DEVELOPMENT OF A METHOD FOR THE LC/MS DETERMINATION OF VICINAL DIKETONES IN BEER

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ABSTRACT

Maxime Blanchette **M.Sc. (Bioresource Eng.)**

Development of an LC/MS Method for the Determination of Vicinal Diketones in Beer

No existent analytical method allowed the determination of the vicinal diketones (VDK), 2,3-butanedione (diacetyl) and 2,3-pentanedione, by liquid chromatography/mass spectrometry (LC/MS). An LC/MS method was developed for the simultaneous determination of diacetyl and 2,3-pentanedione in beer. A method allowing the determination of the amino acids (AA) related to the formation of VDK during fermentation was also developed. VDK were derivatized with o-phenylenediamine (OPDA) to form quinoxaline compounds. The reaction of VDK with OPDA was studied to optimize reaction time. Conversion of the diacetyl precursor, α-acetolactate, was tested using multiple oxidative decarboxylation techniques. Attempts were also made to determine simultaneously the AA, leucine, isoleucine and valine with the VDK. Simultaneous determination was unsuitable for the AA levels found in beer fermentation and separate methods for the determination of AA were developed. Total VDK were measured over a concentration range of 10 μg/L to 10 mg/L with less than 10% variation. These analytical methods were tested using a laboratory scale experiment to assess the impact of fermentation temperature on total VDK production and AA absorption. Samples collected in a local brewery were analyzed for total VDK using the developed method.

RÉSUMÉ

Maxime Blanchette **M.Sc. (Bioresource Eng.)**

Développement d'une méthode de détermination des dikétones vicinaux dans la bière par LC/MS

Aucune méthode analytique existante ne permettait la détermination des dikétones vicinaux (VDK), 2,3 butanedione (diacétyle) et 2,3 pentanedione, par chromatographie liquide/spectrométrie de masse atomique (LC/MS). Une méthode LC/MS fut développée pour la détermination du 2,3-butanedione et du 2,3-pentanedione dans la bière. Une méthode de détermination des acides aminés (AA) reliées à la production des VDK durant la fermentation fut aussi développée. Les VDK furent dérivatisés avec du o-phenylenediamine (OPDA) afin de former des composés quinoxalines. La conversion du précurseur α-acetolactate en diacétyle fut testée par le biais de multiples méthodes de décarboxylation oxydative. Des tentatives ont été effectué de déterminer les dikétones vicinaux simultanément avec les AA, leucine, isoleucine et valine. La détermination simultanée étant inadéquate pour les niveaux d'AA retrouvés durant la fermentation de la bière, des méthodes distinctes de détermination d'AA furent dévelopées. La teneur totale en dikétones vicinaux fut mesurée directement pour des concentrations allant de 10 µg/L à 10 mg/L avec moins de 10% de variation. Les méthodes développées furent testées au cours d'une étude de laboratoire sur l'impact de la température de fermentation sur la production de dikétones vicinaux et sur l'absorbtion d'acides aminés. Des échantillons de fermentations industrielles de lager et d'ale furent collectés dans une brasserie locale et furent analysés pour leur teneur en dikétones vicinaux en utilisant la méthode développée.

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LIST OF ACRONYMS

AA: Amino acids.

APCI: Atmospheric pressure chemical ionization.

DAD : Diode array detector.

ESI : Electrospray ionization.

GC : Gas chromatography.

ILV: Isoleucine-leucine-valine.

Ile: Isoleucine.

Leu : Leucine.

HPLC : High performance liquid chromatography.

MS: Mass spectrometry.

OPA: o-phtalaldehyde.

OPDA: o-phenlynediamine.

RP: Reverse phase.

SPE: Solid phase extraction.

UV: Ultra-violet.

VDK: Vicinal diketone.

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I INTRODUCTION

1.1 Background

Over the last centuries, beer brewing grew from a process based on tradition and very little scientific knowledge into a 147 billion liter per year industry where only ten companies are responsible for more than 57% of the world's production. Optimization of production cost and product consistency are now crucial factors that dictate companies' success in an industry characterized by fierce competition. Over the last century, breweries were involved in much research on the metabolism of brewers yeast in order to get a better understanding of the phenomena taking place in their fermentation vessels and to acquire more control over the brewing process. Diacetyl and 2,3-pentanedione, also referred to as the vicinal diketones (VDK), are produced through the yeast biosynthetic pathway of leucine, isoleucine and valine and are among the fermentation byproducts that are the most concern to brewing scientists. The presence of VDK in the final product is usually unwanted and is very often the limiting factor in beer maturation. Research was conducted in order to understand the kinetics of VDK production and reduction, and to reduce or even eliminate its influence on brewing practices but it still remains a major constraint in implementing new brewing technologies such as continuous fermentation. The analytical techniques developed for the detection of these compounds generally involves a laborious sample preparation process requiring skilled labor, often resulting in poor reproducibility.

Liquid chromatography/mass spectrometry (LC/MS) is a technology that allows the identification and the quantification of sample analytes based on their mass to charge

(m/z) ratio. The high level of selectivity of this detector could potentially represent a significant advantage over the instruments that are generally used to perform VDK analysis. In addition to having lower detection limits and greater reproducibility for testing beer samples with very little sample pre-treatment, LC/MS could potentially allow the simultaneous determination of VDK with the amino acids that are associated with VDK production during beer fermentation.

1.2 Objectives

There were three objectives for this present study:

- To develop a method allowing for the determination of diacetyl, 2,3-pentanedione, isoleucine, leucine and valine by LC/MS.
- To test and optimize the methods for the conversion of VDK precursors.
- To use the developed methods to analyze samples collected during beer fermentations to evaluate the relationship between amino acid consumption and VDK production.

II LITERATURE REVIEW

2.1 Beer production

Beer is defined as an alcoholic beverage obtained from the fermentation of a mixture of malt, cereals, hops and water. An extract of malt and cereals is used as the nutrient source to ensure the production of alcohol by yeast. Hops are introduced prior to fermentation for their aromatic, bittering and antibacterial properties. The beer production process is composed of three steps: wort manufacture, fermentation and post-fermentation processing (7). At the simplest level, brewers are interested in achieving the highest possible alcohol yield from the grain. At the same time, they insist on consistency in all other attributes of their product: foam, clarity, color and flavor (2).

2.1.1 Wort manufacture

Malted barley is the ideal grain for brewing beer because it has a high content of fermentable sugars and a good balance of the other nutrients necessary for yeast growth (25). Other cereals such as corn, wheat and rice, referred to as the *solid adjuncts*, can be incorporated to produce specialty beers and/or reduce production cost. The process of extracting the sugars and other nutrients from the grain is divided into three steps: milling, mashing and lautering.

Milling: The grain husk is cracked open in order to liberate its starchy endosperm.

Mashing: The crushed malt is placed in a vessel and soaked in warm water. During this process, the malt enzymes convert some of the malt saccharides and proteins into fermentable sugars and amino acids that can be readily used by yeast during beer

fermentation. Solid adjuncts must usually be processed separately before being incorporated into the mash (7).

Lautering: Sweet wort, the product of mashing, is clarified and separated from the bed of grain.

The wort is then transferred to the kettle and boiled for one to two hours in order to perform sterilization and to precipitate some unwanted malt constituents. Hops are added at different stages of the boil depending on the targeted aroma profile. It is a common practice in breweries nowadays to add more concentrated sources of carbohydrates to the kettle to reduce the production cost and/or to elaborate new products. Examples of these other sugar sources, also referred to as the *liquid adjuncts*, are syrups, caramel, sucrose, maltose and glucose. Those adjuncts contain high amounts of fermentable sugars but are generally deficient in the other nutrients necessary for yeast growth.

2.1.1 Beer fermentation

Beer fermentation consists of inoculating chilled wort with a culture of brewer's yeast and maintaining it in an anaerobic environment to allow alcohol production. Two different yeast species can be introduced: ale yeast (*Saccharomyces cerevisiae*) or lager yeast (*S. pastorianus*).

Ale fermentations are maintained at higher temperatures (18-22°C) (2). At that temperature, the yeast is very active and will generally take no more than a few days to attenuate the wort to its final gravity. These fast fermentations are generally characterized by yeast producing larger amounts of metabolic byproducts such as esters, ketones and

higher alcohols; some of these products, if concentrated enough, having a definite influence on the final aroma of the ale (38, 61).

Lager fermentations are usually performed slowly at low temperatures (6-15°C) and can sometimes last up to a few months (2, 25). Their organoleptic profiles are cleaner with yeast usually having a minimal influence on the beer flavor (68).

Aging/lagering: Once primary fermentation is finished, most of the yeast is removed or "racked off" and the beer is stored in a vessel for a certain amount of time at a usually lower temperature. This step is necessary to achieve the reduction of unwanted fermentation byproducts.

Yeast separation and filtration: Yeast and any other particles are removed from the product.

Carbonation, sterilization and packaging: The product is injected with carbon dioxide, transferred to a sterile container and packaged.

2.2 Diacetyl and 2,3-pentanedione in beer production

The presence of diacetyl and 2,3-pentanedione in beer has long been solely attributed to infection by micro-organisms such as *Lactobacillus* and *Pediococcus* (68). However, those compounds are normal products of yeast metabolism that are formed in every brewery fermentation (68). Diacetyl and 2,3-pentanedione are important contributors to beer flavor and aroma (11). Organic chemists classify both as ketones, and diacetyl is usually called 2,3-butanedione in the literature (62) (Table 2.2.1). Sometimes these two ketones are grouped and reported as the vicinal diketone (VDK) content of beer (12). They both possess very low flavor thresholds, 0.15 mg/L and 0.9 mg/L respectively, and

impart a generally undesirable "buttery" flavor (40). The desired level in the final beer depends on the particular flavor aimed but, in all types of beer, flavor defects are caused by excessive concentrations of diacetyl and many brewers might be happy to have no diacetyl in the beer (68).

Table 2.2.1.1 Chemical properties of VDK.

Compound	2,3-butanedione	2,3-pentanedione
Molecular formula	$CH_3COCOCH_3$	$CH_3CH_2COCOCH_3$
Molecular weight	86.09	100.12
Boiling point	88°C	110-112 °C
Precursor	α-acetolactate	α-acetohydroxybutyrate

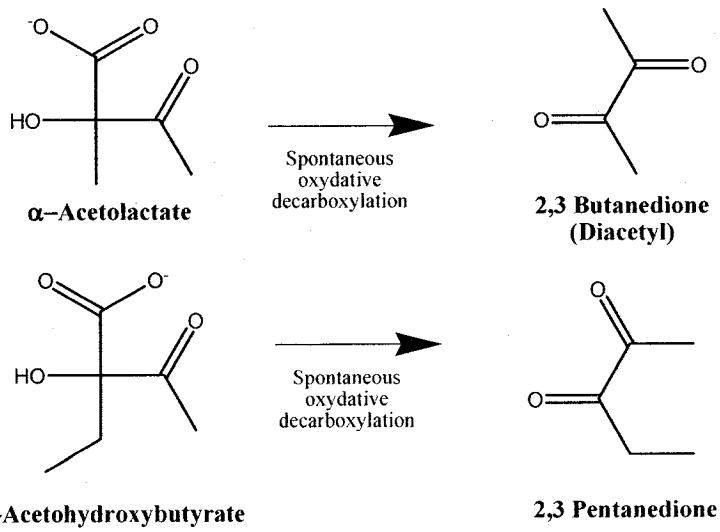


Figure 2.2.1.1 Production of VDK in fermenting wort.

2.2.1 Vicinal diketone formation and reduction by yeast

VDK formation during fermentation is the result of the spontaneous oxydative decarboxylation of α-acetolactate and α-acetohydroxybutyrate in wort (Figure 2.2.1.1) (29). These α-acetohydroxy acids are intermediates in the yeast biosynthetic pathways of

isoleucine, leucine and valine (ILV) and are excreted in the wort by yeast during fermentation (28) (Figure 2.2.1.2).

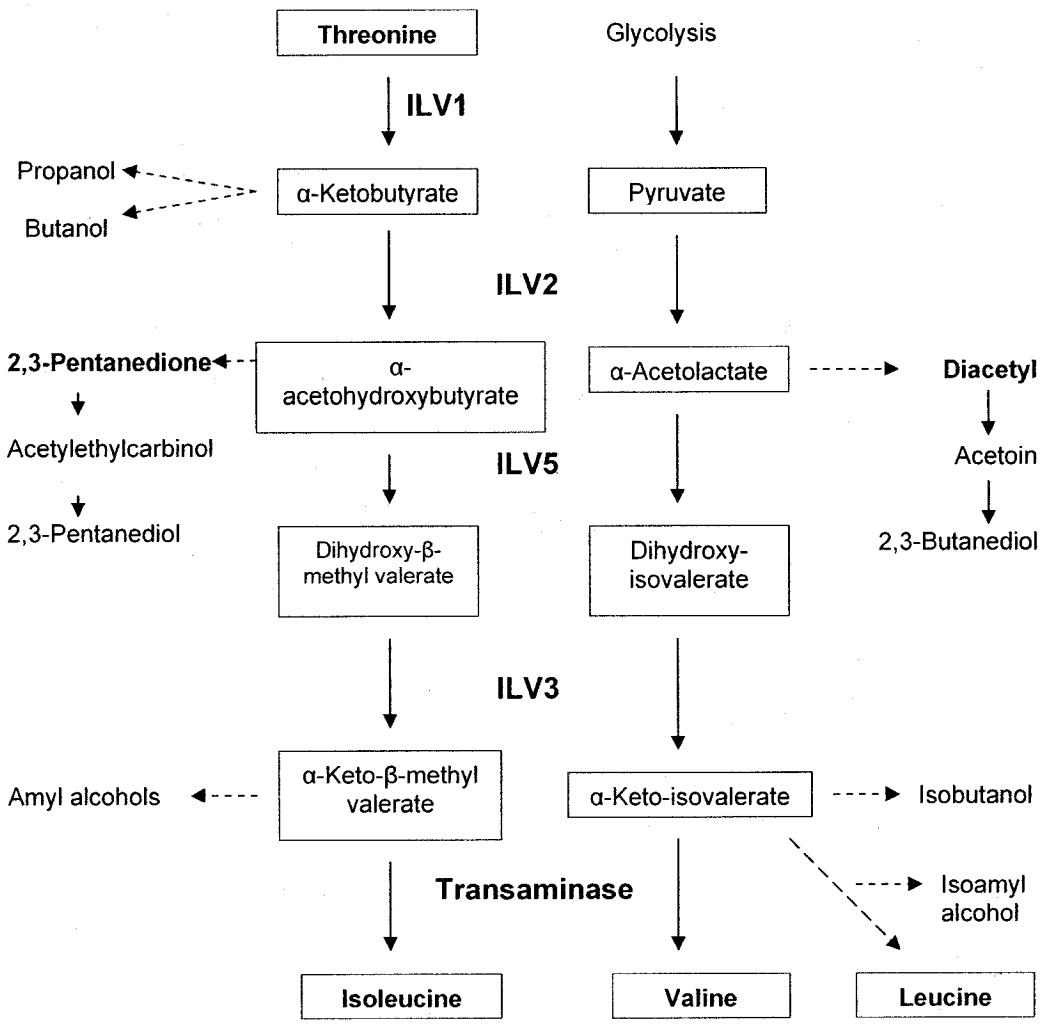


Figure 2.2.1.2 ILV biosynthetic pathway.

The VDK produced in wort are reabsorbed by the yeast where they are reduced enzymatically to form stable compounds that are of less importance in terms of beer flavor (3). Diacetyl is reduced to acetoin and then to 2,3-butanediol and 2,3-pentanedione

is reduced to acetylethylcarbinol and then to 2,3-pentanediol (10, 13, 37) (Figure 2.2.1.3). Previous research showed that the potential capacity of yeast cells to reduce diacetyl far exceeds the normally required activity (65). It is then generally accepted that the spontaneous oxidative decarboxylation of α-acetolactate to form diacetyl is the rate-limiting step in the VDK cycle (8).

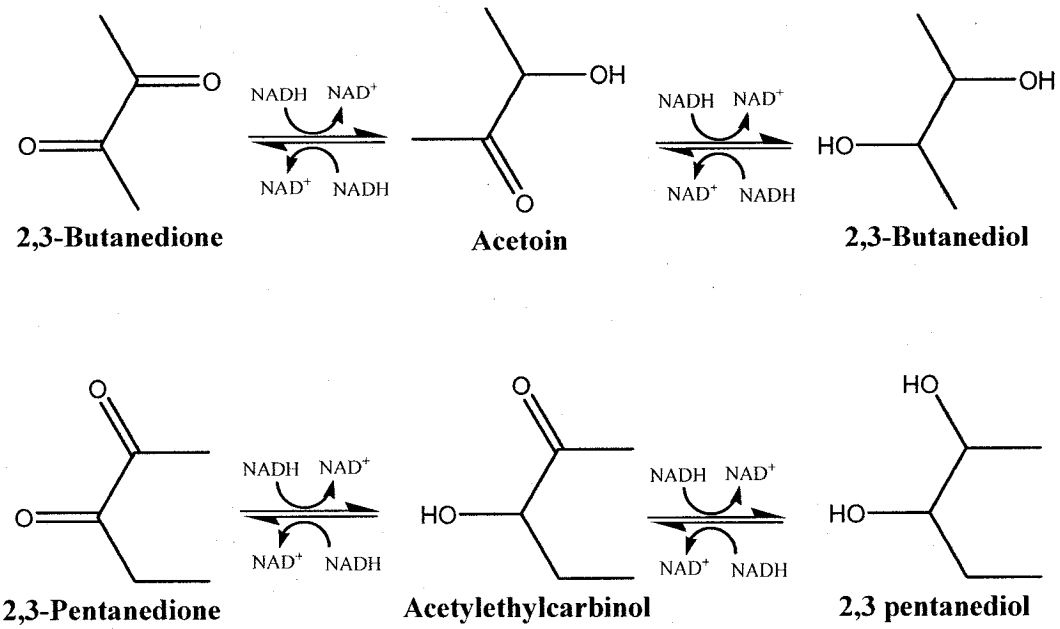


Figure 2.2.1.3 Reduction of VDK by yeast.

2.2.2 Factors affecting VDK production during brewing

Fermentation conditions

The production of VDK precursors is directly proportional to the fermentation rate (45, 68). Therefore, the higher the yeast pitching rate, the wort initial oxygen content and temperature, the earlier the VDK precursors will be produced in the fermentation (45,

54). The rates of oxydative decarboxylation of the precursors in wort and the rate of VDK reduction by yeast are also positively affected, to a certain extent, by the fermentation temperature, the amount of yeast in suspension, the wort oxygen level, and the acidity level of the fermenting broth (8, 21, 28).

Wort composition

Fermentation of higher gravity worts will lead to the production of higher levels of unwanted volatiles (64). However, high levels of sugar will generally tend to ensure sufficient metabolic activity of the yeast late in the fermentation when the VDK have been formed from α-acetohydroxy acids; which will result in faster reduction rates (68).

One of the main factors impacting the production of VDK during beer fermentation is the amino nitrogen content of the wort (54); precursor production being interrelated with the absorption of certain amino acids (AA) during fermentation.

During batch fermentation, the uptake of AA is known to respect a particular absorption priority. AA are classified in four groups (A, B, C and D) that are sequentially used by yeast (32, 33) (Table 2.2.2.1). Leucine, isoleucine and valine are included in group B and are generally transported into the cell after a 12 to 24 hour lag following the start of fermentation (33, 49, 51).

Table 2.2.2.1 AA classification in absorption groups.

