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EVALUATION OF CAPRYLINS BY THIN LAYER CHROMATOGRAPHY

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ABSTRACT

This work aims to demonstrate the applicability of thin layer chromatography (TLC) coupled with the JustTLC 4.0.3 software for the evaluation of caprylins (mono-, di- and tricaprylin) in concentrated organic extracts obtained from the esterification of glycerol (G) with caprylic acid (CA). Caprylins are medium chain fatty acid acylglycerols with applications in food, pharmaceutical and cosmetic industries. Esterification reactions catalyzed by 2.5g of Lipozyme RM IM® or P:1X2-400 (Palatase® immobilized on DOWEX® resin) were conducted at 400 rpm and CA/G molar ratio of 1:1 for 6 h or 10 h. Monocaprylin (50%), dicaprylin (18%) and the total yield (69%) of the esterification catalyzed by Lipozyme IM® were independent of reaction duration. The esterification catalyzed by P:1X2-400 led to a yield of 55% and to the formation of monocaprylin (31%), dicaprylin (24%), and tricaprylin (5%).

KEYWORDS: Thin layer chromatography, monocaprylin, dicaprylin, tricaprylin, caprylic acid.

1. INTRODUCTION

Caprylins are esters of glycerol with caprylic acid comprising the following compounds: 1-monocaprylin and 2-monocaprylin (2,3-dihydroxypropyl octanoate and 1,3-dihydroxypropyl octanoate, respectively), 1,2-dicaprylin and 1,3-dicaprylin (3-hydroxy-2-octanoyloxypropyl octanoate and 2-hydroxy-3-octanoyloxypropyl octanoate, respectively), and tricaprylin (2,3-

di(octanoyloxy)propyl octanoate), all belonging to the class of medium chain fatty acid glycerides.[1]

Caprylins are used in the pharmaceutical, food and cosmetic industries. Monocaprylin is used for food conservation due to its antimicrobial action against *E.coli 0157:H7, Salmonella spp.* and *Lysteria monocytogenes*.[2][3] The application of monocaprylin in textiles aiming to eliminate the proliferation of bacteria and fungi during storage was also suggested.[4] Dicaprylin is used as an emulsifying agent in cosmetic and drug formulations, whereas tricaprylin is used as a supplement in diets for infants and for patients with intestinal disorders.[5][6] Moreover, a possible use of tricaprylin as an adjuvant drug to minimize some deleterious symptoms of Alzheimer's disease was also described.[7]

Caprylins can be obtained mainly by glycerolysis or hydrolysis of triglycerides, and by direct esterification of glycerol with caprylic acid in the presence of chemical or enzymatic catalysts.[8][9][10][11] Independent from the process used, the final reaction medium will contain a mixture of reactants, mono-, di- and tricaprylin. Each component must be evaluated individually.

The quantification of lipids has been determined by thin layer chromatography (TLC), gas chromatography (GC), and high-pressure liquid chromatography (HPLC). All involve the partition of lipid molecules into a mobile phase (gas or liquid), and a stationary phase (liquid or solid). Traditionally, the TLC has been used for detection, whereas GC and HPLC have been used for quantification. However, an interconnection between TLC and GC or HLPC could be obtained by preparative analysis, i.e., the separation of a mixture of lipids by TLC followed by a plate treatment (mainly by drying and color development methods), scratching of the spot from the silica layer, treatment of the scratched material with a proper solvent and, finally, quantification of lipids by GC or HLPC.

TLC is the oldest chromatographic technique used for lipid analysis, mainly for its detection. Notwithstanding, during the last century, GC and HPLC emerged and have developed into major techniques for quantification of a wide range of compounds, including lipids. These techniques have a high sensitivity, accuracy, resolution, and reproducibility, which led TLC to become an obsolete process. Nevertheless, several features of TLC have improved over the last years. Such improvements have caused a return of the use of this technique. Currently, there are high quality adsorbing materials available, such as pre-coated plates, and every

method step – application of samples, running development, and plate revelation – can be automatized.[12] Moreover, TLC is a low-cost and quick analytical tool because it requires less disposable material and a simple equipment when compared to GC and HLPC.

