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Determination of isoflavones in soybean food and human urine using liquid chromatography with electrochemical detection

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Abstract

A highly sensitive high-performance liquid chromatographic method with electrochemical detection (HPLC-ED) was developed for the determination of isoflavones. Electrochemical behaviour of daidzein and genistein was studied on carbon paste electrode (CPE) by adsorptive transfer stripping square wave voltammetry. The obtained electrochemical results were used for the development of HPLC-ED method. Furthermore, isoflavones were separated on an Atlantis dC18 column using a mobile phase consisting of acetonitrile (solvent A) and 0.15 M acetate buffer of pH 5.5 (solvent B) at a flow rate 0.4 mL/min. A linear gradient profile (solvent B) was at 0–2 min 87%; 22 min 60%; 27 min 50%; 31 min 45%; 47 min 87%. Full scan of multi-channel coulometric detection was tested and optimal potential at 450 mV was chosen for our purposes. Calibration curves were linear (daidzein $R^2 = 0.9993$ and genistein $R^2 = 0.9987$). The detection limit for daidzein/genistein was 480/394 pg/mL (1.8/1.5 nM) and per column 2.4/1.9 pg. Isoflavones extracted from soybean products (farina, meat, milk) by the accelerated solvent extraction (ASE) procedure and isoflavones present in human urine were determined by the HPLC-ED method.

Keyword: Isoflavones

1. Introduction

Interest in the biological activity of plant estrogen-like compounds has started during the last several years [1,2]. Phytoestrogens encompass several classes of compounds, including flavonoids, isoflavonoids, coumestrans (coumestrol), and lignans. Isoflavones represent a group of distinct secondary metabolites produced predominantly in leguminous plants [3]. The synthesis of isoflavones in plants is based on a carbon skeleton of 3-phenylbenzpyrone and on different oxidations of the three central atoms. The isoflavone structure differs in the degree of methylation, hydroxylation and glycosylation [4].

Relationship between plants and rhizobial bacteria influences concentration levels of isoflavones in plant cells [5]. Isoflavones (e.g. daidzein) [6] are precursors of phytoalexins and play important role during the plant disease resistance response, anticancer, antiproliferative and antifungal activity (e.g. genistein) [7,8]. Although phytoestrogens proved to be responsible for infertility in animals, recently they have been found beneficial to human health and even to prevent certain diseases [9–11]. The impact of dietary isoflavones, daidzein and genistein, upon the health of adults and infants [12–15] is well known.

A wide range of analytical techniques [16,17] has been applied for determination of polyphenols and phytoestrogens in foods and biological materials. We assumed that electrochemical methods that have been found highly useful for the determination of DNA [18,19], proteins [20,21], antibiotics [22] and metals in biological matrices [23] could be used for our purposes. They are preferable due to their very good reproducibility and low costs. Their serious drawback is lower selectivity, especially for samples with complicated matrices. The disadvantages could be overcome by

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combining separation and electrochemical steps in hyphenated techniques. A HPLC separation coupled with a multi-channel electrochemical detector offers potential to evaluate complex biological samples.

Nurmi and Adlercreutz [24] used a highly sensitive high-performance liquid chromatographic method with coulometric electrode array detection for profiling phytoestrogens. In our study the same technique was applied for highly sensitive and reproducible determination of some major isoflavones (daidzein and genistein) in soybean food and human urine.

2. Experimental

2.1. Chemicals and reagents

Isoflavones were purchased from Karlsroth GmbH (Karlsruhe, Germany). HPLC-grade acetonitrile and methanol from Merck (Darmstadt, Germany) were used. All other analytical reagents of ACS purity were purchased from Sigma Aldrich Chemical Corp. (St. Louis, USA). The stock standard solutions at 10 μg/mL were prepared in ACS methanol and ACS water 1:1 (v/v) (Sigma Aldrich, USA) and stored in the 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 operations.

2.2. Electrochemical measurement

Adsorptive transfer stripping square wave voltammetric (AdTS SWV) measurements were performed using an AUTOLAB analyser (EcoChemie, The Netherlands) connected to a VA-Stand 663 (Metrohm, Zurich, Switzerland). The three-electrode system consisted of a carbon-paste working electrode, an Ag/AgCl reference electrode (3 M KCl) and a platinum wire counter electrode. A borate buffer (0.05 M $Na_2B_4O_7$ + 0.1 M H_3BO_3, pH 9.2) was used as a supporting electrolyte. The AdTS SWV was performed using the following parameters: initial potential 0.1 V, end potential 1.0 V, amplitude 25 mV, step potential 5 mV and frequency 200 Hz. All experiments were carried out at 25 °C. The raw data were treated using the Savitzky and Golay filter (level 2) [25] and the moving average baseline correction (peak width 0.05 mV) of the GPES software [26].

2.3. Preparation of carbon paste electrode (CPE)

The carbon paste was made of 70% graphite powder (Aldrich, USA) and 30% mineral oil (Sigma-Aldrich, USA; free of DNase, RNase and protease). The carbon paste was housed in a Teflon body having a 2.5 mm diameter of active disk surface. The electrode surface was polished with a soft filter paper prior to measurement. The sample volume of 6 μL was used.

2.4. Chromatographic apparatus

2.4.1. HPLC-ED

An HPLC-ED system consisted of two Model 582 ESA solvent delivery pumps (operating range of 0.001–9.999 mL/min) and an eight-channel CoulArray electrochemical detector (Model 5600A). The detector included a low volume flow-through analytical cell (Model 6210) containing a reference electrode, a counter electrode and eight porous graphite working electrodes. An organizer module housed the column, the cells and related components and a thermal chamber for a column and the detector (all parts from ESA Inc., Chelmsford, MA, USA). A sample (5 μL) was injected manually. An Atlantis dC18 column (Waters, USA, 150.0 mm × 2.1 mm, 3.5 μm particle size) was used for separation.

2.4.2. HPLC-UV method

An HP 1100 LC system (Hewlett Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an autosampler (G1313A), a column thermostat (G1316A), and a UV-Vis diode array detector (model G1315A). Spectra were registered in the range of 190–400 nm (SBW 100 nm).

