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A novel methodology for direct esterification of olives optimized through design of experiments

Metodologia para esterificação direta de azeitonas otimizada através do planejamento de experimentos

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Abstract

A novel methodology was proposed, and optimized using the Design Expert software, aiming to enable characterization of the fatty acid profile of olives whilst abolishing the lipid extraction step. Furthermore, the proposed method was proven more efficient whereas requiring less time, and sample and solvent amounts, consequently improving process yield. Optimum conditions obtained after experimental design were as follows: sonication temperature and time of 60 ºC and 8 minutes, respectively, and concentrations for alkaline and acid reactions of 0.70 and 1.5 mol L^{-1}, respectively. Total fatty acid content for olive sample was 172.0 mg g^{-1}, the predicted value was and is in the coefficient of variation range of 11.52%

Keywords: Olive. Design expert. Experimental planning. Fatty acids. Optimization of experiments.

Resumo

Uma nova metodologia foi proposta e otimizada utilizando o software Design Expert, com o objetivo de possibilitar a caracterização do perfil de ácidos graxos das azeitonas e ao mesmo tempo retirar a etapa de extração lipídica. Além disso, o método proposto mostrou-se mais eficiente, exigindo menos tempo, quantidade de amostra e solvente, consequentemente melhorando o rendimento do processo. As condições otimizadas obtidas após o planejamento experimental foram as seguintes: temperatura e tempo de sonicação de 60 ºC e 8 minutos, respectivamente, e concentrações para reações alcalinas e ácidas de 0.70 e 1.5 mol L^{-1}, respectivamente. O teor total de ácidos graxos para a amostra de azeitona foi de 172.0 mg g^{-1}, o valor previsto foi e está no intervalo de coeficiente de variação de 11,52%.

Palavras-chave: Oliveiras. Design expert. Planejamento experimental. Ácidos graxos. Otimização de experimentos.

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Introduction

The olive tree (*Olea europaea* L.) is a medium-sized tree from the Mediterranean basin that produces edible fruits called olives (CASTRO *et al*., 1997). Over 30 different olive varieties exist, all with a remarkable reputation for olive producers, once it is a raw material for olive oil extraction and/or canned olives production (PESTANA-BAUER; DUTRA; ZAMBIAZI, 2011). Among these varieties, the best adjusted to the Brazilian soil and climate are koroneike, arbosana, arbequine, and frantoio (COUTINHO; RIBEIRO; CAPPELLARO, 2009). Aberquine, this study subject, exhibits vegetative vigor, precocity, and high production capacity. Furthermore, despite its fruits being small, they present relatively high oil yields (20.5%) (BAKHOUCHE *et al*., 2013; BRUSCATTO *et al*., 2017).

Both oil and pulp present extremely valued chemical compositions due to the compounds presence contributing to the avoidance of cardiovascular, circulatory, neurological and cancerous diseases (WAHLE *et al*., 2004). Benefits associated with the presence of monounsaturated fatty acids, tocopherols, phenolic compounds, phytosterols and vitamins (BIANCO; RAMUNNO, 2006; ZEB; MURKOVIC, 2013).

Lipid extraction is an essential determination in the characterization of the raw material. Conventional fatty acid profiling methods consist of several steps; among it, lipid extraction with organic solvents, hydrolysis, methylation/derivatization, and, finally, analysis and quantification with gas chromatography (AOAC, 2017). The derivatization step involves converting all fatty acids that may be present in the sample into their corresponding volatile fatty acid methyl esters (FAMEs). This step can be performed by acid catalysis (BONDIA *et al*. 1994), by alkaline catalysis, or by a combination of both procedures (HARTMAN; LAGO, 1973).

The conventional processes hold several disadvantages, such as a high risk of contamination and recovery losses, moreover, these methods become unfeasible for analyses of large sample series, especially when the amount of sample is limited. To overcome such drawbacks, methods that simultaneously extract and derivate the fatty acids have been developed. Direct esterification has been previously used by other authors successfully to define the fatty acid composition of seeds, fruits, and fermented yogurt samples (PICCIOLI *et al*., 2019; SANTOS *et al*., 2018; SINOSAKI *et al*., 2019).

