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Optimisation of Enzyme-Assisted Extraction of Oil Rich in Carotenoids from Gac Fruit (*Momordica cochinchinensis* Spreng.)

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Summary

Gac (*Momordica cochinchinensis* Spreng.) fruit arils contain an oil rich in carotenoids, especially lycopene and β-carotene, which can be enzymatically extracted in water. Response surface methodology was used to determine the factors influencing the enzymatic extraction of oil from gac fruit arils. A central composite design with four independent variables, namely enzyme mass per volume ratio, time, temperature and the stirring speed of the reaction, was carried out. The results show that all of these four factors have a significant effect on the oil yield recovery, with no significant interaction between these factors. Under the optimum conditions obtained (enzyme mass per volume ratio of 14.6 %, incubation time of 127 min, temperature of 58 °C and stirring speed of 162 rpm), it is estimated that the maximum oil recovery and the total carotenoid extraction per dry mass would be 79.5 % and 5.3 mg/g, respectively. There is a strong correlation between the oil recovery and total carotenoid content. The physicochemical properties of the extracted gac oil were characterised. Finally, the Schaal oven test shows that conservation time of gac oil is comparable to that of other edible oils.

Key words: Momordica cochinchinensis Spreng., gac aril oil, enzyme-assisted extraction, carotenoids

Introduction

Carotenoids are natural, mainly lipophilic pigments that are responsible for the colour of fruits and vegetables (*1*). They have recently attracted considerable attention because of epidemiological evidence suggesting that their antioxidant capacity may provide protection against cancer and other degenerative diseases (*2*). The carotenoid content, especially β-carotene and lycopene, in the gac arils was found to be much higher than that in other common carotenoid-rich fruit (*3,4*). The arils of the gac fruit contain significant concentrations of long-chain fatty acids, a key constituent for the efficient absorption and transport of β-carotene and other fat-soluble vitamins (*5,6*).

There are many recommended technologies for extraction of carotenoid-rich oil from natural sources using organic solvents, supercritical CO_2 extraction or enzyme-assisted aqueous extraction. The advantages and disadvantages of these common methods are summarised in Table 1 (*1,2,7–12*). Within these analyses, enzyme-assisted extraction and supercritical CO_2 are the most suitable from a human consumption and environmental point of view. Supercritical CO_2 method requires important technological investments, while enzyme-assisted extraction is a more convenient solution for application in developing countries (*13*).

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Table 1. Carotenoid extraction by different methods (*1,2,7–12*)

Extraction parameters	Organic solvent extraction	CO_2 extraction	Enzyme-assisted aqueous extraction
Yield	high	high	low
Investment cost	medium	high	low
Solvent cost	high	low	low
Environmental impact	high (by-products)	low	low
Intrinsic safety	no	yes	yes
Need to degum the fruit	yes	yes	no
Direct consumption of the product	no	yes	yes
Time	rapid	rapid	medium
Isomeration and degradation of carotenoids	yes	no	no
Trace solvent residues	yes	no	no
Handling	difficult	difficult	simple

Enzyme-assisted extraction is widely used for extraction of various kinds of substances. The role of enzyme is to degrade cell wall constituents and release intracellular contents. Usually, a plant cell wall comprises cellulose, hemicelluloses and pectin, while flesh has a significant content of pectin and proteins. Cellulases and pectinases can therefore be used for degradation of cell structure in the extraction process (*13*). Lipids exist in the cell in the form of oil droplets, or are associated with other components (lipoproteins) in the cell wall and in the cell cytoplasm. Consequently, better results of oil extraction are obtained when cell walls are broken down. Protease destroys lipoprotein envelope and facilitates the oil extraction process. α-Amylase can decrease the viscosity of an emulsion, which can facilitate oil liberation and then increase oil recovery. Dela Cruz *et al.* (*14*) observed that the vutalao (*Calophyllum inophyllum*) oil recovery reached 85 and 79 % of the total oil when using individually α--amylase or cellulase, respectively. Moreover, as reported by many authors, high oil yields were obtained when using α-amylase in combination with other enzymes (*14, 15*). According to Puangsri *et al*. (*16*), when using a combination of three enzymes: α-amylase, pectinase and protease for the extraction of coconut oil, a maximum extraction yield of 80 % was obtained. Enzymes have been employed for the extraction of lycopene from tomato tissues (*2,17*) or tomato paste (*18,19*), extraction of carotenoids from marigold flower (*8,20*) and from chili (*21*), and the extraction of luteolin and apigenin from pigeon pea leaves (*22*). Carotenoids have been extracted from orange peel, sweet potato and carrot using different concentrations of cellulase and pectinase combinations (*23*). Moreover, cell-wall-degrading enzymes have also been successfully used to increase the oil extraction yield from soybean (*24,25*), coconut (*26*), sunflower kernel (*27*), olive (*28*), safflower (*29*) and apricot kernel (*30*). Rosenthal *et al*. (*31*) reported that the usage of a combination of protease and cellulase could increase the oil extraction yield from soybean from 41.8 to 58.7 %. A combination of all four enzymes (α-amylase, protease, cellulase and pectinase) can also be tested for better efficiency.

The enzymatic extraction process is influenced by many factors including enzyme concentration, substrate concentration, temperature, pH, activators and/or inhibitors (*32*). As an enzyme is a protein-based catalyst, its reaction rate depends on its concentration and has an optimum temperature at which its activity is maximum. Stirring speed also facilitates contact and the reaction between the substrate and the catalyst. An optimization of reaction time is important to ensure maximum extraction without degrading product quality. The substrate concentration of the active catalysts and that of the obtained products increases over time, causing changes in the enzymatic reaction rate (*33*). As far as we know, a study of optimum onditions for enzyme-assisted extraction of gac oil has not been reported in the literature.

