CHEMICAL ENGINEERING TRANSACTIONS
VOL. 97, 2022
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Jeng Shiun Lim, Nor Alafiza Yunus, Jiří Jaromír Klemeš

ISBN 978-88-95608-96-9; **ISSN** 2283-9216
DOI: 10.3303/CET2297047

Comparison of Extraction Methods for Recovery of Antioxidant Compounds from White Radish Root (Raphanus sativus L.) and Application as A Natural Preservative in Bottled Beer

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Raphanus sativus L. contains many active ingredients, including phenolic compounds, and has potent antioxidant and radical scavenging activity. This study compared quantity and antioxidant capacity of extracts obtained from 4 methods: Ultrasonic-assisted extraction (UAE), enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE), and Soxhlet. The extract obtained from the best extraction method was then applied to preserve bottled beer. The results showed that the EAE method with Viscozyme L. brought the highest extraction efficiency, so it was conducted to optimize the enzyme extraction parameters. The optimal parameters obtained using EAE consisted of 2 stages: pre-treatment with 0.067 FBG (Fungal Beta - Glucanase Units) / g_{db} (gram of dry basic) of Viscozyme L at 49.3 °C for 66 min, then extraction with ethanol 70 % at 60 °C for 4 h. White radish root powder at 0.5 g_{db} / L was used as a natural antioxidant to preserve bottled beer from oxidation.

1. Introduction

Raphanus saivus L. (Radish) is an annual herb of the family Brassicaceae grown as an edible root. It is a widely consumed root vegetable worldwide due to its high nutritional values and phytochemical content. Several types of research have proved radishes' nutritional and medicinal values due to the contains many bioactive compounds such as saponin, flavonoid, polyphenols, essential oil, vitamin A and C (Grassi et al., 2010). So, it possess the antibacterial, antioxidant activity, and anti-inflammation. Phenolic compounds are plants' most abundant secondary metabolites, which have recently gained considerable attention due to multiple hydroxyl groups and high free radical scavenging potential. They are characterized by one or more phenolic hydroxyl groups in the molecule and classified into four types: phenolic acids, flavonoids, stilbenes, and lignans (Wang et al., 2020). Plant phenolics also show high antioxidant capacity, antitumor, antimicrobial, and antibiotic activity. Synthetic antioxidants have been widely used in the food industry, but they are not safe for health. Modern extraction methods include UAE, MAE, EAE, pulsed electric field, pressurized liquid extraction, and supercritical fluid extraction and others are being replaced by traditional methods such as Soxhlet equipment, exhaustive extraction, and immersion extraction. Increasing extraction capacity combined with new technologies will increase extraction rates (Rifna et al., 2021). Objectives of this study; 1) Extract phenolics from white radish pulp from Soxhlet, UAE, MAE, and EAE processes and compare both quantity and antioxidant capacity 2) Optimisation of extraction efficiency; 3) Use extract as a natural antioxidant to preserve San Miguel bottled beer.

2. Materials and Methods

2.1 Raw material

White radish: Fresh root was harvested from Lam Dong province, Viet Nam. It was washed, cut into rectangular slices, size 6 - 7 cm x 1 - 1.2 cm, slices were dried by convection drying at 50 °C. Dried sample with moisture

Paper Received: 26 April 2022; Revised: 29 June 2022; Accepted: 1 July 2022
Please cite this article as: Nguyen T.M.N., Ngoc P.T.K., 2022, Comparison of Extraction Methods for Recovery of Antioxidant Compounds from White Radish Root (Raphanus sativus L.) and Application as A Natural Preservative in Bottled Beer, Chemical Engineering Transactions, 97, 277-282 DOI:10.3303/CET2297047

content 7 - 9 (% wb) was powdered and sieved through a sifter (40 mesh). Eventually, it was stored in a sealed bag and kept in the dark with a freezer at -20 °C until further analysis. The extracted residue samples were sent to Saigon Hi-Tech Park Center for SEM imaging.
Bottle beer and storage experiments determining the physicochemical properties of beer were conducted at San Miguel Beer Company Vietnam - Nha Trang Branch, Viet Nam.

