A Study into Alternatives to Gas Chromatography for Quantification of Vicinal Diketones in Beer

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# Abstract

The gas chromatography-headspace (GC-HS) analytical technique used for the assay of vicinal diketones (VDK); 2,3-butanedione and 2,3-pentanedione is over 50 years old (1). Although well established and predictable the GC-HS method is not without its shortcomings, with long analysis times and a radioactive detector as two such issues.

The technological advances within the last 50 years in analytical chemistry have mirrored those seen in computers and as such real time analysis and the miniaturisation of hardware are standard advancements. This has culminated in the growth of microfluidics and “Lab on a Chip” technology, especially in the field of bioreactors.

This study aims to assess the use of microfluidics for the electrophoretic separation of VDKs and their precursors, and subsequent detection on an ion mobility spectrometer or mass spectrometry with an intention of superseding the incumbent GC-HS technology in terms of speed and accuracy of assay.

# Introduction and Literature Review

## What is VDK and why is it important

Diacetyl (2,3-butanedione) and 2,3-pentanedione, collectively known as VDK, are normal products of yeast metabolism and are formed in every brewery fermentation. The desired level in the final beer depends on the particular flavour aimed for. However, in many lagers it is seen as an off flavour and due to the low taste threshold concentration (less than 20 parts per billion (ppb) ≈ 20 µg.l-1) many brewers are happy to have a minimum of diacetyl in their beer.

Therefore VDK concentration is an important quality parameter in brewing, potentially only second in importance to that of the alcohol content. The synthesis of diacetyl is well understood and has been described separately and previously by Inoue et al, Engan, and Wainwright (2) (3) (4).

The uptake of amino acids by yeasts plays a large role in the metabolism of diacetyl and in particular the uptake of valine. The uptake is performed in a sequential order of which there are four groups. Acetolactate which is the precursor of valine and in turn is derived from glucose, diacetyl is also produced as a by-product of this reaction. Acetoin is the precursor of diacetyl and when assayed by GC-HS is reduced to diacetyl by extended heating to give “total diacetyl”, “free diacetyl” assay is performed on the same GC column but the heating is for a shorter period and subsequently the acetoin reduction is not accomplished.
Once diacetyl has been excreted it is quickly reduced enzymatically to acetoine and finally to 2,3 butanediol which is excreted from the yeast cell. This last product does not convey any off flavour to the beer (5). The mechanism is shown below:

## Glucose to pyruvate;

C6H12O6 + 2 ATP + 2 NAD+ CH3COCOOH + 4 ATP

glucose pyruvate

## Pyruvate to diacetyl and valine;

CH3COCOOH + CH3CHO acetaldehyde

α-acetolactate CO2 acetoin - 2H diacetyl

CH3C(OH)COOH CH3COCHOHCH3  COCH3

COCH3 COCH3

+ NACH + H+

- NAD+ + H2O

(CH3)2CHCOCOOH (CH3)2CHCHNH3COOH

α-oxovalerate valine

## Diacetyl reduction

NADH + H+ NAD+

CH3COCOCH3 CH3COCHOHCH3

 diacetyl acetoin dehydrogenases acetoin

NADH + H+ NAD+

CH3COCHOHCH3 CH3CHOHCHOHCH3

 acetoin butanodiol dehydrogenases 2, 3-butanodiol

## VDK and Major Beer Component Composition and Properties

Table 2.1 Properties of Selected Beer Components



Data taken from Sigma Aldrich (6). Although there is no data for the pKa of hexanedione, it is likely similar to that of butanedione and pentanedione.

## Current Technology, Gas chromatography

To test for the diacetyl at the part per billion levels required, gas chromatography (GC) head space (HS) analysis has traditionally been employed. (1)

Gas chromatography is the separation of a vaporised sample due to the difference in the partition co-efficient between the gaseous mobile phase and the stationary phase within a column.

The mobile phase does not interact with the components of the analyte other than providing the motive force to move them down the column, the difference in retention is wholly down to the stationary phase and the effect of the temperature of the column on this parameter. As temperature is increased, so the partition co-efficient is forced toward the mobile phase and therefore swifter elution from the end of the column. Generally the components with the lower boiling point exhibit the lower affinity for the stationary phase and likewise the lowest retention times within the column. Therefore gas chromatography is mainly a separation of components based on their boiling point (7).