Group A	Glutamic acid, Aspartic acid, Asparagine, Glutamine, Serine, Threonine, Lysine, Asparagine, Arginine.
Group B	Valine, Methionine, Leucine, Isoleucine, Histidine.
Group C	Glycine, Phenylalanine, Tyrosine, Tryptophan, Alanine.
Group D	Proline

Previous research has shown that more acetohydroxy acids are produced in the fermentations of high α-amino nitrogen compounds (30, 55). This has been explained by the fact that yeast is synthesizing its own group B amino acids (including isoleucine, leucine and valine) during this lag period through which group A amino acids are being used; resulting in the production of VDK precursors. Proposing that worts containing high levels of free amino nitrogen (FAN) contain more group A amino acids, an extended lag period is experienced resulting in a larger production of VDK (55).

However, wort with critically low AA content should again give beer of high α-acetohydroxy acid content after the depletion of group B amino acids (30, 45). In this particular situation, the VDK profile will be characterized by a pause in precursor production; the pause corresponding to the period during which group B amino acids are being removed from the wort. Further research has shown that the individual concentrations of isoleucine, leucine and valine have a direct impact on the production of VDK precursors (26, 30, 46, 54). On addition of leucine, the disappearance of valine and isoleucine and the pauses in precursor formation are delayed (30). It was also found that α-acetolactate formation is strongly suppressed when valine is sufficiently assimilated by

yeast while the formation of α-acetohydroxybutyrate is strongly suppressed when isoleucine is sufficiently assimilated by yeast (30, 46, 52, 54).

2.2.3 Industrial context

VDK production is a major concern in the operation of large breweries. The competition between the large brewing companies is constantly pushing the brewers to review their production process in order to decrease production cost and to maximize brewery throughput. The general trend of the domestic beer industry is to ferment high gravity worts containing large proportions of brewing adjuncts and to minimize fermentation time as much as possible. Although primary fermentation rate is important, examination of brewery records have revealed that for many beer qualities, achievement of an appropriate VDK concentration is the factor that actually limits vessel residence time (8).

Apart from being extremely careful in avoiding bacterial contamination, several alternatives were developed to minimize/eliminate the amount of time beer is left in the vessel to attenuate VDK (19), such as introducing a warm rest (referred to as diacetyl rest) at the end of primary fermentation (34), developing genetically modified yeast strains (17), using immobilized yeast reactors to perform continuous aging (48, 70), using a heat treatment under anaerobic conditions to encourage the direct nonoxidative decarboxylation of α-acetolactate to acetoin (27) and adding microbial α-acetolactate decarboxylase (ALDC) to the pitching wort (15).

2.3 VDK and AA analysis

2.3.1 Analytical methods

The analytical methods recognized by the brewing industry to determine VDK in beer are limited to gas chromatography and spectrophotometry. The spectrophotometric methods only require basic laboratory equipment and offer a cheap alternative in measuring VDK. However, these methods involve laborious sample pretreatment procedure that includes distilling the sample and reacting the VDK with chemical compounds to allow their spectrophotometric detection: examples of those compounds are α-naphtol and creatine (31), o-phenylenediamine (OPDA) (42), and hydroxylamine (14). The spectrophotometric methods have the major limitation of not differentiating between diacetyl and 2,3-pentanedione (14, 42).

The official headspace gas chromatographic method, on the other hand, allows for the individual quantification of diacetyl and 2,3-pentanedione. It consists of heating a filtered beer sample in a closed container at 35°C for 40 minutes, taking a sample from the headspace and injecting it into a gas chromatograph coupled to an electron capture detector (6, 9, 41). Collaborative works to evaluate this method were however not unanimous with regards to reproducibility of the method (5, 9). A disadvantage of this method is the fact that the matrix composition affects the vapor pressure of the analytes (66). The complexation of VDK with sulphite compounds produced during fermentation is another potential source of error (66).

Other gas chromatographic methods were more recently developed using mass spectrometry (MS) detection. Those methods involve the use of reactive compounds (derivatizing agents) that selectively bind to VDK in order create products that are more

readily detectable by MS. *Landaud et al.* (36) derivatized with 4,5-dichloro-1,2-diaminobenzene while *Pejin et al.* (50) derivatized with o-phenylenediamine (OPDA). OPDA was also used by *Rodriguez et al.* (56-58) to detect VDK by pulse polarography and voltametric detection.

Several methods were developed for the detection of VDK in beer with HPLC either using fluorometric or UV spectrophotometric detection. Those methods generally required the capture of VDK derivatives by solid phase extraction (SPE) in order to reduce interference with other beer constituents. *McCarthy* (39) derivatized VDK with 2,3-diaminonaphtalene and analyzed with fluorometric detection. *Yamaguchi et al.* (69) prepared samples by methanol extraction, derivatized VDK with 1,2-diamino-4,5-methylenedioxybenzene and analyzed with fluorometric detection. *Verhagen et al.* (66) and *Barros et al.* (4) used a double SPE sample treatment, derivatized VDK with OPDA and analyzed with UV spectrophotometric detection. *Moree-Testa and Saint-Jalm* (44) used 3-methylisoquinoline as an internal standard for the UV spectrophotometric detection of OPDA derivatives of VDK in cigarette smoke.

2.3.2 OPDA derivatization

The reaction of OPDA, also referred to as 1,2-diaminobenzene, with VDK leads to the formation of non-volatile and rather non-polar quinoxaline compounds that can be detected by multiple detection methods (43, 56-58, 66). The products of diacetyl and 2,3-pentanedione with OPDA are respectively 2,3-dimethylquinoxaline and 2-ethyl-3-methylquinoxaline (figure 2.3.2.1) (Table 2.3.2.1).

Table 2.3.2.1 Chemical properties of OPDA derivatives of VDK.

Compound	o-Phenylenediamine (OPDA)	2,3-Dimethylquinoxaline	2-ethyl-3-methylquinoxaline
Molecular formula	$C_6H_8N_2$	$C_{10}H_{10}N_2$	$C_{11}H_{12}N_2$
Molecular weight	108.14	158.2	172.2
Melting point	100 - 103 C	104-108 °C	n/a

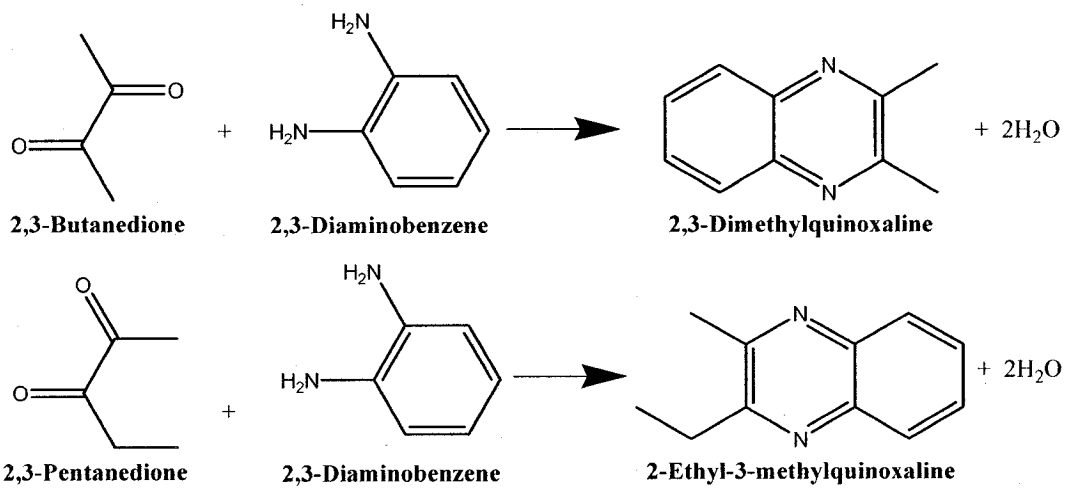


Figure 2.3.2.1 Derivatization of VDK with OPDA.

The conversion of α-dicarbonyl compounds to the corresponding quinoxalines, which can be performed in aqueous phase at room temperature, is quantitative and fast over a broad pH range (pH= 1 to 10) (56); with reaction rates being inversely proportional to pH (66). OPDA was also found to be able to dislodge diacetyl from its complex with sulphite, which has been reported as a cause of underestimation of the VDK content (57, 66).

2.3.3 Oxydative decarboxylation of the VDK precursors

Previous research displayed an absence of detectable amounts of VDK during beer fermentation that was explained by the low redox potential at the active stage, which tends to prevent oxidative decarboxylation of VDK precursors, and by a rate of VDK reduction by yeast being more rapid than that of its formation at later stages (28). Therefore, the VDK precursors accumulate during the active stage of fermentation but do not form VDK until later in the fermentation at which time they are quickly reduced by yeast. It is then essential to perform complete oxydative decarboxylation of the precursors prior to analyzing samples for VDK in order to appropriately monitor VDK production and reduction during beer fermentation.

The conventional way of achieving precursor oxydative decarboxylation is by performing a so-called "autoxidative" treatment which consists of aerating the sample prior to incubating at a high temperature. Different percentages of precursor conversion were reported using this technique. For an incubation temperature of 60°C, some of the results reported in the literature have been 98% α-acetolactate conversion in 75 minutes (14), 99% conversion in 90 minutes (28) and 56% conversion in 48 hours (22). Similar oxydative decarboxylation rates were achieved at that temperature for both α-acetolactate and α-acetohydroxybutyrate (28).

Another method consists in the addition of ferrous sulphate and ferric chloride prior to incubating the sample at 80°C (16). When both were added at an optimal concentration of 0.15 mM, $FeCl_3$ and $FeSO_4$ act as decarboxylation catalysts enabling 100% conversion within 10 minutes (16, 36, 50). Use of higher concentrations of those salts at that temperature leads to equivalent or slower oxydative decarboxylation rates and can

ultimately lead to the oxidation of acetoin to diacetyl (16). It was, however, shown that no acetoin is oxidized to diacetyl when 0.15 mM of each iron salt is used in beer samples (36).

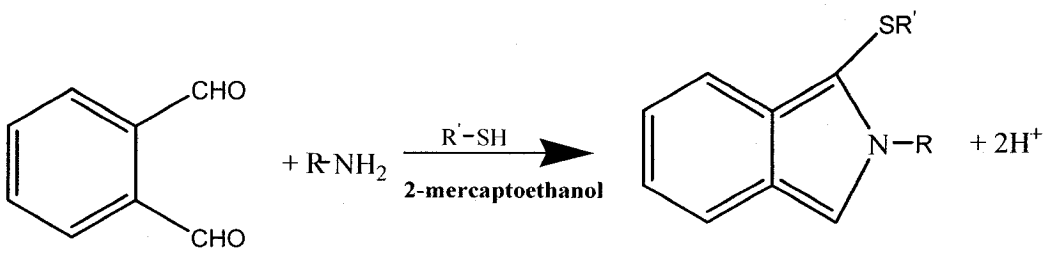
The validity of the results obtained by those methods was, however, questioned by the fact that VDK could be produced from the modification of carbohydrates through the Maillard reaction during the incubation of samples at 80°C (20). So, an alternative method allowing the oxydative decarboxylation of VDK at room temperature using aniline hydrochlroride as a reaction catalyst was proposed. Full conversion of α-acetolactate to diacetyl was achieved by incubating beer samples at room temperature for one hour following the addition of 10.8 mM of aniline hydrochloride (20, 60).

2.3.4 HPLC determination of AA

Numerous methods report the HPLC analysis of o-phtalaldehyde (OPA) derivatives of AA in complex organic media. OPA-2-mercaptoethanol reacts with AA in alkaline conditions to form fluorescent compounds that are stable enough to allow measurements to be made within 5 to 25 minutes (59). *Piepponen et al.* (53) as well as *Soufleros et al.* (63) derivatized with OPA-2-mercaptoethanol and analyzed several AA in wine with fluorometric detection. *Hess et al.* (23) derivatized with *OPA*/isobutyryl-l-cysteine and analyzed unusual AA with both UV detection and electrospray ionization mass spectrometry. Table 2.3.4.1 shows the chemical properties of leucine, isoleucine and valine and Figure 2.3.4.1 shows the reaction of OPA-2-mercaptoethanol with AA.

Table 2.3.4.1 Chemical properties of leucine, isoleucine and valine.

Compound	Leucine	Isoleucine	Valine
Molecular formula	$(CH_3)_2CHCH_2CH(NH_2)CO_2H$	$C_2H_5CH(CH_3)CH(NH_2)CO_2H$	$(CH_3)_2CHCH(NH_2)CO_2H$
Molecular weight	131.17	131.17	117.15
Melting point	>300 °C	288 °C	295-300 °C



o-Phtalaldehyde (OPA) **AA** **Fluorescent derivative**

Figure 2.3.4.1 Derivatization of AA with OPA.

2.4 Principle of liquid chromatography/mass spectrometry (LC/MS)

2.4.1 Reverse phase high performance liquid chromatography (RP HPLC)

HPLC column selection (18, 24, 47)

In liquid chromatography, the separation is based on the interaction of sample analytes between a liquid mobile phase and a stationary phase. The sample is injected by the means of an injector port into a mobile phase stream delivered by a high pressure pump. The heart of the system is the HPLC column in which the stationary phase is contained and through which the mobile phase is pumped. The reverse phase columns are generally packed with non-polar silica gels. Compounds bind to the stationary phase in highly polar aqueous mobile phase and are forced out of the column by a non-polar organic mobile

phase. In RP HPLC, compounds are separated based on their hydrophobic character and elutes from the column in order of decreasing polarity.

The stationary phase is generally composed of hydrophobic alkyl chains ($-CH_2-CH_2-CH_2-CH_3$) that interact with the analyte. There are three common chain lengths: C4, C8, and C18. The longer the chain, the more retention the column will offer. As a general rule, C4 is used for large molecules (e.g. proteins) while C18 is used for small molecules (e.g. AA and peptides). Stationary phases are also available in different particle and pore sizes; particle size generally ranging from 3 to 50 μ and pore size ranging from 100-1000 angstroms. Smaller particle sizes and longer columns offer more surface area hence higher separation efficiency (retention) but also result in higher operating pressures. Thus, larger bore columns allow the use of higher flowrates for the mobile phase.

Since retention is also inversely proportional to temperature, the column and the mobile phase are usually held at constant temperature during analysis in order to ensure reproducibility. When developing a method for HPLC detection and selecting an HPLC column, one must then compromise between sample throughput and separation efficiency. The selection of a high retention column will result in higher operating pressures and hence impose limitations in the selection of mobile phase composition and flowrate, while a column with lower retention might result in insufficient separation of the sample analytes.

Mobile phase selection (18, 24, 47)

Mobile phase is usually composed of a mixture of an aqueous solution and organic solvents. Since the pH of the mobile phase affects column retention and also impacts ionization in the MS spray chamber, it is a general practice to use a buffer solution as the

aqueous phase to ensure method reproducibility. The organic solvents used for HPLC are classified according to their polarity index. Table 2.4.1.1 contains important properties of some usual mobile phase constituents. Parameters that must be taken into account when selecting mobile phase constituents are viscosity, boiling point and miscibility. Mobile phases of high viscosity can lead to operating pressure problems, while using mobile phases that are not miscible can lead to permanent damage of the HPLC column. Finally, heating the mobile phase to temperatures close to or beyond its boiling point is likely to result in permanent damage to the HPLC system.

Table 2.4.1.1 Properties of mobile phase constituents.

Solvent	Viscosity at 20°C (cP)	Polarity index (Snyder)	Boiling Point (°C)
Acetonitrile	0.37	6.2	81.6
Methanol	0.6	6.6	64.7
Tetrahydrofuran	0.55	4.2	66
Water	1	9	100

During the analysis, mobile phase proportions can be held constant (isocratic) or the proportion of organic solvent can be linearly increased, if the HPLC system allows, using a gradient program. It is generally simpler to deal with isocratic conditions although the use of gradient programs is often required to achieve good resolution and optimize sample throughput when many sample analytes have to be determined simultaneously.

2.4.2 Mass spectrometric detection (MSD)

The basis of MSD is the production of ions that are sequentially separated and filtered according to their mass-to-charge ratio (m/z) to be finally detected (47). The resulting

mass spectrum is a plot of the relative abundance of the ions produced in the MS spray chamber as a function of their m/z ratio. The MSD system used in this study had a quadrupole mass analyzer with an electrospray ionization chamber (Figure 2.4.2.1).

Electrospray ionization (18, 47, 67)

Electrospray ionization can be used for high and low molecular weight ionizable solutes. It consists of introducing the mobile phase stream from the outlet of the HPLC column through a very small, charged capillary needle. At the end of the needle, the stream is put into contact with a flow of nitrogen (nebulizing gas). The analyte exists as an ion in solution either in a protonated form or as an anion. As like charges repel, the liquid pushes itself out of the capillary needle and forms a cone shaped mist of small droplets about 10 μm across. The spray is then submitted to a high flow of heated nitrogen to evaporate the water and organic solvents contained in the droplets. The droplets shrink and the sample analyte ions are forced closer to each other. The proximity of the molecules becomes unstable and the droplets will ultimately explode. This phenomenon, referred to as the Coulomb fission, will repeat itself until the analyte is free of solvent and is a lone ion (Figure 2.4.2.2).

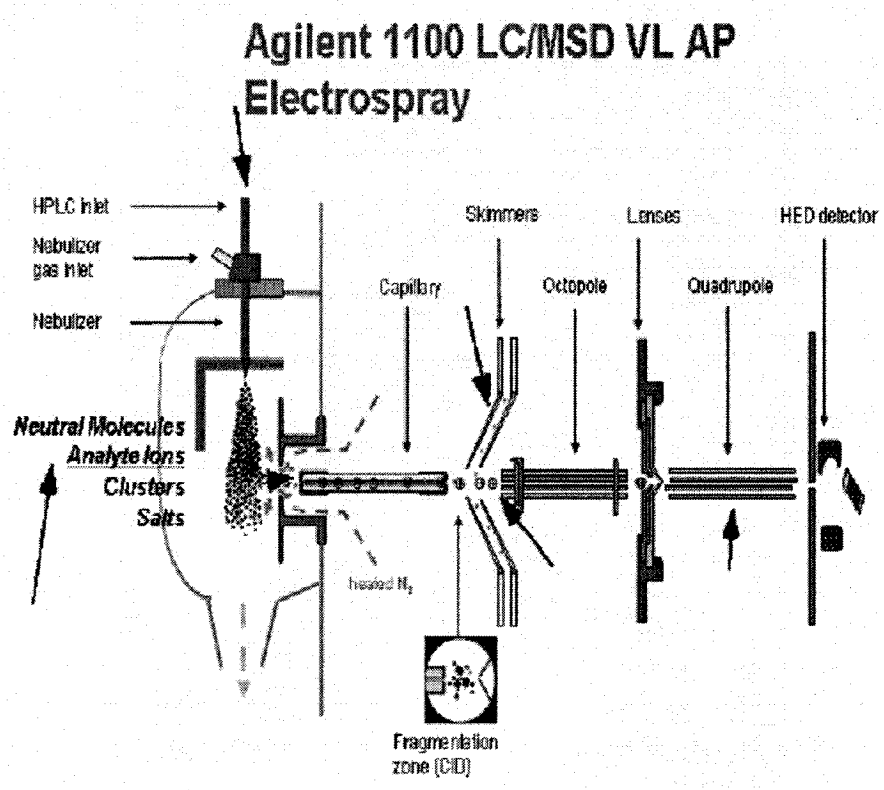


Figure 2.4.2.1 Interior of the Agilent 1100 LC/MSD VL AP electrospray (67).