Response surface methodology (RSM), which is a collection of statistical and mathematical procedures (*34*), has been successfully used for development, improvement and optimisation of biochemical and biotechnological extraction processes (*28–30,35–37*). This method enables the evaluation of the effects of several process parameters and of their interactions on the response variables.

The aim of this work is to investigate the performance of a combination of cellulase, pectinase, protease and α-amylase in the extraction of oil rich in carotenoids from gac aril. After a set of prospective tests, four parameters (enzyme ratio, reaction time, reaction temperature and stirring speed) were optimised in order to improve the yields of carotenoids and oil extraction. A composition analysis and Schaal oven tests were finally used to check the oxidative stability of the obtained oil.

Materials and Methods

Biological material

Four enzymes: pectinase, protease, cellulase and α--amylase were obtained from Novozymes (Bangalore, India) *via* Nam Giang Co., Ltd. (Ho Chi Minh City, Vietnam). Pectinase was Pectinex® Ultra SP-L, produced from a selected strain of *Aspergillus aculeatus*, with an activity of 3800 PG/mL (polygalacturonase activity per mL); protease was Neutrase® 0.8 L, produced from *Bacillus amyloliquefaciens*, with an activity of 0.8 AU/g (Anson

Units per gram); cellulase was Cellusoft-L, produced from *Trichoderma* sp., with an activity of 1500 NCU/g (Novo Cellulase Units per gram) and α-amylase was Termamyl 120®, type L, produced from *Bacillus licheniformis*, with an activity of 120 KNU/g (K Novo Units per gram). The temperature and pH for an optimum activity of pectinase, protease, cellulase and α-amylase were 45–55 °C at 4.5–5.5; 40–60 °C at 6.0–9.5; 40–60 °C at 3.0–7.0 and 50–55 °C at 4.5–5.5, respectively.

Fresh gac fruits were purchased on Pham Van Hai market in Ho Chi Minh City, Vietnam. The fruit arils were dried to about 0.15 g of water per gram of dry mass by using vacuum dryer, and stored in sealed aluminium package until use in the experiments.

Physicochemical characterisation

Tissue structure analysis

Surface tissue structure of dried gac aril was analysed by using a KEYENCE VK-X200 (Keyence Corporation, Brussels, Belgium) series optical laser microscope at magnification of 20, 50 and 150×. A slide of dried gac aril (1×1 cm) was put in the right position under the objective lens and then automatically scanned under the optimal settings.

Proximate analysis

Water, oil, total carotenoid, carbohydrate, protein and crude fibre contents of the materials used for extraction were determined. The total carotenoid content (TCC) was determined by UV-visible spectrophotometer (Thermo Spectronic, model Genesys 20, Thermo Fisher Scientific, Waltham, MA, USA) and expressed as carotene equivalents (in mg/g) following the method of Tran *et al.* (*38*). β-Carotene (synthetic, type I) and lycopene from tomatoes, purchased from Sigma-Aldrich (St. Louis, MO, USA) were used as standards. The method is based on measuring the absorbance of light by the carotenoids applying the Beer-Lambert's law:

$$A=\varepsilon \cdot l \cdot c \qquad /1/$$

where A is the absorbance, ε is the molar absorbance coefficient at the selected wavelength (in L/(mol·cm)), l is the length of optical path (in cm) and c is the concentration of the solution (in mol/L). Absorbance was measured at 473 nm (*38*). The absorbance coefficient, ε, was obtained from a calibration curve. The TCC was then calculated using Eq. 1. Each experiment was carried out in triplicate.

Crude oil extract analysis

Characterisation of crude oil extract quality was evaluated by testing the principal indices used for edible oil: saponification, non-saponification, acid, peroxide and iodine indices, and the physical properties such as viscosity, density, melting point and refraction. Viscosity was determined by using a capillary viscometer (model no. 835, Pioneer Scientific Instrument Corporation, Kolkata, West Bengal, India) according to the Hagen-Poiseuille law (*39*). A 25-mL hydrometer was used for the determination of oil density. Methods used for determination of water content, fatty acid composition, protein, fibre, melting point, saponification, non-saponification, acid, peroxide, iodine and refraction indices were: AOAC 967.19, GC-ISO/CD 5509:94, AOAC 1997, AOAC-973-18c-1990, ISO 6321:2002, AOAC 920.160-2005, AOAC 933.08-2005, ISO 660:2009, ISO 3960:2007, AOAC 920.159-2005 and AOCS Cc7-25, respectively (*40*).

Enzymatic treatment for oil extraction

Dried gac arils were ground by using IKA® MF 10 Basic Micro grinder drive (IKA®, Selangor, Malaysia), with MF 10.1 cutting-grinding head and microfilter of 0.25 mm thickness. Then, 50 g of powder were mixed with 300 mL of water to give a ratio of 1:6 (by mass per volume), which was considered to be the best ratio for the oil extraction procedure (*14,41,42*). The pectinase, protease, α-amylase and cellulase preparations were added separately or in different combinations. The mixture was then thoroughly mixed in a 1000-mL graduated beaker and incubated by placing on a magnetic stirrer Heidolph MR Hei-Standard (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) with a magnetic stirring bar (60.9 mm in length and 11.5 mm in diameter). The enzyme ratio, incubation time, temperature and stirring speed were adjusted for each experiment. The pH was adjusted to a suitable value for the optimum activity of each enzyme by using 0.5 M of either NaOH or HCl. A mixture without the enzyme was prepared as a control sample. Conditions of pH and temperature for the control and the tested sample were modified in each experiment to correspond to the optimum conditions of the considered enzyme. For enzyme mixes, a compromise between the optimal conditions of each enzyme was used (Table 2). At the end of the incubation time, the obtained mixture was an emulsion of oil in water. Distilled water was added and the sample was stirred vigorously in order to separate the oil from the residue (*43*). The sample was then centrifuged using a Hettich EBA 20 (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany) centrifuge operating at 5500 rpm for 30 min, then decanted into a