2.4.3. HPLC-MS method

An HP 1100 chromatographic system (Hewlett-Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an autosampler (G1313A), a column thermostat (G1316A) and a diode array detector (model G1315A). The system was coupled on-line to a mass selective HP MSD detector (G1946A, Hewlett-Packard, Palo Alto, USA). The ChemStation software (Rev. A07.01) controlled the whole liquid chromatographic system.

The column effluent was monitored with diode array detector and directly introduced into the quadruple mass spectrometer operated in positive ESI mode. The mass spectrometer was regularly calibrated with an ESI tuning solution reserpine (m/z = 609.30) from Hewlett-Packard (Palo Alto, CA, USA). The autotune function accomplished calibration of MS detector for m/z = 118.10, 622.04, 921.95, 1521.95 and 2121.95 of the standard mixture Hewlett-Packard (Palo Alto, CA, USA). The nebulizer gas pressure was 50 psi, the drying gas was nitrogen at 12 L/min and the temperature of 300 °C and capillary voltage was 3500 V. The fragmentor voltage was set to 80 eV and the gain was 1. The *m/z* spectra and data for the selected ion-monitoring (SIM) mode were recorded at *m/z* for daidzein 255 → 137, at *m/z* for genistein 271 → 153, at *m/z* for formononetin 269 → 137, at *m/z* for biochanin A 285 → 153, at *m/z* for daidzin 417 → 255, at *m/z* for genistin 433 → 271.

2.5. Chromatographic conditions for sample analysis

Isoflavones were separated on an Atlantis dC18 column using a mobile phase consisting of acetonitrile (solvent A) and 0.15 M acetate buffer of pH 5.5 (solvent B). The linear gradient (solvent B) was as follows 0–2 min 87%; 22 min 60%; 27 min 50%; 31 min 45%; 47 min 87%. Two cleaning cycles were applied between analyses (cleaning potential 900 mV). The flow rate was 0.4 mL/min and the column and the detector temperature was set at 25 °C.

2.6. Extraction and quantification of daidzein and genistein in food

Accelerated solvent extraction (ASE) procedure [27–33] was performed on a PSE (Applied Separations, USA). The sample (0.5 g, ±5 mg) of food products (soybean farina, meat, milk; Ekoprodukt, s.r.o., Czech Republic) was homogenised by an Ika A11 basic grinder (IKA Werke GmbH and Co., Staufen, KG, Germany) and then was wrapped with a filter paper (small envelope like format) and sprinkled with 3.0 g SPE-ed™ matrix, 1.5 g florisil (15.5% MgO, 84% SiO_2, 0.5% Na_2SO_4; pH 8.5; 60–100 μm particle size) and 3.0 g Ottawa sand (Allentown, PA, USA). Flavone was used as an internal standard and was pipetted on the top of the sample. The mixture was placed into a 10 mL stainless steel extraction cell and extracted under controlled conditions in two steps. Step one: pre-heating period (5 min), the solvent hexane (elution of lipophilic compounds); temperature 100 °C, pressure 130 bar; two extraction cycles (5 min), 90 s using pressurized nitrogen. Step two: pre-heating period (5 min), the solvent 60% methanol and 0.3% formic acid; temperature 100 °C, pressure 130 bar; two extraction cycles (5 min), 90 s using pressurized nitrogen. The final extracts were collected in 60 mL glass vials with Teflon coated rubber caps and centrifuged at 3000 rpm (Hettich, Germany). The supernatants were evaporated to dryness in a rotary vacuum evaporator (IKA RV 05-ST) with an HB 4 water bath (both, IKA-Werke GmbH and Co., Staufen, KG, Germany). The residue was reconstituted in 1 mL methanol and filtered through a 0.45 μm Teflon membrane filters (MetaChem, Torrance, CA, USA) prior to injection into the HPLC system.

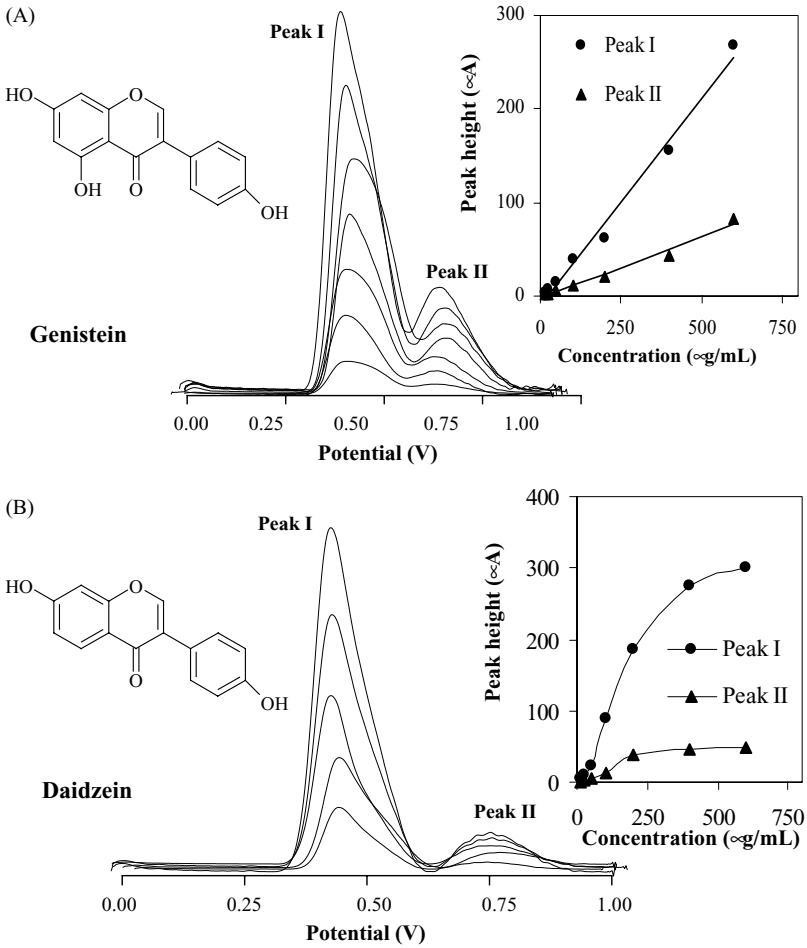

Fig. 1. Chemical formula and AdTS square wave voltammograms of different concentrations on carbon paste electrode of (A) genistein and (B) daidzein. Inset shows concentration dependences in the range 0–600 μg/mL. Borate buffer, pH 9.2, initial potential 0.1 V, end potential 1.0 V; amplitude 25 mV, step potential 5 mV, accumulation time 120 s, and frequency 200 Hz were used.

