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A Sequential Enzymatic Microreactor System for Ethanol Detection of Gasohol Mixtures

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Abstract

A sequential enzymatic double microreactor system with dilution line was developed for quantifying ethanol from gasohol mixtures, using a colorimetric detection method, as a new proposal to the single micro reactor system used in previous work. Alcohol oxidase (AOD) and horseradish peroxidase (HRP) immobilized on glass beads, one in each microreactor, were used with phenol and 4-aminophenazone and the red-colored product was detected with a spectrophotometer at 555 nm. Good results were obtained with the immobilization technique used for both AOD and HRP enzymes, with best retention efficiencies of 95.3 ± 2.3% and 63.2 ± 7.0%, respectively. The two microreactors were used to analyze extracted ethanol from gasohol blends in the range 1–30 % v/v (10.0–238.9 g ethanol/L), with and without an on-line dilution sampling line. A calibration curve was obtained in the range 0.0034–0.087 g ethanol/L working with the on-line dilution integrated to the biosensor—FIA system proposed. The diluted sample concentrations were also determined by gas chromatography (GC) and high-pressure liquid chromatography (HPLC) methods and the results compared with the proposed sequential system measurements. The effect of the number of analysis performed with the same system was also investigated.

Index Entries: Biosensors; ethanol; flow injection analysis; alcohol oxidase; horseradish peroxidase.

Introduction

Flow injection analysis (FIA) has been used as an analytical technique because it is a reliable, reproducible, reagent-saving technique and readily automated. The application of FIA *(1–6)* systems to quantify ethanol as a simple and rapid response method has been reported in many articles.

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Recently, increased applications of integrated biosensors and FIA systems in monitoring and controlling biochemical processes have been reported *(6, 7)*. Among the physicochemical methods developed to identify chemicals, biosensors have been studied in the last 10 yr as analytical instruments that can be applied in clinical, food, and environmental analyses *(8)*. Nowadays, Brazilian government funds have been awarded to a program for a strict control of the physicochemical characteristics of the gasohol blend and hydrated fuel alcohol for combustion machines to prevent adulteration. Biosensors can be used as analytical instruments for quality control, because they have important technical characteristics, such as low response time, high selectivity, stability under the conditions of the analysis, and reproducibility of the measurements *(9,10)*. Different potentiometric, amperometric, and spectrophotometric biosensors have been developed recently, used for quality control and bioprocess monitoring, for ethanol analyses, with microorganisms like *Gluconobacter oxydans*, *Saccharomyces ellipsoideus*, or enzymes as alcohol dehydrogenase or alcohol oxidase, as shown in Table 1. Biosensors can be important instruments for long-distance transmission and data transfer systems to an analytical central station in monitoring and process control program.

The aim of this work is to develop an integrated biosensor–FIA system with two sequential microreactors packed with immobilized alcohol oxidase and horseradish peroxidase, as a new proposal to the system previously used *(6)*, in order to reuse the enzymes for analyses of diluted ethanol samples. The dilution of the extracted ethanol solutions was made with a dilution line adapted to the integrated biosensor–FIA system. The performance of the system, with and without an on-line dilution, was compared to the previous one-microreactor system *(6)*. Some real samples were analyzed and good results were obtained with the dilution line integrated biosensor—FIA system and the results compared with established methods such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) *(7,19)*.

Materials and Methods

Chemicals

Alcohol oxidase, 4-aminophenazone, phenol, glutaraldehyde, aminopropyl glass beads from Sigma Chemical Co. Toyobo of Brazil donated horseradish peroxidase. All other chemicals used were of analytical grade.