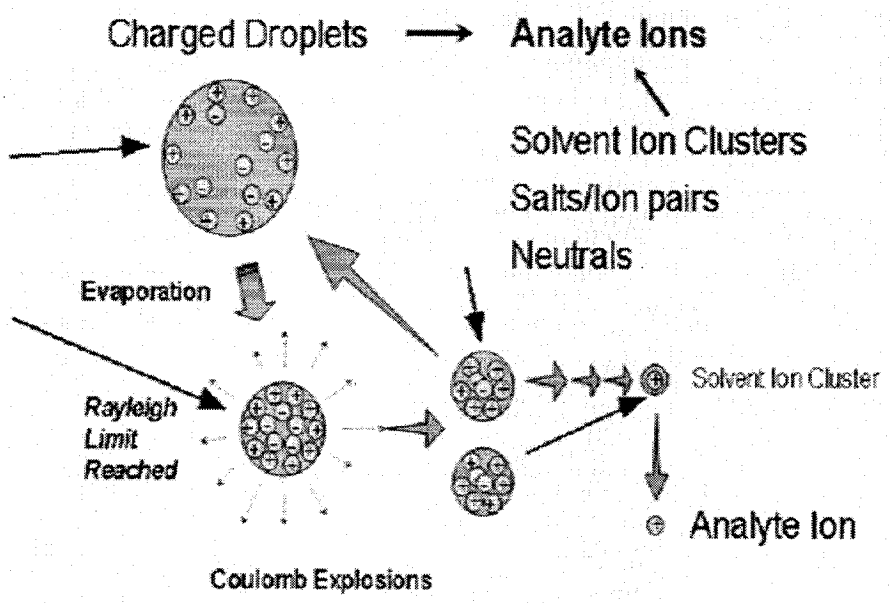


Figure 2.4.2.2 Schematic of electrospray ionization (67).

Quadrupole detection (1, 47, 67)

The quadrupole system is composed of circular bars that are placed parallel in radial array. Opposite rods are charged by an either positive or negative DC potential (U) at which an oscillating radio frequency voltage $V_o\cos(\omega t)$ is imposed. The system is maintained under large vacuum (less than $2*10^{-5}$ torr) that directs the ions formed in the spray chamber through the capillary tube and the fragmentor zone and then through the middle of the quadrupole. In the quadrupole, the charged ions start to oscillate in a plane perpendicular to the rod length. By varying the amplitude U and Vo , the quadrupole only allow ions contained in a determined m/z range to go through the lense while the other ions are deflected out of the system. At the latter stage of the process, the high energy detector (HED) records the relative abundance of the ions that were allowed through the system. Parameter adjustment for the quadrupole system and HED detector is very

complex and laborious and is usually performed automatically by the LC/MSD control software through an autotune procedure. The autotune procedure consists of running a standard solution containing an ion mixture of known concentration through the detector and adjusting the mass axis and electron multiplier voltage to attain a satisfactory level of performance.

MSD control parameters (1)

Drying gas flow: This parameter controls the flow rate of the nitrogen drying gas. The flow required depends on the type of analysis, the HPLC flow rate, and the drying gas temperature. In general, the higher the HPLC flow rate the higher the drying gas flow rate.

Drying gas temperature: This parameter controls the temperature of the nitrogen drying gas. The maximum temperature of 350°C should be used unless a highly volatile solvent is used and/or analyzing thermally unstable analytes.

Nebulizer pressure: This parameter controls the pressure of the nitrogen nebulizing gas. The pressure required is set as a function of the HPLC flow rate, the mobile phase composition, and the ionization mode.

Capillary voltage: This parameter controls the voltage applied to the entrance of the capillary. This voltage is relative to the nebulizer and spray chamber, which are at ground potential. The polarity of Vcap is set automatically depending on the polarity of the ions analyzed. This parameter affects the ion transmission and the fragmentation.

Fragmentor voltage: This voltage is applied in the fragmentation zone at the exit of the capillary. This parameter affects the ion transmission and the fragmentation.

Gain: The MSD signal amplification factor. It is a correlation of signal abundance to electron multiplier voltage of the HED detector. Gain can be used to amplify or reduce the MSD signal. High gain leads to faster deterioration of the HED electron multiplier.

2.4.3 HPLC parameters for ESI-MSD (18, 67)

The first consideration when using electrospray ionization is to make sure that the mobile phase can provide charged analytes. A general rule is to keep the mobile phase pH 1-2 units above or below the pKa of the analytes. Mobile phase flow rate must also be minimized in order to ensure complete evaporation of the mobile phase in the spray chamber. The use of smaller bore HPLC columns is advised in order to achieve satisfactory separation at low flowrates. The salts used in the buffer preparation must form volatile ions to reduce interference and avoid salt coating of the nebulizer/spray chamber apparatus; acetate, propionate, formate, trifluoroacetate, ammonia and diethylamine are examples of volatile buffers. A protic solvent such as methanol should be used when possible for positive ion mode to reduce background noise. Finally, working with higher proportions of solvents in the mobile phase ameliorates evaporation in the spray chamber and hence allows the application of higher flow rates.

III MATERIALS AND METHOD

3.1 Liquid chromatography/mass spectrometric detection (LC/MSD)

3.1.1 Introduction

LC/MS parameters were optimized for the detection of o-phenylenediamine (OPDA) derivatives of diacetyl and 2,3-pentanedione, and o-phtalaldehyde (OPA) derivatives of leucine, isoleucine and valine in beer. The reaction of vicinal diketones (VDK) with OPDA was studied to optimize reaction time. Conversion of the diacetyl precursor, α-acetolactate, was tested using different oxydative decarboxylation techniques. Attempts were made to achieve simultaneous determination of VDK and amino acids (AA) performing a two steps derivatization procedure. Since simultaneous determination of VDK and AA could not be achieved for AA levels generally found in beer, a separate method for the determination of AA by LC/MS was developed; in addition to an alternative method for determination of AA using a different HPLC system coupled to a fluorescence detector was developed. These new analytical methods were tested using a laboratory scale experiment to assess the impact of fermentation temperature on total VDK production and AA absorption. The developed methods were also used to analyze samples collected in a local brewery for total VDK and free AA levels during typical fermentations of both lager and ale.

3.1.2 Instrument description

The system used was a *1100 series LC/MSD type SL* (Agilent Technologies, Palo Alto, CA, USA). The high performance liquid chromatography (HPLC) system is composed of a quaternary pump, an automatic degasser, a standard autosampler, a temperature

controlled column holder and a diode array detector (DAD) connected in series with the MSD. System control, data acquisition and integration were all performed with version 5.0 of the *Agilent Chemstation* software.

The quaternary pump allowed flexible mixing of four mobile phase channels, and flow rate adjustment with three decimal places in a range of 0.2 mL/min to 10 mL/min. Mobile phase and column temperature could be adjusted from 10°C below room temperature up to 80°C. The autosampler had a capacity of a hundred samples and could perform injection over a volume range from 0.1 to 100 µL.

The analyzer part of the Type SL mass spectrometer was composed of an octopole detector and could cover a molecular mass range of 50 to 3000 m/z. Electrospray ionization or atmospheric pressure chemical ionization (APCI) spray chambers were available to install on the system. The system allowed for the simultaneous acquisition of two MS signals that could be set to different polarities and operated in scan or in simultaneous ion monitoring (SIM) modes.

In SIM mode, the signal could be configured to record the abundance of up to 30 different ions simultaneously. The composition of the ion group analyzed could be modified up to 50 times during a same analysis. The fragmentor could be set to a different voltage for each signal and for each ion group analyzed. Scan mode allowed for recording the abundance of all the ions contained in a certain molecular mass range: each signal being limited to analyze one molecular mass range at a time.

Another HPLC system was used to perform part of the AA analysis using flurorescence detection. This system was also a 1100 series HPLC system from Agilent Technologies

mainly composed of the same HPLC components. This system was coupled to a fluorescence detector, a reflective index detector and a UV-VIS detector. Only fluorescence detection was used during this study. Parameter control, data acquisition, integration and calibration using this system were all performed with version 6.0 of the *Agilent Chemstation* software.

The user interface of *Agilent Chemstation* was similar for both versions of the software used. The method and run control part of the software was divided in two parts: method development and sequence programming. The developed methods contained the parameters for all the system components and for data acquisition: mobile phase conditions, sample injection parameters, column temperature, detector settings and integration parameters. The programmed sequences contained the information regarding the samples injected; i.e. type of sample, vial location on the autosampler, number of injection to be performed, method of analysis, name and destination of the signal output. When a sequence was started, the software sets up the operating conditions automatically according to the parameters entered in the method selected, and the autosampler picked up a vial on the autosampler and loaded it on the injector to perform the analysis. Once the method was complete, the software moved to the next line of the sequence to analyze the next sample. Different methods could be used within a same sequence.

The data analysis section of the software allowed for data integration and calibration. The report section prepared an output document that contained selected information about the analysis performed.

Online and offline versions of the software are available upon installation and can be both run simultaneously. Both modes basically contain the same options. It is however

recommended to exclusively use the online version to communicate with the instrument and to use the offline version for data analysis and report production in order to avoid experiencing software crashes and data losses, and having to *reset* communication with the instrument parts.

3.1.3 Operating conditions

3.1.3.1 HPLC columns and parameter

Two different HPLC columns were used throughout method development and sample analysis. Both are 15 cm long *Gemini C-18* analytical columns (Phenomenex, Torrance, CA, USA). One has a 2.0 mm bore and a 3 µ particular size while the second has a 4.6 mm bore and a 5 µ particular size. An interesting particularity of the Gemini column is that it provides good performance with mobile phase conditions ranging from 100% aqueous to 100% of organic solvent in a broad pH range (1.0 to 12.0). The transition to a 4.6 mm bore column was made once the 2.0 mm bore column started showing excessive operating pressures due to gradual clogging and had to be replaced. A larger bore column and a larger particular size were selected to allow the selection of higher flow rates and larger injection volumes, and to provide less retention. An overall longer column life was achieved with the 4.6 mm bore column since satisfactory elution times could be achieved with this column, while still maintaining the operating pressure in a low range (50 to 150 bars).

Both columns have a maximum operating pressure of 245 bars and a maximum operating temperature of 60°C. The suggested maximum sample injection volume is 4 µL on the 2.0 mm bore column and 50 µL on the 4.6 mm bore column.

Both columns were exclusively operated at 35 °C. At that temperature, the maximum achievable flow rates are 0.15 mL/min on the 2.0 mm bore column and 1.5 mL/min on the 4.6 mm bore column. Both columns were stored in HPLC grade methanol overnight and in HPLC grade acetonitrile for long-term storage in order to prevent stationary phase degradation. A guard column (*Phenomenex*) was used to optimize column life. C-18 guard cartridges were replaced at intervals varying from every day to every week, depending on the types and amounts of sample injected, in order to maintain low operating pressures and ensure proper column protection.

3.1.3.2 Mobile phase considerations

Mobile phase selection

Mobile phases were 10 mM acetate buffer solution (Phase A) and HPLC grade methanol (Phase B) (Fisher Scientific, Ont., Canada). Deionized water treated with a *Milli-Q® Ultrapure Water Purification System* (Millipore, Billerica, MA, USA) was used to prepare the buffer solution. Ammonium acetate, a volatile buffer, was used at low concentration in order to avoid salt coating in the MS spray chamber and to reduce background noise. The acetate buffer was prepared by diluting 10 mM of ammonium acetate and adjusting the pH to 5.85 by adding 2 mL/L of 5% acetic acid. The acetate buffer was filtered through a 0.2 μm pore size filter. Methanol was selected as the organic solvent since it provided a cleaner MS signal than acetonitrile: the other common HPLC solvent. To avoid baseline drifting and ease integration, the mobile phase conditions were maintained isocratic during analysis time.

Mobile phase conditions

Different proportions of mobile phase could be used depending on the HPLC column, the concentrations to be measured and the level of interference found in the sample/standard analyzed. The selected isocratic conditions were maintained from sample injection until the elution of the last analyzed peak. After each analysis, the column was rinsed with 2.0 column volumes of pure methanol and equilibrated with 2.0 column volumes of the starting isocratic conditions of the next analysis. Changes in mobile phase proportions were performed over 30 seconds.

An operating flow rate of 0.15 mL/min was maintained on the 2.0 mm bore column to obtain elution times of 5.80 minutes for diacetyl and 8.98 minutes for 2,3-pentanedione using 55% Phase B.

Due to some instability in the laboratory air (nitrogen) supply pressure, the flow rate was limited to 0.5 mL/min on the 4.6 mm bore column in order to ensure complete drying of the mobile phase in the MS spray chamber. Incomplete drying can lead to partial ionization of the sample components and ultimately damage to the detector. In order to find the methanol proportions that would lead to satisfactory elution times at that flow rate, a preliminary run was made by injecting 10 µL of an aqueous standard containing 10 mg/L of the OPDA derivatives of diacetyl and 2,3-pentanedione and linearly increasing the proportion of Phase B from 10% to 100% over 20 minutes (Figure 3.1.3.1).

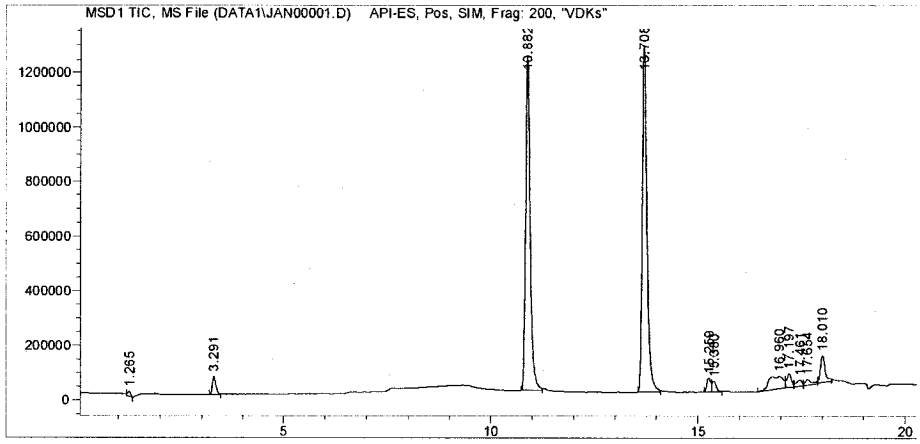


Figure 3.1.3.1 Chromatogram of a mobile phase gradient test for VDK elution on the 4.6 mm bore HPLC column.

The chromatogram showed that OPDA derivatives of VDK would require roughly 60% methanol to be washed out of the column. Tests were made with isocratic conditions ranging from 60 to 70% Phase B and elution times were recorded (Table 3.1.3.1).

Table 3.1.3.1 Elution times as a function of the percentage of methanol in the mobile phase.

	Elution times				
% methanol	**Diacetyl**	**2,3-pentanedione**	**Leucine**	**Isoleucine**	**Valine**
60	7.85	12.51	-	-	-
65	6.75	9.95	13.08	11.83	8.85
70	5.96	8.14	-	-	-

In samples where high VDK concentrations were to be measured and/or where potential interfering peaks were not present, the mobile phase was set to 70% phase B. The amount of organic solvent was decreased to 60% phase B for samples where significant

interference levels were found. Upon the elution of the last peak, once the MS stopped acquiring signal and that the mobile phase stream by passed the MS spray chamber to directly go to waste, the flowrate was raised to 1.0 mL/min in order to accelerate the rinsing and equilibration stages. Table 3.1.3.2 shows a summary of the different isocratic mobile conditions used and Table 3.1.3.3 shows an example of a mobile phase program used for VDK analysis.

Table 3.1.3.2 Isocratic mobile phase conditions for data acquisition.

Column	% A	%B	Flow rate (mL/min)	Type of analysis
2.0 x 150mm (3µ)	55	45	0.15	AA and simutaneous
	45	55	0.15	VDK
4.6 x 150mm (5µ)	40	60	0.5	VDK (high interference)
	35	65	0.5	AA and simultaneous
	30	70	0.5	VDK

Table 3.1.3.3 Example of a mobile phase program.

Time	%A	%B	Flow rate (mL/min)	Stage
0.0	30	70	0.5	Data acquisition
10.0	30	70	0.5	Data acquisition
10.5	0	100	1.0	MeOH rinse
20.0	0	100	1.0	MeOH rinse
20.5	30	70	1.0	Phase equilibration
30.0	30	70	1.0	Phase equilibration

In order to maintain the HPLC column in good working conditions, a flushing routine, consisting of 20 column volumes rinse with water for salt removal followed by a 20 column volumes rinse with 100% organic solvent was performed at the end of each day of analysis.

3.1.3.3 MS parameters

Spray chamber conditions

Since large proportions of organic solvent were required to wash the OPDA derivatives out of the C18 columns, the APCI spray chamber could not be used since it could not be operated with more than 5% organic solvent. Electrospray ionization was therefore selected for method development. Selection of the spray chamber parameters mostly depends on the quantity of mobile phase to be dried, i.e. on the mobile phase flow rate, and different operating conditions had to be used for each HPLC columns (Table 3.1.3.4).

Table 3.1.3.4 Electrospray ionization chamber parameters.

HPLC column	**2.0 x 150 mm (3 µ)**	**4.6 x 150 mm (5 µ)**
Mobile phase flow (mL/min)	0.150	0.500
Drying gas flow (L/min)	10.0	13.0
Drying gas Temperature (°C)	350	350
Nebulizer pressure (psig)	20	37
Capillary voltage (V)	4000	4000

MS signals

A MS scan was first ran on aqueous standards containing large quantities of the compounds to be analyzed in order to validate their molecular masses and to show that the derivatizing agents were binding strongly enough to the VDK and AA to avoid significant fragmentation in the spray chamber.

MS signals were restricted to SIM mode to perform further analysis in order to obtain more sensitivity. The signals were configured so that the MS would be searching for only

one ion at a time. About one minute before the elution of the first peak, the mobile phase was diverted to the spray chamber and the MS was starting to acquire signal. The MS was switched to standby and the mobile phase was diverted to waste about one minute after the elution of the last peak to analyze. This procedure was followed in order to avoid contamination of the MS nebulizer and spray chamber apparatus by sugar compounds and other beer constituents that could be eluting from the column at different times than the compounds analyzed.

Gain was adjusted between 1 and 100 depending on the injection volume and the concentrations to be detected in order to maintain signal response in a range that allows accurate determination. Oversetting gain leads to unnecessary deterioration of the MS electron multiplier and unstable signal response, while insufficient gain leads to a lack in sensitivity. For a capillary voltage setting of 4000 V, maximum signal was obtained when the fragmentor voltage was set to 175 V for AA detection and 200 V for VDK detection. Table 3.1.3.5 shows a summary of the different MS signal settings used for the different types of analysis performed.

Table 3.1.3.5 MS signal settings.

Column	Analysis	Signal 1	Gain	Frag. (V)	Signal 2	Gain	Frag. (V)
2.0*150mm (3µ)	VDK	diacetyl	100	200	2,3 pentanedione	100	200
	AA	val, ile, leu	1	175	-	-	-
	Simult.	VDK	100	200	val, ile, leu	1	175
4.6*150mm (5µ)	VDK	diacetyl	10	200	2,3 pentanedione	10	200
	AA	val, ile, leu	1	175	-	-	-
	Simult.	VDK	10	200	val, ile, leu	1	175

Signal integration

Signal integration was performed by computing the area under the peak. Peak baseline limits were set manually and maintained identical for the integration of all the samples coming from a same beer matrix. The area was designated by drawing a straight line between the baselines levels before and after peak elution. Figure 3.1.3.2 shows an example of how data integration was performed on a peak representing a low diacetyl concentration in a beer sample eluted with 70% Phase B.