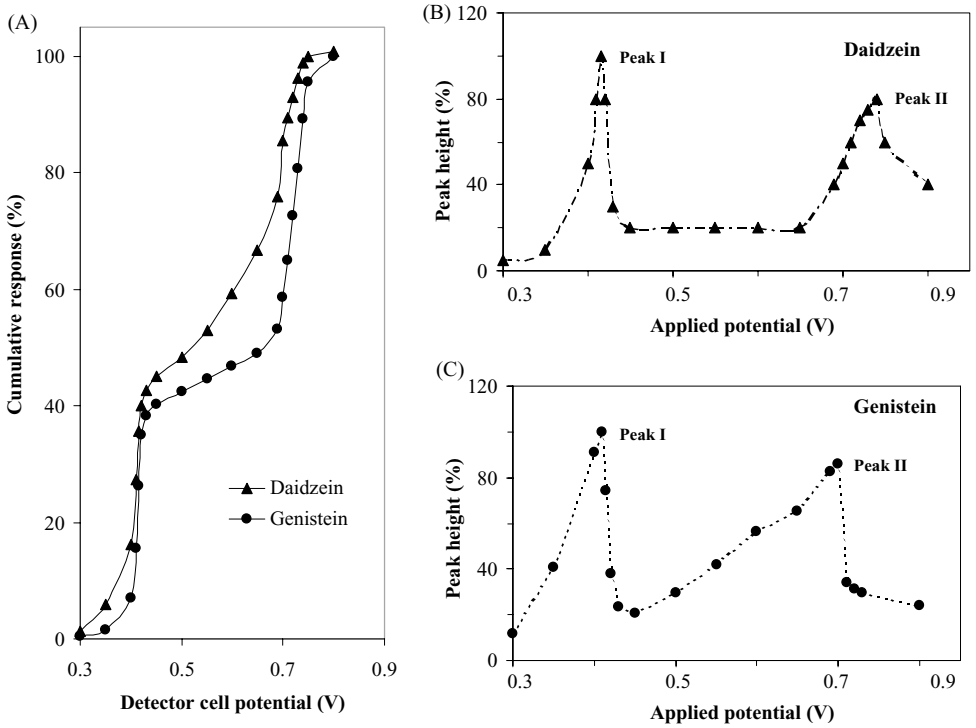

Fig. 2. Genistein and daidzein (A) HPLC-ED hydrodynamic voltammograms (B and C) dependence of peak heights on applied potentials at 10 μg/mL. Atlantis dC18 column (Waters, USA, 150.0 mm × 2.1 mm), isocratic elution, mobile phase 13% (v/v) acetonitrile and 87% (v/v) acetate buffer (pH 5.5), flow rate 0.4 mL/min, column and detector temperature 35 °C, injected volume 5 μL. Peak height of daidzein and genistein at potential 450 mV was equal to 100% (B and C).

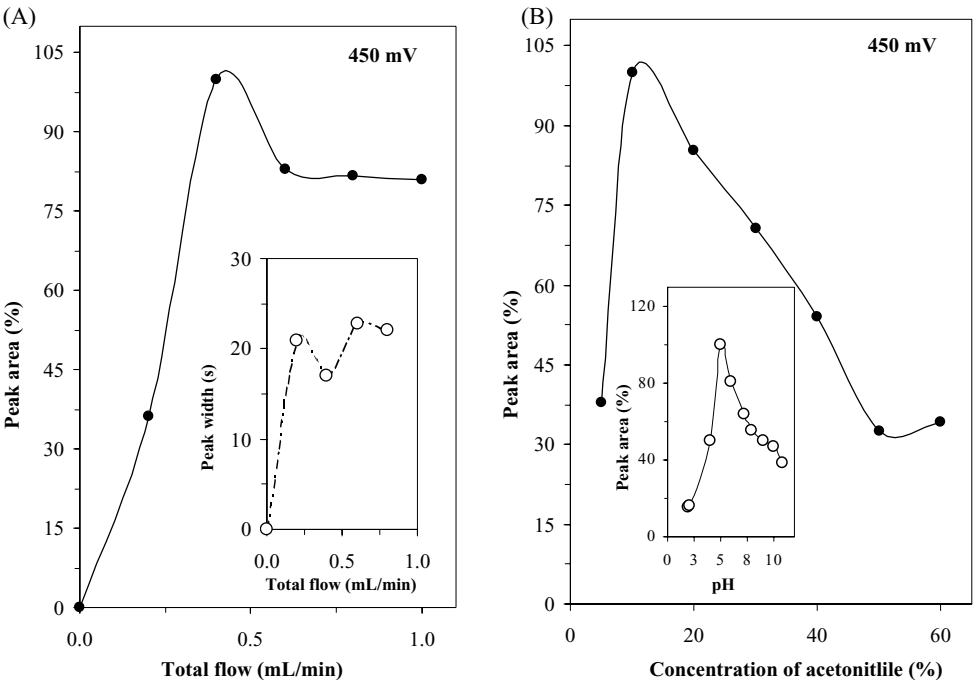

Fig. 3. HPLC-ED dependence of peak area (peak width in inset) on (A) total flow rate and (B) concentration of acetonitrile or pH (in inset). Selected potential 450 mV, 10 μg/mL daidzein, other condition see Fig. 2.

