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DIAGNOSIS OF *HELICOBACTER PYLORI* INFECTION WITH THE ^{13}C-UREA BREATH TEST: ANALYSIS BY MEANS OF GAS CHROMATOGRAPHY WITH MASS SELECTIVE DETECTION

By

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Abstract

Helicobacter pylori infection is a key risk factor in most peptic ulcer diseases and a primary risk factor for gastric cancer. It is current standard practice, that individuals with ulcers be treated to eradicate the organism. It is therefore, evident that accurate and reliable diagnostic tools are required to diagnose patients with a *Helicobacter pylori* infection. Histological examination of biopsy specimens is currently considered to be the gold standard in the diagnosis of *Helicobacter pylori* infection. It is well documented, however, that the non-invasive ^{13}C-urea breath test (^{13}C-UBT) is extremely accurate, when compared to histological examination of biopsy specimens. The ^{13}C-UBT has proved to be a simple, safe, and effective test, which can be performed repeatedly without risk on the same patient, children and pregnant women. The ^{13}C-UBT is also the method of choice to confirm eradication of *Helicobacter pylori* after treatment.

The ^{13}C-UBT was first described in 1987, and a number of optimization studies have since then been reported. Isotope ratio mass spectrometry (IRMS) is used worldwide

to measure the $^{13}CO_2/^{12}CO_2$ ratio in ^{13}C-UBT samples with high precision. The high cost and complexity of this instrument, however, has prevented the widespread implementation of the ^{13}C-UBT. It is documented in literature that gas chromatography coupled to a quadrupole mass spectrometer (GC-MS) can also be applied to accurately measure the $^{13}CO_2/^{12}CO_2$ ratio in breath samples. However, GC-MS is not a technique that is frequently used to obtain isotope ratios of compounds with low molecular weights (e.g. CO_2). Therefore the aim of this research was to develop a GC-MS method for accurate measurement of the $^{13}CO_2/^{13}CO_2$ ratio in breath samples. Furthermore, the ^{13}C-UBT was optimized with regard to the cut-off value ($\Delta\delta$ (‰)), sampling time, sampling frequency, as well as the size of the dose of ^{13}C-urea employed in the test. The optimized ^{13}C-UBT GC-MS method, was implemented in clinical practice, and compared with the histology gold standard, to establish the diagnostic accuracy of the test. Finally, the impact of implementing the ^{13}C-UBT in South Africa was assessed.

Key Words: ^{13}C-urea breath test (^{13}C-UBT), Gas Chromatography-Mass Spectrometry (GC-MS); *Helicobacter pylori (H. pylori)*

Opsomming

Helicobacter pylori infeksie is 'n belangrike risiko faktor vir die meeste peptiese ulkus siekte toestande en 'n primêre risiko faktor vir maagkanker. Huidiglik is dit standaard praktyk om individue met ulkusse te behandel sodat die bakterië vernietig kan word. Derhalwe bestaan daar 'n behoefte aan akkurate en betroubare diagnostiese toetse om *Helicobacter pylori* infeksie te diagnoseer. Histologiese toetsing van biopsie monsters word beskou as die goud standaard vir die diagnose van 'n *Helicobacter pylori* infeksie. In die literatuur is dit egter goed gedokumenteer dat die nie-ingrypende ^{13}C-ureum asemtoets baie akkuraat is, wanneer dit vergelyk word met die histologiese toetsing van biopsie monsters. Dit is bewys dat die ^{13}C-ureum asemtoets eenvoudig, veilig, en effektief is. Boonop kan dit herhaaldelik en sonder enige risiko op dieselfde pasiënt, kinders en swanger vrouens uitgevoer word. Nadat pasiente vir *Helicobacter pylori* behandel is, word die ^{13}C-ureum asemtoets bo al die ander diagnostiese toetse verkies om te bevestig dat die bakterië vernietig is.

Die ^{13}C-ureum asemtoets is vir die eerste keer in 1987 beskryf, en sederdien is 'n groot hoeveelheid optimiseringsstudies gepubliseer. Isotoop verhouding massa spektrometrie word wêreldwyd gebruik vir die akkurate bepaling van die $^{13}CO_2/^{12}CO_2$ verhouding in ^{13}C-ureum asemtoets monsters. Die feit dat die instrument baie duur en kompleks is het egter die uitgebreide implementering van die ^{13}C-ureum asemtoets verhinder. In die literatuur is dit gedokumenteer dat 'n gas chromatograaf, gekoppel aan 'n kwadrupool massa spektrometer (GC-MS), ook gebruik kan word vir die akkurate bepaling van die $^{13}CO_2/^{12}CO_2$ verhouding in asem monsters. Die GC-MS is egter nie 'n tegniek wat op 'n gereelde basis gebruik word om die isotoop verhoudings van verbindings met lae molekulêre massas (bv. CO_2) te bepaal nie. Die doel van die navorsing was dus om die GC-MS metode te ontwikkel sodat die $^{13}CO_2/^{12}CO_2$ verhouding in asemmonsters met hoë akkuraatheid bepaal kan word. Verder, is die

^{13}C-ureum asemtoets geoptimiseer met betrekking tot die afsnypunt ($\Delta\delta$ (‰)), die tyd en die frekwensie van monster versameling, sowel as die minimum ^{13}C-ureum dosis wat aan die pasient toegedien kan word. Die geoptimiseerde ^{13}C-ureum asemtoets is in 'n kliniese praktyk toegepas, en met die histologiese bestudering van biopsie monsters vergelyk, sodat die diagnostiese akkuraatheid van die toets bepaal kon word. Laastens is die moontlike impak wat die implementering van die ^{13}C-ureum asemtoets in Suid Afrika kan hê bepaal.

Kernwoorde: ^{13}C-ureum asemtoets, Gas Chromatografie-Massa Spektrometrie (GC-MS); *Helicobacter pylori (H. pylori)*

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DIAGNOSIS OF *HELICOBACTER PYLORI* INFECTION WITH THE ^{13}C-UREA BREATH TEST: ANALYSIS BY MEANS OF GAS CHROMATOGRAPHY WITH MASS SELECTIVE DETECTION

Abstract.....i

Key Words..... ii

Opsomming.....iii

Kernwoorde.....iv

Acknowledgements.....v

Table of contents..... vi

List of figures.....xiii

List of tables.....xvii

CHAPTER 1

HELICOBACTER PYLORI – AN OVERVIEW

1.1 Discovery..... 1

1.2 Morphology..... 3

1.3 Epidemiology..... 3

1.4 Transmission.....5

1.5 Urease activity of *H. pylori*..... 6

1.6 Diseases associated with *H. pylori*..... 8

1.7 Diagnostic tests to determine the presence of *H. pylori* infection..... 11

1.7.1 Invasive testing.....12

1.7.1.1 Culture..... 12

1.7.1.2 Histology..... 12

1.7.1.3 Rapid urease test.....12

1.7.1.4 Polymerase chain reaction..... 13

1.7.2 Non-invasive testing..... 14

1.7.2.1 Serology.....14

1.7.2.2 Stool antigen testing..... 15

1.7.2.3 Urea breath test..... 16

1.7.2.3.1 ^{14}C-urea breath test..... 16

1.7.2.3.2 ^{13}C-urea breath test..... 16

1.8 Which patients should be tested for *H. pylori*? What test should be used?..... 18

1.9 Conclusion..... 21

1.10 References.....23

CHAPTER 2

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

2.1 Gas chromatography..... 45

2.1.1 Sample introduction techniques..... 46

2.1.1.1 Split injection.....47

2.1.1.2 Splitless injection..... 48

2.1.1.3 Programmed Temperature Vaporization..... 49

2.1.1.4 Multi port valves for gaseous samples.....50

2.1.2 Gas chromatography columns.....50

2.1.2.1 Optimization of column parameters..... 55

2.2 Mass spectrometry..... 56

2.2.1 Sample introduction 57

2.2.3 GC-MS interface..... 58

2.2.4 Sample ionization..... 59
2.2.4.1 Electron impact ionization..... 59
2.2.5 Mass analyzers..... 60
2.2.5.1 Quadrupole mass analyzer..... 62
2.2.6 Vacuum system..... 63
2.2.7 Ion detection..... 65
2.2.8 Recording and presentation of mass spectrometric data..... 66
2.2.9 Performance characteristics of mass analyzers..... 68
2.2.9.1 Sensitivity and efficiency..... 68
2.2.9.2 Accuracy..... 70
2.2.9.3 Mass Range..... 70
2.2.9.4 Mass resolution and mass calibration..... 70
2.2.9.4.1 Resolution..... 70
2.2.9.4.1.1 Resolving power..... 71
2.2.9.4.2 Mass calibration..... 72
2.2.9.5 Isotope dilution..... 73
2.3 Conclusion..... 74
2.4 References..... 77

CHAPTER 3

THE ^{13}C-UREA BREATH TEST

3.1 Introduction..... 79
3.2 Fasting..... 81
3.3 Test meal..... 82
3.4 ^{13}C-urea dose..... 83
3.5 Sampling time and frequency..... 84
3.6 Influence of natural occurring ^{13}C isotope on the ^{13}C-UBT..... 85

3.7 Measurement of $^{13}CO_2$.....86

3.7.1 Isotope ratio mass spectrometry.….....86

3.7.2 Infrared spectrometry.....88

3.7.3 Laser assister ratio analysis.....89

3.7.4 Gas chromatography-mass spectrometry.....91

3.7.5 Comparison of analytical techniques.....92

3.8 Expression of results.....93

3.8.1 Atom percent.....93

3.8.2 Atom percent excess.....93

3.8.3 Delta.....94

3.9 Diagnostic and analytical accuracy.....95

3.10 Cut-off Δδ values.....98

3.11 Applications.....100

3.11.1 Assessment of *H. pylori* status.....100

3.11.2 Research.....100

3.11.3 Follow up after treatment.....101

3.12 Conclusion.....102

3.13 References.....104

CHAPTER 4

EXPERIMENTAL PROCEDURES

4.1 Introduction.....115

4.2 Materials.....116

4.3 Patients.....116

4.4 Calibration standards.....117

4.5 ^{13}C-urea breath test.....117

4.6 Endoscopy.....118

4.6.1 Histology..... 118

4.6.2 Rapid urease test..... 118

4.7 Mass spectrometry..... 119

4.7.1 Gas chromatography-mass spectrometry..... 119

4.7.2 Gas chromatography-isotope ratio mass spectrometry..... 120

4.8 Gas chromatography inlet techniques..... 121

4.8.1 Six-port valve gas inlet..... 121

4.8.2 Gas-tight syringe..... 123

4.8.3 Gas tight syringe with cryogenic pre-concentration..... 123

4.9 Selection of the GC column..... 124

4.10 Validation of the ^{13}C-UBT GC-MS method..... 124

4.10.1 Analysis of $^{13}CO_2$ calibration standards..... 124

4.10.2 Repeatability of the GC-MS in analyzing patient breath samples..... 124

4.10.3 Determination of the optimal cut-off $\Delta\delta$ value..... 125

4.10.4 Determination of the $^{13}CO_2$ excretion profile..... 125

4.10.5 Stability of breath samples..... 125

4.10.6 Amount of ^{13}C-urea dose..... 125

4.10.7 Accuracy of GC-MS compared to GC-IRMS..... 126

4.11 Assessing the diagnostic accuracy of the ^{13}C-UBT GC-MS method..... 126

4.12 Investigation of the existence of the "African enigma"..... 126

4.13 Impact of implementing the ^{13}C-UBT in South Africa..... 127

4.14 Conclusion..... 129

4.15 References..... 130

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Optimization of the gas chromatography system..... 132

5.1.1 Sample introduction system..... 132

5.1.1.1 Six-port valve gas sample introduction system.....132

5.1.1.2 Manual vs. autosampler injections.....133

5.1.1.3 Split/splitless injections vs. Cooled Injection System injections..134

5.1.1.4 Maintenance of the sample introduction system.....136

5.1.2 Selection of the GC column..... 137

5.2 Selected ion monitoring GC-MS..... 139

5.3 Validation of the ^{13}C-UBT GC-MS method.....141

5.3.1 Analysis of $^{13}CO_2$ calibration standards..... 141

5.3.2 Repeatability of GC-MS in analyzing patient breath samples.....144

5.3.3 Receiver Operating Characteristic (ROC) curve analysis..... 144

5.3.4 $^{13}CO_2$ excretion profile..... 145

5.3.5 Stability of breath samples..... 146

5.3.6 Amount of ^{13}C-urea dose.....148

5.3.7 Accuracy of GC-MS compared to GC-IRMS..... 149

5.4 Assessing the diagnostic accuracy of the ^{13}C-UBT GC-MS method.....150

5.5 Investigation of the existence of the “African enigma”.....156

5.6 Impact of implementation of the ^{13}C-UBT in South Africa.....159

5.7 Conclusion..... 162

5.8 References.....164

CHAPTER 6

CONCLUSION

6.1 Optimization of the ^{13}C-UBT..... 167

6.2 Application of the ^{13}C-UBT.....168

6.3 Contribution of the ^{13}C-UBT to the medical field..... 168

6.4 Suggestions for future research.....169

APPENDIX

Appendix A.....170

List of Figures

Figure 1-1 Global distribution of *H. pylori* infection..... 4

Figure 1-2 Prevalence of *H. pylori* infection in developed and developing countries..... 4

Figure 2-1 Diagram indicating the various components of a gas chromatograph..... 45

Figure 2-2 Photo of a modern gas chromatograph: Agilent 6890 Series Gas Chromatograph..... 46

Figure 2-3 Diagram to illustrate the split/splitless injector..... 47

Figure 2-4 Diagram showing how samples are loaded and introduced into the chromatographic system using a six-port valve..... 50

Figure 2-5 Diagram of Porous Layer Open Tubular (PLOT) capillary columns..... 52

Figure 2-6 Diagram of Wall Coated Open Tubular (WCOT) capillary columns..... 53

Figure 2-7 Components of a mass spectrometer..... 57

Figure 2-8 A typical GC-MS interface for fused silica capillary gas chromatography columns. The end of the column enters the ion source of the mass spectrometer..... 58

Figure 2-9 Schematic diagram of the quadrupole mass analyser..... 62

Figure 2-10 Connection of pumps to a mass spectrometer so that samples can be analyzed under vacuum conditions..... 64

Figure 2-11 Schematic diagram of an electron multiplier and the cascade of electrons that results in a 10^6 amplification of the current in a mass spectrometer..... 66

Figure 2-12 Full scan mass spectra for hexane and CO_2, recorded at 70eV.. 67

Figure 2-13 Diagrams illustrating the “10 percent valley” (A) and “Full Width

at Half Maximum (FWHM)" (B) definitions of resolution..... 72

Figure 3-1 Graphical illustration of the ^{13}C-UBT..... 80

Figure 3-2 Michaelis Menten kinetics for urease..... 84

Figure 3-3 Diagram illustrating how the Faraday cup converts the striking ion into a current by temporarily emitting electrons creating a positive charge and the adsorption of the charge from the ion striking the detector..... 87

Figure 3-4 Typical infrared spectrophotometer..... 89

Figure 3-5 The asymmetric stretch modes of the linear CO_2 molecule..... 89

Figure 3-6 Schematic diagram of LARA system for $^{13}C/^{12}C$ isotope ratio determination in gas samples containing CO_2..... 90

Figure 3-7 Graph illustrating the definition of accuracy and precision..... 97

Figure 3-8 Receiver Operating Characteristic (ROC) curves is constructed to determine the discriminative ability of diagnostic tests..... 99

Figure 4-1 Schematic diagram of the gas inlet. The sample loop is loaded with breath sample or calibration gas (A) after which the six port valve is switched to the "INECT" position (B) so that the carrier gas can transfer the sample into the GC column..... 122

Figure 5-1 Diagram illustrating the operation of the GERSTEL Cooled Injection System (CIS)..... 134

Figure 5-2 Levey-Jennings graph illustrating the day-to-day repeatability of the $^{13}CO_2$ calibration standards when using a cryogenic pre-concentration inlet system..... 135

Figure 5-3 The influence of a poorly maintained GC inlet septum on the within batch repeatability of the $^{13}CO_2$ calibration standards..... 136

Figure 5-4 Separation of O_2 and CO_2 on the PoraPLOT QS (530 µm)..... 138

Figure 5-5 (A) Chromatogram (as supplied by the manufacturer) illustrating the ability of the GS-CarbonPLOT (320 µm) to separate CO_2

from N_2 and O_2 and other atmospheric gasses. (B) GC-MS Total Ion Chromatogram illustrating the ability of the GS-CarbonPLOT (320 µm) to separate CO_2 from N_2 and O_2 in air..... 138

Figure 5-6 Total ion chromatogram (A) and full scan mass spectrum (B) of CO_2 recorded at 70 ev..... 139

Figure 5-7 Determining the $^{13}CO_2/CO_{2\ (total)}$ ratio in breath samples using m/z = 44, m/z = 45 and m/z = 46..... 140

Figure 5-8 Levey-Jennings graph illustrating the day-to-day repeatability of the midlevel and high-level $^{13}CO_2$ calibration standards..... 143

Figure 5-9 Receiver Operating Characteristic curve for the ^{13}C-UBT. The cut-off $\Delta\delta$ with highest efficiency was achieved between 3.5 and 5.0 ‰..... 145

Figure 5-10 Typical excretion kinetic patterns after ingesting ^{13}C-urea. Negative (Patient A and B) and positive (Patient C and D) patients display similar excretion patterns..... 146

Figure 5-11 Comparison between GC-MS and GC-IRMS analysis of patient samples after being stored for one- and five weeks at room temperature. Patients with $^{13}CO_2$ enrichment values above 4.5 ‰, as obtained by GC-MS analysis, were considered to be positive for *H. pylori* infection. Histology results are indicated at the bottom of the graph (+ve = Positive and –ve = Negative)..... 147

Figure 5-12 Results of the ^{13}C-UBT when a dose of 35mg was used as the test meal, as compared to a dose of 75mg used as the test meal..... 148

Figure 5-13 Regression plot of the correlation, for $^{13}CO_2$ enrichment, between samples analyzed by GC-IRMS (x-axis) and those analyzed by GC-MS (y-axis). The vertical and horizontal lines

indicate the cut-off Δδ values for GC-IRMS (Δδ > 3.5 ‰) and GC-MS (Δδ > 4.5 ‰)..... 149

Figure 5-14 Distribution of *H. pylori* positive and negative patients as diagnosed with the ^{13}C-UBT. Part (B) of the figure focuses in on the cut-off line to illustrate that the ^{13}C-UBT can easily distinguish between *H. pylori* positive and negative patients..... 152

Figure 5-15 Box-and-Whiskers plot to illustrate the distribution of *H. pylori* positive and negative patients. The mean, median and interquartile range for the negative subjects were 0.60, 0.20 and -0.80 to 1.70 and for the positive subjects were 26.2, 21.0 and 12.1 to 34.4 respectively..... 155

Figure 5-16 Guidelines for the management of dyspepsia..... 158

Figure 5-17 Decision tree of the “test and treat” and endoscopy strategies. The probability of an event occurring is given above branches and the cost associated with it below..... 160

List of Tables

Table 1-1 Indications for diagnosis and treatment of *H. pylori*..... 19

Table 2-1 Application fields of different adsorbents..... 53

Table 2-2 Commonly used gas chromatography stationary phases for WCOT columns..... 53

Table 2-3 Comparison of the performance factors of the different mass analyzers. Ionization modes, resolving power, mass accuracy, mass-to-charge range, and scan speed are important factors qualifying these techniques for various applications..... 61

Table 2-4 Relationship between vacuum conditions and distance of the mean free path..... 65

Table 3-1 Substrates of ^{13}C-breath tests for potential use in routine clinical applications and their diagnostic and metabolic implications..... 80

Table 3-2 Calculation of the diagnostic accuracy of an analytical test…….. 97

Table 5-1 Precision of ^{13}C enrichment measurements of the CIL $^{13}CO_2$ calibration standards using a six-port valve gas inlet..... 132

Table 5-2 Analysis of CIL $^{13}CO_2$ calibration standards using the 100 µL GERSTEL gas tight syringe both manually and with the autosampler..... 133

Table 5-3 Comparison of CIL $^{13}CO_2$ calibration standards by injecting into the CIS and split/splitless inlet..... 135

Table 5-4 GC-MS analyses of CIL $^{13}CO_2$ calibration standards..... 142

Table 5-5 $^{13}CO_2$ enrichment values of certified $^{13}CO_2$ calibration standards, obtained by analysis with IRMS and GC-MS. Samples were analysed in triplicate over a period of 20 days..... 143

Table 5-6 Sensitivities and specificities obtained with the use of different cut-

off $\Delta\delta$ values for the ^{13}C-UBT..... 145

Table 5-7 Regression statistics of the correlation, for $^{13}CO_2$ enrichment, between samples analyzed by GC-IRMS and those analyzed by GC-MS..... 150

Table 5-8 Sensitivities and Specificities of the ^{13}C-UBT with reference to histology "gold standard"..... 151

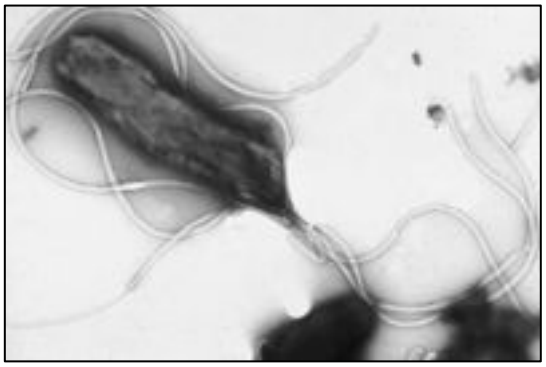
Table 5-9 Descriptive statistics of subjects stratified by age, gender and race 157

Table 5-10 Sensitivity analysis based on multiple regression of the cost and probability inputs used in the Monte Carlo simulation..... 161

CHAPTER 1

HELICOBACTER PYLORI: AN OVERVIEW

Helicobacter Pylori - *"Spiral rod of the lower part of the stomach"*



1.1 Discovery

While the complications of peptic ulcer disease are mentioned in records of ancient and medieval medicine, the first description of gastric ulceration was only reported in 1586 by an Italian physician. In 1688, a report of duodenal ulceration was documented. This was followed by a report of erythema and erosions of the stomach and duodenum, in patients with heartburn and upper abdominal pain, in 1761.[1] The understanding and function of the upper gastrointestinal tract gradually increased during the eighteenth and nineteenth centuries, and the symptomatology of peptic ulceration was first described in detail in 1857.[2] At this time gastric ulceration was a common autopsy finding, especially in females, while duodenal ulceration was rarely reported. The prevalence of duodenal ulceration began to increase dramatically in young and middle-aged males, and by 1900, was more common than gastric ulceration.

Until 1983, various substances were used for the treatment of peptic ulcer disease. Relapse of ulceration after cessation of treatment, however, was frequent, which resulted in the need for long-term maintenance treatment.

It was only after the association between peptic ulceration and the *Helicobacter pylori* bacterium was suggested by Marshall et al,[3,4] that the scientific community came to the realization that this organism needs to be eradicated to enhance the long-term management of peptic disease. The acute effects of the infection were illustrated after deliberate consumption of a suspension of the organisms.[5-7]

Prior to 1983 this "Campylobacter-like organism" were called *Campylobacter pyloridis* since they were micro-aerobic, curved, Gram-negative bacteria and resembled other *Campylobacters* both morphologically and in guanine/cytosine DNA content. The presence of multiple flagellae on these bacteria, differentiating them from other *Campylobacters*, was also noted.

For grammatical reasons, the name was changed to *Campylobacter pylori (C. pylori*) in 1987. [8] It was shown that the *C. pylori* did not belong to the genus *Campylobacter* in 1989 and a new genus name, *Helicobacter pylori*, was suggested.[9] "*Helicobacter*" reflects the two morphological appearances of the organism, helical in vivo but often rod-like in vitro ("bacter", a staff). *Helicobacter pylori* is Latin for "spiral rod of the lower part of the stomach".

The discovery of *H. pylori* and the gradual realization of its importance in upper gastrointestinal diseases represent one of the most important developments in medicine in the past century. It is now possible to cure hitherto chronic diseases with short, safe antibiotic-based treatments. It has also given rise to the potential to prevent a variety of gastroduodenal conditions. Whether this will be done by community based screening programmes or vaccination should become clear in the next decade.

1.2 Morphology

H. pylori organisms are spiral, micro-aerophillic, gram-negative bacteria, that demonstrate bluntly round ends in gastric biopsy specimens. They are 2.5 to 5.0 µm long and 0.5 to 1.0 µm wide.[10]

They resemble the *Campylobacters* morphologically, and in respect of atmospheric requirements and DNA base composition, but their flagellar morphology is not that of the genus *Campylobacter*. *Campylobacters* have a single unsheathed flagellum at one or both ends of the cell, whereas *H. pylori* has four to six unipolar sheathed flagella.[3,10] Each flagella is approximately 30 µm long and 2.5 nm thick. The flagella exhibit a characteristic terminal bulb, which is an extension of the flagellar sheath.

Growth requirements for *H. pylori* include nutrient rich media, an atmosphere enriched in CO_2, high humidity (96 – 100%) and a pH near 7.0.

The bacterium can be characterized in the laboratory using its oxidase, catalase and strong urease positive activity, nitrate reduction, H_2S production in triple sugar iron agar and resistance to nalidixic acid and susceptibility to cephalothin and metronidazole.[9] The strong urease activity and unique carbohydrate profiles also helps to differentiate between *H. pylori* and the genus *Campylobacter*.[11]

1.3 Epidemiology

H. pylori are found in the stomachs of humans in all parts of the world. By some estimates, over one half of the world is infected with this organism (Figure 1-1).[12] It is commonly isolated from non-human primates as well.[10,13]

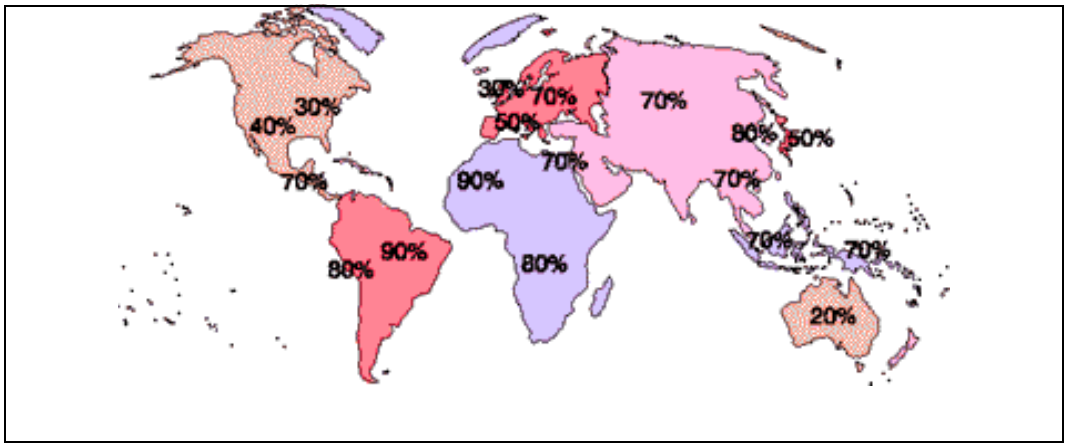


Figure 1-1: Global distribution of H. pylori infection.

The incidence of *H. pylori* infection has been declining with the changes concomitant with industrial development. This will explain why in developing countries, 70 to 90 % of the population carries *H. pylori*, while in developed countries the prevalence of infection ranges from 25 to 50 % (Figure 1-2).[10,13]

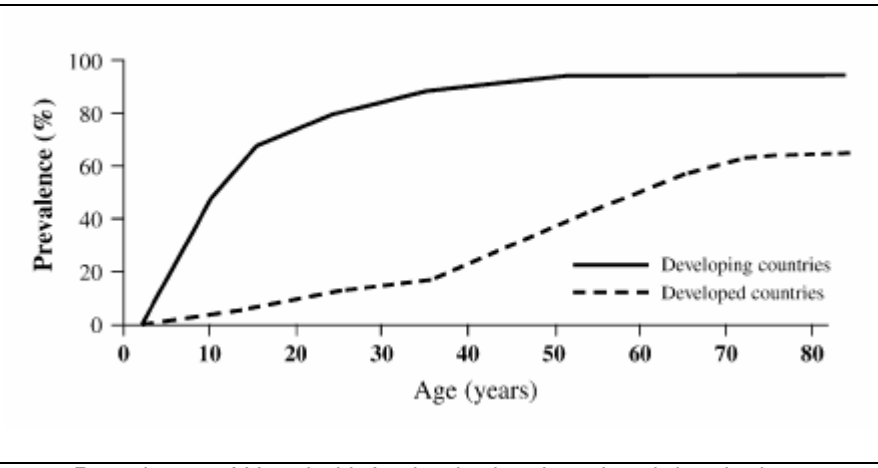


Figure 1-2: Prevalence of H. pylori infection in developed and developing countries.[14]

Although research show that persons with higher socio-economic status have lower infection rates, a study by Mendall et. al. suggested that *H. pylori* infection is more closely associated with childhood living conditions than with current socio-economic status.[15]

Infection is almost always acquired before the age of ten, and once established in the gastric mucosa, the bacterium may persist for life if not treated. It appears though, that in the early years of life, spontaneous clearance of infection might occur.[13] The incidence of *H. pylori* increases with age worldwide. This may possibly be due to a cohort effect with acquisition of *H. pylori* during childhood followed by life-long infection, or due to ongoing acquisition at all ages.[16]

In a study by Graham et. al.[17] it was found that the prevalence of infection was twice as great in blacks than in whites. Males and females, though, are infected at approximately the same rates.[10,13]

1.4 Transmission

H. pylori is mainly harboured in the stomachs of humans. It has been proposed that the acquisition of *H. pylori* occurs via a common environmental source.

Although some groups have successfully isolated *H. pylori* from rhesus monkeys,[18,19] sheep[20-22] and cats,[23] it is still questionable whether animals are important reservoirs for *H. pylori*. Water has also been implicated as a potential source for *H. pylori* infection,[24,25] but findings are controversial.[13]

Since attempts to consistently isolate *H. pylori* from reservoirs other than humans has failed, it has been suggested that direct person-to-person contact is the most likely mode of transmission, and three routes have been described.

Firstly, it might be possible that the bacterium can be transferred on tubes, endoscopes, or specimens in contact with the gastric mucosa of an infected person to another person.[26] As reported by Fantry et al,[27] the incidence of infection with *H. pylori*

through iatrogenic transmission greatly decreases with improved disinfection of endoscopes. Occupational acquired infections have also been reported[28] and universal precautions should be taken when specimens are handled.[10] This method of transmission is the least common.

Secondly, and perhaps most importantly, a faecal-oral mode of transmission has been proposed. Although not common, *H. pylori* has been isolated in the faeces of young children.[29,30] Feacally contaminated water and food may therefore be sources of infection.[10,31]

Thirdly, oral-oral transmission has been identified in the case of African women who premasticate foods given to their infants.[32] *H. pylori* has been detected in dental plague and saliva, but the recovery of *H. pylori* in the mouth is probably intermittent, associated with gastroesophageal reflux, but not with specific oral disease.[33] Olivier et al substantiated that *H. pylori* organisms could not be detected in either dental plague or saliva in all confirmed *H. pylori* positive patients.[34]

Many studies have shown that the prevalence of *H. pylori* infection is significantly higher in families where some of the members, whether it is the children or the parents, are infected.[35-37] There is no identified association of infection with sexual transmission or aspiration of *H. pylori* from vomitus.[10]

1.5 Urease activity of *H. pylori*

In 1924, the presence of urease activity in the human stomach was documented for the first time.[1] It was thought that this urease activity originated in gastric mucosal cells and was not associated with the presence of bacteria. It was only in 1968 that the bacterial source of urease was confirmed when the absence of urease activity was

demonstrated in the stomachs of germ-free animals.[38] Furthermore, urease is not present in mammalian cells; consequently the presence of urease enzyme in the stomach is proof that bacteria are present.[39]

In a study by Dunn et al[40] it was concluded that the *H. pylori* urease enzyme has a molecular mass of 380 ± 30 kDa in crude extracts, and a molecular mass of 680 ± 50 kDa in purified urease extracts, which suggests that aggregation of the protein is taking place. It is a nickel containing, hexameric molecule, consisting of two subunits UreA (30,0 ± 1,0 kDA) and UreB (62,0 ± 2,0 kDa), in a 1:1 molar ratio. Purified urease has a V_{max} of 1100 ± 200 µmol/min/mg of protein and a low Michaelis constant (K_m = 0.3 ± 0.1 mM) which permits this enzyme to be catalytically efficient even at submillimolar concentrations of urea. The isoelectric point (pI) was determined to be 5,99 ± 0.03 in both crude and purified preparations of urease. The amino acid sequence of urease from *H. pylori* shows similarities to that of ureases found in plants (Jack beans) and other bacteria (*Proteus mirabilis* and *Klebsiella aerogenes*).[41-43]

The overall consensus is that urease is located on the surface of *H. pylori*. In a study by Phadnis et al,[44] it was observed that urease is located strictly in the cytoplasm of early log phase cultures of *H. pylori.* However, in late log phase cultures, urease become associated with the bacterial surface in a novel manner: urease and other cytoplasmic proteins are released from the cytoplasm by bacterial autolysis and become adsorbed to the surface of intact bacteria due to the unique characteristics of the outer membrane. The association between urease and the bacterial surface is apparently stabilized by divalent cations such as Ca^{2+} and Mg^{2+}, although other cations can inhibit the activity of the enzyme.[45]

In the absence of urease, *H. pylori* is sensitive to the acidic pH of the stomach. Urease activity is an important colonization factor by generating ammonia in the immediate

bacterial micro-environment, thus protecting *H. pylori* from the deleterious effects of gastric acid. The urease activity is also associated with virulence.[40,45] The high concentrations of ammonia may exert a direct toxic effect upon intercellular junctions, resulting in alteration of gastric mucosal permeability. [11,46,47] Possession of glutamate dehydrogenase activity permits urease to also serve a nutritional function by promoting the uptake of available urea nitrogen by *H. pylori*.[48]

H. pylori's potent urease activity forms the basis for rapid detection of the bacteria in gastric biopsy specimens. In addition, measurement of $^{13}CO_2$ or $^{14}CO_2$ excretion in urea breath test samples can give an indication whether or not the patient is actively infected with the bacterium.

1.6 Diseases associated with *H. pylori*

H. pylori is associated with many of the most important diseases involving gastroduodenal tissue.

In 1984, after the discovery of *H. pylori,* it was clear that the bacterium is strongly associated with both chronic inflammation in the gastric mucosa (chronic superficial gastritis), as well as gastric mucosal inflammation consisting of a polymorphonuclear cell infiltrate (chronic active gastritis).[5,7,10] In addition to chronic active gastritis, Marshall and Warren noted that *H. pylori* was also associated with duodenal and gastric ulceration.[3] It is worthy to note, however, that most individuals infected with *H. pylori* will never suffer any symptoms related to the infection.

A multitude of studies suggested that the eradication of the bacterium was followed by a significant reduction in ulcer relapse rates.[49-52] In 1994, at a consensus conference convened by the National Institutes of Health to evaluate the role of *H. pylori* in peptic

ulcer disease, it was concluded that *H. pylori* is a major cause of peptic ulcer disease, and they recommended that infected individuals with ulcers be treated to eradicate the organism.[10,53] Antibiotic-based regimens are now the recommended treatment for peptic ulceration. In the foreseeable future, it may be possible to offer prophylaxis against these conditions by means of an oral vaccine.

