

# Analysis of the optical properties of bile

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**Abstract.** Invasive bile determination is very useful in the diagnosis of many gastric pathologies. At the moment, this measurement is performed with Bilitec 2000, an optical fiber sensor, that is based on absorption by bilirubin. Nevertheless, erroneous evaluations are possible, due to the different configurations which the bilirubin molecule can adopt. The optical behavior of human samples of pure bile and bile+gastric juice has been examined using an optical fiber spectrophotometer and two suitably modified Bilitec 2000 units. A protocol has been established for the treatment of biological fluids, in order to make it possible to study the behavior of their optical properties as a function of *pH* and concentration without causing any alteration in the samples. The analysis of *pH* dependence evidenced the presence of different calibration curves at different *pH* values: the self-aggregation of the bilirubin molecules observed in pure bile samples was almost totally absent in the gastric samples. Measurements carried out on Bilitec 2000 showed that the most appropriate wavelength for bilirubin detection in the stomach should be 470 nm. © 2000 Society of Photo-Optical Instrumentation Engineers. [S1083-3668(00)00403-2]

Keywords: optical fibers; bile; sensor; enterogastric reflux; gastro-oesophageal reflux; Bilitec 2000.

Paper JBO-224 received Nov. 19, 1998; revised manuscript received Jan. 7, 2000; accepted for publication Jan. 19, 2000.

## 1 Introduction

The presence of enterogastric reflux represents a prerequisite for "alkaline" or "nonacid" gastro-oesophageal reflux. Enterogastric and nonacid gastro-oesophageal refluxes have been believed to be contributing factors in the development of pathological conditions such as gastric ulcer,<sup>1</sup> chemical gastritis,<sup>2,3</sup> upper dyspeptic syndromes,<sup>4</sup> and severe oesophagitis.<sup>5,6</sup> Under particular conditions, enterogastric reflux may also increase the risk of gastric cancer.<sup>7</sup>

Among the different techniques available for their detection, the optical fiber approach, which is based on the optical properties of the bile which is always present in these refluxes, seems to be the most promising one, since it allows an *in vivo* 24 h continuous and direct monitoring of bile-containing refluxes.<sup>8</sup> The optical fiber sensor, Bilitec 2000, utilizes the light emitted at  $\lambda = 470$  (which is heavily absorbed by bilirubin, the main biliary pigment) and 570 nm (reference) by two light-emitting diodes (LEDs), and an optical fiber bundle that transports the light from the sources to the probe (which is actually a miniaturized spectrophotometric cell whose external diameter is 3 mm), and from the probe to the detector.<sup>9</sup> At the moment, Bilitec 2000 is used in many hospitals for the ambulatory monitoring of bile-containing refluxes.<sup>10–15</sup>

The utilization of Bilitec 2000 by many physicians has evidenced a relation, which is still not well defined, between the optical properties of the bile and the *pH*. This uncertainty could lead to a mistaken estimation of the biliary content in gastric and oesophageal fluids. A 30% underestimation in the bilirubin concentration for a *pH* of 3.5 has been reported.<sup>16</sup>

In the literature, the complexity of the body fluids containing bile has been well emphasized. On the basis of these studies, it is understood that, in order to obtain a correct knowledge of the optical properties of the bile mixed with gastric fluids, an exact evaluation of the bile content in enterogastric or gastro-oesophageal refluxes is necessary. The present study is concerned with determining the optical properties of bile in human samples.

An analysis of bilirubin (as a bile marker) in the biological fluids of the stomach and oesophagus can be carried out by following two different approaches: a diagnostic approach, aimed at determining the total bilirubin in the stomach, in order to assess the clinical risks associated with the presence of bile in the stomach; and an analytical approach, aimed at determining the optical properties of the different forms of bilirubin present in the stomach.

As far as the diagnostic approach is concerned, physicians are interested in knowing how much bile and, consequently, bilirubin enters the stomach when an enterogastric reflux occurs. They are not interested in knowing how much bilirubin is dissolved in solution and how much is precipitated. Therefore, the absorbance values measured with the Bilitec 2000 diagnostic device should be related to the total bilirubin concentration at *pH* 7, whichever the *pH* value of the sample is, since this value expresses the total amount of bilirubin (and of bile) which enters the stomach. It is important to recall that the *pH* of the bile is around 7, and that all forms of bilirubin should be dissolved in solution at this *pH* value.

Analytical approach: in this case, the final goal is to determine the optical behavior and solubility of the different forms present in the stomach are the final goal. In the circumstances,

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**Table 1** Typical constituents of bile.

Compound	% of total
H <sub>2</sub> O	85.92
solids	14.08
biliary acids	9.14
bile pigments	2.98
cholesterol	0.26
lipids and fatty acids	0.32

it would be better to perform centrifugation on drawn samples that have a native  $pH \geq 7$ . As a matter of fact, if centrifugation is applied to samples with  $pH < 7$ , nonsoluble precipitated forms are eliminated and are no longer considered.

In the present article, the diagnostic approach was followed, since we were interested in a correct interpretation of the measurements performed with the Bilitec 2000 optical fiber sensor.

