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KILAZA SAMSON MWAIKONO

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VALIDATION OF CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF MELATONIN IN WINE AND GRAPES USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY- FLUORESCENCE DETECTION

Memory of the presentation of the thesis project as a partial fulfilment for the award of the European Masters of Quality in Analytical Laboratories

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Supervisor: Prof. Miguel Palma Lovillo

Supervised by: _____

Date:

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Dedication

To my sons Samson and Wenceslaus

Declaration

I declare that this is my own original work and has never been submitted in any university or institution for whatever purpose.

By: Kilaza Samson Mwaikono

Signature ……

Date: ……..

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List of Acronyms

RSD- Relative standard deviation

SD – standard deviation

LOD- Limit of detection

LOQ- Limit of quantitation

ELISA- Enzyme Linked Immunosorbent Assay

RIA- Radioimmunoassay

WM-Wine Matrix

MWM- Methanol Water Matrix

HPLC – high performance Liquid Chromatography

FD- Fluorescence Detector

Abstract

This masters research project reports two validated method for determination of melatonin in wine using High performance liquid chromatography and Fluorescence detector. The Excitation/Emission condition was 280/310 nm. Retention time of melatonin peak was around 17.70 minutes. Method "A" intended for samples with higher levels of melatonin achieved a LOD and LOQ of 1.661mg/L and 5.537mg/L respectively and linearity of 98.453% with analytical sensitivity of 0.563mV/mg/L. Method "B" intended for samples with lower levels of melatonin achieved LOD and LOQ of 0.027 and 0.090 mg/L respectively; with linearity of 99.15% while analytical sensitivity was 0.009mV/mg/L. Method "A" achieved excellent accuracy when real sample of wine was spiked with melatonin and results compared to when solvent was 50/50 methanol/water. The precision of the method was below 7% and the method was robust to most of the variables which are likely to influence the performance of the method.

CHAPTER 1

INTRODUCTION

1.1 Overview of the research programme

This Masters Research Thesis documents a program, undertaken at the Research Institute "Institutos de Investigacion" in the University Cadiz, between the years of Sept 2009 and February 2010. The research work, presented herein, was part of a larger program of research involving two researchers under the project title: DETERMINACIÓN DE MELATONINA EN UVAS VINOS Y OTROS ALIMENTOS DE ANDALUCÍA: Code: P07-AGR-02480 under the programme: Proyectos de Excelencia with Financing Agency: Junta de Andalucía; who developed this work.

The objective of the overall research program was to develop and validate a chromatographic method for determination of melatonin in wine, grapes and tomatoes. This master research was a subset of that overall program and the specific objective here was for the first time to validate the chromatographic method of High Performance Liquid Chromatography and Fluorescence detector for determination of melatonin in wine and grapes. Method validation which is an essential component of the measure to be implemented so as to produce a reliable and traceable analytical data as per ISO/IEC 17025:2005 requirement has been performed and various analytical variables for melatonin determination were suggested.

1.6 Background

Melatonin is a bioactive food component with a potential influence of dietetic intake to human health pose challenges to the currently available analytical methods; there was a gap in knowledge and insufficiently standardised methods for its analysis. There was no independent validated method for melatonin as the only analyte in any of the available methods. Melatonin in some plants was found to be in microgram/gram while in others much lower; therefore was a need for a method which was sensitive to those variations.

The basic and acidic characteristic of the functional groups of melatonin molecule challenges a selection of the solvent which would provide a complete recovery; also melatonin as a potential antioxidant has a potential reacting quickly with other food constituents if not carefuly handled during sample storange and preparation. Due to those challenges, the currently available analytical methods did not provide adequate justification to rely upon. Reliable analytical method was required which complies with National and International regulation with respect to food analysis. There was a need for information to prove that a method intended for a given purpose was capable of providing adequate confidence at a given analyte concentration in appropriate matrices. Due to the known advantages of melatonin as bioactive component and its availability in most of the foods of plants origin; a validated method for its determination which would enable to come up with food composition table with respect to wine, grape and tomato was wealthy researching.

1.3 Overview of the proposed methodology

Chromatographic technique HPLC-FD method using reverse phase column (RP 18 Lichrospher 250 x 4, 5µm) at flow rate of 1.0mL/min was validated and all the variables likely to influence the quality of the analytical results were taken into consideration. Melatonin standard solution was used in the validation process and real samples of wine were also used along to provide a real matrix performance of the method.

Various validation parameters for performance of HPLC–FD for melatonin analysis were established. The method was tested to real samples and the performance characteristics for applicability, selectivity, calibration, trueness, recovery, working range, limit of quantification, limit of detection, and ruggedness, specificity, accuracy, precision and measurement uncertainty at relevant analyte concentration and in appropriate matrices were established and documented.

1.4 Overview of the experimental procedure

Melatonin standard was used in the method validation; chromatographic peaks of high performance Liquid Chromatography online with PDA (UV-VIS) detector at 3D scanning mode was used to characterise melatonin peak and then identify the best Excitation and Emission properties of Melatonin. The same conditions for mobile phase and instrumental setting were used for HPLC-FD where a good resolution of melatonin peak at a chosen Excitation and Emission was studied. Method validation process was then carried out to two methods namely method "A" intended for samples with higher levels of melatonin and method " B" intended for samples with lower levels of melatonin; due to lack of possibility to make a full range calibration curve when using fluorescence detector. Melatonin standard solution was prepared at different concentration levels, then analysis of method response at different replicates and concentration levels using the previously defined method for the mobile phase proportions, type of column, flow rate, excitation and emission setting for fluorescence detector. Real wine sample was also used to provide real matrix presentation. Validation parameters were analysed, and lastly the method was tested to real samples of wine.

1.5 Perceived specific contribution of the research

Developed and validated method for determination of melatonin in wine and juice will enable to come up with reliable data on the quantity of melatonin in these food products and therefore, for the first time it will possible to come up with food composition tables for bioactive compounds with respect to melatonin, which is currently lacking due to unreliable analytical data.

Melatonin effects are more interesting if the compound is taken regularly than at specific time, therefore it should be better if people can eat foods containing low or high amount of melatonin regularly instead of taking it as a drug. Functional foods

which are interesting source of melatonin normally contain very low levels and therefore needs a very sensitive analytical method for its analysis.

1.6 Thesis structure

Literatures about the current status of analytical techniques, roles of melatonin to human health and the available sources in plants was consulted; the knowledge so far about melatonin availability in both unicellular, multi cellular animals and plants was reviewed. The gaps on the available analytical methods in plants were identified and the Chromatographic method HPLC-FD as an alternative method was developed validated and tested for melatonin determination in spiked wine matrix, Validation report was prepared and the method proposed for further improvement prior to its full application5.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of the literature review process

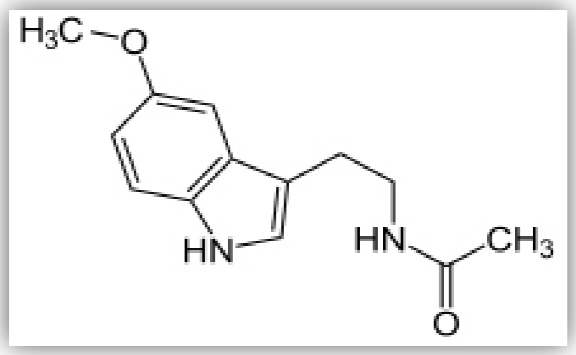


Figure 2: 1 Melatonin chemical structures [C13H16N2O2]

Melatonin (N-acetyl-5-methoxytriptamine) is a hormone and antioxidant which in mammals is synthesised from serotonin in the pineal gland cells, but has also been found in other tissues such as gastrointestinal tract, retina and skin (Joseph et al., 2002; Hardeland R. and SR.Pandi-Perumal, 2005) Melatonin has been found in almost all vertebrates tested. The presence of Melatonin in plants was reported for the first time by (Poeggeler B. and Hardeland R., 1994) in algae and later on (Hattori et al., 1995) reported melatonin at different levels in twenty four different edible plants. Other researchers such as (Dubbels et al.,1995; Arnao and Hernandez-Ruiz, 2007; D.E. Blask, et al., 2004) at different time have also proved the presence of melatonin in various parts of the plants such as leaves, roots, seeds and flowers; these were also proved by (Hattori et al., 1995; Manchester et al., 2000; Badria, 2002). Melatonin was also found in bacteria, unicellular organisms and fungi (Manchester et al., 1995; Hardeland R, 1999; Reiter et al., 2001; G. Chen et al., 2003). With such research findings, it seems that melatonin may have evolved in all evolutionary life forms from simplest bacteria to humans as well as plants. Analysis melatonin to determine the levels in various plant materials gave different levels of melatonin even to the same kind of sample matrix when different analytical techniques were

used; this posed a challenge to be answered as to whether the analytical techniques used are reliable to justify the levels of melatonin found in plants.

2.2 General theory and roles of melatonin in animals and plants

2.2.1 Melatonin in animals

Melatonin is a light-sensitive hormone, meaning that the absence of light stimulates its secretion (Manchester et al., 2000, Oliveira et al., 2009 and M. C. Garcia-Parilla et al., 2009). It is widely distributed in the animal kingdom and other vertebrates including fish, birds and reptiles (Lopez-Olmeda J.F et al., 2009; Arnao and Hernandez-Ruiz, 2007). Among many functions; melatonin synchronizes circadian and circannual rhythms, stimulates immune function, Regulate sleeping disorders, jet lag and may increase life span, inhibits growth of cancer cells in vitro and cancer progression and promotion in vivo, and was recently shown to be a potent hydroxyl radical scavenger and antioxidant. Hydroxyl radicals are highly toxic by-products of oxygen metabolism that damage cellular DNA and other macromolecule (Dubbels R. et al., 1995; Ferrari, 2004; George et al., 1998 and Shazip, 2003) these toxins are rendered harmless by melatonin. Other claims of melatonin functions are its ability to improve health by boosting the immune system, fighting the effects of aging, and providing a safer means of birth control (Vazquez et al., 2009 ; Van Tassel and O'Neill , 2001). Shang and other researchers also reported in Chinese medical journal (Shang et al., 2009) the ability of melatonin to reduce acute lung injury in endotoxemic rats. Researchers have found that; before puberty the pineal gland produces comparatively large amounts of melatonin while with age melatonin production continually decreases (Mary et al., 2000) perhaps explaining why older people either have difficulty in sleeping or sleep less.

Melatonin nowadays available in health food stores and pharmacies is a synthetic version of the melatonin hormone. It is also available in a form that combines synthetic and natural (from sheep and cow pineal glands) melatonin. Both types of melatonin in a bottle mimic the real thing in chemical composition and behaviour. It

has been found that most people prefer synthetic form because it does not carry the risk of contamination that the partially organic form does (Carol et al.,1995); a small dose (0.1 to 0.5 mg) puts most people to sleep. The body secretes between 5 and 25 micrograms of melatonin nightly which is 80 times less than the average 2mg dose that melatonin is sold. Few side effects of melatonin use have been reported (Hardeland and Pandi-Perumal, 2005; Clarke et al., 2003) such as daytime drowsiness, headaches, and unusual dreams and at higher doses (up to 300 mg) reported diarrhoea, abdominal pain, and headaches. Currently researchers propose that melatonin is safe for use to person over 18 years old and healthy, not Pregnant or breast-feeding, and not taking any medications besides minor analgesics (such as aspirin, acetaminophen) and oral contraceptives.

2.2.2 Melatonin in plants

From the early finding of melatonin in algae (Poeggeler and Hardeland, 1994), many research findings have been published proving the presence of Melatonin in plants. Report by (Dubbels et al.,2007; Arnao and Hernandez-Ruiz, 2007; Blask, et al., 2004) shows melatonin in varying concentrations in a variety of plants such as oats, sweet corn, ginger and tomatoes, bananas, grapes, tart cherry, barley and rice. Melatonin reported in one research findings indicated that measured values in nine different plants by radioimmunoassay technique ranged from 0 to 862 pg melatonin/mg proteins (Dubbels et al., 2007). Another group of researchers (Chen et al., 2003) worked on more than hundred Chinese Medicinal herbs by analyzing using the methods of solid phase extraction and HPLC-FD on-line with Mass Spectrometry. The finding was that; Melatonin was detected in majority of plants analysed and sixty-four of them were containing melatonin in excess of 10 ng per gram dry mass while levels in several other herbs were in excess of 1000 ng/g (Chen et al., 2003).

Olive and sunflower oil was also found to contain melatonin when analysed using Enzymes Linked Immunosorbent Assay “ELISA” and Immunoprecipitation techniques (C. de la Puerta et al.,2007), and they found higher levels in extra virgin

oil than refined olive and sunflower oil, this finding justified the account for the health benefits of the Mediterranean diet in which olive oil is the main source of fat and its beneficial influence on diseases associated with oxidative damage such as coronary heart disease, cancer, cardiovascular and neurodegenerative as reported by (Alexandratos, 2006) in the public health nutrition journal. Manchester and other fellows they also examined fifteen seeds of edible plants for the presence of melatonin which was extracted using cold ethanol, Melatonin was identified by radioimmunoassay then quantified and confirmed using high performance liquid chromatography (Manchester et al., 2000). Melatonin levels were found in the concentration range of 2 to 200 ng/g dry weight in the fifteen seeds studied. These higher values of melatonin in seeds may be associated with protecting germ and reproductive tissues of plants from oxidative damage due to ultraviolet light, drought, extremes in temperature, and environmental chemical pollutants. Melatonin was also identified in other phototrophic organisms such as tomatoes, ginger and the marine green macroalga, *Ulva lactuca* (Pape and Luening, 2006), levels of melatonin concentration in ginger was below 5 pg/g fresh weight, in tomatoes about 1200 pg/g fresh weight and in green alga *U. lactuca*, approximately 12 pg/g fresh weight. Okazaki and Ezura researched on the distribution and accumulation of melatonin during leaf and fruit development of tomato (*Solanum lycopersicum* L.), the values of melatonin concentrations in leaves, stems roots flowers and seeds was found to be in the range of 1.5 – 66.6 ng/g fresh weight with seeds having higher melatonin concentration levels (Okazaki and Ezura, 2009). Melatonin values in plants as collected from different literatures sources was reported by (Posmyk and Janas, 2009) as shown in table 1 below.

Table 2:1 Melatonin MEL content in some plant organs

Common name	Scientific name	Organs	MEL ng/g	source
Morning glory	*Pharbitis nil* choisy	Leaf	0.0005	[1]
Banana	*Musa paradisiacal*(L)	Fruit	0.002	[2]
Morning glory	*Pharabitis nil* choisy	Shoot	0.004	[1]
Asparagus	*Asparagus officinalis* (L)	Shoot	0.01	[3]
Beet	*Beta vulgaris* (L.)	Root	0.01	[2]
Wild strawberry	*Fragaria ananassa (Duch.)*	Fruit	0.01	[3]
Onion	*Allium cepa* (L.)	Bulb	0.03	[3]
Cucumber	*Cucumis sativus* (L.)	Fruit	0.03	[3]
Pineapple	*Ananas comosus* (stickm.)	Fruit	0.04	[3]
Apple	*Malus domestica* (Borkh)	Fruit	0.05	[3]
Carrot	*Daucus carota*	Root	0.06	[3]
Chine cabbage	*Brassica chinensis* (Juslen)	Leaf	0.1	[3]
Cabbage	Brassica oleracea (L.)	Leaf	0.1	[3]
Red pigweed	*Chenopodium rubrum* (L.)	Shoot	0.2	[4]
Barley	*Hordeum vulgare* (L.)	Seeds	0.4	[3]
Tomato	*Lycopersicon esculentum*	Fruits	0.5	[2]
Ginger	*Zingiber officinale* (Roscoe)	Root	0.6	[3]
Red radish	*Raphanus sativus* (L.)	Root	0.6	[3]
Turnish	*Brassica compestris* (L.)	Root	0.7	[3]
Rice	*Oryza sativa japonica* (L.)	Seed	1	[3]
Oat	*Avena sativa* (L.)	Seed	2	[3]
St .John's wort	*Hypericum perforatum* (L.)	Leaf	2	[5]
Milk thistle	*Silybum marianum* (L.)	Seed	2	[6]
Fever few	*Tanacetum parthenium* (L.)	Leaf	2	[5]
Corn	*Zea mays* (L.)	Seed	2	[3]
St. John's wort	*Hypericum perforatum* (L.)	Flower	4	[5]
Tall fescue	*Festuca arundinacea*	Seeds	5	[3]
Poppy	*Papaver somniferum* (L.)	Seeds	6	[6]
Celery	*Apium graveolens* (L.)	Seeds	7	[6]
Coriander	*Coriandrum sativum* (L.)	Seeds	7	[6]
Anise	*Pimpinela anisum* (L.)	Seeds	7	[6]
Huang-qin	*Scutellaria biacalensis*	Seeds	7	[6]
Flax	*Linum usitatissimum* (L.)	Seeds	12	[6]
Green	*Elettaria cardamomum*	Seeds	15	[6]
cardamom	*Medicago sativum* (L.)	Seeds	16	[6]
Alfalfa	*Prunus cerasus* (L.)	Fruits	18	[7]
Cherry	*Foeniculum vulgare* (Gilib.)	Seeds	28	[6]
Fennel	*Helianthus annus (*L.)	Seeds	29	[6]
Sunflower	*Prunus amygdalus (Batsch)*	Seeds	39	[6]
Almond	*Trigonella foenum-graecum*	Seeds	43	[6]
Fenugreek	*Lycium barbarum*	Seeds	103	[6]
Wolf berry	*Brassica nigra* (L.)	Seeds	129	[6]
Black mustard	*Sinapis alba* (L.)	Seeds	189	[6]

[1]-Van Tassel et al., 2001, [2] - Dubbels et al., 1995, [3] - Hattori et al., 1995, [4]- Kola´rˇ et al. 1997, [5]- Murch et al., 1997, [6]- Manchester et al.,2000, [7]- Burkhardt et al., 2001

It is noted from table1 there is really huge variation in the levels of melatonin in plants from lowest amount in some to the highest in others. Within the same plant there was also a variation in melatonin levels from one organ to another; for example in seed there was higher levels of melatonin when compared to leaves and fruits and it is suggested that the higher levels in seeds offers protection to germ tissue from the environmental factors (Manchester et al., 2000). When studying Melatonin in plant tissues and its dietary and health implications (Baghurst and Coghill, 2006) reported a variation of melatonin levels in selected edible plants when analysed with different methods as shown in table 2

Table 2-2 Melatonin levels of selected edible plants using different techniques

Plant	Melatonin ng/g plant tissue		
	Hattori (RIA)	Badria (GC-MS)	Dubbels (HPLC-MS)
Banana		0.655	1.0
Barley	0.378	0.873	
Cabbage	0.107	0.309	
Carrot	0.055	0.494	
Corn	1.366	1.878	
Cucumber	0.0246	0.592	
Ginger	0.538	1.423	
Onion	0.032	0.299	
Pineapple	0.036	0.278	
Rice	1.006	1.498	
Strawberry	0.0124	0.136	
tomato	0.0322	0.302	2-8

The variation of melatonin levels to the same plant when analysed by different methods as shown in table 2-2, poses a challenge to the reliability of the analytical methods used hence a need for further research.