2.2 Chemicals

A. aculeatus produced Viscozyme L. from Novozyme. It is a clear brown liquid with a density of approx. 1.2 g / mL activity is 108 FBG / g (Fungal Beta - Glucanase Units). The optimal conditions for pH: 3.3 - 5.5 and temperature: 25 – 55 °C. It is stored in the refrigerator at 4 - 10 °C.
Trolox, ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), ammonium molybdate, acid ascorbic, Folin - Ciocaulteu reagent, potassium peroxydisulfate, acid garlic, quercetin, potassium acetate, aluminum chloride, sodium carbonate, ethanol 99%, sulfuric acid, sodium phosphate were purchased from Merck, Sigma - Aldrich (Germany) and Himedia (India). Ethanol 96 % used for extraction is a food-grade solvent.

2.3 Extraction process

Determined the properties of raw material: 20 g of ground powder were mixed with 162.5 g of deionized water and 17.8 mg of viscozyme L. (Novozymes, Franklinton, NC) enzyme in a closed 1 - liter glass flask. The enzyme concentration in the mixture was 0.108 FBG / g_{db}. The reaction was performed at 35 °C for 60 min. Add 70% ethanol with sample and solvent ratio of 1/30 (g / mL). It was kept for 8 h by horizontal shaking at 100 rpm. Extraction of the phenolic content (TPC) and antioxidant capacity (AC): A sample of 2 g of white radish powder was used for each experiment. The supernatant was collected in a volumetric flask to analyze TPC, TFC, ABTS, and TAC. The specific extraction parameters were as follows: 1) Soxhlet: extracted at 60 °C for 4 h; 2) EAE: after being treated with the viscozyme L, the sample mixture was added 437.5 g of 96 % ethanol to inactivate the enzyme's activity, then extracted with solven at 60 °C for 4 h; 3) UAE and MAE: Mixing with solvent and soaking for 60 min in room temperature, UAE using ultrasonic bath Elmasonic S60H, Germany with the frequency of 37 kHz and power effectively of 150 W, 40 °C for 25 min; 4) MAE: using microwave Sharp R-G371VN-W with levels of 400 W for 6 min. The extraction process on the residue was repeated four times. The total collected extraction was mixed well, then filtered by vacuum filtration with a membrane of 0.45 µm (Whatman No.1) and used as the analytical sample.

2.4 Analytical method

The total phenolic content (TPC) was determined according to Folin – Ciocalteu method reported by Singleton et al. (1999). The aluminium chloride colorimetric method measured the total flavonoid content (TFC) according to Chang et al. (2002). ABTS* radical scavenging activity (ABTS) was determined according to the method described by Re et al. (1999). Total antioxidant capacity (TAC) was determined by the formation of a bluish-green color due to phosphate/Mo (V) complex at acid pH (Nair et al., 2012).
0.5 gdb / L of phenolic extract concentration was added into bottled beer. With this supplement dose, the trial and control bottled beer were kept at room temperature for 4 months and at 40 °C in incubator for 28 d to keep track of oxidation in storage time. The samples were dispensed slowly through the sides of the glasses, avoiding over foaming. Approximately 100 mL of sample was poured into each glass. The flavor of two samples (trial and control) at the same time were tested by descriptive test to evaluate defected flavor (sun struck/ light struck and oxidized flavor) with bipolar rating scale.
Physical-chemical parameters of beer were analyzed by specialized methods from Standard procedures of QA Laboratory, San Miguel Brewery INC.(Kerry and Mallari, 2017), specific description was as follows:
Analyze OG (original gravity), AE (apparent extract), and alcohol content: decarbonated by moving the sample to a large flask and shaking until gases no longer escape from the beer. Filtering beer through dry filter paper if necessary. After decarbonization, the beer temperature should be approximately 20 °C. Beer was poured into the vial with the appropriate cap. The measuring was carried out automatically by DMA analyzer 4500 - Anton Paar.
The beer's clarity was determined by a Hach Turbidimeter 2100 N calibrated with the Formazin haze standard.
Bitterness: the bitter substances were extracted from the beer with iso-octan and are estimated by a UV spectrophotometer. 10 ml of chilled beer was transferred into the bitter tube. 1 mL 3N HCl and 20 mL iso-octan were added. It was covered with a stopper, manually shaken vigorously for 2.5 minutes, and let stand for 30 min. In the end, transferred the iso-octane layer to a 10 mm quartz cuvette and read at 275 nm using the spectrophotometer UV 1800 Shimadzu, Japan.
Foam: According to the NIBEM principle, Foam stability measurement was based on determining the times during which the foam - collar descends 10, 20, and 30 mm. It used Foam meter Nibem T– Haffman.

