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ABBREVIATIONS

6-OH-T	6-β-OH-testosterone
αBA	α-boswellic acid
α-TE	α-tocopherol equivalents
ADR	Adverse drug reaction
ALA	α-linolenic acid
AMD	Age-related macular degeneration
AUC	Area under curve
βBA	β-boswellic acid
BSE	*Boswellia serrata* extract
CAT	Catalase
cDNA	Complementary deoxyribonucleic acid
CV	Coefficient of variation
CVD	Cardiovascular disease
CYP	Cytochrome P450
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
EtOH	Ethanol
FA	Fatty acid
FRTA	Free Radical Theory of Ageing
GPx	Glutathione peroxidase
GRAS	Generally recognized as safe
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
IC_{50}	Inhibitory concentration decreasing the enzyme activity by 50%
KPO	Potassium phosphate buffer
KTZ	Ketoconazole
LLOQ	Lower limit of quantification
MeOH	Methanol
MVT	Multivitamin
n-3 LC-PUFAs	Omega-3 long-chain polyunsaturated fatty acids

NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of NADP
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
P-gp	P-glycoprotein
PIC	Positive inhibitory control
PL	Phospholipid
PT-INR	Prothrombin time – international normalized ratio
PUFAs	Polyunsaturated fatty acids
QC	Quality control solution
R^2	Regression coefficient
RA	Rheumatoid arthritis
RDA	Recommended dietary allowance
RELIS	Regional Drug Information Centers
ROS	Reactive oxygen species
SD	Standard deviation
Se-Met	Selenomethionine
SJW	St. John's wort
SOD	Superoxide dismutase
STD	Standard solution
TAG	Triacylglycerol
VP1	First generation VitaePro
VP2	Second generation VitaePro
VP3	Third generation VitaePro
VP4	Fourth generation VitaePro
WS	Working solution

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SUMMARY IN ENGLISH

Introduction and aims: There has been an almost exponential growth within the nutritional supplement industry, and along with this, an increasing number of reported interactions between supplements and prescribed drugs have been observed. One of Scandinavia's best-selling supplements, VitaePro, has been developed in four "generations" with varying constituents. The aim of this study was to investigate the *in vitro* inhibitory effect of the two latest products towards CYP3A4. Further, the inhibitory potency of the two products was compared. The two first generations have already been investigated in one earlier study of VitaePro and CYP3A4

Materials and methods: The experimental work was performed using human cDNA expressed CYP3A4, an NADPH regenerating system and with testosterone as substrate. After incubation with VitaePro, the formation of the metabolite 6-β-OH-testosterone was quantified using a validated high performance liquid chromatography (HPLC)-method. Based on the inhibition plots, inhibitory concentrations decreasing the enzyme activity by 50%, IC_{50}-values, were calculated by non-linear regression. Ketoconazole (KTZ) was used as positive inhibitory control (PIC).

Results and conclusion: VP3 inhibited CYP3A4 with IC_{50}-values of 5.29±0.72 mg/mL and 3.77±0.83 mg/mL in the first and second experiment, respectively, and the corresponding values for VP4 were 0.14±0.03 mg/mL and 0.17±0.01 mg/L. Both generations proved to inhibit CYP3A4 *in vitro*, but VP4 to a greater extent than VP3. The 95% confidence intervals of the IC_{50}-values showed a significant difference in inhibitory potency between VP3 and VP4, which presumably can be attributed to the *Boswellia serrata* extract added in VP4. Using theoretical approaches, one can hypothesize that VitaePro might be a candidate for *in vivo* effects, and this should be investigated in further trials.

Key words: VitaePro, CYP3A4, inhibition, testosterone, 6-β-OH-testosterone, ketoconazole

SUMMARY IN NORWEGIAN

Introduksjon og målsetning: Det har vært en nesten eksponentiell vekst innenfor kosttilskuddbransjen, og samtidig har man observert et økende antall rapporterte interaksjoner mellom kosttilskudd og forskrevne medikamenter. Et av Skandinavias bestselgende kosttilskudd, VitaePro, har blitt utviklet i fire "generasjoner" med varierende innholdsstoffer. Målsetningen med denne studien var å undersøke inhibisjonseffekten til de to siste produktene mot CYP3A4 *in vitro*. Videre ble inhibisjonspotensialene til de to produktene sammenlignet med hverandre. De to første generasjonene har allerede vært undersøkt i en tidligere studie av VitaePro og CYP3A4.

Materiale og metode: Det eksperimentelle arbeidet ble utført ved bruk av humant cDNA-uttrykt CYP3A4, et NADPH-regenererende system og med testosteron som substrat. Etter inkubering med VitaePro, ble mengden dannet metabolitt, 6-β-OH-testosteron, kvantifisert ved bruk av en validert væskekromatografimetode (high-performance liquid chromatography, HPLC). Basert på inhiberingsplottene ble inhibisjonskonsentrasjoner som reduserer enzymaktiviteten med 50%, IC_{50}-verdier, regnet ut ved ikke-lineær regresjon. Ketokonazol (KTZ) ble brukt som positiv inhiberingskontroll (PIC).

Resultater og konklusjon: VP3 inhiberte CYP3A4 med IC_{50}-verdier på 5,29±0,72 mg/mL og 3,77±0,83 mg/mL i henholdsvis første og andre forsøk, og de tilsvarende verdiene for VP4 var 0,14±0,03 mg/mL og 0,17±0,01 mg/mL. Begge generasjoner viste seg å inhibere CYP3A4 *in vitro*, men VP4 i større grad enn VP3. 95% konfidensintervallene til IC_{50}-verdiene viste en signifikant forskjell i inhibisjonspotensial mellom VP3 og VP4, som antakelig kan tilskrives *Boswellia serrata*-ekstraktet tilført i VP4. Ved bruk av teoretiske tilnærmingsmodeller, er det muligheter for at VitaePro kan gi *in vivo*-effekter, og dette bør derfor undersøkes i videre forsøk.

Nøkkelord: VitaePro, CYP3A4, inhibering, testosteron, 6-β-OH-testosteron, ketokonazol

1 INTRODUCTION

1.1 Nutritional supplements

Good health and an active lifestyle have become symbols of personal wealth and high social status in many parts of the world. This culture of healthy living is particularly seen in industrialized and high-income countries, and along with this sporty trend, many people are willing to optimize their health using simple and easy measures. Promises of better health, better sexual life, slimmer waist and wellbeing in general have contributed to an explosion in the sales of nutritional supplements. The market for supplements is growing larger every day, and the increasing interest has been particularly prominent over the past decades. Today the market is rife with more or less documented supplement products, both regarding exaggerated health claims and quality [1].

It is well known that certain foods and diets have a more favorable impact on our health than others [2]. Vitamins, minerals and other bioactive nutrients, that are abundantly present in fruits and vegetables, are now available in the form of nutritional supplements. New methods for extraction, purification and concentration of nutritional substances have made it possible for us to enjoy a wide variety of such substances in the form of tablets, capsules, portioned powder bags, or in other forms of administration.

The term nutritional supplement has been defined in different ways around the world according to the laws and regulations in force within different countries. A common and recurring definition is that nutritional supplements are “concentrated sources of nutrients or other substances with a nutritional or physiological effect that supplement the normal diet” [3]. In Norway, the Regulation on Nutritional Supplements (Forskrift om kosttilskudd), formulated by the Ministry of Health and Care Services (Helse – og omsorgsdepartementet), is strictly regulating the composition, labeling, marketing and distribution of nutritional supplements in pre-packaged form to the consumer. Other countries have similar laws and regulations, e.g. in the United States of America, where the U.S. Food and Drug Administration regulates both finished nutritional supplement products and dietary ingredients in foods [3, 4]. The purpose of these regulations, whether it is in Norway or elsewhere, is to ensure safe use and honest sales of nutritional

supplements. Unfortunately, there are many unserious actors in the supplement industry that do not respect these regulations [5]. This may have created a bad reputation for the supplement industry, which also affects the serious actors.

As mentioned in the definition above, nutrients or other substances used in nutritional supplements are claimed to have a nutritional or physiological effect. Because of this, vitamins and minerals are frequently occurring components in nutritional supplements. Other ingredients may be antioxidants, amino acids, fatty acids and certain herbs. However, it is of great importance to distinguish between supplements and chemical medicines. Definition-wise and legally, there is a large distinction between nutritional supplements and medicines.

In Norway, nutritional supplements are defined as food and along with the Regulations on nutritional supplements, the industry is under surveillance of the Norwegian Food Safety Authority (Mattilsynet)[6]. Medicines, on the other hand, are regulated by the Norwegian Act of Medicines (Legemiddelloven) and under close surveillance of the Norwegian Medicines Agency (Statens legemiddelverk)[7].

According to the Norwegian Act of Medicines, medicines are defined as "substances, drugs and preparations intended for, or used in, the prevention, treatment or alleviation of illness, symptoms of illness or pain in humans or animals" [8]. These claims about actual prevention, cure and relief of illness are reserved for medications alone, and it is the Norwegian Medicines Agency that provides marketing authorization for all medicines. Remedies that are not covered by this definition, such as nutritional supplements, can be traded far more freely, albeit with the composition, labeling, marketing and distribution requirements established in the Regulations on Nutritional supplements. A product classified as medicine cannot be marketed as a nutritional supplement.

Many nutritional supplements claim to be natural products. A chemical component or substance is traditionally understood as natural when it is produced by living organisms found in nature. In an editorial from the peer-reviewed scientific journal Nature Chemical Biology, the expanding meaning of the term is discussed. The author states that along with the evolvement of new

technologies and new production methods, the "natural product" term has expanded to include all chemical substances as long as they are found in nature. This means that a product can be considered as natural, even though it is produced by total synthesis. "Whether a natural product is isolated from a native organism, synthesized in a laboratory, biosynthesized *in vitro*, or isolated from a metabolically engineered organism, if the resultant compound is chemically equivalent to the original natural product, it is natural [9]." Neither the Regulation on Nutritional Supplements nor the Norwegian Food Safety Authority have given an unambiguous definition of natural products, and the understanding of the term therefore remains vague in Norway. However, worse than a somewhat confusing and unclear definition, is it that many consumers associate natural products with something that is safe and harmless, which we today know is far from the truth [10].

The recommendation from The Norwegian Directorate of Health (Helsedirektoratet) is that most people have no need for supplements at all. The current belief is that the majority of people in Norway get the nutrients they need through their diet. Yet supplements may be beneficial to meet the needs of selected nutrients in certain groups or during certain parts of life. Examples here include vitamin D to elderly and those who are little outside in daylight, folic acid to pregnant women, iron to women with large iron losses, and multivitamin-mineral supplements to those with very low energy intake. However, supplements can never replace the variety of substances that a normal diet provides [11].

1.2 VitaePro

VitaePro is one of the many nutritional supplements on today's huge market. The product, produced by the Norwegian company VitaeLab AS, has been up for sale since 2002, and since then four "generations" of VitaePro have been developed and released to the market.

The "first generation" of VitaePro (VP1) from 2002 contained the three antioxidants astaxanthin, lutein and zeaxanthin. In 2010 a "second generation" of VitaePro (VP2) was released to the market, and in this generation vitamin C, vitamin D, vitamin E and krill oil were added to the three original antioxidants. Only a year later, in 2011, a "third generation" of VitaePro (VP3) was

released. In this product the antioxidants and vitamins from the first two generations had been preserved, but the krill oil had been replaced with calamari oil. Most recently in 2015, a "fourth generation" of VitaePro (VP4) has entered the market. In this product *Boswellia serrata* extract (Indian salai tree) and selenium are added to the ingredients from the preceding generation. Table 1 shows the different VitaePro products and their components in detail. Most of the information is obtained from the package labeling [12], while the information concerning the EPA and DHA content is obtained through personal communication with Ramskjell, CEO at VitaeLab AS [13].

Table 1: An overview of the different VitaePro products and their components.

VP1	VP2	VP3	VP4
2 mg astaxanthin 6 mg lutein 1.2 mg zeaxanthin	2 mg astaxanthin 6 mg lutein 1.2 mg zeaxanthin 25 mg vitamin C 5 µg vitamin D 12 mg α-TE vitamin E 300 mg krill oil (36 mg EPA and 16.5 mg DHA)	2 mg astaxanthin 6 mg lutein 1.2 mg zeaxanthin 100 mg vitamin C 5 µg vitamin D 12 mg α-TE vitamin E 347 mg calamari oil (48 mg EPA and 125 mg DHA)	2 mg astaxanthin 6 mg lutein 1.2 mg zeaxanthin 100 mg vitamin C 10 µg vitamin D 12 mg α-TE vitamin E 250 mg calamari oil (35 mg EPA and 90 mg DHA) 55 µg selenium 100 mg *Boswellia serrata* extract

The ingredients in VitaePro are collected from different locations around the world, and according to Svennevig, who is Research and Development Manager at NutraQ (personal communication), VitaeLab uses several alternative suppliers to ensure continuous availability of the most special ingredients. Astaxanthin comes from the freshwater microalgae *Haematococcus pluvialis*, and was previously bought from Hawaii where the algae were cultured in large pools.

Now the astaxanthin is acquired from the Swedish company BioReal AB, who cultivates *Haematococcus pluvialis* in photobioreactors in Sweden. Lutein and zeaxanthin are extracted from the petals of the Mexican marigold flower, *Tagetes erecta*. This flower is native to Mexico and Central-America, but also cultivated commercially other places. Those providing VitaeLab with lutein and zeaxanthin are grown in vast fields in India, and bought from the Indian company OmniActive Health Technologies. Sorbitol, which is produced from corn and wheat, is the basis for the production of ascorbic acid, vitamin C. Vitamin D and vitamin E are obtained from fat in sheep wool (lanolin) and soybeans, respectively. The krill oil, used only in VP2, was bought from the Norwegian company Aker BioMarine AS, who harvests krill in the Antarctic. The calamari oil, used in both VP3 and VP4, comes from calamari caught in Asia. VitaeLab buys Calamarine® oil from the Norwegian company Pharma Marine AS, who harvests calamari in Korea. *Boswellia serrata* is a tree growing in Africa and Asia, and the *Boswellia serrata* extract (BSE) used in VitaePro is collected from trees grown in India. The company providing VitaeLab with BSE is mainly the American founded company Sabinsa Corporation. The chemical element and trace mineral selenium comes from selenomethionine [14, 15].

The producers of VitaePro have in consultation with the authorities agreed on using the wording "extracted from a natural source" for those of the ingredients that are synthesized; astaxanthin, lutein and zeaxanthin, and also vitamin E [15].

In 2008 VitaePro was launched in Sweden and Finland, and in 2010 it appeared on the Danish market. As previously mentioned, VitaePro is one of the best-selling supplements in Scandinavia, and according to the producer, there are over 100 000 daily users only in Norway [16]. Despite these successful sales figures, the marketing of VitaePro is still massive, and both ordinary people and famous athletes appear regularly in VitaePro TV commercials.

VitaePro is claimed to be favorable for the joints, the musculature, the heart and the immune system, and it is also claimed to protect the cells and reduce tiredness and fatigue [17]. According to the information on VitaeLab's official webpage, *Boswellia serrata* will keep the joints comfortable, vitamin D will maintain normal muscle function, and the omega-3 fatty acids, EPA and DHA, will contribute to the normal heart function. Further, selenium, vitamin C and vitamin

D will contribute to maintain the immune system's normal function and the antioxidants, including selenium, vitamin C and vitamin E, will protect the cells against oxidative stress. Vitamin C is, in addition to its above-mentioned effects, also claimed to reduce tiredness and fatigue [17].

In Norway, the Regulation on Nutrition and Health Claims made on Foods (Forskrift om ernærings – og helsepåstander om næringsmidler) was implemented in 2010. This regulation is based on the corresponding regulation drawn up by the European commission [18]. A health claim is a statement about a relationship between food and health, and according to both the European and the Norwegian regulation, they can be divided into two main categories, "Function Health Claims" and "Risk Reduction Claims". The first group comprises claims that relate to the growth, development and functions of the body, the psychological and behavioral functions, and to slimming or weight-control. The latter group comprises claims that relate to reducing a risk factor in the development of a disease, and to children's development [19, 20].

All producers of nutrients, regardless of whether they operate in the food or supplement industry, will have to apply to the EU commission for approval of health claims within these two categories [18]. VitaeLab claims to be one of the more serious actors in the supplement industry, and have several times emphasized that their production, labeling and marketing of VitaePro is completely consistent with the law. However, when returning to the fundamental law that prohibits food and supplements in claiming prevention, cure or alleviation of illness, symptoms of illness and pain, VitaeLab has been accused several times of having misleading advertisements [21-26].

1.3 Antioxidants

1.3.1 Reactive oxygen species, oxidative stress and antioxidants in general

Oxygen is essential to all aerobic life forms, including human life. However, the formation of harmful oxygen metabolites is a well-known disadvantage of aerobic metabolism. Reactive oxygen species (ROS) encompass a variety of chemical compounds containing molecular oxygen. They are termed "reactive" because some of them are extremely unstable and can initiate

damaging chain reactions. Many of the ROS are so-called free radicals, meaning that they are atoms, molecules or ions with unpaired valence electrons. The reason why these radicals are unstable, is that they react with target molecules to capture an electron and thus become stable molecules with only paired electrons in the outer shell. However, the target molecules left behind these reactions are turning into new free radicals, initiating a chain reaction that continues until two free radicals meet and create a product with a covalent bond. ROS, and the cascades of reactions initiated by ROS, have proven to be harmful to important biological molecules, such as proteins, lipids and DNA [27].

In other words: ROS are natural occurring compounds, continuously generated as a result of normal intracellular metabolism, and with the potential to damage important cellular components. Quantitatively, the most important source of ROS in humans is the mitochondrial electron transport chain, and even though less than 1% of the oxygen used in aerobic metabolism generates ROS, this amount would have been lethal in the absence of protective mechanisms. Other endogenous sources of ROS are peroxisomes, lipoxygenases, NADPH oxidase and cytochrome P450 [28].

Fortunately, nature has created an intricate defense system to neutralize ROS and other free radicals. Antioxidants play an important role in this defense, as they are substances that can be oxidized and thereby neutralize and stop the harmful chain reactions. The antioxidant defense in humans is extremely elaborate and consists of enzymatic systems, comprising catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), and several non-enzymatic antioxidants, including glutathione and the vitamins A, C and E [28].

The balance between ROS production and antioxidant defenses determines the overall degree of oxidative stress. The potential damage caused by increased ROS levels has already been emphasized, and different studies have shown that increasing ROS levels can lead to cell death, acceleration in ageing and age-related diseases. Traditionally, the impairment caused by increased ROS is thought to result from random damage of proteins, lipids and DNA. On the other hand, ROS levels below the homeostatic set point may interrupt the physiological role of

oxidants in cellular proliferation and host defense. Figure 1 shows the fine balance between ROS production and elimination, and what happens when the homeostasis is disturbed [28].

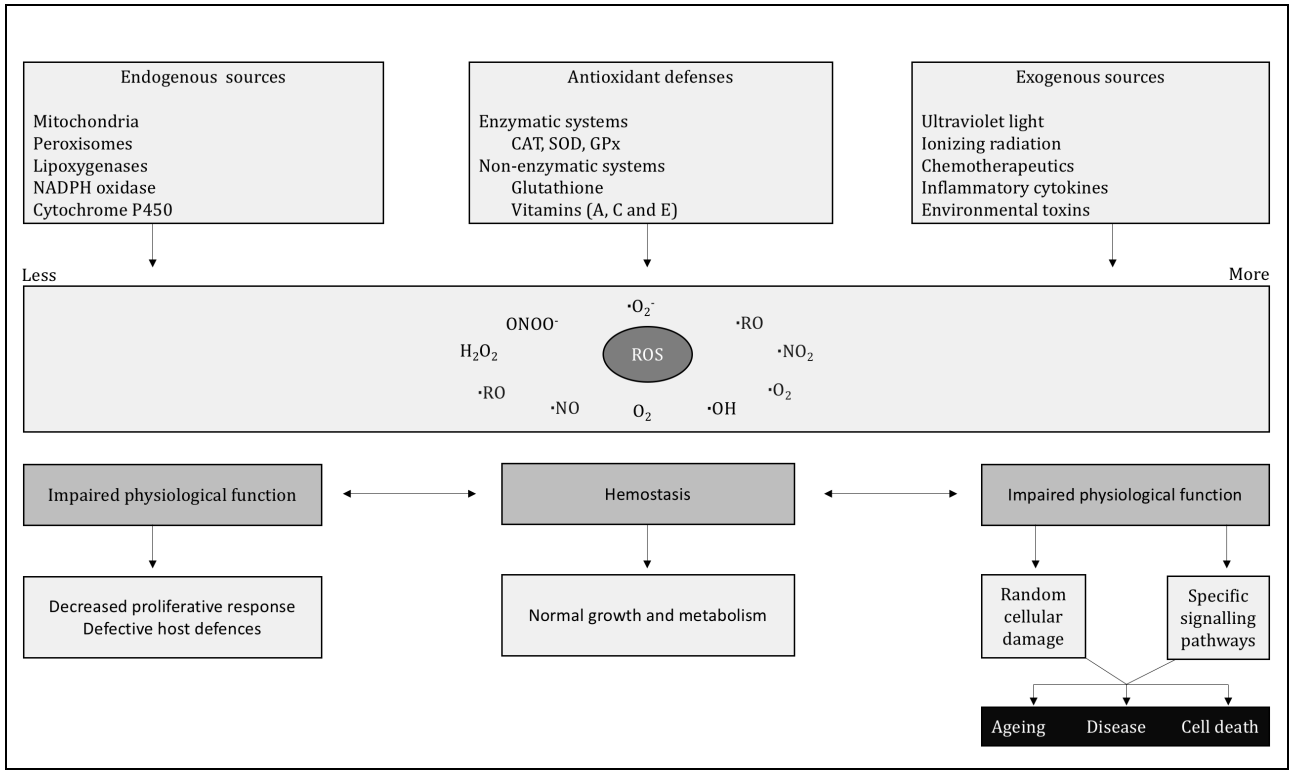

Figure 1: Production and elimination of reactive oxygen species (slightly modified from Finkel and Holbrook, 2000).

Although the physiology of ageing seems to be a multifactorial process, recent studies have made it clearer that the accumulation of molecular damage, caused by ROS and other free radicals, plays an important role in this process. The "Free Radical Theory of Ageing" (FRTA) is now so substantiated that the effect of antioxidant supplements has been studied by comparing the lifespan of organisms provided antioxidant supplements with the lifespan of the same organisms not provided such supplementations. Given that the FRTA is true, antioxidants should be able to slow down the aging process and thus prolong the lifespan. This hypothesis has encouraged a great number of studies aimed to investigate the relationship between supplemented exogenous antioxidants and the lifespan of various organisms. However, despite this deluge of studies, the conclusions have shown that the benefits of antioxidant supplementations are uncertain [29].

