V. Kellner, M. Jurková, J. Čulík, T. Horák and P. Čejka

# Some phenolic compounds in Czech hops and beer of Pilsner type

**The HPLC method coupled with eight-channel coulometric detector (CoulArray) was used for determination of polyphenolic compounds in Pilsner beer and hops cultivated in the Czech Republic. Some important flavonoids and isoflavonoids as well as some phenolic acids were analysed. The CoulArray detector is characterized by its high sensitivity and enables determination in very low concentration. Calibration ranged from 0,001 mg/L to 1 mg/L of each analytes without any clean-up procedure before analysis. The two HPLC gradients with different content of acetonitrile as an organic modifier were used. Compounds with higher polarity, like phenolic acids (gallic, protocatechuic, gentisic, 4-hydroxyphenylacetic, vanillic, caffeic, syringic, and ferulic acid) besides flavan-3-ols (catechin and epicatechin), 4-hydroxycoumarin and derivatives of coumarin (esculin, umbelliferone, scopoletin) could be separated completely with 30 % content of acetonitrile in mobile phase only. 4-hydroxycoumarin, flavonoids or their glycosides (rutin, naringin, myricetin, quercetin, apigenin) and some isoflavonoids (daidzein, genistein, formononetin and biochanin A) had to be eluated with a higher content (50 %) of acetonitrile in the mobile phase due to their higher hydrophobicity. Specific oxidative potencial of each phenolic compound enables higher degree of selectivity of these chromatographic methods. The typical representative compounds from wide spectrum of phenolics were chosen for establishing of composition of Czech hops and beer of Pilsner type.**

Descriptors: beer, CoulArray detector, coulometric detection, hops, phenolic compounds

## 1 Introduction

The importance of polyphenolic compounds in beer and their influence on the physical stability of beer are already known. But the antioxidative features of polyphenols were intensively studied only recently. Polyphenolic compounds protect beer against its oxidation and ageing.

The stability of the flavour is explained by antioxidizing activities of present polyphenols [1, 2].

Beer is a source of many compounds not only for human nutrition but also for human health [3]. The polyphenols in beer due to their antioxidative features could contribute together with other dietary sources of polyphenols to protection from degenerative changes in the human body and so protect from diseases by moderate beer consummation. They have anticarcinogenic and antifungicide activities [3]. There is also a group of isoflavonoids in beers whose phytoestrogenic effects are now studied [4, 5].

The most effective method for separation of these compounds is HPLC method with UV or electrochemical detection. Although the sum of polyphenols in beer could be high, a concentration of individual compounds is often low for its direct analysing without their preconcentration and clean-up procedure before separation process. An overview of analytical methods including mass spectrometric methods for determination of phenolic acids in foodstuffs was published by Robbins [6]. García [7] developed a rapid method for determination of some polyphenols in alcohol-free beers. Polyphenols were isolated on C18 Sep-Pak cartridges with subsequent elution by acetonitrile. Then the SPE organic eluates were evaporated and final extracts were analysed with programmable UV-detector. The higher detection limit is a considerable disadvantage in this case and preconcentration of samples is often necessary. Due to their electrochemical activity polyphenols can be detected electrochemically with higher sensitivity of detection. CoulArray detector enables in one analytical run to detect electroactive compounds with high sensitivity because they have different oxidative potential.

Determination of free phenolic acids with CoulArray detector was carried out by Floridi [8] and Jandera [9]. The samples of beers and worts were on the contrary of methods with UV detection diluted with phase at the same pH as the mobile phase, which enables the high sensitivity of CoulArray detector. Rehova [10] studied separation of wide spectrum of phenolic compounds with using different stationary phases and founded suitable gradient for separation of 25 compounds together in one analysing run. CoulArray analyses of polyphenols in different kinds of beers made Škeřiková [11].

This method was chosen to describe the composition of the Czech hops and beer of Pilsner type due to advantages of CoulArray detection in HPLC. Our attention was also aimed at isoflavonoids but this group of naturally occurring compounds is distributed in plants in limited amount. Nevertheless due to high sensitivity of this detector we were able to detect and in some cases also to determine isoflavonoids together with polyphenols (phenolic acids and flavonoids).

