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Analysis of biodiesel conversion using thin layer chromatography and nonlinear calibration curves

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ABSTRACT

Biodiesel (BD) is a fuel produced by the (trans)esterification reaction between the components of vegetable oil (or animal fat) and an alcohol. The presence of several substrates complicates analytical separation of the mixture, yet understanding of the complex reaction kinetics requires acquisition of a large body of data. The two well-established methods of gas chromatography (GC) and HPLC are time consuming and expensive when analyzing multiple samples. Additionally, it is not always possible to record all the reactants on one elution profile. We examined applicability of thin layer chromatography (TLC) for this purpose, where the detection was based on either flame ionization detector (FID) or a modified staining procedure. The suggested staining method gave no background and appeared well suited for quantitative analysis. The relevant calibrations are presented, and the general principles of analysis of nonlinear responses are discussed. Several experimental samples were produced by enzymatic conversion of rapeseed oil to BD. One reaction step resulted in 85–95% conversion (6 h). The second step (after removal of glycerol and water) increased the yield to 97–98%. All components of the mixtures were separated and quantified. Relation of the BD contents measured by TLC and GC gave the values of 1.03 ± 0.07 (TLC-staining) and 0.95 ± 0.04 (TLC–FID), indicating applicability of the TLC-methods.

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1. Introduction

Biodiesel (BD) is a fuel produced in the (trans)esterification reaction between components of a vegetable oil and an alcohol (typically methanol or ethanol) [1–3]. The substrates in oil are triglycerides (TG), diglycerides (DG), monoglycerides (MG) and free fatty acids (FA). The products obtained are BD, glycerol and water. An efficient conversion requires application of a catalyst, where KOH, $NaOCH_3$, H_2SO_4, etc. are among the most commonly used compounds [1]. Recently, enzymatic production of BD has attracted attention as an environmentally advantageous alternative to chemical conversion [2–4]. Several preparations of immobilized lipases are currently under examination as potential candidates for the industrial application [2–4]. Yet, presence of multiple substrates and products requires a thorough knowledge of the enzymatic reaction kinetics.

Examination of oil and BD samples is often based on gas chromatography (GC) or HPLC [5–9]. Both methods are well established but have a few disadvantages including a relatively long time of analysis (approximately 30 min per sample), high operational costs and difficulties in getting all relevant compounds on one profile. At the same time, monitoring of all reactants over the time is important for a thorough kinetic description of such process, where numerous measurements are required. The two methods discussed below appear, in fact, better suited for this purpose. These are thin layer chromatography (TLC) on chromarods assisted by flame ionization detector (FID) and TLC on plates, where the spots are visualized by staining.

TLC–FID method is described in the literature; though, the results are somewhat contradictory [10–12]. For instance, both linear [12] and nonlinear dependencies [10,11] of the signal on the loaded mass are described, even if the detector settings were the same. Interpretation of nonlinear calibration curves is, however, complicated because a twofold change in the mass gives disproportional signals from different compounds, each of them following its own curve. Likewise, a change in the signal (caused by technical reasons but not the mass) introduces disproportion to the apparent composition of the mixture unless the signal is scaled to the original calibration. Application of an internal standard compensates this error, but the mass of the standard must be exactly the same under all measurements. This approach is not always possi-

Abbreviations: BD, biodiesel; FAEE, fatty acid ethyl ester; FAME, fatty acid methyl ester; FA, fatty acid (free); GC, gas chromatography; m/m, mass per mass; m/v, mass per volume; OA, oleic acid; TG/DG/MG, tri-/di-/mono-glyceride; TO/DO/MO, tri-/di-/mono-oleine; TLC–FID, thin layer chromatography assisted by flame ionization detector.

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Table 1
Composition of the oil samples used to produce calibrations.

Major component	Component BD, %	Component TG, %	Component FA, %	Component DG, %	Component MG, %
BD (rapeseed)	96	0	1	1	2
TG (rapeseed)	0	95	2	2	1
FA (OA)	0	0	98	1	1
FA (mix)	0	29	46	20	5
DG (sunflower)	0	5	1	72	22
MG (OG)	0	2	1	2	95

ble, especially when the standard and the analyte partially overlap. As a partial solution, some authors recommend application of different combinations of linear response factors to different parts of the nonlinear dependencies [11]. All the above issues were not adequately covered in the literature and require a more general approach.

Separation of lipids on TLC plates followed by staining is another potentially convenient procedure [13,14]. The staining is often based on $KMnO_4$ oxidation, yet the described method gives a strong violet background with irregular patterns, which precludes any reliable quantitative analysis [14]. Additionally, the produced spots are not stable and fade over the time. A proper modification is required to combine a low cost and simplicity of this method with the sufficient reliability.

In the current publication we present a modification of the TLC-staining which avoids the problems of background and fading of the spots. A comparison of several methods was carried out, where the oil components under enzymatic biodiesel conversion were separated and quantified. Correct application of the nonlinear calibration curves, scaled with help of the total sample mass, is discussed.

2. Materials and methods

2.1. Materials

All salts and solutions were purchased from Sigma–Aldrich. Chromarods S III were form SES GmbH-Analysesystem (Germany). TLC plates Polygram Sil G 20 cm × 20 cm (gel 0.2 mm) were from Macherey–Nagel (Germany). Lipid standards of high purity (methyl oleate, ethyl oleate, triolein, diolein, monoolein, oleic acid) were from Sigma–Aldrich. Rapeseed oil was from a Danish supermarket. Preparations of MG, DG and FA were from Danisco (Denmark), see Table 1 for details. Immobilized enzyme preparations of lypozyme TL HC and Novozym 435 were kindly provided by Novozymes (Denmark).

