

Comparison of Different Solvents and Extraction Methods for Isolation of Phenolic Compounds from Horseradish Roots (*Armoracia rusticana*)

Lolita Tomsone, Zanda Kruma, Ruta Galoburda

Abstract—Horseradish (*Armoracia rusticana*) is a perennial herb belonging to the *Brassicaceae* family and contains biologically active substances. The aim of the current research was to determine best method for extraction of phenolic compounds from horseradish roots showing high antiradical activity. Three genotypes (No. 105; No. 106 and variety 'Turku') of horseradish roots were extracted with eight different solvents: n-hexane, ethyl acetate, diethyl ether, 2-propanol, acetone, ethanol (95%), ethanol / water / acetic acid (80/20/1 v/v/v) and ethanol / water (80/20 by volume) using two extraction methods (conventional and Soxhlet). As the best solvents ethanol and ethanol / water solutions can be chosen. Although in Soxhlet extracts TPC was higher, scavenging activity of DPPH[•] radicals did not increase. It can be concluded that using Soxhlet extraction method more compounds that are not effective antioxidants.

Keywords—DPPH[•], extraction, solvent, Soxhlet, TPC

I. INTRODUCTION

PLANTS provide abundant natural antioxidants, which are vitally important for human health [1]. Phenolic compounds commonly found in plants are biologically active substances having antiseptic, vitamin activity etc. [2], [3]. It is known that phenolic compounds are very effective antioxidants [4], [5], [6]. Based on these statements, it can be concluded that it is very important to develop the best method for extraction of these compounds from plants.

Horseradish (*Armoracia rusticana*) is a perennial herb belonging to the *Brassicaceae* family and cultivated in temperate regions of the world mainly for the culinary value of its roots. Since horseradish has long been used as a spice for meat and fish products, the Food and Drug Administration (FDA) approved it as seasoning, spice, and flavoring and affirmed it as Generally Recognized As Safe (GRAS) [7]. Scientists are interested in horseradish because it is a rich source of peroxidase, a heme-containing enzyme that utilizes hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds [8]. Also horseradish is rich in other valuable substances – vitamins, minerals, phenolic compounds and also isothiocyanates [9].

Lolita Tomsone, Latvia University of Agriculture, Faculty of Food Technology, Jelgava, LV-3001, Latvia (phone: 0037163005644; fax: 0037163022829; e-mail: lolita.tomsone@llu.lv).

Zanda Kruma Latvia University of Agriculture, Faculty of Food Technology, Jelgava, LV-3001, Latvia (phone: 0037163005644; fax: 0037163022829; e-mail: zanda.kruma@llu.lv).

Ruta Galoburda Latvia University of Agriculture, Faculty of Food Technology, Jelgava, LV-3001, Latvia (phone: 0037163005644; fax: 0037163022829; e-mail: ruta.galoburda@llu.lv).

Several authors reported that horseradish has a high antioxidant activity compared to butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α -tocophere [10], [11].

Many researchers reported influence of different extraction solvents, techniques on the content of natural antioxidants in extracts [12], [13]. Efficiency of solvents and methods are strongly dependent on plant matrix used [14], [15], [13]. Solvents, such as methanol, ethanol, acetone, propanol and ethyl acetate have been commonly used for the extraction of phenolics from fresh product [16], [17]. The properties of extracting solvents significantly affected the measured total phenolics content ($\pm 25\%$ variation) and antioxidant capacity (up to 30% variation) in fruits and vegetables [13]. Very important parameter is solvent polarity – higher the polarity, better the solubility of phenolic compounds [1]. The highest extract yields (up to 22.8%) were obtained with polar alcohol based solvents [12]. Addition of water to ethanol improves extraction rate, but too high water content brought an increased concomitant extraction of other compounds, and, then to lower phenols concentrations in the extracts [15]. For wheat, 50% acetone extracts contained the highest level of total phenolics, whereas ethanol is the least effective solvent for extracting phenolics from wheat bran samples [14]. Literature data shows that extraction efficiency of solvents is strongly dependent on food matrix and the aim of current research was to determine best method for extraction of phenolic compounds from horseradish roots showing high antiradical activity.

