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SOLVING THE PUZZLE THE CSI WAY: THE ROLE OF INSTRUMENTATION

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Abstract

Modern television programmes have highlighted the role of analytical instrumentation in solving forensic dilemmas. Access to such analytical instrumentation is the 'lifeblood' of any researcher. Most modern instruments produce large quantities of raw data that require processing and interpretation. Examples of these instruments include gas chromatography coupled to mass spectrometry, liquid chromatography coupled to diode-array or pulsed amperometric detectors and nuclear magnetic resonance spectroscopy. Instruments are often named in reports, research and SASTA papers with many of them being seen as 'black boxes' by the sugar technologist. To widen the understanding of the technologists, this paper reviews the instrumental techniques and their application by presenting a number of consulting analyses with which the SMRI has been associated. These include the mystery of the substituted costly pharmaceutical active ingredient, the rapid determination of a toxic substance to help save lives and helping to resolve the case of the 'unaccounted tequila'.

Keywords: instrumentation, chromatography, spectroscopy

Introduction

Forensic science has been the backbone of mystery stories from Edgar Allan Poe's Dupin adventures to Sir Arthur Conan Doyle's Sherlock Holmes tales to Jack Klugman's Quincy television series to today's successful television shows such as CSI: Crime Scene Investigation. These programmes depict specialised analyses using sophisticated equipment being performed in less than five minutes. The reality is that analysis time is often longer and can require from hours to days. There are very few scientific instruments that give a result within the short period of time as shown on television. The operator is shown as being a 'Jack of all trades' – instrument operator, crime scene examiner and detective. Generally, specialist scientists are required to run the instruments and arenot necessarily experts in every field. The specialist forensic laboratories would employ separate specialists for flammable liquid residues, DNA, drug analysis, documents, and fingerprints.

The positive value of these modern programmes has been in highlighting the role of analytical instrumentation in solving forensic mysteries. Many of the scientific techniques shown are based on reality and many of the results obtained are also possible. The televisionclose-ups of items shown are also usually realistic. Access to this same

analytical instrumentation is the 'lifeblood' of any researcher. Examples of necessary instrumentation include gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography coupled to photodiode-array (LC-PDA) or pulsed amperometric detectors (LC-PAD) and nuclear magnetic resonance spectroscopy (NMR). Without results from these instruments and the necessary interpretative skills of the researcher, the problem solving and research outputs expected of the modern researcher would be difficult to attain.

Explanation of instrumentation

Gas chromatograph coupled to mass spectrometry (GC-MS)

A GC-MS consists of four components (Figure 1), the external GC and the three parts of the MS. A sample containing a mixture of compounds of interest is separated in a GC column. As the separated volatile compounds elute from the column they are bombarded with electrons within an ion-source in a process termed 'ionisation'. These energetic electrons cause the compound to disintegrate and produce a mixture of positively charged ions in the gas phase. Provided the ionisation occurs under the same conditions (constant ionisation potential), the disintegration will be reproducible. The resultant mixture of ions is accelerated into an analyser where they are separated and sorted from each other according to their mass-to-charge (m/z) ratio by manipulation of electrical fields. The sorted ions are collected by a detector and converted to a proportional electrical current. The data system records the magnitude of these electrical signals as a function of m/z ratio. The data is stored in a three dimensional format – chromatographic retention time, m/z and abundance. The three MS components (ion source, analyser and detector) are housed within a vacuum system to minimise interaction with other molecules.

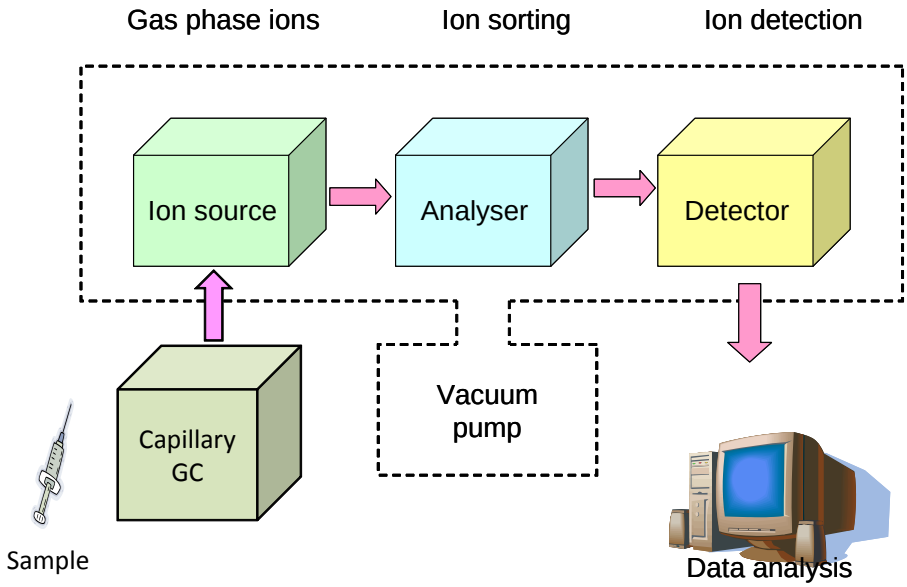

Figure 1.Diagram of a gas chromatograph (GC) mass spectrometer.

A mass spectrum is a graph of ion intensity (abundance) as a function of m/z ratio. Mass spectra are often depicted as simple histograms. Figure 2 shows the theoretical mass spectrum of carbon dioxide (CO_2). Since a molecule of carbon dioxide is composed of only three atoms, its mass spectrum is relatively simple. The fragmentation products of the ionisation are the molecular ion CO_2^+ (also the base peak), a fragment with one

oxygen removed CO^+ (m/z=28), O^+(m/z=16) and C^+. This record of ions and their intensities serve to establish the molecular weight and structure of the compound being analysed.

