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The Effect of Refining Process on the Conjugated Dienes in Soybean Oil

Z. Bachari-Saleh[1]*, H. Ezzatpanah[1], M. Aminafshar[2], and H. Safafar[3]

ABSTRACT

The present study was carried out to investigate the effect of various refining processes' conditions on conjugated dienes in soybean oil. Conjugated dienes contents of soybean oil were found to undergo reduction in neutralization and bleaching steps while being increased in deodorization. It was observed that alkali refining (neutralization) through 12 and 14 °Bé, bleaching with either acid activated clay or a mixture of acid activated clay plus activated carbon at 110˚C along with deodorization at 220˚C and at 4 or 8 mbar had the highest effect on reduction of conjugated dienes in soybean oil. Conjugated dienes were in good correlation with peroxide value, free fatty acids, *Totox* value and as well with *p*-anisidine value in each step. Overall, alkali refining proved to be the most effective step in reduction of conjugated dienes, free fatty acids and oxidative stability index. Peroxide value, *p*-anisidine and *Totox* values changed during refining and got significantly decreased in deodorization.

Keywords: Conjugated dienoic acid, Edible oil, Oil processing, Peroxide value, *p*-Anisidine value.

INTRODUCTION

Oils and fats have been consumed by humans in their food, and have a variety of other applications since prehistoric times. Soybean, an annual oil seed crop with low oil content, still contributes the largest share to vegetable oil production throughout the world. Soybean oil is obtained from soybeans (*Glycine max)* grown in some several countries of the world. Soybean oil, high in Polyunsaturated Fatty Acids (PUFAs), both linoleic and linolenic, has become a popular vegetable oil for use in foodstuffs due to its nutritional qualities, abundance, economy, and desirable functionality (O'Brien, 2004).

Autoxidation or oxidative rancidity is the major cause of quality loss in crude and as well in refined oils during their storage. Oxidative stability and as well a deterioration of oils depend on their initial composition, concentration of minor compounds of either antioxidant or prooxidant characteristics, degree of processing, and their storage conditions. The outcome of oxidation is the development of unpleasant tastes and odors accompanied by a degradation of functional and nutritional properties (Crapiste *et al.,* 1999). One of the methods employed in improvement of quality of oils is their chemical refining, including neutralization, bleaching and deodorization that help remove the undesirable compounds, providing a product acceptable to consumers.

During the autoxidation process, lipid free radicals are formed with the original pentadienoic double bond systems being

[1] Department of Food Science and Technology, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Islamic Republic of Iran.
*Corresponding author; e-mail: Zahra.Saleh1@yahoo.com
[2] Department of Animal Science, Faculty of Agriculture and Natural Resources, Science and Research Branch, Islamic Azad University, Tehran, Islamic Republic of Iran.
[3] Technical Manager, Olive Oil Section, Techno Azma Laboratory, Adl Blvd, Poonak Sq., Tehran, Islamic Republic of Iran.

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shifted into two isomeric derivatives containing two conjugated double bonds. Edible fats and oils do not absorb ultraviolet radiation at wavelengths higher than 210 nm because they either do not contain any conjugated dienoic systems or only in trace amounts. The conjugated dienoic compounds dissolve in isooctanes, absorb ultraviolet radiation of a maximum at 233 nm (Pokorný *et al.,* 2005). These intermediate components react with molecular oxygen to form conjugated hydroperoxides. From linoleic acid, three isomeric hydroperoxides can be formed. In this mixture of 9, 11, and 13 hydroperoxides, the conjugated ones occur in greatest quantity because of their more stable forms. Six isomeric hydroperoxides can be expected from the oxidation of linolenic acid (Deman, 1999). The Conjugated Dienes (CDs) method measures primary oxidation products that occur during the oxidation of fats and oils, with the value being fairly well in correlation with Peroxide Value (PV). The *CDs* method offers several advantages over the PV method as it is faster and simpler, requiring no chemical reagents. It does not depend upon a chemical reaction or color development, and can be conducted on small (about 100 mg) sample sizes. The CDs method can also be employed to measure changes in oils held at frying temperatures (about 180˚C). Unlike peroxides, which are very unstable at high temperatures and cannot be used as a progressive measure of high-temperature oxidation, *CDs* form and accumulate rapidly in oils containing PUFAs.