The roles of melatonin in plants is thought to contribute to the antioxidative protection against free radicals and pro oxidative substances such as H_2O_2 or O_2^- which are generated by side reactions of photosynthesis (Pape and Luening, 2006; Dubbels et al., 1995), is also responsible with conveyance of photo periodical information and signaling of darkness (Kolár et al.,1997; Van Tassel et al., 2001); another physiological roles are cytoprotector, cytoskeletal modulator, growth promoter and rhizogenesis, cellular expansion and stress protection (Arnao and Hernandez-Ruiz, 2007; Hernández-Ruiz et al., 2004; Hernandez-Ruiz et al., 2005 ; Hernández-Ruiz and Arnao 2008). It was also found to be growth promoting effects in aerial organs (epi- and hypocotyls, coleoptiles) and growth inhibitory effects on roots (Arnao and Hernandez-Ruiz, 2007), with the same researchers, it was also reported in the journal of pineal research that; melatonin was also found to activate generation of lateral and adventitious roots.

2.3.0 Analysis of melatonin in plants

Analysis of melatonin in foods of plants origin presents some challenges, first; the content of melatonin in some plants is in the microgram per gram range, while the amount found in others is as much lower as in picogram per gram range. Thus, any analytical method meant for analysis of melatonin in plants must be sensitive to these variations. Secondly; the amphiphilic (a molecule containing both polar and non polar portions) as shown in figure 2.1 characteristic of the molecule makes difficult to choose a solvent yielding a complete recovery and accurate results (Hardeland and Pandi-Perumal, 2005; Maria D. Maldonado et al, 2009) and thirdly; melatonin is a potent antioxidant (Tan et al., 2007; Arnao and Hernandez- Ruiz, 2007, 2008; Garcia-Parilla et al., 2009) and reacts quickly with other food constituents during sample preparation. Melatonin can also be easily destroyed by oxidants during extraction (Poeggeler and Hardeland, 1994; Hardeland and Pandi-Perumal, 2005) thus false positive and false negative data are easily obtained due to the presence of secondary plant metabolites, either mimicking melatonin or interfering with it in the

assays. The analytical method is therefore challenged by these constraints. Despite of much being known about melatonin presence in foods; but the analytical methods suitable for assay in food matrices and the potential influence of dietetic intake on human health is the ongoing research. Various analytical methods have been devised to detect and quantify the levels of melatonin in food of plants origin but their reliability is still questionable.

2.3. Trends of analytical techniques for analysis of melatonin in plants

2.3.1 Immunological techniques (RIA and EIA)

"Radio Immunoassay" (RIA) technique has successfully been applied to determine quantitatively melatonin in biological samples such as human serum Plasma, and saliva as reported by (M.C. Garcia-Parrilla et al., 2009; Ian M. McIntyre et al., 1987). The method is based on the competition principle and works by providing a limited amount of specific antibody (Ab) which reacts with the corresponding antigen (*Ag) labelled with ^{125}I; and upon addition of an increasing amount of the Ag (sample) a correspondingly decreasing fraction of *Ag is bound to the antibody, bound and free *Ag can be separated by centrifugation. The bound radioactivity is subsequently determined in a Gamma counter, and results are obtained directly using a standard curve. When Van and O'Neill worked on Morning glory (*Pharbitis nil)* and tomato (*Lycopersicon esculetum)* to determine whether melatonin levels increased during the night (Van Tassel D L. and O'Neill S.D, 2001) reported their concerns when trying to validate RIA to tomato and *P. nil* samples; There was an over estimation of melatonin determined by RIA to that determined by GC-MS. The degradation process in GC-MS analysis may also produce the same result, however after consulting the literature the most probable problem is related with the cross-reactivity process with RIA antiserum. In a similar approach cross- reactivity of with co-extractives was reported by (Pape and Lüning, 2006; Hardeland et al., 2007).

Enzyme immunoassay (EIA) which is also immunological technique is based on competitive principle; where by unknown amount of antigen present in the sample and a fixed amount of enzyme- labelled antigens competes for the binding sites of the antibodies coated on the wells. Then, after incubation the wells are washed to stop the competition reaction, then; once the substrate reaction has occurred, the intensity of the colour generated is inversely proportional to the amount of antigen in the sample (Iriti et al., 2006). Several researchers have applied EIA for melatonin determination, for example (Pape and Luening , 2006) when researching on quantification of melatonin in phototrophic organisms such as tomatoes, ginger and the marine green macroalga used Enzyme Linked Sorbent Assay (ELISA) techniques for Melatonin determination after the sample has been purified by High performance liquid chromatography. Melatonin was also determined in olive and sunflower oil by (C. de la Puerta et al., 2007) using ELISA techniques. The same kit was also used to determine melatonin in grape skin extracts of different *Vitis vinifera* cultivars (Iriti et al., 2006). Okazaki and Ezura also used ELISA technique when worked on profiling of melatonin in the model tomato (Okazaki and Ezura, 2009). Plant physiologists tried to directly adopt methods from vertebrate melatonin research to plants but the chemical complexity of the vegetal extracts can interfere with determinations giving false-positive or overestimation as most researchers reported (Baghurst and Coghill, 2006; Pape and Lüning, 2006 and Van Tassel et al., 2001)

2.3.2. Chromatographic techniques

Chromatographic techniques as reported by (M. Carmen Garcia-Parilla et al, 2009) in their review about analysis of melatonin in fooods; the concerns about specificity and sensitivity of the immunological techniques led to develeopment of LC-RIA technique where by the separation was done by Liquid chromatography and quantification by RIA with limit of detection 5pg/ml and limit of quantification 15pg/ml reported by (Simonin et al., 1999). The techniques was not sensitive enough and could not work for samples with very low levels of melatonin. As an alternative, Simonin et al,. reported GC-MS as the most sensitive technique and it involved solid

phase extraction or liquid-liquid extraction followed by derivatization of the indolic moiety of melatonin by pentafluoropropionic anhydride which allowed a spirocyclic derivative to be obtained which is specific to melatonin and then gas chromatographic separation and lastly quantification by MS. The quantification limit was found to be 1pg/ml for melatonin in human plasma and saliva, other researchers who used GC-MS technique are (D. González-Gómez et al., 2009) when working on detection and quantification of melatonin and serotonini in sweet cherry cultivars. GC-MS method was recommended over LC-RIA due its wider working range for most sample variety and a possibility for chromatographic techniques to use both retention and detection properties for analyte identification, hence absence of overestimation errors despite of the method being time consuming and instrument dependent.

Later on, a highly sensitive method for determination of melatonin by reverse phase high performance liquid chromatography by fluorescence detector which could be applicable to both biological samples and vegetal food matrix was reported by (Vitale et al., 1996), this method had sensitivity possible to detect melatonin in single pineal cell with detection limit of 3pg/mg. (Arnao and Hernandez-Ruiz, 2007) also reported that when ethyl acetate is used as a mobile phase it provided a wide range of detection due to its low fluorescence quenching properties and further sited that a good sensitivity was also achieved if water: methanol (60:40) was used as a mobile phase with excitation at 280nm and emission at 345nm. It was also reported that an excitation at 280nm and emission at 350nm under isocratic condition with mobile phase of water : Acetonitrile (50:50) v/v with HPLC-FD was used by (Arnao and Hernandez-Ruiz, 2009) when assessing different sample processing procedure applied to the determination of melatonin in plants. When working on chinese medicinal herbs (Chen et al., 2003) used a mobile phase of a mixture of 40:60 v/v of Methanol:50mM Na_2HPO_4/H_3PO_4 (pH 4.5) and HPLC with fluorescence detector on-line with MS for the determination of melatonin levels as well. (Mercolini et al., 2009) aslo reported using HPLC-F when analysing melatonin and resveratrol isomers

in red and white wine with mobile phase composition 79% phospahate buffer at pH 3.0 and 21% ACN.

Several reports are also available where HPLC-FD was used for melatonin determination in biological samples (José L.P. Muñoz et al., 2009; V. Rizzo et al., 2002) due to its high sensitivity and no need for sample derivatisation. Other researchers have also reported using HPLC with diiffrerent detectors for melatonin determination for example; when working on melatonin in walnuts (Reiter et al., 2005) used HPLC-ECD system consisting of C_{18} reverse-phase column and a mobile phase made of 0.1mM potassium phosphate buffer (pH4.5) with acetonitrile 20%. But this method required high electrochemical potential for oxidation or reduction of melatonin which resulted into background current which affects the detector sensitivity as reported by (José L.P. Muñoz et al., 2009); with such high sensitivity of melatonin to temperature it is likely that the high electrochemical potential would denature melatonin hence a likely error for the test resulsts. HPLC with DAD using reverse phase R_{18} column with Methanol: Water (28:78) v/v as mobile phase was aslo reported for quantitative determination of melatonin in *Lamium album flos* by (Wójciak-Kosior and Woźniak, 2008).

2.3.3 Other techniques for analysis of Melatonin

Some reserachers have reported other techniques for analysis of melatonin such as chemiluminescence method (Lu et al., 2002; M. C. Garcia-Parilla et al., 2009) which was based on the observation that melatonin can greatly enhance the ultra-weak chemiluminescence between H_2O_2 and acetonitrile in alkaline solution where light emitted was intense and long lived resulting to high sensitivity. The method was based on the Singlet oxygen produced by the reaction between H_2O_2 and acetonitrile and was responsible for the Chemiluminescence of melatonin. Due to the basis of the method relying on the formation of singlet oxygen in the reaction implied that apart from melatonin, other compounds which can results in singlet oxygen formation such as phenolics and terpenes which are wide spread in plants; then can interfere with the analysis therefore disqualifying the method for melatonin analysis in vegetal matrix.

Melatonin was also determined spectrophotometrically after reaction with $KMnO_4$ and formaldehyde in acidic medium with grape skin extract due to its ability to generate chemiluminescence in acidic potassium permanganate solution and formaldehyde as reported by (Iriti et al., 2006; M. C. Garcia-Parilla et al., 2009) where UV spectra were detected and quantified. The method was found doubtful because under such condition there was possibility of other compounds present in vegetal matrix to react in similar pattern as well and interfere with the actual melatonin determination.

Electrophoresis techniques has also been used for melatonin determination by some researchers; for example determination of melatonin and its precursors and metabolites using capillary electrophoresis with UV and fluorometric detection has been reported by (Poboz˙y et al., 2005) when a micellar electrokinetic chromatography method has been developed for simultaneous determination of melatonin and its precursors and metabolites, in this work fluorometric detection provided about two-fold improvement over UV in the detection of melatonin when excitation was at 275nm and emission at 350nm; to both detectors melatonin was found to be in ng/g levels. Another research by (Ali et al., 2007) also used Capillary electrophoresis when analyzing melatonin in pharmaceutical dosage formulation, the limits of detection and quantification achieved were 10 and 15 mg/mL respectively when UV detection at 214nm was used. Similar approach was also reported by (G.P Carton et al., 2000) when they developed a rapid analytical method for determination of melatonin in pharmaceutical tablets by capillary electrophoresis with UV detector. It was also reported by (Pesek and Matyska, 1998) having developed a capillary electrophoresis method for the analysis of melatonin as the only analyte or along with serotonin and tryptamine using a High performance capillary electrophoresis (HPCE) and capillary electro-chromatography (CEC). Melatonin was also used as internal standard where a well resolved peak was possible as reported by (V.Pucci et al., 2005) when analysing lamotrigine and its metabolites in human plasma and urine by micellar electrokinetic capillary chromatography. Higher detection levels reported

by capillary electrophoresis was good for biological samples but could work better for vegetal matrix where values ranges from picogram levels in some plants up to microgram per gram of sample analysed in others; probably being the reason as to why this technique is not developed for melatonin determination to date.
The following section covers the method validation steps and parameters that must be documented for objective evidence on the method performance.

2.4 Validation of analytical method

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use (Huber, 2007). US Food and Drugs Authority 1987 guideline (US Food and Drug Administration, 1987) defines validation as "establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes". Harmonised guideline for single laboratory validation of a method of analysis (Thompson et al., 2002) pointed out a need for method validation to use a set of test that both test any assumption on which the analytical method is based and establish documented performance characteristics of a method thus demonstrating whether the method is fit for a particular purpose. (ISO/IEC 17025:2005) recommends analytical method to be validated and has given a list of parameters for validation of the methods; (ICH, 1996) also has developed a consensus text on the validation of analytical procedures.
(Huber, 2007) pointed out the situation under which analytical methods need to be validated or revalidated as follows: - before their introduction into routine use, whenever the conditions change for which the method has been validated such as an instrument with different characteristics or samples with a different matrix and whenever the method is changed and the change is outside the original scope of the method. The IUPAC technical report (Thompson et al., 2002) for harmonised guide line for single laboratory validation of methods of analysis sited the following requirement for which validated method should provide documented evidence.

2.4.1. Applicability of the method

After the validation of the analytical method; the documentation should provide performance specification, the identity of analyte, the concentration range covered by the validation, a specification of the range of the matrices of the test materials covered by the validation, a protocol describing the equipment, reagents, procedures, calibration and quality procedures and special safety precaution required and also to provide intended application and its critical uncertainty requirement.

2.4.2 Selectivity of the method

Selectivity is defined as the degree to which the method can quantify the analyte accurately in the presence of interference (Thompson et al., 2002) and the method should be evaluated for any important interference likely to be present. IUPAC guide line gave a selectivity index as one of the quantitative measure and is given by b_{an}/b_{int} where b_{an} is the sensitivity of the method (slope of the calibration function) and b_{int} the slope of the response independently produced by a potential interferent; and can be determined by execution of the procedure on a matrix blank and the same blank when spiked with a potential interferent at appropriate concentration.

2.4.3 Calibration and linearity of the method

The calibration model gives the mathematical relationship between the signal of the measuring system and the concentration of the analyte in the sample. Several authors have published guidelines concerning calibration in analytical chemistry where a simple regression model is used (Miller, 1991; D. MacTaggart, 1992). The guiding questions should be whether the calibration function is linear, passes through the origin and is unaffected by the matrix of the test material. The linearity and intercept shall be tested by examination of the plot of residuals produced by linear regression of the response on the concentration in an appropriate calibration set. Where any curved pattern shall suggests lack of fit due to non linear calibration function. IUPAC guideline (Thompson et al., 2002) suggested the use of both of a test of significance by comparing the lack of fit variance with that due to pure error in conjunction with the residual plot. Testing the significance of R can also be done

using the t-test as suggested by (Alfassi et al., 2005) where t is calculated from equation $\mathbf{t} = \left[\frac{R^2(n-2)}{\sqrt{(1-R^2)}}\right]^2$ ..... [1]

Then the calculated value of t is compared with the tabulated value for two tailed test with (n-2) degrees of freedom. The null hypothesis to be tested in this case will be that there is no correlation between x (independent) and y (dependent) variables. If the calculated value of ***t*** is larger than the critical tabulated value then the null hypothesis is rejected and the conclusion is that within a given level of confidence there is a correlation.

The guide discourages the wide spread current use of correlation coefficient as an indication of quality of fit and judged it as misleading and inappropriate. This was the case with most of scientific report reviewed in which correlation coefficient was the only indicator used for linearity justification such as work by (L. Mercolini et al., 2008; Arnao and Hernandez-Ruiz., 2009; Chen et al., 2003). The guide also demands for univariate linear calibration the need for six or more calibration standards which should be evenly spaced over the concentration range of interest and the range to encompass 0 -150% of the concentration likely to be encountered and lastly the calibration standard to be run at least in triplicate in random order.

The test for general matrix effect on the calibration curve shall be made by applying the method of analyte addition also known as standard addition to a test solution derived from a typical test material. If the slope of the calibration is linear then the slope of the usual calibration function and the analyte addition plot can be compared for significant difference; where the lack of significance will imply the absence of detectable general matrix effect on the calibration function.

2.4.4 Trueness of the method

Trueness is defined by IUPAC guideline (Thompson et al., 2002) as the closeness of agreement between a test result and the accepted reference value of the property being measured. Trueness shall be stated quantitatively in terms of bias; where a smaller bias indicates greater trueness. It is determined by comparing the response of

the method to a reference material with a known value assigned to the material. In this case significance testing is recommended and the uncertainty in the reference material is taken into consideration. The mean of repeated analyses of the reference material in several runs estimates the combined effect of method and laboratory bias. Certified reference materials (CRMs) are suggested as reference value for trueness experiments because they are traceable to the international standards with a known uncertainty (ICH, 1996; Eurachem guide, 1998). Trueness experiments generate a mean response on reference materials, when interpreting results the uncertainty associated with a certified value should also be taken into consideration along with the uncertainty arising from statistical variation in the laboratory. There is a challenge to the availability of certified reference materials especially when the analyte in question is still under research condition as is the case with most scientific research laboratory when the method is developed for the first time. In all reviews of the melatonin determination there is no available certified reference material for melatonin. In this case; typical material is analysed by the method under validation both in its original state and after spiking of a known amount of the analyte to the test portion where by recoveries significantly different from unit indicates that a bias is affecting the method.