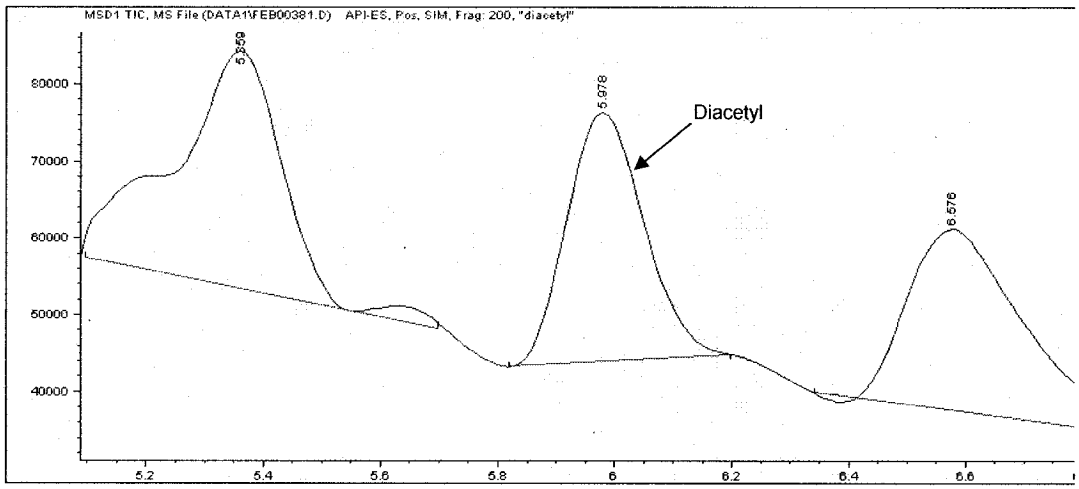


Figure 3.1.3.2 Chromatogram of a beer sample containing 61µg/L of diacetyl.

3.2 Statistical design

3.2.1 Procedures

All statistical analysis was performed on true replicates. Standards analyzed for derivatization and decarboxylation tests were prepared from concentrated stock solutions and were treated individually. In order to account for variation in the MS response and in

the yield of acetolactate synthesis, reaction yields were calculated by dividing the signal obtained at the tested reaction conditions by the signal obtained by analyzing an aliquot of the same standard using the optimal conditions described in this section.

3.2.2 Method of analysis

Calibration curves were obtained by performing linear regression analysis on the data obtained from standard analysis. Regression was performed using the points obtained from all replicates analyzed. In the case of calibration curves performed on aqueous standards, the intercept was set to 0.

3.3 VDK derivatization

3.3.1 Selection of a suitable derivatizing agent

Diacetyl and 2,3-pentanedione could not be detected directly by LC/MS. The first difficulty resided in the fact that both compounds possess a high level of polarity and hence are poorly retained on reverse phase HPLC columns. Secondly, the fragmentation pattern of both compounds yields ions of molecular masses that are below the mass detection limit of the MSD used. OPDA was selected as a derivatizing agent for VDK because it reacts specifically with dicarbonyl compounds in aqueous conditions. The products of the derivatization of VDK with OPDA are strongly retained on the C18 columns and are easily detectable with the MS. Purchased in the dihydrochloride form (Sigma, WI, USA), OPDA is a cheap reagent that is soluble in water and that reacts quickly with diacetyl and 2,3-pentanedione. OPDA binds strongly to VDK and the resulting compounds are resistant to product degradation and fragmentation in the MS spray chamber. The final molecular masses of the derivatized compounds detected by the

MS detector are 159.1 m/z for diacetyl and 173.1 m/z for 2,3-pentanedione. These masses correspond to the theoretical products of OPDA derivatization: 2,3-dimethylquinoxaline and 2-ethyl-3-methylquinoxaline.

3.3.2 Optimization of the reaction of OPDA with VDK

Optimization of the reaction of OPDA with VDK was based on three parameters: the ratio of sample to reagent, the pH conditions and the OPDA concentration. The ratio of sample to reagent was fixed to 4:1 in order to achieve a detection limit of 10 µg/L in aqueous standards. The reaction of OPDA with VDK was evaluated at two different pH conditions. Buffer solutions were prepared with deionized water treated with a *Milli-Q® Ultrapure Water Purification System* (Millipore). The buffer solutions were passed through a 0.2 µm pore size filter. Buffered solutions of OPDA were prepared daily and were kept at 4°C to ensure good product reactivity.

The first buffer solution was a 0.1 M phosphate buffer adjusted to pH 7 (Fisher Scientific). This buffer solution was prepared by mixing 5.38 g/L of potassium phosphate monobasic with 8.66 g/L potassium phosphate dibasic. The reaction at pH 7 was mostly studied with the intention of performing simultaneous determination of AA and VDK: the optimal pH for the reaction of AA with OPA being in the alkaline range. The goal was to use a buffer solution that would contribute to an increase in the pH of the sample analyzed. However, α-diketones are known to degrade in alkaline conditions (56). It was therefore decided to limit the OPDA reagent pH to 7.

The second buffer solution used was a 0.04 M acetate buffer solution adjusted to pH 3.5. The buffer was prepared by diluting 3.08 g/L of ammonium acetate and adjusting with 16

mL/L of glacial acetic acid. This buffer solution was used to perform VDK analysis since OPDA reacts faster with VDK in acidic conditions.

Derivatization tests were performed on aqueous standard solutions containing 10 mg/L of diacetyl and 2,3-pentanedione. OPDA solutions of two different concentrations (0.375 g/L and 3.75 g/L) were prepared. Standards were incubated at room temperature and 1 µl of the final mixture was injected on the HPLC column. The amount of derivatization occurring over a certain period was estimated by comparing the signal obtained by injecting the standard after a certain incubation time with the signal obtained by re-injecting the same standard once the reaction was complete. Selected conditions for other tests and for sample analysis were confirmed by performing tests on commercial lager spiked with the same amount of VDK. The pH of the 4 to 1 beer/reagent mixture was 3.55 for the acetate buffer and 4.65 for the phosphate buffer, while the original beer pH was 4.15.

Selected derivatization conditions

The derivatization procedure selected for further tests and sample treatment, except where other conditions are described, was the following:

1. Add 100 µL of a solution containing 3.75 g/L of OPDA dihydrochloride in the acetate buffer to 400 µL of sample;
2. Incubate for a minimum of 5 minutes at room temperature.
3. Inject 10µL on the HPLC.

3.3.3 Detection of OPDA derivatives

3.3.3.1 Reproducibility test

In order to assess the reproducibility of signal response and to ensure consistency in the preparation of standard solutions, a first test was performed by preparing nine different aqueous standards containing 1 mg/L of VDK. Separate diacetyl and 2,3-pentanedione stock solutions were prepared by diluting 50 mg in 50 mL of water. Diacetyl and 2,3-pentanedione were weighed directly into water to minimize evaporation. Three different stock solutions were prepared and each stock was used to produce three different standards. The produced standards were derivatized according to the selected conditions described previously. An overall coefficient of variation of 1.75% for diacetyl and 5.5% for 2,3-pentanedione was obtained while the mean variation for dilution of a same stock was 1.90% for diacetyl and 1.34% for 2,3-pentanedione.

3.3.3.2 External vs. internal calibration

The potential of using 3-methylisoquinoline as an internal standard for calibration was assessed by performing three replicate dilutions of a VDK stock solution and comparing the variation using both internal and external calibration. Calibration was performed in three points: 0.1 mg/L, 1 mg/L and 10 mg/L. A solution of 20 g/L of 3-methylisoquinoline was first prepared in methanol. This solution was diluted in water to a concentration of 400 mg/L. This aqueous solution was used to spike the OPDA solution with 5 mg/L of 3-methylisquinoline. The aqueous standards were derivatized using the spiked OPDA reagent according to the selected conditions. The molecular mass detected

by the MS for 3-methylisoquinoline was 144.4 m/z and the compound was conveniently eluting between the OPDA derivatives of diacetyl and 2,3-pentanedione (Figure 3.2.3.1).

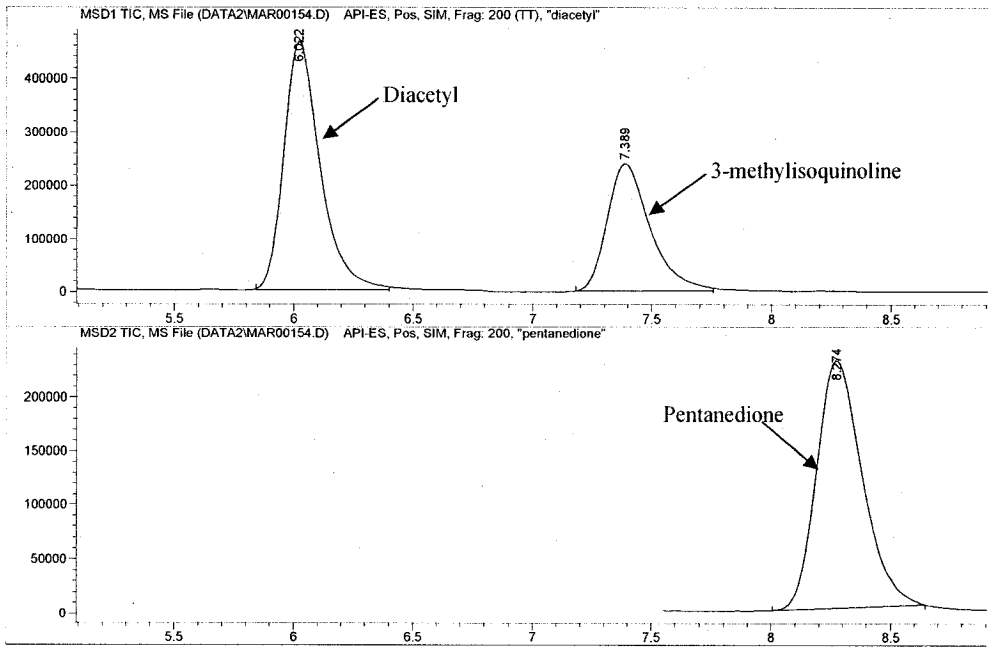


Figure 3.3.3.1 Chromatogram of an aqueous standard containing 1 mg/L of diacetyl, 2,3-pentanedione and 3-methylisoquinoline eluted with 70% Phase B.

The mean variation for external calibration was 5.25% for diacetyl and 6.25% for 2,3-pentanedione, while values for internal calibration were 5.71% for diacetyl and 5.73% for 2,3-pentanedione. Irregularities in signal response were, however, observed between the different stocks of 3-methylisoquinoline prepared. Since both calibration methods showed similar levels of variation, it was judged simpler to use external calibration for most of the analyses performed.

3.3.3.3 Calibration with commercial beer

In order to show that the relationship between signal response and sample analyte concentration was the same in beer samples as in aqueous standards, commercial beer samples spiked with various amounts of VDK were used to perform a calibration curve. Aliquots of a commercial imported beer were degassed, any precursors present were decarboxylated according to the selected procedure described later in this chapter and the beer was finally spiked with the following VDK concentrations: 0.050 mg/L, 0.1 mg/l, 0.5 mg/L and 1 mg/L. Beer without any spiked VDK was also analyzed upon precursor oxydative decarboxylation to determine the beer original content in VDK. Standards were derivatized according to the selected conditions. Since high levels of interference were found in the tested beer, analysis was performed using 60% Phase B.

3.3.3.4 Evaluation of long term product/signal stability

In order to evaluate product stability, a 4.0 mL solution containing 1 mg/L of diacetyl and 2,3-pentanedione was prepared in commercial imported lager. The solution was derivatized using selected conditions. Eight aliquots of the derivatized solution were placed into closed vials and left on the autosampler at room temperature. Samples were shaken before analysis and 10 µL was injected on HPLC at different intervals over four days. Signal variations of 9.0% for diacetyl and 9.2% for 2,3-pentanedione were recorded over 103 hours.

3.4 Oxydative decarboxylation of the VDK precursor

3.4.1 Synthesis of α-acetolactate

Ethyl 2-acetoxy-2-methyl-acetoacetate (Sigma, WI, USA) was transformed to α-acetolactate, ethanol and acetate by the addition of NaOH (35). The procedure consisted of adding four equivalents of NaOH to one equivalent of ethyl 2-acetoxy-2-methyl-acetoacetate and incubating the mixture for 30 minutes in a closed vial at room temperature with constant stirring. In order to obtain a final stock solution that would theoretically contain 11.63 mM of α-acetolactate, 47.0 mg of 2-acetoxy-2-methyl-acetoacetate were added to 10 mL of water. Aliquots of 600 µL of the diluted ester were stored at -50°C until their use. Cold storage of the aqueous ester solution did not appear to affect the amount of α-acetolactate ultimately produced since similar yields were obtained by performing the saponification on a fresh stock. Once thawed, 500 µL of the diluted ester was transferred to a vial with 500 µl of 100 mM sodium hydroxide. The vial was closed and the mixture stirred for 30 minutes at room temperature. An average reaction yield of 83.9±2.8% was obtained over 5 samples leading to a final mean α-acetolactate concentration of 9.76 mM.

3.4.2 Optimization of decarboxylation parameters

3.4.2.1 Standard preparation

100 fold dilutions of the α-acetolactate ester stock were prepared with commercial imported lager to achieve a concentration of 97.6 µM, which is equivalent to a diacetyl potential of 8.39 mg/L. This concentration was selected because it represented roughly ten times the maximum concentration usually encountered in beer fermentations. Tests

with the precursor of 2,3-pentanedione in beer, α-acetohydroxybutyrate, were not performed since the behavior of this compound was often reported to be the same as α-acetolactate (28).

3.4.2.2 Decarboxylation catalysts and heat treatment

Different methods for α-acetolactate oxydative decarboxylation were tested in order to find the one that would lead to the fastest reaction rate and most complete conversion. The influence of three different catalysts was assessed by incubating α-acetolactate standards at different temperatures for different amounts of time. 1 mL of α-acetolactate standard was placed in a 1.5 mL disposable centrifuge tube, the reagent solutions were added, the tube was closed, the mixture was vigorously agitated and left to incubate. Incubation at temperatures higher than room temperature was performed in a water bath. After heat treatment, the reaction was immediately stopped by immersing the standards into liquid nitrogen and storing them at -50°C. The mixture was thawed prior to analysis, derivatized according to selected conditions and 1 µL of the final mixture was injected on the HPLC column.

The catalysts tested were aniline hydrochloride, OPDA, and the combination of ferrous sulphate and ferric chloride (Sigma, WI, USA). Aqueous solutions of 0.227 M and 2.27 M aniline hydrochloride were prepared and added at a concentration of 50 µL/mL to α-acetolactate standards. 20.7 mM and 207 mM OPDA solutions were prepared in 0.04 M acetate buffer solution adjusted to pH 3.5 and added at a concentration of 250 µL/mL to α-acetolactate standards. Standards treated with OPDA were injected on HPLC immediately after they were thawed. New solutions of aniline hydrochloride and OPDA

were prepared daily and kept at 4°C. 10 mM solutions of ferrous sulphate and ferric chloride were also prepared and added to α-acetolactate standards at a concentration of 15 µL/mL to reach a final concentration of 0.15 mM or at a concentration of 60 µL/mL to reach a final concentration of 0.60 mM. Ferric chloride and ferrous sulphate solutions were prepared weekly and kept separate to avoid precipitation.

Selected decarboxylation conditions

After evaluation of the test conditions, the oxydative decarboxylation procedure selected for total VDK analysis of beer samples was the following:

1. Add 15 µL/mL of 10 mM ferric chloride and ferrous sulphate solutions;
2. Incubate at 80°C for 10 minutes;
3. Chill for 10 minutes on ice and vortex prior to sample derivatization.

3.5 Free amino acids by HPLC

3.5.1 Optimization for AA detection in beer matrix

3.5.1.1 AA derivatization

In order to increase their retention on the reverse phase HPLC column and to be able to detect them by MS and fluorescence, amino acids were derivatized prior to their analysis. The selected derivatization reagent was a premixed Fluoraldehyde reagent solution (Pierce Biotechnology Inc., Rockford, IL, USA) containing 2-mercaptoethanol and 0.8 mg/L of OPA in a pH 10 borate buffer solution. The 1 L reagent bottle was kept at 4°C and was spiked with 500 µl of 2-mercaptoethanol after 1 year of storage to maintain its reactivity.

Once the OPA derivatives of leucine, isoleucine and valine had been individually detected and identified on HPLC, calibration was made using an AA standard solution

(Pierce Biotechnology Inc.) containing 2.5 mM of each the following compounds: L-alanine, L-leucine, ammonium sulphate, L-lysine•HCl, L-methionine, L-arginine, L-phenylalanine, L-aspartic acid, L-proline, L-cystine, L-serine, L-glutamic acid, L-threonine, glycine, L-tyrosine, L-histidine, L-valine , and L-isoleucine. The main purpose of using this AA mixture was to separate OPA derivatives of leucine, isoleucine and valine from the OPA derivatives of other AA which are also detected by fluorescence. This mixture was used for calibration to ensure that enough OPA was present to perform complete derivatization in a sample containing a complex amino acid profile. Since preparing amino acid solutions can often appear to be imprecise and laborious, the purchased amino standard solution provided a simple tool to perform accurate calibration.

3.5.1.2 Leucine, isoleucine and valine determination by LC/MS

AA were eluted from the 2.0 mm bore column using a flow rate of 0.150 mL/min and 45% Phase B. A flow rate of 0.500 mL/min and methanol concentration of 65% were selected for the 4.6 mm bore column. Phase B proportion was generally maintained to lower values than for separate VDK determination in order to properly separate leucine and isoleucine, since the OPA derivatives of these AA have identical molecular masses and very close elution times.

The masses of the OPA derivatives detected were respectively 294.1 for valine and 308.1 for leucine and isoleucine. AA samples analyzed by MS were prepared manually by mixing 25 µl of sample with 500 µl of OPA reagent. Samples were incubated at room temperature for 5 to 10 minutes prior to injecting 4 µL on the 2.0 mm bore column or 10 µL on the 4.6 mm bore column. Because the OPA derivatives were only temporarily

stable, longer incubation times than 10 minutes had to be avoided. Respecting the sample dilution rate mentioned above, accurate determination of amino acids was performed for a concentration range of 8 µM to 2.5 mM.

3.5.1.3 Fluorometric determination of AA

The HPLC column used for AA analysis by flurorescence detection was a Eclipse XDB-C18 column (150 X 4.6mm, 5µ) (Agilent Technologies). 0.05 M potassium phosphate buffer adjusted to pH 6.6 (Phase A) and HPLC grade methanol (Phase B) were used for mobile phases at a constant flowrate of 1 mL/min. Buffer solutions were prepared with deionized water treated with a *Milli-Q® Ultrapure Water Purification System* (Millipore). The buffer solutions were passed through a 0.2 µm pore size filter. The following gradient was used to separate OPA derivatives of valine, leucine and isoleucine from the interference and the OPA derivatives of other amino acids: starting with 40% Phase B, the concentration was raised linearly to 62% Phase B in 24 minutes. The column was washed with methanol and re-equilibrated to 40% Phase B prior to the next injection. Injections were prepared automatically on the autosampler by the means of a programmed routine. The routine was the following: draw 0.5 µl from sample and 9.5 µl from a vial containing the OPA reagent; move the injector needle up and down 6 times in the air to allow mixing; wait for 3 minutes and inject the needle content on the HPLC column. Because the OPA reagent was degradable at room temperature, the reagent vial left on the autosampler to perform the analyses was replaced with one containing fresh reagent every 24 hours. The excitation and emission wavelengths of the fluorescence detector were respectively set to 340 nm and 455 nm.