Double bonds can also shift to form *CDs* during the hydrogenation or deodorization of oil if the temperature rises above 245˚C (Dutton, 1995). In hydrogenation 0.2-1.9% *CDs* can be produced in margarines and shortenings (Chow, 2007). However, some incoherent cases have also been detected, for example the increasing and sometimes decreasing of *CDs* in bleaching of olive oil (Boskou, 2008). In this study the *CDs* of soybean oil were evaluated under several conditions of refining. Then for the step with the lowest *CDs* content, other parameters like FFAs, *PV*, *p-AV*, *Totox* value, Oxidative Stability Index (OSI) and *Cox* value (Calculated oxidizability) were assessed, with correlation between *CDs* and these factors evaluated. This is a first study reporting the changes of *CDs* in soybean oil during the process refining.

MATERIALS AND METHODS

Crude degummed soybean oil was obtained from a commercial refinery and subjected to different processing steps in sequence. The processed oil samples were stored at -8˚C in an incubator until later analysis. The needed solvents and chemicals were purchased from Merck, Germany. Refining processes were carried out at refinery pilot lab of the Agro-industry Complex and Veg. Oil of Mahidasht, Kermanshah, Iran.

Neutralization

For the neutralization process, oil samples were mixed with 12, 14 and 16 °Bé (degree Baume) NaOH solution at 85-90˚C. For the removal of residual soaps, the samples were washed with distilled water and then dried at 100˚C in an oven (Farhoosh *et al.,* 2009).

Bleaching

Neutralized oil sample of the lowest *CDs* content was subjected to bleaching. Bleaching was carried out with two types of bleaching earth: Acid Activated (AA) clay and a mixture of AA plus activated carbon (AC) (1% w/w) at three temperatures (90, 100 and 110˚C) at 750 mmHg.

Deodorization

Neutralized and bleached oil samples of the lowest CD contents were subjected to deodorization process at three temperatures of 220, 240 and 260˚C, and three pressure

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conditions of 2, 4 and 8 mbar, respectively, for 1 hour .

Fatty Acid (FA) Composition

Methyl esters of fatty acids were prepared according to the AOCS Official Method Ce 2-66 (Firestone, 1994). Solution of oil in hexane (0.2 g in 7 mL) with 0.2 mL of methanolic potassium hydroxide was shaken and refluxed at 50˚C for 20 minutes. FA methyl esters were separated and dried over anhydrous sodium sulfate then analyzed through Agilent 6790N gas chromatograph equipped with a FID detection and a BPX70 column (120 m length×0.25 mm id) coated with 70% cyanopropyl polysilphenylene-siloxane(Agilent Technologies, USA). Nitrogen was used as a carrier gas with a flow rate of 1 mL min^{-1} and a pressure of 310×10^6 Pa. The injector, oven and detector temperatures were maintained at 250, 188 and 300˚C, respectively. The injection volume amounted to 1 µL.

p-Anisidine Value (p-AV)

p-AV was determined according to the AOCS Official Method Cd18-90 (Firestone, 1994). The oil sample (0.5–4.0 g) was diluted to 25 mL with isooctane in a volumetric flask. The oil solution (5 mL) was mixed with 1 mL of 0.25% *p*-anisidine in glacial acetic acid and, after 10 minutes, the absorbance at 350 nm was red using a spectrophotometer UV-2550 (Shimadzo, Japan) against a reagent blank. The *p-AV* is given by the formula:

$p\text{-}AV= 25\times(1.2A_s-A_b)/m$

Where, A_s= Absorbance of the oil solution after reaction with the *p*-anisidine reagent, A_b= Absorbance of the oil solution alone, and *m*= Mass of the sample (g)

Conjugated Dienes (CDs)

CDs were determined according to the AOCS Official Method Ti 1a-64 (Firestone, 1994). The sample (90–130 mg) was dissolved in 75 mL of purified isooctane. The flask was rotated and warmed to have the sample completely dissolved. After the sample being cooled to room temperature, isooctane was added to volume with thorough mixing and to give a final concentration of about 0.5 g L^{-1} to soybean oil. The absorbance of the solution (at 233 nm) was determined using a matching cell containing only pure solvent for the blank. The *CD* value was reported as the percentage of conjugated dienoic acid and was calculated from the formula:

Conjugated dienoic acid (%)= 0.84 (A_s /bc–K_0)

Where, K_0= Absorptivity by acid or ester groups, A_s= Observed absorbance at 233 nm, *b*= Cell length (cm), and *c*= Concentration of sample in g L^{-1} of the final dilution used for the absorption measurement.