## 2 Materials and methods

### Chemicals

Gallic acid, 3,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, esculin, 4-hydroxyphenylacetic acid, vanillic acid, chlorogenic acid, (+)-catechin, caffeic acid, syringic acid, vanillin, umbilliferone, (–)-epicatechin, scopoletin, ferulic acid, sinapic acid, 4-hydroxycoumarin, rutin, naringin, myricetin, quercetin, apigenin, biochanin A, daidzein, genistein, formononetin (Fluka).

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Tables and figures see Appendix

Acetonitrile gradient grade (Merck), water HPLC grade (Millipore), ammonium acetate, formic acid, methanol gradient grade (Merck).

#### Samples

6 samples of hops were obtained from Hop Research Institute Co., Ltd., Žatec.

3 samples of beer were obtained from Plzensky Prazdroj (Brewery Plzen, hopped with hops from Žatec area).

#### Apparatus

Apparatus for HPLC consists of solvent delivery module model 582 (ESA, U.S.A.), autosampler model 542 (ESA, U.S.A.) with 100 µl sample loop and CoulArray detector model 5600A (ESA, U.S.A.) with eight electrochemical cells in series consisting of porous graphite electrodes and palladium reference electrode and platinium counter electrode.

Analytes were separated on Zorbax SB-C18 column, 3,0 x 150 mm, 3,5 µm particle size (Agilent).

#### Chromatographic methods

Two chromatographic methods were generated. Mobile phases consist of buffer (0,005M ammonium acetate) and acetonitrile, pH of all phases was adjusted to 3,0 with formic acid.

I. Method with using phase A with 5 % content of acetonitrile and phase B with 30 % content of acetonitrile. Gradient 0–30 min 0–30 % B. After the end of analysing run in 30th min the column was washed for 10 minutes with 100 % of phase B. Equilibrium time was 15 minutes. Using this method made possible to analyse more polar compounds as free polyphenolic acids and catechin, epicatechin and coumarin and its derivatives. Flow was 0,6 mL/min.

II. Method with using phase A with 14,5 % content of acetonitrile and phase B with 50 % content of acetonitrile. Gradient 0–15 min 100 % A (= 0 % B), 15–70 min 0–76 % B. After the end of analysing run in 70th min phase B increased to 100 % in five minutes and from 75th to 76th min changed to 100 % of A. Flow was 0,6 mL/min.
Equilibrium time was also 15 minutes. This method was useful for less polar flavonoids and isoflavonoids.

The analytes eluated from column went through the array of electrodes with potentials ordering uplink 250, 300, 400, 500, 600, 700, 800 and 900 mV and are oxidized and conformable currents are measured as peaks on all potentials. The highest response (dominant peak) was used for calibration and determination of each analyte in its retention time.

#### Standard and sample preparation

Mixed stock solutions were prepared about 10 mg accurately weighted to 0,1 mg of each compound and dissolved in 100 mL methanol. The stock solutions were stored at –4 °C for a maximum of 3 months. The calibration standard solutions were prepared on four concentration levels (1,0; 0,5; 0,1 and 0,01 mg/L) by diluting stock solution in 100 mL beakers by phase A in both methods.

Beer samples: beer degassed by ultrasonification was diluted by phase A in the ratio 1:4 (V/V).

Hop samples: 1,25 g of milled hops is boiled in 200 mL water (Millipore) under reverse cooler for 30 minutes. The hot extract was filtered through the folded filter, cooled and filled to 250 mL by water and then diluted by mobile phase A in the ratio 1:4 (V/V). Dilutions of samples in starting mobile phase A were carried out according to Jandera [9].

#### Data collection and evaluation

The data were collected and evaluated by CoulArray for Windows software. Beyond retention time each compound is characterised by its oxidative potential (dominant potential) on which the highest response is measured by passing through of array of cells. The important qualitative indices are also ratios of responses on adjacent potentials to dominant potential respectively. Calibration relations are constructed from responses on dominant potentials for every compound. For the evaluation of the high and narrow peaks in method I it is more suitable to measure their heights which agree with the current, and in method II it is preferable to measure areas which agree with the passed charge. Calibration curves are constructed as relations of concentration vs. current (method I) and relations of concentration vs. electrical charge (method II). Retention times and parameters of calibration including limits of detection and estimation are given in tables 1 and 2.