II. MATERIALS AND METHODS

A. Materials

Three genotypes (No. 105; No. 106 and variety 'Turku') of horseradish roots (*Armoracia rusticana*) were collected in Pure (latitude 57° 03' N, longitude 22° 91' E) during the period from September to November, 2011. For analyses the average sample of 300 grams was taken from 3 roots. Fresh roots were washed, peeled and homogenized (for 5 minutes). All samples of one type of horseradish were homogenized together in order to obtain representative sample.

B. Chemicals

Gallic acid, Folin-Ciocalteu phenol reagent was purchased from Sigma-Aldrich (Switzerland). All other chemicals used in the research were obtained from Acros Organic (USA). Eight different solvents were used: n-hexane (HE), ethyl acetate

(EA), diethyl ether (DI), 2-propanol (PR), acetone (AC), ethanol (95%) (ET), ethanol / water / acetic acid (80/20/1 v/v/v) (EWA), and ethanol / water (80/20 v/v) (EW).

C. Extraction procedure

For extraction of phenolic compounds the conventional extraction and Soxhlet extraction was used.

1. Conventional extraction (CONVE)

Five grams of homogenized sample were extracted with 50 ml of an appropriate solvent in a conical flask with magnetic stirrer (magnet size 4.0 x 0.5 cm) at 700 rpm for 1 h at room temperature (20 ± 1 °C). The root extracts were then filtered (paper No. 89). The extraction process was done in triplicate.

2. Soxhlet extraction (SOXE)

Three grams of the sample were placed in the filter cartridge (paper No. 89) in a classical Soxhlet apparatus and extracted with 170 ml of an appropriate solvent for 2 h. Extracts were cooled to room temperature. The extraction process was performed in triplicate.

D. Analytical methods

For all extracts total phenolic content and DPPH[•] radical scavenging activity were determined.

1. Determination of total phenolic content (TPC)

The TPC of the roots extract was determined according to the Folin-Ciocalteu spectrophotometric method [18] with some modifications. To 0.5 ml of extract 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) was added and, after 3 minutes 2 ml of sodium carbonate (Na_2CO_3) (75 g/L) was added. The sample was mixed. The control sample contained all the reaction reagents except the extract. After 2 h of incubation at room temperature, the absorbance was measured at 765 nm using a spectrophotometer JENWAY 6300 (Baroworld Scientifid Ltd., UK). Total phenols were expressed as gallic acid equivalents (GAE)/100 g dry weight (DW) of the horseradish.

2. Determination of DPPH[•] radical scavenging activity

Antioxidant activity of the plant extracts was measured on the basis of scavenging activities of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) radical as outlined by Yu et al. [19]. The antioxidant reaction was initiated by transferring 0.5 ml of plant extract into a sample cavity containing 3.5 ml of freshly prepared DPPH[•] methanol solution (0.004 g DPPH[•] to 100 ml methanol). After 30 min of incubation in the dark at room temperature, the absorbance was measured at 517 nm using a spectrophotometer JENWAY 6300. Inhibition of DPPH[•] in percent (I%) of each extract sample was calculated from the decrease of absorbance according to the formula:

$$I\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100,$$

where

A_{blank} - absorbance of control (methanol-water with DPPH[•]);

A_{sample} - absorbance of the tested samples.

Lower absorbance of the reaction mixture indicates higher free radical scavenging activity [20].

Additionally for all horseradish roots moisture content was determined according to standard ISO 6496:1999 and all results are expressed to dry basis.

