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Article

Production of Additives with Antimicrobial Activity via Tandem Hydroformylation-amine Condensation of Soybean FAME Using an Ionic Liquid-Based Biphasic Catalytic System

Hugo F. Ramalho, Karlla M. C. Ferreira, Paula M. A. Machado, Tamyris B. Silva, Ellen T. Rangel, Marcos J. Prauchner and Paulo A. Z. Suarez*

Laboratório de Materiais e Combustíveis, Instituto de Química, INCT-CATÁLISE, Universidade de Brasília, P.O. Box 4478, 70919-970 Brasília-DF, Brazil

A biphasic catalytic system based in the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate was employed for tandem hydroformylation-amine condensation reactions of soybean FAME using $HRhCO(PPh_3)_3$ as the catalyst precursor and *n*-butylamine. Using a tenfold excess of the ligand PPh_3, the presence of the ionic liquid increased the selectivity for imine if compared to the reaction carried out under similar conditions, but in homogeneous media.The yield for imine reached 75% after 24 h. On the other hand, in the absence of a PPh_3 excess, the effect of using the ionic liquid was opposite and the selectivity for imine decreased. This supposedly occurred due to the generation of *N*-heterocyclics carbenes, which would coordinate to Rh to form species active for parallel and/or consecutive reactions. When an excess PPh_3 is used, it suppress the carbenes coordination, maintaining the Rh complex in a form active for hydroformylation. The obtained imine products presented remarkable antimicrobial activity towards a set of fungi and bacteria commonly present in fuel storage tanks.

Keywords: biodiesel, ionic liquids, hydroaminomethylation, antimicrobial additives

Introduction

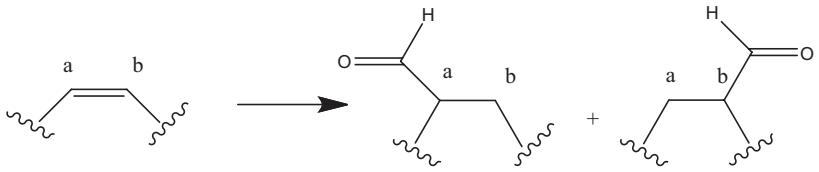
The production and use of fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE) obtained from vegetable or animal fats and oils, usually known as biodiesel, has increased abruptly over the last decade due to the double threats of oil shortage and climate changes. Whilst the biodiesel production was 265 million gallons in 2001, in 2011 it increased to 5,651 million gallons.[1] Biodiesel is foremost used blended to conventional diesel. For example, the addition of 7% of biodiesel in the commercialized diesel (B7 blend) is nowadays mandatory in Brazil and is the maximum percentage permitted in Europe. However, the use of even higher percentages is currently under discussion. The replacing of petroleum derivatives by biodiesel is ecologically advantageous because the biofuel gives rise to an equalized CO_2-balance, besides proportionating lower emission of particulate matter into the atmosphere and no release of aromatic and sulfur-containing compounds.[2,3]

In Brazil, soybean oil is the major feedstock for biodiesel production.[4] This oil consists mainly of unsaturated fatty acids esters, such as oleate (24%), linoleate (54%) and linolenate (6%).[5] The presence of double bonds promotes the positive effect of reducing the freezing point. On the other hand, it impairs the biofuel oxidative stability. Another technical problem verified in the use of biodiesel concerns the propensity to microbial contamination in storage tanks, which occurs mainly due to the hygroscopic nature of biodiesel.[6]

One approach to overcome the low stability of biodiesel without impairing the fuel cold properties is to functionalize their double bonds by means of hydroformylation. This reaction consists in the addition of a formyl group (H–C=O) and a hydrogen atom in a *cis*-manner (Scheme 1).[7,8] The resulting aldehydes are highly reactive and versatile compounds that can be used as building blocks for a wide range of chemicals such as alcohols, carboxylic acids, amines, and ethers.[9]

In a previous work,[9] an ionic liquid-based biphasic catalytic system was used by the first time to hydroformylate a FAME sample, which was obtained through the transesterification of soybean oil. In accordance with the above-mentioned constitution of the soybean oil, the resulting FAME is majority constituted by unsaturated fatty

*e-mail: psuarez@unb.br



Scheme 1. Hydroformylation reaction.

acids. The employed ionic liquid (IL) and catalyst precursor were 1-butyl-3-methylimidazolium hexafluorophosphate ($BMI{\cdot}PF_6$) and $HRhCO(PPh_3)_3$, respectively. Remarkably, the results suggest that the IL stabilized the metal complex in an active form for hydroformylation and prevented side reactions, leading to high reaction yields for aldehydes. Further, the addition of an excess of PPh_3 increased the conversion rate and the selectivity for aldehyde, supposedly because the ligand excess shifts the equilibrium toward the formation of the catalytic complex active for hydroformylation and prevents the occurrence of other forms active for side and consecutive reactions.

If hydroformylation is carried out in the presence of primary or secondary amines or ammonium, condensation reaction takes place to render nitrogen-containing products, as depicted by Eilbracht *et al.*[10] The nucleophilic addition of ammonium or amine to the formed aldehyde yields an O,N-semiacetal that undergoes subsequent dehydration to form imine (if ammonium or primary amine was used) or enamine (if secondary amine was used). Since hydroformylation is carried out in the presence of H_2 and the employed catalyst is also active for hydrogenation, the imine or enamine are usually reduced to primary, secondary, or tertiary amines. The global conversion of the alkene into amine is usually called hydroaminomethylation; the condensation and reduction steps are represented in Scheme 2.

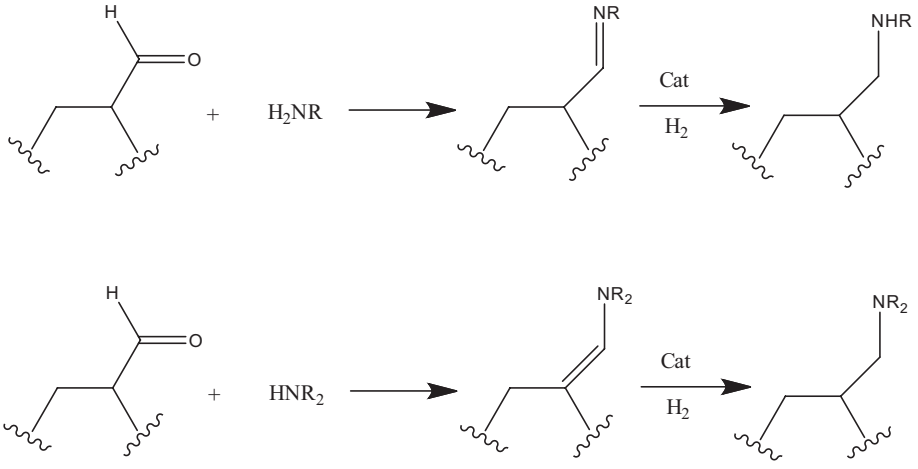
At this point, it is worth to mention that the literature makes reference to a plenty of nitrogen-containing molecules with biological activity, much of them derived from natural compounds. For example, Graebin *et al.*[11] reported that some products of the (+)-limonene hydromethylamination demonstrated *in vitro* activity against *Leishmania (V.) braziliensis*. Further, they identified two promising new anti-*Trypanosoma cruzi* limonene derivatives. In turn, Ferrel and Merkler[12] have published a review about the biological activity of fatty acid amides that they have isolated from mammalian sources.

