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Analytical Methods

Determination of resveratrol in grains, hulls and leaves of common and tartary buckwheat by HPLC with electrochemical detection at carbon paste electrode

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ABSTRACT

A reverse-phase high-performance liquid chromatographic method for the determination of *trans*-resveratrol with spectrophotometric detection (306 nm) and amperometric detection at carbon paste electrode (E = + 1,2 V) was developed and tested on real samples of grains, hulls and leaves of six varieties of common buckwheat (*Fagopyrum esculentum* Möench) and two varieties of tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.). Optimal conditions for the determination of *trans*-resveratrol were as follows: column Kromasil C-18 (7 μm), 125 × 4 mm; mobile phase acetonitrile: diluted BR buffer pH 7 (50:50, 30:70 for grains and hulls and 20:80 for leaves); flow rate 1 ml min^{-1}. Under these conditions, the limit of detection of *trans*-resveratrol (L_D) was 3.5×10^{-8} mol l^{-1} (R^2 = 0.9986) for electrochemical detection and 3.2×10^{-8} mol l^{-1} (R^2 = 0.9993) for spectrophotometric detection.

1. Introduction

Trans-reveratrol (*trans*-3,5,4′-trihydroxystilbene) is a polyphenol phytoalexin produced by plants in response to exogenous stimuli like UV light, ozone exposition, mechanical damage or fungal infection (Šmidrkal et al., 2001). Resveratrol exists in two isomeric forms *trans*-resveratrol and *cis*-resveratrol. The *trans*-isomer is the more stable form, with *trans* to *cis* isomerisation facilitated by UV light and high pH, while *cis* to *trans* conversion is facilitated by visible light, high temperature, or low pH (Trela & Waterhouse, 1996). Both isomers can be present in variable amounts in plants, but amount of *trans*-resveratrol usually predominates. Resveratrol was first detected in the roots of white hellebore (*Veratrum grandiflorum*) in 1940 (Shakibaei, Harikumar, & Aggarwal, 2009). Up to now it was found in more than 72 plants, which are often components of a human diet, for example in wine grapes (Fan, Zhang, Jinag, Yan, & Bai, 2008), with the result of average concentrations of 2.57 mg l^{-1} in red wine (Souto et al., 2001) and average concentration of 0.36 mg l^{-1} in white wine (López-Hernández, Paseiro-Losada, Sanches-Silva, & Lage-Yusty, 2007), in peanuts (Sobolev, Horn, Potter, Deyrup, & Gloer, 2006), cabbage, beetroot, broccoli, cranberries, blueberries (Rimando & Cody, 2005), etc. At present, a large body of evidence from *in vitro* and animal studies indicates that resveratrol may be beneficial to many aspects of human health. The remarkable interest in resveratrol is mainly due to its observed anti-cancer activities (Harikumar & Aggarwal, 2008), cardio-protective properties (coronary artery protection cumulating in the so called "French paradox") (Pirola & Fröjdo, 2008), anti-inflammatory, inhibition of platelet aggregation, antioxidation, neuroprotective (Sönmez, Sönmez, Erbil, Tekmen, & Baykara, 2007), antidiabetic and phytoestrogenic properties (Sharma, Anjaneyulu, Kulkarni, & Chopra, 2006) and anti-ageing properties. The quantitative determination of *trans*-resveratrol is mainly done by HPLC with UV/VIS (Piñeiro, Palma, & Barroso, 2006), MS (Stecher, Huck, Popp, & Bonn, 2001) and electrochemical detection (McMurtrey, Minn, Pobanz, & Schultz, 1994), by GC/MS (Luan, Li, & Zhang, 2000) or capillary zone electrophoresis (Gu, Chu, ÓDwyer, & Zeece, 2000). The HPLC with electrochemical detection is sufficiently sensitive and cost-effective alternative method to HPLC with UV/VIS or MS detection or capillary zone electrophoresis. In some matrices, electrochemical detection is superior to spectrophotometric one because of a large number of substances which are not electrochemically active and thus do not interfere with the detection of electrochemically active analyte. For HPLC-ED determination of resveratrol a glassy carbon electrode has been previously used (McMurtrey et al., 1994) at +0.5 V in 0.05 mol l^{-1}