Oxidative Stability Index (OSI)

The *OSIs* of the oil samples were studied according to the AOCS Official Method Cd 12b-92 (Firestone, 1994) with the Metrohm Rancimat model 679 (Switzerland). The oil sample (2 g) was placed in a reaction cell and kept at a constant temperature (120˚C) with an air flow of 20 L h^{-1} through the oil. The purged air stream was bubbled into distilled water (60 mL) in a bottle equipped with a conductivity electrode. The conductivity data of the heated oil sample was plotted against time. This data was stored electronically while the conductivity being periodicaally checked. A baseline slope was generated when a rapid change in conductivity was detected. After the conductivity rise plateaus, a second slope was established. The intercept of these two slopes, derived automatically by the Rancimat software, was reported as the induction period or OSI in hours for the oil.

Peroxide Value (PV)

PV of oil samples was determined according to the AOCS Official Method Cd 8-53 (Firestone, 1994). The assay included the accurate weighing of the sample usually 5 g, dissolution of oil in mixture of acetic

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acid–chloroform (3:2, v/v), addition of saturated potassium iodide, incubation for exactly 5 minutes, and titration with sodium thiosulfate using starch solution as indicator. *PV* was calculated from the formula:

PV (meq) 1000/g sample = (S – B)(N) 1000/g of sample

Where, *S*= Titration figure of the sample, *B*= Titration figure of the blank, *N*= Normality of sodium thiosulfate solution.

Free Fatty Acids (FFAs)

FFAs were determined according to the AOCS Official Method F 9a-44 (Firestone, 1994). The oil sample (5 g) was mixed with 30 mL of neutral ethyl alcohol in a conical flask and titrated against standard KOH solution using phenolphthalein as an indicator. FFA was calculated through the following formula;

FFA (% as Oleic acid)= [(Volume of KOH used×Normality of KOH×Molecular weight of oleic acid×100)/(Weight of sample in g×1000)].

Calculated Oxidizability (Cox) value

The *Cox* value for the oil samples was calculated as based on the percentage of unsaturated C18 fatty acids and as follows (Farhoosh *et al.,* 2008):

Cox value (Calculated oxidizability)= (1(18:1%)+10.3(18:2%)+21.6(18.3%))/100

Totox Value

Totox value= p-Anisidine value+2×(Peroxide value)

Statistical analysis

All the determinations were carried out in quintuple, the data being subjected to Analysis of Variance (ANOVA). Analysis of variance and regression analyses were performed, applying SPSS16 software. Significant differences between means were determined through Duncan's Multiple Range Tests. *P* values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Table 1 shows the effect of various refining conditions on the content of *CDs*. The neutralization process was found to be the most effective step in the removal of *CDs* (about 42%). Neutralization treatments with 12 *vs.* 14 °Bé of alkali did not differ significantly and rendered the most effective result in reduction of *CDs*, but, an increase in the alkali concentration led to the

Table 1. Conjugated dienes (%) in refining steps of soybean oil [a].

Step		Conjugated dienes	Step		Conjugated dienes
Crude	--	0.68±0.01		T_1V_1	0.94±0.05b
	°Bé $_1$	0.39±0.02a		T_1V_2	0.81±0.01a
Neutralized	°Bé $_2$	0.41±0.03a		T_1V_3	0.81±0.02a
	°Bé $_3$	0.44±0.01b	Deodorized[b]	T_2V_1	0.96±0.04b
	T_1, AA	0.39±0.08a		T_2V_2	0.94±0.24b
Bleached	T_2, AA	0.37±0.03a		T_2V_3	0.93±0.04b
	T_3, AA	0.34±0.01b		T_3V_1	1.11±0.07c
	T_1,AC	0.37±0.01a		T_3V_2	1.06±0.05c
	T_2, AC	0.36±0.02a		T_3V_3	1.10±0.08c
	T_3, AC	0.34±0.02b			

[a] Mean±Standard deviation, within each step, with the same lowercase letters are not significantly different at P< 0.05. °Bé $_1$= 12 degree Baume, °Bé $_2$= 14 degree Baume, °Bé $_3$= 16 degree Baume. In the bleached step T_1 = 90˚C, T_2=100˚C, T_3=110˚C; *AA*= Acid Activated clay, *AC*= Mixture of AA and Carbon active. In the deodorized step T_1= 220˚C, T_2 = 240˚C, T_3= 260˚C; V_1= 2 mbar, V_2= 4 mbar, V_3 = 8 mbar.