3.5.1.4 Simultaneous determination of VDK and amino acids

Attempts to achieve simultaneous determination of leucine, isoleucine and valine with the VDK were made using a two step derivatization process. Samples were first spiked with OPDA diluted in a pH 7 phosphate buffer, left to incubate until completion of VDK derivatization, spiked with a certain quantity of OPA reagent, left to incubate until completion of AA derivatization and then injected immediately on the HPLC column. The main problem faced in achieving simultaneous determination was based on the fact that VDK and AA are found in very different concentrations during beer fermentation. The challenge consists of providing enough OPA to assure complete derivatization of the AA, which are generally present in higher concentrations in beer (0.008 to 2.5 mM), without diluting the trace amounts of VDK produced during fermentation to concentrations below their detection limits.

Sample preparation was performed following this procedure:

1. Add 100 µl of OPDA diluted in the pH 7.0 phosphate buffer to 400 µl of sample.
2. Stir and incubate for a minimum of 10 minutes at room temperature.
3. Add 100 µl of OPA reagent.
4. Stir and incubate for exactly 5 minutes at room temperature.
5. Inject 4 µL on the 2.0 mm bore column or 10 µL on the 4.6 mm bore column.

The purchased reagent contained only 0.8 mg/l of OPA, so accurate determination was only possible for an AA concentration lower than 0.008 mM. Attempts were made to increase the OPA concentration of the reagent to 20 mg/l in order to achieve AA determination at the concentrations normally found in beer (Sigma, WI, USA). OPA crystals were first dissolved in methanol at a concentration of 200 mg/mL. A tenfold

dilution was performed with the OPA reagent and the final mixture was spiked with 25 µL/mL of 2-mercaptoethanol to ensure good reactivity of the OPA. Attempts to increase the OPA concentration of the reagent appeared to lead to an over saturation of the MS detector; which overall yielded a decrease in sensitivity for VDK determination due to increased background noise, inaccurate peak shape for AA and accelerated deterioration of the MS electron multiplier due to analyzer flooding. Separate determination of VDK and AA was therefore performed on beer samples.

3.6 Method application

3.6.1 Evaluation of VDK and precursor degradation through storage of the samples at low temperature

The objective of these tests was to evaluate the potential degradation of VDK through freezing, cold storage and thawing cycles. Preliminary tests were made on commercial lager spiked with 97.6 µM of α-acetolactate. Aliquots of 1 mL were frozen at different steps of the sample preparation procedure by storing them in a freezer where the temperature was maintained at -50°C. Total diacetyl was analyzed by LC/MS. Aliquots of the same solution were frozen after α-acetolactate synthesis, α-acetolactate oxydative decarboxylation and after diacetyl derivatization. The aliquots were thawed and identical decarboxylation and derivatization treatments were done prior to LC/MS analysis, if they have not been already performed prior to freezing. The obtained signal response was compared to measure potential losses in final product (Table 3.5.1.1). The signal response did not vary significantly for any of the conditions tested.

Table 3.6.1.1 Results of degradation test.

Frozen post	Signal	% Original
-	3287561	100
Decarboxylation	3136077	95
Derivatization	3178336	97
Synthesis	3133672	95
Synthesis	3064522	93
Synthesis	3311132	101

The second degradation test was performed on samples collected from 1.5 L lager fermentations. Following their collection, the samples were centrifuged at 8000 rpm for 10 minutes and the supernatant was filtered through 0.45 μm disposable filters to remove yeast. A portion of the clarified supernatant was immediately processed through precursor oxydative decarboxylation, sample derivatization and total VDK analysis, while the remaining portion was stored at -50°C for at least one month. Frozen samples were thawed, submitted to identical decarboxylation and derivatization procedures and analyzed for total VDK. The values obtained for total VDK were compared between the samples that were freshly analyzed and the ones that were stored at -50°C (Table 3.5.1.2). Variation for diacetyl was generally below 10% except for two samples while higher degrees of variation were detected for 2,3-pentanedione. The variation in signal could not, however, be attributed to degradation through cold storage of the samples since some of the standards stored at -50°C provided higher signal. The difference in signal could more likely be attributed to sample handling and variation in MS response.

Table 3.6.1.2 Signal response difference between samples analyzed freshly and samples conserved at -50°C.

Sample	Diacetyl (µg/L)				2,3-pentanedione (µg/L)			
	Frozen	Fresh	Mean	% diff	Fresh	Frozen	Mean	% diff
A1	94	87	91	7.1	155	153	154	1.1
A2	139	125	132	10.8	246	190	218	25.9
A3	198	192	195	3.3	302	273	287	10.1
A4	222	220	221	0.8	305	296	301	3.2
A5	269	236	252	13.1	321	304	313	5.4
A6	297	295	296	0.6	513	513	513	0.0
B1	180	193	186	-7.2	180	0	90	-
B2	102	98	100	4.6	102	130	116	-23.4
B3	152	142	147	6.8	152	186	169	-19.9
B4	251	183	217	31.3	251	287	269	-13.4
B5	303	288	296	5.1	303	316	310	-4.2
B6	318	305	312	4.3	318	330	324	-3.5
B7	328	330	329	-0.8	328	388	358	-16.8

3.6.2 Effects of fermentation temperature on VDK profile

Computer controlled 3.7 L BioEngineering (Wald, Switzerland) bioreactors were filled with 2 L of high gravity lager wort (16°P), sterilized in place at 121°C for 15 minutes and adjusted to their respective fermentation temperatures. Three trial fermentations, performed with an industrial lager brewing strain (S. pastorianus), were started under identical initial conditions and maintained at 12, 14 and 16°C for twelve days without agitation. Yeast slurry collected from the repitching vessel of a commercial brewery was used for inoculum and included in the wort at a rate of 1 x 10^7 cells/mL. Mild agitation (200 rpm) was provided for roughly 2 minutes before collecting the samples. Samples of 10 mL were collected every 24 hours, centrifuged at 8000 rpm for 10 minutes and the supernatant was filtered through 0.45 µm pore size disposable cartridges. The clarified supernatant was used for subsequent analysis.

pH was monitored online throughout the fermentation. Specific gravity was measured on fresh clarified supernatant using an Anton Paar DMA-4600 densitometer (Anton Paar, Ashland, VA, USA). The remaining portion of the supernatant was stored at -20°C prior to HPLC analysis. VDK and AA analysis were performed separately by LC/MS using the 2.0 mm bore HPLC column and an injection volume of 4 µL. VDK precursor oxydative decarboxylation was performed by adding 10.8 mM of aniline hydrochloride and incubating at room temperature for one hour. VDK derivatization was performed by incubating 400 µl of the mixture for a minimum of 10 minutes at room temperature with 100µl of a solution containing 3.75 g/L of OPDA in a 0.1M phosphate buffer solution adjusted to pH 7.0.

3.6.3 Industrial samples

3.6.3.1 Sample collection and LC/MS analysis

Samples from two lager and two ale large scale industrial fermentations were collected in a local commercial brewery. Lagers were fermented at 14°C while ales were fermented at 20°C. Both ales were fermented in 700 hL rectangular vessels. Lager A was fermented in a 5000 hL cylindroconical vessel while Lager B was fermented in a 1400L rectangular vessel. The initial sample was collected after the final wort addition in the fermentation vessel and samples were collected at different time intervals during fermentation. Upon collection, samples of fermenting beer were immediately immersed into liquid nitrogen and were stored in dry ice until they were carried to the McGill laboratory where they were stored at -80°C prior to their analysis. Samples were then thawed and filtered through 0.45 µm disposable cartridges. The clarified supernatant was analyzed for

specific gravity, VDK and for AA. VDK analysis of the filtrate was performed by LC/MS using the 4.6 mm bore column, an injection volume of 10 µL and an isocratic mobile phase conditions of 70% Phase B. AA analysis was performed by HPLC and fluorometric detection.

3.6.3.2 Headspace gas chromatography analysis

Six of the samples analyzed by LC/MS were sent to the laboratories of a local brewery to be analyzed for total VDK by headspace gas chromatography (GC). The detector used for analysis was an electron capture detector. Samples were filtered and disposed on the GC autosampler. Beer samples were then held at 60°C for 35 minutes after which the temperature was raised by 10 °C/min to 70°C and held at that temperature for 4 minutes prior to injection. Results provided by the brewery technicians were compared to the ones obtained with the newly developed LC/MS method for total VDK analysis.

IV RESULTS

4.1 Vicinal diketone (VDK) derivatization

Faster derivatization rates were achieved in acidic conditions (pH 3.5) (Table 4.1.1). Satisfactory times to achieve complete derivatization were recorded using reagent solutions containing an o-phenylenediamine (OPDA) concentration of 3.75 g/L. At that concentration, similar amounts of derivatization were achieved in commercial beer and aqueous standards (Tables 4.1.2). The minimum derivatization time for the analysis of beer sample was determined to be 5 minutes with the pH 3.5 acetate buffer and 10 minutes with the pH 7.0 phosphate buffer.

Table 4.1.1 Derivatization tests performed at room temperature on VDK aqueous standard containing 10 mg/L.

OPDA	Buffer			Diacetyl		2,3-pentanedione		
Conc. (g/L)	Type	Conc. (M)	Rx time (min.)	Deriv. (%)	Stdev* (%)	Rx time (min.)	Deriv. (%)	Stdev* (%)
0.375	PO_4	0.1	5	51.9	1.1	5	46.5	0.3
0.375	PO_4	0.1	10	79.7	1.7	10	73.9	0.9
0.375	PO_4	0.1	15	92.7	1.9	15	88.1	0.2
3.75	PO_4	0.1	2	91.2	0.9	2	90.5	0.7
3.75	PO_4	0.1	5	100.1	1.8	5	100	1.8
0.375	NH_4Ac	0.04	5	101.1	1.7	5	98.2	2.9
3.75	NH_4Ac	0.04	2	95.1	0.03	2	94.5	2.1

*: results were based on triplicates.

Table 4.1.2 Derivatization tests performed at room temperature on commercial lager spiked with 10 mg/L.

OPDA	Buffer			Diacetyl		2,3-pentanedione		
Conc. (g/L)	Type	Conc. (M)	Rx time (min.)	Deriv. (%)	Stdev* (%)	Rx time (min.)	Deriv. (%)	Stdev* (%)
3.75	PO_4	0.1	5	96.8	0.7	5	98.5	0.4
3.75	NH_4Ac	0.04	2	92.3	4.8	2	89.3	4.3

*: results were based on triplicates.

4.2 VDK detection and calibration

4.2.1 Detection and calibration using the 2.0 mm bore *Gemini* column

Detection of diacetyl and 2,3-pentanedione was achieved down to 5 µg/L with a variation coefficient of less than 10% for aqueous standards on the 2.0 mm bore column. Signal response was linear for VDK concentrations up to 10 mg/L (Figures 4.2.1.1, 4.2.1.2). A calibration curve was performed with aqueous standards containing the following concentrations of diacetyl and 2,3-pentanedione: 0.005, 0.05, 0.1, 0.2, 1, 2, 10 mg/L. An injection volume of 4 µL was used and VDK derivatization was performed by incubating 400 µl of the standard for a minimum of 10 minutes at room temperature with 100µl of a solution containing 3.75 g/L of OPDA in a 0.1M phosphate buffer solution adjusted to pH 7.0.

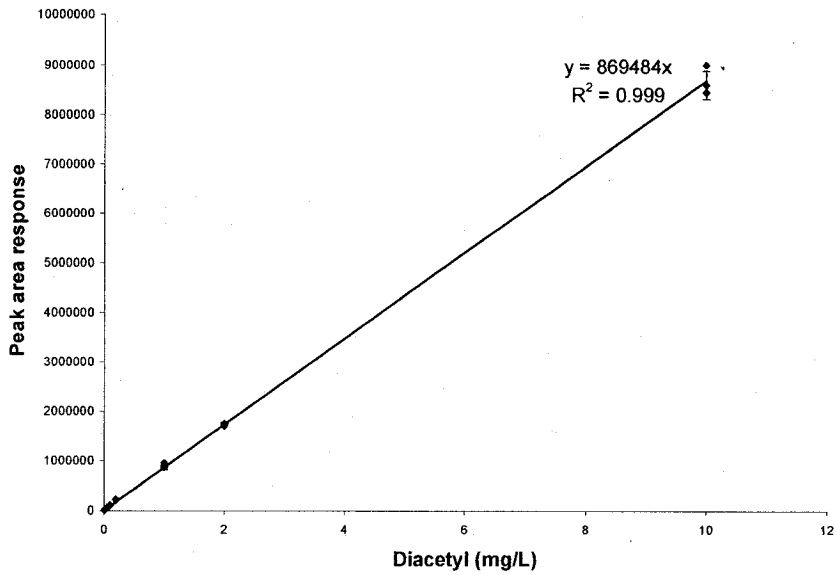


Figure 4.2.1.1 Diacetyl calibration curve using the 2.0 mm bore Gemini column.

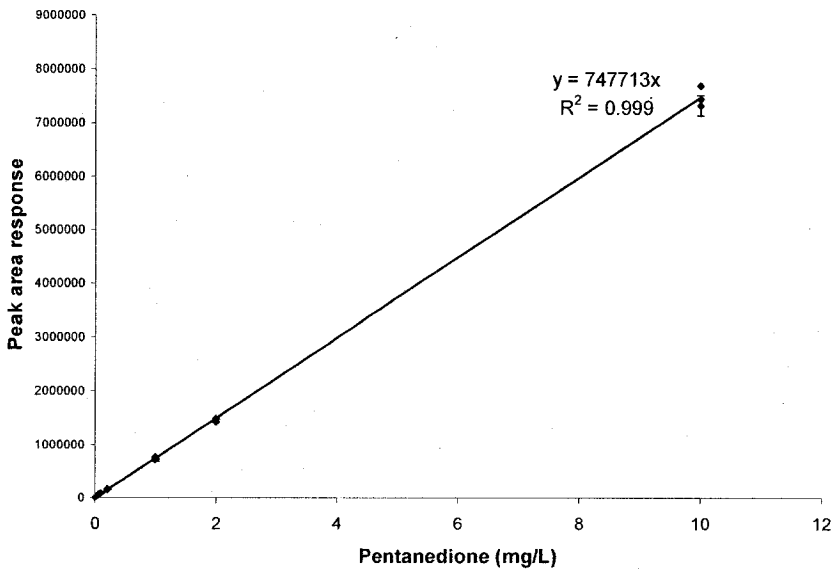


Figure 4.2.1.2 2,3-pentanedione calibration curve using the 2.0 mm bore Gemini column.

4.2.2 Detection and calibration using the 4.6 mm bore *Gemini* column

Aqueous standards

Detection of diacetyl and 2,3-pentanedione was achieved down to 10 µg/L with a variation coefficient of less than 10% for aqueous standards with the 4.6 mm bore column. Signal response was linear up to 10 mg/L. Figure 4.2.2.1 and 4.2.2.2 contain the calibration curve for the standards prepared according to the procedure mentioned in section 3.2.3.2.

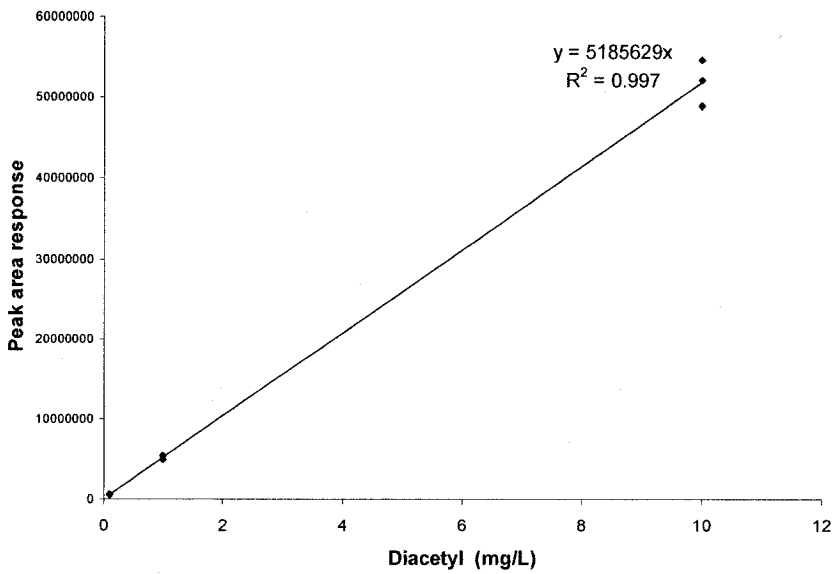


Figure 4.2.2.1 Diacetyl calibration curve in aqueous standards with 70% Phase B on the 4.6 mm bore Gemini column.

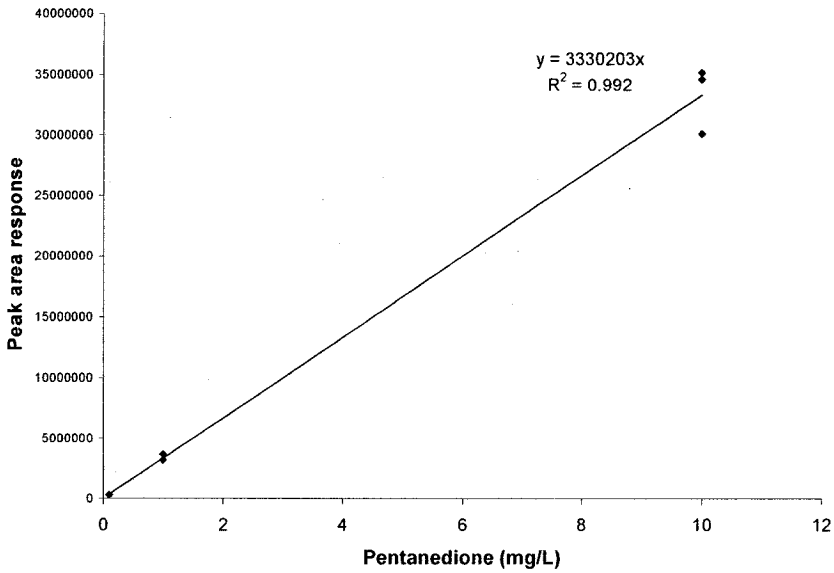


Figure 4.2.2.2 2,3-pentanedione calibration curve in aqueous standards with 70% Phase B on the 4.6 mm bore Gemini column.

Beer standards

The calibration curve for the standards prepared according to the procedure mentioned in section 3.2.3.3 using a commercial larger beer is displayed on Figure 4.2.2.3. The level of interference being too high in that particular commercial beer, 2,3-pentanedione detection could not be detected at low levels. For diacetyl, a difference of 7.3% in slope was noted between the calibration curves of aqueous and beer standards using 60% Phase B.

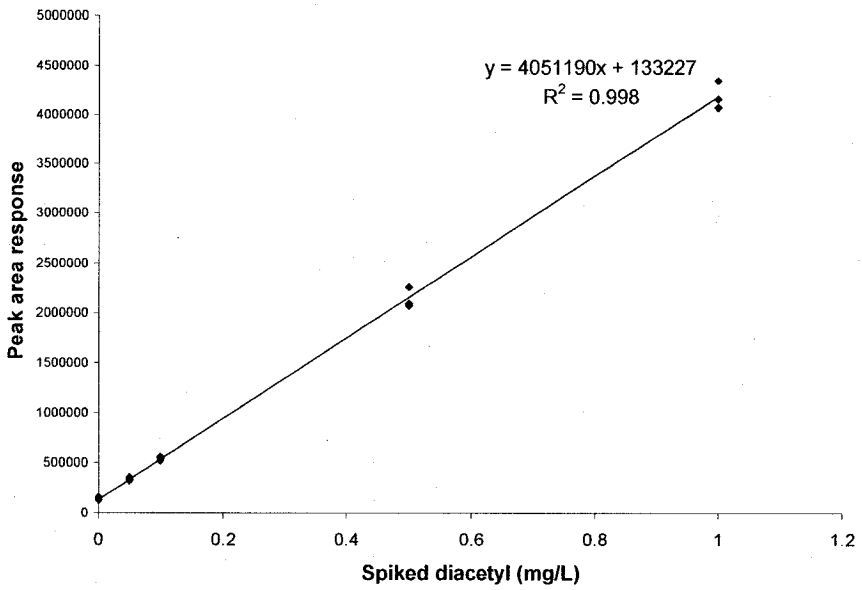


Figure 4.2.2.3 Diacetyl calibration curve in commercial lager with 60% Phase B.