2.2. Methods

2.2.1. Enzymatic preparation of the calibration mixtures

An FA-enriched mixture was prepared in the following way. Rapeseed oil was incubated with 20% (v/v) water and 4% (m/v) Lypozyme TL HC for 3 h (35 °C, 200 rpm). The contents of FA after hydrolysis was determined by titration.

BD samples of 96% purity (FAME or FAEE) were prepared from rapeseed oil and MeOH or anhydrous EtOH as described below for "98%" analytical sample except for the absence of molecular sieves at the second step and the incubation time shortened to 12 h.

The above samples were used either separately or in the mixture with commercial preparations of TG, DG and MG.

2.2.2. Enzymatic preparation of BD-containing samples

The test mixtures were notated according to the detected level of BD, e.g. "24%". They were produced by incubation of the below components with 3% (m/v) of Lypozyme TL HC at 35 °C, 200 rpm (all compounds in relative volumes v): (1) 0.92 v oil, 0.04 v water, 0.04 v ethanol, incubated for 6 h ("24%" sample); (2) 0.88 v oil, 0.04 v water, 0.12 v ethanol, incubated for 6 h ("65%" sample); (3) 0.82 v oil, 0.007 v water, 0.17 v ethanol added in two steps at 0 h and 3 h over 6 h ("85%" sample). Two more samples "97%" and "98%" were produced according to a separate procedure, where two incubation steps were involved. Step 1 generally followed the method for "85%" sample except for supply of 96% ethanol added at 0 h, 2 h, 4 h as three portions (each of 0.09 v). The process ended by removal of enzyme glycerol, water and ethanol. Enzyme particles were separated by filtration, glycerol was settled as a separate phase by gravity (2 h), and excessive ethanol and water were evaporated under vacuum (1 mbar, 1 h). The obtained product was subjected to the second reaction (step 2) with 4% (m/v) Novozym 435, 8% (v/v) anhydrous ethanol, and 8% (m/v) of molecular sieves. The conversion was continued for 21 h ("98%" sample) and 90 h ("97%" sample) at 35 °C, 200 rpm.

The more detailed kinetic records of the reaction were produced for step 1 with 1 v of oil and 5% (m/v) of Lypozyme TL HC (35 °C, 200 rpm, 6 h). The supply of 96% ethanol was as follows: 0.045 v was added at the beginning of reaction, whereupon 0.2–0.23 v was continuously added over 1.5–4 h (see Section 3.7). The reaction was continued for 6 h, and small samples were collected at time intervals. They were centrifuged to precipitate glycerol and after evaporation of ethanol and water subjected to GC or TLC analysis.

2.2.3. GC-analysis

Quantification of FAEE (%, m/m) was performed with methyl heptadecanoate as internal standard according to the EN14103 standard method on a Varian Chrompack CP-3800 gas chromatograph (GC) equipped with a Varian "Select Biodiesel for FAME" (30 m, 0.32 i.d.) column.

2.2.4. TLC–FID analysis

The general experimental procedure followed the method described earlier [11,12]. Separation was carried out on Chromarods S III cleaned by two sequential runs on Iatroscan (see below). The lipid samples were diluted in hexane and loaded on each rod at the total mass of 1–15 μg. The developing mixture of 16% diethyl ether and 0.04% formic acid in hexane was used and provided a good separation of all components (Fig. 1). The rods were dried at 120 °C for 3 min before scanning. Detection was performed on Iatroscan MK 6 s using the below settings: air flow 2 L/min, hydrogen flow 160 mL/min, scan speed 30 s per rod. The recorded profiles were analyzed by Crom-Star 6.0 program, see an example in Fig. 1. The full experimental proceeding of 10 samples on one chromarod frame took approximately 2 h.

2.2.5. Separation on TLC-plates and the staining procedure

Silica-gel TLC-plates (Polygram Sil G) on plastic support were used. Aluminum support is not recommended because it crucially hinders soaking in water at the later step. The size of the plates was 20 cm × 20 cm, 10 cm × 20 cm or 10 cm × 10 cm (height × width). The lipid samples (0.1–4 μL per lane) were loaded without dilution at a distance of 1.8–2.5 cm from each other and 1–2 cm from the bottom. The development was carried out in either 10% ethyl acetate in hexane (20 cm × 20 cm plates, 1.5 h) or 15% ethyl acetate