E. Statistical methods

Experimental results were means of three parallel measurements and were analyzed by Microsoft Excel 2010 and SPSS 17.00 for Windows. Analysis of variance (ANOVA) and differences among samples were tested by post hoc Dunnett test, Independent samples t-test was used to compare any significant differences between one genotype roots of the two types of extraction. A linear correlation analysis was performed in order to determine relationship between TPC and antiradical activity. Differences were considered significant at $p < 0.05$.

III. RESULTS AND DISCUSSION

A. Total phenolic content (TPC)

Phenolic composition of plants extracts is affected by different factors – variety, climate, storage, processing etc. Extracts of horseradish roots were prepared using conventional and Soxhlet extraction, and TPC was determined using Folin-Ciocalteu reagent, that reacts nonspecifically with phenolic compounds; it can also be reduced by a number of non-phenolic compounds, e.g., vitamin C, Cu(II), etc. The TPC determined in different solvent extracts of horseradish roots is shown in Fig. 1 and Fig. 2. For horseradish root No. 106, TPC determined in extracts made by conventional and Soxhlet extraction depending on used solvent ranged from 20.73 to 307.52 mg GAE/100 g DW and from 169.77 to 985.87 mg GAE/100 g DW, respectively. In the case of horseradish root 'Turku', TPC ranged from 23.02 to 334.29 mg GAE/100 g DW using conventional extraction and from 68.68 to 743.49 mg GAE/100 g DW using Soxhlet extraction. While TPC of horseradish root No. 105 TPC ranged from 19.21 to 327.49 mg GAE/100 g DW. Results of multivariate dispersion analyses showed that both used solvent and extraction method are significant factors affecting TPC ($p < 0.05$). Mainly, results of TPC obtained using a Soxhlet extraction is higher compared to a conventional extraction. TPC in plants grown over the world differ significantly. Malaysian researchers reported that in dates TPC ranged from 2.89 to 141.35 mg GAE/100 g DW [21], and Italian researchers reported that in ginger flour TPC ranged from 14.30 to 71.00 mg GAE/100 g DW [22]. It can be concluded that in some plants content of phenolics is similar or slightly lower compared to horseradish. But also many investigations showed higher TPC in plants, compared to horseradish. Škerget et al. [23] in their studies found that plant material contains different amount of total phenols: laurel – 9970 mg GAE/100 g, oregano – 18600 mg GAE/100 g, olive tree leaves – 14400 mg GAE/100 g. While other researchers found that TPC leaves of *Crithmum maritimum* L., *Eryngium maritimum* L. and *Cakile maritima* Scop. ranged from 1644 to

3193 mg GAE/100 g DW [24], but TPC in the 13 dry spice extracts analyzed ranged from 1970 mg GAE/100 g for dahurian angelica root up to 7950 mg GAE/100 g DW for clove [25]. Algerian researchers reported, that TPC varied in some Algerian medicinal plants and ranged from 310 to 3230 mg GAE/100 g of dry material [26]. In fresh pistachios TPC ranged from 801 mg GAE/100 g DW to 1620 mg GAE/100 g DW [27].

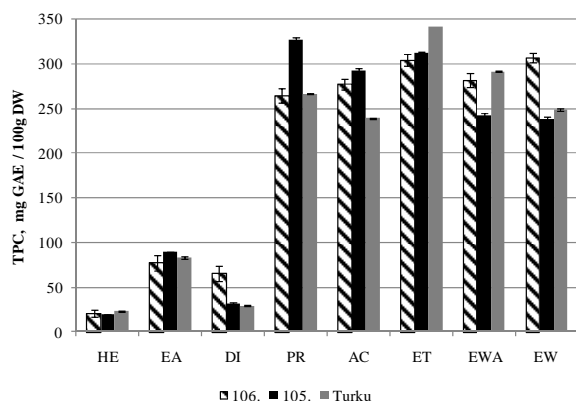


Fig. 1 TPC in horseradish depending on solvent using conventional extraction

The recovery of polyphenols from plant materials is influenced by the solubility of the phenolic compounds in the solvent used for the extraction process [28]. In the current research eight solvents with different polarity were used, and they can be arranged as follows (starting from more unpolar solvents): HE < EA < DI < PR < AC < ET < EWA < EW. From selected solvents the lowest polarity is for hexane, but the highest for EWA and EW.