Table 2. Extraction conditions using different enzyme combinations

Enzymatic treatment	t / °C	pH
protease	50	7.5
cellulase	50	5.0
pectinase	50	5.0
α-amylase	50	5.0
protease+cellulase (1:1 by volume)	50	6.0
protease+pectinase (1:1 by volume)	50	6.0
protease+α-amylase (1:1 by volume)	50	6.0
cellulase+pectinase (1:1 by volume)	50	4.5
cellulase+α-amylase (1:1 by volume)	55	5.0
pectinase+α-amylase (1:1 by volume)	50	5.0
protease+pectinase+cellulase (1:1:1 by volume)	50	5.5
protease+pectinase+α-amylase (1:1:1 by volume)	55	5.5
protease+cellulase+α-amylase (1:1:1 by volume)	55	5.5
pectinase+cellulase+α-amylase (1:1:1 by volume)	50	5.5
four-enzyme mix (1:1:1:1 by volume)	50	5.5

separator funnel and allowed to separate into oil and water layers. The oil was recovered, and then dried in an oven at 60 °C under vacuum conditions. Finally, the oil was filtered through a filter paper and dried over anhydrous sodium sulphate in order to eliminate all traces of water in the extracted oil. Due to the filtration, a smaller quantity of oil was recovered, which led to a systematic error. Experiments were carried out in triplicate.

The mass of extracted oil $m_{extracted}$ was measured by weighing the dried oil. The oil recovery, H (in %), was calculated by Eq. 2:

$$H=\frac{m_{extracted}}{m_{total}} \qquad /2/$$

where m_{total} is the mass of oil obtained by Soxhlet extraction (*38*).

Experimental design and statistical analysis

Prospective experiments

A scale range considered for enzyme combinations, enzyme ratios and incubation time was determined in prospective experiments. Pectinase, protease, α-amylase and cellulase were added separately or in different combinations of 2–4 types of enzymes in equal proportion at 10 % mass per volume ratio. Control samples for each experiment at the same pH and temperature were designed (Table 2). According to the literature, an experiment of 7 levels of enzyme ratios (2, 4, 8, 12, 16, 20 and 24 % by volume per mass) and 8 levels of incubation time (30, 60, 90, 120, 150, 180, 210 and 240 min) were carried out. The stirring speed for these prospective experiments was 150 rpm. Experiments were done in triplicate.

Principal experiments

After these prospective experiments, the RSM coupled with central composite design (CCD) was used for experiment design. The software JMP v. 9.0 (SAS, Cary, NC, USA) was employed to generate the experiment designs, statistical analysis and regression model. The independent variables were: enzyme ratio X_1 (5–25 %, by volume per mass), time X_2 (60–180 min), temperature of reaction X_3 (40–80 °C) and stirring speed X_4 (50–250 rpm). Each variable had five regularly spaced levels (Table 3). The CCD contains an imbedded factorial design with central points which is augmented with a group of star points that allow the estimation of curvature (*44*). A total of thirty-one combinations of independent variables were realized. The response of the model, which is the oil recovery H (in %), is modelled following a quadratic regression model in Eq. 3:

$$H=a_0+\sum_{n=1}^{4}a_nX_n+\sum_{n=1}^{4}a_{nn}X^2+\sum_{n<m}^{4}a_{nm}X_nX_m \qquad /3/$$

where a_0 is the value of the fixed response at the central point, while a_n, a_{nn} and a_{nm} are the linear, quadratic and cross product coefficients, respectively.

The data were examined for the analysis of variance (*45*) and regression models using a commercial statistical package Statgraphics v. 7.0 (Statpoint Technologies, Inc, Warrenton, VA, USA). Multiple range test and LSD (least significant differences) were used for comparing the mean values in an analysis of variance. All the statistical tests were realized with a confidence interval of 95 % ($p<0.05$).

Table 3. The central composite experimental design (in coded level of 4 variables) and their levels employed for the extraction

Independent variables	Coded variable level				
	–2	–1	0	1	2
X_1: enzyme ratio/%	5	10	15	20	25
X_2: reaction time/min	60	90	120	150	180
X_3: reaction temperature/°C	40	50	60	70	80
X_4: stirring speed/rpm	50	100	150	200	250

Determination of oxidative stability (storage life)

The Schaal (oven) test was used for evaluating the oxidative stability of gac oil. The oven test at (63±5) °C was useful for revealing oil rancidity. A mass of 50 g of oil was put in a 250-mL brown jar closed with a lid (*42*). Tests were done with the pure sample or with the addition of 0.1 % (by mass per volume) butyl hydroxyltoluene (BHT). The peroxide formation, considered as an indicator of odour and flavour changes, was measured every 3 days for 45 days. The oil freshness was classified according to O'Brien (*46*). A sample of oil extracted using *n*-hexane/propanol (8:2, by volume) (*47*) was also tested under the same conditions for 30 days. Each combination was tested in three repetitive experiments.

Results and Discussion

Microscopic analysis of tissue structure

The microscopic observation of dried gac aril tissues showed the cellular structure arrangement of the material (Fig. 1). The diameter of the cellule was in the range of 100 μm. Carotenoids are most probably restricted to cell wall and/or membrane structures (*48*). Based on the tissue structure analysis, the cellular structure of the gac aril is confirmed, which supports the use of enzymatic agents mentioned in this paper to break the gac aril's wall.