The quantitative aspect of thin layer chromatography has also been improved mainly by the improvement of reading methods that allow the measurement of spot intensity directly from the plates. For instance, it is possible to use a scanner coupled to an adequate software to perform the reading.[13] Recently, the software JustTLC 4.0.3,[14] developed to treat images obtained from TLC plates, became available in the market. This software has been used to evaluate carbidopa/levodopa and oleic acid derivatives (mono-, di- and triolein).[15][16]

Considering such improved features, TLC has become an important alternative technique when a high volume of qualitative and quantitative analyses is needed, for instance, during an analytical screening procedure.

JustTLC 4.0.3 treats digital images (tif, jpg, jpeg, among others) considering the color intensity and the area occupied by each spot printed on the TLC plate. As the software works only with monochromatic images, the spots are transformed into a gray tone scale. It is possible to analyze plates either with a clear background and dark spots or with a dark background and clear spots. After choosing the desired type of image, the software draws the borders, and then provides the RF, the area and the volume of the spots.

The aim of this study is to demonstrate the possibility of detecting and quantifying caprylins obtained by esterification of glycerol with caprylic acid catalyzed by immobilized lipases using the software JustTLC 4.0.3. In addition, the establishment of a correlation between TLC and titration of residual caprylic acid data is also an objective because both techniques are easily handled and require low sophisticated apparatus.

1. MATERIAL AND METHODS

1.1. Reagents: Materials were obtained from SIGMA® (caprylic acid; silica-gel plate 995700-25EA) and AccuStandard® (monocaprylin, dicaprylin and tricaprylin). Immobilized lipases Lipozyme®RM IM ($25.34x10^{-3}$ U/mg protein) and P:1X2-400 ($22.13x10^{-3}$ U/mg protein) were obtained from Novozymes (Bagsvaerd, Denmark) and the Laboratory of Enzyme Biocatalysis (University of São Paulo, Brazil), respectively. All other reagents used were of analytical grades.

1.2. Enzymatic Esterification

Esterification assays were carried out using a 125 mL batch-type reactor to which a 50 g mixture of glycerol and caprylic acid was added at a molar ratio of 1:1. An amount of 2.5 g of immobilized lipase (Lipozyme RM IM or P:1X2-400) was added to the reactor. The reactions catalyzed by Lipozyme were conducted at 30^{o}C, and agitation of 400 rpm for 6 h or 10 h, whereas the reaction catalyzed by P:1X2-400 was performed for 6 h under the same conditions. The reaction was interrupted by filtration under low pressure, and the filtrate was collected for the separation of caprylins. The separation consisted on vortex-stirring the filtrate for 10 min, followed by introducing 5 g of the mixture into a separation funnel containing 45 mL of petroleum ether and 30 mL of distilled water. The aqueous layer was discharged, and the organic phase was concentrated by a rotary evaporator operated at low pressure, 70^{o}C and 60 rpm for 20 min. Samples from the concentrates were used for titration with 0.3M NaOH and determination of TLC.

1.3. Analytical Methods

1.3.1. Determination of caprylic acid (titration method)

In an Erlenmeyer flask, 0.5 g of concentrated organic extract was mixed with 10 mL of absolute ethanol. Titration was carried out by using 0.3M NaOH in presence of phenolphthalein 1%. The volume of sodium hydroxide consumed was converted into the amount of caprylic acid in a standard linear curve. The curve was obtained by titrating solutions containing caprylic acid at concentrations between 0.63 mmol and 5.05 mmol. The caprylic acid was dissolved into 1 mL of petroleum ether and 10 mL of absolute ethanol. The minimum square linear regression is represented by the equation.

$V_{NaOH} = 0.383 + 3.256\ X\ (r = 0.998)$ (Eq. 1)

Where V_{NaOH} = volume of sodium hydroxide consumed and X = mmol of caprylic acid.