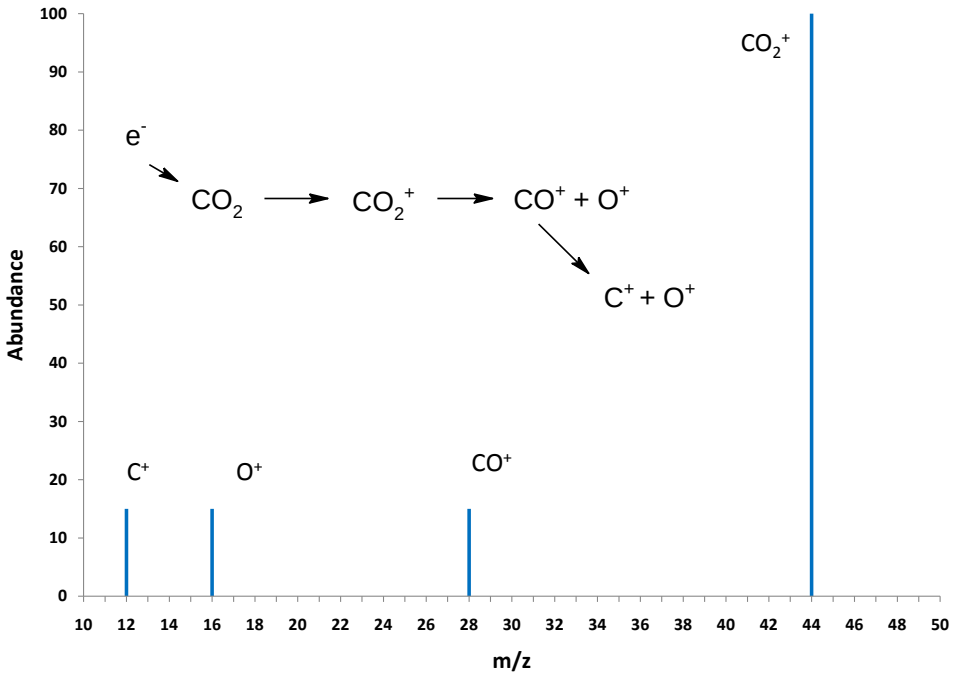

Figure 2.Mass spectrum of carbon dioxide.

GC-MS is an established analytical tool for obtaining both compound separation within a mixture and structural information on the separated organic compounds. It is used routinely in environmental analysis, forensics (profiling for drugs of abuse), arson analysis, food contamination and for identification and characterisation in the development of new pharmaceuticals. The coupling of a mass spectrometer as a detector to a gas chromatograph was developed during the 1950s and 1960s (Gohlke, 1959; Ryhage, 1964; Gohlke and McLafferty, 1993). However the type and size of the mass spectrometers available precluded their use in routine analysis. It was not until the early 1980's, with the advent of the personal computer, that 'benchtop' instruments became available and the routine use of GC-MS became commonplace.

Liquid chromatograph coupled to photodiode-array (LC-PDA)

An LC-PDA consists of a binary or quaternary liquid chromatography separation system coupled to anUltraviolet-visible Photodiode Array detector (UV-Vis PDA) (Figure 3). The binary pump allows for the generation of an eluent that changes in composition with time (known as a gradient). An example would be starting at 95% of solvent A and 5% solvent B to 0% of solvent A to 100% solvent B over 30 minutes. A sample of a mixture is injected into the system by the autosampler and the gradient aids the separation of the components of the mixture across the column. The PDA detector generates a UV-Vis spectrum of the eluting compounds as a function of time and the resultant three dimensional data (chromatographic retention time, absorbance and wavelength) is processed by the data system (Figure 4). An example of the application of the technique is the separation and identification of sugarcane flavonoids (Walford,*et al*,2009).

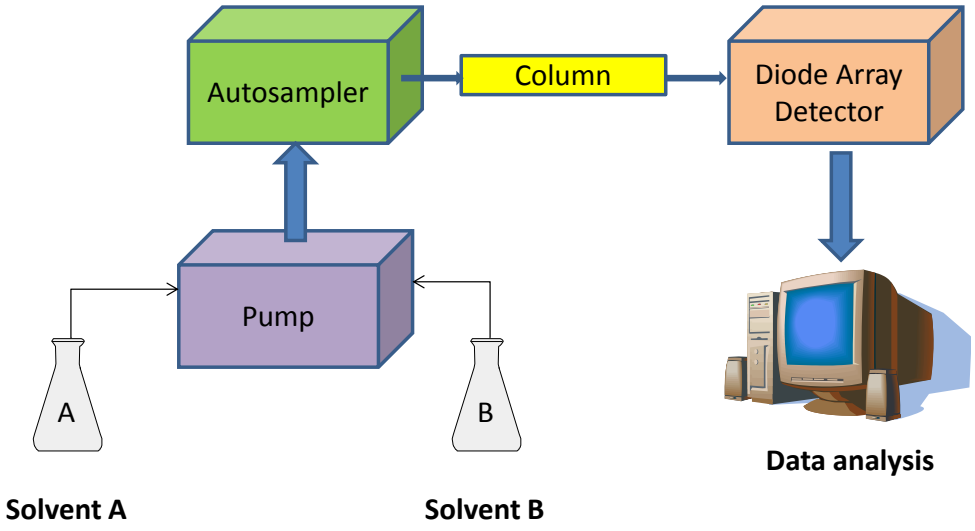

Figure 3. Diagram of a binary liquid chromatograph coupled to a photodiode array detector.