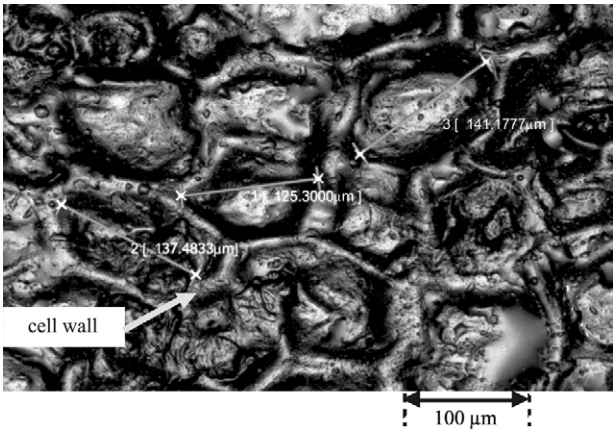

Fig. 1. Tissue structure of gac aril

Chemical composition of the fresh gac aril is presented in Table 4. The gac aril has high water content (76.8 %), while its oil content is about 17.3 %, measured on fresh and dry mass basis, respectively. Variability of water and oil content is related to maturity, variety and cultivating conditions of gac fruit. The TCC in gac aril is

Table 4. Chemical composition of gac arils

Composition	Value
w(water)/%	76.8±3.3
w(oil)/%	17.3±2.6
w(TCC)/(mg/g)	6.1±0.2
w(crude protein)/%	8.2±0.2
w(fibre)/%	8.7±1.4
w(carbohydrate)/(g per 100 g)	10.5*
w(starch)/(g per 100 g)	0.14*
w(pectin)/(g per 100 g)	1.25*
w(cellulose)/(g per 100 g)	1.8*

Results are expressed as mean values±SEM (standard error of the mean), N=3; *the result of Vietnamese Nutritional Institute presented by Vuong *et al.* (*5*)

about (6.1±0.2) mg per g of dry mass. The results suggest that minor quantities of other nutritional components, such as proteins, carbohydrates and crude fibre, are also present. Unsaturated fatty acids are the major parts of fatty acids present in the gac arils. Saturated acids such as palmitic, myristic and lauric acids are also found in the gac arils (Table 5). Moreover, 0.1 % of starch, 1.25 % of pectin and 1.8 % of cellulose were found in the aril (*5*).

Table 5. Fatty acid composition of gac aril

Fatty acid	*w*/%	Fatty acid	*w*/%
lauric (C12:0)	0.04	oleic (C18:1)	59.50
myristic (C14:0)	0.22	linoleic (C18:2)	13.98
palmitic (C16:0)	17.31	α-linoleic (C18:3)	0.52
palmitoleic (C16:1)	0.18	arachidic (C20:0)	0.32
margaric (C17:0)	0.14	eicosa-11-enoic (C20:1)	0.17
stearic (C18:0)	7.45	erucic (C22:1)	0.10

Recovery of oil from gac arils

Effect of enzyme combinations

Single-factor ANOVA analysis shows that the use of any enzymatic treatment presented here has a significant effect on oil recovery when compared to the control sample (Fig. 2). For individual enzyme use, multiple range test and LSD show that each enzyme has a significantly different impact on the oil yield, except for pectinase and protease. Protease and pectinase give a higher yield than cellulose, followed by α-amylase. This result

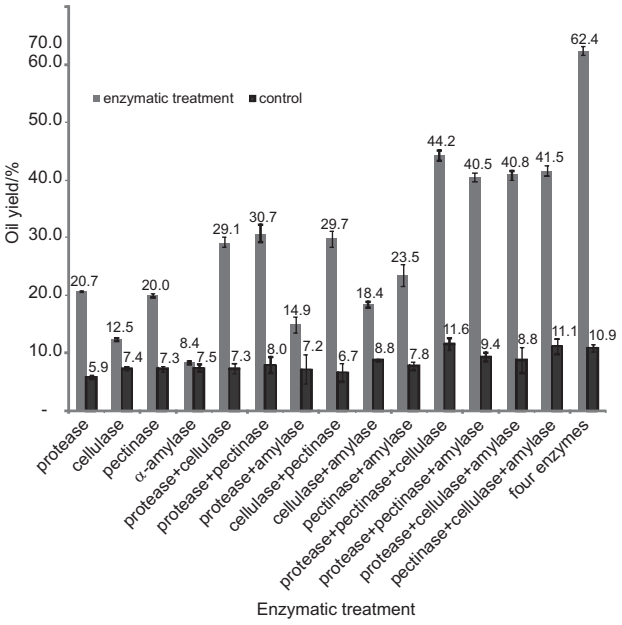

Fig. 2. Effect of the enzymatic treatment on the oil extraction yield

is slightly at variance with the result of Puangsri *et al.* (*16*) showing that the highest percentage of extracted papaya seed oil was obtained with protease, followed by pectinase, α-amylase and cellulase. This might be correlated with different vegetal matrix structures. Using a combination of two or three different enzymes, a higher yield of oil was obtained during the extraction. The highest extraction efficiency of 62.4 % was obtained with a mixture of the four enzymes in the ratio of 1:1:1:1 (by volume). A mixture of enzymes has a better effect on the oil release from the cell and on breaking of an emulsion than individual enzymes. This result is similar to the previous reports showing that a mixture of four enzymes (pectinase, protease, cellulase and α-amylase) in an equal proportion gives the highest yield of oil extraction from avocado (*42*) and from *Moringa oleifera* seeds (*43*).

The results of the effects of different enzymes on TCC extraction are presented in Table 6, showing that a mixture of the four types of enzymes at the ratio of 1:1:1:1, by volume, has a stronger effect on the carotenoid extraction than individual enzymes or mixtures of two or three enzymes.

Taking these results together, we have chosen a mixture of the four types of enzymes in an equal proportion for the next experimental process in this paper. However, further experiments with different ratios of these enzymes should be conducted in the future.