The conversion of V_{NaOH} into total residual caprylic acid (mmol) in the reaction medium after esterification was performed by the equations 2 and 3:

$F = (M_T/M_E).(M_C/M)$ (Eq. 2)

$M_R = X.F$ (Eq. 3)

Where F = conversion factor; M_R = mmol of residual caprylic acid in esterification medium; M_T = total mass of esterification medium (g); M_E = mass of esterification medium submitted to extraction (g); M_C = total mass of concentrated organic extract (g); M = mass of

concentrated organic extract (g) titrated with 0.3M NaOH. Calculation with experimental data is presented in the Appendix.

1.3.2. Determination of caprylins and caprylic acid by TLC

Samples of 10 µL containing a mixture of standard caprylic acid and caprylins dissolved in n-hexane at concentrations between 10 and 45 mg of acylglycerols/g of n-hexane were applied to TLC plates in duplicate (silica gel 60TLC Merck, 20x20 cm, 0.25 mm). The mobile phase runs 14 cm from the bottom to the top of the plate, and comprised n-hexane/ethyl ether/acetic acid (80:20:1 v/v/v). For visualization of spots, the plates were dried at 30°C, followed by a 5-second immersion into 200 mL of distilled water containing 48 mM $KMnO_4$, 470 mM Na_2CO_3 and 15.6 mM NaOH. After drying at 30°C, the revealed plates were photographed with a 5-pixel resolution digital camera. The images were analyzed using the software JustTLC 4.0.3.[14]

2. RESULTS AND DISCUSSION

First, the correlation between the area obtained by the software and the concentration of caprylic acid, monocaprylin, dicaprylin and tricaprylin of high purity was determined. Figure 1 shows the plates analyzed by the software, and Table 1 shows the area of each spot *versus* concentration.

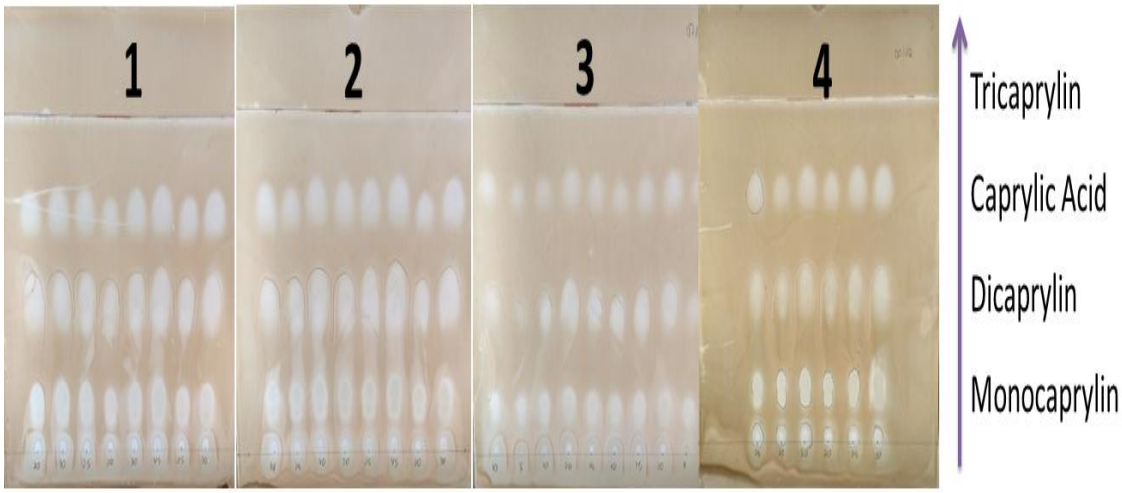

Figure. 1: Plates analyzed by Just TLC 4.0.3 to stablish the correlation between spot areas and concentrations of caprylic acid (RF= 0.47), monocaprylin (RF = 0.12), dicaprylin (RF = 0.23) and tricaprylin (RF = 0.75). Each plate refers to one out of four replicated measurements.