To properly develop a new methodology, investigating which parameters strongly influence the sample preparation is imperative. Therefore, it is essential to use an experimental design to achieve an accurate analysis through the assessment of interaction factors and a mathematical model enabling the optimization of the process by reducing steps, time, and cost.

Therefore, this work aimed to develop and optimize a methodology for direct esterification of olives from the arbequine variety through the usage of the Design Expert *software*. The proposed method efficiency was gauged through gas chromatography coupled with a flame ionization detector (GC-FID).

Materials and methods

Materials

Olives from the arbequine variety were used for analyses. Samples were acquired in March from Coxilha dos Cunhas (Canguçu, Rio Grande do Sul, Brazil, 31º23'42" S, 52º40'32" W). The selected fruits were ripe and free of spots and cracks. These olives were then cleansed under running water, milled, vacuum packed, and kept at -18 °C for further analysis.

Chloroform, methanol, n-heptane, sulfuric, and hydrochloric acids solvents and reagents were applied without further purification and purchased from Millipore Sigma (Darmstadt, Germany). Fatty acid methyl esters (FAMEs 189-19) standard mixture and methyl tricosanoate (PI 23:0me) were also purchased from Millipore Sigma (St. Louis, MO, USA).

Experimental design

A central composite design was built utilizing the Design Expert® 7 *software* to assess the influence of sonication temperature [(-) 40 °C, (+) 60 °C], acid and alkaline concentration [(-) 0.7, (+) 1.50 mol L^{-1}], and time to complete acid and alkaline reactions [(-) 5.0, (+) 8.0 min] on the fatty acid composition of Olives directly esterified. The design contains 47 experiments, from which 5 are central points required to define experimental errors, see Table 1.

Optimized Methodology (OM)

Approximately 100 mg of olives were weighed and added in 10 cm test tubes with 2 mL of $NaOH/CH_3OH$, placed in an Eco-Sonics Q 5.9/25 ultrasound bath (Unique, São Paulo, Brazil) with 100 W of power and 35 kHz.

Table 1 – Fatty acid content for each experimental run of the design.

Experiments	Temp. °C	Acid Conc. (mol. L^{-1})	Basic Conc. (mol. L^{-1})	Basic Time (min) (min)	Acid Time (min)	Concentration (mg g^{-1} of sample)
1	40.0	0.70	0.70	5.0	5.0	133.45
2	40.0	0.70	1.50	5.0	5.0	61.55
3	60.0	0.70	1.50	5.0	5.0	11.10
4	40.0	1.50	0.70	5.0	5.0	181.45
5	60.0	1.50	0.70	5.0	5.0	167.50
6	40.0	1.50	1.50	5.0	5.0	132.00
7	60.0	1.50	1.50	5.0	5.0	175.05
8	40.0	0.70	0.70	5.0	5.0	156.75
9	60.0	0.70	0.70	8.0	5.0	84.35
10	40.0	0.70	1.50	8.0	5.0	28.40
11	60.0	0.70	1.50	8.0	5.0	7.35
12	40.0	1.50	0.70	8.0	5.0	188.20
13	60.0	1.50	0.70	8.0	5.0	172.10
14	40.0	1.50	1.50	8.0	5.0	165.90
15	60.0	1.50	1.50	8.0	5.0	171.70
16	40.0	0.70	0.70	8.0	8.0	155.95
17	60.0	0.70	0.70	5.0	8.0	136.95
18	40.0	0.70	1.50	5.0	8.0	23.20
19	60.0	0.70	1.50	5.0	8.0	11.45
20	40.0	1.50	0.70	5.0	8.0	199.55
21	60.0	1.50	0.70	5.0	8.0	184.00
22	40.0	1.50	1.50	5.0	8.0	170.15
23	60.0	1.50	1.50	5.0	8.0	160.20
24	40.0	0.70	0.70	5.0	8.0	168.45
25	60.0	0.70	0.70	8.0	8.0	137.80
26	40.0	0.70	1.50	8.0	8.0	81.40
27	60.0	0.70	1.50	8.0	8.0	2.35
28	40.0	1.50	0.70	8.0	8.0	194.40
29	60.0	1.50	0.70	8.0	8.0	186.10
30	40.0	1.50	1.50	8.0	8.0	212.85
31	60.0	1.50	1.50	8.0	8.0	171.20
32	40.0	0.70	0.70	8.0	8.0	138.90
33	26.2	0.70	0.70	6.5	6.5	155.60
34	73.8	0.70	1.50	6.5	6.5	90.20
35	50.0	0.15	0.15	6.5	6.5	50.35
36	50.0	2.05	2.05	6.5	6.5	9.30
37	50.0	1.10	1.10	6.5	6.5	150.75
38	50.0	1.10	1.10	6.5	6.5	176.05
39	50.0	1.10	1.10	2.93	6.5	168.65
40	50.0	1.10	1.10	10.1	6.5	154.05
41	50.0	1.10	1.10	6.5	2.9	170.70
42	50.0	1.10	1.10	6.5	10.1	151.20
43	50.0	1.10	1.10	6.5	6.5	153.40
44	50.0	1.10	1.10	6.5	6.5	129.30
45	50.0	1.10	1.10	6.5	6.5	168.50
46	50.0	1.10	1.10	6.5	6.5	154.85
47	50.0	1.10	1.10	6.5	6.5	168.65