Color: using the spectrophotometer was based on measuring the absorbance at 430 nm.
NBS in beer was determined by heating of sample to 60 °C for seven days, and then the chill haze was measured after cooling at 0 °C for 24 h by Turbidimeter.

2.5 Experimental design

Determined the best extraction method: TPC and AC of raw material were used as the standard to compare and calculate the recovery efficiency. The best method of the four methods was chosen to optimize its factors.
Using the extract as a natural antioxidant in San Mig Light bottled beer: After the extraction treatments at optimum conditions, the extract was filtered by filter paper (Whatman No.1) 2 times and evaporated under vacuum (220 - 250 mmHg and the temperature was lower than 55 °C) to constant weight. It was a crude phenolic paste stored in a glass bottle at 40 °C, covered by aluminum paper to avoid light.
Prepare trial beer: after filler, unpasteurized botted beer was taken out of the conveyor. The beer was cold; it was about 4 °C to prevent the foaming. First, the crown was opened in a sterile microbiology room. The extract had already been weighed in the glass Bucher with the required weight for 330 ml (0.099; 0.132; 0.165 and 0.198 g_{db} / 330 mL). Second, 20 mL of beer was poured into Bucher to dissolve the extract, and then all of them were rushed back into the bottle. A new crown has capped the bottle again. This process required the sterile condition and quick operation (less than 2 min) to prevent contamination and the loss of CO_2. Finally, bottled beer, which was added to the extract, was put in a plastic crate and pasteurized usually.

2.6 Data analysis

The measurements were performed three times, and the data reported were mean ± SD. Statistical analysis was performed by using Stagraphic centurion XV.I. Data were analyzed by analysis of variance, and the mean values were considered significantly different when $P < 0.05$. The optimal extraction conditions were estimated through RSM (Response Surface Method) using Modde 5.0 software.

3. Results and Discussion

3.1 TPC and AC of raw material

The high content of phenolic and flavonoid compounds, as shown in Table 1, indicated the high antioxidant activity of white radish root.

Table 1: TPC and AC of raw white radish powder

Parameters	Result
Total phenolic content (TPC)	68.529 ± 2.064 mg GAE / g_{db}
Total flavonoid content (TFC)	12.595 ± 0.289 mg QE / g_{db}
ABTS* radical scavenging activity (ABTS)	61.551 ± 0.744 mg TEAC / g_{db}
Total antioxidant capacity (TAC)	10.806 ± 0.376 mg AAE / g_{db}

The TFC and ABTS have closely related, so the ABTS value is also very high (61.551 ± 0.744 mg TEAC / g_{db}). The TPC and TFC of Viet Nam white radish were higher than Thailand *Raphanus sativus* L. (Jakmatakul et al., 2009) but lower than Bulgary radish, *Raphanus sativus* var. Radicular (160 mg GAE / 100 g fresh sample) (Ribarova and Atanassova, 2005).

3.2 Phenolic and flavonoid extraction from white radish powder by four extraction methods

In this research, ethanol was chosen because it was a suitable solvent for polyphenols extraction and safe for human health. Table 2 showed that the extraction yield of TPC and TFC by the EAE method were 60.77 ± 1.259 (mg GAE / g_{db}) and 10.97 ± 0.76 (mg QE / g_{db}), better than other methods. The same results happened with antioxidant activity (ABTS and TAC) due to the pre-treatment with Viscozyme L. before Soxhlet extraction.