2.4.5 Precision of the method

Precision is defined as the closeness of agreement between independent tests results obtained under stipulated conditions and normally expressed as standard deviation or relative standard deviation. Under single laboratory validation; two sets of conditions for precision are relevant; the first is the precision under repeatability condition which describes variation observed during a single run (σ_r **)** and precision under run to run conditions describing variation in the run bias (σ_{run}), both resulting into combined precision $\boldsymbol{\sigma_{total}} = \left(\frac{\sigma_r^2}{n} + \boldsymbol{\sigma_{run}}^2\right)^{1/2}$ …….[2]

Where n is the number of repeat results averaged within a run for the reported result. The separate variance components can be calculate by application of one-way

analysis of variance into which each replicate analysis must be an independent execution of the procedure applied to separate test portion. It is important that the precision values are representative of the likely test condition. As opposed to the most scientific research laboratory; in an accredited laboratory where routine analysis is done; the measured standard deviation is divided into repeatability, intermediate precision, and reproducibility for the purpose of traceability and global comparability of analytical results (ICH, 1996; ISO/IEC 17025, 2005)

Repeatability is obtained when the analysis is carried out in a laboratory by an operator using a piece of equipment over a relatively short time span; where at least 6 determinations of 3 different matrices at 2 or 3 different concentrations should be performed, and the RSD calculated.

Intermediate precision as defined by defined by (ICH, 1996) is the long-term variability of the measurement process. It is determined by comparing the results of a method run within a single laboratory over a number of weeks. A method's intermediate precision may reflect discrepancies in results obtained from different operators; from inconsistent working practice of the same operator; from different instruments; with standards and reagents from different suppliers; with columns from different batches. The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

Reproducibility represents the precision obtained between different laboratories. The objective is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts, and by using operational and environmental conditions that may differ from, but are still within the specified parameters of the method. Such a study is known as inter

laboratory test. Validation of reproducibility is important if the method is to be used in different laboratories. (Huber, 2007) Outlined variables for measurements of Precision, Intermediate precision, and Reproducibility as shown in the table 3.

0-1Table 2:3 Variables for Precision, intermediate precision, and reproducibility

variables	Precision	Intermediate Precision	Reproducibility
Instruments	Same	Different	Different
Batches of accessories	Same	Different	Different
Operators	Same	Different	Different
Sample matrix	Different	Different	Different
Concentration	Different	Different	Different
Batches of materials e.g. reagents	Same	Different	Different
Environments e.g. temperature, Laboratory	Same	Different	Different
	same	same	Different

From (Huber, 2007)

Harmonised guidelines for single laboratory validation of method of analysis (Thompson et al.,2002) IUPAC report; pointd out the awareness of the property of precision to be varying with analyte concentration; but the assumption used is that there is no change in precision with analyte level or the standard deviation is proportional to the analyte level, thus for the assumption to hold there is a need to check if the analyte level is expected to vary substantially more than 30% from its central value prior to data treatment for method validation.

2.4.6 Working range

Working range is the interval of the analyte concentration within which the method is regarded as validated. (Huber, 2007) defined it as the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy, and linearity using the method as written. Working range is also known as the range of analyte concentration where the instrument error is below the assumed value. Working range is expressed in the same units as the test results (e.g., percentage, parts per million) obtained by the analytical

method. It ranges from the LOQ value to the highest analyte concentration for which the instrument displays an increase of the analytical signal. When the use of the method focuses on a concentration of interest well above the detection limit; validation near that one critical level would be appropriate but in this case, the validation study report should state the range around the critical value in which validation is carried out.

2.4.7 Limit of detection (LOD)

Detection limit is the smallest amount of concentration of the analyte in the test sample that can be reliably distinguished from zero (IUPAC Recommendation, 1995). According to (Huber, 2002 and ICH, 1995); in chromatography the detection limit is the injected amount of analyte that results in a peak with a height at least two or three times as high as the baseline noise level. Besides this signal/noise method, the (ICH, 1995) described three more methods for determining detection limits as follows:-

a) *Visual inspection:* The detection limit determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.
b) *Standard deviation of the response based on the standard deviation of the blank:* Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses. The precision estimate used (S_o) for limit of detection should be based on at least six independent complete determination of analyte concentration in typical matrix blank or low level materials with no zero or negative results and the approximate detection limit calculated as $3S_0$.
c) *Standard deviation of the response based on the slope of the calibration curve:* A specific calibration curve is studied using samples containing an analyte in the range of the limit of detection. The residual standard deviation

of a regression line, or the standard deviation of y-intercepts of regression lines, may be used as the standard deviation.

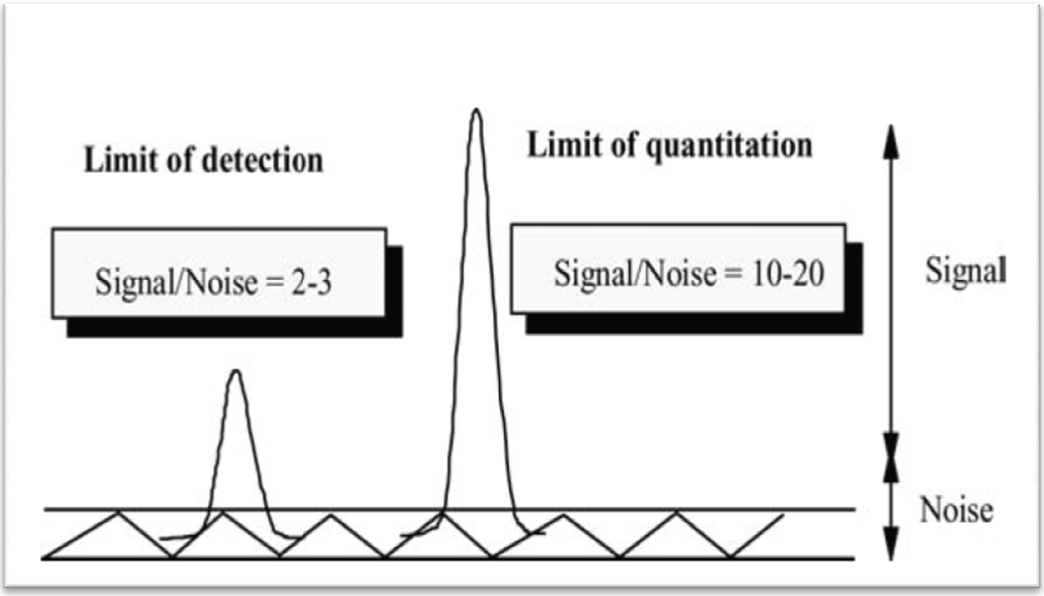


Figure 2:2 Limit of detection and limit of quantitation via signal to noise ratio

From (Huber, 2007)

IUPAC Technical report (Thompson et al., 2002) pointed out the challenges associated with the Limit of detection:- if not excessive amount of data is collected the estimate of detection limit will be subject to large amount of variation; also the estimate of detection limit are often biased on low side due to operational factors; another challenge is associated with the statistical interferences related to detection limit which depend on the assumption of normality which is questionable at low concentration levels. It is important for method validation process to be aware of these challenges so as to get the correct limit of detection.

2.4.8 Limit of quantitation or determination (LOQ)

The limit of quantification is the minimum injected amount that produces quantitative measurements in the target matrix with acceptable precision (Huber,

2007; ICH, 1996; Chan et al., 2004). In chromatography LOQ typically requires peak heights 10 to 20 times higher than the baseline noise as shown in figure 1. (EURACHEM Guide, 1998) gave an approach for LOQ determination whereby; if the required precision of the method at limit of quantification has been specified; the approach to be used is by using by analysing samples with decreasing amounts of the analyte injected six times; then the calculated RSD percent of the precision is plotted against the analyte amount. The amount that corresponds to the previously defined precision is equal to the limit of quantification.

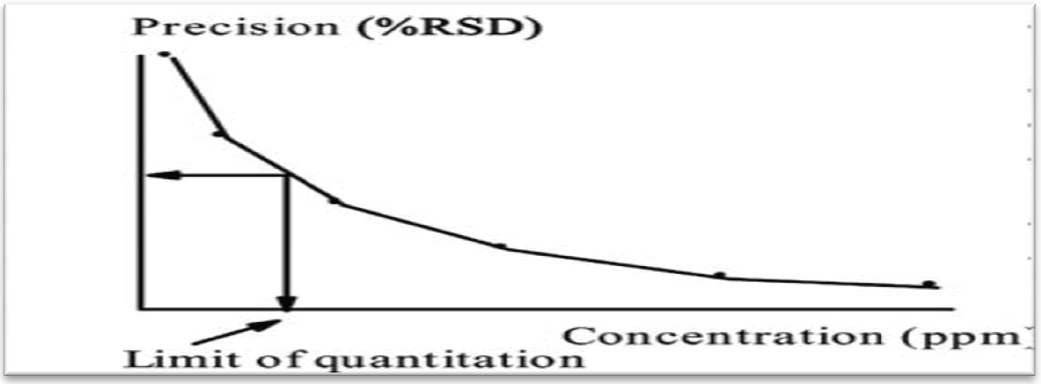


Figure 2:3 Limit of quantitation with the Eurachem method

Source (Huber, 2007; EURACHEM Guide, 1998)

Two types of approaches can also be used to determine the quantitation limit as suggested by (Chan et al., 2004) as follows:-

a) *Signal-to-Noise Approach:* - where limit of quantitation is defined as the concentration of analyte substance in the sample that will give signal-to-noise (S/N) ratio of 10: 1. The quantitation limit of a method is affected by both the detector sensitivity and the accuracy of sample preparation at such a low concentration.
b) To investigate the effect of both factors; solutions of different concentrations are prepared by spiking known amounts of analyte substances. Each solution is

prepared according to the procedure and analyzed repeatedly to determine the S/N ratio. The average S/N ratio from all analyses at each concentration level is used to calculate the LOQ. The following equation can be used to estimate the QL at each concentration level.

LOQ at each concentration = 10 x $\frac{\text{Concetration of analyte}}{\frac{S}{N}(\text{average at each concetration})}$ …… [3]

Since different concentration levels give different LOQ; the worst-case will be reported as the LOQ

c) *Standard Deviation Approach:* **-** the following equation shall be used for limit of quantification determination: - LOQ = 10 x $\frac{SD}{S}$ ……[4]

Where SD is the standard deviation of the response and S is the slope of the linearity curve.

Two approaches have been suggested by (Chan et al., 2004) on determination of the Standard deviation used for limit of detection calculation as follows:-

1. Using experiments for the signal-to-noise approach then determine the standard deviation of the responses by repeat analysis of a solution.
2. Construction of the calibration curve; then determining the residual standard deviation of the regression line of calibration or determining the standard deviation of the y-intercept

It is important to use spiked matrices that closely represent the unknown samples apart from pure standard so as to get a representative result.

2.4.9 Ruggedness

The ruggedness of an analytical method is the resistance to change in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the procedure (Thompson et al., 2002). Ruggedness is also defined by USP as the degree of reproducibility of results obtained under a variety of conditions such as different laboratories, analysts, instruments, environmental conditions, operators and materials; is a measure of

reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst (Huber, 2007).The limits for experimental parameters should be prescribed in the method protocol and permissible deviations, separately or in combination should produce no significant change in the results produced. The aspects of the method that are likely to affect results should be identified, and their influences on method performance are evaluated by using ruggedness tests.

The ruggedness of a method is tested by introducing small changes to the procedure and examining the effect on the results. A number of aspects of the method are considered, it is possible to vary several variables at once, and an experiment based on fractional factorial designs is used where several combinations of variables can be varied. Univariate approaches are also possible where only one variable is changed at time. Some of the factors that ruggedness test could address are: changes in the instrument, operator, or brand of reagent; concentration of a reagent; pH of a solution; temperature of a reaction and time allowed for completion of a process.

2.4.10 Fitness for purpose

According to the harmonised guideline for the single laboratory validation of the method of analysis (Thompson et al., 2002); Fitness for purpose is defined as the extent to which the performance of a method matches the criteria, agreed between the analyst and the end-user of the data. This is a common case for the accredited laboratories where test results must be comparable and traceable to SI units. For that matter the errors in data should not be of a magnitude that would give rise to incorrect decisions more often than a defined small probability, but should also not be so small that the end-user is involved in unnecessary expenditure. Fitness-for-purpose shall be based on the combined uncertainty.

2.4.11 Stability

Many analyte decompose prior to chromatographic investigations such as during preparation of sample solutions, extraction, cleanup, phase transfer and storage of prepared vials in refrigerators or automatic samplers. Due to noted sensitivity of

melatonin to environmental changes such as light and temperature; method validation should investigate and document the stability study of the system for a given method. (Huber, 2007) defined system stability as the stability of the analyte in a sample solution. It is determined as a bias in assay results generated during a pre-selected time interval. System stability shall be determined by replicate analysis of the sample solution; and is considered appropriate if when RSD calculated on the assay results obtained at different time interval does not exceed 20% of the corresponding value of the system precision. When plotting the assay results as a function of time, the maximum duration of the usability of the sample solution can be calculated.

2.4.12 Matrix variation

Matrix variation is one of the most important sources of error in analytical measurements. When the analytical system is validated there must be a specified matrix of the test material and there may be scope for considerable variation within the defined class into which the method can be used. Matrix variation uncertainties need to be quantified separately; the information is acquired by collecting a representative set of the matrices likely to be encountered within the defined class all within analyte concentrations in the working range of the method. The materials are analyzed according to the protocol, and the bias in the results estimated. If the test materials are not certified reference materials, the bias estimate will be determined by means of spiking and recovery estimation. The uncertainty is estimated by the standard deviation of the biases. This estimate will also contain a variance contribution from the repeat analysis with a magnitude $2\sigma^2_r$ if spiking has been used (Thompson et al., 2002). For strict uncertainty budget and avoiding double counting, the variance due to repeatability should be deducted from the variance due to matrix variation.

2.4.13 Measurement uncertainty

Uncertainty is defined as a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand (Eurachem guide, 2000; ISO, 1993). Uncertainity function replaces traditional features namely accuracy, linearity, precision, recovery, selectivity and sensitivity (Thompson and Wood 2006) and it tells how the uncetainity changes with concetration. Uncertainty of measurement comprises many components; some of the components may be evaluated from the statistical distribution of the results of series of measurements and can be characterised by standard deviations. The other components, which also can be characterised by standard deviations, are evaluated from assumed probability distributions based on experience. The ISO Guide refers to these different cases as Type A and Type B estimations respectively. Thompson and Wood (Thompson and Wood, 2006) sited two ways by which uncertainty function can be used; first as "fitness function" which describes the uncertainty that is fit for purpose and secondly as "characteristic function" that describes the performance of a defined method applied to a defined range of test materials.

Eurachem guide identified various sources of uncertainties such as sampling, matrix effect and interferences, environmental conditions, uncertainties of masses and volumetric equipments, reference values, approximations and assumptions incorporated in the measurement method and random variation. In estimating the overall uncertainty, it is necessary to take each source of uncertainty and treat it separately to obtain the contribution from that source. Each of the separate contributions to uncertainty is known as uncertainty component and when expressed as a standard deviation is known as standard uncertainty. For a measurement result Y, the total uncertainty, termed combined standard uncertainty and denoted by $\mathbf{U}_c$ (Y), is an estimated standard deviation equal to the positive square root of the total variance obtained by combining all the uncertainty components evaluated, using the law of propagation.

2.5 General views on the available analytical techniques

Several methods have been used for analysis of melatonin in foods as shown in the above review. Most of the methods that have been adopted from biological sample analysis such as RIA and ELISA could not work better in vegetal matrix. The chromatographic technique which was developed as an alternative showed some improvement to the reliability of the analytical results. GC-MS which was the immediate alternative technique to immunological techniques needed derivatisation step of the sample prior to analysis which was time consuming while Liquid chromatography technique does not need derivatisation and therefore economical and time saving. Despite of the advantages by HPLC techniques, there was evident variation on reliability of the results when different detectors were used for melatonin detection and quantification. When compared to all detectors used, Fluorescence detector despite of the disadvantage of not having spectral properties for reference; the chromatographic properties and checking responses at different excitation/ emission conditions which may be used to overcome the limitation was found to be the most sensitive and versatile to quantify melatonin in a complex vegetal matrix and reaching lowest limit of detection and quantification. Most of the HPLC method reviewed have used reverse phase column RP_{18} or RP_8 for melatonin separation where's methanol and water was in most of the techniques used as one of the mobile phase

There is no report for an independent chromatographic method that has been developed and fully validated for determination of melatonin in grapes, wines and tomatoes. Most of the methods used for melatonin analysis has been for melatonin along with other analyte; such as a work by (Mercolini et al., 2008) where the analytes were cis-resverastrol, trans-resverastrol and melatonin, (D. González-Gómez et al., 2009) where melatonin was determined along with serotonin. Due low levels of melatonin when compared to other analyte raises questions on the reliability of the test results by a given methods when two analytes are determined at the same time.

2.6 Research direction

Due to the gaps identified in the method for determination of melatonin in wine, grapes and the challenges on handling of melatonin prior to its determination; this research will validate a chromatographic method for determination of melatonin in wines and grapes. High Performance Liquid chromatography will be used where detection will be done by Fluorescence detector; PDA UV/ Vis detector will be used for 3D mode scanning to identify the excitation and emission wavelength of melatonin; reverse phase column LiChroCART 250-4 Lichropher RP 18 (5µm) will be used for analyte separation; Flow rate 1ml/min; Mobile phase A: 93% water, 5% methanol and 2% Acetic acid and B: 88% Methanol, 10% water and 2% Acetic acid.