Table. 1: Areas of spots calculated by Just TLC 4.0.3, and respective concentrations of caprylic acid and caprylin standards.

Concentration (mg/g)	Spot Area				
	Caprylic Acid	Monocaprylin	Dicaprylin	Tricaprylin	Total Area
10	29000	1688	14950	30714	76352
15	33514	2239	17778	33571	87102
20	37500	2800	21000	37143	98443
25	41081	3351	24028	40714	109174
30	44865	3950	27500	44500	120815
35	48500	4450	30500	47857	131307
40	52300	5054	33750	51429	142533
45	55500	5622	36528	55000	152650

Figures 2 and 3 shows the spot areas of each compound in function of caprylic acid, mono-, di- and tricaprylin concentrations after plotting the data shown in Table 1. The correspondent minimum square linear equations are

$A_{ca} = 753. X_{ca} + 22078$ (r = 0.9993) (Eq. 4)

$A_m = 112. X_m + 556$ (r =0.9990) (Eq. 5)

$A_d = 626. X_d + 8542$ (r = 0.9997) (Eq. 6)

$A_t = 703. X_t + 23286$ (r = 0.9996) (Eq. 7)

Where A_{ca}, A_m, A_d and A_t are spots areas corresponding to caprylic acid, monocaprylin, dicaprylin and tricaprylin, respectively; Xca, Xm, Xd and Xt are concentrations (mg/g) of caprylic acid, monocaprylin, dicaprylin and tricaprylin, respectively.

Linear correlation coefficients over 0.999 indicate that the thin layer chromatography analyzed using the software JustTLC 4.0.3 is a valuable tool to determine caprylins and caprylic acid at concentrations varying between 10 mg/g and 45 mg/g.

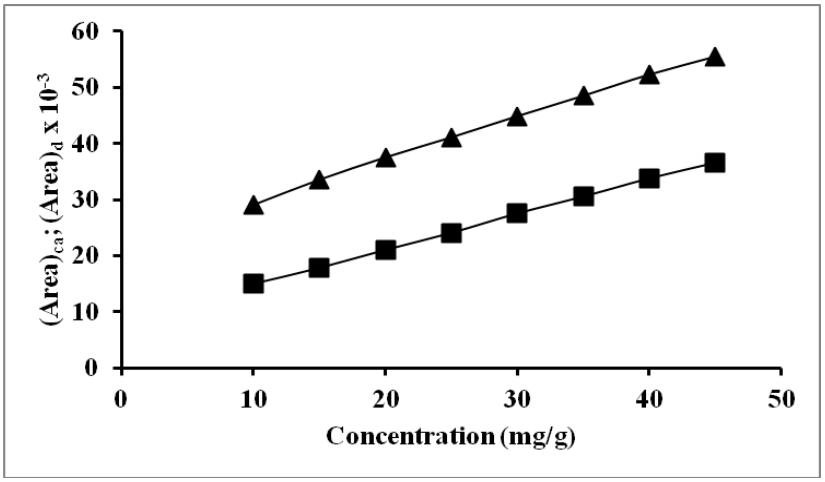

Figure. 2: Spot areas in function of caprylic acid (▲) and dicaprylin (■) concentrations.