4.3 α-Acetolactate oxydative decarboxylation

The official heat treatment method of 60°C for 90 minutes without catalyst was primarily used as the basis for comparison to evaluate the yield during oxydative decarboxylation. At that incubation temperature, complete oxydative decarboxylation of the standard was achieved in one hour (Table 4.3.1.1).

4.3.1 $FeSO_4$/FeCl addition

Complete oxydative decarboxylation was achieved in 10 minutes by incubating standards at 80°C with 0.15 mM of $FeSO_4$ and $FeCl_3$; showing a yield increase of 25% over a standard containing no catalysts heated at the same temperature. Heating standards at the same temperature for longer times than 10 minutes without catalyst did not result in any substantial increases in yield. Attempts to increase the concentration of the iron catalysts

did not have any positive effect, while decreasing the incubation temperature to 60°C resulted in a major drop in reaction rate.

Table 4.3.1.1 Results of α-acetolactate oxydative decarboxylation tests with FeSO4 and FeCl3 catalysts.

FeSO4 / $FeCl_3$ (µM)	Inc. Temp (°C)	Inc. Time (min.)	Decarb. (%)	Stdev* (%)
0	60	10	47.0	0.1
0	60	30	84.8	2.2
0	60	60	103.3	3.5
0	60	90	100.0	4.9
0	60	120	98.0	2.4
150	60	10	58.7	1.7
150	60	20	82.7	0.7
150	60	30	90.7	6.3
0	80	10	76.5	1.5
0	80	20	73.2	6.2
0	80	30	75.0	3.8
150	80	5	73.2	0.8
150	80	10	100.0	2.1
150	80	15	98.1	4.8
600	80	5	69.8	2.8

*: results were based on duplicates.

4.3.2 Aniline hydrochloride addition

The technique consisting of the addition of aniline hydrochloride as a catalyst followed by incubation at room temperature was also tested. The addition of the concentration prescribed in the literature (20) (10.8 mM) resulted in only 22.2 % increase in oxydative decarboxylation after one hour of incubation compared to a standard in which no catalyst was added. Higher reaction yields were obtained 67.8% when the aniline hydrochloride concentration was increased to 108 mM. Longer incubation times resulted in an increase in diacetyl yield but the reaction was not complete after 3 hours for all the conditions tested.

Attempts to incubate standards at higher temperatures with aniline HCL did not benefit the reaction compared to standards incubated at the same temperatures without the catalyst.

Table 4.3.2.2 Results of α-acetolactate decarboxylation tests with aniline HCl catalyst.

Aniline HCl (mM)	Inc. Temp (°C)	Inc. Time (min.)	Decarb. (%)	Stdev* (%)
0	RT	60	10.4	-
0	RT	120	16.4	-
0	RT	180	24.1	-
10.8	RT	60	32.6	1.9
10.8	RT	120	56.9	1.0
10.8	RT	180	63.3	2.4
108	RT	60	67.8	0.7
108	RT	120	81.7	1.7
108	RT	180	72.4	1.6
10.8	60	10	68.0	0.1
10.8	60	20	73.9	0.8
10.8	60	30	74.2	0.3
108	60	10	46.2	0.1
10.8	80	10	77.3	3.6
108	80	10	49.9	2.1

*: results were based on duplicates.

4.3.3 OPDA addition

Trials were made to perform both precursor oxydative decarboxylation and VDK derivatization by the single addition of OPDA. OPDA did not appear to have any effect on the rate of oxydative decarboxylation at room temperature while heating standards with OPDA led to lower yield.

Table 4.3.3.3 Results of α-acetolactate decarboxylation tests with OPDA.

OPDA (mM)	Inc. Temp (°C)	Inc. Time (min.)	Decarb. (%)	Stdev* (%)
4.14	RT	60	11.8	-
4.14	RT	120	15.3	-
4.14	60	10	50.2	5.9
4.14	80	10	42.3	3.9
41.4	60	10	18.3	-

*: results were based on duplicates.

4.4 Amino acid (AA) determination

4.4.1 LC/MS determination

AA calibration was performed using the 2.0 mm bore HPLC *Gemini* column with aqueous standards containing the following concentrations of the AA standard solution: 4.17 μM, 8.33 μM, 41.7 μM, 0.208 mM, 0.417 mM and 1.25 mM (Figure 4.4.1.1). Signal variation was less than 10% over this range. Table 4.4.1.1 contains the slopes and R^2 values of the calibration curves.

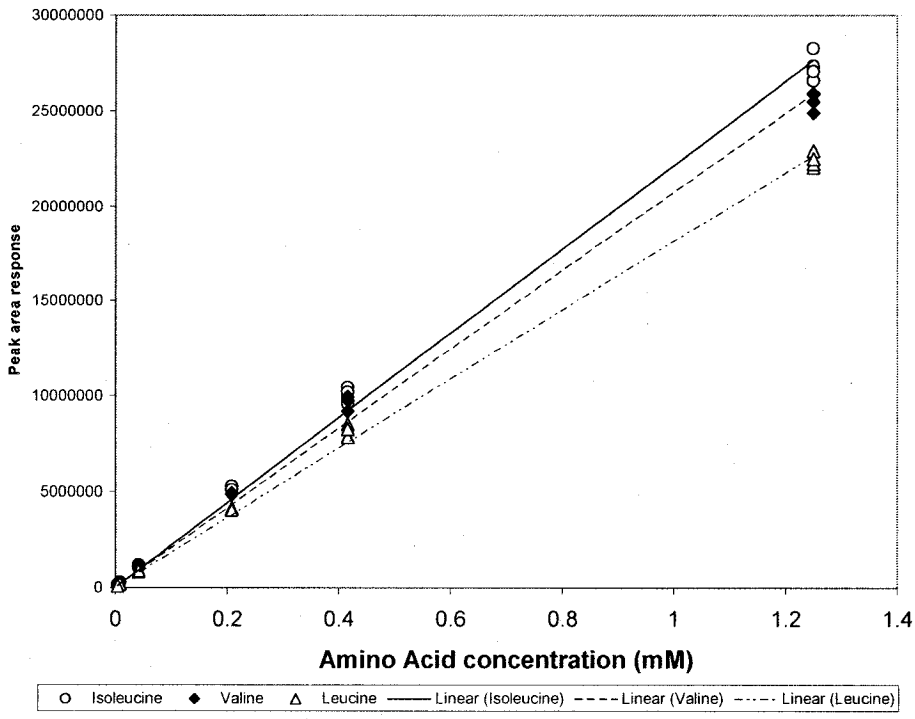


Figure 4.4.1.1 AA calibration curves of aqueous standards using the 2.0 mm bore Gemini column.

Table 4.4.1.1 Properties of AA calibration curves displayed in Figure 4.4.1.1.

Amino acid	Slope	R^2
Valine	22132975	0.997
Isoleucine	20728795	0.997
Leucine	18122433	0.998

4.4.2 Fluorometric determination of AA

AA calibration was performed with aqueous standards containing the following concentrations of the AA standard solution: 0.10 mM, 0.25 mM, 0.50 mM. (Figure 4.4.2.1). Signal variation was less than 5% over this range. Table 4.4.2.1 contains the slopes and R^2 values of the calibration curves.

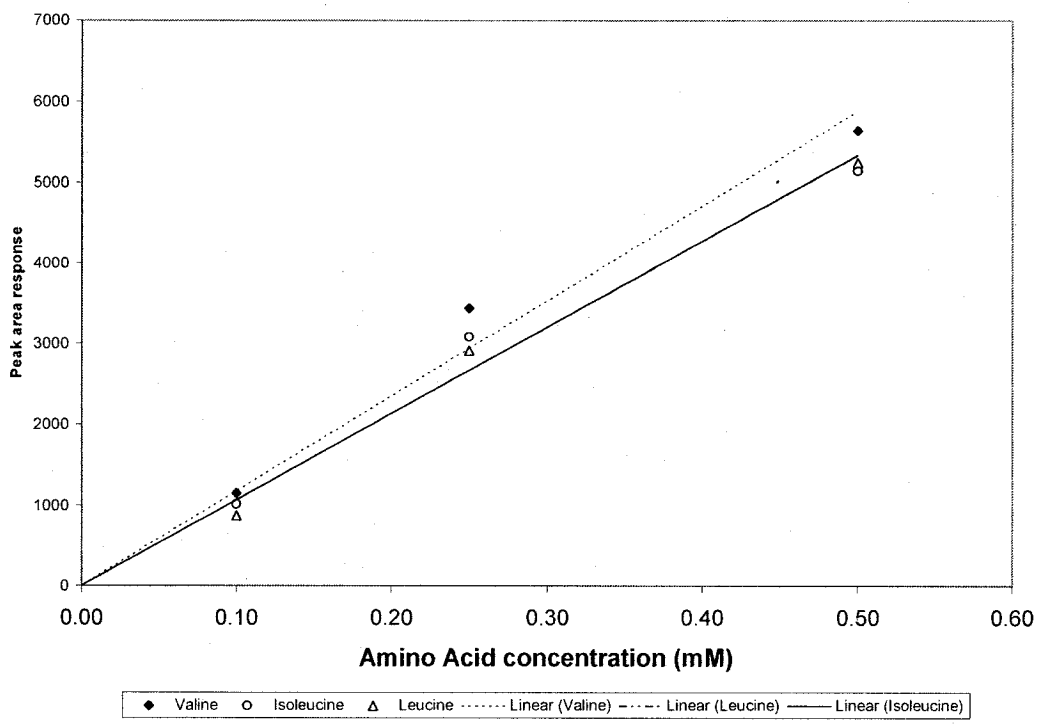


Figure 4.4.2.1 AA calibration curve for fluorometric determination.

Table 4.4.2.1 Properties of AA calibration curves displayed in Figure 4.4.2.1.

Amino acid	Slope	R^2
Valine	11765	0.970
Isoleucine	10673	0.975
Leucine	10667	0.989

4.5 Simultaneous determination

Figure 4.5.1 shows a chromatogram of the simultaneous determination of an aqueous standard containing 0.5 mg/L of diacetyl and 2,3-pentanedione and 10 mg/L of leucine, isoleucine and valine. A calibration curve was performed, using the sample preparation procedure mentioned in Section 3.4.1.4, with aqueous standards containing the following concentrations of isoleucine, leucine and valine: 0.83 µM, 8.3 µM, 83 µM (Figure 4.5.2).

Samples were injected on the 4.6 mm column. Signal variation was less than 10% over this range.

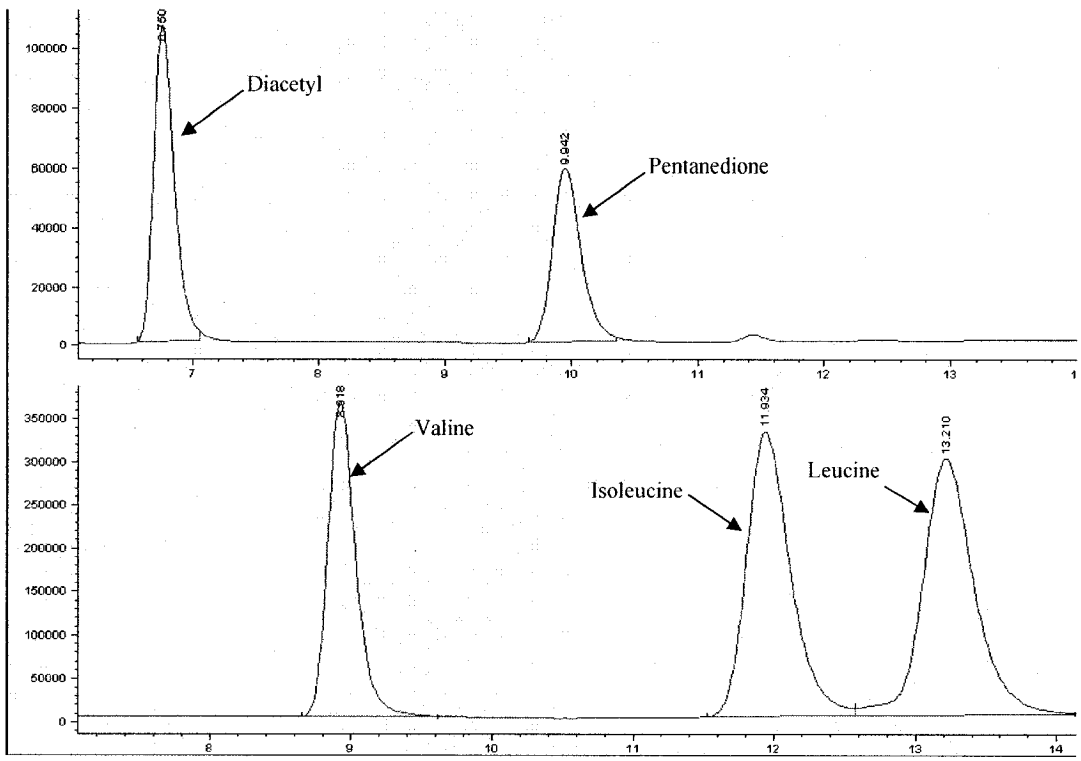


Figure 4.5.1 MSD signals from simultaneous determination of aqueous standard containing 0.5 mg/L of VDK and 10 mg/L of isoleucine, leucine and valine.

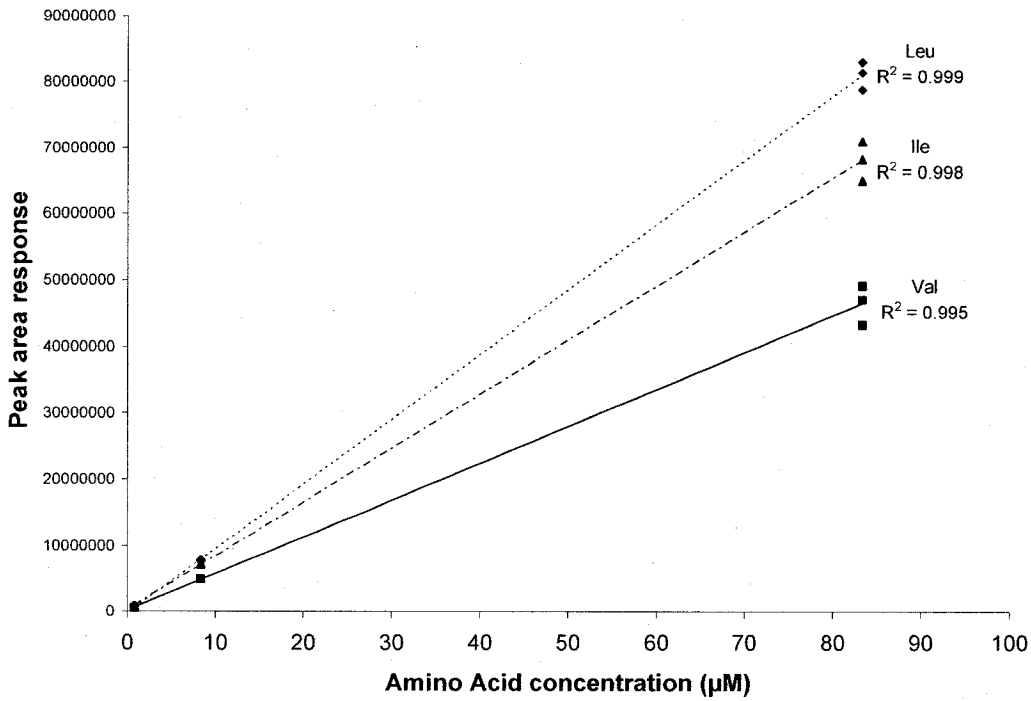


Figure 4.5.2 Amino acids calibration curve in aqueous standard.

4.6 Method application

4.6.1 Impact of fermentation temperature on VDK production.

Fermentation performance

Increases in the fermentation temperature led to smaller decrease in wort pH (Table 4.6.1.1). After 24 hours of fermentation, the apparent gravity of the wort maintained at 12 °C dropped by 0.26 °Plato while wort maintained at 16C° dropped by 3.33 °Plato; which is equivalent to 2.0% and 26.4% of the total amount of wort attenuation achieved during the experiment (Figure 4.6.1.2). Final gravity of the wort was 4.1 °Plato for the 12°C fermentation, 4.05 °Plato for the 14°C fermentation and 3.37 °Plato for the 16°C fermentation. One degree Plato is equivalent to 1% of sucrose or 4% of alcohol potential in sugars.

Table 4.6.1.1 Fermentation pH.

	pH		
Time (hours)	12°C	14°C	16°C
0	5.45	5.1	5.11
24	4.42	4.24	4.22
31	4.34	4.12	4.1
47.5	4.16	3.92	4.09
72	3.92	3.89	4.15
96	3.83	3.96	4.21
117.5	3.8	4.01	4.25
144	3.83	4.04	4.27
167	3.84	4.07	4.29
191	3.9	4.09	4.31
216	3.9	4.11	4.32
287	3.99	4.15	4.36

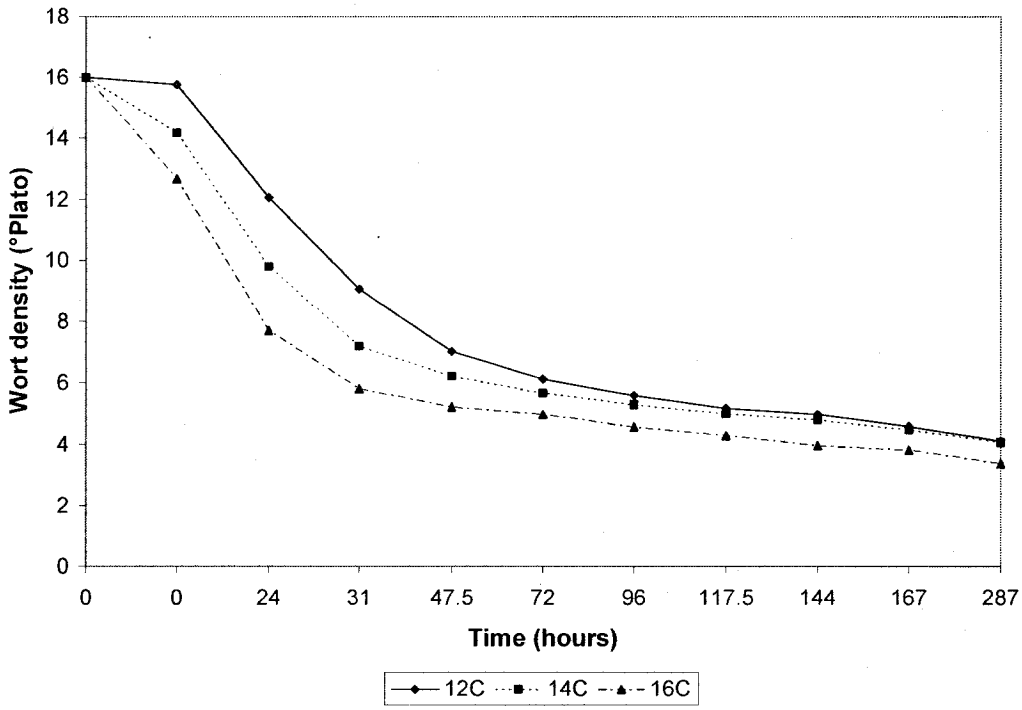


Figure 4.6.1.1 Wort attenuation profiles of three different fermentations temperatures.