Two methods will be validated; the first will be for liquid sample analysis where direct analysis without pre processing to avoid losing melatonin; in this case the highest response from the detector should be used by increasing the time in the photomultiplier, i.e. gain= 16 as the expected levels of melatonin is very low and the other method will be for solids samples which will need sample processing prior to analysis; in this case the expected levels of melatonin are higher due to the concentration process during preparation and therefore the working time for the photomultiplier in the fluorescence detector should set at the minimum value, i.e. gain=1

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Instrumentation and Materials

3.1.1 Instrumentation

High performance liquid chromatography

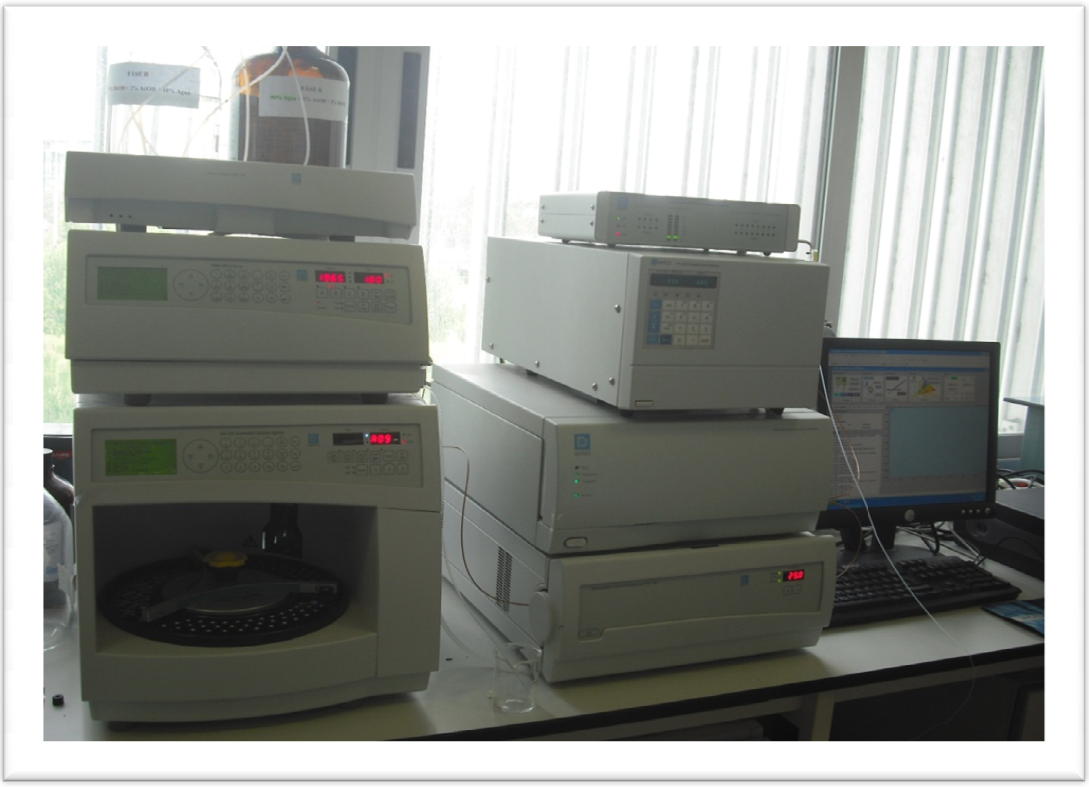


Figure 3:1 High Performance Liquid Chromatography, Fluorescence detector RF 2000 and Photo Diode Array detector –PDA 100

High performance Liquid Chromatography composed of the following: Dionex P680 HPLC Pump; Dionex ASI 100 Automated sample injector; Dionex RF2000 Fluorescence Detector; Dionex PDA-100 Photodiode Array Detector. Dionex UCI-50 Universal Chromatography Interface; Dionex Thermostatted Column Compartment TCC-100; both components are from Dionex Corporation, 1228 Titan Way, Sunnyvale, California 94088-3603 U.S.A. Separation column: Reverse phase RP 18 Lichrospher column (LiChroCART 250 x 4 (5µm) Merck KGaA, 64271

Darmstadt, Germany, Mobile phase Filtration Vacuum pump brand 2C - VACUUBRAND GMBH + CO KG by Wertheim, German ; Filter paper 0.45µm MAGNA nylon supported, Weighing analytical balance-Mettler Toledo AG 135-Switzerland; Refrigerator (15 to -32^{0}C) Liebherr Premium and Micropipette – Eppendorf (10-100µl, 100-1000µl and 1000-5000µL).

3.1. 2 Reagents

Melatonin standard M 5250, SIGMA ALDRICH- Germany; Methanol-HPLC Gradient, Assay 99.9%, PANREAC QUIMICA SA, Barcelona, Spain; Acetic acid (Glacial) 100%- Assay 99.8% Merck KGaA Darmstadt Germany; Mill Q water from Mill Q System

3.1.3 Mobile phase preparation

The mobile phase was made of two solvents i.e. Phase A which was composed of Methanol 5%, Water 93% and Acetic acid 2%. While mobile phase B was composed of Methanol 88%, water 10% and Acetic acid 2%. Both solvents were filtered using vacuum pump and filter paper 0.45µm to remove any impurities and excess gases which would interfere with the system.

3.1.4. Instrumental set up

Instrument was set as follows: Gradient elution at flow rate of 1.0 mL/Minute, Excitation and Emission wavelength was set at 280/310 based on the established excitation and emission wavelength by 3D scanning mode of UV-VIS spectrum for melatonin. PDA-100 Photodiode Array Detector for UV-VIS and 3D mode was set at collection rate of 1.0 Hz, Rise time 1.0 second, 3D wavelength scan range 250 – 600 nm, 3D bunch width 1nm and band width 50nm. The sensitivity of the FD was set at either Gain 1 or 16 depending on the method under validation; whether the method was for lower melatonin concentration levels when gain 16 was used or higher levels where gain 1 and response 1 second was used. Column compartment thermostat was

set 25°C. Auto sampler injection volume was set to 10 µl for the gain 1 and 40µl for gain 16.

3.1.5 Gradient elution profile for mobile phase

The gradient elution profile for phase A and B was established and used throughout the method validation process as follows

Table 3-1 Gradient elution profile

Time (min)	0	5	12	14	20	21	27	29	32
Flow rate (Ml/min)	1	1	1	1	1	1	1	1	1
%B	15	30	30	50	50	100	100	15	15
%A	85	70	70	50	50	0	0	85	85

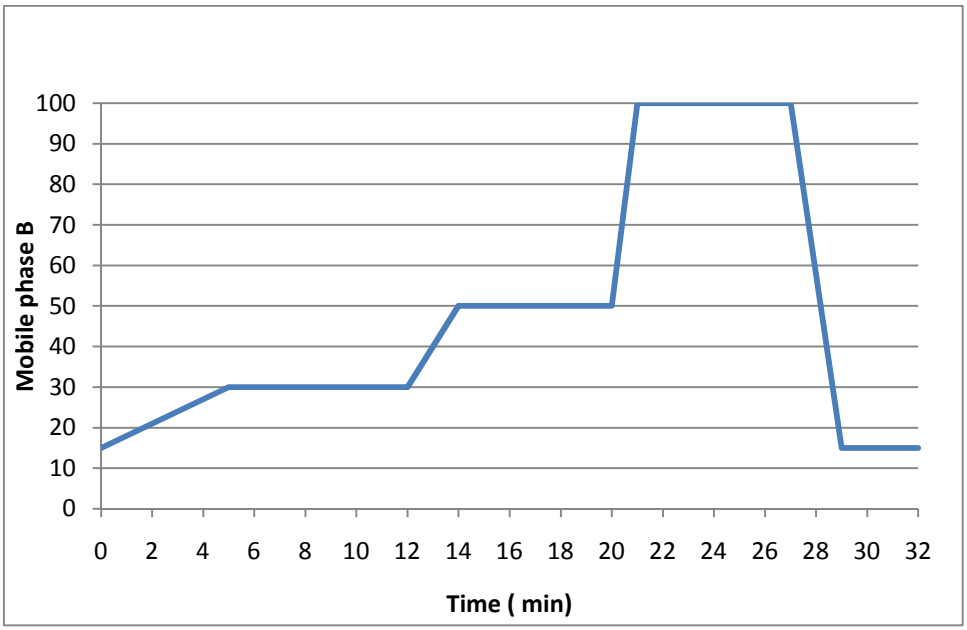


Figure 3:2 Gradient elution profiles

3.2. Validation steps

3.2.1 Melatonin peak identification

Melatonin peak purity was identified by injecting Melatonin standard solution under gradient elution profile in figure 3.2. The UV-VIS spectrum under 3D scan mode between 250 and 600nm wavelength was used. Excitation at 280nm and emission at 310nm wavelength was found to be appropriate for melatonin analyte in the standard solution. Fluorescence Detector connected in-line with Photo Diode Array detector was therefore used to quantify Melatonin at excitation of 280nm and emission at 310nm.

3.2.2 Specificity of the method

3.2.2.1 Blank solution

Specify of the method was assessed by injecting blanks solvents which are used in the assay i.e. Methanol which was used as a mobile phase, Ethanol which was expected to be present in wine and lastly water which was used both as mobile phase and was also present in wine. These solvents are also expected to be used for both solid and liquid samples as extracting solvents if an extraction step is needed. Three injections of those blanks were injected at 5, 50 and 100µl along with melatonin standard with 0.5mg/L to monitor the performance of the HPLC system. The objective was to check for the peak which could elute at the same retention time with melatonin due to solvents used.

3.2.2.2 Degraded melatonin by-product co-elution

Degradation by product of melatonin which may be co-eluted with melatonin peak was assessed by analysing wine samples spiked with melatonin standard 0.5mg/L. Eight spiked samples were prepared and then analysed as they were allowed to degrade for 0hr, 24 hrs, 72hrs and 7days. In each of the time schedule two vials were analysed and checked for the co-elution of the degradation products at the retention time of melatonin.

3.2.3 Calibration curve and Linearity

Calibration curves were established by injecting melatonin standard solution at two different ranges; the first was at higher concentration level ranging from 0.5 mg/L to 20.5 mg/L with eleven data points at gain 1 intended for samples which need preparation step which increases its concentration prior to analysis, and the second was at lower concentration levels ranging from 0.008mg/L to 0.600 mg/L with eleven data points intended for liquid samples which are for direct injection without prior concentration at gain 16. Three replicates were made at each concentration level.

The homogeneity of the analytical results collected was tested to assess whether they all belonged to the same family according to Hartley's test (Hartleys, 1950) where the analysis of variance is used to verify whether different groups belongs either to the same or different family by computing the ratio of the largest group variance to the smallest group variance and the resulting F ratio, F_{max} is compared to the critical value from a table of sampling distributions as shown in the attached annex A.2. Calibration curve was then established from the relation of the concentration to the signal area at a given concentration for each gain. Residue plots for the relation of the analyte concentration and the response deviation from linearity was used to test for linearity. Alamin statistical package (Campana et al.,1997) and JMP statistical package (Freund et al., 2003) were used to generate regression parameters.

3.2.4 Limit of Detection and Limit of Quantification

Limit of detection and Limit of quantification were established using Alamin statistical package (Campana et al.,1997), where the linearity and method sensitivity were also estimated.

3.2.5 Accuracy

Accuracy of the method was tested by analysing melatonin standard in two sets with different matrix composition. The first five dilutions of melatonin were made of 50/50 (Methanol/Water) and the second set of five dilutions was made of 100%

wine. The dilutions were made such that they covered the full linear range for the methods established. The samples were then analysed with dilution run in triplicate; then the relative areas for the different matrices were compared to establish the accuracy. Statistical comparison of areas at a given concentration levels was done to check for significant difference between the two matrices using JMP statistical package (Freund et al., 2003) thereby judging the accuracy of the method.

3.2.6 Precision

3.2.6.1 Intermediate precision

Repeatability of the method was determined analysing the sample which represented the real matrix composition. Wine sample was spiked with melatonin standard and the final composition was over 99% wine matrix. Three different concentration levels within the working range of the method were prepared and injected three runs from each vial. Then the repeatability in terms relative standard deviation (RSD) of injections from the same sample was determined.

3.2.6.2 Intraday precision

Reproducibility of the method was assessed by analysing the same sample for three days while varying the solvents (mobile phase) used. The sample was prepared in similar approach used in repeatability testing. Three concentration levels within the working of the methods were also chosen i.e. 6 mg/L 12mg/L and 18mg/L, and then the samples at each level were prepared and divided into three vials. The first vial at each level was analysed the first day while the rest of the vials were kept in the refrigerator below 0^{o}C and taken for analysis in the second and third day respectively after the Samples being exposed to reach room temperature before injection. The reproducibility measures as relative standard deviation was calculated at each concentration level for the three days analysis. The overall reproducibility for the three days was determined and statistical comparison on whether there was any difference on analysing the sample on different days due to changing mobile phase was made to judge the reproducibility of the method.

3.2.7 Robustness of the method

Four important variables which are likely to suffer changes during analyses and also likely can influence the performance of the method were varied out of the working range of the method to test for robustness. Factorial experimental design using Mintab15 statistical package (MINITAB, 2002) was used to generate variable combination from which the sample was analysed and the signal output (Area mV) was statistically compared to normal experimental condition to check for the variables under which the method was robust. Full factorial design 2^4 with the following variable changes was established:-

i. HPLC Column oven increased by + 2^oC
ii. Solvent B with 2% higher amount of methanol
iii. The FD excitation and detection condition increased by +6nm
iv. Solvent A and B with 2% higher amount of acetic acid

Table 3-2 Variable combinations for robust test

Run Order	Blocks	Oven Temperature (oC)	Solvent B %Methanol	Excitation/ Emission (nm)	Solvent A and B Acetic acid (mL)
1	1	27	88	280/310	20
2	2	25	90	280/310	20
3	3	25	88	280/310	20.4
4	1	25	88	280/310	20
5	3	27	88	280/310	20.4
6	2	25	90	286/316	20
7	3	27	88	286/316	20.4
8	4	25	90	280/310	20.4
9	2	27	90	286/316	20
10	2	27	90	280/310	20
11	4	27	90	280/310	20.4
12	4	25	90	286/316	20.4
13	1	27	88	286/316	20
14	1	25	88	286/316	20
15	3	25	88	286/316	20.4
16	4	27	90	286/316	20.4

CHAPTER 4

EXPERIMRNTAL RESULTS

4. 0 Experimental results

4.1 Melatonin peak identification

4.1.1 Melatonin peak Purity

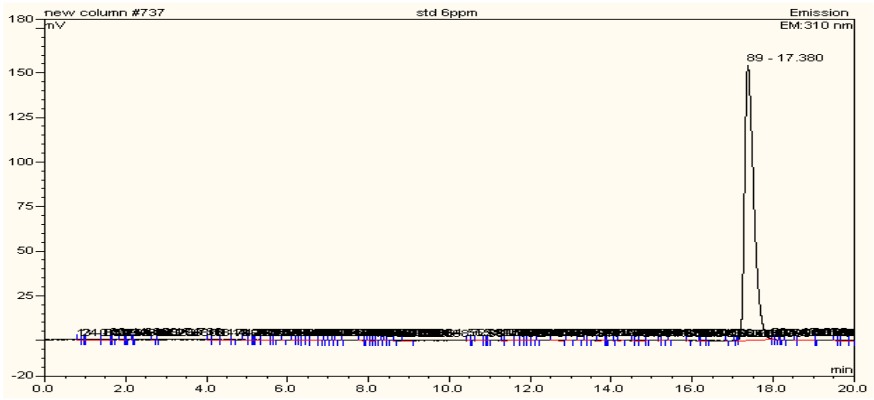


Figure 4:1 Melatonin standard chromatogram Rt=17.38

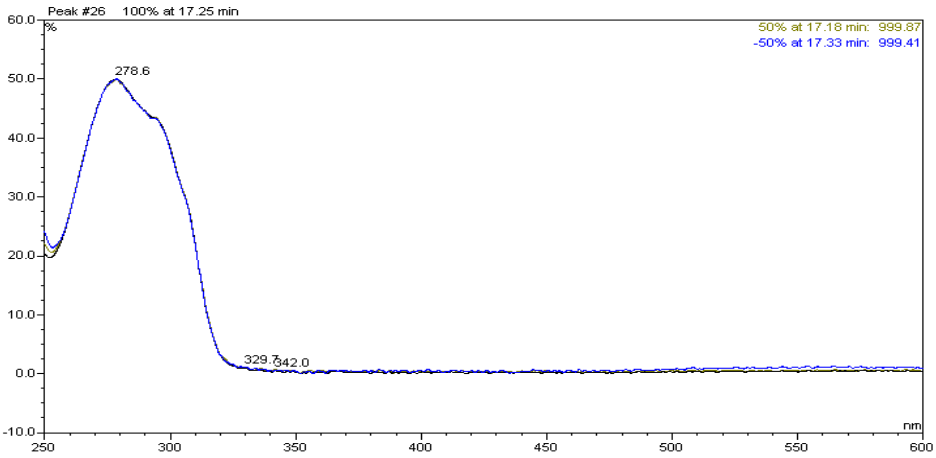


Figure 4:2 UV-Vis spectrum for peak in chromatogram shown in figure 4:1(Rt =17.380)

Melatonin peak identification by Fluorescence detection using area at different excitation/emission condition

Table 4-1 Area ratios for different excitation/emission condition of the fluorescence detector

Melatonin levels (mg/L)	280/310 (A)	290/320 (B)	Ratio
	signal(area mV)		B/A
0.5	1.786	6.470	3.624
0.5	1.782	7.209	4.047
0.5	1.774	7.209	4.063
5	12.469	53.886	4.322
5	12.792	50.411	3.941
5	12.364	51.345	4.153
20	50.236	229.591	4.570
20	55.061	228.228	4.145
20	58.069	223.008	3.840
		MEAN	4.078