## 3 Results and discussion

Six samples of hops and three samples of beer were analysed. Structural forms of studied phenolic compounds correspond to their different oxidative potentials as it is shown on figure 1 and 2. Two types of substituents are on aromatic scaffold by studied polyphenolics: –OH and $–OCH_3$. If, in the same position two compounds are different from each other only by this substituent, compound with $–OCH_3$ substituent has higher oxidative potential than compound with –OH substituent regulary: gallic acid 400 mV and syringic acid 600 mV, protocatechuic acid 500 mV and vanillic acid 700 mV, caffeic and chlorogenic acid both 400 mV and ferulic acid 600 mV, genistein 700 mV and bichanin A 900 mV, daidzein 700 mV and formononetin 900 mV.

Isoflavon genistein has lower oxidative potential (700 mV) then its isomer flavon apigenin (800 mV). The structural difference affects various values of oxidative potentials.

The stability of compounds is caused by conjugations of electrones of aromatic rings and electrones of substituents. The position and the type of substituent contribute to stability of molecule. In a group of coumarins 4-hydroxycoumarin and umbelliferone have oxidative potential 900 mV while scopoletin has only 700 mV.

We evaluated the recovery by using of the spiked samples of some analysed polyphenols on two spiking concentration levels (concentration levels of each analysed compound increased approximately about 5 and 10 mg/L respectively). Recoveries and measuring potencials are given in table 1. We found that the recoveries were decreasing indirectly with increasing measuring potential (table 3).

Contents of polyphenols in hops calculated from their concentrations measured in water extracts (diluted by mobile phase A 1:4) are given in table 4. Concentrations of polyphenols in beers are presented in table 5.

Obtained results showed that hops could be a rich source of (+)-catechin, (–)-epicatechin, rutin, 4-hydroxycoumarin, naringin and also a source of quercetin, esculin, chlorogenic and syringic

acid. Derivatives of benzoic acid (gallic, protocatechuic, gentisic, 4-hydroxyphenylacetic acids, vanillic acid and vanilline) and derivatives of cinnamic acid (caffeic, ferulic acids) occur in both matrices regularly. Umbelliferone and scopoletin were found in hops and in beer in concentrations near to detection limits and their importance from the point of quantity is not significant. But umbelliferone and scopoletin are the widespread coumarins in nature and are probably very effective also in low concentrations. It was described that biosynthesis of these coumarins is induced by tubers (e.g. potatoes) as a defense factor after attack by fungi deceases [12, 13]. If it is a defensive reason for formation of these two coumarins umbelliferone and scopoletin in hops were not studied yet.

The chromatographic method II is not suitable for estimation of apigenin due to tailing of peak and too high limits of estimation. In comparison with other phenolics analysed a higher limit of estimation of this compound was also calculated by Jandera [9]. Assymetry of this peak and long retention time make worse quantitive evaluation of this compound.

Phytoestrogens were detected in both matrices in the Czech hops and in Pilsner beer. But their quantification was near to limits of detection. Neither dilution nor direct analysing of samples of beer or hop extracts can solve problem of quantifications of these compounds. The dilution of samples is optimal in case of rich contents of some flavonoids as was published by other authors [9, 10, 11]. Analytical columns are not overloaded and separation and quantification are good. But for low concentrations of isoflavonoids it is necessary to find another way of sample preparation with suitable clean-up procedure, e.g. SPE.

## 4 Conclusion

The electrochemical reactions occuring on CoulArray detector enable the specific detection of polyphenols and due to high sensitivity of detection neither clean-up procedure nor preconcentration of samples are not necessary. The samples are simply diluted by mobile phase in contrast to other methods with UV detector often requiring preconcentration of polyphenols and clean-up procedure of samples. Also the oxidative potential is specific for each compound (dominant potential). The studied spectrum of polyphenolic compounds in the hops or beer is known simultaneously in one analysing run. We obtain good results to describe a composition of polyphenols in hops and beers using this unique detection technique of electrochemical CoulArray detector coupled with high performance liquid chromatography.

Typical compounds found in hops are also found in beers: (+)-catechin, (–)-epicatechin, rutin, 4-hydroxycoumarin, naringin and also quercetin, esculin, chlorogenic and syringic acid.

Determination of isoflavonoids together with other polyphenols in the same samples can cause some problems. The study showed that the analysing of polyphenols from group of phytoestrogens (daidzein, genistein, formononetin and biochanin A) asks a different manner of sample preparation, due to their very low concentrations in both matrixes in hops and consequently in beers and due to their higher detection limit (formononetin and biochanin A).