[b] Bleached sample by AA is used for deodorization.

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formation of more *CDs*. Conjugated dienes are more stable than unconjugated ones (Deman, 1999) and do not decompose with neutralization temperatures. The reduction in the *CDs* contents might be attributed to the use of alkali solution and removal of soap stock. The alkaline treatment of linoleic and linolenic acids may cause positional isomerization and could increase the level of *CDs* (Dutton, 1995). The selection of suitable concentrations of alkali solutions exerts a great effect on oil quality. Therefore, during alkali refining, a minimum level of alkali along with a low temperature are more effective in the reduction of *CDs*. In this research, alkali refining took 35 minutes; however, alkali refining when continuous takes a shorter time (3-10 minutes); so a shortening of the process time of neutralization may be also a valuable approach in this regard (Shahidi, 2005).

The neutralized soybean oil of alkali with 12 °Bé which resulted in a minimum level of *CDs*, was subjected to bleaching process. In the bleaching step, the temperature treatment of 110˚C differed significantly ($P \leq 0.05$) (Firestone, 1994) as compared with 90 and 100˚C causing a maximum reduction of *CDs*. However, the use of different types of bleaching earths employed exerted no significant ($P \geq 0.05$) effect on the content of *CDs*. In general, the bleaching step reduced *CDs* by about 7% (Table 1). In the bleaching step, color, soaps, phospholipids, and metals are assumed removed through the adsorption, but the reduction occurring in the level of peroxides might be attributed to their decomposition (Taylor, 2005). *CDs* have hydroperoxide-like structure so their content is decreased in the same way as occurred in peroxide reduction. This can account for ineffectiveness of bleaching earth on *CDs*' contents. An upper temperature intensifies decomposition, resulting in the reduction of *CDs*, but at temperatures above 150˚C isomerization occurs with *CDs* being increased. Moreover, *p-AV* begins to increase at temperatures above 110˚C as an indication of oxidative damage to oils (O'Brien, 2004). Because the effect of different types of bleaching earth had no significant effect on the content of *CDs* and also because of the high cost of AC, bleached samples through AA clay were selected. Oštrić-Matijašević *et al.* (1979) demonstrated the effect of joint bleaching and deodorization steps on the refining process of sunflower oil and found that when the bleaching earth was utilized, the oil was of a higher stability, tocopherol preserved, with formation of *CDs* at a minimum.

The neutralized and bleached soybean oil at 110˚C through AA bleaching earth was subjected to the deodorization process to investigate the effect of different processing conditions on the reduction of *CDs*. In the deodorization step an increasing trend in the formation of *CDs* (Table 1) was observed. The high temperature utilized in physical refining and deodorization can damage the oil by causing positional trans-isomerization and by increasing the level of *CDs* (Dutton, 1995; Taylor, 2005). The temperature 220˚C caused significant difference, ($P \leq 0.05$) increasing *CDs* in soybean oil to a minimal extent. Deodorization treatments under 4 and 8 mbar vacuums did not differ significantly as compared with 2mbar and caused the minimum increasing in CDs content(table 1). Therefore, it is concluded that the deodorization of soybean oil at 220˚C and at either 4 or 8 mbar can lead to the most desirable result for a minimum formation of *CDs*. Although temperature increase leads to an increase in positional isomerization and increase in *CDs*' content, an increase in vacuum leads to further removal of oxidation products, and hence a decrease in *CDs*.

Table 2 shows the *PV*, FFAs, *p-AV*, *Totox* value and *OSI* of the soybean oil following each processing step. The neutralization was done under atmospheric pressure and due to the occurrence of oxidation, all the parameters, except FFAs, increased. In the bleaching process, *PV* was considerably reduced (about 80%) and reached zero after deodorization. The reasonable reduction in such oxidation parameters as *PV, p-AV*, and

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Table 2. Oxidation factors of the soybean oil during the refining steps [a].

Process	*PV* [b]	FFA [c]	*p-AV* [d]	*Totox* value	*OSI* [e]
Crude	1.79±0.09	0.918±0.008	4.50±0.10	8.10±0.30	16.84±0.32
Neutralized	2.89±0.11	0.027±0.004	10.59±0.18	16.36±0.39	3.28±0.30
Bleached	0.23±0.21	0.044±0.003	6.20±0.30	6.67±0.71	8.99±0.27
Deodorized	0.00±0.00	0.037±0.008	2.72±0.22	2.72±0.22	8.98±0.15

[a] Mean±Standard deviation of quintuplet determinations; [b] Peroxide Value (meq O_2 kg^{-1} oil). [c] Free Fatty Acids (% as oleic acid); [d] *p*-Anisidine Value (mmol kg^{-1} oil), [e] Oxidative Stability Index (h).