Taking into account the above-depicted context, the aim of this work was to synthesize biodiesel-based molecules with biocide activity. For that, nitrogen-containing molecules were synthesized through the hydroformylation of soybean FAME in the presence of *n*-butylamine. Therefore, the initially formed aldehydes promptly condensed with *n*-butylamine to form the nitrogenated compounds whose antimicrobial activities were evaluated using standard tests.

Experimental

Reagents

The catalyst precursor $HRhCO(PPh_3)_3$ (Sigma-Aldrich, USA), methanol (Cromoline, Brazil), potassium hydroxide (Vetec, Brazil), triphenylphosphine (Sigma-Aldrich, USA), H_2 (Praxair, 99.9%, Brazil) and CO (Praxair, 99%, Brazil) were used as received. The refined soybean oil (Bunge, Brazil) was dried under reduced pressure prior to the use. The *n*-butylamine (Sigma-Aldrich, USA) was previously distilled. The IL employed was 1-butyl-3-



Scheme 2. Condensation and reduction steps in hydroaminomethylation reactions.

methylimidazoliumhexafluorophosphate (BMI·PF_6), synthesized via halide-free route according to the literature.[13]

Equipment

The conversion of soybean oil into FAME was measured by means of high performance liquid chromatography(HPLC) using a Shimadzu CTO-20A chromatograph (Japan), equipped with an UV-Vis detector (205 nm) and a Shim-pack VP-ODS column (Japan, C18, 250 mm, 4.6 mm of internal diameter).[14] Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained using two equipment: (*i*) an Anasazi EFT-60 MHz instrument (USA) using no solvent; (*ii*) a Bruker Advance III HD 600 MHz (USA), using $CDCl_3$ as solvent. Chemical shifts were reported in ppm (δ) relative to tetramethylsilane (TMS). ^{13}C Attached-Proton-Test (APT) spectra were recorded at 75.5 MHz in a Varian Mercury 300 instrument (USA) using $CDCl_3$ as solvent. Fourier transform infrared (FTIR) spectra were obtained using Shimadzu FTIR Prestige equipment (Japan), equipped with an ATR cell, model ATR Miracle. The Rh leaching from the ionic phase to the organic phase was evaluated by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The analyses were carried out in a Termo Scientific ICAP 6000 equipment (USA) using the external calibration method. Atomic force microscopy (AFM) was performed using a SPM-9600 (Shimadzu, Japan) operating in dynamic mode (height images). The instrument was equipped with a 125 × 125 μm scanner and standard silicon cantil ever tip PPP-NCHR (Nanosensors, Switzerland) with approximately 125 nm length, curvature radius < 10 nm and resonance frequency of 250 kHz was applied. Images were acquired with 512 × 512 lines resolution and scanning area of 10 μm^2 at the rate of 1 Hz. The images were processed by automatic plane fitting plus average horizontal line wise correction procedures and displayed as 2D top view images.

Soybean FAME synthesis

The FAME synthesis was performed according to a previous work, using KOH, methanol and dried soybean oil.[9] The FAME content in the final product was measured by HPLC, resulting in a conversion of 98%.

Tandem hydroformylation-amine condensation of soybean FAME

Soybean FAME was submitted to hydroformylation conditions in the presence of *n*-butylamine. The reaction was carried out in an IL-based biphasic catalytic system. The catalyst precursor was $HRhCO(PPH_3)_3$. In some experiments, an excess of the ligand PPh_3 or its sulfonated form (TPPTS) was added.

The desired amounts of catalyst precursor and, if it was the case, the excess of PPh_3 were added to a 100 mL stainless steel homemade autoclave. After that, the reactor was sealed and purged with 3 cycles of vacuum/N_2. Then, the IL and the liquid reactants (FAME and *n*-butylamine) were added into the reactor using a syringe under N_2 flux. Finally, the reactor was pressurized with 40 bar of CO/H_2 (2:1) and the reaction was carried out in an oil bath (100 °C) under magnetic stirring. After the desired reaction time, the system was cooled down, the gas pressure was released, the volatiles were removed under reduced pressure. Then, the upper phase, corresponding to the nitrogenated products, was collected.

Conversion, selectivity and yield calculations

The calculations of conversion, selectivity and yield were performed using the data obtained from the 60 MHz ^{1}H NMR spectra, based on a method described in the literature.[15] The 60 MHz spectra were chosen because no solvent is needed to perform the analyses, so that effects of hydrogen exchange are prevented. The first step for all calculations was to determine the number of FAME double bonds (double bonds number, DBN, equation 1), which is calculated by dividing the half peak area in the region between 5 and 5.5 ppm (Figure 1a, peak B), assigned to the olefinic hydrogens, by 1/3 of the peak area at 3.6 ppm (Figure 1a, peak A), related to the three hydrogens of the methoxy group, which are used as internal standard.

$$DBN = \frac{B/2}{A/3} \quad (1)$$

The amount of consumed double bonds (ΔDBN) was determined by the difference between the initial number and the final number of double bonds (DBN_i and DBN_f, respectively, equation 2). In turn, the aldehyde number of hydrogens (AHN) was obtained by dividing the peak area of aldehydic hydrogens at 9-9.5 ppm (Figure 1b, peak C) by 1/3 of the peak area relative to the methoxy group hydrogens (Figure 1a, peak A), according to equation 3. The conversion, selectivity and yield for aldehydes are given by equations 4 to 6, respectively.

$$\Delta DBN = DBN_i - DBN_f \quad (2)$$

$$AHN = \frac{C}{A/3} \quad (3)$$

$$Conversion = (\Delta DBN/DBN_i) \times 100 \quad (4)$$

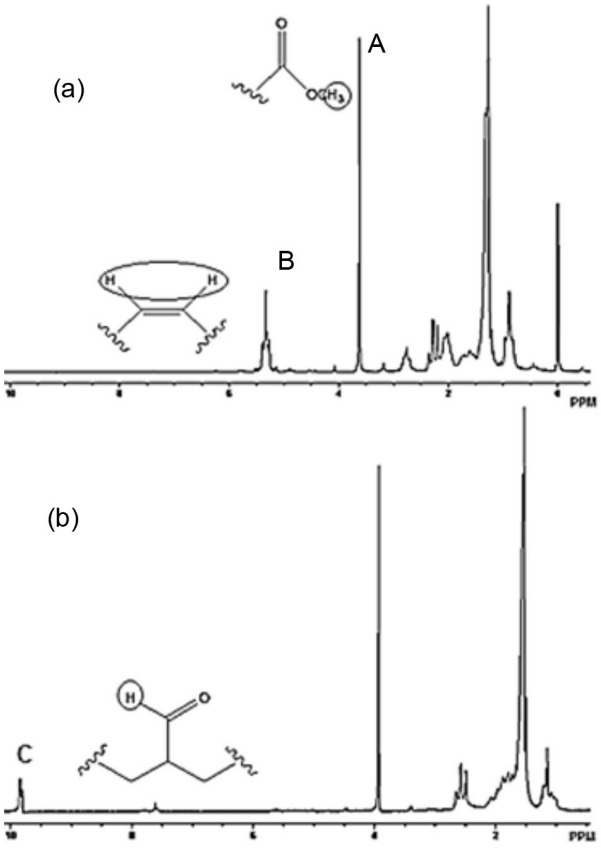


Figure 1. Main ^{1}H NMR spectra attributions for (a) FAME (60 MHz) and (b) the product of its hydroformylation in absence of *n*-butylamine.