It has been argued that antioxidant mixtures, such as those found in VitaePro and other natural products, are better than simple antioxidant formulas due to synergism between the different antioxidants. Positive effects on the lifespan of model organisms have been reported using such mixtures, but other studies have shown no significant effect. An example of the latter is a study published in 2013, where simple and complex nutraceuticals, with antioxidative and anti-inflammatory properties, failed to affect the lifespan of long-lived, male, F1 mice. In the same article the authors discuss an earlier conducted study that found significant antioxidant effects in worms and flies, and suggest that screening drugs and supplements in lower eukaryotes may be confounded by high false discovery rates [30].

In summary, nutrition has been recognized as a key factor to overall longevity and antioxidants have become objects of extensive scientific research. However, their biochemical mechanisms of protection against oxidative stress and antiaging effects are not fully understood, and until now, no prospective clinical intervention studies have been able to show a positive association between antioxidant supplementation and increased survival. While beneficial effects of antioxidant supplements seem convincing in theory, more clinical trials with clear clinical and human endpoints are needed before a definite conclusion can be drawn.

1.3.2 The antioxidants in VitaePro: astaxanthin, lutein and zeaxanthin

Carotenoids are lipid soluble organic pigments found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms, including algae, some bacteria and some fungi. In plants and algae, the carotenoids exert two important functions: they absorb light energy for use in the photosynthesis and they protect the chlorophyll from photo damage. The latter feature is due to their well-known antioxidative properties, and recent studies have also suggested that these organic pigments possess antiapoptotic and anti-inflammatory properties as well [2]. All carotenoids are characterized by a conjugated π-electron system meaning that they have alternating single and double bonds in their hydrocarbon chain. This feature decreases the total energy of the molecule and provides it with more stability, and the conjugated double-bond structure is the main reason why carotenoids are such strong scavengers of ROS and other free radicals.

As mentioned above, carotenoids are only synthesized by photosynthetic organisms, meaning that animals and humans totally rely on dietary uptake of these compounds. At the beginning of 2014 about 700 different carotenoids had been defined, but only 50 of these were reported to be part of the human diet. The carotenoids are divided into two groups: xanthophylls, which contain oxygen, and carotenes, which are purely hydrocarbons. The three strong antioxidants found in VitaePro, astaxanthin, lutein and zeaxanthin, are all xanthophylls. The different carotenoids, whether they are xanthophylls or carotenes, absorb wavelengths ranging from 400-550 nm (violet to green light), and this causes the compounds to be deeply colored in the spectrum yellow to red [2]. Figure 2 shows the chemical structure of the 3 antioxidants astaxanthin, lutein and zeaxanthin.

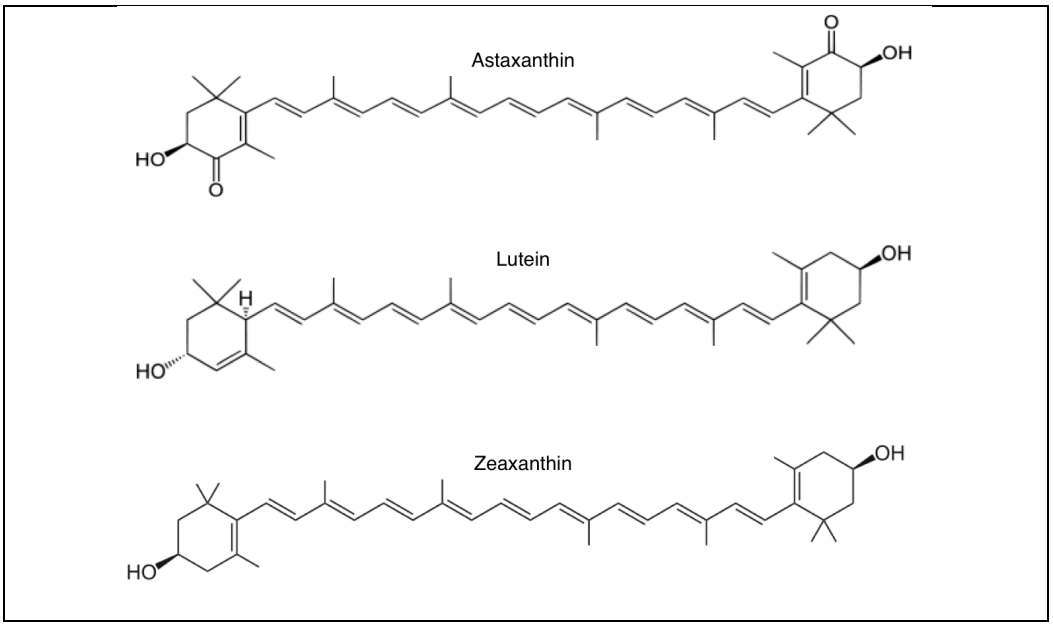

Figure 2: The chemical structure of astaxanthin, lutein and zeaxanthin. Note the characteristic π-electron system with alternating single and double bonds in all the compounds.

Astaxanthin is a deeply red-colored carotenoid pigment that naturally exists in a great variety of living organisms, including microalgae, plants and marine species. To most people it is probably best known for its pigmentation of seafood, including salmon, trout, krill, shrimp and many other aquatic organisms. Astaxanthin has proven to be one of nature's most powerful antioxidants, with greater ability to prevent lipid peroxidation in biological membranes than other naturally

occurring antioxidants. It is commercially cultivated from the microalgae *Haematococcus pluvialis* and the yeast *Phaffia rhodozyma* [31].

Astaxanthin has demonstrated greater antioxidative properties than both vitamin E and the famous β-carotene, and is said to play an important role in UV-light protection and prevention of age-related diseases. Numerous studies have also suggested that astaxanthin has the potential to reduce cardiovascular risk, improve the immune function, especially in the eye, and prevent or treat a wide variety of chronic inflammatory diseases, including cancer, asthma and rheumatoid arthritis (RA). Potential prevention and treatment of metabolic syndrome, diabetes and diabetic nephropathy are also suggested, as inflammation and oxidative stress seem to be major components in these diseases. Further, astaxanthin has in some studies shown a protective bioactivity against *Helicobacter pylori*, and it is thought to have preventive effect against gastrointestinal and hepatic diseases as well [32]. Another widely studied effect is the effect against acute brain injury and neurodegenerative diseases, and several studies have shown that astaxanthin can prevent neuronal damage, improve memory and be preventive against common neurodegenerative diseases, such as Alzheimer's and Parkinson's disease [31, 33].

Lutein and zeaxanthin are carotenoid isomers, and together with astaxanthin, they belong to the xanthophyll group of the carotenoids. Lutein and zeaxanthin are responsible for the yellow or orange color in many common foods, including cantaloupe, pasta, corn and egg yolks, and they are also abundantly present in green vegetables, such as kale, spinach, broccoli, peas and lettuce. Despite this presence in many dietary products, marketed lutein and zeaxanthin are mainly extracted from marigold flowers [34]. Like other carotenoids, lutein and zeaxanthin also exert antioxidative effects, but studies have revealed that these are of special importance in the human eye, protecting the retina. The two isomers constitute the main pigments in the yellow spot, *macula lutea*, of the retina and protect it from damage by blue light. Several studies have shown that lutein and zeaxanthin reduce the risk of age-related macular degeneration (AMD) and development of cataracts. The association between AMD prevention and these pigments is now so well-established that lutein, zeaxanthin and the conversion isomer of zeaxanthin, *meso*-zeaxanthin, are termed macula pigments (MP) [35, 36]. Recent studies have also suggested that

lutein and zeaxanthin might have a direct impact on brain functions and cognition, as they are the most abundant carotenoids not only in the retina, but also in the brain [37].

In addition to the above-mentioned features, lutein and zeaxanthin have been investigated for possible anticarcinogenic properties and a possible effect against chronic inflammatory conditions, including cardiovascular diseases [38]. Prevention of oxidative damage, regulation of immune response, inhibition of angiogenesis, modulation of apoptosis and induction of cell differentiation have been the main arguments for a possible anticarcinogenic effect, while the antioxidative and anti-inflammatory properties have been emphasized when it comes to the prevention of chronic inflammatory conditions [39].

According to the FDA, astaxanthin, lutein and zeaxanthin are substances generally recognized as safe (GRAS) when administrated in recommended doses [40-42].

1.4 Vitamins

1.4.1 Vitamins in general

Vitamins are organic compounds that are essential to serve specific biochemical functions in an organism. Further, vitamins are typically required only in limited amounts, but they are usually not synthesized in sufficient amounts within the organism. With these characteristics in mind, it is understandable that an excessive intake of vitamins through the diet might cause toxicity, while marginal availability and uptake can result in vitamin deficiencies. Up to date, 13 vitamins have been identified, and despite their diverse chemical structures, they can roughly be divided into two groups: fat-soluble (A, D, E and K) and water-soluble (B and C) vitamins [43].

Even though vitamins are not energy sources themselves, they play essential roles in the body, and vitamin-related activity includes hormone-like functions, catalyst or enzyme cofactor roles, antioxidant effects, and more. Table 2 gives an overview of the 13 vitamins, their role in the human body, recommended daily allowance (RDA) and what deficiencies might lead to [44].

Table 2: Overview of the vitamins, their role in the human body, RDA and deficiency consequences (slightly modified from Binder and Reuben, 2009).

Vitamin	Role	Recommended Dietary Allowance	Deficiency
A (retinol)	Retinal pigment	Male: 1000 µg Female: 800 µg	Follicular hyperkeratosis, night blindness
B_1 (thiamine)	Coenzyme in decarboxylation of pyruvate and α-keto acids	Male: 1.5 mg Female: 1.1 mg	Beriberi
B_2 (riboflavin)	Coenzymes flavin adenine dinucleotide and flavin mono-nucleotide, H carriers in mitochondria	Male: 1.7 mg Female: 1.3 mg	Hyperemia of nasopharyngeal mucosa, normocytic anemia
B_3 (niacin, nicotinic acid)	Coenzymes NAD and NADP, H carriers in mitochondria	Male: 19 mg Female: 15 mg	Pellagra
B_5 (pantothenic acid)	CoA; necessary for carbohydrate and fat metabolism involving acetyl-CoA amino acid synthesis	4-7 mg	Abdominal pain, vomiting, neurological signs
B_6 (pyridoxine)	Coenzyme in transamination for synthesis of amino acids	Male: 2 mg Female: 1.6 mg	Stomatitis, glossitis, normocytic anemia
B_7 (biotin)	Coenzyme in carboxylation reactions	30-100 µg	Neurological changes
B_9 (folate, folic acid)	Backbone used to synthesize purines and thymine	Male: 200 µg Female: 180 µg Pregnancy: 400 µg	Megaloblastic anemia
B_{12} (cobalamin)	Coenzyme in reduction of ribonucleotides to deoxyribonucleotides; promotion of formation of erythrocytes, myelin	2 µg	Pernicious anemia (megaloblastic anemia)
C (ascorbic acid)	Coenzyme in formation of hydroxyproline used in collagen	60 mg	Scurvy

D (1,25-dihydroxy-cholecalciferol)	Ca^{2+} absorption	5-10 µg	Rickets
E (α-tocopherol)	Antioxidant: thought to prevent oxidation of unsaturated fatty acids	Male: 10 mg Female: 8 mg	Peripheral neuropathy
K (K_1 = phylloquinone, K_2 = various menaquinones)	Clotting: necessary for synthesis of prothrombin and factor VII, IX and X in the liver	Male: 70-80 µg Female: 60-65 µg	Hemorrhagic disease

RDAs denote the daily nutrient intake that meets the requirements of 97-98% of all healthy individuals. Vitamin deficiency may be asymptomatic or present with wide range of clinical manifestations. Several studies have investigated the possible benefits of vitamin supplementation, and as earlier mentioned, the current belief is that healthy people get all the nutrients they need, including vitamins, through the diet. Elderly constitutes a special group, with greater risk of nutritional deficiencies, but also here, the benefits from multivitamin (MVT) supplementation remains debated [43]. However, in the case of specific comorbidities or settings that predispose for deficiency, it is probably appropriate to provide marginal vitamins through supplementations.

1.4.2 The vitamins in VitaePro: vitamin C, vitamin D and vitamin E

Vitamin C is a water-soluble vitamin and an important cofactor in many hydroxylation reactions, including the biosynthesis of collagen. It also serves as an antioxidant, and promotes the absorption of iron in the intestines [45]. The antioxidative effect from vitamin C has been investigated widely, and many studies conclude that vitamin C possesses immunostimulant, anti-inflammatory, antiviral and antibacterial features. These features are thought to be beneficial in many chronic diseases, including certain cancers and cardiovascular disease (CVD) [46]. However, vitamin C is never preferable as monotherapy in those conditions, rather as adjuvant treatment in addition to more specific drugs [47].

Despite the above-mentioned features of vitamin C, the effect of excessive doses remains uncertain, and vitamin C overdoses can cause nausea, vomiting, abdominal pain and diarrhea. Large doses over time are also known to promote the formation of kidney stones, as vitamin C breaks down to oxalate and is excreted through the urine [45].

Fruits and vegetables are generally rich sources of vitamin C, with particularly high content in tomatoes, potatoes and citrus fruits. Other sources include fortified cereals, bell peppers, broccoli and strawberries [48]. The absorption of vitamin C is 80-90% by oral administration, and the RDA of vitamin C is 60 mg. One capsule of VP3 and VP4 contains 100 mg, which is 167% of the RDA.

Vitamin D is a fat-soluble vitamin with hormone-like functions. It plays an important role in the bone metabolism, promoting calcium and phosphate absorption from the intestines, and stimulating bone formation [49]. Because of these anabolic effects on the skeleton, the benefits of vitamin D in bone health have been object to extensive research, and today vitamin D is recommended as treatment for osteoporosis and as a preventive measure to those who are little outside in daylight. However, along with this well-established association between vitamin D and bone health, there is a growing awareness of potential benefits of vitamin D in other chronic diseases, including diabetic conditions and CVD [50].

A toxic overdose of vitamin D is unlikely despite large isolated exposures. However, an excessive intake over time may cause hypercalcemia resulting in symptoms of fatigue, nausea, vomiting, decreased appetite, weight loss, thirst, polyuria, kidney failure, ECG changes and arrhythmias [49].

Vitamin D exists in two major forms: vitamin D_2 (ergocalciferol), mainly from dietary sources, and vitamin D_3 (cholecalciferol), mainly synthesized in the skin during exposure to UVB radiation. Both forms have to undergo two hydroxylation reactions, first in the liver and then in the kidneys, to become the biologically active form, calcitriol (1,25-dihydroxy-cholecalciferol) [50]. Far fewer food products contain vitamin D than vitamin C, but fatty fish and fortified products, such as milk and cereals, contribute to the daily intake [48]. The absorption of vitamin

D is normally good after oral administration, and the RDA of vitamin D is 5-10 μg. One capsule of VP3 and VP4 contains 5 μg and 10 μg vitamin D, respectively, which corresponds to 50-100% and 100-200% of the RDA.

Vitamin E is another fat-soluble vitamin, and this vitamin actually exists in several different chemical forms. However, α-tocopherol is the form that is preferentially found in the blood and the most studied form in clinical trials [51]. The best-known physiological properties of vitamin E include antioxidative effect, especially against polyunsaturated fatty acids, inhibition of inflammation and inhibition of protein kinase C. Vitamin E has also proven to increase the release of prostacyclin, thus promoting blood vessel dilatation and reducing platelet aggregation [48].

There is virtually no toxic hazard associated with excessive consumption of vitamin E, but large doses can cause gastrointestinal distress and give nausea, diarrhea and abdominal pain. It should also be used with caution when co-administrated together with anticoagulants such as vitamin K antagonists [52].

Vitamin E is usually obtained through dietary intake of nuts, seeds, vegetable oil, green leafy vegetables and fortified cereals [48]. The absorption of vitamin E varies a lot when administrated orally, ranging from 15-70%, and the RDA is 8-10 mg. One capsule of VP3 and VP4 contains 12 mg vitamin E, which is 120-150% of the RDA.

Figure 3 shows the chemical structure of all the three vitamins found in VitaePro: vitamin C, the vitamin D analogs and vitamin E.

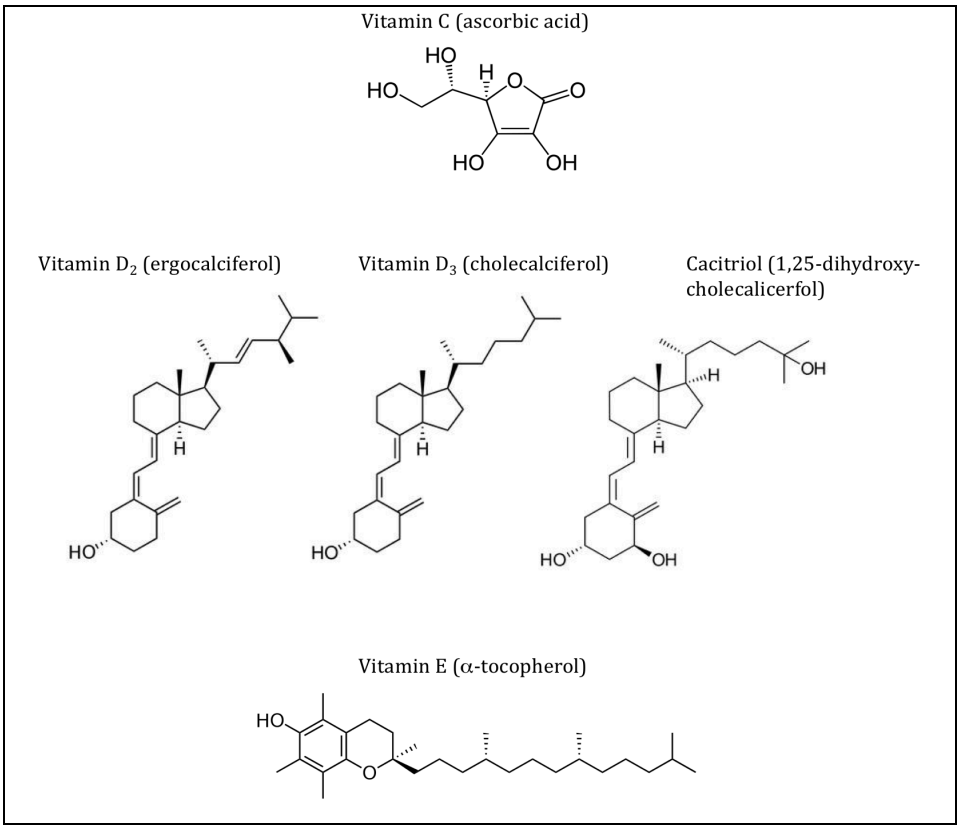

Figure 3: The vitamins in VitaePro and their chemical structure.

1.5 Omega-3

1.5.1 Omega-3 fatty acids in general

Omega-3 fatty acids are polyunsaturated fatty acids (PUFAs) with well-known effects against several inflammatory and degenerative diseases [53]. They are termed omega-3, eventually ω-3 or n-3, because they have a double bond at the third carbon atom in the hydrocarbon chain, and they exist as both long-chained and short-chained acids. The best known short omega-3 fatty acid, α-linolenic acid (ALA), comes from plants, while the more commonly recognized long omega-3 fatty acids mainly come from marine sources [54]. The group of long-chained omega-3 fatty acids (n-3 LC-PUFAs) is the most studied, and this group includes eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are the two most relevant n-3 LC-PUFAs in human physiology [54]. Figure 4 shows the chemical structure of EPA and DHA.

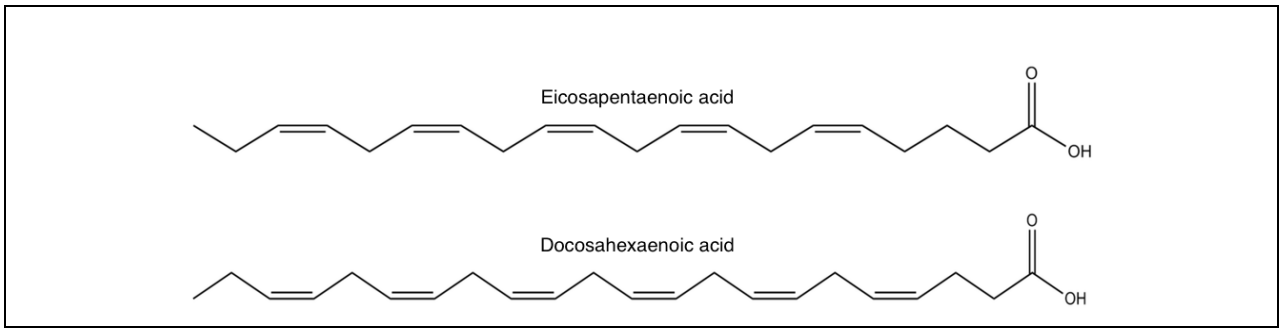

Figure 4: The chemical structure of eicosapentaenoic acid and docosahexaenoic acid.

There are several well-established health promoting effects of dietary n-3 LC-PUFAs, and EPA and DHA have been objects to extensive research and clinical trials. Many studies have evidenced important anti-inflammatory and immunomodulatory properties of EPA and DHA, and consumption of these fatty acids have been particularly associated with cardiovascular protection and decreased cardiovascular death [55]. The clinical effect appears to be caused by suppression of fatal arrhythmias, stabilization of atherosclerotic plaques, and improvement of cardiovascular risk factors. At doses of 3 g/d, EPA + DHA have shown to decrease plasma triacylglycerol levels, lower the blood pressure, prevent platelet aggregation and inflammation, and improve vascular reactivity [56].

Along with the effects on cardiovascular health, EPA and DHA have shown promising effects on many other chronic inflammatory diseases. Some data from clinical trials have suggested positive effects on inflammatory bowel disease (IBD), rheumatoid arthritis (RA), diabetic conditions and some types of cancer [53, 54]. The relationship between n-3 LC-PUFAs and neurodegenerative diseases and mental health has also been investigated, and it is well-known that DHA is present at higher concentrations in the brain than elsewhere in the body. Due to this fact, n-3 LC-PUFAs, particularly DHA, are thought to be essential for proper development and functioning of the brain [57]. A study conducted on mice in 2008 suggested an association between n-3 LC-PUFAs and delay in cognitive decline, while a meta-analytic review from 2007 found significant antidepressant efficacy of n-3 LC-PUFAs [57, 58].