VDK production and reduction

Fermentations performed at lower temperatures generally took longer to both produce and reduce VDK (Figures 4.6.1.2, 4.6.1.3). The maximum diacetyl concentrations recorded were 0.618 mg/L for the 16°C fermentation, 0.533 mg/L for the 14°C fermentation and 0.454 mg/L for the 12°C fermentation. The maximum 2,3-pentanedione concentration recorded were 0.518 mg/L for the 16°C fermentation, 0.481 mg/L for the 14°C fermentation and 0.370 mg/L for the 12°C fermentation. Diacetyl concentrations below the taste threshold value of 0.150 mg/L were recorded after 96 hours in the 16°C fermentation, 118 hours in the 14°C fermentation and 144 hours in the 12°C fermentation.

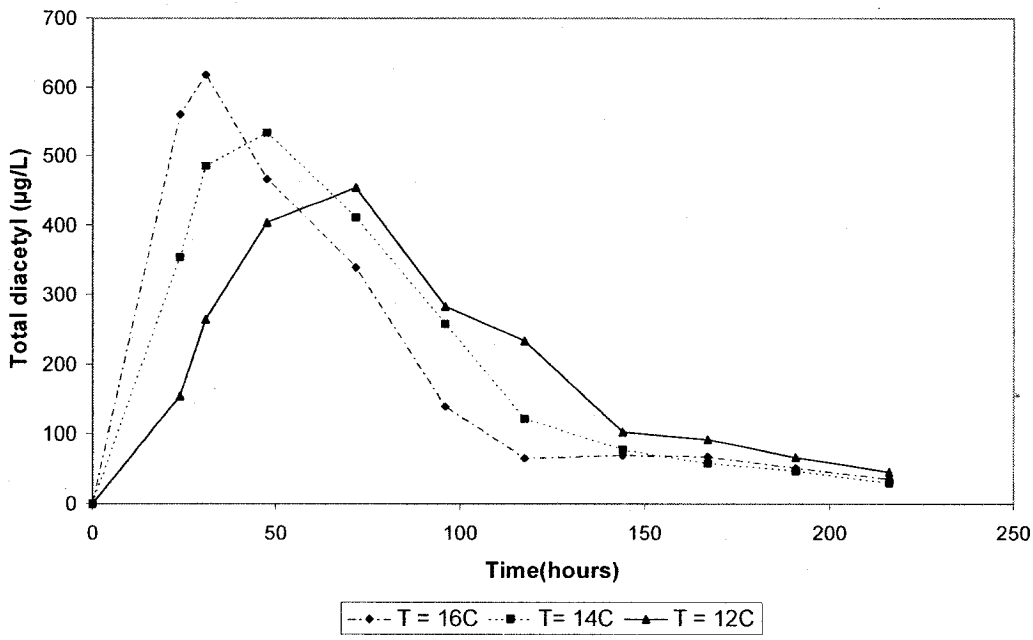


Figure 4.6.1.2 Total diacetyl profile for three fermentation temperatures.

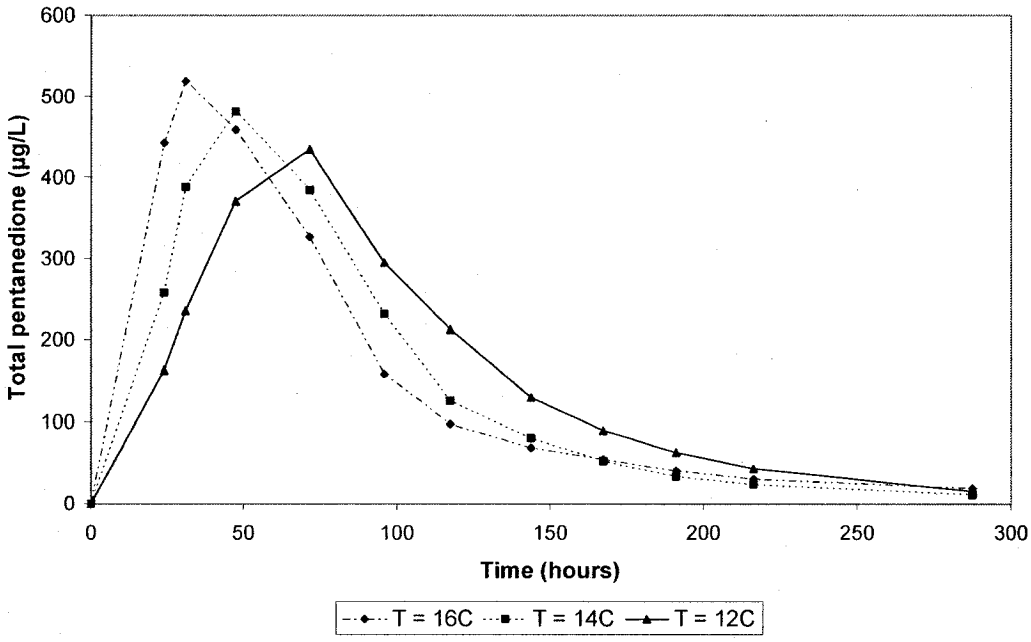


Figure 4.6.1.3 Total 2,3-pentanedione profile for three fermentation temperatures.

AA absorption

The initial wort concentrations in isoleucine, leucine and valine were respectively 0.59 mg/L, 1.25 mM and 0.92 mM. Faster uptake of AA was observed in fermentations performed at higher temperatures. The residual AA content of the beer was higher by 131 µM of valine, 45 µM of isoleucine and 43 µM of leucine in the fermentation performed at 12°C compared to the one performed at 16°C.

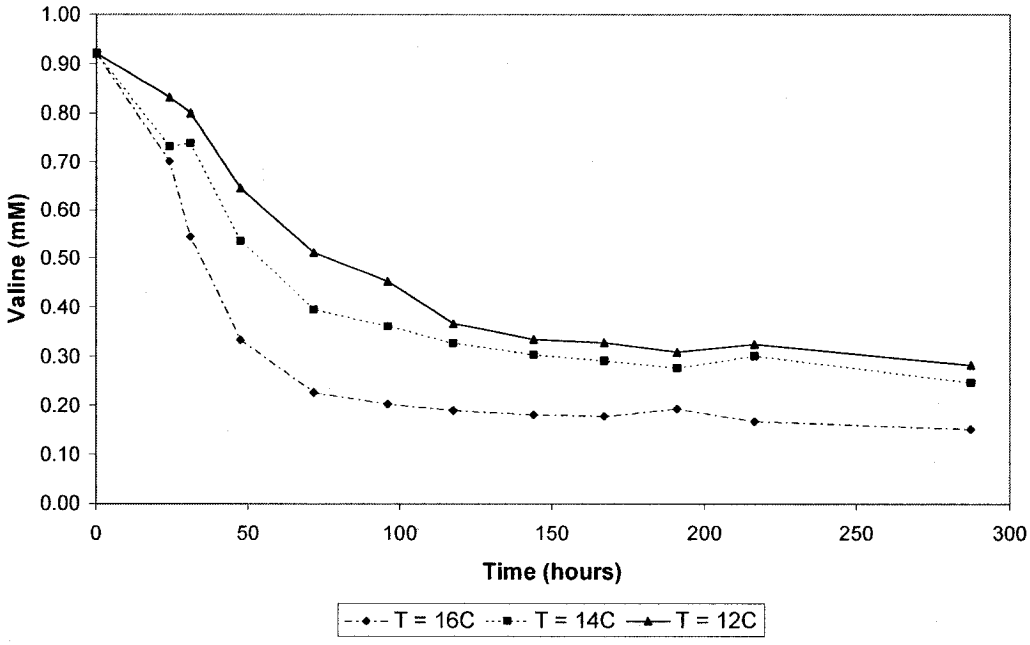


Figure 4.6.1.4 Valine uptake for three fermentation temperatures.

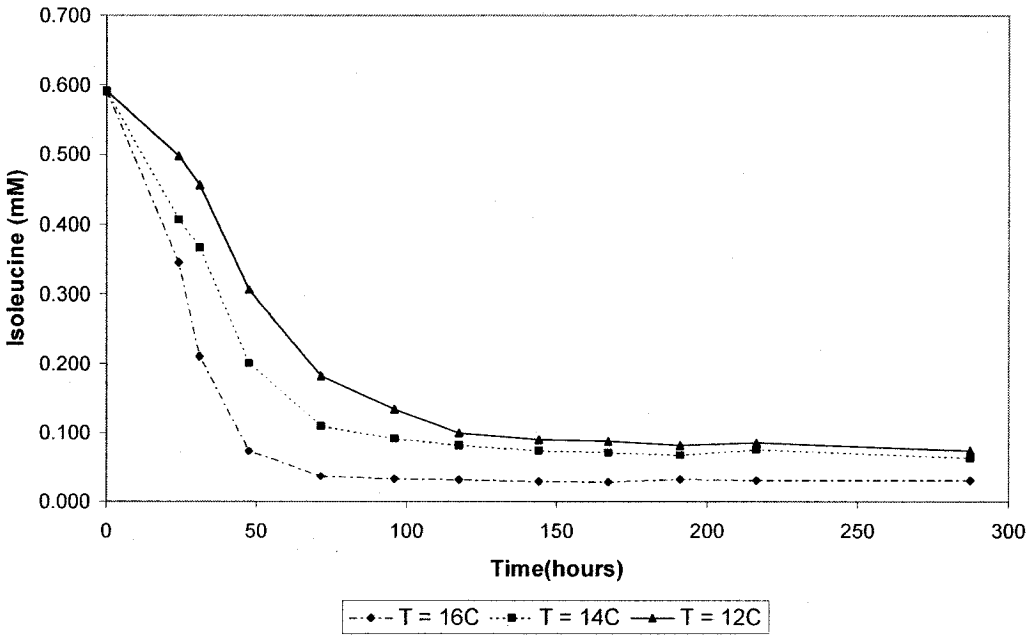


Figure 4.6.1.5 Isoleucine uptake for three fermentation temperatures.

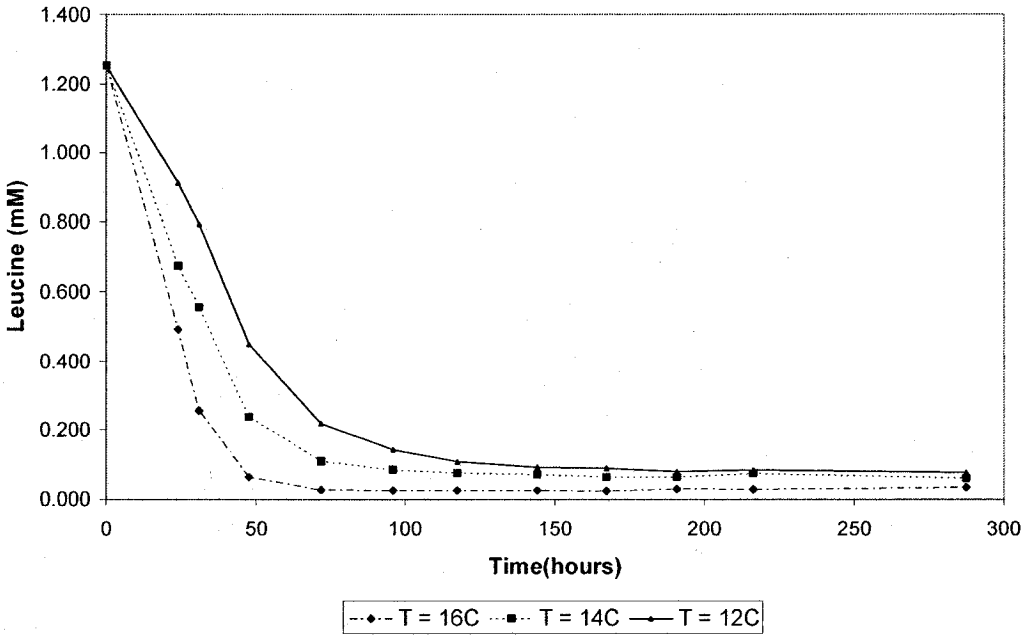


Figure 4.6.1.6 Leucine uptake for three fermentation temperatures.

4.6.2 Analysis of industrial samples.

Fermentation performance

Table 4.6.2.1 shows the initial wort gravity of the four batches studied. Ale yeast was able to quickly attenuate wort gravity. After 48 hours of fermentation, more than 95% of the total attenuation was achieved in both ale batches. Lager fermentations were characterized by much slower attenuation rates than ales; with one of the batches (Batch A) taking more than 4 days to achieve an equivalent drop in gravity. The final wort gravity was 2.99 °Plato in ale Batch A (130 hours), 2.97 °Plato in ale Batch B (130 hours), 3.39 °Plato in lager Batch 1 (202 hours) and 2.39 °Plato in lager Batch 2 (173 hours).

Table 4.6.2.1 Initial gravity of the industrial worts.

Wort	Gravity (°Plato)
Ale A	16.23
Ale B	16.2
Lager 1	17.79
Lager 2	16.88

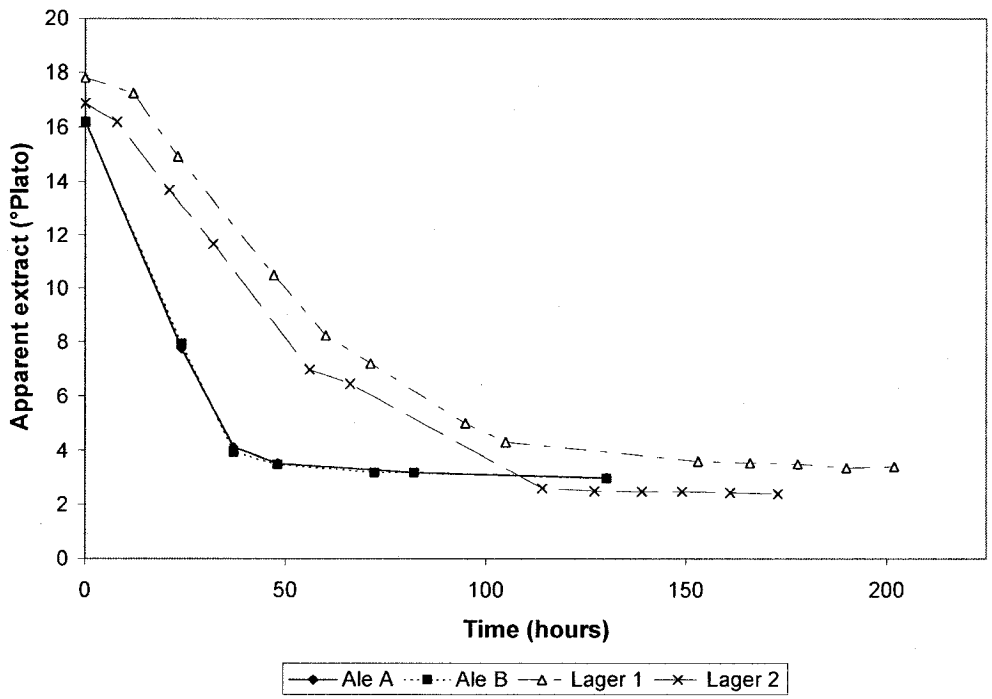


Figure 4.6.2.1 Wort attenuation profiles in two ale and two lager industrial fermentations.

VDK production

The VDK profiles obtained for the two lager batches were quite different in terms of abundance and peak shape. In fact, maximum concentrations of 0.522 mg/L for diacetyl and 0.601 mg/L of 2,3-pentanedione were recorded at 47 hours after the start of fermentation for Batch 1 while Batch 2 showed maximum values of 0.755 mg/L of diacetyl and 0.745 mg/L of 2,3-pentanedione at 66 hours. Ale batches showed more

similar VDK profiles with maximum values recorded at 24 hours. Maximum concentrations of 1.304 mg/L of diacetyl and 0.604 mg/L of 2,3-pentanedione were recorded for Batch 1 while Batch 2 showed maximum values of 1.137 mg/L of diacetyl and 0.560 mg/L of 2,3-pentanedione. The diacetyl concentration in the two ale batches reached values below the taste threshold within 72 hours of fermentation while it took more than 140 hours to reduce it to this level in the lager batches.

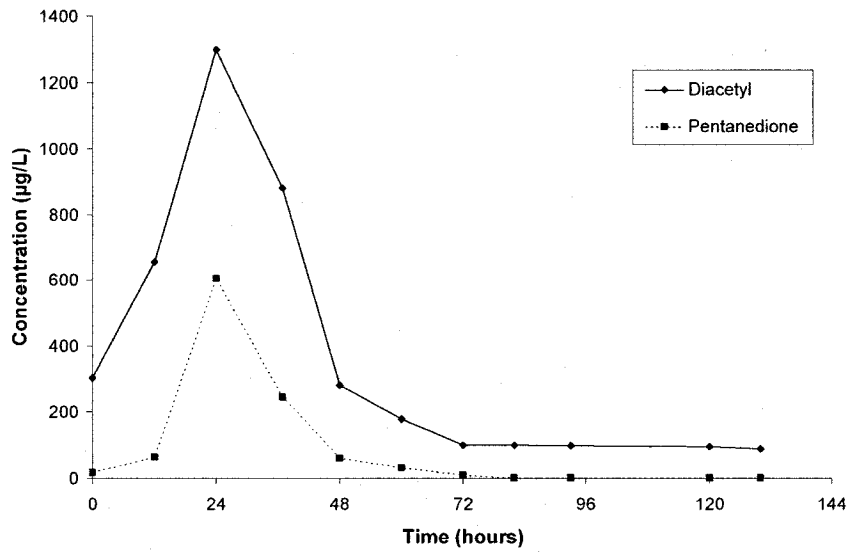


Figure 4.6.2.2 Total VDK profile for ale Batch A.

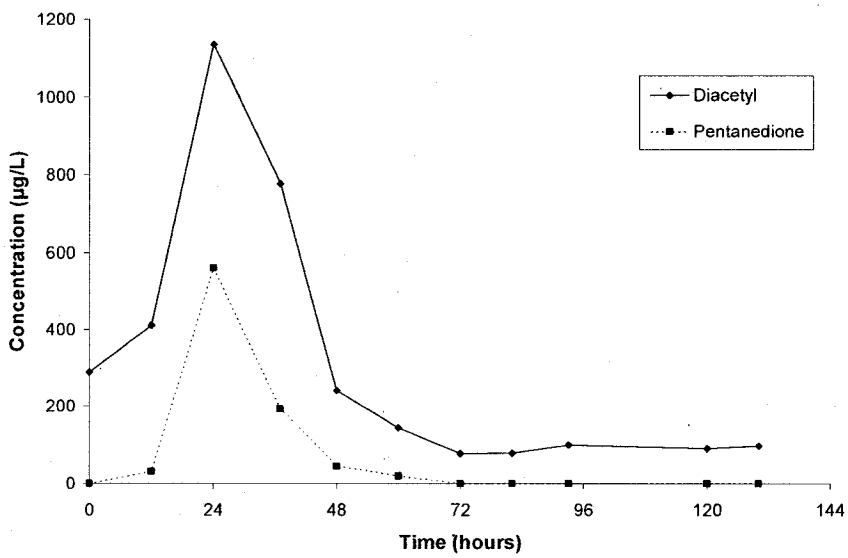


Figure 4.6.2.3 Total VDK profile for ale Batch B.