4.1.2 Melatonin peak purity for real wine sample

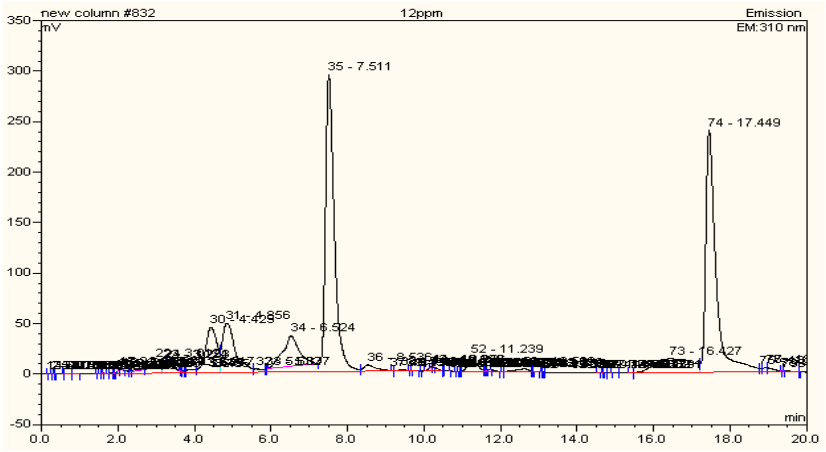


Figure 4:3 Melatonin peak in wine spiked with 12ppm of melatonin

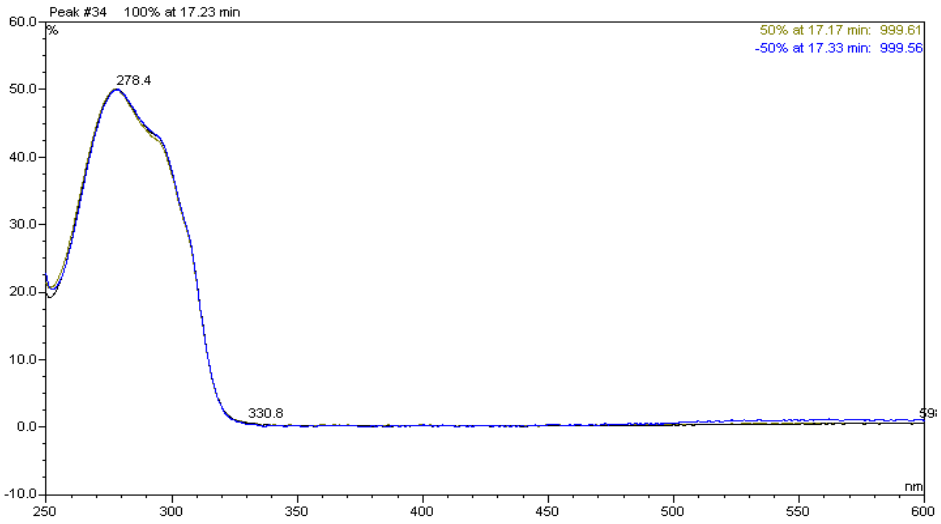


Figure 4:4 UV-Vis spectrum for peak in chromatogram shown in figure 4.3 (RT=17.449)

4.2 Specificity of the method

4.2.1 Blank test

Status of the blank elution at the retention time of melatonin

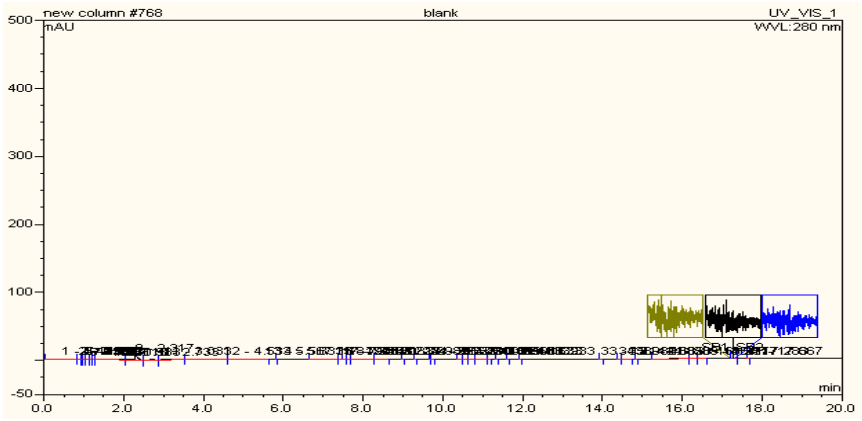


Figure 4:5 Chromatograms for the blank solution

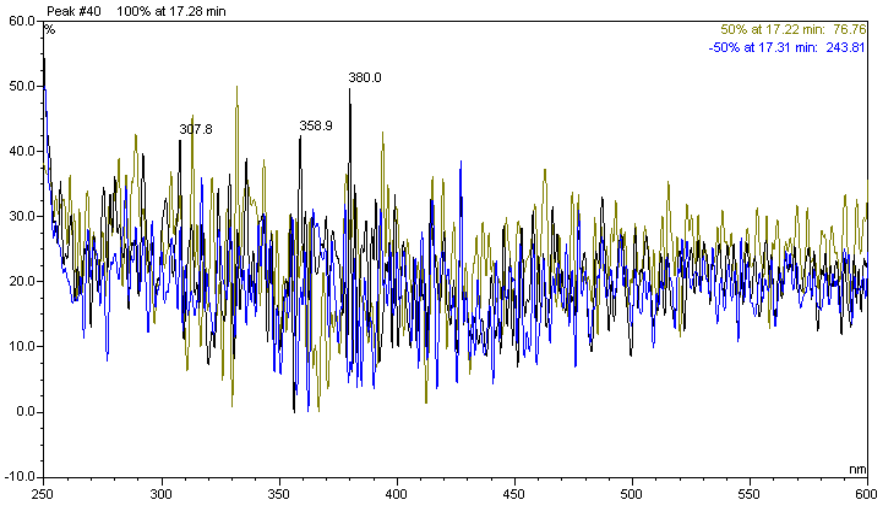


Figure 4:6 UV-Vis spectrum for peak in chromatogram shown in figure 4.5 (Rt=17.420

4.2.2 Baseline check with blanks solution at retention time of melatonin

Table 4-2Baseline check for solvents

	Baseline height (mV)				
Solvent	a	b	c	Average	SD
Water	0	0.2	0.2	0.13	0.12
Methanol	0	0	0	0	0.00
Ethanol	-0.16	0	0	-0.05	0.09
				Mean	0.07

4.3 Calibration curve and linearity

4.3.1 Method "A" for higher concentration levels

The data were subjected to homogenity check to assess if they belonged to the samee family using Hartlelys test (Hartleys, 1950) as shown in table 4.3 below

Table 4-3 Homogeneity test for standard calibration curve values of higher concentration level

Replicate	Calibration standard levels (mg/L)										
	0.5	**2.5**	**4.5**	**6.5**	**8.5**	**10.5**	**12.5**	**14.5**	**16.5**	**18.5**	**20.5**
1	3.0337	14.186	21.847	34.392	43.0167	57.8212	64.067	71.0517	80.593	90.27	108.62
2	3.01	15.96	23.06	34.68	48.64.54	53.13	64.860	71.18	78.72	95.07	110.00
3	2.82	15.60	22.86	34.28	45.91	53.52	64.080	69.33	79.01	92.71	107.24
Mean	2.953	15.248	22.587	34.452	44.462	54.824	64.336	70.520	79.441	92.68	108.62
SD	0.1192	0.937	0.648	0.205	2.0442	2.602	0.4547	1.037	1.007	2.399	1.3756
CV%	4.0362	6.1489	2.873	0.596	4.597	4.746	0.7068	1.4706	1.268	2.588	1.266
F_{max}	106.126931										
F_{mo} (α=0.05, K=11, n=3)	626										

Where $F_{Max} = \frac{CV_{max}^{\ 2}}{CV_{min}^{\ 2}}$

Calibration curve was generates by JMP statistical package (Freund et al., 2003) and is as shown in figure 4.3

4.3.1.1 Calibration curves for higher concentration levels

Bivariate fit of Area (mV) by Concentration (mg/L)

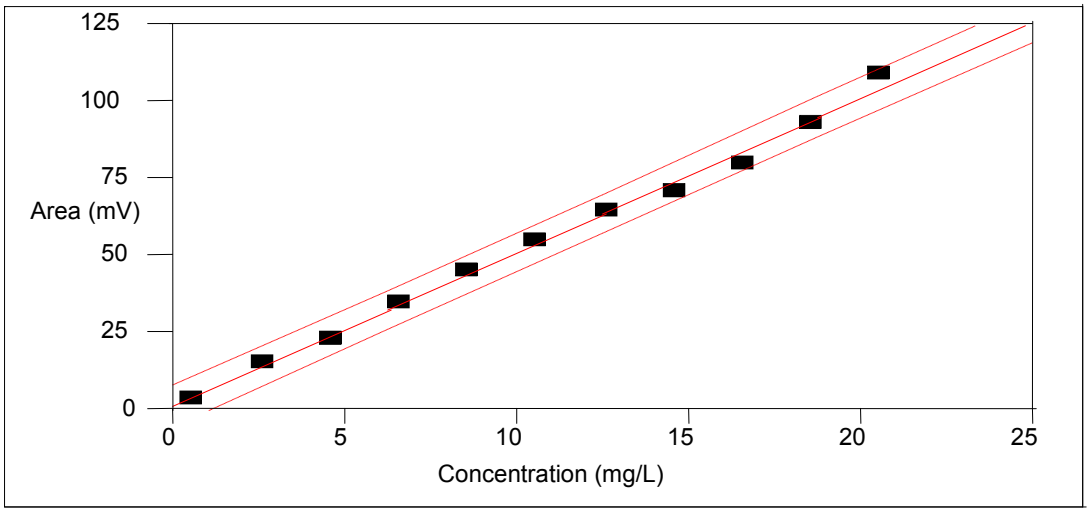


Figure 4:7 Calibration curves for higher concentration levels

Linear Fit equation: Area (mV) = 1.118 + 5.003 Concentration (mg/L)

Table 4-4 Summary of Fit for calibration curve of higher levels

RSquare	0.994
RSquare Adj	0.994
Root Mean Square Error	2.592
Mean of Response	53.648
Observations (or Sum Wgts)	11

Residual plot

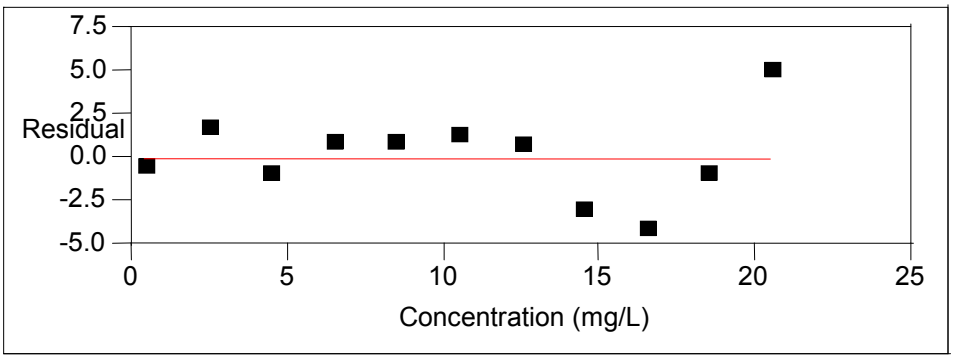


Figure 4:8 Residuals plot for the calibration curve of higher concentration

4.3.1.2 Limit of detection and limit of quantification for higher concentration levels

ALAMIN statistical package (Campana et al., 1997) was used to establish the regression parameters as well as Limit of Detection (LOD), Limit of Quantitation (LOQ) and analytical sensitivity of the method as shown in table 4.5

Table 4-5 Performance of the method for the higher concentration levels

parameter	values
Limit of Detection (LOD) (mg/L)	1.661
Limit of Quantitation (LOQ) (mg/L	5.537
Linearity of the method (%)	98.452
Analytical sensitivity of the method mV/mg/L	0.562
standard deviation slope	0.097
standard deviation intercept	0.0948

4.3.1.3 Working range of the method for higher concentration levels

The working range or linearity range will be the values from the limit of quantitation to the highest values of the standard used in the calibration curve, thus 5.537 to 20.5mg/L

4.3.2 Lower concentration levels

4.3.2.1 Calibration curve and linearity

Homogeneity check using Hartley's test (Hartleys, 1950) to asses if they all belonged to the same family as

Table 4-6 Homogeneity test for standard calibration curve values of higher concentration level

	Calibration standard levels (mg/L)										
Levels	**0.008**	**0.015**	**0.030**	**0.050**	**0.075**	**0.100**	**0.125**	**0.150**	**0.300**	**0.450**	**0.600**
1	4.837	9.471	16.899	23.650	30.356	44.440	55.517	59.307	136.969	217.494	271.389
2	4.619	8.275	16.604	20.307	32.330	46.398	57.400	60.342	139.690	212.325	278.546
3	3.878	9.230	19.107	22.718	30.992	47.316	57.144	59.342	137.144	211.455	273.555
Mean	4.445	8.992	17.537	22.225	31.226	46.051	56.687	59.663	137.934	213.758	274.497
SD	0.503	0.633	1.368	1.725	1.007	1.469	1.021	0.588	1.523	3.264	3.670
%CV	11.317	7.035	7.800	7.762	3.226	3.189	1.802	0.985	1.104	1.527	1.337
F_{max}				131.892							
F_{maxo} (α= 0.05, K =11, n=3)				626							

Where $\boldsymbol{F_{Max}} = \frac{CV_{max}^{2}}{CV_{min}^{2}}$ Since $F_{max} < F_{maxo}$, then the data are homogeneous and can be used to establish calibration curve. Calibration curve was generated by JMP statistical package (Freund et al., 2003) and is as shown in figure 4.5

4.3.2.1 Calibration curve for higher values

Bivariate Fit of Signal (Area mV) by Concentration (mg/L)

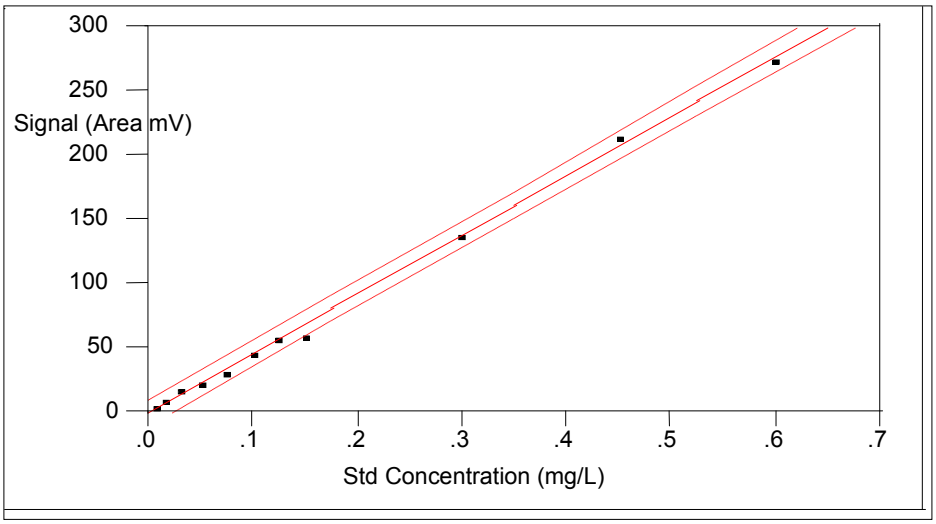
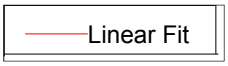


Figure 4:9 Calibration curves for lower concentration levels



Linear Fit equation

Signal (Area mV) = -0.623 + 462.357 Std Concentration (ppm)

Table 4-7 Summary of Fit for calibration curve of lower levels

RSquare	0.998
RSquare Adj	0.998
Root Mean Square Error	4.261
Mean of Response	79.365
Observations (or Sum Wgts)	11

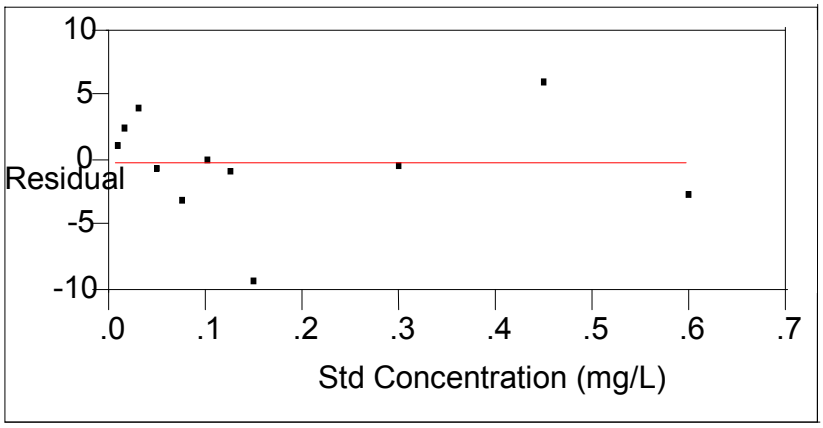


Figure 4:10 Residuals plot for the calibration curve of lower concentration levels

4.3.2.2 Limit of Detection and Limit of Quantification

Alamin statistical package (Campana, 1997) was used to calculate the statistical values for Limit of detection, Limit of Quantitation, Analytical sensitivity and Linearity of the calibration curve for the lower concentration levels of melatonin as shown in table 4.9

Table 4-8 Performance of the method for the lower concentration levels

parameter	values
Limit of Detection (LOD) (mg/L)	0.027
Limit of Quantitation (LOQ) (mg/L)	0.09
Linearity of the method (%)	99.15
Analytical sensitivity of the method mV/mg/L	0.009
standard deviation slope	6.905
standard deviation intercept	1.754

4.3.2.3 Working range of the method for liquid samples

From the Alamin statistical output, the working range was established as the concentration level from the LOQ to the maximum calibration level in the linear range hence 0.09 to 0.60mg/L

4.4 Accuracy of the method

Results of the comparison of the relative areas at four levels within the working range of the method was done using JMP Statistical package (Freund et al., 2003) for areas of 50/50 Methanol /water matrix and for spiked wine matrix.

Table 4-9 Results for methanol/water 50/50 matrix (MWM)

50/50 methanol: water			
Levels	Signal (Area in mV)		
(mg/L)	a	b	c
7.00	39.687	36.195	37.414
10.00	51.030	49.971	51.368
14.00	61.610	64.080	64.140
18.00	94.526	83.130	93.572

Table 4-10 Results for spiked wine matrix (WM)

Spiked wine			
Levels	Signal (Area in mV)		
(mg/L)	a	b	c
7.00	29.326	27.579	28.453
10.00	43.812	43.308	43.590
14.00	52.197	61.140	56.669
18.00	81.542	77.671	79.670

JMP statistical package output for the comparison of the areas was as shown below in figure 4:11

One-way Analysis of Signal (Area, mV) By Matrix

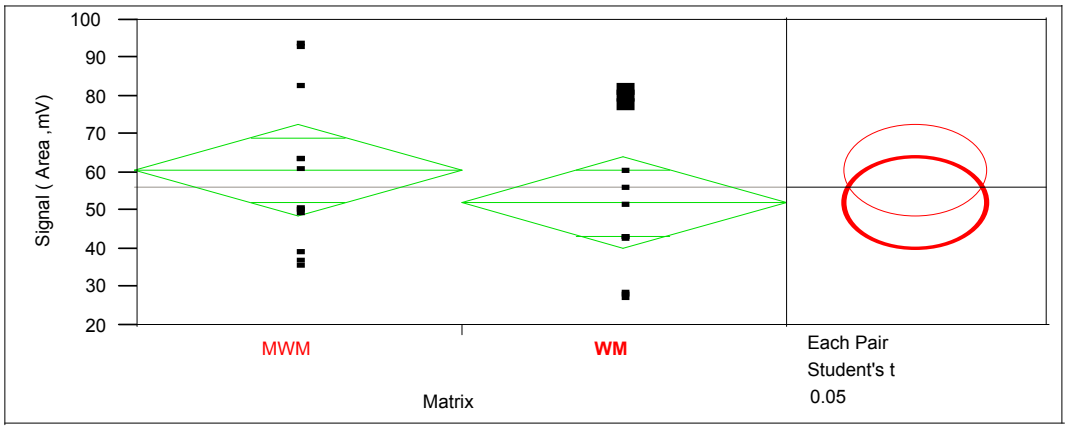


Figure 4:11Comparison of Melatonin signal response between Methanol-Water Matrix (MWM) and Wine Matrix

Means Comparisons

Table 4-11 Comparisons for each pair of means using Student's t

t	Alpha	
2.07387	0.05	
Abs(Dif)-LSD	MWM	WM
MWM	-17.038	-8.550
WM	-8.550	-17.038

Positive values show pairs of means that are significantly different.