All mammals, including humans, are unable to synthesize omega-3 fatty acids, and must rely on their diet to ensure sufficient supply of these vital compounds. However, humans have the ability

to convert approximately 5% of ingested short-chained ALA into EPA and DHA, but this conversion is considered too small to cover the total need of EPA and DHA. A diet rich in n-3 LC-PUFAs is therefore recommended [54]. In nature, ALA is found in several plants, while only plankton and cold water algae seem to synthesize EPA and DHA. Because these organisms are located at the bottom of the food chain, EPA and DHA are abundantly present in fish and fish oil. Fish and other marine organisms are therefore the main sources of n-3 LC- PUFAs in the human diet [56].

1.5.2 Krill and calamari as sources of omega-3

Krill are small shrimplike crustaceans found in all oceans of the world, yet more abundantly in the Arctic and Antarctic [59]. As consumers of phytoplanktons, krill is a rich source of marine omega-3 fatty acids [60]. It is particularly rich in EPA and DHA, but, in contrast to the corresponding n-3 LC-PUFAs in traditional fish oil, these fatty acids are mainly bound in phospholipids (PLs) instead of triacylglycerols (TAGs) [59, 61]. This distinction is of great importance both biologically and therapeutically, as PLs are the main components of human cell membranes. PL-bound n-3 LC-PUFAs seem to be better absorbed from the intestine than TAG-bound n-3 LC-PUFAs, and the structural similarity with the cell membrane is most likely the reason for this [62]. Based on the same reasoning, it is suggested that PL-bound n-3 LC-PUFAs also have a more potent effect in peripheral tissues. A study published in 2010 states that the clinical effect of krill oil is presumably the same as the effect of fish oil, but at lower doses of EPA and DHA [63]. The authors also reviewed three earlier conducted trials on newborns, and all of them concluded that fatty acids in dietary PLs might be better absorbed than those from TAGs [64-66]. Moreover, compared to fish oil, krill oil also contains high amounts of astaxanthin and choline [59, 63]. Astaxanthin is, as previously discussed, a strong antioxidant, while choline is a conditionally essential nutrient, as it is needed in the synthesis of neurotransmitters (acetylcholine). The commercial krill oil is extracted from the largest of the krill species, the Antarctic *Euphausia superba* [59].

Calamari is the culinary name for squid, and in recent years calamari oil has been recognized as another source of marine n-3 LC-PUFAs [67, 68]. The producers of calamari oil consider their

production as more sustainable and ecofriendly than the production of both fish and krill oil. This is because trimmings from squid food production, that otherwise would have been thrown away, are purified and refined to high-concentrated omega-3 oil [69]. Because the interest in calamari as a source of omega-3 is so recent, there has been little research in this field. When searching in the databases PubMed and Cochrane Library, combining the terms "calamari", "calamari oil" and "omega-3", no scientific articles were found (latest search done November 24th, 2015). However, despite the lacking of scientific articles and reviews, the producers, including Pharma Marine, states that the n-3 LC-PUFAs from calamari are superior to n-3 LC-PUFAs from fish and krill. The arguments are not only the sustainable production methods, but also the beneficial ratio between EPA and DHA in calamari oil. While most fish – and krill based omega-3 products happen to be richer in EPA, the dominant component in calamari oil is DHA [70]. Since DHA has proven to be the most abundant n-3 LC-PUFA in most tissues, including the brain, the retina and the myocardium, it is thought that DHA is more beneficial than EPA, and this is emphasized by both Pharma Marine and VitaeLab [54, 68, 70]. Regarding the bioavailability, the n-3 LC-PUFAs from calamari are not available bound to PLs, only to TAGs.

Neither the Norwegian Food Safety Authority nor the Norwegian Directorate of Health has quantified their recommendations of EPA and DHA. On the other hand, the Food and Agriculture Organization of the United Nations (FAO) and the European Food Safety Authority (EFSA) recommend a daily intake (RDA) of 250 mg EPA + DHA [71, 72].

As earlier emphasized, the absorption of n-3 LC- PUFAs depends on whether they are bound in PLs or TAGs. One capsule of VP2 contains 52 mg EPA + DHA, which is 21% of the RDA. One capsule of VP3 contains 173 mg EPA + DHA, which is 69% of the RDA. One capsule of VP4 contains 125 mg EPA + DHA, which is 50% of the RDA.

1.6 *Boswellia serrata*

1.6.1 Medicinal plants and herbal medicine in general

Throughout the ages, humans have relied on Nature for all their basic needs, including food, clothing and medicines. The beneficial properties of plants and herbs in preventing and treating

various illnesses have been recognized since ancient times, and have formed the basis of sophisticated traditional medicine systems. Today, molecules from natural products and their derivatives represent more than 50% of all drugs in clinical use.

Although some therapeutic properties attributed to plants have proven to be erroneous, plant and herbal medicine are based on the empirical discoveries through millennia, and will most likely continue to provide humans with new remedies in the future. Examples of potent and authorized drugs derived from plants are the opioid analgesic morphine from *Papaver somniferum*, the analgesic, anti-inflammatory and antiplatelet agent acetylsalicylic acid from *Salix* spp., the antiarrhythmic agent digoxin from *Digitalis* spp., and the antipsychotic and antihypertensive agent reserpine from *Rauwolfia* spp. [73, 74]

Despite the tremendous development in modern medicine, the World Health Organization (WHO) has stated that 80% of the world's population still relies on plant-derived medicines today. This is mainly people from developing countries who do not have the financial ability to buy modern medicines, but in recent years there has been an increasing interest in plant and herbal remedies also in Western countries [73]. As previously mentioned, many people erroneously associate natural products with something safe, but such remedies have repeatedly shown to cause adverse effects like any other medication. Another issue is undiscovered interactions, both between different herbal products, but also between herbs and prescribed medications. This is addressed in section 1.9 "Metabolic interactions".

1.6.2 *Boswellia serrata* extract

Frankincense, also called olibanum, is an aromatic resinous extract from the trees of the genus *Boswellia*. The Indian species *Boswellia serrata* has gained particular attention for its promising medicinal properties, and *Boswellia serrata* extract (BSE) has been object to both *in vitro*, preclinical, pharmacokinetic and clinical trials in recent years. The resin is obtained by making incisions in the trunk and branches of the tree and after collection and air-drying, the remaining resin consists of typically 30% boswellic acids [75].

There are many different compounds in BSE, and a study has reported the following: monoterpenes, diterpenes, triterpenes, pentacyclic triterpenic acids and tetracyclic triterpenic acids. It is the pentacyclic triterpenic acids, also called boswellic acids, that are the supposed bioactive components [76]. The major boswellic acids found in BSE are: 11-keto-β-boswellic acid (KBA), acetyl-11-keto-β-boswellic acid (AKBA), α-boswellic acid (αBA), acetyl-α-boswellic acid (AαBA), β-boswellic acid (βBA) and acetyl-β-boswellic acid (AβBA). BSE contains quite similar amounts of KBA and AKBA, and almost up to present, these components have been considered the active ingredients of *Boswellia serrata*. The anti-inflammatory effect has been attributed to KBA and AKBA, as they have shown to suppress leukotriene formation in several studies. However, results from recent studies have questioned this, as new data have revealed very low plasma concentrations following oral administration. β-boswellic acid (βBA), which is present at larger concentrations, and has proven to inhibit prostaglandin synthesis, is now the proposed bioactive component. Various pharmacological studies indicate that the β-configurated derivatives exert stronger bioactivities than the respective α-derivatives [75]. Figure 5 shows the chemical structure of the major boswellic acids found in BSE.

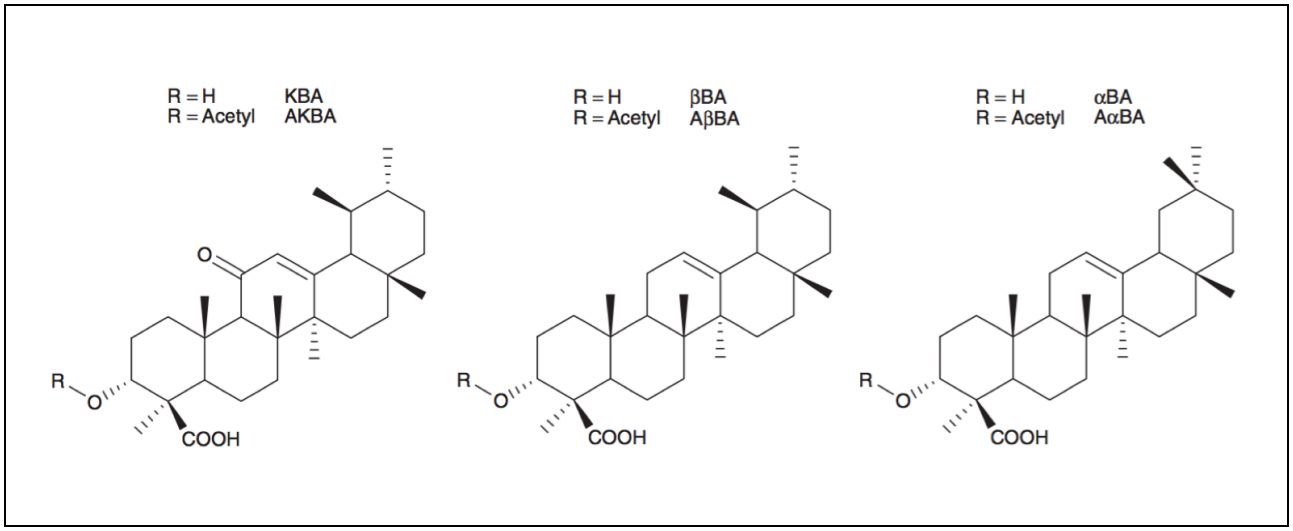

Figure 5: The major boswellic acids in Boswellia serrata extract, and their chemical structure.

Now that we have gained more knowledge on biochemical pathways of leukotrienes, prostaglandins and other pro-inflammatory cytokines, most scientists consider the statements about anti-inflammatory properties, provided by BSE, as reliable. It is also highly understandable

that frankincense from different *Boswellia* species have been used in African and Asian cultures since ancient time in the prevention and treatment of various inflammatory diseases. The obtained data from experiments with BSE suggests promising beneficial effects in the treatment of chronic inflammatory diseases like inflammatory bowel disease (IBD), rheumatoid arthritis (RA), osteoarthritis (OA) and asthma. Moreover, BSE has shown remarkable effects on reducing peritumoral brain oedema, and is classified as an orphan drug for treating this condition by the European Medicines Agency [75, 76]. Based on the current evidence, BSE might be regarded as a promising alternative to non-steroidal anti-inflammatory drugs (NSAIDs) in the future.

Regarding the bioavailability of BSE, sparse studies conclude that the plasma concentration of boswellic acids after oral administration varies a lot between subjects and are dependent on the pharmaceutical preparation and the conditions of intake. Among the factors influencing on the bioavailability, poor absorption and extensive metabolism may play an important role. Currently, there are no approved and authorized medicinal products of BSE, meaning that there is no official pharmacovigilance data on adverse effects. However, based on observations from clinical trials, adverse effects were found to be rarely occurring, not casually related to the treatment, and not significant different from those observed in the placebo group. The adverse effects were also of little severity, with nausea, abdominal pain and diarrhea being the only effects reported in more than one study [75].

Depending on the different commercial *Boswellia* products and the recommendations from various manufacturers, the daily intake of boswellic acid may vary from 18.5 mg to 109.5 mg [75]. One capsule of VP4 contains 100 mg BSE, and this amount is thus kept within the upper limit reported in a review article by Abdel-Tawab *et al*. In Norway, *Boswellia serrata* belongs to the group of herbs classified as food, but the Norwegian Food Safety Authority has not quantified their recommendations of BSE, neither has the Norwegian Directorate of Health [77].

1.7 Dietary trace minerals

1.7.1 Trace minerals in general

Trace minerals, or trace elements, are minerals that are required in amounts between 1 to 100 mg/day. Essential trace minerals in humans serve a wide variety of important biological functions. Chromium (Cr) functions as a coenzyme in many metabolic reactions. Copper (Cu) has diverse roles in the electron and oxygen transport, as well as being an important coenzyme. Fluoride (F^-) has an important role in preventing dental caries and it also appears to promote osteoblast activity, thereby increasing bone density. Some consider fluoride beneficial rather than essential. Iodine (I) is primarily important for the metabolism and homeostasis of the thyroid gland and its hormones. Iron (Fe) constitutes an important component of heme proteins such as hemoglobin (the oxygen transport protein), myoglobin (the oxygen storage protein), cytochrome P450, and peroxidases. Moreover, a small percentage of the iron presents as crucial metalloenzymes. Manganese (Mn) is present in manganese superoxide dismutase, arginase, glutamate synthetase and pyruvate carboxylase. The former enzyme has attracted particular attention because of its antioxidative effect. Zinc (Zn) interacts with many organic ligands, especially certain amino acids, and plays an important role in DNA metabolism and gene expression. Moreover, zinc is found in many proteins and crucial enzymes. Selenium (Se) serves many biological functions. It is incorporated in several proteins, and plays a particularly important role in the antioxidant defense [78].

1.7.2 Selenium

Selenium (Se) is a trace element of great importance for many regulatory and metabolic functions in animals and humans. Although selenium salts are toxic in large amounts, trace amounts of selenium are essential for many biological functions, including the antioxidant defense system, the adaptive and acquired immune system, the thyroid hormone metabolism, and the prevention of certain cancers. Recent studies have also suggested that selenium plays an important role for optimal functioning of the cardiovascular system [79, 80].

The biological effects of selenium are mainly mediated through incorporation of the chemical element into different proteins, selenoproteins. Selenium dependent enzymes are responsible for increasing antioxidant capacity, regulation of immune responses through proliferation and

differentiation of immune cells, and regulation of inflammation through influence on inflammatory signaling pathways [81]. Glutathione peroxidase (GPx) is considered the most important protein within the selenoproteins due to its ability to neutralize both ROS and reactive nitrogen species [82]. Another well known selenoprotein is thioredoxin reductase that regulates numerous intracellular redox processes, as well as DNA metabolism and repair [83]. Moreover, selenium functions as a cofactor for three of the four known types of thyroid hormone deiodinases, which are crucial to activate and deactivate thyroid hormones [84].

The selenium content in food and nutritional supplements exists in different chemical forms, including selenomethionine (Se-Met), selenocysteine, selenite, selenious acid and sodium selenite. The bioavailability varies among the different selenocompounds, but the Se-Met used in VP4 (shown in figure 6), is one of the most biological available forms. Organic forms of selenium are more frequently preferred than inorganic, because the risk of acute toxicity is lower [80]. Selenium deficiency is rare in healthy, well nourished individuals, but impaired immune response, skeletal muscle dysfunction, cardiomyopathy and Keshan disease are known consequences of chronic inadequate supply. Selenium toxicity is even more rare, but it is typically caused by chronic over-supplementations. Signs and symptoms of selenosis include brittle nails, hair loss, garlic odor from the breath, gastrointestinal disorders, fatigue, irritability and neurological affection. In the case of acute overdose, the symptoms are more unspecific such as vomiting, dizziness and pulmonary edema [80, 85].

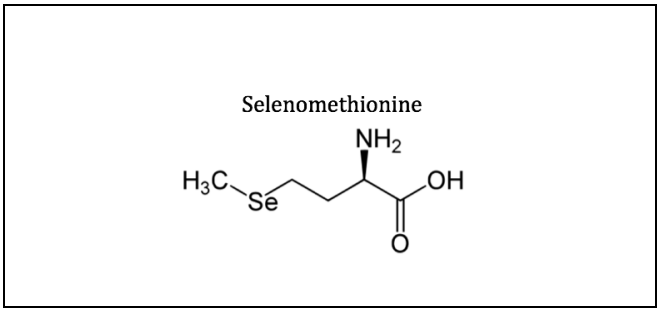

Figure 6: The chemical structure of the organic selenocompound, selenomethionine.

Seafood, kidney, liver and meat are rich in selenium. Plants also represent a dietary source, but the selenium content of grains and seeds varies a lot, and depends on the selenium content of the soil. Drinking water usually contains small amounts of selenium. As earlier emphasized, the

absorption of selenium following oral administration is deeply dependent on the type of selenocompound. The RDA of selenium are 55 μg, and one capsule of VP4 contains exactly 55 μg, which corresponds 100% of the RDA [85].

1.8 Cytochrome P450 metabolism

1.8.1 The cytochrome P450 system

The cytochrome P450 (CYP450) system comprises a superfamily of haem-containing isoenzymes responsible for the metabolism of a large number of endogenous and exogenous compounds [86]. The enzymes are believed to have originated from an ancestral gene for probably more than 3.5 billion years ago, and are now found in animals, plants, fungi and bacteria as well as in humans. The number "450" refers to the characteristic spectrophometric absorption peak at 450 nm that appears when these enzymes are bound and reduced by carbon monoxide. The genes encoding the enzymes, as well as the enzymes themselves, are designated with the abbreviation CYP. The classification system then divides the enzymes into families and subfamilies according to the gene sequence similarity. Enzymes with more than 40% identical gene sequences are placed in the same family, designated by an Arabic number (e.g. CYP3), and enzymes that exceed 55% equality are further placed in the same subfamily, designated by a capital letter (e.g. CYP3A). Finally, each individual enzyme is designated by another Arabic number (e.g. CYP3A4). Up to date, 18 families and 44 subfamilies of CYP enzymes have been recognized in humans [87].

Studies have revealed several endogenous functions of CYP enzymes in humans, including synthesis of steroid hormones, bile acids, arachidonic acid and prostaglandins [87]. However, the ability to metabolize endogenous and exogenous substances is the most investigated feature. The impact on medicinal drugs and other xenobiotics has been particularly studied, and despite the many CYP families identified, CYP1, CYP2 and CYP3 seem to be the most important regarding number of drugs metabolized by these families. Studies have suggested that CYP-enzymes account for approximately 90% of all drug metabolism in humans [88].

Drug metabolizing CYP enzymes have a distinct, but often overlapping substrate specificity. However, despite this overlap, many drugs and other xenobiotics are only metabolized by one or a few enzymes [89]. In humans, the distribution of different CYP families and subfamilies varies greatly among different tissues and organs. Although CYPs are predominantly present in the liver, they are also found in extrahepatic locations such as the small intestine, pancreas, brain, heart, lung, adrenal gland, kidney, bone marrow, skin, ovary and testis [87, 90].

Drug metabolism can be divided into two phases. In phase I, enzymes, such as the CYPs, contribute in making the substrates more hydrophilic. The CYPs are capable of catalyzing many types of reactions, including oxidation, reduction, hydrolysis, cyclization, decyclization and many more. However, monooxygenation is the predominating process, and CYP3A4 contributes particularly through hydroxylation of the substrates. The mechanism proceeds through reduction of cytochrome-bound oxygen, coupled with oxidation of NADPH. During the reaction molecular oxygen is activated and one atom is incorporated in the substrate while the other is converted to H_2O. The typical monooxygenation reaction can be summarized as follows: $NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + R\text{-}OH$, where R represents a lipophilic substrate, such as a steroid or a fatty acid. The oxidation exerted by CYP enzymes can result in either activation or inactivation of a drug, depending on whether the active substance is the substrate itself or the metabolite (prodrug) [87].

In phase II, other transferase enzymes, such as glutathione S-transferase, conjugate the substrates to polar compounds by binding them to larger hydrophilic compounds. Both phases facilitate urinary excretion as the compounds are getting more water-soluble. After these two phases, the metabolites may be further processed before getting pumped out of the cells by efflux transporters, and this processing are by many recognized a third phase of metabolism [91].

Because of genetic polymorphisms in CYP genes there is a great interindividual variability in the amount and efficiency of different CYP enzymes. The polymorphisms are caused by single nucleotide variability, and this variability explains why some people are poor or intermediate metabolizers, while others are classified as extensive or ultrarapid [92]. CYP2D6 is one of the best known examples of polymorphic enzymes, and studies have revealed that about 5-10% of

Caucasians metabolize CYP2D6 substrates markedly slower than the average population. This has proven to be clinical significant, for example in analgesic treatment with codeine. Codeine is converted to the active compound morphine by CYP2D6, and people who are poor metabolizer may obtain a lower concentration of morphine, and subsequently less analgesic effect, than those metabolizing codeine at a normal rate. On the other hand, extensive or ultrarapid metabolizers may obtain harmful doses of morphine [93]. Studies have also revealed that the polymorphisms are distributed differently in various ethnical groups. While 5-10% of Caucasians are poor metabolizers of CYP2D6-depend drugs, only less than 1% of Asians seem to metabolize CYP2D6 substrates at a decreased rate [87]. In summary: interindividual pharmacokinetic differences, provided by polymorphisms, can cause either treatment failure or toxic overdose in the opposite case. The growing knowledge concerning CYP enzyme polymorphisms should therefore be taken into account by physicians in order to optimize the treatment [87, 92].

1.8.2 CYP3A4

As already stated, the CYP1, CYP2 and CYP3 families are those responsible for the majority of all CYP-mediated reactions. However, the CYP3 family, which consists of only one subfamily - CYP3A, is the most dominant both in terms of hepatic expression and in terms of number of drugs metabolized [94]. Further, CYP3A4 has been revealed as the most abundant CYP enzyme found in both the liver and gastrointestinal tract [87, 95]. It is probably the most important contributor to drug metabolism, as it metabolizes about 30% of the clinically most common drugs [96]. In fact, CYP3A4 is believed to metabolize 50% or more of all marketed drugs [97]. It should be noted that some individuals polymorphically express CYP3A5, which metabolizes the same drugs as CYP3A4, thus contributing to these statistics [96].

As the major expressed CYP enzyme in both the intestinal wall and the liver, CYP3A4 contributes substantially to both first-pass metabolism of orally administrated drugs, and the systemic metabolism regardless form of administration. Figure 7 gives an overview of the most clinical relevant CYP enzymes, and their contribution to drug metabolism [96].

