Increased-rate stability studies for St John's wort (*Hypericum perforatum*), *Ginkgo biloba* and Kava Kava (*Piper methysticum*) under unfavourable environmental conditions.

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PREFACE

I hereby confirm that this is my own work, and that it has not been submitted to any other institution.

Andre Marais

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CHAPTER 3. Results and discussion

SUMMARY

This was a chemical laboratory study. The main focus was to evaluate the chemical stability of *Hypericum perforatum* (St John's wort), *Ginkgo biloba* and *Piper methysticum* (Kava Kava) under unfavourable environmental conditions. Different dosage forms representing the same amount of active ingredients for each were used. Some of the dosage forms were self manufactured according to Good Manufacturing Practice. Samples of the dried powder of each plant was also exposed to a series of gamma-radiation.

Acetone was used as an extractant for all three plants, after evaluating and discarding the extraction method stipulated in the British Herbal Pharmacopoeia. Identification of the different plants were carried out by means of Thin Layer Chromatography. The in-house developed mobile phases EMW, BEA and CEF, showed better separation and visibility compared to the mobile phases used in the British Herbal Pharmacopoeia. The plates were sprayed with either vanillin or *p*-anisaldehide for optimal visualization of the separated compounds.

After the specified period of 6-months, comparative TLC was performed on all samples. This was achieved for each plant by applying all samples stored at a specific condition i.e.25°C, on the same plate. The samples were stored at low temperature after exposure to the specific time interval.

Quantitative analysis was performed by spectrophotometry, and high pressure liquid chromatography. The data obtained from these analytical methods, were used to evaluate the relative chemical stability of each dosage form. The relationship between the quantitative data and the qualitative changes in the TLC fingerprints, were compared, hoping to achieve a common pattern relating to the stability.

The order of the reaction as well as the reaction rate constant (*k*) for each dosage form was calculated, except for kava kava. The shelf-life (t_{90}) was calculated using the analyzed data obtained by spectrophotometry or HPLC. The relevance of conventional pharmaceutical calculations in the prediction of shelf-life, by means of accelerated stability tests, was investigated for the possible application to herbal products.

The effects of gamma radiation on the degradation of the chemical compounds present in each plant, was evaluated.

After an evaluation of all the relevant data, it seemed that the tablet-dosage forms were equally effective regarding stability, compared to the capsules. Liquid extracts appeared to be less stable than the extract capsules. The extract capsules seemed to degrade more rapidly than the herbal tablets or herbal capsules. Exposure to low dose radiation (4.4 kGy) did not seem to have an influence on the stability. It was evident that some herbs were more sensitive to sunlight or heat than others.

In general, all three of the chosen plants seemed to be relatively stable if stored in the specified conditions. It seemed valid for the shelf-life to be expressed as two years.

OPSOMMING

Die hoof klem van hierdie projek was om die chemiese stabiliteit van drie van die mees algemeen gebruikte natuurlike medisynes in Suid-Afrika te ondersoek. Verskillende doseervorme van St John's wort (*Hypericum perforatum*), *Ginkgo biloba,* en Kava Kava (*Piper methysticum)*, is vir die ondersoek gebruik. Uitsluitend Kava Kava, het die verskillende doseervorme elkeen oor dieselfde hoeveelheid aktiewe plant materiaal beskik. Waar geskikte doseervorme nie beskikbaar was nie, is dit self vervaardig. Alle vervaardiging het geskied onder sogenaamde Goeie Vervaardigings Praktyk (GMP), 'n vereiste gestel deur die medisyne- beheer- raad tydens vervaardiging van alle etiese produkte. Monsters van die gedroogde poeier van elke plant is ook blootgestel aan verskillende dosisse van gamma-bestraling.

In al drie plante was asetoon die gekose ekstraheermiddel, nadat daar besluit is om nie die ekstraksie metode, soos vervat in die British Herbal Pharmacopoeia, te implementeer nie. Identifikasie van die verskillende plante is ook uitgevoer deur middel van dunlaag chromatografie. Met die gebruik van ons eie ontwikkelde mobiele fases, EMW, CEF en BEA in die plek van die mobiele fases soos vermeld in die BHP. Die skeiding en visualisering van die bande in die verskillende plante was meer duidelik waarneembaar met ons eie metodes. Die verskillende dunlaagplate is gesproei met vanillien of anysaldehied vir die optimale visualisering van die geskeide komponente.

Na die verstryk van die gespesifiseerde 6-maande, is die verskillende dunlaagplate met mekaar vergelyk. 'n Goeie vergelyking kon getref word deur elke monster wat by dieselfde kondisie onderworpe was, op dieselfde plaat aan te wend bv. Al die monsters van kava kava wat by 25°C gestoor was, is op dieselfde plaat aangewend. Na die onttrekking van die monsters by elke gekose tydsinterval, is it by 'n lae temperatuur gestoor totdat analises daarop gedoen kon word.

Kwantitatiewe analise is uitgevoer deur gebruik te maak van spektrofotometrie sowel as hoë-druk vloeistof chromatografie. Die data wat deur hierdie analitiese metodes verkry was, is gebruik om die chemiese stabiliteit in elke doseerform te evalueer. Die verwantskap tussen die ge-analiseerde data en die dunlaag-identifikasie profiele is ondersoek, met die hoop dat daar 'n sekere mate van ooreenstemming getoon kon word, of 'n waarneembare patroon wat 'n moontlike toepassing op die stabiliteit kon hê.

Die orde van die chemiese afbraak, sowel as die reaksie snelheids-konstante (*k*) is ook vir elke produk, behalwe Kava Kava bepaal. Die rakleeftyd (t_{90}) is ook vir elke produk bepaal deur die waardes uit die analises verkry uit spektrofotometrie en hoë-druk vloeistof chromatografie te gebruik. Die toepaslikheid van konvensionele farmaseutiese vergelykings in die skatting van 'n rakleeftyd, deur gebruik te maak van versnelde stabilitietstoetse, is ook ondersoek. Dit is uitgevoer met die hoop van 'n moonlike toepassing in natuurlike medisyne.

Na evaluering van al die relevante data het dit geblyk dat die tablette net so effektief, betreffende die stabiliteit, is in vergelyking met die kapsules. Verder het dit ook geblyk dat die vloeistof ekstrakte minder stabiel was as die ekstrak kapsules. Die ekstrak kapsules toon 'n vinniger afbraak as die tablette of die fyn-kruie kapsules. Blootstelling aan 'n lae dosis bestraling (4,4 kGy) het geen noemenswaardige invloed op die stabiliteit getoon nie.

Dit was duidelik dat sekere produkte meer sensitief teenoor blootstelling aan sonlig en hoë temperature was, as ander. Oor die algemeen het dit geblyk dat al drie hierdie plante oor 'n aanvaarbare stabiliteit beskik, tensy dit onder die regte bewaringstoestande gestoor word. 'n Vervaldatum van twee jaar op hierdie produkte blyk aanvaarbaar te wees.

LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
BEA	Benzene/Ethanol/Ammonium hydroxide [18/2/0.2 v/v/v]
BHP	British Herbal Pharmacopoeia
BP	British Pharmacopoeia
CEF	Chloroform/ethyl acetate/formic acid [10/8/2 v/v/v]
DI water	De-Ionized water
EC	Extract capsule
EMW	Ethylacetate/methanol/water [10/1/35 v/v/v]
GABA	Gamma Amino Butyric Acid
GMP	Good Manufacturing Practice
G-protein	Gluco-protein
HC	Herbal capsule
HIV	Human Immune deficiency Virus
HPLC	High Pressure Liquid Chromatography
HT	Herbal Tablet
LIQ	Liquid Extract
MCC	Medicine Control Councel
MAO	Monoamine oxidase
MeOH	Methanol
OTC	Over the Counter
PAF	Platelet activation factor
Rf	Retention factor
TLC	Thin layer Chromatography

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Chapter 1

Background and Literature review

1.1 INTRODUCTION AND PROBLEM STATEMENT

The herbal market is becoming a multi-million dollar industry, with more people tending to use herbal supplements instead of conventional western medicine. It is estimated that one out of every three persons in America had used at least one form of alternative medicine in 1990 (Eisenberg et al., 1993). This can be seen in the average spend on the leading herbal products available in the United States (Johnston, 1997). A similar pattern can be assumed for South Africa.

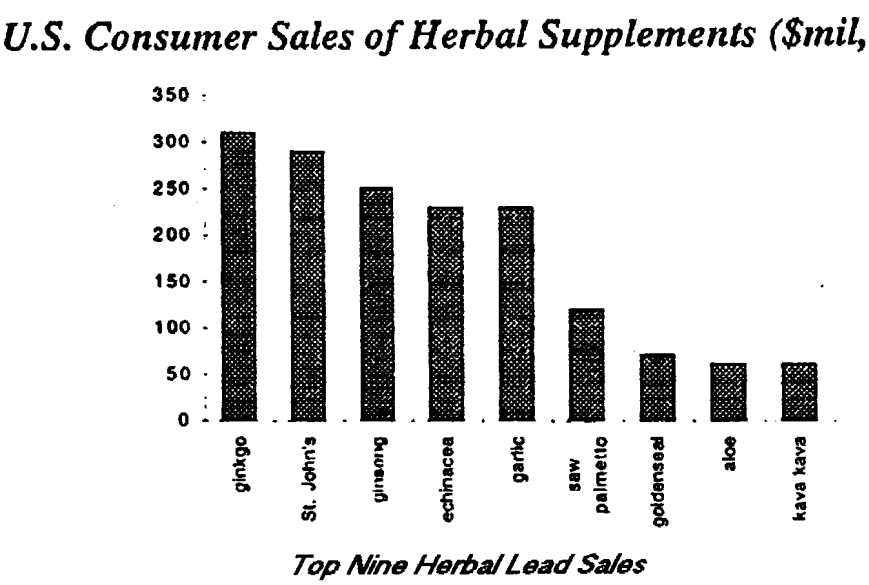


Figure 1.1 Lead Herbal sales in the United States for 1998 (Anon., 1999)

We generally accept that freshly harvested material is the best, and that an increase in the amount of chemical changes that occur, can be seen as an indication of a lower quality. This hypothesis has not been proven.

Taking this into account and considering the most commercially available herbal products in South Africa, it was decided to use *Hypericum perforatum* (St John's wort), *Ginkgo biloba* (Maidenhair tree) and *Piper methysticum* (Kava Kava) as subject for this study. The stability of different dosage forms currently available e.g. capsules, tablets and drops will

be examined. The stability will be evaluated after exposure to the following parameters: increased temperature, moisture (humidity), and light. Investigation will be carried out by analyzing the chemical components present in the extracts by making use of Thin Layer Chromatography, Spectrophotometry and High Pressure Liquid Chromatography.

No comparative information regarding stability tests could be found on any one of the above mentioned dosage forms. A similar study was however done on *Echinacea sp.*, but the investigators only compared the stability between the flowering herb powder vs. the dry root extract (Halloran, 2000).

Many herbal, nutritional and combination products are available in pharmacies today. An ever increasing number of patients use medicinal herbs or seek advice from their physician or pharmacist regarding their use. The popular use of medicinal herbs make it essential for health care professionals to become aware of the health benefits, risks and uncertainties, so that they can educate their patients about these issues (Tyler, 1996). Doctors and pharmacists often lack accurate information about the safety, efficacy and stability of herbal remedies. A common problem is that they are unaware of important similarities and differences between medicinal herbs and the Medicines Control Council's (MCC) approved medications. For example, some mistakenly think of herbs as "natural" alternatives to chemicals, which can do no harm or has no toxic effect.

Manufacturers market their herbs as nutritional supplements- a legislative loophole that allows products with no proven efficacy or established quality, to be marketed and sold as long as no claims of efficacy are printed on the product label. Herbal and nutritional (vitamin) supplements are available as high-dose preparations and patients can obtain them without the consent of a doctor. This adds to the concern of herb-drug and nutrient-drug interactions, about which little is known. The most frequent health problems treated with alternative remedies are chronic pain (37%), anxiety (31%), chronic fatigue (31%), sprains and muscle strains (26%), addiction (25%), arthritis (25%) and headaches (24%) (Austin, 1998). If people treat themselves, or if they are treated by professionals lacking accurate information regarding the safe use of alternative medicine, concern should be raised about the unsafe use of herbals.

Data about the safety and efficacy of medicinal herbs are limited in many ways. Clinically important information is particularly sparse in the literature, regarding the results of negative trials, drug interactions, effects on pregnant or lactating woman, children and the elderly, and the stability of, and quality control on the preparations. Unlike the MCC

approved Over the Counter (OTC) and prescription medications, medicinal herbs are not required at this stage to demonstrate safety or efficacy prior to marketing, nor are they regulated for quality. This is a problem in many parts of the world, including South Africa. Unlike the MCC approved drugs, which are defined pure standardized substances, herbs contain a great variety of constituents. Usually there is a great difference in concentration of the active compound(s) depending on the growing conditions, plant parts used, time of harvesting, preparation and storage (Liberti and DerMaderosian, 1978). We often do not know the active compounds or interaction of biological active principles. These variables can have an influence on the effectivity and stability of the preparations, and need to be investigated.

Although not a requirement, quality control and stability has become an issue in the herbal medicine trade, due to the interest in these alternative sources of medicine by the public. To ensure consistent results with herbal supplements, attention must be given to the processing and preparation of these products. The use of standardized herbal preparations will help to create reliable and predictable results for the persons utilizing these medicines, as well as performing clinical trials in a reproducible scientific setting (Seligman, 1998).

Aspects such as the uncontrolled conditions of use and sale of herbal products need to be addressed. Storage conditions are hardly ever mentioned on the product labels, and a lack of accurate and well-detailed information in the form of package inserts is often omitted. Herbal products are seldom stored in the dispensary of e.g. a pharmacy, where the temperature is supposed to be regulated below 25°C. Instead it is often exhibited on separate shelves near the windows where it is often subjected to direct sunlight and a large variation in temperatures.

During the manufacturing of herbal products, the active ingredients often have to undergo a series of radiation exposures in order to decrease the microbial load in the product. One has to ask the question if the radiation has any effect on the active ingredients itself?

Recognizing which companies have a commitment to quality control and standardization, is an important aspect of herbal medicine. Adequate quality control may become a strong marketing focus, depending on the proposals drafted in the new SAMDRA act. The objective of this new legislation requirements must be to demonstrate stability throughout the proposed shelf-life when stored under the proposed storage conditions (Cameron, 1998). Very few herbal companies provide information concerning their manufacturing and

processing techniques. Care should therefore be taken when considering the use of their products (Hardley, 1999).

1.2 ST JOHN'S WORT (*Hypericum perforatum*)

Figure 1.2 *Hypericum perforatum* flowering plant

1.2.1 History

St John's wort is a common roadside plant, which grows wild in most parts of the world (Anton, 1997). St John's wort is believed to have originated in Europe, Western Asia and North Africa. The plant was brought to the North-eastern United States by European Colonists (Pickering, 1879 cited O'Hara et al., 1998).

It is a perennial plant belonging to the family *Clusiaceae* that grows to a height of 28 to 70 centimeters. This shrub is characterized by it's 5-petalled yellow flowers (Tyler, 1998). Visual examination of the flowers reveal small black dots. These black dots produce a red stain when it is rubbed between one's fingers (Hobbs, 1989). According to Orth et al. (1998), this red pigment contains the active constituent hypericin.

The leaves of the plant display a number of bright translucent dots when held up to the light. This perforated look led to the Latin name *perforatum* (Brown, et al., 1998). It's scientific name derives from the Greek word *yperikon,* meaning, "to overcome an apparition". This relates to the ancient belief in the plant's ability to ward off evil spirits (Hobbs, 1989). There are many explanations as to how the common name St John's wort

was derived. One belief is that the red spots appeared on the leaves during the anniversary of St John's beheading, symbolizing his blood. Another refers to the fact that the flowers bloom around St John's day (June 24), and another belief refers to the ancient English tradition of throwing the flowers into a bonfire on the eve of St John's day (Anton, 1997).

St John's wort has been used for thousands of years for many conditions. This includes the topical use for healing wounds, as a diuretic and the treatment of neuralgic conditions such as sciatica and hip pain. It was also used as a folk remedy for kidney and lung ailments as well as depression (Lindle, et al., 1996).

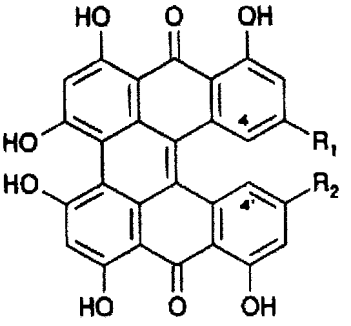
St John's wort is by far the most common antidepressant used in Germany. Here it is prescribed 4 times more often than Prozac® (Blumenthal, et al., 1998). Sales in the United States increased 20-fold between 1995 and 1997, from $10 million to $200 million annually (Anon., 1999).

1.2.2 Chemistry

St John's wort contains numerous compounds with biological activity. Although many of the compounds have been isolated, the active antidepressant principles have not been identified (Tyler, 1998). Most researchers consider its effects to be due to a variety of constituents rather than any single component (Lindle, et al., 1996).

Constituents which have been identified include the naphthodiantrones (0.1-0.3% hypericin and pseudohyperiscin), a broad range of flavonoids (0.5-1% rutin and hyperoside), essential oil (0.05-0.3% a-pinene and cineole), antraquinones, carotenoids and cumarine (Muller, et al., 1997).

A standardized extract containing 0.3% hypericin (LI160) is used in clinical studies to make results comparable (Holzl, et al., 1989).



4-4': Hypericin
$R_1 = R_2 = CH_3$
4-4': Pseudohypericin
$R_1 = CH_3$, $R_2 = CH_2OH$

Figure 1.3 Chemical structure for hypericin and pseudohypericin

1.2.3 Mechanism of Therapeutic Action

The mechanism of St John's wort 's antidepressant action is not fully known. Some early *in vitro* studies demonstrated monoamine oxidase inhibition, but only at concentrations unattainable *in vivo* (Anton, 1997). It also showed an activation of dopamine receptors, but a reduction of serotonin receptors resulting in the impaired uptake of serotonin by post-synaptic neurons. Hypericin has a high affinity for gamma-aminobutyric acid (GABA), (Muller and Russol, 1994) with a median inhibitory concentration of 60 ng/ml for $GABA_A$ and 9 ng/ml for $GABA_B$ (Cott, 1997). Stimulation of gamma-aminobutyric acid is known to have antidepressant effects (Sanders-Bush and Mayer, 1996). St John's wort extract also inhibits the action of interleucin-6, a cytokine that may contribute to the increased cortisol production noted in major depression (Brown, et al., 1999).

A common feature of antidepressant drugs, including St John's wort, is a lag phase of up to eight weeks before the full therapeutic effect becomes noticeable. For prescription drugs this lag phase has been postulated to be due to long-term adaptations within the central nervous system that correct imbalances in the brain amine neurotransmitter receptors which is then translated into a therapeutic effect (Brown, et al., 1999).

1.2.4 Clinical studies

Many studies have been performed on this herb, mostly in Germany (Fugh-Berman, 1999). A recent meta-analysis evaluated 23 randomized trials (20 were double blind) of St John's wort in a total of 1757 outpatients with mild to moderate depression (Lindle, et al., 1996). Improvement in depressive symptoms was observed in all groups. In 15 placebo-

controlled trials, St John's wort was found to be significantly more effective than placebo. In eight treatment-controlled trials, clinical improvement in those receiving St John's wort did not differ significantly from those receiving tricyclic antidepressants.

Most trials were 4 to 8 weeks in duration. The trials in this meta-analysis were heterogenous and used various diagnostic criteria and dosages of the herb. In 20 trials, single-herb preparations were tested; the remainder tested combination herb products. Thirteen trials compared a single hypericum preparation with placebo and provided data on treatment responders; of these, 55.1% of those receiving the herb improved, compared with 22.3% of those receiving placebo. No significant differences in treatment effect were found between single-herb preparations of St John's wort and standard antidepressants. Combination products (containing both hypericum and the sedative herb valerian) also were not significantly different from standard antidepressants. Side effects were reported less often with St John's wort; 19.8% of those on St John's wort reported symptoms, compared with 52.8% of those on tricyclic antidepressants (Fugh-Berman, et al., 1999).

One 6-week trial from the United Kingdom (Wheatley, 1977) using DSM-IV criteria found that the proportion of patients responding to a daily dose of 75 mg of amitriptyline (34 of 78) was similar to those responding to 900 mg of hypericum (37 of 87), but by the end of the study, the total decrease in depression scores favored amitriptyline.

1.2.5 Clinical indications

The standardized extract containing 0.3% hypericin is used as a mild to moderate antidepressant in a dose of 300 mg three times per day (Blumenthal, et al., 1998). Recent studies concentrate on it's antiviral (including HIV/AIDS) effects (Lavie, et al., 1995).

Hypericum has also been widely used for the treatment of enuresis (bed-wetting) in children due to nerve irritation in the bladder (Blumenthal, et al., 1998).

It has been administered as an overnight retention enema in patients with inflammatory bowel syndrome (Hahn, 1992). It has also been used for gastric conditions, including gastric ulcer and functional gastritis (Krylof, 1993 cited Anton, 1997).

Topical forms of hypericum are used for bites, bruises, burns, frostbite, hemorrhoids, myalgia, scrapes and sunburn (Blumenthal, et al., 1998).

1.2.6 Adverse effects and toxicity

The side effects are mild, but gastrointestinal symptoms, dry mouth, nervousness and fatigue have been reported (Lindle, et al., 1996). The most predictable side effect is photosensitization, mainly in fair-skinned people (Brockmoller, et al., 1997). Photosensitization is generally mild and transient, disappearing within a few days of drug discontinuation. St John's wort should not be used during pregnancy or in combination with other psycho-active agents (Gordon, 1998).

1.3 *GINKGO BILOBA*

Figure 1.4 ***Ginkgo biloba*** **leaves**

1.3.1 History

The Ginkgo tree, also known as the Maidenhair tree, is the only remaining species of the family Ginkgoaceae. Fossil records exist which date back more than 200 million years. These records prove that the Ginkgo tree is the oldest surviving tree species in the world (Kleijnen and Knipschild, 1992). It is a decorative tree often planted in cities because of its resistance to pollution, insects and disease (Fugh-Berman, et al., 1999). Ginkgo trees are native to China, and were reportedly brought to Europe in 1730 and to the USA in 1784 (Kleijnen and Knipschild, 1992).

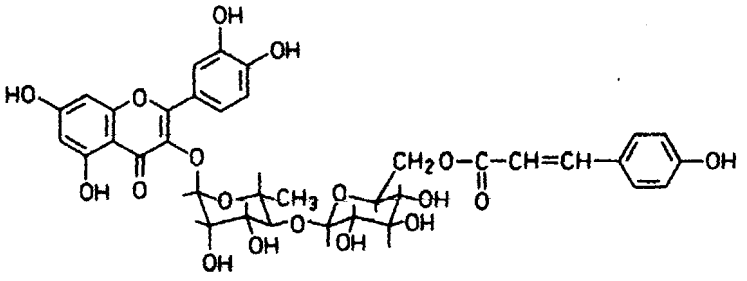
(center) and CEF (bottom) and sprayed with *p*-anisaldehide (left) and vanillin (right). Each plate shows extraction with Methanol (left), Acetone (center), and n-Hexane (right). R_f values for hypericin are indicated.

Ginkgo trees can easily be identified by their fan shaped leaves. They can grow as high as 40 meters and individual trees can live as long as 1000 years (Brown, 1998). For 5000 years traditional Chinese medicine have used ginkgo to treat asthma and as a memory enhancer (Busse, 1994). The herbal remedy prepared from its leaves today has been developed in the last twenty years, and is relatively new (Tyler, 1994). Germany was the first to produce a concentrated extract of the leaves. This extract known as EGb 761 was standardized on 24 % ginkgo flavone glycosides and 6 % terpene lactones (Drieu, 1988).

Most of the research on *Ginkgo biloba* has been conducted in Germany, and their federal health authorities have concluded that treatment with EGb 761 is safe and effective for peripheral and cerebral circulatory disturbances, including claudication and memory impairment (Blummenthal, et al., 1998). Sales in the United States reached 240 million dollars in 1997 (Anon, 1999) and by the end of 1998, more prescriptions were written (5.4 million) for *Ginkgo biloba* extract by physicians in Germany than for any other drug (Tyler, 1994). In many countries, including South Africa, it is also available as an over-the-counter (OTC) drug (Robbers, 1999). Large plantations of Ginkgo trees have been established to supply the market with adequate quantities of the leaves. These planted trees are pruned to shrub height allowing easy mechanical picking. After the leaves are picked, they are dried and shipped to Europe for processing (Tyler, 1994).

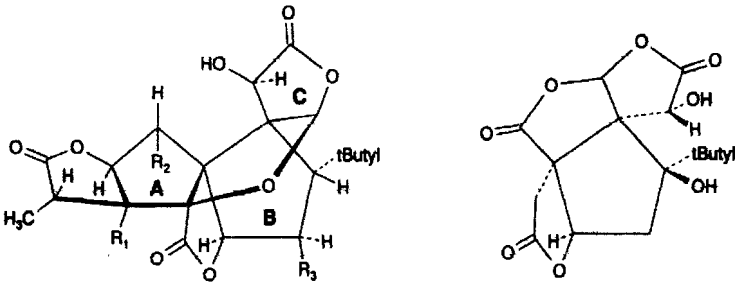
1.3.2 Chemistry

The dried leaves are milled and treated with an acetone-water mixture. This extract is then adjusted to a standardized strength containing 22% to 27% flavonoid glycosides (including quercetin, kaempferol and isorhamnetin glycosides) and 5% to 7% terpene lactones (concisting of 2.8-3.4% ginkgolides A,B and C and 2.6-3.3% bilobalide) (DeFeudis, 1998). The extract also contains rutin and some organic acids (6-hydroxykynurenic, shikimic acid, protocatechuic acid, vanillic acid and *p*-hydroxybenzoic acid) (Wagner and Bladt, 1996).



Quercetin-α-rhamnopyranosyl-4"-O-β-D-(6"'-trans-p-coumaroyl)-glucopyranoside

Figure 1.5 Chemical structure for quercetin



Ginkgolide	R_1	R_2	R_3
A	OH	H	H
B	OH	OH	H
C	OH	OH	OH

Bilobalide

Figure 1.6 Chemical structure for ginkgolide A,B,C and bilobalide

1.3.3 Mechanism of Therapeutic Action

The mechanism of Ginkgo's therapeutic effects are not fully understood. It is believed to be attributed to the synergistic effects of it's constituents, particularly the flavonoids, terpenoids and organic acids (O'Hara, et al., 1998).

The flavone glycosides (quercetin, kaempferol and isorhamnetin) are responsible for the anti-oxidant properties by functioning as scavengers of free-radicals, and thus preventing oxidative modification of cellular proteins (Maitra, et al., 1995 and Alaoui-Youssefi, et al.,

1999). The rutin-type flavonoids can reduce capillary fragility and increase the threshold of blood loss from the capillary vessels, and thus prevent structural (ischemic) brain damage (Drieu, 1988).

Platelet-activating-factor (PAF) mimics many of the features seen in the allergic response. It induces aggregation of the blood platelets. It also causes bronchoconstriction, cutaneous vasodilation, chemotaxis of phagocytes, hypotension and the release of inflammatory compounds such as leukotrines, prostaglandin, liposomal enzymes and superoxide from phagocytes. PAF further stimulates G-protein linked cell-surface receptors, which causes activation of phospholipase A_2. This results in the formation of arachidonic acid, which is converted to prostaglandins, tromboxane A_2 and leukotrines. The binding of PAF to its receptor on platelets unmasks cell-surface binding sites for fibrinogen that promote platelet aggregation and trombus formation (Vander, 1994).

The terpene lactones (ginkgolide A,B and C) block all of these actions, with the most significant effect being an increase in blood fluidity, thereby improving circulation. Bilobalide acts synergistically with the ginkgolides to improve the tolerance of brain tissue to hypoxia and to increase cerebral circulation (Krieglstein, 1994).

1.3.4 Clinical studies

A standardized extract known as Egb761 is used in clinical studies (DeFeudis, 1998).

A randomized, double blind, placebo controlled trial of 216 patients with Alzheimer's or multi-infarct dementia found that 240 mg/d for 6 months of a standardized ginkgo extract resulted in significant improvements in memory, attention, psyco-phatology and behavior compared with placebo (Kanowski, et al., 1996).

Another randomized, double blind, placebo controlled trial of 40 Alzheimer's patients, half of whom were given 240 mg/d of a standardized ginkgo extract for 3 months, found significant improvements in memory, attention and psycho-pathology in the group receiving ginkgo after 1 month (Hofferberth, 1994).

A double-blind study of 31-patients older than 50 years with mild to moderate memory impairment found a beneficial effect with 120 mg/d on some tests (e.g. digit copying and speed of response in a classification task) (Rai, et al., 1991).

1.3.5 Clinical indications

The most common uses include the treatment of intracerebral and peripheral vascular insufficiency, including intermittent claudication (O'Hara, et al., 1998). This condition is caused by sclerosis of the arteries of the leg and is characterized by a constant or cramping pain in the calf muscles brought on by walking a short distance (Ernst, 1996). The recommended dosage for this indication is 120 to 160 mg daily in divided doses taken with meals (Tyler, 1994).

It is also used to improve memory and social functioning in patients with mild to moderate dementia (Alzheimer disease or multi-infarct dementia), where a divided dosage of 240 mg daily is recommended (Hindemarch and Subhan, 1984).

Ginkgo biloba extract is also useful in the treatment of stroke patients and persons with transient ischemic attacks. It is also indicated for persons recovering from brain trauma and injury (Stein and Hoffman, 1992).

It can be considered an alternative for the treatment of depression in the elderly who is resistant to standard drug therapy. This is because depression is often an early sign of cognitive decline and cerebrovascular insufficiency in elderly patients (Brown, et al., 1999).

It is currently being investigated for use against mountain sickness (Roncin, et al., 1996), resulting from hypoxia at altitudes greater than 2000 meters (Cohen, 1997).

1.3.6 Adverse effects and toxicity

Therapeutic doses of ginkgo extract may cause mild gastro-intestinal irritation, headache allergic skin reactions, nausea, anxiety and sleep disturbances, but no serious adverse effects have been noted in clinical trials, including no mutagenicity or teratogenicity (O'Hara, et al., 1998).

Because of its anti-coagulant effects, it has been associated with bleeding problems, especially in patients taking warfarin. A similar problem is also seen with aspirin (Blumenthal, et al., 1999).

Ginkgo biloba seeds can cause fatal neurologic and allergic reactions and are not used medicinally (Woerdenbag and Van Beck, 1997).

1.4 KAVA KAVA (*Piper methysticum)*

Figure 1.7 Leaves of *Piper methysticum*

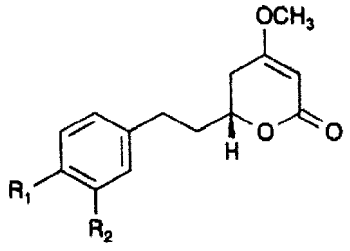
1.4.1 History

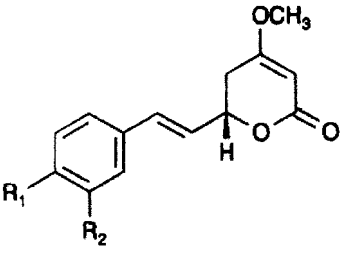
This scrub grows in the islands of the South Pacific, is about 2-2.5 meters tall and is usually located at altitudes of 150-300 meters above sea level in stony ground. Seventy-two different varieties, which range in color from white to black, green and red has been described by traditional crop growers.

Kava is made from the dried roots of the pepper plant, *Piper methysticum*, family Piperaceae, found in Polynesia, Melanesia and Micronesia (Singh, 1992). The Latin name, *Piper methysticum,* meaning intoxicating pepper, was given to the plant in 1777 by Johann Georg Forster (Steinmentz, 1960). The roots of this plant have been extensively used by the indigenous people of these Pacific islands in the preparation of an intoxicating beverage. Besides being the social beverage for chiefs and noblemen, it was also used to welcome visitors at formal gatherings, at initiation and completion of work, in preparing for a journey or an ocean voyage, celebration of birth, marriage and death, to cure illnesses and to remove curses (Singh, 1992). Kava is ritually prepared by pounding or grating the root into a bowel to which coconut milk is added. This muddy looking liquid is then rapidly drunk from coconut shells. It first causes a numbing effect in the mouth, followed by a relaxed sociable state in which fatigue and anxiety are lessened. Eventually a deep restful sleep ensues from which the user awakes refreshed and without a hangover (Morrison, 1935).

1.4.2 Chemistry

Kava is one of the few herbs for which active constituents are well delineated (Fugh-Berman, et al., 1999). These are the kavapyrones, including kawain, dihydrokawain, methysticin and dihydromethysticin (Schulz, et al., 1998).





Dihydrokawain	(0,6–1%)	$R_1 = R_2 = H$	Kawain	(1,8–2.1%)	$R_1 = R_2 = H$
Dihydromethysticin	(0,5–0,8%)	$R_1, R_2 = -OCH_2O-$	Methysticin	(1,2–2%)	$R_1, R_2 = -OCH_2O-$

Figure 1.8 Chemical structure for kawain, di-hydrokawain, methysticin and di-hydromethysticin

Roots of good quality contain between 5.5 and 8.3 percent of kavalactones, which is responsible for the central nervous system activity of the plant (Tyler and Robbers, 1999).

1.4.3 Mechanism of Therapeutic Action

Animal studies demonstrated the ability of kava extracts to induce sleep and produce muscle relaxation and analgesia, as well as anti-convulsive protection against strychnine and electroshocks. At low doses kavalactones resemble the pharmacological action of benzodiazepines although they do not bind to benzodiazepine binding sites in rat or mouse brain membranes (Davies, et al., 1992).

There are no significant interactions with gamma amino butyric acid (GABA) and the mechanism of action is still unknown. Considering the many pharmacological activities shown by kava, it may remain in the lipid membranes and act non-selective on a variety of receptors rather than interacting with one specific receptor (Heiligenstein and Guenther, 1998). *In vitro* studies have shown that the kava pyrones can inhibit monoamine oxidase-B (MOA-B) enzymes. This might also be an important mechanism by which kava exerts its

psychotropic activity (Uebelhack, et al., 1998). Evidence exists that the spasmolytic, analgesic and anti-convulsant activity might be explained by the inhibition of the current through both voltage-dependent calcium and sodium channels in the brain. This suppression reduces the excitability of neurons and causes a calming effect that may reduce seizure activity and decrease the release of pain-provoking chemicals (Gleitz, et al., 1996).

1.4.4 Clinical studies

For research and medicinal purposes, the root is extracted with organic solvents to yield the lipid soluble components. In this process a preparation standardized to 70 % kava lactones is created which is called WS1490 (Seligman, 1998).

Lehmann, Kinzler and Friedeman conducted a randomized double-blind placebo controlled trial in 1996 with 58 patients suffering from neurotic disorders. Randomly selected patients received 70 mg WS1490 three times per day, and others placebo for 4 weeks. By the end of the first week, the kava-group demonstrated a significant reduction in anxiety. Reported side effects were minimal.

In a placebo controlled double-blind clinical trial, 38 patients with neurotic or psycho-somatic disorders were treated with DL-kavain or oxazepam. The anxiolytic effectiveness of the two preparations was judged by means of the Anxiety Status Inventory and the Zung self-rating Anxiety scale to be equivalent. No adverse reactions were reported (Lindenberg and Pitule-Schodel, 1990).

1.4.5 Clinical indications

It seems to be a safe herb for short-term relief from stress and anxiety. Kava has been approved in Germany for conditions of nervous anxiety, stress and restlessness. The recommended dosage is 70 mg two or three times per day given in the standardized extract form.

A dose of 180-210 mg of kavalactones can be taken one hour before bedtime as a sleeping aid.

It is also conjectured to have sedative, aphrodisiac and stimulant activity, and the resin has been used as a local anaesthetic and urinary antiseptic (Murray, 1995).

Traditional uses include treatment of rheumatologic illnesses, and to promote muscle relaxation (Singh, 1992).

Pharmacological properties such as half-life and metabolism are not known.

1.4.6 Adverse effects and toxicity

Therapeutic doses may result in mild gastro-intestinal complaints or allergic skin reactions (Schulz, et al., 1998). Chronic use of kava results in kava dermatophy, and is often accompanied by eye irritation (Norton and Ruze, 1994). Although this scaly dermatitis is similar to that seen in pellagra, niacin deficiency is not the mechanism. A randomized controlled trial of 100 mg nicotinamide in 29 habitual kava drinkers with dermatophy showed no difference between the treatment and the placebo groups (Ruze, 1990). Abstaining from kava results in a complete resolution of symptoms.

Kava may interact with benzodiazepine metabolism. A 54 year old man on daily doses of alprasolam, cimetidine and terazosin was hospitalized after experiencing an acute change in mental status 3 days after taking kava (Almeida, 1996).

Kava shows no additive effects with alcohol (Herberg, 1993).

