A flow injection procedure based on solenoid micro-pumps for spectrophotometric determination of free glycerol in biodiesel

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ABSTRACT

A flow system designed with solenoid micro-pumps is proposed for fast and greener spectrophotometric determination of free glycerol in biodiesel. Glycerol was extracted from samples without using organic solvents. The determination involves glycerol oxidation by periodate, yielding formaldehyde followed by formation of the colored (3,5-diacetil-1,4-dihidrolutidine) product upon reaction with acetylacetone. The coefficient of variation, sampling rate and detection limit were estimated as 1.5% (20.0 mg L^{-1} glycerol, $n = 10$), 34 h^{-1}, and 1.0 mg L^{-1} (99.7% confidence level), respectively. A linear response was observed from 5 to 50 mg L^{-1}, with reagent consumption estimated as 345 µg of KIO$_4$ and 15 mg of acetylacetone per determination. The procedure was successfully applied to the analysis of biodiesel samples and the results agreed with the batch reference method at the 95% confidence level.

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1. Introduction

Biodiesel is an important alternative fuel, produced from animal fats or natural vegetable oils from different varieties of feedstock (*e.g.*, soybean, cottonseed, palm, canola, peanut, and castor beans). This biofuel can be used in the pure form or blended with petroleum diesel in different proportions [1,2]. Biodiesel production is generally carried out by catalytic transesterification in the presence of methanol or ethanol. Besides the fats conversion into fatty acid methyl esters [2,3], the reaction yields glycerol (1,2,3-propanetriol) as a side product, and this species is usually removed by water flush. Residual amounts of glycerol may cause occlusion of fuel filters, often in the co-presence of fatty acids soaps, thus impairing the engine motors. Glycerol may also lead to emission of hazardous substances (*e.g.* acrolein) during combustion, thus causing pollution if catalytic converters for exhaust treatment are absent [4]. Consequently, the maximum content of free glycerol allowed in biodiesel is 200 mg kg^{-1} (0.02% (m/m), according to the regulations established in Brazil (ANP 42) [5], European Community (EN 14214:2008) [6] and United States (ASTM D 6751-02) [7].

Considering these regulations and the increasingly use of this fuel, there is a need for fast, precise and reliable analytical methods for the determination of free glycerol in biodiesel. The Brazilian, European and American norms recommend gas chromatography

[8] for this analysis, but the procedure is prone to baseline drift and overlapped signals. In some samples, the presence of trace of volatile hydrocarbons as additives may interfere in the determination and EN 14214 Standard suggests an alternative method to solve the problem [6]. Alternative procedures involving enzymatic reactions [9], HPLC [10], LC–GC [11], CG–MS [12] and CE [13] have also been presented, but they usually require high-cost instrumentation, skilled analysts and complex and time-consuming sample pretreatments. Moreover, the relatively low sample throughput is less compatible with the increasing demand for this analysis and toxic wastes are often generated. Regarding this later aspect, ASTM recommends analysis by GC after analyte derivatization with N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA), in pyridine medium.

Bondioli and Della Bella [14] proposed a spectrophotometric procedure based on oxidation of free glycerol by periodate, after extraction with a 1:1 (v/v) ethanol:water mixture. Hexane (4 mL per sample) was used to make feasible phase separation. The method was based on glycerol oxidation yielding formaldehyde that reacted with acetylacetone. Heating was required to increase the reaction rate, and a rigid control of temperature and time were then essential for obtaining reliable results.

Implementing this analytical method in a flow system circumvents the above mentioned limitations due to the ability for sample handling under very reproducible conditions. The versatility of the flow systems is improved by exploiting multicommutation. Additionally, diminution of operational costs and minimization of reagent consumption are attained [15]. The flow manifolds are

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Table 1
Switching course of the solenoid micro-pumps for free glycerol determination[a].