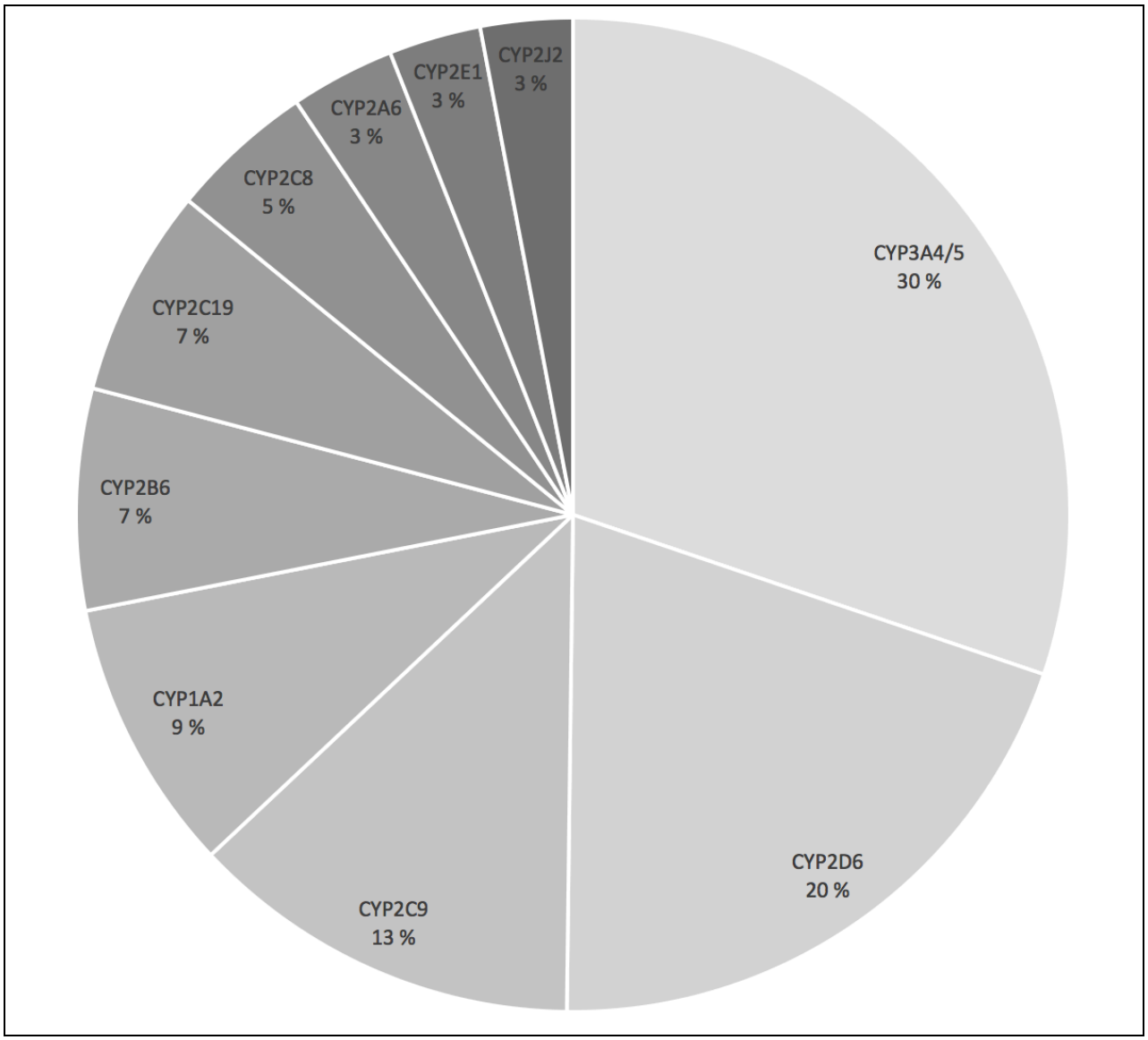

Figure 7: Fraction of clinically used drugs metabolized by cytochrome P450 isoenzymes (slightly simplified from Zanger and Schwab, 2013).

The extensive list of CYP3A4-metabolized drugs is overwhelming, and still growing. The enzyme is responsible for drug metabolism in several classes of drugs, including analgesics, antiarrhythmics, antibacterial agents, anticonvulsants, antidepressants, antihypertensive drugs, benzodiazepines, calcium channel blockers, chemotherapeutic agents, H_1 antihistamines, HMG CoA reductase inhibitors, immunosuppressive drugs, protease inhibitors and many others [98]. Table 3 shows some of the most common drugs metabolized by CYP3A4.

Table 3: Some of the most common drugs metabolized by CYP3A4 (modified and extended from Shapiro and Shear, 2002).

Analgesics	Benzodiazepines	HMG CoA reductase
Codeine	Alprazolam	inhibitors
Fentanyl	Diazepam	Atorvastatin
Lidocaine	Midazolam	Cerivastatin
Lignocaine	Triazolam	Lovastatin
Paracetamol	Calcium channel blockers	Simvastatin
Ropivacain	Amlodipine	Immunosuppressive drugs
Antiarrhythmics	Diltiazem	Corticosteroids
Amiodarone	Felodipine	Ciclosporin
Digoxin	Isradipine	Dapsone
Propafenone	Nifedipine	Tacrolimus
Quinidine	Verapamil	Protease inhibitors
Antibacterial agents	Chemotherapeutic agents	Indinavir
Erythromycin	Busulfan	Nelfinavir
Rifampicin	Cyclophosphamide	Ritonavir
Anticonvulsants	Docetaxel	Saquinavir
Carbamazepine	Doxorubicin	Miscellaneous
Ethosuximide	Etoposide	Bexarotene
Antidepressants	Ifosfamide	Estrogens
Amitriptyline	Imatinib	Flutamide
Doxepin	Paclitaxel	Pimozide
Imipramine	Tamoxifen	Omeprazole
Sertraline	Vinblastine	Oral contraceptives
Antihypertensive drugs	Vincristine	Retinoic acid
Enalapril	H_1 antihistamines	Sildenafil
Losartan	Fexofenadine	Theophylline
	Loratidine	Warfarin (R)
		Zileuton

As shown in table 3, CYP3A4 is responsible for the metabolism of several drugs used together in anesthesia: opioids (e.g. codeine and fentanyl), benzodiazepines (e.g. diazepam and midazolam) and local anesthetics (e.g. lignocaine, ropivacaine). Thus, individual variation in clearance of these commonly co-administrated agents, and interactions (discussed below), may cause unforeseen and unfortunate events in surgery or other situations where adequate anesthesia is required [87]. A study conducted back in 1997 reported that the systemic clearance of midazolam was decreased by 30%, and the $T_{1/2}$ was prolonged by 49%, in surgically patients after co-administration with fentanyl [99].

Moreover, in addition to the anesthetics there are many drugs listed in table 3 that can have severe consequences in case of unintended plasma concentrations. Both too low and too high concentrations of antiarrhythmics can cause fatal arrhythmias, while insufficient concentrations of antibacterial or antiretroviral agents can cause treatment failure and development of microbial resistance. For cancer patients receiving cytostatic treatment, insufficient concentrations of chemotherapeutic agents can cause fatal therapeutic failure, while too high concentrations can be severely toxic and cause unacceptable adverse drug reactions (ADRs). Interactions between different drugs, or interactions between drugs and other remedies, are some of the main issues in pharmacology, and will be addressed in the next chapter.

1.9 Metabolic interactions

1.9.1 Drug-drug interactions and interactions between drugs and dietary substances

Many substances are able to induce or inhibit CYP enzyme activity and thereby give rise to metabolic interactions. The alteration in drug metabolism can affect the concentration of CYP-metabolized drugs, and thus be of clinical significance [100]. The inducer or inhibitor can be either another drug, giving rise to drug-drug interactions, or it can be another kind of xenobiotic, or even a dietary product [101]. Herb-drug interactions have attracted particularly attention the last decades and are now widely documented [102, 103].

When multiple drug therapies are prescribed, drug interactions become an important consideration for patients and physicians, and studies have shown that the likelihood of ADRs

increases exponentially with each drug co-administrated [104]. Drugs with a narrow therapeutic range are especially vulnerable to interactions, and the anticoagulant warfarin is probably one of the best known examples in this regard. The far most biological active form of warfarin, s-warfarin, is metabolized by CYP2C9 only, and inhibitors of CYP2C9 have repeatedly proven to increase the plasma concentration of warfarin. Broad-spectrum antibiotics, NSAIDs, salicylic acid, other antiplatelet drugs, cimetidine, phenytoin, oral antidiabetics, and many other commonly prescribed drugs have proven to increase warfarin concentrations and subsequently the risk of bleeding [105]. Other examples of clinical significant interactions range from severe rhabdomylysis caused by co-administration of statins and macrolide antibiotics (CYP3A4) to symptoms of Parkinsonism caused by co-administration of risperidone and fluoxetine (CYP2D6) [106, 107]. During the past decades it has gotten increasingly clear that interactions can occur virtually in all aspects of pharmacology, involving all kinds of drug, and resulting in a correspondingly wide variety of clinical outcomes. However, there are some vulnerability factors particularly predisposing for interactions. Along with the number of co-administrated drugs, ages of 60 or older seem to be associated with clinical relevant drug interactions [98]. Table 4 shows observed patient risk factors for drug interactions.

Table 4: Patient risk factors for drug interactions (modified from Shapiro and Shear, 2002)

Multiple medications	Pharmacogenetic risk factors	Endocrine risk factors
Medicines ≥ 3	Slow acetylator	Hypothyroidism
Age	phenotype	Other medical issues
Age > 60	Other genetic	Hypothermia
Major organ dysfunction	polymorphisms	Hypotension
Liver dysfunction	Metabolic risk factors	Dehydration
Renal dysfunction	Obesity	
Congestive heart	Hypoproteinemia	
failure		

As briefly mentioned, there has been a growing awareness of drug interactions caused by herbal remedies and nutritional substances during the past decades. Several herbs have proven to alter CYP enzyme activity, both *in vitro* and *in vivo*, and induction of CYP3A4 by St. John's wort

(*Hypericum perforatum*, SJW) is to date probably the best known herb-drug interaction [108, 109]. Examples of well-documented herb-drug interactions, regarding CYP3A4, are the ability of SJW to reduce the concentration of oral contraceptives (induction of CYP3A4, thereby increasing the ethinyl estradiol-norethindrone metabolism) [110]; the ability of SJW to reduce the concentration of ciclosporin (induction of CYP3A4, thereby increasing the ciclosporin metabolism) [111]; and the ability of grape fruit juice to increase the concentration of ciclosporin (specific inhibition of intestinal CYP3A4, thereby decreasing the the first pass metabolism of ciclosporin) [112].

The four main topics within pharmacokinetics are absorption, distribution, metabolism and excretion [98]. Having discussed the significance of CYP-mediated metabolism, it is also important to be aware of the contribution of intestinal P-glycoprotein (P-gp) in the absorption. P-gp is an ATP-dependent plasma membrane glycoprotein encoded by the *multidrug resistance gene 1 (MDR1)*. The protein is abundantly found in the mucosal epithelial lining of the small intestine, and is capable of pumping a wide variety of substances back into the intestinal lumen, thereby preventing absorption. In other words, intestinal P-gp is an important efflux transporter and has proven to play a significant role in the first-pass elimination of certain drugs. Intestinal P-gp is thought to be a "gatekeeper" for xenobiotics being absorbed, and P-gp shares substrate specificity with the many of the CYP enzymes [98]. Moreover, P-gp and CYP enzymes also share many of the same inducers and inhibitors, and this can be of clinical significance. The above-mentioned case where SJW lead to decreased concentrations of ciclosporin was an example of this. SJW is an inducer of both P-gp and CYP3A4, causing ciclosporin to be pumped more effectively back to the intestinal lumen as well as causing CYP3A4 to metabolize the remaining ciclosporin at a higher rate [111].

1.9.2 Interactions and VitaePro?

In the period 2002-2009, before former NTNU student Martha Vorkinn conducted the first inhibition study on VitaePro and CYP3A4, 11 cases of possible ADRs or interactions had already been reported to the Regional Drug Information Centers (RELIS) in Norway. In two of the cases elevated prothrombin time – international normalized ratio (PT-INR) was reported in patients treated with warfarin after initiation of VitaePro. A third case was a patient treated with the

antiepileptic drug carbamazepine, who experienced increased seizure frequency after initiation of VitaePro. The seizure frequency decreased after discontinuation, and serum samples showed that the concentration of carbamazepine had been lower than expected during co-administration of VitaePro. All the reported cases in this period were with VP1 [39, 113]. The two incidences of elevated PT-INR could theoretically be explained by inhibition of CYP2C9, as it metabolizes s-warfarin. The increased seizure frequency, on the other hand, was more likely caused by CYP3A4 inhibition, as carbamazepine is a known CYP3A4 substrate.

At present a total number of 14 incidences have been reported to RELIS where VitaePro is suspected to have triggered an ADR or interacted with a drug [113]. One of the more recent cases was a hemodialysis patient with kidney failure who got hyperkalemia after initiation with VitaePro. The potassium levels reached 7 mmol/L, but normalized after discontinuation. No other medical or dietary changes were done during the same period. The case of hyperkalemia was most likely with VP3 [113, 114]. RELIS state that no evidence in the literature have been found to explain the hyperkalemia.

Other adverse event reports have referred suspected adverse reactions with rash, itching, obstipation, insomnia, irritable excitation, restlessness, worsening of joint pains, dizziness, nausea, discomfort, syncopal attack, bleeding gastric ulcers, elevated liver enzymes, decreased INR, chills and hot flashes [113]. A complete summary of all reported ADRs and interactions related to VitaePro is shown in section 8.3 in the appendices. The information presented in section 8.3, as well as here, was obtained through personal communication with Aa, cand. pharm. at RELIS, and is presented with permission from the Norwegian Medicines Agency.
RELIS state that the effect, risk and interaction profile of VitaePro still is insufficiently investigated, and emphasize that all reports are on suspected ADRs and interactions, not definitively proven [113]. Today, there is not enough available documentation that can explain whether VitaePro caused the symptoms or not. However, the lack of evidence does not exclude causality, and finally, it is also important to remember pharmacodynamic mechanisms when evaluating ADRs.

Up to date it seems like there has been only one earlier study on VitaePro and CYP3A4 metabolism, the *in vitro* interaction experiment conducted by Vorkinn in 2010. As mentioned in the summary, this study investigated the inhibitory effect on CYP3A4 provided by VP1 and VP2. Nevertheless, the experiments revealed that both VP1 and VP2 were inhibitors of CYP3A4 *in vitro*, but to different extent; VP1 being a significantly more potent inhibitor than VP2. Vorkinn estimated the IC_{50}-values to 25.97±3.92 mg/mL and 162.44±27.07 mg/mL for VP1 and VP2, respectively, and the 95% confidence intervals showed significant difference in inhibitory potency between these generations [39].

2 AIMS OF THE THESIS

VitaePro is a high-selling nutritional supplement, while CYP3A4 is the predominant CYP enzyme in humans. CYP3A4 is dominant both in terms of expression in the liver and gastrointestinal tract, but also in terms of number of common drugs metabolized by this enzyme [87, 95]. With this in mind, it is of great interest to investigate whether VitaePro exerts any inhibitory effect on CYP3A4. There are good reasons to believe that a considerable proportion of the population uses VitaePro along with prescribed medicines, and possible interactions have already been reported [115].

The following questions are explored in this student thesis:

- Does VitaePro exert an inhibitory effect on the CYP3A4 enzyme activity *in vitro*, here demonstrated by affected conversion of testosterone to 6-β-OH-testosterone?
- Given the findings of inhibition, is there a significant difference in inhibitory potency between the third and fourth generation product of VitaePro?
- In case of a significant difference in inhibitory potency, could this difference be caused by different contents in the two VitaePro products?
- Is it theoretically possible that the CYP3A4 inhibition provided by VitaePro could be of significance for patients using VitaePro together with CYP3A4 metabolized drugs?

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Monopotassium dihydrogen phosphate (KH_2PO_4; batch no. A673073) and dipotassium monohydrogen phosphate (K_2HPO_4; batch no. A878304) were both purchased from Merck, Darmstadt, Germany. Methanol (CH_3OH, chromatographic grade; ord. no. C17C11X, batch no. 1408507) was purchased from Fisher-Scientific, Loughborough, UK. Ethanol (C_2H_5OH; art. no. 600051, batch no. 0607046829) was purchased from Arcus Kjemi, Vestby, Norway. Water was prepared by a Milli-Q® Advantage A-10 Water Purification System purchased from Mitron O. R., Melhus, Norway.

3.1.2 Enzymes and cofactors

Human CYP3A4 SUPERSOMES™ (192 pmol/mg protein; cat. no. 456202, lot no. 2116771), containing cytochrome b_5 (790 pmol/mg protein) and cDNA-expressed human P450 reductase, was purchased from BD Biosciences, Woburn, MA, USA. The CYP3A4 SUPERSOMES™ was expressed from human CYP3A4 cDNA prepared from a baculovirus-infected insect cell system. The solution was stored at -80℃.

The NADPH regenerating system was obtained from BD Biosciences, Woburn, MA, USA. The system consisted of two solutions, solution A (31 mM $NADP^+$, 66 mM glucose-6-phosphate, and 66 mM $MgCl_2$ solved in H_2O; cat. no. 451220, lot no. 2180641) and solution B (40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate; cat. no. 451200, lot no. 56568). Both solutions were stored at -20℃.

3.1.3 Substrates, metabolites and inhibitors

Testosterone ($C_{19}H_{28}O_2$, purity ≥99 %; cat. no. 86500, lot no. 1166233, CAS. no. 58-22-0) was obtained from Fluka, while 6-β-OH-testosterone ($C_{19}H_{28}O_3$, purity ≥ 97 %; ref. no. 132404, TB0519, CAS. no. 62-99-7) and ketoconazole ($C_{26}H_{28}O_{12}N_4O_4$, purity ≥98%; cat. no. K186000, lot no. TKC-30030112, CAS. no. 65277-42-1) were obtained from Sigma-Aldrich.

3.1.4 VitaePro

VitaePro® was generously donated from VitaeLab AS (Enebakkveien 117, 0680 Oslo). Both the third generation product (batch no. 30015735) and the fourth generation product (batch no. 30015879) were delivered directly from the manufacturer.

The recommended daily dose of VP3 is 1-2 capsules (à 914 mg) administrated together with food, and the recommended daily dose of VP4 is 1-2 capsules (à 918 mg) administrated together with food.

3.1.5 Composition of the HPLC system

The HPLC system used in this experiment was an Agilent 1200 series liquid chromatography portfolio consisting of a quaternary pump (G1311A), a degasser (G1322A), a well-plate handler and an auto sampler (G1367C), a Waters Eclipse XBD-C18 5 µm 4.6 x 150 mm column and a UV detector (G1365D) for detecting multiple wavelengths. The system was purchased from Matriks AS, kjemisk teknologi, Oslo, Norway.

3.2 Methods: Preparing, handling and stability of experimental solutions

3.2.1 KPO-buffer

A 0.1 M potassium phosphate buffer (KPO-buffer) was made by adding approximately 50 mL 0.5 M KH_2PO_4 to 200 mL 0.5 M K_2HPO_4 adjusting the pH to 7.4. The 0.5 M KPO-buffer was further diluted to the final 0.1 M KPO-buffer by adding deionized water. The KPO-buffer was adjusted to pH 7.4 using 0.1 M KH_2PO_4 and 0.1 M K_2HPO_4. The final buffer was stored at 4℃ for maximum 4 weeks.

3.2.2 CYP3A4 and NADPH

50 µL CYP3A4 SUPERSOMES™ were thawed and diluted with 950 µL KPO-buffer shortly before the incubation to make a 50 nM CYP3A4 solution.

Both solutions A and B from the NADPH regenerating system were thawed shortly before the incubation. 140 µL of solution B was diluted by adding 560 µL KPO-buffer. This resulted in 700 µL 8 U/ml solution B. Approximately 700 µL of solution A was thawed without any modifications. Both the CYP3A4 solution and the two NADPH solutions were kept on ice until shortly before the incubation assay was initiated.

3.2.3 Testosterone and 6-β-OH-testosterone

A 4.0 mM testosterone stock solution was dissolved in 39% acetonitrile and stored at 4℃. Shortly before the initiating of the incubation assay, 1600 µL KPO-buffer was added to 400 µL of the 4.0 mM testosterone stock, which resulted in a 0.8 mM testosterone working solution.

The 1.0 mM 6-β-OH-testosterone (6-OH-T) stock solution was diluted with 50% MeOH into 7 non-zero working standard solutions (WS-STDs), ranging from 312.5 nM to 100000 nM. A separate 1.0 mM 6-OH-T was used to make 4 quality control working solutions (WS-QCs) with the following concentrations: 312.5 nM, 1000 nM, 18000 nM and 40000 nM. All working solutions were diluted 1:5 before HPLC analysis, as shown in table 11 and 12 in section 8.1.1 in the appendices.

3.2.4 Ketoconazole

Different concentrations of ketoconazole (KTZ) was incubated together with CYP3A4 in a separate inhibition experiment, in order to find the KTZ IC_{50}-value. This KTZ concentration was then used in the VitaePro experiments as a positive inhibitory control (PIC).

A 1.0 mM KTZ stock solution was diluted into 7 working solutions with concentrations ranging from 0.20 µM to 20.00 µM. Table 13 in section 8.1.2 in the appendices shows how the KTZ working solutions were made along with the final KTZ concentrations in the incubations. Note that the MeOH content in all the solutions was adjusted to the same level.

The IC_{50}-value of KTZ was determined to 0.34±0.06 µM, and because of the 1:4 dilution during the incubation procedure, a 4 times more concentrated solution was prepared. Because the

VitaePro content from VP3 and VP4 were dissolved with different amounts of ethanol (EtOH), it was necessary to make two PIC solutions; each of them being adjusted for the EtOH content in VP3 and VP4, respectively. Table 14 and table 15 in section 8.1.2 in the appendices show how the two PIC solutions were prepared.

3.2.5 VitaePro

Three VitaePro capsules from VP3 were weighted separately. The contents of the capsules were removed using a syringe (1 mL) and a needle, and the VitaePro-liquid was transferred from the capsules to a plastic tube. The empty capsules were reweighted in order to calculate the weight of the removed liquid. The same procedure was done with 3 capsules from VP4. The 2 plastic tubes, containing liquid from VP3 and VP4, respectively, were centrifuged at 1000 rpm for 2 minutes, and the volume in each tube was determined. Based on these measurements, a "concentration of VitaePro" was estimated (see table 6 in section 4.1).

VitaePro was then diluted 1:2 with 96% EtOH. The VitaePro-EtOH-solution was used to make 9 working solutions with VitaePro concentrations ranging from 0.04 mg/ml to 40 mg/ml. This is shown in table 16 in section 8.1.3 in the appendices together with the final incubation concentrations.

3.2.6 Incubation assay

50 µL 0.8 mM testosterone were added to the test tube, together with 20 µL 8 U/ml NADPH B, 50 µL 0.1 M KPO-buffer, 160 µL 50 nM CYP3A4, 20 µL NADPH A and 100 µL VitaePro in varying concentrations (0 mg/ml in the reference sample). All these experiments were performed in triplicates, and in addition each assay also included incubations (one of each) without CYP3A4 (00), with ketoconazole (KTZ) and without testosterone (Ctr. 9).

All the solutions, except NADPH A, were added to the test tubes, and pre-incubated for 5 minutes in a shaking water bath at 37℃. After 5 minutes of pre-incubation, the reaction was initiated by adding 20 µL NADPH A, as a "start"-solution, to the test tubes. After 10 minutes of incubation, 200 µL ice-cold 100 % MeOH were added to the tubes, to stop the reaction, and they

were consecutively placed on ice. Both before adding the "start"-solution and after adding the "stop"-solution, the contents in the tubes were blended adequately using a vortexer. Table 5 gives an overview of the different solutions in the test tubes.