## 2 Bile Composition

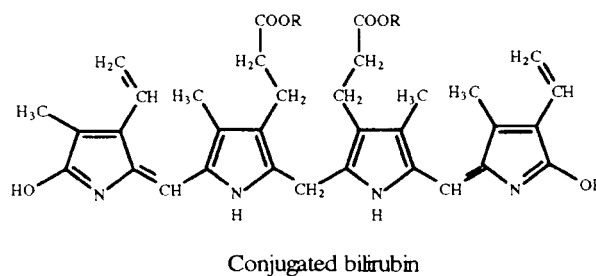
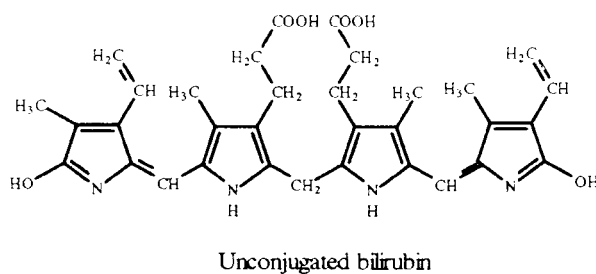
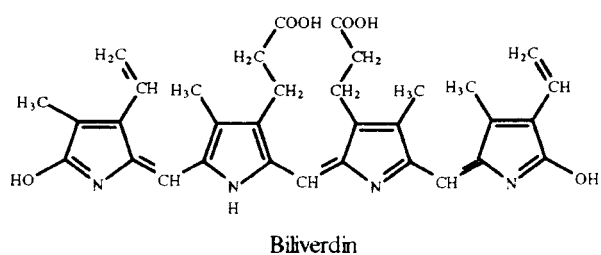
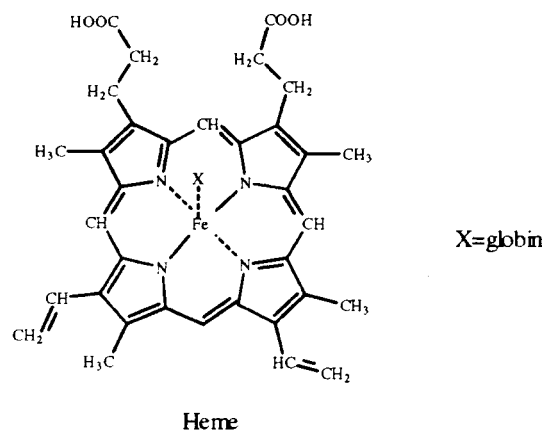
Bile is secreted by the liver, and is an isotonic and isotropic aqueous solution of inorganic and organic compounds. Typical constituents of bile are shown in Table 1.<sup>17</sup> Many of the inorganics in bile are salts consisting of anions, such as bicarbonates and chlorides, and cations, such as Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup>. Due to the presence of bicarbonates, bile is alkaline with a value of between 7.5 and 9.5 pH units. The organic content is mainly cholesterol, bile salts, and biliary pigments.

Cholesterol is present in bile in a free form, and is maintained in solution, thanks to the presence of micelles consisting of biliary acids and phospholipids.

Bile acids are catabolites of cholesterol. In bile, they are present as salts, and are very important factors for the digestion of lipids. They are surfactants, and are able to emulsify lipids.

Biliary pigments are catabolites of heme (see Figure 1). The heme of erythrocytes is first oxidized to an intermediate, which is green in color and contains globin and Fe<sup>2+</sup>. This intermediate is converted to biliverdin, which is easily reduced to bilirubin.

Bilirubin is the major organic product of heme catabolism: it is a yellow tetrapyrrole pigment that possesses two propionic side chains, which might be expected to render it highly polar and, therefore, water-soluble. However, the bilirubin molecule can adopt a variety of configurations, due to internal hydrogen bonding that render bilirubin poorly soluble in water. It is solubilized by interactions with micelles of bile salts and other lipids in bile. Furthermore, bilirubin is conjugated with glucuronic acid, giving rise to the formation of mono- and diglucuronides. In bile, the unconjugated bilirubin is normally 5% of the total bilirubin. Bilirubins are also susceptible to oxidation, and are photosensitive, thus leading to a wide variety of derivatives. Due to the above consideration, it is not easy to completely understand the composition of and interactions among, all the species present in bile. A complete

**Fig. 1** Chemical structure of heme and biliary pigments.

analysis of bile in biological specimens would involve specific measurements for each individual compound. Considering the potential complexity of bile, such a goal could be achieved only by combining the results of a number of methods, each of which would permit the determination of a specific compound or group of compounds.

Bilirubins have a strong and broad main absorption band in the visible region, with an absorption peak of between 390 and 460 nm and a molar absorption coefficient of approxi-

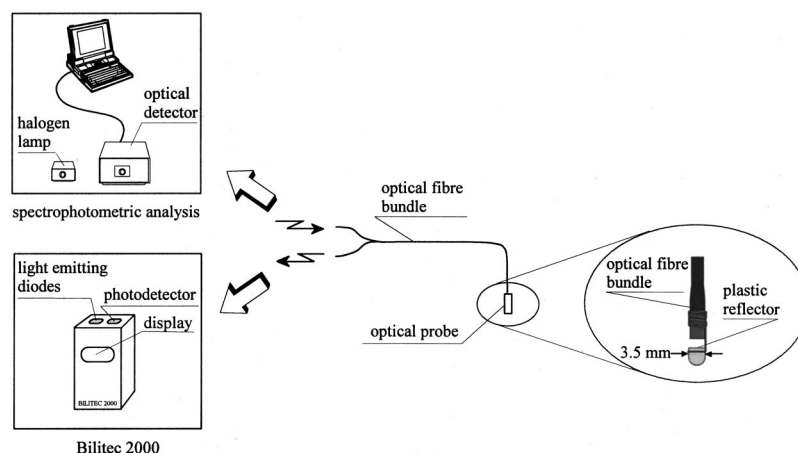
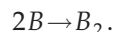


Fig. 2 Scheme of the optical instrumentation used for the optical characterization of bile.

mately  $50 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$  in aqueous solvents. Usually, this absorption band in the visible region is not Gaussian in shape, but exhibits a more or less pronounced shoulder on either the long- or the short-wavelength side. The position of  $\lambda_{\text{max}}$ , the shape of the spectrum, and the molar absorption coefficient of individual bilirubins appears to depend greatly on a conformational structure, which in turn is determined mainly by the nature and extent of intramolecular hydrogen bonding.<sup>18</sup>

Studies on bile are complicated by the fact that, with the exception of unconjugated bilirubin, natural bilirubins are unavailable in pure form. Moreover, spectrophotometric analysis may be further complicated by the fact that Beer's law is not always followed by some bilirubins, including bilirubin diglucuronide, due to the dimerization and formation of aggregates.