2.7. Standard extraction procedure for quantification of daidzein and genistein in food

A magnetic stirrer (Ikamag RCT basic) was used for the stirred extraction at 30 °C for 60 min. A computer controlled commercially available device related to Soxhlet apparatus "fex Ika Werke 50" was used for the extraction [30,34] using a temperature program (first step: temperature of cooling/heating block 130 °C for 30 min, cooling/heating block to 30 °C for 5 min; second step: temperature of 120 °C for 30 min, cooling to 30 °C for 5 min). An extraction solvent was 90% aqueous methanol. Flavone was used as an internal standard and was pipetted on the top of the sample.

2.8. Preparation of human urine

Human urine (obtained from healthy laboratory staff) was filtered through a Teflon disc filter (0.45 μm and 13 mm diameter, Alltech Associates, Deerfield, IL, USA) and 1, 10, 100 times diluted with 0.15 M acetate buffer (pH 5.5) before measurements.

2.9. Accuracy, precision and recovery

Accuracy, precision and recovery of the determinations of individual isoflavones (daidzin, genistin, daidzein, genistein, formononetin and biochanin A) were evaluated with samples spiked with 100 μL isoflavone standards (concentrations varying from 10 to 50 μg/mL) and 100 μL flavone. Coefficients of variation (CVs) of intra-day assay were performed in six homogenates. Inter-day precision was determined by analysing six homogenates over a 5-day period. Homogenates were assayed blindly and isoflavone concentrations were calculated from the calibration curves. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of the individual isoflavones.

2.10. 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. Electrochemical behaviour

Electrochemical behaviour of both isoflavones, daidzein and genistein, was studied by AdTS square wave voltammetry (SWV) on the carbon paste electrode (CPE) in borate buffer of pH 9.2 under the same conditions. Two typical electrochemical signals were present in SW voltammograms of both compounds (see Fig. 1). Peaks I probably correspond to an oxidation signal at potentials $E_p = 0.41$ and 0.44 V for daidzein and genistein (Fig. 1A and B), respectively. They correspond to a two-electron quasi-reversible electrode reaction [35,36]. An additional irreversible oxidation, peaks II at potentials $E_p = 0.74$ and 0.66 V for daidzein and genistein,

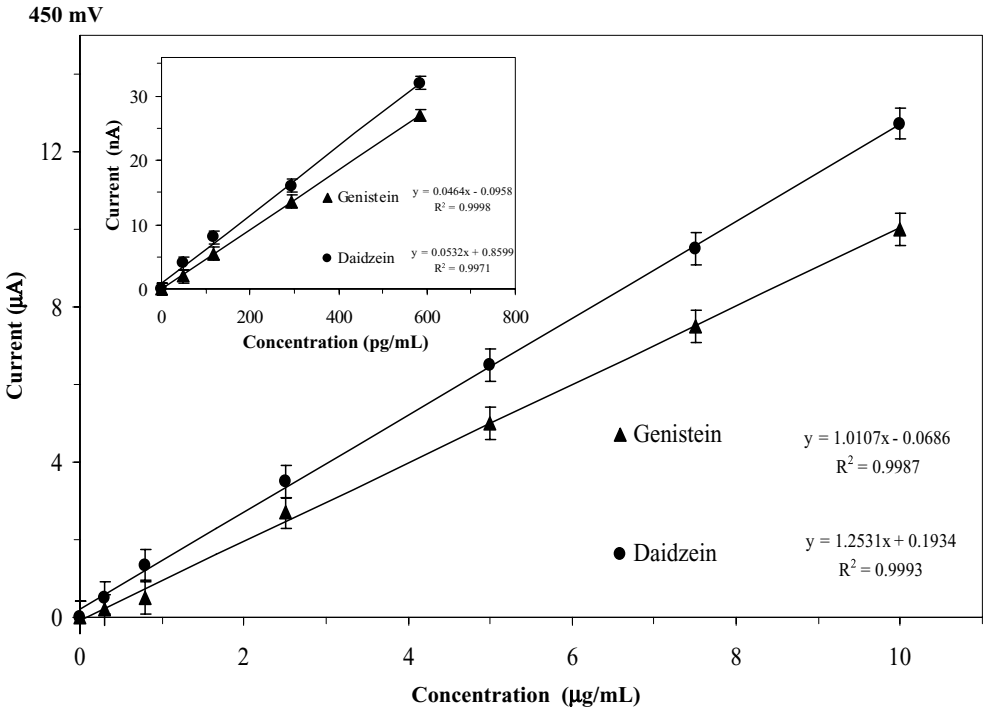

Fig. 4. HPLC-ED dependence of peak height on concentration of daidzein and genistein in the range 0–10 μg/mL and 0–600 pg/mL (inset). Other condition see Fig. 3.