1.5 AIM OF THE STUDY

The effects of unfavourable environmental conditions, including direct sunlight, high humidity or increased temperatures, on the stability of *Hypericum perforatum, Ginkgo biloba* and *Piper methysticum* will be evaluated. In order to predict a shelf-life for each of the products:

- Dosage forms (if available) will be obtained from registered herbal manufacturers. Unavailable products will be self-manufactured according to Good Manufacturing Practice (GMP).

- Different extractants will be used on the contents of the different dosage forms, e.g. tablets, capsules and herbal drops, to determine which extractant extracts the most compounds.

- The chemical composition of each sample will be analyzed by TLC, and compared with the original fingerprint obtained from the fresh raw material taken as time = 0 months.

- High Pressure Liquid Chromatography and spectrophotometry will be used to validate and quantify the use of TLC on the stability of these herbal preparations.

- Conventional pharmaceutical calculations, including increased rate stability tests will be used to predict a relevant shelf-life for each dosage form.

- Industrial relevance might be gained by developing some standard during the manufacturing process.

Chapter 2

Materials and methods

2.1 MATERIALS

Neosorb® (sorbitol) was purchased from Roquette, Lestrem – France.
Aerosil® (colloidal silica) was purchased from Degussa-Hüls, Halfway house- South Africa.
Magnesium stearate was purchased from Dr Paul Lohmann GmbH KG, Emmerthal-Germany.
St John's wort extract was purchased from Delatrade, Esplanade- Hamburg.
St John's wort dried herb powder was purchased from Caesar & Loretz GmbH, Hausarschrift- Hilden.
Kava Kava extract and dried root powder, as well as Ginkgo extract and dried leaf powder was purchased from Blue California Corp. Santa Margarita – CA.
Empty size 0 clear-clear hard gelatin capsules were purchased from the Su-Hueng Capsule Co. Seoul-Korea.
Empty securitainers were purchased from Console plastics, Wadeville, Gauteng, South Africa.
Silika bags were purchased from Moss Packaging, Johannesburg, South Africa
Vanillin and all analytical and HPLC reagents were purchased from MERCK-Schucard, München,Germany.
p-anisaldehide , hypericin standard and quercetin standard was purchased from Sigma-Aldrich, St Louis, USA.
Rutin was purchased from Warren Chem, Johannesburg-South Africa.
TLC plates were purchased from Macherey-Nagel GmbH & Co, Germany
HPLC columns, vials and 0.45um filters were purchased from Anatech (Pty) Ltd., Johannesburg.

2.2 METHODS

2.2.1 Manufacturing of tablets

A standard formula was used. It was decided to produce tablets containing 350 mg of the active ingredient, since other preparations on the market varied between 120 mg and 500 mg. The formula included 30% sorbitol, which was used for it's bulking and binding properties, 1.3% magnesium stearate as a lubricant, and colloidal silica 0.9 % (Aerosil®) as a free-flowing and moist-regulating agent (Rubenstein, 1995). All raw materials were generously supplied by Biomox Pharmaceuticals, Pretoria.

Due to its many benefits, the process of direct compressibility was used. These benefits include increased stability, the cost-effectiveness of the process, as well as increased particle release after disintegration. It is furthermore beneficial in situations where the active ingredient or the secondary ingredients are sensitive to moist or heat (Rubenstein, 1995).

The raw materials were weighed on a Sartorius balance. Tablets were manufactured on a Manesty B3B tabletting machine, under normal humidity and room temperature. Medium sized round tablet dies and punches (diameter = 10 mm) were used. Compression was set at 3.0 tons.

Each product was packed into securitainers (35 mm x 52 mm) containing 10 tablets, and a 0.5 g silica bag as desiccant. The securitainers were labeled according to the sample type, storage condition and test date.

2.2.1.1 Hypericum herbal tablets.

Rx	***Hypericum perforatum*** herb powder	140 g
	Sorbitol	42 g
	Magnesium stearate	2.4 g
	Aerosil®	1.6 g

Hypericum perforatum herb powder was sieved by hand and mixed for 5 minutes with Aerosil® after which the sorbitol and magnesium stearate was added and again mixed

thoroughly for another 5 minutes. The 86 g of mixed powder yielded 295 tablets with an average mass of 476.9 mg. The test for disintegration of uncoated tablets (BP, 1998) was performed on a Pharma-Test Type PTZ disintegration machine. Disintegration time was 8 minutes 10 seconds. The test for uniformity of weight (BP, 1998) was performed on a Sartorius balance. Not more than two tablets weighed below 453.05 mg and above 500.75 mg. Not more than one tablet weighed below 429.21 mg and above 524.59 mg.

2.2.1.2 *Ginkgo biloba* herbal tablets.

Rx	***Ginkgo biloba*** leaf powder	123 g
	Sorbitol	36.75 g
	Magnesium stearate	2.1 g
	Aerosil®	1.4 g

Ginkgo biloba leaf powder was sieved by hand and mixed with Aerosil® for 5 minutes after which the sorbitol and magnesium stearate was added and again mixed thoroughly for another 5 minutes. The 164.25 g of mixed powder yielded 272 tablets with an average mass of 465.7 mg. The test for disintegration of uncoated tablets (BP, 1998) was performed on a Pharma-Test Type PTZ disintegration machine. Disintegration time was 8 minutes 50 seconds. The test for uniformity of weight (BP, 1998) was performed on a Sartorius balance. Not more than two tablets weighed below 442.4 mg and above 488.98 mg. Not more than one tablet weighed below 419.13 mg and above 512.27 mg.

2.2.1.3 Kava Kava herbal tablets.

Rx	***Piper methysticum*** root powder	140 g
	Sorbitol	42 g
	Magnesium stearate	2.4 g
	Aerosil®	1.6 g

Piper methysticum root powder was sieved by hand and mixed with Aerosil® for 5 minutes after which the sorbitol and magnesium stearate was added and again mixed thoroughly for another 5 minutes. The 86 g of mixed powder yielded 321 tablets with an average mass of 461.2 mg. The test for disintegration of uncoated tablets (BP, 1998) was

performed on a Pharma-Test Type PTZ disintegration machine. Disintegration time was 11 minutes 36 seconds. The test for uniformity of weight (BP, 1998) was performed on a Sartorius balance. Not more than two tablets weighed below 438.15 mg and above 484.26 mg. Not more than one tablet weighed below 415.08 mg and above 507.32 mg.

2.2.2 Manufacturing of Capsules

All manufacturing took place at normal humidity and room temperature.

2.2.2.1 Extract capsules:

The formula for the capsules containing the extract of the different plants was also decided on 350 mg, except for Kava Kava, which was 400 mg. The reason being that during pre-formulation it seemed difficult reaching a mass of only 350 mg per capsule, since the extract root powder appeared much more dense than the extract powders of Hypericum and Ginkgo. No additional non-active ingredients were added.

The raw materials were weighed on a Sartorius balance. Manufacturing was performed on a PAM AT 90 Manual Capsule-filling machine. Clear-Clear, size 0, hard gelatin capsules were used in the production. All raw materials and empty capsules were supplied by Biomox Pharmaceuticals, Pretoria.

Each product was labeled in the same way as described in section 2.2.1.

2.2.2.1.1 Hypericum extract capsules.

Seventy grams of *Hypericum perforatum* extract (standardized to 0.3% hypericin according to certificate of analysis) yielded 199 capsules with an average weight of 448.83 mg, where 96.7 mg is accountable by the empty gelatin capsule.

The test for disintegration of hard capsules (BP, 1998) was performed on a Pharma-Test Type PTZ disintegration machine. Disintegration time was 10 minutes 42 seconds. The test for uniformity of weight (BP, 1998) was performed on a Sartorius balance. Not more than two capsules weighed below 426.38 mg and above 471.27 mg. Not more than one capsule weighed below 403.94 mg and above 493.71 mg.

2.2.2.1.2 Ginkgo extract capsules.

Seventy-two grams of *Ginkgo biloba* extract yielded 197 capsules with an average weight of 432.35 mg, where 96.7 mg is accountable by the empty gelatin capsule.

The test for disintegration of hard capsules (BP, 1998) was performed on a Pharma-Test Type PTZ disintegration machine. Disintegration time was 9 minutes 22 seconds. The test for uniformity of weight (BP, 1998) was performed on a Sartorius balance. Not more than two capsules weighed below 410.73 mg and above 453.96 mg. Not more than one capsule weighed below 389.11 mg and above 475.58 mg.

2.2.2.1.3 Kava Kava extract capsules.

Seventy-nine grams of *Piper methysticum* extract yielded 196 capsules with an average weight of 498.3 mg, where 96.7 mg is accountable by the empty gelatin capsule.

The test for disintegration of hard capsules (BP, 1998) was performed on a Pharma-Test Type PTZ disintegration machine. Disintegration time was 13 minutes 23 seconds. The test for uniformity of weight (BP, 1998) was performed on a Sartorius balance. Not more than two capsules weighed below 475.95 mg and above 526.78 mg. Not more than one capsule weighed below 451.53 mg and above 551.87 mg.

2.2.2.2 Dried herb capsules:

Bioharmony CC supplied samples of the dried herbal capsules. The individual batches were manufactured by Biomox Pharmaceuticals, Pretoria. Manufacturing was performed on a Kwang DaH Semi Automatic capsule-filling machine. The laboratory staff of Biomox Pharmaceuticals carried out the tests for disintegration and uniformity of weight.

Each product was labeled in the same way as described in section 2.2.1.

2.2.2.2.1 Hypericum herbal capsules.

According to the master-manufacturing document of this batch, each capsule contained 350 mg of the active ingredient, *Hypericum perforatum* dried herb. No secondary ingredients were included in the formula. Disintegration time was 12 minutes 50 seconds. The batch had an average mass of 453.5 mg. The empty hard size 0 gelatin capsule accounted for 96.8 mg of the total capsule weight. The original batch size produced by Biomox Pharmaceuticals was 160 kg.

2.2.2.2.2 Ginkgo herbal capsules.

According to the master-manufacturing document of this batch, each capsule contained 350 mg of the active ingredient, *Ginkgo biloba* dried leaf. No secondary ingredients were included in the formula. Disintegration time was 13 minutes 10 seconds. The batch had an average mass of 457.85 mg. The empty hard size 0 gelatin capsule accounted for 97.2 mg of the total capsule weight. The original batch size produced by Biomox Pharmaceuticals was 100 kg.

2.2.2.2.3 Kava Kava herbal capsules.

According to the master-manufacturing document of this batch, each capsule contained 350 mg of the active ingredient, dried Kava Kava root. No secondary ingredients were included in the formula. Disintegration time was 11 minutes 19 seconds. The batch had an average mass of 448.45 mg. The empty hard size 0 gelatin capsule accounted for 98.1 mg of the total capsule weight. The original batch size produced by Biomox Pharmaceuticals was 100 kg.

2.2.3 Liquid extracts.

Bioharmony CC also supplied alcoholic extracts used as oral herbal drops. Each supplied sample had a total volume of 50 ml. No strengths regarding the active ingredient were stipulated on the label. Certificate's of analysis was unobtainable and the extractants used was unknown.

Four and a half milliliters of each sample was decanted into smaller plastic bottles and labeled in the same way as mentioned in section 2.2.1.

2.3 STORAGE CONDITIONS OF SAMPLES.

Initially it was decided to use five parameters for this stability study. The parameters used included storage under different temperatures in the absence of light and moist i.e. normal room temperature, (25°C), 40°C, and 50°C. The other two parameters included storage in direct sunlight and the combination of direct sunlight and high humidity. The samples were exposed to these conditions for a period of six months.

The hypericum, Ginkgo and Kava Kava samples were represented by dried herbal tablets, dried herbal capsules, extract capsules, and extract liquid.

It was later decided to discard the samples stored at 50° since the oven in which it was kept did not heat evenly due to an electrical problem. In-stead two new parameters were added to the study. This included storage at 60°C and 80°C. The samples used for this part of the study were identical to the samples used in the six month part, except that this time the liquid extract was not added. The samples stored under these conditions were analyzed after two weeks, four weeks and again after six weeks.

2.3.1 Radiated samples

In the herbal pharmaceutical industry, raw materials are regularly exposed to microbiological contamination from a wide variety of sources. This contamination cannot be effectively removed by the practices of washing or drying under low temperatures. Alternatively ionization using the radiation energy of gamma rays, X-rays or electron beams is a new technique used in the sterilization of herbal raw materials prior or after manufacturing.

Dried herbal powder of *Hypericum perforatum*, *Ginkgo biloba* and *Piper methysticum* was send to Gammaster® South Africa (Pty) Ltd, a company situated in Isando, Gauteng. This company specializes in the sterilization of foods, medical disposables and pharmaceutical raw materials. The samples were exposed to a Cobalt-60 source in a dose of 4.6 kGray

13.8 kGray and 27.9 kGray. The distance between the radiation source and the sample, as well as the time of exposure determines the dose.

2.4 EXTRACTION

2.4.1 Extraction of raw materials

Five hundred mg of the raw materials used in the manufacturing of the different dosage forms were weighed into polyethylene centrifuge tubes. It was subsequently extracted with 5 ml of each of the following extractants: (the polarity and selectivity group according to Snyder and Kirkland, 1979 given in brackets) methanol [5.1,II], acetone [5.1,VIa] and n-hexane [0.1,0]. This was carried out on all raw materials, except for the purchased oral herbal drops. The centrifuge tubes were then tightly closed and vigorously shaken in a Labotec model 232 test tube mixer for 10 minutes. The extracts were centrifuged at 4500-x g in a Heraeus sepatech labofuge model 200 centrifuge for 5 minutes and decanted into small pre-weighed containers. The extraction process was repeated three times with the originally weighed material and the extracts were combined in the same containers. A stream of cold air at room temperature was used to remove the different extractants. In the case of the methanol extract, complete dryness did not occur after 24 hours. It was then placed in a dessicator and a vacuum pump was used to remove the methanol by vacuum evaporation. The dry extracts were then redissolved in acetone, to yield a known concentration of extract. The extracts were clearly labeled and stored at 7°C.

2.4.2 Extraction according to the British Herbal Pharmacopoeia.

2.4.2.1 *Hypericum perforatum*

One gram of *Hypericum perforatum* dried herb powder was weighed in a measuring beaker. Ten ml of methanol was added, and heated on a water-bath for 15 minutes at 60°C. The extract was then left to cool down and filtered. A standard rutin solution was also prepared. Ten ml of methanol was added to 2.5 mg of rutin to yield a concentration of 0.025% (m/v). The solution was shaken until all the rutin dissolved in the methanol.

2.4.2.2 Ginkgo biloba

The method for extracting Ginkgo leaf was performed in the same way as the method used for extracting *Hypericum perforatum* [see section 2.4.2.1].

2.4.2.3 *Piper methysticum*

One gram of Kava Kava dried root powder was weighed in a measuring beaker. Ten ml of chloroform was added and heated under reflux for 15 minutes at 60°C. It was left to cool and filtered.

2.4.3 Extraction of dosage forms

Since the mass of the individual dosage forms (tablets and capsules) were less than 500 mg, it was decided to take a combination of the different dosage forms and weigh 500 mg of the combined powder. The capsules were opened by hand and the contents directly weighed into centrifuge tubes, where as the tablets had first to be crushed using a mortar and pestle, and then weighed into centrifuge tubes. In the case of the capsule-extracts stored at 60°C and 80°C, the heat caused the once fine, free flowing extract powder to form a hard unbreakable solid mass, which also had to be crushed with a mortar and pestle. Acetone in a ratio of 10 ml for each 1 g of powder was added as the extractant. The centrifuge tubes were tightly closed and vigorously shaken in a Labotec model 232 test tube mixer for 10 minutes. The extracts were then centrifuged at 4500-x g in a Heraeus sepatech labofuge model 200 centrifuge for 5 minutes and decanted into pre-weighed containers. The extraction process was repeated three times with the originally weighed material and the extracts were combined in the same containers. A stream of cold air at room temperature was used to remove the acetone.

One gram of each of the oral herbal drops was weighed into small pre-weighed containers. The containers were directly placed in a stream of cold air at room temperature, allowing the unknown extractant to evaporate.

All of the dry extracts were then redissolved in acetone after 24 hours, to yield a known concentration of extract. The extracts were clearly labeled and stored at 6-7°C. This procedure was repeated after 3 months and again after 6 months with each sample stored at 25°C, 40°, direct sunlight and direct sunlight in combination with high humidity.

The samples exposed to 60°C and 80°C for 2, 4 and 6 weeks were stored at 6-7°C. Analysis was performed simultaneously after the six- week period had passed.

All the extractants used were of analytical grade.

2.5 THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatography is a technique that makes use of a relatively thin layer of material that is coated on a glass, plastic or metal surface. Here the mobile phase moves through the stationary phase by capillary action allowing separation of compounds. A thin layer plate is prepared by placing a known quantity of the compound under investigation, onto the pre-coated surface about 1 cm from the bottom. The mobile phase is typically prepared in a transparent chromatographic tank made out of thick glass plates 20 cm long and with a ground glass fitting lid. The plate is then inserted into the tank and allowed to develop by ascending chromatography for about 8-10 cm. A pencil line is drawn on the plate, marking the distance the mobile phase run. This is useful in calculating the Retention factor (R_f) a compound.

$$R_f = \frac{\text{Distance of Compound (mm)}}{\text{Distance of Mobile phase (mm)}} \qquad \textbf{Equation 2.1}$$

The advantages of TLC are the speed and low-costs of these experiments. It can also be used to develop optimal conditions for separations by column liquid chromatography (Skoog, et al., 1996).

2.5.1 THIN LAYER CHROMATOGRAPHY (TLC) ANALYSIS OF THE EXTRACTS

Alugram® SIL G/UV_{254} TLC plates with a layer of 0.20 mm pre-coated silica gel were used. A known volume representing 50 μg of each extract prepared in section 2.4.1 was applied to the plates with a Socorex micropipet. The atmosphere inside the tank was saturated by placing filter paper soaked in the mobile phase against its walls.

Three different mobile phases (solvent systems) were used:

EMW: Ethylacetate/Methanol/Water in the ratios 10:1.35:1.
BEA: Benzene/Ethanol/Ammonia in the ratios 18:2:0.2.
CEF: Chloroform/Ethylacetate/Formic acid in the ratios 10:8:2.

Each TLC plate was duplicated. The one plate was subsequently sprayed with a vanillin spray reagent [see section 2.5.1.1] and the other one with a *p*-anisaldehide (4-methoxybenzaldehyde) solution [see section 2.5.1.2]. The plates were heated in an oven at 105°C for about 2-3 minutes until the colour of the bands were optimally visible. The plates were then scanned in with a Hewlett Packard Colour scanner and saved on computer for reference.

2.5.1.1 Preparation of the vanillin spray reagent:

Vanillin (0.1 g) was weighed in a flask and dissolved in 28 ml of analytical grade methanol. One ml of sulfuric acid (H_2SO_4) was added to the solution and shaken. When not in use, the reagent was stored in a refrigerator for not more than 72 hours.

2.5.1.2 Preparation of the *p*-anisaldehide spray reagent:

p-Anisaldehide (1 ml) was added to 18 ml of analytical grade ethanol. After this solution had been mixed thoroughly, 1 ml of sulfuric acid was added and stirred a few times. When not in use the reagent was stored in a refrigerator for not more than 72 hours.

The above in-house developed TLC methods were then validated against the methods stipulated in the British herbal Pharmacopoeia regarding the TLC identification of the three different plant species, *Hypericum perforatum, Ginkgo biloba,* and *Piper methysticum* [See section 2.6].

2.5.2 IDENTIFICATION (TLC) ACCORDING TO THE BRITISH HERBAL PHARMACOPOEIA

2.5.2.1 *Hypericum perforatum*

A solvent system containing a mixture of ethyl acetate:formic acid:glacial acetic acid:water in a ratio of 100:11:11:27 was prepared in a chromatographic tank [See section 2.5]. The

walls of the tank were lined with filter paper and left to stand for one hour at room temperature to achieve saturated conditions.

Twenty µl of the extract prepared in section 2.4.2.1 was applied to the plate. Five µl of the standard rutin extract was applied to the plate to serve as a reference. After the system was allowed to develop, the plate was sprayed with a 1% (w/v) solution of diphenylboric acid 2-aminoethyl ester in methanol, followed by a separate application of a 5% (w/v) solution of polyethylene glycol 4000 in ethanol (Bradley, 1996). The plates were then examined under ultraviolet light (366 nm) with a Camac Universal UV lamp TL-600, photographed with a digital camera and stored on computer [See figure 3.2.2].

2.5.2.2 Ginkgo biloba

Identification of the extract prepared in section 2.4.2.2 was carried out in the same way as for *Hypericum perforatum.* [Section 2.5.2.1]

2.5.2.3 *Piper methysticum*

A solvent system consisting only of di-ethyl ether was placed in a glass chromatographic tank. The walls of the tank were lined with filter paper and left to stand for one hour at room temperature to achieve saturated conditions.

Twenty µl of the extract prepared in section 2.4.2.3 was applied to the plate. After the system was allowed to develop, the plate was sprayed with the vanillin spray reagent. The plate was heated in an oven at 105 °C until the major bands were visible. The plate was examined in visible light [See figure 3. 4.2] and again scanned in and stored on computer for reference.

2.5.3 THIN LAYER CHROMATOGRAPHY (TLC) ANALYSIS OF THE SAMPLE DOSAGE FORMS

TLC was applied to the samples stored under the conditions mentioned in section 2.3 and extracted according to the method described in section 2.4.3. This was done after comparing the in-house developed methods using EMW, BEA and CEF [section 2.5.1] against the methods contained in the British Herbal Pharmacopoeia [section 2.5.2]. All of the in-house developed methods gave the same or better results when compared to the

methods of the British Herbal Pharmacopoeia. Based on this evidence it was decided to implement the in-house methods due to their good reproducibility and relative simplicity compared to the British Herbal Pharmacopoeia.

For hypericum and Ginkgo it was decided to use all three solvent systems (mobile phases), EMW, BEA and CEF as good separation could be seen by all of them. BEA and CEF were used for the Kava Kava. The choice between either vanillin or *p*-anisaldehide as spraying reagents was based upon their ability to visualize the most bands present in the sample under investigation. For Ginkgo it was decided to use vanillin as a spray reagent [see figure 3.3.1] and for Hypericum [figure 3.2.1] and Kava Kava [figure 3.4.1] the *p*-anisaldehide as spray reagents.

Another difference in the TLC on the raw materials between the TLC on the dosage forms was the adjustment of the applied 50 μg on the plate to only 25 μg. Application of less extract gave better separation and visibility of the bands.

These TLC methods were performed at 0 months, 3 months and again at 6 months. After the six-month period all the different plates were compared to each other. No definite conclusions could be made from these results since the conditions at t=0 months and t=6 months might not have been the same.i.e. the temperature and humidity in the laboratory, TLC plates purchased from different sources and the contamination of the *p*-anisaldehide solution.

It was then decided to repeat all the samples, stored at low temperature, taken after each specific time interval at the relevant temperature or condition. This time it was on the same plate i.e for all the samples stored at 25°C, there would be only one plate containing all the samples taken at t=0 months, t=3 months and t=6 months. The same was applied to the excelerated samples with t=0 weeks, t=2 weeks, t=4 weeks and t=6 weeks. Polygram® Sil /G plates were used this time in stead of the Alugram® SIL G/UV_{254} plates used previously.

2.6 SPECTROPHOTOMETRY

A spectrophotometer is an instrument that is used for absorption measurements with ultraviolet or visible radiation. Spectrophotometers employ a grating prism monochromator to provide a narrow band of radiation for measurements. Depending on the model, spectrophotometers can cover a range of wavelengths between 100-800 nm (Skoog, et al., 1996).

A Beckman model DU 65 spectrophotometer connected to a Seikosha SP 2000 dot matrix printer was used for the quantitative analysis of the hypericin content in each of the samples prepared from *Hypericum perforatum*. All samples were analyzed in a 1 cm. Quartz cuvette, at a wavelength of 590 nm (Anton, 1997).

2.6.1 Hypericin assay by Spectrophotometry.

The method for determining the hypericin content of *Hypericum perforatum* was based on the method described in the American Herbal Pharmacopoeia (Anton, 1997).

2.6.1.1 Standard Preparation:

A standard stock solution of hypericin was prepared in methanol. Stock solution A was prepared by weighing 0.99 mg of the hypericin standard purchased from Sigma, into a measuring beaker. Exactly 16.50 ml of methanol was added to give a known concentration of 0.06 mg/ml. Stock solution A was diluted 1:5(B), 1:10(C), 1:20(D) and 1:25(E) giving known concentrations of 0.012 mg/ml, 0.006 mg/ml, 0.003 mg/ml and 0.0024 mg/ml respectively.

A blank sample containing only methanol was also prepared. Standard solutions B-E together with the blank sample were used to create a five-point standard curve [Figure 3.2.10].

Optimal settings for the spectrophotometer were determined with stock solution B (0.012 mg/ml). The scan speed was set at 750 nm per minute. The upper limit was set at 700 and the lower limit was set at 400 nm [Fig 3.2.9].

2.6.1.2 Sample preparation:

2.6.1.2.1 *Herbal capsules:*

Approximately 500 mg of the powder contained in the herbal capsules were accurately weighed and placed in a 25 ml volumetric flask. Fifteen ml of methanol was added to the flask and shaken for a few seconds, after which it was placed in an ultrasonic bath for 15 minutes. The solution was left to cool and then diluted to volume with methanol. Ten ml of this solution was then filtered and 5 ml of this filtrate was then pipetted into a 25 ml volumetric flask and diluted to volume with methanol. The cuvette was then rinsed with methanol and refilled with about 3 ml of the sample after which it was placed in the spectrophotometer.

2.6.1.2.2 *Herbal tablets:*

The tablets had first to be crushed using a mortar and a pestle. Approximately 665 mg of the crushed tablet powder representing 500 mg of the active hypericum herb were then weighed and treated the same as for the herbal capsules.

2.6.1.2.3 *Extract capsules:*

The extract capsules were treated the same as the herbal capsules.

2.6.1.2.4 *Radiated samples:*

Approximately 500 mg of the raw material which was send to Gammaster® and radiated at different doses [See section 2.3.1] was extracted and treated the same as for the herbal capsules [Section 2.6.1.2.1].

The percentage hypericin contained in each sample were then calculated according to equation 2.2.

$$\% \text{ Hypericin} = \frac{x \text{ (mg/ml)} \cdot 125 \text{ (Dilution factor)}}{\text{Quantity weighed (mg)}} \times 100 \qquad \textbf{Equation 2.2}$$

Where x = Concentration obtained from Standard Curve. [Figure 3.2.10]

2.6.2 Validation of method

Exactly 500 mg of powdered herb was weighed and treated as described in section 2.6.1.2.1. This sample was analyzed 5 times on the spectrophotometer without changing any settings or changing cuvettes [See table 3.2.1]. A standard deviation of the method (data obtained from spectrophotometry) was calculated according to equation 2.2.

$$s = \sqrt{\frac{\sum_{i=1}^{N} x_i^2 - \frac{\left(\sum_{i=1}^{N} x_i\right)^2}{N}}{N \quad 1}}$$

Equation 2.3

Where x_1 = values of individual samples

X_1^2 = Square of individual samples

N = Number of samples

The standard deviation of the data obtained by extraction extraction was also determined. Exactly 500 mg of powdered herb was weighed off 5 times and treated the same as in section 2.6.1.2.1. The standard deviation on the quantity extracted [See table 3.2.2] was also calculated according to equation 2.2

2.7 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a type of chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rates, the liquid must be pressurized to several hundred kilograms per square centimeter. A HPLC system consists of a pump, a column (stationary phase) into which a sample is injected, a detector system measuring the absorbance of the compounds separated by the column, and a integrator to report the data measured by the detector (Skoog, et al., 1996).

The HPLC system used for the quantitative analysis for *Ginkgo biloba* and Kava Kava consisted out of a Milton Roy Constametric III metering pump, a Spectraphysics AS3000 Autosampler, a Varian 2050 UV/VIS variable wavelength detector and a Hewlett Packard 3390A Reporting Integrator.

2.7.1 Ginkgo Flavonol Glycoside (Quercetin) assay by HPLC

2.7.1.1 Chromatographic Conditions:

A SGE U5 250x4.6 mm ODS2-30/5 column, purchased from Anatech (Pty) Ltd., with a set column temperature of 25°C, and isocratic mobile phase containing methanol: 0.5% phosphoric acid in DI water (50:50) with a flow rate of 0.7 ml/min. was used. After preparation, the mobile phase was sonicated for 10 minutes. This ensures the absence of any air bubbles that might enter the pump and alter the pressure causing inconsistency of the retention times. The injected sample volume was 10 µL and detection was set at 270 nm.

2.7.1.2 Standard Preparation:

A standard stock solution of quercetin dihydrate was prepared in methanol. Stock solution A was prepared by weighing 0.0407 g of quercetin dihidrate, purchased from Sigma, into a 50 ml volumetric flask and made up to volume with methanol, giving a known concentration of 0.814 mg/ml. Stock solution A was diluted 1:5 (B), 1:10 (C), 1:20 (D) and 1:25 (E)

A blank sample containing only methanol was also prepared and injected into the column.

Standard solutions A-E were used to create a five-point standard curve [Figure 3.3.10].

Optimal settings for the integrator were determined with standard solution C (0.06 mg/ml) [Figure 3.3.9].

Attenuation = 6 units
Chart speed = 0.5 cm/min
Peak width = 0.80 cm
Threshold = 4 units
Area rejected = 0
Run time = 40 min
Integration set on Peak height rather than Peak area.

The standard solutions were stored in a refrigerator at -8°C when not in use. Standard C (1:10 dilution) was analyzed at the beginning of each day to ensure consistency of the

retention time of the quercetin reference compound. Any precipitants formed on storage were redissolved by warming the container in hot running tap water for several minutes.

A test sample of both the extract and leaf powder were prepared as described in section 2.7.1.3. A single injection was made and then spiked with a few drops of the stock standard C to confirm the retention time of the quercetin contained in the samples.

2.7.1.3 Sample Preparation.

2.7.1.3.1 *Herbal Capsules:*

Approximately 500 mg of the powder contained in the herbal capsules were accurately weighed and placed in a 25 ml volumetric flask along with 5 ml of ethanol, 2 ml de-ionized water and 1 ml of concentrated hydrochloric acid. It was then heated on a waterbath at 67°C for 2.5 hours after which it was cooled to room temperature. Two ml of methanol was added to the hydrolysis flask and sonicated for 30 minutes in a Bransonic® Ultrasonic bath. The solution was then filtered and transferred to a 10 ml flask and made up to volume using methanol. An aliquot of about 0.5 ml was filtered through a 0.45 µm Septa NSF 2500-1 filter into a HPLC vial and placed in the Spectraphysics autosampler.

2.7.1.3.2 *Herbal Tablets:*

The tablets first had to be crushed again using a mortar and pestle. Approximately 664 mg of the crushed tablet powder representing 500 mg of the powdered ginkgo leaf were then accurately weighed. This powder was then treated the same as for the herbal capsules.

2.7.1.3.3 *Extract Capsules:*

Approximately 25 mg of the extract contained in the capsules were accurately weighed into a 25 ml volumetric flask. Five ml of ethanol and 2 ml of de-ionized water was added and sonicated for 5 minutes. One ml of concentrated hydrochloric acid was added after the sonification and placed in a waterbath for 2.5 hours at 67°C. The solution was left to cool to room temperature, and transferred to a 10 ml volumetric flask. Methanol was used to make the solution up to volume. An aliquot of about 0.5 ml was filtered through a 0.45 µm Septa NSF 2500-1 filter into a HPLC vial and placed in the Spectraphysics autosampler.

2.7.1.3.4 *Radiated samples:*

Approximately 500 mg of the raw material which was send to Gammaster® and radiated at different doses [See section 2.3.1] was extracted and treated the same as for the herbal capsules. [Section 2.7.1.3.1]

The percent quercetin present in each sample was then calculated according to equation 2.4

$$\% \text{ (w/w) Quercetin} = \frac{(C)(FV)(F)}{(W)} \times 100 \qquad \text{Equation 2.4}$$

Where C = Sample's quercetin concentration (mg/ml) from linear regression.

FV= Final volume of sample preparation (ml)

F = Correction factor (2.504) according to www.nutraceuticalinstitute.com/methods

W= Sample weight in mg

2.7.1.4 Validation of method

Exactly 500 mg of powdered herb was weighed and treated as in section 2.7.1.3.1. This sample was injected 5 times in the column without changing any settings or changing mobile phases [See table 3.2.1]. A standard deviation of the method was then calculated according to equation 2.2.

A standard deviation of the extraction was also performed. Exactly 500 mg of powdered herb was weighed off 5 times into pre-weighed glass beakers, and prepared as in section 2.7.1.3.1. The beakers were then placed in a room with a low humidity and a temperature above 25°, allowing the methanol to evaporate. The beakers were then weighed again and the difference in the quantity extracted calculated [See table 3.2.2]. The standard deviation on the quantity extracted was also calculated according to equation 2.2.

2.7.2 Kava lactone assay by HPLC

2.7.2.1 Chromatographic Conditions:

A Spherisorb 250x4.6 mm S5-ODS1 column, supplied by Anatech (Pty) Ltd., with a set column temperature of 25°C, and isocratic mobile phase containing 0.1% phosphoric acid in DI water: isopropyl alcohol: acetonitrile (64:16:20 v/v) with a flow rate of 0.99 ml/min.

was used. After preparation, the mobile phase was sonicated for 10 minutes ensuring the absence of any air bubbles that might enter the pump and alter the pressure, causing inconsistency of the retention times. The injected sample volume was 5 µL and detection was set at 220 nm.

2.7.2.2 Standard Preparation:

No standards regarding any of the known kavalactones i.e. methysticin, di-hydromethysticin, kavain, di-hydrokavain, desmethoxyyangonin or yangonin was available in South Africa. Obtaining standard samples elsewhere was prohibitively expensive. The method used was described in detail on the internet site http://www.nutraceuticalinstitute.com/methods/kava. This site also provided an example of the chromatograms obtained from this method. Since all the conditions described in this site were replicated, the different kava lactones could be identified with relative accuracy. No validation of this method was done with regard to the actual kava-lactone content since no kava-lactone standard could be obtained. A standard deviation on the method as well as the extraction was however done [See section 2.7.2.4].

Optimal settings for the integrator was determined with the injection of 75 mg of powdered herb as described in section 2.7.2.3 [Figure 3.4.9]

Attenuation = 9 units
Chart speed = 0.5 cm/min
Peak with = 0.80 cm
Threshold = 7 units
Area rejected = 0
Run time = 40 min
Integration set on Peak height rather than Peak area.

2.7.2.3 Sample Preparation.

2.7.2.3.1 *Herbal Capsules:*

Approximately 75 mg of the powder contained in the herbal capsules were accurately weighed and placed in a 5 ml volumetric flask along with 4 ml of methanol/water (70:30). It was sonicated for 60 minutes in a Bransonic® Ultrasonic bath and allowed to cool to room temperature after which it was diluted to volume with methanol/water (70:30). An aliquot of

about 0.5 ml was filtered through a 0.45 μm Septa NSF 2500-1 filter into a HPLC vial and placed in the Spectraphysics autosampler.

2.7.2.3.2 *Herbal Tablets:*

The tablets first had to be crushed again using a mortar and pestle. Approximately 99 mg of the crushed tablet powder representing 75 mg of the powdered Kava Kava herb was then accurately weighed. This powder was then treated the same as for the herbal capsules [see section 2.7.2.3.1].

2.7.2.3.3 *Extract Capsules:*

Approximately 11 mg of the extract contained in the capsules were accurately weighed into a 5 ml volumetric flask. This powder was then treated the same as for the herbal capsules [see section 2.7.2.3.1].

2.7.2.3.4 *Radiated samples:*

Approximately 75 mg of the raw material which was send to Gammaster® and radiated at different doses [see section 2.3.1] was extracted and treated the same as for the herbal capsules [section 2.7.2.3.1].

2.7.2.4 Standard deviation determination

Exactly 75 mg of the powdered root was weighed and treated the same as in section 2.7.2.3.1. This sample was injected 5 times into the column without changing any settings or changing mobile phases. A standard deviation on the method was the calculated according to equation 2.2.

A standard deviation of the extraction was also performed. Exactly 75 mg of powdered herb was weighed of 5 times and treated the same as in section 2.7.2.3.1. The standard deviation on the quantity extracted was also calculated according to equation 2.2.

Chapter 3

Results and Discussion

3.1 EXTRACTION

The first step was the comparison between the in-house developed extraction methods and the methods contained in the British Herbal Pharmacopoeia.