The mechanism by which *H. pylori* induces peptic ulcer disease is not completely understood but most likely involves a combination of genetic predisposition of the host, virulence factors of the organism (e.g. VacA and CagA proteins), mechanical damage to the mucosa, and alterations of the gastric and duodenal secretions.[54] The high levels of ammonium ions that are produced by the characteristic strong urease activity are toxic to the gastric epithelial cells. It has been suggested though, that urease is only a virulence factor and not the cause of the inflammatory response since animals infected with other spiral bacteria that are urease positive do not manifest such damage.[55] VacA, a vacuolating cytotoxin produced by *H. pylori*, is responsible for massive vacuolization in mammalian cell lines as well as in gastric epithelia of patients with chronic gastritis.[56] Clinical reports have indicated that patients suffering from duodenal ulcers or gastric cancer show a much higher probability of harbouring cytotoxin producer strains as compared to only a fraction of those patients suffering only from gastritis.[57] This supports the vacuolating cytotoxin factor as a potential mechanism for gastritis.

Many reports have now shown associations between *H. pylori* infection and the presence,[58] or the development[59-62] of gastric cancer. The typical course of disease in infected patients begins with chronic superficial gastritis, eventually progressing to atrophic gastritis. This progression appears to be a key event in the cellular cascade that results in the development of gastric carcinoma. It is not surprising then that *H. pylori* carriage is also associated with adenocarcinoma of the distal stomach. Infection

is associated with both the intestinal and diffuse histologic types of tumours. This association is important since, in total, gastric cancer is the second leading cause of cancer death in the world.[10,12] In 1994 the International Agency for Cancer Research, an arm of the World Health Organization, declared *H. pylori* a grade I (definite) carcinogen of humans.[63]

H. pylori infection has been associated with the development of gastric non-Hodgkin's lymphomas,[64] with another lymphoproliferative disorder, gastric mucosa-associated lymphoid tissue (MALT) lymphoma (MALToma),[65,66] and with Ménétrièr's disease, a rare gastric disease in which the gastric folds are hypertrophic.[10]

Nonulcer dyspepsia has been linked to *H. pylori* infection, however, infection with this bacterium is only a single part of the multifactorial aetiology of the disease.[67] Some other controversial associations have also been made between *H. pylori* infection and cardiovascular disease,[68] gastroesophageal reflux disease,[69,70] and iron deficiency anaemia.[71]

In Africa and other developing countries, a discrepancy exists between the occurrence of *H. pylori* infection and the expression of clinically significant disease – the "African enigma". This has led some to postulate that, apart from gastritis, this bacterium does not play a major role in the aetiology of gastric cancer or any other upper gastrointestinal disease.[72-75] The evidence for these views was derived largely from sero-epidemiological studies in asymptomatic individuals combined with anecdotal evidence about the prevalence of various disease entities.[72-74,76,77] The differences in the clinical consequences of *H. pylori* infection in developed and developing countries might be due to factors such as the pattern of infection, age of acquisition, environmental, dietary, and host and bacterial genetic influences.[72,78,79] The very low incidence of gastric cancer in Africa may have more to do with the average life

expectancy of 40 years in many parts of sub-Saharan Africa than with a possible protective effect provided by *H. pylori* infection.

Conversely, the "African Enigma" has recently been investigated in a review of prospective endoscopic studies on African populations.[80] Based on 40 studies the authors rejected the existence of this enigma and concluded that the prevalence of peptic ulcer disease in patients infected with *H. pylori* is similar to that in the developed world. Evidently, further research into the "African enigma" is needed to resolve this position.

1.7 Diagnostic tests to determine the presence of *H. pylori* infection

Since *H. pylori* is involved in several clinical conditions, the demand for treatment of the infection, and thereby also the interest for reliable diagnostic tests have increased tremendously. The choice of test depends on issues such as cost, availability, clinical situation, population prevalence of infection, pre-test probability of infection, and factors such as the use of proton pump inhibitors and antibiotics, which may influence the test results.

Tests for diagnosing *H. pylori* infection can be categorized as either invasive or non-invasive. Invasive tests involve all those in which gastric biopsies are taken and include culture, histology, rapid urease test, and polymerase chain reaction (PCR) of biopsy samples. Non-invasive tests include serology, ^{13}C- and ^{14}C-urea breath tests, stool antigen assays, and stool -, dental plague -, and saliva PCR assays.

1.7.1 Invasive testing

1.7.1.1 Culture

H. pylori can be cultured in a variety of selective and non-selective commercially available media.[10,13] Criteria for culture should be followed exactly as sensitivity can be compromised by sampling problems, delayed transport of biopsy samples, exposure to air, and suboptimal laboratory conditions.[81] To culture *H. pylori* is complicated, time-consuming and costly, and requires trained and motivated personnel. It is the only method though, which allows for antimicrobial susceptibility testing, the study of growth factors and metabolism of the organism.[1]

1.7.1.2 Histology

Histological examination of biopsy samples is accepted all over the world as the "gold standard" for diagnosing *H. pylori* infection. Accept for definitive diagnosis of infection, it also allows for the determination of the degree of inflammation or metaplasia, and the presence/absence of MALT-lymphoma or other gastric cancers in high-risk patients.[67,81] Limitations can arise if an inadequate number of biopsy specimens are obtained, or if there is failure to obtain specimens from different areas of the stomach. Processing times and costs depend on the staining technique used.[67]

1.7.1.3 Rapid urease test

The rapid urease test is based on the fact that *H. pylori* produces a large amount of urease. Urease is not present in mammalian cells under normal circumstances; therefore the urease in the stomach is almost always due to the presence of *H. pylori* organisms.[38,39] Swallowed urease from mouth bacteria is denatured below pH 4.0, so it is quickly destroyed by gastric acid and cannot interfere with the test.[82] When urease

is produced beneath the gastric mucus layer, as in the case of *H. pylori* infection, it is protected from luminal acid and remains fully active. A biopsy specimen is introduced into a medium containing urea. If present, the urease in the specimen will split the urea into ammonia and carbon dioxide. The ammonia released results in an increase in pH, which is detected by a colour change in a pH indicator. Commercially available tests e.g. CLOtest (developed by Marshall), Hpfast, and PyloriTek, all operate on this principle. The sensitivity of the test depends on the bacterial load in the stomach.[10,81] The test is inexpensive, fast, widely available, accurate and easy to perform.[83] If the test is read after 24h, or if other urease-producing bacteria are present in the stomach, false positive results will occur.[81,84] Alternatively, antibiotic agents, bismuth compounds, proton pump inhibitors, or bile reflux can cause decreased urease activity, which will result in false negative results.[13]

1.7.1.4 Polymerase chain reaction

With the polymerase chain reaction (PCR), *H. pylori* can be identified in small samples with only a few bacteria present.[67] DNA primers are directed against specific target sequences in the *H. pylori* genome. The primers, together with the enzyme *Taq* polymerase and DNA nucleotides are added to double stranded DNA. The reaction mixture goes through a series of temperature changes to allow melting, annealing and polymerase copying of the original DNA. If the target sequence is present in the sample tissue, *H. pylori* DNA will be amplified 2^{30} times after 30 cycles.[85] PCR assays have mostly been based on DNA fragments from the urease A (*ureA*)[86], urease C (*ureC*)[87], 16S rRNA[88], and phosphoglucosamine mutase (*glmM*)[89] genes, all of which show high sensitivities and specificities. PCR allows typing of the strains by performing restriction fragment length polymorphisms on the amplified DNA fragments.[13,83] Limitations of the test is that only few laboratories have the capability to run the test, and test accuracy is easily affected by the choice of primers and target

DNA, specimen preparation, bacterial density, and technical issues regarding the PCR procedure.[10]

1.7.2 Non-invasive testing

1.7.2.1 Serology

Serology tests are based on the fact that infection of the gastric mucosa with *H. pylori* results in systemic as well as local immune responses, which include elevation of specific IgG and IgA levels in serum and elevated levels of secretory IgG and IgM in the stomach.[10,90,91] The utility of these tests for the detection of *H. pylori* specific antibodies is dependent on the antigen preparation used.[10,92,93] A wide variety of serological methods for the detection of *H. pylori* have been described in the literature.[13,94] Most tests available commercially are enzyme-linked immunosorbent assays (ELISA).[85,86] Serology tests can also be used for "office-based" or "near-patient" testing, where either serum or fingerstick whole blood are used as samples. They are, however, generally less reliable and are not recommended for clinical use.[93,94] Although serology tests are fast, simple, and relatively inexpensive, it is important to note that none of the tests for IgA and IgM to diagnose *H. pylori* infection have been approved by the U.S. Food and Drug Administration. The sensitivity and specificity of these tests have generally been too low for them to be recommended either alone, or in combination with an IgG test.[13] Non-specific cross-reactions of the antigen preparations with other organisms[90] and IgG antibodies that tend to persist for months after eradication of *H. pylori,*[13] will contribute to false positive results. The lower positive predictive value with serological testing has led to concerns that antibiotics may be administered unnecessarily after serology testing. However, this is the traditional view in Western countries and it is not entirely applicable in countries with a high *H. pylori* prevalence. In a low-prevalence area, serology works less well, so

that a negative test has more value than a positive test. In a high-prevalence area, a positive serology test may be acceptable. It is proposed that antibody tests should be used only as screening tests, and that a positive or negative result, depending on the state of the patient being examined, should be confirmed.[10]

1.7.2.2 Stool antigen testing

Determining the presence of *H. pylori* infection from stool samples is relatively new methodology. Most commonly, *H. pylori* infection is identified by detecting *H. pylori* protein antigens in stool (HpSA); however, *H. pylori* organisms can also be cultured, or detected with PCR assays. In vitro qualitative enzyme immunoassay kits (Premier Platinum HpSA and FemtoLab *H. pylori*) are available for the detection of the protein antigens in stool.[95-104] Both tests show comparable sensitivities and specificities to the urea breath test and biopsy based methods; however, the discrimination between positive and negative results was enhanced for the FemtoLab test.[104] The stool antigen test (SAT) also showed to be of great value in the diagnosis of *H. pylori* infection in children because it is easy to perform, reliable and noninvasive.[98,99,105] Further studies are necessary to determine whether the SAT can be performed on patients with diarrhoea or severe constipation, and to evaluate whether the test is accurate in patients with subtotal gastrectomy, where urea breath test seems to be less precise. Local test validation is important since appropriate cut-off points are still a matter of debate.[95] Several publications have stated that the SAT might be useful for the diagnosis of *H. pylori* infection ≥ 4 weeks after the end of eradication therapy.[95,96,106-108] In contrast, it has also been reported that at 6 weeks and at 6 months post-treatment, the SAT lacks accuracy as compared to the urea breath test for evaluating the outcome of the eradication treatment.[100]

H. pylori culture from stool has proven to be difficult, with a low yield.[13] The bacterium is known to be sensitive to bile, and as bile is present in the duodenum and the colon, the organism may not survive transit through the alimentary tract in association with the feces.[106]

The sensitivity of PCR makes it especially suitable for diagnosis when the organism is present in low numbers. A major drawback of this procedure is the high rate of false negative results caused by inhibition factors (complex polysaccharides probably of plant origin in the diet) that is present in stool.[13,106] Several procedures have been implemented to overcome this inhibition,[106,109] however, care must be taken not to compromise sensitivity.

1.7.2.3 Urea breath test

Similar to the rapid urease test, the urea breath test (UBT) is based on the presence of urease produced by *H. pylori*. In order to detect gastric urease, the patient swallows either ^{14}C- or ^{13}C- labelled urea. If urease (*H. pylori*) is present, urea is split into $^{13}CO_2$ or $^{14}CO_2$ and NH_4^+ at the interface between the gastric epithelium and lumen. Ammonium ions are sequestered in the gastric acid, and isotope labelled CO_2 enters the bloodstream where it is carried to the lungs and rapidly expired.

1.7.2.3.1 ^{14}C-Urea breath test

For the ^{14}C-UBT, an exact amount of expired CO_2 (usually 1 or 2 mmol) is collected in a solution consisting of hyamine hydroxide, a carbon dioxide trapping agent, dissolved in either ethanol or methanol, with thymolphthalein added as a pH indicator.[110-114] A drying chamber can also be incorporated into the breath collection tubing.[115-118] Samples are measured on a liquid scintillation counter that is readily available in major

medical centers.[114,116] According to early reports it is less costly and even more sensitive than the ^{13}C-UBT,[110,111] however, although the radiation dose is relatively low,[119,120] it remains unsuitable for children and pregnant females.[10,13,121]

1.7.2.3.2 ^{13}C-Urea breath test

Since the first ^{13}C-UBT was described by Graham et al in 1987,[122] many adaptations have been made to simplify and optimise the test, but the basic principle stayed the same.[123-133] After an overnight fast, and baseline breath sample, 75 to 200 mg of ^{13}C-urea is given to patients together with a test meal. Breath sampling is done at various time intervals and no further sample preparation is needed. The amount of labelled CO_2 in breath samples, liberated as a result of the urease activity in the stomach, is quantitated as an increase in the natural $^{13}CO_2/^{12}CO_2$ ratio. The volume of breath collected is therefore irrelevant.[133,134] The UBT has a very high sensitivity and specificity compared to the other non-invasive tests,[134-136] and it is the only non-invasive test that can detect active *H. pylori* infection.[137] It is the preferred test to determine if treatment was successful in eradicating the organism.[125,128,129,133,135,138,139] To avoid false negative results, it is recommended to wait at least 4 weeks after treatment before performing the UBT. Unlike serology, the UBT is very accurate in children.[140-142] The amount of false positive results in children can be reduced by lowering the cut-off value.[137,143,144] For the assessment of eradication in children, a waiting period of at least 6 weeks is recommended for best results.[139]

Carbon-13 (^{13}C) comprises only 1.1 % of all carbon. In the case of the ^{13}C–UBT in the diagnosis of *H. pylori*, a change of less than 8 ‰ needs to be detected reproducibly in the $^{13}CO_2$ concentration. This is complicated by the presence of bulk $^{12}CO_2$. Determining the $^{13}CO_2/^{12}CO_2$ ratio in gasses by isotope dilution mass spectrometry can be potentially very difficult, due to the high precision that is required. Gas

chromatography coupled to an isotope ratio mass spectrometer (GC-IRMS) is trusted worldwide to accurately analyse the $^{13}CO_2/^{12}CO_2$ ratio in breath samples. Other techniques based on infrared spectroscopy, laser optogalvanic spectroscopy and gas chromatography with mass selective detection have been suggested as cheaper and more economic alternatives.

1.8 Which patients should be tested for *H. pylori*? What test should be used?

The issue of which patients to test, when to test them, and what test to use is still debatable. Diagnostic procedures feasible in one part of the world is not necessarily so in another. Factors such as patient preference, cost, availability of different tests, and positive and negative predictive values of the different tests (which depend on the individual patient population, including the prevalence of disorders caused by *H. pylori* infection in the community) needs to be taken into consideration. Furthermore, testing for *H. pylori* should be performed only if the clinician plans to offer treatment for positive results. No single test can be considered the gold standard for the diagnosis of *H. pylori* infection, therefore, deciding which test to use in which situation relies heavily upon whether a patient requires evaluation with upper endoscopy and an understanding of the strengths and weaknesses, and costs of the individual tests.

Although the majority of people infected remain clinically silent, there are a number of well-established clinical conditions that have been associated with *H. pylori* infection. One of the problems with diagnosing *H. pylori* is that there are several conditions that can be responsible for generating such symptoms. A rigorous process of identification and exclusion is required. Testing for *H. pylori* infection is indicated in patients with active peptic ulcer disease, a past history of documented peptic ulcer, or gastric MALT lymphoma. The "test-and-treat" strategy for *H. pylori* infection is a proven management strategy for patients with uninvestigated dyspepsia who are under the age of 55 and

have no “alarm features” (bleeding, anemia, early satiety, unexplained weight loss, progressive dysphagia, odynophagia, recurrent vomiting, family history of GI cancer, previous esophagogastric malignancy).[145-149] The indications for the diagnosis and treatment of *H. pylori* infection are summarized in Table 1-1.[145]

Table 1-1: Indications for diagnosis and treatment of H. pylori

Established
• Active peptic ulcer disease (gastric or duodenal ulcer) • Confirmed history of peptic ulcer disease (not previously treated for *H. pylori*) • Gastric MALT lymphoma (low grade) • After endoscopic resection of early gastric cancer • Uninvestigated dyspepsia (depending upon *H. pylori* prevalence)
Controversial
• Nonulcer dyspepsia • Gastroesophageal reflux disease • Persons using nonsteroidal anti-inflammatory drugs • Unexplained iron deficiency anemia • Populations at higher risk for gastric cancer

Non-invasive “test-and-treat” strategies have to be balanced with clinical factors and an estimate of the possible cancer risk in each patient. A non-invasive "test-and treat" strategy in patients with uninvestigated dyspepsia is supported by both the American Gastroenterological Association[150] and the American College of Gastroenterology.[151] The National Institute for Clinical Excellence (NICE) has issued similar guidelines that recommends that all dyspeptic patients without alarm symptoms, irrespective of age, should be managed initially with a full dose PPI therapy for 1 month. If patients relapse, NICE suggests that they should be offered "test-and treat" for *H. pylori*. "Test-and treat" is more effective than antacids at reducing dyspeptic symptoms, cures those patients with peptic ulceration and reduces the need for endoscopy, providing significant cost savings without increasing the risk of missing neoplasia.[152] It is common consensus that, for the "test-and–treat" approach to be effective, patients need to be fully informed, reassured and participate in their health care. It is recommended that the non-invasive UBT and SAT should be used for the diagnosis of *H. pylori* infection. When the prevalence of *H. pylori* infection in the population of interest is high, e.g South Africa, negative results should be viewed with caution. The

UBT with it's high sensitivity and specificity then becomes particularly useful to diagnose *H. pylori* infection. The PPV of serological testing is reasonably good in regions where the prevalence of *H. pylori* is high, and therefore also provides an acceptable means of screening for *H. pylori* infection. Serology should especially be considered in situations where other diagnostic tests tend to give false negative results, such as in patients with bleeding ulcers, gastric atrophy or MALT lymphoma, or in the case of recent or current use of PPI's and antibiotics.[148]

For patients aged 55 and older, endoscopy to look for an upper gastrointestinal malignancy and testing for *H. pylori* infection if a malignancy is not found is the logical approach. Endoscopic testing should be carried out in younger patients in countries with a high risk of gastric cancer. In developing countries where ulcer rates or cancer rates are high, an empirical "test-and-treat" approach or endoscopy would be more appropriate than starting treatment with a PPI.[146] If a patient's clinical presentation requires the need for endoscopy (as indicated above), biopsy based diagnostic tests are the most appropriate. The RUT offers the desirable combination of accuracy and low cost, provided that the patient have not been on recent PPI's, antibiotics or bismuth preparations. If any mucosal abnormalities were identified during endoscopy, it is recommended that biopsy specimens be retained for histological examination. For patients who have been taking PPI's, antibiotics or bismuth preparations, endoscopic testing should include histological examination of biopsy specimens, with or without the RUT, or the patient should be referred to do a UBT or SAT at a later stage after withholding medication for at least two weeks. In the case of an active ulcer bleed, negative RUT or histology results should be confirmed with either the UBT or SAT. With all biopsy based tests it is important to note that, as the prevalence and density of *H. pylori* varies throughout the stomach, particularly in the face of medications that may reduce the density of *H. pylori*, multiple biopsies are needed for accurate diagnosis.[153]

This is the case particularly in high prevalence countries, where inadequate sampling can cause the rate of false negative results to be very high.

When confirmation of eradication is necessary, testing should be performed no sooner that 4 weeks after the completion of eradication therapy. Because of its high cost, endoscopic tests should only be used if endoscopy is clinically indicated for other reasons. In such case, histology, or a combination of histology with RUT, is recommended. When endoscopic follow-up is unnecessary, testing to prove successful eradication of *H. pylori* is best accomplished with the UBT.[145,148,154]

1.9 Conclusion

H. pylori infects approximately one half of the world's population, with infection being almost ubiquitous in Africa and some other developing countries. Infection with the bacterium occurs predominantly in childhood. *H. pylori* is the cause of most peptic ulcer disease and a primary risk factor for gastric cancer. The global burden of gastric cancer is considerable but varies geographically, with the incidence in Africa being relatively low. Eradication of the bacterium results in ulcer healing and has the potential to reduce the risk of gastric cancer development.

Management of *H. pylori* infection must be done with thorough investigation into the clinical symptoms and social circumstances of patients since widespread testing and treatment is neither justified nor cost-effective. It is recommended that endoscopy be reserved for use in patients with alarm symptoms for ulcer complications and gastric cancer, or those who do not respond to treatment. Research suggests that the "test-and-treat" strategy before endoscopy referral is cost-effective, although it will take time before the benefits to become evident. The effectiveness of a "test-and-treat" strategy appears to be the same as for endoscopy management.

The RUT and/or histological examination of gastric biopsy specimens are accurate and cost-effective tests to diagnose *H. pylori* infection when endoscopy is required. In the "test-and treat" approach, however, it is recommended that the highly accurate and non-invasive ^{13}C-UBT or SAT be used as diagnostic tests. Serological testing is feasible in high prevalence populations or in situations where other diagnostic tests tend to give false negative results.

H. pylori infection can be eradicated by antimicrobial therapy, but no treatment regimen is 100 % effective. After completion of treatment, is must be determined whether or not the bacterium has been successfully eradicated. Histology, serology (provided that a sufficient time interval was allowed after cessation of treatment) and the ^{13}C-UBT are accurate enough to be considered as follow up tests.

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CHAPTER 2

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

2.1 Gas Chromatography

"Chromatography is one of the most truly chemical methods for manipulating and understanding the natural world. It is an enabling technology for a wide range of products and discoveries, including those of more than a half-dozen Nobel Prize winners." – ***Mark S Lesney***

Gas chromatography is the principle method of analysis for thermally stable and volatile organic compounds.[1] Instrumentation for gas chromatography comprises of well-defined components, each of which contributes to the overall chromatographic performance. A basic gas chromatograph consists of an *injector* for introducing the sample onto the column; a *chromatographic column* (which contains the stationary phase) to separate the analytes being measured; a column *oven* to heat the column; an online *detector* to detect the separated analytes as they elute from the column; a *gas reservoir* and *flow control apparatus* to force the carrier gas (mobile phase) through the injector, column, and detector; and a *data recording and processing device* to capture and process the data generated by the detector. Figure 2-1 shows the arrangement of these components in a block diagram. This configuration is more or less standard for all gas chromatographs.

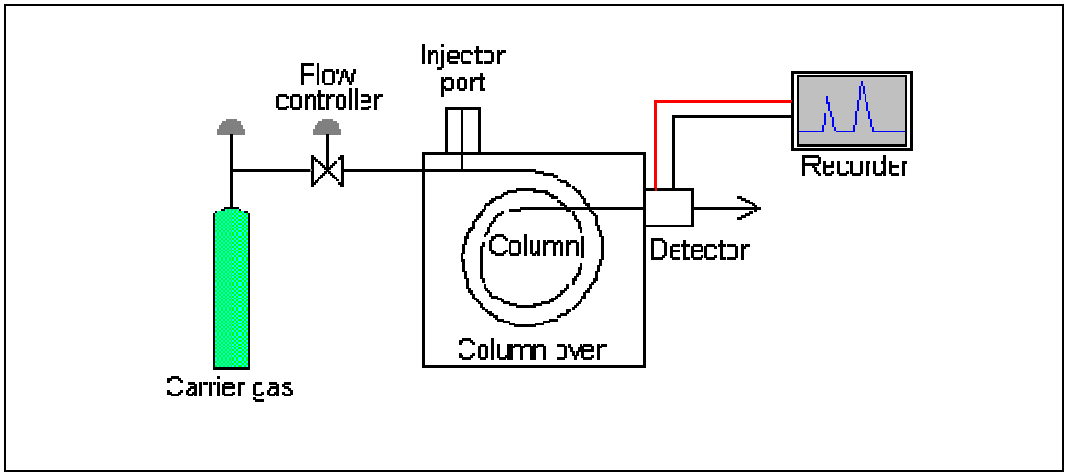


Figure 2-1: Diagram indicating the various components of a gas chromatograph.

Figure 2-2: Photo of a modern gas chromatograph: Agilent 6890 Series Gas Chromatograph.

Gas chromatography is the method of choice when qualitative and quantitative information regarding the composition of a mixture/sample needs to be known. Different components are separated by their distribution between the stationary phase (column inner coating) and mobile phase (carrier gas), either by adsorption, partition, or a combination of the two. Therefore, the chemical properties of the sample must be carefully matched to that of the column stationary phase. In addition, all of the parameters of the other components of the gas chromatograph have to be optimised to achieve maximum resolving power and sensitivity.

2.1.1 Sample introduction techniques

The most common method for introducing liquid samples into the gas chromatograph is by means of an online injector (autosampler) using a micro-syringe.[1,2] Typically this syringe consists of a glass barrel with a close-fitting metal plunger, which is used to dispense a chosen volume of sample through the syringe needle. Gases and vapours are injected with gas tight syringes or gas sample loops. Gas tight syringes have Teflon-tipped plungers for improved sealing of the plunger with the barrel in the face of the backpressure created by the inlet pressure of the injector. The needle of the syringe is inserted through the septum of the injector and into the heated injection port. Once injected, the volatile analytes and solvent are flash vaporised in the injection port

(200 to 250 ºC) and swept onto the column by the flow of the carrier gas. The accuracy of quantitative injection depends on the rate of sample introduction, syringe dead volume, heating of the syringe needle by the injector, and sample handling techniques.

In capillary gas chromatography, special techniques have to be used to inject an aliquot of a sample onto the column because of the low sample capacities of the column and of the low flow rates (1 to 2 ml/min) of the carrier gas required for optimum performance. For liquid samples the split/splitless injection mode are the most common technique developed for use with capillary columns. Programmed Temperature Evaporation is a modification of the split/splitless technique.

2.1.1.1 Split injection

During split injection the evaporated sample is mixed with carrier gas and divided between two streams of different flow, one part entering the column (carrier gas flow) and the major part is vented to the outside (split flow) (Figure 2-3).

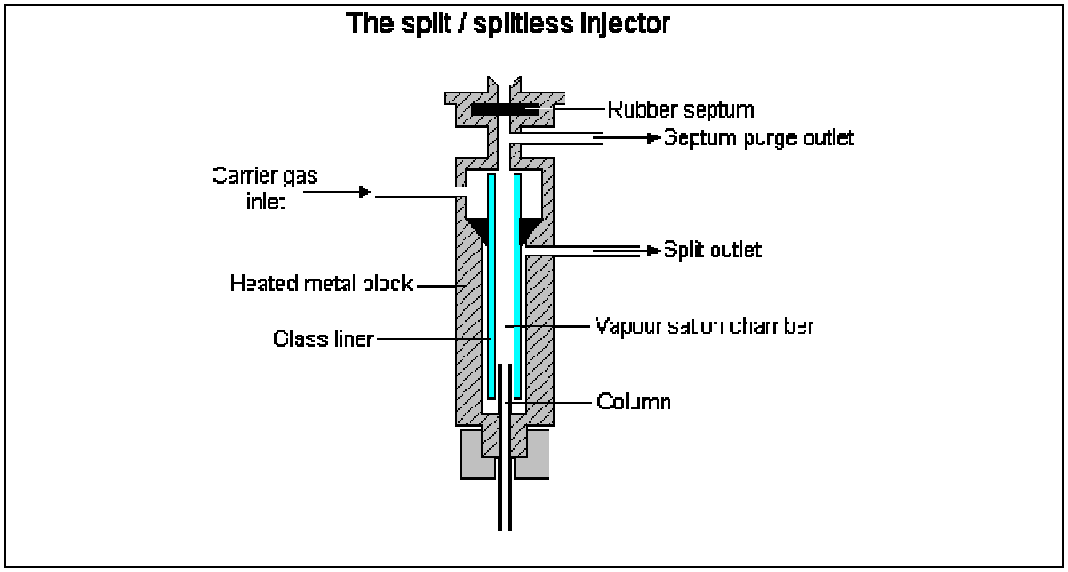


Figure 2-3: Diagram to illustrate the split/splitless injector.

Split injections are typically used when the concentration of the analytes are relatively high to avoid overloading of the column which will lead to a decrease in chromatographic resolution. The split ratio is an indicator of the amount of sample that is introduced into the column, and can typically be anywhere between 1:10 and 1:1000. The higher the split ratio, the smaller the fraction that is injected onto the column. The split ratio is calculated from the following equation:

$$\text{Split ratio} = \frac{\text{Column flow rate}}{\text{Column flow rate + split vent flow rate}} \qquad \text{[Eq. 2-1]}$$

In samples containing analytes of different boiling points, split injection discriminates against the less volatile components due to selective vaporization from the syringe needle, incomplete sample vaporization, and non-homogeneous mixing of sample vapours with the carrier gas in the vaporization chamber. When the sample is vaporised it generates an instantaneous pressure pulse and rapid change in the viscosity of the carrier gas/sample mixture, altering the flow of gas between the column and split line in a manner that is hardly reproducible. Therefore, split injection has been used as the preferred sampling technique for qualitative analysis under high-resolution conditions and less frequently for quantitative analysis.

2.1.1.2 Spitless injection

Splitless injection has the advantage that the quantitation of trace components can be achieved by introducing a relatively large sample volume onto the column. Due to the large volume of the injector relative to the column, excessive tailing will result if the split valve remains closed for too long. The split valve is opened typically after 0.5 to 1 min, to allow the “tails” (especially that of the solvent) to be blown away. Splitless injection of large volumes of dilute samples should be performed slowly through a hot needle. This will ensure that no discrimination occurs, and that the pressure in the injector does

not become too large (due to the evaporating solvent) to cause back streaming into the supply lines.

The velocity of the gas flow through the splitless injector is relatively low, and the sample is introduced onto the column over a comparatively long time. The vaporized sample components can be concentrated at the head of the column prior to separation by either cold trapping or by the solvent effect.[3] This yields less peak broadening i.e. better resolution and narrower peaks, which maintains maximum column efficiency and enhances the detection limits. With the cold trapping approach the column inlet is maintained at least 150 ºC below the boiling point of the analytes being separated. This ensures condensation of the analytes at the top of the column. When the column temperature is subsequently raised, the analytes will revaporize and migrate through the column in a narrow band.[2] The solvent effect occurs when the column is 20 to 30 ºC lower than the boiling point of the injection solvent. This will cause the solvent to condense at, and saturate, the stationary phase at the top of the column. This region of the stationary phase will subsequently act as a barrier to the sample components, which will condense in the solvent-saturated region. Heating of the column will first cause the solvent to vaporise, after which the sample components will revaporize to migrate through the column.

2.1.1.3 Programmed Temperature Vaporization

The Programmed Temperature Vaporization (PTV) injector is a split/splitless injector with the ability to rapidly heat to 300 ºC. This rapid heating capability enables large volume liquid injections. The PTV injector can be used as a thermal desorber for volatiles and semi volatiles, online or offline.

Samples (1 to 200 µL) are introduced into the vaporizing chamber, which is kept at a temperature below the boiling point of the solvent. After retraction of the needle, the temperature of the injector is raised from ambient to 300 °C in less than 30 seconds. The carrier flow transfers the analytes onto a cool column. This promotes condensation and focussing of the analytes and helps prevent smearing and excessive tailing.

The precision and accuracy of the PTV technique is generally superior to those of the hot split/splitless technique. However, many parameters have to be considered and PTV injection still lacks optimization.[1]

2.1.1.4 Multi-port valves for gaseous samples

Gaseous samples can be injected with gas-tight syringes. However, better efficiencies of separation and more reliable and reproducible quantitative data are obtained with gas sample loops based on multi-port devices (Figure 2-4).

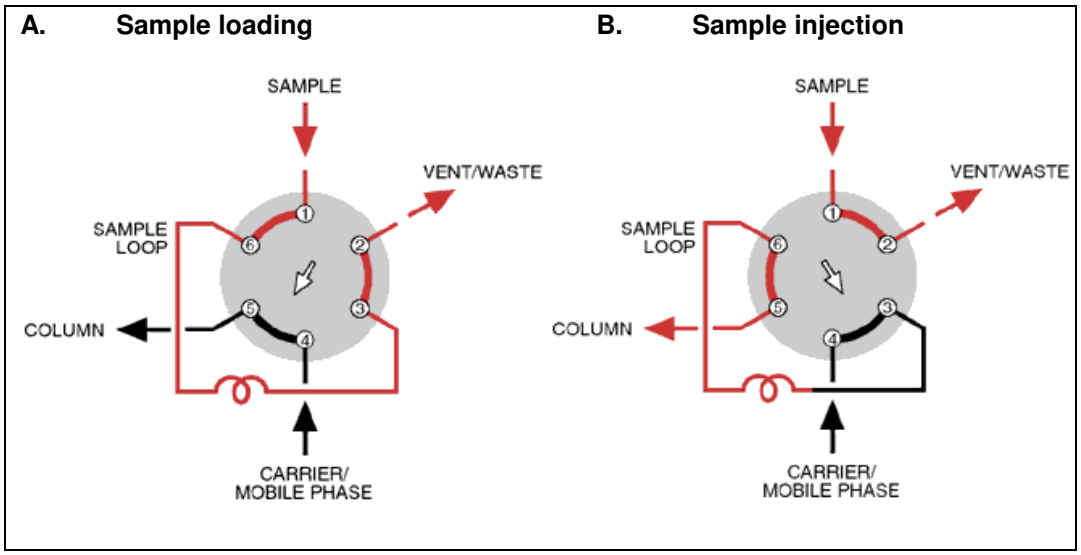


Figure 2-4: Diagram showing how samples are loaded and introduced into the chromatographic system using a six-port valve.

Gas samples can either be transferred from their container to an evacuated sample loop, or the loop can be filled with sample by flushing it with the sample, which must be available at large volumes under an overpressure. Samples are loaded into the loop and, with a change in the valve position, are subsequently swept onto the column under flow of the carrier gas. The amount of sample introduced can be deduced from the measured pressure inside the loop.[4]

Cryogenic freeze-out techniques are used more frequently where samples from flasks or canisters are concentrated by reducing the volume, which is then subsequently rapidly heated and injected onto the gas chromatographic column.[5] This focusing step improves resolution for more volatile samples.

2.1.2 Gas chromatography columns

Many types of columns exist but in practise mainly two types of columns are used: packed columns and capillary (open tubular) columns. Packed columns are typically a glass or stainless steel coil (typically 1 to 5 m total length and 5 mm inner diameter) that is filled with the stationary phase, or a packing coated with the stationary phase. The advantage of packed columns is that they have higher sample capacity than capillary columns. However, the difference has been greatly reduced by the large-bore 530 µm capillary columns and improvements in detector sensitivity have also reduced the need for large samples. Today they are mainly used for preparative separations, but can also be applied for many practical analyses of less complicated samples. The one area in which packed columns may have an advantage is in analysis of gas samples. This practice, however, is also declining.[5]

With the introduction of open tubular or capillary columns by Marcel Golay in 1958 it became possible to separate solutes with high chromatographic efficiency and

resolution.[6] Initially capillary columns were made of metal material. Later, different types of glass material were used which were very fragile. In 1979, high-purity silica columns with thin walls (40 to 45 µm) were introduced. Fused-silica columns are protected from mechanical damage by a polyimide protective coating and can be bent and coiled without the risk of breakage. These columns typically have inner diameters of 0.53 mm or less and can be 1 to 100 m or more in length. For gases, the most commonly used columns are 0.32 mm and 0.53 mm in diameter. The stationary phase is coated on the inside of the tube wall as a very thin (0.05 to 10 µm) continuous layer which in most cases are polymerised or cross linked to provide a thermo stable layer, provided oxygen is kept out of the column and the maximum specified temperature is not exceeded.

Capillary columns most frequently used are Porous Layer Open Tubular (PLOT) and Wall Coated Open Tubular (WCOT).[7] In PLOT columns, the inner surface of the column has an embedded layer of the adsorbent stationary phase (Figure 2-5).