Effect of enzyme ratio

Statistical analysis shows that the effect of enzyme ratio on the oil yield is significant. The results in Fig. 3 show that oil yield reached the highest value at 16 % (by volume per mass) of the enzyme ratio, and it declined with further increase of the enzyme ratio above the optimal values. Under the same extraction conditions, the enzyme ratio thus has a significant influence on the re-

Table 6. Mass fraction and yield of total carotenoids extracted using different enzymatic treatments

Enzymatic treatment	*w*(carotenoids, oil)	Carotenoid extraction yield
	mg/g	%
protease	1.66±0.46	27.2
cellulase	1.49±0.54	24.4
pectinase	1.52±0.57	24.9
α-amylase	1.28±0.39	21.0
protease+cellulase (1:1 by volume)	2.26±0.76	37.0
protease+pectinase (1:1 by volume)	2.35±0.90	38.5
protease+α-amylase (1:1 by volume)	2.14±0.90	35.0
cellulase+pectinase (1:1 by volume)	2.06±0.34	33.8
cellulase+α-amylase (1:1 by volume)	2.89±0.54	47.4
pectinase+α-amylase (1:1 by volume)	2.98±0.75	48.9
protease+pectinase+cellulase (1:1:1 by volume)	3.55±0.89	58.0
protease+pectinase+α-amylase (1:1:1 by volume)	3.28±0.63	53.8
protease+cellulase+α-amylase (1:1:1 by volume)	3.02±0.80	49.5
pectinase+cellulase+α-amylase (1:1:1 by volume)	3.28±0.67	53.8
four-enzyme mix (1:1:1:1 by volume)	4.25±0.76	69.7

Results are expressed as mean values±SEM (standard error of the mean), *N*=3

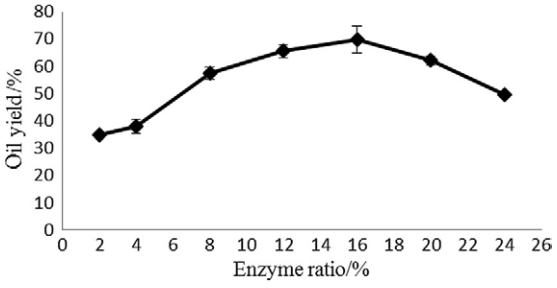

Fig. 3. Effect of the enzyme ratio on the oil extraction yield

action yield. This might be caused by the accumulation of intermediate products that inhibit the enzyme activity or react with the oil. Moreover, increasing the ratio of an enzyme increases the water content in the reaction mixture because the commercial enzyme is conditioned in an aqueous phase. The addition of water can reduce the concentration of the substrate and slow the reaction rate down. According to Nguyen (*41*), the decrease of oil yield extracted at high enzyme ratios might be due to the stabilisation of a fat emulsion in water by the enzymes, which act as protein surfactants.

Effect of reaction time

The results presented in Fig. 4 show that the reaction time has an effect on the oil extraction yield. The yield reached the maximum value at 120 min. During the process, the substrate and catalyst concentration were progressively reduced and possibly the concentration of intermediate inhibitory products increased, which as a consequence had the decrease of reaction rate. Moreover, the decrease of the reaction rate may be caused by a partial inactivation of the enzyme. The observed decrease of oil quantity in the long term might also be attributed to the adsorption of oil on the remaining solid fraction. Furthermore, long reaction time implies a risk of oil damage by hydrolytic or oxidation reaction (*41*). These phenomena could also cause a decrease in the oil yield because the short-chain fatty acids could not be extracted with oil. This can also lead to a decrease of the oil

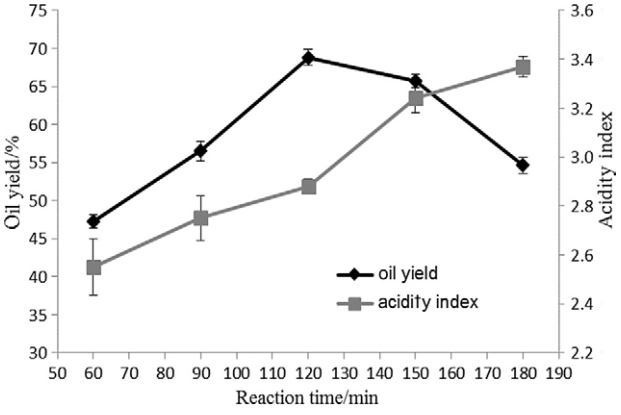

Fig. 4. Effect of the reaction time on oil extraction yield and acidity index

quality. Effect of reaction time on the acidity index are also presented in Fig. 4. The results show that the acid index of gac oil extracts increases, following a linear trend with the reaction time.

Optimisation of extraction conditions by RSM

Statistical analysis of the extraction conditions

The variance analysis shows that all of the four factors have a significant effect on the oil extraction yield. For the quadratic regression model, all the first- and second-order terms of the four factors are significant (Table 7). The cross product coefficients are not significant, meaning that there is no significant interaction between these factors. The fitting of the quadratic regression model leads to the following expression:

$$H=78.88-2.047X_1+2.62X_2-2.12X_3+1.78X_4- \\ -12.097X_1^2-5.958X_2^2-5.72X_3^2-3.876X_4^2 \qquad /4/$$

The significance of the model is confirmed by the Fisher's *F*-test (*F*=24.8691) with a very low probability value [(p>*F*)<0.001]. Moreover, the determination coeffi-

Table 7. Coefficient estimation in quadratic model for the oil recovery response

Term	Estimation	Standard error	*t* ratio	Prob>|*t*|
Intercept	78.882143	1.549458	50.91	<0.0001
X_1: enzyme ratio/%	–2.046958	0.836803	–2.45	0.0264
X_2: reaction time/min	2.6238333	0.836803	3.14	0.0064
X_3: temperature/°C	–2.121167	0.836803	–2.53	0.0221
X_4: stirring speed/rpm	1.776125	0.836803	2.12	0.0498
$X_1 \cdot X_1$	–12.09725	0.766617	–15.78	<0.0001
$X_2 \cdot X_2$	–5.958004	0.766617	–7.77	<0.0001
$X_3 \cdot X_3$	–5.720754	0.766617	–7.46	<0.0001
$X_4 \cdot X_4$	–3.875567	0.766617	–5.06	0.0001

Probability values below 0.05 are significant

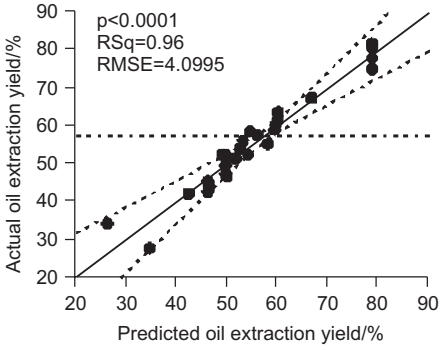

Fig. 5. Comparison of predicted and actual values of oil extraction yield

cient (R^2=0.956) indicates a good fitting quality. The value of the adjusted determination coefficient (R^2_{adj}=0.918) is also sufficiently high to advocate a high significance of the model. Comparison of the actual and predicted values of the response for oil recovery is presented in Fig. 5.

