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| | |

A Infrared Spectroscopy method to detect ammonia in gastric juice

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Abstract

Ammonia in gastric juice is considered a potential biomarker for *H. pylori* infection and as a factor contributing to gastric mucosal injury. High ammonia concentrations were also found in patients with chronic renal failure, peptic ulcer disease and chronic gastritis. Rapid and specific methods for ammonia detection are then urgently required by the medical community. Here we present a method to detect ammonia directly in gastric juice based on Fourier Transform Infrared Spectroscopy (FTIR). The ammonia dissolved in biological liquid samples as ammonium ion was released in air as a gas by shifting the pH equilibrium of ammonium/ammonia reaction and in line detected by FT-IR set up equipped with a gas cell for the quantification. The method here developed provided high sensitivity and selectivity in ammonia detection both in pure standard solutions and in a simulated gastric juice matrix over the range of diagnostic concentrations tested. Preliminary analysis were also performed on real gastric juice samples of patients with gastric mucosal injury and with symptoms of *H. pylori* infection, and the results were in agreement with the clinical-pathological information. The whole analysis, performed in less than ten minutes, can be directly applied on the sample without extraction procedures and it assures high specificity of detection due to the ammonia fingerprint absorption bands in the infrared spectrum. This method could be easily interfaced with endoscopy instrumentation providing information in real time and enables the endoscopist to improve and integrate gastroscopic examinations.

keywords: ammonia, gastric juice, FTIR spectroscopy, *H. pylori*, gastric mucosal injury

46 Introduction

47 Gastric juice is a colorless, watery, acidic digestive fluid that is secreted by various glands in the
48 mucous membrane of the stomach and consists chiefly of hydrochloric acid, pepsin, rennin, and
49 mucin. The resulting highly acidic environment in the stomach lumen causes proteins food
50 denaturation during digestion and is able to inhibit microorganisms growth, which is helpful to
51 prevent infection. Gastric juice is commonly thrown away during upper endoscopy and, if properly
52 analyzed, may represent a valuable source of clinical-pathological information, especially about
53 *Helicobacter pylori* infection and atrophic gastritis of the oxyntic mucosa (AGOM), which cannot
54 be detected by simple endoscopic examination [1]. Invasive antral biopsies need to be applied for
55 collecting specimen samples which are further analyzed by several methods such as histology
56 assays, *H. pylori* culture, molecular methods (PCR) or urease test [2]. Each of these techniques is
57 laborious and requires several hours to days before the test result is known [3]. Moreover, in
58 patients with normal/mild endoscopic findings, endoscopists take only few antral samples or do not
59 perform biopsies making sometimes impossible the detection of such diseases [1]. Several papers in
60 literature have indicated ammonia in gastric juice as a potential biomarker and as a factor
61 contributing to gastric mucosal injury. Elevated concentrations of ammonia have been frequently
62 found in patients with chronic renal failure [4], peptic ulcer disease [5] and chronic gastritis [6]. It
63 has been shown that ammonia accelerates apoptosis in gastric mucosa [7], inhibits proliferation and
64 cell cycle progression at S-phase [8], impairs cell migration and proliferation of gastric mucosa [9].
65 Ammonia is mainly produced by *Helicobacter pylori* who may contribute to gastric mucosal injury.
66 *H. pylori* has several adaptations for an acid milieu of the stomach. One of them is the urease
67 enzyme, which converts urea into ammonia and carbon dioxide to establish a locally neutralizing
68 surrounding against penetrating acid [10]. This is one of the features that make it possible for the
69 bacterium to survive in the human stomach and enables the colonization of gastric mucosa.
70 Ammonia is transformed to NH_4^+ ion (ammonium) and the relative concentration of these two
71 forms is pH dependent. Gastric juice ammonia has been successfully related to *H. pylori* infection

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3 72 and the severity of gastritis in several studies [3, 6, 11-14]. Medical community is considerably
4
5 73 interested in ammonia analyzers that can be applied for measuring ammonia levels in gastric juice
6
7 74 for the diagnosis of certain diseases especially related to *H. pylori* infection. Development of
8
9 75 diagnostic tests that may be performed at the time of the endoscopy and rapidly interpreted by the
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11 76 endoscopist is important. At the present, quantitative estimation of ammonium in biological fluids is
12
13 77 usually based on spectrophotometric procedures, potentiometric ion selective electrodes or on
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15 78 amperometric enzyme electrodes [15-17]. Although many of these procedures have been reported
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17 79 and improved over the years, all these methods suffer some limitations in terms of electrode low
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19 80 sensitivity, low selectivity in a complex matrix, enzymes expensive cost and frequent or long
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21 81 calibration procedures.
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25 82 The method developed in this work is a cost effective, in-line and no contact analysis which is able
26
27 83 to detect ammonia present in gastric juice by analyzing the saturated vapor. Ammonium (liquid
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29 84 phase) in gastric juice is forced to be released as ammonia (gas phase) in air by varying the pH of
30
31 85 the solution which moves the equilibrium of the reaction to the gas phase. Ammonia in gas phase
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33 86 has a typical absorption bands in the infrared spectrum that can be rapidly detected in real time. The
34
35 87 goal of this study is to determine the sensitivity of this FTIR based method over the diagnostic
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37 88 ammonia concentrations range and to evaluate the role of interferences in a gastric juice simulated
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39 89 matrix. Moreover, gastric juices of patients with gastric mucosal injury and with symptoms of *H.*
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41 90 *pylori* infection were analyzed in order to assess the feasibility of this test in a real sample.
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98 **Material and Methods**

99 *Reagents*

100 Ammonium chloride (NH₄Cl), Sodium chloride (NaCl), hydrochloric acid (HCl, 37%) and sodium
101 hydroxide (NaOH) were purchased from Carlo Erba (Milan, Italy). Pepsin 1:10000 was purchased
102 from Farmalabor (Farmacia Specchiulli). All chemicals were of analytical grade and used without
103 further purification.