Table 2: TPC and AC of raw white radish powder in four extraction methods

Parameters	Soxhlet	EAE	MAE	UAE
TPC (mg GAE / g_{db})	34.69^{d} ± 1.49	60.77^{a} ± 1.26	45.49^{c} ± 2.26	49.03^{b} ± 2.45
TFC (mg QE / g_{db})	6.25^{d} ± 0.253	10.97^{a} ± 0.76	8.26^{c} ± 0.46	9.05^{b} ± 0.46
ABTS (mg TEAC / g_{db})	28.95^{d} ± 2.10	50.18^{a} ± 0.33	38.63^{c} ± 0.66	42.26^{b} ± 0.70
TAC (mg AAE / g_{db})	4.93^{c} ± 0.08	8.59^{a} ± 0.86	6.76^{b} ± 0.13	7.09^{b} ± 0.19

(Means with different superscripts in the same row are significantly different at $P < 0.05$ by LSD)

MAE also improved the extraction compared with Soxhlet, but its efficiency was lower than EAE and UAE.

Viscozyme L. was a multi-enzyme complex (including arabanase, cellulase, beta-glucanase, hemicellulase, and xylanase). These enzymes could destroy the plant cell wall structure, improving the extraction yield from plants because they hydrolyzed cell wall components and increased cell wall permeability (Puri et al., 2012).
Using UAE induced a greater penetration of solvent into the cell wall of plants and increased mass transfer. It could also disrupt biological material and release bioactive compounds easily. However, the efficiency of the method depended on the nature of the plant matrix. MAE was a process utilizing microwave energy to facilitate moving bioactive compounds from the material into the solvent with a short extraction time. MAE offered energy delivery to a total solvent volume and sample efficiently and homogeneously. Water in the selection absorbed this energy, and internal superheat promotes cell disruption, promotes chemical desorption from the matrix and improves recovery (Wang and Weller, 2006).
The recovery efficiency of the four trial methods in Figure 1a showed that the percent of phenolic and flavonoid were received from white radish powder. The higher recovery was, the more efficient the method was. Similarly, the loss of antioxidant activities was noted in Figure 1b.

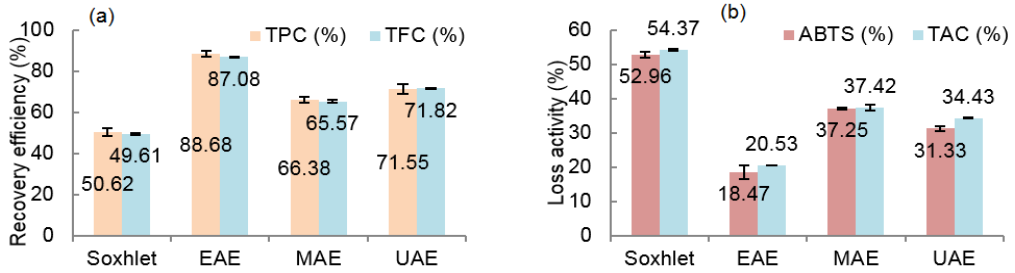

Figure 1: Comparison of extraction methods (a) Recovery efficiency; (b) Loss activity

The ratio of antioxidant activity was lost in the extraction because polyphenols were easily destroyed at high temperatures for a long time (Dai and Mumper, 2010). The lower this value was, the better the method was. It could be observed that EAE was the best method for extracting phenolic compounds from this material to keep the highest AC. With the same type of solvent, ratio sample/solvent, although MAE used more time than the others, it brought the best efficiency both in quantity and quality. It could receive 87.08 % of TPC, and 87.68 % of TFC from the material. UAE and MAE were also better than Soxhlet and only needed a short time (1 h 25 min for UAE and 1 h 6 min for MAE), but the efficiencies were not good as EAE. Therefore, the EAE method was used for the next optimal experiment.
A relation between extraction methods and distinguishable physical change in the sample (see Figure 2a. In the extraction methods, cell walls were destroyed, and released chemical components from the matrix of plants (Dahmoune et al., 2015). In Figure 2a the extraction process relied on the diffusion of the solvent into the solid matrix and solubilization of components under heating for a long time. In this picture, the surface is wrinkled, but rupture was rarely observed. For MAE, the surface of the sample was significantly disrupted. The electromagnetic waves increase temperature and internal pressure because of the formation of the high vapor pressure inside the sample leading to cell rupture; bioactive compounds in the cell-matrix were rapidly released into the solvent (2b). Severe damage on cell walls observed is similar to UAE due to the formation of acoustic cavitation (2c). Solvents could penetrate the cell wall, and the bubbles generated by acoustic cavitation stimulate cell wall destruction, and chemical components were released. In addition, more damage was seen in 2 d after EAE: most cells were entirely destroyed, and the cell walls were finally broken and damaged.