Step	Description	P_1	P_2	P_3	P_4	Pulses
1	Sampling and mixing of S, R_1 and R_2[b]	0	1/0	0	0	3
		0	0	1/0	0	1
		0	0	0	1/0	2
2	Zone stopping interval (60 s)	0	0	0	0	–
3	Transport, measurement and cleaning	1/0	0	0	0	150
4	Sample replacement[c]	0	1/0	0	0	20
		1/0	0	0	0	20

[a] Codes 1/0 and 0 indicate actuation of the solenoid micro-pumps and that the devices remain inactive, respectively.
[b] The sequence of pulses 3:1:2 was 10-fold repeated in each measurement cycle.
[c] Solenoid valve (V) switched on.

built up with discrete computer-controlled commuting devices (solenoid valves or micro-pumps), which permits manifold re-configuration by software. Solenoid micro-pumps can replace the injection and propulsion devices, yielding compact manifolds. Other advantages are low reagent consumption and better mixing conditions due to the inherent pulsed flow [16,17].

In this work, a flow system designed with solenoid micro-pumps was developed for spectrophotometric determination of free glycerol in biodiesel, exploiting in-line analyte oxidation by periodate. An alternative extraction procedure was also proposed to avoid the use of organic solvents.

2. Experimental

2.1. Apparatus

The flow system comprised four solenoid micro-pumps of 20 (P_1), 10 (P_2 and P_3) and 5 (P_4) μL per pulse (Biochem Valve Inc., Boonton, NJ, USA), one three-way solenoid valve (NResearch, West Caldwell, NJ, USA), 0.8-mm i.d. polyethylene tubes and a Perspex connector. A parallel port of a Pentium IV microcomputer was used for controlling the active devices through a power drive based on an ULN2803 integrated circuit [18]. Measurements were carried out by a multi-channel CCD spectrophotometer (USB2000, Ocean Optics, Dunedin, FL, USA) with a tungsten-halogen light source (LS-1, Ocean Optics). A glass flow cell with 1-cm optical path and 80 μL internal volume (Hellma, Plainview, NY, USA) was used with two 100 μm i.d. optical fibers. The control software was developed in Visual Basic 6.0 (Microsoft) and the software furnished by the spectrophotometer manufacturer was used for data acquisition.

2.2. Solutions

All solutions were prepared with analytical grade chemicals and deionized water. A 1.5 mol L^{-1} ammonium acetate stock solution was prepared by dissolving appropriate amounts of the salt in water and adjusting the pH to 4.5 with hydrochloric acid. The 1.5 mol L^{-1} acetylacetone (2,4-pentanedione, Across Organics) and 15 mmol L^{-1} potassium periodate (Fluka) solutions were daily prepared in 0.8 mol L^{-1} ammonium acetate, pH 4.5). Working solutions were prepared by dilution of a 1000 mg L^{-1} glycerol (Sigma–Aldrich) stock in water.

2.3. Flow diagram and procedure

The flow system for determination of free glycerol in biodiesel is shown in Fig. 1. The system was operated as described in Table 1, and exploited binary sampling [15] for solutions handling. The solenoid micro-pumps were actuated at 5 Hz, yielding flow rates ranging from 1.5 to 6.0 mL min^{-1}. The sample aliquot (3 pulses, P_2) was inserted in tandem with the reagents: 1 pulse of periodate (P_3)

and 2 pulses of acetylacetone (P_4) solutions, step 1. The sample zone was stopped by 60 s into the reactor coil B (step 2) before transport towards detection at 412 nm (step 3). Measurements were based on peak height and carried out in triplicate.

A three-way solenoid valve (V) was used when solutions were replaced (step 4), thus avoiding the unnecessary passage through the flow cell, saving time and minimizing risks of contamination.

2.4. Extraction procedure

The extraction of free glycerol was carried out with about 1 g of biodiesel samples directly weighted into 15 mL graduated tubes. After addition of 4 mL of deionized water, the mixture was vigorously shaken in orbital platform for 30 min, with the tubes positioned horizontally in relation to the platform table. The mixture was then centrifuged for 5 min at 3000 rpm, the biodiesel phase was removed with a Pasteur pipette and the free glycerol content was determined in the aqueous phase.