104

105 *Preparation of standard solutions*

106 Ammonium chloride stock solution was prepared by accurately dissolving 0.943 g of NH₄Cl in 1 l
107 of deionized (DI) water to reach a concentration of 943 ppm. Ammonium chloride standard
108 solutions were prepared by subsequent dilutions of the stock solution in DI water to reach the
109 following concentrations: 153, 70, 60, 50 and 17 ppm. Pure ammonium chloride standards were
110 used to set up the analytical procedure. Aliquots of the NH₄Cl standards were mixed in a 9:1 ratio
111 with ISA solution (Ionic strength adjuster, NaOH 20% in DI water), mixed with a magnetic stirrer
112 and the evolved gas phase was analyzed by Fourier Transform Infrared Spectroscopy equipped with
113 a 6.4 m gas cell. Setup and measurements details are explained in the paragraph Measurement
114 Apparatus.

115 Simulated gastric fluid (SGF) was prepared by dissolving 2.0 g of sodium chloride and 3.2 g of
116 purified pepsin, that is derived from porcine stomach mucosa, with an activity of 800 to 2500 units
117 per mg of protein (or 250 mg of 1:10000 pepsin), in 7.0 ml of hydrochloric acid and sufficient water
118 to make 1 l (NOTE – Pepsine activity is described in the Food Chemical Codex specification under
119 General Tests and Assays). This test solution has a pH of about 1.2. Specification of 1:10000 pepsin
120 means 1g pepsin contains 10000 pepsin units, we can calculate that 1mg contains 10 units. If the
121 pepsin is not purified (when it is not marked "purified"), it consists of protease, amylase and lipase.
122 So, when it is not purified the units calculated is, indeed, a smaller number. *NF Unit*: Pepsin digests

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2
3 123 not less than 3000 and not more than 3500 times its weight of coagulated egg albumin. (1 pepsin
4
5 124 unit will digest 3000 units coagulated egg albumin at 52°C, pH 2-3, no time involved.)
6
7 125 Ammonium chloride standard solution in simulated gastric fluid was prepared by dissolving 0.943 g
8
9 126 of standard in 1 l of SGF to reach a concentration of 943 ppm. Ammonium chloride standard
10
11 127 solutions were prepared by subsequent dilutions of the stock solution in SGF to reach the following
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13 128 concentrations: 153, 60, 50 and 17 ppm respectively. Aliquots of standards solutions in SGF were
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15 129 mixed with ISA (9:1 ratio) and the evolved gas phase was analyzed by FT- IR equipped with a 6.4
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17 130 m gas cell. Setup and measurements details are explained in the paragraph Measurement Apparatus.
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23 132 *Gastric juice samples*

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25 133 Real gastric juice samples were provided by the Gastroenterology Division of Mauriziano Hospital
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27 134 (Turin, Italy). Three different samples were provided: sample A was obtained from a diluted
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29 135 solution used for endoscope cleaning. This sample was chosen as negative control because it
30
31 136 contains a very low concentration of ammonia as well as a healthy patient. Sample B was from a
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33 137 celiac patient with symptoms of *Helicobacter pylori* infection and Sample C from a patient with
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35 138 stomach ulcer. All these samples have been stored in sterile tubes at 4° C in the dark and analyzed
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37 139 in the same day of the endoscopy.
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41 141 *Measurement Apparatus*

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43 142 The measurement apparatus is described in figure 1. Briefly, 4.5 ml of sample solution (NH₄Cl
44
45 143 standard solution in water/SGF or real gastric juice samples) are injected in a two neck round
46
47 144 bottom flask with magnetic stirring to continuously mix the solution. The flask is immersed in a
48
49 145 water bath at 30° C to keep the temperature stable. One neck is used for solution injection and is
50
51 146 subsequently safely sealed with a cap. The other one is connected through a Teflon tube to a MARS
52
53 147 Series long path gas absorption cell for FTIR gas analysis which is located and aligned inside the
54
55 148 FTIR spectrophotometer. A stopcock valve (valve n.1 in the scheme) is positioned between the
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3 149 flask and the gas cell to regulate the communication between these two compartments. The MARS
4
5 150 series cell gas has an optical path of 6.4 m and a volume of 0.75 l assuring high sensitivity with
6
7 151 potential gas detection in the ppb range. Internal body of the cell contains gold reflecting mirrors
8
9 152 and the internal surface is covered with passivated stainless steel to avoid corrosion and
10
11 153 contaminations when a corrosive gas is used. A thermocouple connected to a programmable
12
13 154 temperature control is located inside the cell which is externally enveloped with an insulator sheet
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15
16 155 to keep the temperature constant at 30° C. On the upper side of the gas cell two separate valves
17
18 156 allow to control the inlet and the outlet of the gas. The inlet is connected with the Teflon tube to the
19
20 157 flask while the outlet is connected to a vacuum pump which is able to create a depression between
21
22 158 the gas cell compartment and the solution containing flask. As soon as the standard solution is
23
24 159 injected into the flask, a depression between these two compartments is applied by opening the inlet
25
26 160 and outlet valves on the gas cell while keeping the stopcock valve above the flask closed. When a
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28 161 pressure of about 1013.25 Pa (10^{-2} atm) is achieved, the outlet valve is closed and the stopcock
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30 162 valve on the top of the flask is opened allowing the gas phase to be injected inside the gas cell. An
31
32 163 infrared spectrum of the gas sample is then collected and it is used as a background for the FTIR
33
34 164 measurement. When the FTIR background is collected, the stopcock valve on the flask is closed and
35
36 165 the outlet valve is opened in order to remove the gas sample from the cell by means of the pumping
37
38 166 system. The optical path is further cleaned before and after every measurement by injecting
39
40 167 nitrogen gas through the stopcock valve n.2 which allows to remove any gas residues from the
41
42 168 system. For ammonia detection, 0.5 ml of ISA solution (Ionic strength adjuster, NaOH 20% in DI
43
44 169 water) is pipetted inside the flask and allowed to react for 5 minutes. The ISA solution changes the
45
46 170 pH of the standard solution to alkaline values (pH around 11) and promotes the transition of
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48 171 ammonium (liquid phase) into ammonia (gas phase). Using the same measurement procedure just
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50 172 described, an infrared spectrum is collected and the ammonia can be quantified.
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175 *FTIR Measurements*