Effect of extraction conditions on oil yield

The results obtained by RSM show that the highest oil yield was obtained when using 15 % (by volume per mass) of enzyme. The use of hydrolytic enzymes can release the oil locked within the matrix of macromolecules present in the gac aril. Under the same reaction conditions, enzyme ratio had a strong influence on the reaction yield. In the first phase, when increasing the enzyme ratio, oil extraction yield increased. Then, proceeding with higher enzyme ratio led to a reduction of extraction efficiency. Possibly, the enzyme proteins combined with the oil, producing a stable emulsion that inactivated the enzyme and as a consequence inhibited the liberation of oil. In parallel, the increase of enzyme ratio indirectly caused the increase of water content in the reaction mixture because the commercial enzymes were conditioned in an aqueous solution. The additional water caused difficulties for oil separation from the mixture, consequently decreasing oil extraction yield. Furthermore, the more enzymes are added, the more intermediate products are accumulated, and then they can inhibit the catalytic activity of the enzymes. Thus, we consider that an enzyme ratio of 15 % is optimal for obtaining the highest yield of reaction.

The highest yield was obtained at about 127 min. Longer incubation time can increase oil recovery. However, above the critical value of incubation time, intermediate products may accumulate, which can cause a decrease of reaction rate. Further experiments with longer reaction time should be performed in order to confirm the trend of this factor.

The maximum value of oil yield was obtained at about 58 °C. When increasing the temperature above this value, a slight decrease of the oil extraction yield was observed. Starting at lower temperature, with its increase, the enzymatic reaction rate also increased. However, when the temperature was further increased, the enzyme proteins were denaturized and inactivated. This is especially true for pectinase, which is inactivated at 65 °C. α-Amylase, cellulase and protease activities also decreased at this temperature, but were not totally inactivated in this temperature range.

When increasing the stirring speed, extraction yield increased and reached its highest value at about 150 rpm. Above this value, extraction efficiency tends to decrease because oil recovery from the emulsion is difficult at a high stirring speed. An optimum speed makes the contact and reaction between the substances more easy. Moreover, oil droplets achieve bigger size at the optimum stirring speed, enabling easier oil recovery.

The graphical representation of the 3D response surfaces for each couple of independent variables is presented in Fig. 6. The optimum conditions could also be selected using contour plots presented in Fig. 7. The effect of two independent variables out of the four on the oil extraction yield was plotted while the remaining two were held at zero level.

Optimisation of extraction conditions

The optimisation of the extraction conditions using RSM indicated that maximum oil yield (79.5 %) was obtained under the following conditions: enzyme ratio of 14.6 % (by volume per mass), reaction time of 127 min, reaction temperature of 58 °C and stirring speed of 162 rpm. The best experimental oil yield achieved was 81.8 % and the calculated amount of oil extraction yield with these parameters using the regression model was 79.5 %. This confirmed that these conditions were optimal for maximum oil extraction yield.