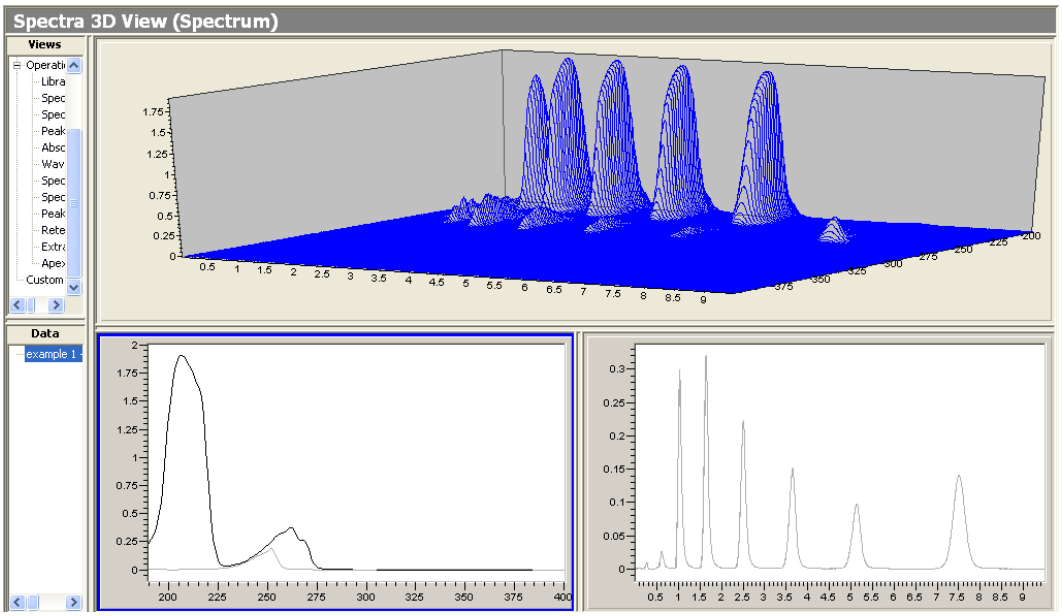

Figure 4. PDA screen output showing in the *top pane* the three dimensional representation of time (x-axis), absorbance (y-axis) and wavelength (z-axis), the *bottom left pane* two of the spectra collected and *bottom right pane* the chromatogram at 250 nm.

Liquid chromatograph coupled to pulsed amperometric detector (LC-PAD)

AnLC-PAD is equivalent to the LC-PDAwith the PDA being replaced by a PAD. PAD is a sensitive detection technique for compounds that can be oxidised at a working electrode (typically gold or carbon) such as sugars. The detector generates two-dimensional data (retention time and response) which is processed by the data system.Examples of the use of this technique include the analysis of sucrose, glucose and fructose in factory products (ICUMSA, 2009) and chromatographic oligosaccharide profiling as a differentiator of cane and beet white sugars(Morel du Boil, 1997).

LC-PAD has been used in the SMRI as a research tool since the early 1980s and been used to determine the sucrose, glucose and fructose in final molasses for factory control since the late 1980s. However, it was used almost exclusively in the isocratic mode (keeping the eluent concentration constant throughout an analysis). The power of the system for research purposes is enhanced when coupled to a gradient pumping system (changing the eluent concentration throughout an analysis). Analysis of mono-, di-, tri-saccharides and oligomers then becomes possible.

Nuclear magnetic resonance spectroscopy (NMR)

The NMR instrument consists of a magnetic field into which the sample of interest is placed and a pair of radio frequency coils (transmitter and receiver) (Figure 5). Atoms with odd masses (^{1}H, ^{13}C, ^{31}P, etc.) have a nuclear spin allowing the atoms to align with an applied magnetic field. When a radio frequency (RF) signal is applied to the sample the nuclei resonate. Depending on the position of the atom within the molecule the frequency emitted by the atom will shift. This shift in frequency is measured and plotted (Figure 6). The number of atoms giving rise to the spectrum can also be calculated by integrating the peaks. This analysis is frequently used to help determine the molecular structure of compounds including carbohydrates (Lopez*et al*, 2003; McIntyreand Vogel, 1993). SMRI researchers have used the technique to elucidate the structure of a novel biopolymer(du Clou, 2010).

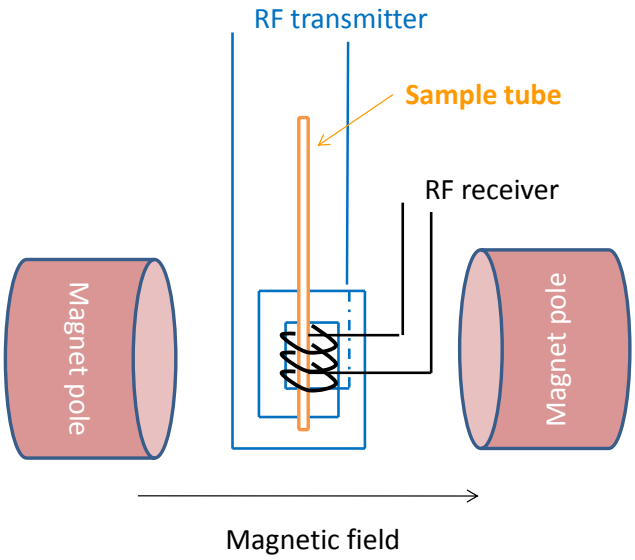

Figure 5. Diagram of a nuclear magnetic resonance (NMR) system.

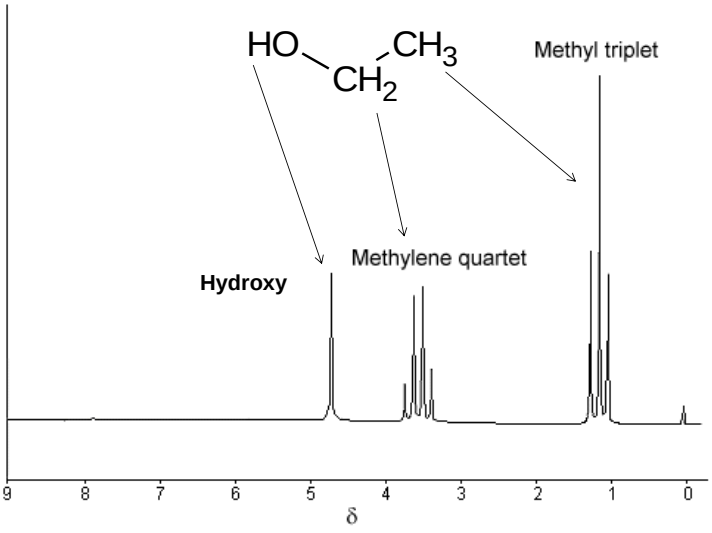

Figure 6. ^{1}H nuclear magnetic resonance (NMR) spectrum of ethanol with the x-axis showing the chemical shift (δ)

Examples of the use of selected techniques

GC-MS

In the medical environment the identification of metabolites and poisons can literally be a matter of life and death. Not only is the positive identification of the particular poison important but the speed with which the information is provided to medical personnel can be critical. This was demonstrated with a recent request to the SMRI for the analysis of an unknown liquid in a branded alcohol bottle. A number of people had drunk some of the contents; two individuals had died and the remainder were in a critical condition. Other laboratories were unable to analyse the sample at the time. The SMRI was called upon to analyse the 'unknown liquid' and identify the toxic component (if any). A simple check on the solubility of the contents in water showed that it was immiscible.The sample was therefore diluted in a non-polar solvent and analysed by GC-MS using a generic set of conditions that would cover non-polar and polar compounds. The resultant total ion chromatogram is shown in Figure 7.