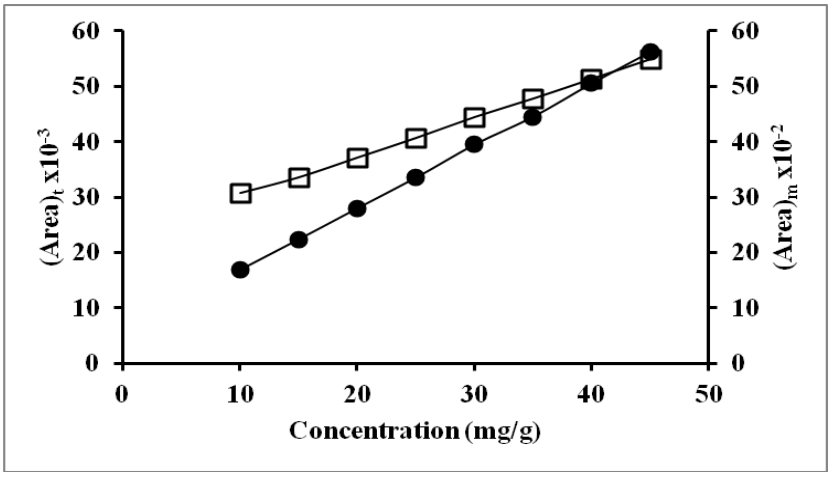

Figure. 3: Spot areas in function of monocaprylin (●) and tricaprylin (□) concentrations.

As each 10 μL-drop, added on the starting point of the plate, contained an equal concentration (from 10 mg/g to 45 mg/g) of caprylic acid, mono-, di- and tricaprylin, the total area of the four spots (A_{tot} = 100%) would theoretically be ¼ of each compound. However, due to particular features of each spot – diffusion through silica layer and molecular interactions among the substances by weak chemical bonds –, the areas were not equally distributed.

Therefore, correction factors ($F_{25\%}$) were defined as follows

$F_{25\%} = (0.25. A_{tot})/A$ (Eq.8)

Where A = area of a single spot.

By applying Eq. 8 to the data shown in Table 1, the $F_{25\%}$ of caprylic acid, monocaprylin, dicaprylin and tricaprylin are 0.67, 7.62, 1.11 and 0.68, respectively. An example of calculation is presented in the Appendix.

The determination of caprylic acid and caprylins in the organic extracts obtained from esterification reactions catalyzed by immobilized lipases (Lipozyme® and P:1X2-400) resulted in the data presented in Table 2. An example of calculation to evaluate the percentage of each component is shown in the Appendix.

Table 2: Percentage of caprylins and residual caprylic acid in concentrated organic extracts obtained from the esterification of glycerol with caprylic acid catalyzed by Lipozyme [reactions I and II] and P:1X2-400 [reaction III]. The conditions of reaction were 2.5 g of Lipozyme [I;II], 2.5 g of P:1X2:400 [III], 30°C, 400 rpm, molar ratio of caprylic acid/glycerol of 1:1, 6 h [I;III] and 10 h [II]. The areas of each substance were evaluated by the software JustTLC 4.0.3.

Reaction	Monocaprylin		Dicaprylin		Tricaprylin		Caprylic acid		CA*
	Area	(%)	Area	(%)	Area	(%)	Area	(%)	(%)
I	9347	50.34	23234	17.92	nd**	-	71092	31.84	34.49
II	8771	49.61	23470	19.01	nd**	-	64197	31.38	31.79
III	5408	30.67	28641	23.68	9806	4.97	81479	40.68	44.45

*CA: Percentage of residual caprylic acid present in the concentrated organic extract determined by titration. **The volume of tricaprylin caused smaller spots areas than the sensitivity of the method was incapable of detecting.

Table 2 shows that the residual caprylic acid differed less than 9% between determination by TLC and titration, which can be considered well fitted values considering the great conceptual differences of both methods. The titration method applied in this work was chosen to compare it with TLC – the simplest chromatographic analytical method available, as already mentioned – because it is largely used by fat and oil industries to assess the changes in lipid levels during its production.[16]