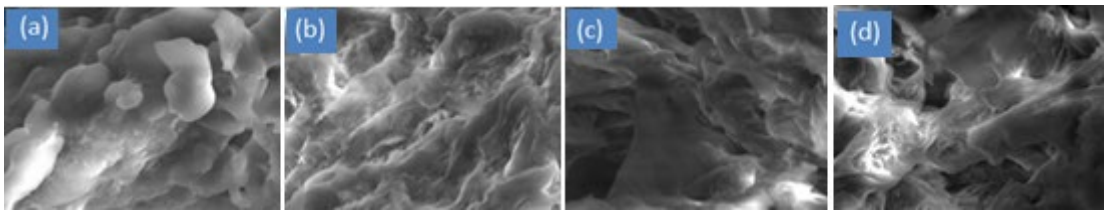

Figure 2: SEM image of residue in extraction process of methods (a)Soxhlet; (b) MAE; (c) UAE; and (d) EAE

3.3 Optimisation of the enzyme treatment conditions of the EAE method by RSM

The central points in the optimal experiment were determined in the preliminary period. Three independent variables and a rotatable central composite face (CCF) design was used to optimize the extraction process in Table 3. The response was the TAC of the extract and the obtained regression equation as per Eq(1):

$$Y\ (TAC) = 8.741 + 0.13x_1 - 0.128x_2 + 0.188x_3 - 0.221x_1^2 - 0.754x_2^2 - 0.212x_3^2 - 0.145x_1x_3 - 0.184x_2x_3 \quad (1)$$

where x1: Enzyme concentration (EC, FBG / g_{db}); x_2: Temperature (°C); and x_3: Time (min).

The determination coefficient (R^2) was 0.975, implying that the sample variation of 97.5 % for the TAC of extraction was attributed to the independent variables. The model could not explain only 2.5 % of the total variations. However, a large value of R^2 did not always indicate that the regression model was a sound one (Karazhiyan et al., 2011). In a good statistical model, R^2 and R^2adj values for the model didn't differ significantly.

Table 3: Experimental design with the observed responses of TAC yield from white radish powder using EAE

Run	EC, x_1 (FBG / g)	Temperature, x_2 (°C)	Time, x_3 (min)	TAC (mg AAE /g_{db})	Run	EC, x_1 (FBG / g)	Temperature, x_2 (°C)	Time, x_3 (min)	TAC (mg AAE /g_{db})
1	0.043	45	45	7.123	10	0.086	50	60	8.674
2	0.086	45	45	7.625	11	0.0645	45	60	8.023
3	0.043	55	45	7.212	12	0.0645	55	60	7.832
4	0.086	55	45	7.725	13	0.0645	50	45	8.124
5	0.043	45	75	8.13	14	0.0645	50	75	8.814
6	0.086	45	75	7.946	15	0.0645	50	60	8.791
7	0.043	55	75	7.379	16	0.0645	50	60	8.887
8	0.086	55	75	7.415	17	0.0645	50	60	8.787
9	0.043	50	60	8.246	Q^2 = 0.825;		R^2 = 0.825;	R^2_{adj} = 0.944	

The optimal conditions for extraction (by Moddle 5.0) were: enzyme concentration of 0.067 FBG / g_{db}, 66 min of enzyme treatment time and 49.3 °C of temperature. At these conditions, the theoretical maximum TAC value was 8.802 mg / g_{db}. The experiment was repeated five times by using the above selected optimal conditions to validate and verify the predictive model. Actual TAC was 8.700 ± 0.142 (mg AAE / g_{db}). Other parameters of the extract were analyzed as followed: TPC: 62.267 ± 1.893 (mg GAE / g_{db}); TFC: 11.287 ± 0.482 (mg QE / g_{db}) and ABTS*: 52.318 ± 0.515 (mg TEAC / g_{db}). The predicted TAC was 8.802 (mg AAE / g_{db}) and it was similar to the experimental yield of 8.700 ± 0.142 (mg AAE / g_{db}). The predicted value was in close agreement with the observed value, and the results showed no significant difference (Hossain et al., 2012). The predicted response values slightly deviate from the experimental data. The expected probability at the residuals shows no anomalies in the applied methodology. Zhang et al. (2013) mentioned that the regression model was sufficient to reflect the expected optimisation through strong correction between the actual and predicted results.