Some authors have postulated the existence of an equilibrium of self-aggregation of bilirubins that would lead to a variation in the shape of the spectrum and a shift of the maximum, due to the fact that the two forms (monomer and dimer) have different  $\lambda_{\text{max}}$ . At a first approximation, the degree of dimerization can be considered independent of  $p\text{H}$ , since hydrogen ions are not involved in the related equilibrium:



However,  $p\text{H}$  is important, since bilirubin and its conjugates are characterized by different dissociation constants ( $pK_a$ ). The  $pK_a$  of bilirubin glucuronide (BG), which predominates in bile, is more than 4.0, but  $pK_a$  values are more than 8.0 for unconjugated bilirubin (UCB) and less than 1.5 for bilirubin ditaurate (BDT). Therefore, in the  $p\text{H}$  range of gastric juice, BDT is ionized and soluble, whereas UCB and BG are predominantly protonated and insoluble. Variable precipitation of bilirubins will occur, depending on the  $p\text{H}$  and bile salt concentration.

All the previous considerations demonstrate that it is important to understand all the shifts and differences that may be encountered in bile samples. Such an understanding can be obtained only by means of an accurate spectrophotometric study.

### 3 Materials and Methods

#### 3.1 Optical Instrumentation

Optical characterization was performed with an optical fiber spectrophotometer (Ocean Optics PS 1000), and with the optical fiber sensor Bilitec 2000 suitably modified so as to allow optical measurements with a signal source emitting at 420, 450, and 470 nm (reference was always taken at 570 nm).

All instruments were coupled to optical fiber plastic bundles which ended with the same optical cell: a plastic reflector placed in front of the fibers at a fixed distance (1.9 mm) by means of a thin stainless steel wire.

The configuration of the optical fiber spectrophotometer makes use of a halogen lamp as source, an optical detector consisting of the combination of a 600 lines/mm grating, and a linear charge-coupled device (CCD) with 1024 elements for the wavelength discrimination, and a personal computer. Absorption spectra in the range 350–800 nm were recorded.

Bilitec 2000 makes use of two light-emitting diodes as sources, with their emission peak in the blue region of the visible spectrum (420, 450, or 470 nm) and at 565 nm for the signal and the reference, respectively. The detecting system consists of a simple photodiode which converts the light in electrical signals. After amplification by operational amplifiers, the signals coming from the two light-emitting diodes were processed by an integrated microcomputer.<sup>9</sup> The scheme of the optical instrumentation is shown in Figure 2.

It is important to point out that Bilitec 2000 does not evaluate a true absorbance, which is defined at a definite wavelength, since the light emitted by the sources at all wavelengths is considered. The following value is calculated:

$$M = \log \left[ \frac{\int I_o(\lambda) d\lambda}{\int I(\lambda) d\lambda} \right]_{\lambda_{\text{sig}}} - \log \left[ \frac{\int I_o(\lambda) d\lambda}{\int I(\lambda) d\lambda} \right]_{\lambda_{\text{ref}}}, \quad (1)$$

where  $I_o$  and  $I$  are the light intensities transmitted in the absence and presence of the absorbing sample, and the integral is evaluated on all the wavelengths emitted by the signal and the reference LED. The choice of the emitting wavelengths is related to the availability on the market of the LED, the emission spectrum of which overlaps with the absorption band of

bilirubins. LED emitting at 470 nm is the source mounted on the commercialized Bilitec 2000 since it was the most reliable LED at the shortest wavelength available on the market when the instrument was designed and manufactured.

### 3.2 Biological Samples

Two different types of biological samples were analyzed: pure bile (PB) samples and bile+gastric juice (BGJ) samples, drawn directly from the gallbladder and from the stomach, respectively. If not immediately analyzed, the samples were kept at a temperature  $T \leq 0^\circ\text{C}$  during the time period between the drawing from the patient and the optical analysis, in order to avoid any possible modification.

Each sample drawn was properly modified in order to study its optical behavior with a change of  $p\text{H}$  and bilirubin concentration. Its  $p\text{H}$  value was adjusted with a drop (or a few drops) of either hydrochloric acid or potassium hydroxide in the 1–7  $p\text{H}$  units range, which is the physiological range of the stomach and oesophagus content. The amount of HCl or KOH used was so small that the bilirubin concentration could be considered unaltered.

Dilution of the biological samples was performed with artificial gastric juice (KCl 2 g, pepsin 32 g, HCl 1 N 80 ml to a final volume of 1 l with distilled water; final  $p\text{H}$ : 1.3). However, dilution with the artificial gastric juice would have led to samples with uncontrolled  $p\text{H}$  values. The use of buffered (e.g., phosphate-based) solutions would have assured controlled  $p\text{H}$  values, although the buffer capacity of bile should have been considered. On the other hand, this would have caused a perturbation of the biologic samples by introducing substances which were not present in the stomach content.

In order to carry out *in vitro* tests on samples as close to the physiological situation as possible, six nonbuffered solutions were prepared by adding different amounts of either 1 N HCl or 0.1 N KOH to the artificial gastric juice. In this way  $p\text{H}$  values similar to those of the treated biological samples were obtained and the solutions were used to dilute the biological samples. Different dilutions were performed according to the type of sample (PB or BGJ).