The extraction of 500 mg dried herb powder of *Hypericum perforatum, Ginkgo biloba and Piper methysticum* with methanol, acetone and n-hexane respectively was repeated three times [Section 2.4 for procedure]. There were hardly any differences between the repetitions and average values. The average of these results are shown in figure 3.1

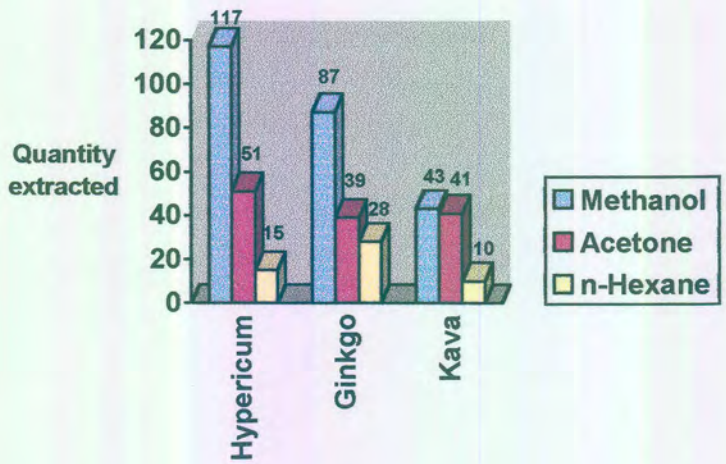


Figure 3.1 Total quantity of compounds extracted with 3 different extractants

3.1.1 Discussion

Methanol extracted the most compounds from especially *Hypericum perforatum* and *Ginkgo biloba*. This can be seen when considering figure 3.1. Chloroform is a good solvent for the extraction of the non-polar kava lactones from *Piper methysticum* as described in the British Herbal Pharmacopoeia, but because it does not mix with water, and the fact that some of the extracts had to be stored for up to 6 months, the use of this method was decided against. In each three plants, methanol extracted the most material compared to acetone and n-hexane. Although methanol gave the best extraction, it was

still decided to use acetone as extractant in the rest of this study. The reason being that the compounds under investigation in this study are mainly organic acids, naphthodiantrones, flavonoids, lactones and pyrones, all of which have a relatively non-polar chemical structure. [See section 1.4.2, section 1.5.2 and section 1.6.2]. Methanol extracts relatively polar compounds such as polysaccharides, polyphenols and tannins. Many pharmaceutical companies are not interested in these compounds because most polysaccharides break down to glucose on oral administration. Polyphenols and tannins is also largely bound to serum and thus not very bio-available (Balick, 1994).

3.2 *HYPERICUM PERFORATUM*

3.2.1 RESULTS

3.2.1.1 Thin layer chromatography

From the visual examination of the plates prepared in section 2.5, it was decided to use all three of the mobile phases, and *p*-anisaldehide as the spraying reagent. The reason for this was that with EMW a total of six distinct bands were observed with the *p*-anisaldehide spray, and only 5 bands (absence of band at R_f=0.06) with vanillin. BEA revealed 6 bands for both the *p*-anisaldehide and the vanillin, and CEF showed 7 bands with *p*-anisaldehide and only 5 bands (absence of bands at R_f=0.06 and 0.2) with the vanillin [See figure 3.2.1]. As mentioned in section 2.6 the official method was discarded because of the simplicity and good reproducibility of our in-house developed methods compared to the British Herbal Pharmacopoeia. [See figure 3.2.2] TLC was done on all samples, except the dried hypericum herb powder exposed to gamma radiation.

Ten µl of the hypericin standard solution was also applied to separate plates, and allowed to develop using all three mobile phases as described in section 2.5.1. Looking at the plate developed in CEF, it seemed that hypericin presents with a R_f value of about 0.47. TLC with EMW showed hypericin to have a R_f value of 0.73. TLC with BEA seemed to indicate a R_f value of 0.13 for the hypericin standard. It is important to note that, as mentioned in chapter 1, there is still no conclusive data showing hypericin as the only or main active ingredient in *Hypericum perforatum*. Based on this it was decided to look at all the compounds as a whole, and not only hypericin.

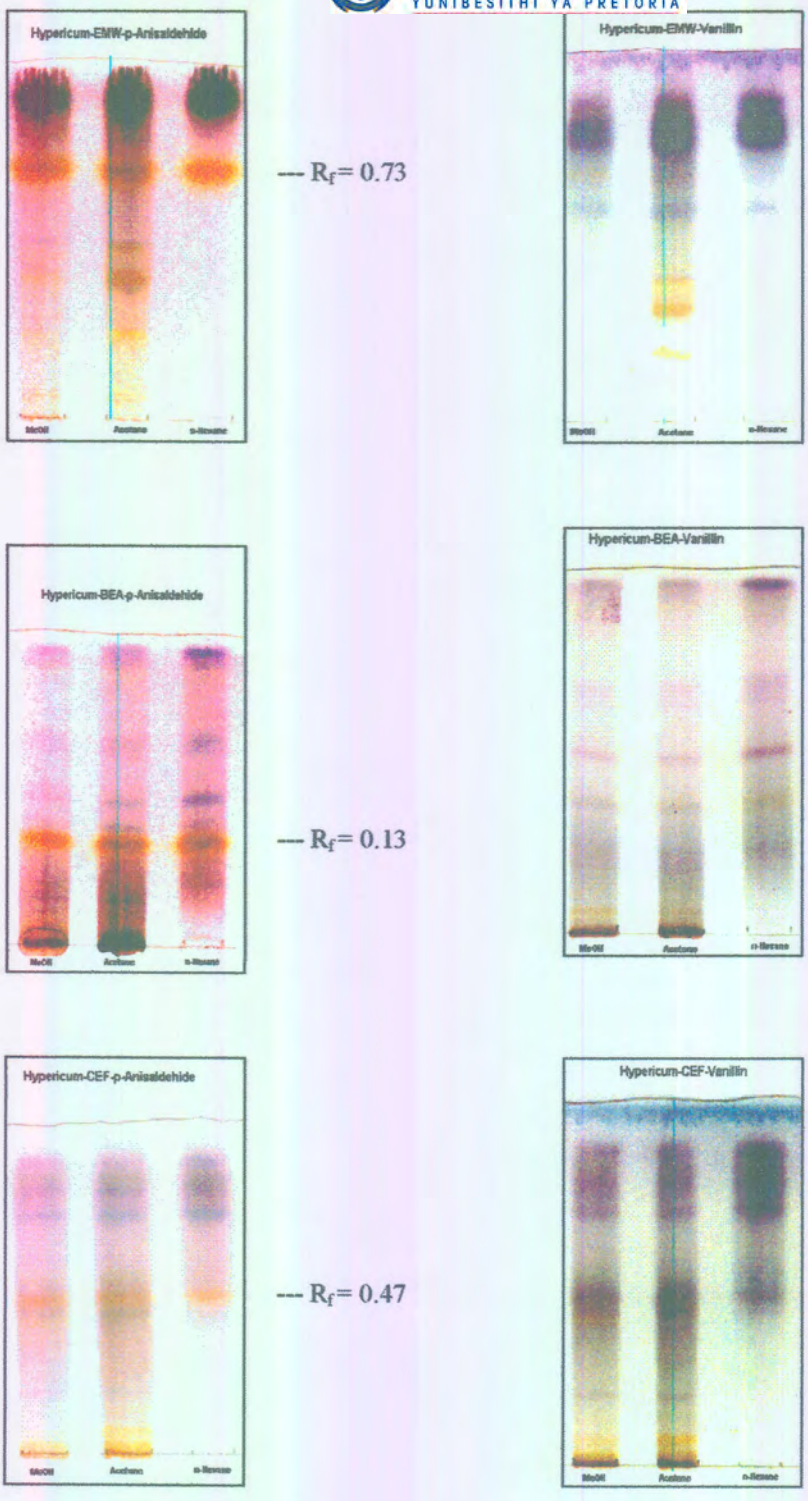


Figure 3.2.1 TLC of Hypericum root powder with mobile phase EMW (top), BEA (center) and CEF (bottom) and sprayed with *p*-anisaldehide (left) and vanillin (right). Each plate shows extraction with Methanol (left), Acetone (center), and n-Hexane (right). R_f values for hypericin are indicated.

Figure 3.2.2 ***Hypericum perforatum*** **identification according to the British Herbal Pharmacopoeia. (Hypericum left and Rutin right)**

3.2.1.1.1 Hypericum samples at 25°C. [See figure 3.2.3]

- Herbal tablets

  TLC showed no obvious change with EMW, but with BEA the colour of the bands at R_f = 0.65 and R_f = 0.42 decreased from t=0 – t=6 months. TLC with CEF showed the colour intensity of the band at R_f = 0.2 to decrease from t=0- t=6 months.

- Herbal capsules

  TLC showed no obvious change with EMW. There was a gradual decrease in colour intensity of the bands at R_f=0.65 from t=0 to t=6 months with BEA as well as a decrease in colour of the compound at R_f=0.42. The compound at R_f =0.42 was totally invisible at t=6 months. TLC with CEF showed the colour intensity of the band at R_f = 0.2 to decrease from t=0- t=6 months.

- Extract capsules

  With EMW as mobile phase, a decrease of the colour intensity of the band at R_f = 0.24 could be seen when comparing t=0 with t=3 and t=6 months. TLC with BEA showed that the intensity of the bands at R_f=0.34 and R_f=0.48 also decreased from t=0 – t=6 months. TLC with CEF showed no obvious change.

- Liquid extracts

  A slight decrease in colour at the compounds present at R_f = 0.24 and R_f= 0.44 could be seen from t=0 – t=6 months when using EMW as the mobile phase. TLC with BEA showed an intense colour of the band at R_f=0.65 which significantly decreased in intensity from t=0 – t=6 months. The colour of the band at R_f= 0.34 also decreased from t=0 – t=6 months. TLC with CEF showed a decrease in intensity of bands at R_f=0.20 and R_f=0.71 from t=0 – t=6 months.

3.2.1.1.2 **Hypericum samples at 40°C.** [See figure 3.2.4]

- Herbal tablets

  Using EMW as eluent, showed a decrease from t=0-t=6 in the intensity of the colours in the bands with R_f=0.24 and R_f=0.44. A massive decrease was seen at the band with R_f=0.84 from t=0-t=6 months. The compound with R_f=0.65 was only visible at t=0 when using BEA as mobile phase. The band at R_f=0.13 decreased in colour from t=0 – t=6 months. TLC with CEF showed no obvious change.

- Herbal capsules

  TLC with EMW showed the same massive decrease of the compound at R_f=0.84. A decrease from t=0-t=6 months in the intensity of the colours at the bands with R_f=0.24 and R_f=0.44. R_f=0.65 was only visible at t=0 with BEA as the mobile phase. The compound present at R_f=0.13 also decreased in colour from t=0 – t=6 months. This compound was much clearer for the capsules than for the tablets. TLC with CEF showed a decrease in colour intensity of the band at R_f=0.20 from t=0-t=6 months.

- Extract capsules

  TLC with EMW showed a decrease of intensity at the band with R_f=0.44 from t=0-t=6 months. Using BEA as eluent showed a decrease in intensity of bands at R_f=0.65 and R_f=0.48 from t=0-t=6 months. CEF as eluent showed no obvious change.

- Liquid extracts

  The compound at R_f=0.53 only decreased at t=6 months, with no obvious change for 0 and 3 months with EMW as the mobile phase. TLC with BEA showed much more defined bands compared to the tablets, capsules and extract at the band with

R_f=0.65, which also decreased from t=0-t=6 months. A decrease of bands with R_f=0.48 and R_f=0.13 from t=0 – t=6 months could also be seen. Using CEF as the mobile phase showed a colour increase at the band with R_f=0.39 from t=0-t=6 months, but a decrease in the colour intensity of the band at R_f=0.20. The compound present at R_f=0.47 was darker at t=3 than t=0 and t=6 months.

3.2.1.1.3 **Hypericum samples at 60°C.** [See figure 3.2.5]

- Herbal tablets

  TLC with EMW showed a slight decrease in the bands with R_f=0.53 and R_f=0.24. The band present at R_f=0.44 decreased slightly from t=0-t=6 weeks. BEA as eluent also showed a slight decrease in bands with R_f=0.07 and R_f=0.65 from t=0-t=6 weeks. TLC with CEF showed no obvious change although all the bands were very light.

- Herbal Capsules

  Using EMW as eluent showed no obvious change in the bands present at R_f=0.53 and R_f=0.24. The colour of the band present at R_f=0.44 decreased slightly from t=0-t=6 weeks. The colour of the bands was much more defined for the capsules than for the tablets. TLC with BEA showed a slight decrease in colour for the bands present at R_f=0.07 and R_f=0.65 from t=0-t=6 weeks. With CEF as mobile phase, the band with R_f=0.17 showed no obvious change but the band with R_f=0.20 decreased from t=0-t=6 weeks.

- Extract capsules

  TLC with EMW showed an absence of the compound at R_f=0.53, with a decrease in intensity of band with R_f=0.24. BEA as mobile phase showed a slight decrease in the colour intensity of the band present at R_f=0.65. The compounds present at R_f=0.07 and R_f=0.42 decreased from t=0-t=6 weeks, with a total absence of the compound present at R_f=0.43 at t=6 weeks. The compound present at R_f=0.48 is totally absent in the herbal tablets and herbal capsules. TLC with CEF showed a decrease in colour intensity of the compound at R_f=0.17 from t=0-t=6 weeks. The band with R_f=0.2 is absent.

3.2.1.1.4 **Hypericum samples at 80°C.** [See figure 3.2.6]

- Herbal tablets

  With EMW as the mobile phase, the intensity of the bands at R_f=0.24, R_f=0.53 and R_f=0.44 decreased from t=0-t=6 weeks. TLC with BEA showed no obvious change of the compound present at R_f=0.07. The compound with R_f=0.65 showed a dark band at t=0 weeks, but was absent at t=2,4,6 weeks. CEF as eluent showed a distinct band with R_f=0.81 for t =0 weeks, but was absent at t=2,4,6 weeks. A gradual decrease in colour intensity from t=0-t=6 weeks could be seen at the compounds presenting R_f values of 0.17, 0.21 and 0.71.

- Herbal capsules

  TLC with EMW showed the intensity of the bands at R_f=0.24, R_f=0.53 and R_f=0.44 to decrease from t=0-t=6 weeks. TLC with BEA showed no obvious change in the band with R_f=0.07. The compound present at R_f=0.65 showed a dark band at t=0 weeks, but was absent at t=2,4,6 weeks. Using CEF as mobile phase, showed a distinct band at R_f=0.81 for t =0 weeks, but was absent at t=2,4,6 weeks. A gradual decrease in the colour intensity from t=0-t=6 weeks could be seen at the compounds present at R_f=0.17, R_f=0.21 and R_f=0.71. In all cases the bands were more distinct for the capsules than for the tablets.

- Extract capsules

  TLC with EMW showed the band with R_f=0.44 to decrease more rapidly from t=0-t=6 weeks when compared to the herbal tablets and herbal capsules. Using BEA as the eluent, showed no band at R_f=0.48 at t=0 weeks, but increased in visibility from t=2-t=6 weeks. The compound with R_f=0.42 was only visible with the extracts and not with the tablets or herbal capsules and decreased from t=0-t=6 weeks. TLC with CEF showed the bands present at R_f=0.17 and R_f=0.81 to decrease in colour intensity from t=0-t=6 weeks.

3.2.1.1.5 **Hypericum samples at direct sunlight.** [See figure 3.2.7]

- Herbal tablets

  Using EMW as mobile phase showed no obvious change except for the band at R_f=0.84 which decreased from t=0-t=6 months. TLC with BEA showed the band with R_f=0.65 to decrease in colour intensity from t=0-t=6 months. The compound present at R_f=0.13 showed no obvious change. TLC with CEF showed the bands

with R_f=0.20 and R_f=0.81 to decrease in colour intensity from t=0-t=6 months. The compound present at R_f=0.17 showed no obvious change when using CEF as the mobile phase.

- Herbal capsules

  TLC with EMW showed the band with R_f=0.84 to decrease in colour intensity from t=0-t=6 months. The compound present at R_f=0.44 decreased with the capsules but showed no obvious change with the tablets. TLC with BEA showed the band with R_f=0.65 to be more distinct at the capsules than with the tablets, but also decreased with t=0-t=6 months. The compound with R_f=0.48 was only visible at t=0 months for the capsules and not for the tablets. The band with R_f=0.13 decreased in colour intensity from t=0-t=6 months, but showed no obvious change at the tablets. Using CEF as the mobile phase, showed the bands with R_f=0.20, R_f=0.17 and R_f=0.81 to decrease in colour intensity from t=0-t=6 months, but showed little or no obvious change at the tablets.

- Extract capsules

  Only the band with R_f=0.84 showed a decrease in colour intensity when using EMW as the mobile phase. TLC with BEA showed no obvious change except for the band with R_f=0.44 which decreased from t=0-t=6 months. With CEF only the band with R_f=0.81 decreased from t=0-t=6 months.

- Liquid extract

  The compound present at R_f=0.24 was only visible at t=0 months with EMW as the eluent. The band with R_f=0.84 decreased from t=0-t=6 months but the band with R_f=0.37 showed an increase in colour intensity from t=0-t=6 months. TLC with BEA showed a drastic decrease in coulour intensity for the bands with R_f=0.13, R_f=0.42 and R_f=0.65, which was totally invisible at t=6 months. TLC with CEF also showed a drastic decrease in the colour intensity of the compounds present at R_f=0.20 and R_f=0.81 which was invisible at t=3 and t=6 months. The compound presenting with a R_f=0.71 decreased from t=0-t=6 months.

3.2.1.1.6 **Hypericum samples at high humidity and direct sunlight.** [See figure 3.2.8]

- Herbal tablets

  TLC with EMW showed only the band with R_f=0.53 to decrease from t=0-t=6 months. BEA as eluent showed R_f=0.13 to decrease from t=0-t=6 months. The compound with R_f=0.48 was only slightly visible at t=0 months. TLC with CEF showed a presence of the compound at R_f=0.20 only at t=0 months. The compound with R_f=0.81 was very distinct at t=0 months, but decreased at t=3 and t=6 months.

- Herbal capsules

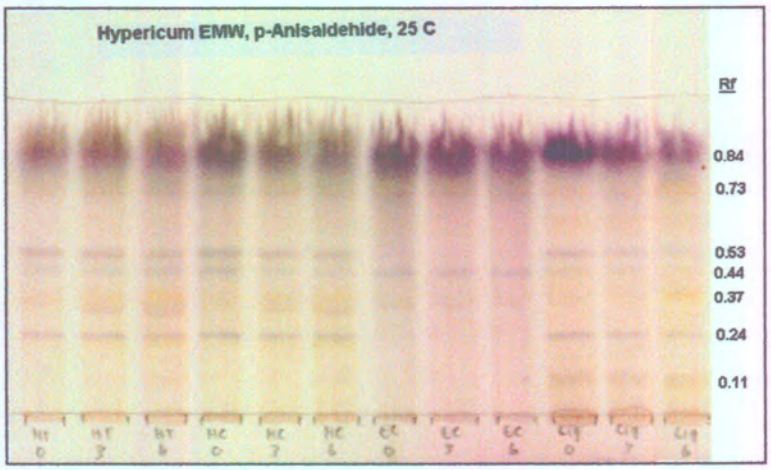
  TLC with EMW showed the intensity of the band with R_f=0.11 to increase in colour from t=0-t=6 months, when compared to the tablets which showed no obvious change. The compound present at R_f=0.24 showed no obvious change, but was more distinct with the capsules than with the tablets. The compound present at R_f=0.53 showed the same decrease as with the tablets. TLC with BEA showed the band with R_f=0.13 to decrease in colour intensity from t=0-t=6 months. The band at R_f=0.48 was only visible at t=0 months. CEF as the mobile phase showed the band with R_f=0.81 to be very distinct at t=0 months, but decreased at t=3 and t=6 months.

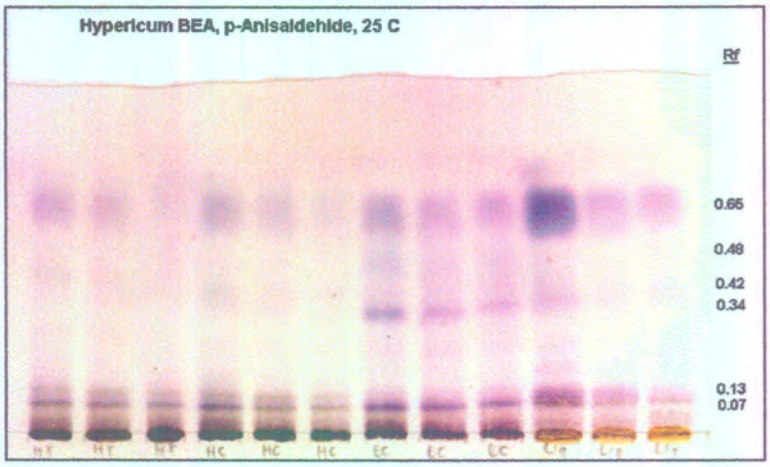
- Extract capsules

  TLC with EMW only showed a decrease in the colour intensity of the band with R_f=0.53 from t=0-t=6 months. BEA as eluent showed the compounds with R_f=0.13, R_f=0.48 and R_f=0.65 to decrease in colour intensity from t=0-t=6 months. TLC with CEF only showed a slight decrease in the colour intensity of the band at R_f=0.20 from t=0-t=6 months.

- Liquid extract

  The bands with R_f=0.24 and R_f=0.53 were only visible at t=0 months with EMW as the mobile phase. The compounds present at R_f=0.13, R_f=0.48 and R_f=0.65 were only visible at t=0 months with BEA as the eluent. TLC with CEF showed a decrease in the colour intensity of the band at R_f=0.81 from t=0-t=6 months.





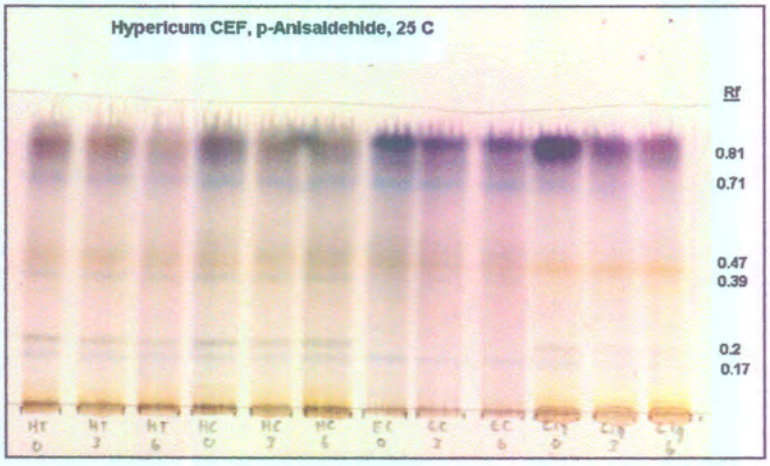


Figure 3.2.3 Hypericum samples stored at 25°C. [Sprayed with *p*-anisaldehide with mobile phase EMW (top), BEA (center) and CEF (bottom). From left :Herbal Tablets (HT) 0,3,6 months. Herbal Capsules (HC) 0,3,6 months. Extract Capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months]

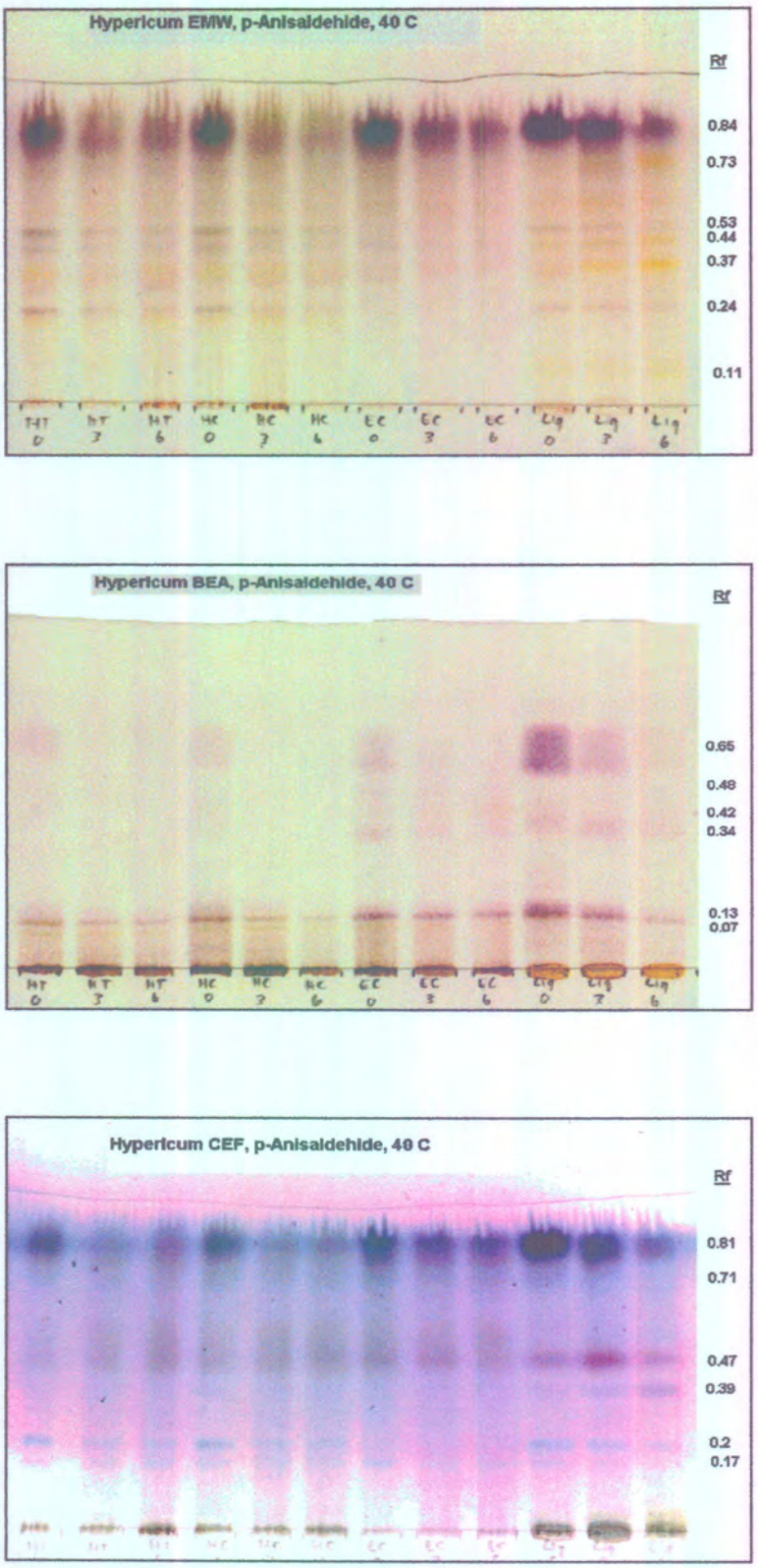


Figure 3.2.4 Hypericum samples stored at 40°C [Sprayed with *p*-anisaldehide with mobile phase EMW (top), BEA (center) and CEF (bottom). From left :Herbal Tablets (HT) 0,3,6 months. Herbal Capsules (HC) 0,3,6 months. Extract Capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months]

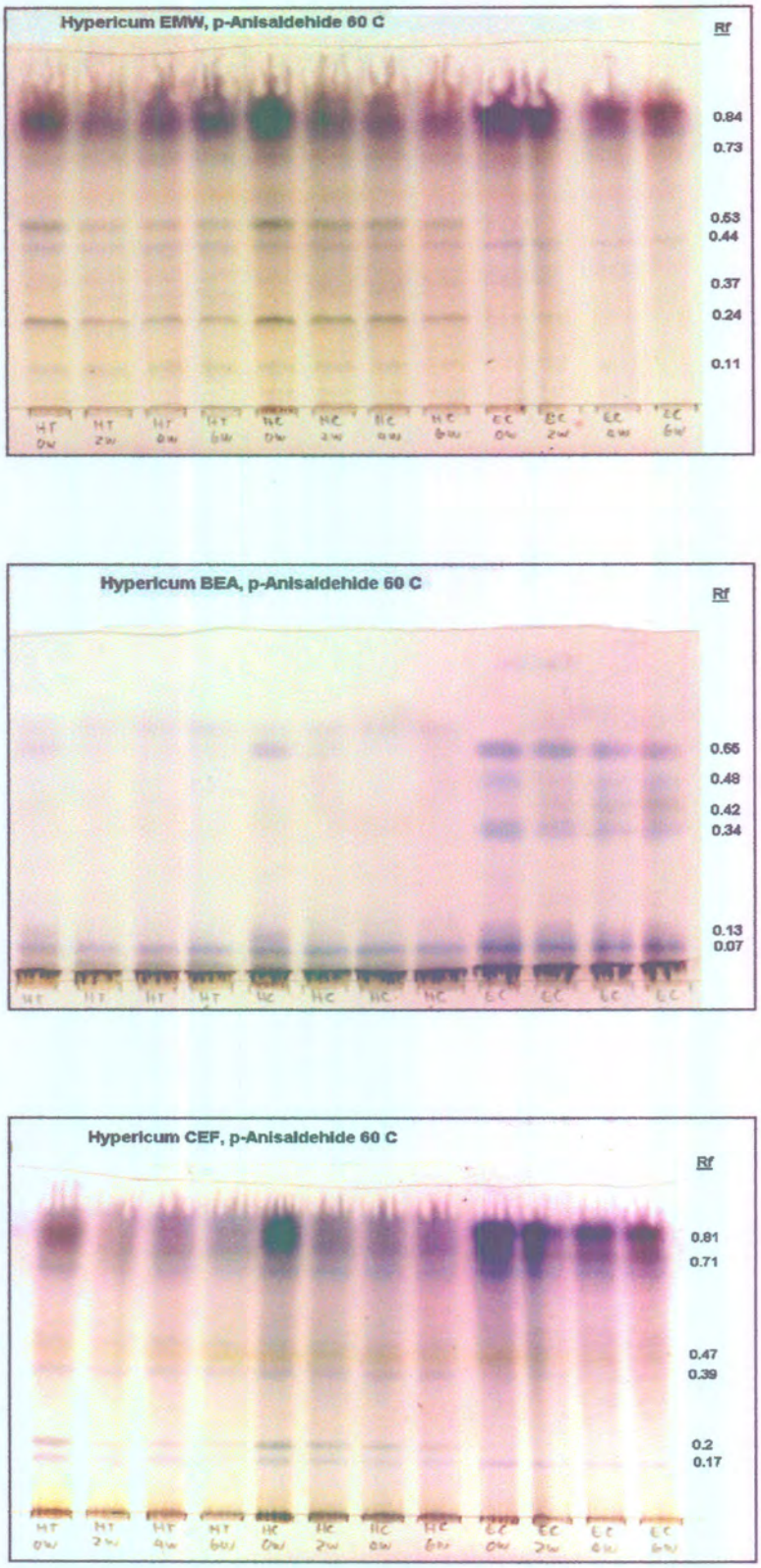


Figure 3.2.5 Hypericum samples stored at 60°C [Sprayed with *p*-anisaldehide with mobile phase EMW (top), BEA (center) and CEF (bottom). From left :Herbal Tablets (HT) 0,2,4,6 weeks. Herbal Capsules (HC) 0,2,4,6 weeks. Extract Capsules (EC) 0,2,4,6 weeks.]

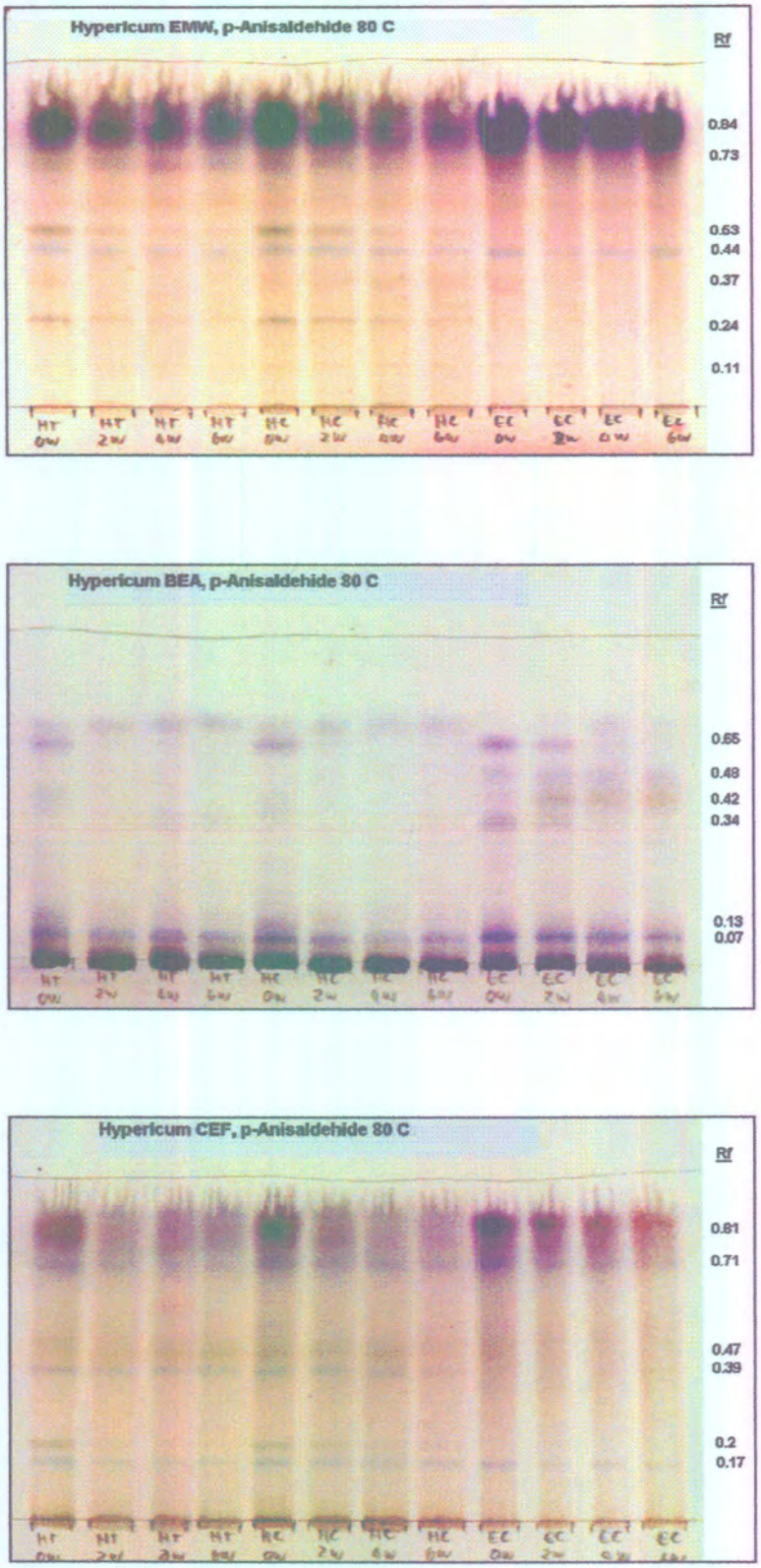


Figure 3.2.6 Hypericum samples stored at 80°C [Sprayed with *p*-anisaldehide with mobile phase EMW (top), BEA (center) and CEF (bottom). From left :Herbal Tablets (HT) 0,2,4,6 weeks. Herbal Capsules (HC) 0,2,4,6 weeks. Extract Capsules (EC) 0,2,4,6 weeks.]