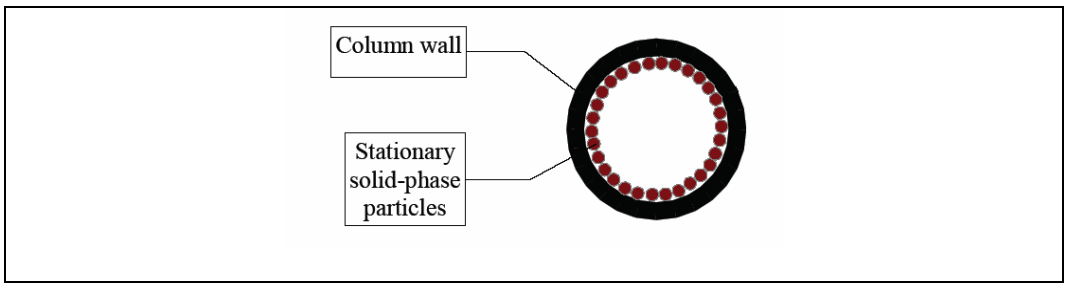


Figure 2-5: Diagram of Porous Layer Open Tubular (PLOT) capillary columns

Molecular sieves, porous polymers, aluminum oxides, carbosieves, and silica are the current adsorptive materials that are commercially available in PLOT columns. Each adsorbent has its own specific application field as shown in Table 2-1.

Table 2-1: Application fields of different adsorbents.

Adsorbent	Applications
Molecular sieves	Permanent gases; Hydrogen isotopes; CO; N_2O
Porous polymers	Volatile polar and non-polar compounds; Samples containing water; CFC's; Solvents
Alumina	Hydrocarbon impurities in $C_1 - C_5$ hydrocarbons
Carbon	CO and CO_2 in air; Impurities in ethylene; volatiles in acidic matrix
Silica	$C_1 - C_5$ hydrocarbons; Sulphur gases; Hydrocarbon and semi-polar impurities; Samples containing moisture

The application field of PLOT columns cover the permanent gases and volatiles up to C_{16} hydrocarbons with boiling points up to about 225 °C. Recent developments in stationary phase technology have enhanced the applicability of PLOT columns for volatile compounds.

WCOT is the most widely used capillary columns in gas chromatography. A thin liquid film of organic stationary phase is bonded directly onto the inner surface of the column (Figure 2-6).

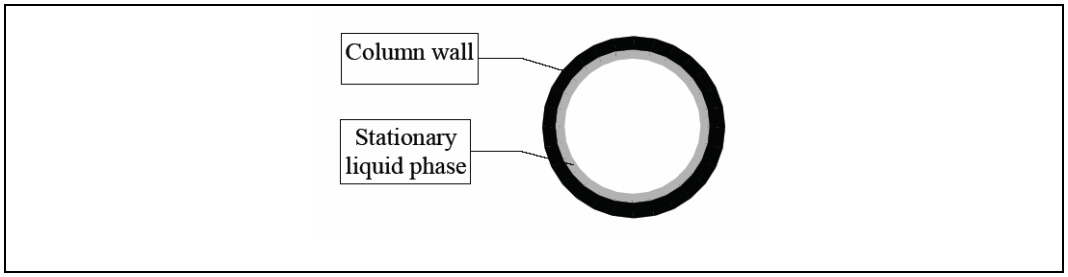


Figure 2-6: Diagram of Wall Coated Open Tubular (WCOT) capillary columns.

Usually the polarity of the stationary phase is matched to that of the sample analytes as to achieve the best separation. The most common stationary phases for WCOT columns are listed in Table 2-2.[2]

Table 2-2: Commonly used gas chromatography stationary phases for WCOT columns.

Composition	Polarity	Applications
100 % dimethyl-polysiloxane	Non polar	Drugs, amino acid derivatives
5% diphenyl – 95% dimethyl-polysiloxane	Non polar	Drugs
50% diphenyl – 50% dimethyl-polysiloxane	Intermediate	Drugs, steroids, glycols
50% cyanopropylmethyl – 50% phenylmethyl-polysiloxane	Intermediate	Fatty acid methyl esters, carbohydrate derivatives
Polyethylene glycol	Polar	Acids, alcohols, glycols, ketones

The most fundamental difference between PLOT and WCOT columns can be traced directly to the type of chromatography that occurs in each. In PLOT columns the solute molecules partition between the stationary phase and mobile phases in accordance with the principles of gas-solid-chromatography. The degree to which solutes are retained by PLOT column phases depends upon solute vapour pressure, the number of sites available for adsorption, and the strength of solute stationary phase attractions. In WCOT columns, solute molecules partition between the stationary and mobile phases in accordance with the principles of gas-liquid chromatography. The degree to which solutes are retained is governed almost exclusively by their latent vapour pressures and solubilities in the stationary phase. Modern PLOT columns offer higher efficiency, greater sensitivity, and the improved inertness of WCOT columns, while retaining the ability to separate highly volatile solutes and permanent gases.

In general, a more polar stationary phase will lower the maximum operating temperature of the column. All stationary phases slowly decompose upon use, or extended storage, which will result in column bleed (stripping of the stationary phase). This is seen as an increase in the baseline as the column temperature rises to values close to the maximum operating temperature of the column. New columns should be carefully conditioned before use for the same reason. It should be noted that column bleed could significantly lower the sensitivity of detectors due to an increased noise level, and therefore a decrease in the signal to noise ratio of the detected peaks. As a result, most manufacturers now supply special “low bleed” columns. Although baseline drifts such as this can be corrected for by most data systems the quantitative result is less than optimum and therefore not recommended. Advances in capillary column technology has led to the stationary phase being integrated on the inside wall of the capillary column. With this method, the column contains no particles, since the stationary phase is present as one, integrated layer. Particle elution is not possible

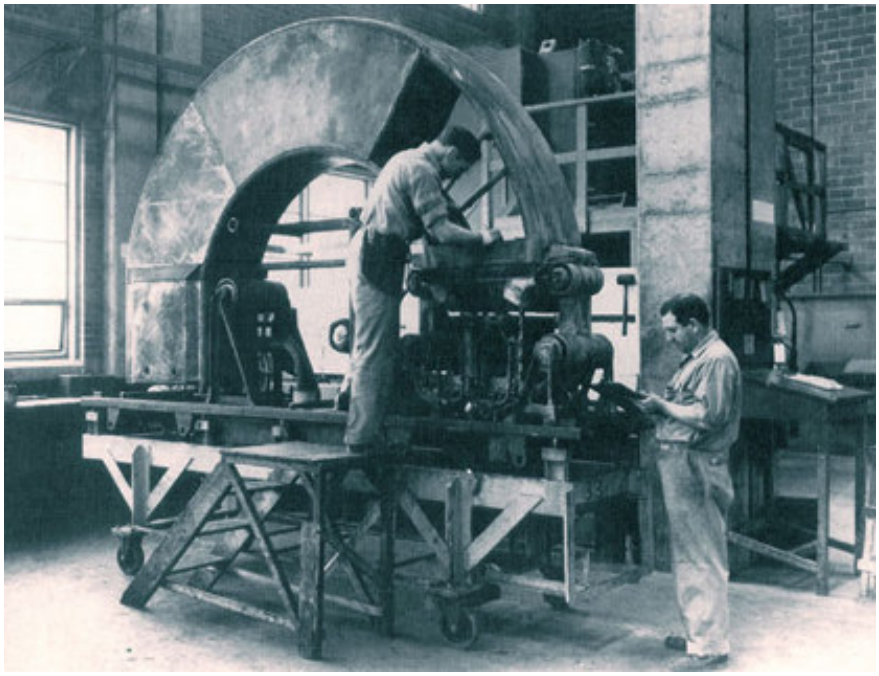
because there are no particles present. These columns offer significantly better mechanical - and temperature stability, reproducibility, and inertness.[8]

For routine use the less polar bonded phase columns are more robust and less prone to contamination. Furthermore, they can be baked out, rinsed or deactivated, thereby extending their useful life by a few hundred injections or more. Most separations in capillary gas chromatography are performed on methylpolysiloxane stationary phases in 10 to 50 m fused silica columns of less than 0.53 mm inner diameter and film thickness between 0.1 and 5 µm. In general this is the column that complies with the largest number of applications.

2.1.2.1 Optimization of column parameters

The primary goal of gas chromatographic analysis is to separate a mixture into its individual components. When all the information regarding the sample to be analysed is known, there is still several factors that have to be considered in order for columns to perform optimally. Column performance can be evaluated on either its resolution or speed of separation. If a detector with universal selectivity would be used, good chromatographic resolution is a necessity to secure analytical selectivity. If the separation is speeded up in a particular column, resolution is traded for speed. A selective detector however, will pose fewer constraints on chromatographic resolution. This means that “poorer resolution” can be tolerated since the detector will respond only towards those compounds for which the detector is selective.

2.2 Mass Spectrometry



"Mass spectrometry is the art of measuring atoms and molecules to determine their molecular weight. Such mass or weight information is sometimes sufficient, frequently necessary, and always useful in determining the identity of a species. To practice this art one puts charge on the molecules of interest, i.e., the analyte, then measures how the trajectories of the resulting ions respond in vacuum to various combinations of electric and magnetic fields.

Clearly, the sine qua non of such a method is the conversion of neutral analyte molecules into ions. For small and simple species the ionization is readily carried by gas-phase encounters between the neutral molecules and electrons, photons, or other ions. In recent years, the efforts of many investigators have led to new techniques for producing ions of species too large and complex to be vaporized without substantial, even catastrophic, decomposition."

-John B. Fenn, the originator of electrospray ionization for biomolecules and the 2002 Nobel Laureate in Chemistry

The concept of mass spectrometry was first introduced by Sir JJ Thomson in 1913[9] and further modified and developed by his protégé FW Aston[10,11] and later by AJ Dempster.[12] The modern commercial mass spectrometer consists of the following basic components: an *ion source* to create the gas-phase ions, a *mass-selective analyser* to separate the ions in space or time based on their mass-to-charge ratio, and an ion *detector* to measure the quantity of ions of each mass-to-charge (*m/z*) ratio (Figure 2-7). In addition, a device to introduce the sample into the mass spectrometer and a data-recording device is needed, and since the mass spectrometer must operate under vacuum conditions, a high vacuum pump is also necessary.

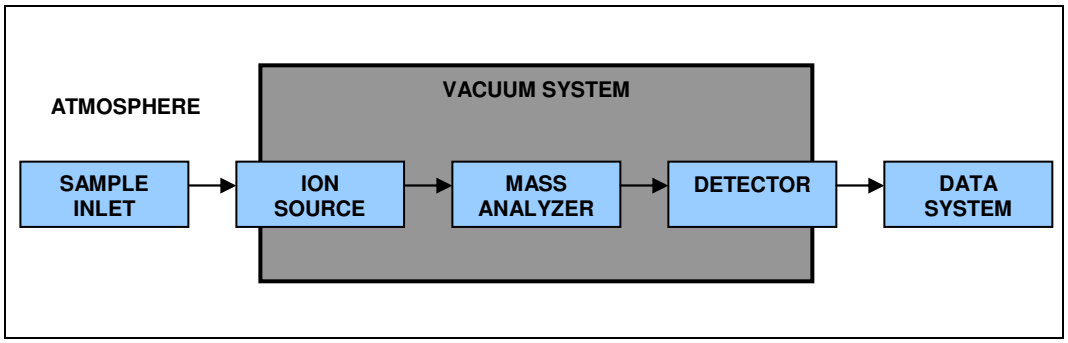


Figure 2-7: Components of a mass spectrometer.

2.2.1 Sample introduction

The method of sample introduction to the ionization source often depends on the ionization method being used, as well as the type and complexity of the sample. The sample can be inserted directly into the ionization source, or can undergo some type of chromatography *en route* to the ionization source. This latter method of sample introduction usually involves the mass spectrometer being coupled directly to a gas chromatograph - or high-pressure liquid chromatography separation column. The sample is now separated into a series of components and enters the mass spectrometer sequentially for individual analysis.

2.2.3 GC-MS interface

The biggest problem encountered in gas chromatography-mass spectrometry (GC-MS) interfacing is that the outlet pressure of the gas chromatograph is 760 Torr (1 Atm), whereas the inlet pressure of the mass spectrometer should be at a vacuum of 10^{-5} Torr or less. In addition, the carrier gas dilutes the samples. Therefore, the interface system (transfer line) should be able to reduce the pressure of the carrier gas by approximately eight-fold while still delivering a useful quantity of sample to the mass spectrometer.

In practice, most GC-MS interfacing is done by simply inserting the capillary column directly into the ion source. Figure 2-8 is a diagram of one such system.

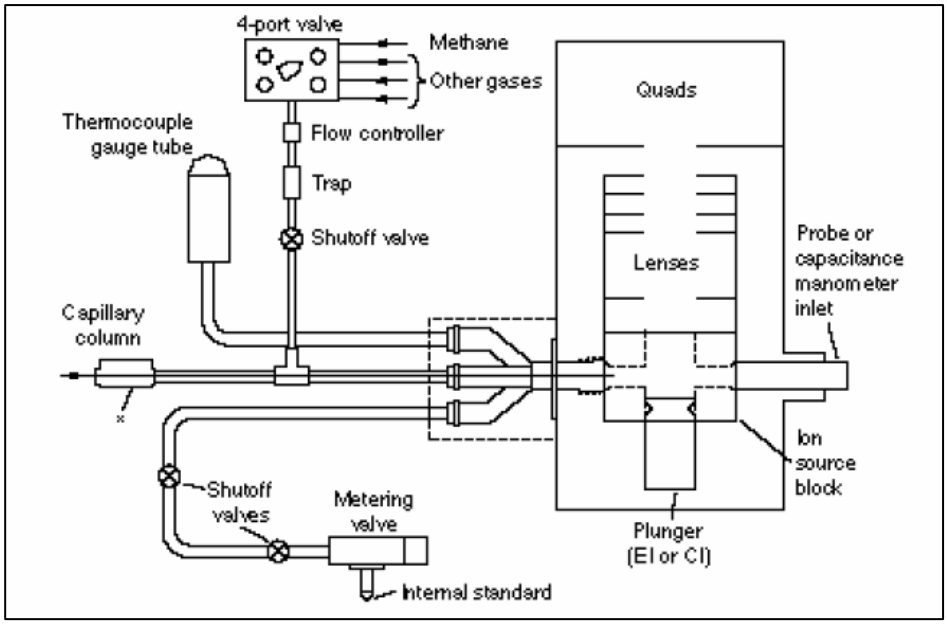


Figure 2-8: A typical GC-MS interface for fused silica capillary gas chromatography columns. The end of the column enters the ion source of the mass spectrometer.

The capillary column runs through a 1/16-in.-diameter tube directly into the ion source. Other gases, such as methane for chemical ionization, are brought into the ion source

by a T-joint around the capillary column. One of the other two lines into the ion source is used for a thermocouple vacuum gauge tube so that the pressure in the ion source can be roughly measured; the other is for the delivery of the mass spectrometer calibration standard, perfluorotributylamine (PFTBA). Most joints are welded together to avoid leaks when this inlet system is thermally cycled or vented. The only removable fitting is the nut, which secures the column in the transfer line (marked with an asterisk in Figure 2-8). This fitting uses Vespel ferrules. Once the ferrules are on the gas chromatographic column, and the column is in the ion source, it is desirable to cut off a few centimetres of the column, if possible. This eliminates the possibility of fine particles partially occluding the end of the column. The transfer line temperature is typically around 280 ℃ to avoid adsorption.[13-15]

2.2.4 Sample ionization

Many approaches to forming ions, both in high vacuum and near atmospheric pressure, have been described. These include field desorption, fast atom bombardment, plasma desorption, laser desorption, and electrospray. These techniques have evolved as a result of the need to efficiently ionize high-molecular weight polymers and proteins. However, for most molecules of interest in clinical chemistry, electron impact ionization (EI) and chemical ionization (CI) remain to be the most important ionization methods.[13-15] For the purpose of this research, EI will be described in more detail.

2.2.4.1 Electron impact ionization

In EI, sample molecules are introduced, as a vapour, into the source, where they pass through a beam of electrons. This beam is emitted from a heated filament and is attracted toward a positively charged plate or trap. If an electron strikes the sample

molecule, sufficient energy may be imparted to remove an electron from the sample molecule, resulting in a positively charged radical molecular ion:

$$M + e^- \rightarrow M^{+\cdot} + 2e^- \qquad \text{[Eq. 2-2]}$$

The electron trap is held at a potential that is positive with respect to the filament, and it is conventional to set the electron beam energy at 70 eV. The energy of the ionizing electrons and the stability of the chemical bonds in the molecule will determine whether the molecular ions subsequently undergo decomposition by internal bond cleavage and atomic rearrangements to give a plasma of ions, radicals and neutral species. The electron beam energy in excess of that required to ionize the sample molecule is dissipated by fragmentation of the molecular ion. The extent to which the molecular ion fragments is usually relatively large, and it is therefore sometimes not possible to detect an appreciable abundance of the molecular ion. Multiple fragmentations occur with even simple molecules, and the pattern of fragmentations as reflected by the mass spectrum is consistently characteristic of that particular sample. Consequently, the mass spectrum of a sample can be considered to be analogous to its "fingerprint" for purposes of identification.[13,14]

2.2.5. Mass analysers

A variety of approaches have been used to separate the stream of ions produced by the ion source. In all of them, the ions travel along different paths as a result of their interaction with a magnetic or electric field. It is of utmost importance that the mass analyser is operated under vacuum conditions to prevent the ions from colliding and interacting with each other, and with air molecules, as this will render the analyses inaccurate. The mass analyser is also usually maintained at an elevated temperature (200 to 300 °C) to prevent gas molecules of organic compounds from adsorbing onto the inner surfaces of the mass analyser.

Many mass analyzers exist, e.g., Magnetic Sector, Time of Flight, Quadrupole, Quadrupole ion-trap, Linear quadrupole ion trap, and Fourier transform ion cyclotron resonance (FT-ICR). Table 2-3 compares the ionization modes, resolving power, mass accuracy, mass/charge range, and scan speed of these mass analyzers.[13-15]

Table 2-3: Comparison of the performance factors of the different mass analyzers. Ionization modes, resolving power, mass accuracy, mass-to-charge range, and scan speed are important factors qualifying these techniques for various applications.

Mass Analyser	Common Ionization Modes	Resolving Power (FWHM)*	Mass Accuracy	Mass/Charge Range	Scan Speed
Magnetic Sector	ESI **	5 000 to 100 000	0.0001 Da	1 000 to 15 000	≈ a second
Time-of-flight (reflection or Q-TOF)	MALDI *** or ESI	2 000 to 10 000	0.001 Da	10 to 1 000 000 (200 to 4 000 for Q-TOF)	≈ milliseconds (Reflection)
Quadrupole	ESI	1 000 to 2 000	0.1 Da	200 to 3 000	≈ a second
Quadrupole ion trap	ESI	1 000 to 2 000	0.1 Da	200 to 4 000	≈ a second
Linear quadrupole ion trap	ESI	1 000 to 2 000 (5 000 to 10 000 in zoom scan mode)	0.1 Da	200 to 4 000	≈ a second
Isotope Ratio Mass Spectrometer (IRMS)	ESI	100 to 200 (10 % valley definition)	N/A	2 to 100	≈ a second
FT-ICR	ESI or MALDI	5 000 to 5 000 000	0.0001 Da	200 to 20 000	≈ a second

**FWHM = Full width at half maximum; **ESI = Electrospray ionization; ***MALDI = Matrix assisted laser desorption ionization.*

The sophisticated magnetic sector and time of flight (TOF) analyzers are examples of high-resolution mass analyzers. The quadrupole and ion trap analysers are the most popular of the low-resolution analyzers. Low-resolution instruments are generally sufficient for the analysis of simple and low molecular weight compounds. Both can only achieve unit resolution and has an upper limit of 600 to 4 000 amu. For the purpose of this study, the quadrupole mass analyser will be discussed in more detail.

2.2.5.1 Quadrupole mass analyzer

Quadrupole mass analyzers have been used with EI sources since the 1950's and are still the most common mass analyzers in existence today. Quadrupole mass spectrometers offer three main advantages: (1) they tolerate relatively high pressures; (2) they have a significant mass range with the capability of analyzing up to a *m/z* of 3000 amu; and (3) they are relatively low cost instruments.

The quadrupole consists of four mutually parallel, high mechanical precisioned, electrically isolated electrodes, oriented such that the electric field between them is hyperbolic (quadrupolar) (Figure 2-9).

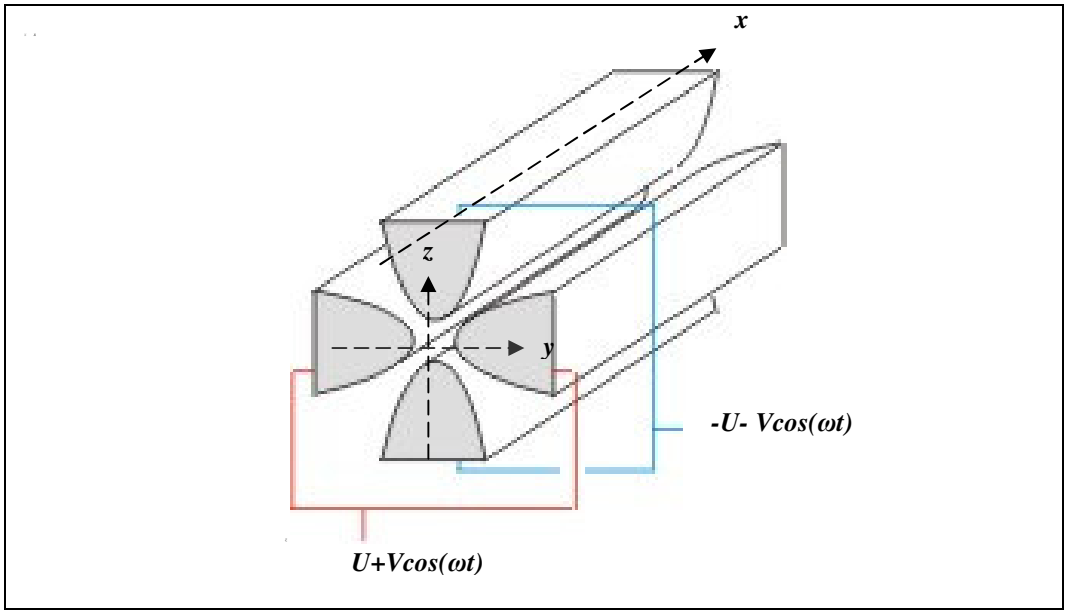


Figure 2-9: Schematic diagram of the quadrupole mass analyser.

A constant dc voltage (U) and a radio frequency potential (*Vcos(ωt)*) are applied between opposite pairs of rods so that the applied potential of one pair is *(U+Vcos(ωt))* and that of the other pair is *-(U+Vcos(ωt))*.

Ions are directed from the ion source into the quadrupole analyser along the *x*-axis, and are acted upon by a variable field in the *y- and z*-plane. The applied voltages affect the trajectory of ions travelling down the flight path centred between the four rods. The alternating electric field makes the ions go off into spirals as they pass down the quadrupole, while the constant voltage forces them in one constant direction, towards one pair of electrodes. For a given set of voltages (*U,V*), frequency (ω), and rod separation, a stable condition exists so that the trajectory of an ion with a specified *m/z* ratio remains within the bounds of the electrodes. Such ions emerge at the exit from the quadrupole and are detected in the usual way. This stable condition applies to ions having a range of *m/z* ratios and in practice this range is made sufficiently narrow to transmit only ions of the same nominal *m/z* value. Ions of other *m/z* ratios undergo unstable oscillations and are removed in the *y* and *z* directions, either by impinging on the electrodes or by escaping between the rods. It is essential that the rods are correctly spaced (to within a few microns) to ensure adequate mass resolution and efficient transmission of ions.[15]

2.2.6 Vacuum system

For a charged particle to behave as directed by the electric and magnetic fields, it must not be subject to forces from collisions with other molecules. Since one ion's collision with another ion, or with a molecule of oxygen, nitrogen, carbon dioxide, or water (i.e. air) would alter its path, mass spectrometry requires a vacuum system such that, on average, a charged particle will undergo no more than a few low-energy collisions between the time of ionization and detection. If such collisions do occur, the instrument will suffer from reduced resolution and sensitivity. Higher pressures may also cause high voltages to discharge to ground, which can damage the instrument, its electronics, and/or the computer system running the mass spectrometer. An extreme leak, basically an implosion, can seriously damage a mass spectrometer by destroying

the electrostatic lenses, coating the optics with pump oil, and damaging the detector. In general, maintaining a good vacuum is crucial to obtaining high quality spectra. For proper operation the ion source, mass analyser, and detector are all kept under vacuum.[15]

Achieving a vacuum sufficient for effective mass spectrometric operation generally requires two pumps. A high vacuum is achieved by connecting a low-vacuum pump to the output of a high-vacuum pump. Three pump combinations are frequently used: (1) a diffusion pump(s) connected to a rotary-vane rough pump, (2) a turbomolecular pump(s) connected to a rotary-vane rough pump, and (3) a cryogenic pump connected to a rotary-vane rough pump (Figure 2-10). The rotary-vane (mechanical) pump serves as a general workhorse for most mass spectrometers and allows for an initial vacuum of about 10^{-3} Torr to be obtained. Once a 10^{-3} Torr vacuum is achieved, the other pumping systems, such as diffusion, cryogenic and turbomolecular can be activated to obtain pressures as low as 10^{-11} Torr.

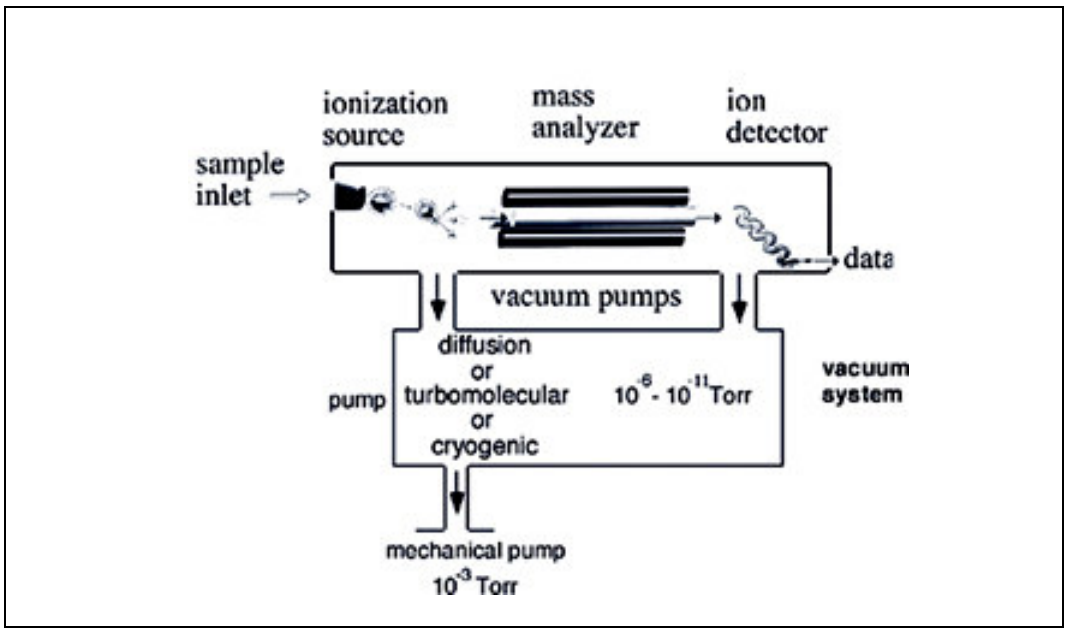


Figure 2-10: Connection of pumps to a mass spectrometer so that samples can be analyzed under vacuum conditions.

The exact vacuum requirements vary with the type of mass analyser, but the mean collision-free path of the ions must be at least as long as the path through the analyser, i.e. greater than the distance between ionization and detection (Table 2-4).

Table 2-4: Relationship between vacuum conditions and distance of the mean free path.

Pressure (Torr)	Mean Free Path (Meters)
760	6.0×10^{-8}
1	4.5×10^{-5}
10^{-3}	4.5×10^{-2}
10^{-5}	$4.5 \times 10^{(0)}$
10^{-7}	4.5×10^{2}
10^{-9}	4.5×10^{4}

2.2.7 Ion detection

After mass analysis, the ions arrive at the collector where they are detected, measured and recorded. The current carried by the total number of ions of each mass is small (10^{-10} to 10^{-18} Ampere) and varies considerably depending on their fluctuation and on the mode of operation of the instrument. The accuracy of ion current measurements depends on the total number of ions arriving at the detector. The electron multiplier is the most frequently employed detector in mass spectrometers capable of detecting these low currents, and to amplify them with a gain of approximately 10^6 (Figure 2-11).

The electron multiplier consists of a series of dynodes electrically connected through a resistive network. Secondary ions are produced when the ion beam strikes the first dynode and these electrons are accelerated towards the second dynode where further electrons are emitted. This process continues down each stage of the multiplier and

the final output current is converted into a voltage suitable for further amplification and recording. Recording of the signal received from the electron multiplier has to be very fast in order to enable rapid scanning.[15]

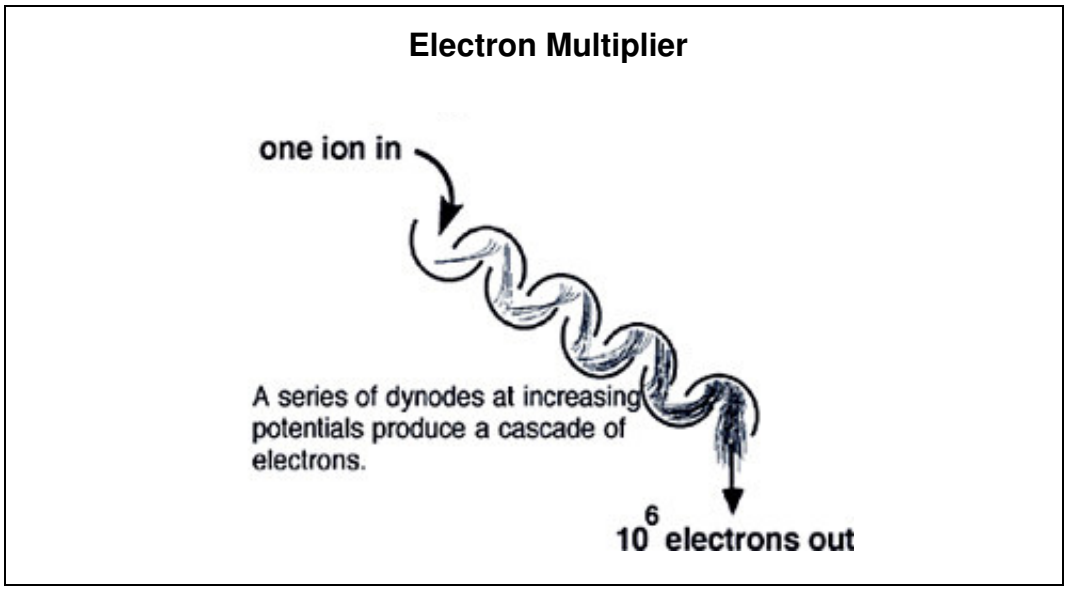


Figure 2-11: Schematic diagram of an electron multiplier and the cascade of electrons that results in a 10^6 amplification of the current in a mass spectrometer.

2.2.8 Recording and presentation of mass spectrometric data

The computer data system is integral to the present generation of mass spectrometers with control function in the operation of the instrument together with its role in collecting and processing mass spectral ion and intensity information. The signals from the electron multiplier are passed through an analogue filter before passing to the analogue-to-digital converter. The digitization rate is selected to obtain a sufficient number of data points across each ion signal to permit definition of the peak and the accurate determination of its position and intensity.[13]

It is customary for a low-resolution mass spectrum (i.e., a mass spectrum in which only the integral *m/z* values have been resolved) to be presented in the form of a bar graph, in which the *m/z* values are plotted horizontally and the ion abundance vertically. Bar

graphs for the 70 eV mass spectra for hexane and CO_2, as well as the fragmentation patterns giving rise to these particular spectra, is given in Figure 2-12.

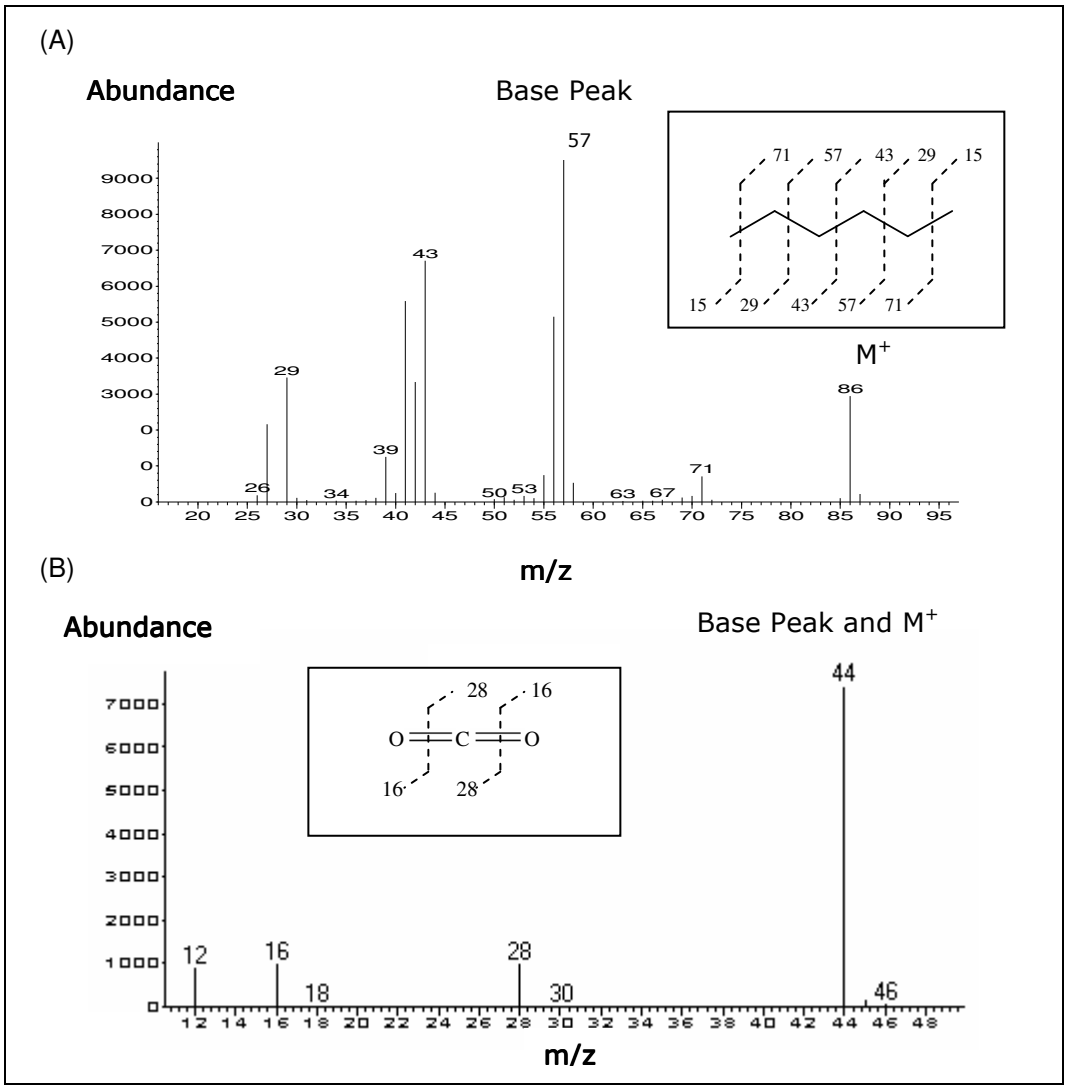


Figure 2-12: Full scan mass spectra for hexane (A) and CO_2 (B), recorded at 70eV.

The most abundant ion is arbitrarily assigned an abundance of 100 percent and is called the base peak. A recording device sensitive to very different ion-abundance ratios is essential in mass spectrometry, since ions of very low abundance will occasionally be of great importance in deducing a molecular formula or structure.

2.2.9 Performance characteristics of mass analyzers

To use any detector to its full potential it is necessary for the analyst to be aware of the characteristics of the detector. The performance of a mass analyser can typically be defined by the following characteristics: sensitivity and efficiency, accuracy, mass range, resolution and mass calibration, and scan speed.