## 3. Results and discussion

3.1. System optimization

Free glycerol in biodiesel samples can be oxidized by periodate forming formaldehyde (Eq. 1) that reacts with acetylacetone in presence of ammonium acetate (Hantzsch reaction), forming a yellow product, 3,5-diacetyl-1,4-dihydrolutidine (Eq. 2). The reaction product can be quantified by fluorimetry or spectrophotometry (absorption maxima at 412 nm).
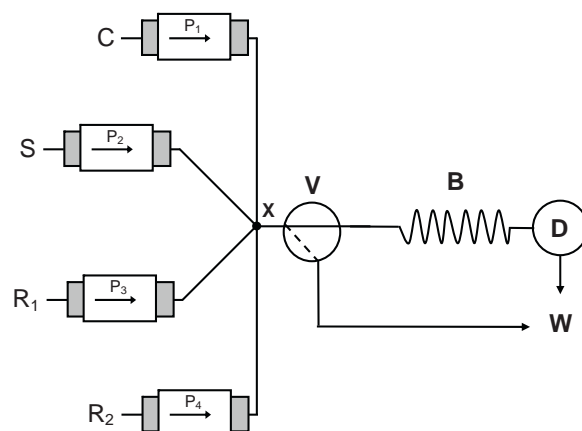

Fig. 1. Flow diagram of the system for free glycerol determination. P_1–P_4: solenoid micro-pumps; V: three-way solenoid valve; S: sample; C: carrier (H$_2$O), R_1: 15 mmol L^{-1} periodate in 0.8 mol L^{-1} ammonium acetate, pH 4.5, R_2: 1.5 mol L^{-1} acetylacetone in 0.8 mol L^{-1} ammonium acetate, pH 4.5. B: 120-cm long reaction coil; D: flow cell coupled to the CCD spectrophotometer; W: waste vessel.
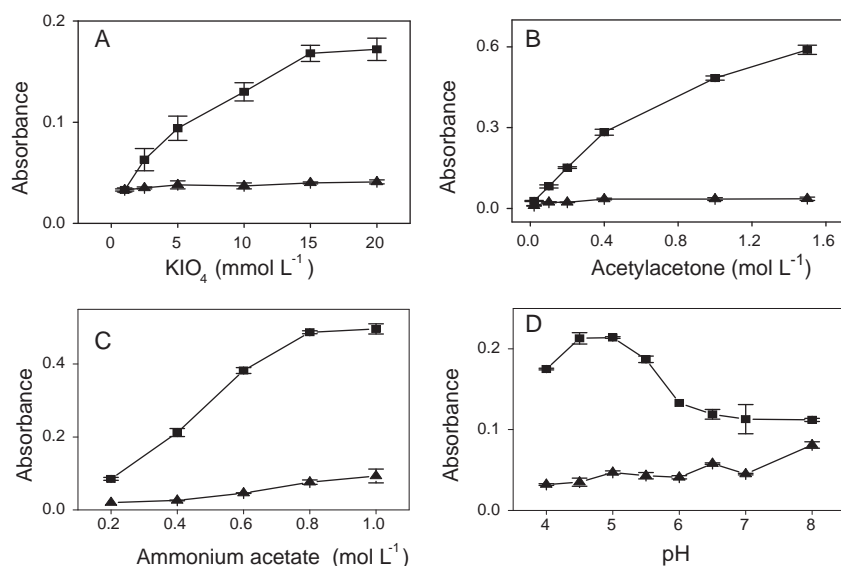
Fig. 2. Effect of concentration of (A) periodate; (B) acetylacetone; (C) ammonium acetate and (D) pH in the analytical (■) and blank (▲) signals. Reagent concentration: (A) 0.2 mol L^{-1} acetylacetone and 0.4 mol L^{-1} ammonium acetate; (B) 15 mmol L^{-1} periodate and 0.4 mol L^{-1} ammonium acetate; (C) 1.5 mol L^{-1} acetylacetone and 15 mmol L^{-1} periodate and (D) 1.5 mol L^{-1} acetylacetone, 15 mmol L^{-1} periodate and 0.8 mol L^{-1} ammonium acetate. Glycerol concentration: 400 mg L^{-1} (A and B) and 100 mg L^{-1} (C and D).
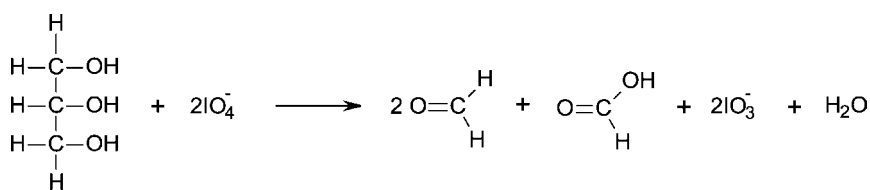
$$(1)$$
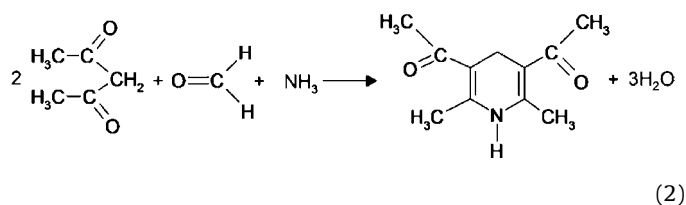
$$(2)$$