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$NH_4H_2PO_4$ in 25% (v/v) aqueous acetonitrile. A carbon paste electrode used in this work for detection of resveratrol has (especially in more complicated matrices such as plant extracts) several advantages, namely the ease of renewal of the electrode passivated surface and lower background current. Resveratrol was identified in buckwheat amongst several other flavonoids (Qian, Mayer, & Kuhn, 1999). Buckwheat is pseudocereal but its grains belong to cereals because of their similar use. There are two buckwheat (*Fagopyrum*) species used for food around the world. Common buckwheat (*Fagopyrum esculentum* Möench) originates from Southwest China, while tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) is grown and used in the mountainous regions of northern India, Bhutan and Nepal (Christa & Soral-Śmietana, 2008). Flavonoid content in tartary buckwheat is higher than that of common buckwheat. Historically, buckwheat was a very important crop in Europe. In many regions, buckwheat was very popular and it was included in many daily meals. But its importance in the course of years decreased, yet recently, it has been observed to increase because of the health-promoting properties of its grains.

The aim of this work was to develop an HPLC method with UV spectrophotometric detection and amperometric detection on carbon paste electrode (CPE) for the determination of trace amounts of *trans*-resveratrol in samples of common and tartary buckwheat. The well-known advantages of CPEs (Zima, Švancara, Barek, & Vytřas, 2009; Švancara, Vytřas, Barek, & Zima, 2001) are broad potential window, low background current, possibility of chemical or biological modification of the carbon paste and ease of renewal of working surface of the carbon paste electrode (Barek, Muck, Wang, & Zima, 2004). To the best of our knowledge CPEs have not yet been used as amperometric detector in HPLC-ED determination of resveratrol. Their main advantage is ease of preparation and ease of regeneration of the working electrode surface. CPE based detectors present less expensive, comparatively sensitive and more selective alternative to spectrophotometric detection.

2. Materials and methods

2.1. Instruments

The HPLC system consisted of the high-pressure piston pump HPP 5001 (Laboratorní přístroje Praha, Prague, Czech Republic), injection valve D with 20 µl sample loop (Ecom, Prague, Czech Republic), spectrophotometric detector Sapphire 800 UV/VIS (Ecom, Prague, Czech Republic), electrochemical detector CHI 802B (CH Instruments, Austin TX, USA) with three-electrode system consisting of reference silver/silver chloride electrode RAE 113 (Monokrystaly, Turnov, Czech Republic) filled with 3 M KCl, working CPE made of a Teflon body (active part 3 mm in diameter) (Vytřas & Švancara, 1994) and platinum wire auxiliary electrode. Column Kromasil C-18 (7 µm), 125 × 4 mm (Prochrome, Mumbai, India) and precolumn Gemini C-18, 4 × 3 mm (Phenomenex, Torrance CA, USA) were used. The amperometric detector, employing electrochemical oxidation of phenolic hydroxy groups, was placed behind the UV/VIS detector operating at 306 nm (or 286 nm for *cis*-resveratrol). CPE based electrochemical detector was working in a wall-jet configuration. The system was operated by Clarity 2.3.0 programme (DataApex, Prague, Czech Republic) and CHI 6.26 programme (CH Instruments, Austin TX, USA) working in the Windows XP system (Microsoft, Redmond WA, USA). The mobile phase was acetonitrile: Britton-Robinson buffer pH 7, 10 times diluted by deionised water (50:50, 30:70 and 20:80 v/v), the flow rate was 1 ml min^{-1}. For the preparation of the concentrated ethanolic samples of buckwheat seeds a vacuum evaporator Büchi B-480, R-114 (Büchi, Flawil, Switzerland) was used. For preparation of samples of leaves a freeze dryer (Martin Christ, Osterode, Germany) was used. An ultrasonic bath PS02000A (Powersonic, San Diego CA, USA) was used to facilitate the dissolution of the analytes. The pH of the solutions was measured with a pH meter Jenway 4330 (Jenway, Felsted, UK) with a combined glass electrode. Spectrophotometer Agilent 8453 (Agilent, Santa Clara CA, USA) was used to study the stability of the stock solution of *trans*-resveratrol. All experiments were carried out at a laboratory temperature.