Level		Mean
MWM	A	60.561
WM	A	52.072

Levels not connected by same letter are significantly different

4.5 Precision of the Method

4.5.1 Intermediate precision

Table 4-12Results of the intermediate precision of the method

levels(mg/L)	Signal (Area in mV)				Precision	
	a	b	c	mean	SD	RSD
6	31.868	31.393	30.533	31.265	0.677	2.1
12	60.189	63.491	62.929	62.203	1.766	2.8
18	82.767	86.011	87.869	85.549	2.582	3.0

4.5.2 Intraday precision

Results of the RSD for Melatonin when solvents were varied over three day's time

Table 4-13 Day 1 intraday precision

levels(mg/L)	Signal (area mV)					
	a	b	c	mean	SD	RSD
6	31.868	31.394	30.533	31.265	0.677	2.1
12	60.189	63.491	62.929	62.203	1.766	2.8
18	82.767	86.011	87.869	85.549	2.582	3.0

Table 4-14 Day 2 Intraday precision

Level(mg/L)	Signal (Area in mV)					
	a	b	c	mean	SD	RSD
6	28.516	28.386	26.948	27.950	0.870	3.1
12	59.321	58.870	55.330	57.840	2.186	3.8
18	80.847	86.135	86.329	84.437	3.111	3.7

Table 4-15 Day3 Intraday precision

Levels(mg/L)	Signal (Area in mV)					
	a	b	c	Mean	STD	RSD
6	27.479	26.256	30.524	28.087	2.198	7.8
12	57.592	59.187	63.574	60.118	3.098	5.2
18	69.854	80.523	88.747	79.708	9.473	11.9

Table 4-16 Intra-day precision for three days data

Levels (mg/L)	Signal (Area mV)									Precision		
	Replicates for three days when changing mobile phase									Mean	SD	RSD
6	31.868	31.394	30.533	28.516	28.386	26.948	27.479	26.256	30.524	29.10	2.04	7.00
12	59.189	63.491	62.929	57.592	59.187	63.574	59.321	58.870	55.330	59.94	2.83	4.72
18	82.767	86.011	87.869	80.847	86.135	86.329	69.854	80.523	88.747	83.23	5.81	6.98

Statistical comparison for the precision for individual days and the overall data for three days was done using JMP statistical package (Freund et al., 2003), the summary output results is as shown in figure 4.8

Intraday precision comparison

One-way Analysis of Precision (RSD) by time of analysis (Day)

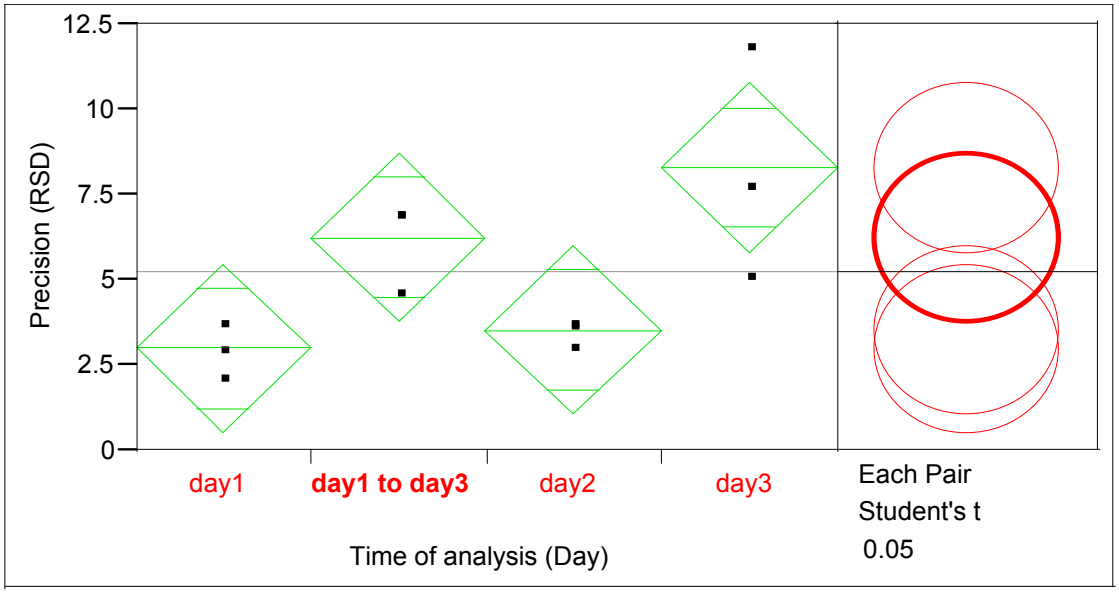


Figure 4:12 intraday precision student t test comparisons for different days

Table 4.15 Comparisons for each pair using Student's t

Table 4-17 Comparisons for each pair using Student's t

t	Alpha
2.30600	0.05

Abs(Dif)-LSD	day3	day1 to day3	day2	day1
day3	-3.511	-1.445	1.255	1.789
day1 to day3	-1.445	-3.511	-0.811	-0.278
day2	1.255	-0.811	-3.511	-2.978
day1	1.789	-0.278	-2.978	-3.511

Positive values show pairs of means that are significantly different.

Analysis day			Mean
day3	A		8.300
day1 to day3	A	B	6.233
day2		B	3.533
day1		B	3.000

Levels not connected by same letter are significantly different

From the comparison of the RSD values for the three different days of analysis as the mobile phase was changing, there was no significant difference in the precision for the overall results of day 1 to day3 with the intermediate precision, thus the method reproducible and there is no difference in precision by analysing either the first or the third day.

4.6 Robustness of the method

Table 4-18Variable combination for method robustness test

Serial number	Experimental Condition	Temp ^{o}C	% Methanol	Excitation /Emission (nm)	Acetic Acid (mL)
1	A	25	88	280/310	20
2	B	27	88	280/310	20
3	C	25	90	280/310	20
4	D	25	90	286/316	20
5	E	27	90	286/316	20
6	F	27	90	280/310	20
7	G	27	88	280/310	20.4
8	H	27	88	286/316	20.4
9	I	25	88	286/316	20.4
10	J	25	90	280/310	20.4
11	K	27	90	280/310	20.4
12	L	25	90	286/316	20.4
13	M	27	90	286/316	20.4
14	N	25	88	280/310	20.4

Wine sample spiked with melatonin at three levels within the linearity range i.e. 6 mg/L, 12mg/L and 18mg/L was analysed in three runs from each vial at various experimental conditions to assess for robustness of the method

Table 4-19 Results for various experimental conditions for robust test

S/N	Exp code	6mg/L	12mg/L	18mg/L	S/N	Exp code	6 mg/L	12mg/L	18 mg/L
		Signal (Areas mV)					Signal (Areas mV)		
1	A	31.867	59.189	82.767	22	H	104.977	170.004	294.4432
2	A	31.393	63.491	86.011	23	H	105.053	170.833	281.291
3	A	30.533	62.928	87.868	24	H	105.047	172.213	278.733
4	B	39.229	52.505	80.223	25	I	126.936	178.353	279.829
5	B	34.118	64.627	93.657	26	I	128.361	177.496	280.392
6	B	35.819	64.885	101.060	27	I	110.197	178.452	279.678
7	C	28.249	58.946	78.128	28	J	30.587	59.674	98.528
8	C	29.569	60.328	90.331	29	J	30.183	63.478	105.560
9	C	31.087	59.065	94.480	30	J	27.502	62.584	110.159
10	D	102.080	154.166	237.476	31	K	26.953	59.265	101.766
11	D	102.780	154.655	246.135	32	K	26.971	60.913	99.326
12	D	104.080	156.226	235.053	33	K	27.576	60.739	99.993
13	E	83.509	148.831	232.206	34	L	93.101	168.112	273.764
14	E	98.733	146.654	247.763	35	L	92.400	170.815	271.780
15	E	85.250	147.896	242.742	36	L	91.624	169.742	281.816
16	F	31.693	54.009	89.034	37	M	89.831	158.770	270.708
17	F	30.163	57.450	88.352	38	M	86.726	160.436	258.612
18	F	33.206	57.408	88.693	39	M	88.137	159.548	267.071
19	G	46.391	53.917	93.472	40	N	29.258	65.079	109.533
20	G	48.377	53.799	103.464	41	N	30.187	71.123	114.154
21	G	36.876	66.417	114.121	42	N	29.039	68.768	114.066

Robust comparison of RSD of different experimental conditions

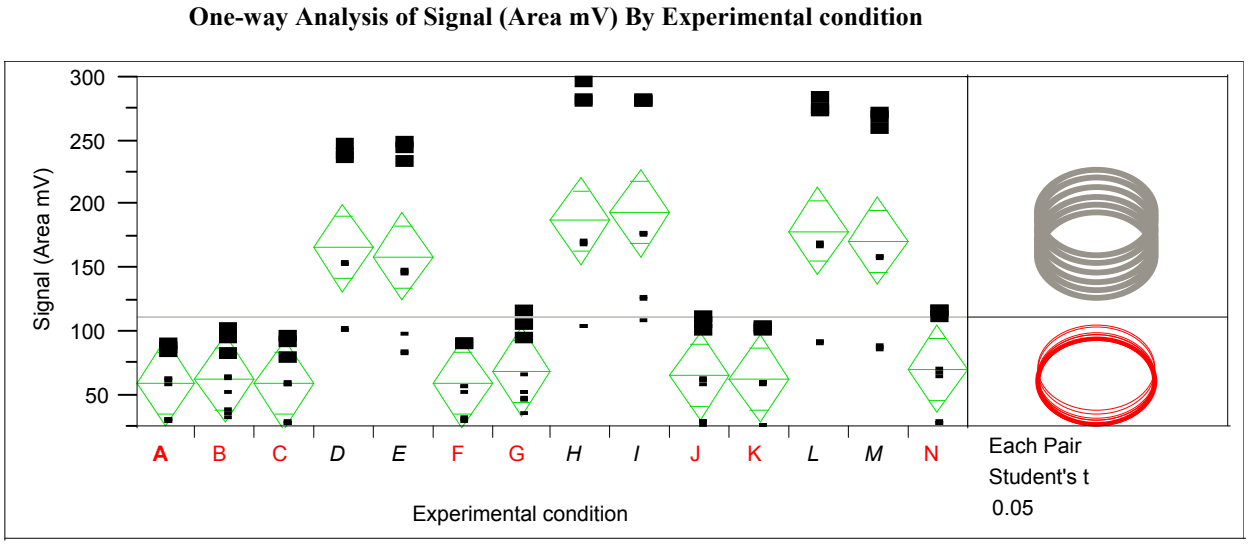


Figure 4:13 Robust comparison of RSD of different experimental conditions

Table 4-20 Experimental conditions comparison

Experimental condition			Mean
I	A		193.299
H	A		186.955
L	A		179.239
M	A		171.093
D	A		165.850
E	A		159.287
N		B	70.134
G		B	68.537
J		B	65.362
B		B	62.902
K		B	62.611
A		B	59.561
C		B	58.909
F		B	58.889

Experimental conditions not connected by same letter are significantly different

The reference experimental condition was coded as “A”, thus all experimental conditions connected by the same letter with reference condition are statistically having no significant difference; such as :- N,G,J, B, K, A, C and F (both connected by letter “A”) thus the method is robust under these experimental variation. While the experimental conditions connected by different letter in this case by letter “B” they statistically perform differently from the reference method, thus the method is not robust to these experimental conditions.

CHAPTER 5

DISCUSSION

5.0 Discussion

5.1 Over view of the significance of the experimental results

Having identified the challenges associated with analytical method for determination of melatonin in foods of plants origin, and the methods currently available being questionable on their reliability, this research work came up with the following findings towards solving these challenges. It was possible to bring confidence to the purity of melatonin peak when both Fluorescence detector and UV-Vis detector was used in-line for melatonin standard detection. Further reliability on the peak identified was also confirmed when real wine matrix was spiked with 12 mg/L of melatonin standard in the matrix which was 99% wine. With the available experimental data it was possible for the first time to come up with the method which was specific only for melatonin as the only analyte and be able to establish the limit of detection, limit of quantitation, working range of the method as well as the precision and robustness of the method. There is also a chance of coming up with two methods, one to be used for direct injection of liquid samples without major prior sample pre-treatment to liquid samples which would avoid analyte degradation along the way, and another method for solid samples which would require sample treatment in advance.

5.2 Validation parameters

5.2.1 Melatonin peak purity

Melatonin peak purity was proved in chromatogram figure 4.1 when melatonin standard was injected, this was also supported by the UV-Vis spectrum at the same retention time as shown in figure 4.2 where there is only one maximum wavelength at which the melatonin standard was excited i.e. 278.1nm which is within the range of 280±2nm working range condition of the detector (Chung et al, 2004). This excitation wavelength was found to be in agreement with several other authors work such as (Arnao and Hernandez-Ruiz, 2009 and Iriti et al., 2006) when they worked on melatonin in plants.

The purity of melatonin peak was also evident when real sample with 99% of wine matrix was spiked with 12mg/L melatonin standard as shown in figure 4.3 for fluorescence detection and figure 4.4 for UV-Vis spectrum of the same peak. It was evident on a significant variation on the areas of the melatonin standard signal when was analysed by fluorescence detector at difference excitation/ emission condition; at excitation/ emission condition of 290/320nm the signal was over four times higher than the signal at excitation/ emission condition of 280/310 nm as shown in table 4-1 suggesting that probably what is detected at excitation/ emission of 290/320 is not just melatonin.

5.2.2 Specificity of the method

The experimental results show that the method developed was specific for melatonin analyte as justified by the findings when blanks solution composing of mobile phase constituents was injected and assessed the chromatogram pattern at the retention time of melatonin around 17.70 minutes. Figures 4.5 and 4.6 both shows the blanks chromatogram for Fluorescence detection and UV-Vis spectrum at the retention time melatonin, in this case there is evidence that there is no peak due to the mobile phase composition at the retention of melatonin as seen in the UV-Vis spectrum where only noise was seen in figure 4.6. This finding justifies the quality of mobile phase composition i.e. methanol, acetic acid and water with respect to specificity of the method for melatonin analysis.

The baseline assessment results in table 4-1 shows that both solvents composition which are likely to be present in the analyte during analysis such as water, methanol both from the mobile phase and ethanol from wine, gave the overall baseline standard deviation at the retention time of melatonin of about 0.070mV. This implies that these components will not have any significant influence in the melatonin peak at its retention time during its determination using fluorescence detector.

5.2.3 Calibration and linearity

The method managed to come up with two calibration curves for melatonin determination in plants. Method "A" shown in figure 4.7 gave a linear fit equation: Signal Y = 1.118 + 5.003X (where Y= Signal (area mV) and X = melatonin concentration in ppm) with linearity of 98.452% and analytical sensitivity of the method of 0.563 mV/mg/L, standard deviation of the slope and the origin as 0.097 and 0.948 respectively as shown in table 4-4.The residual plot in figure 4.8 indicates most of the levels of standards used had residuals near zero implying a high linearity. Method "B" which was intended for samples with lower levels of melatonin gave a calibration curve with linear fit equation: Y = - 0.623 + 462.356X and linearity of 99.150%, standard deviation for slope and intercept as 6.905 and 1.754 respectively, and the analytical sensitivity of the method 0.009 mV/ppm as shown in table 4.7 and a fairly good residual for each level as shown in figure 4.10 These findings are quite good at this method development stage where there has never been any method specific for melatonin determination in wine. No data were found in the literature review regarding a similar method, so it was not possible to compare these results with other ones

5.2.4 Limit of detection and limit of quantitation

The limit of detection and quantification for method "A" intended for samples with higher levels of melatonin was found to be 1.661mg/L and 5.537mg/L respectively as shown in summary table 4-4, while the method "B" intended for samples with lower levels of melatonin was found to have a limit of detection of 0.027mg/L and limit of quantitation of 0.090 mg/L as shown in table 4.7. Method "A" which was intended for use in samples with higher levels of melatonin or samples which has undergone pre-treatment process such as solid phase extraction and liquid-liquid extraction as reported by (Reiter et al., 2005; De la Puerta et al., 2007 and Van Tasel et al., 2001). In both cases method "A" and "B" the levels of limit of detection and limit of quantification was higher than that reported in literature such as limit of detection of 0.01ng/mL in wine

reported by (Mercolini et al., 2008) when used HPLC-FD for melatonin analysis in wine, detection limits of 0.017, 0.015 and 0.055ng/g in plants when different sample processing procedure was employed then analysed by HPLC-FD (Arnao and Hernandez-Ruiz, 2008) and 4.3 ng/g melatonin detection limit in sweet cherry cultivars reported by (D. González-Gómez., 2009). Both literature reports limits below those established by these two methods. However it has to be in mind that the reported limits were calculated for the complete analytical method, including the extraction step. Therefore, if the extraction/concentration step is taken in mind, better results would be achieved in the method which is under validation. For example, the paper by (Mercolini et al 2009) reported a limit of detection =0.01 ng/mL but the concentration ratio was 4:1 so, the limit of detection for the chromatographic method should be around 0.04 mg/L., i.e. 1.5 fold higher than the limit of detection found for the method "B". The important note here is that, all the other methods the sample was pre-treated either by liquid-liquid extraction or solid phase extraction thus concentrating the analyte and removing most of the interfering substances. Another observation is the use o simulated wine instead of real wine sample during method validation in which case the matrix was representative of the real wine also reported by Mercolini. Further improvement on the limit of detection is possible if wine sample is pre-treated before injection into HPLC-FD system thereby reducing the interference.

