),
e … Relative standard deviations.

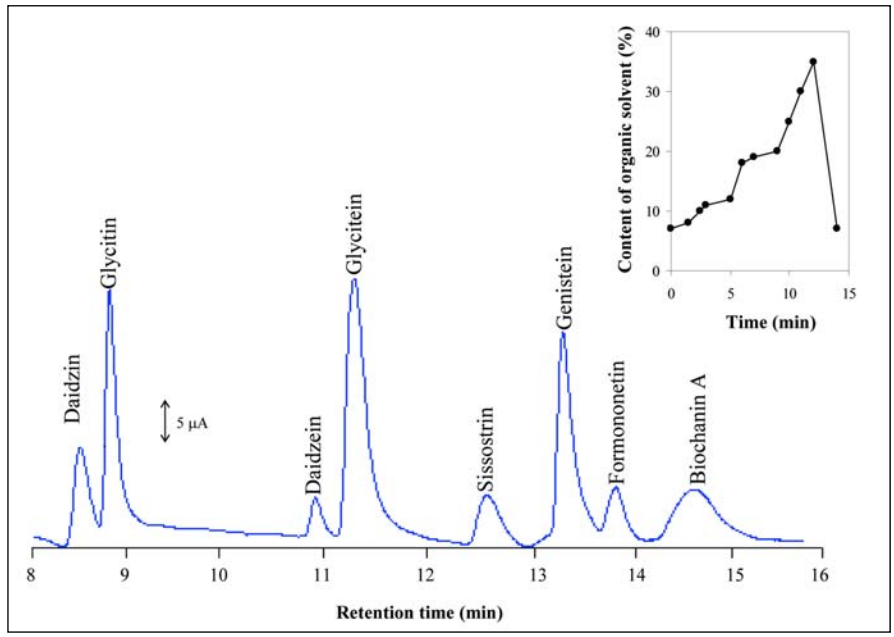

Figure 5. HPLC-ED chromatogram of biochanin A, formononetin, sissotrin, daidzin, daidzein, glycitin, glycitein and genistein, concentration 10 μmol L^{-1}. The applied mobile phase gradient is shown in the inset.

3.5. HPLC-ED

To ensure the separation of isoflavones in 30 min, an addition of organic solvent in the mobile phase was needed, not exceeding 35% (*v*/*v*) due to a decrease in sensitivity. The separation and sensitivity of the method are satisfactory (Figure 5).

4. Conclusion

We present a simple, easy-to-use and low-cost technique enabling us to determine eight various isoflavones (biochanin A, formononetin, sissotrin, daidzin, daidzein, glycitin, glycitein and genistein) within 15 min and with satisfactory detection limits. This technique could be used for routine analysis, as almost no sample preparation is needed and due to the low associated costs.

5. Acknowledgement

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Povzetek

Med biološko pomembnimi funkcijami izoflavonov je tudi njihov učinek na karcinogenezo. Za simultano analizo nekaterih izoflavonov (biohanin A, formononetin, sisotrin, daidzin, daidzein, glicitin, glicitein in genistein) smo uporabili pretočno analizo in tekočinsko kromatografijo v povezavi z elektrokemijsko detekcijo. Kot najprimernejšo mobilno fazo predlagamo 0,2 mol L^{-1} acetatni pufer (pH 5,0) pri pretoku 2,0 mL min^{-1}, temperaturi kolone in detektorja 26 °C in potencialu na detektorju 800 mV. Pri optimalnih pogojih je meja določljivosti nekaj ng mL^{-1}.