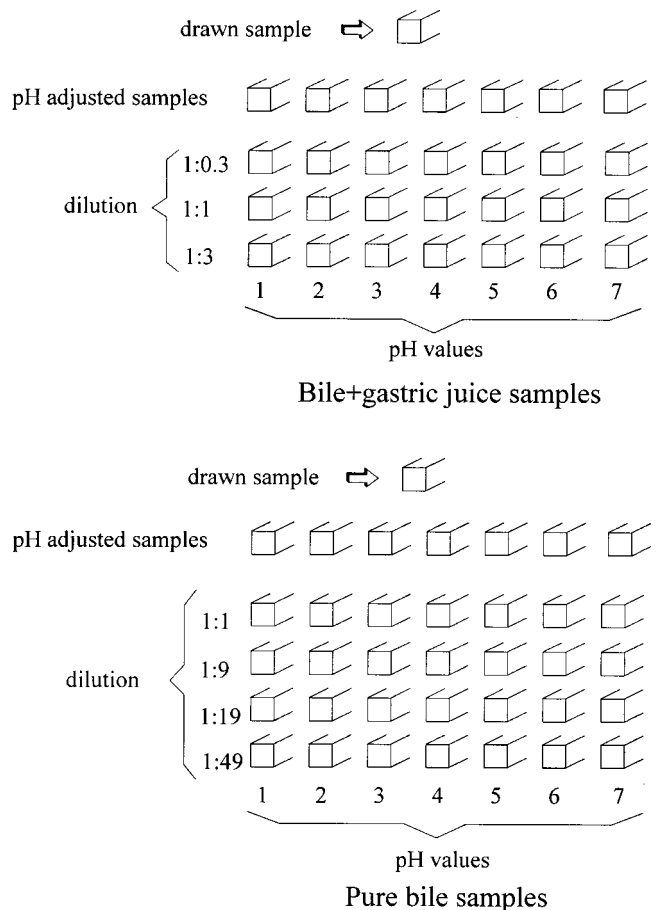
**BGJ samples:** bilirubin was already in the range of physiological interest, since the samples were drawn from the stomach. Therefore, only small dilutions were utilized.

**PB samples:** greater dilutions were used, in order to obtain the concentrations of total bilirubin that are normally found in the stomach.

All the diluted samples were contained in a 5 mm plastic cuvette (Figure 3).

Many of the BGJ samples were highly viscous, and in some cases contained solid particles. This prevented a homogeneous subdivision of the drawn samples among the cuvettes, which was a necessary procedure for analyzing the sample.

Homogenization alone and homogenization followed by centrifugation were considered as possible procedures to be followed in order to make the samples analyzable. The drawn sample, which was always protected from light and was kept at low temperature, was homogenized with 15–20 strokes, using a glass-teflon homogenizer. The sample obtained was divided in aliquots (5–6 ml) the  $p\text{H}$  of which was adjusted to

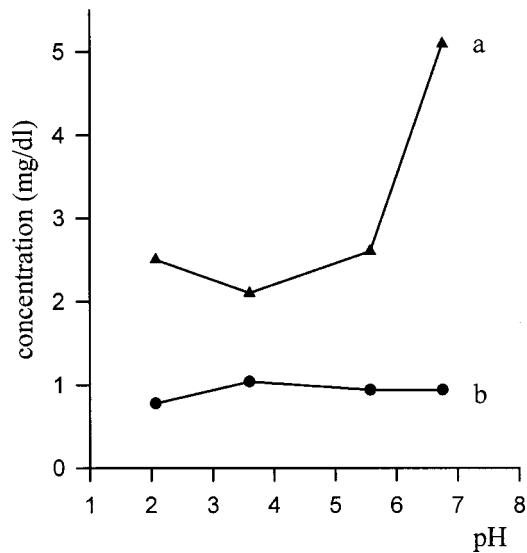


**Fig. 3** Sketch of the procedure for *in vitro* optical analysis of the drawn samples; typical utilized dilutions are also given.

the following values: 1, 2, 3.5, 5.5, and 7. The  $p\text{H}$  adjustment was performed by adding an amount between 10 and 200  $\mu\text{l}$  of HCl 3 N or KOH 4.5 N. Half of each  $p\text{H}$ -adjusted sample was centrifugated at 1000 rpm for 5 min. The homogenized samples and the supernatant were used for bilirubin determination.

Total bilirubin concentration was determined by a colorimetric method based on the Van der Berg reaction. Conjugated and unconjugated bilirubin, in the presence of detergents, such as caffeine, couple with diazotized sulphanilic acid, forming a blue color, detectable at 600 nm in the presence of alkaline  $p\text{H}$  (Sigma chemicals, Diagnostic kit, 605-C).

A comparison between homogenization and homogenization+centrifugation procedures is provided in Figure 4: the concentration of total bilirubin at different  $p\text{H}$  values is shown for the homogenized only and the homogenized+centrifugated samples obtained from the same gastric content that were treated following the two procedures. Completely different values of concentration are determined in the two cases. Homogenization assured that modification of the sample was minimized, and made it possible to divide and to mix finely all the material contained in the sample. This, in turn, made possible the homogeneous subdivision of the drawn sample and the subsequent analysis. Centrifugation, even at a low velocity, caused considerable alter-



**Fig. 4** Total bilirubin concentration as a function of  $pH$  for the homogenized (a) and centrifuged (b) samples obtained from the same drawn gastric content.

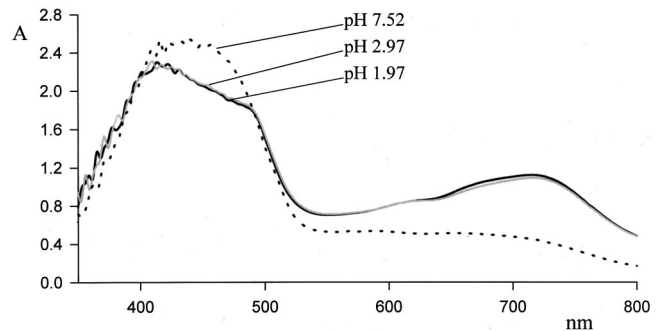
ation in the drawn sample. In fact, the viscous material separated by means of centrifugation dragged down smaller materials and also molecules dissolved in solution which might be entrapped and/or adsorbed in it. This aspect should be considered if the analytical approach is followed in studying of the optical behavior and different solubility of the forms present in the stomach. In this case, centrifugation would be necessary for separating the different species. As already emphasized in the introduction, the aim of the present work was the correct interpretation of the measurements performed using the Bilitec 2000 optical fiber sensor. In this case, it is more correct to consider homogenized samples, since they better reproduce the situation in the stomach. In fact no matter, which material is precipitated, it remains suspended in the gastric content.