Enzymatic Reactions

The ethanol determination is based on the enzymatic reactions of alcohol oxidase (AOD) and horseradish peroxidase (HRP):

$$\text{Ethanol} + O_2 \xrightarrow{\text{AOD}} \text{acetaldehyde} + H_2O_2$$

Table 1
Ethanol Biosensors

Transductor	Enzyme/microorganism	Detection range
Amperometric *(2–3)*	Alcohol oxidase and Horseradish peroxidase	5.0 m*M*; 0.1– 4.0 m*M*; 0.1–2 m*M*
Amperometric *(11)*	Catalase	0.05–1 m*M*
Potentiometric *(12)*	Alcohol dehydrogenase	0.77 *M*
Amperometric *(5,13–15)*	Alcohol dehydrogenase	0.2–0.3 m*M*; 0.5 m*M* 1–250 (*M*; 0.1–10 m*M*
Amperometric *(7)*	*Gluconobacter oxydans*	2–270 m*M*
Potentiometric *(16)*	*Saccharomyces ellipsoideus*	0.02–50 m*M*
Spectrophotometric *(1,6**,17*,18**)*	Alcohol oxidase and horseradish peroxidase	1.1–21.7 m*M*; 0.011–0.043 m*M* 1.1–16.3 m*M*; 0.11–32.6 m*M*

* Not immobilized
** With AOD immobilized and free HRP.

$$2H_2O_2 + 4-\text{aminophenazone} + \text{phenol} \xrightarrow{\text{HRP}} \text{monoimino - p - benzoquinone - 4 - phenazone} + 4\,H_2O$$

The second reaction gives a colored product that can be detected in the spectrophotometer at 555 nm.