$$\text{Selectivity (aldehyde)} = (\text{AHN}/\Delta\text{DBN}) \times 100 \quad (5)$$

$$\text{Yield} = \text{conversion} \times \text{selectivity} \quad (6)$$

As will be discussed in section "Formation of aldehyde *vs.* imine *vs.* amine", hydroformylation reactions in the presence of *n*-butylamine did not cause significant formation of amines, but only imines, i.e., no considerable reduction thereof. Therefore, the selectivity and yield for the tandem hydroformylation-amine condensation were calculated for imine. For that, the imine hydrogen numbers (IHN) were obtained by dividing the signal area at 7.4 ppm (Figure 2, peak D) by 1/3 of the peak area of the methoxy group hydrogens (Figure 1a, peak A), accordingly to equation 7. Thus, the conversion, selectivity and yield for the imine formation are given by equations 8 to 10, respectively.

$$\text{IHN} = \frac{\text{D}}{\text{A}/3} \quad (7)$$

$$\text{Conversion} = (\Delta\text{DBN}/\text{DBN}_i) \times 100 \quad (8)$$

$$\text{Selectivity (imine)} = [\text{IHN}/(\Delta\text{DBN})] \times 100 \quad (9)$$

$$\text{Yield} = \text{conversion} \times \text{selectivity} \quad (10)$$

Antimicrobial tests

Agar diffusion test

The agar diffusion test consists in depositing the substance to be tested in a reservoir (hole) made in a Petri dish containing a culture medium. During incubation, the substance diffuses from the holes to the culture medium. After incubation, the diameter of the inhibited area (no microorganism growth) was measured. The colonies that grow on the inhibition zone were considered resistant.

The diameters of the inhibited area were measured after 48 h of incubation at 32-37 °C using an optical projector, ruler or caliper. The tests were carried out in triplicate on Sabouraud dextrose agar and nutrient agar culture. American type culture collection (ATCC) standard strains of bacteria and fungi were used. Common environment microorganisms which have been isolated from fuels such as diesel/biodiesel were tested (e.g., fungi *C. krusei*,

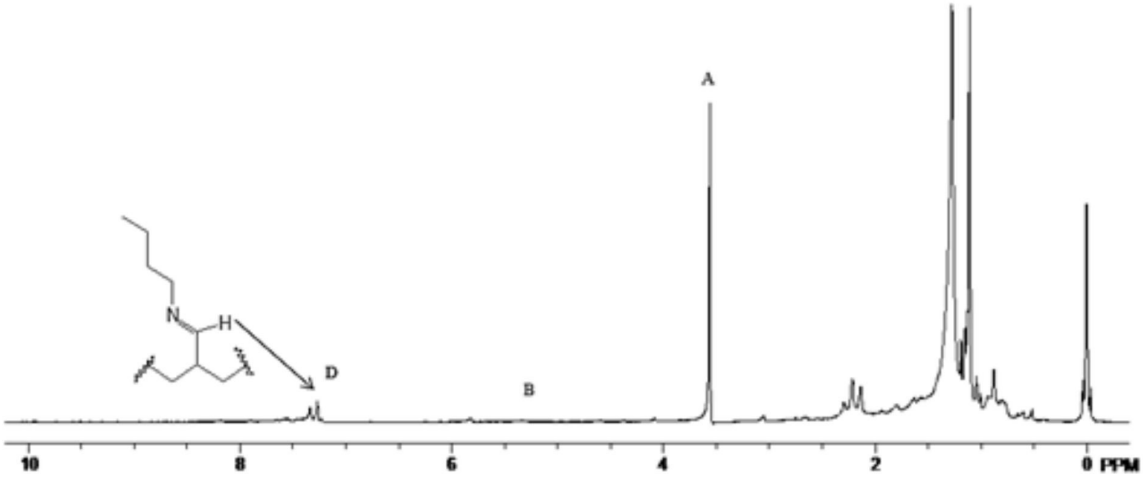


Figure 2. Main ^{1}H NMR spectra attributions for the product of soybean FAME hydroformylation in the presence of *n*-butylamine.

C. parapsilosis, *C. albicans*, *A. niger*, *A. fumigatus* and *S. cerevisae,* and the bacteria *E. coli*, *B. subtillis* and *S. aureus*).[16]

Antimicrobial susceptibility testing

The antimicrobial susceptibilities of microorganisms to modified biodiesel and to modified biodiesel/diesel blends were evaluated through the microdilution tests with standard Clinical and Laboratory Standards Institute (CLSI) broth. The concentrations of the test samples varied from 1 to 512 μg mL^{-1}. Due to the relatively high viscosity of biodiesel/diesel blends, they were previously solubilized in dimethylsulfoxide. The microorganisms studied in this test were the same studied in the agar diffusion test.

The culture medium used was RPMI 1640 with L-glutamine without sodium bicarbonate, buffered with 0.165 mol L^{-1} 4-morpholinepropanesulfonic acid buffer (MOPS) to pH 7.0, and sterilized. The inoculum was prepared from yeast colonies grown on Sabouraud dextrose agar for 48 h at 35 °C. A slurry was prepared in an aqueous NaCl 0.85% solution, sterilized, and the cell density measured by spectrophotometry at a transmittance of 85% at a wavelength of 530 nm. This suspension was subsequently diluted 1:100 and then 1:20 in RPMI1640 culture medium to achieve a final inoculum concentration of 0.5 to 2.5 × 10^3 cells mL^{-1}.

For filamentous fungi, the inoculum was prepared from mycelium grown in cultures derived from potato dextrose agar for five days at 35 °C. A suspension was prepared with spores of filamentous fungi with sterile NaCl 0.85% solution and the cell density was measured with an absorbance spectrophotometer at a transmittance of 85% for a wavelength of 530 nm. This suspension was then diluted in RPMI 1640 medium at a ratio of 1:5 affording 0.4 to 5.0 × 10^4 colony forming units (CFU) mL^{-1}. The bacterial inoculum was prepared from the colonies of bacteria grown on nutrient agar. After incubated at 35 °C for 24 h, the colonies were selected and isolated with a bacteriological loop and transferred to a tube containig NaCl 0.85%. The bacterial suspension was then compared with the standard 0.5 McFarland scale. The tube was shaken immediately prior to use. The next step was to add 100 μL of the inoculum to each tube containing the sample already diluted on the plate. The plates were incubated at 35 °C for 48 h, 72 h and up to 5 days for the tests with bacteria, yeasts and filamentous fungi, respectively. The readings were performed by viewing the turbidity in the culture medium.