Therefore, if not otherwise specified, any sample considered was obtained from the homogenization of the drawn biological fluid, and no centrifugation of the sample was carried out.

#### 4 Results and Discussion

The absorption spectrum of each diluted sample was measured in the 400–800 nm range; measurements on all the samples obtained were also performed with a modified Bilitec 2000. The stability of the samples during the optical analysis (which took approximately 1 h) was checked by repeating the subsequent cycle of measurements. No relevant changes were observed in the absorption spectra.

The optical properties of the PB and BGJ samples did not change after having been kept at a temperature  $\leq 0^\circ\text{C}$ . This was checked both by measuring the total bilirubin concentration and by recording the absorption spectrum of the fresh sample and of the same sample after having been kept at a temperature  $\leq 0^\circ\text{C}$  for a few days. No substantial differences were observed.

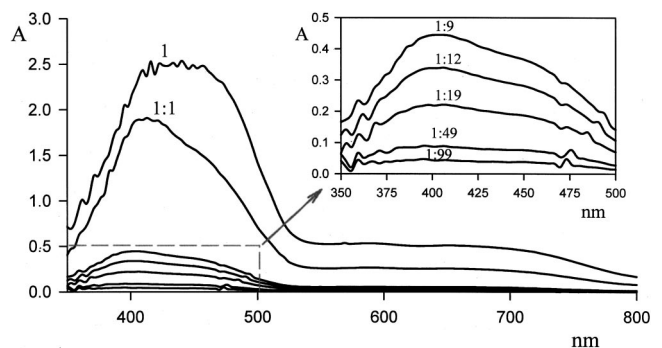


**Fig. 5** Absorption spectra of a PB sample at different  $pH$  values.

In all the samples, a precipitate was observed in acidic solutions (generally for  $pH \leq 3.5$ ). This behavior was due to the change in solubility of the different forms of bilirubin following the formation of intramolecular hydrogen bonding, as a consequence of a  $pH$  decrease, as well as to the different  $pK_a$  value of the various forms of bilirubin.

Typical absorption spectra of a PB sample at different  $pH$  values are shown in Figure 5. With the decrease in  $pH$ , a decrease in the absorption at the highest wavelength band-side was observed; for  $pH \leq 4$ , oxidation of bilirubin to biliverdin took place, as testified to by the appearance of an absorption peak at  $\approx 700$  nm.

The strong absorption in a broad region (in practice, from 400 to 500 nm) observed in PB samples could be ascribed to the phenomenon of the self-aggregation of bilirubins, which caused a broadening of the absorption band. In this case, a linear relation between absorbance and concentration, as predicted by Beer's law, was no longer valid. The effect of dilution in the PB sample is shown at  $pH 7.5$  in Figure 6 (the concentration was normalized to the concentration of the drawn sample); similar results were obtained for the other  $pH$  values. By diluting the sample, the absorption band became narrower, and the absorption peak became centered at  $\approx 405$  nm. In Figure 7, the absorbance evaluated at  $\lambda = 405$  nm as a function of concentration, normalized to the concentration of the drawn sample, is shown for the different  $pH$  values: after the dilution 1:1, the relationship became linear, testifying to the disappearance of aggregates. It is worthwhile observing a different curve for each  $pH$  value; at different  $pH$  values, the forms or the configurations of bilirubin



**Fig. 6** Absorption spectra of a PB sample at  $pH 7.5$  for different dilutions.

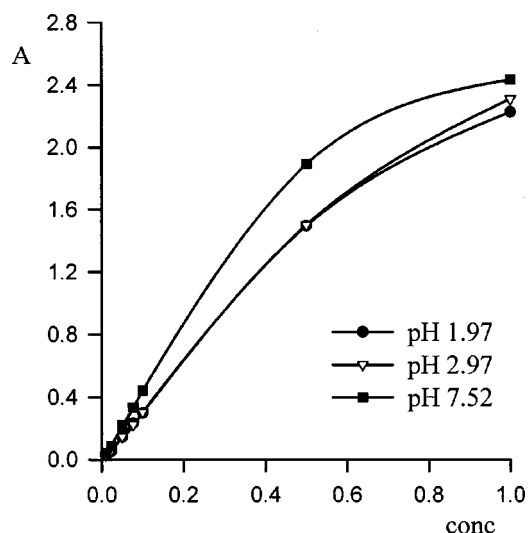


Fig. 7 Absorbance (at  $\lambda = 405$  nm) vs normalized concentration at different pH values for the PB sample shown in Figure 5.

which were dissolved in the sample, were different. Therefore, hypothesizing a change of absorbance with pH is quite obvious, since these forms and configurations should be characterized by a different value of the molar absorption coefficient. Figure 8 shows the absorption spectra of a BGJ sample at different pH values. In this case, the absorption peak was better defined, and was generally centered between 400 and 420 nm. A slight blue-shift (smaller than the one observed in the PB samples) was observed, while the oxidation of bilirubin to biliverdin occurs at  $pH < 4$ , as in the PB samples. Figure 9 shows the relationship between absorbance and concentration at different pH values. In this case, the relationship was perfectly linear, which excluded the presence of aggregates.

It is important to point out that the self-aggregation phenomenon is strictly related to the concentration of a defined biological compound: the higher the concentration, the higher the possibility of self-aggregation. Although bilirubin has quite a high concentration (25–1000 mg/dl) in the bile, which explains the formation of aggregates, it is progressively diluted to its final concentration in the refluxate by means of pancreatic juice, duodenal secretion, and finally, by gastric content.