## Electron capture detector

Electron capture detectors (ECD) are used in conjunction with gas chromatograms in order to detect highly electronegative compounds such as 2,3-butanedione and 2,3-pentanedione. The volume of the detector is kept as small as practicable and is lined with a fairly highly active ß-emitter (high speed electrons) usually Ni-63. The inlet tube and main body are insulated from one another and used as opposing electrodes. When the carrier gas of the GC enters the detector it is ionised by the radiation, these ions migrate to the anode causing an ionisation current to be produced. However in the presence of a component capable of capturing electrons, this ionisation current will be reduced as the ionised electrons are absorbed by the electronegative components and do not reach the anode. This change in current is detected as the electronegative components pass through the detector (8).

Compared to other detectors ECD is difficult to use, requires a relative high level of maintenance and stable temperature, has a small linear dynamic range, due to the nature of the radioactive ionisation source, ECD also have to be moved, serviced and disposed of by specialists (8).

## Head Space Assays

Head space analysis is basically a preparation separation technique where the more highly volatile portions of the matrix are extracted from the less volatile components within a heated, sealed vial. After a certain amount of time at a certain temperature the head space (the vapour about the liquid) is injected into the GC.

GC-HS is utilised when the matrix is extremely complex with components which are not required to be assayed or may interfere with the GC analysis and potentially some of which would be damaging to the stationary phase of the column if injected.

Due to the higher volatility of the components of the headspace, the time taken for the assay is reduced; this is due to the components of the matrix with the higher boiling points and therefore a higher affinity for the GC column being left within the matrix within the vial. However, what time is gained within the assay is often lost in the heating and equilibrating the vial to the required temperature and this is especially true of the methodology utilised for the total diacetyl assay.

The partition co-efficient of the components under assay also plays a role on the limit of detection. By definition, any compound with a co-efficient < 1 will not be fully present in the vapour phase within the vial and therefore will reduce the amount injected in to the instrument. However the limit of detection (LoD) will be enhance by the reduction in noise from other compounds which remain within the matrix, the LoD will largely depend on the method specificity in regards to the temperature and partition co-efficient for the component.

## Future Technologies: Microfluidics, Ion Mobility Spectrometry and Mass Spectrometry

Microfluidics is a miniaturisation of analytical instrumentation to the size of printed circuit board, hence the term lab on a chip. This has offered automation and a high throughput of screening whilst also operating at low reagent and consumable use, thereby reducing both per assay cost and waste production. Microfluidics also has the distinct potential to reduce turnaround times vastly increasing the number of assays which can be performed within a set time (9).

Lab on a chip technology has seen some market use for biological screening and could also be extended to free amino nitrate but little development in the analytical chemistry field has been performed. However, the miniaturised pumps and channels lends itself partially well to the use as an electrophoretic column (7).

Electrophoresis is a separation method which separates on the basis of the different migration rate of charged species within a direct current electric field. To perform a separation a small volume of sample is introduced to a narrow tube or slab of semisolid medium. There is an aqueous buffer within the tube and a high voltage is applied across the tube to create a motive force on the charged particles within the sample. The rate at which the particles move is governed by the charge and size of the component (7).

It was developed by Tiselius in the 1930s to study serum proteins for which he gained the Nobel Prize in 1948. The advancement of electrophoresis as a quantitative analytical method was realised in the 1980s with the introduction of a fine bore tube as the drift medium. This lead to small sample volumes, high-speed separations and high resolution separations. Due to the extremely low volume to surface area of the capillary the issue of heating when the high voltage is applied are negated. In addition, the analytes are eluted from the end of the fine bore tube meaning that liquid chromatography detectors such as IMS or MS can be employed with minimal changes (7).

Ion Mobility Spectrometry (IMS) is the method and instrumentation involved in the separation and detection of chemicals due to their ion velocity through an electric field operating in the gas phase (10). When a gaseous ion is placed in an electric field it will be accelerated until it collides with a neutral gas molecule, over a (relatively) large distance this acceleration and collision will be averaged out to a drift velocity (11). It allows the analyte ions to be distinguished not only upon their mass but also charge and cross sectional area. This separation by multiple factors has also allowed the study of the structure of the chemicals involved, especially biomolecules (12).