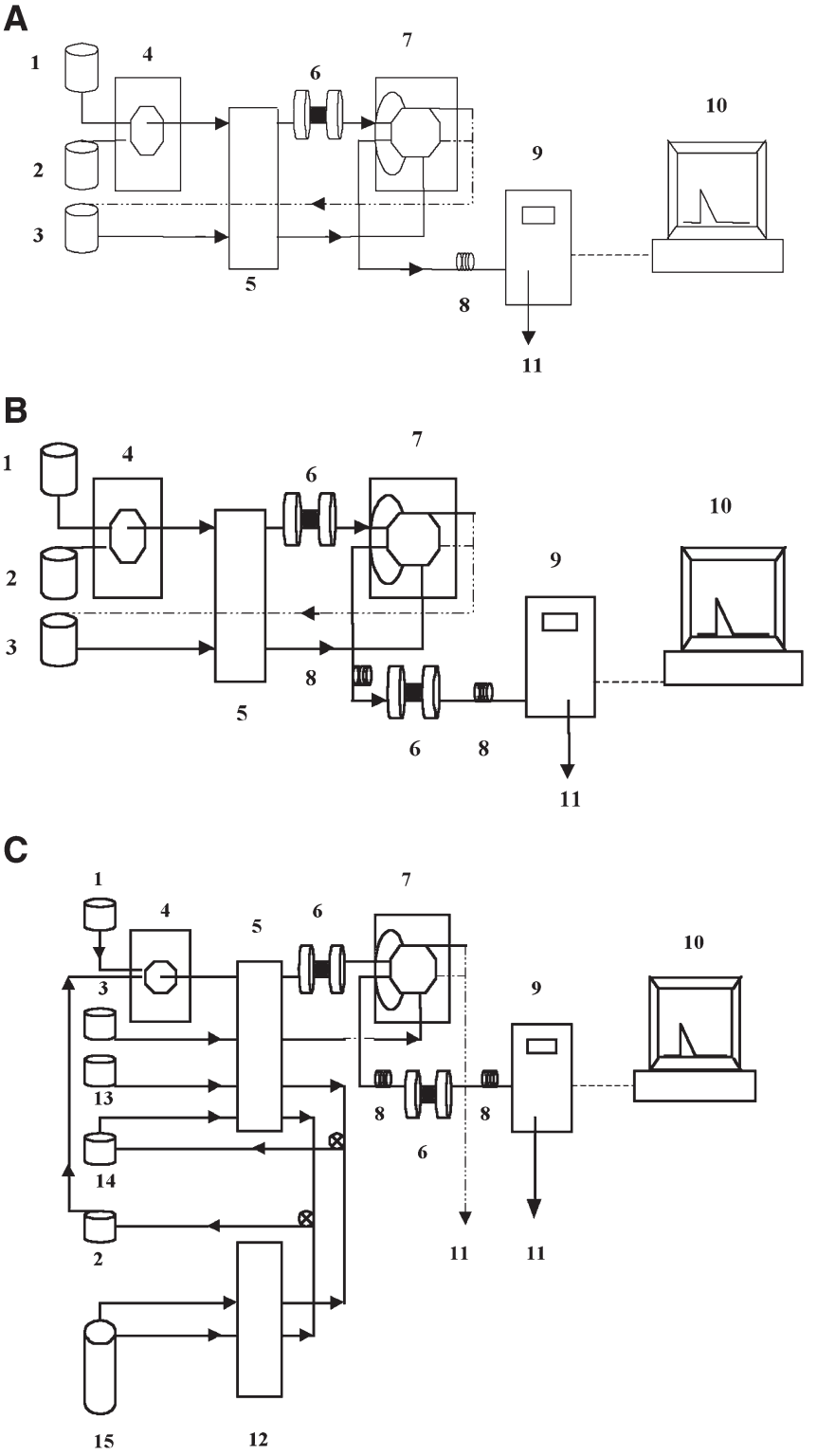
Immobilization

Alcohol oxidase and horseradish peroxidase were immobilized separately on aminopropyl glass beads, which were first treated with 2.5 % (v/v) glutaraldehyde, in a shaker incubator at 30 ºC and 50 rpm for 24 h. The retention efficiencies of AOD and HRP immobilizations were calculated as in previous work *(6)*.

The first integrated biosensor FIA system used the immobilized AOD in a microreactor, and worked with a phosphate buffer pH 7.0 as carrier, free HRP (0.444 g/L), and reagent solution: 0.875 g phenol/L and 0.305 g 4-aminophenazone/L, as described in a previous work *(6)*. In the second system proposed, both enzymes were immobilized in two microreactors in sequence, with 0.4 g of glass beads, with 0.0140 mg of alcohol oxidase/mg of glass beads and 0.0036 mg of horseradish peroxidase/mg of glass beads, respectively. The reagent solution (phenol and 4-aminophenazone) was prepared with the same concentration used in the previous system.

The Integrated Biosensor Flow Injection Analysis Systems

The FIA system consisted of TMI modules (Técniques Mesura Instrumentació), a five-channel peristaltic pump, an eight-channel injec-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tion valve, an eight-channel distributed valve, and a colorimeter, connected to an interface and to an IBM-PC microcomputer. The two microreactors were made of acrylic, each one with 0.91 mL of void volume, 3:1 of length to diameter ratio. Both were packed with the enzyme immobilized on glass beads (80–120 mesh). The beads were retained in the microreactor with a 110-mesh nylon screen and two rubber O-rings with 11.4 mm of external diameter. The lids were attached to the microreactor with four stainless-steel screws. Flexible tubes with 2.4 mm internal diameter were connected to each side of the microreactor as sampling lines of the integrated FIA system. In the first system as described in previous work *(6)*, HRP was used in solution with the other two reagents, and injected in to the FIA line after ethanol samples reacted with the immobilized AOD. In the second system both enzymes were immobilized separately and packed in two microreactors that work in sequence. Figure 1 shows the scheme of the integrated biosensor FIA system with one (12A) and two (12B) packed enzyme microreactors, respectively. In these experiments the extracted ethanol solutions were diluted off-line and the sample bottle changed in each analysis. Figure 1C shows the proposed integrated biosensor system with two reactors adapted with a dilution line where diluted samples were obtained continuously before each analysis. This new system was developed to improve the control and automate the process for an on-line monitoring program. In this last system another peristaltic pump was adapted which permitted the dilution of the real concentrated extracted ethanol solution to the appropriate sensitive range of the analysis concentration.

Actuator and Monitoring Equipment

In all proposed integrated biosensor–FIA systems, with one, with two microreactors, with and without the dilution line, an automatic analysis was coupled to the system and carried out with a specific programmed software for data acquisition and pump and valves control. The computer program controlled the analysis timing sequence, scheduling the signals to the distribution and injection valves to introduce the sample and enzyme–reagent solutions into the system. The difference between the peak heights formed and the base line were referenced to the absorbance values detected.