## 5 Acknowledgement

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# Appendix

**Table 1 Retention times and calibration parameters in method I**

| No. of analyte | Polyphenol | Retention time [min] | Dominant potential [mV] | Slope [-] | Intercept [µA] | Correlation coefficient [-] | Detection limit [µg/L] | Estimation limit [µg/L] |
|---|---|---|---|---|---|---|---|---|
| 1 | Gallic acid | 2,14 | 400 | 1,056 | 0,004 | 0,9996 | 0,8 | 2,7 |
| 2 | Protocatechuic acid | 4,69 | 500 | 0,861 | 0,003 | 0,9998 | 0,8 | 2,6 |
| 3 | Gentisic acid | 7,95 | 300 | 0,595 | 0,001 | 0,9998 | 0,9 | 3,2 |
| 4 | Esculin | 10,01 | 800 | 0,494 | 0,010 | 0,9998 | 5,0 | 16,8 |
| 5 | 4-Hydroxyphenylacetic acid | 10,87 | 800 | 1,310 | 0,007 | 0,9997 | 1,9 | 6,3 |
| 6 | (+)-Catechin | 11,83 | 400 | 0,254 | 0,001 | 0,9998 | 3,3 | 11,0 |
| 7 | Vanillic acid | 12,45 | 700 | 0,550 | -0,001 | 0,9998 | 4,1 | 9,7 |
| 8 | Chlorogenic acid | 12,82 | 400 | 0,221 | 0,000 | 0,9999 | 3,8 | 12,7 |
| 9 | Caffeic acid | 13,38 | 400 | 0,466 | 0,002 | 0,9998 | 1,8 | 60, |
| 10 | Syringic acid | 15,88 | 600 | 0,498 | 0,001 | 0,9998 | 2,6 | 8,8 |
| 11 | Vanillin | 18,17 | 800 | 0,688 | -0,006 | 0,9997 | 11,9 | 20,4 |
| 12 | (-)Epicatechin | 19,56 | 400 | 0,252 | 0,001 | 0,9998 | 3,3 | 11,1 |
| 13 | Umbelliferone | 22,82 | 900 | 0,582 | 0,012 | 0,9935 | 13,5 | 45,0 |
| 14 | Scopoletin | 26,34 | 700 | 0,583 | -0,005 | 0,9999 | 10,5 | 15,8 |
| 15 | Ferulic acid | 26,87 | 600 | 0,509 | 0,001 | 0,9998 | 2,6 | 8,6 |

**Table 2 Retention times and calibration parameters of method II**

| No. of analyte | Polyphenol | Retention time [min] | Dominant potential [mV] | Slope [-] | Intercept [µA] | Correlation coefficient [-] | Detection limit [µg/L] | Estimation limit [µg/L] |
|---|---|---|---|---|---|---|---|---|
| 16 | Rutin | 7,15 | 400 | 1,789 | 0,015 | 0,9997 | 40 | 132 |
| 17 | 4-Hydroxycoumarin | 14,70 | 900 | 1,419 | -0,032 | 1,0000 | 284 | 884 |
| 18 | Naringin | 16,30 | 900 | 0,941 | 0,091 | 0,9906 | 384 | 1200 |
| 19 | Myricetin | 17,97 | 300 | 3,059 | -0,150 | 0,9999 | 69 | 79 |
| 20 | Daidzein | 26,22 | 700 | 4,304 | 0,534 | 0,9912 | 34 | 113 |
| 21 | Quercetin | 31,23 | 400 | 4,363 | -0,082 | 0,8201 | 74 | 115 |
| 22 | Genistein | 37,74 | 700 | 4,127 | 0,260 | 0,9763 | 34 | 121 |
| 23 | Apigenin | 40,10 | 800 | 1,924 | -0,052 | 0,8846 | 406 | 1354 |
| 24 | Formononetin | 47,74 | 900 | 2,033 | -0,455 | 1,0000 | 303 | 786 |
| 25 | Biochanin A | 61,19 | 900 | 3,330 | -0,070 | 1,0000 | 147 | 489 |