2.2. Materials

Trans-resveratrol was purchased from Sigma–Aldrich (Saint Louis MO, USA). Its stock solution (1×10^{-3} mol l^{-1}) was prepared by dissolving an accurately weighed amount of the substance in p.a. methanol (Lach-Ner, Neratovice, Czech Republic) and stored in dark at 4 °C. Solutions of lower concentrations were prepared by dilution of the stock solution with methanol. Stock solution of *cis*-resveratrol was prepared from solution of *trans*-resveratrol (1×10^{-4} mol l^{-1}) by UV-irradiation for 48 h on daylight. Optimum conditions were determined by our spectrophotometric measurements of the isomerisation on daylight for a total period of 48 h (conversion 85%) which were based on previous findings in the literature (Trela & Waterhouse, 1996). A spectrophotometric study of the stability of the *trans*-resveratrol stock solution was measured in solution 1×10^{-3} mol l^{-1} in 1 mm silica cuvette for two absorption maxima (306 and 217 nm) and it demonstrated that it was stable for at least 1 year, if it is stored in dark at 4 °C. Britton-Robinson (BR) buffers were prepared in a usual way, by mixing 0.04 mol l^{-1} phosphoric acid, 0.04 mol l^{-1} acetic acid and 0.04 mol l^{-1} boric acid with an appropriate amount of 0.2 mol l^{-1} sodium hydroxide. Ethanol was used for extraction of resveratrol from buckwheat. All the chemicals used were of analytical reagent grade (Lachema, Brno, Czech Republic). The mobile phase for HPLC contained acetonitrile for HPLC (Merck, Darmstadt, Germany) and aqueous BR buffer diluted 10 times. Carbon paste consisted of 250 mg of spherical micro particles of glassy carbon with a diameter of 0.4–12 µm (Alpha Aesar, Ward Hill MA, USA) and 90 µl of mineral oil (Fluka Biochemica, Buchs, Switzerland). All aqueous solutions were prepared using deionised water obtained from a MilliQ Plus system (Millipore, Molsheim, France).

2.3. Samples of buckwheat

Six samples of common buckwheat and two samples of tartary buckwheat (Table 1) were supplied by Crop Research Institute, Department of Gene Bank (Prague, Czech Republic).

2.4. Extraction of buckwheat

At first, we tried a simple extraction 17 g of grinded grains in 50 ml of ethanol (ratio 1:3) in the refrigerator overnight in the dark (Kolouchová, Melzoch, Šmidrkal, & Filip, 2005). Then we tried another method (Qian et al., 1999) for extraction of antioxidant compounds from buckwheat flour (4 g of buckwheat flour with 50 ml of ethanol extracted under reflux for 1 h and concentrated under reduced pressure to 5 ml). But the extracts from the two above mentioned extractions contained resveratrol very near to the limit od detection. For these reasons, we modified published method (Qian et al., 1999) to extract resveratrol from buckwheat grains, hulls and leaves. Grains were separated from hulls except of two samples of tartary buckwheat (with tartary buckwheat the separation of hulls from grains is impossible) and grains and hulls were grinded by kitchen mixer. The samples of leaves were a mixture of leaves picked before and during flowering, which was immediately frozen to the temperature below −18 °C, lyophilised on freeze dryer (Martin Christ, Osterode, Germany) during

Table 1
List of plant material of buckwheat samples.

NS[a]	ECN[b]	Name	Origin	Flower colour[c]	Stem colour[c]	Leaf colour[c]	Seed shape[d]	Plant height[e] (cm)	1000-seed weight[e] (g)
1	01Z5100007	Unnamed	Bhutan	5	6	4	3	99	15.82
2	01Z5100014	Unnamed	USA	5	6	4	3	102	14.33
3	01Z5000070	Špačínska 1	Slovakia	2	6	4	2	106	25.01
4	01Z5000076	Zelenocvetkovaya 90	Ukraine	5	6	4	2	115	25.70
5	01Z5000111	Emka	Poland	1	6	4	1	91	27.90
6	01Z5000123	Kara-Dag	Ukraine	2	6	4	1	100	25.61
7	01Z5000134	Rubra	Russian Federation	3	3	3	3	77	25.96
8	01Z5000127	Jana	Ukraine	1	6	4	1	90	32.81

[a] Number of a sample.
[b] National accession number.
[c] 1-White, 2-pink, 3-red, 4-green, 5-greenish yellow, 6-green-red.
[d] 1-Triangular, 2-ovate, 3-conodial.
[e] Mean from 3 years.