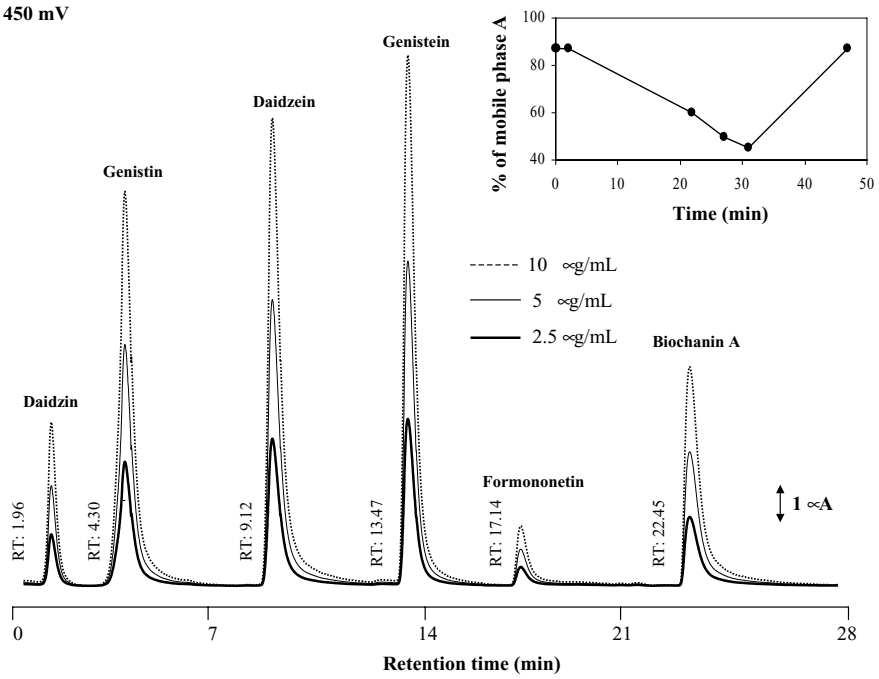

Fig. 5. HPLC-ED chromatograms of the isoflavone standard mixture (A) of daidzin, genistin, daidzein, genistein, formononetin and biochanin A (at three different concentration 2.5, 5.0 and 10 μg/mL each). Inset: changes in the composition of the mobile phase during chromatography of isoflavones. Other condition see Fig. 3.

respectively, is probably connected with a strong adsorption of the oxidation products of isoflavones at the CPE [35,36]. Our hypothesis was confirmed by cyclic voltammetry measurements (not shown).

Relationships between the peak heights and concentrations of daidzein and genistein are shown in Fig. 1. The electrochemical signals (peaks I and II) of genistein increased nearly linearly. A shape of the curve similar to adsorption isotherm was obtained for daidzein probably due to the saturation of the surface of electrode by the analyte [36].

4. Optimisation of determination of daidzein and genistein by HPLC-ED

4.1. Effect of the potentials applied to the working electrodes

The abovementioned parameters were studied under dynamic (flow injection–FIA) conditions to find the highest sensitivity of electrochemical detection of the isoflavones (see Fig. 1). Isocratic elution using mobile phase consisting of 13% ACN and 87% acetate buffer (for other conditions of chromatographic separation see Section 2) was used in the experiments to simplify the optimisation.

At least two distinct peaks at 400 mV (peak I) and 700 mV (peak II) could be expected. Thus, potentials from 90 to 720 mV were applied to the porous graphite electrodes; 20 mV increments were used at potentials near the expected peak maxima (see in Fig. 1A and B).

Compiling data from the series of injections at these potentials, we constructed the hydrodynamic voltammograms shown in Fig. 2A for daidzein and genistein. Dependence of peak height on applied potential (Fig. 2B and C) exhibited two distinct maximum at 450 and at 700 mV for daidzein and genistein (10 μg/mL each) at pH 5.5. The higher peak heights were obtained at applied potential of 450 mV (Fig. 2). The value of the peak height II increased gradually and reached about 60–80% of the electrochemical signal for peak I. Then it rapidly decreased (see Fig. 2B and C). The results are in good agreement with the results

Table 1
Limit detection of isoflavones

Isoflavones	LOD (3 S/N) on column		LOQ (10 S/N) on column	
	pg	fmol	pg	fmol
Daidzin	6.4	15.4	21.1	50.6
Genistin	2.9	6.7	9.6	22.2
Daidzein	2.4	9.5	7.9	31.1
Genistein	1.9	7.3	6.3	23.3
Formononetin	20.8	77.7	68.6	255.9
Biochanin A	5.1	17.9	16.8	59.2

Table 2
Concentration of isoflavones in several food samples

Food	Content (μg/g)/mean ± S.D. ($n = 5$)	
	Daidzein	Genistein
Dried soybeads	158.2 ± 18.5	295.6 ± 25.5
Soy farina	189.3 ± 15.6	345.2 ± 22.2
Soy meat	45.5 ± 7.8	78.5 ± 15.7
Soy milk	15.5 ± 6.9	56.8 ± 9.5

Table 3
Recovery of isoflavone from soy farina homogenate in triplicate

Extraction procedure	Isoflavone content	Homogenate (μg/mL)[a,b,c]	Spiking isoflavone (μg/mL)[a,b,c]	Homogenate + spiked isoflavone (μg/mL)[a,b,c]	Recovery (%)
Soxhlet	Daidzein	3.9 ± 0.2	10.1 ± 0.5	14.5 ± 0.6	104
	Genistein	2.5 ± 0.3	10.2 ± 0.4	11.8 ± 0.6	93
	Daidzin	29.7 ± 0.2	10.7 ± 0.6	37.8 ± 2.9	94
	Genistin	22.6 ± 1.6	10.3 ± 0.9	31.3 ± 2.7	95
	Formononetin	ND	9.9 ± 0.2	9.2 ± 0.3	93
	Biochanin A	ND	9.5 ± 0.7	9.3 ± 0.8	98
ASE	Daidzein	3.4 ± 0.3	8.9 ± 0.5	12.9 ± 0.9	103
	Genistein	2.6 ± 0.2	9.3 ± 0.4	11.1 ± 0.6	94
	Daidzin	49.5 ± 3.6	9.1 ± 0.6	60.2 ± 5.7	103
	Genistin	30.4 ± 1.6	9.5 ± 0.8	39.5 ± 2.8	99
	Formononetin	ND	9.4 ± 0.6	9.0 ± 0.7	96
	Biochanin A	ND	9.7 ± 0.5	10.1 ± 0.7	104

ND: not detected.
[a] Isoflavone amounts per 1 mL of extract.
[b] Results expressed as a mean ± S.D.
[c] Internal standard (flavon) was added.