Figure 10 shows the distribution of the concentration of

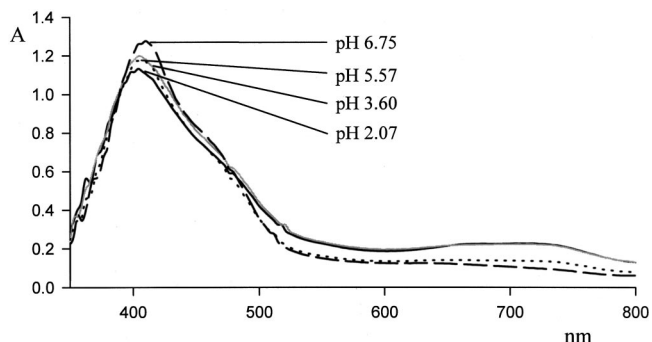


Fig. 8 Absorption spectra of a BGJ sample for different pH values.

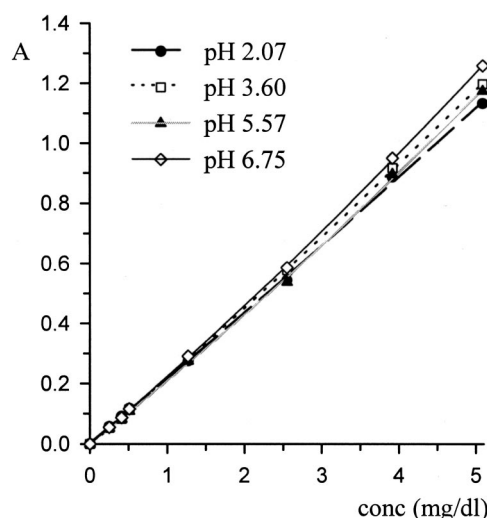


Fig. 9 Absorbance (at  $\lambda = 405$  nm) vs total bilirubin concentration at different pH values for the BGJ sample shown in Figure 8.

total bilirubin in the stomach as a result of 30 samples drawn from different patients. For all the samples with a concentration up to 20 mg/dl the relationship between absorbance and concentration is perfectly linear. On the basis of these results, it is possible to affirm that, in most cases, the degree of dilution of bile was sufficient to avoid the formation of aggregates in the gastric samples. Aggregates could be observed for bilirubin concentrations above 20 mg/dl, a value of concentration which is seldom found in the stomach.

In Figure 11, the results obtained with Bilitec 2000 are shown. In all the graphs the relationship between  $M$ , the quantity measured with Bilitec and defined in Sec. 3.1, and total bilirubin concentration is given. In Figure 11(a), this relationship is for different pH values, obtained on a BGJ sample with the optical fiber sensor utilizing the LED emitting at 420

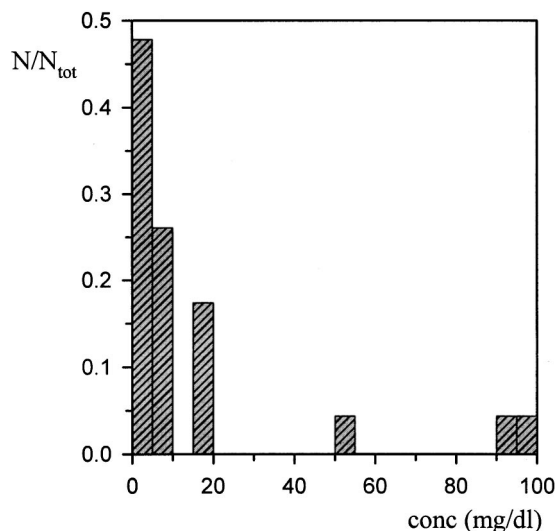
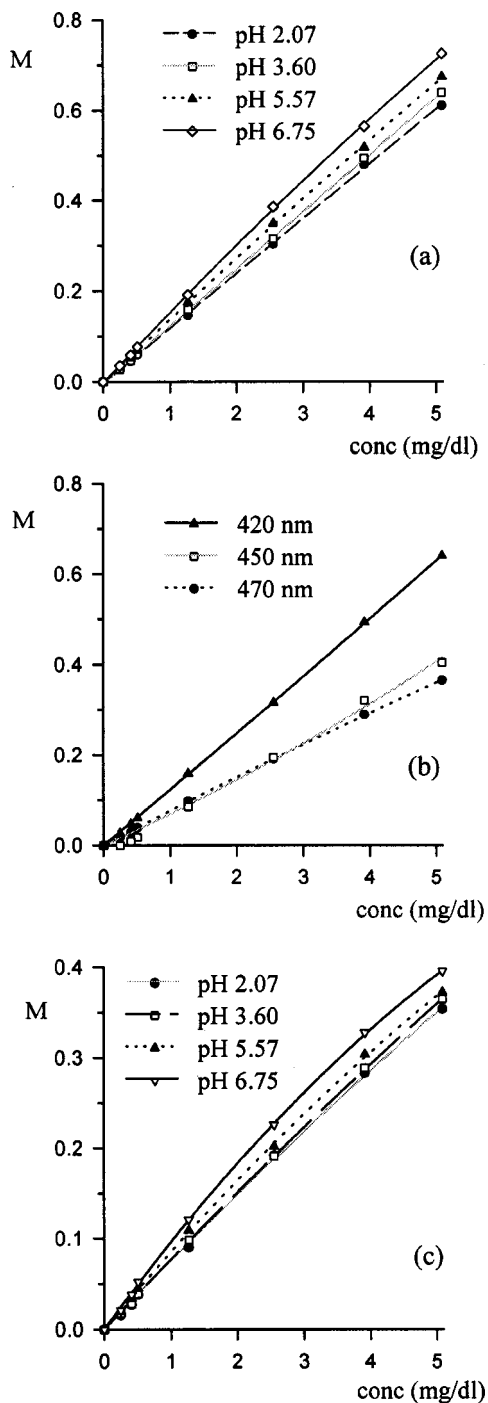


Fig. 10 Distribution of total bilirubin concentration in the samples drawn from the stomach. On the Y axis the number of samples ( $N$ ) within a definite interval ( $= 5$  mg/dl), is normalized to the total number of drawn samples ( $N_{tot}$ ), as reported.