Source: The authors.

Different temperatures, and alkaline concentration and reaction times were evaluated, according to the experimental model. Upon completing the alkaline reaction, 2 mL of H_2SO_4/CH_3OH were added to the mixture and temperatures, and acid content and reaction times were assessed based on the experimental design. Lastly, 1 mL of hexane and 500 μ L of an internal standard (PI, 23:0) were added. The tubes were then shaken for 30 s and centrifuged at 2000 rpm for 1 min. The organic phase was collected for further analysis in the GC-FID.

Gas chromatography analysis with flame ionization detector

Chromatographic analysis was performed on a Thermo Scientific GC equipped with FID, split/splitless injector and CP-7420 fused silica capillary column (Select FAME, 100.0 m long, 0.25 mm internal diameter and 0.25 μm thin flam of cyanopropyl as stationary phase 165 °C. The operation parameters were as follows: column temperature was set at 165 °C for 18 min, ramped to 235 °C (4 °C min^{-1}), and kept stable for 20 min. The injector and detector temperatures were kept at 230 and 250 °C, respectively. Gas flows were 1.2 mL min^{-1} for carrier gas (H_2), 30.0 mL min^{-1} for make-up gas (N_2), and to feed the FID 30.0 and 300.0 mL min^{-1} of H_2 and synthetic air, respectively. The samples were injected in split mode, with a 1:40 ratio. The injection volume was 1.0 μL. FAMEs were identified by comparing the retention times for the samples' constituents with those of a Sigma FAME standard (FAMEs 189-19). A theoretical correction factor was used and calculated to obtain FA concentrations according to Visentainer *et al*. (2014). FA content was calculated in mg $g^{-1}$of sample. FID correction is necessary due to the signal magnitude being related to the number of C+ bonded to hydrogen atoms (PICCIOLI *et al*., 2019).

Optimized Validation Conditions

To validate the proposed method, a numerical optimization technique was applied to define the optimal conditions and linear regression equation for the proposed methodology. Accuracy and precision were calculated to evaluate the analytical method following the International Conference on Harmonization guidelines using the five central point replications.

Results and discussion

Model optimization

Optimization of the method proposed for direct methylation for arbequine olives was performed gauging the effect of five independent variables: Temperature, acid and alkaline concentration, and time for both reactions. For that matter, a central compound experimental design was generated to define which conditions would improve the total fatty acid content. It can be seen from data in Table 1 that experiment 30 provided the highest FA sum, fatty acids sum, (212.85 mg $g^{-1}$of sample) hence indicating the optimal conditions for the method.