3.4 Application as a natural antioxidant in bottled beer

The oxidation in beer may be due to dissolved oxygen and air in the headspace. Dai and Mumper (2010) argued that phenolic compounds could inhibit or prevent the oxidation by scavenging free radicals or interact with oxygen and produce quinones and superoxide anion as a prooxidant. Therefore, the phenolic content in beer was added; they acted as free radical acceptors and chain breakers and thus, a new chain reaction was broken or not quickly initiated. However, phenolic substances can interact with other beer constituents. Among them, polyphenol–protein interactions have been researched most thoroughly due to their involvement in the beer haze (Wannenmache et al., 2018). Physical-chemical analysis was analyzed to quantify the difference between the two samples and results as display at Table 4.

Table 4: Physical – chemical analysis result of beer

Parameters	Unit	Fresh beer		After 4 months at room temperature	
		Control	Trial	Control	Trial
OG	°P	8.82^b ± 0.005	8.85^a ± 0.005	8.82^a ± 0.006	8.84^b ± 0.005
AE	°P	-0.59^b ± 0.010	-0.57^a ± 0.006	-0.59^b ± 0.006	-0.57^a ± 0.01
Alcohol content	w/w	3.83^a ± 0.006	3.82^a ± 0.006	3.82^a ± 0.006	3.81^a ± 0.006
Clarity	EBC	0.41^b ± 0.006	0.43^a ± 0.01	0.42^b ± 0.006	0.45^a ± 0.006
Color	SRM	3.42^b ± 0.00	3.46^a ± 0.006	3.42^b ± 0.006	3.47^a ± 0.015
pH	-	4.10^a ± 0.01	4.11^a ± 0.006	4.10^b ± 0.006	4.12a ± 0.006
TPC	ppm	65.57^b ± 0.046	92.55^a ± 0.035	63.89^b± 0.021	90.17^a ± 0.04
bitterness	spectro	9.45^a ± 0.01	9.453^a ± 0.006	9.427^a ± 0.006	9.40^a ± 0.01
NBS	EBC	1.05^b ± 0.006	1.22^a ± 0.01	1.08^b ± 0.026	1.34^a ± 0.042
Foam	Sec	252^a ± 1.53	250^a ± 2.00	249^a ± 1.00	244^a ± 3.79

(Means with different superscripts in the same row are significantly different at P < 0.05 by LSD)
Notes: NBS (non-biological stability), EBC: the unit of measure for clarity and NBS, SRM: the unit of measurement for color

At 40 °C heating condition, the flavour of 28 d age trial samples was the same as that of 14 d age control samples. There was a similarity between the 4-months age trial samples and 2 months age control samples when they were stored at room temperature. This results in the integrity of the beer's flavor for a longer time. Some parameters (OG, clarity, color, and TPC) were increased, others didn't change much with adding extract. Besides, NBS, chill haze, and foam were affected as their consequences. However, all of them were to the manufacturer's specifications. These changes were not considered to have a significant impact on beer quality.

4. Conclusions

Extraction of phenolic compounds from white radish pulp by EAE method with two stages: the first pretreatment with 0.067 FBG / g_{db} of Viscozyme L at 49.3 °C for 66 min, and then extraction with 70 % ethanol at 60 °C for 4 h was used as a natural antioxidant to preserve bottled beer from oxidation, preventing defects in beer flavor for 4 months under normal conditions and 28 d under accelerated conditions at 40 °C. Results showed that adding the extract to bottled beer halved the oxidation process and prevented the flavor of aging beer but did not affect the overall quality of the beer.

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