2.2.9.1 Sensitivity and efficiency

The sensitivity and efficiency of a mass spectrometer is a function of the number of ions striking the detector per molecule introduced into the ion source and the gain of the detection system. The lowest amount any detector can detect is called the limit of detection. The limit of detection is influenced by the way the sample is collected and prepared, the GC inlet-, column-, and detector parameters, the way the signal is integrated, and random noise generated by the system.

To obtain the full mass spectrum of a compound, the abundance of every *m/z* value is determined within the selected mass range. Many of the peaks introduced into the ion source are only a few seconds wide and the mass spectrometer needs to make a few scans per second to maintain the integrity of the data. The increased scan speed reduces the time that individual mass values are sampled during a scan, with the statistical chance of detection and recognition of an ion peak finally reaching a limit. The small elution volume of a capillary peak, however, results in a higher maximum concentration of the sample peak in the source and has a favourable effect on the sensitivity of detection of a given amount of compound. However, the low loading capacity of capillary columns places an increased general demand on the instruments' sensitivity.

In quadrupole analysers scanning over a mass range of 100 in 1 second, only 1% of the ions of each mass will be detected. The time spent by the analyser at each mass (dwell time) will be 1/100th of a second. The rest of the time will be spent analysing other masses causing loss of the ions at all the masses not analysed for at that specific time. As capillary gas chromatographic peaks can be extremely narrow the scan time of the mass spectrometer should therefore be selected such that at least 10 scans (spectra) can be obtained during the time the peak elutes to maintain the integrity of the data. In addition, to obtain maximum sensitivity, the range of masses to be scanned should be kept as narrow as possible. In doing this the dwell time at each mass is increased and consequently the number of ions allowed through the detector. The quadrupole mass spectrometer has a sensitivity of around 1 ng in the full scan mode. One must keep in mind however, that if the number of ions available for detection is too high the detector becomes saturated resulting in distorted mass spectra or in the worst-case temporary lack of sensitivity of the detector. Utilizing the full scan mode to record complete mass spectra has the unique ability to qualitatively identifying substances.

In the selected ion monitoring (SIM) mode, one or a few ion masses are monitored continuously, where each is detected in sequence for a fixed period of time. Operating the mass spectrometer in SIM mode increases the dwell time on the selected ions dramatically. The sensitivity achievable in this way is greatly increased (10 pg) over a mass scan where each ion in the spectrum is recorded for only a short time (dictated by the scan speed and resolution used). For this reason, data is acquired in SIM mode when sensitive and quantitative detection of specific compounds is necessary. Quadrupole instruments are particularly suitable to operate in the SIM mode since the rod voltages to transmit selected ions can be changed accurately and rapidly.[13]

2.2.9.2 Accuracy

This is the ability with which the analyser can accurately provide *m/z* information and is largely a function of an instrument's stability and resolution. The difference, $\Delta m_{accuracy}$, between the true mass (m_{true}) and the measured mass ($m_{measured}$) of an ion may be expressed in terms of mill mass units (0.001 Da) or in ppm, which is calculated as $(\Delta m_{accuracy}/m_{measured})\ 10^6$. For example, an instrument with 0.01 % accuracy can provide information on a 1000 Da peptide to ± 0.1 Da or a 10,000 Da protein to ± 1.0 Da. The accuracy varies dramatically from analyser to analyser depending on the analyser type and resolution.[15]

2.2.9.3 Mass range

This is the *m/z* range of the mass analyzer. For instance, quadrupole analyzers typically scan up to a *m/z* of 3 000 amu. A magnetic sector analyzer typically scans up to a *m/z* of 10 000 amu, while the time-of-flight analyzers have a virtually unlimited *m/z* range.[13]

2.2.9.4 Mass resolution and mass calibration

2.2.9.4.1 Resolution

Not all the ions of a specific mass has the same energy and some spreading occurs as it moves through the analyser, resulting in a peak with a Gaussian shape for each mass ion detected. This is known as the mass profile. The width of this signal is normally one amu wide, centred on the mass of the ion detected. The former is known as resolution and the latter calibration. Both parameters should be optimised as over-resolution decreases sensitivity and incorrect calibration will compromise identification.

The mass resolution of a mass spectrometer is its ability to separate ions of different mass. The mass resolution achievable by a mass spectrometer depends on both the type of analyser and the experimental conditions. Simple MALDI-TOF instruments may only achieve unit mass resolution over a limited mass range. High performance FTMS systems can achieve resolving powers of several hundred thousand.

The mass resolution for a quadrupole is controlled via the application of a certain ratio of DC and RF voltages.[16]

2.2.9.4.1.1 Resolving Power

The degree by which two masses, *M* and "*M* + Δ*M*", can be separated, is defined as the resolving power (RP) of the mass spectrometer, where RP = *M*/Δ*M*. The extent to which the ions are separated has to be specified. Resolving power can either be defined by the "10 Percent Valley" definition or by the "Full Width At Half Maximum" definition.[15]

The "10 percent valley" definition is mostly applied to magnetic sector instruments. With this definition, ions of the same intensity are considered to be resolved when the overlap is no more than 10% of the maximum intensity of either peak. An example of resolution measurement is shown in Figure 2-13 where the peak has an *M* of 500 and a *ΔM* of 1. The resulting resolution is *M/ΔM* = 500/1 = 500. Alternatively, when a peak *M* = 500 has a *ΔM* of 0.1, the resulting resolution will be *M/ΔM* = 500/0.1 = 5 000. On the basis of this definition, single-focussing magnetic instruments can attain resolving powers in the region of 300 to 3 000, or sometimes as high as 9 000. Double focussing instruments give resolving powers from about 10 000 to 100 000. The quadrupole mass spectrometer is capable of analyzing CO_2 samples with a resolving

power of about 880 ($M/\Delta M$ = 44/0.05). Using the 10 percent valley definition, the IRMS has a reported resolving power of about 100 to 200.

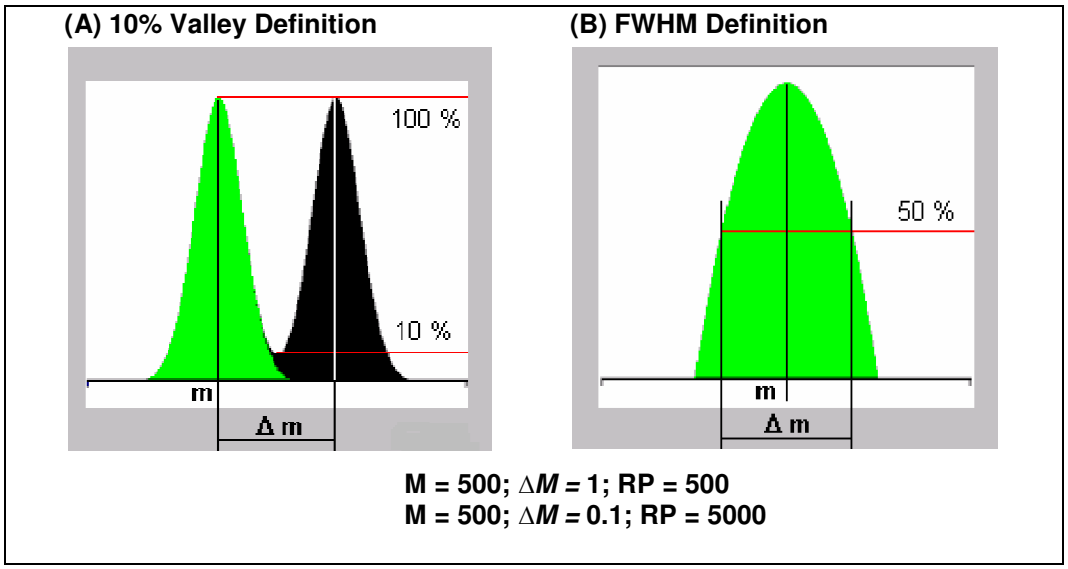


Figure 2-13: Diagrams illustrating the "10 percent valley" (A) and "Full Width at Half Maximum (FWHM)" (B) definitions of resolution.

The Full Width at Half Maximum (FWHM) definition for resolution is mostly applied to quadrupoles, ion traps and TOFs. This definition states that for a single peak made up of singly charged ions at mass M in a mass spectrum, the resolution may be expressed as $M/\Delta M$, where ΔM is the width of the peak at half the height of the maximum peak height. The example given for the 10 percent valley definition is also applicable for the FWHM definition. The quadrupole is considered to be a low-resolution analyser since it only has a resolving power of 1 000 to 3 000 (FWHM), however this is more than adequate for quantitative and qualitative analysis of a wide range of chemical compounds.[13,15]

2.2.9.4.2 Mass calibration

The primary stage in processing acquired scan data is to determine a mass calibration. The computer develops a list of reference masses obtained by scanning a calibration

compound, and uses this to assign mass values to sample ions. For the quadrupole, the reference masses are related to a voltage scale derived from the quadrupole rod voltage. The calibration reference standard can be any compound that gives a sufficient number of ions of high relative intensity regularly spaced across the mass range of interest. In practice a volatile liquid is usually selected that is chemically inert and can be easily pumped away from the ion source. Perfluorotributylamine (PFTBA) is frequently used as a calibrant for quadrupole instruments, and masses 69, 219, and 502 are selected for calibrating the instrument for ions with masses up to ≈ 800.[13]

2.2.9.5 Isotope dilution

During the pre-instrumental part of the analysis, and even during the gas chromatographic run, an unknown amount of the analyte under investigation may be lost. To compensate for these losses, internal standards are introduced into the samples to improve the reliability of quantitative analysis. Internal standards are usually compounds whose structure and chemical properties are similar to that of the analyte to be analysed. The ideal internal standard would therefore be an isotopically labelled variant of the measured analyte since they are expected to exhibit the same properties as the unlabelled analyte during extraction, purification, and gas chromatography.

The general principle of isotope dilution mass spectrometry (IDMS) is as follows. At the beginning of the sample preparation phase, the labelled internal standard is added to a known volume of a biological sample containing an unknown amount of the analyte. It is also added to standard samples, which contains known amounts of the pure unlabelled analyte. The labelled and unlabelled analytes is then usually removed from the biological matrix by extraction with an appropriate organic solvent or by utilizing solid phase chromatography. Depending on the gas chromatographic and

mass spectrometric requirements, it might be necessary to form a derivative of the labelled and un-labelled analytes. Furthermore, since the concentration of most analytes present in biological fluids is relatively low, it is also necessary to up-concentrate the analytes prior to introduction onto the gas chromatographic column. On a chromatogram, the retention time of the labelled and unlabelled compounds will be more-or-less the same, however their mass spectrums will be different. The isotope ratio of the non-labelled and labelled compounds is measured by mass spectrometry, and the concentration in the unknown sample is then calculated from a calibration curve developed from the isotope ratios of the standards.

The most frequently used non-radioactive isotopes are ^{2}H (Deuterium), ^{13}C, ^{15}N, and ^{16}O, of which the deuterium isotope is the most popular. The use of radioactive isotopes, e.g. ^{14}C and ^{3}H (Tritium), for internal standard purposes are very limited. When selecting an isotope it is important to keep in mind the possibility that the isotope label can be exchanged with naturally occurring isotopes present in the sample, e.g. $H \leftrightarrow {}^{2}H$. The possibility of exchange obviously depends on the position of the label in the molecule. Fortunately, ^{13}C and ^{15}N does not have this disadvantage. It is also important to consider the mass difference between the labelled and unlabelled compounds so that interference from naturally occurring isotopes is limited.[13,15]

2.3 Conclusion

Gas chromatography is an excellent technique for the analysis of low molecular weight, organic molecules. Because volatility is an inherent requirement for separation by gas chromatography, compounds either have to be intrinsically volatile or be converted to a volatile form by chemical derivatization. The primary limitation of gas chromatography is that the sample, or sample-derivative, must be thermally stable at the temperature required for its volatilization. The factors affecting the ability of a gas chromatographic system to yield reliable and repeatable results relate to the sample

introduction technique, the mobile and stationary phases, and the detector employed. No other analytical technique can provide equivalent resolving power and sensitivity to sample components at low concentrations when all of the above mentioned parameters are fully optimized.

Quadrupole mass spectrometry, simply stated, is a powerful instrumental method in which molecules are ionized, separated, and finally detected, based on their *m/z* ratio. Mass spectrometry can typically be used for low molecular weight compounds that are of moderate to high volatility, and generally produce fragments over a narrow mass range. In the full scan mode, mass spectrometry can be utilized to accurately identify unknown compounds by the mass of the compound molecules or their fragments, to determine the isotopic composition of the elements in a compound, and to determine the structure of a compound by observing its fragmentation pattern. The quadrupole mass spectrometer offers high resolution (≈ 2 000 FWHM) for masses up to 4 000 amu, and can identify masses with an accuracy of 0.01 % in the SIM mode. The high accuracy offered by isotope dilution mass spectrometry in the SIM mode makes quantitative analysis of biological samples much more reliable. In addition, the low cost and ease of switching between positive and negative ions makes the quadrupole mass spectrometer particularly suitable for routine analysis in clinical and research laboratories.

The combination of gas chromatography with mass spectrometry is a powerful analytical technique that combines the resolving power of the gas chromatograph with the specificity and sensitivity of the mass spectrometer. GC-MS has the following advantages:

- The mass spectrometer is a universal detector, therefore selective for all compounds.
- Determination of peak purity and deconvolution of multi-component peaks.

- The ability to separate isomers, including chiral mixtures.
- The use of stable isotopically labelled internal standards for accurate quantitative measurements.

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CHAPTER 3

^{13}C-UREA BREATH TEST

3.1 Introduction

Improvements in analytical technologies and the availability of suitable tracers has led to considerable advances in the utilization of $^{13}CO_2$ breath tests in diagnostic testing. If the metabolic reaction to be investigated yields CO_2, sampling for isotope analysis becomes particularly simple, because the patient has only to breathe into a bag or a sample vial. In addition, the widespread availability of isotope ratio mass spectrometers made the analysis of breath samples very simple. The fact that ^{13}C-breath tests are easy to perform, and that they are safe and convenient for the patient, makes them very useful.

Today, ^{13}C-breath tests are used as tools to investigate numerous metabolic processes, and for diagnosing metabolic and infectious diseases. The appearance of excess $^{13}CO_2$ in expired breath provides at least three types of information: (1) it indicates the presence of enzymatic activity, (2) it gives an indication of the level of enzyme present in the whole body from the rate and extent of label appearance over time, and (3) it may reflect the rate of a physiological process or indicate the presence of a foreign agent e.g. bacterial or enzyme. Appendix A lists nearly fifty substances that can be used as substrates for ^{13}C–breath tests, and their application in clinical research and diagnosis.[1] The question, however, is whether or not these tests are viable in a routine clinical practice. Fischer et al[2] evaluated them with respect to: (1) the abundance and significance of the metabolic disorder or infection to be investigated, (2) sensitivity and specificity, (3) the speed of the test, (4) the price of substrate to be applied, (5) the number of breath samples to be collected and analyzed, and (6) topicality. Based on these criteria, they pointed out that only about ten of the approximately fifty tests has a high potential to successfully enter into routine clinical practice (Table 3-1). Furthermore, for a breath test to be implemented, it must

be compared with an established or predictive method of diagnosis, and the efficacy of the breath test must be established.

Table 3-1: Substrates of ^{13}C-breath tests for potential use in routine clinical applications and their diagnostic and metabolic implications.

SUBSTRATE	DIAGNOSTIC CAPABILITY
^{13}C-Octanoic acid	Gastric emptying of solids
^{13}C-Acetate	Gastric emptying of liquids
^{13}C-Urea	*H. pylori* infection
^{13}C-Trioctanoin	Fat metabolism; cystic fibrosis; steatorrhea
^{13}C-Aminopyrine	Activity of microsomal monoxygenases
^{13}C-Caffeine	Hepatic microsomal biotransformation
^{13}C-Galactose	Liver fibrosis in chronic hepatitis B
^{13}C-Glucose	Glucose absorption and utilization (especially in children and diabetes mellitus patients)
^{13}C-Phenylalanine	Hepatocyte functional capacity; cytosolic enzyme activity
^{13}C-Lactose	Lactase deficiency; lactose assimilation

The ^{13}C-urea breath test (UBT) is a prime example of a test that has successfully made the transition from being a research probe to being an established clinical test. It is considered worldwide to be the most important non-invasive test to detect the presence of *H. pylori* infection.

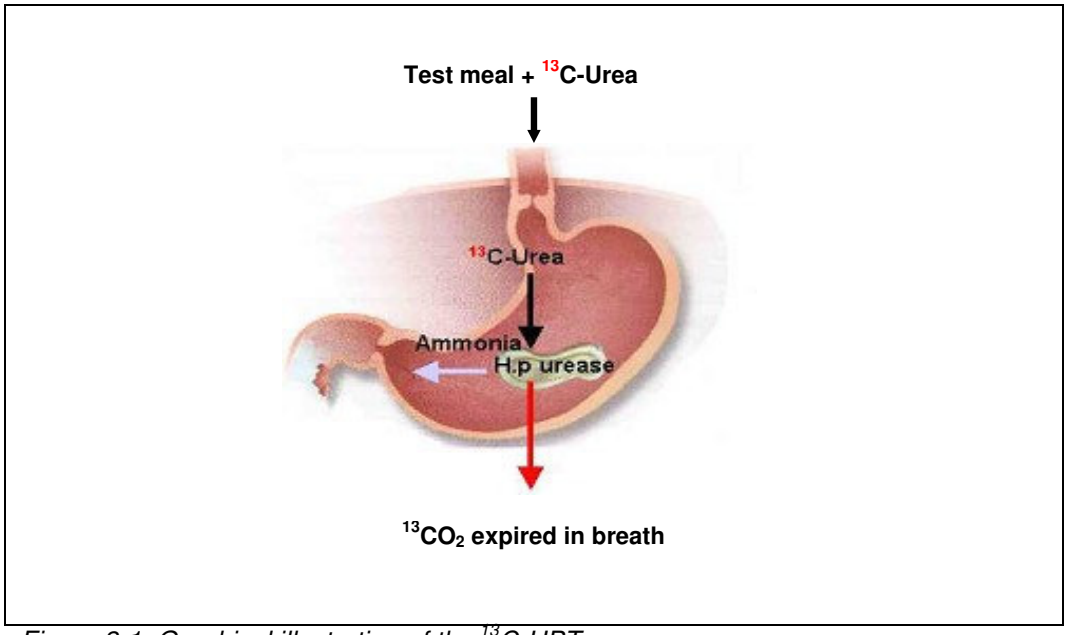

Figure 3-1: Graphical illustration of the ^{13}C-UBT.

Graham et al first described the ^{13}C-UBT in 1987.[3] In his protocol, baseline samples were obtained after subjects fasted overnight. A test meal, consisting of one can of "Sustacal" pudding or 120 mL of 25 % glucose polymer (Polycose) was consumed to delay gastric emptying. This was followed 10 min later by a polycose solution containing 5 mg ^{13}C-urea/kg bodyweight. Breath samples were then obtained every 10 min for 180 min and analyzed for excess $^{13}CO_2$ by gas chromatography with isotope ratio mass spectrometry (GC-IRMS). Since then, almost every variable of the ^{13}C-UBT have been optimised to improve the accuracy and practicality of the test. The following aspects needs to be carefully validated and optimized before performing the test:

- Fasting before the test
- The type of test meal to be used
- Amount of isotope to be consumed (i.e. dose)
- Cut-off value
- Sampling time and frequency

3.2 Fasting

The question as to whether or not patients should fast before undergoing the ^{13}C-UBT is still under debate. Many protocols recommend that patients should fast for at least four hours before testing since it is the assumption that the test meal for delaying gastric emptying wouldn't be as effective in non-fasting subjects, and that the presence of food in the stomach might contribute to variability in $^{13}CO_2$ enrichment values.[4,5] Studies by Perri et al[6] and Moayyedi et al[7] have shown that there is no statistical difference between the $^{13}CO_2$ enrichment values of fasting and non-fasting patients. Contrary to this, Epple et al[8] and Savarino et al[9] have reported a significant decrease in the 30 min $^{13}CO_2$ enrichment values of non-fasting patients as well as an increase in false negative results. While these contradictions continue to exist it is recommended that patients fast before undergoing the ^{13}C-UBT.

3.3 Test Meal

The purpose of the test meal is to slow gastric emptying and to maximize the distribution of the substrate within the stomach, so that the area and time of contact between the bacteria and substrate can be increased.[10,11] This allows for increased sensitivity of the test, and for the dose of isotope used to be reduced.[5] The test meal also aid in reducing false negative results, especially in patients with a low *H. pylori* count, and in those where *H. pylori* is confined to the fundus of the stomach.[4]

Carbohydrates, proteins, fats (or a mixture of these) and citric acid are capable of delaying gastric emptying and are therefore suitable test meals. Furthermore, they are palatable, have long shelf lives, and doesn't affect the patient negatively or add to initial symptoms in any way. When using high-energy test meals, in contrast to low energy non-carbohydrate test meals, it must be kept in mind that these can increase the proportion of $^{13}CO_2$ in the expired breath.[12]

Citric acid has been found to be superior to fatty or mixed-nutrient meals and has become the test meal of choice for the following reasons:

- Due to the low pH of citric acid, it directly influences the duodenal receptors to slow gastric emptying by reducing antral motility and relaxing the gastric fundus.[13]
- It has been shown in vitro that urease activity of *H. pylori* biopsy specimens is markedly increased at an acidic pH.[14] Solutions containing citric acid have the ability to reduce the pH at locations where *H. pylori* resides and therefore increases the urease activity.[10,15] The increased urease activity causes an increase in the $^{13}CO_2$ enrichment values and therefore an increased discrimination between positive and negative results.[5,12]

- Citric acid produces an increase in urease activity within 10 min, without increasing the amount of false positive results.[16] Therefore, the use of citric acid allows for a shorter test period and possible elimination of the fasting step.
- Citric acid reduces contamination by urease that may be present in the mouth due to other bacterial sources without reducing gastric urease activity.[13,17]

Orange juice has been suggested as a possible substitute for citric acid, but it has been found that the sensitivity isn't as high and gastric emptying is increased as compared to citric acid.[18]

Whether or not the test meal can be omitted is still debatable. It has been reported by Atherton et al[19] that without the test meal, the UBT may reflect only the urease activity of bacteria colonizing the antrum, which may lead to false negative results. Another report suggests that sensitivity and specificity may not be affected at all.[20] Until further research has been done on whether or not the test meal can be omitted from the ^{13}C-UBT, it is recommended that the test meal should continue to be part of the ^{13}C-UBT protocol.

3.4 ^{13}C-urea dose

The ^{13}C-urea is one of the major cost limiting factors of the ^{13}C-UBT, therefore it is preferable to keep the amount of dose given as low as possible, without compromising the sensitivity of the test.

The rate at which urease catalyse the ^{13}C-urea is directly proportional to the ^{13}C-urea concentration. As illustrated in Figure 3-2, the rate at which urease catalyses the conversion of ^{13}C-urea to $^{13}CO_2$ and ammonia increases exponentially until a maximum (V_{max}) is reached. This happens when all of the active enzyme sites are

saturated with the ^{13}C-urea. Purified urease has a V_{max} of 1100 ± 200 µmol/min/mg of the urease and a low Michaelis constant (K_m = 0.3 ± 0.1 mM).[17,21,22] For the ^{13}C-UBT to be sensitive enough it is necessary for the quantity of the substrate to be sufficient to saturate the enzyme.[17] Compared to the high doses (100 to 700 mg (10 mg/kg)) used in earlier protocols,[3,16,23] it has been shown that a single dose of 75 mg of ^{13}C-urea for adults[5,13,20] and 50 mg ^{13}C-urea for children[24,25] is sufficient. Hamlet et al[26] and Everts et al[27] reported that it is possible to further reduce the dose to 35 mg by giving it as a tablet or rapidly dissolving capsule. The tablet or capsule based formulation give greater separation between positive and negative results at early time points, and the background ^{13}C-urea hydrolysis due to urease producing bacteria in the mouth is close to zero.

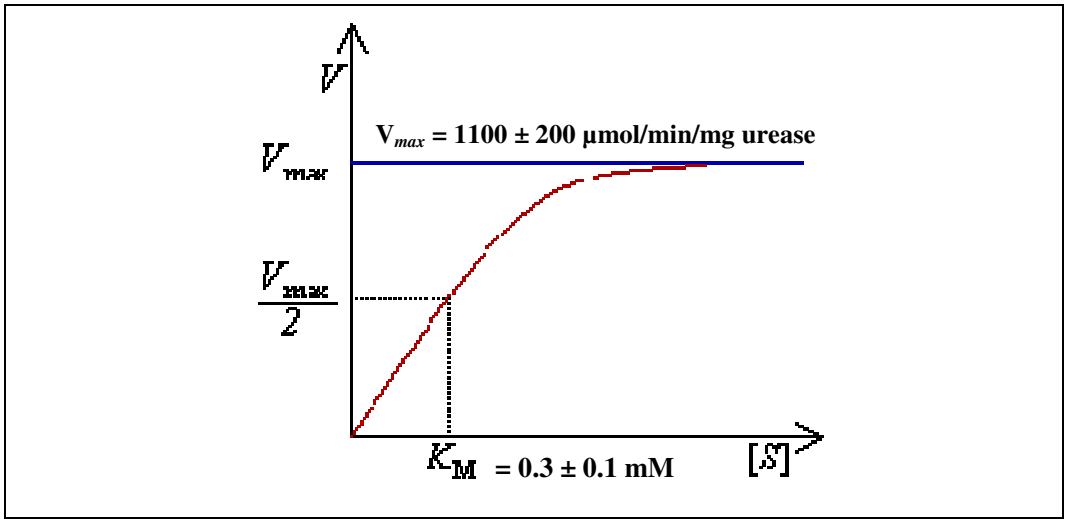

Figure 3-2: Michaelis Menten kinetics for urease.

3.5 Sampling time and frequency

Sampling time and frequency depend entirely on the protocol that is used for the ^{13}C-UBT. Usually duplicate samples are taken at baseline after which the patients will drink the test meal containing the ^{13}C-urea. When urea is administered in powder form together with a test meal, optimal sampling time will be 15 to 40 min after ingestion of

the ^{13}C-urea.[5,13,25,28] However, when the label is ingested as a tablet or capsule the sampling time can be as early as 10 min.[26,27] Inaccurate sampling time will lead to either false positive or false negative results.[12] When the ^{13}C-urea is administered together with the test meal, false positive results might occur due to urease producing bacteria in the mouth. False negative results may occur when sampling too early, as the residence time of the ^{13}C-urea wasn't enough for maximal $^{13}CO_2$ to appear in the breath, as well as when sampling too late, as the ^{13}C-urea is cleared from the stomach.

Sampling at short intervals (every 10 min) for a prolonged period of time will help to determine the $^{13}CO_2$ excretion profile. At least 3 different kinds of excretion profiles where observed for adults by Kasho et al.[29], with maximal excretion occurring at 30 to 40 min. Therefore, in practice, only a single breath sample at 30 min is sufficient to accurately distinguish between *H. pylori* positive and negative subjects.[20]

Sampling time may vary between adults and children. Kalach et al[30] found that for the same dose of citric acid and ^{13}C-urea, the optimal sampling time for children was later (40 min) than for adults. This might be due to lower counts of *H. pylori* in children or due to higher retention of ^{13}C-urea in the stomach.

3.6 Influence of natural occurring ^{13}C isotope on the ^{13}C-UBT

One of the major constraints on the ^{13}C-UBT arises from the large natural abundance of ^{13}C (1.1% of all carbon).[31] In humans natural variations in the $^{13}CO_2/^{12}CO_2$ ratio have been reported between geographically distinct populations, between individuals in the same population, and within single individuals. The exact abundance of $^{13}CO_2$ in breath is influenced by an individual's CO_2 production and the degree of $^{13}CO_2$ dilution within the body's CO_2 and bicarbonate pools. Carbon dioxide production is known to be influenced by several factors such as age, gender, weight and height, but also by

variations in the diet and physical activity.[32,33] Amino acids produce the earliest $^{13}CO_2$ response followed in order by medium chain triglycerides, carbohydrates, and long chain triglycerides. Fasting before the UBT will minimize the baseline shift. For fasting individuals at rest, it has been reported that the natural abundance of $^{13}CO_2$ in expired breath varies with a standard deviation of 0.79 ‰.[34] This natural variation can be a source of error in the $^{13}CO_2$ breath analysis especially if the final breath ^{13}C enrichment is small because of the low dosage of the substrate or because it is only partially oxidized. Taking baseline breath samples will correct for a $^{13}CO_2/^{12}CO_2$ ratio that might deviate from the natural $^{13}CO_2/^{12}CO_2$ ratio. It has been suggested that the age dependence relating to the ^{13}C-UBT can be eliminated if results were normalized for the estimated individual CO_2 production rate.

3.7 Measurement of $^{13}CO_2$

The conventional and gold standard method of measuring the $^{13}CO_2/^{12}CO_2$ ratio in breath samples involve the use of an IRMS.[3,10,16,30,35-38] However, due to the high cost and complexity of the instrument, technologies different from the IRMS have been introduced. Techniques based on infrared spectroscopy,[9,39-44] laser optogalvanic spectroscopy,[45-47] and mass selective detection[29,48] have been proven to measure the $^{13}CO_2/^{12}CO_2$ ratio just as accurately as the IRMS.

3.7.1 Isotope ratio mass spectrometry

The dual-inlet, multiple collector, GC-IRMS, inherently possess a low mass range (2 to 100), a resolving power of 100 to 200 (10 percent valley definition), but extremely high sensitivity (0.2 A ion current $mbar^{-1}$). The IRMS has been designed specifically for the purpose of measuring isotope abundance ratios of pure, low molecular weight, permanent gases, such as H_2, CO_2, N_2, and SO_2. IRMS measures the abundance of two selected isotopically analogous ions from a standard gas (e.g. Pee Dee Belemnite

(PDB)) and the same two ions from a test gas (e.g. breath sample). The intensity ratios of these two ions obtained from the standard and test samples are compared to determine the isotopic abundance of the test sample. For the ^{13}C-UBT, the ratio of $^{12}CO_2$ (m/z = 44) and $^{13}CO_2$ (m/z = 45) is measured.

Gas samples are introduced into the ion source with a steady flow for minimal population fluctuation. The two gases are alternately introduced into the ion source with the aid of switching by magnetically operated valves. $^{12}CO_2$- and $^{13}CO_2$ ions are separated by means of a magnetic field. Ion currents corresponding to the different CO_2 ions are collected in Faraday cups and integrated to yield isotope ratios.

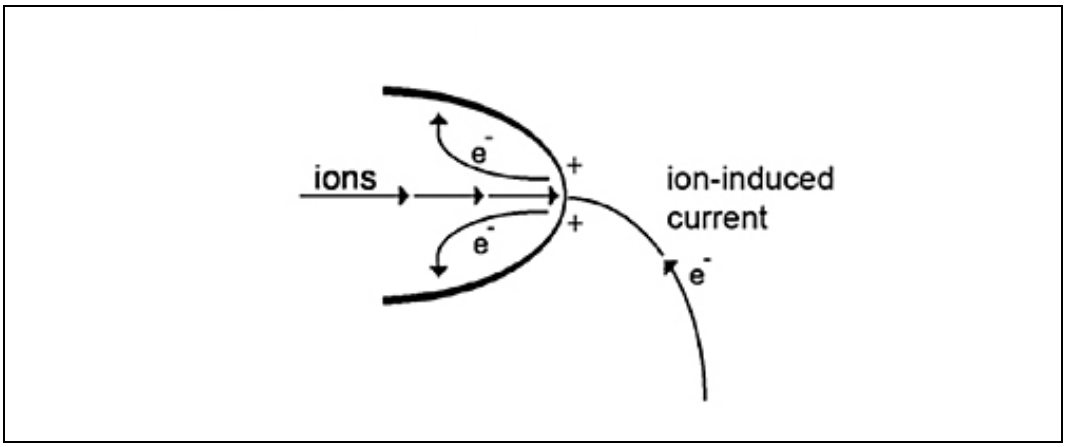

Figure 3-3: Diagram illustrating how the Faraday cup converts the striking ion into a current by temporarily emitting electrons creating a positive charge and the adsorption of the charge from the ion striking the detector.

A Faraday cup (Figure 3-3) involves an ion striking the dynode (BeO, GaP, or CsSb) surface, which causes secondary electrons to be ejected. This temporary electron emission induces a positive charge on the detector and therefore a current of electrons flowing toward the detector. This detector is not particularly sensitive, offering limited amplification of signal (≈ 10), yet it is tolerant of relatively high pressure.

Peak scanning is not used during mass spectrometric measurements, rather, the ion beam is held steady and two or more ion signals are measured simultaneously with separate detectors.

This double-inlet/double-collector configuration provides extremely high precision measurements, which allows for the detection of natural variations in stable isotopic ratios.[49] IRMS is a suitable technique to detect the enrichment of stable isotopes in breath samples with high accuracy. Changes as small as 0.1 ‰ (one part in 10 000) in the $^{12}CO_2/^{13}CO_2$ isotope ratio can be detected.[13] To ensure accurate measurements, a high quality vacuum and extremely low levels of gas impurities are required. A gas chromatograph, which will separate carbon dioxide from other breath gasses, is therefore coupled to the IRMS system.

3.7.2 Infrared spectrometry

The infrared spectrum lies on the immediate low energy side of the visible spectrum. The region of the infrared spectrum, which is of greatest interest to organic chemists, lies in the 4 000 to 600 cm^{-1} region. Absorption of radiation in this region by a typical organic molecule results in the excitation of vibrational, rotational and bending modes, while the molecule itself remains in its electronic ground state. Organic molecules experience a wide variety of vibrational motions, characteristic of their component atoms. Consequently, virtually all organic compounds will absorb infrared radiation that corresponds in energy to these vibrations. Infrared spectrometers permit chemists to obtain absorption spectra of compounds that are a unique reflection of their molecular structure. Figure 3-4 illustrates a typical infrared spectrophotometer. Infrared light is passed through a sample. The transmitted light energy at each wavelength is compared to a reference. The resulting spectrum identifies the composition of the sample.

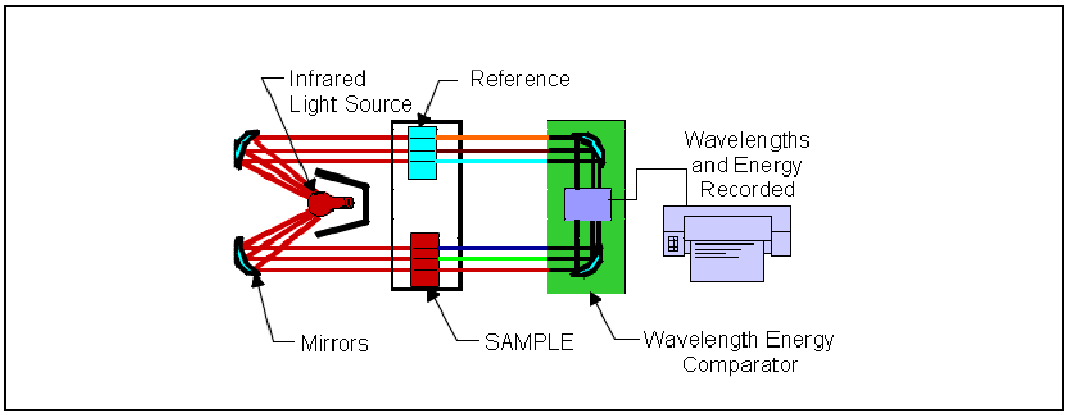

Figure 3-4: Typical infrared spectrophotometer.