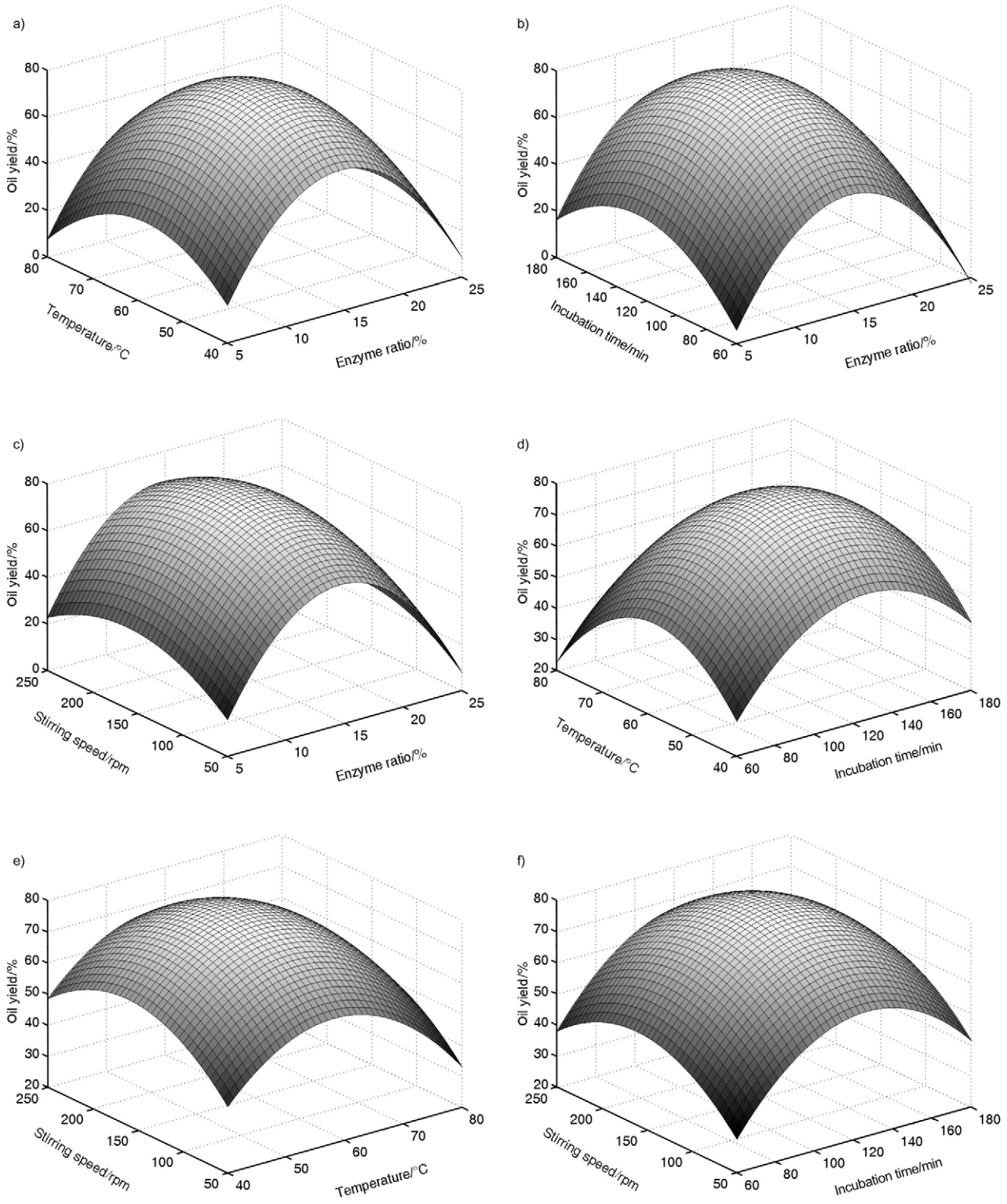

Fig. 6. Response surface for oil extraction yield showing the effect of different factors: a) enzyme ratio and temperature, b) enzyme ratio and incubation time, c) enzyme ratio and stirring speed, d) incubation time and temperature, e) temperature and stirring speed, and f) incubation time and stirring speed

Correlation between the oil recovery and carotenoid content in the oil

Fig. 8 shows the correlation between the oil yield and the total carotenoid content in the oil extracted by enzymatic hydrolysis. The results show that the total carotenoid content increases linearly with the oil recovery. The value of the coefficient of determination (R^2) is 0.90. The Pearson's correlation coefficient of 0.95 demonstrates a strong correlation between the oil yield and carotenoid

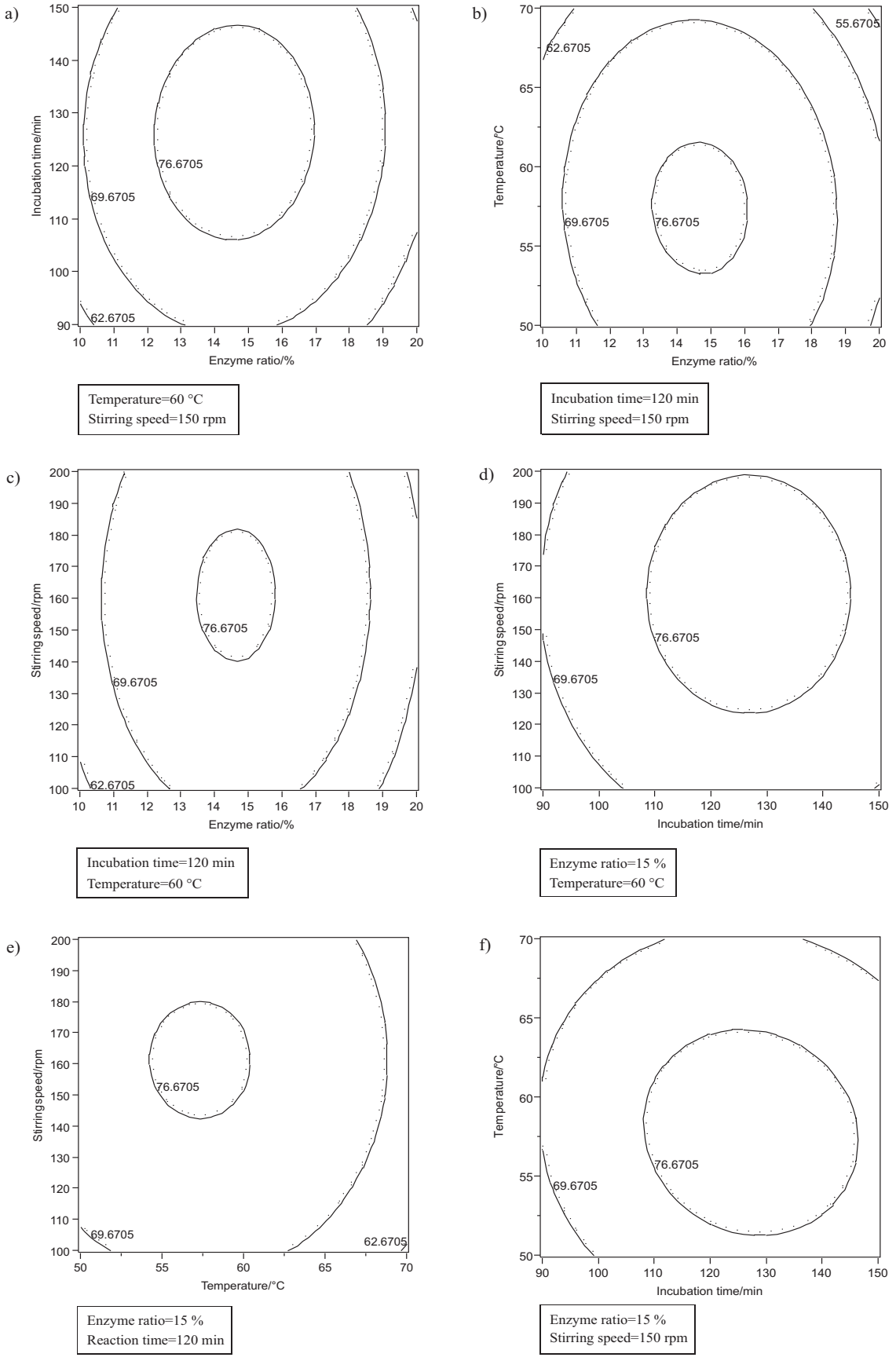

Fig. 7. Contour plots for oil yield as a function of different factors: a) enzyme ratio and incubation time, b) enzyme ratio and temperature, c) enzyme ratio and stirring speed, d) incubation time and stirring speed, e) temperature and stirring speed, and f) temperature and incubation time