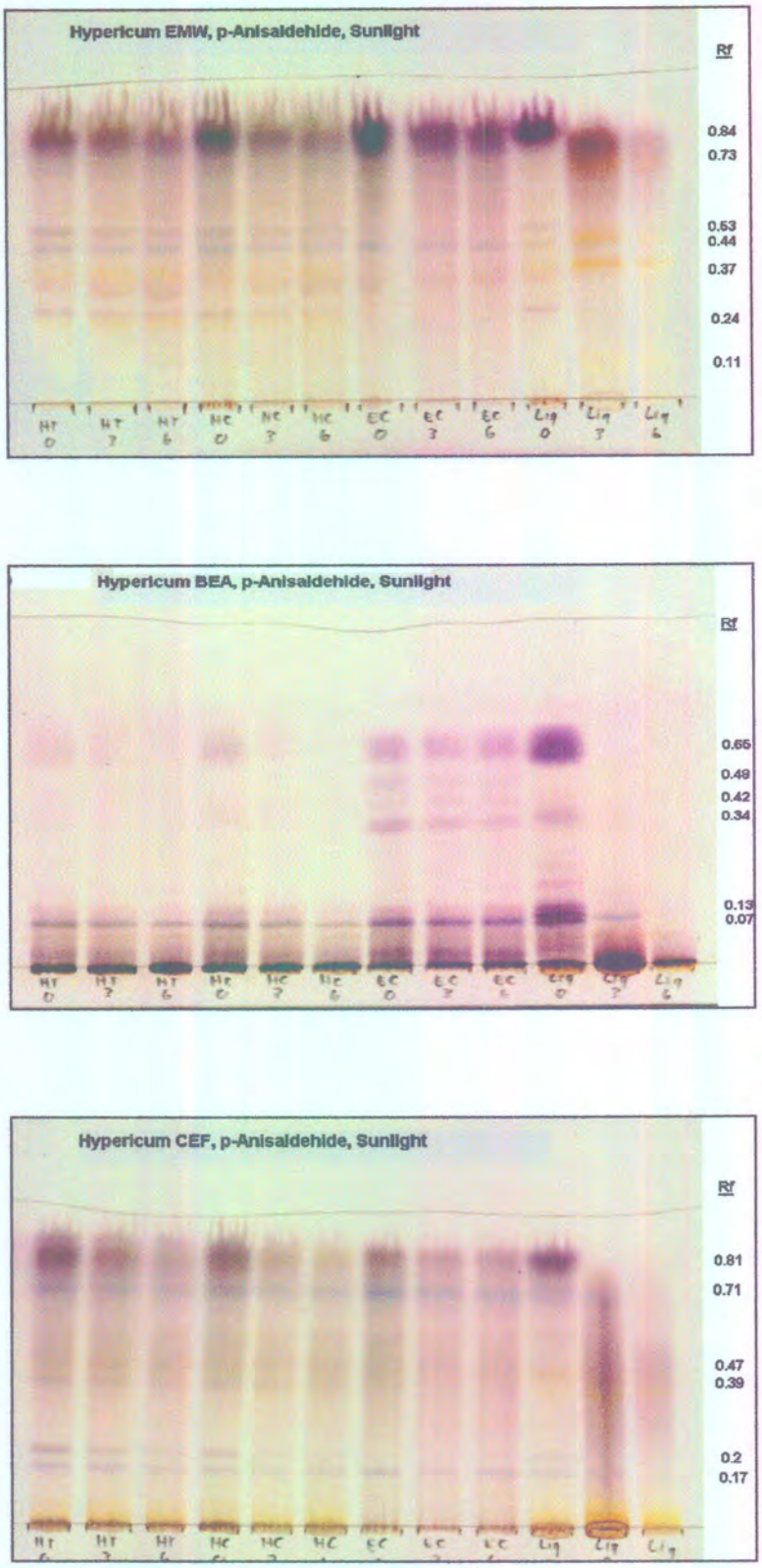
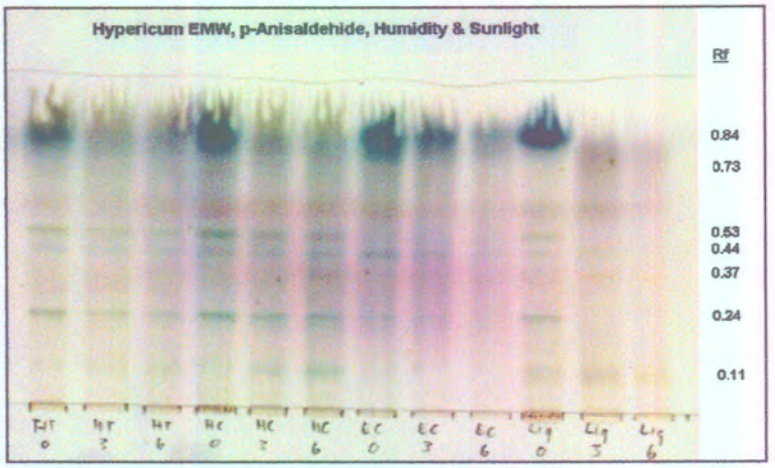
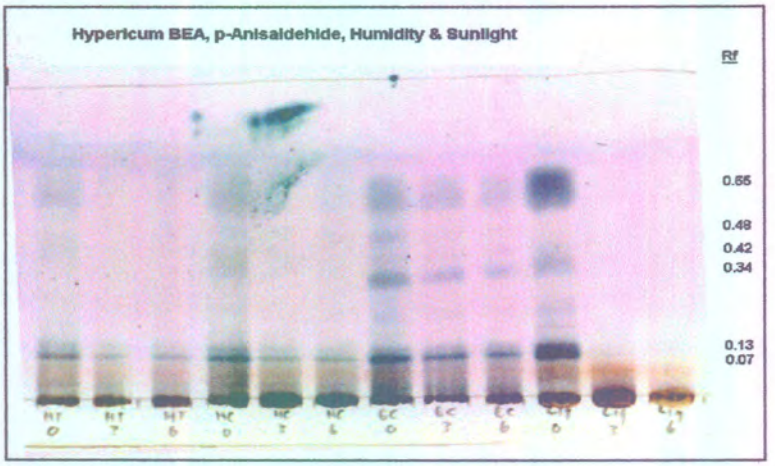


Figure 3.2.7 Hypericum samples stored at direct sunlight [Sprayed with *p*-anisaldehide with mobile phase EMW (top), BEA (center) and CEF (bottom). From left :Herbal Tablets (HT) 0,3,6 months. Herbal Capsules (HC) 0,3,6 months. Extract Capsules (EC) 0,3,6 months. Liquid extract (LIQ)0,3,6months.]





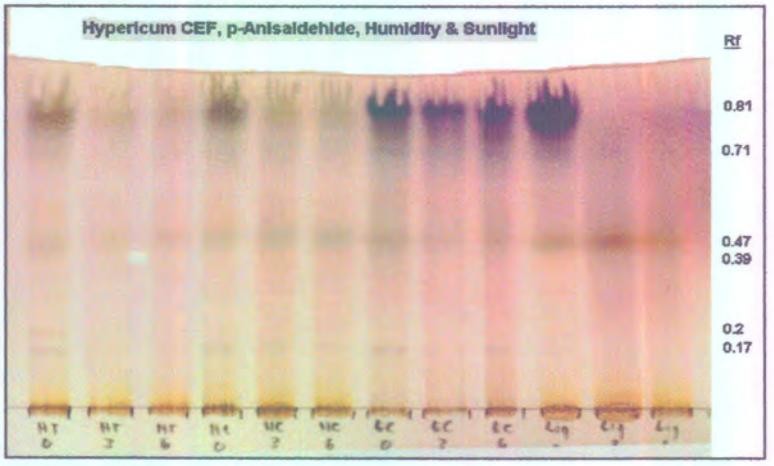


Figure 3.2.8 Hypericum samples stored at direct sunlight and high humidity [Sprayed with *p*-anisaldehide with mobile phase EMW (top), BEA (center) and CEF (bottom). From left :Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]

3.2.1.2 Spectrophotometry

Only samples of the herbal tablets, herbal capsules, extract capsules and radiated dried herb were analyzed by spechtrophotometry, as the quantity required for the liquid extracts was not sufficient for the sample preparation described in section 2.6.1.2

The absorption spectrum of each hypericum sample was printed as mentioned in section 2.6. Maximum absorbance for hypericin was in the vicinity of 590 nm as can be seen in figure 3.2.9. The absorbancy at 590 nm was read of the printout generated by the spectrophotometer [see figure 3.2.9].

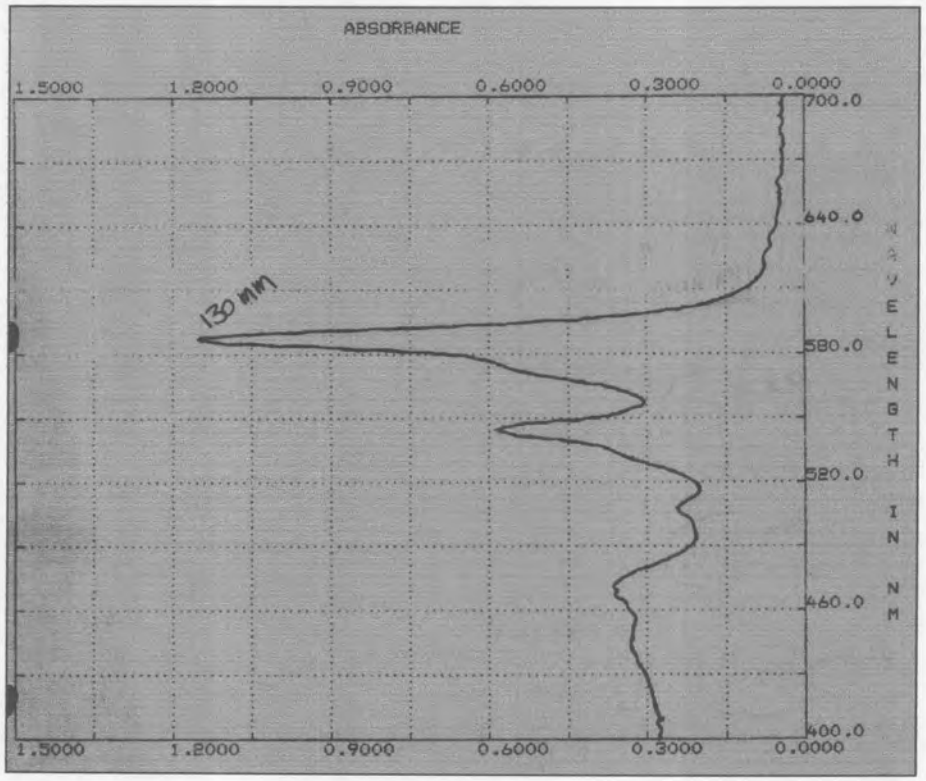


Figure 3.2.9 Absorption spectrum of the hypericin standard-solution B prepared in section 2.6.1.1

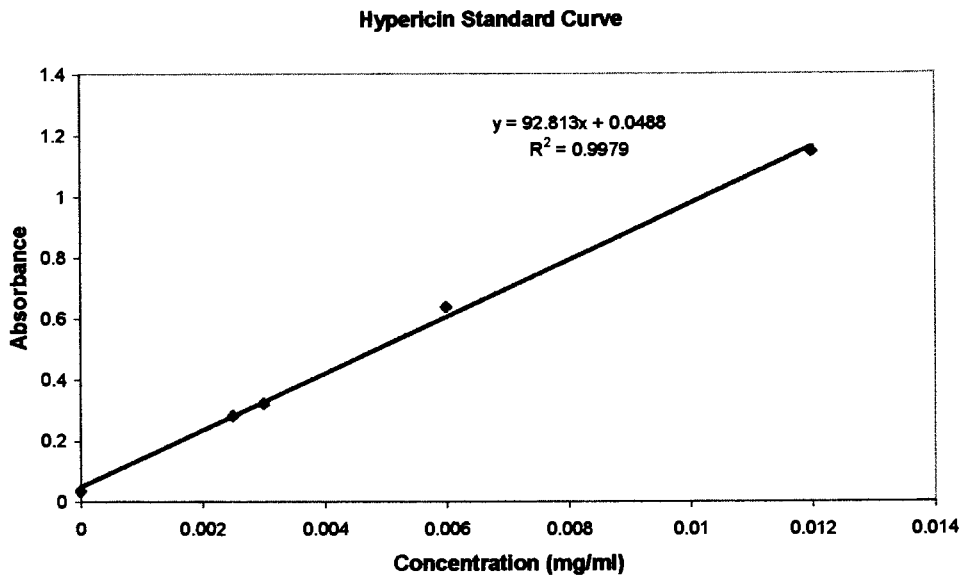


Figure 3.2.10 Hypericin standard curve [Section 2.6.1.1 for preparation]

The standard deviation on the spectrophotometric method for determining the hypericin content of the different samples, was calculated according to equation 2.3.

Table 3.2.1 Analysis of one hypericum sample repeated 5 times

Sample	X_1	X_1^2
1	0.697	0.486
1	0.692	0.479
1	0.697	0.486
1	0.692	0.479
1	0.705	0.497
Sum	3.483	2.426

$$\frac{(3.483)^2}{5} = \frac{12.131289}{5} = 2.426$$

Substituting into equation 2.3 leads to a standard deviation in absorbance of 0.0053. Using the equation for a straight line (y=mx) and substituting m with a value of 92.813 (from linear regression curve, figure 3.2.10), a standard deviation of 0.058 µg/ml hypericin is calculated. Substituting this value in equation 2.2 leads to a standard deviation of 0.00145% hypericin. According to the spectrophotometric

analysis [Table 3.2.4] there was 0.166% hypericin present at t=0 weeks = 100%. The standard deviation of 0.00145% hypericin therefore represents a variability coefficient (% standard deviation) of 0.87%.

The standard deviation in the quantity hypericin extracted from exactly 500 mg of the different hypericum dosage forms was also calculated according to equation 2.3.

Table 3.2.2 Analysis of 5 hypericum samples with exact weight of 500mg

Sample	X_1	X_1^2
1	0.666	0.444
2	0.661	0.437
3	0.679	0.461
4	0.684	0.468
5	0.705	0.497
Sum	3.395	2.306

$$\frac{(3.395)^2}{5} = \frac{11.526025}{5} = 2.305$$

Substituting into equation 2.3 leads to a standard deviation in absorbance of 0.017. Using the equation for a straight line (y=mx) and substituting m with a value of 92.813 (from linear regression curve, figure 3.2.10), a standard deviation of 0.0183 µg/ml hypericin is calculated. Substituting this value in equation 2.2 leads to a standard deviation of 0.00457% hypericin. According to the spectrophotometric analysis [Table 3.2.4] there was 0.166% hypericin present at t=0 weeks = 100%. The standard deviation of 0.00457% hypericin therefore represents a variability coefficient (% standard deviation) of 2.76%. This in other words relates to the deviation seen due to the extraction method, rather than the spectrophotometric method.

To limit the labour involved, only 5 analysis were made to determine the variability coefficient. For the spectrophotometric determination yielding a variability coefficient of 0.87% this was acceptable. For the extraction technique a value of 2.76% indicates a high variability, which might be minimized by analyzing more samples.

A summary of the actual data regarding the calculated hypericin content of each sample can be seen in table 3.2.3, 3.2.4 and 3.2.5. The following graphs are a summary of the results with the different dosage forms, with regard to their stability at each condition mentioned in section 2.3. In each graph the hypericin content at t=0 weeks was expressed as 100%. The values at 2,4,6,8,10 and 12 weeks are expressed in terms of a increase or decrease relative to the value of 100% at t=0 weeks.

Differences in the actual hypericin content calculated according to equation 2.2 will be compared to the value of the standard deviation of the extraction method (2.76%)

3.2.1.2.1 **Hypercum herbal tablets** [Table 3.2.3 for actual values]

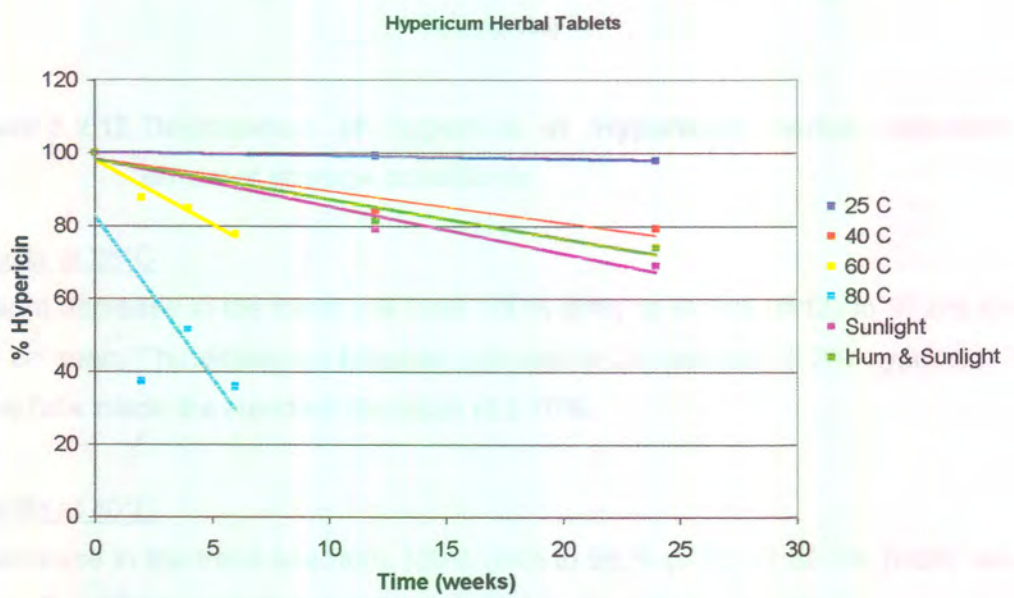


Figure 3.2.11 Degradation of hypericin in Hypericum herbal tablets at different storage conditions

Results at 25°C

A slight decrease in the trend line from 100% (t=0) to 99.2% (t=12) to 97.9% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 2.1% hypericin. This value falls inside the standard deviation of 2.76%.

Results at 40°C

A decrease in the trend line from 100% (t=0) to 84.2% (t=12) to 79.1% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 20.9% hypericin.

Results at 60°C

A noticeable decrease in the trend line from 100% (t=0) to 87.7% (t=2) to 84.8% (t=4) to 77.6% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 22.4% hypericin.

Results at 80°C

A massive decrease in the trend line from 100% (t=0) to 37.4% (t=2) to 51,7% (t=4) to 35.9% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 64.1% hypericin.

Results at direct sunlight

A decrease in the trend line from 100% (t=0) to 79.1% (t=12) to 69% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 31.% hypericin.

Results at direct sunlight and high humidity

A decrease in the trend line from 100% (t=0) to 81.2% (t=12) to 74.% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 25.9% hypericin.

3.2.1.2.2 Hypercum herbal Capsules [Table 3.2.4 for actual values]

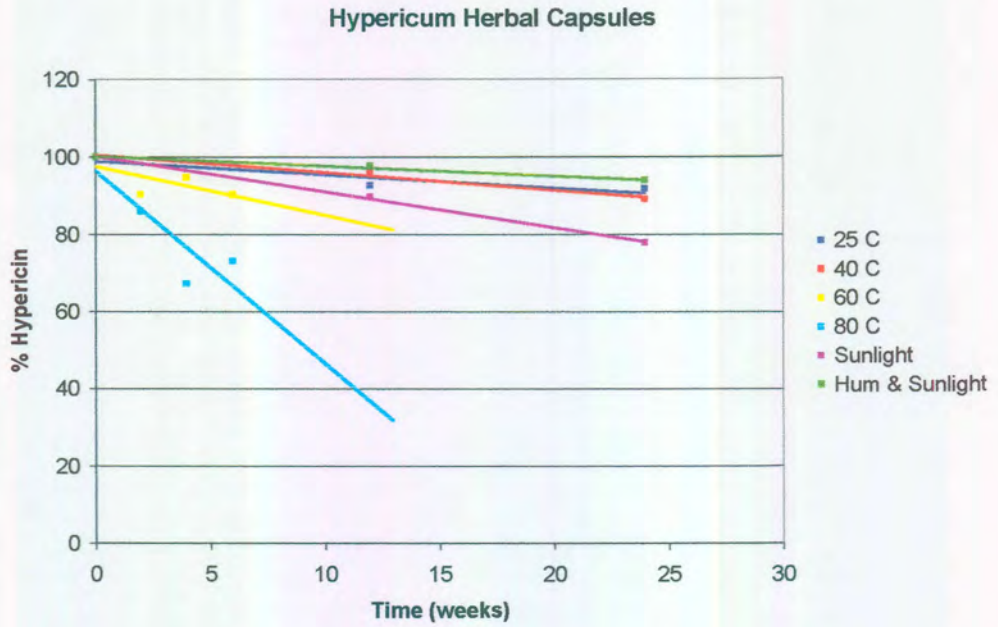


Figure 3.2.12 Degradation of hypericin in Hypericum herbal capsules at different storage conditions

Results at 25°C

A slight decrease in the trend line from 100% (t=0) to 98.9% (t=12) to 97.8% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 2.2% hypericin. This value falls inside the standard deviation of 2.76%.

Results at 40°C

A decrease in the trend line from 100% (t=0) to 96.% (t=12) to 88.9% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 11.% hypericin.

Results at 60°C

A decrease in the trend line from 100% (t=0) to 90.3% (t=2) to 94.6% (t=4) to 90.2% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 9.8% hypericin.

Results at 80°C

A noticeable decrease in the trend line from 100% (t=0) to 85.9% (t=2) to 67.2% (t=4) to 73% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 32.8% hypericin.

Results at direct sunlight

A noticeable decrease in the trend line from 100% (t=0) to 89.6% (t=12) to 77.9% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 22.2% hypericin.

Results at direct sunlight and high humidity

A decrease in the trend line from 100% (t=0) to 84.5% (t=12) to 79.4% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 20.6% hypericin.

3.2.1.2.3 Hypercum Extract Capsules [Table 3.2.5 for actual values]

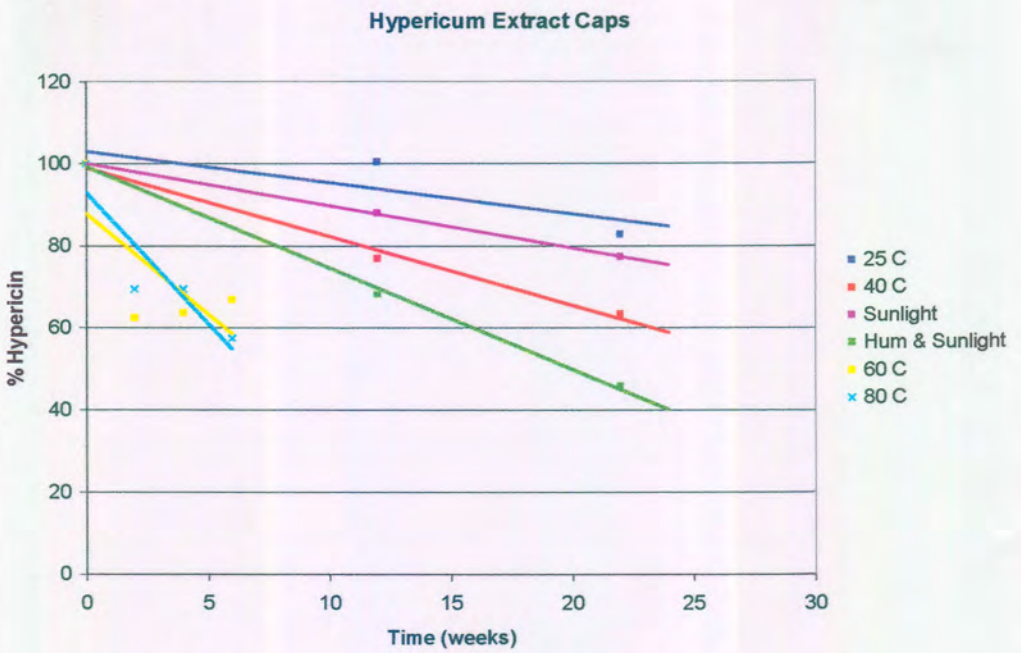


Figure 3.2.13 Degradation of hypericin in Hypericum extract capsules at different storage conditions

Results at 25°C

A slight decrease in the trend line from 100% (t=0) to 100.4% (t=12) to 94.6% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 5.4% hypericin.

Results at 40°C

A decrease in the trend line from 100% (t=0) to 76.9 (t=12) to 63.2% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 36.8% hypericin.

Results at 60°C

A decrease in the trend line from 100% (t=0) to 62.3% (t=2) to 63.6% (t=4) to 66.9% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 33.1% hypericin.

Results at 80°C

A strong decrease in the trend line from 100% (t=0) to 69.4% (t=2) to 69.4% (t=4) to 57.4% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 57.4% hypericin.

Results at direct sunlight

A noticeable decrease in the trend line from 100% (t=0) to 87.9% (t=12) to 60.% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 39.9% hypericin.

Results at direct sunlight and high humidity

A massive decrease in the trend line from 100% (t=0) to 68.1% (t=12) to 45.8% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 54.2% hypericin.

3.2.1.2.4 Hypericum root powder radiated with Cobalt-60 source

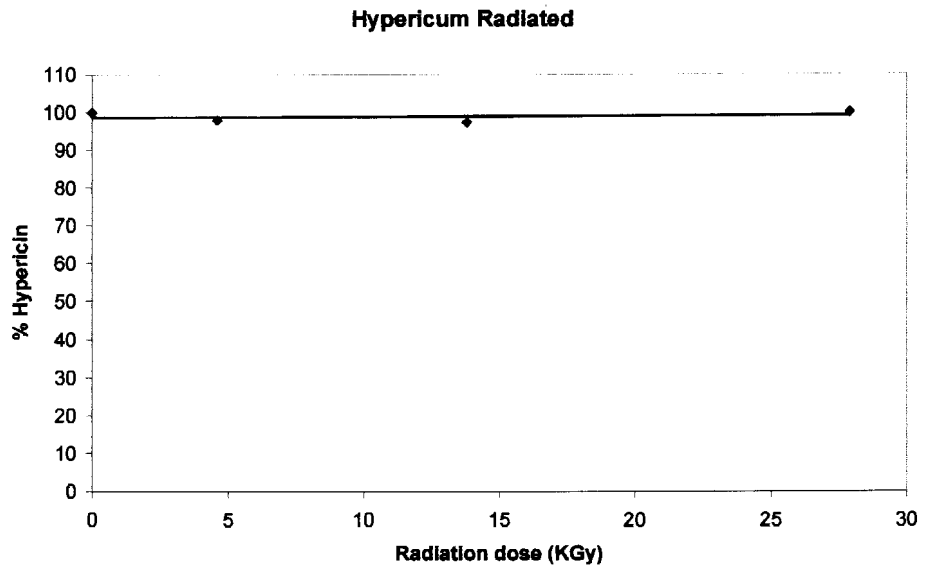


Figure 3.2.14 **Degradation of hypericin in hypericum herbal root powder at different radiation doses**

There seem to be no change in the trend line comparing the actual hypericin content at 0 kGy (100%) to a dose of 4.6kGy (97.98%) to a dose of 13.8 kGy (97.31%) and to a dose of 27.9 kGy (100.04%). The maximum difference in hypericin content is 0.0037% which is well within the range of 0.0045% hypericin calculated from table 3.2.2.

Table 3.2.3 Summary of data obtained from Spectrophotometric analysis of Hypericum Herbal tablets.

Condition	Time (weeks)	Absorbance	% Hypericin	% relative to t=0
25°C	0	0.613	0.165	100.0
25°C	12	0.608	0.164	99.2
25°C	24	0.6	0.162	97.9
40°C	12	0.516	0.139	84.2
40°C	24	0.485	0.131	79.1
60°C	2	0.538	0.145	87.7
60°C	4	0.52	0.140	84.8
60°C	6	0.476	0.128	77.6
80°C	2	0.229	0.062	37.3
80°C	4	0.317	0.085	51.7
80°C	6	0.22	0.059	35.9
Sun	12	0.485	0.131	79.1
Sun	24	0.423	0.114	69.0
Sun+Hum	12	0.498	0.134	81.2
Sun+Hum	24	0.454	0.122	74.0

Table 3.2.4 Summary of data obtained from Spectrophotometric analysis of Hypericum Herbal Capsules.

Condition	Time (weeks)	Absorbance	% Hypericin	% relative to t=0
25°C	0	0.615	0.166	100.0
25°C	12	0.608	0.164	99.0
25°C	24	0.601	0.162	97.8
40°C	12	0.591	0.159	96.1
40°C	24	0.547	0.147	88.9
60°C	2	0.556	0.150	90.3
60°C	4	0.582	0.157	94.6
60°C	6	0.555	0.149	90.2
80°C	2	0.529	0.142	85.9
80°C	4	0.414	0.111	67.2
80°C	6	0.45	0.121	73.1
Sun	12	0.551	0.148	89.6
Sun	24	0.617	0.166	77.9
Sun+Hum	12	0.519	0.140	84.5
Sun+Hum	24	0.488	0.131	79.4

Table 3.2.5 Summary of data obtained from Spectrophotometric analysis of Hypericum Extract Capsules.

Condition	Time (weeks)	Absorbance	% Hypericin	% relative to t=0
25°C	0	1.067	0.287	100.0
25°C	12	1.072	0.289	100.4
25°C	24	1.009	0.272	94.6
40°C	12	0.82	0.221	76.9
40°C	24	0.675	0.182	63.2
60°C	2	0.666	0.179	62.3
60°C	4	0.679	0.183	63.6
60°C	6	0.714	0.192	66.9
80°C	2	0.741	0.199	69.4
80°C	4	0.741	0.199	69.4
80°C	6	0.613	0.165	57.4
Sun	12	0.939	0.253	87.9
Sun	24	0.640	0.172	60.0
Sun+Hum	12	0.727	0.196	68.1
Sun+Hum	24	0.489	0.132	45.8

3.2.1.3 Summary of TLC and spectrophotometric results.

3.2.1.3.1 Herbal tablets

25°C

The hypericin content decreased by 2.1% over a 24-week period according to the spectrophotometric results. With TLC, the only significant change was with BEA as eluent where the compound present at R_f=0.65 decreased in colour

40°C

The hypericin content decreased by 20.9% over a 24-week period according to the spectrophotometric results. TLC revealed that 2 bands decreased with EMW as the mobile phase (R_f=0.24 and 0.44), and 1 band (R_f=0.65) with BEA as the mobile phase, changed. The change seen in this band is more severe than the change at 25°C.

60°C

The hypericin content decreased by 22.4% over a 6-week period according to the spectrophotometric results. TLC revealed that 3 bands decreased with EMW, and 2 with BEA as eluent. This change was still more noticeable than at 25°C and 40°C.

80°C

The hypericin content decreased by 64.1% over a 6-week period according to the spectrophotometric results. TLC revealed that 4 out of the 6 visible bands with EMW, 3 out of the 4 with BEA and 4 out of the 6 bands with CEF as mobile phases, decreased.

Sunlight

The hypericin content decreased by 31% over a 24-week period according to the spectrophotometric results. TLC revealed that 1 out of the 6 visible bands with EMW, 1 out of the 4 with BEA, and 2 out of the 6 bands with CEF as mobile phases, changed.

Sunlight and Humidity

The hypericin content decreased by 26% over a 24-week period according to the spectrophotometric results. TLC revealed that 1 out of the 6 visible bands with EMW, 1 out of the 4 with BEA, and 2 out of the 6 bands with CEF decreased.

3.2.1.3.2 Herbal capsules

25°C

The hypericin content decreased by 2.23% over a 24-week period according to the spectrophotometric results. With TLC, a slight decrease in colour of two bands could be seen with BEA as the eluent, and one change of a band with CEF as the mobile phase.

40°C

The hypericin content decreased by 11% over a 24-week period according to the spectrophotometric results. TLC revealed that 3 bands visible with EMW, and BEA as mobile phases, decreased in colour. CEF as eluent showed a decrease in only 2 bands. In all cases the decreases seen with the capsules were more noticeable that the decreases observed with the tablets.

60°C

The hypericin content decreased by 9.8% over a 6-week period according to the spectrophotometric results. TLC showed that 1 band decreased with EMW and 2 bands with BEA as eluents. This seems to contradict the decrease observed at 40°C. TLC with CEF also showed a decrease of 2 bands.

80°C

The hypericin content decreased by 27% over a 6-week period according to the spectrophotometric results. TLC showed that 3 bands with both EMW and BEA decreased. TLC with CEF showed a decrease of 4 bands. All of the decreases observed at 80°C was more prominent that the changes seen at 60°C and 40°C

Sunlight

The hypericin content decreased by 22.2% over a 24-week period according to the spectrophotometric results. TLC revealed that 2 bands decreased with EMW. BEA

and CEF both showed a decrease in colour of 3 bands. The changes observed were more pronounced than the changes seen at 25°C and 40°C.

Sunlight and Humidity

The hypericin content decreased by 20.6% over a 24-week period according to the spectrophotometric results. TLC showed that only 1 band decreased with EMW as well as BEA. TLC with CEF showed a decrease in two bands.

3.2.1.3.3 Extract capsules

25°C

The hypericin content decreased by 5.4% over a 24-week period according to the spectrophotometric results. TLC showed a decrease in colour intensity of 1 band with EMW, and 2 bands with BEA as the mobile phases.

40°C

The hypericin content decreased by 36.8% over a 24-week period according to the spectrophotometric results. TLC revealed the same decrease in colour of the bands as seen at 25°C.

60°C

The hypericin content decreased by 33.1% over a 24-week period according to the spectrophotometric results. TLC showed a decrease of 1 band and a total absence of 1 band with EMW. BEA also showed an absence of 1 band and a decrease of colour in 2 bands. TLC with CEF revealed a decrease in 1 band and a total absence of 1 band.

80°C

The hypericin content decreased by 42.6% over a 24-week period according to the spectrophotometric results. The same decreases were observed with TLC than those at 60°C. BEA aslo showed an increase in colour intensity at one band (R_f=0.48).

Sunlight

The hypericin content decreased by 40% over a 24-week period according to the spectrophotometric results. TLC revealed a decrease of 1 band with EMW, BEA and CEF.

Sunlight and Humidity

The hypericin content decreased by 54.2% over a 24-week period according to the spectrophotometric results. TLC revealed 1 band for both EMW and CEF, and 2 bands with BEA to decrease in colour.

3.2.1.3.4 Liquid extract

25°C

TLC with EMW showed a decrease in 2 bands. Three out of three with BEA and four out of the five bands with CEF decreased.

40°C

Two bands decreased with EMW as the mobile phase, all three bands with BEA and four bands with CEF decreased. The decrease was more noticeable than the decrease seen at 25°C

Sunlight

TLC showed all the possible bands to change with EMW, BEA and CEF.

Sunlight and Humidity

Five out of the 6 bands changed with EMW as eluent, all the band with BEA and three with CEF changed.

3.2.1.3.5 Radiated dried herb

Spectrophotometric analysis showed a maximum difference in the actual hypericin concentration of 0.0037%. This value falls within the range of the standard deviation (0.0045%) [See section 2.6.2 for calculation].

3.2.2 BACKGROUND OF STABILITY PRINCIPLES

The theory of chemical kinetics is based upon the law of mass action. In accordance with this law, the rate of chemical change in a reaction varies directly as the active molar concentrations of the reacting substances. For the reaction :

$$mA + nB = \text{products}$$

in which the active concentrations of A and B are equal to their actual concentrations in the system the rate of reaction is expressed as:

$$-\frac{d[A]}{dt} = k[A]^m[B]^n \qquad \textbf{Equation 3.1}$$

This mathematical equation is known as the rate expression or general rate equation. The proportionality constant *k* is the rate constant for the reaction.

Before any calculation regarding the reaction-rate or reaction-rate constant can be made, the order of the reaction must firstly be determined (Lund, 1994). Chemical reactions are classified in three different orders. Zero-order reactions are independent of the concentration of the substance under investigation and are characterized by a constant reaction-rate.

$$k = \frac{(C_0 - C)}{t} \qquad \textbf{Equation 3.2}$$

Where $C_0 - C$ = Change in concentration

T = Time in which the change took place

In a first order reaction the rate is proportional to the concentration of one of the reactants.

$$k = \frac{2.303}{t} * \log \frac{C_0}{C} \qquad \textbf{Equation 3.3}$$

In a second order reaction the rate is proportional to the concentrations of the two reactants. The expression rate is:

$$k = \frac{1}{t} * \left[\frac{(C_0 - C)}{C_0.C}\right] \qquad \textbf{Equation 3.4}$$

A detailed explanation of the derivation of equation 3.3 and 3.4 can be found in The Pharmaceutical Codex, 12th edition, page 278.

The method of substitution was used in this study for determining the order of reaction. An example of the way *k* for the hypericum herbal capsules at 25°C follows:

3.2.2.1 Example for calculating the reaction order

[Intact Hypericin concentration] (%)	100	98.95	97.77
Time (weeks)	0	12	24

Substituting these values into equation 3.2, 3.3 and 3.4 leads to:

	Zero-order	First-order	Second-order
T= 12:	0.08725%.weeks^{-1}	0,00087 weeks^{-1}	0.000088169%$^{-1}$.weeks^{-1}
T= 24:	0.09294%.weeks^{-1}	0.03345 weeks^{-1}	0.000009488%$^{-1}$.weeks^{-1}

The average values are 0.090095%.weeks^{-1}, 0.01716 weeks^{-1}, 0.000048828%$^{-1}$.weeks^{-1} respectively. Considering the values above, it seems that the calculations according to the zero-order is the most constant. Performing linear regression analysis by plotting concentration (%) over time (weeks) the slope (*k*) is calculated at 0.0929, which seems to be consistent with the values obtained for the zero-order calculations. It will therefore be assumed that hypericin follows a zero-order degradation.

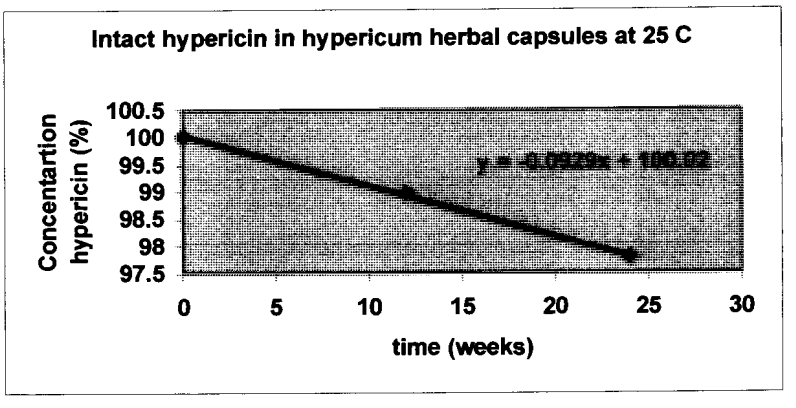


Figure 3.2.15 Using linear regression analysis to calculate *k* for Hypericum herbal capsules at 25°C.