176 The infrared spectra of gas samples were collected using a Nicolet Nexus Thermo Fischer
177 spectrophotometer equipped with a DTGS detector in transmission mode; 64 scans with a resolution
178 of 4 cm^{-1} were registered for each spectrum.

179 MARS Series long path gas absorption cell (Gemini scientific instruments) was located into the
180 main compartment of the instrument and an alignment procedure was performed. Background
181 spectra were collected on every samples before adding ISA solution. A cleaning procedure is
182 applied after every measurement using nitrogen purging and vacuum by means of a piston pump, as
183 described in measurement apparatus.

184

185 *Calibration procedure*

186 Quantitative analysis of levels of ammonia in FTIR measurements is based on the Lambert-Beer's
187 law which relates the absorbance at a specific wavelength with the standard concentration. For each
188 standard solutions of ammonium chloride, both in pure water or in SGF, the peak height of the
189 absorption band at 966 cm^{-1} (N-H symmetric deformation of ammonia) was calculated. Hence, a
190 regression curve was fitted to the intensities, providing therefore the calibration curve of the
191 spectrophotometer for the ammonia, as the basis for subsequent quantification analyses. A weighted
192 least-squares (WLS) regression was applied to the data, that is able to deal with uncertainty in the
193 values of the dependent variable, i.e., the absorbance values. The algorithm used for the WLS
194 regression is a MATLAB®-based tool for calibration problems (Calibration Curves Computing –
195 CCC Software, Release 1.2), recently developed at INRIM in the framework of the Joint Research
196 Project “Novel mathematical and statistical approaches to uncertainty evaluation”, within the
197 European Metrology Research Program.

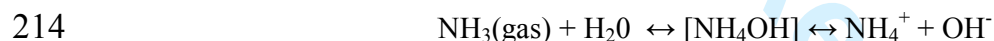
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200 Results and Discussion

201 High concentrations of ammonia have been found in several gastric diseases, especially the ones
202 related to *H. pylori* infection. New methods for detecting ammonia in gastric fluid are then required
203 in order to make rapid diagnosis and to prevent the aggravation of such diseases. The proposed
204 method represents an alternative way to detect ammonia in biological fluids and in particular in
205 gastric juice. Infrared spectroscopy is a very sensitive and selective technique for gas detection
206 since these molecules have strong absorption bands related to the roto-vibrational structure of the
207 gas molecules in the medium infrared region that can be rapidly identified due to their specific
208 fingerprint. In order to develop a method to detect ammonia (gas) in gastric juice by FTIR
209 spectroscopy, the ammonium ions in solution must be released in air as gas phase. The
210 ammonium/ammonia equilibrium is very well known in chemistry and the direction is governed by
211 the pH of the solution. Ammonium hydroxide is a weak base that is partially ionized in water
212 according to the equilibrium:

213



215 When the pH is equal to the pKa (9.25 pH), half of the ammonia will be un-ionized (NH_3) and half
216 will be ionized (NH_4^+). At pH 10.25 and 11.25, 90% and 99% of the ammonia will be un-ionized,
217 respectively. The volatility of ammonia increases with increasing pH; therefore, it volatilizes freely
218 from solution at high pH values. This condition can be forced by adding a proper amount of sodium
219 hydroxide (ISA solution) in the gastric juice to shift the pH to a value around 11. As soon as the
220 ammonia evolves from the solution in the gas phase and when it reaches the vapour saturated
221 condition, the gas phase can be rapidly injected into the FTIR gas cell (see Fig. 1 and Measurement
222 apparatus in Material and methods for details) and the ammonia can be specifically detected by
223 functional group identification in the infrared spectrum.