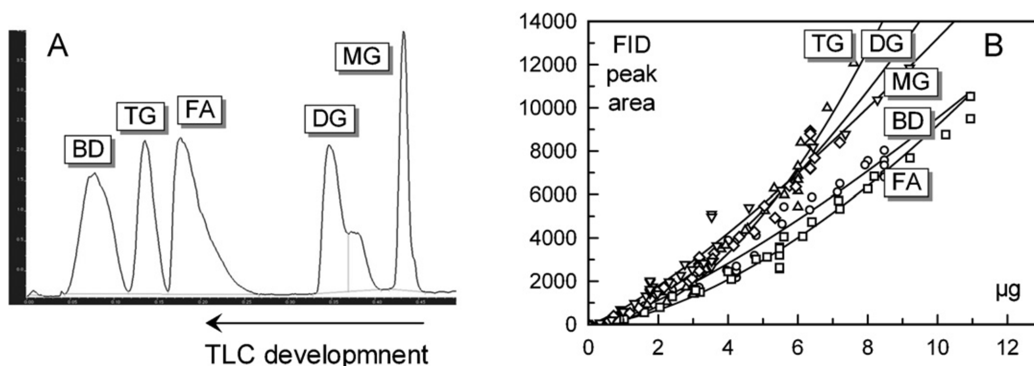

Fig. 1. TLC–FID calibration. (A) Separation of a five-component mixture on chromarods developed in 16% diethyl ether and 0.04% formic acid in hexane. (B) Calibration curves constructed from the profiles as the one in panel A. Calibration coefficients of power approximation are presented in Table 2. Coefficients of determinations (R^2) for all the curves varied in the interval of 0.97–0.98.

in hexane (10 cm × 20 cm, 10 cm × 10 cm plates, 15 min) until the solvent front reached the top of the plate. After a brief drying, the plates were either subjected to second development in water (1–2 h) or slowly immersed into a water bath by tilting the plate (1–2 min). Development in water is relatively slow but gives a more homogeneous saturation of the gel. The wet plate was stained in 100 mL of 1% solution of $KMnO_4$ in 4% NaOH for approximately 20 s with constant agitation. It is not recommended to continue this procedure over 60 s, because a slight background starts to appear after a prolonged staining. The staining liquid can be stored and repeatedly applied to approximately 10 plates (20 cm × 20 cm), before the results start to deteriorate. The stained plate should be extensively washed with water (3–4 changes for 3–4 min) and dried on an even surface to prevent cracking of the gel. Experimental proceeding of 10 samples put on one plate takes approximately 4 h (20 cm × 20 cm plate, two developments), 2 h (20 cm × 20 cm plate, one development plus immersion) or 30 min (10 cm × 20 cm plate, one development plus immersion).

2.2.6. Quantitative analysis of the samples after TLC-staining

A semi-quantitative analysis can be done alone by visual inspection of the spots and comparison to the standards of known masses either on the same plate or on a separate plate. More advanced procedure requires scanning or photographing of the plate followed by measurement of optical density in the areas of different spots. The below equipment and settings were used when acquiring the images: scanner CanoScan 8800F; TWAIN compatible driver ScanGear set to resolution (300 dpi), contrast (+22), adjust tone (Master 0, 266, 255), reduce dust (high), grain correction (high), backlight correction (high). The obtained image was additionally processed using the program PhotoStudio 5.5 and the below settings of Tone Adjustment: highlights between +5 and +7, Midtone −5, Shadow −5.

The optical density of the spots was measured using the image editing features of the data analysis program KyPlot 5.0 (KyensLab Inc., Japan). An image file was imported into an empty figure, and Image, Edit Region functions were sequentially selected. The option Rectangular region was chosen. A rectangle was drawn, which covered all the spots of one lane. The menu Analyze, Densitogram was activated and a profile of the optical density was produced (see an example in Fig. 2). Density area of each peak was evaluated by commands Measure, Consecutive Areas by Mouse Clicks. A table of data was produced, where the column area represented optical density of each peak. Then, exactly the same rectangular region was applied to other lanes. The obtained measurements were used to create a calibration curve (if the masses were known) or to measure the mass of a component in an experimental sample, see Section 3 for details.

An alternative method of quantification is based on measurement of the area of each spot, proportional to the loaded mass. Measurement of the area can be done either using a computer program or just by a ruler, where two diameters (A and B) of an approximating ellipse describe the value of Area = $1/4 \cdot \pi \cdot A \cdot B$. We used a computer based approach within the Image, Edit Region functions of KyPlot 5.0. The areas were measured in pix^2 using options select an elliptic region or select a region by free hand. The collected data were use to either prepare a calibration curve or measure the composition of the samples.

2.2.7. Nonlinear regression analysis

The approximation of nonlinear curves was done using the computer program KyPlot 5 (KyensLab Inc., Japan) with the selection of quasi-Newton method of least squares. Goodness of fit is indicated as coefficients of determination R^2 in the corresponding figure legends.

3. Results and discussion

3.1. Preparation of the standards and separation by TLC on chromarods

As the first step, the high purity standards of biodiesel (methyl oleate, ethyl oleate), triolein, diolein, monoolein and oleic acid were applied to chromarods (see Section 2.2.4). A typical separation profile is shown in Fig. 1A. The recorded intensities of FID-signal (area of the peaks) were plotted as functions of the standard masses (not shown). These data were used to produce a preliminary calibration chart to verify composition of heterogeneous mixtures of lower purity: rapeseed oil, biodiesel (FAME, FAEE) from rapeseed oil, mono- and diglycerides from sunflower oil, etc. A separate mixture enriched by natural FA (46% according to titration) was prepared by enzymatic hydrolysis of rapeseed oil (see Section 2.2.1). Composition of the prepared samples (Table 1) agreed with the specifications known for TG, FA, DG and MG. Both the individual preparations and their mixtures (e.g. a five-component mixture of 20% BD, 20% TG, 20% OA, 18% DG, 23% MG, m/m, Fig. 2A) were used to calibrate FID. The described approach was chosen to take into account a potentially existing difference in FID-responses of the olein-based pure compounds and the heterogeneous components of natural oil. This assumption was not corroborated though (see Section 3.2).