Cox value in the bleaching and deodorization steps might be due to the vaccum-steam distillation during deodorization in the process of which the volatile oxidation products are removed. Bleaching under vacuum and AA bleaching earth eliminated hydroperoxides, generating secondary oxidation products (O'Brien, 2004). Hopia (1993) reported that *PV* of vegetable oils practically reached zero following bleaching, whereas deodorization had no effect on the *PV* in the bleached oil. Our findings are contrary to the results of Farhoosh *et al.* (2009) who reported that *PV* increased significantly in commercial refining of soybean oil upon neutralization, but did not chang significantly in the bleaching step. In the present research, *PV* did not reach zero upon bleaching, because a complete vacuum was not reached in this step.

The crude soybean oil FFAs were 0.92% and reached their lowest level of 0.027% after neutralization (about 98% reductions) and being much not changed in the other steps (Table 2). The reason for this reduction in the neutralization is that FFAs are saponified with alkali solution and separated along with the soap stock. In the bleaching process there was a little increase observed in the FFAs, because AA clay splits soap residues promoting hydrolysis and increasing FFAs. In the deodorization step FFAs decreased *via* vacuum-steam distillation.

Crude oil is of a high oxidative stability as a result of the high level of natural antioxidants and phosphatides that have synergistic effects on antioxidants (Taylor, 2005). However, upon neutralization, these compounds are removed and OSI reduced significantly ($P \leq 0.05$) (Table 2). Upon bleaching, *OSI* increased significantly because of the decomposition of hydroperoxides and *CDs* along with a removal of compounds that reduce oxidative stability (e.g. heavy metals). In the deodorization process *OSI* did not change much because most compounds affecting oxidative stability of oil were removed during the bleaching step.

Table 3 shows the fatty acid composition and *Cox* value of soybean oil upon the refining process. During refining a little change in fatty acids composition was observed. A slight decrease in linoleic and linolenic acids, as a result of oxidation, led to a slight relative increase in oleic acid. Although *Cox* value differs significantly throughout the refining steps but these changes are minor, thus changes in *CDs* did not much affect the *Cox* value. The greater the level of PUFAs in the oil, the greater the potential rise in *CDs*. PUFAs not being changed considerably, the changes of *CDs* were considered as not related to PUFAs during refining.

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Table 3. Fatty acids profile in soybean oil in the refining steps[a].

Step				
Fatty acid (%)	Crude	Neutralized	Bleached	Deodorized
C14:0	0.07±0.00a	0.07±0.00a	0.07±0.01a	0.08±0.01a
C16:0	10.3±0.02a	10.3±0.00a	9.90±0.10a	10.2±0.19a
C16:1	0.11±0.01a	0.11±0.00a	0.09±0.01a	0.10±0.00a
C18:0	4.23±0.11a	4.27±0.07a	4.20±0.10a	4.72±0.02b
C18:1	21.4±0.02a	21.5±0.02a	23.2±0.15b	22.0±0.74c
C18:2	54.2±0.01a	54.2±0.05a	54.5±0.04b	53.9±0.77a
C18:3	8.79±0.01a	8.79±0.00a	8.74±0.04a	7.86±0.04b
C20:0	0.36±0.01a	0.36±0.00a	0.42±0.02a	0.38±0.03b
Others	0.55±0.07a	0.42±0.07ab	0.37±0.13b	0.53±0.18c
Cox value[b]	7.69±0.00a	7.69±0.00a	7.72±0.01b	7.61±0.16c

[a] Mean±Standard deviation of quintuplet determinations within a row with the same lowercase letters are not significantly different at P< 0.05, [b] Calculated oxidizability.