Table 5: Overview of the different constituents in all the test tubes before and during incubation.

No.	Testosterone	NADPH B	VitaePro	KTZ	KPO-buffer	CYP3A4	NADPH A
00	50 µL	20 µL	100 µL WS9	---	210 µL	---	20 µL
Ref.	50 µL	20 µL	100 µL WS0	---	50 µL	160 µL	20 µL
1	50 µL	20 µL	100 µL WS1	---	50 µL	160 µL	20 µL
2	50 µL	20 µL	100 µL WS2	---	50 µL	160 µL	20 µL
3	50 µL	20 µL	100 µL WS3	---	50 µL	160 µL	20 µL
4	50 µL	20 µL	100 µL WS4	---	50 µL	160 µL	20 µL
5	50 µL	20 µL	100 µL WS5	---	50 µL	160 µL	20 µL
6	50 µL	20 µL	100 µL WS6	---	50 µL	160 µL	20 µL
7	50 µL	20 µL	100 µL WS7	---	50 µL	160 µL	20 µL
8	50 µL	20 µL	100 µL WS8	---	50 µL	160 µL	20 µL
9	50 µL	20 µL	100 µL WS9	---	50 µL	160 µL	20 µL
KTZ	50 µL	20 µL	---	100 µL	50 µL	160 µL	20 µL
Ctr. 9		20 µL	100 µL WS9	---	100 µL	160 µL	20 µL

The test tubes were centrifuged at 3500 rpm for 12 minutes, and the supernatants, approximately 100 µL, were pipetted to HPLC vials for quantification of 6-OH-T.

3.2.7 HPLC-analysis of 6-β-OH-testosterone

A validated HPLC-method, slightly modified from the one described by Zhao *et al.* (2012), was used for quantification of 6-OH-T [116].

40 µL of the sample were injected to the column and eluted in a mobile phase consisting of 50% MeOH and 50% water. The flow rate was 1.0 mL/min. After 7.0 min the MeOH concentration in

the mobile phase was gradually increased over the next 0.5 min until reaching a concentration of 80% at 7.5 min. This concentration of MeOH was then maintained for the next 5.5 min in order to elute the testosterone in the samples. At 13 min the MeOH concentration was decreased back to 50%, and continued for the remaining 3.0 min. Total runtime was 16 min. Peak areas of 6-OH-T were detected using UV-light at 240 nm. The retention time for 6-OH-T was 7.8 min and the lower limit of quantification (LLOQ) was 62.5 nM.

A slightly less advanced and shorter method was used to analyze the STD and QC samples: 40 µL of the sample were injected to the column and eluted in a mobile phase consisting of 50% MeOH and 50% water. The flow rate was 1.0 mL/min with a total runtime of 9 min.

3.2.8 Standard curves and quality controls

When an incubation series was performed, 7 standard solutions (STDs), with known concentrations ranging from 62.5 nM to 20000 nM, were analyzed with HPLC for each experiment. The measured AUCs (area under curve) from these STDs were plotted against the concentrations, and a linear standard curve was adapted using linear regression to estimate the unknown concentrations of formed 6-OH-T.

In addition to the STDs, 3 quality control solutions (QCs), with the concentrations 200 nM, 3600 nM and 8000 nM, were used. The QCs were analyzed in duplicates, and these results provided the basis for accepting or rejecting the analytical series. The QC concentrations were back calculated, using the corresponding standard curve equation, and compared with the nominal values in order to estimate accuracy and precision. Neither the STD samples, nor the QC samples, were incubated, but analyzed directly using the HPLC-method.

3.3 Analytical acceptance criteria

The validity of the analytical method in this study was assured by intra-run and inter-run validations as described by international standards [117]. Pre-run validation was considered unnecessary as the current method had been explored and validated before [118].

Intra-run validation was performed by making a standard curve and analyze QCs for each of the incubation series, as described in section 3.2.8. The acceptance criteria for each individual run were as follows: a regression coefficient $R^2 \geq 0.995$, and at least four out of six back calculated QC concentrations within an accuracy of 85%-115% from the nominal value, given that the two deviating values were from different concentrations.

Inter-run validation was assessed using the accumulated data of all standard curves and QCs for all experiments. The acceptance criteria for inter-run validation also required at least four out of six back calculated QC concentrations within an inter-run accuracy of 85%-115% from the nominal value, given that the two deviating values were from different concentrations. Further, an inter-run precision of both STDs and QCs was required within 15% CV (coefficient of variation) for acceptance.

3.4 Metabolic acceptance criteria

In addition to the acceptance criteria concerning the STDs and QCs, the basic CYP3A4 activity (reference) was required to have an inter-run variation of less than 20%. Further, the ketoconazole solutions, used as positive inhibitory controls (PICs), were also required to have an inter-run variation of less than 20%. Finally, when analyzing the chromatograms, the peaks were only accepted if they had uniform configurations.

3.5 Calculations and statistics

The raw data from all STDs and QCs in this experiment consisted of peak areas (AUC) obtained from HPLC-analysis. Calculations were performed in Excel (Microsoft) and SigmaPlot 12.5 (Systat Software 2013) was used for graphs and regressions. All linear regression curves were expressed as $y = ax + b$. The back calculated values of STDs and QCs were used to estimate accuracy and precision. For IC_{50} curves, best-of-fit non-linear regression was used.

After doing two incubation experiments with VP3 and two incubation experiments with VP4, the 95% confidence intervals of the IC_{50}-values were calculated in Excel, based on the three incubation parallels in each experiment. Statistical significance was set a priori to 0.05.

The enzyme activity is given as pmol formed metabolite per pmol enzyme per minute, where the formed metabolite is the mean measured concentration from three incubation parallels. The basic activity of CYP3A4 was estimated in the presence of 0.92% EtOH and 0.82% EtOH in the VP3 and VP4 incubations, respectively, and based on two experiments (à three parallels) for each generation. In contrast, the basic activity of CYP3A4 in the ketoconazole experiment was estimated in the presence of 0.25% MeOH, and based on only one single experiment (à three parallels).

4 Results

4.1 Measurements and calculations of "VitaePro concentrations"

In order to determine the most exact "concentration of VitaePro", it was necessary to measure the weight and volume of the capsule content as accurately as possible, the results are presented here in table 6.

Table 6: Measurements and calculations of "VitaePro concentrations".

VP3	Weight before emptying (mg)	Weight after emptying (mg)	Weight of VP liquid (mg)	Volume (mL)	Concentration (mg/mL)
Capsule 1	917.7	325.7	591.9	0.57	1044.6
Capsule 2	901.4	291.9	609.5	0.57	1075.6
Capsule 3	923.3	346.7	576.6	0.57	1017.5
Average	914.1 ± 11.4	321.4 ± 27.7	592.7 ± 16.5	0.57	1045.9 ± 29.1
VP4	Weight before emptying (mg)	Weight after emptying (mg)	Weight of VP liquid (mg)	Volume (mL)	Concentration (mg/mL)
Capsule 1	918.1	298.5	619.7	0.53	1161.9
Capsule 2	912.9	290.0	622.9	0.53	1167.9
Capsule 3	922.5	290.6	631.9	0.53	1184.8
Average	917.9 ± 4.8	293.0 ± 4.7	624.8 ± 6.3	0.53	1171.5 ± 11.9

4.2. Validation of standard curves and quality controls

4.2.1 Ketoconazole experiment

In the separate ketoconazole experiment, conducted before the VitaePro inhibition experiments, all QCs were within acceptable limits from the standard curve, and the acceptance criteria regarding accuracy were complied. The standard curve is shown in figure 8, and the QC data from this experiment are presented in table 7.

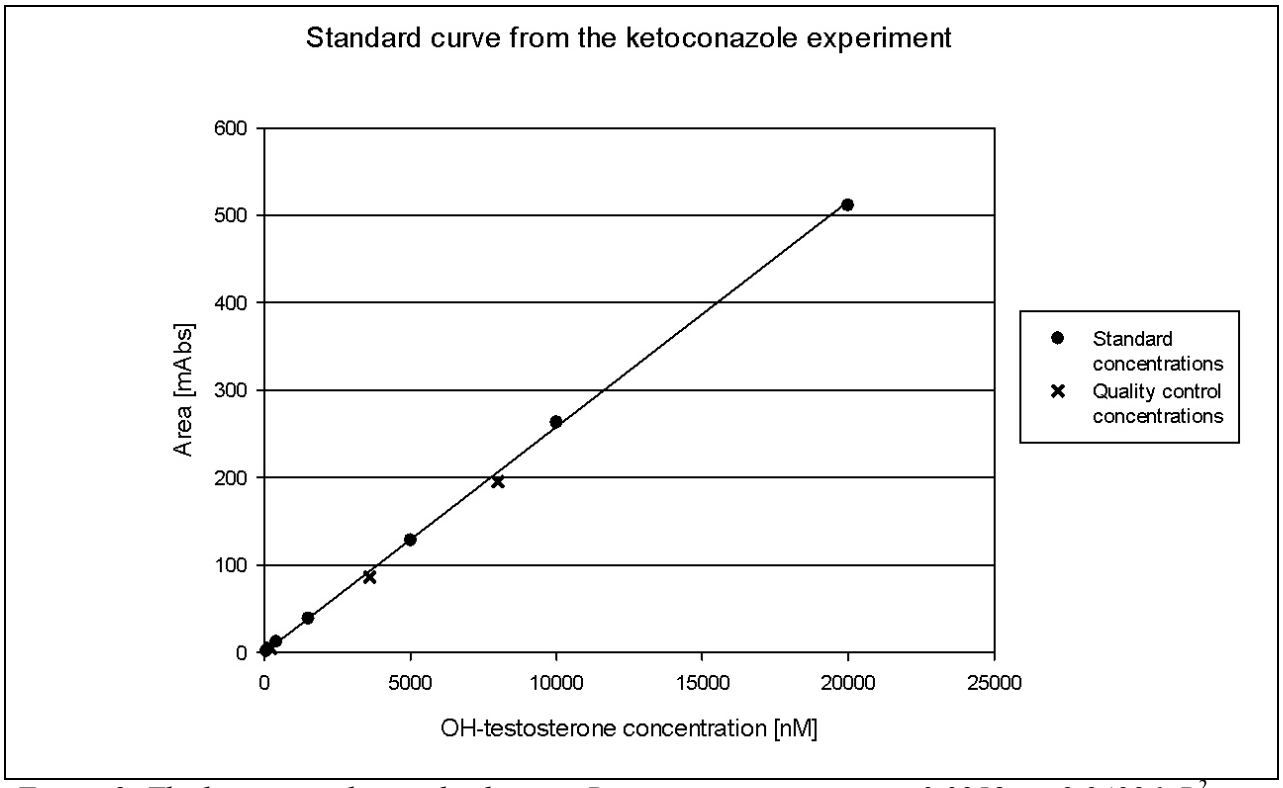

Figure 8: The ketoconazole standard curve. Regression equation: y = 0.0258x + 0.04326, R^2-value: 0.9995.

Table 7: Data from the ketoconazole experiment showing the accuracy for all QCs.

QC	Nominal concentration [nM]	Calculated concentration [nM]	Accuracy (%)
1	200	188.7	94.4
1	200	177.0	88.5
2	3600	3316.6	92.1
2	3600	3312.7	92.0
3	8000	7564.6	94.6
3	8000	7560.8	94.5

4.2.2 Intra-run validation

The intra-run validation revealed that all 4 individual VitaePro experiments (2 x VP3 and 2 x VP4) had QCs within acceptable limits from the corresponding standard curves, and the earlier described acceptance criteria (section 3.3) regarding QC accuracy were complied.

The standard curves from all 4 runs are shown in figures 20-23 in section 8.2.1-8.2.4 in the appendices, while the corresponding QC accuracies are presented in tables 17-20 in the same sections. Note that the variation between two duplicated QCs is to small for the individual points to be distinguished.

4.2.3 Inter-run validation

As described in section 3.3, the inter-run validation was assured by looking at the accumulated data of all standard curves and QCs throughout the entire experiment, meaning the accumulated data from the 4 VitaePro experiments. The inter-run QC accuracy requirement was complied, and so were the inter-run precision requirement of both STDs and QCs.

Figure 9 shows the standard curve based on accumulated data from all 4 VitaePro experiments. Table 8 presents the calculated precision of all the STDs, and table 9 presents the calculated accuracy and precision of all the QCs.

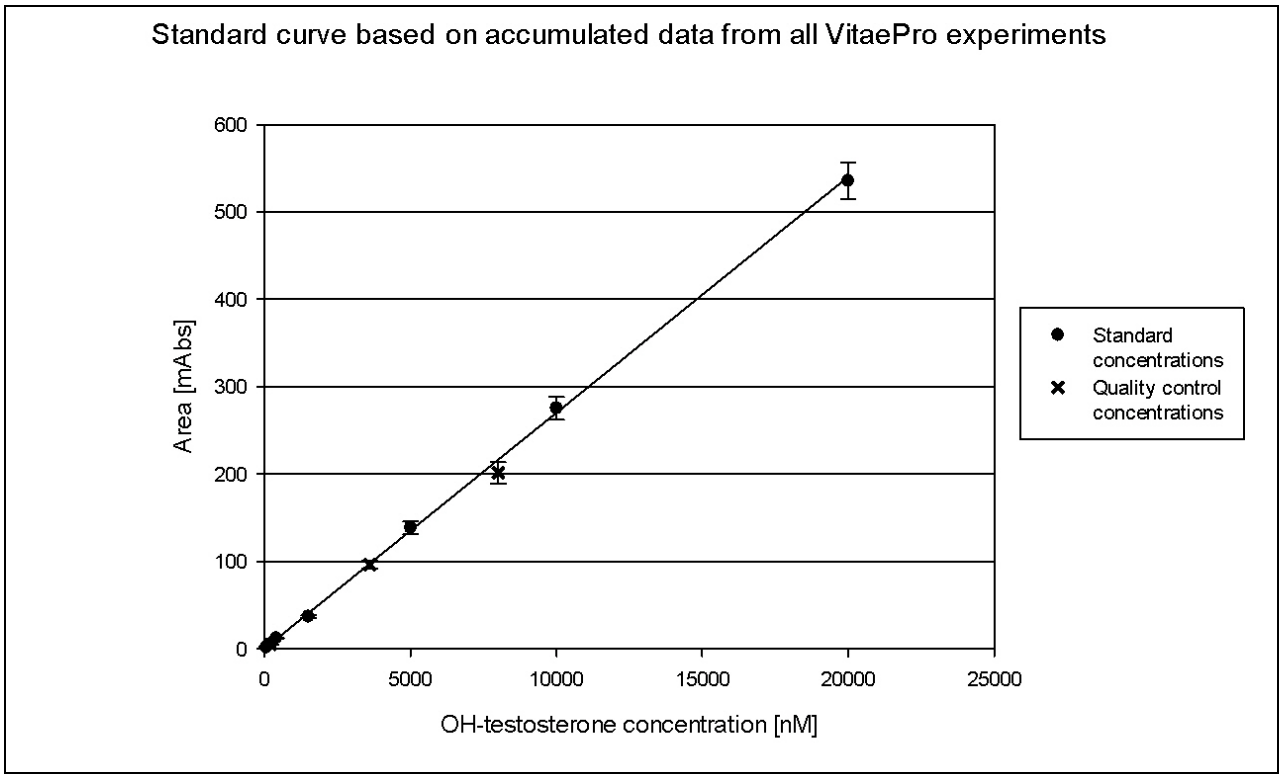

Figure 9: The standard curve based on accumulated data from all VitaePro experiments. Regression equation: y = 0.0270x + 0.5054, R^2-value: 0.9992. The vertical bars represent the ±SD of the mean values obtained from the four experiments.

Table 8: Accumulated data from all 4 VitaePro experiments showing the inter-day precision of all STDs.

STD	Nominal concentration [nM]	Mean calculated concentration [nM]	CV (%)
1	62.5	54.4	10.2
2	125	142.4	6.5
3	500	433.1	4.1
4	1500	1356.3	3.4
5	5000	5117.4	5.4
6	10000	10180.4	4.8
7	20000	19819.2	3.9

Table 9: Accumulated data from all 4 VitaePro experiments showing the inter-day accuracy and precision of all QCs.

QC	Nominal concentration [nM]	Mean calculated concentration [nM]	Accuracy (%)	CV (%)
1	200	181.3	90.6	4.4
1	200	176.7	88.3	6.0
2	3600	3560.0	98.9	4.4
2	3600	3557.2	98.8	4.4
3	8000	7449.8	93.1	6.0
3	8000	7443.3	93.0	6.1

4.3 Validation of metabolic activity

As described in section 3.4, the basic CYP3A4 activity (reference) should not vary more than 20% between the two experiments with VP3 and the two experiments with VP4. This requirement was met as the basic activity of CYP3A4 was estimated to 144.09±1.93 pmol x $pmol^{-1}$ x min^{-1} and 144.89±3.64 pmol x $pmol^{-1}$ x min^{-1} in the incubations with VP3 and VP4, respectively, and the inter-run variation did not exceed 20% in neither of the experiments. The basic activity of CYP3A4 in the separate ketoconazole experiment was 169.62 pmol x $pmol^{-1}$ x min^{-1}.

Further, the ketoconazole solutions, used as positive inhibitory controls (PICs) in the VitaePro experiments, were also required to have an inter-run variation of less than 20%. This requirement was met as the PICs gave a CYP3A4 inhibition of 39.37±5.96% and 38.24±3.44% for VP3 and VP4, respectively, and the inter-run variation did not exceed 20% in neither of the experiments.

Finally, all the chromatograms showed uniform peaks and were thereby accepted for analysis.

4.4 Chromatograms

In this section, some selected and representative chromatograms from the VitaePro experiments are presented. Figures 10a, b and c show chromatograms of increasing QC concentrations. Figures 11a, b and c show chromatograms from the first VP3 incubation with one of the reference solutions, one of the lowest and one of the highest concentrations of VitaePro, respectively. Figures 12a, b and c show the same chromatograms, but from the second VP3 incubation, figures 13a, b and c from the first VP4 incubation, and figures 14a, b and c from the second VP4 incubation.

Note that the values on the Y-axis vary among the figures, and that there is no visible peak of 6-OH-T in the incubation samples containing 0.10 mg of VP4. The other chromatograms show peaks of varying sizes at the 7.8 min, which is the specific retention time for 6-OH-T.

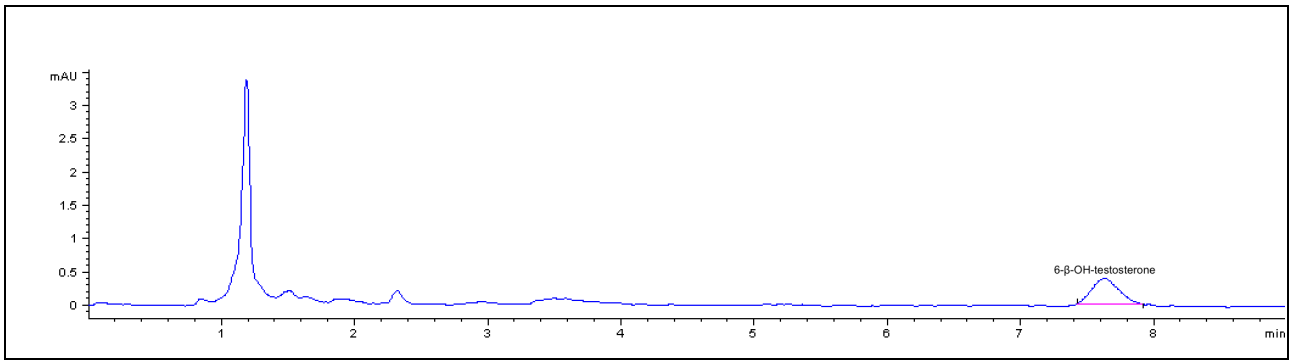

Figure 10a: Chromatogram of a quality control solution containing 200 nM 6-β-OH-testosterone (QC1). Area: 5.5 mAbs x minutes.

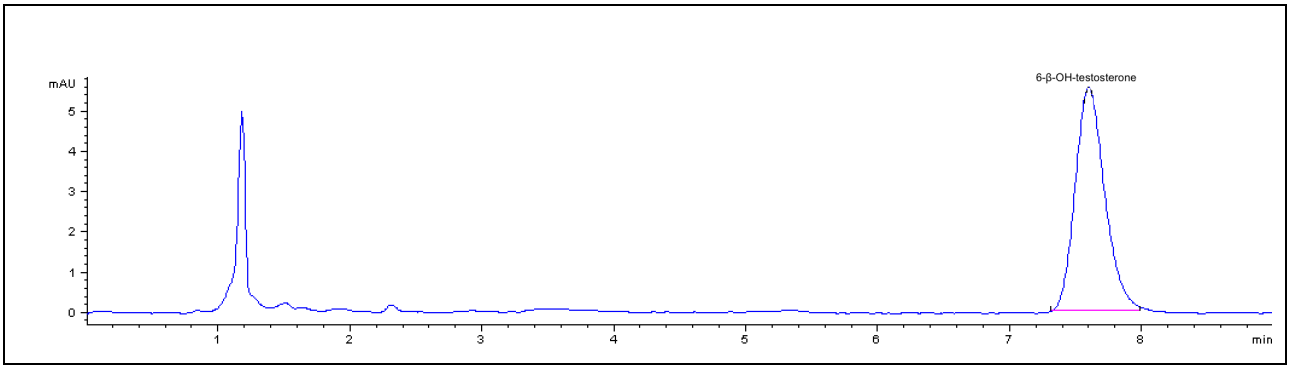

Figure 10b: Chromatogram of a quality control solution containing 3600 nM 6-β-OH-testosterone (QC2). Area: 86.5 mAbs x minutes.

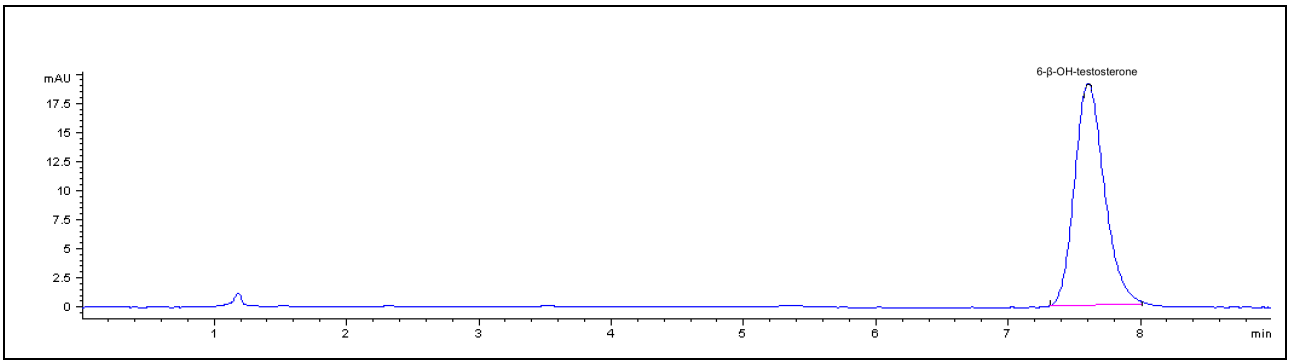

Figure 10c: Chromatogram of a quality control solution containing 8000 nM 6-β-OH-testosterone (QC3). Area: 302.3 mAbs x minutes.