All tests were performed in triplicate. The lowest concentration of the tested samples that prevented growth represented the minimal inhibitory concentration (MIC). When verified a MIC less than 256 μg mL^{-1}, the microorganisms were considered susceptible to the corresponding sample being tested.

Microbial growth inhibition test with the weighing of fungal biomass

An Erlenmeyer flask containing the sterilized sample to be tested and another one containing pure biodiesel were incubated (the late was used for sake of comparison). The content of both Erlenmeyer flasks were inoculated with the fungus *A. fumigatus* at a concentration of 10^6 CFU mL^{-1}. The microbial growth was followed by taking aliquots at 7, 14 and 28 days of incubation and weighing the dry fungal biomass.

Results and Discussion

The conversion, selectivity and yield data concerning FAME hydroformylation in the presence of *n*-butylamine are presented in Table 1. The reactions were carried out in the presence or absence of an excess of PPh_3 ligand. As general rule, the reactions were performed in an IL-based biphasic catalytic system; however, for comparison reasons, some reactions were also performed in homogeneous media (that is to say, without the use of IL).

Aiming to make the discussions easier, the reactions and respective products were labeled using 4 fields separated each other by a dot as it follows. Field 1: "Ho" or "IL", depending on if the reaction proceeded in homogeneous media or in the IL-based biphasic system. Field 2: "P" or "0" for the reactions performed in the presence of a tenfold excess of PPh_3 or not, respectively. Field 3: "6" or "24", in order to indicate the reaction time in hours. Field 4: "750" or "2000", in order to indicate the DBN/Rh molar ratio. This way, the label IL.0.24.750 corresponds to the reaction carried out using the IL-based biphasic system, without the addition of an excess of PPh_3, for 24 h, using a DBN/Rh molar ratio of 750. Also regarding nomenclature, the IL.P.24.750 product, which showed high yield for imine, will be hence forth referred to as IFAME (imine functionalized FAME).

Formation of aldehyde *vs.* imine *vs.* amine

As stated in the Introduction section, hydroformylation conducted in the presence of primary amines generates imines as a result of the condensation of aldehydes formed in the hydroformylation stage with the added amine. Indeed, for all hydroformylations carried out in the presence of *n*-butylamine, aldehydes were not detected in the products. For example, the signal relative to the

Table 1. Conversion, selectivity and yield data for soybean FAME hydroformylation in the presence of *n*-butylamine

Reaction condition				Reaction/Product	Conversion / %	Selectivity[e] / %	Yield[e] / %
Ionic liquid[a]	PPh_3/Rh[b]	t[c] / h	DBN/Rh[d]				
No	0	6	750	Ho.0.6.750	77	43	33
	10	6	750	Ho.P.6.750	79	77	60
Yes	0	6	750	IL.0.6.750	65	76	49
			2000	IL.0.6.2000	45	64	28
	10	6	750	IL.P.6.750	84	98	82
			2000	IL.P.6.2000	55	84	46
	0	24	750	IL.0.24.750	90	68	61
			2000	IL.0.24.2000	56	66	37
	10	24	750	IL.P.24.750 (IFAME)	94	80	75
			2000	IL.P.24.2000	72	60	43

[a]BMI.PF_6; [b]PPh_3/Rh molar ratio; [c]reaction time; [d]DBN/Rh molar ratio; [e]for imine.

hydrogen of the aldehyde group (at 9.0 ppm) was not observed in the ^{1}H NMR spectra of the reaction product (Figure 2). Moreover, the absorption relative to C–H stretching in the C(=O)H group, which can be seen at 2710 cm^{-1} in the spectrum of a typical hydroformylation product obtained in absence of amine (Figure 3b), is not observed in the infrared spectra of the product obtained in the presence of *n*-butylamine.

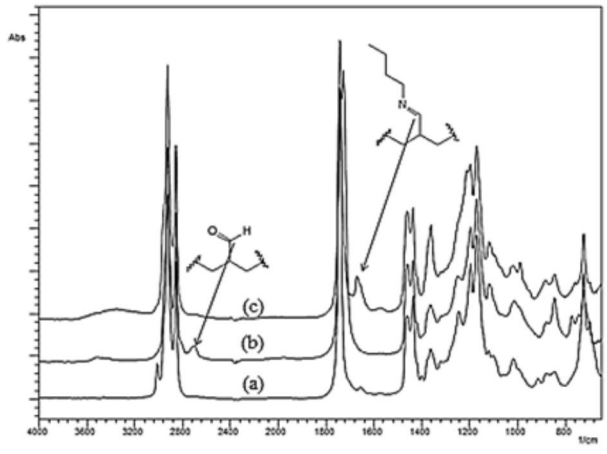


Figure 3. Infrared spectra of (a) soybean FAME and the products of its hydroformylation (b) in absence or (c) in presence of *n*-butylamine.

As already reported in the Introduction section, imines are usually hydrogenated under the conditions employed for hydroformylation, leading to the respective amines. Nevertheless, the infrared and ^{1}H NMR spectra evidence a pronounced presence of imine in the products of the hydroformylation reactions carried out in the presence of *n*-butylamine, whereas the evidence of the presence of amines were very faint or nonexistent. In the infrared spectra, the disappearance of the C–H stretching absorption relative to sp^2 carbon (indicative of the presence of C=C bonds in the FAME sample) was accompanied by the appearance of an absorption at 1670 cm^{-1} relative to the C=N stretching of imines (Figure 3c). In turn, in the 60 MHz ^{1}H NMR spectra, the appearance of a characteristic peak of the hydrogen attached to the carbon sp^2 of the imine was observed at 7.4 ppm (Figure 2).

Regarding to the ^{1}H NMR spectra, the only evidence of amine formation was the appearance of a signal with low intensity and undefined multiplicity at 2.42 ppm for some of the reaction products. Even though, this signal could be identified only in the 600 MHz spectra because it is overlapped by other peaks in the 60 MHz spectra. As it will be detailed in the section Reduction of imine products with Pd/C, this signal was assigned to the alpha-hydrogens of the $CHCH_2NHCH_2CH_2$ group; however, since there are strong evidences that the intensity of this signal is underestimated, it was not used to calculate amine selectivity and yield.

As shown throughout this section, imine hydrogenation was not effective under the reaction conditions employed in the present study. Therefore, these reactions were termed hydroiminomethylation instead of hydroaminomethylation.

Hydroiminomethylation *vs.* hydroaminomethylation

Briggs *et al.*[17] performed the hydroformylation of 1-pentene in the presence of piperidine using $Rh(CO)_2(acac)$ as the catalyst precursor and biphosphite and phosphite as auxiliary ligands. When the reaction was carried out using equimolar amounts of Rh and biphosphite, the yield was low in amine. However, if the ratio of biphosphite to rhodium was decreased below the stoichiometric level, the yield of amine abruptly increased. The authors interpreted

these results as an evidence that the hydrogenation of the enamine intermediate is predominantly catalysed by a Rh complex which does not contain the biphosphite ligand (supposedly $HRH(CO)_3$). Thus, if an equimolar amount of ligand and Rh is used, the hydrogenation would be suppressed because virtually all Rh would be coordinated to the bidentade ligand to form a very stable complex. On the other hand, the use of an excess of Rh would ensure the presence of biphosphate-free catalyst in sufficient quantity to promote hydrogenation.