3.2. FID calibration curves and nonlinear responses

The produced calibration curves present areas of FID-peaks (Fig. 1A) plotted vs. the mass of each compound (Fig. 1B). The

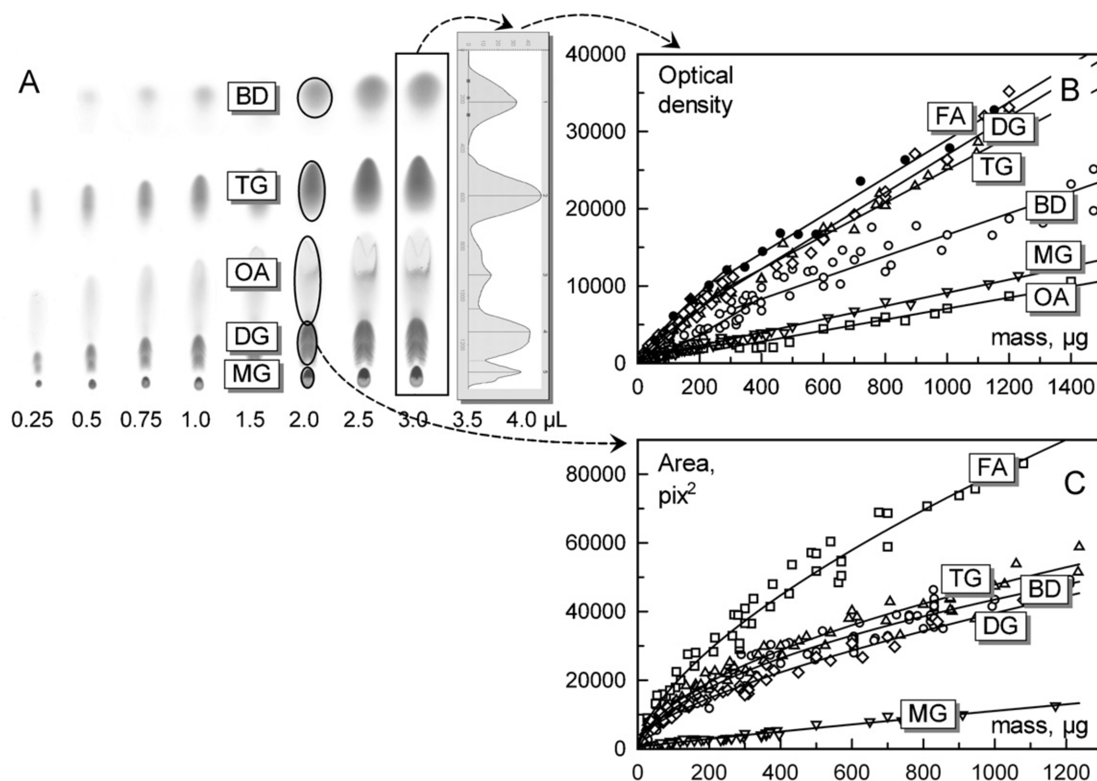

Fig. 2. TLC-staining calibration. (A) Separation of a five-component mixture on a TLC-plate. Optical densities of the individual spots were determined within the same frame, sequentially applied to all lanes. Optical density areas were used to create the calibration curves in panel B. Elliptic or free hand areas, delineating each spot, were used to create the calibration curves in panel C. See main text for details. (B) Optical densities of the spots plotted as the functions of the corresponding masses. The calibration chart was separated into the two regions of 0–300 μg and 150–1500 μg, which were fitted independently by power and linear functions, respectively. The fitting coefficients are presented in Table 3. Coefficients of determinations (R^2) for different curves varied from 0.92 (BD) to 0.99 (DG). (C) Areas of the spots plotted as the functions of the corresponding masses. The calibration chart was separated into the two regions of 0–300 μg and 150–1500 μg, which were fitted independently by power and linear functions, respectively. The fitting coefficients are presented in Table 4. Coefficients of determinations (R^2) for all the curves varied in the interval of 0.96–0.98.

curves are clearly nonlinear, which contradicts the data from Ref. [12]. It should be, however, mentioned, that in the latter publication all intersection coefficients b of linear calibrations ($y = a{\cdot}x + b$) were negative. This result is expected if a linear fit approximates a power function $y = a{\cdot}x^b$ with $b > 1$. In our case, we have chosen to fit calibrations in Fig. 1B by the power function, and the corresponding coefficients of best approximation are presented in Table 2. Responses of the pure oleins (preliminary calibrations) were pooled into the final dataset because of a general overlap between oleins and mixed compounds (not shown).

Analysis of nonlinear calibrations with different power coefficients (b) presents some difficulty, because the relative positions of the curves are changing along x-axis. This means that the same slope coefficients (a) or response factors ($RF = 1/a$) cannot be used throughout the whole mass scale. In other words, any global shift in the signal intensity introduces an error if using the calibration curves directly. Relation of the sample peaks to an internal standard partially compensates this error; but a nonlinear proportion between the samples and the standard requires a constant standard mass in all samples. Different combinations of the response factors for several typical mixtures were also suggested as a roundabout solution [11]. Yet, such approach is practically inconvenient.

Table 2
Calibration coefficients connecting the FID-signal (y) with the mass (x, μg), $y = a{\cdot}x^b + c$ (c assigned as 0 in all cases).