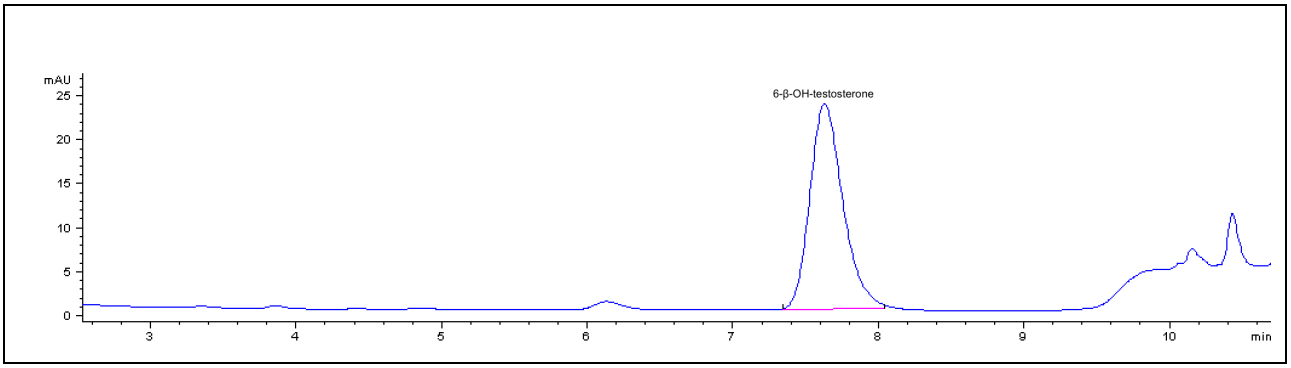

Figure 11a: Chromatogram of an incubated sample with a reference solution from the first VP3 experiment. Area: 368.7 mAbs x minutes.

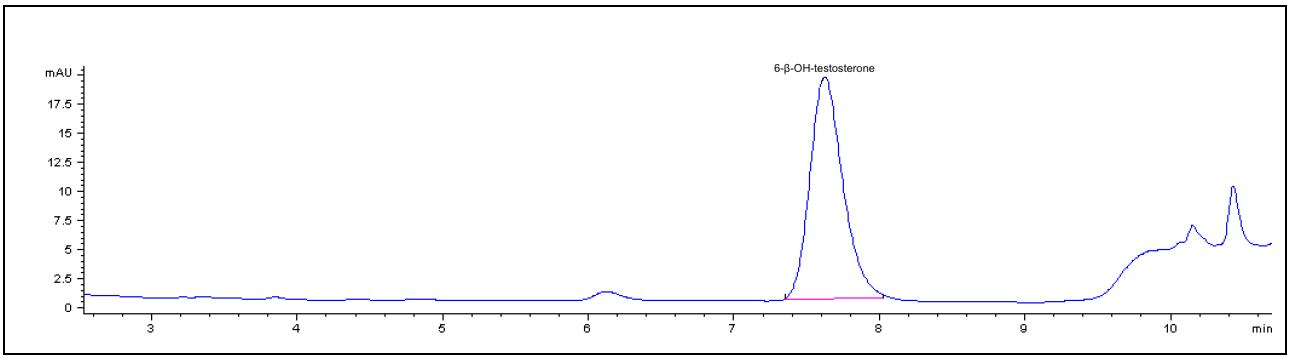

Figure 11b: Chromatogram of an incubated sample with 0.01 mg/mL VitaePro from the first VP3 experiment. Area: 303.5 mAbs x minutes.

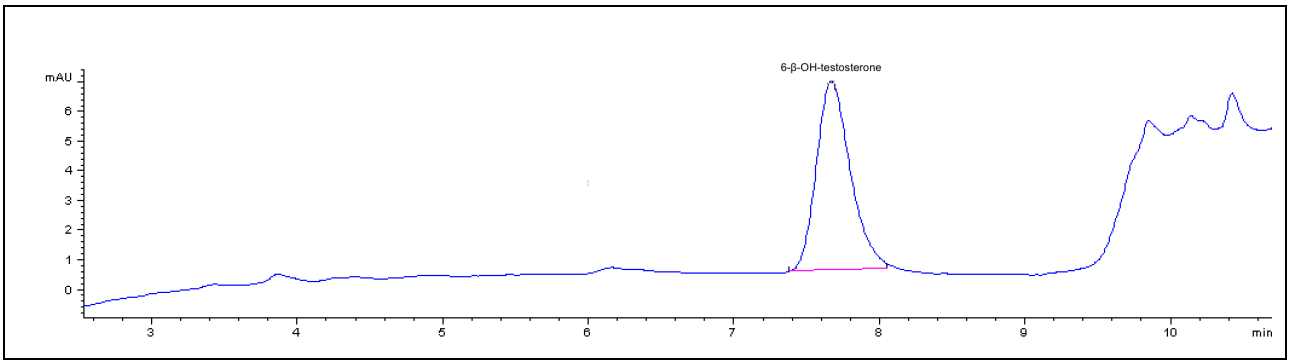

Figure 11c: Chromatogram of an incubated sample with 0.10 mg/mL VitaePro from the first VP3 experiment. Area: 103.9 mAbs x minutes.

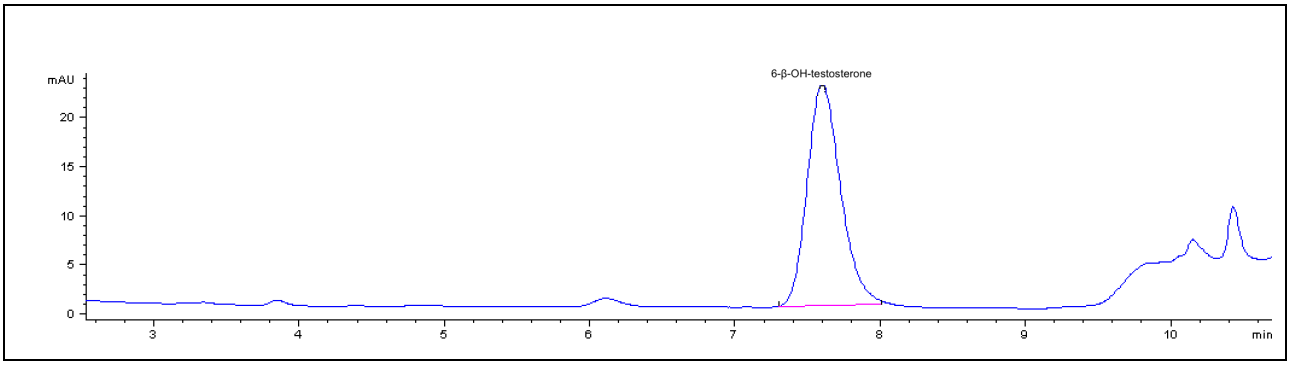

Figure 12a: Chromatogram of an incubated sample with a reference solution from the second VP3 experiment. Area: 363.1 mAbs x minutes.

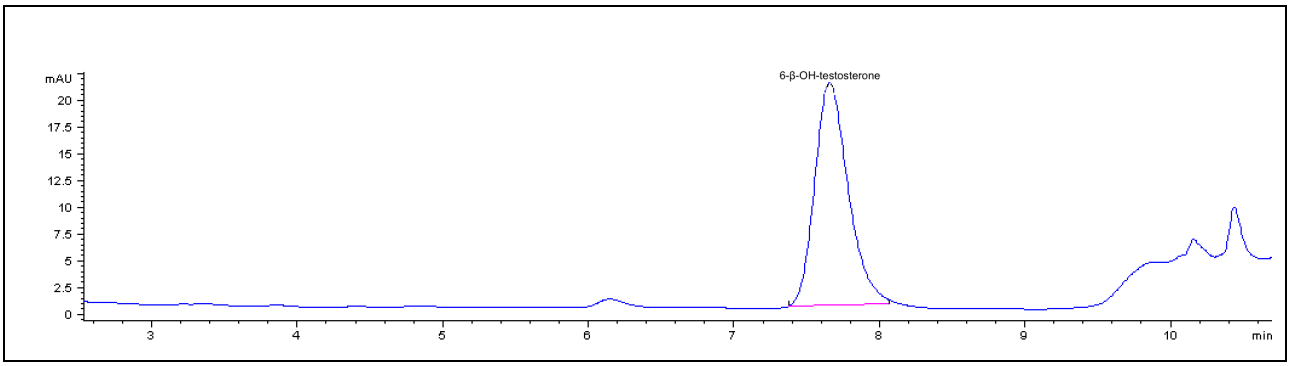

Figure 12b: Chromatogram of an incubated sample with 0.01 mg/mL VitaePro from the second VP3 experiment. Area: 336.1 mAbs x minutes.

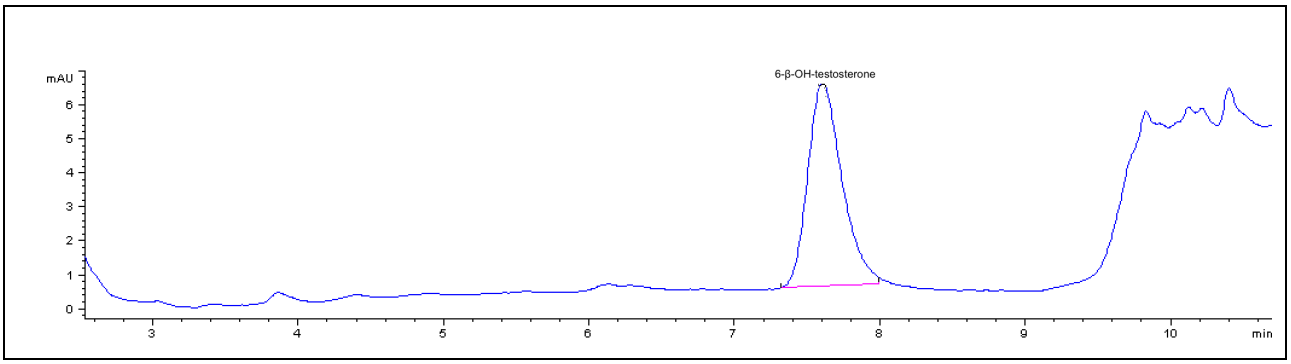

Figure 12c: Chromatogram of an incubated sample with 0.10 mg/mL VitaePro from the second VP3 experiment. Area: 97.6 mAbs x minutes.

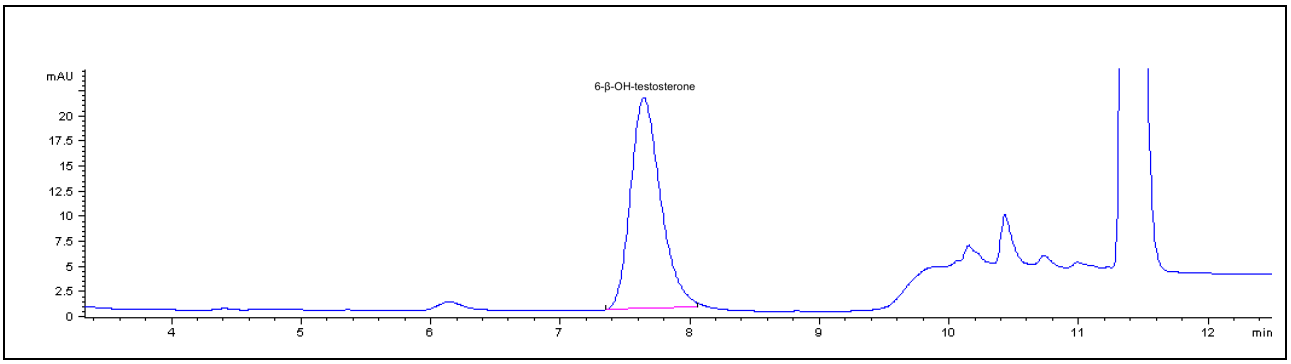

Figure 13a: Chromatogram of an incubated sample with a reference solution from the first VP4 experiment. Area: 341.0 mAbs x minutes.

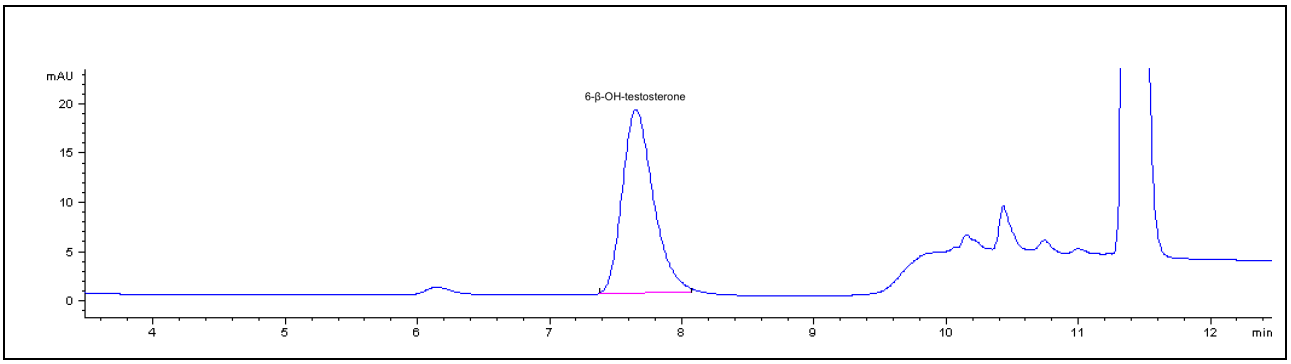

Figure 13b: Chromatogram of an incubated sample with 0.01 mg/mL VitaePro from the first VP4 experiment. Area: 306.0 mAbs x minutes.

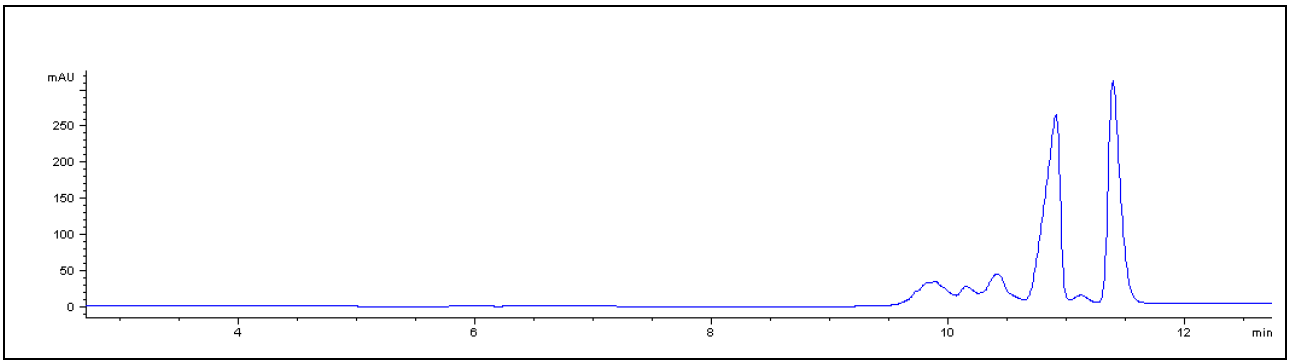

Figure 13c: Chromatogram of an incubated sample with 0.10 mg/mL VitaePro from the first VP4 experiment. Area: below LLOQ, no visible peak.

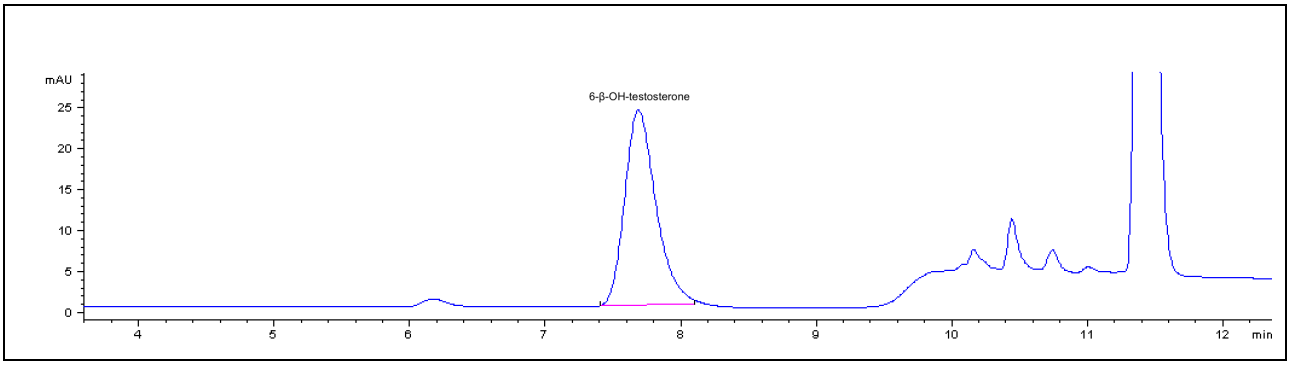

Figure 14a: Chromatogram of an incubated sample with a reference solution from the second VP4 experiment. Area: 385.7 mAbs x minutes.

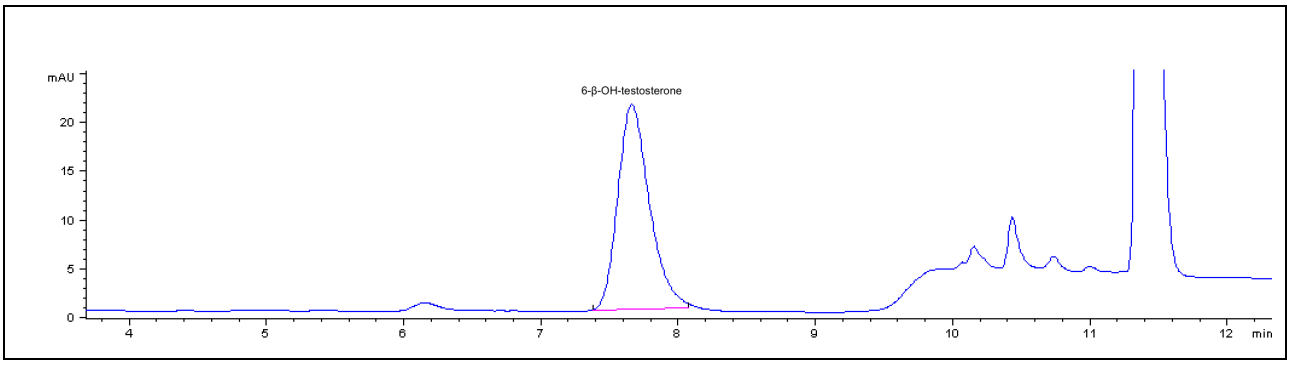

Figure 14b: Chromatogram of an incubated sample with 0.01 mg/mL VitaePro from the second VP4 experiment. Area: 337.0 mAbs x minutes.

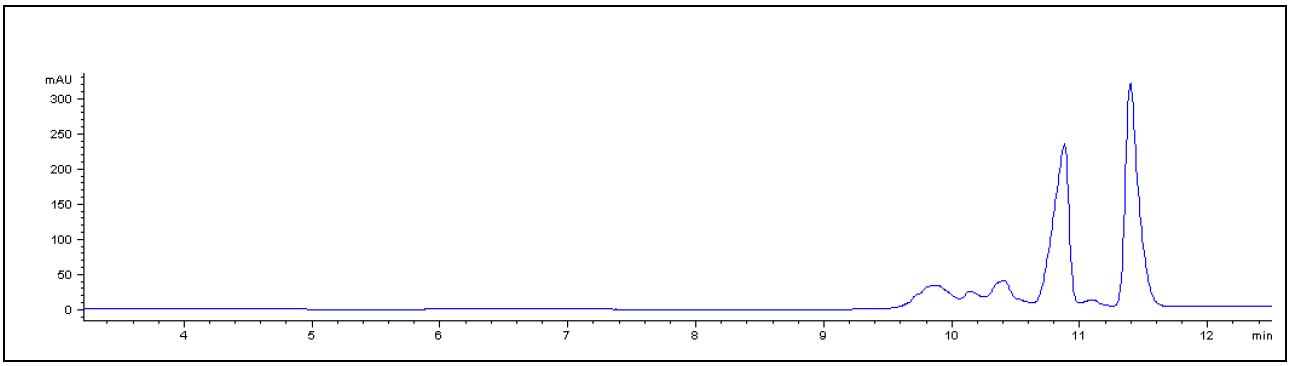

Figure 14c: Chromatogram of an incubated sample with 0.10 mg/mL VitaePro from the second VP4 experiment. Area: 0.4 mAbs, no visible peak.

4.5 Determination of IC_{50}-values

4.5.1 Ketoconazole inhibition

Figure 15 shows the IC_{50} inhibition curve for CYP3A4 with increasing concentrations of KTZ. This separate experiment was conducted before the VitaePro incubations in order to determine the KTZ concentration decreasing the CYP3A4 enzyme activity with 50% for later use as PIC. Also this experiment was performed with triplicate parallel incubations, and the following concentrations (μM) were used: 0.01, 0.05, 0.1, 0.15, 0.3, 0.5, 1.0 and 5.0. The curve was well adapted to the experimental points with a regression coefficient R^2 of 0.9884.