5.2.5 Working range of the method

Method "A" which was intended for higher melatonin levels analysis had a working range of 5.537 mg/L to 20.500 mg/L while method "B" which intended for lower levels had a working range of 0.090 mg/L to 0.600 mg/L. These results agrees with levels of melatonin reported by others researchers where their results for melatonin in plants falls within these ranges; Example; is a report of 189 ng/g of melatonin in white mustard seeds, 103 ng/g of melatonin in wolf berry both reported by (Manchester et al., 2000), 7110 ng/g in huang qin plant reported by (Reiter et al., 2000), 3771ng/g in chantui plant

reported by (Zhang et al., 2003) when used HPLC-FD for analysing melatonin in chines medicinal plant. Other report within the same working range was 1610ng/g in scullcap plant and 1690ng/g in feverfew plant as reported by (Murch et al, 1997). Therefore the methods compared better with the available literature and having an advantage of very sensitive Fluorescence detector, it is possible to establish several calibration curves with different sensitivity setting to accommodate the varying levels of melatonin in plants. Sample pre-treatment such as solid phase extraction as reported by (Mercolini et al., 2008) with melatonin detection level of 0.01 ng/mL and (Guerrero et al., 2008) who reported melatonin levels of 50-80pg/m in red wine when sample extraction was done using methanol and C-18 SPE would make the method achieve much lower detection level, hence increase the working range.

5.2.6 Accuracy of the method

Analytical results for accuracy of the method when comparison of the method response was made between using 50/50 methanol/water matrix which makes most of the mobile phase and wine spiked with melatonin within the working range of the method "A"; the results as shown in figure 4.11 and table 4.10, there is no significant difference in the method when it was used in either Methanol-water matrix (MWM) and wine matrix (WM) for melatonin determination. Method accuracy determination was only possible to method "A. while the fluorescence detector setting for lower level detection in method "B" the spiked wine sample produced saturation in the fluorescence detector due to other components in the matrix which brought higher signals in the detector at such very sensitive setting thus interfering with melatonin peak identification. This would be possible if wine sample is prior treated for the method for the method to work better.

5.2.7 Precision of the method

Intermediate precision of the method expressed as relative standard deviation for three replicates of wine spiked with melatonin at three concentration levels within the working

range of the method was found to be less than 3% as shown in table 4.11.while the intraday precision for nine samples of three days was less than 7% as shown in figure 4.12 and table 4-16 This performance of the method compares better with the earlier report by (Mercolini et al., 2008) where the intermediate precision was 3.7% and repeatability was less than 3.5% when recovery approach was used for precision determination. Since this method used a real wine sample spiked with melatonin for precision determination as opposed to the Mercolini who used simulated blank wine; thus the method precision was more representative of the wine matrix.

5.2.8 Robustness of the method

Statistical analysis of the results obtained when experimental variables which are likely to affect method performance shows that there is some variable combination under which the method was robust and in other variable combination where the method was not robust. Summary results in figure 4.13 and table 4.20 into which a reference experimental condition "A" (column oven temperature 25^{o}C, Solvent B % methanol 88, Excitation/emission wavelength 280/310 nm, solvent A and B acetic acid content 20mL) was found to be statistically the same to experimental conditions B, C, F, G, J and K, while significantly different to experimental conditions D, E, H, I, L and M.

The method was found to be robust against more easily changing working variables such as those which can be influenced by the personnel i.e. reagents preparation; while the main variables which affected the method robustness was an instrumental variable such excitation/ emission condition when was changed from 280/310 nm to 286/316 nm. In this case to maintain the method robustness it would be important to establish a control system to check the fluorescence detector accuracy. Fluorescence detector accuracy can be checked regularly along with the entire HPLC system using a stable chemical compound of a mixture of Acridine and Anthracene under gradient elution where mobile phases: Solvent A: acetonitrile (HPLC grade) Solvent B: water (HPLC grade), Column

(4.6mm ID X 150mm).The acceptance criteria for the %RSD should be: Retention time: 0.5% or less; Peak area: Acridine 5.0% or less, Anthracene 2.0% or less; Peak height: Acridine 5.0% or less, Anthracene 2.0% or less (It is necessary that changes of room temperature are less than 2°C) as recommended by Dionex operating manual for RF-2000 (Dionex, 2000) as shown in annex 7. Another approach to maintain the instrumental performance is by establishing a performance verification schedule for the entire HPLC-FD system as reported by (Chung et al., 2004) where all the components of the system are verified their performance as seen in attached annex 8.

5.2.9 Summary of the significance and context achieved

The main objective of this work was to come up with an independent validate method for analysis of melatonin in foods of plants origin specifically in wine. Due to nutritional value of melatonin reported by several authors and challenges addressed on its determination; a need for sensitive and validated analytical method for its determination so as to come up with a reliable data from which food composition tables could be established was the objective of this research. This work managed to come up with methods with a working range which complies with many other authors who worked on melatonin determination in plants. The working range of 5.537 to 20.5 ppm (5537ng/g to 20500ng/g) for method "A" and 0.090 to 0.600 ppm (90 to 600 ng/g) for method "B" are within the range of some melatonin reports in plants such as 189 ng/g of melatonin in white mustard seeds, 103 ng/g of melatonin in wolf berry both reported by (Manchester et al., 2000), 7110 ng/g in huang qin plant reported by (Reiter et al., 2000), 3771ng/g in chantui plant reported by (Zhang et al., 2003) when used HPLC-FD for analysing melatonin in chines medicinal plant. Other report within the same working range was 1610ng/g in scullcap plant and 1690ng/g in feverfew plant as reported by (Murch et al, 1997).

The methods validated could not meet some levels reported in literature such as 28ng/g of melatonin in cherry seed reported by (Manchester et al., 2000), strawberry with 0.01 (Hattori et al., 1995), 0.5ng/g of melatonin in tomato by (Dubbels et al., 1995) and detection limit of 0.010 ng/g melatonin in wine reported by (Mercolini et al., 2008) and cherry with 18ng/g reported by (Burkhardt et al., 2001). Since the validation process involved using wine sample without pre- treatment, there is a chance that once pre treated sample was used much lower levels of melatonin would be achieved. Usually pre-treatment concentration/cleaning steps are applied on these samples, so, the validated method could be used for most of them.

Since this work reports for the first time validated chromatographic methods for determination of melatonin in wine at higher levels and lower levels using spiked wine matrix, there is a chance to improve the detection limits by using pre-treated wine sample where melatonin will be concentrated before injection

The variables under which the method was validated were the most relevant for the performance of the chromatographic techniques hence differences in performance of the method, if at all exist will be minor ones. The robustness test of the method has provided confidence on the method performance as the method was robust to all normal chromatographic operation conditions.

The objective has not been achieved in the sense that the working range of the method does not cover the levels of melatonin found in wine. The achievement was that the method covers most of the higher levels of melatonin found in plants. With improvement on the method lower values of melatonin found in wine can be achieved.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.0 Conclusion and Recommendations

6.1 Conclusion

1. This master research thesis has managed to come up for the first time with a validated method for determination of melatonin in wine at developmental stage..Method "A" was for samples with higher levels of melatonin; such as solid samples which requires extraction or concentration steps which produces higher levels in the final chromatographical sample. This method performed better in all the parameters validated. Method "B" which was intended for samples with lower levels was possible to establish calibration curve, working range, limit of detection and limit of quantification. Spiked wine sample could not work to method "B" due to high levels of interference in the wine matrix at high sensitivity setting of the detector.

2. Method "A" has achieved detection limit of 1.661mg/L and quantitation limit of 5.537 mg/L and method "B" limit of detection of 0.027 mg/L and limit of quantification of 0.090 mg/L.

3. The intermediate and intraday precision was 3% and 7% respectively while analytical sensitivity achieved by method "A" was 0.563 mV/mg/L and linearity 98.542%. Method "B" analytical sensitivity was 0.009mV/mg/L and linearity of the method was 99.150%.

4. The method validated was found to be robust to the following variables when subjected to changes:

    a. Increase of column oven temperature from $25^{o}C + 2^{o}C$

    b. Solvent B increase of %Methanol 88% + 2%

    c. % Acetic acid in solvent A and B from 2% increase by 2%

5. The method was not robust to changes in excitation/emission condition i.e. changes from 280/310 to 286/316nm,

6.2 Recommendation

1. Further research on improvement of the variables is highly recommended and validation of the parameters such as robustness for more variable such as different instruments, analyst and laboratory in classical approach; and establishment of the measurement uncertainty are important for the method to be fully operational in routine laboratory especially those aspiring accreditation.
2. Most wines have several compounds which can interfere with melatonin peak. Therefore cleaning step such as solid phase extraction and liquid-liquid extraction to be done prior to sample injection.
3. A research on real wine samples with melatonin content is recommended so at to get the real performance of the method as opposed to the spiked wine. However a real wine containing melatonin was not found, so it is still pending.
4. For the method to be extended to other laboratories the performance assay of the method should be done within the robust limit and only when proved okay should be fully implemented

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ANNEXES

Annex 0-1A.1 Analysis programme

TempCtrl =	On
Temperature.Nominal =	25.0 [°C]
Temperature.LowerLimit =	5.0 [°C]
Temperature.UpperLimit =	85.0 [°C]
EquilibrationTime =	0.5 [min]
ReadyTempDelta =	1.0 [°C]
HumidityLeakSensor =	Standard
GasLeakSensor =	Standard
ActiveColumn =	NotUsed
Pressure.LowerLimit =	200 [psi]
Pressure.UpperLimit =	4000 [psi]
%A.Equate =	"%A"
%B.Equate =	"%B"
%C.Equate =	"%C"
%D.Equate =	"%D"
DispSpeed =	20.00 [µl/s]
DrawSpeed =	5.00 [µl/s]
SampleHeight =	2.00 [mm]
SyringeDelay =	5 [s]
Up Speed =	10.00 [mm/s]
DownSpeed =	10.00 [mm/s]
Radial Speed =	20.00 [mm/s]
SyncWithPump =	On
Pump Device =	"pump"
Pump_Pressure.Average =	On
Emission.ExWavelength =	280 [nm]
Emission.EmWavelength =	310 [nm]
Emission. Gain =	1.0
Emission. Response =	0.1 [s]
Emission. Sensitivity =	Med
Emission. Average =	On
Emission. Step =	Auto
Data_Collection_Rate =	1.00 [Hz]

	Rise_Time =	1.0 [s]
	UV_VIS_1.Wavelength =	280 [nm]
	UV_VIS_1.Bandwidth =	1 [nm]
	UV_VIS_1.RefWavelength =	Off
	UV_VIS_1.RefBandwidth =	1 [nm]
	UV_VIS_1.Recorder_Range =	1.0000 [AU]
	UV_VIS_2.Wavelength =	260 [nm]
	UV_VIS_2.Bandwidth =	1 [nm]
	UV_VIS_2.RefWavelength =	Off
	UV_VIS_2.RefBandwidth =	1 [nm]
	UV_VIS_2.Recorder_Range =	1.0000 [AU]
	UV_VIS_3.Wavelength =	320 [nm]
	UV_VIS_3.Bandwidth =	1 [nm]
	UV_VIS_3.RefWavelength =	Off
	UV_VIS_3.RefBandwidth =	1 [nm]
	UV_VIS_3.Recorder_Range =	1.0000 [AU]
	UV_VIS_4.Wavelength =	293 [nm]
	UV_VIS_4.Bandwidth =	1 [nm]
	UV_VIS_4.RefWavelength =	Off
	UV_VIS_4.RefBandwidth =	1 [nm]
	UV_VIS_4.Recorder_Range =	1.0000 [AU]
	UV_VIS_5.Wavelength =	306 [nm]
	UV_VIS_5.Bandwidth =	1 [nm]
	UV_VIS_5.RefWavelength =	Off
	UV_VIS_5.RefBandwidth =	1 [nm]
	3DFIELD.RefWavelength =	300 [nm]
	3DFIELD.RefBandwidth =	50 [nm]
	3DFIELD.MinWavelength =	250 [nm]
	3DFIELD.MaxWavelength =	600 [nm]
	3DFIELD.BunchWidth =	1 [nm]
0.000	Emission.Autozero	
	UV.Autozero	
	Wait	AZ_Done
	Flow =	1.000 [ml/min]
	%B =	15.0 [%]
	%C =	0.0 [%]
	%D =	0.0 [%]
	Wait	ColumnOven.Ready and Sampler.Ready

	Inject	
	Pump_Pressure.AcqOn	
	Emission.AcqOn	
	UV_VIS_1.AcqOn	
	UV_VIS_2.AcqOn	
	UV_VIS_3.AcqOn	
	UV_VIS_4.AcqOn	
	UV_VIS_5.AcqOn	
	3DFIELD.AcqOn	
	Flow =	1.000 [ml/min]
	%B =	15.0 [%]
	%C =	0.0 [%]
	%D =	0.0 [%]
5.000	Pump_Pressure.AcqOff	
	%B =	30.0 [%]
12.000	%B =	30.0 [%]
14.000	%B =	50.0 [%]
20.000	UV_VIS_1.AcqOff	
	UV_VIS_2.AcqOff	
	UV_VIS_3.AcqOff	
	UV_VIS_4.AcqOff	
	UV_VIS_5.AcqOff	
	3DFIELD.AcqOff	
	%B =	50.0 [%]
	Emission.AcqOff	
21.000	%B =	100.0 [%]
27.000	%B =	100.0 [%]
29.000	%B =	15.0 [%]
32.000	Flow =	1.000 [ml/min]
	%B =	15.0 [%]
	%C =	0.0 [%]
	%D =	0.0 [%]

Annex A.2 Hartley's Statistical table

Table Critical values for the Hartley test (right-sided)

Level of significance α = 0.01

	k										
n - 1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2	199	448	729	1036	1362	1705	2069	2432	2813	3204	3605
3	47.5	85	120	151	184	216*	249*	281*	310*	337*	361*
4	23.2	37	49	59	69	79	89	97	106	113	120
5	14.9	22	28	33	38	42	46	50	54	57	60
6	11.1	15.5	19.1	22	25	27	30	32	34	36	37
7	8.89	12.1	14.5	16.5	18.4	20	22	23	24	26	27
8	7.50	9.9	11.7	13.2	14.5	15.8	16.9	17.9	18.9	19.8	21
9	6.54	8.5	9.9	11.1	12.1	13.1	13.9	14.7	15.3	16.0	16.6
10	5.85	7.4	8.6	9.6	10.4	11.1	11.8	12.4	12.9	13.4	13.9
12	4.91	6.1	6.9	7.6	8.2	8.7	9.1	9.5	9.9	10.2	10.6
15	4.07	4.9	5.5	6.0	6.4	6.7	7.1	7.3	7.5	7.8	8.0
20	3.32	3.8	4.3	4.6	4.9	5.1	5.3	5.5	5.6	5.8	5.9
30	2.63	3.0	3.3	3.4	3.6	3.7	3.8	3.9	4.0	4.1	4.2
60	1.96	2.2	2.3	2.4	2.4	2.5	2.5	2.6	2.6	2.7	2.7
∞	1.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

*The third-digit figures for n - 1 = 3 are uncertain.

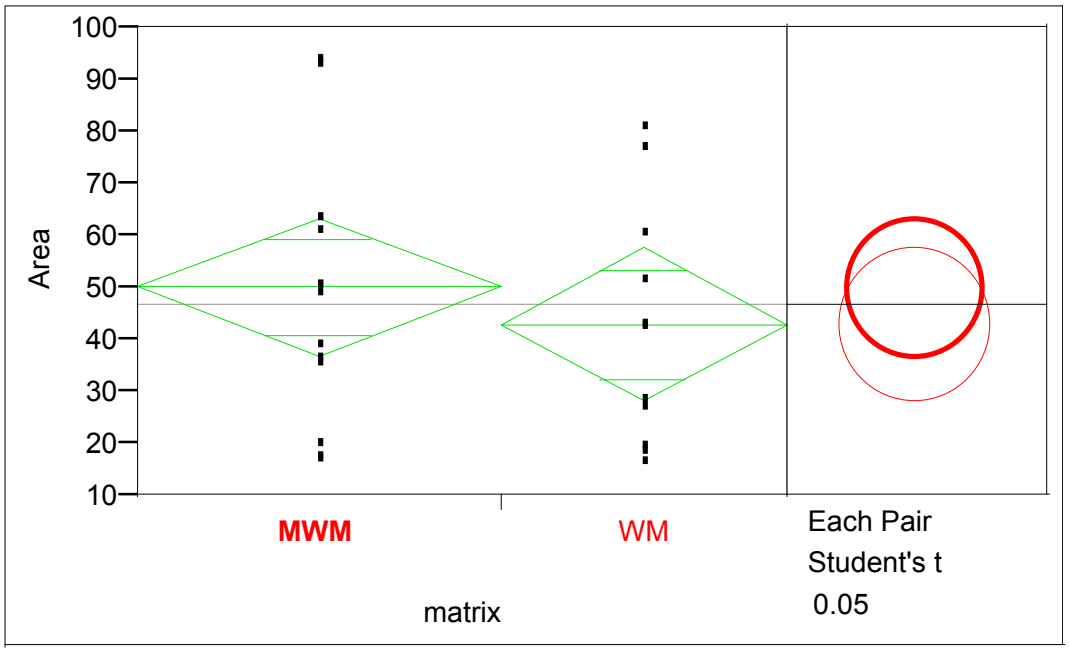
Level of significance α = 0.05

	k										
n - 1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2	39.0	87.5	142	202	266	333	403	475	550	626	704
3	15.4	27.8	39.2	50.7	62.0	72.9	83.5	93.9	104	114	124
4	9.6	15.5	20.6	25.2	29.5	33.6	37.5	41.1	44.6	48.0	51.4
5	7.15	10.8	13.7	16.3	18.7	20.8	22.9	24.7	26.5	28.2	29.9
6	5.82	8.38	10.4	12.1	13.7	15.0	16.3	17.5	18.6	19.7	20.7
7	4.99	6.94	8.44	9.70	10.8	11.8	12.7	13.5	14.3	15.1	15.8
8	4.43	6.00	7.18	8.12	9.03	9.78	10.5	11.1	11.7	12.2	12.7
9	4.03	5.34	6.31	7.11	7.80	8.41	8.95	9.45	9.91	10.3	10.7
10	3.72	4.85	5.67	6.34	6.92	7.42	7.87	8.28	8.66	9.01	9.34
12	3.28	4.16	4.79	5.30	5.72	6.09	6.42	6.72	7.00	7.25	7.48
15	2.86	3.54	4.01	4.37	4.68	4.95	5.19	5.40	5.59	5.77	5.93
20	2.46	2.95	3.29	3.54	3.76	3.94	4.10	4.24	4.37	4.49	4.59
30	2.07	2.40	2.61	2.78	2.91	3.02	3.12	3.21	3.29	3.36	3.39
60	1.67	1.85	1.96	2.04	2.11	2.17	2.22	2.26	2.30	2.33	2.36
∞	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Kanji, Gopal K. **100 Statistical Tests**. London : SAGE Publication Ltd., 1993.