In addition, the reactions I and II, carried out for 6 h and 10 h, respectively, led to similar caprylic acid consumption, as well as to the formation of monocaprylin and dicaprylin independently of the duration of reaction. By comparing the reactions I with III (which differed only in the type of immobilized lipase used), the reaction III resulted in less monocaprylin formed and less caprylic acid consumed (38% and 22% less, respectively, when compared to the reaction I). However, in reaction III, more dicaprylin and tricaprylin were formed. The formation of tricaprylin is an unexpected result considering that both immobilized lipases are sn,1-3 specific. The carrier used to immobilize the lipases could have interfered with the enzyme mechanism, favoring the formation of tricaprylin. It is possible that the low diffusion of substrates and products through the DOWEX resin (P:1X2-400 lipase) played a role in tricaprylin formation. This is because a slow molecule movement can favor the occurrence of internal migration of the octanoyl group from the position C1 or C3 to the position C2 of glycerol backbone, freeing the hydroxyl group from one extremity for the esterification process with a third caprylic acid molecule.[1] Naturally, further studies are

needed to clarify this point. Notwithstanding, the effects of chemical and structural aspects of the carrier on the intrinsic mechanism of enzymes positional specificity should open a new insight in the immobilization field.

3. CONCLUSIONS

The data indicate that thin layer chromatography coupled with the software JustTLC 4.0.3 is a suitable tool for an early scanning of the composition of concentrated organic extracts containing caprylins and caprylic acid. Lipozyme (commercial immobilized lipase) and P:1X2-400 lipase (Palatase® immobilized on DOWEX® resin) have a comparable performance in esterification. By comparing two simple analytical methods, TLC and titration with 0.3M NaOH, there were convergent values for esterification yields (between 55% and 69%).

APPENDIX A

Calculation of $F_{25\%}$

Table A1 shows the values used for calculating F25% of caprylic acid (considered an example). The values for A and Atot were taken from Table 1.

Table A1: Application of equation 8 [$F_{25\%} = (0.25 \times A_{tot})$, A] to calculate $F_{25\%}$ of caprylic acid.

Concentration (mg/g)	A_{tot}	0.25 x A_{tot}	A	$F_{25\%}$
20	98443	24610.75	37500	0.66
25	109174	27293.50	41081	0.66
30	120815	30203.75	44865	0.67
35	131307	32826,75	48500	0.68
40	142533	35633,25	52300	0.68
45	152650	38162.50	55500	0.69
Average				**0.67**

Calculation of percentage of caprylins and caprylic acid by TLC

The data of reaction III was taken to demonstrate the calculation of the final percentage (FP%) of caprylins and caprylic acid present in the concentrated organic extract (Table A2).

Table A2: Calculation of the final percentage (FP%) of caprylins and caprylic acid present in the concentrated organic extract obtained from reaction III (catalyzed by lipase P:1x2-400).

Compound	A	$(A/A_{tot}).10^2$	$F_{25\%}$	$(A/A_{tot}).F_{25\%}.10^2$	$FP_{\%}$
Monocaprylin	5408	4.31	7.62	32.84	30.67
Dicaprylin	28641	22.85	1.11	25.36	23.68
Caprylic acid	81479	65.01	0.67	43.56	40.68
Tricaprylin	9806	7.82	0.68	5.32	4.97
A_{tot}	125334	-	-	-	-
$S(A/A_{tot}).10^2.F_{25\%}$	-	-	-	107.08	-

The final percentage (FP%) for each compound can be calculated directly using the equation A1: $FP\% = 10^4.[(A/A_{tot}).F25\% \div \Sigma(A/A_{tot}).10^2.F25\%]$ (Eq. A1)

Calculation of residual caprylic acid by titration

By using the experimental data from reaction III: VNaOH = 3.25 mL, MI = 211.57 mmol, MT = 50.01g, ME =5.005g, MC = 4.668g, and Mt = 0.437g, the equations 1, 2 and 3 were applied successively, obtaining the following results:

X = 0.881 mmol; F = 106.7; MR = 94.03 mmol.

Therefore, the percentage of residual caprylic acid is 44.4% [100.(MR/MI) = 100.(94.03/211.57)]. Conversely, the percentage of caprylic acid consumed, which is the yield of esterification (Y%), is 55.6%, and can be calculated by the equation:

Y% = [1 – (MR/Mi)].102 (Eq. A2)

Where MR = total mmol of residual caprylic acid in the esterification medium; Mi = total mmol of caprylic acid at the beginning of esterification.

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