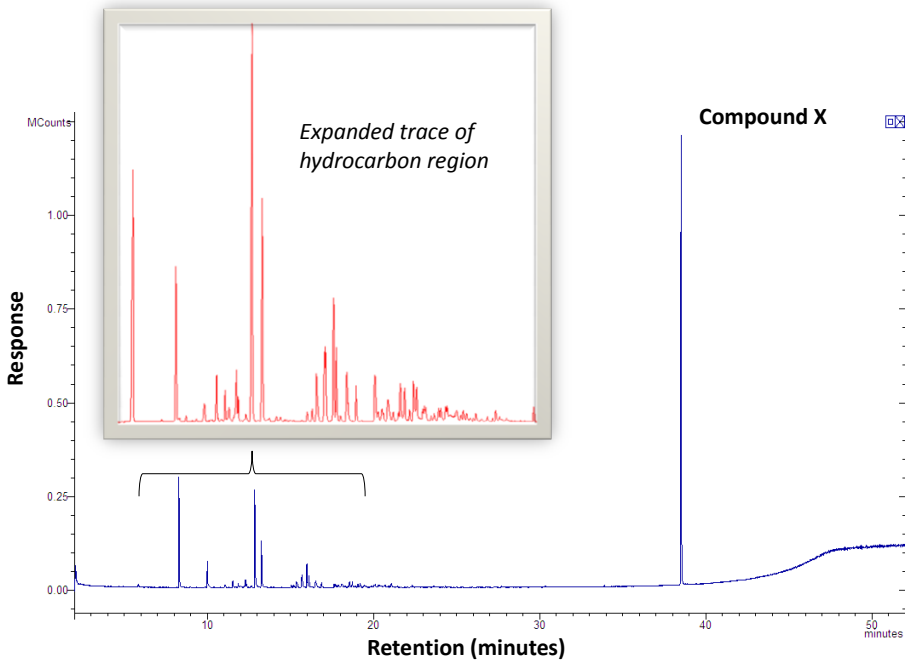

Figure 7. Total ion chromatogram of liquid in the 'unknown' sample.

Extraction of the mass spectra of the peaks showed that the compounds eluting in the 7.5 to 20 minutes range were components of diesel. The mass spectrum of the large peak at 39 minutes was extracted and compared to the National Institute of Standards and Technology (NIST) library that comes with the GC-MS software. This library contains the mass spectra of over 300 000 compounds.The extracted spectrum showed a match of greater than 90% probability to a known veterinary product known as Amitraz (Figure 8). The preliminary results were available in under an hour and the necessary life saving medical procedures could be started. This example shows the usefulness of the NIST library. However, for the SMRI carbohydrate projects, the library excludes many of the derivatised sugars relevant to the researcher. It therefore requires the development of a spectrum library specific for the compounds of interest (du Clou and Walford, 2010).

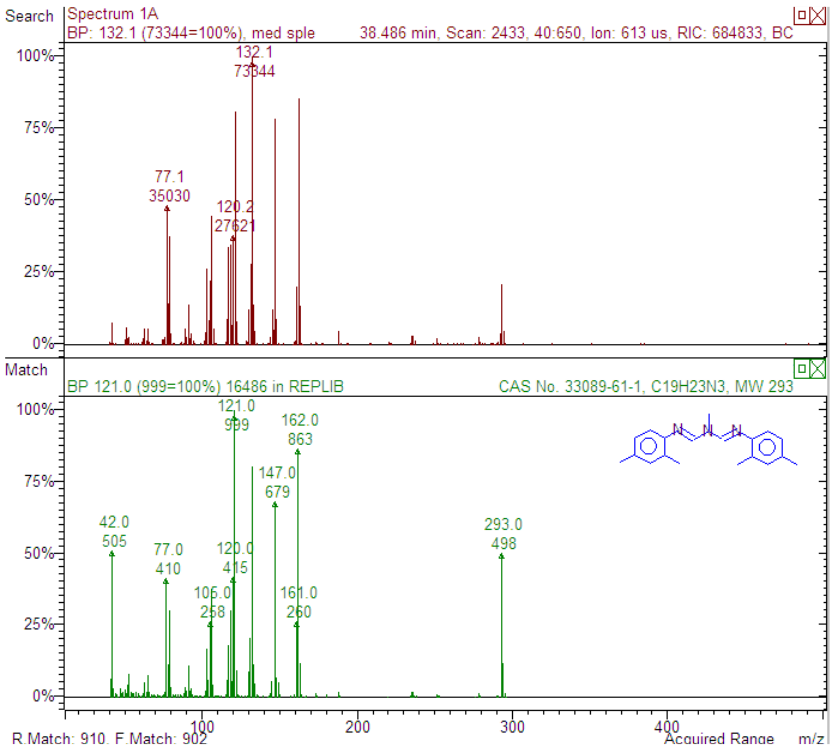

Figure 8. Comparison of the spectra extracted from Compound X (upper) and the NIST library spectrum (lower) for Amitraz.

GC-MS is also used to verify the authenticity and adulteration of foodstuffs, chemicals and pharmaceuticals. Habit-forming drugs such as heroin are also used medicinally to alleviate pain and are therefore legally imported into South Africa by pharmaceutical companies, but under strict controls. In this particular case a consignment of drugs had been substituted with sugar in the delivery chain from Europe. Police had isolated the swopping of the articles to two locations and the type of sugar would highlight the likely location – if substituted in Europe with local sugar it would more likely be of a beet origin, whist if it was substituted in South Africa the sugar would more likely be cane sugar. The SMRI was requested to analyse the sugar and give an opinion as to whether it was beet or cane sugar.