Compound	a	b
BD	448	1.33
TG	223	1.94
FA	218	1.62
DG	449	1.53
MG	750	1.25

The mass of each compound (x, μg) can be calculated from the FID-signal (y) using equation $x = ((y - c)/a)^{1/b}$ (see the main text for details).

We suggest another strategy, where the known total mass (e.g. 10 μg) of a sample helps to scale an arbitrary signal within the limits of the calibration. A sample should be separated into the individual peaks as shown in Fig. 1A, and the area of each peak should be evaluated according to standard procedures. Then, a "trial" mass of each component (as well as their sum) should be calculated using parameters from Table 2 and the equation $x = ((y - c)/a)^{1/b}$. If FID-responses in the experimental sample are completely identical to those of the shown calibration, the calculated total masses of the analyzed sample will correspond to its correct value of 10 μg. This outcome is not very probable, however; because properties of different detectors are not absolutely identical (the same counts for the same detector over a long time period). If a deviation is observed (e.g. the sum is equal to 7 μg instead of 10 μg), all FID-area should be uniformly multiplied by an approximate correcting coefficient d, which brings the calculated total mass closer to its true value. This is an iterative process which should be repeated until the calculated total mass is brought within ±5% of the expected value. Afterward, the mass % of each component in the mixture can be estimated as e.g. TG mass % = $m_{TG}/m_{total} \times 100$.

The described method facilitates application of nonlinear calibrations to the samples of any arbitrary composition analyzed on different detectors. There is, however, a requirement that the recorded profile covers the whole mass (the latter being the internal standard).

Table 3
Calibration coefficients of TLC-staining connecting the optical density (y) with the mass (x, μg) if working on the plate 20 cm × 20 cm or 10 cm × 20 cm ([a]).

Compound	10–300 μg, $y = a{\cdot}x^b + c$			150–1500 μg, $y = a{\cdot}x + c$	
	a	b	c	a	c
BD	7.84 (255[a])	1.17 (0.68[a])	−155 (0[a])	13.9	2778
TG	52.4 (255[a])	0.932 (0.68[a])	−201 (0[a])	21.4	3648
OA	82.7	0.607	−45.1	7.11	−43.1
FA	130 (255[a])	0.803 (0.68[a])	−86.9 (0[a])	24.4	4456
DG	85.2 (229[a])	0.834 (0.645[a])	114 (0[a])	24.6	2342
MG	56.6 (310[a])	0.701 (0.483[a])	−15.9 (0[a])	8.54	579

The mass of each compound (x, μg) can be calculated from the optical density (y) by equation $x = ((y - c)/a)^{1/b}$ (see the main text for details).
[a] Calibration for 10 cm × 20 cm plate, the suitable region of masses is within the limits of 0–500 μg.

The correcting coefficient d can be calculated directly if a standard (one of the measured compounds or a mixture of them) is run on a separate rod and its response is quantified. The below equation explains the correlation between the experimental and theoretical FID-responses:

$$d{\bullet}y_2 = y_1 = a{\bullet}x_2^b + c; \quad \rightarrow d = \frac{y_1}{y_2} \tag{1}$$

where x_2 is the mass of the applied standard (e.g. μg of TG); a–c are the calibration coefficients from Table 2 (Tables 3 and 4 for TLC-staining); y_1 is the theoretical FID-response (or staining intensity) predicted for x_2 by the calibration coefficients; y_2 is the actual FID-response (or staining intensity); d is the correction coefficient used to get the experimental measurements within the scale of calibration in Fig. 2B (Fig. 3B and C for staining). If only one component of the reaction mixture is monitored, this approach becomes preferable because evaluation of the mass of other irrelevant compounds becomes unnecessary.

3.3. Separation on TLC plates and staining

A good separation of the components of a BD–oil mixture (0.2–4 μL samples without dilution) was achieved when using 10% ethyl-acetate in hexane and silica-based TLC-plates 20 cm × 20 cm (see Section 2.2.5). It is recommended to use the plates on a plastic support instead of aluminum because this facilitates the second development in water. As an additional advantage one can mention partial transparency of a plastic plate, where the dark spots of separated compounds can be seen even without staining if holding the plate against the light. The second development in water (or slow tilting of the plate into a water bath for 2–4 min) displaces the remaining organic solvents from the gel and prevents development of a heavy irregular background under the following staining (see Section 2). Finally, the stained plate should be washed with water to remove the excessive permanganate. An example of separation and staining is shown for a five-component mixture in Fig. 2A.

The detected spots are stable for at least 2 years and do not fade over the time. This makes possible to store a calibration plate and use it as a visual standard for comparison to the experimental samples (particularly relevant under the field analysis of oils without electronic equipment).

3.4. Calibration of the staining response

The mixtures of known compositions from Table 1 were separated on TLC-plates (see an example in Fig. 2A) and used to produce the staining calibrations, where either optical densities of the spots (Fig. 2B) or their area (Fig. 2C) were plotted as functions of the corresponding masses. Optical densities were determined using a densitometric utility of the program KyPlot 5 (see Section 2.2.6), where all tracks were sequentially covered by a rectangular frame of the same size (Fig. 2A). Areas of the spots were measured in pix^2 (300 dpi scan, 139.5 pix^2 = 1 mm^2) using a utility for evaluation of different shapes (ellipse or free hand).