24 h at −58 °C and grinded by laboratory mill (IKA A11 Basic). The buckwheat grains (12 g), hulls (4 g) or leaves (12 g) were weighted into a distillation flask and 300 ml of ethanol were added, and extracted under reflux for 2 h, the solid substances were separated by filtration through filtration paper FN 1/90 g cm^{-2} (Filtrak, Bärenstein, Germany). The filtrates were concentrated under reduced pressure at 50 °C to the volume less than 5 ml, and then refilled to 5 ml with ethanol and filtrated through microfilter ProFill Plus PVDF/0.45 μm (Fischer Scientific, Pardubice, Czech Republic). The filtrates were then used for consequent analyses.

3. Results and discussion

First of all, optimum conditions for amperometric detection on CPE were found. The mobile phase containing acetonitrile and diluted BR buffer (1:1) allowed us to separate these two isomers in 4.5 min (with resolution of 1.6). The separation of *trans*-resveratrol and *cis*-resveratrol was found to be independent of the pH of the mobile phase within pH 3 to 7. Therefore, optimum pH was determined from hydrodynamic voltammograms at pH values compatible with the used column. Hydrodynamic voltammograms in BR buffers of pH 3, 5 and 7 in the potential range from 0 to +1.4 V are shown in Fig. 1. The highest and best developed peaks were obtained with diluted BR buffer pH 7: acetonitrile (1:1) at a potential of +1.2 V. The calibration curves were measured in the concentration range of 4×10^{-8}–1×10^{-4} mol l^{-1}. Calibration curves were measured in triplicate and evaluated by a linear regression method. The limit of detection of *trans*-resveratrol (L_D) was 3.5×10^{-8} mol l^{-1} ($R^2 = 0.9986$) for electrochemical detection (E = +1.2 V) and 3.2×10^{-8} mol l^{-1} ($R^2 = 0.9993$) for spectrophotometric detection (306 nm). The limit of detection of *cis*-resveratrol (L_D) was 1.8×10^{-8} mol l^{-1} ($R^2 = 0.9998$) for electrochemical detection (E = +1.2 V) and 6.5×10^{-8} mol l^{-1} ($R^2 = 0.9995$) for spectrophotometric detection (286 nm). The limit of detection was calculated as the amount of resveratrol, which gave the signal three times higher than the background noise (3 *S*/*N*). It can be seen that the electrochemical detection on carbon paste electrode gives comparable results as the UV/VIS detection. However, in many cases HPLC-ED chromatograms are simpler because of lower number of electrochemically active organic compounds as compared to number of compounds absorbing in UV region.

After optimisation of detection conditions, optimum conditions for separation of *cis*- and *trans*-resveratrol in concentrated ethanolic extract of grains, hulls and leaves of buckwheat were found. Composition of a mobile phase acetonitrile: diluted BR buffer (50:50, 40:60, 35:65, 30:70, 20:80) was tested first. The optimisation of chromatographic separation consisted in changing pH of the mobile phase from 3 to 7, the composition of mobile phase from 50% to 80% of acetonitrile in isocratic mode. Optimum conditions for the determination of *trans*- and *cis*-resveratrol with amperometric detection using CPE were found to be: a mobile phase acetonitrile: diluted BR buffer (30:70 for samples of grains and hulls and 20:80 for samples of leaves of buckwheat) and a potential of +1.2 V. The peak of *trans*-resveratrol was separated from other compounds in the samples of grains and hulls in the used ratio of buffer and acetonitrile. However, for the separation of peak of resveratrol from other compounds in the sample of leaves the ratio of BR buffer and acetonitrile had to be changed. *Trans*-resveratrol was detected and determined in all buckwheat samples, but *cis*-resveratrol was not found in any real sample analysed. Chromatograms of concentrated ethanolic extracts of grains are shown in Fig. 2 for spectrophotometric detection. Chromatograms