Theanderose (6-α-glucopyranosyl-sucrose), a trisaccharide, has been identified as a constituent of cane sugar but not beet sugar,as reported by Morel du Boil (1992) and others.Research has shown that chromatographic oligosaccharide profiling can be used as a means of differentiating cane and beet white sugars (Morel du Boil, 1997; Eggleston *et al.*, 2004). The separation techniques may not totally separate the closely related trisaccharides but the chromatographic profiles are useful in classifying a sugar, and can be used to determine adulteration of beet with cane white sugar at between 10 and 20%. The trisaccharides commonly measured include raffinose, 1-kestose, 6-kestose, neokestose and theanderose. Whilst theanderose has not been detected in white beet sugar, other unknown trisaccharides were found to co-elute at the same retention time.

The use of GC-MS for this type of profiling is not common. A total ion chromatogram from the GC-MS analysis of a typical cane refined sugar showing the regions of elution is shown in Figure 9. The trisaccharide region is of importance in fingerprinting the sugar.

Raffinose elutes at approximately 26 minutes whilst theanderose elutes at about 28 minutes. The other oligosaccharides elute around these two peaks.

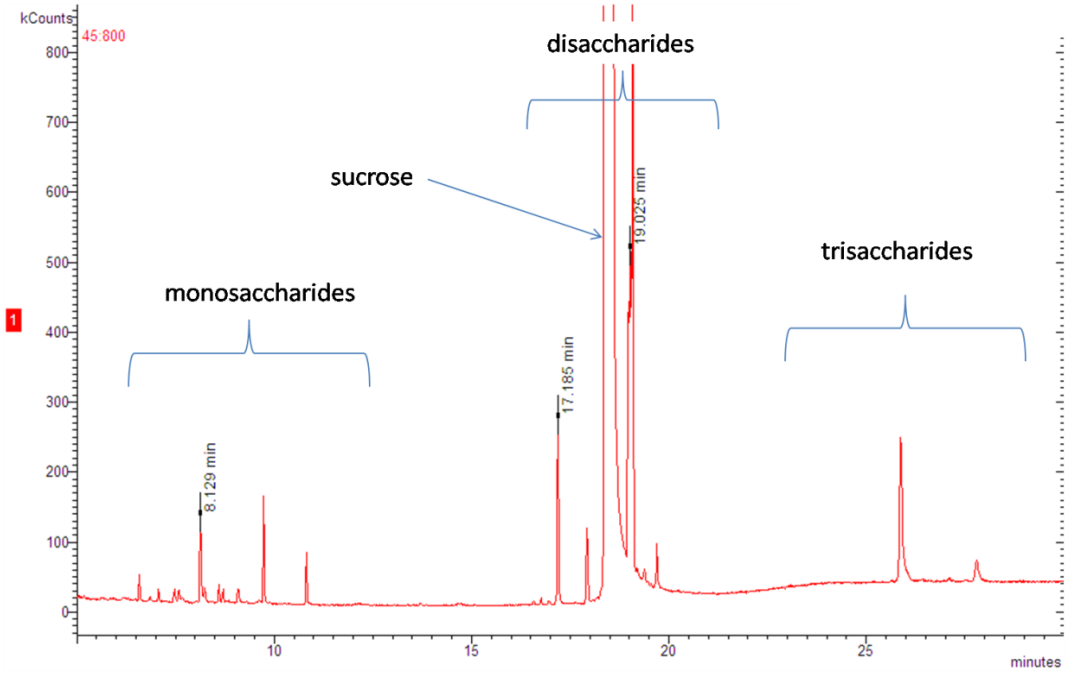

Figure 9. Chromatogram showing the GC separation of a typical refined cane sugar sample showing the regions of separation of mono-, di- and trisaccharides.

Figure 10 shows a close-up of the trisaccharide region for two typical cane sugar samples (chromatograms B and D), a beet sample (chromatogram A) and the unknown sample (chromatogram C). The trisaccharides are present around the raffinose peak (approximately 26 minutes). The silylated trisaccharides give similar mass spectrums. The power of the GC-MS in profiling is the resolving power of the GC to separate the trisaccharides followed by the MS to confirm the structure of the peak. Thus the combination of the unique retention time and associated mass spectrum gives confirmation of the known trisaccharide peaks. A typical mass spectrum for the silylated trisaccharides is shown in Figure 11. The main features are the peak at m/z 437, indicative of the trisaccharide, the peak at m/z 361, indicative of a disaccharide (from the breakdown of the trisaccharide) and the peaks at m/z 217,204 and 147, indicative of the monosaccharides (from the breakdown of the disaccharide). From these chromatograms it can be seen that the unknown sugar profile (C) more closely resembles the beet sugar profile (A) rather than the two cane sugar profiles (B and D).