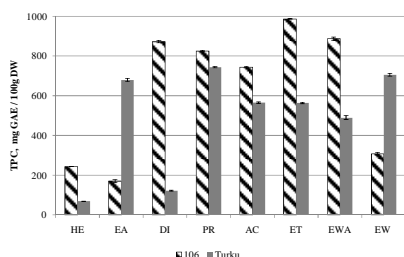


Fig. 2 TPC in horseradish depending on solvent using Soxhlet extraction

Solvent polarity plays a key role in increasing phenolic solubility [1]. Obtained results showed that TPC generally increased by increasing a polarity of solvents, and a tendency is more pronounced in the conventional extraction. Results of Tukey's test showed that using Soxhlet extraction all solvents can be classified in two groups that differ significantly ($p < 0.05$) – the first HE and EA (with lower TPC), and the

second with solvents DI, PR, AC, ET, EWA, EW. Polarity of phenolic compounds differs therefore; it is hard to develop a standard extraction procedure suitable for the extraction of all plant phenols.

In a conventional extraction influence of solvent is more significant, and there are no significant differences ($p < 0.05$) only between EW, AC, EWA and AC, EWA and PR. The results of analyses showed that the highest TPC of horseradish was extracted using 95% ethanol by both extraction methods. Ethanol and water mixtures are commonly used for the extraction of phenols from plant materials [16], [17]. Nićiforović, [28] studied Soxhlet extraction, where the highest TPC was found in *H. sendtneri* (Boiss.) extracted using 96% ethanol, which agrees with horseradish results. This is due to the wide range of phenols that the aqueous ethanol mixtures can dissolve. Furthermore, ethanolic mixtures have acceptability for human consumption models [17]. Contrary results can be found in literature. Fresh leaves of *C. siliqua* extracts presented the best TPC with solvents hexane and ethyl acetate [29]. Literature data shows that acetone–water mixtures are good solvent systems for the extraction of polar antioxidants [30], [31], [32]. Results of the current research show that acetone comparing to other solvents is good solvent but it is not the best. Literature describes that acetone and water extracts of fresh lychee (*L. chinensis* Sonn.) flowers presented the best total phenolic content [33]. Malaysia researchers reported that the highest TPC was in 70% ethanol honey pineapple extract, 90% acetone banana *pisang mas* extract and 90% acetone guava extract, respectively [17]. Whereas for Spanish white onions 100% acetone showed the lowest results [34].

B Radical scavenging activity (DPPH')

The scavenging activity of DPPH' radicals has been widely used to determine the free radical-scavenging activity. DPPH' is a stable free radical that is dissolved in methanol and its colour shows a characteristic absorption at 517 nm. Antioxidant molecules scavenge the free radical by hydrogen donation and the color from the DPPH' assay solution becomes light yellow resulting in a decrease in absorbance. Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation [35].

There are variations of antioxidants contained in horseradish roots. The results showed differences in DPPH' scavenging activity between horseradish roots obtained using conventional extraction (Fig. 3) and Soxhlet extraction (Fig. 4).

Results of multivariate dispersion analyzes showed that solvent significantly ($p < 0.05$) influence DPPH' scavenging activity, but extraction methods does not have significant ($p > 0.05$) influence.

For horseradish root No.106, DPPH' determined by conventional extraction and Soxhlet extraction ranged from 8.46 to 20.56% and from 3.16 to 14.72%, respectively. In the case of horseradish root 'Turku', DPPH' scavenging activity ranged from 1.98 to 6.99% and from 1.84 to 13.28%,

respectively. While horseradish roots No.105 DPPH[•] scavenging activity ranged from 1.16 to 12.07%.