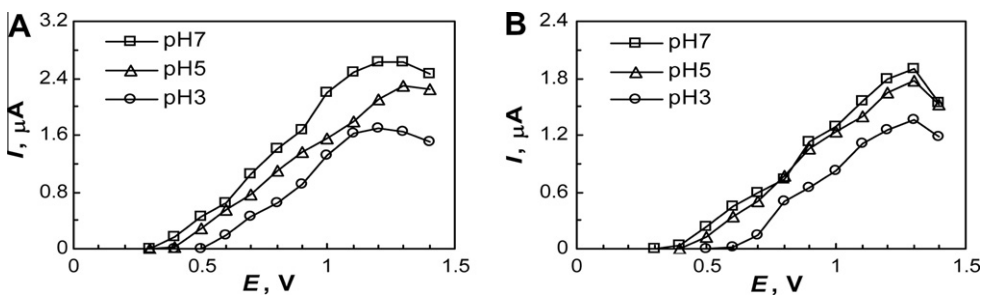

Fig. 1. Hydrodynamic voltammograms of *trans*-resveratrol **A** and *cis*-resveratrol **B** (injected 20 μl of 1×10^{-4} mol l^{-1} solutions) on CPE in the mobile phase diluted BR buffer: acetonitrile (1:1).

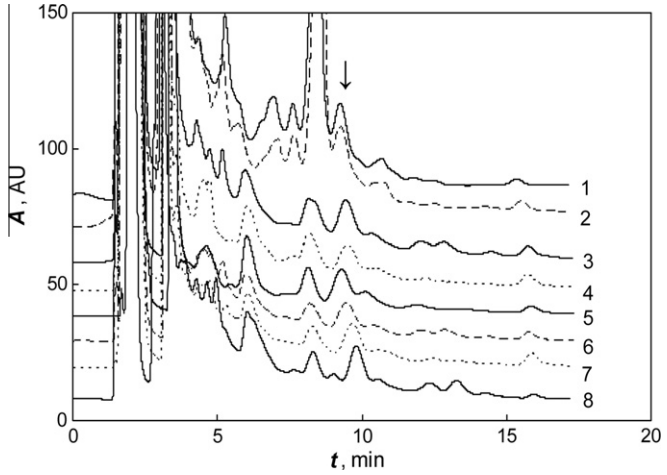

Fig. 2. HPLC chromatograms with spectrophotometric detection (306 nm) of concentrated ethanolic extracts of buckwheat samples of grains, numbers of curves correspond to NS (number of sample) from Table 1, mobile phase acetonitrile: diluted BR buffer pH 7 (30:70), flow rate 1 ml min^{-1}, injected 20 μl.

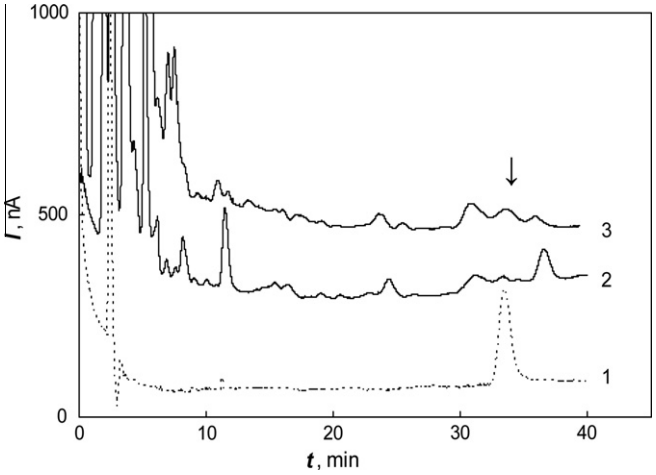

Fig. 3. HPLC chromatograms with electrochemical detection on CPE (E = +1.2 V) of 1×10^{-5} mol l^{-1} of *trans*-resveratrol (1), concentrated ethanolic extracts of buckwheat samples of leaves NS 2 (2) and NS 8 (3), mobile phase acetonitrile: diluted BR buffer pH 7 (20:80), flow rate 1 ml min^{-1}, injected 20 μl.

of concentrated ethanolic extracts of leaves are shown in Fig. 3 for electrochemical detection, an arrow shows the peaks of *trans*-resveratrol. Standard addition method has been applied for the determination of *trans*-resveratrol in concentrated ethanolic extracts and obtained contents are shown in Table 2. Three measurements were replicated for each sample to obtain the average values and standard deviations (mean ± SD). Obtained results of electrochemical and spectrophotometric detection were tested by paired *t*-test and Youden plots. Used statistic methods confirmed that both detection methods give comparable results of content of *trans*-resveratrol.