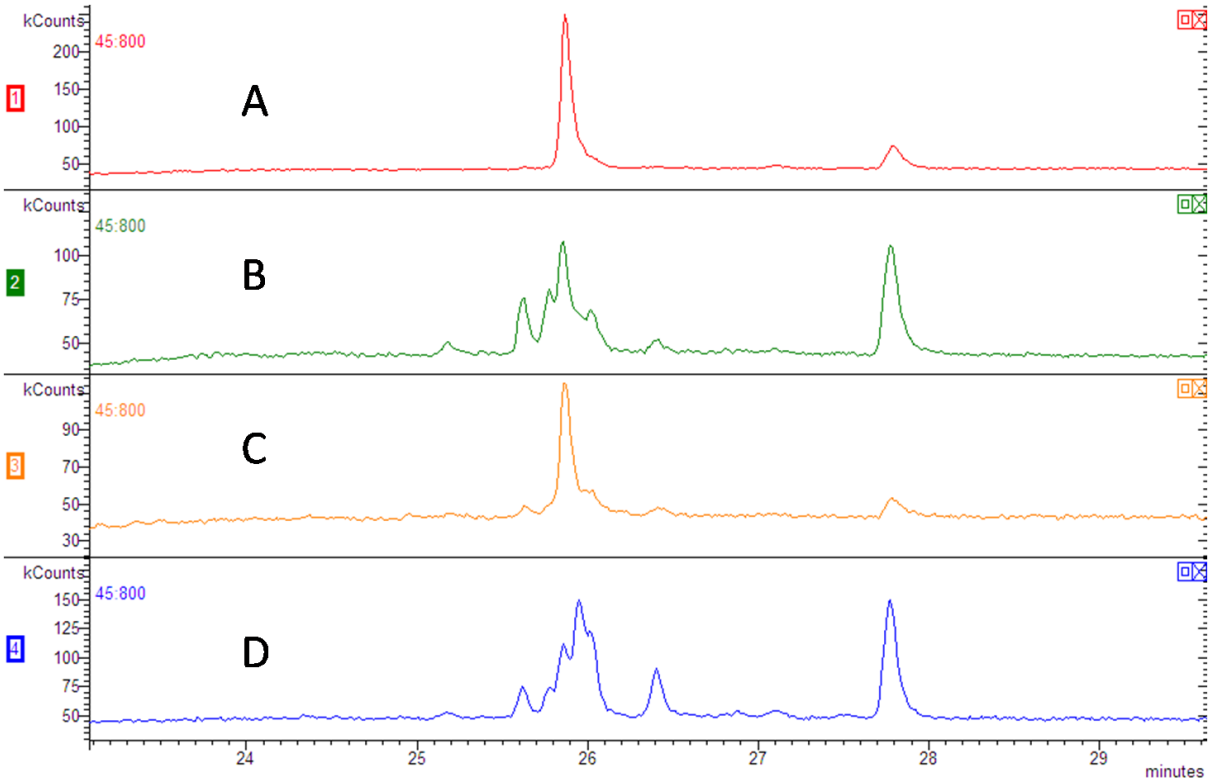

Figure 10. Close-up of the trisaccharide region showing the raffinose (26 minutes) and theanderose (27.8 minutes) and other oligosaccharides present in cane sugar. Samples A - beet sugar; B and D - cane sugars; C - unknown sample.

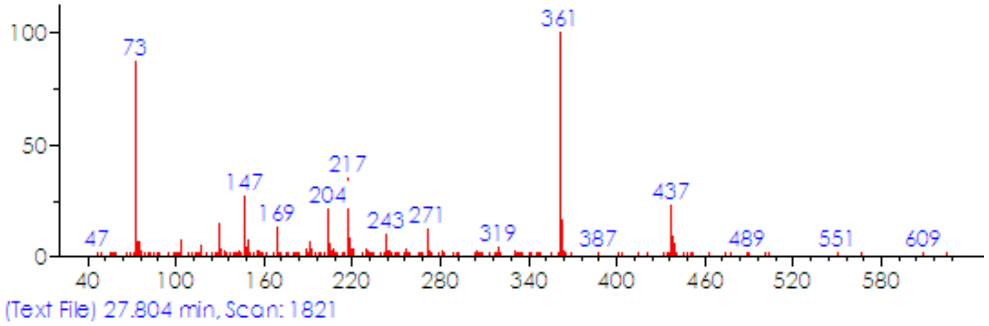

Figure 11.The mass spectrum of theanderose as an example of a typical silylated trisaccharide.

The MS allows the researcher to extract the ions at a particular m/z ratio for a range of compounds of interest, in this case m/z of 437 to look at the trisaccharides (Figure 12). In this figure the extracted scan is shown below each of the normal chromatograms. The beet sugar (Sample A) in Figure 12shows a clean extracted chromatogram around the raffinose peak, whereas the cane sugar (Sample B) shows other peaks around the raffinose peak indicating the presence of the other trisaccharides. The extracted unknown sample showed a profile similar to the beet sugar indicating that there are no other trisaccharides present in the sample.In profiling, the ratio of peak heights is useful to indicate similarities. The peak ratios of raffinose totheanderose type compounds are

shown in Table 1. The unknown sample has a raffinose:theanderose type ratio similar to the beet sample and quite different to the cane sugars.

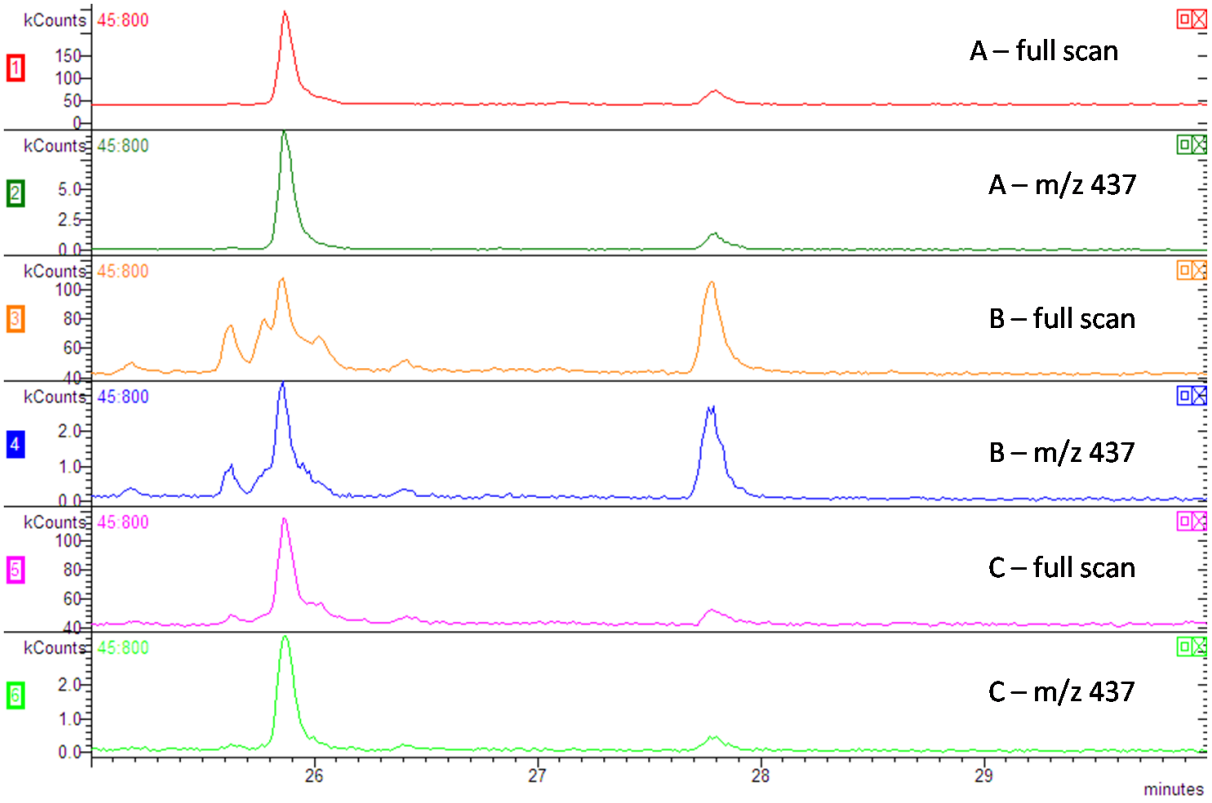

Figure 12. Trisaccharide region showing the full scan and extracted chromatograms (m/z 437). A - beet sugar, B- cane sugar, C- unknown sugar.