Fig. 1. Proposed integrated biosensors FIA systems: (A) one microreactor packed with immobilized AOD and free HRP, (B) two microreactors in sequence, packed with AOD and HRP, and (C) two microreactors with dilution line, respectively: 1, buffer solution; 2, diluted sample solution; 3 (A), free horseradish peroxidase and reagents solution; 3 (B) and (C), reagents solution; 4, eight-channel distribution valve; 5, peristaltic pump; 6, micro reactor; 7, eight-channel injection valve; 8, coil; 9, colorimeter; 10, computer; 11, waste; 12, peristaltic pump; 13, concentrated sample; 14, first dilution sample; 15, phosphate buffer for sample dilutions.

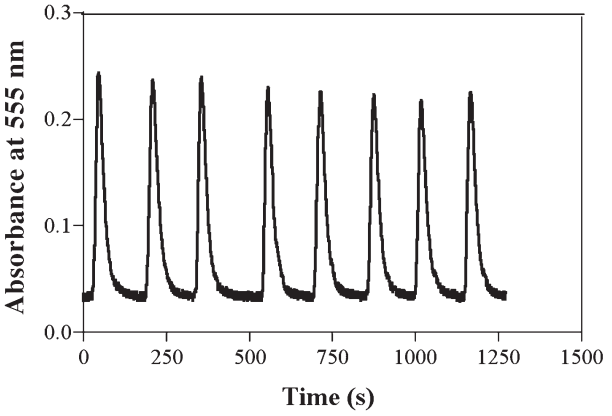

Fig. 2. The reproducibility of the two microreactor system for 0.2 g ethanol/L extracted and diluted samples.

Results and Discussion

Good results were obtained with the immobilization technique used in this work with a mean retention efficiency of 95.3 ± 2.3% for alcohol oxidase, the best results for horseradish peroxidase were around 63.2 ± 7.0 %.

Extracted ethanol solutions from gasohol blends [1–30 % (v/v)] were obtained using the ABNT (Associação Brasileira de Normas Técnicas) method and confirmed by HPLC analysis as described in previous work *(6)*. The working features used for the system of one microreactor, Fig. 1A, were: constant loop volume (69.4 (L), scheduled time control program (cycles of 174 s) and sample volume (1.0 mL): 51 s for sampling, 113 s for flowing, 10 s for injecting the reagent solution. The analysis total response time was 5.0 min for each sample in one operation cycle. For the range 0.05 –1.5 g ethanol/L, a hyperbolic correlation was obtained for the calibration curve, with correlation coefficient of 0.9909 as concluded in previous work *(6)*.

For the system with two microreactors, operated with the same scheduled time control program, constant loop volume (69.4 (L) for reagent solution, and sample volume (1.0 mL), good sample frequency analysis and high reproducibility were obtained. Figure 2 shows the signals registered for 0.2 g ethanol/L of extracted and diluted samples. For eight consecutive analyses, the mean of the maximum pick value observed was 0.228 ± 0.0074 with 2.2 % of relative error, and the stability of the signals was verified with the delta of absorbance mean value of 0.1975 ± 0.0062, with an error of 3.2 %.

The new system shown in Fig. 1B was tested with standard ethanol solutions in phosphate buffer, pH 7.0, and diluted extracted ethanol solutions were prepared from only one gasohol mixture with 20% v/v ethanol. The calibration curve obtained for the extracted ethanol solutions permit-

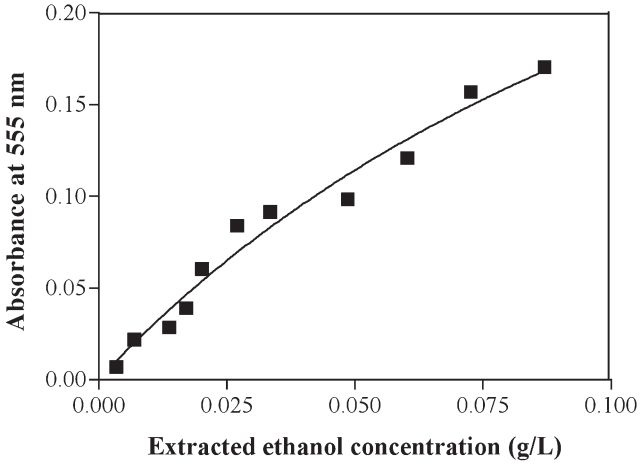

Fig. 3. Calibration curve for the two microreactor biosensor—FIA integrated system with the on-line dilution.