**Fig. 11**  $M$  vs total bilirubin concentration for a BGJ sample: (a) at different  $pH$  values with the signal LED emitting at 420 nm; (b) at  $pH=3.60$  for the three different LEDs, and (c) at different  $pH$  values with the LED emitting at 470 nm.

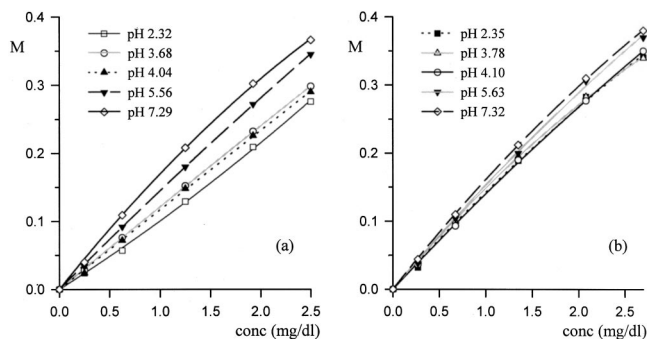
nm as signal source. As for the results obtained with the spectrophotometer, different calibration curves at different  $pH$  values could be distinguished. A comparison of the results obtained with the three different signal sources was also interesting. In Figure 11(b), the relationship is shown between  $M$  and total bilirubin concentration at a  $pH$  of 3.60 for the three different LEDs. A higher sensitivity was obtained for the LED with the emission peak centered at 420 nm: this

could be expected, since there was a better overlapping between the emission band of the LED and the absorption spectrum of bilirubin. A comparable sensitivity was observed at 450 and 470 nm. From these results, it could be concluded that the 420 nm LED is the most appropriate source for bilirubin detection. On the other hand, with this LED, higher interferences could derive from hemoglobin, since this pigment is characterized in the visible region by a strong absorption band centered at 400–420 nm (Soret band), with a molar absorption coefficient higher than that of bilirubin. Therefore, traces of blood, which can be found in operated patients or in patients affected by gastric ulcers, could alter the measurements. From this point of view, 470 nm LED could still be the best source for total bile determination in the refluxes, since the interferences coming from blood should be reduced in this case.

Another point in favor of the use of the 470 nm LED as signal source is the lesser dependence of absorbance on  $pH$  which occurs at this wavelength. The absorption spectra in Figure 8 show that the absorbance at 470 nm was practically the same for all the  $pH$  values, while the same was not true in correspondence with the absorption peak around 400 nm. This aspect is quite visible if Figure 11(a) is compared with Figure 11(c), where the relationship between  $M$  and the total bilirubin concentration for different  $pH$  values is shown for the optical fiber sensor utilizing the LED emitting at 470 nm as signal source. The maximum error in the evaluation of the bilirubin concentration coming from the  $pH$  effect could be considered as the horizontal distance between the calibration curves at  $pH$  6.75 and 2.07; at 5 mg/dl, this underestimation was 13%, against the 18% if 420 nm was used as signal source and similar or lower underestimations were found in all the other tested samples.

On the basis of these results, it is possible to affirm that the error in the measurement of bilirubin concentration due to the change of  $pH$  was not as strong as outlined in previous works,<sup>16</sup> in which an underestimation of 30% for  $pH < 3.5$  is affirmed. It is important to point out that a higher dependence on  $pH$  (and, consequently, a higher underestimation) was observed on the centrifugated samples: in this case, the bilirubin forms which precipitated were separated from the sample tested. On the contrary, in the homogenized samples, bilirubin precipitate remained suspended in solution, as occurs in the stomach where the precipitate remains suspended in the biological juice, due to the continuous movements of the gastric content. The precipitate was still “seen” by the optical probe in the stomach, and this implies that the change of bilirubin concentration as a function of  $pH$  caused by the precipitation of some forms of bilirubin was noticeably attenuated. In Figure 12, the relationship between  $M$  and the total bilirubin concentration for different  $pH$  values is shown for the optical fiber sensor utilizing the LED emitting at 470 nm as signal source in the case of both centrifugated (a) and homogenized (b) samples obtained from the same drawn gastric material. At about 2.5 mg/dl, the errors were 11% and 27% for the homogenized and centrifugated samples, respectively. This signifies that an inappropriate treatment of the samples drawn from the stomach could lead to completely wrong conclusions.

The experimental data shown in Figures 11 and 12 were obtained from the same sample. The error in the measurement could be only related to the error associated to the reading of



**Fig. 12**  $M$  vs total bilirubin concentration at different  $pH$  values for both centrifugated (a) and homogenized (b) BGJ samples (from the same drawn gastric material) obtained with the signal LED emitting at 470 nm.

Bilitec 2000. This error was sufficiently small so as not to be visible in the figures.