Literature data showed that DPPH[•] scavenging activity differs depending on used solvent and food matrix. Researchers studied selected tropical fruits from Malaysia and stated that DPPH[•] scavenging activity of pineapple ranged from 12.7% to 93.7%, banana ranged from 32.8% to 79.1%, but guava ranged from 67.5% to 94.6% [17].

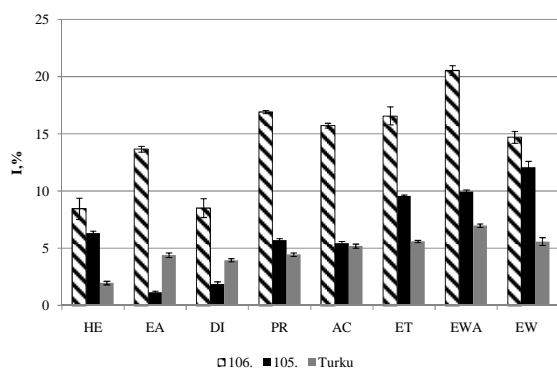


Fig. 3 Scavenging activity of DPPH[•] radicals of horseradish depending on solvent using conventional extraction

Also antiradical activity of horseradish differed significantly depending on solvents used and the highest activity was determined in EWA (Fig. 3) and EW extracts of horseradish root type No. 106 (Fig. 4).

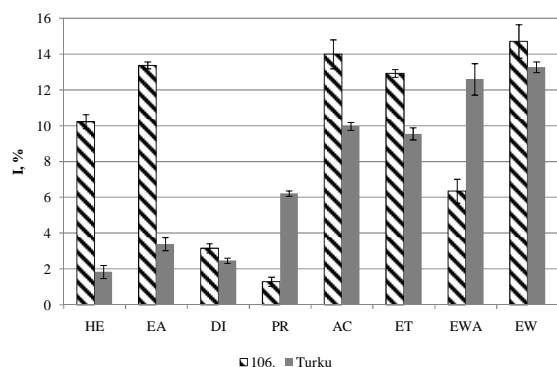


Fig. 4 Scavenging activity of DPPH[•] radicals of horseradish depending on solvent using Soxhlet extraction

Using both extraction methods it is possible to see increase in DPPH[•] scavenging activity by an increased polarity of solvent. López [36] reported that the highest activity was observed in the aqueous algae extract. The selected tropical fruits from Malaysia showed the highest DPPH[•] scavenging activity for pineapple and guava 90% acetone extract [17]. Whereas Allothman [17] reported that the highest DPPH[•] antiradical activity for bananas showed 70% ethanol extract.

C Correlation between total phenolic content and radical scavenging activity

Phenolic compounds have radical scavenging activity. Regression and correlation analysis were performed to

determine relationship between these parameters (Fig. 5, Fig. 6). Stronger correlation was found in TPC of extracts obtained from roots No. 106 and 'Turku' using a conventional extraction.

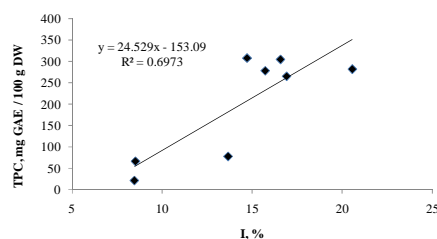


Fig. 5 Correlation between TPC and DPPH[•] scavenging activity of horseradish roots No. 106 extracted with conventional extraction ($n = 8$)

There was no correlation between TPC and DPPH[•] antioxidant activity, since the estimated coefficient of determination, R^2 values were less than 0.5 at $p < 0.05$.