Table 4
Precision and recovery of isoflavones for soy farina sample analysis

Isoflavone	Isoflavone content[b,c]	Homogenate (μg/mL)[a]	Spiking isoflavone (μg/mL)[a]	Homogenate + spiked isoflavone (μg/mL)[a]	Recovery (%)
Daidzein	Intra-day ($n = 6$)	3.4 ± 0.2	8.9 ± 0.5	13.1 ± 0.6	107
		6.9 ± 0.5	51.2 ± 3.1	59.3 ± 3.6	102
	Inter-day ($n = 30$)	3.3 ± 0.5	9.5 ± 0.8	13.2 ± 1.0	103
		7.0 ± 0.7	54.2 ± 5.4	61.9 ± 4.9	101
Genistein	Intra-day ($n = 6$)	2.6 ± 0.2	9.3 ± 0.4	11.3 ± 0.5	95
		5.4 ± 0.3	50.8 ± 3.0	55.4 ± 3.5	99
	Inter-day ($n = 30$)	2.5 ± 0.6	9.8 ± 0.7	11.7 ± 0.9	95
		5.8 ± 0.8	53.8 ± 4.9	57.3 ± 6.1	96
Daidzin	Intra-day ($n = 6$)	49.6 ± 3.5	9.1 ± 0.6	56.5 ± 4.5	96
		93.5 ± 5.6	51.5 ± 3.9	149 ± 9	103
	Inter-day ($n = 30$)	49.1 ± 4.3	9.8 ± 0.9	59.9 ± 3.9	102
		97.5 ± 8.1	54.9 ± 5.9	149 ± 11	98
Genistin	Intra-day ($n = 6$)	30.5 ± 1.5	9.5 ± 0.8	38.6 ± 3.7	97
		61.4 ± 3.6	50.2 ± 2.9	111 ± 10	99
	Inter-day ($n = 30$)	33.3 ± 3.1	10.1 ± 1.1	45.4 ± 4.1	105
		65.9 ± 5.6	53.3 ± 4.8	115 ± 8	96
Formononetin	Intra-day ($n = 6$)	ND	9.4 ± 0.6	9.8 ± 0.7	104
		ND	52.0 ± 3.8	52.5 ± 4.1	101
	Inter-day ($n = 30$)	ND	10.0 ± 0.7	9.7 ± 0.9	97
		ND	55.2 ± 5.7	57.2 ± 6.7	104
Biochanin A	Intra-day ($n = 6$)	ND	9.7 ± 0.5	10.1 ± 0.7	104
		ND	50.0 ± 2.5	48.5 ± 3.5	97
	Inter-day ($n = 30$)	ND	10.2 ± 0.6	10.0 ± 0.9	98
		ND	53.5 ± 5.4	51.6 ± 5.9	96

ND: not detected.
[a] Isoflavone amounts per 1 mL of extract.
[b] Results expressed as a mean ± S.D.
[c] Internal standard (flavon) was added.

obtained in the study of determination of phenolic compounds in flax seeds and nettle roots [37,38]. The applied potential of 450 mV was selected as the optimum for development of further analytical method for determination of daidzein and genistein (Fig. 2).

4.2. Effect of the flow rate, concentration of acetonitrile and pH on HPLC-ED signal

Total flow rate of the mobile phase and its composition were selected since they are the very important factors influencing the electrochemical response of the detector. The highest response was obtained at the flow rate of about 0.4 mL/min (see Fig. 3A). The analytical signal decreased up to 90–70% at the higher flow rates due to shorter time of preconcentration (i.e. shorter accumulation time). The rapid decrease of analytical signal at the lower flow rates could be explained by partial or total saturation of active surface of the electrode at any concentration of analyte. Surprisingly the narrower peaks (about 5%) were observed at the highest analytical signal (see inset 3A).

An increasing content of acetonitrile in the mobile phase affected negatively the electrochemical response. The highest response was obtained at about 10–20% (v/v) ACN (Fig. 3B). The final pH of the buffer, added into the mobile phase, seriously influenced the resulting analytical signal of the electrochemical detector (see inset in Fig. 3B). On the basis of previous results for ruthin and qurcetin [35] and other isoflavones [24], the highest electrochemical response was expected in slightly acidic medium at pH 5. The highest analytical signal was obtained in the pH range 4.5–5.5 at applied potential of 450 mV. Acetate buffer 0.15 M at pH 5.5 was selected for further experiments (Fig. 3B inset).

4.3. Calibration curves

A highly sensitive HPLC-ED method was evaluated for the determination of daidzein and genistein based on the abovementioned selected experimental conditions. Calibration curves (see Fig. 4) were strictly linear (daidzein $y = 1.2531x + 0.1934$, $R^2 = 0.9993$; genistein $y = 1.0107x - 0.0686$, $R^2 = 0.9987$). The detection limit (LOD for 3 S/N criterion) LOD = 480 pg/mL (1.8 nM, 254 MW) and the on column detection limit 2.4 pg/9.5 fmol were obtained. The corresponding values (LOD = 390 pg/mL, and an on column detection limit 1.9 pg/7.3 fmol) were better for genistein (230 MW) due to less beneficial electrochemical properties (see Fig. 1).

4.4. Determination of standard isoflavones in acetate buffer

For electrochemical determination of isoflavones in foods and biological samples it was necessary to evaluate and