**Table 3 Recovery of some polyphenols**

| Polyphenol | Origin beer [mg/L] | I. level Increment [mg/L] | I. level Spiked beer [mg/L] | I. level Recovery [%] | II.level Increment [mg/L] | II.level Spiked beer [mg/L] | II.level Recovery [%] | Dominant potential [mV] |
|---|---|---|---|---|---|---|---|---|
| Gallic acid | 0,00 | 5,30 | 5,30 | 100 | 10,60 | 9,95 | 94 | 400 |
| Protocatechuic acid | 0,38 | 5,65 | 6,35 | 105 | 11,30 | 11,65 | 100 | 500 |
| Gentisic acid | 0,14 | 5,15 | 5,00 | 94 | 10,30 | 11,10 | 106 | 300 |
| Esculin | 1,59 | 5,05 | 4,31 | 54 | 10,10 | 8,10 | 64 | 800 |
| 4-Hydroxyphenylacetic acid | 2,11 | 5,30 | 6,20 | 77 | 10,60 | 10,50 | 79 | 800 |
| (+)-Catechin | 1,36 | 5,25 | 7,60 | 119 | 10,50 | 14,15 | 122 | 400 |
| Vanillic acid | 2,20 | 5,20 | 6,75 | 88 | 10,40 | 11,00 | 85 | 700 |
| Chlorogenic acid | 0,17 | 5,30 | 5,25 | 96 | 10,60 | 10,30 | 96 | 400 |
| Caffeic acid | 0,30 | 5,45 | 5,70 | 99 | 10,90 | 11,40 | 102 | 400 |
| Syringic acid | 0,47 | 6,10 | 5,60 | 84 | 12,20 | 12,05 | 95 | 600 |
| Vanillin | 1,13 | 5,45 | 4,45 | 61 | 10,90 | 8,55 | 68 | 800 |
| (-)Epicatechin | 0,83 | 5,25 | 6,00 | 98 | 10,50 | 11,30 | 100 | 400 |
| Umbelliferone | 0,33 | 5,50 | 3,84 | 63 | 11,00 | 6,80 | 59 | 900 |
| Scopoletin | 0,51 | 5,10 | 4,96 | 87 | 10,20 | 9,70 | 90 | 700 |
| Ferulic acid | 3,50 | 5,15 | 8,50 | 97 | 10,30 | 15,25 | 114 | 600 |
| Sinapic acid | 0,84 | 5,25 | 7,15 | 120 | 10,50 | 14,25 | 128 | 500 |
| Rutin | 0,05 | 5,20 | 5,10 | 97 | 10,40 | 9,90 | 95 | 400 |
| 4-Hydroxycoumarin | 0,67 | 5,25 | 4,00 | 63 | 10,50 | 6,35 | 54 | 800 |

**Table 4 Content of polyphenols in hops [mg/kg]**

| Polyphenol | Hop 1 | Hop 2 | Hop 3 | Hop 4 | Hop 5 | Hop 6 |
|---|---|---|---|---|---|---|
| Gallic acid | 35 | 25 | 9,1 | 11 | 8,5 | 12 |
| Protocatechuic acid | 59 | 47 | 44 | 42 | 45 | 24 |
| Gentisic acid | 3,0 | 4,6 | 2,0 | 0,3 | 4,4 | 5,7 |
| Esculin | 110 | ND | ND | 130 | 140 | ND |
| 4-Hydroxyphenylacetic acid | 4,7 | 6,4 | 5,4 | 6,5 | 4,5 | 3,8 |
| (+)-Catechin | 850 | 840 | 800 | 820 | 960 | 970 |
| Vanillic acid | 56 | 53 | 59 | 53 | 78 | 93 |
| Chlorogenic acid | 170 | 160 | 100 | 110 | 61 | 77 |
| Caffeic acid | 36 | 35 | 20 | 26 | 33 | 27 |
| Syringic acid | 100 | 91 | 110 | 110 | 130 | 150 |
| Vanillin | 30 | 23 | 19,0 | 8,1 | 16 | 17 |
| (-)Epicatechin | 360 | 450 | 530 | 220 | 320 | 300 |
| Umbelliferone | 4,3 | 0,3 | 1,3 | 0,1 | 3,7 | 2,8 |
| Scopoletin | 1,8 | 8,3 | 4,3 | 19 | 4,5 | 1,9 |
| Ferulic acid | 28 | 26 | 28 | 5,9 | 34 | 33 |
| Rutin | 190 | 180 | 170 | 140 | 100 | 120 |
| 4-Hydroxycoumarin | 830 | 850 | 780 | 650 | 550 | 630 |
| Naringin | 1080 | 1240 | 1020 | 1020 | 770 | ND |
| Myricetin | ND | ND | 160 | 150 | ND | 83 |
| Daidzein | 3,5 | ND | 4,6 | 6,2 | 6,5 | 9,0 |
| Quercetin | 18 | 15 | 130 | 130 | 16 | 14 |
| Genistein | 18 | ND | 18 | 8,0 | ND | ND |
| Apigenin | ND | ND | 110 | 140 | 89 | 92 |
| Formononetin | 41 | ND | ND | ND | 100 | 30 |
| Biochanin A | 14 | ND | 350 | 140 | ND | ND |