3.2.2.2 Effect of temperature on the Rate constant

A rise in temperature increases the rate for most chemical reactions. The effect of temperature on the rate constant of a reaction can be described by the Arrhenius equation. For application to the stability of drug substances this equation is more usefully expressed in a logarithmic form:

$$\log k_T = \log A - \frac{E_a}{2.303RT} \qquad \text{Equation 3.5}$$

Where T = Absolute temperature (kelvin) for the particular order of reaction

A = Arrhenius frequency factor

Ea = Activation energy of the reaction (J/mol)

R = Universal gas constant (8.314 $J.mol^{-1}$)

The Arrhenius equation is widely applied to the results of accelerated stability tests in order to predict the stability at room temperature from that at elevated temperatures. A graph of the logarithm of the rate constant against the reciprocal of temperature is rectilinear with a slope of –Ea/2.303.R. The activation energy and the frequency factor can thus be calculated and the rate constant (k_T) at a particular storage temperature can be predicted (Lund, 1994). An example of the Arrhenius graph using data obtained from the hypericum herbal capsules is given below:

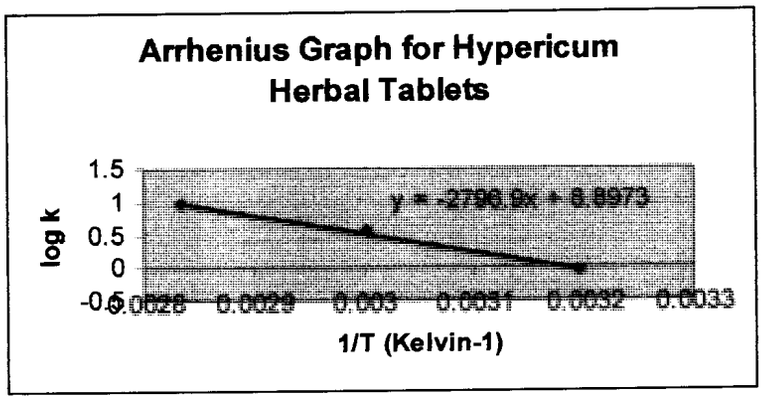


Figure 3.2.16 Arrhenius graph for data obtained for Hypericum herbal tablets.

Knowing the activation energy and the rate constant at a certain temperature, the rate constant at any given temperature i.e. 25°, can then theoretically be calculated.

An old concept based on Q_{10} is sometimes useful for estimating the shelf-life at room temperature of a product. The Q_{10} value is the factor by which a rate constant increases for a 10° rise in temperature for rough estimates (Gibaldi, 1991). It is assumed that the activation energy for the breakdown of most pharmaceutical drugs are in the order of 12-24 $kkal.mol^{-1}$. Using a minimum value of 12.2 $kkal.mol^{-1}$ for the activation energy, it can be assumed that $Q_{10} = 2$ (for an optimistic estimate) or using a maximum value of 24.5 $kkal.mol^{-1}$ so that $Q_{10} = 4$ (for a pessimistic estimate).

The Q_{10} approach is not suitable for the more precise predictions of shelf-life required in the development of a new product. It should therefore be used with caution because values of Q_{10} may not be 2 to 4 and may vary with the particular 10° range of temperature chosen. It's use may give misleading results, for example where the mechanism of action changes with temperature or where the manufacturer bases shelf-life on physical or microbiological stability, which is currently the norm for herbal products (Lund, 1994 and Stoklosa and Ansel, 1991).

The stability of *Hypericum perforatum, Ginkgo biloba* and *Piper methysticum*, will be quantitatively expressed in terms of their shelf-life. This is defined as the time during which the product is predicted to remain fit for it's intended use under specified conditions of storage. The shelf life of a medicinal product kept in it's closed container under specified conditions is commonly defined as the time from manufacture until the original potency or concentration of active constituent has been reduced by 10%. This time is known as the $t_{10\%}$ or t_{90}. For calculating t_{90} for zero-order reactions the following equation can be used:

$$t_{90} = \frac{0.1C_0}{k} \qquad \textbf{Equation 3.6}$$

Where k = reaction rate constant

C_0 = Concentration at t=0

Derivation of this equation can be found in the Pharmaceutical Codex.

As calculated in section 3.2.2.1 above, hypericin seems to follow a zero-order degradation. A summary of the k value of the hypericin concentration present in each dosage form and different storage condition is given in table 3.2.6, 3.2.7 and 3.2.8. This k values was obtained from the linear regression analysis from the graphs of hypericin concentration (%) plotted against time (weeks)

Table 3.2.6 *k*-values for hypericin in Hypericum Herbal tablets

Condition	*K* – value ($\%.weeks^{-1}$)
25°C	0.0888
40°C	0.8704
60°C	3.5065
80°C	8.9015
Sunlight	1.2921
Sunlight & humidity	1.0817

Table 3.2.7 *k*-values for hypericin in Hypericum Herbal capsules

Condition	*K* – value ($\%.weeks^{-1}$)
25°C	0.0929
40°C	0.4617
60°C	1.2555
80°C	4.974
Sunlight	0.9229
Sunlight & humidity	0.86

Table 3.2.8 *k*-values for hypericin in Hypericum Extract capsules

Condition	*K* – value ($\%.weeks^{-1}$)
25°C	0.2263
40°C	1.5317
60°C	4.906
80°C	6.392
Sunlight	1.6666
Sunlight & humidity	2.2588

The t_{90} values for each dosage form at different storage conditions were calculated according to equation 3.5. A summary of these values are given in table 3.2.9 below.

Table 3.2.9 t_{90} values for Hypericum Herbal tablets, herbal capsules and extract capsules.

Condition	Herbal tablets(weeks)	Herbal capsules(weeks)	Extract capsules(weeks)
25°C	113	108	44
40°C	11	22	7
60°C	3	8	2
80°C	1	2	2
Sunlight	8	11	6
Sunlight & humidity	9	12	4

Using the Arrhenius graph to plot log *k* over 1/T the activation energy for the herbal tablets was calculated at 53.552 kkal/mol, the herbal capsules at 48.321 kkal/mol and the extract capsules at 33.189 kkal/mol.

3.2.2 DISCUSSION

It seemed that the herbal tablets were very stable at 25°C. The slight decreases of colour seen with some TLC plates might be the result of the incomplete reaction between the spraying reagent and the separated compound. The fact that all the values obtained from spectrophotometry fell within the standard deviation confirms this assumption. Exposure to high temperatures seems to cause a noticeable decrease in the hypericin concentration of the tablets. It also seems that the higher the temperature, the more the decrease. This hypothesis appears to be confirmed by the noticeable pattern between the decrease calculated from spectrophotometry, and the decreases in colour observed with TLC. Having calculated the activation energy at 53.552 kkal/mol from the Arrhenius graph, and substituting this value into a corrected form of equation 3.5, the estimated k_{25} was then calculated at 0.308%.weeks^{-1} for the herbal tablets. This leads to a estimated t_{90} value of 32 weeks. Comparing this value to the actual value calculated from the data acquired over the six month storage period for the herbal tablets at 25°C (113 weeks), showed no similarity.

The herbal capsules further appeared to be a very stable dosage form. As with the herbal tablets, the decrease in the calculated hypericin concentration at 25°C fell

within the standard deviation. A noticeable difference however, was the slight decrease seen with the herbal capsules, but not with the tablets at 25°C. A possible explanation of the less noticeable decrease in colour intensity at 60°C, when compared to 40°C, could be the result of an incomplete reaction between the spraying reagent and the separated compounds in some cases. This seems to be verified by the spectrophotometric calculation of the actual hypericin concentration at 60°C, which is less than calculated at 40°C. As with the herbal tablets, the activation energy was calculated at 48.321 kkal/mol, which lead to an estimated t_{90} value of 21.61 weeks. There was also no comparison between this value, and the actual value calculated at 107.6 weeks for the herbal capsules at 25°C.

A rise in temperature seemed to decrease the hypericin concentration in the herbal capsules in a similar pattern as for the tablets. Again TLC confirmed this predicament.

The extract capsules seem to be less stable at 25°C in comparison to the herbal tablets and herbal capsules. This can be supported by the measured decrease of hypericin concentration. The noticeable decrease in colour intensity observed with TLC also supports this notion. As seen with the herbal tablets and herbal capsules, a rise in temperature also seems to cause a decrease in hypericin concentration. TLC patterns seems to verify this decrease in concentration. The increase in colour intensity at 80°C for R_f=0.48 might be the formation of a by-product, but this is not certain. As with the herbal tablets and herbal capsules, the activation energy was calculated at 33.18 kkal/mol. This leads to an estimated t_{90} value of 14 weeks. Again there was no comparison between this value, and the actual value calculated at 44.18 weeks for the extract capsules at 25°C.

A general pattern can be observed. The stability regarding the herbal tablets and capsules seems to decrease in the following order: 25°C ➔40°C➔Direct sunlight & Humidity ➔ Sunlight➔60°C➔80°C. The extract capsules decrease in the same way, with only one possible difference. Here it seems that the combination of direct sunlight and high humidity have a greater effect on the degradation of hypericin, compared to sunlight alone. This is supported by the TLC chromatograms, were a decrease of colour in 2 bands are observed with exposure to high humidity and sunlight, compared to the decrease of only one band exposed to sunlight alone.

Keeping this in mind, and taking into consideration the small differences calculated between the hypericin concentration for the tablets, as well as the herbal capsules at direct sunlight, compared to the combination of sunlight and high humidity, [see section 3.2.1.3 and 3.2.1.4] it may be dangerous to speculate on which combination causes the most degradation. The effect on the degradation of hypericin after exposure to direct sunlight, and the combination of sunlight and high humidity should be further investigated.

It seems that the stability of the herbal capsules supercedes both the tablet and extract capsule- dosage forms. This is due to smaller differences in the calculated hypericin concentration measured at each exposed condition, as well as the longer t_{90} values [see table 3.2.9]. This pattern is observed in all the different environmental conditions, except for the herbal tablets at 25°C. At 25°C the herbal tablets shows a slightly longer t_{90} value, than the value for the herbal capsule. This slightly longer t_{90} value might only be the result of a variation in determining the regression line in the graph used to calculate the *K* value, and is probably not significant.

Assuming the longer t_{90} value was not the result of a mathematical miscalculation, several possibilities can be given. One reason might be the additional effects of some of the non-active ingredients, perhaps contributing to the stability at a temperature where it is less likely for degradation to occur. However, exposure to temperatures higher that 25°C seems to have a more negative effect on the stability when compared to the herbal capsules. The effects of non-actives should be further investigated.

According to Rubenstein (1995), the process of direct compressibility in the manufacturing of tablets has a positive effect on the stability of the product. Since this method was used in the manufacturing of these tablets, it could be a possible explanation of the seemingly superior stability. The effect of high pressure on the stability of hypericin should be further investigated.

A possible reason for the herbal capsules showing an increased stability at higher temperatures and unfavourable environmental conditions, compared to the herbal tablets, might be due to the additional stability provided by the empty gelatine capsule. Taking the above into consideration, it seems that the herbal tablets provide more stability at 25°C than the empty gelatine capsule alone. This however still needs to be confirmed.

The extract capsules show the least stability [Table 3.2.9]. One reason might be the fact that, in general, chemical reactions in solution is faster than in the dry state. Another reason might be because of the extraction and purification during the manufacturing process of the standardized 0.3% extract. Some of the compounds present in the milled dried herb, are removed during this extraction process. The removal of compounds can clearly be seen when considering the TLC fingerprints between the extract and the whole herb. These compounds might have an additional effect on the stability and degradation of hypericin by acting as anti-oxidants and thus protecting the active(s) against oxidation. Another possible reason could be the stability provided by the intact cell-membranes in the whole herb, which is broken or destroyed during the preparation of the extract. This however needs to be further elucidated.

When considering all of the above dosage forms, it seems that sunlight, or the combination of sunlight and high humidity, has a seemingly negative effect on the stability. This effect seems to be more noticeable with the extract capsules, than for the herbal tablets or capsules.

A comparison between the different dosage forms reveal in all cases that the extract capsules and the liquid extracts do not contain all of the compounds present in the whole herb. It is however uncertain that those compounds absent in the extract dosage forms, contribute to a negative effect on the stability and/or a positive synergistic effect on the whole herb. This needs to be investigated.

The fact that the liquid extracts were not quantitatively analyzed, made it more difficult in evaluating the stability compared to the herbal tablets, herbal capsules and extract capsules. As mentioned in the above discussion, certain differences and similarities between the intensity of the colours varied in a few instances because of the possible incomplete reaction between the spraying reagent and the separated compounds. This added to the concern of not making a true and meaningful assumption on the stability of the liquid extracts.

The only compounds that were present in all of the dosage forms seemed to be R_f=0.44 and R_f=0.37 (with EMW), R_f=0.07 and R_f=0.65 (with BEA), R_f=0.47 and R_f=0.17 (with CEF) [See figure 3.4]. Looking only at these compounds, and making a judgement upon the intensity of the colours of these bands, the liquid extracts seem

to follow the same pattern as the herbal tablets, herbal capsules and extract capsules. It also seemed that the concentration of the compounds present in the liquid extract at t=0 weeks (although only 25 μg applied in all cases) [see section 2.5.3], are higher than for t=0 weeks compared to the extract capsules. A much more intense decrease of this colour intensity is however observed at t=12 and t=24 weeks, compared to the extract capsules. It might therefore be considered that the extract capsules are more stable than the liquid extracts.

When looking at figure 3.15 it can be assumed that gamma radiation has very little effect on the stability of hypericin since the maximum change in concentration was only 0.0038% which falls within the standard deviation of 0.0045%

Certain differences in the TLC fingerprints of the different dosage forms was evident, but with the correct mobile phase and spraying reagent, TLC certainly proved to be beneficial in confirming the results obtained from the quantitative analysis performed by spectrophotometry. Although TLC doesn't give any quantitative data, it is still useful in the qualitative analysis of samples. The TLC provides an interesting picture of all the changes (whatever the chemical compound) that took place in each respective time interval.

Where quantitative data is not required for expressing the stability of a dosage form containing hypericum, TLC could possibly be used alone. This might be considered if a sample of the dosage form at t=0 is available, or if a known standard concentration hypericin is placed on the same plate, thus comparing the sample of unknown stability with the sample at t=0 (or the hypericin standard)

A disappointing conclusion seems to be the fact that the Arrhenius equation for calculating the estimated shelf-life at 25°C, could not be applied to hypericum herbal tablets, herbal capsules or the extract capsules. One of the reasons might be because of the high activation energy displayed in all three dosage forms. The activation energy for most medicinal substances is in the order of 12.2 kkal/mol - 24.5kkal/mol (Gibaldi, 1991). The activation energy as mentioned earlier, was 53.552 kkal/mol, 48.32 kkal/mol and 33.18 kkal/mol for the herbal tablets, herbal capsules and extract capsules respectively. The activation energy for these hypericum samples thus seems much higher than the average values of the activation energy of medicinal substances for which the Arrhenius equation is usually applicable.

Another reason to be considered could be the unlikeliness that the estimates of log *k* at different temperatures will be known with equal precision because of the reproducibility worsening as degradation proceeds (Lund, 1994). The regression line of the graph plotted in accordance with the linear form of the Arrhenius equation could then be incorrect.

The Arrhenius equation is mostly used for substances in aqueous solution. The kinetics of degradation of drugs in solid dosage forms is usually complex. The use of the Arrhenius equation can therefor not be applied with the same level of confidence than with drugs in aqueous solution; for example: moisture loss from tablets or capsules at higher temperatures may lead to deviations from the Arrhenius equation (Lund, 1994).

3.2.3 SUMMARY

In summarizing the above discussion, it seems that hypericum herbal tablets and herbal capsules display an almost equal stability at 25°C. The higher the temperature, the faster the degradation of hypericin. Capsule dosage forms seem to be slightly more stable than the tablets in this scenario. Both the tablets and the herbal capsules seem to exhibit a superior stability profile over the extract capsules and the liquid extracts. Extract versions of hypericum seems to degrade faster that the whole herb. Moist and sunlight seem to have a negative effect on the stability of all the dosage forms. Hypericin generally seems to be unaffected by radiation.

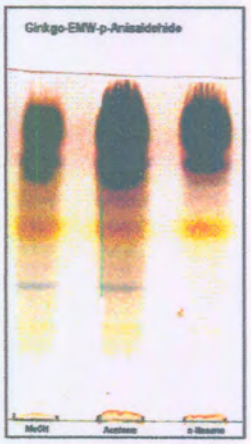
3.3 GINKGO BILOBA

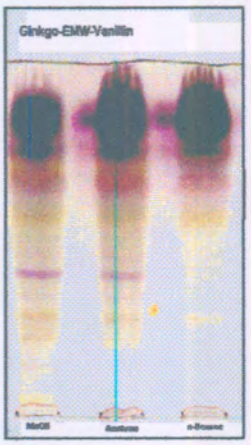
3.3.1 RESULTS

3.3.1.1 Thin layer chromatography

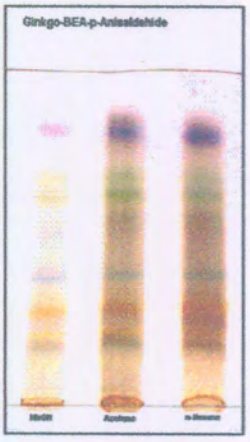
From the visual examination of the plates prepared in section 2.5, it was also decided to use all three of the mobile phases, excluding the official method (section 2.5.3) and using only vanillin in stead of *p*-anisaldehide as spraying reagent. With vanillin EMW showed 5 distinct bands and *p*-anisaldehide only 3 (absence of bands at R_f=0.51 and R_f=0.24). BEA revealed 8 bands with vanillin but only 7 (absence of band at R_f=0.08) with *p*-anisaldehide CEF showed 3 bands for both vanillin and *p*-anisaldehide [See figure 3.3.1]. The official method was also discarded as mentioned in section 2.6 because of the simplicity of our methods compared to the British Herbal Pharmacopoeia. [See figure 3.3.2] TLC was done on all samples, except for the dried ginkgo leaf powder exposed to gamma-radiation.

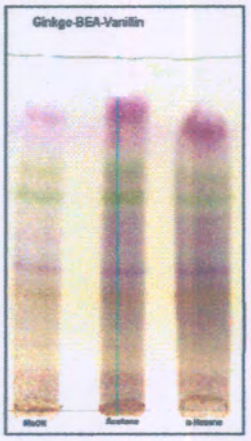
Ten µl of the quercetin standard solution was also applied to separate plates, and allowed to develop using all three mobile phases as described in section 2.5.1. Looking at the plate developed in CEF, it seemed that quercetin presents with a R_f value of about 0.45. TLC with EMW showed quercetin to have a R_f value of 0.77. TLC with BEA seemed to indicate a R_f value of 0.09 for the quercetin standard. It is important to note that, as mentioned in section 1.5.3, the mechanism of ginko's therapeutic effects is believed to be attributed to the synergistic effects of it's constituents. Based on this it was decided to look at all the compounds as a whole, and not only quercetin.



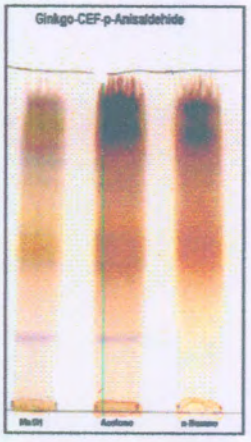


---R_f = 0.77





---R_f = 0.09



---R_f = 0.45

Figure 3.3.1 TLC of Ginkgo leaf powder with mobile phase EMW (top), BEA (center) and CEF (bottom) and sprayed with *p*-anisaldehide (left)and vanillin (right). Each plate shows extraction with Methanol (left), Acetone (center), and n-Hexane (right). R_f values for quercetin are indicated.

Figure 3.3.2 Ginkgo identification according to the British Herbal Pharmacopoeia. (Ginkgo left and Rutin right)

3.3.1.1.1 Ginkgo samples at 25°C [See figure 3.3.3]

- Herbal tablets

  There was no obvious change with EMW or BEA as the mobile phases. TLC with CEF showed no clear distinction between any bands, but the colour at t=3 months was more intense than at t=0 and t=6 months.

- Herbal capsules

  TLC with EMW showed no obvious change. TLC with BEA showed a slight decrease of the bands at R_f=0.09 and R_f=0.18. The compound presenting with R_f=0.75 was only visible at t=0 and not at t=3 of t=6 months. Using CEF as the mobile phase showed no clear distinction between any bands, but the colour still decreased from t=0-t=6 months.

- Extract capsules

  TLC with EMW showed only the compounds presenting with R_f=0.37, R_f=0.54, R_f=0.62, R_f=0.73 and R_f=0.77 to be present at t=0 months, and not at t=3 or t=6 months. Using BEA as eluent only showed a band with R_f=0.03 which had a slight decrease in intensity from t=0-t=6 months. TLC with CEF showed a very light band at R_f=0.46 and only at t=0 months. No bands were visible at t=3 or t=6 months.

- Liquid extracts

  The bands at R_f=0.48 and R_f=0.77 were more distinct at t=3 months than at t=0 and t=6 months with EMW as the mobile phase. The compound present at R_f=0.83 showed no obvious change in time. TLC with BEA only showed the bands with R_f=0.05 and R_f=0.18, which seemed to be equally intense at t=0 and t=6 months, but more intense at t=3 months. CEF as the eluent showed no bands at t=0 or t=6 months, but the bands with R_f=0.46 and R_f=0.68 were visible at t=3 months.

3.3.1.1.2 **Ginkgo samples at 40°C.** [See figure 3.3.4]

- Herbal tablets

  TLC with EMW showed a band only at R_f=0.83, but no obvious change in the intensity of this band. TLC with BEA showed no obvious change in any bands. Using CEF as the mobile phase showed a slight decrease in the intensity of the band at R_f=0.14 from t=0-t=6 months.

- Herbal capsules

  TLC with EMW also showed the band with R_f=0.83 without any change. The compound with R_f=0.77 was only visible at t=0 and only visible with the capsules. Using BEA as the eluent showed a decrease in the colour intensity of the band with R_f=0.09 from t=0-t=6 months. TLC with CEF showed the band with R_f=0.14 to be more visible for the capsules than for the tablets, but the decrease in intensity from t=0-t=6 months seemed more pronounced that with the tablets.

- Extract capsules

  TLC with EMW showed bands with R_f=0.37, R_f=0.54, R_f=0.62, R_f=0.73, and R_f=0.77 only to be visible at t=0 months, and not at t=3 or t=6 months. These bands was only visible with the extract capsules and not with the tablets or herbal capsules. TLC with BEA showed a decrease in colour intensity for the band with R_f=0.09 after t=3 months, and disappeared at t=6 months. CEF as the mobile phase showed a strong decrease in colour intensity of the bands at R_f=0.18 and R_f=0.45 from t=0-t=6 months. The compounds presenting with R_f=0.27, R_f=0.35, R_f=0.54 and R_f=0.68 were only visible at t=0.

- Liquid extracts

  The compound with R_f=0.77 was only visible at t=0 and t=3 months with EMW as the mobile phase, but almost disappeared after t=6 months. TLC with BEA showed the bands at R_f=0.05 and R_f=0.09 to decrease from t=0=t=3 months. These bands were almost invisible at t=6 months. TLC with CEF showed a slight decrease in the colour intensity of the bands with R_f=0.14, R_f=0.45 and R_f=0.68.

3.3.1.1.3 Ginkgo samples at 60°C. [See figure 3.3.5]

- Herbal tablets

  TLC with EMW showed no bands except for the one with R_f=0.83 which showed no obvious change from t=0-t=6 weeks. BEA as mobile phase showed the band with R_f=0.18 to decrease from t=0-t=6 weeks. The band with R_f=0.09 was only visible at t=0 weeks. TLC with CEF showed no clear separation of bands, but what seemed to be a compound with R_f=0.74, showed a decrease from t=0-t=6 weeks.

- Herbal capsules

  TLC with EMW showed no bands except for one with R_f=0.83 which showed no obvious change from t=0-t=6 weeks. BEA as the eluent showed the band with R_f=0.18 to decrease in colour intensity from t=0-t=6 weeks, but this change in colour intensity was more definite with the capsules than with the tablets. The band with R_f=0.09 also showed a drastic decrease, but was still visible at t=2,4 and 6 weeks. TLC with CEF showed no clear separation of the bands although the colour was more visible compared to the tablets. What seemed to be a compound with R_f=0.74, also showed a decrease from t=0-t=6 weeks.

- Extract capsules

  TLC with EMW showed the band with R_f=0.77 only to be visible at t=0 and t=2 weeks, but disappeared at t=4 and t=6 weeks. No bands could be observed with BEA as the mobile phase. TLC with CEF showed a decrease in colour intensity from t=0-t=6 weeks for the bands with R_f=0.45 and R_f=0.18.

3.3.1.1.4 Ginkgo samples at 80°C. [See figure 3.3.6]

- Herbal tablets

  Only the band with R_f=0.83 was visible with EMW as the mobile phase, but showed no obvious change. TLC with BEA showed the band with R_f=0.18 to

decrease in colour intensity from t=0-t=6 weeks. The compound with R_f=0.09 was only visible at t=0 weeks and not at t=2,4 or 6 weeks. TLC with CEF showed a decrease in colour intensity from t=0-t=6 weeks for the band with R_f=0.74.

- Herbal capsules

  TLC with EMW also showed no obvious change for the band with R_f=0.83, but the band with R_f=0.77, which was more visible for the capsules than for the tablets, showed a slight decrease from t=0-t=6 weeks. BEA as the mobile phase showed the band with R_f=0.18 to decrease in colour intensity from t=0-t=2 weeks, but was invisible at t=4 and t=6 weeks. The compound with R_f=0.09 was only visible at t=0 and t=2 weeks. TLC with CEF showed a decrease in colour intensity from t=0-t=6 weeks for the band with R_f=0.74.

- Extract capsules

  The bands with R_f=0.37 and R_f=0.54 was only visible at t=0 weeks, but not at t=2,4 and 6 weeks, when using EMW as the mobile phase. The compounds with R_f=0.77, R_f=0.73 and R_f=0.62 were clearly visible at t=0 weeks, almost invisible at t=2 and t=6 weeks, but more visible at t=4 weeks. TLC with BEA showed no change. TLC with CEF showed the bands with R_f=0.18 and R_f=0.54 only to be visible at t=0 weeks. The bands with R_f=0.45 and R_f=0.74 increased in colour intensity from t=0-t=6 weeks.

3.3.1.1.5 **Ginkgo samples at direct sunlight.** [See figure 3.3.7]

- Herbal tablets

  TLC with EMW showed no obvious change for the bands with R_f=0.83 and R_f=0.77. Using BEA as the eluent showed the band with R_f=0.75 to increase in colour intensity from t=0-t=6 months, otherwise there was no obvious change. TLC with CEF showed no obvious change for the bands with R_f=0.74 and R_f=0.54.

- Herbal capsules

  TLC with EMW showed no obvious change of the band present at R_f=0.77, but showed a decrease in colour intensity of the band at R_f=0.83 from t=0-t=6 months. BEA as the mobile phase showed a decrease in the intensity of the colour at the bands with R_f=0.38 and R_f=0.75. The compound present at R_f=0.75 had a drastic decrease from t=0-t=6 months. CEF as the eluent showed no obvious change of

the band with R_f=0.54, but the compound present at R_f=0.74 showed a noticeable decrease in colour intensity from t=0-t=6 months.

- Extract capsules

  TLC with EMW showed the compounds present at R_f=0.77, R_f=0.73, R_f=0.62, R_f=0.54 and R_f=0.37 to decrease noticeably in colour intensity from t=0-t=6 months. BEA as the mobile phase showed a slight decrease in colour intensity of the band with R_f=0.09 from t=0-t=6 months. TLC with CEF showed a drastic decrease in the colour intensity of the band at R_f=0.45 from t=0-t=6 months. The compound present at R_f=0.18 also decreased dramatically from t=0-t=6 months, but was only visible with the extract capsules, and not with the herbal capsules, tablets, or liquid extract.

- Liquid extracts

  With EMW there was a seemingly increase of the intensity of the colour at bands R_f=0.83 and 0.77. TLC with BEA and CEF showed no obvious change.

3.3.1.1.6 **Ginkgo samples at high humidity and direct sunlight.** [See figure 3.3.8]

- Herbal tablets

  With EMW no obvious change could be seen. The compound with R_f=0.38 had no obvious change, but the band with R_f=0.75 was more intense at t=3 months, than at 0 or 6 months when looking at BEA as the eluent. TLC with CEF showed no obvious change.

- Herbal capsules

  All of the bands at t=0 months are clearly visible, even more visible than compared to the TLC plate at 25°C, but at t=3 and t=6 months none of these bands were present except for the band at R_f=0.83, when using EMW as the eluent. The same pattern is observed with BEA and CEF as the mobile phases, but not as intense. TLC with BEA showed a decrease in the bands at R_f=0.75 and R_f=0.38. CEF as the mobile phase showed the bands with R_f=0.18, 0.45 and 0.54 to drastically decrease in colour intensity from t=0-t=6 months.

- Extract capsules

  TLC with EMW showed a slight decrease in the colour intensity of the band with R_f=0.37. TLC with BEA showed no obvious change. The compounds with R_f=0.18 and R_f=0.45 showed a decrease in colour intensity with CEF as the mobile phase.

- Liquid extract

  TLC with EMW showed no obvious change. Using BEA as the eluent, a decrease in the colour intensity of the band at R_f=0.75 was seen. The compound with R_f=0.68 was only present at t=0 months when using CEF as the mobile phase.

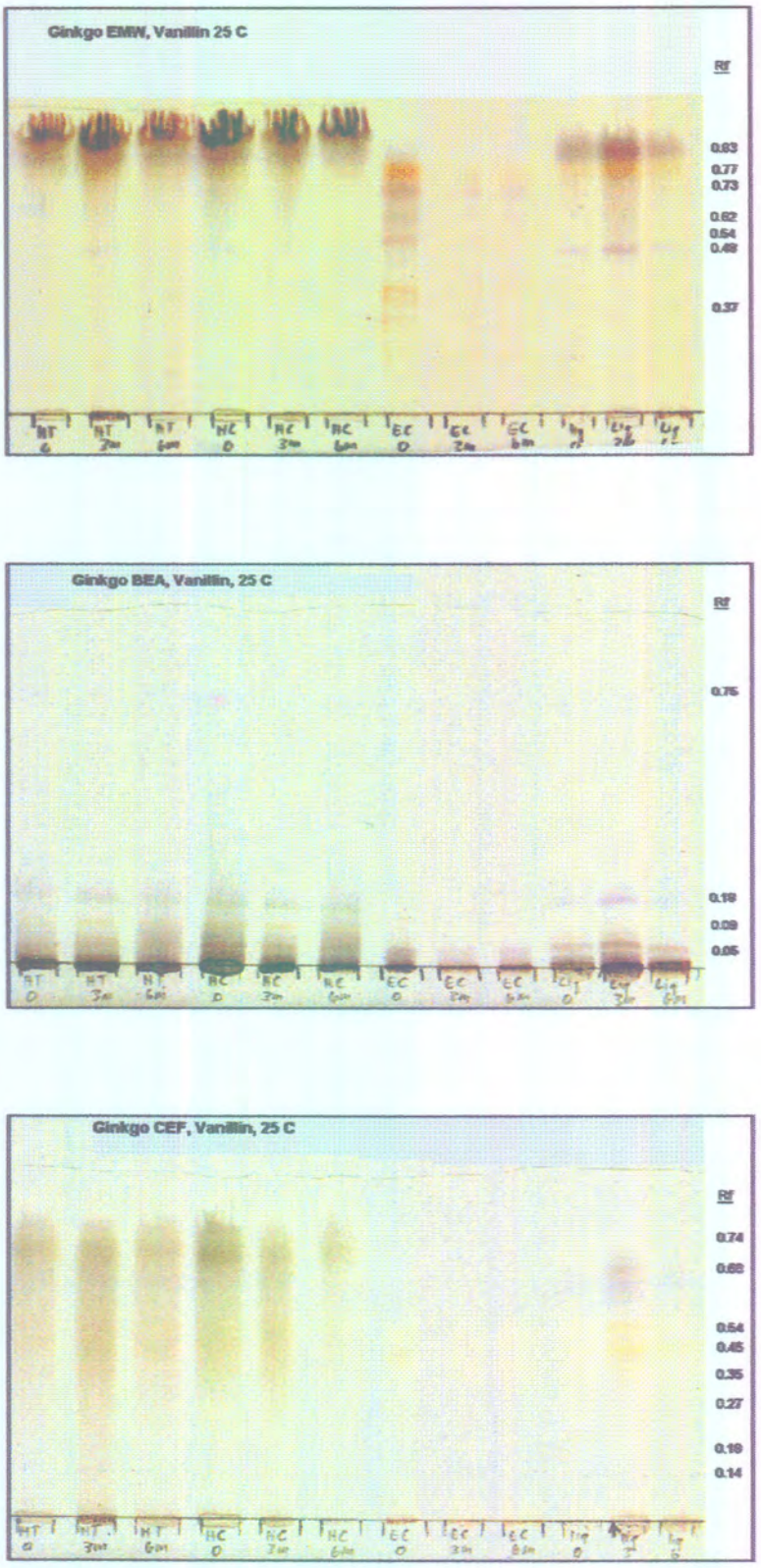
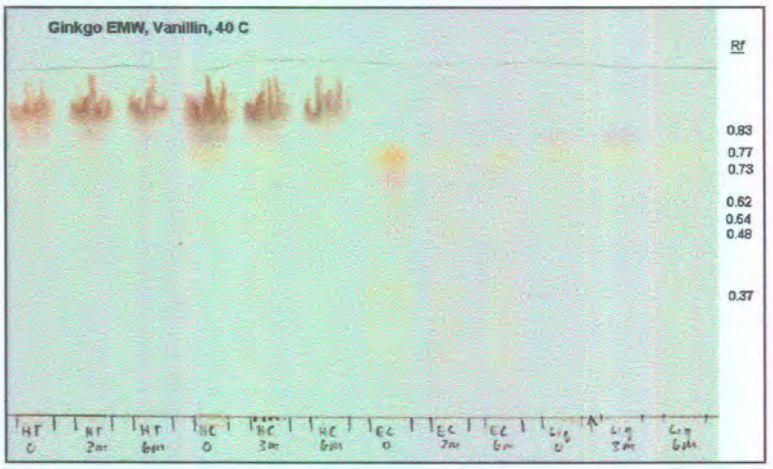
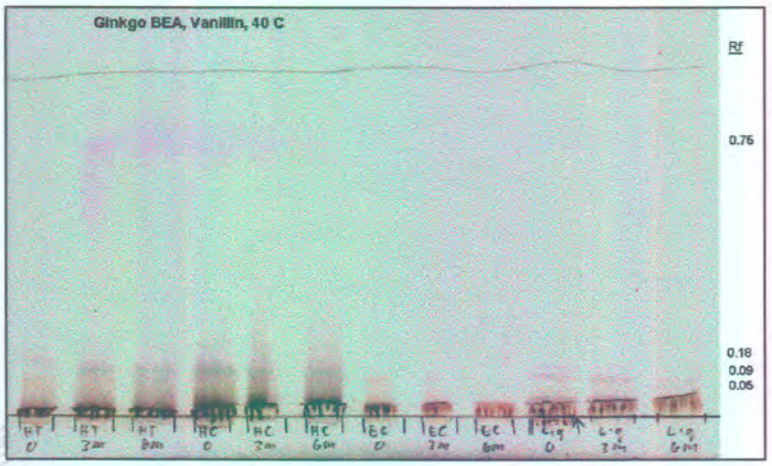


Figure 3.3.3 Ginkgo samples stored at 25°C [Sprayed with vanillin with mobile phase EMW (top), BEA (center) and CEF (bottom). From left: Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]





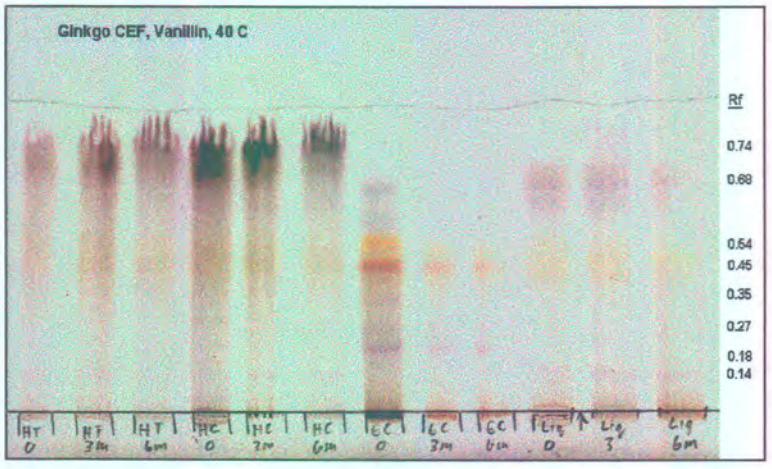


Figure 3.3.4 Ginkgo samples stored at 40°C. [Sprayed with vanillin with mobile phase EMW (top), BEA (center) and CEF (bottom). From left: Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]

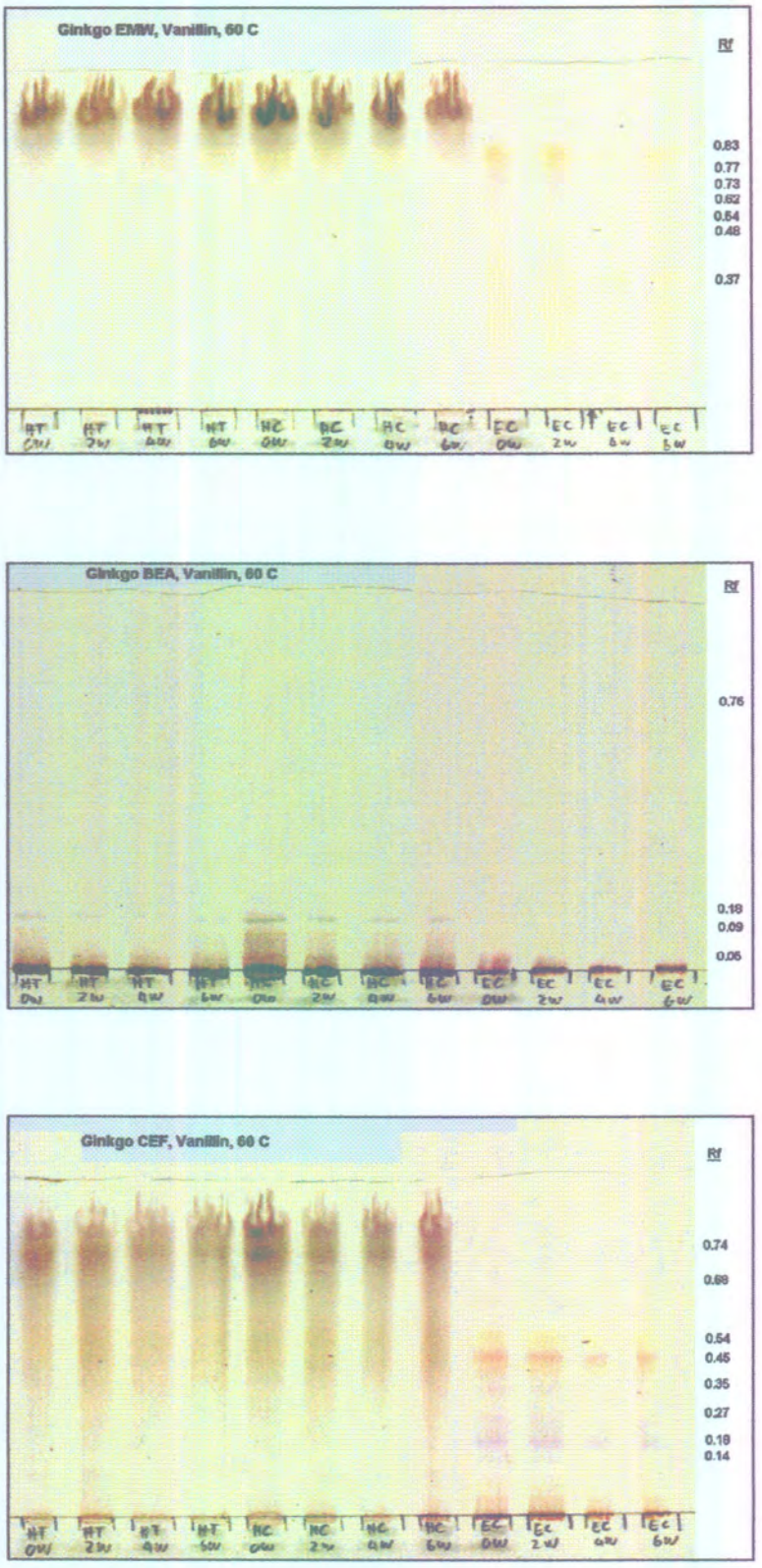


Figure 3.3.5 Ginkgo samples stored at 60°C. [Sprayed with vanillin with mobile phase EMW (top), BEA (center) and CEF (bottom). From left: Herbal tablets (HT) 0,2,4,6 weeks. Herbal capsules (HC) 0,2,4,6 weeks. Extract capsules (EC) 0,2,4,6 weeks.]