There are no big differences in *trans*-resveratrol amounts within analysed varieties. But the content of *trans*-resveratrol in grains of tartary buckwheat is almost three times higher than in samples of common buckwheat. On the other hand content of *trans*-resveratrol in leaves of common buckwheat is almost ten times higher than that in tartary buckwheat. The lowest level of *trans*-resveratrol was found in hulls of common buckwheat, therefore, we are confident that high level of *trans*-resveratrol in grains of tartary buckwheat is not due to the presence of hulls in samples (with tartary buckwheat the separation of hulls from grains is impossible). We cannot compare obtained results with any previous data because there are no literature data available regarding the content of resveratrol in buckwheat.

4. Conclusion

An HPLC method with electrochemical detection on carbon paste electrode and spectrophotometric detection was developed to determine *trans*-resveratrol in samples of grains, hulls and leaves of common and tartary buckwheat. Optimal conditions for the determination of *trans*-resveratrol were a mobile phase acetonitrile:

Table 2
Content of *trans*-resveratrol in concentrated ethanolic extracts of grains **A** hulls **B** and leaves **C**, obtained using HPLC with spectrophotometric (306 nm, from height of peak) and electrochemical (E = +1.2 V, from height of peak) detection, mobile phase acetonitrile: diluted BR buffer pH 7 (30:70 for **A**, **B** and 20:80 for **C**).

NS[a]	HPLC–UV/VIS		HPLC-ED	
	c in extract[b] (μmol l^{-1})	m[b] (mg kg^{-1})	c in extract[b] (μmol l^{-1})	m[b] (mg kg^{-1})
A				
1	36.1 ± 3.6	3.43 ± 0.35	36.5 ± 4.8	3.47 ± 0.46
2	36.5 ± 4.2	3.47 ± 0.40	36.8 ± 4.7	3.50 ± 0.45
3	17.7 ± 2.5	1.68 ± 0.24	17.3 ± 1.8	1.64 ± 0.17
4	11.0 ± 1.9	1.05 ± 0.18	11.3 ± 2.5	1.07 ± 0.24
5	13.8 ± 1.3	1.31 ± 0.12	14.3 ± 2.9	1.35 ± 0.28
6	10.8 ± 1.1	1.02 ± 0.10	10.3 ± 2.4	0.98 ± 0.23
7	13.0 ± 1.2	1.23 ± 0.11	13.0 ± 3.4	1.23 ± 0.32
8	18.3 ± 3.3	1.72 ± 0.31	15.5 ± 2.6	1.47 ± 0.24
B				
3	2.4 ± 0.3	0.66 ± 0.08	2.2 ± 0.3	0.62 ± 0.09
4	1.7 ± 0.2	0.46 ± 0.05	1.5 ± 0.3	0.43 ± 0.07
5	0.8 ± 0.1	0.22 ± 0.02	0.9 ± 0.2	0.25 ± 0.06
6	0.6 ± 0.1	0.16 ± 0.03	0.8 ± 0.3	0.21 ± 0.07
7	1.3 ± 0.2	0.38 ± 0.04	1.2 ± 0.2	0.33 ± 0.06
8	0.8 ± 0.2	0.21 ± 0.04	0.9 ± 0.3	0.25 ± 0.08
C				
2	2.1 ± 0.2	0.20 ± 0.01	2.0 ± 0.2	0.19 ± 0.02
8	19.2 ± 1.1	1.82 ± 0.10	19.2 ± 1.7	1.81 ± 0.16

[a] Number of sample (see Table 1).
[b] Means (of three measurements) ± SD.

diluted BR buffer pH 7 (30:70 for grains and hulls and 20:80 for leaves) with spectrophotometric detection at 306 nm and electrochemical detection at CPE at +1.2 V. The content of *trans*-resveratrol was 3.43–3.50 mg kg^{-1} of grains of tartary buckwheat, 0.98–1.68 mg kg^{-1} of grains of common buckwheat, 0.66–0.21 mg kg^{-1} of hulls of common buckwheat, 0.19–0.20 mg kg^{-1} of leaves of tartary buckwheat and 1.81–1.82 mg kg^{-1} of leaves of common buckwheat for both detection methods. Electrochemical detection on carbon paste electrode for *trans*-resveratrol gives comparable limits of detection as the UV/VIS detection for *cis*-resveratrol is electrochemical detection more sensitive than UV/VIS detection which enables fast and sensitive detection of light induced changes in resveratrol samples or analysis of samples containing both isomers.

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