224

Figure 1

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3 225 A standard solution of 943 ppm ammonium chloride was first tested to set up the procedure and to
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5 226 carry out infrared qualitative analysis of ammonia. A FTIR spectrum of the pure solution was first
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7 227 collected without adding ISA in order to register the signal background. As Fig. 2a shows, this
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9 228 spectrum, in the range of 800 cm^{-1} to 4000 cm^{-1} , is mainly characterized by water absorption bands
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11 229 with O-H stretching vibrations at $3600\text{--}3800\text{ cm}^{-1}$, H-O-H bending vibrations at $1500\text{--}1700\text{ cm}^{-1}$
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13 230 and C-O stretching vibration at 2350 cm^{-1} due to the presence of carbon dioxide (CO_2). No other
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15 231 signals can be distinguished throughout the spectrum. However, as soon as the ISA solution is
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17 232 added into the flask to move the reaction equilibrium from ammonium to ammonia, specific
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19 233 ammonia absorption bands show up. FTIR spectra in Fig. 2b, c, d clearly show in the three different
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21 234 spectral regions the presence of the N-H symmetric stretching vibration at 3300 cm^{-1} (ν_1), the
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23 235 degenerate deformation at 1640 cm^{-1} (ν_4) and the symmetric deformation with a double peak at 966
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25 236 and 934 cm^{-1} (ν_2), respectively [18].
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30 237 Figure 2
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33 238 In order to investigate the sensitivity and the dynamic range of the FTIR based method, ammonium
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35 239 chloride standard solutions with a concentration of 17, 50, 60, 70, 153 ppm respectively were then
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37 240 analyzed. Since the absorption band at 966 cm^{-1} has the highest intensity and is free of water
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39 241 interference (Fig. 2d), it was the frequency band selected signal for ammonia quantification. As
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41 242 Fig. 3a shows, the ammonia absorption band at 966 cm^{-1} raises proportionally with the ammonium
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43 243 concentrations.
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46 244
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48 245 Figure 3
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51
52 246 Calibration of the infrared equipment was performed by using the Lambert-Beer's law that is
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54 247 expressed by the equation:
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$$56 248 A(\nu) = \epsilon(\nu) l C$$

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58 249 where
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3 250 $A(\nu)$ is the absorbance at wave number ν ,

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5 251 $\epsilon(\nu)$ is the molar absorptivity at wave number ν ,

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7 252 l is the path length,

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9 253 C is the molar concentration.
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14 255 Thus, it was possible to correlate the absorbance values at 966 cm^{-1} with the ammonium chloride

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16 256 concentrations (Fig. 3b). The relative standard uncertainty associated with the absorbance values

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18 257 (reported as y error bars in Fig. 3b) was considered equal to 10 % in the considered ammonia range.

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20 258 This value was evaluated on previous experiments aimed at assessing the reproducibility of the FT-

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22 259 IR signals. Notice that calibration uncertainty associated with the standard concentrations were

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24 260 proved to be negligible with respect to the absorbance variability, hence they were not take into

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26 261 account in the present analysis. A 1st order polynomial fit $y = a + bx$ was used for fitting the data

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28 262 (Fig. 3b), and the relevant normalized χ^2 value (i.e., the sum of the weighted squared residuals

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30 263 normalized by the number of degrees of freedom) showed a high goodness of fit for ammonia in

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32 264 water, being lower than the expected unit value.
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35 265 According to the literature, the 60 ppm concentration of ammonia has been established as a cut off

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37 266 to determine the infection of *H. pylori*. This threshold value has been calculated by Tucci et al.,

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39 267 2007 [1] during the validation of the Mt 21-42 device, an ion selective electrode that measures

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41 268 ammonia in gastric juice. This analysis have stated 60 ppm of ammonia as a cut off value for

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43 269 patients infected with *H. pylori* by comparing the obtained results with traditional analytical

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45 270 methods such as histology, breath test analysis and serological test. In order to assess whether the

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47 271 FTIR based method can be potentially used for ammonia detection and for the identification of

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49 272 patients infected with *H. pylori*, concentrations around the threshold value of 60 ppm were chosen.

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51 273 The results shown in Fig. 3a-b have indicated that our methodology display high sensitivity at low

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53 274 concentration of ammonia such as 17 ppm and that is able to discriminate ammonia concentration
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3 275 with a good resolution around the cut off value of 60 ppm with a linear correlation along the whole
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5 276 range tested.

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7 277 After the method was calibrated with pure standard solutions in water, we decided to test ammonia
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9 278 detection in a simulated gastric juice matrix. SGF solution contains several interferents frequently
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11 279 found in a real gastric juice sample such as hydrochloric acid, pepsin, enzymes and ions. Specific
12
13 280 amounts of ammonium chloride were spiked into the simulated gastric juice to calibrate the FTIR
14
15 281 based method in presence of such interferents. The diagnostic concentrations tested were 0, 17, 50,
16
17 282 60 and 153 ppm, respectively. FT-IR spectra were collected for these standards before and after
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19 283 adding ISA solution and the absorbance values were plotted over the cited concentrations (Fig. 4).
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24 284 Figure 4