In turn, dos Santos[18] investigated the hydroformylation of monoterpenes in the presence of amines using $[Rh(cod)(\mu\text{-}OMe)]_2$ as catalyst precursor. They found that the addition of PPh_3 to the reaction system prevents side reactions, but it also hinders the hydrogenation of the imines/enamines formed as intermediates. The authors interpreted these results as follows: due to equilibrium issues, the higher the amount of PPh_3 in the reaction system, the higher the concentration of coordinated PPh_3-rhodium species, which are active for hydroformylation but few active for side reactions and imine/enamine hydrogenation. On the other hand, at low or zero concentration of PPh_3, there is a relatively high concentration of PPh_3-free rhodium species, which are active for the conversion of imine/enamine intermediates into amines but also for side reaction such as double bonds hydrogenation.

The results reported by Briggs *et al.*[17] and dos Santos[18] can be related to the work of Clarke *et al.*,[19] who proposed that electron-donating ligands are less active than electron withdrawing ligands for enamine hydrogenation. Namely, at the same time that electron-donating ligands such as biphosphite and PPh_3 increase the activity of Rh complexes for hydroformylation, they reduce the activity for hydrogenation reactions. In turn, Rh species coordinated only to electron withdrawing ligands, such as CO, are active for promoting the hydrogenation of enamine/imine to render amine, but the reaction selectivity is reduced due to the occurrence of side reaction such as the hydrogenation of double bonds.

Regarding the results reported in Table 1, it is worth to stress that, unlike in the works of Briggs *et al.*[17] and dos Santos[18] the catalyst precursor used in the present work ($HRhCO(PPh_3)_3$) already contained a PPh_3/metal molar ratio of 3:1. Thus, even without the addition of an excess of PPh_3, it is plausible to assume that there was no considerable occurrence of PPh_3-free Rh species in the reaction media, so that the imine hydrogenation was not favored.

At this point, one could postulate that, in the present work, the lack of PPh_3-free Rh species and consequent inability to convert the imines into amines would be due to the presence of the ionic liquid. Indeed, as previously discussed,[9] the ionic liquid contributes to stabilize PPh_3-coordinated Rh species. However, it is necessary to point out that the presence of amines was not significant even in the products of the reactions carried out in homogeneous media (Ho.0.6.750 and Ho.P.6.750 reactions).

Reactions in homogeneous media

The hydroformylation in the presence of *n*-butylamine performed for 6 h in homogeneous medium, with a DBN/Rh = 750, without the use of an excess of PPh_3 (Ho.0.6.750), led to a conversion of 77% and a selectivity for imine of 43%. In turn, in the presence of PPh_3, the conversion was similar (79%) but the selectivity increased to 77%. Thus, it is possible to infer that the use of an excess of PPh_3 favors the formation of PPh_3-coordinated Rh species active for hydroformylation to the detriment of species that are active for hydrogenation. It is worth to mention that hydrogenation can occur at the C=C bonds present in the starting material (parallel reactions) and/or at the C=N bonds of the formed imines (consecutive reactions), both of which result in selectivity loss.

The use of the IL-based biphasic system

By comparing reactions carried out in homogeneous or biphasic medium under similar conditions (Ho.0.6.750 and IL.0.6.750; Ho.10.6.750 and IL.10.6.750), it can be seen that the presence of the ionic liquid increased the selectivity. It is valid to highlight that this increase was verified for the reactions carried out in the presence as well as in the absence of an excess of PPh_3 (from 77 to 98% and from 43 to 76%, respectively). These results are attributed to the "ionic liquid effect",[20] which can act stabilizing the complex in an active form to hydroformylation (PPh_3-coordinated Rh species) and/or stabilizing the activated complex and/or intermediaries of the reaction of interest.

Regarding the conversion, a comparison of the reactions Ho.10.6.750 and IL.10.6.750 shows that, in the presence of an excess of PPh_3, the use of the biphasic system increased the conversion from 79 to 84%, which is in accordance with the aforementioned ionic liquid effect. Contrarily, the comparison of the reactions Ho.0.6.750 and IL.0.6.750 shows that, in the absence of an excess PPh_3, the use of the biphasic system decreased the conversion from 77 to 65%. These different behaviors suggest that, in the absence of PPh_3, the addition of the ionic liquid promotes the formation of less active species, but that are more selective for hydroformylation. One possibility is that the *n*-butylamine reacts with the imidazolium cation to form

N-heterocyclic carbenes, which could complex with Rh, being that the verified selectivity increase would be related to the electron-donating character of carbene ligands.[21] In the case of the reactions carried out in the presence of an excess of PPh_3, the ligand excess would suppress the complexation of the carbenes, so that the addition of the ionic liquid did not decrease the conversion.

The effect of the catalyst proportion

A comparison of the reactions carried out for 6 h under identical conditions but using different FAME/catalyst ratios (compare the reactions IL.0.6.750 with IL.0.6.2000 and IL.10.6.750 with IL.10.6.2000) shows that, as expected, the use of a lower proportion of the catalyst reduced the conversion. Moreover, there was also significant loss of selectivity, which dropped from 76 to 64% and from 98 to 84% for the reactions performed in the absence and presence of a PPh_3 excess, respectively. A possible explanation for this selectivity loss is that, the lower the catalyst proportion, the higher the number of cycles to which the catalyst is submitted, which makes it more likely to be converted into forms that are active for side and/or consecutive reactions.

The effect of the reaction time

The comparison of reactions carried out under identical conditions for different periods of time and using the higher proportion of catalyst (DBN/Rh = 750) demonstrates that, as would be expected, the higher reaction times led to conversion increases (compare the reactions IL.0.6.750 with IL.0.24.750 and IL.10.6.750 with IL.10.24.750). On the other hand, there was a significant loss of selectivity for imine: from 76 to 68% and from 98 to 80% for reactions performed in the absence and presence of an excess of PPh_3, respectively.

The loss of selectivity over the reaction time leads to the conclusion that consecutive reactions consumed the formed imine. One possibility is that the catalytic species containing electron withdrawing ligands, active for hydrogenation, start to convert the formed imines into amines. However, this hypothesis could not be confirmed because, as already mentioned in section Formation of aldehyde *vs*. imine *vs*. amine and it will be deeply discussed in section Reduction of imine products with Pd/C, there was great difficulty in detecting the formation of amines. Other possibility of imine consecutive reaction would be the cleavage of the carbon-carbon bond in the C–C=N group, in a similar way to decarbonylation reactions of aldehydes reported in the literature.[22]

One cannot rule out the possibility that the occurrence of consecutive reactions is related to the formation of rhodium nanoparticles, which were detected in the products of the reactions carried out for longer periods of time, especially when an excess of PPh_3 was not used. The presence of the nanoparticles (10 nm average diameter) was evidenced by the darkening of the ionic liquid and by atomic force microscopy analysis (Figure 4).