ND – not detected

**Table 5 Concentration of polyphenols in beer [mg/L]**

| Polyphenol | Beer 1 | Beer 2 | Beer 3 |
|---|---|---|---|
| Gallic acid | 0,36 | 0,31 | 0,40 |
| Protocatechuic acid | 0,51 | 0,49 | 0,49 |
| Gentisic acid | 0,30 | 0,24 | 0,07 |
| Esculin | ND | ND | ND |
| 4-Hydroxyphenylacetic acid | 0,46 | 0,46 | 0,47 |
| (+)-Catechin | 4,62 | 2,04 | 4,77 |
| Vanillic acid | 0,76 | 1,10 | 0,76 |
| Chlorogenic acid | 0,15 | 0,24 | 0,23 |
| Caffeic acid | 0,60 | 0,20 | 0,74 |
| Syringic acid | 1,13 | 0,42 | 0,52 |
| Vanillin | 0,38 | 0,38 | 0,36 |
| (-)Epicatechin | 1,44 | 0,67 | 1,72 |
| Umbelliferone | 0,01 | 0,01 | 0,01 |
| Scopoletin | ND | ND | ND |
| Ferulic acid | 1,44 | 1,24 | 1,52 |
| Rutin | 0,34 | 0,27 | 0,36 |
| 4-Hydroxycoumarin | 0,26 | 0,31 | 0,39 |
| Naringin | 1,14 | 2,63 | 0,70 |
| Myricetin | 0,15 | 0,16 | 0,15 |
| Daidzein | 0,36 | 0,23 | 0,35 |
| Quercetin | 0,59 | 0,60 | 0,57 |
| Genistein | 0,06 | 0,08 | 0,07 |
| Apigenin | 0,81 | 0,81 | 0,80 |
| Formononetin | 1,30 | 0,17 | ND |
| Biochanin A | ND | ND | ND |