The effects of concentrations and volumetric fractions of periodate, acetylacetone and ammonium acetate as well as pH were evaluated aiming at optimizing sensitivity. Magnitude of the blank signal, precision and reagent consumption were also taken into account. The effects of reagent concentrations were evaluated by the merging zones approach (micro-pumps P$_2$–P$_4$ were simultaneously actuated for inserting sample and reagent aliquots into the analytical path). Other experimental conditions were: one pulse of each solution, 10 sampling cycles and a 60-s zone stopping period.
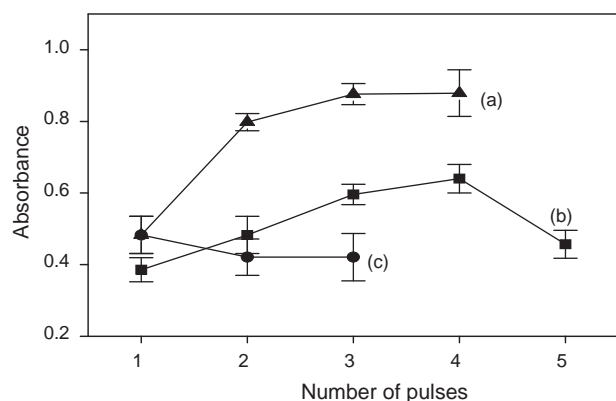
The analytical signal increased with KIO$_4$ concentration up to 15 mmol L^{-1}, without affecting the blank value (Fig. 2A). This concentration was then selected for subsequent studies. The effect of the acetylacetone concentration was evaluated from 0.2 to 1.5 mol L^{-1} (Fig. 2B) and the general tendency was analogous to that related to KIO$_4$ concentration. Best results were observed for 1.5 mol L^{-1}, the maximum evaluated concentration due to limitations of solubility of the ketone in water (1.6 mol L^{-1} at 25 °C [19]).

The ammonium acetate concentration in the 1.5 mol L^{-1} acetylacetone and 15 mmol L^{-1} potassium periodate reagents was varied from 0.2 to 1.0 mol L^{-1} (Fig. 2C). Higher concentrations resulted in higher analytical signals due to the increase in the amount of free ammonia (ca. 3.2×10^{-6} mol L^{-1} and 1.1×10^{-5} mol L^{-1} for 0.2 and 0.8 mol L^{-1} ammonium acetate), as pH was maintained for all the tested concentrations. On the other hand, the blank value increased with ammonium acetate concentration due to Schlieren effect [20]. For subsequent studies, a 0.8 mol L^{-1} ammonium acetate solution was adopted, given the compromise between reagent consumption and analytical signal. The dual wavelength strategy was exploited to compensate the Schlieren effect [20], so that the difference in absorbances at the maximum absorption (412 nm) and reference (780 nm) wavelengths was considered as the net analytical signal.