The main application of IMS however is to be found in the military, forensic and counterterrorism fields (13). This is due to the intrinsic properties of IMS; high sensitivity, fast response and ease of portability due to small size means that it is ideally suited for the detection of trace amounts of compounds including the highly electronegative nitro groups of many explosives and high proton affinity of the amide groups of many illegal drugs (13). This is however extendable to the electronegative VDK compounds and precursors.

An IMS instrument is composed of a sample inlet, an ionisation region which is separated from the drift region by a shutter grid. Detection is performed by faraday plate which is again separated from the drift region by shutter or aperture gate (11).



Figure 2.1 Schematic of an IMS instrument. (12)

Not shown on the diagram is the drift gas. This enters the instrument at the detection end of the drift region, flowing against the electric field and the ions of the sample (11).

The sample is introduced to the ionisation region via a number of methods, head-space injection, use of a nickel wires or laser ablation or even electrospray ionisation (12). The sample is then ionised (if not already) in the first compartment or ionisation region, this is performed by β radiation emitted from radioactive source (normally 63Ni), which reacts with the drift gas which in turn reacts with the sample (11). These sample ions are then injected into the drift region by the opening of the shutter grid and accelerated towards the faraday plate by the electric field. Different compound ions will exhibit different mobility and therefore will take varying times to navigate the drift region. Once the ions hit the detector they are neutralised causing an electric charge to flow, this detector response is measured against the drift time producing a mobility spectrum (10). The IMS drift tube can be used as the ion separator and coupled to a mass spectrometer for detection.

Mass spectrometry’s (MS) inherent features have raised it to an elevated position within analytical methods. Due to the unequalled limits of detection, sensitivity and wide range of applications with applicable mass ranges from the atomic to large protein assay, together with the large number of instruments with which it can be coupled, the profile of mass spec continues to increase and means that MS is possibly the most widely applicable analytical technique giving information about elemental composition, molecular structures and, both quantitative and qualitative composition (14) (7).

Developed by the petroleum industry to measure the mixture of hydrocarbons within catalytic crackers in the 1940s and 50s, MS as commercial instruments were first introduced in the 1950s but due to the improvement of data acquisition and more importantly the ionisation techniques in the 1980s and 90s the use and range of applications has seen a dramatic increase (7).

The analysis of a sample by MS requires the ionisation of the component, conversion of this ionised component to a stream of ions, separation of this stream by their mass to charge ratio (*m/z*, where *m* is the mass number of the ion in atomic mass units and *z* is the number of fundamental charge units it bears) and finally measuring the ion current produced when these ions contact the detector (7).

An ion trap mass analyser is a device in which gaseous ions are confined for an extended period within electric and magnetic field. This analyser was first introduced in 1953 by Paul but the use and development by Paul and Dehmelt led to them being awarded the Nobel Prize for physics in 1989 (15) (7). Ion traps are now routinely used for the detector on chromatographic separations.

When used as a mass spectrometer, a stable region is created within the electrodes in much the same way as the quadrupole. Ions are produced by electron impact or chemical ionisation in a burst but are not accelerated as in the quadrupole or time of flight spectrometers and a wide range of ion masses can be stored for extended periods within the instrument. A technique called mass-selective ejection is then used to release the trapped ions onto the detector. This is achieved by the increase in the voltage of the ring electrode when the ions become unstable they are forced out of the trap and onto the detector.

Ion trap detectors have the advantages of being rugged, compact, exhibits a low detection limit and are the most inexpensive mass spectrometers they also have the advantage of not requiring a large vacuum pump in order to operate due to the small volume required for the stable area within the ring and endcap electrodes. They can also be interfaced with a large number of other applications and utilise electrospray ionisation for the source of ions. Another advantage is that the ion trap does not require a large separate high vacuum pump as required by the large volumes quadrupole and time of flight instruments reducing running costs.