The experimental data were analyzed in different models (cubic, interactive, linear, and quadratic) to verify which would provide the finest result. It can be seen from the data in Table 1, the cubic model best represents the concentration of the fatty acids in olives.

To assess experimental results and interactions between factors, data were submitted to analysis of variance (ANOVA) and a 3-D response surface generated automatically by Design Expert® 7 *software* using data from Table 1. Table 2 presents the intercorrelations among factors used to build the experimental design. F-value for the model is significant. The probability that acquired results occurred due to noise is 0.01%.

The second order polynomial equation for FA sum, defined by equation (1):

$$\begin{aligned}\text{FA sum} = {} & 157.45 + 29.75(\text{C}) + 9.30(\text{AC}) + 21.75(\text{BC}) \\ & - 15.75(\text{B}^2) - 14.00(\text{C}^2) - 6.85(\text{ABE}) \\ & - 20.50(\text{A}^2\text{B}) + 19.25(\text{A}^2\text{C}) - 16.35(\text{AB}^2), \qquad (1)\end{aligned}$$

was employed to predict results by multiple regression analysis of data. Good values were obtained for the correlation coefficient (R^2, 0.98) and coefficient of variation (11.50%).

The adjusted coefficient value (R^2, 0.94) is close to the correlation coefficient indicating an optimal relationship between predicted values and experimental response (MARAN; PRIYA, 2016). Furthermore, the terms C, AC, BC, B^2, C^2, ABE, A^2 B, A^2 C, and AB^2 had values for "Prob > F" inferior 0.0500 indicating that the model is significant. Values greater than 0.100 indicate that model terms are not significant. Concerning the pure error, the F value (0.79) for lack of adjustment indicates that it is not significant and there is a 60.75% of chance that the lack of adjustment of F value may be greater due to noise.

Table 2 – ANOVA test results for statistical significance of the model for Optimized Methodology (OM).

	Square aum	LD[a]	Square	F value	Prob>F[b]	
model	1.669x10^{+5}	35	4769.31	7.35	0.0100	S[f]
C	10004.56	1	10004.56	24.13	0.0100	-
AC	2391.55	1	2391.55	10.13	0.0079	-
BC	13053.97	1	13053.97	24.77	0.0100	-
ABE	1291.74	1	1291.74	5.47	0.0375	-
B2	13221.00	1	13221.00	6.93	0.0100	-
C2	10480.61	1	10480.61	44.38	0.0100	-
A2B	3376.05	1	3376.05	14.30	0.0026	-
A2C	2981.22	1	2981.22	12.62	0.0040	-
AB2	2149.54	1	2149.54	9.10	0.0107	-
Residue	2833.74	12	236.15	-	-	-
Lack of adjustment	1253.06	6	208.84	0.79	0.6074	N[g]
Pure error	1580.69	6	263.47	-	-	-
Total core	1.698x10^{+5}	47	-	-	-	-
Model	1.669x10^{+5}	35	4769.31	7.35	0.0100	S[f]

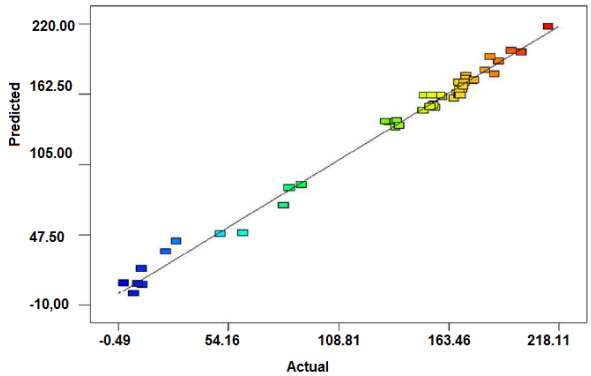
[a]LD: Degree of freedom;
[b]Prob>F: Probability value associated with F value;
[c]A: NaOH concentration;
[d]B: H2SO4 concentration;
[e]ACD: Interaction between the concentration of NaOH, reaction time of NaOH, and reaction time of H_2SO_4, respectively;
[f]S: significant;
[g]NS: non-significant.