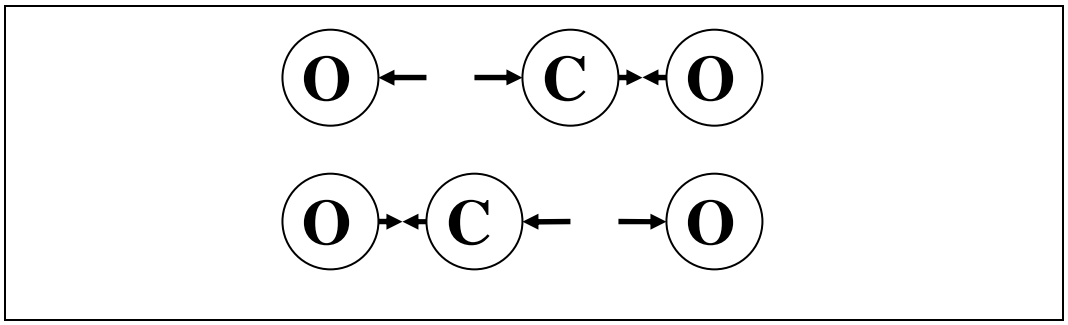

Figure 3-5: The asymmetric stretch modes of the linear CO_2 molecule.

The asymmetric stretch modes of the linear CO_2 molecule are illustrated in Figure 3-5. The heavier ^{13}C atom vibrates with a lower frequency than the ^{12}C atom. This causes an isotope shift of approximately 66 cm^{-1}, which almost completely separates the vibrational bands of $^{12}CO_2$ and $^{13}CO_2$. As no other compounds in breath samples absorb near the wavelength of CO_2 (2 240 to 2 290 cm^{-1}), no gas purification is necessary for infrared methods.

3.7.3 Laser assisted ratio analysis

Laser assisted ratio analysis (LARA) is based on large isotope shifts in molecular spectra, the use of fixed frequency isotopic lasers, and sensitive detection via the laser optogalvanic effect. Figure 3-6 is a schematic representation of a basic measurement

system used for $^{13}C/^{12}C$ isotope ratio determination. Two or more samples, a standard and one or more unknowns, are maintained in low power radio frequency gas discharges and irradiated by fixed frequency carbon dioxide isotopic lasers. Mirrors (M1 to M3) combine the laser beams and direct the light through cells to beam stops (BB). Pressure sensors (PS), are used to measure the pressure in the cells. Electrical discharges are controlled by excitation circuits which also feedback the signals (S). A computer with digital signal processing (DSP) capability, controls the system via analog to digital and digital to analog conversion (A/D). The samples are at low pressure controlled by vacuum pumps and adjustable valves.[50]

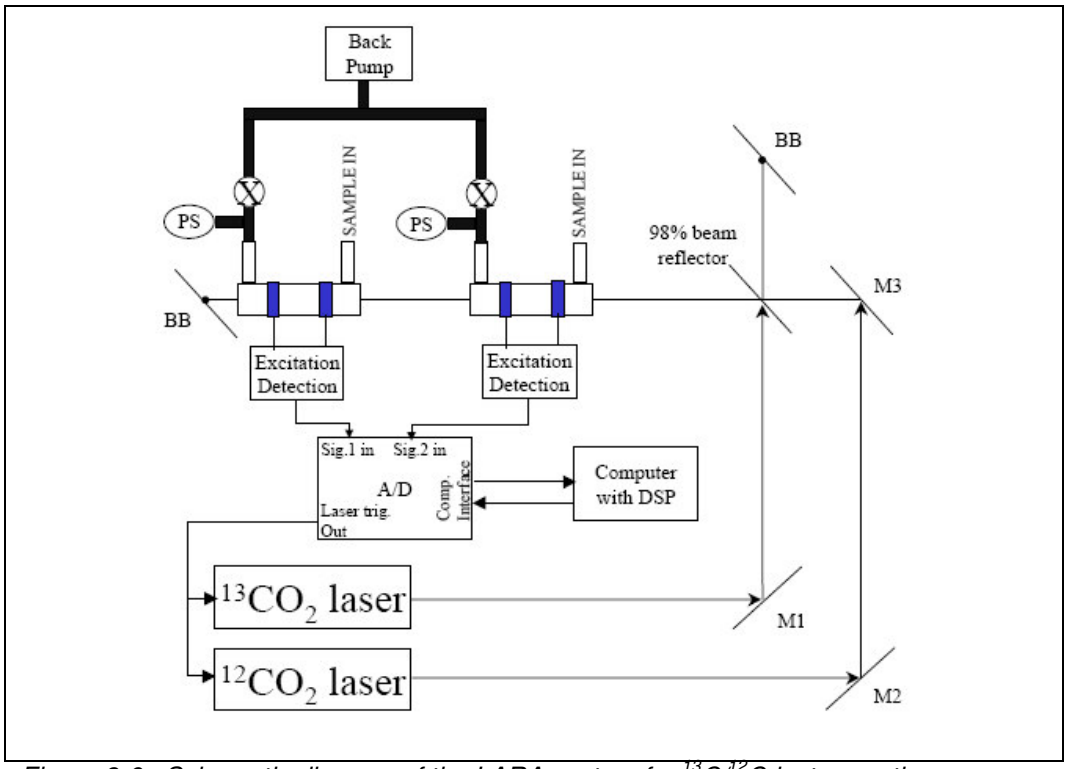

Figure 3-6: Schematic diagram of the LARA system for $^{13}C/^{12}C$ isotope ratio determination in gas samples containing CO_2.

The transitions chosen for $^{12}CO_2$ and $^{13}CO_2$ must be well separated in wavelength and are automatically in resonance with the same molecular transitions in the samples, providing the specificity required for the isotope ratio analysis. Purification or up-concentration of the samples is generally not required. The laser intensity provides a

gain factor so that signals for dilute isotopes can be amplified relative to the majority species. The LARA system is able to measure the $^{13}CO_2$ enrichment even if the isotope ratio change is in the parts per 100 000 range. Another way to enhance sensitivity is by choosing optimal discharge conditions. An optimum discharge has been found to consist of less than 5% CO_2 in nitrogen. A mixture of CO_2 in nitrogen greatly enhances the optogalvanic effect due to the almost resonant exchange between electron excited N_2 and the upper laser level of CO_2. This is the same reason a CO_2 laser operates with a majority N_2 gas fill.

3.7.4 Gas chromatography-mass spectrometry

The mass spectrometer is a device designed to produce a mass spectrum of a sample to find out its composition. The sample is introduced into the ion source, where it is ionized by a metallic filament to which a voltage is applied. This filament discharges electrons upon collision with the sample molecules, which causes ionization. The stream of ions is then passed through the mass analyzer into the detector. A series of ion lenses is used to focus the ions onto the mass analyser. The mass analyzer separates all the ions of a compound by their mass-to-charge (*m/z*) ratios. For enhanced sensitivity, the mass analyzer is operated in the selected ion monitoring (SIM) mode, where it focuses only on one or a few ions. The mass spectrometer is capable of determining the isotopic composition of a sample with very high precision. For the analysis of ^{13}C-UBT samples, it is operated in the SIM mode for ions 44, 45 and 46 to determine the $^{13}CO_2/^{12}CO_2$ ratio. To further improve the accuracy of the analysis, the mass spectrometer is connected to a gas chromatograph so that CO_2 can be separated from the other components that are present in the breath samples.

3.7.5 Comparison of analytical techniques

With the IRMS, only a small volume (10 ml) of sample is required and, as already mentioned, the precision of measurements is reported to be as high as 0.01 %. However, the cost and complexity of this instrument prevents the widespread implementation of the ^{13}C-UBT.

Non-dispersive infrared spectrometry (NDIRS) and LARA have been proposed as alternative methods for detecting $^{13}CO_2$ in breath. With regards to analytical sensitivity and specificity, both of these show results comparable to the conventional IRMS. NDIRS is the least expensive, but it requires large sample volumes (at least 500 mL). In addition, the NDIRS analyser must be close the breath sample collection site, since the breath bags can't be transported over long distances. Compared to IRMS and NDIRS, the LARA system has the quickest analysis time and a highest throughput of samples.

Kasho et al and Lee et al demonstrated that a gas chromatograph coupled to a quadrupole mass spectrometer (GC-MS) could be applied to accurately measure the $^{13}CO_2/^{12}CO_2$ ratio in breath samples. Resolution and precision, comparable to GC-IRMS, can be achieved by operating the mass spectrometer in the SIM mode, and by using calibration standards with certified isotope ratios. With the use of GC-MS, which is available in many analytical and biomedical laboratories, the ^{13}C-UBT will become more readily available, and at reduced costs.

3.8 Expression of results

Studies involving the use of stable isotopes make use of one of the following three measurement notations when reporting results: “Atom Percent”, “Atom Percent Excess” and “Delta”.

3.8.1 Atom percent

Results from environmental and agricultural studies using isotopically enriched tracers are usually reported in units of atom percent (At%). This value gives the absolute number of atoms of a given isotope in 100 atoms of total element. For example:

$$At\%^{15}N = \frac{^{15}N}{^{14}N + ^{15}N} \times 100\ At\% \qquad \text{[Eq. 3-1]}$$

or,

$$At\%^{13}C = \frac{^{13}C}{^{12}C + ^{13}C + ^{14}C} \times 100\ At\% \qquad \text{[Eq. 3-2]}$$

Note, for the At%^{13}C calculation, the amount of naturally present ^{14}C is usually treated as negligible and the sum of ^{12}C and ^{13}C are used as the total carbon concentration.

3.8.2 Atom percent excess

Medical tracer studies of human physiology are most often reported in units of atom percent excess (APE). This specifies the level of isotopic abundance above a given background reading, which is considered zero. The background reading in At% is subtracted from the experimental value to give APE.

3.8.3 Delta

Studies examining stable isotopes at or near natural abundance levels are usually reported as delta (δ), a value given in parts per thousand or per mil (‰).

Delta values are not absolute isotope abundances but differences between sample readings and one or another of the widely used natural abundance standards which are considered delta = zero (e.g. air for N, At%^{15}N = 0.3663033; Pee Dee Belemnite (PDB) calcium carbonate for C, At%^{13}C = 1.1112328). Absolute isotope ratios (R) are measured for sample and standard, and the relative measured delta is calculated:

$$\Delta\delta\ ^{15}N \text{ vs. (std)} = \frac{R_{(sample)} - R_{(std)}}{R_{(std)}} \times 1000\ (‰) \qquad \text{[Eq. 3-3]}$$

$$\text{Where,} \quad R = \frac{At\%^{15}N}{At\%^{14}N + At\%^{15}N} \qquad \text{[Eq. 3-4]}$$

For the ^{13}C-UBT, the amount of $^{13}CO_2$ excreted is also measured as "δ $^{13}CO_2$ (‰)". This obviates the need for a defined amount of CO_2 to be collected, as is the case with the ^{14}C-UBT. Due to the presence of natural occurring $^{13}CO_2$ in the breath, results are measured against the PDB reference standard. When *H. pylori* is present in the stomach, the relative amount of $^{13}CO_2$ exhaled increases considerably and exceeds that in the standard. Therefore results are expressed as the "excess" (Δ) δ $^{13}CO_2$ relative to the PDB standard according to the following equation:[31]

$$\Delta\delta\ ^{13}CO_2 = \frac{R_{(sample)} - R_{(reference)}}{R_{(reference)}} \times 1000\ (‰) \qquad \text{[Eq. 3-5]}$$

Where $R_{(reference)}$ is At% of the PDB standard. It is possible to measure the increase of $^{13}CO_2$ relative to a patient's baseline breath sample. Then $R_{(reference)}$ will be the mean

of the zero time point breath samples i.e., the patient's baseline abundance of $^{13}CO_2$ on the day of the test.

3.9 Diagnostic and analytical accuracy

Any given laboratory test has not one but two kinds of sensitivity and specificity: analytical and diagnostic. Understanding the different meanings of these terms is key to properly requesting and interpreting diagnostic test results.[51]

The *analytical sensitivity* of an assay is the ability of the assay to detect a low concentration of a given substance in a biological sample. The lower the detectable concentration, the greater the analytical sensitivity. "Limit of detection" is often used as a synonym for analytical sensitivity. Analytical sensitivity may also be expressed in terms of an assay's ability to detect a change in concentration (as is the case with the ^{13}C-UBT). The smaller the detectable change, the greater the analytical sensitivity.

When an assay is applied to a population to detect a condition or disease, *diagnostic sensitivity* becomes relevant. The diagnostic sensitivity of a test is the ability of the test to accurately identify persons with the condition of interest. Diagnostic sensitivity often has more to do with the ability to obtain the target substance in a processed sample from a person who has the condition, than with the ability to detect very low concentrations of a substance. If the target substance is not in the processed sample, because of vagaries of sampling or processing, an assay with perfect analytical sensitivity still fails to give a positive result.

Analytical specificity is the ability of an assay to exclusively identify a target substance, rather than a similar but different substance, within a sample or specimen. When an assay is analytically non-specific, it often produces a positive result when the specimen

is truly negative for the exact agent being sought. This problem will diminish *diagnostic specificity*, which is the ability of an assay to accurately identify a person who does not have the disease in question.

Sensitivity and specificity define the operating characteristics of an assay, but it is the predictive value (positive or negative) of the assay that is generally of diagnostic importance. The positive predictive value (PPV) is the probability that a person whose test result is positive truly has the disease or condition of interest. The negative predictive value (NPV) is the probability that a person, whose test result is negative, truly does not have the disease of interest. Strong diagnostic sensitivity improves negative predictive values, and strong diagnostic specificity improves positive predictive values (regardless of analytical sensitivity and analytical specificity).

The accuracy of a given test is the degree of conformity of a measured or calculated quantity to its actual (true) value (Figure 3-7). Accuracy is closely related to precision, which is the degree to which repeat measurements or calculations show the same or similar results. The results of calculations or a measurements can be accurate but not precise; precise but not accurate; neither; or both. A valid result is one that is both accurate and precise. The accuracy and precision of an analytical test is usually established by repeatedly measuring a certified reference standard. Precision is usually characterised in terms of the standard deviation of the measurements, sometimes called the measurement process's standard error.

Precision is sometimes stratified into: (1) repeatability, which is the variation arising when all efforts are made to keep conditions constant by using the same instrument and operator, and repeating during a short time period; and (2) reproducibility, which is the variation arising using the same measurement process among different instruments and operators, and over longer time periods.

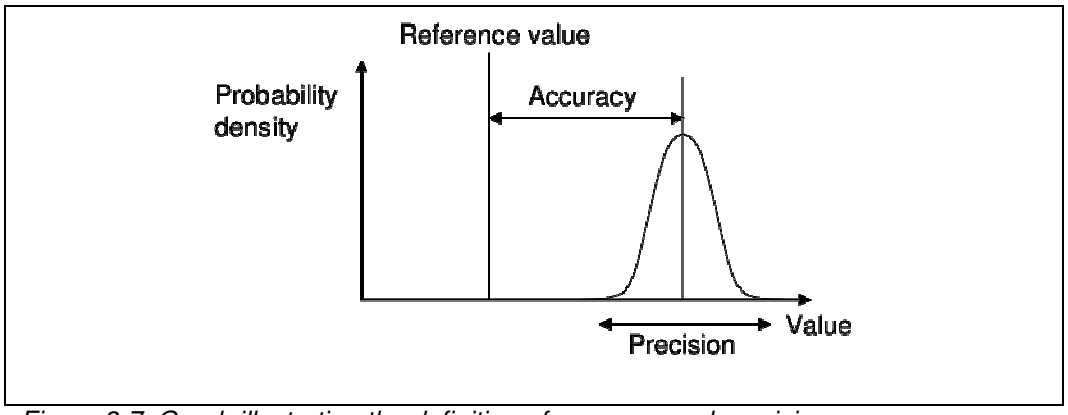

Figure 3-7: Graph illustrating the definition of accuracy and precision.

Calculation of the diagnostic accuracy of an analytical test is shown in Table 3-2.

Table 3-2: Calculation of the diagnostic accuracy of an analytical test.

		Gold standard (Histology)		
		Positive	*Negative*	
^{13}C-UBT	*Positive*	True Positive	False Positive	**PPV** [c]
	Negative	False Negative	True Negative	**NPV** [d]
		SENSITIVITY [a]	**SPECIFICITY** [b]	Total

[a] *Sensitivity = TP/(TP + FN) ;* [b] *Specificity = TN/(TN + FP) ;* [c] *PPV = TP/(TP + FP)*
[d] *NPV = TN/(TN + FN) ; Accuracy = (TP + TN)/Total*

The diagnostic sensitivity and specificity of the ^{13}C-UBT may vary slightly according to the protocol that is used, but is typically around 95% and 98% respectively for adults, in both the pre- and post-treatment setting.[4,13,18,23,26,28,36,37,52,53] The average sensitivity and specificity values reported for children are 95% and 97% respectively,[24,25,30,38,59] which compare well with those reported for adults. Some authors have even proposed that the ^{13}C-UBT is more accurate than the currently accepted gold standard of histology, which is susceptible to sampling errors. This provokes the thought that the sensitivities and specificities reported in the past might be inaccurate. Epple et al[8] have shown that several apparently false positive ^{13}C-UBT results were in fact correct

findings, as clearly shown by additional biopsy samples taken from the same patients who underwent second gastroscopies. The average PPV and NPV reported for adults are 97% and 94% respectively,[23,28,36,52] and those reported for children are 91% and 99% respectively.[30,38]

3.10 Cut-off Δδ values

The cut-off Δδ value is a fundamental element of the ^{13}C-UBT. This value is not fixed but varies with different protocols according to the type of test meal used, the quantity of ^{13}C-urea used, and the time of breath collection. For each new protocol, the ^{13}C-UBT must be evaluated against the gold standard, which is currently accepted as the histological examination of biopsy samples, and sensitivities and specificities must be determined for different cut-off Δδ values. The best cut-off Δδ value is then determined by constructing a receiver operating characteristic (ROC) curve in which the sensitivity is plotted against "1-specificity". In such plots, a rectilinear curve rising steeply to a maximum and then maintaining a plateau is indicative of a test with good discriminative ability (Figure 3-8). The optimal cut-off Δδ value corresponds to the upper, most left hand corner of the curve.

The cut-off Δδ value was originally determined to be 5 ‰ based on the normal distribution of $^{13}CO_2$ enrichment values for *H. pylori* negative subjects who have never been infected. At three SD above the mean, the upper limit for a normal range gave a cut-off value of Δδ = 4.9 ‰.[36,54] Reanalysis of data from several large clinical trials of *H. pylori* eradiation have allowed construction of ROC curves to set the optimum cut-off value according to sensitivity and specificity. On the basis of ROC analysis, a cut-off value of Δδ = 3.5 ‰ changes sensitivity from 96.8% to 98.5% and the specificity from 99.3% to 97.0%. This suggests that this lower Δδ value might be better to use as the cut-off for the ^{13}C-UBT.[55,56]

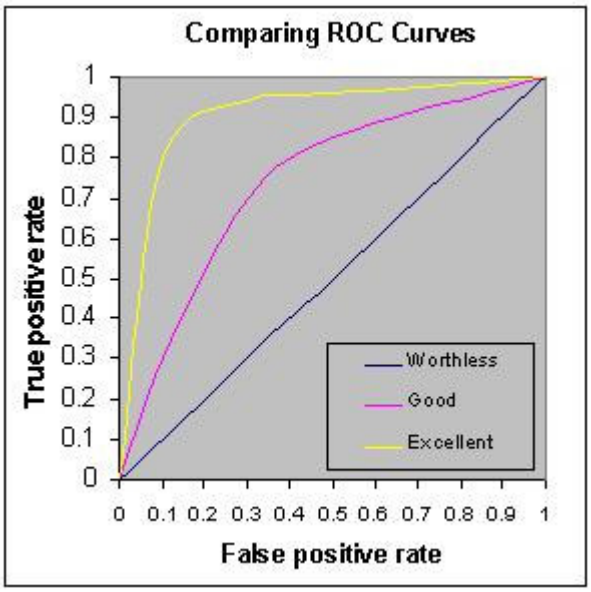

Figure 3-8: The Receiver Operating Characteristic (ROC) curve is constructed to determine the discriminative ability of diagnostic tests.

In cases where greater discrimination exists between positive and negative results, such as when citric acid is given as the test meal, or when the dose is given in tablet form, it might be necessary to further reduce the cut-off point. Hamlet et al reported the optimal cut-off point to be as low as $\Delta\delta$ = 1.8 ‰ for the tablet based ^{13}C-UBT.[26]

When using the ^{13}C-UBT with children, it might be necessary to use different cut-off points for different age groups to minimize the risk of false positive and false negative results. Caution should be taken when interpreting positive results in children younger than 2 years.[57,58] The ^{13}C-UBT has been validated in children giving sensitivity and specificity above 97% using a cut-off of $\Delta\delta$ = 3.5 ‰.[25,59]

The proposed cut-off values are derived from data collected using unique protocols for each ^{13}C-UBT. Any proposed "improved" cut-off value is not valid and can only be used as a guideline until the hypothesis has been tested with new samples.

3.11 Applications

3.11.1 Assessment of *H. pylori* status

Both the ^{13}C-UBT and serological examination by enzyme-linked immunosorbent assay (ELISA) are accurate non-invasive tests for the diagnosis of *H. pylori* infection. The difficulty with serology, however, is that the test may remain positive for months, even years, after the bacterium has been eradicated, and therefore has difficulty in distinguishing between past and present infection. Consequently, the ^{13}C-UBT is the only non-invasive test that can give information regarding the current *H. pylori* status. This unique feature of the ^{13}C-UBT makes it particularly useful in the following clinical situations: (1) all instances where the knowledge of *H. pylori* status is required; (2) when an ulcer is detected on ingestion of a barium meal; (3) during endoscopy, where biopsies cannot be obtained owing to the patient being on anticoagulant therapy; and (4) in children, before endoscopy is performed, as an alternative to serological testing.[28]

3.11.2 Research

During the last century, extensive research has been done on the subject of *H. pylori*. The ^{13}C-UBT has become the method of choice in epidemiological studies since it is easy to perform, relatively cheap, and capable of assessing current *H. pylori* status.[28] It is progressively replacing the serological test (which requires a blood sample), especially in studies that involve children. The ^{13}C-UBT is considered to be the best non-invasive test to accurately determine the success of eradication therapy, therefore forms an integral part of the diagnostic tests utilized in clinical trials involving eradication therapies.[4]

Some authors have suggested that the ^{13}C-UBT are capable of accurately quantifying the extent of bacterial colonization, and therefore the severity of the disease.[30,37,60,61] In an ideal UBT, *H. pylori* urease would be saturated and a steady state of urea hydrolysis would be reached which would accurately reflect total gastric urease activity.

In contrast to this, Atherton et al[12] suggested that accurate assessment of bacterial load by the ^{13}C-UBT is unlikely for the following reasons: (1) carbon dioxide production varies widely between subjects and is further varied by the test meal; (2) the contribution of endogenous urea to the substrate pool is unknown and presumably may vary between patients; (3) not all of the carbon dioxide produced in the stomach is excreted by the lungs; (4) there is wide variation in urease activity between *H. pylori* strains in vitro[22]; and (5) other bacterial strains like *Helicobacter Heilmanii* can also contribute to the observed urease activity. Therefore, the ^{13}C-UBT can only be applied as a quantitative tool when repeated in the same individual, since the same strain of *H. pylori* is present, and the above-mentioned inaccuracies are likely to be constant between tests.

3.11.3 Follow up after treatment

Once diagnosed with an *H. pylori* infection, patients are treated to eradicate the bacterium. There is currently no therapy that is 100% effective. Therapy for *H. pylori* infection (80 to 85% cure rate) typically consists of 10 days to 2 weeks of one or two effective antibiotics, such as amoxicillin, tetracycline (not to be used for children < 12 yrs.), metronidazole, or clarithromycin, plus either ranitidine bismuth citrate, bismuth subsalicylate, or a proton pump inhibitor (PPI). Acid suppression by the H_2 blocker or proton pump inhibitor in conjunction with the antibiotics helps alleviate ulcer-related symptoms (i.e., abdominal pain, nausea), helps heal gastric mucosal inflammation, and may enhance efficacy of the antibiotics against *H. pylori* at the gastric mucosal surface.

After treatment, follow up tests are needed to confirm that the bacterium has been successfully eradicated. Currently, only the ^{13}C-UBT and endoscopy with histological examination of biopsy specimens are tests accurate enough to confirm eradication. The follow-up required depends on the nature of the underlying disease. The ^{13}C-UBT can be used as the sole method for follow-up in dyspeptic patients, in those with a family history of gastric cancer, and in cases of uncomplicated duodenal ulcer, because cure of *H. pylori* infection is associated with resolution of histological gastritis and prevention of relapse of duodenal ulcer. Patients who have had previous duodenal ulcer complications, gastric ulcers, and ulcers associated with non-steroidal anti-inflammatory drugs should be followed up by endoscopy.[28]

All the drugs that are active against *H. pylori* in vivo can reduce the sensitivity of the ^{13}C-UBT. It might happen that the bacterium may not have been eradicated, but only temporarily suppressed, thus producing false negative results. Therefore, before interpreting a negative result it is necessary to know the class, dose, and duration of drug intake. It is also recommended that the test must be performed at least four weeks after therapy comprising of antibiotics with PPI's or bismuth preparations,[11,24,35] and two weeks after therapy with only PPI's or H_2 Blockers.[4,62,63,64]

3.12 Conclusion

The ^{13}C-UBT is a non-invasive, extremely simple and safe test, which can be performed repeatedly without risk on the same patient, children and pregnant women. The limiting factor, preventing the widespread implementation of the ^{13}C-UBT, is principally due to the high cost of the IRMS needed for $^{13}C/^{12}C$ isotope ratio analysis. This, however, has been virtually eliminated with the recent introduction of cheaper and just as accurate alternative instrumentation for isotope ratio analysis.

Overall, the ^{13}C-UBT offers considerable savings for the patient, both in terms of money and discomfort. It provides excellent accuracy for both the initial diagnosis of *H. pylori* infection and for the confirmation of its eradication after treatment.

3.13 References

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CHAPTER 4

EXPERIMENTAL PROCEDURES

"*An investigator starts research in a new field with faith, a foggy idea, and a few wild experiments. Eventually the interplay of negative and positive results guides the work. By the time the research is completed, he or she knows how it should have been started and conducted. "* - ***Donald Cram***

4.1 Introduction

Isotope ratio mass spectrometry (IRMS) is considered to be the gold standard in isotope ratio measurement. The high cost and complexity of IRMS guided the development of alternative techniques for measurement of the $^{13}CO_2/^{12}CO_2$ ratio in ^{13}C-UBT samples.

Kasho et al.[1] and Lee et al.[2] have suggested the use of gas chromatography-mass spectrometry (GC-MS) as one such alternative for analyzing ^{13}C-urea breath test (^{13}C-UBT) samples. GC-MS, however, is not a technique that is frequently used to obtain isotope ratios of compounds with low molecular weights (e.g. CO_2). Rather, the more general uses of GC-MS include: (1) identification and quantitation of volatile and semi-volatile organic compounds in complex mixtures; (2) determination of molecular weights and (sometimes) elemental compositions of unknown organic compounds in complex mixtures; and (3) structural determination of unknown organic compounds in complex mixtures, both by matching their spectra with reference spectra and by a priori spectral interpretation.

The purpose of this research was: (1) to develop and optimize a GC-MS method for accurate measurement of the $^{13}CO_2/CO_{2\ (total)}$ ratio in breath samples; (2) to apply the optimized GC-MS ^{13}C-UBT method in a clinical setting, and to compare the results obtained with that of the gold standard, histology, to evaluate it's ability to accurately diagnose *H. pylori* infection; (3) to utilize the clinical and demographic data from the

patient population to investigate the existence of the "African enigma"; and (4) to use this information to outline what the possible impact of implementation of a "test-and-treat" strategy for *H. pylori* in South Africa would be.

4.2 Materials

Headspace vials and caps were purchased from Gerstel (Mulheim an der Ruhr, Germany). Sigma Aldrich (Steinheim, Germany) supplied the citric acid. Urea enriched with ^{13}C (99%), and $^{13}CO_2$ calibration standards containing 5% CO_2 in air, were purchased from Cambridge Isotope Laboratories (CIL) (Andover, Massachusetts, USA). Argon (99.99%) and Carbon dioxide (3.6% in air) were obtained from AFROX (Pretoria, South Africa). Tedlar bags (1 L) were acquired from Supelco (Bellefonte, USA).

4.3 Patients

One hundred and eleven (111) patients, presenting with dyspepsia, referred to the Gastroenterology Department of the Pretoria Academic Hospital, participated in the study. None of these patients used any antibiotics, proton pump inhibitors (PPI's) or bismuth preparations in the four weeks prior to endoscopy. Eight of the 111 patients were excluded (five patients with previous major upper gastro-intestinal tract surgery, two patients with incomplete records, and one patient with a non-representative histology sample). Histological examination, the rapid urease test, and the ^{13}C-UBT were used to obtain the *H. pylori* status of the remaining 103 patients. Ethical approval for this study was obtained from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria, South Africa (Reference number: S17/2004).

4.4 Calibration standards

Certified $^{13}CO_2$ calibration standards containing 5% CO_2 in air were used to determine the accuracy with which the GC-MS can determine the $^{13}CO_2$ enrichment in breath samples. The Baseline Calibrant Gas was certified to have a delta value (δ) of –22.8 against the Pee Dee Belemnite (PDB) Standard. The $^{13}CO_2$ enrichment of the mid- and high-level calibrant gases was certified to be 9.58 ‰ (SD = 0.12 ‰) and 15.5 ‰ (SD = 0.9 ‰) respectively above the baseline calibrant gas.

Headspace vials were completely purged with Argon and capped immediately to remove as much as possible of the atmospheric gasses. The vials were then loaded with a small amount of calibrant gas to be analyzed by GC-MS.

4.5 ^{13}C-urea breath test

All patients had to be fasting from 10 pm the previous night. At the onset of the procedure, duplicate baseline breath samples (T+0) were taken. Patients were asked to take a deep breath and to exhale through a straw into a glass vial. The vial was capped immediately and extreme care was taken not to lose any of the breath sample in the capping process. Patients then swallowed the test meal: citric acid (50 mL; 0.1 M) containing ^{13}C-urea (75 mg). The cup was rinsed with the citric acid solution (50 mL; without ^{13}C-urea) to ensure that all of the ^{13}C-urea was consumed. Thirty and forty minutes after the dose was consumed, patients were asked to provide the "T+30" and "T+40" breath samples.

4.6 Endoscopy

Gastroscopies were performed after completion of the ^{13}C-UBT. Endoscopies, with local sedation, were performed with a PENTAX EG-2770K endoscope (PENTAX Medical Company, Montvale, NJ, USA). Single use biopsy forceps (MTW, Germany) were used to take the biopsy specimens. Due to the patchy nature of *H. pylori* infection, two biopsy specimens were obtained from each patient: one from the antrum and the second from the corpus on the greater curvature. Biopsy specimens were tested for the presence of *H. pylori* with histology and the rapid urease test.

4.6.1 Histology

The staining technique utilized is based on the one employed by Genta et al.[3] Paraffin sections of biopsy specimens were dewaxed in xylene and transferred to distilled water. Sections were pre-rinsed in acid water (pH = 4.2) and incubated in 1% $AgNO_3$. *H. pylori* staining were developed in a Gelatius-Silver Nitrate-Hydroquinone solution to a light yellow-brown color. Sections were toned in $AuCl_4$ and staining was fixed in $Na_2S_2O_3$. Sections were counter stained in a Haematoxylin-Eosin sequence, dehydrated and mounted. The *H. pylori* organisms appear black against a pink background, and the cell nuclei are blue. Biopsy sections were interpreted by an independent pathologist, blinded to results of the rapid urease test and ^{13}C-UBT.

4.6.2 Rapid urease test

Biopsy samples taken during the gastroscopy were also tested for the presence of *H. pylori* by a rapid urease test (HpOne, GI Supply, Camp Hill, Pennsylvania). The biopsy is placed in a well and allowed to react with reagent containing urea, a pH

indicator and hydrogen peroxide. This solution is yellow in color. The following reaction takes place in the presence of *H. pylori* urease:

$$\underset{\textit{Urea}}{(NH_2)CO} + 2H_2O + H^+ \rightarrow \underset{\textit{Ammonium}}{2NH_4^+} + \underset{\textit{Bicarbonate}}{HCO_3^-}$$

The ammonium ion causes a decrease in pH, which in turn will cause the color of the reaction medium to change from yellow to blue. If *H. pylori* organisms were present, the reagent in the tray well turned blue. An unchanged yellow color indicates the absence of the urease enzyme, and a negative result for *H. pylori*. For interpretation of a negative test, the readings should be taken at not more than an hour after the addition of the reaction medium.

4.7 Mass spectrometry

Duplicate breath samples were obtained from eighteen, randomly selected patients. For comparison of accuracy, one set of samples was analyzed by means of GC-MS and the other by GC-IRMS.

4.7.1 Gas chromatography-mass spectrometry

A HP6890 GC system fitted with a Gerstel MPS2 Auto sampler and a HP5973 *inert* mass-selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) was used for GC–MS analysis. Data collection and integration was performed with HP Chem Station software. A GS-Carbon PLOT (J & W Scientific, Agilent Technologies, Palo Alto, CA, USA) (30 m X 320 µm; d_f 3 µm), capillary column was used. Tuning of the MSD was performed with perfluorotributylamine (PFTBA) for masses 69, 219, and 502 using the auto tune option.

A 100 µL volume of breath sample was injected in the splitless mode with a gas tight syringe. Helium was used as the carrier gas at a constant flow-rate of 2 mL/min (52 cm/s). The inlet temperature was set at 50 °C and the oven was kept isothermal at 40 °C. The total chromatographic time was 3 min. The transfer line temperature was set at 280 °C and that of the quadrupole at 106 °C. The source temperature was 230 °C. A solvent delay time of 1.75 min was used to allow for elution of oxygen before the source was turned on. Mass spectra were recorded at 70 eV. Quantitation was performed in the selected ion monitoring (SIM) mode with a dwell time of 50 ms. Ions with m/z = 44, 45 and 46 were employed to calculate the $^{13}CO_2/CO_{2\ (total)}$ ratio (Eq. 4.1).

$$^{13}CO_2/CO_{2\ (total)} = \frac{45}{(44+45+46)} \times 100 \qquad \text{[Eq. 4.1]}$$

The $^{13}CO_2$ enrichment in the "T+30" and "T+40" breath samples, relative to the baseline breath samples, were calculated as follow (Eq. 4.2):

$$\Delta\delta\ ^{13}CO_2 = \frac{R_{(sample)} - R_{(reference)}}{R_{(reference)}} \times 1000\ (‰) \qquad \text{[Eq. 4.2]}$$

Where, $R_{(sample)}$ is the $^{13}CO_2/CO_{2\ (total)}$ ratio in the "T+30" or "T+40" breath samples, and $R_{(reference)}$ is the average of the $^{13}CO_2/CO_{2\ (total)}$ ratio in the duplicate baseline breath samples.

4.7.2 Gas chromatography-isotope ratio mass spectrometry

The duplicate set of breath samples were sent to the Erasmus Medical Centre (Rotterdam, The Netherlands) to be analyzed by IRMS (ABCA, SerCon Ltd, Crewe, UK). A 5 mL volume of breath sample, from septum capped containers, was purified by a simple chemical water trap and gas chromatograph before flowing directly into

the mass spectrometer for measurement of the $^{13}CO_2$ enrichment. The GC was kept isothermal at 50 ℃ and the helium flow rate was 20 mL/min. The mass spectrometer contains universal Faraday triple collectors for the simultaneous collection of adjacent masses 44, 45 and 46. Enrichment of $^{13}CO_2$ in the breath samples were reported as "excess delta" ($\Delta\delta$) against the Pee Dee Belemnite (PDB) international reference standard, which are considered to have a $\Delta\delta$ = 0 ‰. Equation 4.2 was also applied to calculate the $^{13}CO_2$ enrichment in IRMS breath samples. In these measurements, $R_{(sample)}$ is the $^{13}CO_2/CO_{2\ (total)}$ ratio in the baseline, "T+30" or "T+40" breath samples, and $R_{(reference)}$ is the $^{13}CO_2/CO_{2\ (total)}$ ratio of the PDB standard. The average $^{13}CO_2$ enrichment in the two baseline samples is subtracted from that of the average of the "T+30" and "T+40" samples to calculate the $^{13}CO_2$ enrichment. Breath samples were considered to be positive if $\Delta\delta \geq 3.5$ ‰.