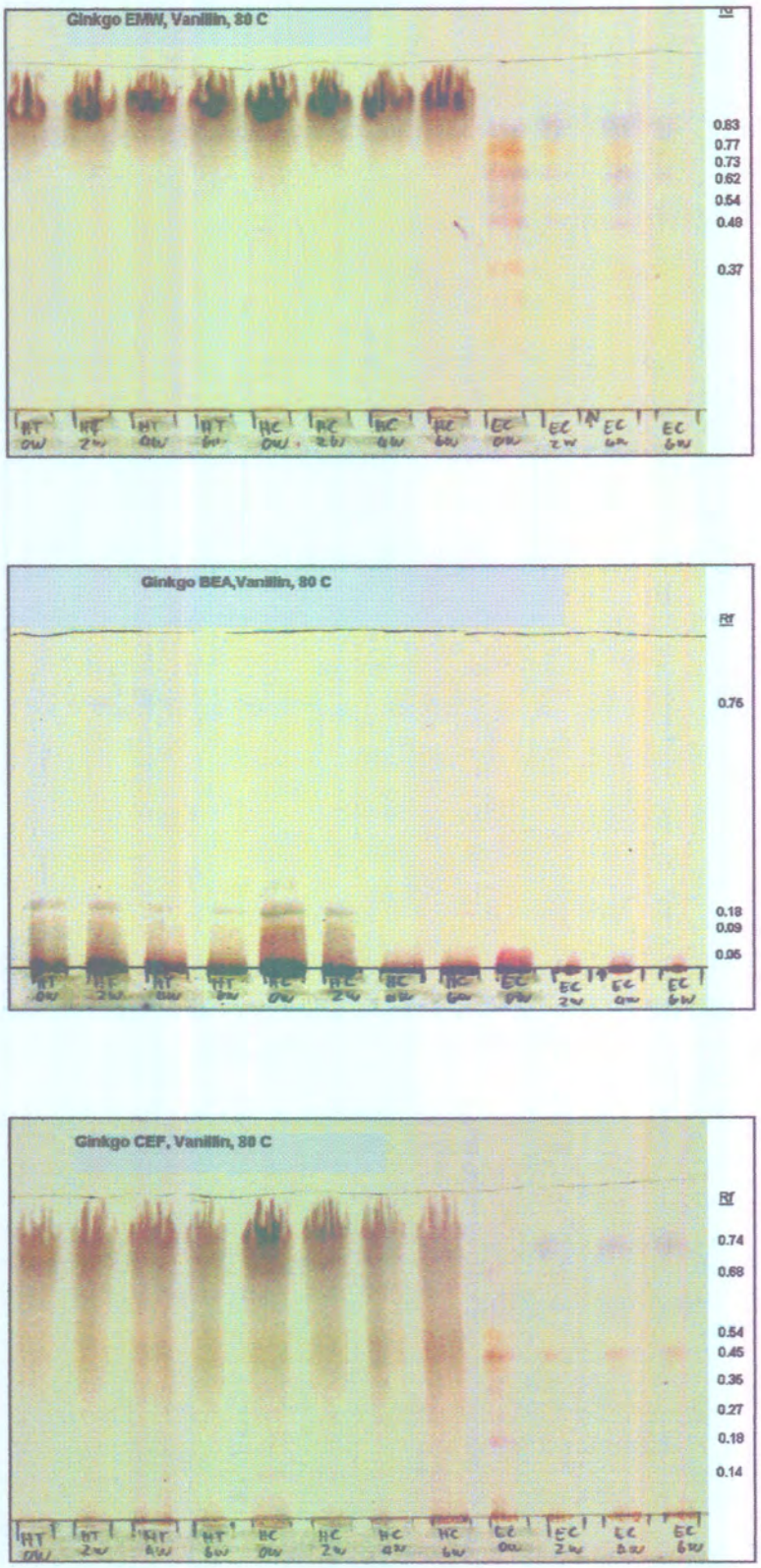


Figure 3.3.6 Ginkgo samples stored at 80°C. [Sprayed with vanillin with mobile phase EMW (top), BEA (center) and CEF (bottom). From left: Herbal tablets (HT) 0,2,4,6 weeks. Herbal capsules (HC) 0,2,4,6 weeks. Extract capsules (EC) 0,2,4,6 weeks.]

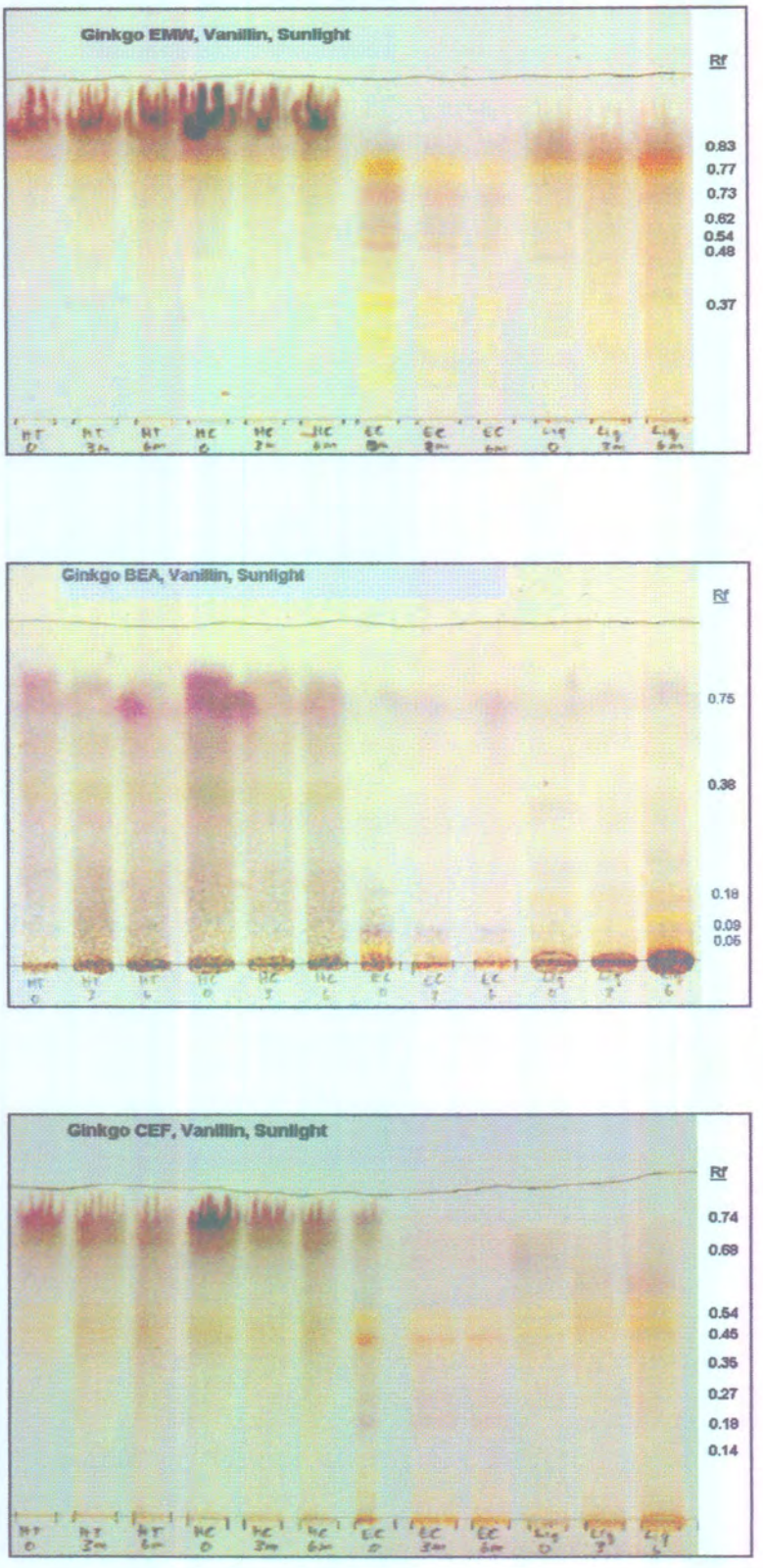


Figure 3.3.7 Ginkgo samples stored at direct sunlight. [Sprayed with vanillin with mobile phase EMW (top), BEA (center) and CEF (bottom). From letf: Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]

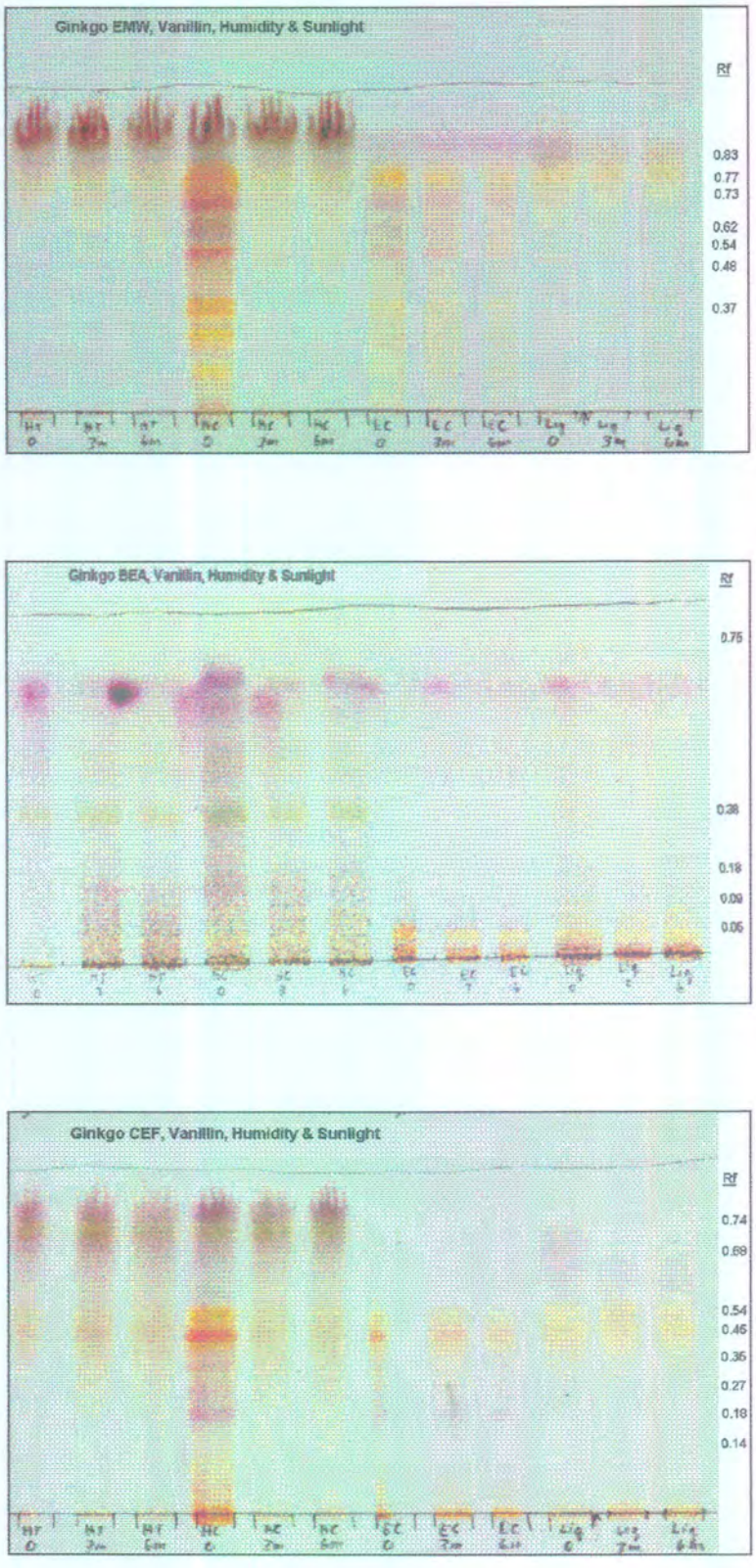


Figure 3.3.8 Ginkgo samples stored at direct sunlight and high humidity. [Sprayed with vanillin with mobile phase EMW (top), BEA (center) and CEF (bottom). From letf: Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]

3.3.1.2 High Pressure Liquid Chromatography (HPLC)

Only samples of the herbal tablets, herbal capsules, extract capsules and radiated dried ginkgo leaf powder were analyzed by HPLC, as the quantity required to be injected into the column, of the liquid extracts, was not sufficient for a sample preparation as described in section 2.7.1.3.

The absorbance of the known compound, quercetin, was measured in each sample and integrated in terms of the peak height on the chromatogram. The quercetin reference peak showed a retention time of ~14 minutes and a peak height of 373737 integrated units. Regression analysis was performed on the 5 diluted stock solutions. R^2 showed to be 0.9999. As discussed in section 2.7.1.3, the quantity of quercetin present in each sample was calculated according to equation 2.4.

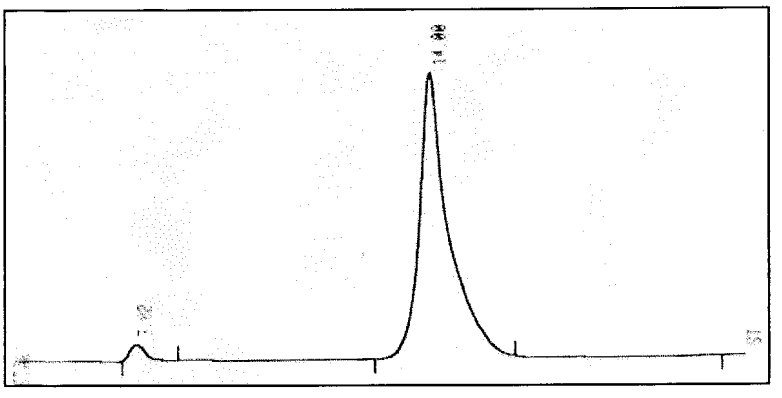


Figure 3.3.9 HPLC chromatogram of quercetin standard solution C (0.06mg/ml) prepared in section 2.7.1.2

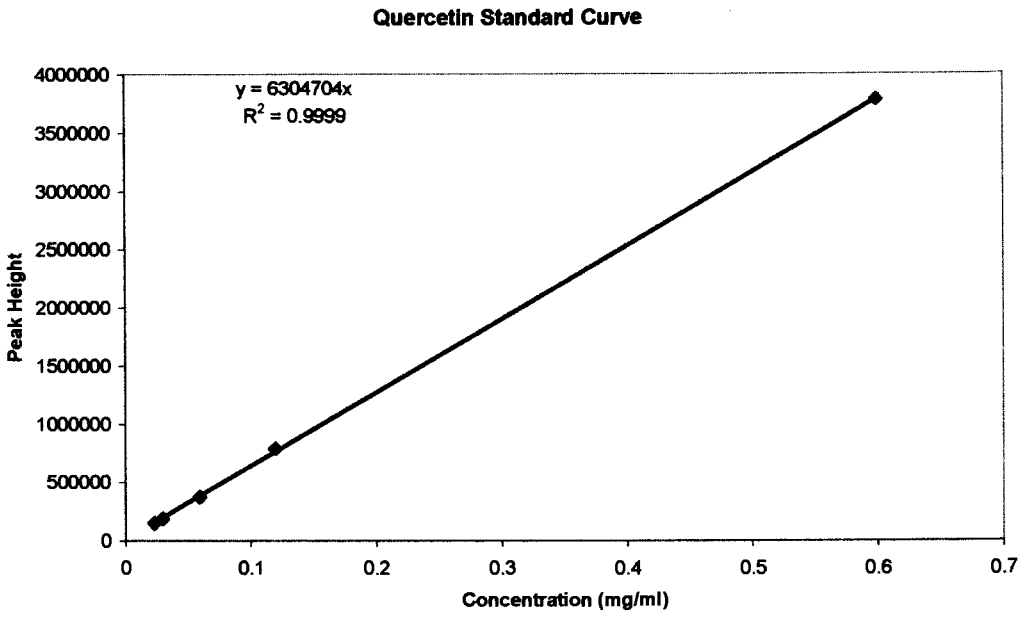


Figure 3.3.10 **Quercetin standard curve.** [Section 2.7.1.2 for preparation]

The standard deviation on the HPLC method was calculated as described in section 2.7.2.

Table 3.3.1 **Analysis of one Ginkgo sample repeated 5 times**

Sample		X_1	X_1^2
1		130169	1.6943×10^{10}
1		96959	0.9401×10^{10}
1		126399	1.5976×10^{10}
1		125435	1.5733×10^{10}
1		107498	1.1555×10^{10}
	Sum	586460	6.961×10^{10}

$$\frac{(586460)^2}{5} = \frac{1.439 \times 10^{11}}{5} = 6.878 \times 10^{10}$$

Substituting into equation 2.3 leads to a standard deviation in peak height of 14356 integrated units. Using the equation for a straight line (y=mx) and substituting m with a value of 6304704 (from linear regression curve, figure 3.3.10), a standard deviation of 0.0023 mg/ml quercetin is calculated. Substituting this value in equation 2.4 leads to a

standard deviation of 0.0115% quercetin. According to the HPLC analysis [Table 3.3.4] there was 0.178% quercetin present at t=0 weeks = 100%. The standard deviation of 0.0115% quercetin therefore represents a variability coefficient (% standard deviation) of 6.46%.

The standard deviation in the quantity of quercetin extracted from exactly 500 mg of the dried leaf powder was also calculated according to equation 2.3

Table 3.3.2 Analysis of Ginkgo samples with exact weight of 500mg

Sample	X_1	X_1^2
1	229.4	52624.36
2	238.2	56739.24
3	247.2	61107.84
4	236.5	55932.25
5	230.9	53314.81
Sum	1182.2	279718.5

$$\frac{(1182.2)^2}{5} = \frac{1397596.84}{5} = 279519.36$$

Substituting into equation 2.3 leads to a standard deviation of 7.05mg in the quantity quercetin extracted for each dosage form. This is a total of 1.41% on the assumption that 500mg contains 100% of active. This relates to the deviation seen due to the extraction method.

A summary of the actual data regarding the calculated quercetin content of each sample can be seen in table 3.3.3, 3.3.4 and 3.3.5. The following graphs are a summary of the different dosage forms, with regard to their stability at each condition mentioned in section 2.3. In each graph the quercetin content at t=0 weeks was expressed as 100%. The values at 2,4,6,8,10 and 12 weeks are expressed in terms of a increase or decrease relative to the value of 100% at t=0 weeks.

Differences in the actual quercetin content calculated according to equation 2.4 will be compared to the value of the standard deviation of the HPLC method (6.46%) rather than the deviation in extraction as used with the hypericin [See 3.2.1.2].

3.3.1.2.1 Ginkgo herbal tablets [Table 3.3.3 for actual values]

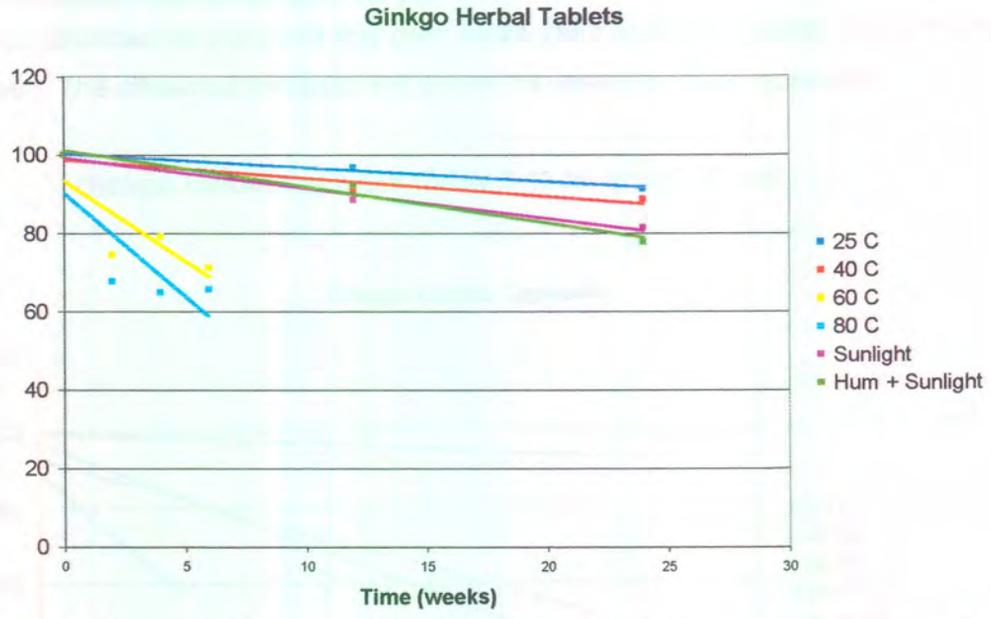


Figure 3.3.11 Degradation of quercetin in Ginkgo herbal tablets at different storage conditions

Results at 25°C

A small decrease in the trend line from 100% (t=0) to 96.7% (t=12) to 91.4% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 8.6% quercetin

Results at 40°C

A slight decrease in the trend line from 100% (t=0) to 90.7% (t=12) to 88.7% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 11.3% quercetin.

Results at 60°C

An obvious decrease in the trend line from 100% (t=0) to 74.6% (t=2) to 79.7% (t=4) to 71.2% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 28.7% quercetin.

Results at 80°C

A massive decrease in the trend line from 100% (t=0) to 67.7% (t=2) to 64.9% (t=4) to 65.7% (t=6) can be seen. The difference between t=0 and t=6 weeks is 34.3% quercetin.

Results at direct sunlight

A noticeable decrease in the trend line from 100% (t=0) to 88.5% (t=12) to 81.4% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 18.6% quercetin.

Results at direct sunlight and high humidity

An obvious decrease in the trend line from 100% (t=0) to 92.3% (t=12) to 77.7% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 22.3% quercetin.

3.3.1.2.2 Ginkgo herbal capsules [Table 3.13 for actual values]

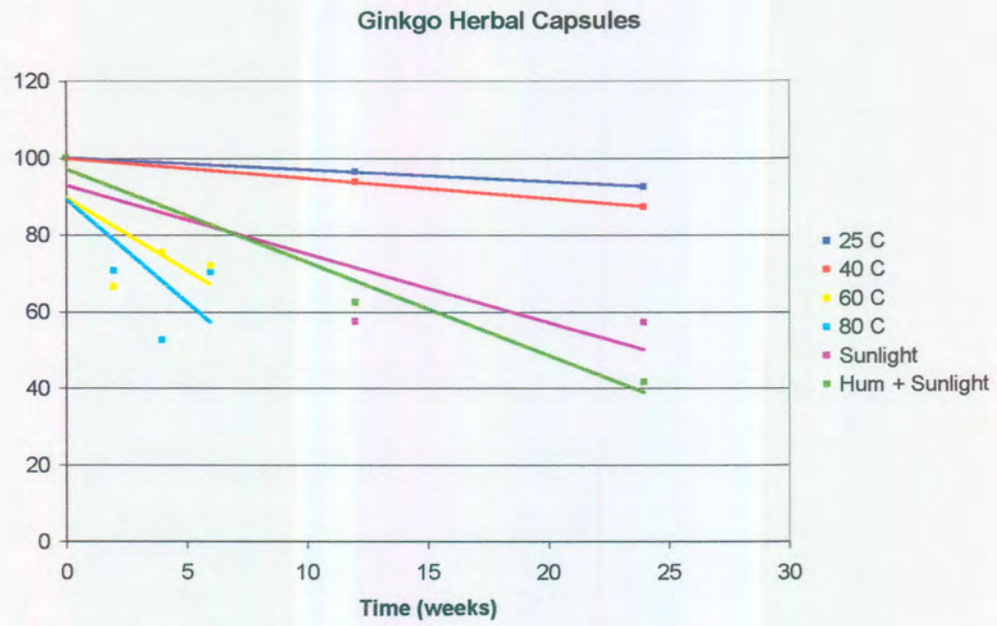


Figure 3.3.12 Degradation of quercetin in Ginkgo herbal capsules at different storage conditions

Results at 25°C

A small decrease in the trend line from 100% (t=0) to 98.5% (t=12) to 92.5% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 7.5% quercetin.

Results at 40°C

A slight decrease in the trend line from 100% (t=0) to 93.7% (t=12) to 87.2% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 12.8% quercetin.

Results at 60°C

An obvious decrease in the trend line from 100% (t=0) to 66.5% (t=2) to 75.9% (t=4) to 72% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 28.% quercetin.

Results at 80°C

A massive decrease in the trend line from 100% (t=0) to 70.6% (t=2) to 52.5% (t=4) to 70.2% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 29.8% quercetin.

Results at direct sunlight

A massive decrease in the trend line from 100% (t=0) to 57.4% (t=12) to 57.2% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 42.8% quercetin.

Results at direct sunlight and high humidity

A massive decrease in the trend line from 100% (t=0) to 62.4% (t=12) to 41.7% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 58.3% quercetin.

3.3.1.2.3 Ginkgo Extract Capsules [Table 3.3.4 for actual values]

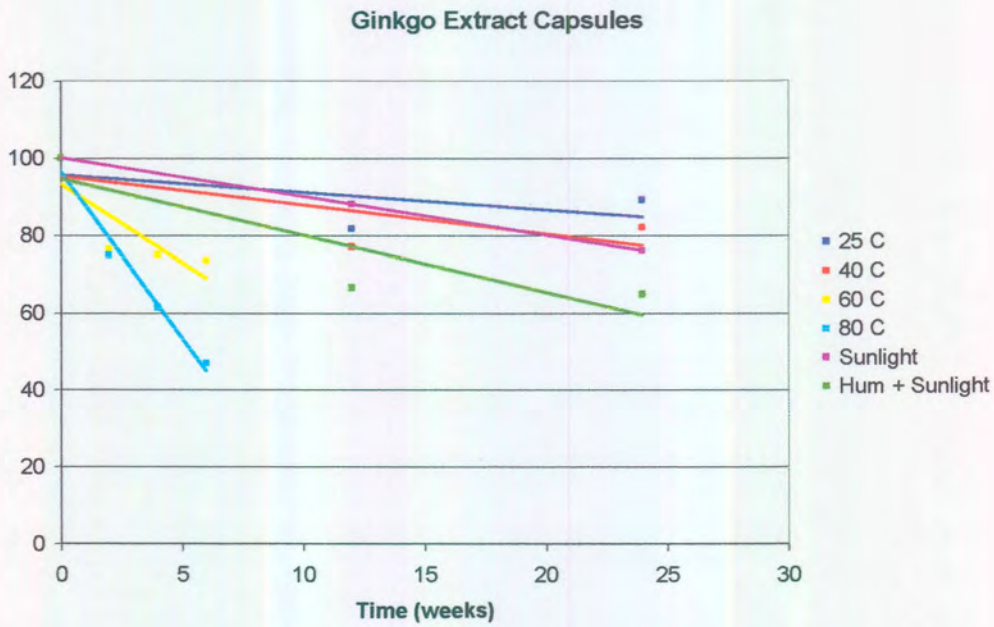


Figure 3.3.13 Degradation of quercetin in Ginkgo extract capsules at different storage conditions

Results at 25°C

A slight decrease in the trend line from 100% (t=0) to 81.6% (t=12) to 89.1% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 10.9% quercetin.

Results at 40°C

A noticeable decrease in the trend line from 100% (t=0) to 77% (t=12) to 82% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 18% quercetin.

Results at 60°C

An obvious decrease in the trend line from 100% (t=0) to 76.4% (t=2) to 75% (t=4) to 73.5% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 26.5% quercetin.

Results at 80°C

A massive decrease in the trend line from 100% (t=0) to 75% (t=2) to 61.4% (t=4) to 47% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 53% quercetin.

Results at direct sunlight

An obvious decrease in the trend line from 100% (t=0) to 88.1% (t=12) to 75.9% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 24.1% quercetin.

Results at direct sunlight and high humidity

A massive decrease in the trend line from 100% (t=0) to 66.5% (t=12) to 64.8% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 35.2% quercetin.

3.3.1.2.4 Ginkgo leaf powder radiated with Cobalt-60 source

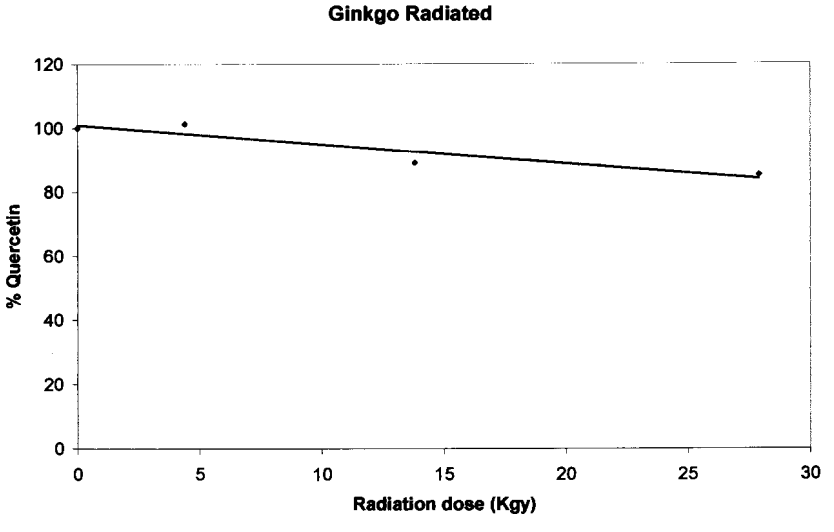


Figure 3.3.14 Degradation of quercetin in ginkgo dried leaf powder at different radiation doses

There seems to be no obvious change in the quercetin concentration at a dose of 4.4 kGy (101.2% quercetin) when compared to the concentration at 0 kGy (100%). A noticeable difference could however be seen at higher radiation dosages. A dose of 13.8 kGy decreased the quercetin concentration from 100% to only 88.9%. This is a decrease of 11.1%. Exposure to 27.9 kGy induced a change of 14.8% when comparing the starting concentration (100%) to the measured concentration (85.2%).

Table 3.3.3 Summary of data obtained from HPLC analysis of Ginkgo Herbal tablets.

Condition	Time (weeks)	Peak Height	% Quercetin	% relative to t=0
25°C	0	137904	0.109	100.0
25°C	12	132636	0.105	96.7
25°C	24	124833	0.099	91.4
40°C	12	124762	0.099	90.7
40°C	24	122232	0.097	88.7
60°C	2	102889	0.082	74.6
60°C	4	108842	0.086	79.1
60°C	6	97983	0.078	71.2
80°C	2	93410	0.074	67.7
80°C	4	89435	0.071	64.9
80°C	6	90807	0.072	65.7
Sun	12	121783	0.097	88.5
Sun	24	111655	0.089	81.4
Sun+Hum	12	126693	0.101	92.3
Sun+Hum	24	107280	0.085	77.7

Table 3.3.4 Summary of data obtained from HPLC analysis of Ginkgo Herbal Capsules.

Condition	Time (weeks)	Peak Height	% Quercetin	% relative to t=0
25°C	0	227055	0.178	100.0
25°C	12	216525	0.171	96.4
25°C	24	209492	0.165	92.5
40°C	12	212277	0.167	93.7
40°C	24	196773	0.155	87.3
60°C	2	149514	0.119	66.5
60°C	4	168122	0.134	75.5
60°C	6	162734	0.128	72.0
80°C	2	159100	0.126	70.6
80°C	4	118575	0.094	52.5
80°C	6	158492	0.125	70.2
Sun	12	128900	0.102	57.4
Sun	24	128344	0.102	57.2
Sun+Hum	12	140039	0.111	62.4
Sun+Hum	24	93861	0.074	41.7

Table 3.3.5 Summary of data obtained from HPLC analysis of Ginkgo Extract Capsules.

Condition	Time (weeks)	Peak Height	% Quercetin	% relative to t=0
25°C	0	301746	0.465	100.0
25°C	12	247234	0.379	81.6
25°C	24	269979	0.414	89.1
40°C	12	231414	0.358	77.0
40°C	24	242562	0.381	82.0
60°C	2	234043	0.355	76.4
60°C	4	222574	0.348	74.9
60°C	6	220911	0.341	73.5
80°C	2	226874	0.348	74.9
80°C	4	185137	0.285	61.4
80°C	6	137913	0.218	47.0
Sun	12	266957	0.409	88.1
Sun	24	221901	0.353	75.9
Sun+Hum	12	204577	0.309	66.5
Sun+Hum	24	200061	0.301	64.8

3.3.1.3 Summary of TLC and HPLC results

3.3.1.3.1 Herbal tablets

25°C

TLC showed no obvious change in the colour of any bands with EMW or BEA as the eluent. TLC with CEF didn't show a definite separation of bands, but it seemed that the colour of the whole profile was more distinct at t=3 than t=0 or t= 6 months. The quercetin content decreased by 8.6% over a 24-week period according to the HPLC results.

40°C

TLC showed no obvious change with either EMW or BEA as the mobile phases, but a slight decrease in colour of one band could be observed with CEF. The quercetin content decreased by 11.3% over a 24-week period according to the HPLC results.

60°C

TLC showed a decrease in one band for both EMW and BEA as the mobile phases, there was also a absence of one band with BEA. Again TLC with CEF didn't show a clear distinction between the individual bands, but the same darker appearance seen at 25°C and 40°C for t=3 months, was also prominent here. The quercetin content decreased by 28.8% over a 6-week period according to the HPLC results.