Figure 2.4 Ion Trap Mass Spectrometer Schematic (16)

## Applications of Lab on a Chip, Electrophoresis, Ion Mobility Spectrometry and Mass Spectrometry.

Virdi *et al* describes a low cost micro capillary electrophoresis lab on a chip devise (17). The chip incorporated electrodes for sample injection, separation and detection electrodes so that no external mechanical hardware would be required in order that the separation could be performed. The manufacture of the chip utilised proven methodologies thereby reducing the unit cost of the chip. The chip was proven to be able to separate and detect glucose, hydrogen peroxide, and uric and ascorbic acid in under a minute. However, the chip used an electrical chemical detector, not the spectrometric detection proposed by this research.

The coupling of electrophoretic separations of to mass spectrometry has been demonstrated previously by Gómez-Caravaca, Daniel and Oedit (18) (19) (20).

In addition to the coupling of electrophoresis to MS, Gómez-Caravaca *et al* also demonstrates that the polar compounds in walnuts can be separated by electrophoresis and subsequently detected by a MS (18). The work demonstrates that phenolic and other polar compounds can be separated, quantified and identified by the coupling of electrophoresis to a mass spectrometer. Although the bulk mass of the analytes were phenolic compounds, there were also acids and esters within the components of the walnuts. The method was also able to identify a previously unidentified compound found within walnuts. To couple the electrophoresis column to the MS an electrospray instrument was used. Although the MS instrument was a different type to that proposed by this research the ionisation method is the same.

Daniel *et al* demonstrated the uptake of amines in beer and wine by separation by electrophoresis and the subsequent detection by tandem mass spectrometers (19). This work was of a similar nature to that undertaken by Gómez-Caravaca *et al* but took a more in depth method development approach and gave an indication as to the limits of detection for the components which are as low as 1 µg.L-1 (ppb).

Oedit meanwhile describes how to couple lab on a chip electrophoresis to mass spectrometry (20). Although not a direct analytical investigation, the paper describes the process of coupling electrophoresis on a chip to mass spectrometry, noting that the two methods are particularly well matched to one another. The paper documents the development of lab on a chip coupled with MS for a period of 2.5 years ending September 2014. It states that electrophoresis was the 3rd most common separation method with electrospray ionisation as the most utilised ionisation method. Although issues regarding electrophoretic separations with MS have been encountered, to a large extent these have been overcome.

Ion mobility spectrometry is a much less developed analytical method however there are examples of its application to relevant compounds. Kotiaho described a method for in-line testing of alcohol concentration of beer using an IMS instrument (21). This work is now over 20 years old but was able to accurately measure ethanol content to 0.05 % v/v. A novel membrane method was developed for the inlet into the instrument to counter the challenge of a liquid sample which has to be tested in the gas phase.

Arce describes the ability to couple electrophoresis to ion mobility spectrometry (22). Like Oedit, this is a review of the applications rather than an analytical investigation. It is however useful to demonstrate that the coupling of electrophoresis to IMS has occurred previously. Again the authors advocate the use of electrospray ionisation.

Therefore with these papers and articles it has previously been demonstrated that each of the individual challenges for the proposed project have been encountered and overcome on an individual basis.

# Research Hypothesis

The time taken to assay rough beer for the presence of VDK and their precursors by GC-HS can be in the region of 90 minutes for a single total diacetyl. With advances in analytical chemistry, this 50 year old methodology could be updated and the assay time vastly improved, potentially by an order of magnitude and certainly down to 15 - 20 minutes.

The use of an electrophoretic separator and drift detector or ion trap mass spectrometer could provide a three dimensional separation, more accurate, easier to set up, calibrate and with a cheaper unit cost in regard to both the assay, reagents and analytical equipment.

The viability of microfluidics will be assessed as a separation method of VDKs as will a desk-top ion trap mass spectrometer and ion mobility spectrometer. The assessment will encompass but not be limited to:

* Specificity,
* Linearity,
* Accuracy,
* Precision (repeatability and reproducibility),
* Range,
* Quantitation limit and
* Detection limit.

As specified by the Food and Drug Administration (FDA) (23) and in comparison to the GC-HS methodology currently employed.

# Materials and Methods

The most important part of the project would be the separation of the components of beer and this will therefore be the initial focus.

In order to simplify initial experiments a surrogate “beer” containing known amounts of water, ethanol, sugars and VDK components and an internal standard will be tested. Once the separation is proven with this surrogate, the method for rough beer with the inherent multitude of chemicals, proteins and yeasts will be developed from the surrogate methodology.