The calibration curves for Plates 20 cm × 20 cm in Fig. 2B and C had complex shapes and were separated into two regions covering the masses of 10–300 μg and 150–1500 μg. Two sets of the fitting coefficients were obtained (one set for each region). They are shown in Table 3 (optical density vs. mass) and Table 4 (area pix^2 vs. mass). Separation on a shorter TLC plate (10 cm × 10 cm or 10 cm × 20 cm, development in 15% ethyl acetate in hexane) is also possible but shows more overlaps at increasing masses. Yet, the time of development is much shorter (15 min vs. 1.5 h). The calibration coefficients differ from those for 20 cm × 20 cm plate because of different stretching of the spots, e.g. responses of BD and MG are amplified in comparison to other compounds. We present the calibration coefficients for optical density on a short plate in Table 3 (masses of 0–500 μg).

Principles of calculation of the mass for nonlinear calibrations were already covered in Section 3.2. In short, after measuring optical density or/and area of the spots, the coefficients from Table 3 or/and Table 4 should be used to calculate the "trial" individual masses and the total mass of the whole experimental sample. The calculated total mass will most probably deviate from its expected value because of a difference in the image acquisition. Therefore, all the peaks should be multiplied by a correction coefficient d to make a concurrent change of their intensities. This iterative correction should be repeated until the calculated total mass corresponds

Table 4
Calibration coefficients of TLC-staining connecting the area (y, pix^2) with the mass (x, μg) if working with 20 cm × 20 cm plate.

Compound	10–300 μg, $y = a{\cdot}x^b + c$			150–1500 μg, $y = a{\cdot}x^b + c$		
	a, pix^2	b	c, pix^2	a, pix^2	b	c, pix^2
BD	964	0.585	−3234	1166	0.528	−1172
TG	2741	0.415	−5386	1035	0.560	−555
FA = OA	3508	0.436	−6852	1034	0.628	470
DG	13,732	0.170	−18,910	446	0.642	1489
MG	318	0.447	−524	22.0	0.895	404

The mass of each compound (x, μg) can be calculated from the area (y, pix^2) by equation $x = ((y - c)/a)^{1/b}$ (see the main text for details). Area units can be recalculated using the transformation 139.5 pix^2 = 1 mm^2.

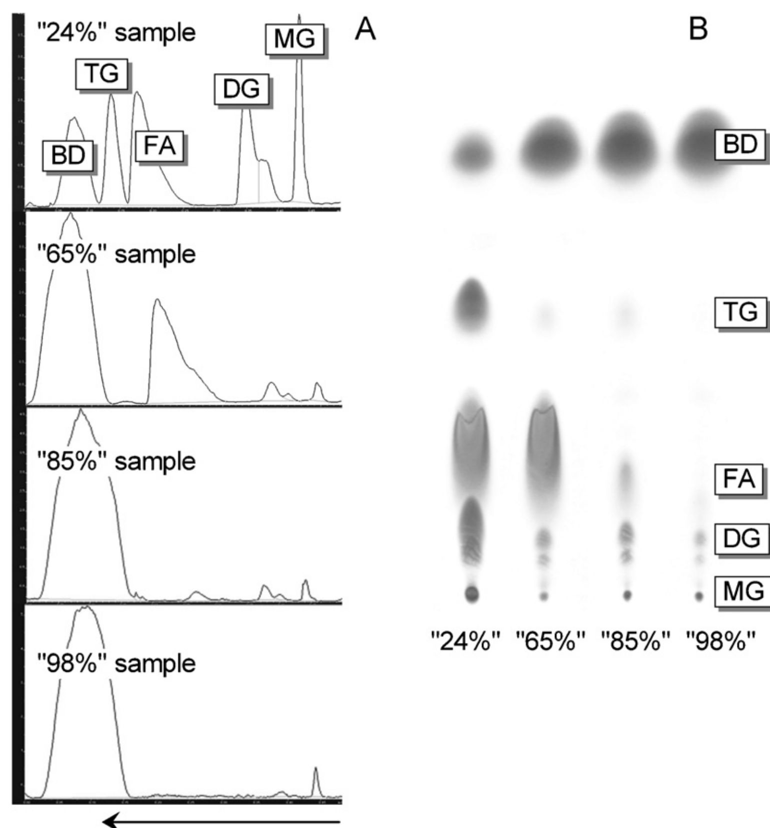

Fig. 3. Analysis of the test samples. (A) Several examples of TLC–FID profiles recorded for the tested mixtures. (B) TLC-plate after separation of the tested mixtures (1 μL of each sample per lane) and staining. All the results of analysis are shown in Table 5.

to the expected mass ± 5%, which means the correct scaling of the data within the limits of calibrations in Figs. 2 and 3. Alternatively, a mixture of standards can be applied to an individual lane and used to calculate the coefficient *d* by Eq. (1) (see above Section 3.2).

3.5. Particular features of staining and analysis of the TLC plates

Color intensities of olein-based glycerides and mixed natural glycerides from rapeseed/sunflower oil were similar within the tested limits, despite a difference in the composition of stainable double bonds. This similarity is, probably, caused by a very high optical density per area unit in the spots of TG, DG and MG. In other words, the absorbance of light was above the limit, where detection of a difference was possible. Decreased sample amount caused shrinking of the spot area, while the optical density remained relatively high (Figs. 2A and 3B). On the other hand, staining of oleic acid (OA) and mixed fatty acids (FA) from rapeseed oil was remarkably different, the intensity of OA-spots being weaker by factor 4. This difference can be ascribed to large areas occupied by both OA and FA spots on a TLC-plate (Fig. 2A). This spread distribution of material made the optical density relatively low, which exposed variations of intensity.