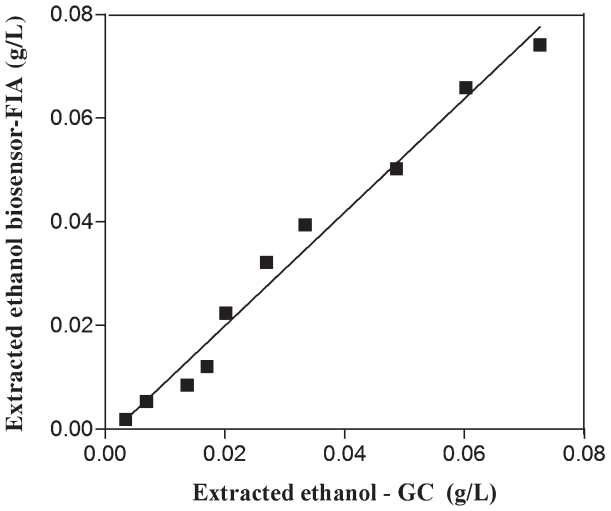

Fig. 4. Ethanol concentration solution analysis results by GC compared to the proposed two microreactor integrated biosensor—FIA system with dilution line.

ted analyses into a lower range of ethanol concentrations, 0.001–0.066 g ethanol/L, as compared to the previous one microreactor system *(6)* and also with an a hyperbolic fit, with a correlation coefficient of 0.9908.

Figure 3 shows the calibration curve for the system, with the on-line dilution arrangement as in Fig. 1C. A correlation coefficient of 0.9706 was obtained for the range of 1–30% v/v ethanol in real samples diluted in a proportion of 1:2150 to a range of 0.0034–0.087 g ethanol/L. Figure 4

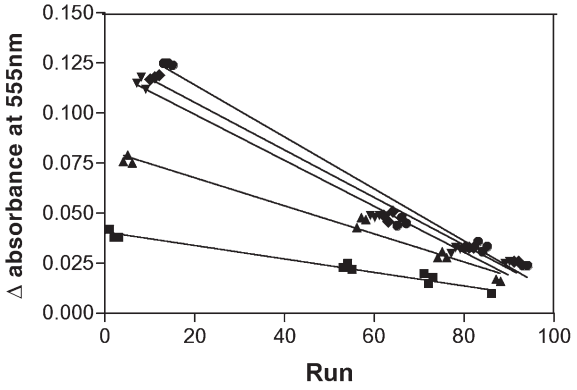

Fig. 5. The numbers of previous analyses effects with different extracted ethanol concentration solutions: (■) 0.01 g ethanol/L, (▲) 0.03 g ethanol/L, (▼) 0.05 g ethanol/L, (◆) 0.08 g ethanol/L, (●) 0.1 g ethanol/L.

shows the results of the measurements obtained by GC method compared to the integrated biosensor–FIA system with dilution line, with a correlation coefficient of 0.9817, and a 5 % error.

The proposed integrated biosensor–FIA system showed higher sensitivity for the range of 0.0034–0.087 g ethanol/L of diluted extracted real samples when compared to the results obtained in other works with AOD and HRP free in solution *(17)* or with only the first one immobilized by covalent binding on aminopropyl glass beads *(6, 18)*. The results obtained in this work were also compared to the ones obtained with both AOD and HRP immobilized onto chitosan to quantify ethanol in alcoholic beverages using a fluorometric determination FIA system *(1)*. Other amperometric systems showed a sensitivity range similar to the one obtained in this work for AOD and HRP immobilized in composite graphite–Teflon electrodes *(3, 4)* or cross-linked to a redox polymer *(1)*.

After several sequential analyses, the number of these previous runs was seen to affect the measurement signal. The effects observed were similar to those reported in the literature *(20)*, where immobilized AOD was used in successive colorimetric determinations. Although the system showed good stability and reproducibility, the authors suggested that the immobilized AOD could be re-used for only 20 successive determinations *(20)*. Immobilized HRP was also reused in polyphenol detection *(21)*, and the activity decreased 50% after 100 successive determinations working with an amperometric biosensor.