The initial separation will require an electrophoretic instrument (capillary zone electrophoresis (CZP)) and associated apparatus, the use of CZP is simpler for swift method development. The capillary will also likely require some form of guard column if rough beer is to be directly analysed. Direct analysis of rough beer is advantageous as it will negate the need for pre-assay centrifugation, significantly reducing sample preparation time.

The VDK components under investigation will be 2,3-butanedione and 2,3-pentanedione and the 2,3-butanedione precursor acetoin. In order that both the total and free diacetyl can be reported without the lengthy heating/conversion step required in GC-HS analysis, the conversion relationship between acetoin and total diacetyl as observed within the GC-HS assay will be required to be modelled especially if it is not a direct 1:1 molar conversion.

Each of the VDK components has an UV absorption centre and therefore an initial detection by UV spectroscopy may be sufficient to meet the investigation requirements. However, to ensure that co-elution of components within peaks is not occurring, a mass spectrum on the rough beer separation would be necessary in order to determine this. It is also likely that UV spectroscopy will not be sufficiently sensitive for the amount of VDK within the sample and therefore mass spectrometry or ion mobility spectrometry will be used as a detection and further separation method, to provide the further dimension to the assay. This separation should be readily transferred to the lab on a chip.

The second part of the project will focus on the detection by MS and IMS. MS which will be used as the detection method for the initial part of the project will be compared to IMS, once the two methods have been optimised. The proposed separation has the inherent advantage for “soft” electrospray ionisation of the components from the CZE column, due to the acidity of beer. This should negate the use of a radioactive source to ionise the sample and the intrinsic difficulties found with using this type of ionisation source.

The comparison will be performed to the specifications outlined in the section 3. Research Hypothesis, with the more appropriate or better-performing then being compared to the current GC-HS method again to the specification within the hypothesis and with additional consideration given to the material costs of equipment purchase, consumables, and standards as well as analysis times including preparation.

Requirements will include:

* CZE equipment,
* Ion trap mass spectrometer and associated equipment,
* Ion mobility spectrometer and associated equipment,
* Chemicals for surrogate standards mobile phases etc.,
* Computer with appropriate software to control apparatus and collate data.
* Access to beer in fermentation,
* GC set up for total and free diacetyl analysis.

A project such as this with appropriate funding should be completed within 24 months as a post-doctoral research project.

# Conclusion and Justification

The use ion mobility spectrometry or mass spectrometry coupled to capillary zone electrophoresis on a chip will enhance and can supersede the outdated and time consuming GC assay methodology. The swift electrophoretic separation will require minimum preparation time, use fewer consumables, energy and reagents, whilst adding an extra analytical dimension to the assay.

The minimum deliverable advancement will be an IMS with a headspace measurement similar to that of GC-HS which will give a single diacetyl results within twenty minutes. However due to the extremely short assay time (milliseconds for the IMS against a 15 minute runtime for the GC,) once analysis is commenced, and so long as the heating regime has been completed for the head space, many samples could be run within the same frame of time as the GC single assay.

The overarching target for the research is the integration of a CZE on a lab on a chip coupled to either an IMS or MS instrument with the negation of a need for pre-assay centrifugation by employing a guard column for solids within the beer. A rapid electrophoretic separation separating the pre-cursor acetoin, diacetyl and pentanedione and detection by the better performing of the spectrometers giving simultaneous results for both a free and total diacetyl. If achieved, this would represent an order of magnitude reduction in assay time from up to 120 minutes to less than 10.

If this method is proven effective for the VDK assay, the other volatile flavour compounds present in beer such as acetaldehyde, propanol, iso-butanol, iso-amyl alcohol, ethyl acetate and iso-amyl acetate could subject to similar research and method development.

The technology could be further applied to other biological reactors other than the fermentation vessels found in breweries. Biodiesel, vaccines, foods and various other biological products have all recently been prepared by biological reactors and a proof of separation on beer volatiles my prove of interest in other fields.

I envisage that this would be a 24 – 30 month post-doctoral project or a 48 month masters project (an MSc by research taking 12 to 18 months). Most likely at a university laboratory.

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