ND – not detected

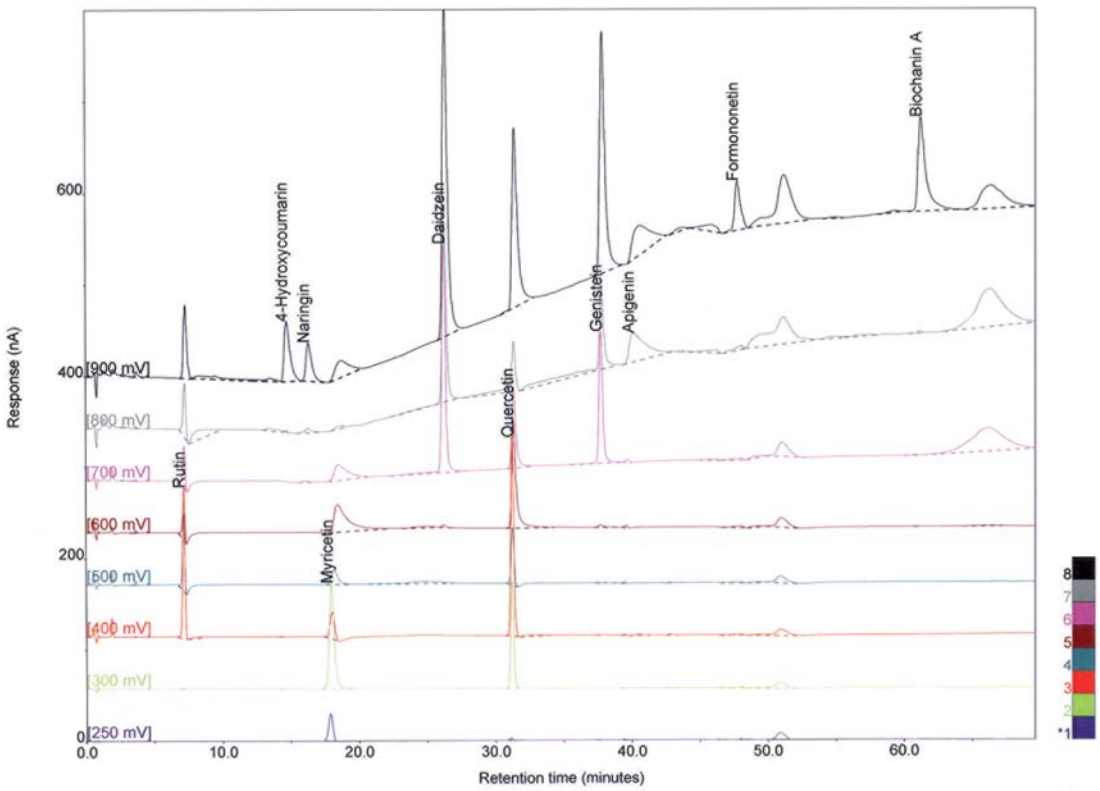


**Fig. 1 Chromatogram of standard solution of polyphenols in chromatographic method I. Concentration 1mg/L of each polyphenol**

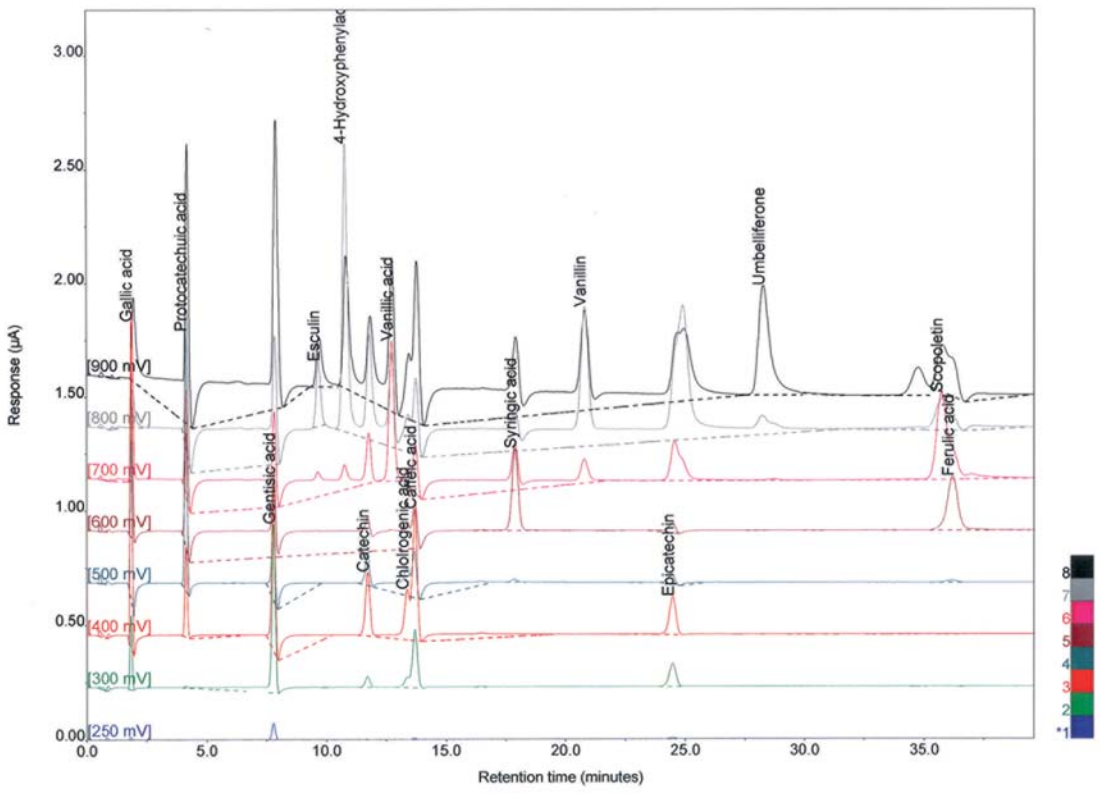


**Fig. 2 Chromatogram of standard solution of polyphenols in chromatographic method II. Concentration 1 mg/L of each polyphenol**