Large areas of weak stain of OA were sometimes prone to discolorations. In such cases, measurement of the area in pix^2 and its comparison to a standard appears to be a better alternative to the optical density determination.

TLC-mobilities of two BD variants (FAME and FAEE from rapeseed oil) were slightly different, FAEE moving a little faster than FAME. No significant difference in their staining was found, however.

Area measurements become less precises if working on the short plates of 10 cm × 10 cm or 10 cm × 20 cm because of a smaller size of all the spots. The corresponding calibrations were therefore omitted.

3.6. Examination of the experimental samples after enzymatic BD-conversion

Five experimental samples were prepared from rapeseed oil and EtOH. They were called "24%", "65%", "85%", "97%" and "98%" according to the biodiesel conversion levels (m/m, %) determined by GC-analysis. Biodiesel was produced in one-step or two-steps enzymatic reactions "oil + EtOH + water + enzyme" or "oil + EtOH + enzyme" with different schemes of ethanol and water supply (see Section 2.2.2). Contents of FA in all the samples were determined by titration. The "97%" preparation was additionally sent for full EN-14214 analysis to ASG laboratory (Analytik-Service Gesellschaft, Neusäss, Germany).

The four samples "24%", "65%", "85%" and "98%" were subjected to a blind analysis using TLC–FID and TLC-staining. The sample "97%" was excluded as essentially resembling "98%" except for a lower FA-contents in the first one. The comparative results are shown in Table 5 and Fig. 3. Both TLC–FID and TLC-staining methods (optical density) showed agreeable results when compared to the GC – data and the FA titration experiments. It should be mentioned that the higher dispersion of GC – measurements if compared to TLC methods (Table 5) was caused by isolated determination of BD, related not to other components of the reaction mixture but to the internal standard. This occasionally caused unreasonable values, e.g. 101% BD in the "true 98%" mixture.

A few contradictions between the different methods should be discussed. For example, the true contents of BD in the sample of "65%" seems to be lower (59%) according to both TLC methods. In the same sample, TLC-staining probably gave an overestimated

Table 5
Comparative analysis of the experimental reaction mixtures (mean ± SD, $n = 5$–7).

Sample	BD, mass %	TG, mass %	FA, mass %	DG, mass %	MG, mass %
"24%"	24 ± 1.2[a]	n.d.	30[b]	n.d.	n.d.
TLC–FID	22.6 ± 1.2	16.5 ± 0.6	31.0 ± 1.2	19.3 ± 1.0	10.6 ± 1.6
TLC staining	26.6 ± 0.7	16.3 ± 0.6	30.6 ± 1.9	17.3 ± 1.1	9.2 ± 0.6
"65%"	65 ± 3.0[a]	n.d.	30[b]	n.d.	n.d.
TLC–FID	58.9 ± 1.5	2.2 ± 0.3	32.5 ± 1.8	4.7 ± 0.5	1.6 ± 0.3
TLC staining	58.8 ± 1.8	1.2 ± 0.1	36.5 ± 1.2	2.1 ± 0.2	2.1 ± 0.5
"85%"	85 ± 4[a]	n.d.	3.7[b]	n.d.	n.d.
TLC–FID	85.2 ± 0.5	2.2 ± 0.6	5.6 ± 0.4	4.7 ± 0.5	2.3 ± 0.2
TLC staining	85.0 ± 0.2	2.4 ± 0.1	4.3 ± 0.2	4.4 ± 0.1	3.6 ± 0.2
"98%"	98 ± 4[a]	n.d.	0.5[b]	n.d.	n.d.
TLC–FID	94.0 ± 0.6	0.7 ± 0.7	1.5 ± 0.5	1.9 ± 0.5	1.8 ± 0.4
TLC staining	96.8 ± 0.2	0.54 ± 0.03	0.46 ± 0.08	0.79 ± 0.06	1.46 ± 0.09
"97%" (ASG)	97.3[c]	0.44[c]	0.16[c]	1.01[c]	1.14[c]

Samples "98%" and "97%" are kindred, except for higher contents of FA in "98%".
[a] BD contents measured by "in house" GC-analysis ($n = 2$).
[b] FA contents measured by titration.
[c] Results of full EN14214 performed by ASG laboratory.

result for FA because of discolorations (discussed in Section 3.5). Measurement of BD in the "98%" sample by TLC–FID demonstrated lower contents of BD (94.0%), whereas contaminants were seemingly overestimated. Such result was probably caused by an erroneous automatic positioning of the baseline performed by Chrom-Star software, where background fluctuations were interpreted as the individual peaks (Fig. 3A, bottom panel "98%"). Manual positioning of the baseline reduced the levels of TG, FA and DG, which corrected BD to its more probable value of 96.5%.

Relation of the biodiesel contents determined by TLC-methods and GC-analysis (Table 5 and Fig. 4B, mean GC-values) gave the values of 1.03 ± 0.07 (TLC-staining) and 0.95 ± 0.04 (TLC–FID), indicating sufficient accuracy of the TLC-methods.