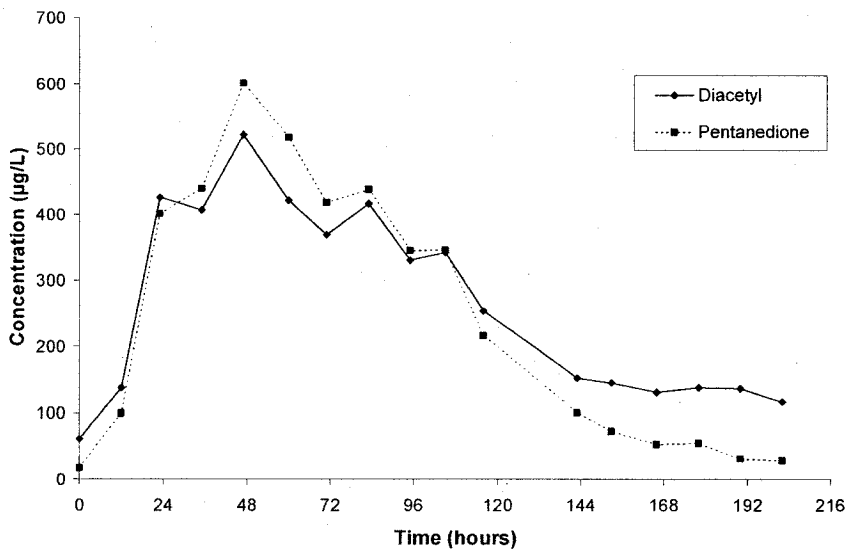


Figure 4.6.2.4 Total VDK profile for lager Batch 1.

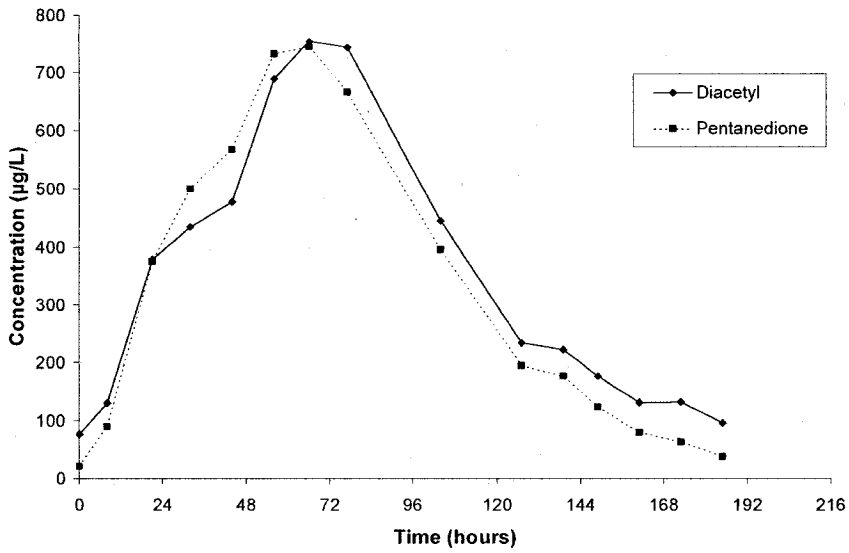


Figure 4.6.2.5 Total VDK profile for lager Batch 2.

AA absorption

Table 4.6.2.2 shows the wort initial and final content in leucine, isoleucine and valine for the four batches studied. Much faster wort AA absorption was observed in ale fermentations (Figure 4.6.2.6, 4.6.2.7, 4.6.2.8).

Table 4.6.2.2 Wort initial and final AA content.

	Initial conc. (mM)			Final conc. (mM)		
Batch	Isoleucine	Leucine	Valine	Isoleucine	Leucine	Valine
Ale A	0.57	1.21	0.90	0.00	0.04	0.02
Ale B	0.55	1.20	0.84	0.00	0.036	0.00
Lager 1	0.65	1.37	1.02	0.17	0.20	0.48
Lager 2	0.51	1.07	0.80	0.03	0.00	0.07

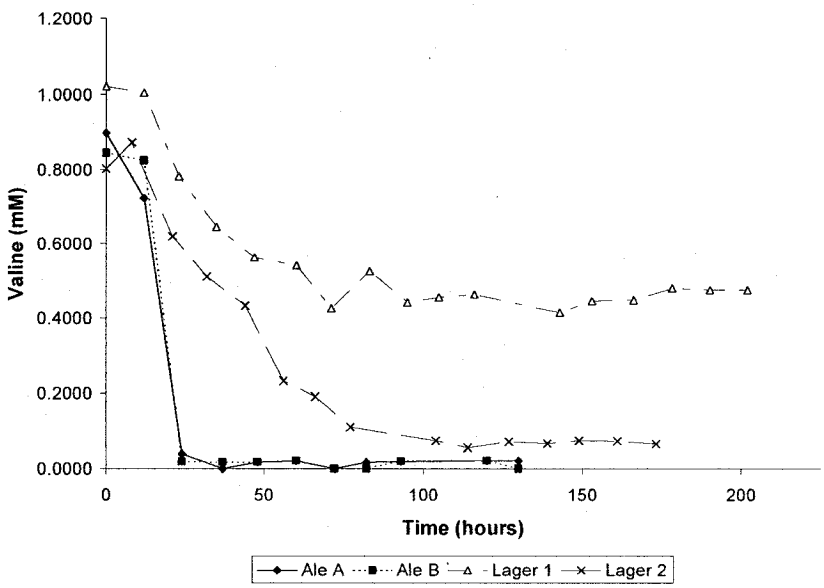


Figure 4.6.2.6 Valine absorption profile in four industrial fermentations.

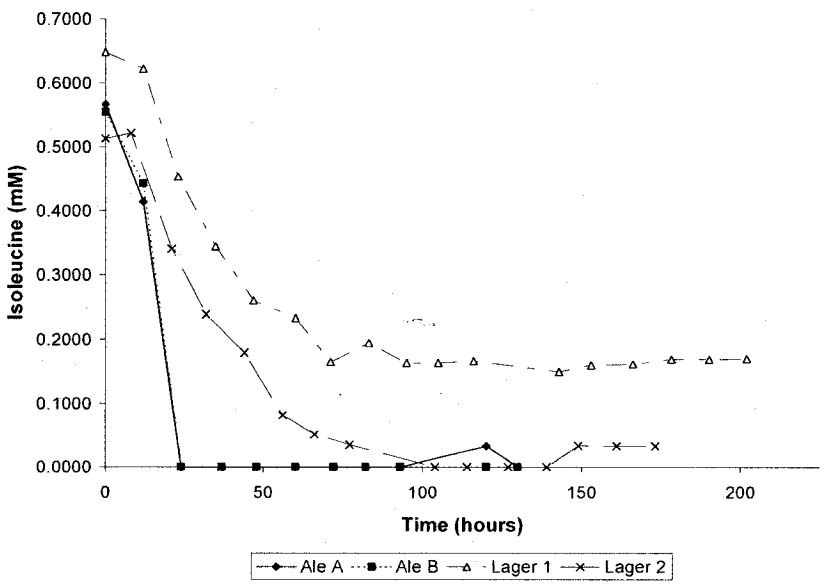


Figure 4.6.2.7 Isoleucine absorption profile in four industrial fermentations.

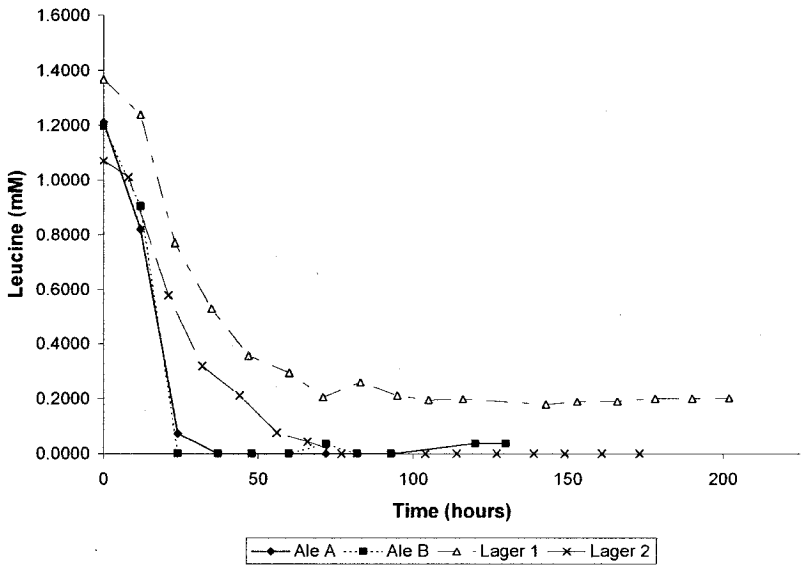


Figure 4.6.1.8 Leucine absorption profile in four industrial fermentations.

Comparison of VDK results obtained by headspace GC and LC/MS (Table 4.6.2.3)

Table 4.6.2.3 Comparison of total VDK results obtained by LC/MS analysis with the ones obtained by GC.

Diacetyl comparison			
LC/MS (µg/L)	**GC (µg/L)**	**Difference (µg/L)**	**% difference**
1300	1699	399	31
1137	1584	447	39
522	738	216	41
755	871	116	15
233	301	68	29
132	118	-14	-11
2,3-pentanedione comparison			
LC/MS (µg/L)	**GC (µg/L)**	**Difference (µg/L)**	**% difference**
604	788	184	30
560	797	237	42
601	751	150	25
745	817	72	10
194	281	87	45
63	104	41	65

V DISCUSSION AND CONCLUSION

5.1 Method development and performance

5.1.1 Detection of vicinal diketones (VDK)

The derivatization of diacetyl and 2,3-pentanedione with (OPDA) allowed their separation and detection by LC/MS. The quinoxaline compounds formed showed long term stability and were resistant to fragmentation in the MS spray chamber allowing their determination by SIM mode. Although acidic conditions were selected for sample analysis in order to achieve faster reaction rates, complete derivatization was achieved at room temperature in satisfactory times for all pH conditions tested.

Identification and separation of the derivatized VDK was achieved and optimized using two C18 HPLC columns of different specifications. The application of larger flow rates and larger injection volumes on the larger bore column allowed achieving an overall longer column life and a larger sample throughput while maintaining a satisfactory level of resolution.

Both VDK were accurately determined in beer samples with the single addition of OPDA solution prior to injection on the HPLC. The relationship between VDK concentration and signal abundance was equivalent for beer samples and aqueous standards; allowing calibration to be performed using aqueous standards. The detection limits obtained in aqueous standards were satisfactory compared to taste threshold concentrations. However, the level of interference found in some beers was too significant to allow accurate determination at threshold concentrations. Performing pre-column sample separation techniques such as solid phase extraction (SPE) and solvent extraction could

likely contribute to a decrease in interference, achieving lower detection limits and hence reducing analysis time. Techniques such as the double SPE treatment proposed in some HPLC methods for VDK detection by spectrophotometric detection (4, 66), could potentially allow the removal of non-polar compounds responsible for interference while allowing for an increase in the concentration of the sample analytes prior to their injection on HPLC. MSD being more specific than the type of detector used by these authors, accurate detection of VDK at levels lower than 10µg/L could probably be achieved using those sample preparation techniques.

5.1.2 Precursor oxydative decarboxylation

The methods yielding the most oxydative decarboxylation of α-acetolactate were the incubation at 60°C without catalyst and the incubation at 80°C with ferrous sulphate and ferric chloride. The results obtained with the latter method were in accordance with literature with 100% conversion being achieved in 10 minutes. This method provided the fastest conversion rate and was selected for total VDK analysis of beer samples.

The fact that no increase in yield was observed after 10 minutes of incubation at 80°C without any catalyst could be explained by a lack of oxygen present in the sample to complete the reaction. The same reason could explain the results obtained by the incubation of standards at 60°C with aniline hydrochloride, since very little change in conversion was observed after 10 minutes of incubation. Further tests should be performed to evaluate whether aerating the samples prior to their incubation would result in higher conversion rates.

Tests performed at 80°C with aniline hydrochloride showed that the addition of this compound was detrimental to α-acetolactate conversion at high temperature. This is also supported by the results obtained after 30 minutes of incubation at 60°C that showed 10.6% less conversion when aniline hydrochloride was added to the standards.

The poor conversion rates obtained with aniline hydrochloride at room temperature could be explained by the fact that research reported on this method was mostly performed on samples where the total VDK concentration was lower than 1 mg/L (20, 60). The catalytic effect of the added concentrations of aniline hydrochloride was not sufficient to achieve complete oxydative decarboxylation of the concentration used during the tests (i.e. 10 mg/L of diacetyl potential).

Finally, decarboxylation tests made with OPDA at room temperature showed that this compound had little impact on the conversion rate when added at the concentration used to derivatize the VDK. It could be assumed, therefore, that the 5 minutes incubation period necessary to complete diacetyl derivatization had no significant effect on the results obtained in the precursor conversion tests.

5.1.3 AA and simultaneous determination

Accurate HPLC determination of the o-phtalaldehyde (OPA) derivatives of leucine, isoleucine and valine was achieved with both mass spectrometric (MSD) and fluorometric detection. Lower degrees of interference were found and a lower detection limit was achieved with MSD. Both methods provided sufficient sensitivity to detect AA levels found during beer fermentation. The HPLC system coupled to the fluorescence detector had a functional autosampler and was selected to analyze the industrial samples

collected in order to automatically derivatize the sample using a programmed routine instead of performing the task manually; this allowed for a faster sample throughput and to limit the errors due to sample manipulation.

Simultaneous determination of VDK and AA was achieved in aqueous standards over a AA concentration range of 0.83 to 83.3 µM. However, attempts to achieve simultaneous determination of VDK and AA for concentrations normally found in beer were unsuccessful due to limitations of the derivatization method and, therefore, separate determination had to be performed in beer samples. Also, the introduction of large quantities of OPA in the MS detector appeared to cause significant damage to the detector apparatus and only limited tests were performed in attempt to achieve simultaneous determination. The analysis of VDK and AA could, however, be accomplished successively using the same LC/MS system on a sample of beer previously derivatized with OPDA. A portion of the sample could first be mixed and diltuted with the OPA reagent by the autosampler and injected for AA analysis while another portion could be directly injected afterward for VDK analysis.

5.1.4 Calibration and MSD operational problems

Several problems were encountered during the utilization of the MSD system. The main problem was a gradual loss in signal abundance experienced during periods of intensive sample analysis. Upon the repetitive injection of beer samples containing high levels of VDK, it was found that the signal abundance decreased gradually from one injection to another (up to 10% over 10 injections in the worst cases recorded). The abundance would continuously keep decreasing until the machine would be left inactive for many hours.

After leaving the detector to rest over a few days, the signal abundance for a same concentration was back to the original value and signal stability was re-established.

Trials to avoid this problem by performing internal calibration were unsuccessful since the magnitude of signal loss appeared to be dependant on the m/z ratio. This problem was partly counteracted by performing intensive external calibration with one standard being injected for every two beer samples in order to keep track of any loss in signal abundance and to adjust the calibration curve. However, the best method to avoid this problem, apparently due to flooding of the detector, was to limit the amount of beer samples injected on one day to about 15 and to be extremely careful not to inject large quantities of analyte on the machine by decreasing the injection volume when using standards of high concentrations. When significant signal loss was experienced, the only alternative was to leave the system to rest for a few days and to perform an autotune before injecting more samples.

5.2 VDK and AA fermentation profiles

5.2.1 Impact of temperature on fermentation performance

Yeast metabolism appeared to slow down dramatically with the decrease in fermentation temperature from 16 to 12° C. Yeast maintained at lower temperatures took longer to start attenuating the wort carbohydrates, showed slower attenuation rates and left wort with more residual sugars after 12 days of fermentation. It took eight days for the 16°C fermentation to attenuate the gravity below 4° Plato while the gravity in the other fermentations was still slightly over that value after twelve days. From an industrial perspective, VDK reduction would not have been a concern in these beer fermentations

since the VDK concentration had reached threshold values before wort had been attenuated to its final gravity.

Leucine was the AA used most quickly over the first 24 hours of fermentation followed successively by isoleucine and valine. These findings corroborate with research suggesting that leucine is absorbed before the two other AA; some research even classifying leucine as part of absorption group A during lager fermentation (30, 49). Residual AA levels were monitored in the three fermentations, making inapplicable the theory of VDK production caused by AA depletion. No correlation was evident between wort attenuation, VDK production and reduction, and AA absorption during this experiment. This could be due to the fact that samples were collected at time intervals that were probably too large to record the subtle changes that are taking place during the first 48 hours of fermentation. Further research with lower fermentation temperatures and more exhaustive sample collection could help in achieving more resolution in the VDK and AA consumption profiles and help to establish if indeed any correlation between the parameters occurs.

5.2.2 Industrial beer fermentations

Ale fermentations were characterized by fast wort attenuation rates, high and early peak VDK values, fast reduction of VDK, and fast and complete absorption of wort amino acids. Very little change in the recorded parameters was observed after 72 hours of fermentation; suggesting that according to the recorded fermentation performance indicators, both batches would have been ready to bottle after 3 days of fermentation.

Both ale batches were brewed in vessels of similar geometries using worts of similar nutrient contents; which resulted in very little difference in the profiles generated.

Lager fermentations were characterized by slower attenuation rates, larger VDK levels, and generally slower VDK reduction and AA absorption rates. A large difference was recorded between the profiles obtained from the two lager batches: VDK peak values were lower and were recorded at an earlier stage in Batch 1 while faster and more significant AA consumption was recorded in Batch 2. These differences could be attributed to the fact that the yeast was probably more active in Batch 1 at the time at which the initial sample was collected. The commercial brew house possessed a capacity of 900 hL, so six fills were required to achieve Batch 1 while less than two complete fills were required for Batch 2. Taking into account that one third of the yeast was pitched following every second fill in Batch 1, at least six hours of metabolic activity took place in Batch 1 before the initial sample was collected. Another explanation for this divergence could be that the wort utilized for Batch 1 had a richer nutrient content; with 25% more AA and a 6 % higher specific gravity. Finally, because the samples were handled by different employees at the brewery, the mixing due to CO_2 evolution is not as efficient in a rectangular fermenter and the location of the sampling port was different could also explain the irregular shape of the profiles generated for Batch 1 and the overall difference in concentration recorded between both batches.

The AA absorption order was similar in the four industrial batches, with leucine being used first followed by isoleucine and valine. Significant residual levels of the three amino acids were found in lager Batch 1.

It is also interesting to note that the overall production of 2,3-pentanedione by ale yeast was much smaller than the diacetyl production, while equivalent concentrations of both compounds were recorded during lager fermentation. By considering that both isoleucine and valine were depleted by the ale yeast after 24 hours, it could be assumed, because of the greater amount of diacetyl produced, that the ale yeast used for these fermentations showed a larger need for valine than for isoleucine.

Comparison of VDK results obtained by headspace GC and LC/MS

A large difference was recorded between the results obtained by LC/MS and those obtained by the brewery's laboratory technicians using a headspace GC technique. One potential explanation for this difference is the fact that the technique used by the brewery was different than the official technique recognized by the brewing industry; e.g. samples were incubated at 40°C instead of 35°C and higher injection temperatures were applied. Another explanation could be that their detector was calibrated to detect VDK at concentrations lower than 300 µg/L in the finished product while the samples analyzed were fermentation samples (higher specific gravity) containing VDK concentration mostly higher than 300 µg/L. It is known that the headspace GC techniques are sensitive to the vapour pressure of VDK, which tend to vary with wort composition. Finally, the fact that sample fractions were frozen and stored without prior removal of the yeast could also be a reason for divergence. It might be possible that the yeast cells contained VDK and their precursors and that some could have been released prior to sample filtration in the fractions that were analyzed by GC. This comparison highlights the difficulties in comparing analytical data obtained from different laboratories using different techniques.

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