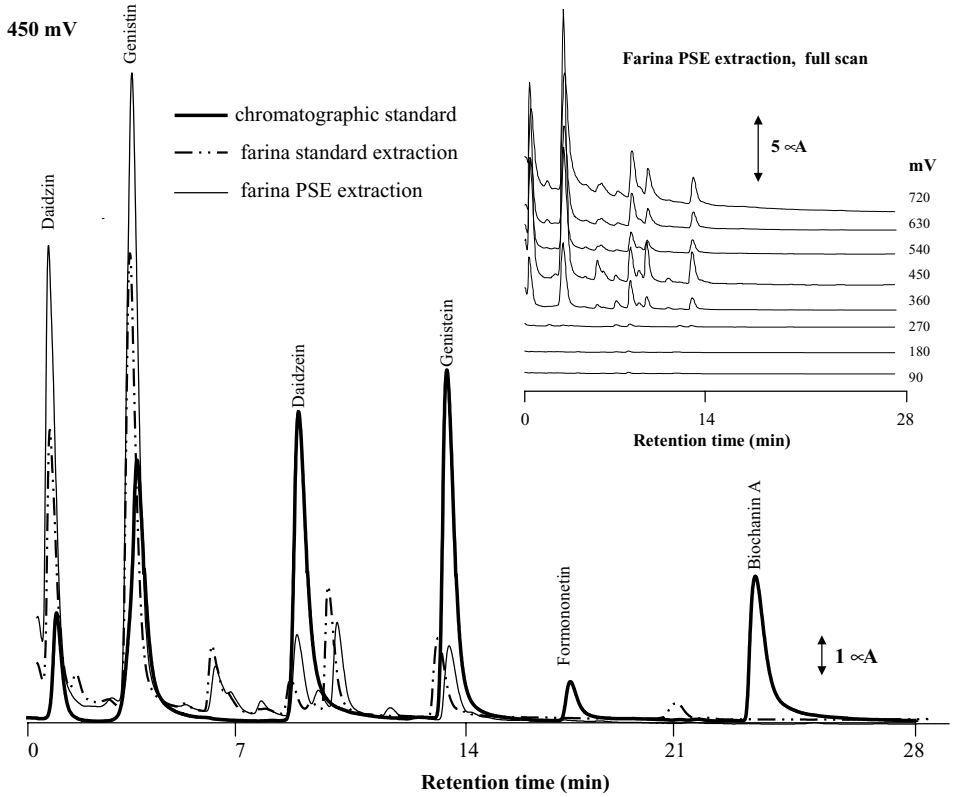

Fig. 6. HPLC-ED chromatograms of the accelerated solvent extract of soya farina. Control chromatographic standards: daidzin, genistin, daidzein, genistein, formononetin and biochanin A at concentration 10 μg/mL without sample; farina (0.5 g) standard extraction; farina (0.5 g) ASE extraction. Inset: response of individual electrodes at different applied potentials. Other conditions see Fig. 5 and Section 2.

partly modify the chromatographic conditions. Gradient elution profile was optimised (the linear gradient was as follows 0–2 min 87%; 22 min 60%; 27 min 50%; 31 min 45%; 47 min 87%) for an efficient separation (see inset of Fig. 5). A typical HPLC-ED chromatogram (at 450 mV) on the Atlantis dC18 column for separation of daidzin, RT 1.96 min; genistin, RT 4.30 min; daidzein, RT 9.12 min; genistein, RT 13.47 min; formononetin, RT 17.14 min and biochanin A, RT 22.45 min (each at three different concentration) is given in Fig. 5. The limits of detection and quantification of studied isoflavones are presented in Table 1. The retention times of individual compounds were comparable with those obtained by HPLC-UV [34,39,40]. The differences were lower than 5%.

4.5. Accelerated solvent extraction and Soxhlet extraction used for preparation of food samples

Classical solvent extraction methods have been described for isolation of daidzein and genistein [30,41] but a quantitative extraction of any isoflavone is still problematical. ASE is a new extraction technique that uses organic solvents at high pressures and temperatures [27–29,31–33,42,43]. We applied the techniques for the extraction of daidzein and genistein from foods (soybean farina and meat). The two steps extraction protocol was applied [44].

4.6. Determination of isoflavones in soybean foods

A lot of chromatographic procedures for more or less efficient separation of isoflavones have been described in the literature [17,28,45–49], but only part of them applied an HPLC-ED hyphenated technique [24,37,38,50–54]. Recently, we have published highly efficient and sensitive methods for separation of particular isoflavones in several plant species [34,39,40]. For electrochemical determination of isoflavones in foods and biological samples it was necessary to evaluate and partly modify the chromatographic conditions. Samples of soybean farina and soybean meat were prepared using the ASE procedure and analysed by the HPLC-ED method (Fig. 6). HPLC-ED chromatograms of food samples on the Atlantis dC18 column using eight electrodes with different applied potential are shown in inset of Fig. 6. The typical HPLC-ED chromatograms allowed identification of daidzin, genistin, daidzein and genistein in soybean foods. The concentrations of daidzein and genistein in the studied soybean foods are presented in Table 2.