Source: The authors.

Proper accuracy measures the signal to noise ratio, a ratio greater than 4 is desirable, for this design the ratio of 16.50 indicates that the signal is adequate and the model was significant for direct esterification.

Figure 1 illustrates the relationship between predicted values and actual values of the experimental design. Moreover, the figure shows the proximity of actual values to predicted values, exhibiting the model authenticity.

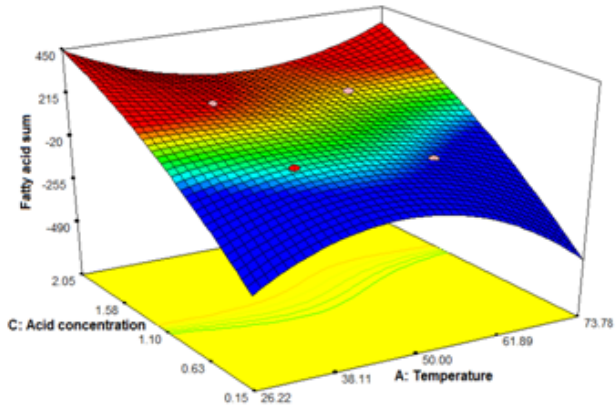
Figure 1 – Predicted model values vs. experimental values for the proposed delineated experiment.



Source: The authors.

Figure 2 presents a three-dimensional surface graph that illustrates the relationship between temperature and acid concentration used in the derivatization to demonstrate which combination provides the largest FA sum, equation (1).

Figure 2 – Effect of the interactions between variables A (temperature) and C (Acid concentration) on the yield of the concentration of fatty acids.



Source: The authors.

A closer inspection of Figure 2 demonstrates that at 40 ºC there is a minimum and a maximum related to experiments 26 and 30, respectively. For these experimental runs, the acid concentration is modified, as shown by Table 1.

The alkaline catalysis using NaOH/MeOH aims to activate transesterification by transforming the free fatty acids possibly present in the samples into fatty acid methyl esters since this initial procedure does not cause polyunsaturated fatty acids oxidation and acid catalysis in the next step causes the transesterification of the remaining acylglycerols and the high concentration was strictly important to obtain the largest FA sum, equation (1). Likewise, at 60 ºC it can be observed, mainly between experiments 27 and 31, that the high acid concentration was primordial to acquire the highest fatty acid concentration. Thus, upon evaluation of the results for the 47 experimental runs generated by the experimental design, it was defined that the conditions used for experiment 30 provided the optimal results. The following conditions were used: Temperature of 40 °C, alkaline and acid concentrations of 1.50 mol L^{-1}, a sonication time of 8 min for acid and basic reactions.

Optimization of methylation variables

Through the design expert software, it was possible to optimize the methylation variables. The ideal conditions for obtaining the olive's fatty acid composition through the methylation reaction were selected with a desirability value of 0.98, using the following parameters: sonication temperature of 60 °C, acid/basic concentration of 1.50 mol L^{-1}, and acid and basic time of 8.0 min. The predicted concentration value was 171.55 mg g^{-1} of sample. From Table 3 we establish that the result obtained for OM under these conditions was 171.86 mg g^{-1} of sample, thus, the result is within the limit of the coefficient of variation.

Accuracy values ranged from 99.00 % to 108.80 % and are within acceptable values. The $RSD_{intra-day}$ (2.60%) and $RSD_{intertra-day}$ (5.10%) values show that the optimization had good accuracy and are within the allowable limits (RIBANI *et al*., 2004).

Application of the methodology to other olive varieties

Table 4 demonstrates the application of the optimized methodology for four other olive varieties: arbequine, arbosana, koroneiki, and frantoio.

Table 4 presents the results for fatty acid composition of each olive variety. Critical evaluation of this table indicates that the optimized method provided the highest relative fatty acid percentage, which corresponds to that encountered in the literature, signaling the efficiency of the proposed method.

Table 3 – Fatty acid quantification from optimized methodology (OM).