4.8 Gas chromatography inlet techniques

Gas chromatography is most often applied to liquid samples, which have to be volatilized before being introduced into the GC. Breath samples have the advantage of not having to be volatilized, however, when analyzing CO_2, extra caution must be applied to ensure that atmospheric CO_2 does not interfere with the sample. Therefore, several modes of sample introduction were explored. In addition, all of the components of the sample introduction system were optimized to ensure accurate analysis of the breath samples.

4.8.1 Six-port valve gas inlet

A six-port valve sample introduction system was customized to introduce gaseous samples into the GC (Figure 4-1).

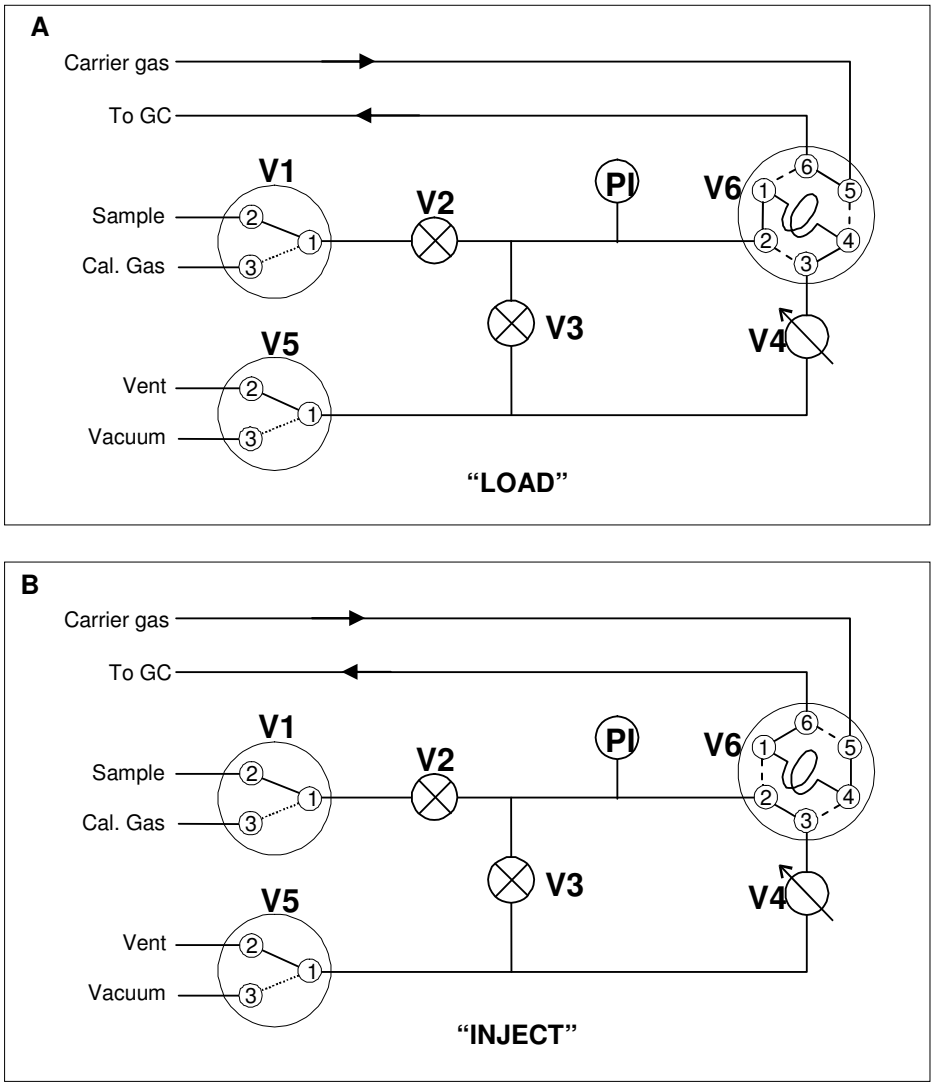

Figure 4-1: Schematic diagram of the gas inlet. The sample loop is loaded with breath sample or calibration gas (A) after which the six-port valve is switched to the "INECT" position (B) so that the carrier gas can transfer the sample into the GC column.

The gas inlet allowed for both static and dynamic sampling at, and below, atmospheric pressure. To ensure that there was no interference from atmospheric gasses, the system was completely evacuated. When breath samples were loaded, the six-port valve was in the "LOAD" position, the three-way valves V1 and V5 were switched to the sample and vacuum positions respectively, and the two-way valves V2, V3 and V4 were opened. Once the entire manifold was evacuated, V5 was turned to a position mid-way between "vacuum" and "vent". The manifold was filled with sample by opening the sample container slowly and then closing it immediately. Thereafter the

manifold was evacuated again. For critical quantitative analyses, as is the case with the ^{13}C-UBT which requires very high precision measurements, the process was repeated several times to ensure that the system was completely flushed with the sample. When the system was ready for the sample to be injected, valve V3 was closed to ensure that the sample went through the sample loop. The pressure at which the sample was injected was regulated by the vent valve, for samples above ambient pressure, or by the vacuum valve, for samples below ambient pressure. The six-port valve was switched to the "INJECT" position, and the GC carrier gas transferred the sample into the GC.

4.8.2 Gas-tight syringe

A 100µL GERSTEL gas-tight syringe was used to inject breath samples into the GC. The split/splitless inlet was used. The syringe was operated both manually and with the autosampler.

4.8.3 Gas tight syringe with cryogenic pre-concentration

Breath samples were injected with the 100 µL gastight syringe through a Cooled Injection System (CIS) with a septumless sampling head. Temperature programmed sample injection provides increased versatility, reproducibility and lowered detection limits to GC analysis. The CIS is equipped with liquid nitrogen, which allows for fast operation cycle time.

4.9 Selection of the GC column

The PoraPLOT QS (d_f = 530µm), GS-CarbonPLOT (d_f = 530µm) and GS-CarbonPLOT (d_f = 320µm) were employed in an attempt to separate CO_2 from other atmospheric gases.

4.10 Validation of the ^{13}C-UBT GC-MS method

4.10.1 Analysis of $^{13}CO_2$ calibration standards

The $^{13}CO_2$ calibration standards containing 5% CO_2 in air were employed to determine the accuracy with which the GC-MS can measure the $^{13}CO_2$ enrichment in breath samples. Each of the three calibration standards were injected multiple times so that the within batch repeatability and precision of GC-MS measurements could be determined. The standard deviation (SD) and coefficient of variation (CV) for repeated measurements of the $^{13}CO_2/CO_{2\ (total)}$ ratio in the $^{13}CO_2$ calibration standards were calculated. Whether or not the GC-MS can reproduce the certified enrichment values of $^{13}CO_2$ in the mid- and high-level calibration standards were also examined.

4.10.2 Repeatability of GC-MS in analyzing patient breath samples

Multiple injections from the breath samples of two patients were performed to determine the within-batch repeatability. The SD and CV for repeated measurements of the $^{13}CO_2/CO_{2\ (total)}$ ratio were calculated.

4.10.3 Determination of the optimal cut-off Δδ value

Sensitivities and specificities obtained by using different cut-off Δδ values (Δδ = 0 – 10 ‰) were used to construct a Receiver Operating Characteristic (ROC) curve. The curve was then used to determine the optimal cut-off Δδ value that discriminates best between *H. pylori* positive and negative patients.

4.10.4 Determination of the $^{13}CO_2$ excretion profile

The excretion profile of $^{13}CO_2$ in breath, after ingestion of ^{13}C-urea, was determined by taking breath samples every 10 minutes for an hour. Knowledge of the $^{13}CO_2$ excretion profile assisted in obtaining the optimal sampling time and to minimize the number of false negative results.

4.10.5 Stability of breath samples

Stability of the breath samples were determined by storing them at room temperature for one week, and then analyzing them for the first time. The accuracy of these results was confirmed by comparing them with the histology results of the same patients. Stability of the samples was also determined by re-analyzing them 5 weeks after the first analyses were performed.

4.10.6 Amount of ^{13}C-urea dose

^{13}C-urea is very expensive in South Africa, and is one of the major cost limiting factors of the ^{13}C-UBT. Currently, the smallest dose accepted to give accurate results is 75 mg ^{13}C-urea for adults, and 50 mg ^{13}C-urea for children. In protocols where the dose is administered in a tablet or capsule form, as little as 35 mg ^{13}C-urea is used.[4,5] It would

be beneficial if the dose can be reduced considerably below the accepted 75 mg without compromising the accuracy of the test. The ^{13}C-UBT was performed with a ^{13}C-urea dose of 75 mg as well as with a dose of 35 mg.

4.10.7 Accuracy of GC-MS compared to GC-IRMS

GC-IRMS is considered to be the gold standard for measuring the $^{13}CO_2/CO_{2\ (total)}$ ratio in breath samples. Duplicate breath samples from eighteen, randomly selected patients, were analyzed by GC-MS and GC-IRMS respectively. Least squares regression statistics were used to compare the results obtained by GC-MS to those obtained by GC-IRMS. Comparison of the results from these two methods gave a good indication of the accuracy with which GC-MS could perform ^{13}C-isotope analysis.

4.11 Assessing the diagnostic accuracy of the ^{13}C-UBT GC-MS method

The same GC-MS method used to analyze the $^{13}CO_2$ calibration standards was also used to analyze the ^{13}C-UBT samples. Results obtained from the ^{13}C-UBT of 103 patients were compared with those obtained from the histology and the rapid urease tests. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the ^{13}C-UBT and rapid urease test, relative to the histology results, were obtained.

4.12 Investigation of the existence of the "African enigma"

Studies on the "African enigma" pertain largely to black Africans. In order to investigate its relevance in non-black Africans, the study participants were stratified according to race as either black or non-black. Further stratification was according to age, gender, *H. pylori* status and pathology. Descriptive statistics were compared to published

statistics, and a logistic regression was performed to calculate the odds ratios of the stratification factors for the development of either peptic or duodenal ulcers.

4.13 Impact of implementing the ^{13}C-UBT in South Africa

A decision tree was used to assess what the financial impact would be if a "test-and-treat" strategy was to be implemented in patients with a low risk for gastric cancer. The costs of the "test-and-treat" and early endoscopy strategies were calculated by multiplying the cost components with their respective probabilities of occurring. All of the probabilities were calculated from the data of patients younger than 50 years of age, except for the probabilities associated with a failed "test-and-treat" strategy, which could not be calculated from the data. These probabilities would have to be based on patient satisfaction with the treatment received, and not on the probability of successful eradication of *H. pylori*, or cure of the pathological process. It will depend on the parameters used to measure satisfaction and the time over which the follow up is conducted. Estimates of these were conservative and similar to that of previous cost models.[6] To keep the number of branches manageable, the tree ends when approximate equivalence in treatment received is achieved between the two strategies. For example: Consider two patients, A and B. Patient A underwent an endoscopy with subsequent histology testing, after failed triple therapy. Patient B initially underwent an endoscopy with histology testing, and subsequently received triple therapy. These two patients are considered to have received approximately the same treatment, since it is only the order of events that is not the same. The model does not take account of the occurrence of gastric cancer since the probability of its occurrence in young patients is extremely low and the delay in treatment caused by a "test-and-treat" strategy may not change the outcome.[7] All costs were obtained from the private sector. The cost of endoscopy includes all costs, as well as the physicians' fee. The cost of histology was kept separate. The average prices of all drugs were based on proprietary drugs

(generic equivalents were not used). The cost saving was calculated as the difference between the endoscopy strategy and the ^{13}C-UBT strategy.

The benefit of *H. pylori* eradication, in terms of symptom alleviation, is well established in patients with peptic ulcers and gastritis. At the gastroenterology unit of Pretoria Academic Hospital patients presenting with upper gastrointestinal symptoms requiring endoscopy, are tested for *H. pylori*, only if inflammation is present. Patients with no macroscopic findings are not tested. The decision tree reflects this practise and no distinction were made between ulcers and inflammatory/atrophic pathology.

The probabilities and cost estimations of the decision tree were subjected to a Monte Carlo simulation. For each of the probabilities and costs in the decision tree a random alternative was generated from a specified underlying distribution. The underlying distributions were assumed to be normally distributed with parameters obtained from the study sample data (the means are the calculated probabilities of the decision tree and the standard deviations are the standard error of the probabilities calculated as $[p*(1-p)/n]^{0.5}$, where p is the calculated probability and n is the number of subjects used in the calculation). The standard deviations of the failed "test-and-treat" strategy probabilities were set arbitrarily to 40% of the mean, which should account for the uncertainty related to these probabilities. The standard deviations of all costs were set arbitrarily to 20% of the proposed cost. All generated probabilities associated with a specific event were mutually exclusive, collectively exhaustive, and limited to values between zero and one. The simulation was run for a total of 5 000 iterations. The distribution of the 5 000 calculated cost savings provides information on the robustness of the "test-and-treat" strategy as the preferred strategy to errors in the estimated probabilities and costs. Multiple regression, using the randomly generated inputs as independent variables and the cost saving as the dependant variable, were performed to estimate the sensitivity of the cost saving to the various inputs.

4.14 Conclusion

The purpose of this research was to develop a GC-MS method, and accurately apply it to measure the $^{13}CO_2/CO_{2\ (total)}$ ratio in breath samples. Furthermore, the ^{13}C-UBT was optimized with regards to the ^{13}C-urea dose, sampling time and frequency, and cut-off points. This will allow our laboratory to aid in the identification of *H. pylori* infected patients. More importantly, since the ^{13}C-UBT is considered to be the most accurate non-invasive test in the post-treatment setting, it will also assist in identifying whether or not treatment to eradicate the organism was successful. Investigating the existence of the African enigma, and indicating the cost saving of the ^{13}C-UBT strategy, will shed some light on the feasibility of utilizing the ^{13}C-UBT in a non-invasive "test and treat" strategy in South Africa.

4.15 References

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CHAPTER 5

RESULTS AND DISCUSSION

5.1 Optimization of the gas chromatography system

Measurement of the $^{13}CO_2/CO_{2\ (total)}$ ratio in breath samples can be very complicated due to interferences from atmospheric CO_2. It was therefore imperative to carefully develop and optimize all of the GC-MS parameters to ensure that measurements were accurate.

5.1.1 Sample introduction system

5.1.1.1 Six-port valve gas sample introduction system

A customized six-port valve gas sample introduction system (Figure 5-1) was initially used to introduce a "3.6 % CO_2 in air" gas mixture, and the Cambridge Isotope Laboratories (CIL) $^{13}CO_2$ calibration standards, into the GC. Before analysis, the system was evacuated completely (as described in Section 4.8.1) and flushed with nitrogen. This process was repeated at least twice to ensure elimination of atmospheric interferences.

Ten injections of the 3.6 % CO_2 gas mixture gave very repeatable results. An average $^{13}CO_2/CO_{2\ (total)}$ ratio of 1.58, with a standard deviation (SD) of 0.008, and a coefficient of variation (CV) of 0.54 % was obtained. Analysis of the $^{13}CO_2$ enrichment in the calibration gases, however, indicated the analytical accuracy of the gas inlet to be very poor (Table 5-1).

Table 5-1: Precision of ^{13}C enrichment measurements of the CIL $^{13}CO_2$ calibration standards using a six-port valve gas inlet.

	CIL $^{13}CO_2$ calibration standards (IRMS)	CIL $^{13}CO_2$ calibration standards (GC-MS using a six port valve gas inlet)
Midlevel Calibration Gas ($\Delta\delta$ (‰) ± SD)	9.58 ± 0.12	11.34 ± 9.06
High-level Calibration Gas ($\Delta\delta$ (‰) ± SD)	15.5 ± 0.9	24.32 ± 3.37

The CV for the midlevel and high-level calibration standards was 79.88 % and 13.88 % respectively.

Even if good accuracy could have been achieved with the gas inlet, there is still the disadvantage of it requiring large sample volumes. Breath samples would typically have to be collected in 1 L Tedlar bags that can be connected to the gas inlet. Using breath bags is expensive as well as uncomfortable (if not impossible) to ship over long distances. In addition, the increased utilization of the $^{13}CO_2$ calibration standards, which are very expensive, would also have reduced the cost effectiveness of the ^{13}C-UBT.

5.1.1.2 Manual vs. autosampler injections

The $^{13}CO_2$ calibration standards were also introduced into the GC with the 100 µL gas tight syringe. Sampling was done both manually (from the Tedlar bags), and with the autosampler (from the headspace vials). The probability of atmospheric interferences when using the Tedlar bags is little, since the manufacturer already evacuates them. The headspace vials, however, were purged with Argon to remove interfering atmospheric gases, after which they were loaded with the calibration standards. A comparison of the performance of the injection techniques from these two sample containers is given in Table 5-2.

Table 5-2: Analysis of CIL $^{13}CO_2$ calibration standards using the 100 µL GERSTEL gas tight syringe both manually and with the autosampler.

	CIL $^{13}CO_2$ calibration standards (Manual sampling from Tedlar bags)	CIL $^{13}CO_2$ calibration standards (With autosampler from headspace vials)
Midlevel Calibration Gas (Δδ (‰) ± SD)	9.11 ± 2.23	9.21 ± 0.20
High-level Calibration Gas (Δδ (‰) ± SD)	15.81 ± 0.36	15.52 ± 0.31

It can be seen that accurate results were obtained by operating the syringe both manually and with the autosampler. The type of container in which the breath samples are collected does not seem to influence the accuracy of GC-MS analysis. Using the gas tight syringe with the autosampler has the advantage that samples can be analyzed overnight without the analyst being present. Samples have to be collected, however, in a container that is compatible with the autosampler. It might happen that breath samples is collected in a container unsuitable for use with the autosampler, in this case, injections will have to be done manually.

5.1.1.3 Split/splitless injections vs. Cooled Injection System injections

The $^{13}CO_2$ calibration standards were also injected with a 2.5 mL gas tight syringe through a cryogenic pre-concentration system - the GERSTEL Cooled Injection System (CIS). A large volume of the calibration gas is injected into the CIS, which is at an initial temperature of – 50 °C. This causes the sample to form a "plug" in the inlet. The inlet is then rapidly heated to a temperature of 120 °C so that the sample can be released onto the column (Figure 5-1). The practice of cooling and rapid heating of the inlet minimizes peak broadening when large sample volumes are injected.

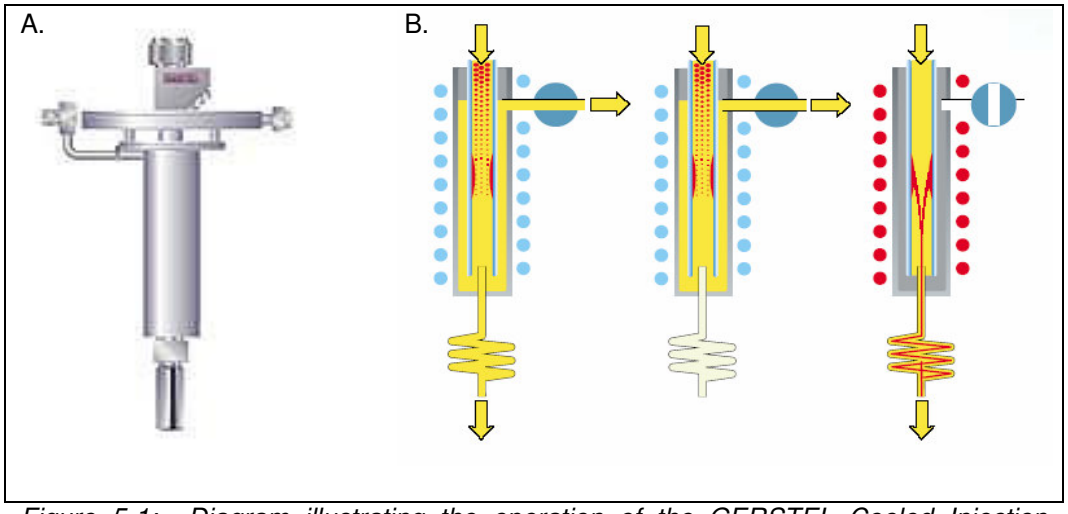

Figure 5-1: Diagram illustrating the operation of the GERSTEL Cooled Injection System (CIS).

Fifteen injections using the gas tight syringe with the CIS were made over a period of 5 days (Figure 5-2). Analysis of the CIL $^{13}CO_2$ calibration standards using the CIS compared well with those where the split/splitless inlet was utilized (Table 5-3). The CV was 9.41 % and 6.56 % for the midlevel and high-level calibration standards respectively. Results can be reported with a 95 % confidence of 0.49 ‰.

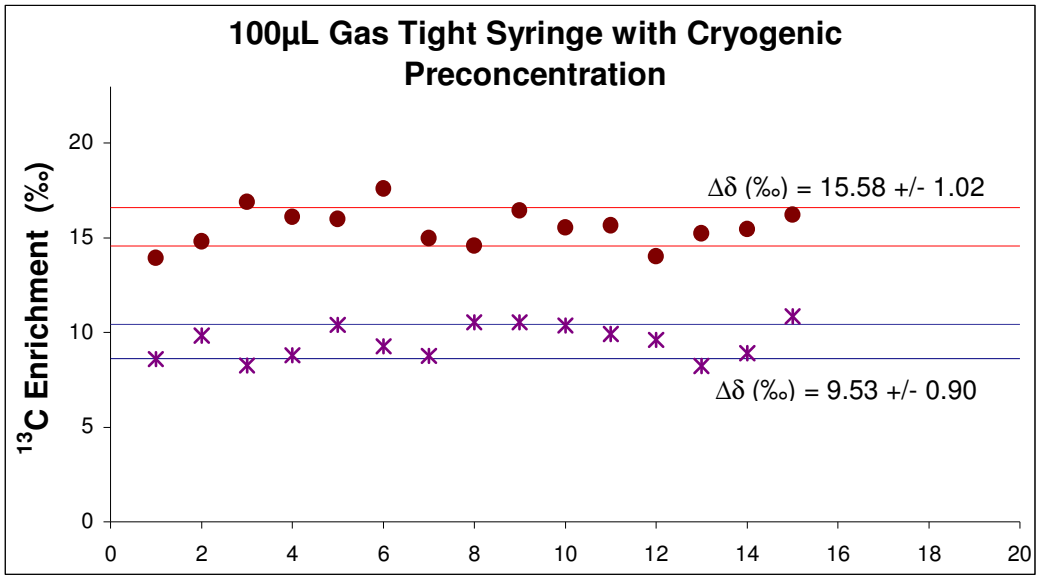

Figure 5-2: Levey-Jennings graph illustrating the day-to-day repeatability of the $^{13}CO_2$ calibration standards when using a cryogenic pre-concentration inlet system.

Table 5-3: Comparison of CIL $^{13}CO_2$ calibration standards by injecting into the CIS and split/splitless inlet.

	$^{13}CO_2$ calibration standards (CIS)	$^{13}CO_2$ calibration standards (split/splitless inlet)
Midlevel Calibrant Gas (Δδ (‰) ± SD)	9.53 ± 0.90	9.44 ± 0.78
High-level Calibrant gas (Δδ (‰) ± SD)	15.58 ± 1.02	15.34 ± 0.75

The initial thought behind using the CIS was to obtain a higher signal-to-noise ratio than by just using the normal split/splitless inlet. This however was not the case. In fact, the difference in the accuracy of measurements observed between using the CIS and using the split/splitless inlet is negligible. Therefore, since not all GC-MS

instruments are equipped with a CIS, it was decided to rather do the $^{13}CO_2$ measurements through the usual split/splitless inlet.

5.1.1.4 Maintenance of the sample introduction system

Several additional components of the sample introduction system need to be carefully maintained to ensure accurate $^{13}CO_2/CO_{2\ (total)}$ ratio measurements. Air leaks into the system can be introduced by the GC inlet septum in the split/splitless inlet, the graphite ferrule securing the column in the inlet and the plunger of the gas tight syringe.

Multiple injections through the same GC inlet septum and very hot inlet temperatures will cause the septum to wear out. As a result, the atmospheric CO_2 that diffuses through the septum with each injection will eventually result in inaccurate measurements of the $^{13}CO_2/CO_{2\ (total)}$ ratio. Figure 5-3 compares the accuracy of injections done with a "worn out" and with a new septum. It is clear that the GC inlet septum can be a major source of interference if not replaced at regular intervals.

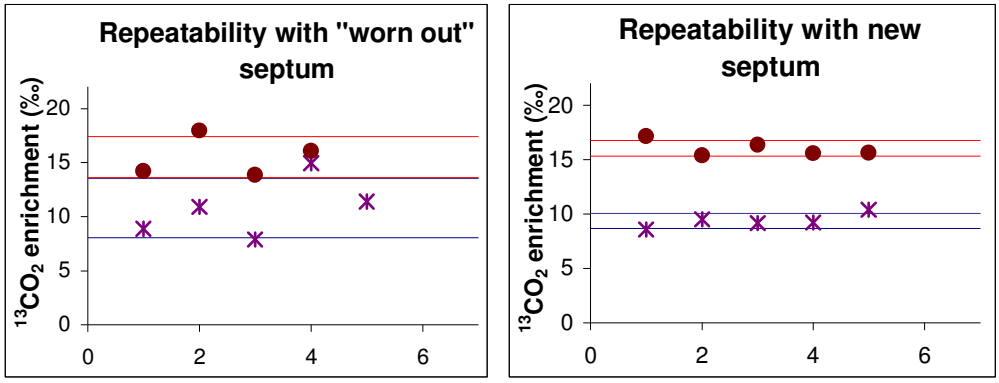

Figure 5-3: The influence of a poorly maintained GC inlet septum on the within batch repeatability of the $^{13}CO_2$ calibration standards.

Furthermore, it would make sense that if the Teflon tip of the plunger doesn't make an airtight seal with the glass walls of the syringe, or if the graphite ferrule doesn't make an airtight seal between the column and the column nut, interference from atmospheric

CO_2 will once again be the source of inaccuracy. It is important that these two variables are carefully examined at regular intervals to ensure that they are still in proper working condition.

Hence, optimizing sample introduction is crucial to eliminate all interferences. Samples can be injected either manually or with the autosampler, depending on what the circumstances necessitate. From the results obtained with the gas inlet, it is clear that any interference from atmospheric gases will yield inaccurate results. It is therefore extremely important that the sample introduction system must either be completely evacuated and/or purged with a non-interfering gas to ensure accurate measurements. The same goes for the vials in which the $^{13}CO_2$ calibration standards and patient breath samples are being loaded, as well as for the syringes with which the samples are being injected into the GC.

5.1.2 Selection of the GC column

Three columns, PoraPLOT QS (d_f = 530 µm) and GS-CarbonPLOT (d_f = 530 µm and d_f = 320 µm), were initially tested. Typical components analyzed with the PoraPLOT QS include alcohols, free fatty acids, gases, glycols, halogenated compounds, C1 to C6 hydrocarbons, ketones, solvents, sulfur compounds. However, the PoraPLOT QS performed chromatographically worse than the GS-CarbonPLOT (Figure 5-4). O_2 and CO_2 could not be separated completely, and peaks were tailing. In addition, the large internal diameters of the PoraPLOT QS (530 µm) and GS-CarbonPLOT (530 µm) require high flow speeds, which also makes it less suitable for GC-MS analysis.

The GS-CarbonPLOT (320 µm) capillary column provided good separation of CO_2 from the other gasses in the breath samples (Figure 5-5), which reduces interferences from the other gases.

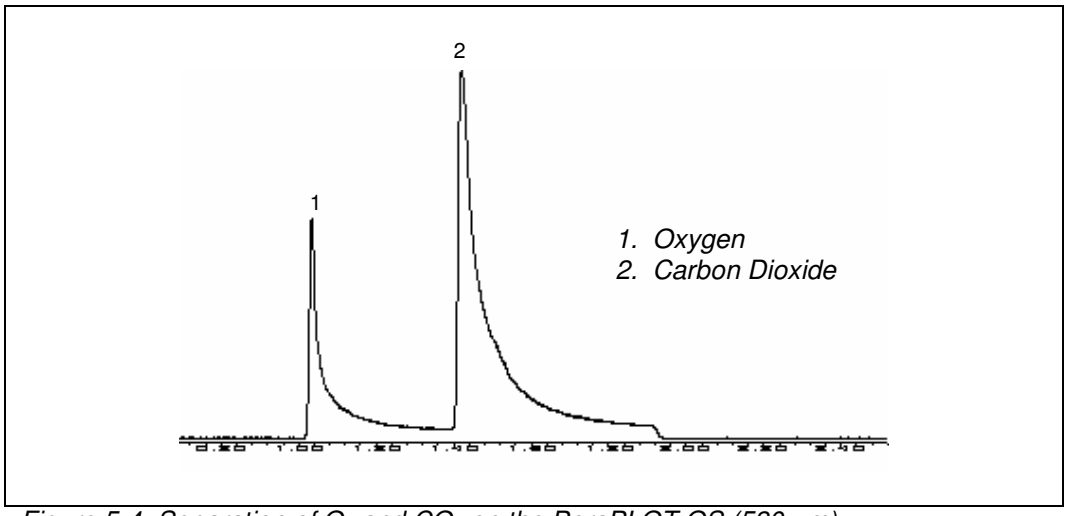

Figure 5-4: Separation of O_2 and CO_2 on the PoraPLOT QS (530 µm).

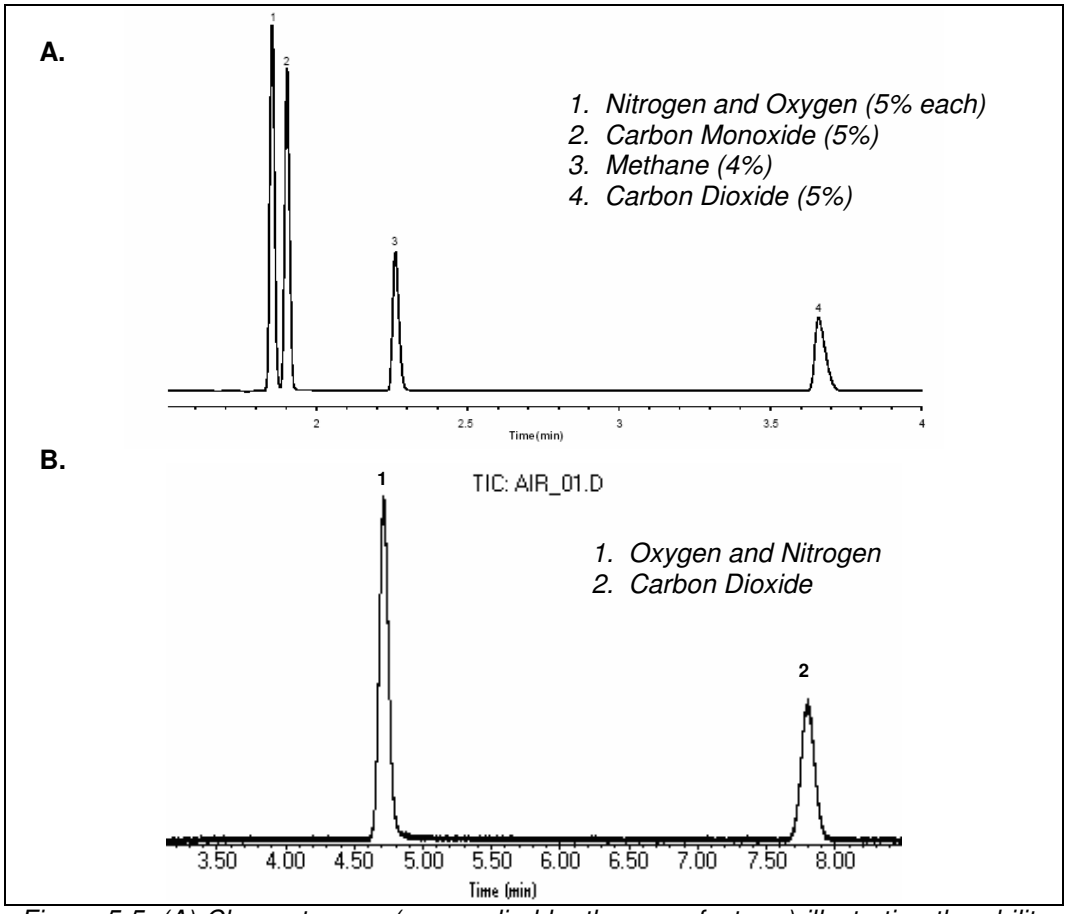

Figure 5-5: (A) Chromatogram (as supplied by the manufacturer) illustrating the ability of the GS-CarbonPLOT (320 µm) to separate CO_2 from N_2 and O_2 and other atmospheric gasses. (B) GC-MS Total Ion Chromatogram illustrating the ability of the GS-CarbonPLOT (320 µm) to separate CO_2 from N_2 and O_2 in air.

The GC-Carbon-PLOT is also suitable to be used with the mass spectrometer. In addition, O_2 could be eluted from the column by introducing a solvent delay before the source was turned on. Other advantages of the GS-CarbonPLOT include: (1) the high stability of the carbon layer stationary phase; (2) the unique selectivity for inorganic and organic gases; and (3) an extended temperature limit of + 360 ℃ (which is not required in this case). No complicated oven temperature programming is necessary when using the GS-CarbonPLOT. With an isothermal temperature of 40 ℃, CO_2 can still be sufficiently retained in the stationary phase to separate it from N_2, O_2, and other atmospheric gasses.

5.2 Selected Ion Monitoring GC-MS

The GC-MS was operated in SIM mode to determine the $^{13}CO_2$ enrichment in the calibration standards and breath samples. The total ion chromatogram and a typical mass spectrum of CO_2 recorded at 70 eV are given in Figure 5-6.

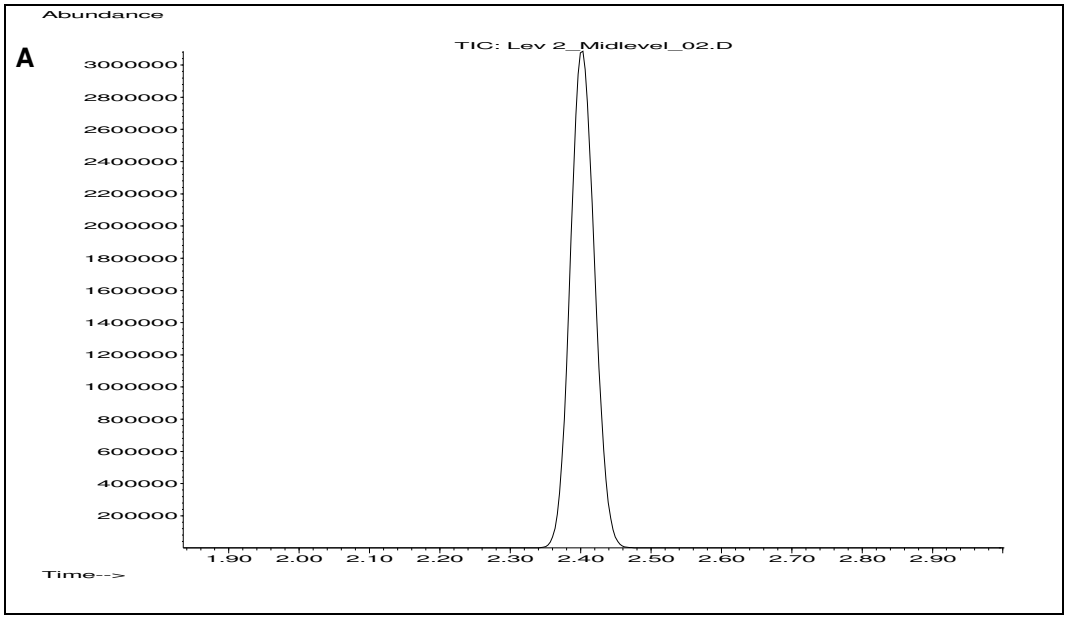

Figure 5-6: Total ion chromatogram (A) and full scan mass spectrum (B) of CO_2 recorded at 70 ev.