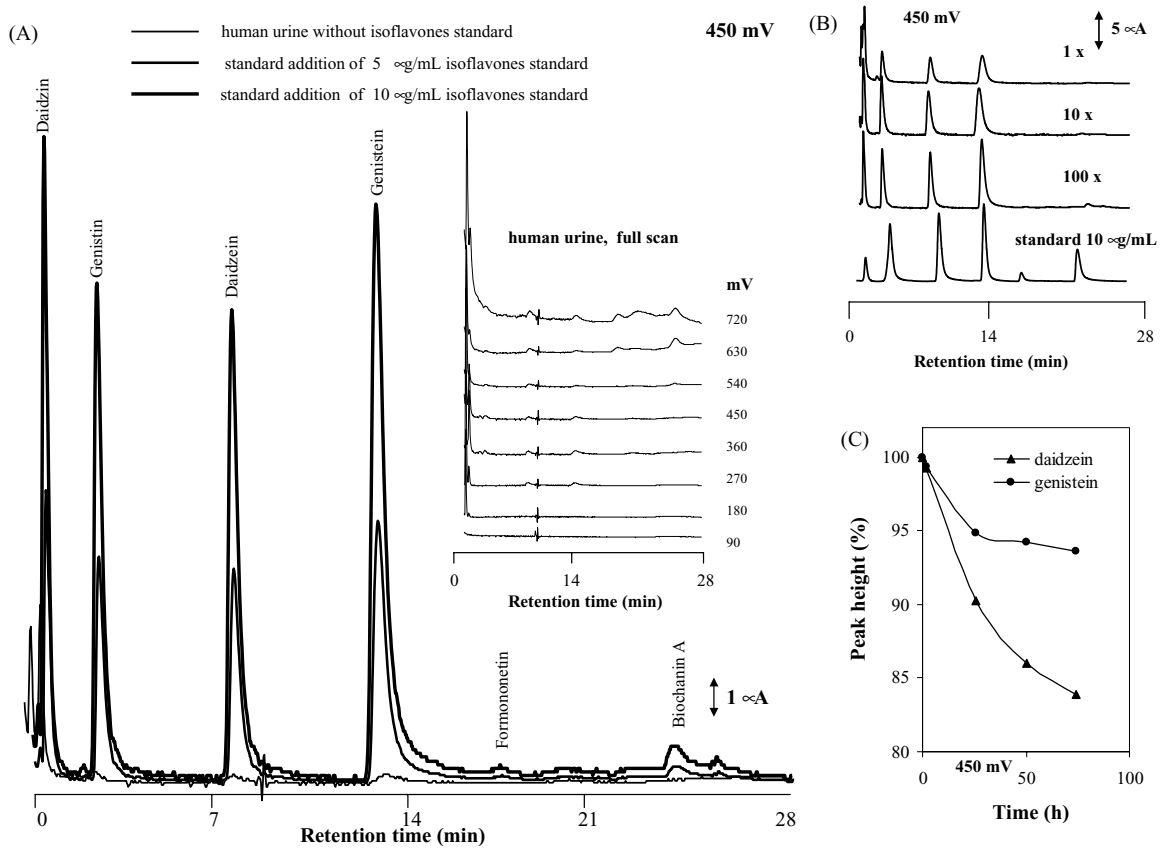

Fig. 7. HPLC-ED chromatograms of human urine. (A) Chromatographic standards added in human urine: daidzin, genistin, daidzein, genistein, formononetin and biochanin A at concentration 5 and 10 μg/mL. Inset: response of eight electrodes at different applied potential (human urine without isoflavones). (B) Effect of urine dilution on electrochemical response. (C) Stability of daidzein and genistein during 0–72 h period stored at 4 °C. Other conditions see Fig. 5 and Section 2.

Table 5
Recovery of isoflavone from human urine homogenate in triplicate

Isoflavone	Urine peak height (μA)[a,b]				Spiking isoflavone peak height (nA)[a,b,c]	Homogenate + spiked isoflavone peak height (μA)[a,b]			Recovery (%)			
	WD	D_1	D_{10}	D_{100}		WD	D_1	D_{10}	D_{100}	WD	D_1	D_{10}	D_{100}
Daidzein	ND	ND	ND	ND	10.1 ± 0.5	6.7 ± 0.6	7.5 ± 0.6	8.6 ± 0.5	9.6 ± 0.4	66	74	85	95
Genistein	ND	ND	ND	ND	14.3 ± 0.7	9.7 ± 0.1	10.7 ± 0.8	12..6 ± 0.8	13.9 ± 0.9	68	75	88	97
Daidzin	1.7 ± 0.2	0.81 ± 0.08	0.16 ± 0.02	ND	4.1 ± 0.3	4.4 ± 0.4	3.9 ± 0.5	3.8 ± 0.6	3.9 ± 0.5	76	79	89	95
Genistin	1.4 ± 0.2	0.71 ± 0.05	0.12 ± 0.01	ND	8.5 ± 0.5	7.0 ± 0.4	7.3 ± 0.3	7.6 ± 0.3	8.7 ± 0.6	71	79	88	102
Formononetin	ND	ND	ND	ND	1.5 ± 0.2	1.0 ± 0.3	1.2 ± 0.4	1.3 ± 0.2	1.5 ± 0.3	69	77	86	100
Biochanin A	ND	ND	ND	ND	5.5 ± 0.5	3.7 ± 0.4	4.3 ± 0.3	4.8 ± 0.8	5.4 ± 0.6	68	79	88	98

ND: not detected; WD: human urine without dilution; D_1: human urine diluted by 0.15 M acetate buffer of pH 5.5 in ratio 1:1 (v/v); D_{10}: human urine diluted by 0.15 M acetate buffer of pH 5.5 in ratio 1:10 (v/v); D_{100}: human urine diluted by 0.15 M acetate buffer of pH 5.5 in ratio 1:100 (v/v).

[a] Results expressed as a mean ± S.D..
[b] Internal standard (flavon) was added.
[c] Concentration of the spiking isoflavones were 10 μg/mL.

4.7. Recovery and precision of the isoflavone extraction and its determination in soybean foods

Recovery was checked by the addition of the known amounts of the isoflavones to homogenates (Tables 3 and 4). The recoveries 93–104% for Soxhlet extraction and 94–104% for ASE extraction were obtained for the different concentrations of the individual isoflavones (see Table 3). The reproducibility of the procedure was determined using six repetitive analyses of representative samples over 5 days (Table 4). Good precision was obtained for isoflavones in food samples (see Table 4).

4.8. Determination of isoflavones in human urine

It is well known that higher animals excrete isoflavones mainly by urine. Thus the method was also tested for direct and highly sensitive determination of isoflavones in human urine (Fig. 7). A full HPLC-ED scan (90–720 mV) of raw human urine is given in an inset in Fig. 7A. No significant interfering signals were observed near the retention times corresponding to individual isoflavones. The HPLC-ED chromatograms of the same urine samples spiked with 5 and 10 μg/mL of standards exhibited strong signals with retention times corresponding to the individual isoflavones in 0.15 M acetate buffer (Fig. 7A). Signals of formononetin and biochanin A in different diluted human urine were significantly reduced compared to the standard solutions (Fig. 7B). The effect could be explained by the presence of the very complicated matrix and the differences in electrochemical behaviour of the compounds (not shown). The relatively acceptable decrease (about 2–5%) of daidzein and genistein signals was observed during the first 24 h. Then the signals steadily decreased with time (0–72 h) at all tested concentrations of human urine stored at 4 °C (Fig. 7C). The matrix effect could be explained by the uncontrolled interactions among compounds presented in such complicated matrix. Thus all measurements have to be done as soon as possible after sample collection.

Recoveries of standard additions of the individual isoflavones are in Table 5 for human urine samples. From the obtained results follow that the height of the isoflavone electrochemical signals increase with increasing dilution of the human urine sample by 0.15 M acetate buffer of pH 5.5. The increase of the electrochemical response well correlates with amount of sample dilution. We used this phenomenon for the determination of the isoflavone concentration in human urine. The well detectable amounts of daidzin and genistin were present in the human urine, and their concentrations were 6.5/2.6 μg/mL for daidzin/genistin, respectively.

As it can be seen from the results, the sensitivity is high enough to determine isoflavone substances in urine without any complicated pre-treatment of samples. Our HPLC procedure with electrochemical determination of the compounds was much more sensitive than the described

immunochemical and radio-immunochemical methods [55,56].

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