80°C

TLC didn't show any pronounced change, with EMW as the eluent, as could possibly be seen at 60°C. TLC with BEA seemed to follow the same decrease, and absence of the bands seen at 60°C. Again no clear distinction between any bands could be seen wit CEF as the mobile phase. The quercetin content decreased by 34.26% over a 6-week period according to the HPLC results.

Sunlight

TLC revealed no obvious change for EMW and CEF as eluents, but a slight increase of only one band was seen with BEA as the mobile phase. The quercetin content decreased by 18.6% over a 24-week period according to the HPLC results.

Humidity and sunlight

TLC showed no significant change with any of the mobile phases. The quercetin content decreased by 22.3% over a 24-week period according to the HPLC results.

3.3.1.3.2 Herbal capsules

25°C

TLC showed no obvious change with EMW as the eluent. TLC with BEA showed a slight decrease in the colour intensity of two bands. No clear separation could be seen using CEF as the mobile phase. The quercetin content decreased by 7.5% over a 24-week period according to the HPLC results.

40°C

TLC with EMW showed no obvious change and BEA showed the same change as seen at 25°C. TLC with CEF revealed a band to show a more pronounced decrease, than the same band present in the herbal tablets. The quercetin content decreased by 12.8% over a 24-week period according to the HPLC results.

60°C

TLC revealed the same changes as seen with 40°C. These changes however seemed more intense than the same changes seen with the herbal tablets. The quercetin content decreased by 28.2% over a 6-week period according to the HPLC results.

80°C

TLC showed a drastic decrease in colour intensity of 2 bands with both BEA and CEF as the mobile phases. With CEF there was an absence of one band after the 6-week analysis. This seemed to be verified by the massive decrease of 29.8% quercetin measured by HPLC.

Sunlight

TLC with BEA showed a drastic decrease of one band (R_f=0.75). A noticeable decrease of one band was also observed with CEF as the eluent. The quercetin content decreased by 42.7% over a 24-week period according to the HPLC results.

Humidity and sunlight

TLC showed a very distinct visibility of all bands present at t=0 with all the mobile phases (even more visible than the bands observed on the 25°C plate), but only a few of these bands were present at t=3 or t=6 months. The quercetin content decreased by 58.3% over a 24-week period according to the HPLC results.

3.3.1.3.3 Extract capsules

25°C

TLC revealed that most bands present at t=0, disappeared at t=3 and t=6 months with all three of the mobile phases. HPLC confirmed with a decrease in quercetin concentration of 10.9%.

40°C

TLC revealed the same changes as seen at 25°C. A decrease of 18.3% was shown with HPLC.

60°C

No bands seemed to be observed with BEA as the eluent. TLC with EMW and CEF both showed bands at t=0, which disappeared at t=2,4 and 6 weeks. The quercetin content decreased by 26.5% over a 6-week period according to the HPLC results.

80°C

The same observation was made with TLC, than the one at 60°C. The change in colour intensity also seemed more severe with 60°C and 80°C, than with 25°C and 40°C. The quercetin content decreased by 53% over a 6-week period according to the HPLC results.

Sunlight

TLC revealed a marked decrease in most bands seen with EMW, BEA and CEF as the mobile phases. This observation was more definite than the change in bands for the herbal tablets and herbal capsules. The quercetin content decreased by 24.1% over a 24-week period according to the HPLC results.

Humidity and sunlight

TLC with both EMW and CEF showed a decrease in colour intensity of one band. TLC with CEF showed no obvious change. The quercetin content decreased by 35.2% over a 24-week period according to the HPLC results.

3.3.1.3.4 Liquid extracts

25°C

TLC with BEA, CEF and EMW showed a stronger presence of colour intensity of some bands at t=3, than for t=0 and t=6 months.

40°C

All mobile phases showed a gradual decrease of colour intensity from t=0-t=6 months.

Sunlight

TLC with EMW showed an increase in the colour intensity of 2 bands. This was also observed with the extract capsules.

Humidity and sunlight

No obvious change could be seen with EMW as the eluent, but TLC with CEF showed a decrease of one band.

3.3.1.3.5 Radiated dried leaf powder

No obvious change in the quercetin concentration could be seen at low radiation doses. A decrease of 14.8% in the quercetin concentration was however measured at high radiation doses.

3.3.2 Kinetics of Quercetin

The determination of the chemical reaction order of quercetin was also calculated by making use of the substitution method [See section 3.2.2.1]. Average values for the zero-order reaction was 0.31583 %.weeks^{-1} , first-order = 3.263829 x10^{-3} weeks^{-1} , and for the second order = 3.373796 x 10^{-5} %$^{-1}$.weeks^{-1}. The difference between the individual values divided by the average values however gave an almost unacceptable result. For the zero-order reaction, the difference was 27.43%, for the first order reaction 30.199% and for the second order reaction 33%. Linear regression analysis performed by plotting the concentration (%) quercetin over time (weeks) for the ginkgo herbal tablets at 25° revealed a slope of 0.3592%.weeks^{-1} . The difference between this value and the one calculated for the zero-order reaction was 13.7%. Again it was assumed that quercetin followed a zero-order degradation, but with this noticeable inconsistency between the

average values, this assumption could not be made with relative confidence compared to the calculation of the hypericin reaction order.

The *k* values for the different dosage forms stored at each different condition were obtained from the linear regression analysis from the graphs of quercetin concentration (%) plotted against time (weeks). A summary of these *k* values are given in table 3.15, 3.16 and 3.17.

Table 3.3.6 *k*-values for quercetin in Ginkgo Herbal tablets

Condition	*K* – value (%.weeks^{-1})
25°C	0.3592
40°C	0.472
60°C	4.0892
80°C	5.2794
Sunlight	0.7742
Sunlight & humidity	0.9279

Table 3.3.7 *k*-values for quercetin in Ginkgo Herbal capsules

Condition	*K* – value (%.weeks^{-1})
25°C	0.3131
40°C	0.5301
60°C	3.7551
80°C	5.3719
Sunlight	1.7817
Sunlight & humidity	2.4284

Table 3.3.8 *k*-values for quercetin in Ginkgo Extract capsules

Condition	*K* – value (%.weeks^{-1})
25°C	0.4539
40°C	0.751
60°C	4.0484
80°C	8.6272
Sunlight	1.0045
Sunlight & humidity	1.4669

The t_{90} values for each dosage form at different storage conditions was also calculated according to equation 3.5. A summary of these values is given in table 3.3.9 below.

Table 3.3.9 t_{90} values for Ginkgo herbal tablets, herbal capsules and extract capsules.

Condition	Herbal tablets(weeks)	Herbal capsules(weeks)	Extract capsules(weeks)
25°C	28	32	22
40°C	22	19	13
60°C	2.	3	2
80°C	2	2	1
Sunlight	13	6	10
Sunlight & humidity	11	14	7

3.3.3 DISCUSSION

The ginkgo herbal tablets seem to be slightly less stable (t_{90}=27.83 weeks), compared to the hypericum herbal tablets at 25°C (t_{90}=112.6 weeks). The overall stability of the ginkgo herbal tablets also seems to be inferior compared to the hypericum herbal tablets. This hypothesis might not be an accurate depiction of the true stability, since the standard deviation seemed to be relatively high. TLC showed no confirmation of the data obtained from HPLC. In spite of the large standard deviation, the pattern of degradation seems to follow the same trend as seen with the hypericum herbal tablets. A rise in temperature

seemed to cause an obvious decrease in the quercetin concentration. This decrease might be more pronounced at 60°C and 80°C, that at 40°C or 25°C. The effect of direct sunlight and the combination of humidity and sunlight, also appeared to have a negative effect on the stability.

The pattern of degradation for the herbal capsules seemed to be similar to the herbal tablets. The TLC of both the herbal capsules and herbal tablets seem to support this assumption. The presence of more colourful bands seen only with the TLC plates at high humidity and sunlight, were different in comparison to other plates. One possible reason might be the possible contamination of this specific sample. When comparing the HPLC data of the quercetin concentration between the herbal tablets and the herbal capsules, only a small difference is seen. The effects of increased temperature seem to be both the same for the tablets as well as the capsules. The effect of sunlight and humidity in combination with sunlight, however seems to have a more obvious decrease in quercetin concentration for the herbal capsules, than for the herbal tablets. This however could not be confirmed with TLC. Again, the precise effect of sunlight, seemed difficult to compare to the effect of the combination of sunlight and high humidity. As mentioned in section 3.2.3, this should be further researched.

The extract capsules seem to be slightly less stable at 25°C in comparison to the herbal tablets and herbal capsules, considering the rate of decrease. (0.35%-tablets; 0.31%-capsules; 0.45%-extract capsules). The noticeable decrease in colour intensity observed with TLC further contributes to this notion. As seen with the herbal tablets and herbal capsules, a rise in temperature also seems to cause a decrease in quercetin concentration. TLC patterns seems to verify this decrease in concentration, but not with the same state of confidence as seen with the hypericum.

Although it might be difficult to determine the effect of sunlight, to the effect of humidity in combination with sunlight, on the degradation of quercetin, the pattern observed here is more constant than seen with hypericum. Considering the data obtained by HPLC, it might appear that the extract capsules is less prone to degradation caused by sunlight or humidity, compared to the herbal capsules. Unfortunately this observation could not be verified with TLC.

The same obvious pattern of degradation can be seen in all of the above mentioned dosage forms. The general observation is that quercetin degrades as follows: 25°C→40°C→Sunlight→Humidity and sunlight→60°C→80°C. As mentioned before, the

samples stored in a combination of humidity and sunlight might also be slightly more stable than samples stored in sunlight alone, due to the large standard deviation.

Having calculated the activation energy at 56.269 kkal/mol for the ginkgo herbal tablets, from the Arrhenius graph, this value seemed noticeably similar to the activation energy calculated for the hypericum herbal tablets. Subsequently the activation energy for the ginkgo herbal capsules was calculated at 55.360 kkal/mol, and 56.432 kkal/mol for the extract capsules. These values appeared to be similar to the activation energy calculated for hypericum herbal capsules and hypericum extract capsules.

Taking this into consideration, and assuming that quercetin also follows a zero-order degradation [see section 3.3.2], the application of the Arrhenius equation in predicting a possible t_{90} value seems not applicable. A rough calculation (not shown in this thesis) confirmed the same misleading values as seen with hypericum.

Having only the qualitative data from TLC, and lacking the quantitative results from HPLC, again it seemed more difficult in commenting on the stability of Ginkgo extract liquid. CEF showed the most consistent colours associated with either an increase or a decrease in the intensity. BEA and EMW were of little value when looking at the liquid extract alone.

Low radiation seems to have no effect on the quercetin concentration. According to the HPLC measured data, a possible small decrease might exist in doses exceeding 5 kGy. This supposition needs to be further investigated due to the influence of the large standard deviation.

TLC did not support all the changes calculated with HPLC in the same supportive way as with the hypericum. EMW showed to be a poor choice for the separation of compounds from ginkgo. BEA seemed to be the mobile phase of choice when identifying *Ginkgo biloba* leaf powder. CEF showed to be more suitable in separating compounds in the dried extract powder. HPLC provided useful data, but it seemed that the method for determining the quercetin concentration should be refined, and the standard deviation minimized.

The fact that only quercetin was measured, also raises concern on the evaluation of the stability of *Ginkgo biloba*. As seen in the literature, the effects of ginkgo are not the result of only one component, but rather the synergistic effect of the combination of a few compounds. These other compounds might have a totally different effect on the stability

as discussed in this part of the chapter. The effects of the other components present in *Ginkgo biloba* should be further investigated

SUMMARY

It seems that the herbal tablets exhibit a slight advantage regarding the stability over the herbal capsules. Both herbal tablets and herbal capsules seem to be stable at 25°C. Degradation is faster for the extract capsules as well as the extract liquid, compared to the tablets or herbal capsules. Radiation at low doses doesn't seem to have much of a negative effect on the degradation of quercetin. TLC seems to be less accurate, if used alone, in evaluating the stability of Ginkgo-products.

3.4 *PIPER METHYSTICUM*

3.4.1 RESULTS

3.4.1.1 Thin layer chromatography

From the visual examination of the plates prepared in section 2.5, it was decided to use only BEA and CEF as mobile phases, and *p*-anisaldehide as the spraying reagent. The reason being that with EMW a complete separation of the bands did not occur, possibly because of the non-polar properties of the kava-lactones, and the fact that EMW is optimal for separating relatively polar components. BEA revealed six bands for both the vanillin- and the *p*-anisaldehide spray reagent. When using CEF, only 4 bands were optimally visible when sprayed with vanillin, compared to the five visible bands with *p*-anisaldehide (absence of band at R_f=0.68). [See figure 3.4.1] As mentioned in section 2.6 the official method was discarded because of the simplicity and good reproducibility of our in-house developed methods compared to the British Herbal Pharmacopoeia. [See figure 3.4.2] TLC was done on all samples, except for the dried kava kava root powder exposed to gamma radiation.

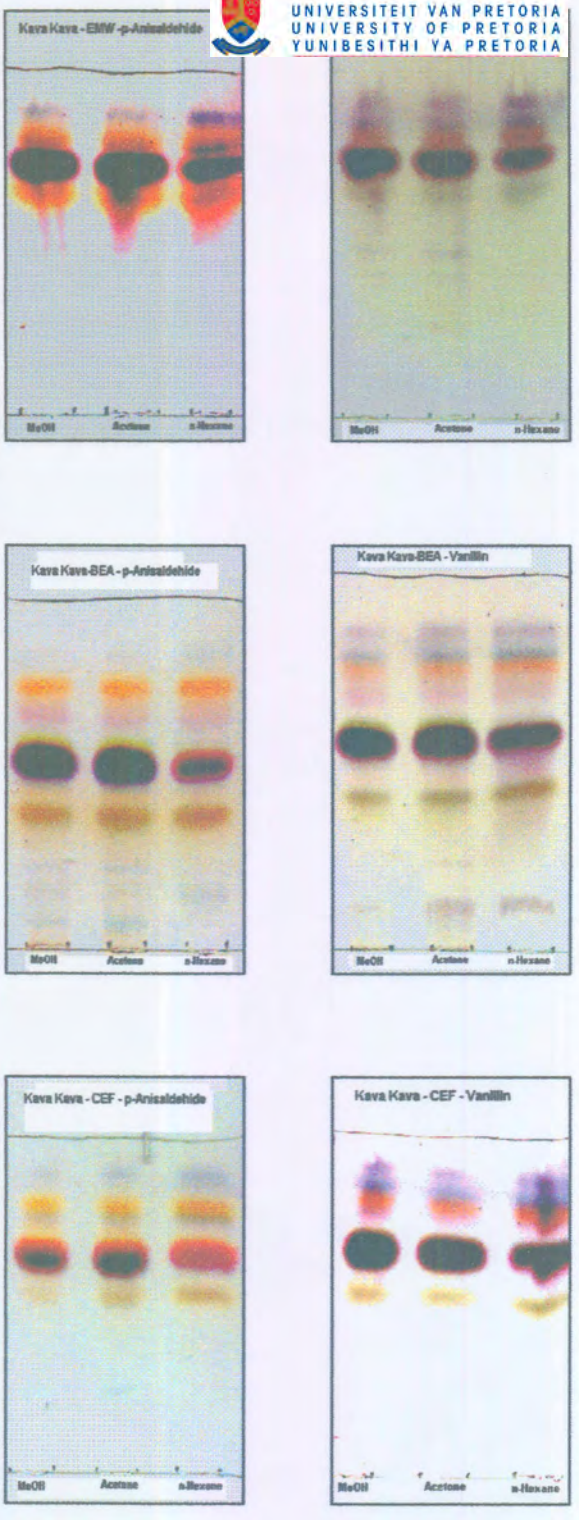


Figure 3.4.1 TLC of Kava Kava root powder with mobile phase EMW (top), BEA (center) and CEF (bottom) and sprayed with *p*-anisaldehide (left) and vanillin (right). Each plate shows extraction with Methanol (left), Acetone (center), and n-Hexane (right).

Figure 3.4.2 Kava Kava identification according to the British Herbal Pharmacopoeia

3.4.1.1.1 Kava Kava samples at 25°C [See figure 3.4.3]

- Herbal tablets

  TLC with BEA showed the bands with R_f=0.07, R_f=0.16 and R_f=0.27 to possible increase slightly in colour intenstity from t=0-t=6 weeks. A small increase in the colour intensity of the band with R_f=0.57 was observed with CEF as the mobile phase, from t=0-t=6 months.

- Herbal capsules

  TLC with BEA showed a slight decrease in the colour intensity for the band at R_f=0.07. CEF as the mobile phase showed a decrease in the colour intensity for the band at R_f=0.14. The compound present at R_f=0.14 is more visible with the capsules, than with the tablets.

- Extract capsules

  BEA as the eluent showed no obvious change. TLC with CEF revealed a slight decrease in the colour intensity of the band present at R_f=0.44 from t=0-6=6 months. This was not seen with the tablets or herbal capsules. The compound with R_f=0.83 was only present with the extract capsules and not with tablets or herbal capsules. A possible increase in the colour intensity could again be seen at the band with R_f=0.57.

- Liquid extracts

  A slight decrease in colour intensity from t=0-t=6 months could be seen with BEA as the mobile phase, for the compound with R_f=0.27. TLC with CEF showed a noticeable increase in colour intensity for the bands with R_f=0.44 and R_f=0.57. This increase was the same as seen with the herbal capsules.

3.4.1.1.2 Kava Kava samples at 40°C. [See figure 3.4.4]

- Herbal tablets

  TLC with BEA and CEF showed no obvious change in any of the bands

- Herbal capsules

  TLC with BEA showed a slight decrease in the colour intensity for the band at R_f=0.07. The compound with R_f=0.76 seemed to have an increase in colour intensity from t=0-t=6 months. This increase is not seen with the tablets. TLC with CEF showed a decrease in colour intensity for the band at R_f=0.14. The compounds present at R_f=0.57 and R_f=0.69 also appeared to have an increase in the colour intensity from t=0-t=6 months. Again this is not seen with the tablets.

- Extract capsules

  TLC with both BEA and CEF showed no obvious change

- Liquid extracts

  A slight decrease in the colour intensity from t=0-t=6 months could be seen with BEA as the eluent for the band with R_f=0.27. TLC with CEF showed a noticeable increase in colour intensity for the bands at R_f=0.44, R_f=0.57 and R_f=0.69. This increase was the same as seen with the herbal capsules

3.4.1.1.3 Kava Kava samples at 60°C. [See figure 3.4.5]

- Herbal tablets

  TLC with BEA and CEF showed no obvious change in any of the bands

- Herbal capsules

  TLC with BEA showed a minimal, but constant increase of colour intensity at the band with R_f=0.16. This increase did not occur when looking at the tablets. CEF as the mobile phase showed a slight increase of colour intensity for the bands at R_f=0.75 and R_f=0.57.

- Extract capsules

  TLC with BEA showed a definite decrease in the colour intensity of the band with R_f=0.07 from t=0-t=6 weeks. CEF as eluent showed no obvious change.

3.4.1.1.4 **Kava Kava samples at 80°C.** [See figure 3.4.6]

- Herbal tablets

  TLC with BEA showed a slight increase of colour intensity at the band with R_f=0.16. TLC with CEF also showed a minimal increase in the colour intensity of the band with R_f=0.44 from t=0-t=6 weeks.

- Herbal capsules

  An increase in colour intensity for the bands with R_f=0.16 and R_f= 0.76 was seen with BEA as the mobile phase. TLC with CEF showed both an increase of the intensity of colours for the bands at R_f=0.57 and R_f=0.69. This was only observed with the capsules and not the tablets.

- Extract capsules

  TLC with BEA showed a decrease in the colour intensity of the band with R_f=0.07 from t=0-t=6 weeks. CEF as the mobile phase showed no obvious change. The change was more obvious than the one observed at 60°C

3.4.1.1.5 **Kava Kava samples at direct sunlight.** [See figure 3.4.7]

- Herbal tablets

  Again no obvious change with either BEA or CEF as the mobile phases were observed.

- Herbal capsules

  TLC with BEA and CEF showed no obvious change.

- Extract capsules

  Again only BEA as the eluent, showed a slight decrease in the colour intensity of the band at R_f=0.07. The compound present at R_f=0.69 decreased in colour intensity from t=0-t=6 months with CEF as the mobile phase.

- Liquid extracts

  TLC with BEA showed no obvious change. CEF as the eluent showed an increase of the intensity of colours at the bands with R_f=0.44, 0.57 and 0.76.

3.4.1.1.6 Kava Kava samples at high humidity and direct sunlight. [See figure 3.4.8]

- Herbal tablets

  TLC with BEA showed no obvious change in any band's colour intensity. With CEF as the eluent, it seemed that the band with R_f=0.57 might have had a slight decrease of intensity at t=3 and t=6 months.

- Herbal capsules

  TLC with BEA showed no obvious change, but a slight possible increase in colour intensity of the band at R_f=0.69 was observed with CEF as the mobile phase. This change did not appear with the tablets.

- Extract capsules

  TLC with BEA and CEF showed no obvious change.

- Liquid extract

  TLC with BEA showed no obvious change. TLC with CEF showed a slight increase of the colour intensity of the band with R_f=0.69, but to a lesser extent as seen only at sunlight.

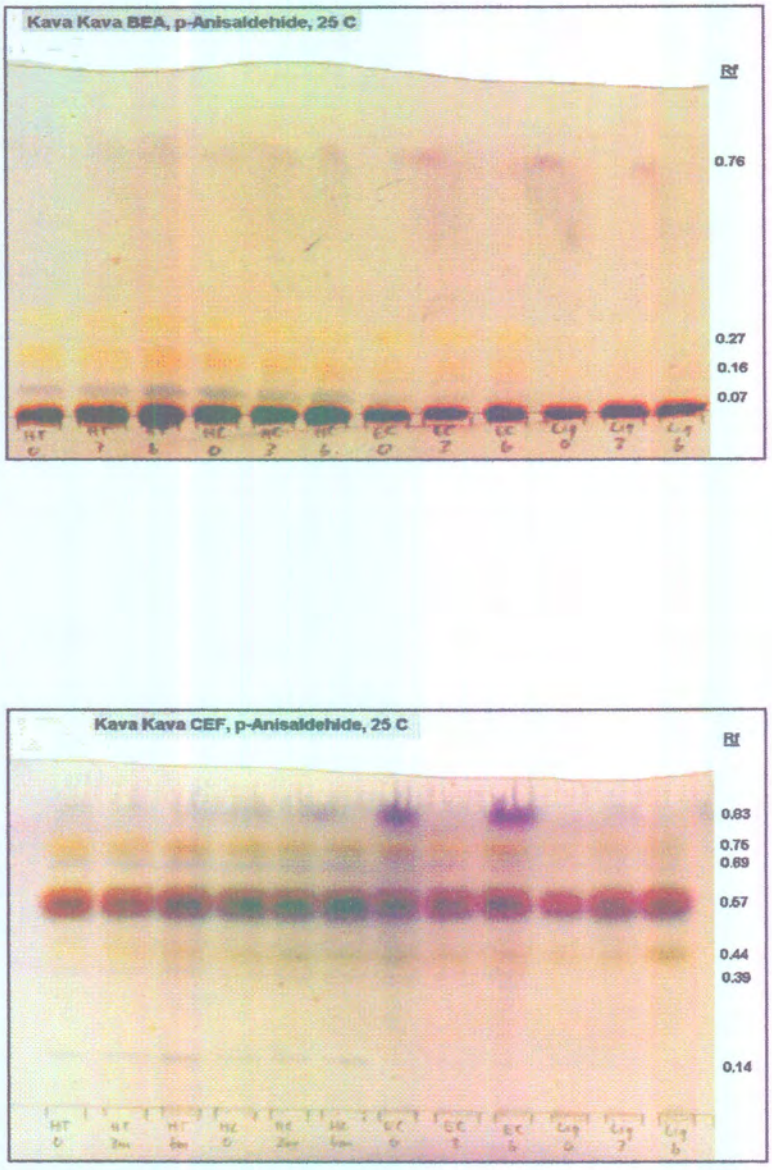
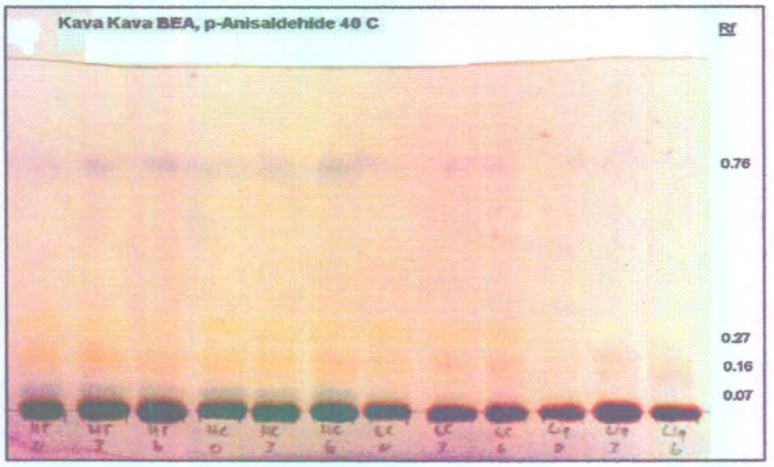


Figure 3.4.3 Kava Kava samples stored at 25°C. [Sprayed with *p*-anisaldehide with mobile phase BEA (top) and CEF (bottom). From left: Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]



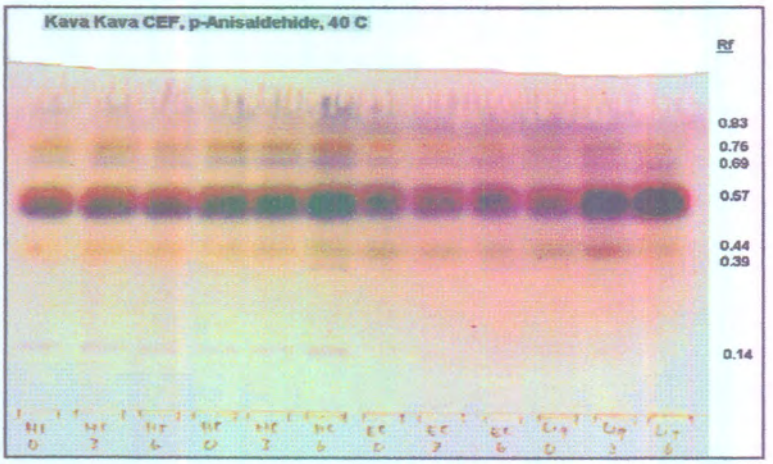
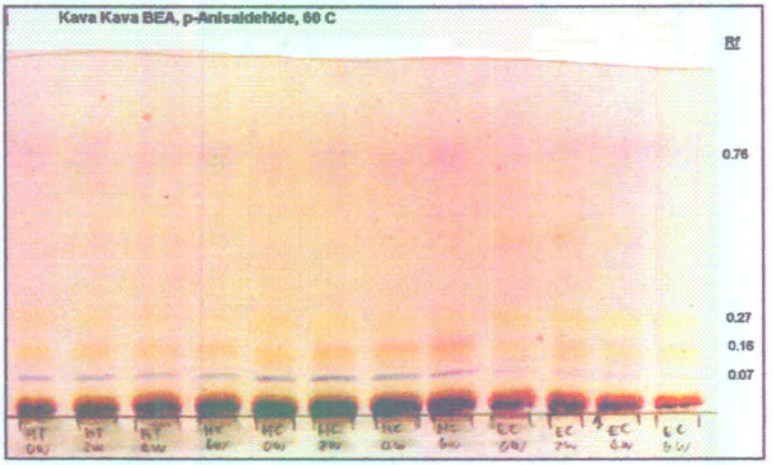


Figure 3.4.4 Kava Kava samples stored at 40°C. [Sprayed with *p*-anisaldehide with mobile phase BEA (top) and CEF (bottom). From left: Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]



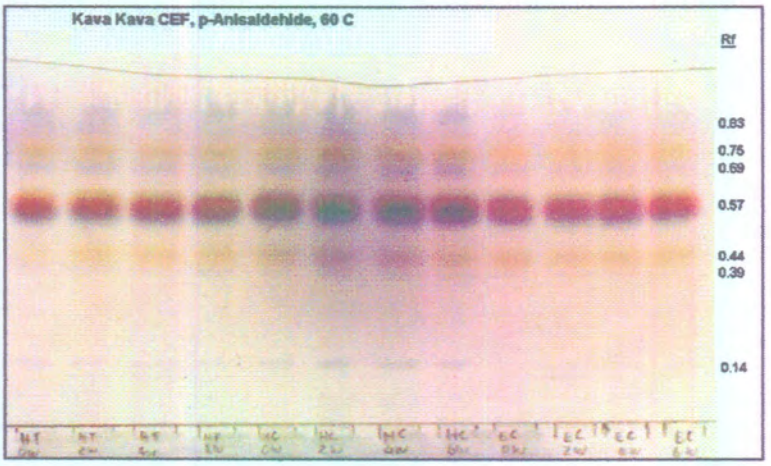
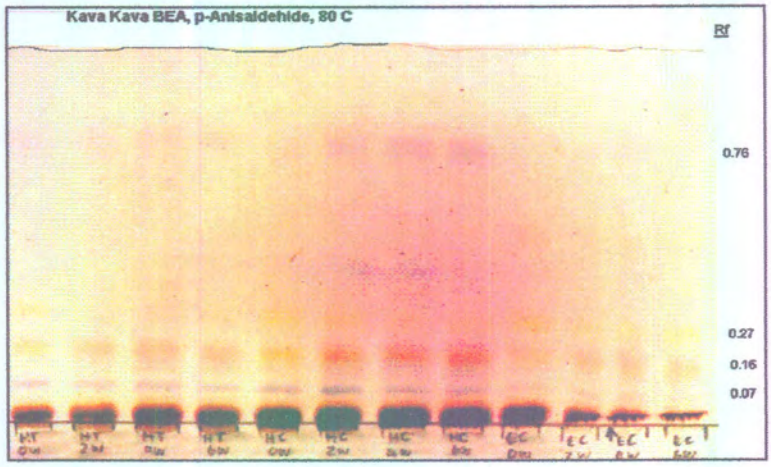


Figure 3.4.5 Kava Kava samples stored at 60°C. [Sprayed with *p*-anisaldehide with mobile phase BEA (top) and CEF (bottom). From left: Herbal tablets (HT) 0,2,4,6 weeks. Herbal capsules (HC) 0,2,4,6 weeks. Extract capsules (EC) 0,2,4,6 weeks.]



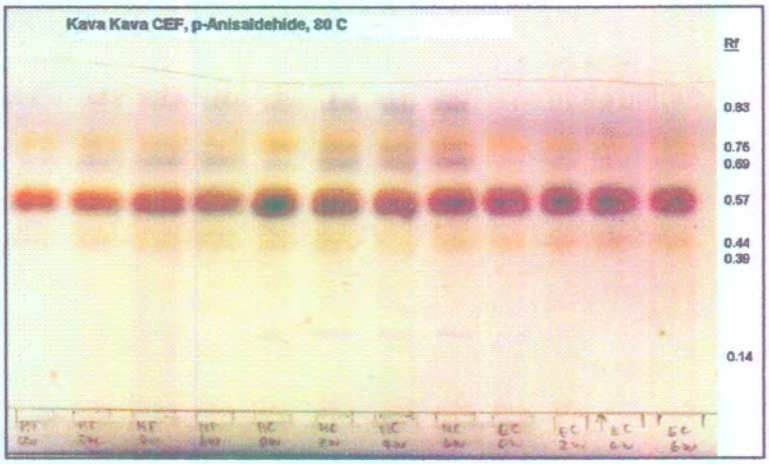
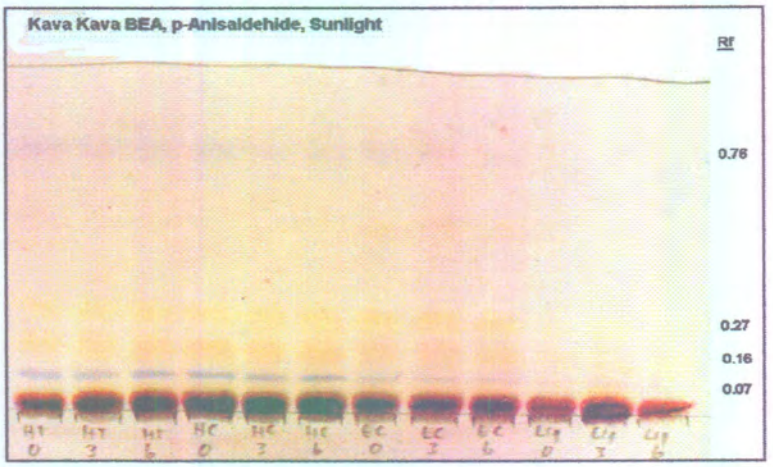


Figure 3.4.6 Kava Kava samples stored at 80°C. [Sprayed with *p*-anisaldehide with mobile phase BEA (top) and CEF (bottom). From left: Herbal tablets (HT) 0,2,4,6 weeks. Herbal capsules (HC) 0,2,4,6 weeks. Extract capsules (EC) 0,2,4,6 weeks.]



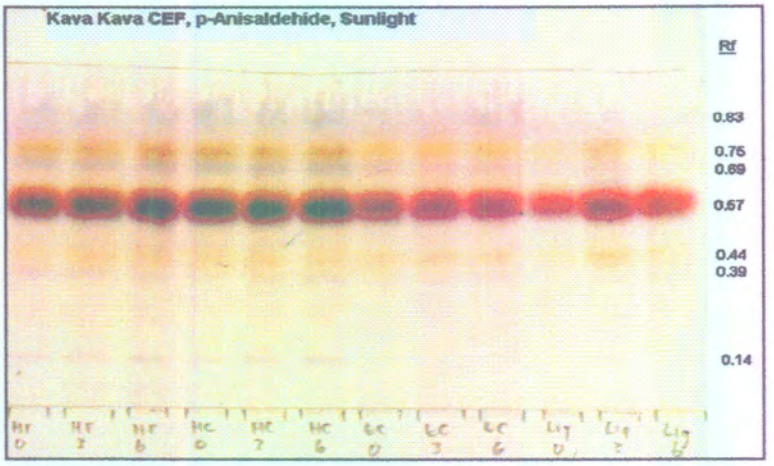
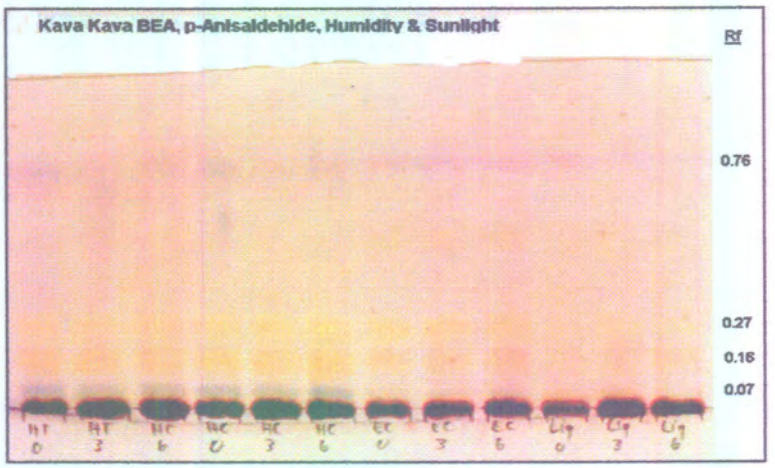


Figure 3.4.7 Kava Kava samples stored at direct sunlight. [Sprayed with *p*-anisaldehide with mobile phase BEA (top) and CEF (bottom). From left: Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]



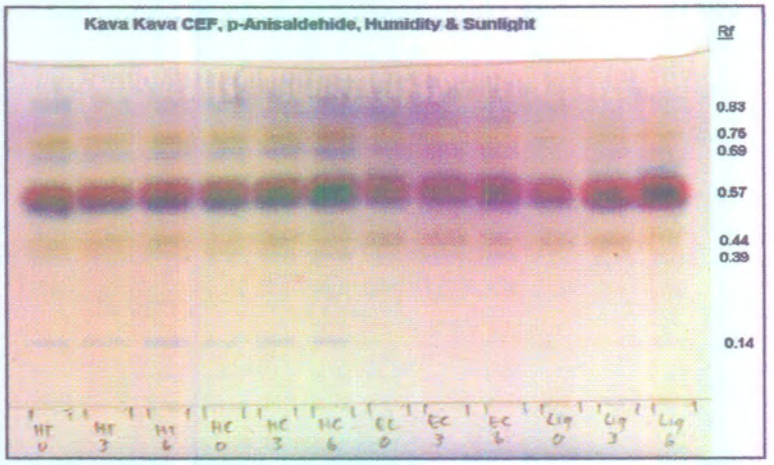
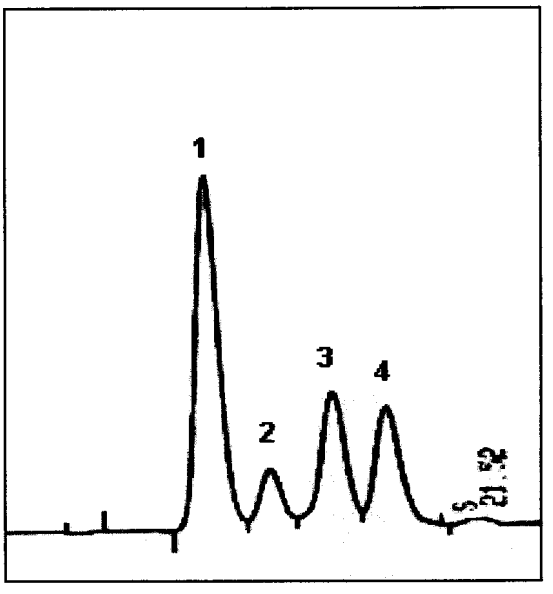


Figure 3.4.8 Kava Kava samples stored at direct sunlight and high humidity. [Sprayed with *p*-anisaldehide with mobile phase BEA (top) and CEF (bottom). From left: Herbal tablets (HT) 0,3,6 months. Herbal capsules (HC) 0,3,6 months. Extract capsules (EC) 0,3,6 months. Liquid extract (LIQ) 0,3,6 months.]