Regarding influence of pH of the reaction medium, best analytical response was obtained in the pH range 4.5–5.0 (Fig. 2D). In the batch procedure [14] a buffer solution at pH 5.5 was used, but the effect of acidity in the determination of glycerol was not reported. Li et al. [21] exploited the reaction with acetylacetone in a flow injection procedure for spectrophotometric determination of formaldehyde in water samples and best sensitivity was reported for pH within 6.5 and 7.5. The influence of the pH on the formation of 3,4-diacetyl-1,4-dihydrolutidine was then evaluated with a 3 mmol L^{-1} formaldehyde solution and a constant analytical signal was observed within pH 4.5 and 8.0. In this way, ammonium acetate buffer pH 4.5 was used in subsequent studies.

Different sampling strategies as well as variation of the volumetric fractions were evaluated to maximize the sensitivity for glycerol determination. The merging zones approach (10 simultaneous pulses of sample and reagent solutions) yielded an analytical signal of 0.281 ± 0.032 for a 100 mg L^{-1} glycerol solution. The influence of the number of pulses for each solution was thereafter investigated by the univariate method and binary sampling [15], which allowed changing the volumetric fractions and provided better analytical response. The analytical and blank signals both increased with the acetylacetone volume up to four pulses. By

Fig. 3. Influence of the number of pulses of (a) sample, (b) acetylacetone and (c) periodate. Concentrations defined in Fig. 2D.

comparing the results obtained with two and four pulses, the analytical signal was increased by 43%, whereas the blank value was 68% higher (0.318 ± 0.025 for four pulses of acetylacetone). In this way, two reagent pulses were selected. The increase in the periodate volume diminished both analytical and blank signals as a consequence of sample dilution, indicating that this reagent was in excess even when only one pulse was added to the sample zone. According to Fig. 3a, three sample pulses yielded the highest sensitivity. In these conditions, the periodate concentration in the sample zone was similar to that of the analogous batch-wise procedure (ca. $3.0\,mmol\,L^{-1}$) [14], but the ketone concentration was 5.5-fold higher. As the reaction of formaldehyde with acetylacetone is relatively slow and measurements were carried out without achieving the steady state condition, a large reagent excess favored the product formation.

The effect of the reaction coil length was evaluated from 70 to 220 cm. The number of sampling cycles was increased proportionally to the reactor volume aiming compensation of the effect of sample dispersion. The analytical signal increased with the reaction coil length due to the longer residence time. For 70 and 120-cm reactors, the analytical signals were 45 and 18% lower in comparison to those obtained with a 220-cm coil, respectively. A 120-cm coil was chosen for subsequent studies to reduce the reagent consumption and the washing time.

The effect of the number of sampling cycles is shown in Fig. 4A. The higher analytical signals resulted from diminution of the sample dispersion and ten sampling cycles were selected by taken into account the sampling rate and reagent consumption. This corresponds to a 500-μL sample zone for a 600-μL reactor.

Formation of 3,4-diacetyl-1,4-dihydrolutidine is relatively slow and heating in a water bath (70 °C, 1 min) is required in the batch procedure [14]. In view of drawbacks such as formation of gas bubbles and formation of refractive index gradients, heating was avoided in the flow-based procedure. The increase of the sample residence time in the reactor was then evaluated to improve the analyte conversion (Fig. 4B) and best analytical signal was obtained with 60 s. For longer stopping times, the rate of product formation did not compensate the effect of analyte diffusion. Table 2 shows a summary of the investigated parameters and selected values in the optimization step.