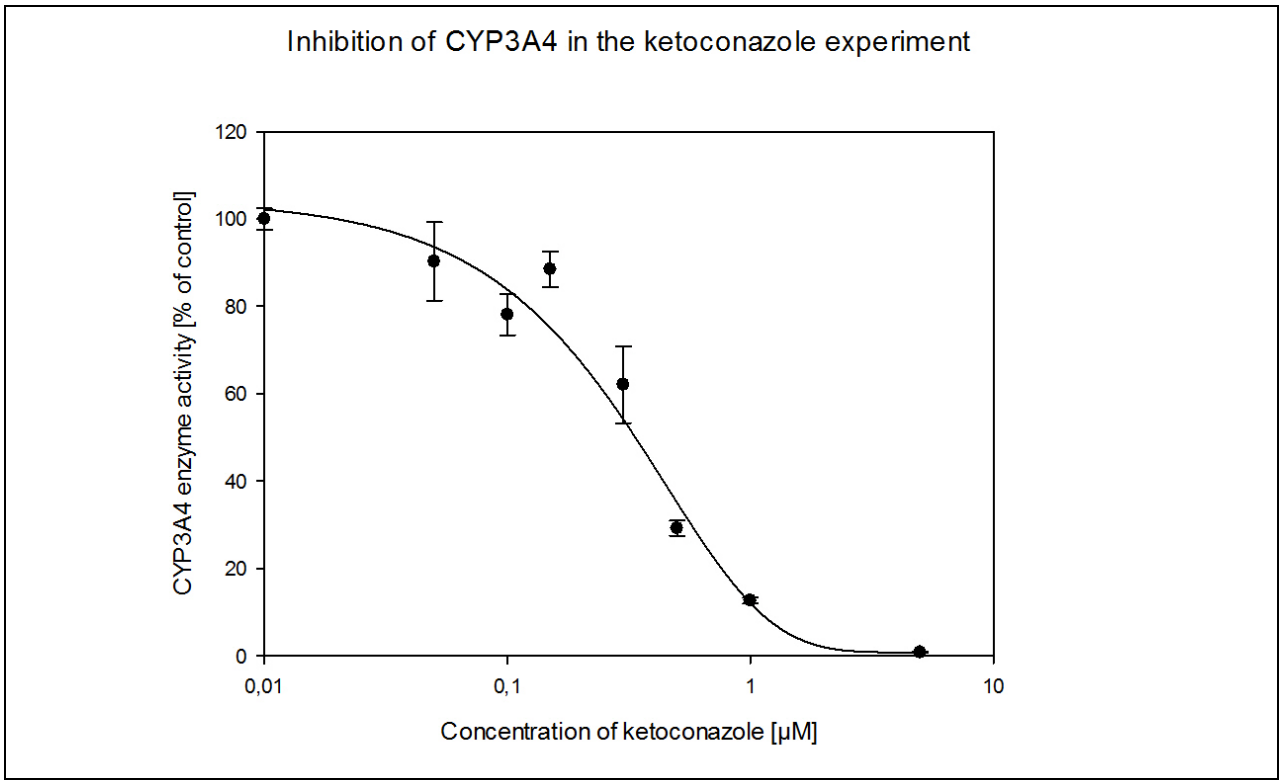

Figure 15: Inhibition of CYP3A4 activity by KTZ. The CYP3A4 enzyme activity is presented as % of control, and the vertical bars represent the ±SD of three parallels. Regression equation: $y = 0.8230 + 103.6290 * e^{-2.2157x}$*,* R^2*-value: 0.9884.*

The estimated IC_{50}-value of KTZ was 0.34±0.06 µM, with a 95% confidence interval of 0.27-0.41 µM.

4.5.2 Third generation VitaePro (VP3) inhibition

Figure 16 and 17 show the IC_{50} inhibition curves for CYP3A4 with increasing concentrations of VP3 in the first and second experiment, respectively. Both incubations were performed in triplicate. The following VitaePro concentrations (mg/mL) were used in both experiments: 0.001, 0.01, 0.03, 0.1, 0.3, 0.5, 1.0, 3.0, 7.0 and 10.0. The curves were well adapted to the experimental points with a regression coefficient R^2 of 0.9257 and 0.9612 for the first and second experiment, respectively.

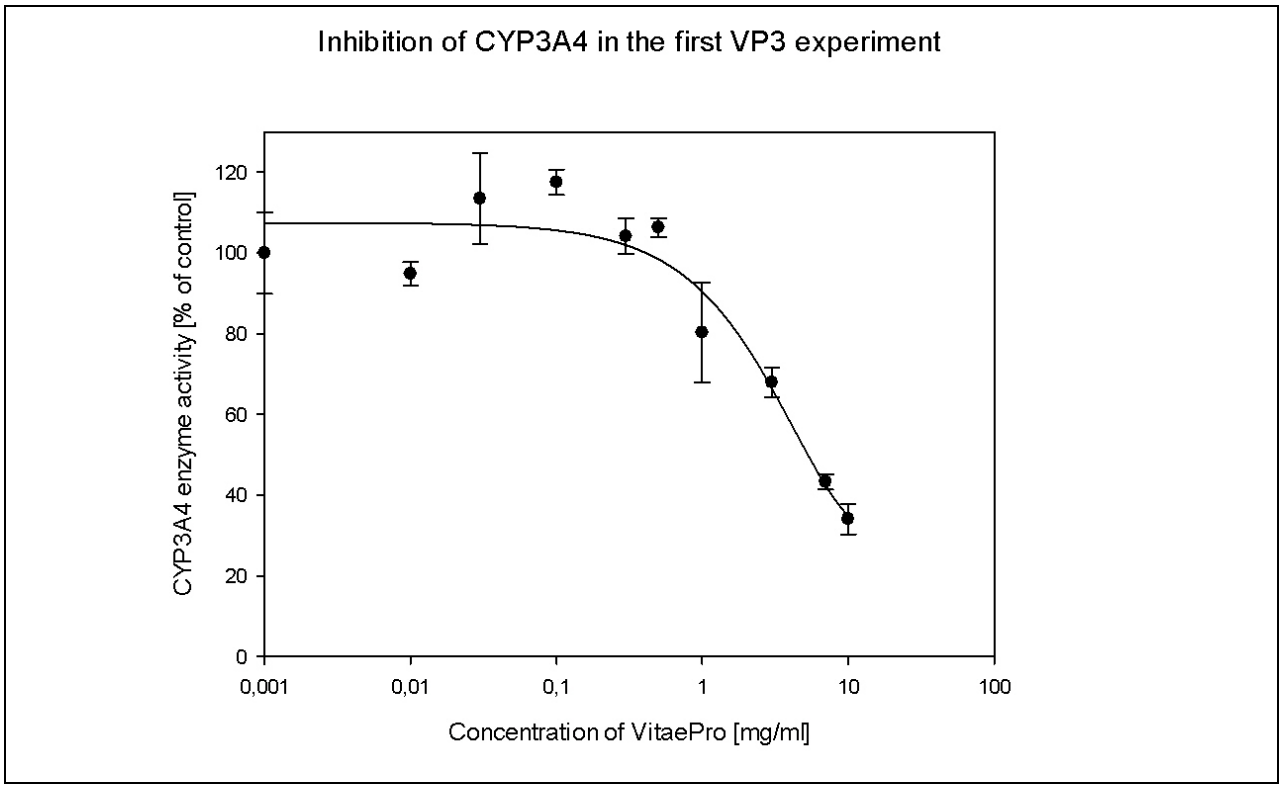

Figure 16: Inhibition of CYP3A4 activity by VP3 in the first experiment. The CYP3A4 enzyme activity is presented as % of control, and the vertical bars represent the ±SD of three parallels. Regression equation: $y = 27.4859 + 80.0317 * e^{-0.2398x}$*,* R^2*-value: 0.9257.*

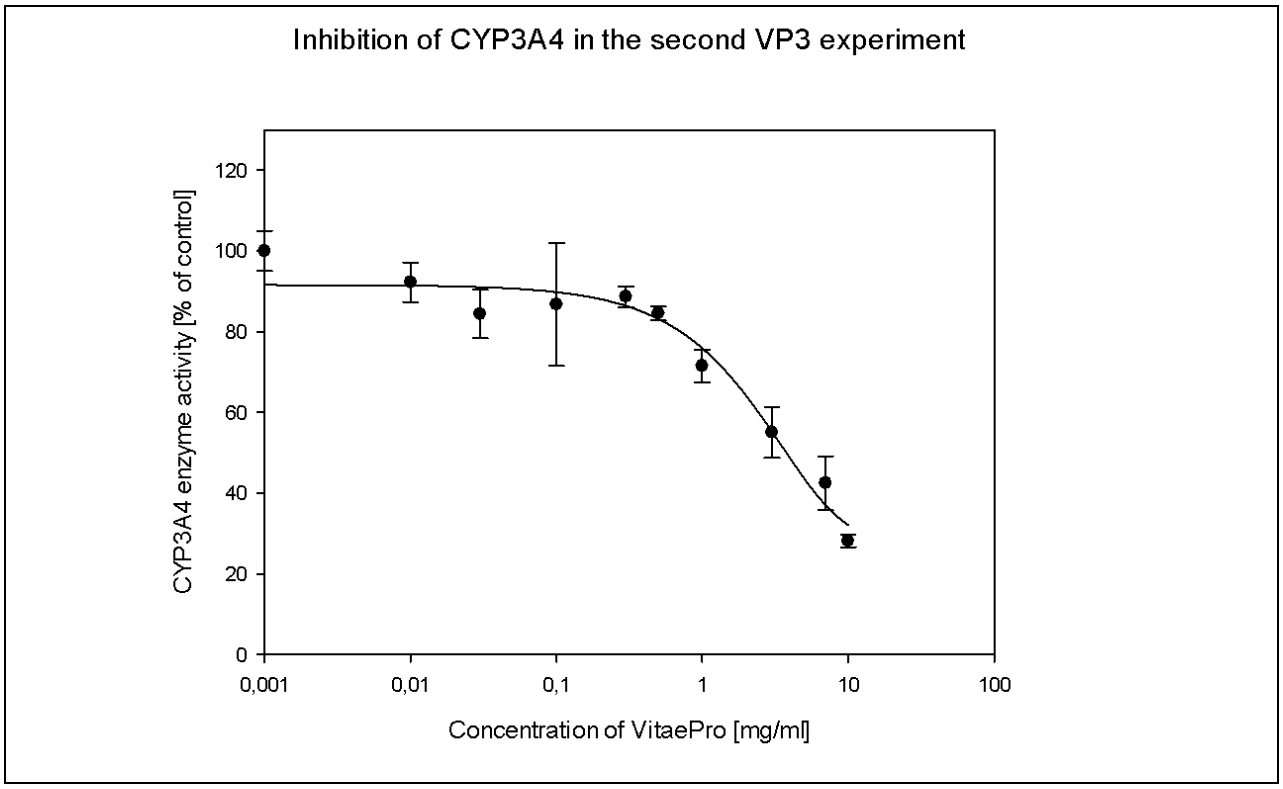

Figure 17: Inhibition of CYP3A4 activity by VP3 in the second experiment. The CYP3A4 enzyme activity is presented as % of control, and the vertical bars represent the ±SD of three parallels. Regression equation: $y = 28.4727 + 63.1325 * e^{-0.2854x}$*,* R^2*-value: 0.9612.*

The estimated IC_{50}-values of VP3 were 5.29±0.72 mg/mL and 3.77±0.83 mg/mL in the first and second experiment, respectively. The 95% confidence intervals of the two IC_{50}-values were 4.48-6.10 mg/ml and 2.83-4.71 mg/ml, respectively, and thereby overlapping.

4.5.3 Fourth generation VitaePro (VP4) inhibition

Figure 18 and 19 show the IC_{50} inhibition curves for CYP3A4 with increasing concentrations of VP4 in the first and second experiment, respectively. Both incubations were performed in triplicate. The following VitaePro concentrations (mg/mL) were used in both experiments: 0.001, 0.01, 0.03, 0.1, 0.3, 0.5, 1.0, 3.0, 7.0 and 10.0. The curves were well adapted to the experimental points, a regression coefficient R^2 of 0.9467 and 0.9801 for the first and second experiment, respectively.

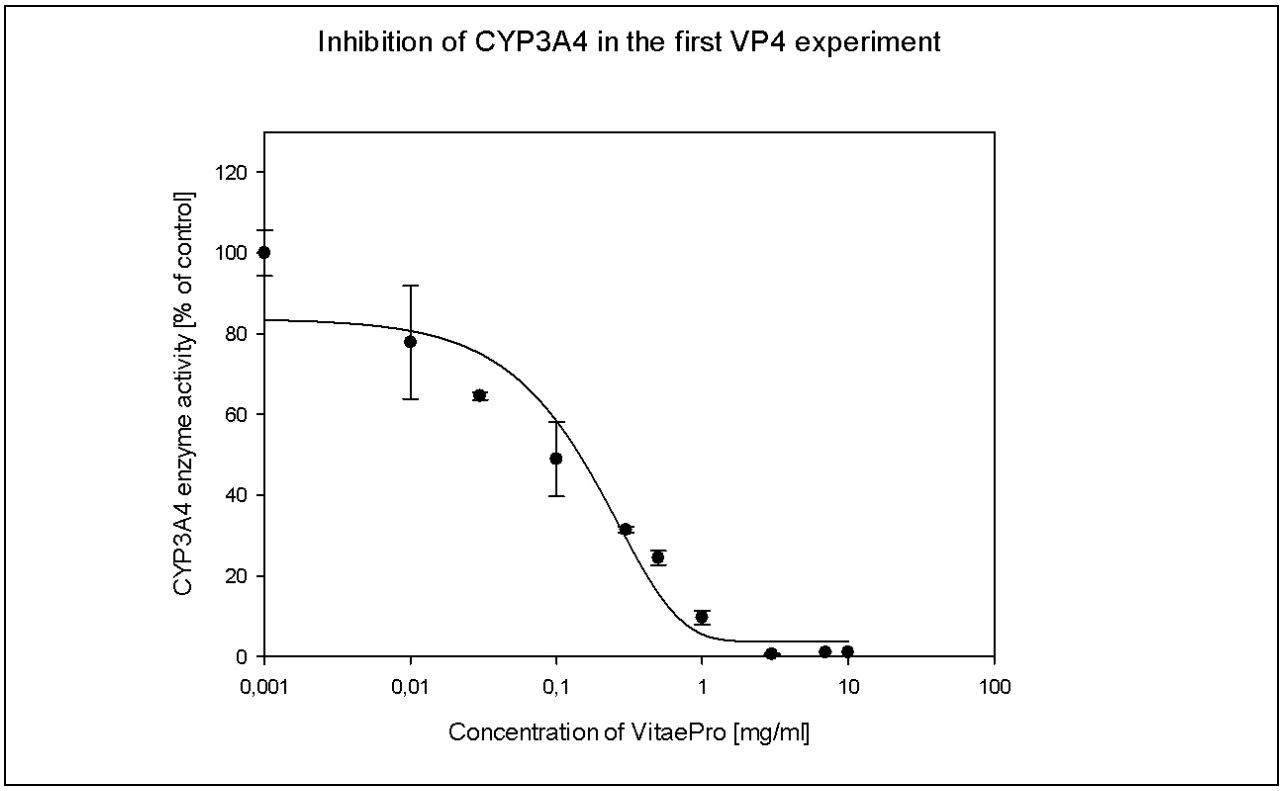

Figure 18: Inhibition of CYP3A4 activity by VP4 in the first experiment. The CYP3A4 enzyme activity is presented as % of control, and the vertical bars represent the ±SD of three parallels. Regression equation: $y = 3.7569 + 79.9384 * e^{-3.8039x}$, R^2*-value: 0.9467.*

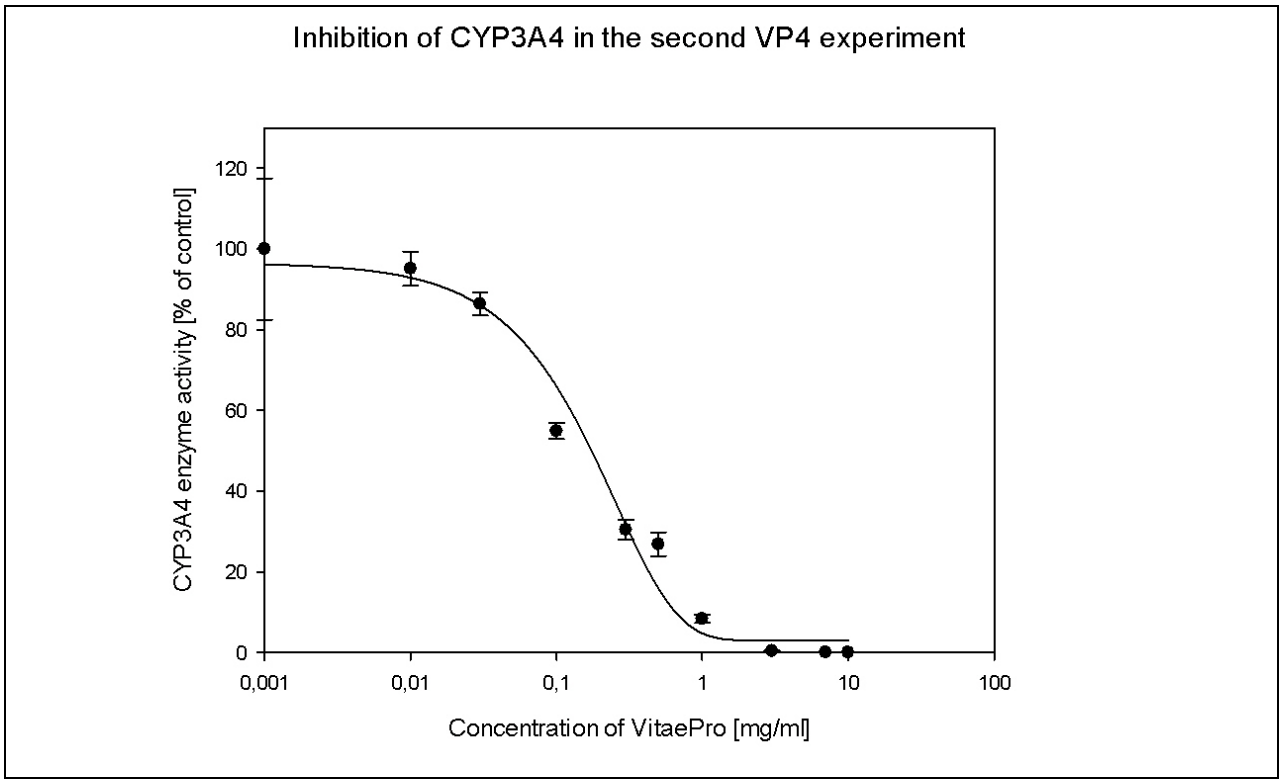

Figure 19: Inhibition of CYP3A4 activity by VP4 in the second experiment. The CYP3A4 enzyme activity is presented as % of control, and the vertical bars represent the ±SD of three parallels. Regression equation: $y = 3.0035 + 93.5360 * e^{-3.9541x}$, R^2*-value: 0.9801.*

The estimated IC_{50}-values of VP4 were 0.14±0.03 mg/mL and 0.17±0.01 mg/mL in the first and second experiment, respectively. The 95% confidence intervals of the two IC_{50}-values were 0.11-0.17 mg/ml and 0.16-0.18 mg/ml, respectively, and thereby overlapping.

4.5.4 Summary

The results from all incubation experiments, including the one with KTZ, can be summarized as presented in table 10.

Table 10: Inhibitors, IC_{50}-values, basic CYP3A4 activity and PIC activity.

	KTZ	VP3	VP4
IC_{50}-value	0.34±0.06 μM	5.29±0.72 mg/mL and 3.77±0.83 mg/mL	0.14±0.03 mg/mL and 0.17±0.01 mg/mL
CYP3A4 basic activity	169.62 pmol x $pmol^{-1}$ x min^{-1}	144.09±1.93 pmol x $pmol^{-1}$ x min^{-1}	144.89±3.64 pmol x $pmol^{-1}$ x min^{-1}
PIC activity		39.37±5.96%	38.24±3.44%

5 Discussion

5.1 Methodological considerations and quality aspects

5.1.1 Nutritional supplements as composite products

The nutritional supplement used in this study, VitaePro®, is easily available as a commercial over-the-counter item in various health care shops and pharmacies, and can also be purchased online from different retailers. *In vitro* studies conducted on some of the individual constituents of VitaePro have shown that some components exert distinct pharmacological properties (section 5.3). However, despite these examples of individual influences, there are certain advantages of investigating an entire product instead of isolated compounds. The newest VitaePro generation, VP4, contains several compounds, which are all consumed together, and an investigation of the overall "VitaePro effect" might thus be of greater public relevance than studies on isolated compounds. Studies on isolated compounds must, however, also be acknowledged, and it is only possible to differentiate safe compounds from potentially harmful when the exact composition of a product is known.

As emphasized in the introduction part of this study, nutritional supplements do not meet the same strict documentation requirements as authorized drugs. The Norwegian Food Safety Authority has several times revealed misleading information regarding the content in products from unserious actors, and despite all implemented regulations, many nutritional supplements can have constituents that vary highly in amount from producer to producer, even from batch to batch. Therefore, when investigating one commercial nutritional supplement, the results are only relevant to the product used in the trial, and cannot be extrapolated to other similar products.

5.1.2 Capsule content and solubility of VitaePro

In order to determine the most exact "concentration of VitaePro", it was necessary to measure the weight and volume of the capsule content as accurately as possible. The procedure is rendered in section 3.2.5, and the results shown in section 4.1. As shown in table 6, the weight of the extracted VitaePro liquid varied slightly between the capsules, and so did also the original capsule weight before emptying.

One of the challenges in obtaining accurate VitaePro concentrations was to extract the highly viscous liquid from the capsules, and thereafter transfer it from the syringe to the plastic tube without too much wastage. Moreover, another challenge was dissolving the capsule content properly in EtOH. Vorkinn reported in 2011 that VitaePro could not be dissolved in water, and this observation is supported by other studies claiming that astaxanthin, lutein and zeaxanthin have poor aqueous solubility in the native state [119, 120]. With the knowledge that even more lipophilic components had been added in the two latest generations (n-3 LC-PUFAs and fat-soluble vitamins), the organic solvent ethanol, which possesses both polar and non-polar characteristics, was used to dissolve the capsule content. However, this solvent did not work optimally either, as the VitaePro liquid and the EtOH seemed to separate spontaneously in room temperature. Because of this, the VitaePro stock solution and the VitaePro working solutions needed to be blended adequately with a vortexer shortly before each pipetting when performing the dilution series, and the same blending was also done before the working solutions were transferred into the test tubes used in the incubation assays.

5.1.3 Possible analytical interferences between VitaePro and 6-β-OH-testosterone

In order to rule out a possible analytical interference between VitaePro and 6-OH-T, a pre-test on co-chromatography was conducted. In this pre-test 200 μL of the strongest VitaePro solution (40 mg/mL) was mixed with 200 μL of the weakest 6-OH-T solution (STD1) and 400 μL 50% MeOH, and analyzed by HPLC. The purpose was to see if any of the constituents in VitaePro would affect the 6-OH-T top during HPLC analysis before initiating analyses of the IC_{50}-experiments in which the 6-OH-T metabolite is analyzed in the presence of VitaePro constituents. This pre-test was done for both VP3 and VP4, but neither of the tests showed any co-chromatographicity between VitaePro and 6-OH-T, thereby eliminating this possible interference problem.