3.4.1.2 High Pressure Liquid Chromatography (HPLC)

Only samples of the herbal tablets, herbal capsules, extract capsules and radiated dried kava kava root powder were analyzed by HPLC, as the quantity required for the liquid extracts to be injected into the column was not sufficient for a sample preparation described in section 2.7.2.3.

Due to the fact that no standard of any one of the kava-lactones could be obtained, and only a reference chromatogram given on the internet [http://www.nutraceuticalinstitute.com/methods/kava] was available, it seemed unrealistic to express any values in a concentration format. In stead the sum-total of the peak heights of the four identifiable kava-lactones, methysticin, di-hydro-methysticin, kavain and di-hydro-kavain were compared to the values measured at t=0 months for each product. The sum-total of the individual peaks measured at t=0 months was given a value of 100% for each product.



1. **Methysticin**
2. **Di-hydro-methysticin**
3. **Kavain**
4. **Di-hydro-kavain**

Figure 3.4.9 HPLC chromatogram of Kava Kava root powder prepared in section 2.7.2.3.

The standard deviation on the HPLC method as mentioned in section 2.7.2.4 was calculated using the data in table 3.4.1

Table 3.4.1 Analysis of one Kava Kava sample repeated 5 times

Sample	X_1	X_1^2
1	27441456.46	7.53034E+14
1	24852828.5	6.17663E+14
1	30754084.43	9.45814E+14
1	27740015.83	7.69508E+14
1	30916453.83	9.55827E+14
Sum	141704839.1	4.04185E+15

$$\frac{(141704839.1)^2}{5} = \frac{2.00803 \times 10^{16}}{5} = 4.01605 \times 10^{15}$$

Substituting into equation 2.3 leads to a standard deviation in peak height of 2539371.9integrated units. Assuming that a peak height of 20705666.2 integrated units represents a total of 100% kava-lactones, when using the data for the Herbal tablets at t=0 months, the deviation is then calculated at 12.26 %. Applying the same assumption, the deviation for the herbal capsules calculates to 7.8%, the extract capsules to 1.79% and the radiated root powder to 10.48%

The standard deviation in the quantity of kava-lactones extracted from exactly 75mg of the dried root powder was also calculated according to equation 2.3

Table 3.4.2 Analysis of 5 Kava Kava samples with exact weight of 75mg

Sample	X_1	X_1^2
1	0.0742	0.005506
2	0.0738	0.005446
3	0.0744	0.005535
4	0.0737	0.005432
5	0.0735	0.005402
Sum	0.3696	0.027321

$$\frac{(0.3696)^2}{5} = \frac{0.1366041}{5} = 0.02732111$$

Substituting into equation 2.3 leads to a standard deviation of 0.34 mg in the quantity of kava-lactones extracted from each dosage form. This is a total of 0.45% on the assumption that 75mg contains 100% of active. This relates to the standard deviation seen due to the extraction method.

A summary of the extrapolated data regarding the total kava-lactone content of each sample is given in table 3.4.3, 3.4.4, and 3.4.5. The following graphs are a summary of the different dosage forms, with regard to their stability at each condition mentioned in section 2.3. In each graph the total kava-lactone content at t=0 weeks was expressed as 100%. The values at 2,4,6,8,10 and 12 weeks are expressed in terms of a increase or decrease relative to the value of 100% at t=0 weeks.

The changes in the kava-lactone content were compared to the standard deviation for the method as calculated for each different dosage form, i.e. herbal tablets 12.26%, herbal capsules 7.8%, extract capsules 1.78% and radiated dried root 10.48%.

3.4.1.2.1 Kava Kava herbal tablets [Table 3.4.3 for values]

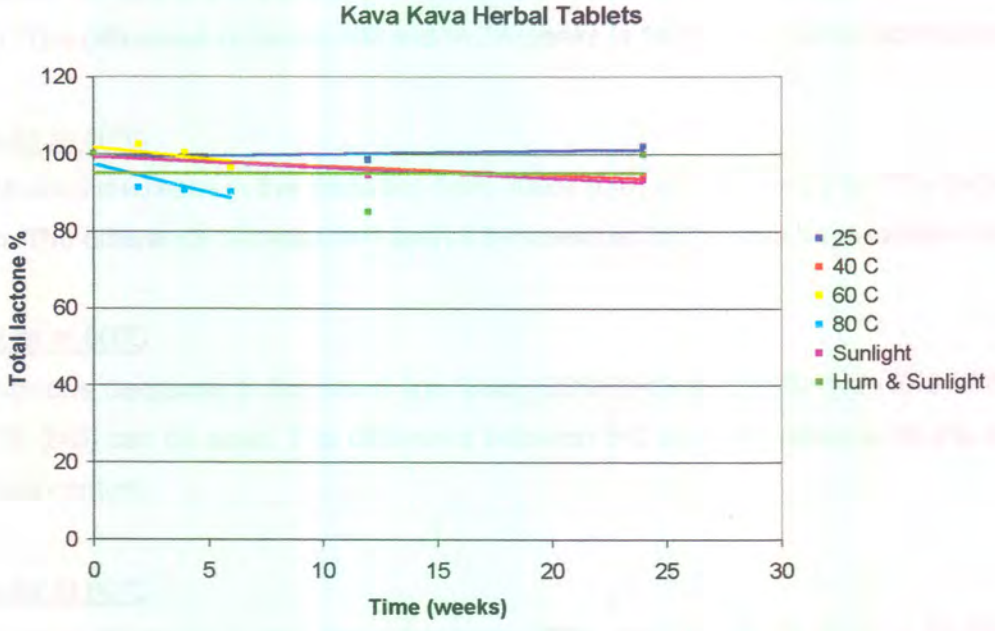


Figure 3.4.10 Degradation of kava-lactones in Kava Kava herbal tablets at different storage conditions

Results at 25°C

A small increase in the trend line from 100% (t=0) to 98.4% (t=12) to 101.9% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 1.9% total kava-lactone content. This value falls inside the standard deviation of 12.3%.

Results at 40°C

A small decrease in the trend line from 100% (t=0) to 95% (t=12) to 94.2% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 5.8% total kava-lactone content.

Results at 60°C

A small decrease in the trend line from 100% (t=0) to 102.5% (t=2) to 100.4% (t=4) to 96.7% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 3.3% total kava-lactone content.

Results at 80°C

A small decrease in the trend line from 100% (t=0) to 91.3% (t=2) to 90.8% (t=4) to 90.2% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 9.84% total kava-lactone content.

Results at direct sunlight

A small decrease in the trend line from 100% (t=0) to 94.5% (t=12) to 93.3% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 6.7% total kava-lactone content. This value falls inside the standard deviation of 12.3%.

Results at direct sunlight and high humidity

A small decrease in the trend line from 100% (t=0) to 84.9% (t=12) to 99.9% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 0.07% total kava-lactone content.

3.4.1.2.2 Kava Kava herbal capsules [Table 3.4.4 for values]

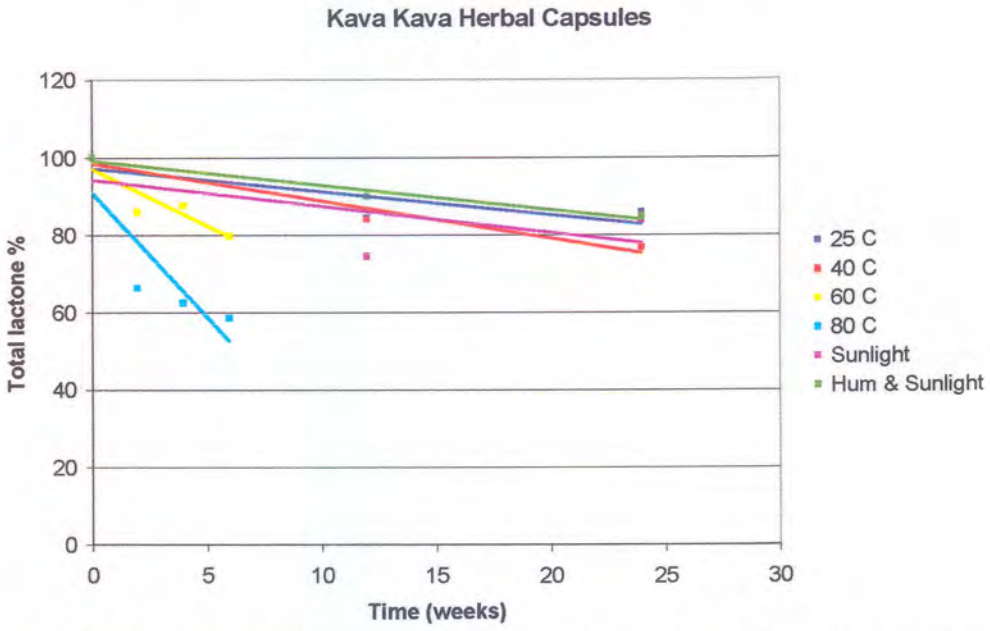


Figure 3.4.11 Degradation of kava-lactones in Kava Kava herbal capsules at different storage conditions

Results at 25°C

A slight decrease in the trend line from 100% (t=0) to 84.3% (t=12) to 85.7% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 14.3% total kava-lactone content.

Results at 40°C

An obvious decrease in the trend line from 100% (t=0) to 84% (t=12) to 77% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 23.3% total kava-lactone content.

Results at 60°C

An obvious decrease in the trend line from 100% (t=0) to 85.9% (t=2) to 87.6% (t=4) to 79.7% (t=6) can be seen. The difference between t=0 and t=6 weeks is 20.3% total kava-lactone content.

Results at 80°C

A massive decrease in the trend line from 100% (t=0) to 66.3% (t=2) to 62.5% (t=4) to 58.5% (t=6) can be seen. The difference between t=0 and t=6 weeks is 41.5% total kava-lactone content.

Results at direct sunlight

A noticeable decrease in the trend line from 100% (t=0) to 74.4% (t=12) to 83.6% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 16.4% total kava-lactone content.

Results at direct sunlight and high humidity

A slight decrease in the trend line from 100% (t=0) to 90% (t=12) to 84.8% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 15.2% total kava-lactone content.

3.4.1.2.3 Kava Kava extract capsules [Table 3.4.5 for values]

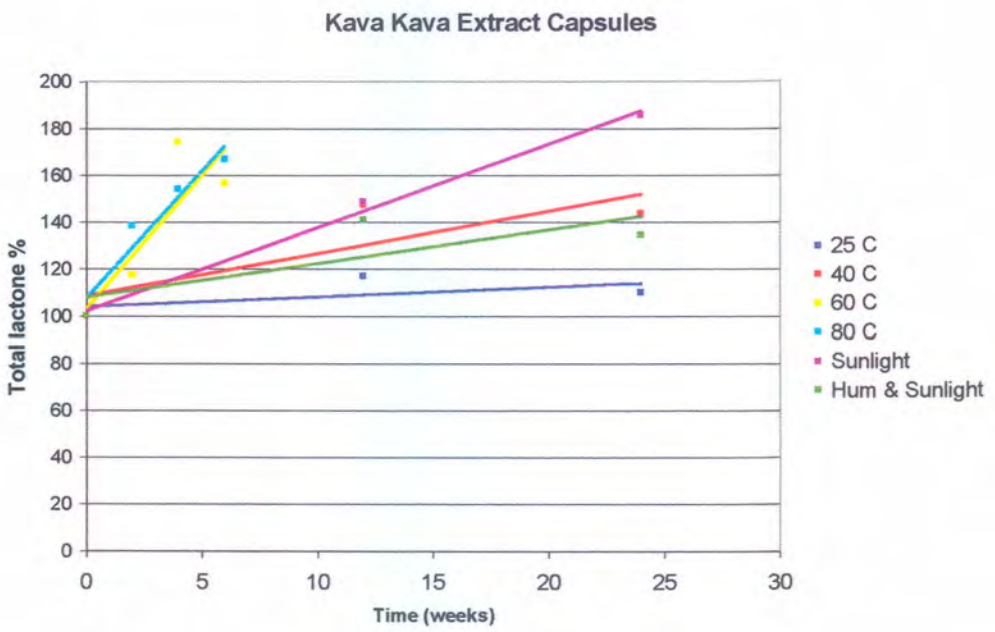


Figure 3.4.12 Degradation of kava-lactones in Kava Kava extract capsules at different storage conditions

Results at 25°C

A small increase in the trend line from 100% (t=0) to 117% (t=12) to 110% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 10% total kava-lactone content.

Results at 60°C

A massive increase in the trend line from 100% (t=0) to 117.6% (t=2) to 174.2% (t=4) to 157.6% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 57.6% total kava-lactone content.

Results at 80°C

A massive increase in the trend line from 100% (t=0) to 138.7% (t=2) to 154.2% (t=4) to 166.8% (t=6) can be seen. The difference between t=0 and t= 6 weeks is 66.8% total kava-lactone content.

Results at direct sunlight

A massive increase in the trend line from 100% (t=0) to 148.7% (t=12) to 185.7% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 85.7% total kava-lactone content.

Results at direct sunlight and high humidity

An obvious increase in the trend line from 100% (t=0) to 141.% (t=12) to 134.5% (t=24) can be seen. The difference between t=0 and t= 24 weeks is 34.5% total kava-lactone content.

3.4.1.2.4 Kava Kava root powder radiated with Cobalt-60 source

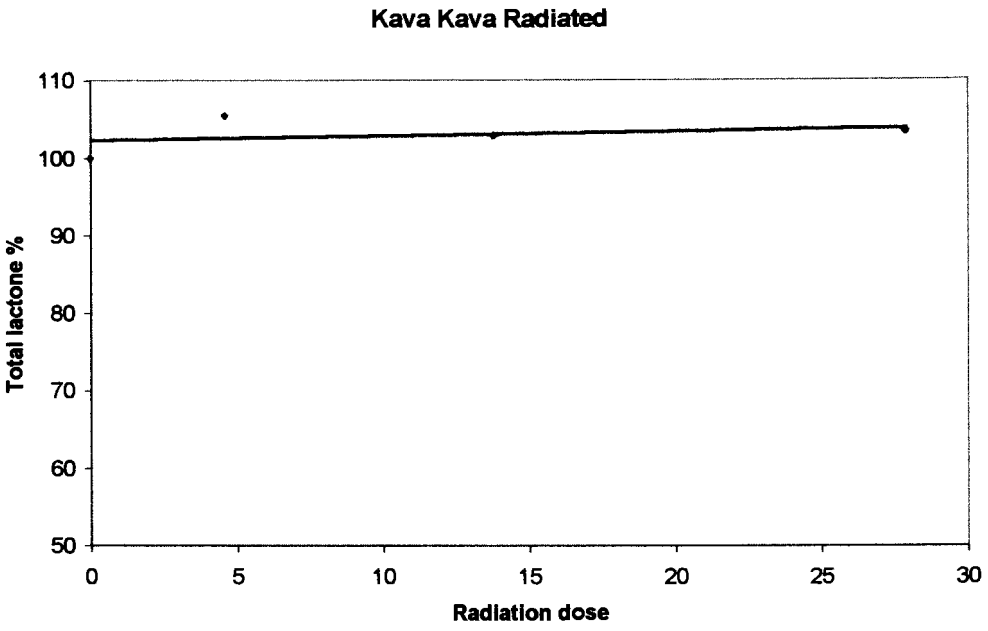


Figure 3.4.13 Kava Kava dried root powder at different radiation doses

There seems to be no obvious change in the kava-lactone content at each radiation dose. A small increase in the trend line was observed where the difference between the content at dose=0 kGy and at dose= 27.9 kGy only came to 3.43%.

Table 3.4.3 Summary of data obtained from HPLC analysis of Kava Kava Herbal tablets.

Condition	Time (weeks)	Total Peak Height	% relative to t=0
25°C	0	20705666.22	100.0
25°C	12	20382229.33	98.4
25°C	24	21117000	102.0
40°C	12	19684170.44	95.1
40°C	24	19502864	94.2
60°C	2	21221320.91	102.5
60°C	4	20789034.67	100.4
60°C	6	20014873.16	96.7
80°C	2	18911418.67	91.3
80°C	4	18792234.98	90.8
80°C	6	18669052.07	90.2
Sun	12	19570668.44	94.5
Sun	24	19323652.87	93.3
Sun+Hum	12	17591306.67	85.0

Table 3.4.4 Summary of data obtained from HPLC analysis of Kava Kava Herbal Capsules.

Condition	Time (weeks)	Total Peak Height	% relative to t=0
25°C	0	32547309.4	100.0
25°C	12	27430536.3	84.3
25°C	24	27901771.9	85.7
40°C	12	27338279.6	84.0
40°C	24	24954095.5	76.7
60°C	2	27967384.8	85.9
60°C	4	28504933.6	87.6
60°C	6	25937113.6	79.7
80°C	2	21601794.9	66.4
80°C	4	20328053.0	62.5
80°C	6	19051872.8	58.5
Sun	12	24229609.5	74.4
Sun	24	27225598.9	83.6
Sun+Hum	12	29279527.0	90.0
Sun+Hum	24	27600769.3	84.8

Table 3.4.5 Summary of data obtained from HPLC analysis of Kava Kava Extract Capsules.

Condition	Time (weeks)	Total Peak Height	% relative to t=0
25°C	0	141356098.2	100.0
25°C	12	165444750	117.0
25°C	24	155487171.2	110.0
40°C	12	208385163.6	147.4
40°C	24	202941090.9	143.6
60°C	2	166202218.2	117.6
60°C	4	246299675.7	174.2
60°C	6	221314285.7	156.6
80°C	2	196085709.1	138.7
80°C	4	217949500	154.2
80°C	6	235782522.5	166.8
Sun	12	210186991	148.7
Sun	24	262486428.6	185.7
Sun+Hum	12	199376714.3	141.1
Sun+Hum	24	190098400	134.5

3.4.1.3 Summary of HPLC and TLC results

3.4.1.3.1 Herbal tablets

25°C

TLC with BEA didn't show any obvious change, but CEF as eluent showed an increase in the colour intensity of 2 visible bands. The kava-lactone content decreased by 1.98% over a 24-week period according to the HPLC results.

40°C

No obvious change was observed with TLC. The kava-lactone content decreased by 5.8% over a 24-week period according to the HPLC results.

60°C

No obvious change was observed with TLC. The kava-lactone content decreased by 3.3% over a 6-week period according to the HPLC results.

80°C

TLC with both BEA and CEF showed a slight increase of colour intensity of one band. The kava-lactone content decreased by 9.8% over a 6-week period according to the HPLC results.

Direct sunlight

No obvious change could be seen with TLC. The kava-lactone content decreased by 6.7% over a 24-week period according to the HPLC results.

High humidity and sunlight

Only TLC with CEF showed a slight decrease in the colour intensity of 1 band. The kava-lactone content decreased by 0.1% over a 24-week period according to the HPLC results.

3.4.1.3.2 Herbal Capsules

25°C

TLC with both BEA and CEF showed a decrease in the colour intensity of one band. The kava-lactone content decreased by 14.3% over a 24-week period according to the HPLC results.

40°C

TLC with BEA and CEF both confirmed an increase in colour intensity of two bands. This is slightly contradicted when looking at the decrease of 23.33% kava lactones measured by HPLC.

60°C

TLC with BEA and CEF both confirmed an increase in the colour intensity of two bands. This increase is more prominent than the same increase observed at 40°C. Again HPLC contradicted these observations, with a measured decrease in the kava lactone content of 20.3% over a 6-week period.

80°C

TLC with BEA and CEF both confirmed an increase in the colour of two bands. This increase is even more observed than the same increase seen at 40°C and 60°C. Again HPLC contradicted these observations, with a massive decrease in the kava lactone content of 41.5% over a 6-week period.

Direct sunlight

No obvious change was seen with TLC. The kava-lactone content decreased by 16.4% over a 24-week period according to the HPLC results.

Humidity and direct sunlight

An increase in the colour intensity of one band was seen with CEF as the mobile phase. The kava-lactone content decreased by 15.2% over a 24-week period according to the HPLC results.

3.4.1.3.3 Extract capsules

25°C

Only TLC with CEF showed a decrease in the colour intensity of one band. The kava-lactone content increased by 10% over a 24-week period according to the HPLC results.

40°C

No noticeable change could be seen with TLC. The kava-lactone content increased by 43.6% over a 24-week period according to the HPLC results.

60°C

TLC was inconclusive with a seemingly decrease observed at only one band with CEF as the mobile phase. The kava-lactone content increased by 57.6% over a 6-week period according to the HPLC results.

80°C

Again only TLC with BEA showed a decrease of colour intensity, which was more obvious than at 60°C. The kava-lactone content increased by 66.8% over a 6-week period according to the HPLC results.

Sunlight

TLC with both BEA and CEF showed a slight decrease in the colour intensity of one band. The kava-lactone content increased by 85.6% over a 24-week period according to the HPLC results.

Humidity and sunlight

TLC showed no obvious change, but an increase of 34.48% total kava-lactone content over a 24-week period was shown with HPLC.

3.4.1.3.4 Liquid extract

25°C

TLC showed one band to decrease with BEA, but CEF showed and increase in colour intensity of 2 bands.

40°C

TLC showed the same decrease with BEA, as well as the increase in intensity of colours with CEF similar to 25°C. The same increase was observed with the herbal capsules.

Direct sunlight

No obvious change could be seen with BEA as a mobile phase, but CEF also showed the increase in colour intensity as seen with 25°C and 40°C.

High humidity and sunlight

TLC with BEA showed no obvious change. CEF showed a slight increase in intensity of only one band, but with a lesser extent as seen in the sunlight alone.

3.4.1.3.5 Radiated dried root

HPLC analysis showed a maximum variation in the total kava-lactone content of 3.43%.

3.4.2 Kinetics of Kava-lactones

The determination of the chemical reaction order of the total kava-lactones present in each dosage form seemed to be a more complex calculation than simply using the substitution method [See section 3.2.2.1], as used for hypericin and quercetin. Substituting data from table 3.4.3, 3.4.4 and 3.4.5 into each different equation (Equation 3.2, 3.3 and 3.4) gave no indication of the actual reaction order. The difference in the calculated values were 74.98% for the zero-order reaction, 75.68% for the first-order reaction and 126% for the second-order reaction. Considering these enormous differences, it seemed unrealistic to even assume a zero-order reaction. Sadly this lead to the conclusion that with the information currently at hand for kava kava, no indication of the reaction-order could be determined, and thus no *k*-values, t_{90}, or estimated shelf-life using the Arrhenius equation.

3.4.3 DISCUSSION

One of the principle reasons of the inability to determine a reaction-order for kava kava might be due to the fact that no quantification of the actual kava-lactones could be calculated by making use of HPLC. This was a direct effect of the unavailability of a quantified standard.

A small value may have a large effect when using the equations necessary for determining the reaction-order of a chemical substance, for example: Assuming that kava kava follows a zero-order degradation, and using the values in table 3.4.3 for the herbal tablets at 25°C, the *k* value is then calculated at 0.02354 $\%.weeks^{-1}$. This leads to a shelf-life of 424.8 weeks. When substituting only the value for the standard deviation of the method (12.26%), a *k* value of 1.02166$\%.weeks^{-1}$ is achieved, which calculates to a shelf-life of 9.78 weeks. Substitution into the first- or second order equations results in the same misleading values.

The implication of this dilemma is clearly the inability of predicting a meaningful shelf-life. Only a general conclusion regarding the stability of each individual product could be made. This was achieved by looking at the trend lines at each different environmental

condition, keeping in mind the massive standard deviation. In other words, a comparison between the stability of the herbal tablets could therefore not be expressed with regard to the herbal capsules, extracts or liquids. TLC provided useful information both confirming and discarding patterns seen with the trend lines.

As with hypericum and ginkgo, it seemed that the kava kava herbal tablets were a very stable dosage form. The slight increases of colour seen with some TLC plates might be the result of the incomplete reaction between the spraying reagent and the separated compound. The fact that all the values obtained from HPLC fell within the standard deviation, confirms this assumption.

Decreases or increases in the colour seen with the herbal capsules, might also be a result of a variation on the TLC-plate preparation. A striking observation was the definite increase in colour of the bands in the capsules stored at 40°C, 60°C and 80°C. In contrast to this phenomenon, was the pronounce decrease in the total kava-lactone content, calculated by HPLC. This decrease was further more definite at 40°C, 60°C and 80°C when compared to the tablets at the same temperatures. No obvious change was observed with the tablets. One has to wonder what the relationship between an increase in colour intensity, seen with TLC, has with a decrease in kava-lactone content, calculated by HPLC. One possible explanation is that increased temperatures might have a more negative effect on the stability of the contents in case of the capsules compared to the tablets. This could possibly be explained by the stabilizing effect of certain non-actives incorporated in the tablets, more so than the empty hard gelatine capsule.

The formation of degradation products that might have similar R_f values than the original compound, should be considered. The composition of the mobile phase used to separate the compounds, might be unsuitable for separating the active from the degradation product, thus enhancing the colour of the bands. When using HPLC, the specific absorbance of the active compound is measured. It could be a possibility that the degradation product has a slightly different absorbency or better separation than the original compound. This could explain the decrease seen with HPLC, compared to the increase with TLC. This supposition requires further investigation.

The observation that no obvious change in the colour intensity of the extract capsules could be seen when compared to the herbal capsules, where the intensity increased, could possibly be explained in light of the extraction method used by the manufacturers of the dry extract powder. The possible effect of the extraction process might have been the

removal of compounds responsible for the intensifying of the colours of the bands in the herbal capsules as mentioned in the above paragraph. This still did not explain the increase calculated in the total kava-lactone content by HPLC. One explanation for this seemingly increase in stability, rather than a decrease, could possibly be due to a concentrating effect of the kava lactones. This could possibly be caused by the loss of water and volatile compounds, still present in the extract powder, evaporating during exposure to extreme temperatures. Weighing the extract capsules stored at each condition, and comparing the mass to the original average mass of 498.3mg, were carried out to test this theory. All of the capsules showed a decreased weight (up to 6%). Compensating for the loss of material, a correction factor was considered in the expression of total kava-lactone content with regard to 100% at t=0. This was however not done because of the uncertainty in the percentage loss due to the extract powder, or the loss from the gelatine capsule.

The decreases seen in certain bands corresponding at the same time, with definite increases in other, favour the hypothesis of degradation products interfering with some of the measurements. The influence of considering the 4-kava lactone peaks as one, probably had an impact, because any decomposition product with a retention time between the four kava-lactones, would have been measured.

Looking only at the TLC chromatograms, it seems that kava kava is also a stable substance, independable of the dosage form. At least, this seems to be the case at normal temperatures. The bands of the tablet dosage forms showed a decrease in intensity to a lesser extend than the same bands in the herbal capsules, when exposed to unfavorable environmental conditions. The tablets appear to be a more stable dosage form than the herbal capsules. The fact that the extract capsules became more concentrated with time as mentioned above, again made it difficult to express it's stability in terms of the herbal tablets or herbal capsules. Taking into account that both the extract capsules of hypericum and ginkgo seemed to be less stable than their herbal capsules, the opinion could be drawn that the stability of kava kava extract capsules might follow the same pattern, and that the increases seen are due to degradation products being formed. This generalized opinion must be further investigated.

Comparing the increases seen in the colour intensity of the bands present in the liquid extracts, and not having any quantitative data to confirm this observation (as with the herbal capsules), no comment regarding the stability of the liquid extracts could be made.

Gamma radiation seemed to have no effect on the kava-lactones present in the dried kava root powder. This has to be investigated in more detail, due to the high standard deviation of this method.

In conclusion to this part of the study, it seemed obvious that the HPLC-method used to determine the total kava-lactones present in each analyzed sample, have to be reviewed. The standard deviation of almost 13% is unacceptable in interpreting data. A reference standard for the kava kava-lactones should also be used in future analysis.

In the case of hypericum, TLC provided an interesting summary of all the changes taking place with each different dosage form. It was however unexplainable why some bands increased in intensity, while others decreased. It might seem that TLC should therefore be used in conjunction with some quantifying analytical method to give possible explanations of this seemingly impossible observations.

3.4.4 SUMMARY

Due to the unrealistic huge standard deviation, it didn't seem logical to evaluate the stability of each product as in the case of hypericum and ginkgo. The general assumption as seen with hypericum and ginkgo, were that an increase in temperature, caused a increase in degradation. Again the specific effect of sunlight and the combination of sunlight and humidity, on the increase in degradation, could not be established. Kava Kava herbal tablets seem to be a more stable dosage form than the capsules, extract capsules or extract liquids. It appears that radiation has little effect on the stability of kava kava root powder.

Chapter 4

Conclusion

In this study, the main focus was the investigation into the chemical stability exhibited by different dosage forms of *Hypericum perforatum*, *Ginkgo biloba* and *Piper methysticum.*

The effects of unfavourable environmental conditions combined with exposure to increased temperatures, were evaluated according to each individual plant, and each different dosage form.

One of the aims were to evaluate the use of non-quantifying analytical methods, for example TLC, in the implementation of stability studies for herbal medicines with respect to St John's word, *Ginkgo biloba* and kava kava.

TLC seemed to be of great value in commenting on the stability of the different hypericum dosage forms. Most of the data obtained from spectrophotometry, seemed to be confirmed by TLC. The mobile phases and spraying reagents chosen, could separate and visualize the different chemical compounds present in each hypericum dosage form, clearly.

The use of spectrophotometry in the analysis of the hypericum dosage forms further seemed to be a suitable choice. This method had the smallest standard deviation compared to the methods used for ginkgo and kava kava. The conclusion could be made that if the method showed a small standard deviation and it was absolutely certain that hypericin was the only active component present in *Hypericum perforatum*, spectrophotometry would seem to be a valuable analytical instrument in determining the stability of the product. This seems to be confirmed by TLC. The only uncertainty remains concerning the question regarding the disputable fact that hypericin is the only active ingredient. If it could be proven that other substances present in *Hypericum perforatum* also contributes to the pharmacological effect, the determination of the stability should be reconsidered.

Thin layer chromatography was of no great value in determining the stability of the ginkgo products. A more suitable mobile phase should be investigated in future. It seemed that EMW was a poor choice in separating the more polar compounds. Adjusting the polarity of

BEA and CEF might prove to be beneficial. HPLC seemed to be a good choice in the analysis of the quercetin concentration present in each dosage form. This technique should however be refined into giving a smaller standard deviation. In spite of the standard deviation of 6%, a pattern could still be seen regarding the stability of the different dosage forms, as well as the effect of different temperatures and unfavorable conditions on the general stability of the ginkgo products. This pattern observed was similar to the pattern seen with the spectrophotometric analysis of the hypericum dosage forms.

Again only one compound, quercetin, was analyzed, and the whole discussion was based on the stability of this single compound. Ginkgo is marketed as a mixture containing 24% flavone glycosides and 6% terpene lactones. If it is assumed that all of these chemical components shows a similar mechanism of degradation as could be seen with quercetin, this interpretation of the chemical stability of the different ginkgo dosage forms, might have some level of accuracy. If these compounds each have it's own unique degradation mechanism, it could be more difficult in determining the actual stability of ginkgo in total.

HPLC in kava kava didn't provide enough reliable data due to the fact concerning the huge standard deviation of the analytical method, a lack of standards and the possible interference of degradation products . A more suitable method should be implemented in the quantification of the kava-lactones. TLC provided a good overall picture of the changes taking place at each different condition. The reason for the unexplained increase in some of the bands, especially in the case of herbal capsules, needs to be investigated by some sort of quantitative analytical method.

The calculation of the reaction order, as well as the reaction-order constant (k), was possible for hypericum and ginkgo, but not for kava kava. This might be explained by the fact that the calculation of the order of reaction was based upon the degradation of only one chemical compound (hypericin in the hypericum dosage forms, and quercetin in the ginkgo dosage forms). In attempting this calculation for kava kava, the sum-total of the individual kava lactones was used, due to the unavailability of a reference standard. This seems to indicate that each individual chemical compound might have it's own unique degradation mechanism.

It seems that an increase in temperature, as to be expected, causes a decrease in stability of all the products. It seems that the tablet-dosage forms are equally effective regarding stability, compared to the capsules. This seems to be observed in a lesser

extent with kava kava, where it seems that the tablets might be more stable that the capsules at room temperature. The effect of tablet exipients, shows to be beneficial in providing additional stability, but needs to be further investigated. In considering the above possible benefits of tablets as dosage forms, it must be emphasized that these tablets were manufactured according to the direct compression method.

In all three plants the liquid extracts appeared to be less stable than the extract capsules. The extract capsules seem to exhibit a more rapid degradation than the herbal tablets or herbal capsules. Faster degradation does not necessarily imply a lesser clinical effectiveness. Considering the fact that the extract powder might contain double or even more of the quantity of "active" compared to the dried harvested powder. This implies that although degradation is faster, it might take longer for the active component to reach such a low concentration as not to show any pharmacological effect, compared to the whole herb, with a slower degradation rate, but with a very low initial concentration of active.

The effect of gamma radiation further seems to be of interest. Exposure to high doses of radiation showed no decrease in the hypericum or kava kava dried herbal powder. High dose radiation however showed a noticeable decrease in quercetin concentration. Exposure to low doses (4.4 kGy) showed to have little effect on the stability. The same pattern was seen by Retief (2001), where the sterol content of some products seemed to decrease after exposure to a high dose radiation, while it seemed unaffected at low doses.

Stability is not only measured in terms of the chemical changes that takes place in a product, but rather a combination of chemical, physical and microbiological changes. It is probably the most difficult to determine the chemical stability. Physical reactions might cause a decreased bio-availability. This may result during the reaction to have a negative effect on the absorption and dissolution. Storage in hard gelatine capsules at high humidity and temperatures, might cause the contents to become compacted or sticky (as was the case with kava kava extract capsules). Tablets may break or show a change in colour especially where the process of wet granulation is used during the manufacturing process.

After considering the relevance of this study and evaluating the observations and conclusions obtained from the various analytical methods, some improvements should be made with regard to future investigations.

In determining the quantity of kava-lactones, a suitable reference standard should be obtained.

All standard deviations should be calculated by the analysis of more than 5 samples.

Spectrophotometry proved to be a reliable and consistent analytical method. The results obtained from this method should still be compared to a HPLC method, confirming all data.

The HPLC methods for determining the quercetin concentration and total kava-lactone content should be refined. Different mobile phases might give better separation of the peaks, and therefore more reliable results.

TLC plates should preferably be done in duplicate, to determine whether a certain observation is not only the result of an incomplete reaction between the spraying reagent and the separated compound.

The actual t_{90} values were calculated using data obtained from only 6-months of storage at 25°C. Analysis should also be done after 12, 18 and 24 months

Considering the use of conventional pharmaceutical calculations in determining the stability with regard to the shelf-life of a product, were one of the major aims of this study. This was probably one of the first studies where the use of the Arrhenius equation was applied to the prediction of a shelf-life for certain herbal products. From the data obtained from excellerated stability studies, it seemed not to be applicable for some herbal products having high activation energies. This was however done on only three plants, and the general conclusion should never be drawn considering only these results. At high temperatures some anti-oxidants present in the herbal powder, might be destroyed. These substances might have a protecting effect on the stability of the active. This phenomenon does not occur with normal allopathic medicines. This might also be a possible reason why it seems that increased temperatures could not be used to predict the stability at normal temperatures.

Some useful industrial application that might be relevant, after evaluating the effect of high temperatures and humidity on the stability of all products, is the possible benefit of direct compression over the use of the traditionally wet granulation process in tablet manufacturing. Granulation involve a series of exposure to granulating liquids followed by

drying under increased temperatures. With direct compression, no heat or moist is used as part of the manufacturing process.

Some herbs are more sensitive to sunlight or heat than others. Considering the possible influence of these parameters on *Hypericum perforatum, Ginkgo biloba* and *Piper methysticum*, regardless of the dosage form, serious implications might be foreseen regarding the storage and packaging of these products. The phrases "Store below 25°C" , "Keep away from moist" and "Keep away from light" should be mentioned on the product label of each product.

To conclude in general, all three of the chosen plants seems to be relative stable, if stored in the specified conditions, and manufactured in a process where degradation is less likely to occur. While there is still no legislation regarding the stability of herbal products, as well as relevant analytical methods in determining the exact shelf-life of a herbal product, the assumption that the shelf-life should not be longer than 2-years seems to be a valid statement.

The End ...(of the Beginning)

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