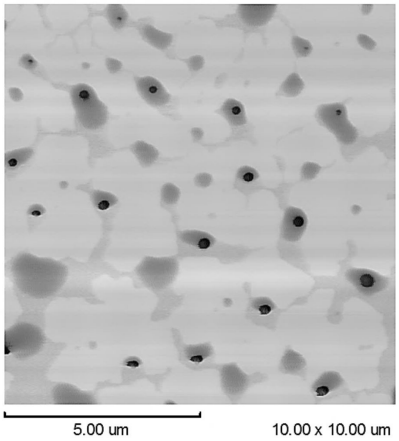
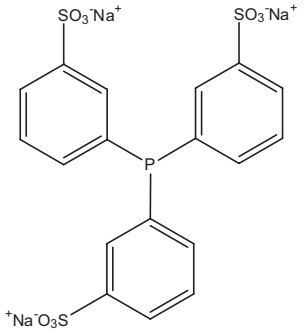


Figure 4. Rh nanoparticles detected by atomic force microscopy of the IL.0.24.750 sample.

Catalyst recycling

One of the most important characteristic of biphasic catalytic systems is the possibility of recycling the phase containing the catalyst. In our methodology, recycle attempts were done by simply removing the organic phase from the reaction medium after completion of the reaction and adding a fresh charge of reagents. However, it was observed that, using the conditions corresponding to the IL.10.24.750 reaction, the system did not show significant activity in the second cycle. Indeed, analyses by inductively coupled plasma optical emission spectrometry (ICP OES) showed that most of the catalyst (approximately 75%) was leached into the organic phase during the first cycle. This behavior can be attributed to the neutral character of the complex used, in addition to the high affinity of the ligand PPh_3 with the organic phase.

In an attempt of avoiding this leaching, the PPh_3 ligand was replaced by the analogue trisulphonated triphenylphosphine (TPPTS, Scheme 3). With the use of this ligand, a reduction of the system activity was observed for the first cycle; but, on the other hand, yields of 42 and 18% were obtained for the second and third cycles, respectively. This improvement was possible due to the better anchoring of the catalyst in the ionic phase. However, this activity is not good enough, so that this is a point that needs to be further investigated in order to improve the results.



Scheme 3. Trisulphonatedtriphenylphosphine (TPPTS).

An attempt of reducing the imine products through a stepwise procedure

A first attempt of promoting the hydrogenation of the imines was carried out following a methodology described in the literature:[23] after hydroiminomethylation, the reactor was depressurized and the excess of unreacted amine was removed under reduced pressure; then, a high pressure of H_2 (80 bar) was applied and the mixture reacted for additional 24 h at 100 °C. The conditions employed in the hydroiminomethylation step were those corresponding to the IL.0.24.750 reaction, because it led to an elevated yield for imine without the employment of an excess of PPh_3, which has shown to prevent imine hydrogenation (see Hydroiminomethylation *vs.* hydroaminomethylation section).

The infrared analysis of the product indicated no conversion of the imine into other products, because the infrared absorption corresponding to the C=N bond of the imine (1670 cm^{-1}) suffered no apparent intensity change (Figure 5b). Furthermore, no considerable reduction in the intensity of the signal relative to the hydrogen of the HC=N group was observed in the 60 MHz ^{1}H NMR spectra. These results indicate that the catalytic species present in the reaction media are inactive for the conversion of imine into amine even under these more severe hydrogenating conditions.

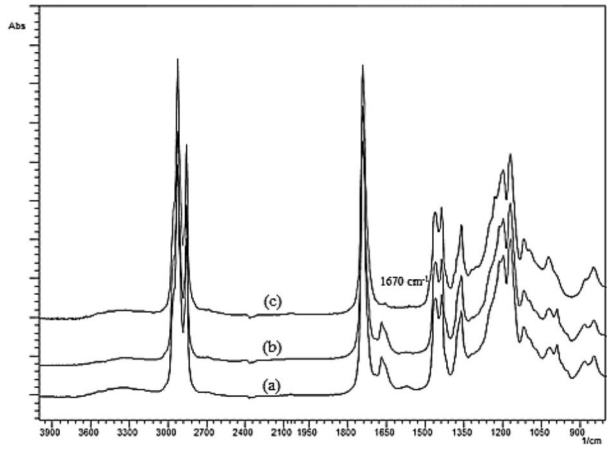


Figure 5. Infrared spectra of (a) IL.0.24.750 sample; (b) the product of its stepwise hydrogenation attempt; (c) the product of is hydrogenation in the presence of the Pd/C catalyst.

Reduction of imine products with Pd/C

In a new attempt of converting the imines into amines, the IL.0.24.750 sample was subjected to a subsequent reaction in the presence of H_2 and palladium deposited on activated carbon (Pd/C), a well-known powerful catalyst for hydrogenation. The products were analyzed and the infrared spectra show that the procedure led to the disappearance of the absorption relative to the C=N stretch at 1670 cm^{-1} (Figure 5c). Furthermore, the 60 MHz ^{1}H NMR spectrum showed that the peak corresponding to the hydrogen of the group HC=N vanished. These results show that the conversion of the imine molecules effectively occurred. However, neither the infrared spectra nor the 60 MHz ^{1}H NMR allowed the identification of any evidence related to amine formation.

In order to obtain better resolved ^{1}H NMR spectra, the analyses were also performed with equipment operating at 600 MHz. In a typical spectrum of soybean FAME, the alpha-hydrogens of the CH_2CH_2COO group renders a triplet centered at 2.3 ppm, with an integration of approximately 2H. However, in the spectrum of the IL.0.24.750 sample (an imine functionalized FAME), this triplet cannot be identified; instead of that, a signal with undefined multiplicity is observed in the corresponding region (Figure 6). This finding shows that there was an overlap of the original triplet, possibly with the signal of the hydrogens of the $C{=}NCH_2$ group of imines. Indeed, after the reduction in the presence of the Pd/C catalyst, the triplet could be observed again (Figure 7), without any overlapping, which is in accordance with the mentioned hypothesis.

A new signal with undefined multiplicity arose at around 2.4 ppm in the 600 MHz ^{1}H NMR spectrum of the product of the hydrogenation of the IL.0.24.750 sample catalyzed by Pd/C. Taking into account its position[22] and the fact that it was not present in the spectrum of the starting material (IL.0.24.750), it is possible to infer that this signal is related to the formation of amines. Namely, it would result from the overlapping between a doublet and a triplet relative to the alpha-hydrogens of the $CHCH_2NHCH_2CH_2$ group (Figure 7). However, it is necessary to point out that integrations are not in accordance with this possibility. Namely, since the integration relative to the hydrogen HC=N in the starting material (the IL.0.24.750 sample) was 0.88 (obtained from the 60 MHz spectrum) and considering a total conversion of imines into amines, thus an integration of 3.54 H (i.e., 4 · 0.88) would be expected for the signal