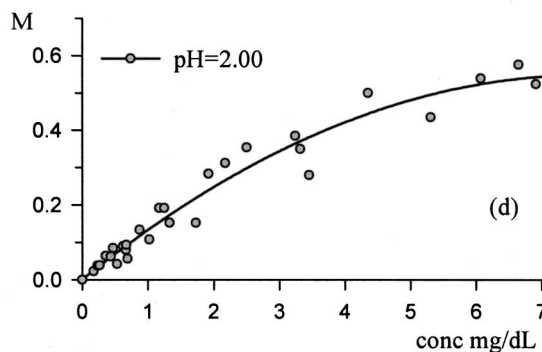
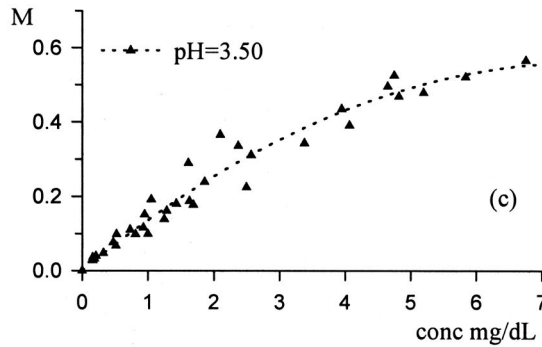
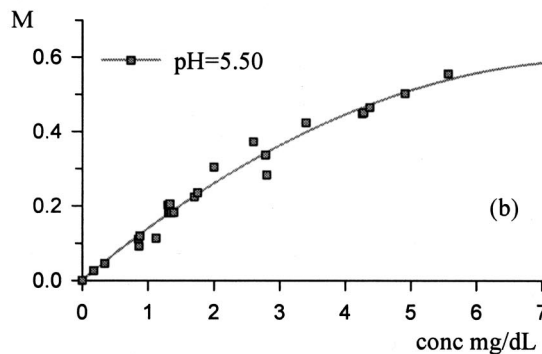
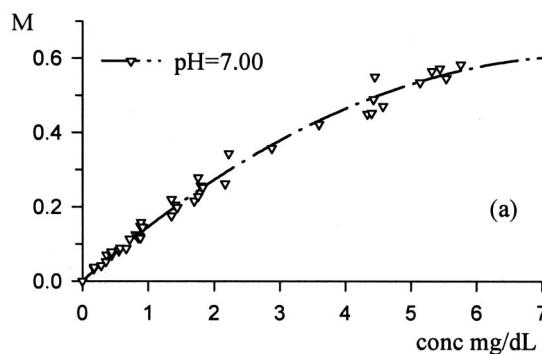
On the other hand, experimental data obtained from different samples had to be considered if Bilitec 2000 was used for a quantitative analysis of bilirubin. It should be noted that Bilitec is considered a reliable tool for the measurement of the exposure time of the mucosa to the bile containing refluxes.<sup>10–12</sup>

Figure 13 shows the relationship between  $M$  and the total bilirubin concentration for different  $pH$  values. Single curves at  $pH$  2.0, 3.5, 5.5, and 7.0 are shown. Data from different samples are reported. Samples with  $pH$  values within  $\pm 0.25 pH$  units were considered, in order to obtain a number of data sufficiently high. The relationship between the measured value  $M$  and concentration should be parabolic, since Bilitec evaluates the absorption which occurs at all the wavelength emitted by the source.<sup>9</sup> For this reason, fitting with parabolic regression was considered. In the case of  $pH$  7.0 and 5.5, the fitting was good with a correlation coefficient of 0.988 and 0.977, respectively. Fitting was a little bit worse in the case of  $pH$  2.0 and 3.5 (0.958 and 0.960, respectively). This behavior could be easily explained with the increase of the scattering due to the precipitation of bilirubin forms which takes place for  $pH \leq 3.5$ . Figure 14 shows all the four curves together; the curves corresponding to different values of  $pH$  could be still distinguished. The effect of  $pH$  at 5 mg/dl implied an error of 18% if the horizontal distance between the calibration curves at  $pH$  2.0 and 7.0 was considered. At 5 mg/dl this underestimation was higher and equal to 22%, if 420 nm was used as signal source. This still demonstrates that 470 nm LED has to be considered the best source for the detection of bile refluxes.

In any case, the main source of error comes from the scattering of the particles suspended in solution, if Bilitec 2000 is used for the *in vivo* determination of the bilirubin content. This represents a “level of noise” which gives rise to a more scattered plot, as can be seen from Figure 14.

### 5 Conclusions

As already pointed out, the conjugated forms of bilirubins, that is, the forms in the bile, are unavailable in pure form. The study performed made possible a preliminary understanding of the spectrophotometric behavior of the bile in the stomach.



**Fig. 13** Parabolic regression for the experimental data coming from different biological samples with the signal LED emitting at 470 nm in the case of (a)  $pH=7.0$ ; (b) 5.5; (c) 3.5; (d) 2.

A protocol for the analysis and study of the optical properties of bile and of gastric juice with bile was established, capable of minimizing the alteration of the samples. Self-aggregation of bilirubin molecules, observed in PB samples, was almost always absent in BGJ samples since it occurred only in the case of concentration of total bilirubin above 20



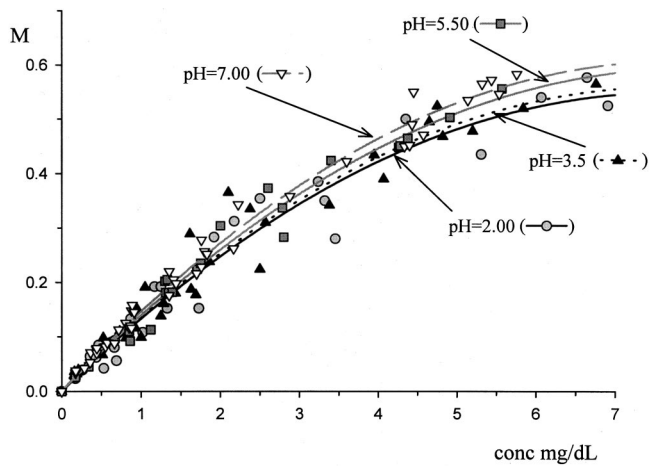


Fig. 14 Comparison among all the curves shown in Figure 13.

mg/dl. The oxidation of bilirubin to biliverdin could be considered negligible for  $pH > 4$ .

The influence of  $pH$  on the bilirubin concentration was smaller if 470 nm was considered as the signal source in the optical fiber sensor. This influence was also attenuated by the presence of precipitate in the gastric content. The evaluation of an error of 30% for  $pH < 3.5$  in the measurement of bilirubin concentration, as outlined in other works,<sup>16</sup> was excessive, and can be quantified in the order of 18%.

It is apparent that the separation of the different forms of bilirubins present in the stomach content and their quantitative determination are fundamental for a complete investigation. On the other hand, this task was complicated by the physical aspects of the drawn sample, characterized by a very high viscosity and by the presence of scattering particles and aggregates which made any quantitative analysis very difficult.

### Acknowledgment

The authors wish to thank Mauro Bacci (Istituto di Ricerca sulle Onde Elettromagnetiche) for his helpful suggestions during the course of this study.

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