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26 285 As in the case of ammonium chloride standard solutions, a relative standard uncertainty equal to
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28 286 10% was associated with the absorbance values (y error bars in Fig. 4b), and calibration uncertainty
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30 287 in the standard solutions was negligible. In this case, a 2nd order polynomial fit $y = a + bx + cx^2$
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32 288 was used for fitting the data (Fig. 4b), and a very good corresponding χ^2 value showed a high
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34 289 goodness of fit for ammonia in SGF. Estimates of the fitting parameters a , b and c and associated
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36 290 covariance matrix were calculated according to the WLS procedure. From such results, it was
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38 291 straightforward to obtain the analysis curve $x = (b - \sqrt{b^2 + 4c(-a + y)})/(-2c)$, by simply
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40 292 inverting the calibration curve. Therefore, given a new measured absorbance value y , corresponding
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42 293 to an unknown concentration x of ammonia, an estimate for such measurand can be derived by the
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44 294 formula above; the corresponding associated uncertainty can be obtained by applying to the analysis
45
46 295 curve either the law of propagation of uncertainty [20, 21]. A Monte Carlo simulation was
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48 296 performed according to latter choice, in which parameter estimates a , b and c were considered
49
50 297 distributed according to a multivariate normal distribution with covariance matrix equal to that
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52 298 determined during the calibration process, and intensity y distributed as a normal distribution with
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54 299 standard deviation equal to the repeatability uncertainty typically associated with y .
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3 300 Comparing these results with those obtained in pure standard solutions, it was shown that
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5 301 absorbance values of ammonia from a gastric juice simulated matrix are lower than those obtained
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7 302 from standard water solutions. Moreover, the linearity is no more preserved. It is possible to infer
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9 303 that a latency phase is maintained from 0 to 40 ppm while above this value a linear response is
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11 304 starting again. Deviation from linearity is probably caused by interferences in solution. Since several
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13 305 charged molecules such as proteins and ions are present in the simulated gastric juice and they are
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15 306 able to interact with ammonium, the shift of the ammonium/ammonia equilibrium could be partially
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17 307 slowed down by association/dissociation and interaction phenomena with the chemical species in
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19 308 solution. This behavior is indeed more evident at low concentrations of ammonium chloride where
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21 309 a latency phase is observed. At higher concentrations, instead, more ammonium molecules are free
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23 310 of interactions with the other species in solution and their release in air as ammonia occurs faster,
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25 311 leading to a linear increase of the signal. Although the linearity is no more preserved and the
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27 312 sensitivity is impaired at lower concentrations, the FTIR based method is still able to sense the
28
29 313 concentrations of ammonia in the whole range tested and to discriminate around the cutoff value of
30
31 314 60 ppm. The relative expanded ($k = 2$) uncertainty associated with an estimate x of the
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33 315 concentration of ammonia in a concentration range from 0 up to 153 ppm appeared to be lower than
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35 316 13%. Thus, the trend line obtained in Fig. 4b was used as a calibration curve in order to quantify
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37 317 ammonia concentrations in real samples. Gastric juices of patients who underwent to upper
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39 318 gastrointestinal endoscopy were analyzed by FT-IR methodology and the results were compared to
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41 319 the clinical-pathological information. As a preliminary study on real samples, three different kind of
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43 320 gastric juices were used for the analysis. Gastric Juice A was obtained from a diluted solution used
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45 321 for endoscope cleaning. This sample was chosen because it contains a very low concentration of
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47 322 ammonia and represents a negative control similar to an healthy patient. Gastric Juice B was from a
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49 323 celiac patient with symptoms of *H. pylori* infection. The last sample (Gastric Juice C) was obtained
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51 324 from a patient with stomach ulcer disease. As Fig. 5 shows, FTIR analysis on real gastric juice
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53 325 samples registered different absorbance values of the N-H symmetric deformation at 966 cm^{-1} , with
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3 326 a lower absorbance for the negative control (0.0015 Abs. for Sample A) and higher values for the
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5 327 patients (0.0098 and 0.03 Abs. for Sample B and C, respectively) which means that different
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7 328 ammonia concentrations were measured by our FTIR based method. Using the calibration curve
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9 329 shown in Fig. 4, the ammonia concentrations of these samples were quantified and compared with
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11 330 the clinical-pathological information (table 1).

13
14 331 Figure 5

16
17 332 Table 1

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20 333 In sample A, a concentration of only 30 ± 5 ppm of ammonia was registered which is consistent
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22 334 with a negative control sample as well as a healthy patient. In sample B and C, instead, high values
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24 335 of ammonia were registered by FT-IR methodology which can be related to the critical clinical
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26 336 conditions of the patients, as it is shown in Table 1. In the celiac patient (Sample B) with symptoms
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28 337 of *H. pylori* infection, a concentration of 97 ± 13 ppm was measured which is higher than the cutoff
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30 338 value of 60 ppm indicated by Tucci et al., to determine the infection of *H. pylori*. Therefore, such
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32 339 amount of ammonia could be reasonably related to the presence of the infection. In the latter case
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34 340 (Sample C), a very high concentration of ammonia, more than 153 ppm, was detected, suggesting
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36 341 that the patient has a severe and critical stomach condition which is in agreement with the clinical
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38 342 information (Table 1). Also in this case it could be inferred an infection of *H. pylori* but histological
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40 343 analysis was not performed on this sample. Even if these analysis only give preliminary information
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42 344 and a complete validation of this methodology in real samples is still missing, these results
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44 345 demonstrated the application of FTIR spectroscopy for the quantification of ammonia in gastric
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46 346 juice. Moreover, since high concentrations of ammonia in blood, saliva and urine were related to
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48 347 inflammatory states, the FTIR method here proposed could be successfully used as screening test
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50 348 for the analysis of biological fluids in the medical field.

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351 **Conclusion**

352 Ammonia is an interesting biomarker that can be found in several biological fluids such as saliva,
353 urine, blood and gastric juice. In the latter case, elevated concentration of ammonia were related to
354 *H. pylori* infection and in general to gastric mucosal injury. In this work we presented a new method
355 to detect ammonia in gastric juice based on FTIR spectroscopy that could be potentially applied to
356 other biological fluids. The ammonia dissolved in biological liquid samples as ammonium ion can
357 be released in air as a gas, by shifting the equilibrium of ammonium/ammonia reaction which is
358 mainly governed by the pH of the solution. Ammonia detection was performed by FTIR
359 spectroscopy which guarantees high sensitivity and specificity both in pure standard solutions and
360 in a simulated gastric juice matrix over the range of diagnostic concentrations tested. Real gastric
361 juice samples were also analyzed and the obtained results demonstrated the variability associated to
362 the patients with clinic-pathological diseases. Our method proved to be very rapid because it does
363 not require extraction procedures, the total analysis time takes less than 10 minutes and it is very
364 easy to be calibrated. Moreover, since ammonia has a specific fingerprint in the infrared spectrum,
365 false positive responses can be minimized.