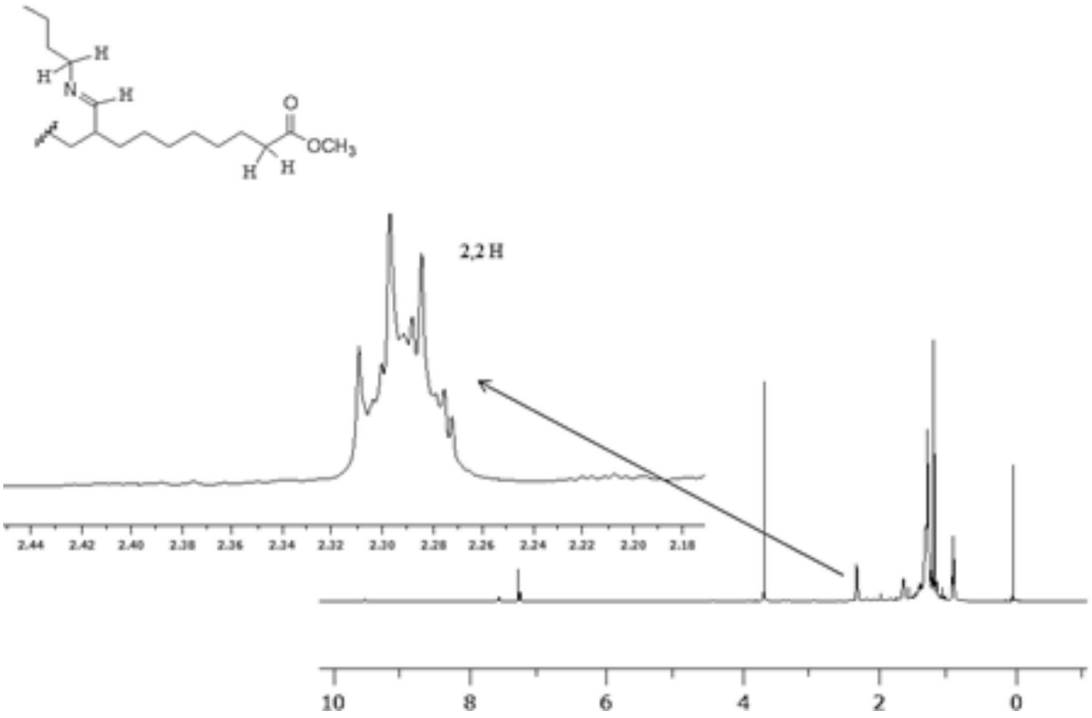


Figure 6. ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of the IL.0.24.750 sample.

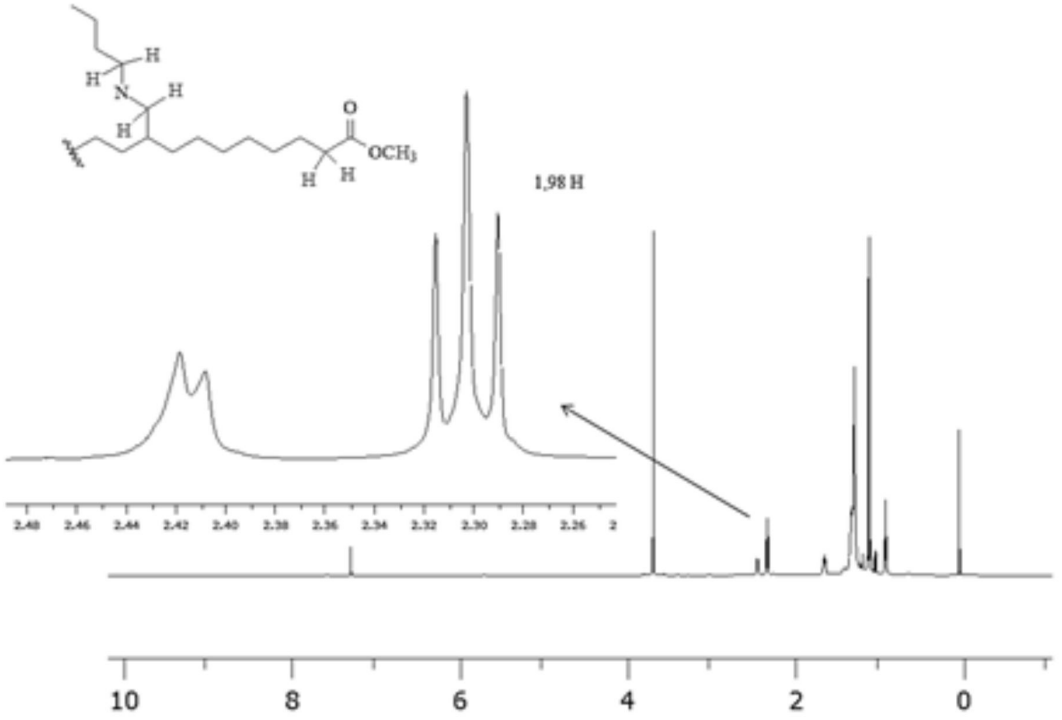


Figure 7. ^{1}H NMR spectrum (600 MHz, $CDCl_3$) of the product of the hydrogenation of the IL.0.24.750 sample with Pd/C.

relative to the four alpha-hydrogens of the amine group, whereas the recorded integration was only 1.3 H.

The above-mentioned low integration verified for the signal at 2.4 ppm in the spectrum of the product of the reaction catalysed by Pd/C could suggest that only part of the imines was converted into amines. However, Krupka and Patera[24] conducted a detailed study about the reduction of aldimines with Pd/C and they verified a quantitative conversion into amines, with no significant formation of other products. Inspired by this study and by the lack of

evidences about the formation of other products, we were led to conclude that the imines were fully converted into amines, being that the intensity of the signal relative to the alpha-hydrogens of amines would be underestimated in the 600 MHz ^{1}H NMR spectrum due to the hydrogen-deuterium exchange with the solvent. This exchange would occur due to the acidity of the alpha-hydrogens of amines, which are subjected to the inductive effect of the nitrogen atom.

^{13}C NMR analysis

In order to further investigate the reduction of the imine functionalized FAME, the IL.0.24.750 sample and the product of its hydrogenation in the presence of Pd/C were analyzed by APT ^{13}C NMR (Figure 8). The main changes verified were that the negative peak initially observed at 163 ppm in the spectrum of the IL.0.24.750 sample, attributed to the carbon of the group HC=N (carbon c), was inverted and shifted to render a positive peak at 45 ppm in the spectrum of the hydrogenation product. This peak was then assigned to the carbon of the H_2C–N group (carbon c'), so that the mentioned changes are in accordance with the proposed conversion of the imines into amines.

Antimicrobial activity tests

Agar diffusion test

The results for agar diffusion tests using different IFAME/FAME blends for bacteria and fungi showed diameters of the inhibition zone larger than 10 mm, which is conventionally adopted as the threshold limit value to characterize a biocide activity. Therefore, the results of Table 2 indicate that a concentration of only 0.75% of IFAME is enough to inhibit the formation of most of the studied fungi. In turn, the biocide activities for bacteria were less pronounced, being that a minimal concentration of 5% of IFAME was necessary to inhibit the growth of *E. coli* and *S. aureus*, and a concentration of 10% to inhibit the growth of *B. subtillis*. The diameters of the inhibition zone were also considerably smaller for the bacteria activity than for the fungi.