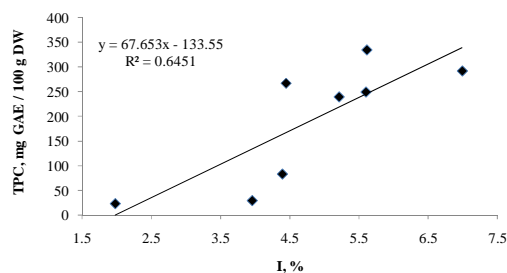


Fig. 6 Correlation between TPC and DPPH[•] scavenging activity of horseradish root 'Turku' extracted with conventional extraction ($n = 8$)

Results of our study showed that there was a medium and weak correlation between TPC and antioxidant activity of the tested extracts (Table 1). High correlation was observed in extracts of 'Turku' and No.106. horseradish root using conventional extraction, allowing the determination of a single indicator, quite accurately predict the other variable parameter. Moderate correlation was determined for horseradish root No. 105. Generally conventional extraction has better positive correlation between these parameters comparing to Soxhlet extraction where weak positive and weak negative correlation was observed depending on the used horseradish type. Totally taking into account all samples no correlation was observed between TPC and DPPH[•] scavenging activity.

These results suggest that the antioxidant activity of some tested extracts might be attributed to the presence of non-phenolic compounds. Even more, simple phenols, although they are not effective antioxidants, react with Folin-Ciocalteu reagent [37]. Also, it should be taken into consideration that different phenolic compounds may show different antioxidant

activities, depending on their structure, as well as synergistic or antagonistic effect of other compounds, which are present in the crude extract [28]. Thus, the total phenolic content can be used to predict their antioxidant activity with reasonable accuracy [25].

TABLE I

PEARSON'S COEFFICIENTS BETWEEN TPC AND DPPH SCAVENGING ACTIVITY

Extracts	R ² A	R ² B	R ² C
106	0.83	-0.391	-0.43
105	0.56	-	-
Turku	0.79	0.49	0.71
Conventional extraction (106, 105, Turku)	-	-	0.42
Soxhlet extraction (106, Turku)	-	-	-0.14
Total	-	-	0.09

^A Correlation coefficient between GAE and DPPH' scavenging activity for conventional extraction

^B Correlation coefficient between GAE and DPPH' scavenging activity for Soxhlet extraction

^C Correlation coefficient between GAE and DPPH' scavenging activity totally

Kubola [38] studied bitter gourd (*Momordica charantia* L.) leaf, stem and fruit fraction and referred that correlation between TPC and antioxidant activity was 0.711 ($p < 0.01$, $n = 12$). Statistical correlations between TPC and antioxidant activity of litchi seed extract were determined ($R^2 = 0.9773$) [39].

IV. CONCLUSION

Analysis of the TPC and free radical scavenging activity of horseradish extracts showed differences depending on extraction method and solvent used. As the best solvents ethanol and ethanol / water solutions can be chosen. Although in Soxhlet extracts TPC was higher, scavenging activity of DPPH' radicals did not increase. It can be concluded that using Soxhlet extraction method more compounds that are not effective antioxidants, but react with Folin-Ciocalteu reagent, are extracted.

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Lolita Tomsone, PhD student in Latvia University of Agriculture, Faculty of Food Technology, was born in Latvia, Bauska in 1974. Main topics of research: biologically active substances and natural antioxidants in foodstuffs.

Zanda Kruma, Dr.sc.ing., leader researcher at the Latvia University of Agriculture, Faculty of Food Technology, was born in Latvia, Aizpute in 1980. In 2008 she defended PhD thesis and obtained doctoral degree in engineering sciences (Food science). Main topics of research: biologically active substances in foodstuffs, food aroma analysis. She has 22 scientific publications and participated in 6 different projects.

Ruta Galoburda, Dr. sc. ing., professor at the Latvia University of Agriculture, Faculty of Food Technology, was born in Latvia, Vainode in 1959. Scientific interests – effect of processing technologies on food quality,

development of new food products. She has 64 scientific publications and participated in 10 different projects. At present R. Galoburda is a leader of the project „Sustainable use of local agricultural resources for development of high nutritive value food products (Food)” within the National Research Programme “Sustainable use of local resources (earth, food, and transport) – new products and technologies (NatRes)” (2010.-2013.)