366 This innovative method could be easily interfaced with endoscopy instrumentation providing
367 information in real time and enables the endoscopist to improve and integrate gastroscopic
368 examinations by comparing the outcome of visual inspection and chemical testing.

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3 476 **FIGURE LEGENDS**
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6 477 **Figure 1** – FTIR gas analysis setup
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9 478 **Figure 2** – (a) FTIR spectrum of a 943 ppm NH_4Cl solution in water (gas phase).
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11 479 (b, c, d) FTIR spectra of a 943 ppm NH_4Cl water solution (gas phase) before (dotted line) and after
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13 480 (black line) adding ISA (20% NaOH in water) in the range 3500-3150 cm^{-1} (b), 2000-1400 cm^{-1} (c)
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15 481 and 1100-850 cm^{-1} (d).
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19 482 **Figure 3** - (a) FTIR spectra of NH_4Cl water solutions (gas phase) after adding ISA: 17, 50, 60, 70
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21 483 and 153 ppm. (b) Calibration curve of NH_4Cl standards in water obtained by plotting the
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23 484 Absorbance at 966 cm^{-1} vs NH_4Cl concentration.
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26 485 **Figure 4** - (a) FTIR spectra of NH_4Cl solutions in simulated gastric fluid (gas phase) after adding
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28 486 ISA: 0, 17, 50, 60, and 153 ppm. (b) Calibration curve of NH_4Cl standards in simulated gastric fluid
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30 487 (SGF) obtained by plotting the Absorbance at 966 cm^{-1} vs NH_4Cl concentration.
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34 488 **Figure 5** – FTIR spectra of real gastric juice samples (gas phase) after adding ISA obtained from
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36 489 an healthy patient (Sample A), a patient with symptoms of *H. pylori* (Sample B) and a patient with
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38 490 stomach ulcer (Sample C).
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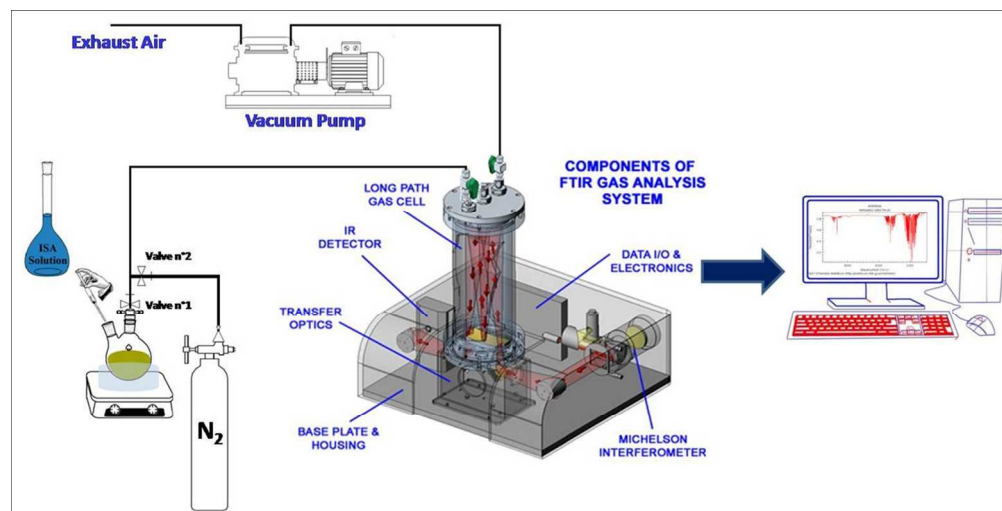


Figure 1 – FTIR gas analysis setup
244x123mm (150 x 150 DPI)

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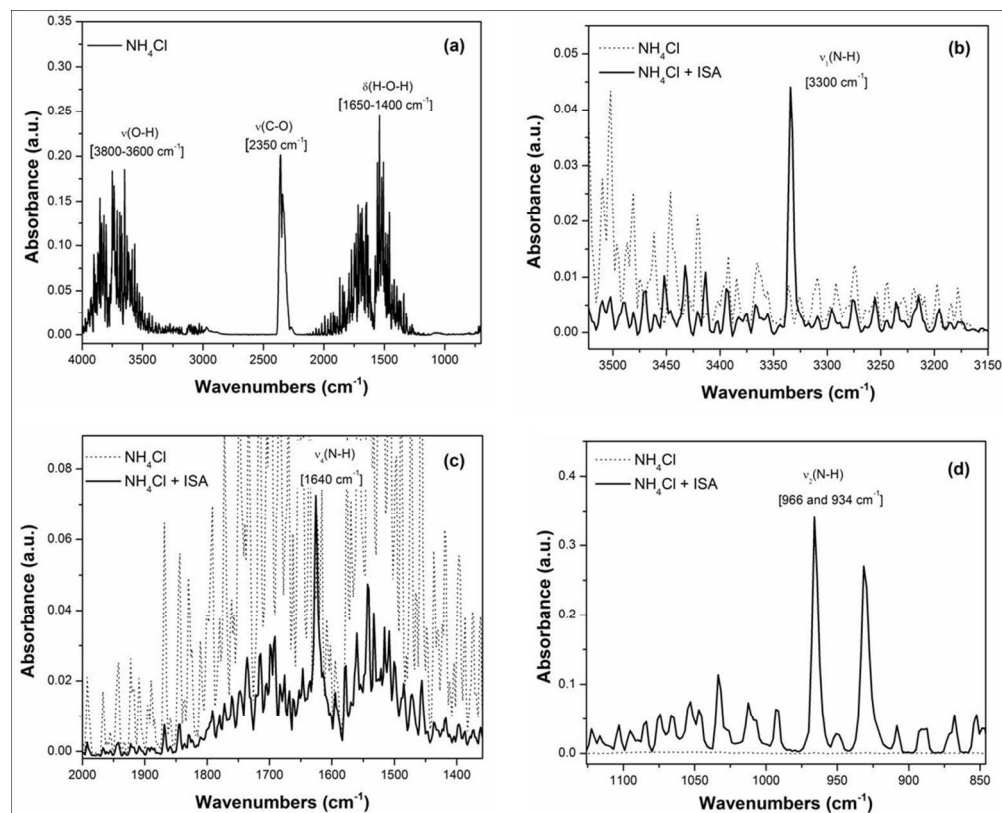