Table 1. Comparison of the raffinose:theanderose type height ratio from Figure 10.

Sample	Raffinose:theanderose height ratio
Beet sample (A)	6.0
Cane sample (B)	1.1
Cane sample (D)	1.0
Unknown (C)	5.4

The region in the chromatogram between the di- and trisaccharide peaks contains trace non-sugar components. A mass spectrum of one of these compounds is shown in Figure 13. The extracted chromatograms at m/z 503 (the highest m/z for this compound shown in the figure) are shown in Figure 14. It can be seen that neither the beet sugar (chromatogram A) nor the unknown sample (chromatogram B) contained either of these compounds whereas the compound can be clearly seen in the two cane samples (chromatograms C and D).

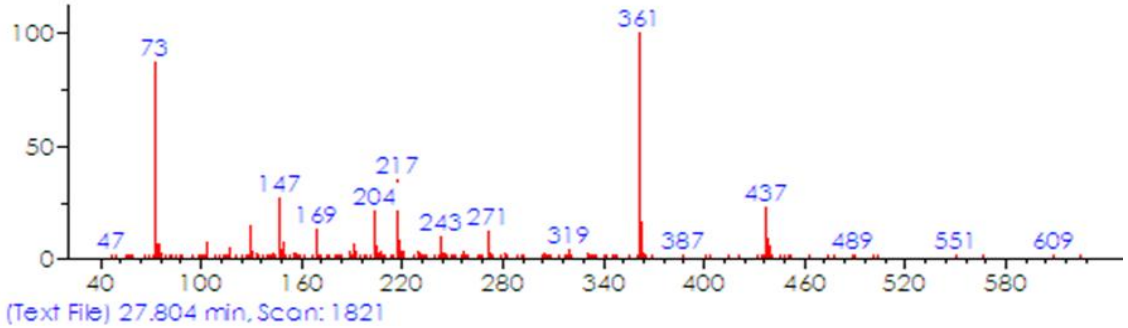

Figure 13. Mass spectrum of silylated trace compound in South African cane sugar.

It was thus concluded that the sample of sugar supplied for analysis that was used to substitute the pharmaceutical was more likely of a beet origin (although it could also have been a mixture) and therefore the substitution was more likely to have taken place in Europe rather than South Africa, although this cannot be proved.

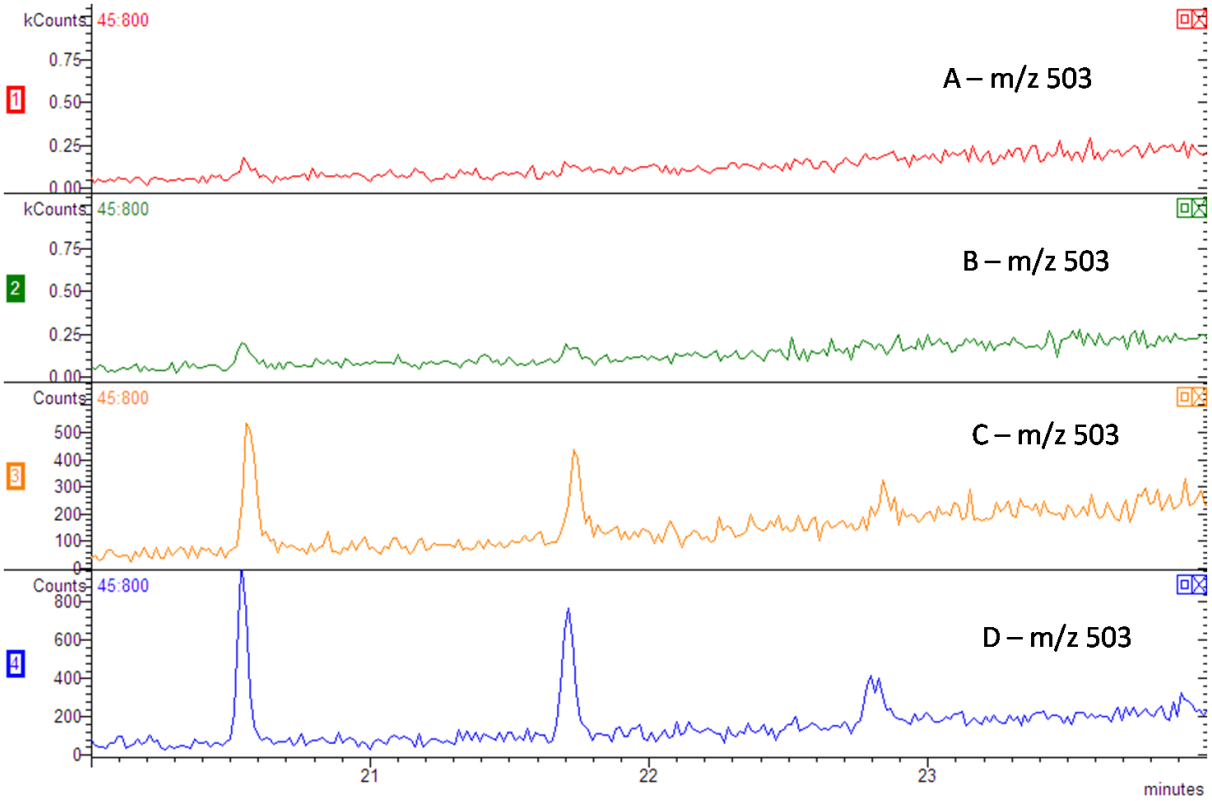

Figure 14. Extracted chromatograms (m/z 503) of trace components. A - beet sugar, B – unknown sample, C and D - South African cane sugar samples.