3.2. Glycerol extraction

Procedures for free glycerol determination in biodiesel generally involve liquid–liquid extraction. As an example, a procedure uses a biphasic system with the biodiesel sample, hexane and a 1:1 (v/v) ethanol/water solution as the polar phase [14]. Alternative procedures are time-consuming. For example, Hájek et al. [22] developed
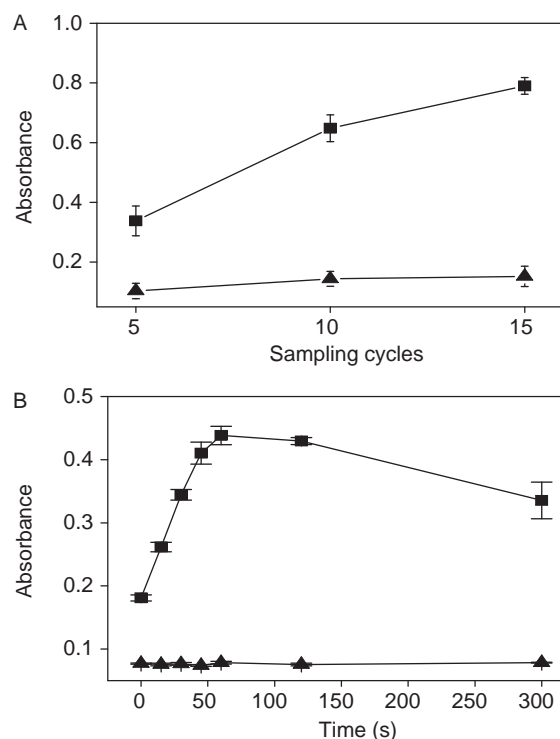
Fig. 4. Effect of the number of sampling cycles (A) and stopping period (B) on the analytical (■) and blank (▲) signals employing 3, 1 and 2 pulses of sample, periodate and acetylacetone solutions, respectively. Concentrations defined in Fig. 2D.

an apparatus for the extraction of glycerol from biodiesel samples with deionized water, in which 4–20 g of sample was mixed with 4.5 mL of deionized water in a glass apparatus. The extraction was carried out by bubbling air through the mixture and total extraction was obtained after 2 h.

In the present work, agitation by vortex for 5 min or in an orbital platform shaker for 30 and 60 min were evaluated for glycerol extraction. In the later, the tubes were positioned horizontally to the platform table, providing better phase interaction. After separation with 4 mL of a 50% (v/v) ethanol solution, the results obtained by both shaking procedures were in agreement (differences lower than 2.5%). However, at least 30 samples can be simultaneously extracted with the orbital platform shaker yielding better analytical productivity. In addition, results obtained with shaking times of 30 and 60 min did not show significant differences.

The efficiency of extraction with water was evaluated to avoid the use of organic solvents. As shown in Table 3, results did not differ significantly of those obtained with ethanol/water and hexane. This result is justified by the high solubility of glycerol in water [4]. In addition to the less toxic residue, water extraction avoids risks of perturbations by Schlieren effect and bubbles formation in the flow system, which would be expected for a 50% (v/v) ethanol extract.

Table 2
Selected parameters of the flow-based procedure for free glycerol determination in biodiesel.

Parameter	Evaluated range	Selected value
Periodate (mmol L^{-1})	1–20	15
Acetylacetone (mol L^{-1})	0.02–1.5	1.5
Ammonium acetate (mol L^{-1})	0.2–1	0.8
pH	4.0–8.0	4.5
Sampling cycles	5–15	10
Zone stopping interval for (s)	0–300	60

Table 3
Free glycerol determination in biodiesel samples. Mean values and uncertainties are based on 3 analytical determinations.

| Samples	Glycerol concentration (mg kg^{-1})		
	Proposed procedure[a]	Batch[a]	Batch[b]
Cottonseed	17.9 ± 0.2	18.5 ± 1.0	18.2 ± 0.5
Soybean (I)	106.5 ± 0.4	98.9 ± 0.7	97.5 ± 1.4
Soybean (II)	56.1 ± 1.8	52.1 ± 1.2	51.0 ± 2.3
Soybean (III)	47.9 ± 0.2	49.2 ± 12.6	47.4 ± 4.8
Soybean (IV)	13.8 ± 1.3	12.0 ± 0.7	–

[a] Glycerol extraction with 4 mL of water.
[b] Glycerol extraction with 4 mL of 50% (v/v) ethanol and 4 mL hexane [14].