Figure 2 – (a) FTIR spectrum of a 943 ppm NH_4Cl solution in water (gas phase). (b, c, d) FTIR spectra of a 943 ppm NH_4Cl water solution (gas phase) before (dotted line) and after (black line) adding ISA (20% NaOH in water) in the range 3500–3150 cm^{-1} (b), 2000–1400 cm^{-1} (c) and 1100–850 cm^{-1} (d). 215x173mm (150 x 150 DPI)

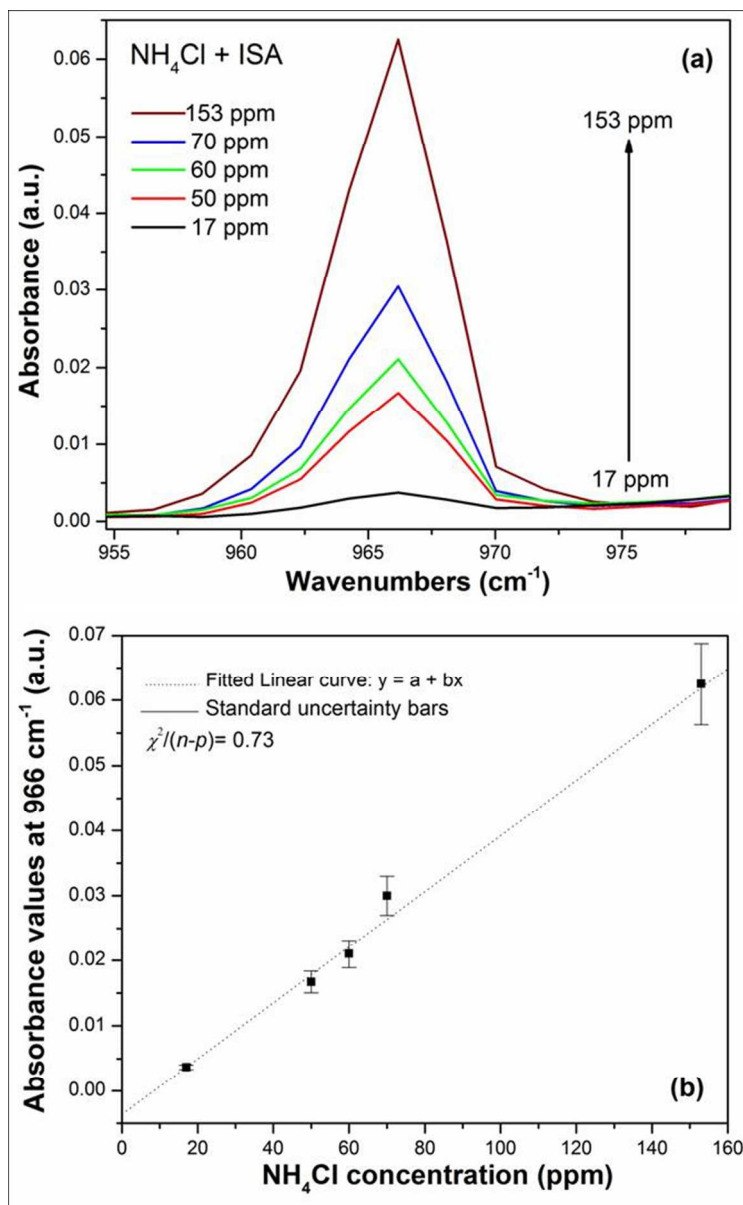


Figure 3 - (a) FTIR spectra of NH_4Cl water solutions (gas phase) after adding ISA: 17, 50, 60, 70 and 153 ppm. (b) Calibration curve of NH_4Cl standards in water obtained by plotting the Absorbance at 966 cm^{-1} vs NH_4Cl concentration.
113x183mm (150 x 150 DPI)