Annex A.3 Accuracy test statistical results

One-way Analysis of Area By matrix



One-way Anova

Summary of Fit

Rsquare	0.022713
Adj Rsquare	-0.01978
Root Mean Square Error	23.86174
Mean of Response	46.91076
Observations (or Sum Wgts)	25

t Test

Assuming equal variances

	Difference	t Test	DF	Prob > |t|
Estimate	7.02916	0.731	23	0.4721
Std Error	9.61417			
Lower 95%	-12.8593			
Upper 95%	26.91758			

Unequal Variances

	Difference	t Test	DF	Prob > |t|
Estimate	7.029	0.737	22.1911	0.4690
Std Error	9.540			
Lower 95%	-12.899			

	Difference	t Test	DF	Prob > |t|
Upper 95%	26.958			

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
matrix	1	304.360	304.360	0.5345	0.4721
Error	23	13095.800	569.383		
C. Total	24	13400.160			

Means for One-way Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
MWM	14	50.0036	6.3773	36.811	63.196
WM	11	42.9744	7.1946	28.091	57.858

Std Error uses a pooled estimate of error variance

Means Comparisons

Dif=Mean[i]-Mean[j]	MWM	WM
MWM	0.0000	7.0292
WM	-7.0292	0.0000

Alpha=0.05
Comparisons for each pair using Student's t

t	Alpha
2.06866	0.05

Abs(Dif)-LSD	MWM	WM
MWM	-18.657	-12.859
WM	-12.859	-21.048

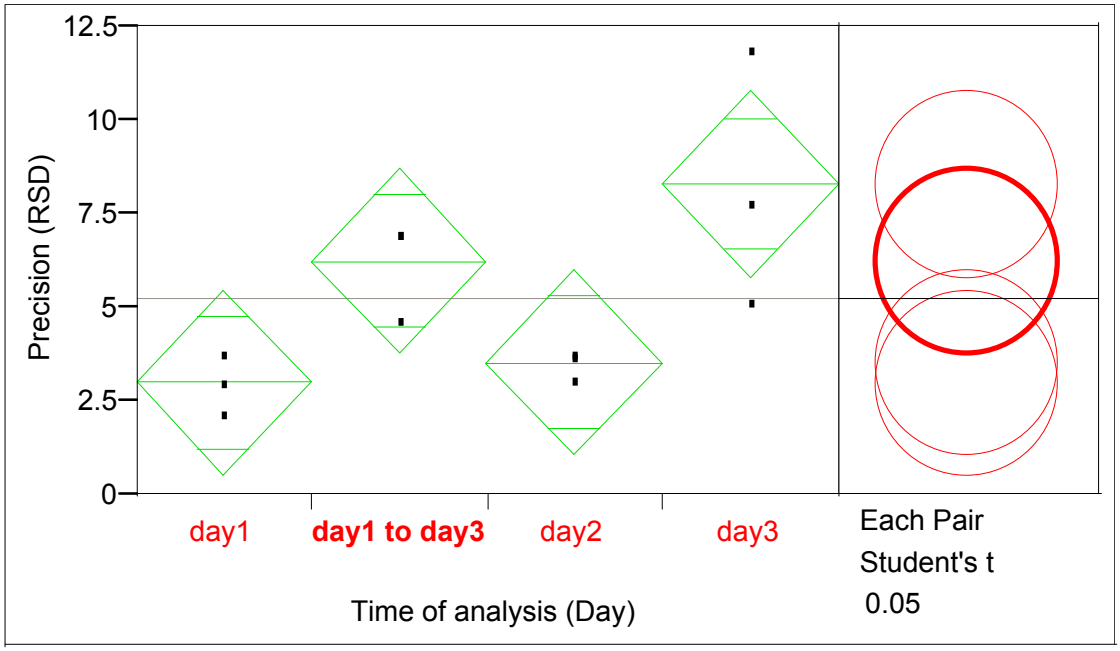
Positive values show pairs of means that are significantly different.

Level		Mean
MWM	A	50.003593
WM	A	42.974436

Levels not connected by same letter are significantly different

Annex A. 4 Intraday precision JMP statistical output

One-way Analysis of Precision (RSD) by time of analysis (Day)



One-way Anova
Summary of Fit

Rsquare	0.663396
Adj Rsquare	0.53717
Root Mean Square Error	1.864877
Mean of Response	5.266667
Observations (or Sum Wgts)	12

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Time of analysis (Day)	3	54.833333	18.2778	5.2556	0.0270
Error	8	27.822133	3.4778		
C. Total	11	82.655467			

Means for One-way Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
day1	3	3.00000	1.0767	0.5172	5.483
day1 to day3	3	6.23333	1.0767	3.7505	8.716

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
day2	3	3.53333	1.0767	1.0505	6.016
day3	3	8.30000	1.0767	5.8172	10.783

Std Error uses a pooled estimate of error variance

Means Comparisons

Dif=Mean[i]-Mean[j]	day3	day1 to day3	day2	day1
day3	0.0000	2.0667	4.7667	5.3000
day1 to day3	-2.0667	0.0000	2.7000	3.2333
day2	-4.7667	-2.7000	0.0000	0.5333
day1	-5.3000	-3.2333	-0.5333	0.0000

Alpha=0.05

Comparisons for each pair using Student's t

t	Alpha
2.30600	0.05

Abs(Dif)-LSD	day3	day1 to day3	day2	day1
day3	-3.5113	-1.4446	1.2554	1.7887
day1 to day3	-1.4446	-3.5113	-0.8113	-0.2779
day2	1.2554	-0.8113	-3.5113	-2.9779
day1	1.7887	-0.2779	-2.9779	-3.5113

Positive values show pairs of means that are significantly different.

Level			Mean
day3	A		8.3000000
day1 to day3	A	B	6.2333333
day2		B	3.5333333
day1		B	3.0000000

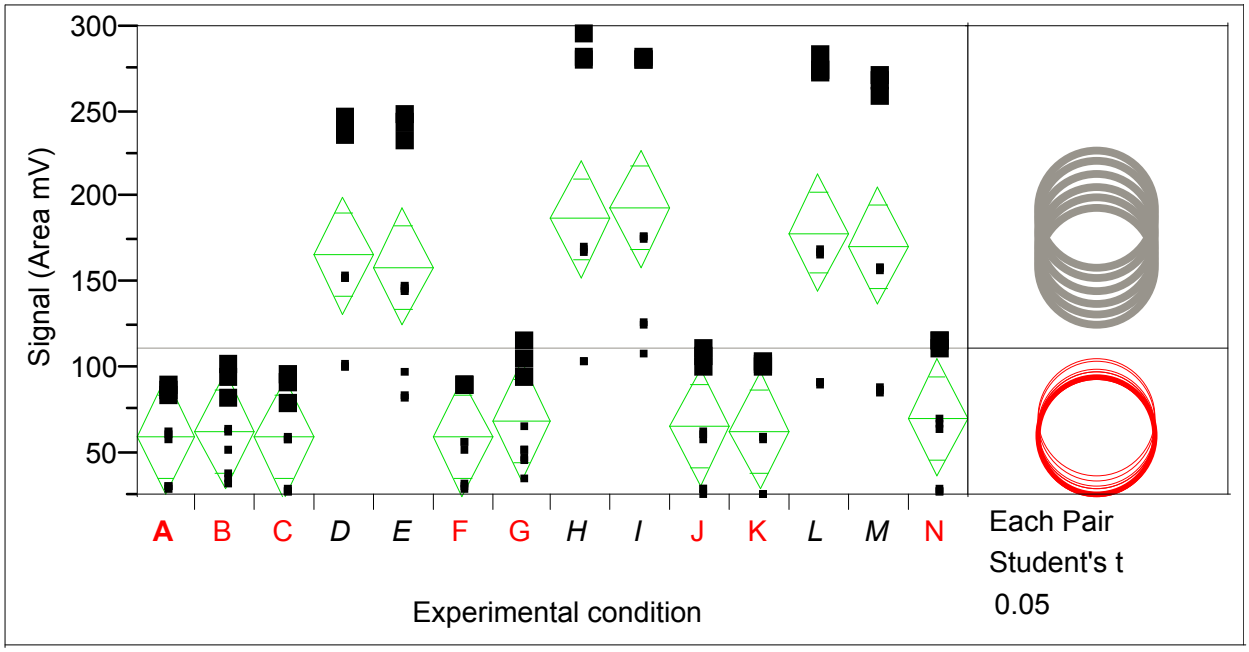
Levels not connected by same letter are significantly different

Annex A.5 Variables combination output by Minitab statistical package

StdOrder	RunOrder	CenterPt	Blocks	Oven temperature (^{o}C)	Solvent B %Methanol	Excitation/ Emission (nm)	Solvent A and B Acetic acid (mL)
2	1	1	1	27	88	280/310	20
3	2	1	2	25	90	280/310	20
9	3	1	3	25	88	280/310	20.4
1	4	1	1	25	88	280/310	20
10	5	1	3	27	88	280/310	20.4
7	6	1	2	25	90	286/316	20
14	7	1	3	27	88	286/316	20.4
11	8	1	4	25	90	280/310	20.4
8	9	1	2	27	90	286/316	20
4	10	1	2	27	90	280/310	20
12	11	1	4	27	90	280/310	20.4
15	12	1	4	25	90	286/316	20.4
6	13	1	1	27	88	286/316	20
5	14	1	1	25	88	286/316	20
13	15	1	3	25	88	286/316	20.4
16	16	1	4	27	90	286/316	20.4

Annex A. 6 Robust comparison of RSD of different variables JMP statistical package output

One-way Analysis of Signal (Area mV) By Experimental condition



One-way Anova

Summary of Fit

Rsquare	0.567989
Adj Rsquare	0.517845
Root Mean Square Error	52.11123
Mean of Response	111.6168
Observations (or Sum Wgts)	126

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Experimental condition	13	399876.26	30759.7	11.3271	<.0001
Error	112	304144.96	2715.6		
C. Total	125	704021.22			

Means for One-way Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
A	9	59.561	17.370	25.14	93.98
B	9	62.903	17.370	28.49	97.32
C	9	58.910	17.370	24.49	93.33
D	9	165.850	17.370	131.43	200.27
E	9	159.288	17.370	124.87	193.70
F	9	58.890	17.370	24.47	93.31
G	9	68.537	17.370	34.12	102.95
H	9	186.955	17.370	152.54	221.37
I	9	193.300	17.370	158.88	227.72
J	9	65.362	17.370	30.94	99.78
K	9	62.611	17.370	28.19	97.03
L	9	179.240	17.370	144.82	213.66
M	9	171.094	17.370	136.68	205.51
N	9	70.135	17.370	35.72	104.55

Std Error uses a pooled estimate of error variance

Means Comparisons

Alpha=0.05

Comparisons for each pair using Student's t

t	Alpha
1.98137	0.05

Positive values show pairs of means that are significantly different.

Level			Mean
I	A		193.29961
H	A		186.95514
L	A		179.23958
M	A		171.09358
D	A		165.85039
E	A		159.28766
N		B	70.13451
G		B	68.53744
J		B	65.36210
B		B	62.90296
K		B	62.61142
A		B	59.56113
C		B	58.90963
F		B	58.88999

Levels not connected by same letter are significantly different

Positive values show pairs of means that are significantly different.

Dif=Mean[i]-Mean[j]	I	H	L	M	D	E	N	G	J	B	K	A	C	F
I	0.00	6.34	14.06	22.21	27.45	34.01	123.17	124.76	127.94	130.40	130.69	133.74	134.39	134.41
H	-6.34	0.00	7.72	15.86	21.10	27.67	116.82	118.42	121.59	124.05	124.34	127.39	128.05	128.07
L	-14.06	-7.72	0.00	8.15	13.39	19.95	109.11	110.70	113.88	116.34	116.63	119.68	120.33	120.35
M	-22.21	-15.86	-8.15	0.00	5.24	11.81	100.96	102.56	105.73	108.19	108.48	111.53	112.18	112.20
D	-27.45	-21.10	-13.39	-5.24	0.00	6.56	95.72	97.31	100.49	102.95	103.24	106.29	106.94	106.96
E	-34.01	-27.67	-19.95	-11.81	-6.56	0.00	89.15	90.75	93.93	96.38	96.68	99.73	100.38	100.40
N	-123.17	-116.82	-109.11	-100.96	-95.72	-89.15	0.00	1.60	4.77	7.23	7.52	10.57	11.22	11.24
G	-124.76	-118.42	-110.70	-102.56	-97.31	-90.75	-1.60	0.00	3.18	5.63	5.93	8.98	9.63	9.65
J	-127.94	-121.59	-113.88	-105.73	-100.49	-93.93	-4.77	-3.18	0.00	2.46	2.75	5.80	6.45	6.47
B	-130.40	-124.05	-116.34	-108.19	-102.95	-96.38	-7.23	-5.63	-2.46	0.00	0.29	3.34	3.99	4.01
K	-130.69	-124.34	-116.63	-108.48	-103.24	-96.68	-7.52	-5.93	-2.75	-0.29	0.00	3.05	3.70	3.72
A	-133.74	-127.39	-119.68	-111.53	-106.29	-99.73	-10.57	-8.98	-5.80	-3.34	-3.05	0.00	0.65	0.67
C	-134.39	-128.05	-120.33	-112.18	-106.94	-100.38	-11.22	-9.63	-6.45	-3.99	-3.70	-0.65	0.00	0.02
F	-134.41	-128.07	-120.35	-112.20	-106.96	-100.40	-11.24	-9.65	-6.47	-4.01	-3.72	-0.67	-0.02	0.00

Annex A.7 Performance check of the HPLC-FD system

Purpose

The purpose of this test is to confirm that the chromatographic data can be obtained with good repeatability. An HPLC System for this inspection consists of the pump, detector, column oven, auto injector, and data system.

Preparation for Inspection

(1) Prepare the following parts and reagents.
(a) Gradient LC system
(b) Mobile phases
Solvent A: acetonitrile (HPLC grade or equivalent)
Solvent B: water (HPLC grade or equivalent)
(c) Column: (4.6mm ID X 150mm)
(d) Sample: Mixture of two components (Acridine and Anthracene)

Method for sample preparation

Place 10mg of Acridine and 10mg of Anthracene in the volumetric flask of 100ml capacity. Add 100ml acetonitrile and shake it to dissolve. Transfer 1ml portion of the sample solution into the 10ml volumetric flask and add acetonitrile, resulting in a total volume of 10ml.

(e) Water (HPLC grade or equivalent)

(f) Isopropyl alcohol

(2) Check connection of the units. Refer to

(3) Before installing the column, check tubing connection of the LC system. Use tubing with 0.3mm I.D. or less from the outlet of auto injector to the column inlet and from the column outlet to the detector inlet. Length of the tubing should be 30cm or less to minimize the extra-column band broadening.

(4) Flush the flow line with appropriate solvents dependent on the operation status of the system. General guideline is shown below. To flush the flow line with solvents, connect the inlet and outlet tubing of the column directly using a proper union.

The column should be connected after flushing.

Procedure

(1) Set the flow rate of the pump to 1ml/min, initial concentration of solvent B to 50%, and column oven temperature to 40°C. Then start pump flow and temperature control. Then, confirm that the solvent comes out from the outlet tubing of the detector and no leak of solvent is observed at the connection.

(2) Set the parameters of RF-2000.
EX (nm) : 360 GAIN : 1
EM (nm) : 450 LAMP : 1
Response: 2 RATIO: 1
Sens: 2

(3) Monitor the baseline. When a stable baseline is obtained, press the zero point adjustment key of the detector. Then, inject 10µl of mobile phase and confirm no peak is observed.

(4) Next, inject 10µl of test sample solution. Repeat measurement at least five times.

(5) Obtain the chromatographic data of retention time, peak area, and peak height from five successive analyses.

(6) Calculate the average (X) of the data and %RSD

The acceptance criteria for the %RSD

Retention time: 0.5% or less

Peak area: Acridine 5.0% or less, Anthracene 2.0% or less

Peak height: Acridine 5.0% or less, Anthracene 2.0% or less

(It is necessary that changes of room temperature are less than 2°C)

Because the fluorescence intensity of Acridine changes more than that of Anthracene by the fluctuation of the room temperature, the %RSD of Acridine is larger than that of Anthracen

Annex A. 2 Performance attribute and test frequency for HPLC modules and test frequency

Module	Performance Attributes	General Expectations	Frequency
Pump	Flow rate accuracy	±2% of the set flow rate	6 months
	Gradient accuracy	±1% of the step gradient composition	6 months
	Pressure test	Proper functioning check valve Pressure decay: <75 psi/min No leak from the pump	6 months
Injector	Precision	1% RSD	6 months
	Linearity	$r \geq 0.999$	12 months
	Carryover	<1%	6 months
Detector	Wavelength accuracy	±2 nm	6 months
	Linearity of response	$r \geq 0.999$	12 months
	Noise and drift	Noise: 10^{-5} AU Drift: 10^{-4} AU/h	12 months
Column compartment	Temperature accuracy	±2°C of the set temperature	6 months