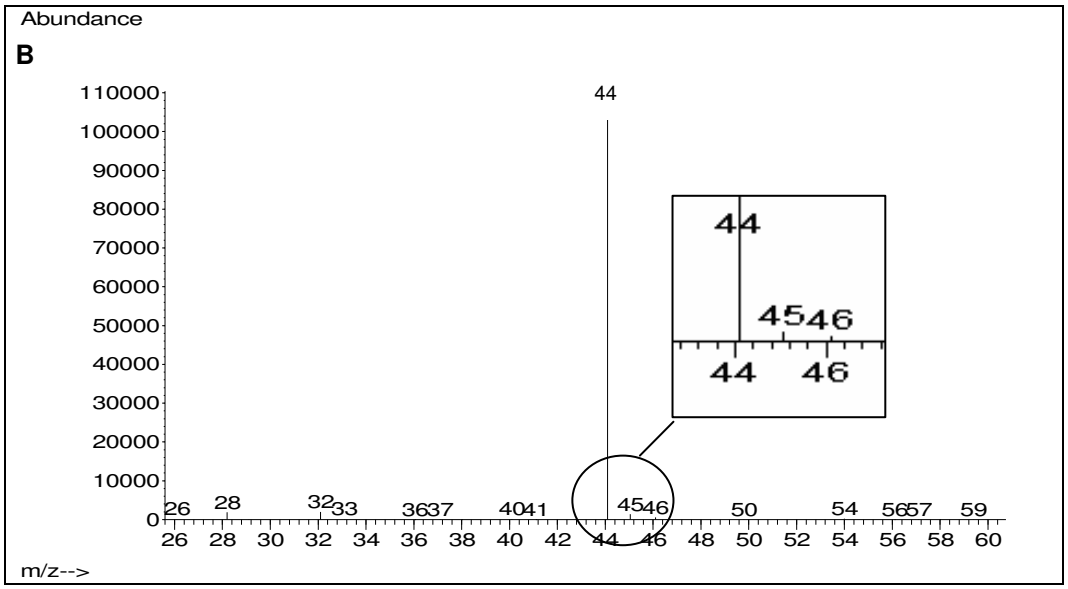

Figure 5-6: (Continued).

The abundance of ions m/z = 44, m/z = 45 and m/z = 46 were used to calculate the $^{13}CO_2/CO_{2\ (total)}$ ratio (Figure 5-7).

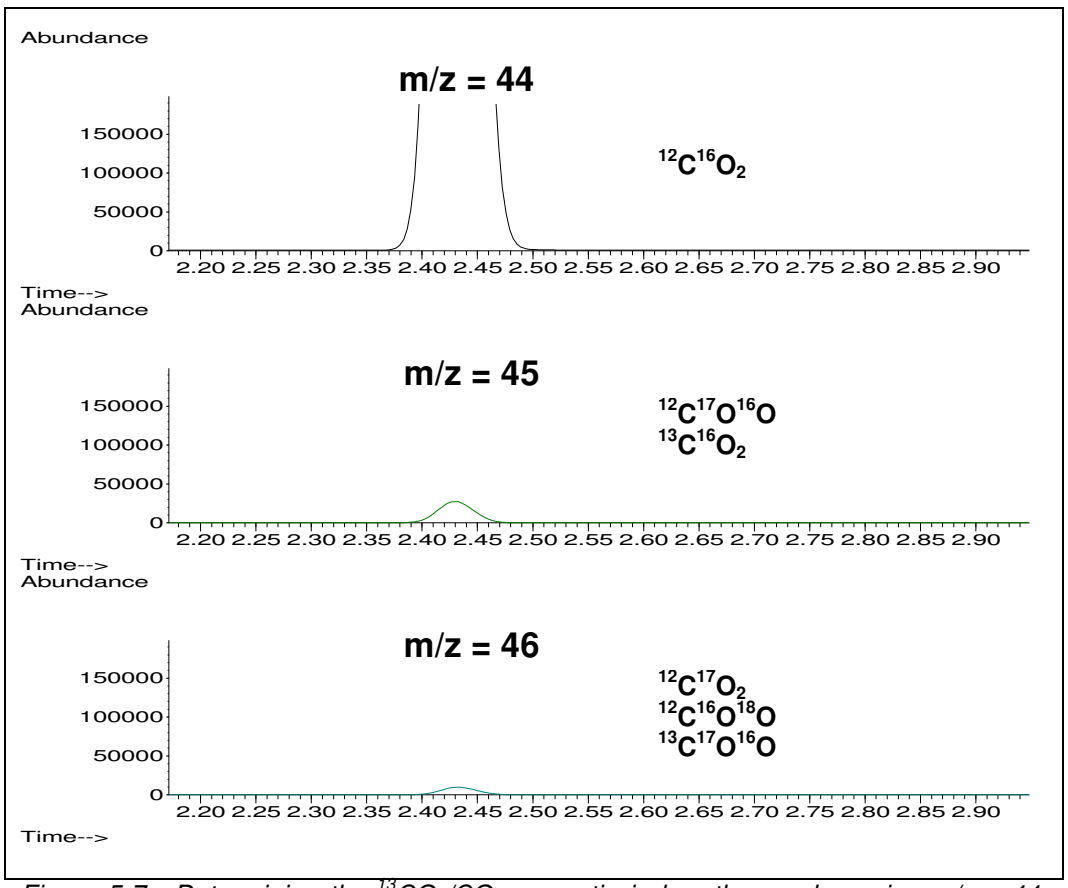

Figure 5-7: Determining the $^{13}CO_2/CO_{2\ (total)}$ ratio in breath samples using m/z = 44, m/z = 45 and m/z = 46.

The amount of $^{13}CO_2$ (m/z = 45) in the CO_2 peak is expressed as a percentage of the total CO_2 (m/z = 44 + m/z = 45 + m/z = 46) in the same peak. The contribution of m/z = 47 ($^{13}C^{17}O^{17}O$) and m/z = 45 ($^{12}C^{17}O^{16}O$) to the ratio were regarded as negligible (0.005 % and 0.038 % respectively), and was therefore not included in the calculations. *H. pylori* also does not effect the distribution of $^{12}C^{17}O^{16}O$.

The use of equation 4.2 in IRMS measurements is different from that of the same equation in GC-MS measurements since, (1) in IRMS, the ratio of mass 45 to mass 44 is measured instead of the relative 45 amu abundance, and (2) in IRMS the reference for all breath measurements is the $^{13}C/^{12}C$ ratio of a calibrated standard gas (Pee Dee Belemnite (PDB)) instead of the baseline breath samples. However, absolute $^{13}CO_2$ enrichment values obtained from samples that were analyzed by both GC-IRMS and GC-MS give similar results. Therefore there is no need to change the symbol with which $^{13}CO_2$ enrichment is reported, as this will only create confusion.

5.3 Validation of the ^{13}C-UBT GC-MS method

5.3.1 Analysis of $^{13}CO_2$ calibration standards

The GC-MS instrument was not calibrated by analyzing PDB or a secondary CO_2 standard certified by the Bureau of Standards, as is done when samples are analyzed with GC-IRMS. The $^{13}CO_2$ calibration standards, however, are certified against the PDB standard to have a certain level of $^{13}CO_2$ enrichment. Patient breath samples can be accurately analyzed by GC-MS provided that these enrichments can be reproduced.

The certified $^{13}CO_2$ calibration standards were analyzed in triplicate over 20 days to evaluate the analytical accuracy of GC-MS to obtain the $^{13}CO_2/CO_{2\,(total)}$ ratio in breath

samples. The accuracy with which the GC-MS could analyze the percentage of $^{13}CO_2$ in the three CIL calibration standards is shown in Table 5-4.

Table 5-4: GC-MS analysis of CIL $^{13}CO_2$ calibration standards.

	Percentage of $^{13}CO_2$ in the Baseline Calibrant Gas	Percentage of $^{13}CO_2$ in the Midlevel Calibrant Gas	Percentage of $^{13}CO_2$ in the High-level Calibrant Gas
Mean (%) ± 95 % CI	1.162 ± 0.001	1.172 ± 0.001	1.179 ± 0.002
SD (%)	0.003	0.003	0.004
CV (%)	0.273	0.281	0.321

The average percentage of $^{13}CO_2$ in the baseline calibrant gas was found to be close that in the PDB standard (1.111 %). However, the accurate measurement of the $^{13}CO_2$ enrichment, rather than the absolute $^{13}CO_2$ concentration, is of prime importance in this case. From Table 5-4 it can be deduced that the GC-MS would be capable of accurately detecting the small differences in the $^{13}CO_2/CO_{2\ (total)}$ ratios of the different breath samples of patients.

The $^{13}CO_2$ enrichment in the midlevel and high-level calibrant gasses was calculated with equation 4.2. When analyzing the calibration standards, $R_{(sample)}$ is the $^{13}CO_2/CO_{2\ (total)}$ ratio in the midlevel and high-level calibrant gasses, and $R_{(reference)}$ is $^{13}CO_2/CO_{2\ (total)}$ ratio in the baseline calibrant gas. The day-to-day repeatability obtained by GC-MS analysis of the $^{13}CO_2$ calibration standards is illustrated in Figure 5-8.

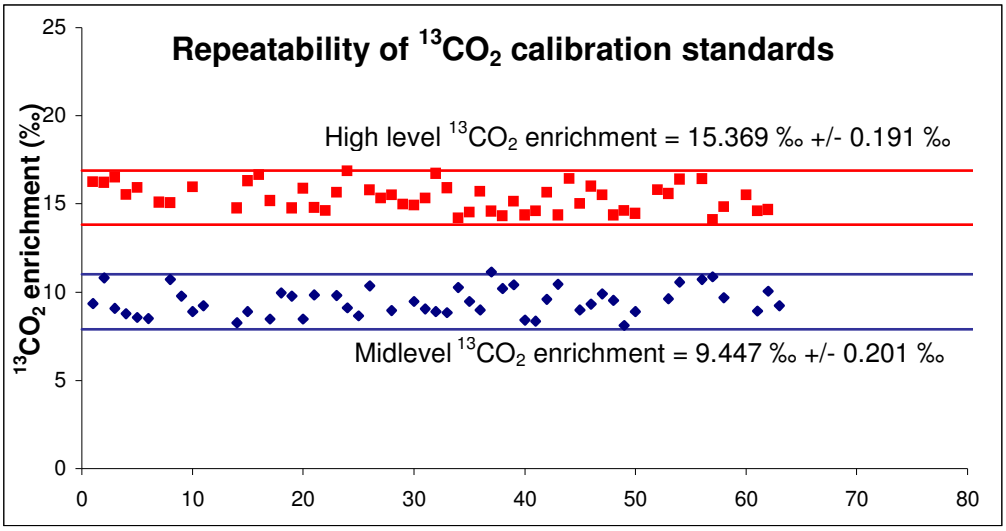

Figure 5-8: Levey-Jennings graph illustrating the day-to-day repeatability of the midlevel and high-level $^{13}CO_2$ calibration standards.

The $^{13}CO_2$ enrichment in the midlevel and high-level calibration gases was found to be 9.447 ± 0.201 ‰ (SD = 0.795 ‰; CV = 8.419 %) and 15.369 ± 0.191 ‰ (SD = 0.756‰; CV = 4.916 %) respectively. These results compared well with the certified $^{13}CO_2$ enrichment values (Table 5-5).

Table 5-5: $^{13}CO_2$ enrichment values of certified $^{13}CO_2$ calibration standards, obtained by analysis with IRMS and GC-MS. Samples were analysed in triplicate over a period of 20 days.

	CIL $^{13}CO_2$ calibration standards (IRMS)	$^{13}CO_2$ calibration standards (GC-MS)
Midlevel Calibrant Gas ($\Delta\delta$ (‰) ± SD)	9.58 ± 0.12	9.45 ± 0.20
High-level Calibrant gas ($\Delta\delta$ (‰) ± SD)	15.5 ± 0.9	15.37 ± 0.19

It was observed that if the vials containing the standards are put on a multi-shaker for a few hours prior to analysis that there was a slight improvement in repeatability. This might be due to the fact that a more homogenized gas mixture is obtained by shaking the vials for a period of time. One must also keep in mind that the vials containing the standards are under pressure, since the calibration standards are loaded into the vials after they have been sealed with a crimp cap. A slightly lower standard deviation was

observed for the calibration standards than for the breath samples. The explanation for this was thought to be the poor diffusion of gases that are under pressure and gases that are at atmospheric pressure (e.g. breath samples). This difference in pressure between the $^{13}CO_2$ calibration standards and patient breath had no influence on the accuracy of the ^{13}C-UBT; therefore, no adjustments in the way the standards are being loaded into the vials were necessary.

5.3.2 Repeatability of GC-MS in analyzing patient breath samples

Patient breath samples were analyzed in the same manner as the calibration standards. Equation 4.2 was once again used for calculating the $^{13}CO_2$ enrichment. For breath samples, $R_{(sample)}$ is the $^{13}CO_2/CO_{2\ (total)}$ ratio in the "T+30" or "T+40" breath samples, and $R_{(reference)}$ is the average of the $^{13}CO_2/CO_{2\ (total)}$ ratios in the duplicate baseline breath samples. The $^{13}CO_2$ enrichment in the "T+30" and "T+40" samples are averaged to report the overall $^{13}CO_2$ enrichment.

The within-batch accuracy with which the $^{13}CO_2/CO_{2\ (total)}$ ratio can be measured in a single breath sample was determined by injecting 100 µL of breath, from two patients, ten times from the same vial. Results for Patient 1 revealed an average $^{13}CO_2/CO_{2\ (total)}$ ratio of 1.151 % (SD = 0.001 %; CV = 0.08 %), and for Patient 2, an average $^{13}CO_2/CO_{2\ (total)}$ ratio of 1.156 % (SD = 0.001 %; CV = 0.06 %).

5.3.3 Receiver Operating Characteristic (ROC) curve analysis

A statistically sound cut-off value had to be obtained with the aid of a receiver operating characteristic (ROC) curve. The sensitivity and specificity data (Table 5-6) obtained with the use of different cut-off $\Delta\delta$ values ($\Delta\delta$ = 0 to 10 ‰) for the ^{13}C-UBT were used to construct the ROC curve. Calculations were made for breath samples

that were analyzed in duplicate, and where the $^{13}CO_2$ enrichment values for "T+30" and "T+40" were averaged.

Table 5-6: Sensitivities and specificities obtained with the use of different cut-off Δδ values for the ^{13}C-UBT.

Cut-off (Δδ (‰))	0	2.5	3	3.5	4	4.5	5	5.5	10
Sensitivity (%)	95.7	95.0	93.33	91.7	91.7	91.7	91.7	90.0	87.7
Specificity (%)	44.2	81.4	86.1	93.0	93.0	93.0	93.0	95.4	97.7

The ROC curve is presented in Figure 5-9. Results with the best diagnostic accuracy were obtained with a cut-off Δδ value of between 3.5 and 5.5 ‰. This correlated well with cut-off Δδ values suggested in literature of between 3.5 and 5.0 ‰.[1-4]

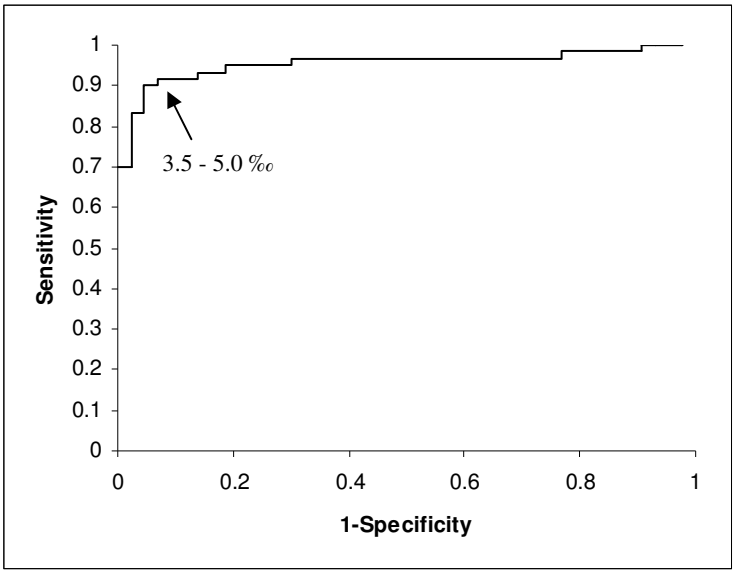

Figure 5-9: Receiver Operating Characteristic curve for the ^{13}C-UBT. The cut-off Δδ with highest efficiency was achieved between 3.5 and 5.0 ‰.

5.3.4 $^{13}CO_2$ excretion profile

The excretion profile of $^{13}CO_2$ in breath after ingestion of ^{13}C-urea were found to be similar amongst most patients. The concentration of $^{13}CO_2$ in breath reached a maximum between 30 to 40 min, irrespective of the patient's *H. pylori* status (Figure 5-

10). This is similar to what is described in literature.[5] A slight increase or decrease in the time where the maximum amount of $^{13}CO_2$ in breath occur might be due to differences in body weight, respiratory volume or severity of *H. pylori* infection. For that reason, sampling at more than one time point is necessary to circumvent this and prevent an incorrect diagnosis. For example, if only one breath sample were to be taken at "T+30", it is possible to make an incorrect diagnosis in those patients that reach maximum excretion 40 min after ingestion of ^{13}C-urea. The probability of this would increase if the $^{13}CO_2$ enrichment were close to the cut-off $\Delta\delta$ value.

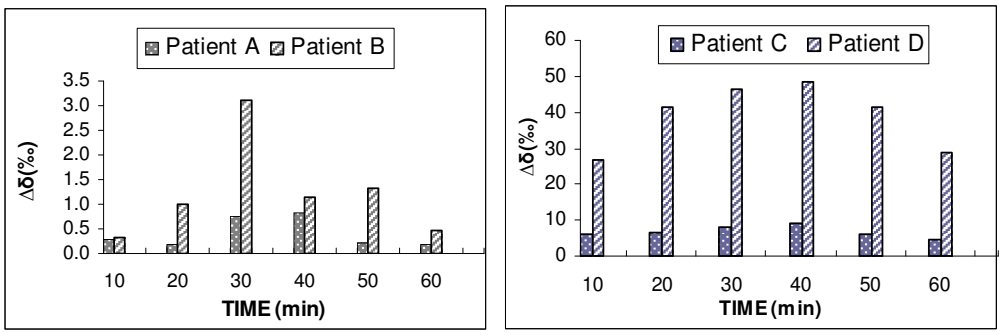

Figure 5-10: Typical excretion kinetic patterns after ingesting ^{13}C-urea. Negative (Patient A and B) and positive (Patient C and D) patients display similar excretion patterns.

5.3.5 Stability of breath samples

Breath samples often need to be shipped over long distances to institutions with the necessary equipment to perform $^{13}CO_2$-isotope analysis. Instruments capable of doing accurate $^{13}CO_2$-isotope measurements are expensive and require trained analysts to operate them, and are therefore not widespread. They are often located only in large medical centers and research institutions. Transporting breath samples to these institutions can delay analysis for days, even weeks. In addition, GC-MS instruments are usually not dedicated for ^{13}C-UBT analyses, and it may happen that breath samples cannot be analyzed immediately, but only after a few days.

To investigate the stability of breath samples over a period of time, the samples of eighteen patients were stored at room temperature for one week. Subsequent GC-MS analyses of these samples revealed that they were correctly diagnosed, with reference to histology. The results are summarized in Figure 5-11. It could be concluded that accurate measurement of $^{13}CO_2$ enrichment in breath samples was still possible after a storage period of one-week.

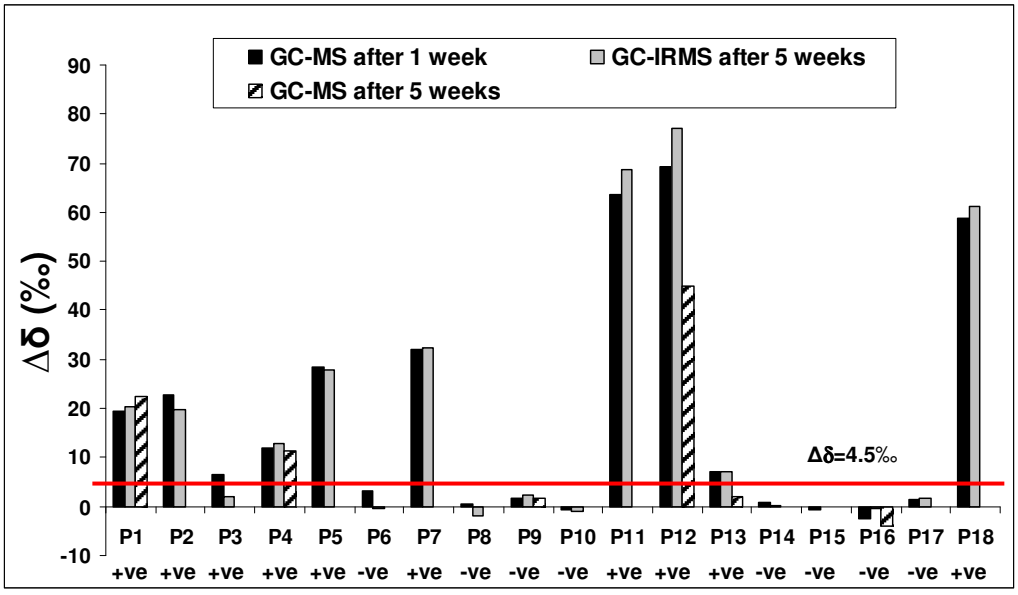

Figure 5-11: Comparison between GC-MS and GC-IRMS analysis of patient samples after being stored for one- and five weeks at room temperature. Patients with $^{13}CO_2$ enrichment values above 4.5 ‰, as obtained by GC-MS analysis, were considered to be positive for H. pylori infection. Histology results are indicated at the bottom of the graph (+ve = Positive and –ve = Negative).

Breath samples of six randomly selected patients (P1, P4, P9, P12, P13 and P16) were re-analyzed by GC-MS after five weeks. The results compared favorably with those obtained after initial analysis (Figure 5-11). A slight decrease in the $^{13}CO_2$ enrichment values was observed for all samples, which resulted in Patient 13 changing from positive to negative after a period of 5 weeks. This may be due to penetration of atmospheric CO_2 through the septum, which was pierced too many times, rather than the samples being stored for too long.

The long-term stability of the samples was also supported by the fact that duplicate samples, from the same eighteen patients, were shipped from South Africa to The Netherlands for analysis by GC-IRMS. This resulted in first time analysis of the breath samples being performed only after 5 weeks. Results obtained with GC-IRMS analysis correlated nearly 100 % with GC-MS and histological examination. This can be depicted from Figure 5-11. Only Patient 3 was incorrectly diagnosed by GC-IRMS (with comparison to histology). It was suspected that the sample vial was not sealed properly.

5.3.6 Amount of ^{13}C-urea dose

The ^{13}C-urea is one of the major cost-limiting factors of the ^{13}C-UBT, and it is therefore preferable to reduce the amount of labeled substrate given with the test meal. The ^{13}C-UBT was performed on a patient with the usual 75 mg dose. From Figure 5-12, it is clear that this patient was positive for *H. pylori* infection.

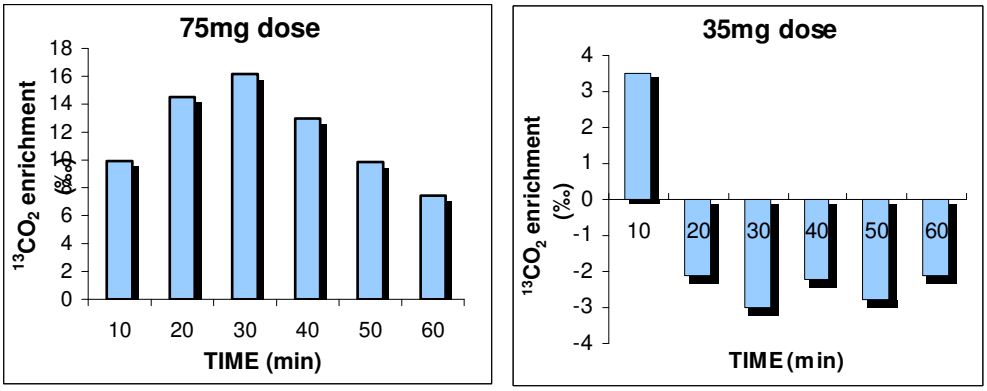

Figure 5-12: Results of the ^{13}C-UBT when a dose of 35 mg was used as the test meal, as compared to a dose of 75 mg used as the test meal.

An attempt was made to perform the ^{13}C-UBT on the same patient, but with a reduced dose of 35 mg ^{13}C-urea. Results obtained subsequently showed the patient being negative for *H. pylori* infection. It is possible that maximal excretion occurred before the 10-minute breath sample was taken. However, the $^{13}CO_2$ enrichment values obtained when using the 35 mg dose are more likely to be due to the variation of the

natural occurring $^{13}CO_2$ in the breath samples. It seems that a dose of 35 mg, mixed with the 0.1 M citric acid test meal, is too low to detect the presence of *H. pylori*. Different results may be obtained if the reduced dose was given in a tablet or capsule, as was done by Hamlet et al[6] and Everts et al.[7]

5.3.7 Accuracy of GC-MS compared to GC-IRMS

Duplicate breath samples of eighteen patients were analyzed with GC-MS and GC-IRMS. The latter is considered worldwide to be the gold-standard in ^{13}C-UBT analyses. IRMS is capable of measuring the $^{13}CO_2/CO_{2\ (total)}$ ratio in ^{13}C-UBT samples with a precision of 0.01 % (0.1 ‰). In developing a new analytical method for analysis of $^{13}CO_2/CO_{2\ (total)}$ ratios, it was necessary to confirm that the accuracy obtained with the GC-MS was just as good, or even better, than the accuracy reported for the GC-IRMS. Least squares regression statistics was used to compare the results of GC-MS with those obtained by GC-IRMS. The regression plot and corresponding statistics are presented in Figure 5-13 and Table 5-7 respectively. The bias at the decision level was found to be 0.915.

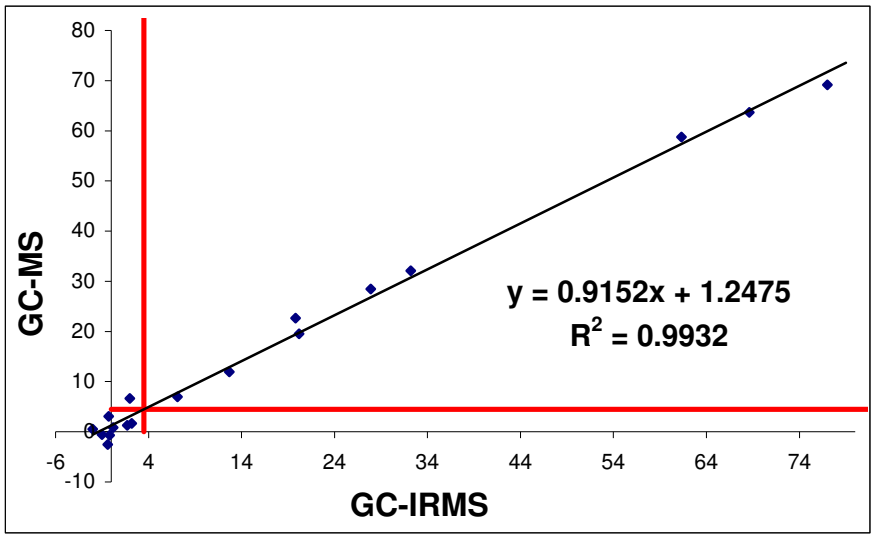

Figure 5-13: Regression plot of the correlation, for $^{13}CO_2$ enrichment, between samples analyzed by GC-IRMS (x-axis) and those analyzed by GC-MS (y-axis). The vertical and horizontal lines indicate the cut-off $\Delta\delta$ values for GC-IRMS ($\Delta\delta$ > 3.5 ‰) and GC-MS ($\Delta\delta$ > 4.5 ‰).

Table 5-7: Regression statistics of the correlation, for $^{13}CO_2$ enrichment, between samples analyzed by GC-IRMS and those analyzed by GC-MS.

Regression statistic	Value (Confidence interval)
Proportional error (slope)	0.92 (0.88 to 0.95)
Constant error (intercept)	1.25 (1.23 to 1.27)
Correlation coefficient (R)	0.993

As already mentioned, in IRMS analysis, the reference for all breath measurements is the $^{13}CO_2/CO_{2\ (total)}$ ratio of a calibrated standard gas (PDB reference standard), whereas with GC-MS analysis, the reference for the "T+30" and "T+40" measurements is the $^{13}CO_2/CO_{2\ (total)}$ ratio of the duplicate baseline samples. It is important that the GC-MS can accurately measure the increase in the $^{13}CO_2/CO_{2\ (total)}$ ratio between the baseline and the "T+30" and "T+40" samples, instead of being able to accurately measure the absolute $^{13}CO_2$ enrichment value (as is done with IRMS measurements). Nevertheless, when looking at Figures 5-11 and 5-13, it is clear that there is an excellent comparison between the absolute $^{13}CO_2$ enrichment values obtained with GC-IRMS and those obtained with GC-MS. The average difference between the $^{13}CO_2$ enrichment values was only 0.3 ‰ (SD = 3.19 ‰). From Figure 5-11 it appears that GC-MS incorrectly diagnosed "P3" as being positive for *H. pylori* infection. This, however, is not the case, since the histological examination of the same patient showed multiple *H. pylori* organisms to be present.

5.4 Assessing the diagnostic accuracy of the ^{13}C-UBT GC-MS method

For the ^{13}C-UBT, $^{13}CO_2$ enrichment in the breath samples was calculated for triplicate, duplicate and single measurements. The sensitivity, specificity, PPV, NPV and accuracy data for the ^{13}C-UBT at different cut-off values are reported in Table 5-8.

Table 5-8: Sensitivities and Specificities of the ^{13}C-UBT with reference to histology "gold standard".

	CUT-OFF (Δδ (‰))	0	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	10
1 X INJECTION AVG T+30 & T+40	SENSITIVITY	0.97	0.97	0.93	0.93	**0.93**	**0.92**	**0.92**	0.90	0.88	0.88	0.88	0.80
	SPECIFICITY	0.40	0.77	0.81	0.88	**0.91**	**0.93**	**0.93**	0.93	0.95	0.95	0.95	0.98
	1-SPECIFICITY	0.60	0.23	0.19	0.12	**0.09**	**0.07**	**0.07**	0.07	0.05	0.05	0.05	0.02
	PPV (%)	69.05	85.29	87.50	91.80	**93.33**	**94.83**	**94.83**	94.74	96.36	96.36	96.36	97.96
	NPV (%)	89.47	94.29	89.74	90.48	**90.70**	**88.89**	**88.89**	86.96	85.42	85.42	85.42	77.78
	ACCURACY (%)	72.82	88.35	88.35	91.26	**92.23**	**92.23**	**92.23**	91.26	91.26	91.26	91.26	87.38
2 X INJECTIONS AVG T+30 & T+40	SENSITIVITY	0.97	0.95	0.95	0.93	0.92	**0.92**	**0.92**	**0.92**	0.90	0.88	0.88	0.78
	SPECIFICITY	0.44	0.79	0.84	0.86	0.91	**0.93**	**0.93**	**0.93**	0.95	0.95	0.95	0.98
	1-SPECIFICITY	0.56	0.21	0.16	0.14	0.09	**0.07**	**0.07**	**0.07**	0.05	0.05	0.05	0.02
	PPV (%)	70.73	86.36	89.06	90.32	93.22	**94.83**	**94.83**	**94.83**	96.43	96.36	96.36	98.04
	NPV (%)	90.48	91.89	92.31	90.24	88.64	**88.89**	**88.89**	**88.89**	87.23	85.42	85.42	76.67
	ACCURACY (%)	74.76	88.35	90.29	90.29	91.26	**92.23**	**92.23**	**92.23**	92.23	91.26	91.26	86.49
3 X INJECTIONS AVG T+30 & T+40	SENSITIVITY	0.97	0.95	0.95	0.93	0.93	0.92	**0.92**	0.90	0.90	0.88	0.88	0.80
	SPECIFICITY	0.44	0.72	0.77	0.88	0.88	0.91	**0.93**	0.95	0.95	0.95	0.95	0.98
	1-SPECIFICITY	0.56	0.28	0.23	0.12	0.12	0.09	**0.07**	0.05	0.05	0.05	0.05	0.02
	PPV (%)	70.73	82.61	85.07	91.80	91.80	93.22	**94.83**	96.43	96.43	96.36	96.36	97.96
	NPV (%)	90.48	91.18	91.67	90.48	90.48	88.64	**88.89**	87.23	87.23	85.42	85.42	77.78
	ACCURACY (%)	74.76	85.44	87.38	91.26	91.26	91.26	**92.23**	92.23	92.23	91.26	91.26	87.38
2 X INJECTIONS Only T+30	SENSITIVITY	0.97	0.95	0.95	0.93	0.93	0.93	**0.93**	**0.93**	**0.93**	**0.93**	**0.93**	0.83
	SPECIFICITY	0.49	0.88	0.88	0.88	0.93	0.93	**0.98**	**0.98**	**0.98**	**0.98**	**0.98**	1.00
	1-SPECIFICITY	0.51	0.12	0.12	0.12	0.07	0.07	**0.02**	**0.02**	**0.02**	**0.02**	**0.02**	0.00
	PPV (%)	72.50	91.94	91.94	91.80	94.92	94.83	**98.21**	**98.21**	**98.21**	**98.21**	**98.21**	100.0
	NPV (%)	91.30	92.68	92.68	90.48	90.91	91.11	**91.49**	**91.49**	**91.49**	**91.49**	**91.49**	81.48
	ACCURACY (%)	76.70	92.23	92.23	91.26	93.20	93.20	**95.15**	**95.15**	**95.15**	**95.15**	**95.15**	90.29
3 X INJECTIONS Only T+30	SENSITIVITY	0.97	0.95	0.95	0.93	0.93	0.92	0.92	**0.92**	**0.92**	**0.92**	**0.92**	0.82
	SPECIFICITY	0.47	0.84	0.88	0.88	0.93	0.93	0.95	**0.98**	**0.98**	**0.98**	**0.98**	1.00
	1-SPECIFICITY	0.53	0.16	0.12	0.12	0.07	0.07	0.05	**0.02**	**0.02**	**0.02**	**0.02**	0.00
	PPV (%)	71.60	89.06	91.94	91.80	94.92	94.83	96.49	**98.21**	**98.21**	**98.21**	**98.21**	100.0
	NPV (%)	90.91	92.31	92.68	90.48	90.91	88.89	89.13	**89.36**	**89.36**	**89.36**	**89.36**	79.63
	ACCURACY (%)	75.73	90.29	92.23	91.26	93.20	92.23	93.20	**94.17**	**94.17**	**94.17**	**94.17**	89.32

All values were calculated against histology results. Only slight differences in accuracy are observed between the $^{13}CO_2$ enrichment values that were determined using single, duplicate and triplicate measurements. Therefore, from a "cost effectivity" point of view, it is adequate to analyze the breath samples in duplicate. It can be seen that it is possible to omit the "T+40" breath sample from the ^{13}C-UBT, in that case a slightly higher cut-off value will have to be used. From these results it can be deduced that the ^{13}C-UBT GC-MS method will be optimal when, (1) the ^{13}C-UBT is performed with the "T+30" and "T+40" samples included in the test, (2) the breath samples are analyzed in duplicate, and (3) that 4.5 ‰ is used as the cut-off Δδ value.

The patient distribution charts in Figure 5-14 shows the $^{13}CO_2$ enrichment values for the 103 patients on which the ^{13}C-UBT was performed. A cut-off Δδ value of 4.5 ‰ was used. Patients were diagnosed as being positive for *H. pylori* infection if the $^{13}CO_2$ enrichment was greater than 4.5 ‰; otherwise they were diagnosed as being negative for the infection.