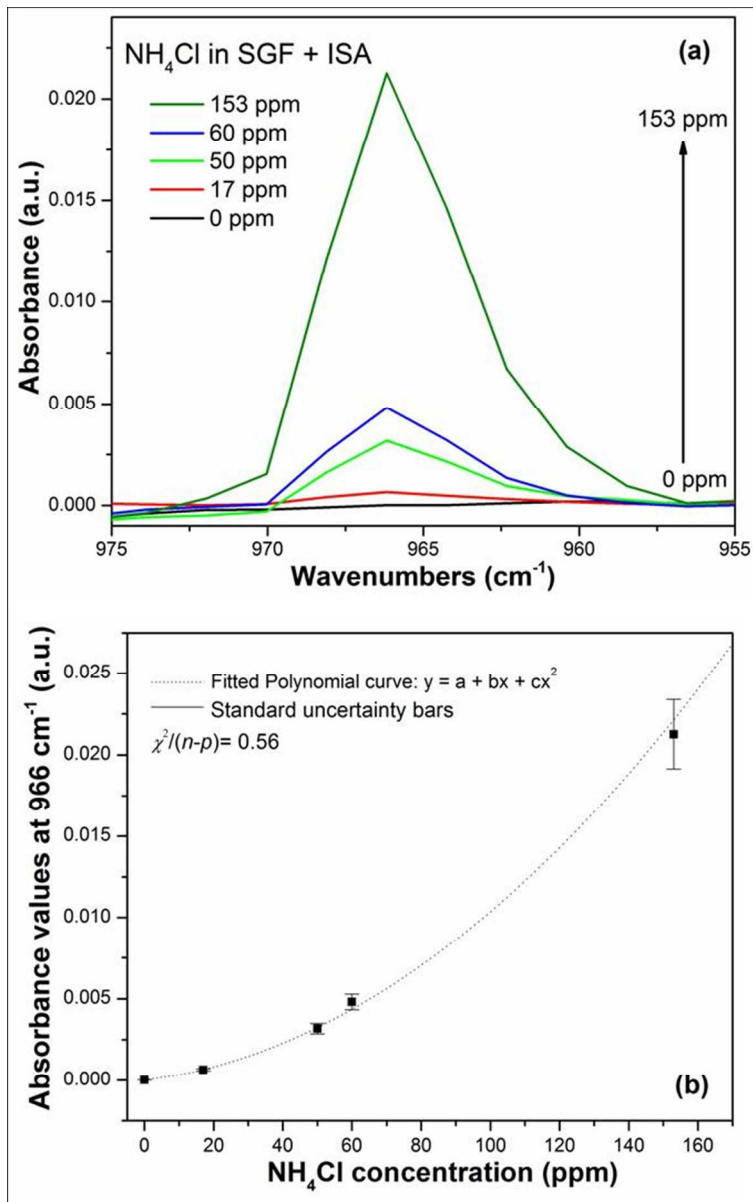


Figure 4 - (a) FTIR spectra of NH₄Cl solutions in simulated gastric fluid (gas phase) after adding ISA: 0, 17, 50, 60, and 153 ppm. (b) Calibration curve of NH₄Cl standards in simulated gastric fluid (SGF) obtained by plotting the Absorbance at 966 cm⁻¹ vs NH₄Cl concentration.
114x183mm (150 x 150 DPI)

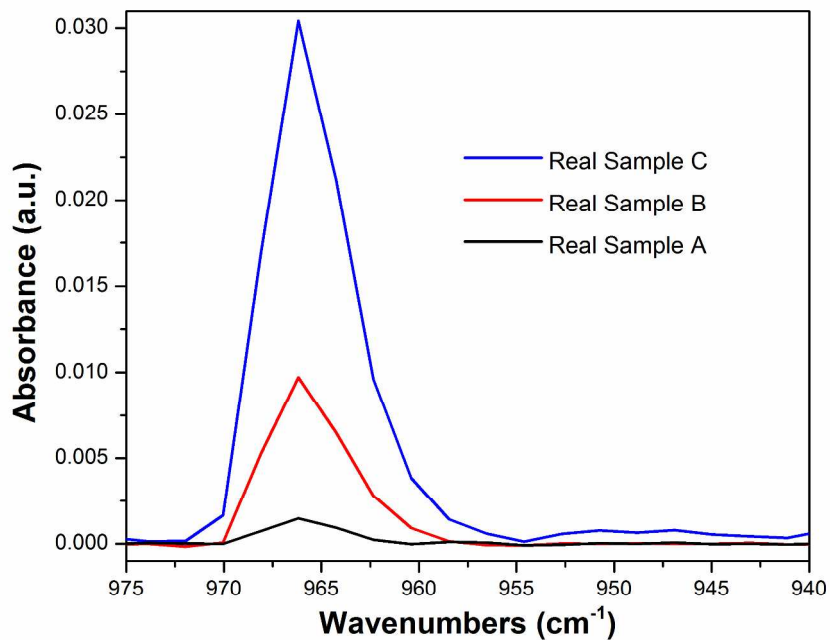


Figure 5 – FTIR spectra of real gastric juice samples (gas phase) after adding ISA obtained from an healthy patient (Sample A), a patient with symptoms of *H. pylori* (Sample B) and a patient with stomach ulcer (Sample C).
273x210mm (300 x 300 DPI)

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| Sample | Clinical-Pathological Information^a | FTIR Quantification^a | Expanded Uncertainty (<i>U</i>) (<i>k</i> = 2)^b |
|---------------|--|--|---|
| A | Healthy Patient | NH ₄ ⁺ = 30 ppm | ± 5 ppm |
| B | Symptoms of <i>H. pylori</i> infection | NH ₄ ⁺ = 97 ppm | ± 13 ppm |
| C | Stomach Ulcer | NH ₄ ⁺ > 153 ppm | N.A. |

^a Ammonia quantification by FTIR in real gastric juices is based on the calibration curve in Fig.4b.
^b Expanded Uncertainty (*U*) with a coverage factor *k* = 2 is reported for ammonia amounts in FTIR analysis.

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Table 1 - Analysis of real gastric juices
189x121mm (96 x 96 DPI)

Review