Table 4 shows the correlation coefficients between *CDs* and oxidation parameters. *PV* showed reasonable correlation with *CDs* in neutralized and bleached oil, but there was no correlation observed as regards the deodorized oil. Correlation coefficient between PV and *CDs* during refining was -0.42. Gomez *et al.* (2004) indicating that *PV* and *CDs* are in high correlation in oxidation of olive oil under accelerated storage conditions (40–60°C) (r= 0.995). Farhoosh and Pazhouhanmehr (2008) demonstrated that *CDs* serve as a good indicator for oxidative stability, showing high correlation with *PV*, as was also observed in this work for each step of the refining process (Table 4). The correlation coefficient during refining was -0.77. Houhoula *et al.* (2002) reported the correlation coefficient of 0.998 between *CDs* and *p*-AV during their evaluation of deep frying of cottonseed oil. The high correlation between these factors indicates that if one factor is inaccessible in the control of the process, the other factor could be utilized. Because of poor correlation between *CDs* and the other factors during refining, a comparison of these indices should be done only individually and within each step.

A high *PV* always means poor quality but a low *PV* is not always indicative of a good quality of oil. Hamilton *et al.* (1998) claimed if significant peroxide breakdown has occurred in oxidation of the oil, the measurement of *PV* alone could give a misleading result; therefore to avoid any mistake it would be preferable to measure *p-AV* and *CDs* levels as well as secondary oxidation products in the oils during and past the induction period. It is also very important to consider such factors as those affecting production, storage and transport of crude oil, condition of soybean growing, damage to the soybean crops, type of the oil

Table 4. Correlation coefficients between *CDs* and oxidation factors in the refining steps.

	Oxidation parameters[a]			
Conjugated dienes	*p-AV*[b]	FFA[c]	*PV*[d]	*TOTOX*
Neutralized	0.908	0.745	0.865	0.924
Bleached	0.908	0.863	0.909	0.931
Deodorized	0.924	0.935	---	0.924
Total(during refining)	-0.77	0.21	-0.42	-0.63

[a] Mean±Standard deviation of quintuplet determinations; [b] *p*-Anisidine Value (mmol kg^{-1} oil); [c] Free Fatty Acids (% as oleic acid), [d] Peroxide Value (meq O_2 kg^{-1} oil).

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extraction, temperature and type of storage tank as well as the materials employed in the construction of the tanks (Codex Standard, Rev.3-2005).

CONCLUSIONS

It can be concluded from the study that the formation of *CDs* is well correlated with major oxidation parameters. The processing conditions involved in different refining steps contribute either directly or indirectly to the aging of oils. These factors and formation of oxidation products in the final material may be controlled by careful handling and optimization of the refining process to minimize the formation of such undesirable compounds as *CDs*.

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اثر فرایند تصفیه روغن بر دی ان های کنژوگه در روغن سویا

ز. باچاری صالح، ح. عزت‌پناه، م. امین‌افشار، و ح. صفافر

چکیده

در این تحقیق اثر شرایط مختلف در مراحل تصفیه بر مقدار دی ان های کنژوگه در روغن سویا مورد مطالعه قرار گرفت. خنثی سازی با محلول سود با ۳ درجه بومه ۱۲ و ۱۴ و ۱۶ انجام شد که بومه ۱۲ و۱۴ کمترین مقدار دی ان کنژوگه را تولید کردند. افزایش غلظت محلول سود باعث افزایش مقدار دی ان های کنژوگه در خنثی سازی شد. در رنگبری با ۳ دمای ۹۰ ، ۱۰۰ و ۱۱۰ درجه سانتیگراد و دو نوع خاک، خاک فعال اسیدی و مخلوط خاک اسیدی و کربن فعال، دمای ۱۱۰ درجه سانتیگراد کمترین دی ان های کنژوگه را تولید کرد اما نوع خاک اثری بر این ترکیبات نداشت. در بی بو با ۳ دمای ۲۲۰، ۲۴۰ و ۲۶۰ درجه سانتیگراد و ۳ مقدار خلا ۲، ۴ و ۸ میلی بار نه تنها کاهشی در مقدار دی ان های کنژوگه دیده نشد بلکه شاهد افزایش این ترکیبات بودیم. دمای ۲۲۰ درجه سانتیگراد و خلا ۴ و ۸ میلی بار باعث ایجاد کمترین افزایش در مقدار دی ان های کنژوگه شد. خنثی سازی موثرترین مرحله در کاهش این ترکیبات بود. دی ان های کنژوگه همبستگی خوبی با اسیدهای چرب آزاد، عدد پراکسید، عدد آنیزیدین و عدد توتوکس داشتند. عدد پراکسید و عدد آنیزیدین در طول فرآیند تصفیه تغییر کرده و در بی بو بیشترین کاهش را نشان دادند.

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