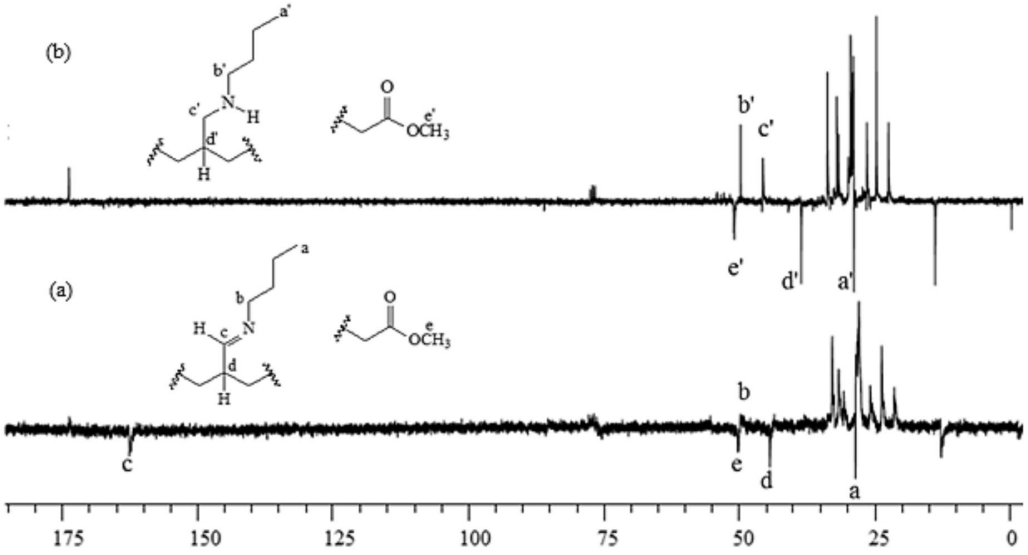


Figure 8. APT 13C NMR spectra of the (a) IL.0.24.750 sample and the (b) product of its hydrogenation with Pd/C.

Table 2. Results of the agar diffusion tests of IFAME/FAME blends for fungus

Fungus	Diameter of the inhibition zone / mm								
	Concentration of the IFAME/FAME blends / (v/v)								
	0.25%	0.5%	0.75%	1%	5%	10%	25%	50%	100%
C. krusei	–	–	11.0	12.0	12.0	13.0	13.0	16.3	14.6
C. parapsilosis	–	–	-	12.5	16.5	16.3	26.0	26.0	35.0
C. albicans	–	–	15.5	15.0	15.2	20.0	18.0	17.5	26.5
A. niger	–	–	12.5	13.0	15.2	15.5	16.0	20.0	24.3
A. fumigatus	–	–	13.4	13.5	14.0	14.5	15.0	18.3	23.5
S. cerevisae	–	–	–	–	14.2	15.5	16.2	16.8	17.3

Table 3. Results of the agar diffusion tests of IFAME/FAME blends for bacteria

Bacteria	Diameter of the inhibition zone / mm								
	Concentration of the IFAME/FAME blends / (v/v)								
	0.25%	0.5%	0.75%	1%	5%	10%	25%	50%	100%
E. coli	–	–	–	–	12.0	14.5	16.5	16.0	17.0
B. subtillis	–	–	–	–	-	13.0	13.5	14.4	15.8
S. aureus	–	–	–	–	11.0	11.3	12.5	14.6	15.3

Table 4. Minimum inhibitory concentrations (MIC) of IFAME/FAME blends for fungi

Fungus	Minimal inhibitory concentration / (μg mL^{-1})						
	Concentration of the IFAME/FAME blends / (v/v)						
	0.75%	1%	5%	10%	25%	50%	100%
C. krusei	512	512	512	256	256	128	128
C. parapsilosis	–	512	512	512	512	512	512
C. albicans	512	256	256	256	256	256	256
A. niger	512	256	256	256	256	128	64
A. fumigatus	256	256	128	128	128	128	64
S. cerevisae	–	–	512	512	256	256	256

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed for the blends that showed positive biocide activity in the agar diffusion test. The measured MIC widely varied according to the fungi and the bacteria studied. The verified results were better for fungi than for bacteria, being that the specie *A. fumigatus* showed the lowest MICs.

Table 5. Minimum inhibitory concentrations (MIC) of IFAME/FAME blends for bacteria

Bacteria	Minimal inhibitory concentration / (μg mL^{-1})				
	Concentration of the IFAME/FAME blends / (v/v)				
	5%	10%	25%	50%	100%
E. coli	512	512	512	512	256
B. subtillis	–	512	512	256	256
S. aureus	512	512	512	256	256

Microbial growth inhibition test with the weighing of the fungal biomass

The microbial growth inhibition test with the weighing of the fungal biomass was carried out for the fungus *A. fumigatus*, which showed the best results in the antimicrobial susceptibility testing. Figure 9 presents a graph of the weight of fungal biomass *versus* the incubation time for the blend containing 5% of IFAME in FAME, along with the data relative to the pure FAME. The results shows that addition of the IFAME decreased by approximately 25% the fungus growth, which evidences the potential of using the modified biodiesel as antimicrobial additive.

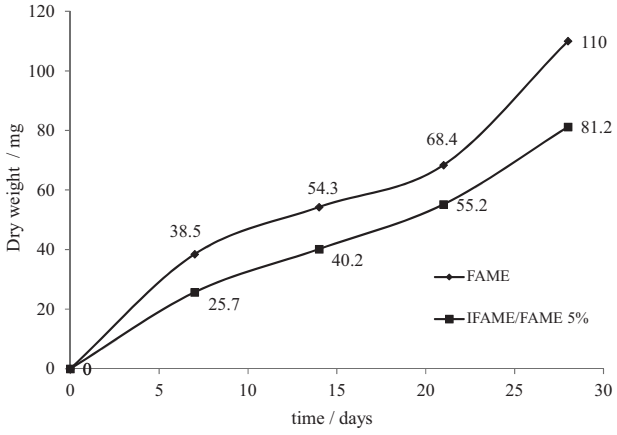


Figure 9. Dry weight of fungal biomass *versus* the incubation time for the samples of pure FAME and the blend IFAME/FAME 5%.

Conclusions

A biphasic catalytic system based in the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate was employed for tandem hydroformylation/amine condensation reactions of soybean FAME using $HRhCO(PPh_3)_3$ as the catalyst precursor and *n*-butylamine. In the presence of a tenfold excess of the ligand PPh_3, the presence of the ionic liquid increased the selectivity for imine if compared to the reaction carried out under similar conditions, but in homogeneous media. The yield for imine reached 75% after

24 h. On the other hand, in the absence of a PPh_3 excess, the effect of using the ionic liquid was opposite and the selectivity for imine decreased. This supposedly occurred due to the generation of *N*-heterocyclics carbenes, which would coordinate to Rh to form species active for parallel and/or consecutive reactions. When an excess of PPh_3 is used, it suppress the carbenes coordination, maintaining the Rh complex in a form active for hydroformylation.

Although adjustments are still necessaries in order to improve the capacity of recycling the ionic phase containing the catalyst, the used system was efficient to produce imine functionalized FAME with remarkable antimicrobial activity towards fungi and bacteria commonly present in fuel storage tanks. These results point out a great potential of using the modified biodiesel as an efficient antimicrobial fuel additive.

Acknowledgements

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