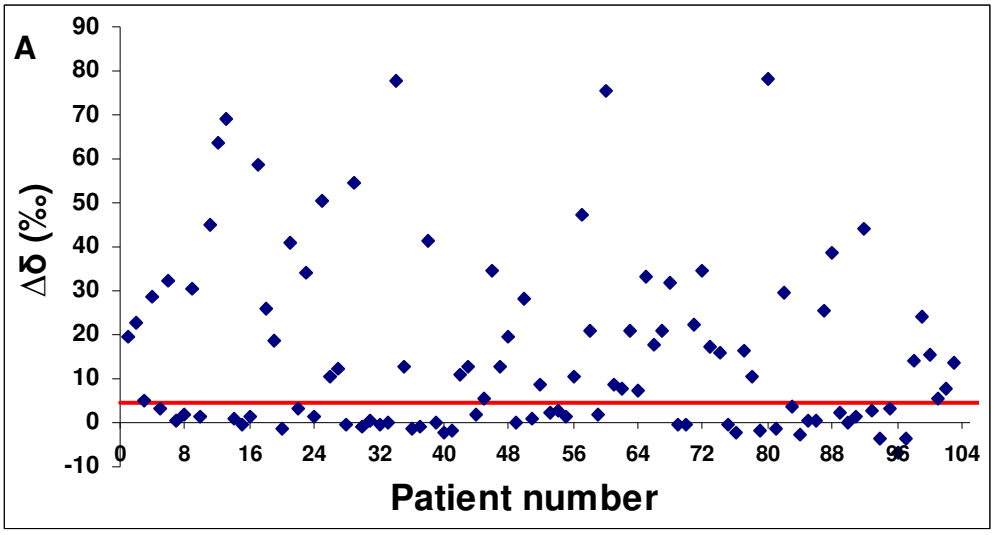

Figure 5-14: Distribution of H. pylori positive and negative patients as diagnosed with the ^{13}C-UBT. Part (B) of the figure focuses in on the cut-off line to illustrate that the ^{13}C-UBT can easily distinguish between H. pylori positive and negative patients.

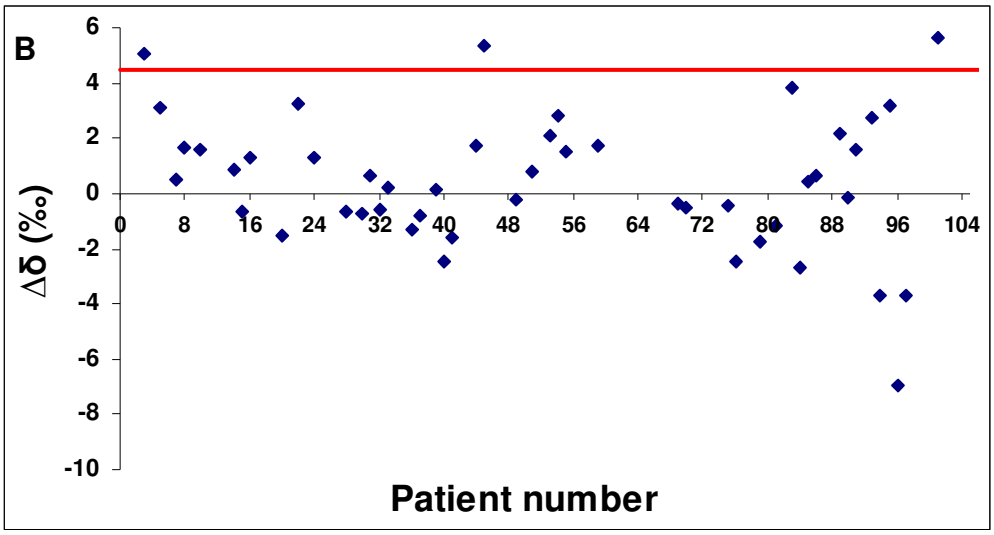

Figure 5-14: (continued).

Histological examination, rapid urease test (HpOne) and ^{13}C-UBT were performed on one hundred and three patients. With histology being considered the gold standard in the diagnosis of *H. pylori* infection, a patient was considered to be positive for *H. pylori* infection when histological examination of biopsy specimens showed *H. pylori* organisms to be present.

Histological examination of all biopsy specimens yielded: sixty (60) positive and forty-three (43) negative results for *H. pylori* infection. It was found that the small amount of citric acid consumed by the patients did not affect the procedure.

With a cut-off Δδ value of 4.5 ‰, the GC-MS ^{13}C-UBT correctly diagnosed 55 of 60 patients as positive (i.e. 55 true positive- and 5 false negative results) and 40 of 43 patients as negative (i.e. 40 true negative- and 3 false positive results). Compared to histology, the ^{13}C-UBT had a diagnostic sensitivity of 92 % (95 % CI = 85 to 99 %) and specificity of 93 % (95 % CI = 85 to 100 %). The PPV, NPV and accuracy were 95 % (95 % CI = 89 to 100 %), 89 % (95 % CI = 80 to 98 %) and 92 % (95 % CI = 87 to 97 %) respectively. The prevalence of *H. pylori* infection was calculated to be 58 %. It is

important to note that the PPV, NPV and prevalence data reported applies to a non-medicated population.

A matter of debate is surely the fact of comparing a new diagnostic test (GC-MS ^{13}C-UBT) with a "gold standard" (histology testing of biopsy samples), which by itself also lacks a one hundred percent performance rate.[8,9] The three false positive results, generated by GC-MS ^{13}C-UBT with regards to histology, all had signs of mild gastritis which can almost be regarded as a prerequisite for *H. pylori* infection. With the limited number of biopsy samples taken during the endoscopy procedure kept in mind, it may well be that the bacteria went undetected by the biopsy and histology procedure. *H. pylori* infection has a patchy nature and is not evenly distributed throughout the stomach. Biopsies can therefore miss the site of infection and thereby produce false negative results. The possibility therefore exists that the three false positive results may actually have been true positives. Speculating about the false positives even further, this could also have been due to the presence of gastric bacterial overgrowth, which might involve urease-producing bacteria other than *H. pylori* (e.g. *Helicobacter Heilmanii*).[10] The occurrence of this bacteria, however, is very rare. Furthermore, if the measurements are taken too soon after urea ingestion, false positives can also occur because of the action of oral flora on the urea. Rinsing the mouth with citric acid before swallowing the citric acid-^{13}C-urea test meal can circumvent this problem.

Four out of the five patients with false negative results had mild chronic gastritis and the fifth had no signs of pathology. The positive results from histology may have been due to sufficient colonization density in the biopsy sample; however, the *H. pylori* load in the stomach was simply too low to be detected by the GC-MS ^{13}C-UBT, resulting in a $\Delta\delta$ value below the cut-off value.

The $^{13}CO_2$ enrichment in patients diagnosed as positive for *H. pylori* infection by GC-MS ^{13}C-UBT, was between 5.073 ‰ and 78.368 ‰, and that for patients diagnosed as negative was between -6.989 ‰ and 3.863 ‰. The large difference in the $^{13}CO_2$ enrichment values suggested a clear distinction between most positive and negative results; however, a statistically sound cut-off $\Delta\delta$ value had to be obtained with the aid of a ROC curve.

Patients diagnosed as being positive for *H. pylori* infection by histology had $^{13}CO_2$ enrichment values between -2.5 ‰ and 78.4 ‰ and those who were diagnosed as being negative had $^{13}CO_2$ enrichment values between -7.0 ‰ and 12.7 ‰. A box and whiskers plot illustrating the distribution of the $^{13}CO_2$ enrichment, as measured by the GC-MS ^{13}C-UBT method for histological classified *H. pylori* positive and negative patients is shown in Figure 5-15. It is clear that the distribution of both the normal and the abnormal population is not Gaussian. Note that the box-and-whiskers plot takes into account the false positive and false negative results of the ^{13}C-UBT as compared with the gold standard.

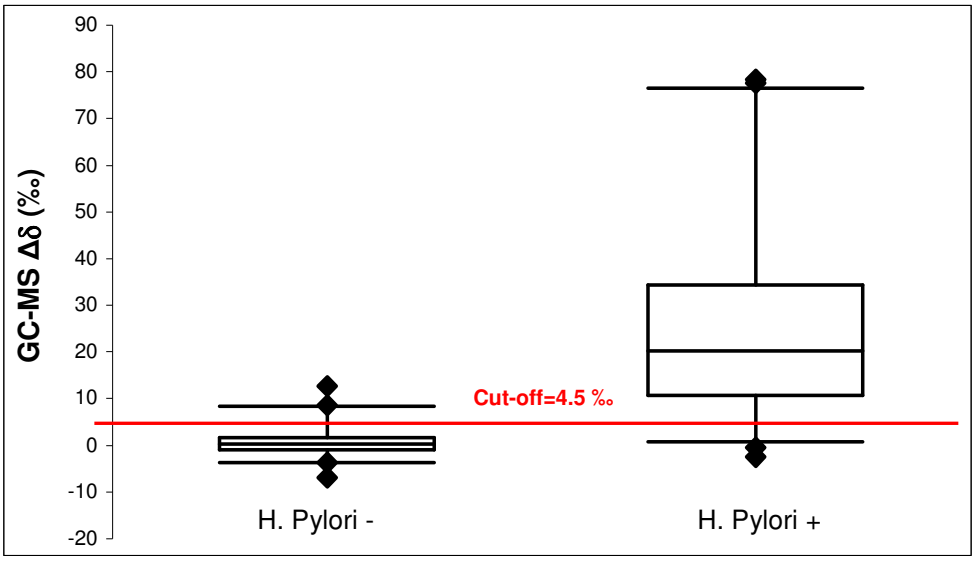

Figure 5-15: Box-and-Whiskers plot to illustrate the distribution of H. pylori positive and negative patients. The mean, median and interquartile range for the negative subjects were 0.60, 0.20 and -0.80 to 1.70 and for the positive subjects were 26.2, 21.0 and 12.1 to 34.4 respectively.

From Figure 5-14 and 5-15 it can be seen that it is likely that *H. pylori* negative patients can have negative $^{13}CO_2$ enrichment values. This is a common phenomenon and is due to the large natural abundance of ^{13}C in the human body, which is influenced by several factors such as age, gender, weight and height, but also by variations in the diet and physical activity. Consequently, in patients who are negative for a *H. pylori* infection, the natural variations in the $^{13}CO_2/CO_{2\ (total)}$ ratio in breath might cause the "T+30" and/or "T+40" breath samples to have a lower delta value than the baseline breath samples.

The average percentage of $^{13}CO_2$ in the baseline breath samples of patients was found to be 1.166 % (SD = 0.009 %), which correlates well with the natural abundance of $^{13}CO_2$ in the body (1.111 %),[11] as well as with the average percentage of $^{13}CO_2$ in the baseline calibrant gas (1.162 %).

When compared to the histological examination of biopsy specimens, the rapid urease test was found to have a diagnostic sensitivity of 82.0 % and a specificity of 100 %. The PPV, NPV and accuracy were 100 %, 78.0 % and 89.0 % respectively. It can be seen that the non-invasive GC-MS ^{13}C-UBT is more accurate than the inherently invasive rapid urease test to diagnose *H. pylori* infection.

5.5 Investigation of the existence of the "African enigma"

The study sample, stratified according to age, gender, race, *H. pylori* status and disease profile is presented in Table 5-9. A peptic ulcer was found in 23 % of all patients, which is in line with the reported 15 to 25 % of that in the developed world.[12] The overall prevalence of *H. pylori* infection was 58 %, which is substantially lower than the reported prevalence in previous studies.[13,14] In the group younger than 50 years of age the highest prevalence was found in black males followed by non-black

females, black females and non-black males. A higher prevalence of *H. pylori* infection was associated with a higher prevalence of a peptic ulcer. It is worthy to note that all subjects that were diagnosed with a peptic ulcer tested positive for *H. pylori.* In contrast, in the group older than 50 years the prevalence of *H. pylori* infection in subjects with a gastric ulcer was similar to the overall prevalence and it was substantially lower in subjects with a duodenal ulcer.

Table 5-9: Descriptive statistics of subjects stratified by age, gender and race.

	Black Male	Non-Black Female	Non-Black Male	Black Female	Total
Subjects younger than 50 years of age					
Mean age (years)	39	38	39	35	37
Number of subjects	6	12	9	23	50
Prevalence of H. pylori	83%	67%	67%	52%	62%
Gastric pathology					
Normal – Minimal[b]	1 (100%)[a]	5 (40%)	5 (100%)	15 (53%)	26 (62%)
Inflammation[c]	2 (50%)	3 (67%)	3 (33%)	8 (50%)	16 (50%)
Ulcer	3 (100%)	4 (100%)	0 (-)	0 (-)	7 (100%)
Cancer	0 (-)	0 (-)	1 (0%)	0 (-)	1 (0%)
Duodenal pathology					
Normal - Minimal	4 (75%)	12 (67%)	6 (67%)	21 (52%)	43 (60%)
Inflammation	0 (-)	0 (-)	2 (50%)	2 (50%)	4 (50%)
Ulcer	2 (100%)	0 (-)	1 (100%)	0 (-)	3 (100%)
Cancer	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Combined pathology[d]					
Normal - Minimal	1 (100%)	5 (40%)	3 (100%)	15 (53%)	24 (58%)
Inflammation	1 (0%)	3 (67%)	4 (50%)	8 (50%)	16 (50%)
Ulcer	4 (100%)	4 (100%)	1 (100%)	0 (-)	9 (100%)
Cancer	0 (-)	0 (-)	1 (0%)[e]	0 (-)	1 (0%)
Subjects 50 years of age and older					
Mean age (years)	59	63	70	61	64
Number of subjects	3	26	10	14	53
Prevalence of H. pylori	67%	69%	20%	50%	55%
Gastric					
Normal - Minimal	0 (-)	11 (64%)	4 (50%)	7 (57%)	22 (59%)
Inflammation	3 (67%)	11 (82%)	3 (0%)	5 (60%)	22 (64%)
Ulcer	0 (-)	4 (50%)	3 (0%)	2 (0%)	9 (22%)
Cancer	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Duodenum					
Normal - Minimal	0 (-)	22 (68%)	9 (22%)	10 (40%)	41 (51%)
Inflammation	3 (67%)	0 (-)	0 (-)	2 (100%)	5 (80%)
Ulcer	0 (-)	4 (75%)	1 (0%)	2 (50%)	7 (57%)
Cancer	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Combined					
Normal - Minimal	0 (-)	10 (70%)	4 (50%)	4 (50%)	18 (61%)
Inflammation	3 (67%)	10 (80%)	2 (0%)	6 (67%)	21 (67%)
Ulcer	0 (-)	6 (50%)	4 (0%)	4 (25%)	14 (29%)
Cancer	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)

[a] *The first figure is the number of subjects in the specified category and the figure in brackets is the percentage that tested positive for H. pylori on histology.*
[b] *This include microscopic inflammation focal abrasions/inflammation/erosions, erythema and superficial ulcers.*
[c] *Inflammation (or atrophy) that was macroscopically visible at endoscopy*
[d] *The most severe pathology between the stomach and duodenum is recorded.*
[e] *A 41 year old patient with adenocarcinoma of unknown primary. The histology was suspicious of a malignancy.*

Stratifying subjects by age is important because of the differences in the management of dyspepsia (Figure 5-16).

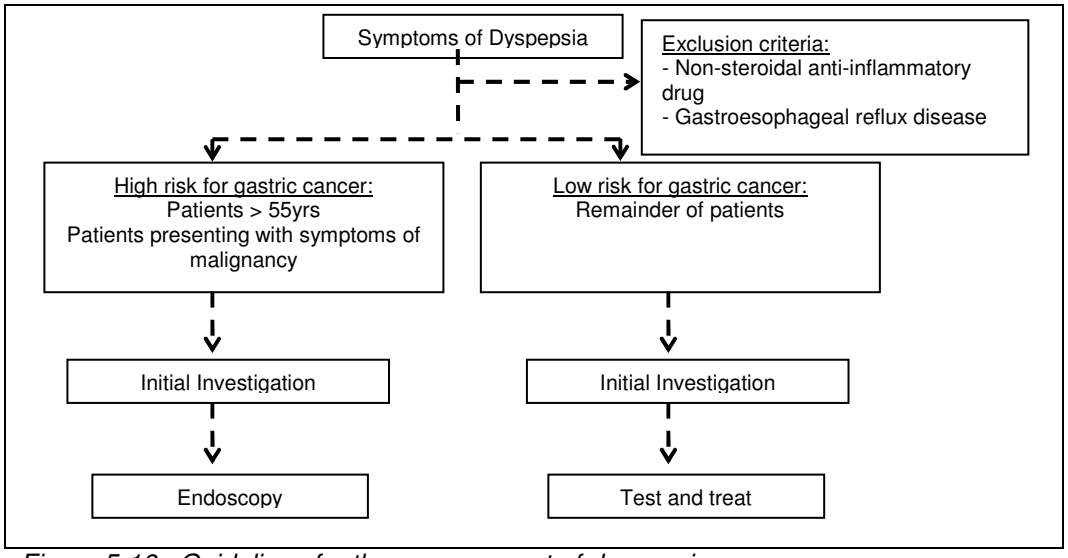

Figure 5-16: Guidelines for the management of dyspepsia

Fifty years was chosen as a cut-off because it provided approximately equal groups. The actual cut-off that would be appropriate for South Africa, if a "test-and-treat" strategy were to be introduced, would have to be based on the risk for malignancy at the chosen cut-off.[15] The odds ratio for a peptic ulcer if a patient is *H. pylori* positive is 13:1 (95 % CI = 1.7 to 101). For age, and the interaction between age and the *H. pylori* status, the odds ratios are 1.02:1 (95 % CI = 0.99 to 1.06) and 0.95:1 (95 % CI = 0.92 to 0.99) per one-year increase in age respectively. Thus, the probability of a peptic ulcer increases with increasing age but decreases for ulcers associated with *H. pylori*. The addition of gender, race and various other interactions including gender with *H. pylori* and race with *H. pylori* were not significant at P = 0.05 and did not improve the model fit.

The interaction effect between age and *H. pylori* status is unexpected. The P value associated with this effect is 0.008, which is of a greater significance than both the *H.*

pylori status (P = 0.014) and age by itself (P = 0.20). A possible explanation for this could be that the effect is not a function of increasing age, but rather a function of the period in history when a person was infected. For example the *H. pylori* strain that was predominant in the fifties may have been less virulent than the strains that predominated in the eighties. This hypothesis is attractive if one considers that the bacterium is mainly acquired during the first two decades of life. If the hypothesis is correct then the effect can be expected to disappear over the next two to three decades. It would also provide further explanation as to why older studies did not find strong associations between *H. pylori* infection and peptic ulcers.

5.6 Impact of implementing the ^{13}C-UBT in South Africa

The cost saving, as calculated with the decision tree (Figure 5-17) is approximately R1 100. The model presented is likely to underestimate the actual cost saving of a "test-and-treat" strategy for the following reasons: (1) it was assumed that all failed "test-and-treat" strategies would be subjected to endoscopy (which is not according to the suggested guidelines); (2) it was further assumed that the prevalence of macroscopic disease did not decrease in patients who received treatment based on the result of the UBT; (3) the use of generic drugs, which will increase the cost saving was not considered; and (4) the model does not account for the higher economic costs of sick leave associated with the direct endoscopy strategy.

The mean and interquartile range of the simulated cost saving distribution was R970 and R810 to R1 160 respectively. The distribution is left skewed which explains why the mean value does not correspond with the average outcome of the decision tree. According to the simulated distribution a cost saving of R0 or less has only a 0.02 % chance of occurring which suggests that the benefit of a "test-and-treat" strategy is robust to the stipulated variations of probabilities and costs.

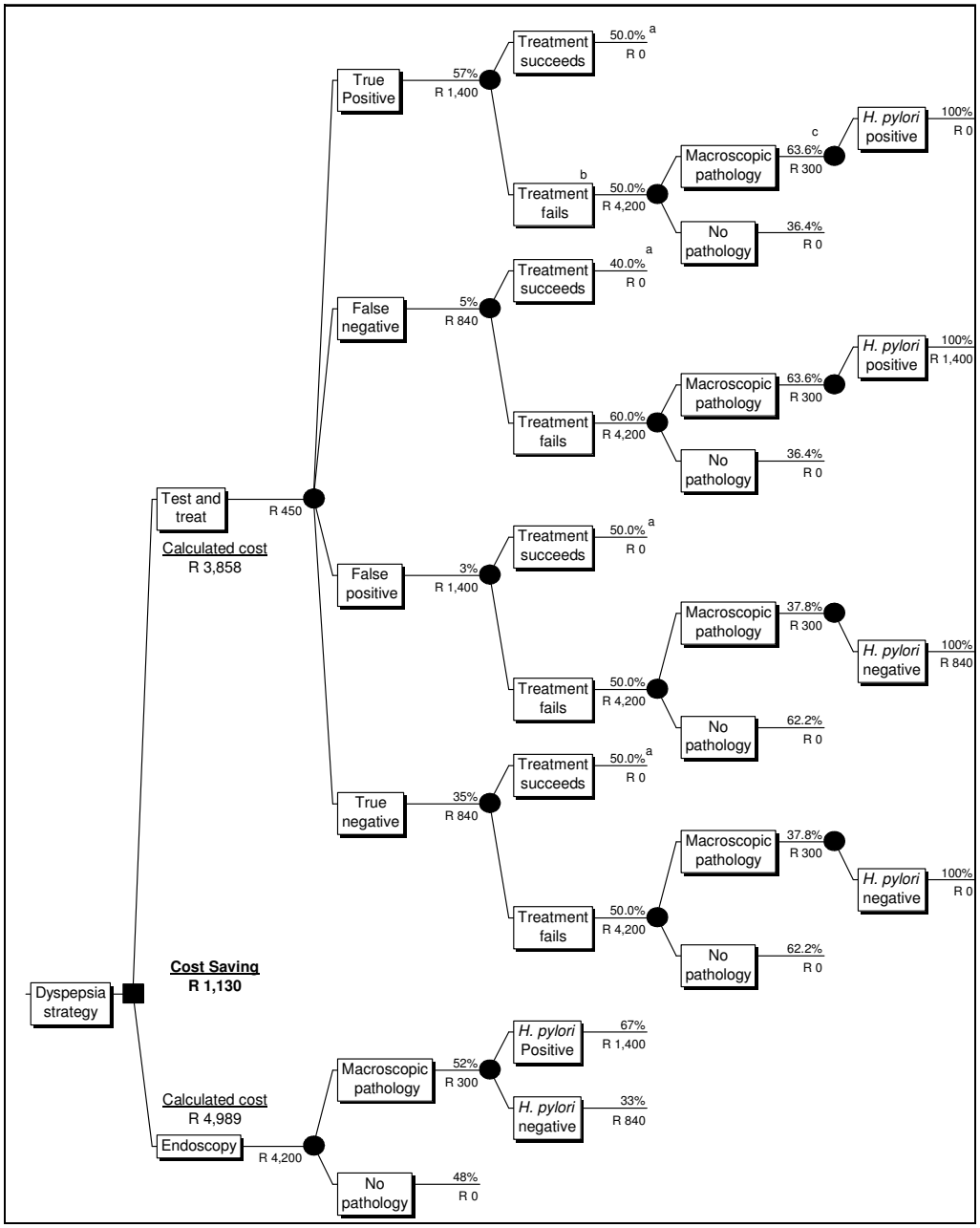

Figure 5-17: Decision tree of the "test-and-treat" and endoscopy strategies. The probability of an event occurring is given above branches and the cost associated with it below.

[a] *Probabilities that were based on literature estimates*
[b] *Whenever treatment fails endoscopy is performed*
[c] *The probability of macroscopic disease in the "test-and-treat" strategy is different from the direct endoscopy strategy because the H. pylori status is taken into account*

The coefficients of the multiple regression equation are presented in Table 5-10.

Table 5-10: Sensitivity analysis based on multiple regression of the cost and probability inputs used in the Monte Carlo simulation.

Parameter	Coefficient (slope)	Standardised Coefficient
Cost of PPI only treatment	-0.22	-0.00006
Cost of biopsy	0.27	0.00008
Cost of triple therapy	-0.28	-0.00008
Cost of endoscopy	0.46	0.00013
Cost of the urea breath test	-1	-0.00028
Probability of normal endoscopy in patients not infected with H. pylori	-107	-0.030
Sensitivity	154	0.043
Probability of failed therapy in patients who tested false negative with the ^{13}C-UBT[a]	-266	-0.075
Specificity	275	0.077
Probability of abnormal endoscopy in patients infected with H. pylori	538	0.15
Prevalence	-603	-0.17
Probability of macroscopic disease	856	0.24
Probability of failed therapy in patients who tested negative with the ^{13}C-UBT[a]	-1574	-0.44
Probability of failed therapy in patients who tested true positive with the ^{13}C-UBT[a]	-2434	-0.68
Constant = 1690		
Adjusted R^2 = 0.97		

[a]*Probabilities associated with a failed "test-and-treat" strategy. Recall that these are based on similar estimations in cost models because it cannot be calculated form the study sample data*

The coefficients can be used to estimate the cost saving under different probabilities and costs given the model assumptions. The standardised coefficients are used to compare the sensitivity of the cost saving to the various probabilities and costs. The Achilles heel of the model is the probabilities associated with a successful "test-and-treat" strategy. This is due to the fact that it is not based on empirical estimates, and the cost saving is highly sensitive to changes in it. Even so, the benefit of the "test-and-treat" strategy is robust to fairly large variations in these probabilities.

5.7 Conclusion

It can be concluded that there are many ways of utilizing the GC-MS instrument to analyze the $^{13}CO_2/CO_{2\ (total)}$ ratio breath samples. It must again be emphasized that a properly maintained system is crucial to ensure accurate results. Using the 100 µL gastight syringe with the autosampler is the most cost-effective way to analyze the samples, which are collected in 20 mL GC headspace vials. It is possible to configure the GC-MS system to accommodate the more commercially available Exetainer™ breath tubes. The use of the GS-CarbonPLOT is optimal since it has the advantage of separating CO_2 from other atmospheric gases, thereby reducing interferences.

Different protocols exist for the ^{13}C-UBT. In this study, patients were asked to consume citric acid (0.1 M; 100 mL) as the test meal, which contained the ^{13}C-urea dose (75 mg). Duplicate baseline breath samples were taken before the test meal was consumed, and one sample was taken at "T+30" and "T+40" respectively. Analysis of the breath samples gave accurate results when samples were analyzed in duplicate, and $\Delta\delta$ = 4.5 ‰ used as the cut-off value. Results obtained using this protocol proved to be very accurate with reference to the gold standard of histology. Unfortunately it was not possible to use a reduced dosage since it leads to the ^{13}C-UBT being inaccurate.

The results obtained from analyzing the CIL ^{13}C calibration standards, as well as from the breath samples from patients on whom the ^{13}C-UBT were performed, showed that SIM GC-MS is capable of producing very accurate results. The analytical precision of the new GC-MS method compared favorably with that of the well-established GC-IRMS method. The high cost and complexity of the GC-IRMS can be overcome by accurate analysis of the $^{13}CO_2/CO_{2\ (total)}$ ratio by GC-MS. This may result in ^{13}C-UBT analyses becoming more widespread, and being performed on a more routine basis.

The average prevalence of *H. pylori* in patients that presented for endoscopy was substantially lower than what was reported previously. *H. pylori* was strongly associated with peptic and duodenal ulcers, especially in younger patients. These findings do not support the existence of the “African enigma”. This warrants the use of the "test-and-treat" approach for the management of dyspepsia as published by the American College of Gastroenterology and the American Gastroenterological Association. The cost analysis indicates that the financial impact of utilizing the ^{13}C-UBT in the "test-and-treat" strategy could be substantial while still providing an approximately equivalent outcome in patient care to the direct endoscopy approach. Consequently, the need for the use of invasive tests in the screening for *H. pylori* infection in South Africa will be eliminated.

The GC-MS ^{13}C-UBT offers considerable savings to the patient, both in terms of money and discomfort. It is non-radioactive and completely safe to be performed repeatedly on the same patient, children and pregnant women. In addition, since the ^{13}C-UBT is considered to be the most accurate non-invasive test in the post-treatment setting, it is also possible to assist in the assessment of the effectivity of eradication treatment.

5.8 References

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CHAPTER 6

CONCLUSION

The discovery of *H. pylori* in 1983, and the realization of its importance in upper gastrointestinal diseases, especially peptic ulcer diseases and gastric cancer, represents one of the most important developments in medicine in the past century. Careful surveying provided evidence that the majority of persons in the world are infected with *H. pylori*. In countries of low socio economic status, infection occurs early in childhood, and if left untreated, persists so that most are infected by their teens. With the prevalence of *H. pylori* infection being so high, the need for an accurate and reliable diagnostic test to diagnose patients with a *H. pylori* infection is apparent.

6.1 Optimization of the ^{13}C-UBT

It is well documented that the non-invasive ^{13}C-urea breath test (^{13}C-UBT) is extremely accurate in diagnosing *H. pylori* infection. The ^{13}C-UBT protocol which was followed in this study involved the consumption of ^{13}C-urea (75 mg) in a citric acid (0.1M) test meal, sampling at baseline, "T+30" and "T+40", and using a cut-off $\Delta\delta$ value of 4.5 ‰. This protocol proved to be optimal to discriminate between *H. pylori* positive and negative patients.

Results obtained with ^{13}C-UBT GC-MS proved to be very accurate with reference to both the diagnostic and analytical gold standards – histology and GC-IRMS. Precise measurements of the $^{13}CO_2/CO_{2\ (total)}$ ratio in breath samples could be made with GC-MS, an instrument that is not commonly used to measure isotope ratios of compounds. The $^{13}CO_2$ enrichment values obtained with GC-MS correlated favourably with those obtained by GC-IRMS analysis (r = 0.993). When compared to histology, the ^{13}C-UBT GC-MS had a diagnostic sensitivity of 92 % and specificity of 93 %.

6.2 Application of the ^{13}C-UBT

The test has the capability to aid in the non-invasive screening and diagnosis of *H. pylori* infected patients. In addition, the ^{13}C-UBT is the only non-invasive test that can give information regarding the current *H. pylori* status. This unique feature of the ^{13}C-UBT makes it particularly useful in the following clinical situations: (1) all instances where the knowledge of *H. pylori* status is required; (2) when an ulcer is detected on ingestion of a barium meal; (3) during endoscopy, where biopsies cannot be obtained owing to the patient being on anticoagulant therapy; and (4) in children, before endoscopy is performed, as an alternative to serological testing.

The ^{13}C-UBT is the diagnostic method of choice in research studies since it is easy to perform, relatively cheap, and capable of accurately assessing current *H. pylori* status.

More importantly, since the ^{13}C-UBT is considered to be the most accurate non-invasive test in the post-treatment setting, it is also possible to assist in the assessment of the effectivity of eradication treatment.

6.3 Contribution of the ^{13}C-UBT to the medical field

Rejection of the "African enigma" warrants the use of the ^{13}C-UBT in the "test and treat" strategy for the management of dyspeptic patients. Screening and diagnosis of *H. pylori* infected patients with the ^{13}C-UBT will reduce the need for the use of invasive tests in the screening for *H. pylori* infection. The ^{13}C-UBT offers considerable savings for the patient, both in terms of money (± R1 100) and discomfort. It is non-radioactive, and completely safe to be performed repeatedly on the same patient, children and pregnant women. The high cost and complexity of the GC-IRMS can be overcome by accurate analysis of the $^{13}CO_2/CO_{2\ (total)}$ ratio

by GC-MS, resulting in ^{13}C-UBT analyses being performed in a more cost effective manner, on a routine basis.

6.4 Suggestions for future research

Further research might include development of the ^{13}C-UBT to incorporate a reduced dosage - which is not in tablet or capsule form. This will aid in further cost reductions of the ^{13}C-UBT. Furthermore, the ^{13}C-UBT GC-MS method must be validated in a clinical practice to determine its utility in assessing the eradication of the organism following treatment. Finally, the cost saving should be confirmed in a randomized control trial.

APPENDIX A: Substances that can be used as substrates for ^{13}C–breath tests, and their application in clinical research an diagnosis

CARBOHYDRATES
Monosaccharides • ^{13}C-glucose, $\delta^{13}C$-glucose - Hexose absorption and utilisation, especially in diabetes mellitus; influence of body training on carbohydrate metabolism hexose absorption and utilisation, especially indiabetes mellitus • ^{13}C-fructose - liver diseases; hepatic microsomal biotransformation; • ^{13}C-galactose, $\delta^{13}C$-galactose - studying galactose–1–phosphate uridyltransferase • ^{13}C-xylose - intestinal bacterial overgrowth **Oligosaccharides** • ^{13}C-sucrose, δ13C-sucrose - sucrase deficiency in children with gastrointestinal symptoms • 13C-lactose, δ13C-lactose - lactase deficiency in children with gastrointestinal symptoms • 13C-maltose - carbohydrate and fat metabolism **Polysaccharides** • ^{13}C-starch - digestion and resorption; pancreatic function; cystic fibrosis • $\delta^{13}C$-glucose polymers - digestion and metabolisation for energy production;studying liver glycogen metabolism
PROTEINS AND AMINO ACIDS
Proteins • $\delta^{13}C$-proteins - protein metabolism • $\delta^{13}C$-casein - steatorrhea; digestion and resorption of proteins • ^{13}C-egg white - protein metabolismus • ^{13}C-labelled egg protein - evaluation of the true ileal digestibility of egg protein • ^{13}C-labelled algal - biomass metabolism **Amino acids** • L–1–^{13}C-leucine - amino acid oxidation; studying phenylketonurea–diet–therapy • $^{13}C2$-glycine - amino acid oxidation; gastric emptying rate • DL–1–^{13}C-phenylalanine - amino acid oxidation • L–1–^{13}C-phenylalanine - amino acid oxidation; evaluation of hepatic function • ^{13}C-tyrosine - rate of tyrosine catabolism **Milk products** • $\delta^{13}C$-milk products – metabolism

LIPIDS
• δ^{13}C-lipids - fat metabolism; fat malabsorption • δ^{13}C-corn oil - fat malabsorption • δ^{13}C-soy oil - fat malabsorption • ^{13}C-and δ^{13}C-triglycerides - defects in lipolysis due to exocrine pancreatic insufficiency • tri–1–^{13}C-octanoyl glycerol - fat malabsorption • 1,3–distearyl–2–1–^{13}C-octanoylglycerol -fat malabsorption • ^{13}C-triolein - fat malabsorption • ^{13}C-tripalmitin - fat malabsorption • ^{13}C-trioctanoin - fat malabsorption
CARBOXYLIC ACIDS
• ^{13}C-fatty acids - myocardial metabolic studies • ^{13}C-acetates - gastric emptying rate; aldehyde dehydrogenase deficiency • ^{13}C-octanoic acid - gastric emptying rate • ^{13}C-ketoisocaproic acid - alcoholic hepatic steatosis • ^{13}C-glycocholic acid - malabsorption of bile acids; intestinal bacterial overgrowth; enterohepatic circulation of bile acids • ^{13}C-valproic acid - studying epileptic diseases
CARBONIC ACID AND ITS DERIVATIVES
• Sodium ^{13}C-hydrogen carbonate - gastrointestinal transit time; gastric emptying rate • ^{13}C-urea - Helicobacter (Campylobacter) pylori infection and gastrointestinal diseases, probably also corona diseases and migraine due to this infection • ^{13}C-glycosyl ureides - intestinal transit time • Lactose^{13}C-ureide - colonic fermentation; orocoecal transit time; coecal retention time • Lactose^{13}C-,^{15}N-ureide - intestinal transit time; studying colonic fermentation
ALCOHOLS
• ^{13}C-ethanol - aldehyde dehydrogenase deficiency
MISCELLANEOUS
• Methyl–^{13}C-methacetin - liver diseases; hepatic microsomal biotransformation • 1,3,7–methyl–^{13}C-caffeine - liver diseases; hepatic microsomal biotransformation • ^{13}C2-aminopyrin - liver diseases; hepatic microsomal biotransformation • ^{13}C-erythromycin - liver diseases