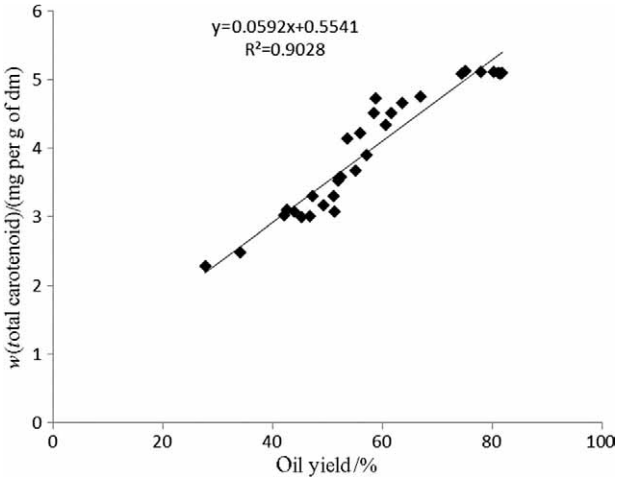

Fig. 8. Correlation between the oil recovery and TCC in the oil

content. This shows that optimisation of the oil extraction and of the total carotenoid content is driven by the same mechanisms.

Physicochemical properties of the extracted gac oil

The physicochemical properties of the extracted oil are presented in Table 8. The acidity index (AI) value of gac oil is lower than that of some common oils such as soybean and sunflower oil (in which AI can rise to 6 and 4, respectively). The low peroxide index (PI) characterizes the purity and stability of this oil at ambient temperature. The iodine index corresponds to a high degree of unsaturation of the oil. This result is in agreement with our proximate analysis. The high saponification index is an indicator that the triglycerides of gac oil are composed of short fatty acids.

Table 8. Physical and chemical properties of the gac aril oil extracted by enzyme-assisted extraction

Property	Value
acidity index/(mg of KOH per g)	2.55±0.57
peroxide index/(meq O_2 per kg of oil)	0.89±0.25
iodine index/(g of I_2 per 100 g of oil)	76.58±1.90
saponification index/(mg of KOH per g)	715.16
non-saponification index/%	0.50
refractive index *nD* at 25 °C	1.47
melting point/°C	12.00
viscosity/(Pa·s)	0.0466±0.0004
density/(g/mL)	0.955±0.012

Results are expressed as mean values±SEM (standard error of the mean), *N*=3

Oil storage study (accelerated oxidation tests)

The result shows that peroxide formation increases with storage time (Fig. 9). There is a difference between the control sample and the sample with the added antioxidant. Peroxide value of the gac oil with and without

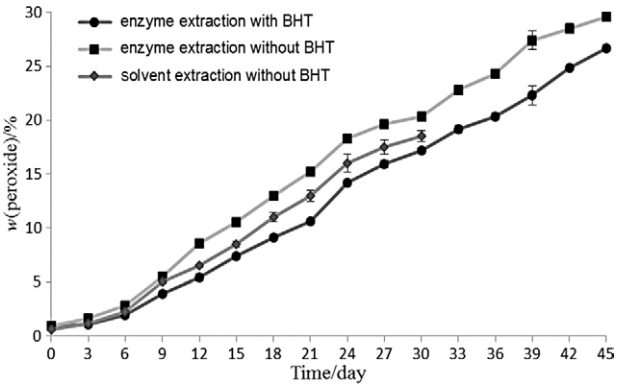

Fig. 9. Evaluation of peroxide formation with storage time

the added BHT at 15 and 12 days of storage, respectively, was less than 10, which is considered as low oxidation level according to the ranking of O'Brien (*46*). After that period, high oxidation (PI>10) is revealed. A very bad odour appears at a very high oxidation (PI>20) from 33 and 27 days for the control and BHT-added sample, respectively. Peroxide value indicates the primary stages of autoxidation and is considered as an important parameter for storage stability of fats and oils. The result obtained from storage stability tests compared with other oil products is presented in Table 9 (*49,50*). This result shows that the stability of the oil extracted by the enzyme is comparable with other common edible oils. The peroxide formation from the oil extracted by the enzyme increased faster than that from the oil extracted by the organic solvent (Fig. 9).

Table 9. Comparison of the stability of oil determined by Schaal oven test

Product	Antioxidant treatment / % by mass per volume	Storage stability to develop peroxide index / day
gac aril oil	control	12
	BHT 0.10	18
cottonseed oil*	control	9
	BHA 0.02	9
soybean oil*	control	6
	BHA 0.02	8
peanut oil**	control	30

*based on Eastman Chemical Company (*49*)
**based on Pokorný *et al.* (*50*)

Conclusion

In this study, a mixture of four enzymes was tested to improve the oil yield and the extraction of total carotenoids from gac aril. The optimisation of the oil recovery led to the optimisation of the extraction of carotenoids. Optimal conditions regarding the enzyme ratio, extraction time, temperature and stirring speed were identified. The oil obtained has conservation properties comparable to other commercial oils. This creates new

opportunities for potential applications of this enzyme-assisted process. However, the high required ratio of the enzyme limits the economic potential. Further improvement might be required by coupling enzymatic degradation to other techniques such as microwave or ultrasonic extraction.

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