LC-PAD

The SMRI was requested to help solve problems related to the fermentation of agave juices used to make tequila. In the normal process, the piña or 'pineapple' of the blue agave plant is harvested, cut and roasted to help hydrolyse the fructans (normally inulin), followed by expression of the juices (containing the partially hydrolysed fructans and

other mono-, di-, oligo- and polysaccharides) and fermentation to produce the alcohol. In this particular problem, the locally harvested agave produced unexpectedly low yields of ethanol. Samples of the expressed juices were prepared and compared to authentic inulin samples (Figure 15) using a gradient eluent system with pulsed amperometric detection.

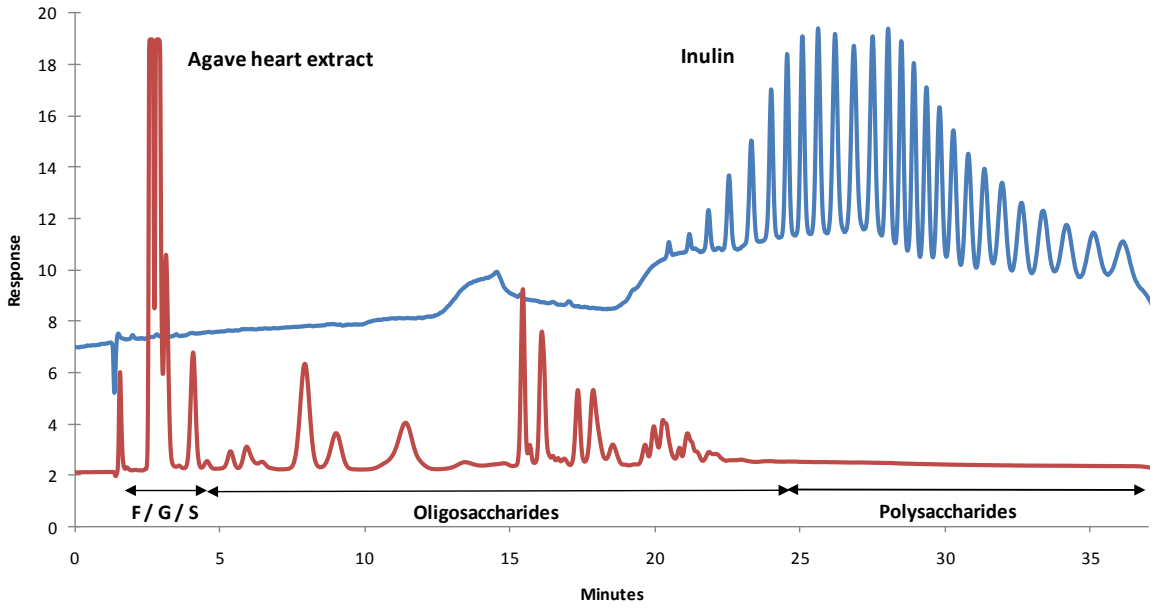

Figure 15. Chromatograms comparing inulin and agave heart extract.

It can be seen that the extract being used to produce the tequila alcohol did not contain any inulin type fructans and contained mainly mono-, di- and oligosaccharides. The fructan levels were thus absent in the extracts and the total carbohydrate content was found to be approximately half of what would normally be expected from the agave plant, thus explaining the low alcohol yields. The company requesting the analysis closed a number of years later partially due to the unfavourable economics at these low yields. Further interest in the oligomeric structure of the fructans present in the locally grown agave plant has been shown by medical researchers and analysis has been provided by the SMRI.

Summary

This paper highlights two case studies that were used to explain a number of modern instruments and demonstrate how the results can be used to solve a variety of problems to widen the understanding of the sugar technologist. Similar carbohydrate based problems have to be solved on a daily basis as part of the SMRI research programme. Access to this modern instrumentation and the development of the necessary skills allows the SMRI to fulfil the required analytical output as part of the SMRI research programme.

REFERENCES

du Clou H (2010). The structural elucidation and characterisation of microbial extracellular polysaccharides. MSc Thesis. University of KwaZulu-Natal (Durban).285 pp.

du Clou H and Walford SN (2010). An introduction to gas chromatography mass spectrometry for the structural elucidation of polysaccharides from sugar processing streams. *ProcS AfrSugTechnol Ass* 83: 392-409.

Eggleston G, Pollach G and Triche R (2004).Differentiating cane white sugar from beet white sugar using ion chromatography profiles.*Proc SPRI Conference on Sugar Processing Research – New Developments*. Atlanta, Georgia. pp 207-214.

Gohlke RS (1959).Time-of-flight mass spectrometry and gas-liquid partition chromatography. *Anal Chem* 31: 535-541.

Gohlke RS and McLafferty FW (1993).Early gas chromatography/mass spectrometry. *J Am Soc Mass Spectrom* 4: 367-371.

ICUMSA (2009).Method GS7/8/4-24.The determination of Glucose, Fructose and Sucrose in Cane Juices, Syrups and Molasses, and of Sucrose in Beet Molasses by High Performance Ion Chromatography – Official.*ICUMSA Methods Book 2009*.

Lopez MG, Mancilla-Margalli NA and Mendoza-Diaz G (2003). Molecular Structures of Fructans from *Agave tequilana*Weber *var. azu*. *J Agric Food Chem* 51: 7835-7840.

McIntyre DD and Vogel HJ (1993).Structural studies of pullulan by nuclear magnetic resonance spectroscopy.*Starch - Stärke* 45: 406-410.

Morel du Boil PG (1992). Theanderose – a contributor to c-axis elongation in cane sugar processing. *IntSug J* 94: 90-94.

Morel du Boil PG (1997). Theanderose – distinguishing cane and beet sugars. *IntSug J* 99: 102-106.

Ryhage R (1964). Use of a mass spectrometer as a detector and analyzer for effluents emerging from high temperature GLC columns. *Anal Chem* 36: 759-764.

Walford SN, Egli S and Martincigh B (2009).Separation, identification and characterisation of flavonoids of extractives from sugarcane (*Saccharum officinarum*) leaf. *Proc S AfrSugTechnol Ass* 82:342-345.