NC/BD	FA (mg g^{-1} of sample)
16:00	26.15 ± 0.5
16:1n-9	2.25 ± 0.05
17:00	0.40 ±0.05
18:00	3.05 ±0.2
18:1n-9	126.90 ±0.10
18:2n-6	9.55 ±0.02
18:3n-3	1.25 ±0.10
20:00	0.75 ±0.5
20:1n-9	0.55 ±0.08
24:00	0.85 ±0.05
SFA	31.20 ± 1.05
MUFA	129.85 ± 0.25
PUFA	10.80 ± 0.15
FA sum	171.85 ± 1.45

FA sum= fatty acids sum;
SFA = saturated fatty acids sum;
MUFA = monounsaturated fatty acids sum;
PUFA = polyunsaturated fatty acids sum;
NC/BD= carbon number/double bond.

Source: The authors.

Ten fatty acids from olive samples were identified, five were SFAs, three MUFAs, and two PUFAs. The high content of monounsaturated fatty acids sum reflects the oleic acid (18:1n-9) value, which is the major acid in all varieties, oscillating from 70.75% to 73.20%. Borges *et al*. (2017) found approximate oleic acid amounts (63.00% - 79.00%) in oil extracted from Arbequina olives. Regarding saturated fatty acids (SFAs), palmitic acid (16:0) represents the highest concentration (16%), mainly in the arbequine and arbosana varieties and this result was comparable to the results found by Mansouri *et al*. (2019) and finally, the polyunsaturated fatty acids (PUFAs) with the lowest concentration, ranging from 7.95% to 10.00%, with linoleic acid (18:3) in greater proportion, mainly in the frantoio variety.

Moreover, the cost/benefit advantages for obtaining the fatty acid composition through the optimized methodology is pronounced, since it is not necessary to perform the extraction for later methylation as was done by Borges *et al*. (2017) and Mansouri *et al*. (2019), that is, sample preparation is unnecessary, hence eliminating one step (extraction), reducing time, solvent, and sample amount.

Table 4 – Relative percentage of fatty acid composition of olive varieties.

Fatty acid composition	Arbequina	Arbosana	Koroneiki	Frantoio
16:0	16.10^{a}	16.20^{a}	14.00^{c}	15.00^{b}
16:1	1.45^{b}	1.60^{a}	1.10^{c}	1.25^{c}
17:0	0.25^{b}	0.25^{a}	0.20^{c}	0.25^{bc}
18:0	1.70^{c}	1.80^{b}	2.10^{a}	1.60^{d}
18:1	71.30^{b}	70.75^{c}	73.20^{a}	71.20^{b}
18:2	6.60^{a}	7.20^{b}	6.60^{c}	9.00^{a}
18:3	1.72^{a}	1.35^{b}	1.35^{b}	1.00^{c}
20:0	0.40^{a}	0.35^{c}	0.35^{c}	0.30^{b}
20:1	0.30^{a}	0.25^{b}	0.30^{a}	0.30^{a}
24:0	0.20^{b}	0.20^{b}	0.20^{a}	0.10^{c}
SFA	18.65^{b}	18.85^{a}	17.45^{c}	17.25^{d}
MUFA	73.07^{b}	72.65^{c}	74.60^{a}	72.75^{c}
PUFA	8.30^{c}	8.55^{b}	7.95^{d}	10.00^{a}

Results expressed as mean ± standard deviation, n=3;
SFA - total saturated fatty acids;
MUFA - monounsaturated fatty acids;
PUFA - polyunsaturated fatty acids.

Source: The authors.

Conclusion

Usage of the design expert software enabled the optimization of a novel direct methylation technique suited for olives, to acquire their fatty acid composition whilst eliminating the need for an extraction step. Besides, the method has proven to be more efficient while decreasing operational costs and time required for sample preparation, consequently improving process yield. The novel method required the following conditions: Temperature of 40 °C, alkaline and acid concentrations of 1.50 mol L^{-1}, a sonication time of 8 min for acid and basic reactions. Under theses condition the maxium yield totaled 171.85 mg g^{-1} of sample.

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