3.7. Kinetics of biodiesel conversion

Time dependencies of rapeseed oil conversion to FAEE by Lipozyme TL HC are shown in Fig. 4 (see also Section 2.2.2). The schemes of 96% ethanol supply slightly varied, but followed the general pattern: some amount added at the very beginning, whereupon the substrate was added continuously over 1.5–4 h (see legend to Fig. 4B). The reactions were monitored by either TLC staining (Fig. 4A and B, closed symbols, all reactants) or GC analysis (Fig. 4B, open symbols, only biodiesel). Both monitoring methods showed the superimposed reaction profiles for biodiesel, where 85–95% of conversion was achieved after 6 h of incubation. The patterns of ethanol supply were slightly different in each experiment. Yet, the conversion process recorded by TLC could be called as "an average reaction" according to its scheme of ethanol feeding (in comparison to three other experiments monitored by GC).

It does not seem to be possible to go above 95% of conversion in one step because of (i) the accumulated products (glycerol and water) and (ii) sensitivity of Novozyme TL HC to high concentrations of alcohol. Different scenarios of the second step with removed glycerol and water are currently under investigation.

3.8. Time expenditure of measurements

The maximal throughput of the data acquisition by TLC-FID corresponded to 40 samples in approximately 6 h, which included dilution of the samples, cleaning of chromarods, application of the samples, simultaneous chromatography of four frames (10 rods in each frame), drying and FID-analysis. TLC on plates required approximately 5 h for separation and staining of 60 points on six 20 cm × 20 cm plates (two developments) or less than 1.5 h on 10 cm × 20 cm plates (development in organic solution followed by immersion in water). The amount of manual labor during this period was considerably less than with TLC–FID. A primary conclusion about the composition of stained samples could be drawn by visual assessment immediately after the final washing. Yet,

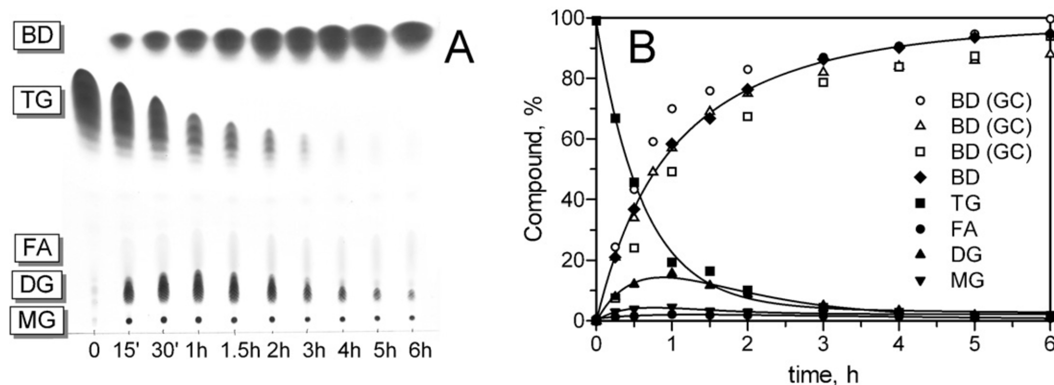

Fig. 4. Reaction kinetics of step 1, rapeseed oil + ethanol + 5% (m/v) Lipozyme TL HC, 35 °C. (A) Reaction record on TLC plate (20 cm × 20 cm). The samples were taken from the reaction mixture at the indicated time intervals and put on the plate in the amount of 1 μL. The plate was developed and stained (Section 2.2.5). (B) Mass % of the reactants over the time. Open symbols correspond to the biodiesel conversion levels (FAEE %) quantified by GC-method. Supply of ethanol followed the schemes: (○) 1 v of oil +0.045 v of 96% ethanol at 0 min, +0.23 v of 96% ethanol continuously over 1.5 h; (Δ) 1 v, +0.031 v at 0 min, +0.22 v over 4 h; (□) 1 v, +0.045 v at 0 min, +0.2 v over 2 h. Closed symbols depict the data calculated from the TLC scan in panel A (see Section 2.2.6 for the details). The scheme of substrate supply corresponded to 1 v of oil +0.045 v of 96% ethanol (0 min), +0.23 v of 96% ethanol continuously over 3 h (all in relative volumes).

additional time was required for manual scanning and accurate quantification of the recorded images (in contrast to the automated analysis using TLC–FID equipment and software).

Under analysis of 3–5 samples, the time expenditure shortened to approximately 2 h (TLC–FID on chromarods), 4 h (TLC Plate 20 cm × 20 cm, two developments), 2 h (TLC Plate 20 cm × 20 cm, one developments + immersion in water) or 30 min (TLC Plate 10 cm × 10 cm, one development + immersion).

The time scale of the above TLC procedures indicates a faster acquisition of data in comparison to GC and HPLC methods, when analyzing a large number of samples. For example, the complete GC-analysis of the components in one biodiesel sample might require up to 3 separate runs (30 min per each run). The GC-procedure is complicated by derivation of MG, DG, FA and change of the columns to separate volatile and heavy fractions independently from each other [6,7]. As a consequence, full analysis of e.g. 10 samples might take up to two days.

4. Conclusions

The presented modifications of TLC–FID and TLC-staining procedures are applicable to analysis of the oil–biodiesel conversion mixtures. The current staining procedure overcomes the persistent heavy background of the earlier methods and allows quantitative evaluation of each compound by analysis of optical density or area. Recording of the whole profile and its scaling by the total mass makes application of an internal standard unnecessary. Both TLC methods appear to be convenient when working with large datasets. The described staining procedure can be recommended for field determination of oil composition because the primary conclusion can be drawn by visual inspection without any electronic equipment.

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