3.3. Analytical features and application

Under the selected conditions, a linear response was observed for 5–50 mg L^{-1} free glycerol, equivalent to 0.002–0.02% (w/w) in biodiesel. The relation between the absorbance (A) and the glycerol concentration (C, mg L^{-1}) can be described by the equation $A = (0.172 \pm 0.009) + (0.014 \pm 0.001)C$, $r = 0.999$. Transient signals for glycerol reference solutions are shown in Fig. 5. The detection limit was estimated as 1 mg L^{-1} (99.7% of confidence level), equivalent to 0.0004% (w/w) in biodiesel, which is 50 times lower than the threshold value established by Brazilian, European and American regulations [5–7] and similar to the obtained by the procedure based on CE [13]. The coefficient of variation and sampling rate for the analysis of the extract were estimated as 1.5% ($n = 10$) and 34 samples/h, respectively. Sample throughput is not significantly hindered by the extraction step because at least 30 samples can be simultaneously processed. The reagent consumption was estimated as 15 mg of acetylacetone and 345 µg KIO$_4$, generating 3.5 mL of waste per determination. Table 4 shows the analytical features obtained in the proposed, batch [14] and FIA [23] procedures for free glycerol determination. The consumptions of acetylacetone and potassium periodate were ca. 3-fold and 8-fold lower than in batch and FIA procedures, respectively. In addition, sensitivity is ca. 20-fold higher than that reported in the previous FIA procedure [23].

The proposed procedure exhibits a good selectivity, as several aldehydes, acetone and methanol did not interfere even in relatively higher concentrations [24]. Horstkotte et al. [25] also verified that inorganic (Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Na$^+$, K$^+$, H$_3$BO$_3$, I$^-$, SO$_4^{2-}$, MoO$_4^{2-}$ and PO$_4^{3-}$) and organic (methanol, ethanol, acetic acid, formic acid, acetone, acetaldehyde, glyceraldehyde and glyoxal) species did not interfere in the formaldehyde determina-
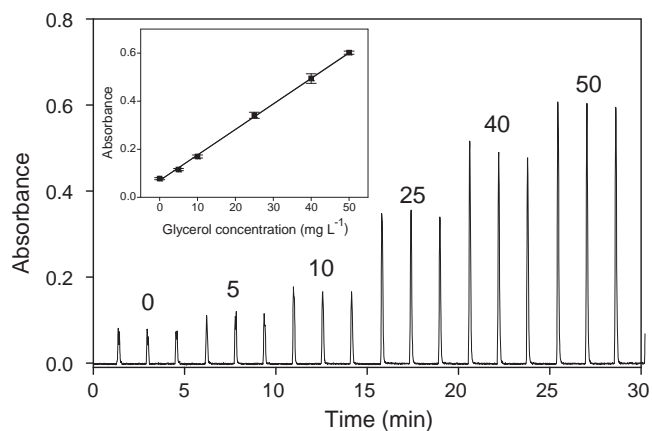
Table 4
Analytical features and reagent consumption of spectrophotometric procedures for determination of free glycerol in biodiesel samples based on Hantzsch reaction.

	Batch [14]	FIA [23]	Proposed
Detection limit (%, w/w)	–	0.0028	0.0004
Coefficient of variation (%)	–	–	1.5
Sampling rate (h^{-1})	–	9	34
Effluent volume (mL)[b]	8	6	3.5
Periodate consumption (mg)[b]	2.7[a]	3.2	0.35
Acetylacetone consumption (mg)[b]	22	40	15

[a] Equivalent concentration – sodium instead of potassium salt was used for glycerol oxidation.
[b] Amounts per determination.

tion. The only positive interferences were observed for Fe(II) and Fe(III), which form yellow complexes with acetylacetone. However, the tolerated iron concentration (1.9 mmol L^{-1}) is ca. 1400-fold higher than the expected in the biodiesel extracts, by considering an iron concentration in biodiesel around 0.3 µg g^{-1} [26].

The reliability of the developed procedure was evaluated by the analysis of biodiesel samples, and the results were compared with those obtained by the reference batch procedure [14]. The results in Table 3 agreed at the 95% confidence level.

4. Conclusions

A flow injection procedure using solenoid micro-pumps was successfully applied for free glycerol determination in biodiesel samples after a simplified off-line extraction. Profitable analytical features were achieved, such as reduction of the reagent consumption and waste generation, reproducibility for handling micro-volumes of solutions, simultaneous extraction of several samples and elimination the need for heating devices and organic solvents. All analyzed samples contained glycerol in concentration lower than the threshold limit specified by the Brazilian, European and American regulations.

Fig. 5. Transient signals for glycerol reference solutions. Numbers indicate concentrations in mg L^{-1} and the inset shows the corresponding analytical curve. Experimental conditions as in Fig. 4.

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