Studying the activity of the system for successive sample determinations, it was observed that the absorbance value decreased with and depended on the extracted ethanol concentration as shown in Fig. 5. The activity decrease could be attributed to other compounds present in the extracted ethanol solutions obtained from gasohol blends.

Table 2
The Integrated Biosensor–FIA Results for Commercial Samples

Sample code Number	Δ abs$_C$	Extracted ethanol [a] biosensor (g/L)	Extracted ethanol [b] HPLC (g/L)	Relative error (%)
1	0.062 ± 0.009	165.61 ± 4.22	157.70 ± 0.65	4.8
2	0.057 ± 0.001	152.53 ± 1.72	156.48 ± 0.09	–2.9
3	0.059 ± 0.002	157.92 ± 4.79	150.42 ± 3.54	4.7
4	0.056 ± 0.001	150.06 ± 2.98	157.01 ± 0.65	–4.6
5	0.063 ± 0.002	169.62 ± 4.55	161.37 ± 0.02	4.9
6	0.063 ± 0.003	169.01 ± 8.44	160.44 ± 2.47	5.1
7	0.068 ± 0.004	183.77 ± 12.65	171.33 ± 5.09	6.8
8	0.066 ± 0.002	179.36 ± 6.33	159.97 ± 0.29	10.8
9 [c]	0.060 ± 0.001	160.45 ± 1.03	161.23 ± 0.01	–0.5

[a] Standard deviation for three replicates.
[b] Standard deviation for three replicates.
[c] Prepared reference sample at 25 % v/v by NBR 13992:1997 (ABNT) method.

In order to re-use the system with the same immobilized enzymes in several and sequential analyses, a linear correlation can be proposed between the number of the previous analyses and the measured absorbance value. A correction factor to compensate these effects is proposed as Eq. (1):

$$\Delta\text{abs}_C = \Delta\text{abs}_\text{M} + \left(K \times N_R\right) \tag{1}$$

The slope of the curves in Fig. 5 shows the dependence of the parameter K with ethanol concentration, and it can be described by Eq. (2) with a correlation coefficient of 0.9904:

$$K = -0.02\text{C}_\text{Et} - 0.0000212 \tag{2}$$

where Δ abs$_\text{C}$ = compensated absorbance, Δ abs$_\text{M}$ = measured absorbance, N_R = number of previous runs, C_Et = ethanol concentration (g ethanol/L), K = correlation coefficient ((abs/number of runs).

A further dilution factor of 1:7000 was used in the integrated biosensor–FIA system with the on-line dilution arrangement for the analysis of eight commercial extracted ethanol samples from gasohol blends of a 25% v/v ethanol declared value. The results obtained and the compensated measured values are shown in Table 2.

The concentrations of the commercial extracted ethanol samples were quantified with HPLC method, and the results compared showed a maximum relative error of 10.8 %. These results show the applicability of the proposed system to quantify ethanol in real samples.

Conclusions

The first proposed biosensor—FIA integrated system that works with one microreactor packed with immobilized alcohol oxidase showed good reproducibility and reliability in the range 0.05–1.5 g ethanol/L. The use of both immobilized enzymes on the sequential microreactors changed the sensitivity of the system to a lower range of 0.001–0.066 g ethanol/L and no significant differences from other analytical procedures (GC). The dilution line introduced in the integrated biosensor—FIA system permitted working directly with a range of 1–30 % v/v of the real samples, and a correlation coefficient of 0.9683 was obtained when they were diluted in the sampling line to a range of 0.0034–0.087 g ethanol/L. High levels for retention efficiencies of immobilization were attained for AOD with a mean of 95.3 ± 2.3%. The best results for HRP retention efficiency were near 63.2 ± 7.0%. Good reproducibility of the registered signals was obtained with both proposed automatic analysis systems with a response time of 5.0 min between each operation cycle of the sampling line. The on-line dilution integrated system developed showed good results and high sensitivity for the range of ethanol blends used in this work. Comparing the commercial extracted ethanol concentrations measures obtained with the proposed integrated sequential enzymatic biosensor–FIA system with the values obtained with HPLC method the maximum relative error observed was 10.8 %.

Acknowledgments

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