5.1.4 The impact of organic solvents on CYP3A4 enzyme activity

EtOH is a biological active substance that has shown to exert inhibition on CYP3A4 activity [121]. Busby *et al*. found the CYP3A4 inhibition to be minor for 1% MeOH and 1% EtOH (4±9% and 3±13% respectively) when metabolizing testosterone [122], and the findings for

EtOH are in accordance with results reported by Hellum and Nilsen [118]. The use of ethanol must be regarded as a compromise between the necessity to dissolve VitaePro and the influence on CYP3A4. However, the final concentrations of EtOH in this study's incubations were 0.92% and 0.82% for VP3 and VP4, respectively, which can be considered a modest amount. When used as a solvent for VitaePro, the added amount of ethanol may potentially affect the enzyme activity during the incubation experiment. Although this influence was considered to be small in this trial, it was still important to adjust all the test solutions to have the same ethanol concentration.

It should also be mentioned that acetonitrile, used as solvent in the testosterone stock solution, exerts inhibitory effect on CYP3A4 [118, 122]. The final acetonitrile concentration in this study's incubations was 0.25% and is regarded as inconsiderable. However, as testosterone was added in the same amount to all the test tubes, the acetonitrile concentration was the same in all experiments.

5.1.5 Quality aspects and validation

The present study was validated consecutively during the experimental period, using intra-run validations, and checked by inter-run validations at the end of all experiments. Both analytical and metabolic international pre-set acceptance criteria were met. The largest deviations observed for QC accuracy were for the lowest concentrations, but still within the requirements of acceptance. The basic CYP3A4 activity had a very low inter-run variation in the four VitaePro experiments, which shows the robustness of the method used. The inter-run variation in PIC activity was somewhat larger, but still within all acceptance criteria.

5.1.6 Choice of supplement to study

Among the huge selection of marketed nutritional supplements, the Norwegian product VitaePro was chosen to this study for several reasons.

Firstly, VitaePro is a popular supplement in Scandinavia with more than 100 000 users only in Norway. The product has since its first appearance in 2002, received massive attention, and been object for both praise and criticism. Further, despite successful sale figures, the marketing of

VitaePro is still massive and many of the commercials and advertisements are geared specifically towards elderly with promises of maintaining normal functions and reduced tiredness/fatigue [17]. This is particularly problematic knowing that the elderly constitutes a group with statistically greater risks of comorbidity, multiple drug treatment, and metabolic interactions.

Having become aware that several interactions, or suspected interactions, possibly involving VitaePro, have been reported, while the effect, risk and interaction profile still is insufficiently investigated, the choice was clear. Moreover, the assumption that there has only been one earlier study on VitaePro and CYP3A4, made it even more interesting, and the fact that this study was conducted by former NTNU and medical student Vorkinn, was considered an advantage, as the procedures and methods already had been explored with earlier generations of VitaePro.

5.1.7 Choice of substrate

Pharmaceutical industry investigators routinely evaluate the potential of new drugs to alter CYP450 activities by conducting *in vitro* metabolic experiments using probe substrates that represent the activity of specific CYPs. The *in vitro* findings obtained with a probe substrate are usually extrapolated to the investigated drug's potential to affect all substrates of the same enzyme. In this study of VitaePro and CYP3A4, testosterone was the chosen substrate, as it is a recommended and suitable probe substrate for *in vitro* CYP3A4 experiments. Other commonly used probe reactions for investigating CYP3A4 activity are midazolam hydroxylation, nifedipine oxidation and erythromycin *N*-demethylation. However, 6β-hydroxylation is the reaction most often used by industry investigators, and was chosen in this experiment with VitaePro [123]. Another advantage of using testosterone was that Vorkinn used the same substrate in 2010, making it easier to compare the two VitaePro studies.

According to Kenworthy *et al.*, one should use a minimum of three different substrates when investigating possible CYP3A4 inhibitors [124]. The thought is that using more than one substrate will correct a possible substrate-dependent inhibition. As the present study only included testosterone, some uncertainty is implied when transferring the results to a general assumption. The results should therefore be approached and interpreted with some caution.

5.1.8 Choice of method

Inhibitory effects on CYP450 are usually screened using human recombinant CYP enzymes, human liver microsomes or cultured primary human hepatocytes [125].

The experimental work in this study was performed using human cDNA expressed CYP3A4. Other systems for heterologous expression of recombinant CYP450 enzymes include bacterial expression in *Escherichia coli*, expression in yeast cells, and expression from mammalian systems [126]. Recombinant enzymes provide an effective way to confirm results obtained from trials with microsomes or hepatocytes, or screen for new interactions, with good prediction reliability [127]. However, there are also disadvantages of using such enzymes. One of the major disadvantages when using recombinant models expressing a single enzyme for the study of metabolic stability of a drug, is the lack of other phase I and phase II enzymes, and other physiological cofactors. The artificial conditions in the test tube differ greatly from those *in vivo* [126, 128].

Microsomes are small vesicles fractioned from rough endoplasmic reticulum when eukaryotic cells are broken-up in the laboratory. Human liver microsomes contain high levels of CYP enzymes as well as other drug-metabolizing enzyme, and are therefore suitable for studies on CYP450 metabolism as well as other intracellular processes, e.g. endoplasmic reticulum-bound protein synthesis. Microsomal fractions are easily prepared from liver tissue by homogenization and centrifugation, and the metabolites identified after incubation with microsomes coincide with those reported as the major metabolites in human *in vivo* studies. However, the major limitation of microsomes is that they lack phase II cytosolic enzymes such as glutathione S-transferases [128].

Human hepatocytes represent a more complete system providing physiological levels of cofactors, natural orientation for linked enzymes and intact membranes assuring intracellular drug concentrations as similar to those *in vivo* as possible. When knowing that the actual concentration of substrates and inhibitors available for CYP450 metabolism depends on processes missing in subcellular models, such as transport mechanisms, cytosolic enzymes, and binding to intracellular proteins, the benefits of human hepatocytes are highly understandable

[125]. Since intact cells more closely reflect the environment to which drugs are exposed in the liver, cultured hepatocytes constitute a more predictive model when investigating CYP interactions [127]. In fact, screenings of CYP inducers cannot be performed using isolated enzymes or microsomes, as it requires a cellular system fully capable of expressing CYP genes. Up to date, cultured hepatocytes remain a unique *in vitro* model for investigating CYP450 metabolism, and is by far the most preferred model for investigating the inductive potential of drugs. The ability to cryopreserve human hepatocytes is a relatively recent discovery, and has countered the earlier restricted accessibility of suitable human liver samples. The restriction has been a limiting factor for widespread use of humane hepatocytes in research, as primary cells are needed to conduct such experiments. However, there are still some remaining disadvantages when using human hepatocytes. The method is regarded more difficult, expensive and time-consuming than the above-mentioned *in vitro* methods. [125, 128].

5.2 CYP3A4 inhibition by ketoconazole

KTZ was used as PIC in all incubation experiments performed in this study, and a separate ketoconazole experiment was conducted before the VitaePro incubations in order to determine the IC_{50}-value of KTZ. As expected, the IC_{50}-experiment with KTZ showed strong inhibition of CYP3A4, as the already well-established inhibitor seemed to decrease CYP3A4 enzyme activity with 100 % at the highest concentration.

Several inhibition experiments on CYP3A4 have been performed in our laboratory using KTZ as PIC, Hellum and Nilsen [118], Djuv and Nilsen [129] and Langhammer and Nilsen [130], giving IC_{50}-values in the range 0.10 μM-0.35 μM. All trials used testosterone as substrate, and the IC_{50}-value found in this study, 0.34±0.06 μM, was within the previous found range. Based on these observations, one single experiment was considered sufficient to determine the IC_{50}-value of KTZ.

5.3 CYP3A4 inhibition by VitaePro

In the current study, the *in vitro* IC_{50}-values of VP3 were found to be 5.29±0.72 mg/mL and 3.77±0.83 mg/mL in the first and second experiment, respectively. The corresponding values for VP4 were 0.14±0.03 mg/mL and 0.17±0.01 mg/mL.

In the student thesis from 2010, VP1 and VP2 were investigated, producing IC_{50}-values of 25.97±3.92 mg/mL and 162.44±27.07 mg/mL, respectively. When compared to the current study, we see that the IC_{50}-values obtained for VP3 and VP4 are smaller. As already described, the two latest products contain additional components compared with the first two generations. The difference in inhibitory potency among all four generations are probably attributed to the various ingredients of the products, since the study methods otherwise have been equal.

VP3 decreased the CYP3A4 enzyme activity with approximately 70% at the highest concentration (0.10 mg/mL), while VP4 decreased the CYP3A4 enzyme activity with nearly 100% at the highest concentration (0.10 mg/mL). Full inhibition was not reached, but could probably have been achieved using higher concentrations of VitaePro, as the ethanol concentration was a limiting factor.

As already mentioned, *in vitro* studies conducted on some of the individual constituents of VitaePro have shown that some components exert distinct pharmacological properties Astaxanthin has been particularly investigated with an *in vitro* study on humane hepatocytes showing that astaxanthin is capable of inducing CYP3A4 [131]. Another *in vitro* study on human liver microsomes has shown no effects of lutein, but weakly inhibitory effects on CYP3A4 provided by zeaxanthin, with an IC_{50}-value of 15.5±1.09 μM. This IC_{50}-value corresponds to 8.82±0.62 μg/mL, and as we can see, the value is much smaller than the IC_{50}-values found for VP4. However, Zheng *et al*., who conducted this trial, claims that this is a weak inhibition, most likely of minor importance *in vivo* [132].

The effect of high-dosage vitamin C on hepatic CYP3A4 activity has been investigated in a clinical trial with fourteen healthy Caucasian adult volunteers. A modest induction of hepatic CYP3A4 was observed in men after the administration of 500 mg vitamin C twice a day for 14

days, but no consistent effect was observed in women. The overall conclusion from this study was that no significant effect of vitamin C was observed [133]. However, the modest induction is consistent with another clinical study that found an overall reduced indinavir plasma concentrations in seven healthy volunteers after concomitant administration of high doses of vitamin C. The authors state that the mechanism may be attributed to induction of CYP3A4 [134].

The other vitamins have been investigated to a lesser extent clinically, but binding of different ligands to the vitamin D receptor (VDR), including the biological active 1,25-dihydroxycholecalciferol, have repeatedly proven to induce CYP3A4 expression in human intestinal Caco-2 cells [135, 136]. Further, several *in vitro* studies and *in vivo* studies on animals, as well as meta-analyses, have suggested that vitamin E may increase the production of hepatic CYP3A4 in human cell lines [137]. An *in vitro* study on HepG2 cells showed up-regulation of CYP3A4 by γ-tocotrienol through binding to the pregnane X receptor (PXR), which in turn is necessary for induction of CYP3A4 expression [138]. Moreover, a recent *in vivo* study on mice revealed that α-tocopherol is a partial agonist of the PXR, thereby increasing hepatic CYP3A4 expression [139].

Regarding omega-3 fatty acids, several studies have investigated the inhibitory effects of n-3 LC-PUFAs towards CYP3A4. An *in vitro* trial, using single enzymes, found that EPA and DHA inhibited CYP3A4 with IC_{50}-values of 54 μM and 34 μM, respectively. This corresponds to 16.33 μg/mL and 11.17 μg/mL, and also these values are smaller than those obtained for VP4 [140]. The results are consistent with the findings of DHA as a competitive inhibitor of intestinal CYP3A4 in rats, which was shown in another study [141].

No scientific articles where found concerning CYP3A4 activity alteration by *Boswellia serrata* or selenium when searching in the PubMed database combining the terms "*Boswellia serrata*", "selenium" and "CYP3A4" (latest search done December 19th, 2015).

In summary, those of the individual VitaePro components with earlier proven inhibitory effects, zeaxanthin, EPA and DHA, all showed smaller IC_{50}-values in their respective studies than the

IC_{50}-values found in the current study. However, it is difficult to draw any certain conclusions based on these observations, as VitaePro is a composite product, with components assumingly exerting both inducing and inhibitory properties.

5.4 Possible reasons for different inhibitory potency between the generations

The *in vitro* IC_{50}-values of VP3 were 5.29±0.72 mg/mL and 3.77±0.83 mg/mL, with 95% confidence intervals of 4.48-6.10 mg/ml and 2.83-4.71 mg/ml, in the first and second experiment, respectively. The corresponding values for VP4 were 0.14±0.03 mg/mL and 0.17±0.01 mg/mL, with 95% confidence intervals of 0.11-0.17 mg/ml and 0.16-0.18 mg/ml, meaning that there is a statistical significant difference in inhibitory potency between the third and fourth generation of VitaePro.

Since all four experiments were performed equally regarding methods, and under the same conditions and circumstances, the only variable factor between the generations seems to be the different constituents of the two products. The amount of the three antioxidants, astaxanthin (2 mg), lutein (5 mg) and zeaxanthin (1.2 mg), were the same in both generations of VitaePro. The contents of vitamin C (100 mg) and vitamin E (12 mg α-TE) were also unchanged, but the vitamin D amount had been doubled from VP3 (5 μg) to VP4 (10 μg). In contrast, the calamari oil had been decreased from VP3 (347 mg) to VP4 (250 mg), with a following decrease in EPA (48 mg to 34 mg) and DHA (125 mg to 90 mg). Further, the selenium (55 μg) and *Boswellia serrata* extract (100 mg) were two new ingredients that appeared in VP4. When looking at these changes, the most likely cause of the observed difference in inhibitory potency seems to be the added *Boswellia serrata* extract (BSE), as selenium is added in a low concentration. The added BSE is the major modification between the two products, and thus the most reasonable explanation why VP4 exerts a significantly stronger inhibitory effect on CYP3A4 than VP3. However, these assumptions should also be interpreted with caution, and more studies are needed in order to rule out which proportions of the total inhibitory effect that can be attributed to the different components of VitaePro.

As earlier mentioned, studies on *Boswellia serrata* and possible alterations of CYP3A4 activity are lacking. Based on the observations from this study, further trials are warranted.

5.5. Theoretical *in vivo* significance

5.5.1 *In vitro* studies and difficulties in extrapolating results

Results from *in vitro* studies of CYP450 metabolism can reveal inhibitory or inducing properties of different substances, but these findings are not necessarily valid for *in vivo* situations. In fact, the majority of herbal intake is believed not to cause any clinically relevant drug interactions, as the herbal concentration giving sufficient alteration in CYP enzyme activity *in vitro* often is too high to represent physiological *in vivo* concentrations [142].

The physiological environment in the human body differs greatly from the controlled circumstances under which *in vitro* trials are performed in the laboratory. The actual concentration of an orally administrated substance is influenced by several factors, e.g. degradation by gastric acid, possible interactions with P-gp or other transport mechanisms, cytosolic enzymes, binding to intracellular proteins and many more. Because of all these factors, there are large uncertainties regarding how much of an inhibitory or inducible substance that really reaches systemic circulation and thus the CYP450 enzymes. *In vitro* studies used to evaluate drug interaction potentials with nutritional supplements possess all the same limitations as seen with conventional or new drugs, but additionally there is often a lack of commercial standards, and more difficult to attribute observed effect to specific compounds in the highly variable mixtures [143].

Another issue when extrapolating *in vitro* results to *in vivo* situation is the significance of administration-related factors, e.g. dosage sizes, dosage intervals and the time aspect of co-administration. While some drug interactions predominantly occur when the inhibitor or inducer is added to an already ongoing treatment regime, others may occur after co-administration for a long time [144]. SJW, for instance, has proven to be a CYP3A4 inhibitor in *in vitro* situations (with pure enzymes or microsomes), whereas it induces CYP3A4 *in vivo* when consumed continuously over weeks [145].

Based on all the arguments discussed in this section, clinical trials are the gold standard for evaluating drug interactions. Nevertheless, *in vitro* studies remain important as a first line tool in selecting which interactions that should be further explored *in vivo*. The most considerable advantages of *in vitro* studies are the time and cost effectiveness, the possibility of providing mechanistic information and the lack of ethical challenges as no patients are involved

5.5.2 Theoretical *in vivo* relevance of the present study

As an introduction to this section concerning *in vivo* relevance, it should be emphasized that the results obtained from this study not are in the position to provide any conclusions regarding *in vivo* or clinical effects. Nevertheless, discussions and reflections concerning our findings using theoretical approaches are desirable, and thus presented throughout this section.

For an *in vivo* significant interaction to take place, two prerequisites have to be fulfilled. Firstly, the concentration of the inhibitor must reach high enough concentrations at the site of action, and secondly, the therapeutic index of the affected drug should be sufficiently narrow so that the change in systemic concentration would cause observable manifestations [146].

In order to determine whether a certain *in vitro* result should be further investigated *in vivo*, Strandell *et al*. have suggested the following method: if the concentration obtained when dissolving the recommended single dose in a volume corresponding to the blood volume exceeds the *in vitro* IC_{50}-value, the clinical inhibitory effects should be investigated [147]. The reasoning behind this method is that the impact on hepatic CYP enzymes is thought to depend upon the systemic plasma concentration. As CYP3A4 is expressed in both the liver and the intestine, the same method can be used in evaluating possible candidates for intestinal inhibition by using the intestinal volume instead of the blood volume to calculate the concentration, and see if it exceeds the IC_{50}-value.

The recommended daily dose of both VP3 and VP4 is 1-2 capsules, corresponding to 592.7-1185.4 mg of VP3 liquid and 624.8-1249.6 mg of VP4 liquid. Supposing a blood volume of 5 liters, complete intestinal absorption and minor protein binding, the maximum plasma concentrations would have reached 0.24 mg/ml and 0.25 mg/ml after ingestion of two VP3

capsules and two VP4 capsules, respectively.

As the in vitro IC_{50}-values of VP3 were 5.29±0.72 mg/mL and 3.77±0.83 mg/mL in the first and second experiment, respectively, and the corresponding values of VP4 were 0.14±0.03 mg/mL and 0.17±0.01 mg/mL, VP3 is not regarded for further trials. For VP4, on the other hand, the IC_{50}-values are slightly lower than the values calculated by the method of Strandell *et al*.

For intestinal evaluation one could use volumes of 250 mL and 530 mL, which have been used in previous studies to calculate a hypothetical intestinal concentration [102, 148]. Supposing a blood volume of 250 mL, the intestinal concentration of VitaePro following oral administration would have reached 4.74 mg/mL and 5.00 mg/mL for VP3 and VP4, respectively. In this case, both VP3 and VP4 exceed the IC_{50}-concentrations obtained from the trial; VP4 to a far greater extent than VP3, and both could be candidates for an *in vivo* trial.

Drugs undergoing intestinal CYP450 metabolism will be converted to more polar metabolites inside the intestinal lumen, which in turn leads to poorer absorption of the drug. This means that a significant inhibition of intestinal CYP3A4 may cause increased bioavailability of CYP3A4-metabolized drugs, which further might lead to unexpected high plasma concentrations and toxicity for drugs with narrow therapeutic range.

Because of the great amount of drugs metabolized by CYP3A4, a significant altering in enzyme activity may be of clinical significance for a wide range of substrates. Drugs with original low bioavailability, e.g. simvastatin, are particularly affected by intestinal CYP inhibition, since these drugs normally undergo extensive first pass metabolism, and a high intake is necessary to achieve therapeutic levels [149]. The result of inhibition is thus decreased metabolism and increased bioavailability.

Clinically observable changes can generally be expected for drugs with wide therapeutic range if the plasma concentration is doubled. However, this varies according to the morphology of the drug's exposure-response curve and the exact therapeutic range. For drugs with narrow therapeutic range, even 50% increase in plasma concentration may cause observable changes.

Warfarin, digoxin, ciclosporin and cytotoxic anticancer agents are examples of drugs within this latter category, and they are thus very vulnerable to drug interactions [150].

Returning to the present VitaePro study, one can theorize that the VitaePro products might have an impact on intestinal CYP3A4, particularly VP4, and it should therefore be a candidate for further trials. However, taking into account the limitations regarding extrapolation of *in vitro* results to *in vivo* situations, these results should be interpreted with cautions. Further studies are warranted, both *in vitro* and *in vivo*.

6 Conclusions

The following conclusions were drawn based on the aims of the thesis:

- Does VitaePro exert an inhibitory effect on the CYP3A4 enzyme activity *in vitro*, here demonstrated by affected conversion of testosterone to 6-β-OH-testosterone?

  Yes, based on the results from this study it seems evident that both the third and the fourth generation product of VitaePro inhibits CYP3A4 *in vitro*.

- Given the findings of inhibition, is there a significant difference in inhibitory potency between the third and fourth generation product of VitaePro?

  The IC_{50}-values of VP3 were determined to 5.29±0.72 mg/mL and 3.77±0.83 mg/mL in the first and second experiment, respectively, and the corresponding values of VP4 were determined to 0.14±0.03 mg/mL and 0.17±0.01 mg/mL. These results, held together with non-overlapping 95% confidence intervals, indicates a significant difference in inhibitory potency between the third and forth generation product.

- In case of a significant difference in inhibitory potency, could this difference be caused by different contents in the two VitaePro products?

  The newly added *Boswellia serrata* extract stands out as the most likely explanation of why the fourth generation product seems to exert stronger inhibition on CYP3A4 than the third generation product.

- Is it theoretically possible that the CYP3A4 inhibition provided by VitaePro could be of significance for patients using VitaePro together with CYP3A4 metabolized drugs?

  The theoretical possibility to exceed IC_{50}-concentrations in plasma, and thus cause significant inhibition in the liver, is present for VP4. The theoretical possibility to exceed IC_{50}-concentrations in the small intestine, and thus cause significant inhibition in the intestinal wall, is present for both VP3 and VP4, the latter in particular.

7 References

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8 Appendices

8.1 Preparation of experimental solutions

8.1.1 6-β-OH-testosterone solutions

Table 11: Preparation of 6-β -OH-testosterone working solutions for the standards.

No.	Conc. (nM)	Conc. working solutions 1-7 (nM)	Procedure
Stock			Stock = 1.0 mM
STD1	62.5	312.5	10 mL = 125 µL WS5 + 9.875 mL 50% MeOH
STD2	125	625	10 mL = 125 µL WS6 + 9.875 mL 50% MeOH
STD3	400	2000	10 mL = 25 µL stock + 9.975 mL 50% MeOH
STD4	1500	7500	10 mL = 75 µL stock + 9.925 mL 50% MeOH
STD5	2500	25000	10 mL = 250 µL stock + 9.750 mL 50% MeOH
STD6	5000	50000	10 ml = 500 µL stock + 9.500 mL 50% MeOH
STD7	10000	100000	10 ml = 1.0 mL stock + 9.000 mL 50% MeOH

Table 12: Preparation of-β-OH-testosterone working solutions for the quality controls.

No.	Conc. (nM)	Conc. working solutions (nM)	Procedure
Stock			Stock = 1.0 mM
QC_{LLOQ}	62.5	312.5	10 mL = 173.6 µL QC_2 + 9.8264 mL 50% MeOH
QC_1	200	1000	10 mL = 0.5555 µL QC_2 + 9.4445 mL 50% MeOH
QC_2	3600	18000	10 mL = 180 µL stock + 9.820 mL 50% MeOH
QC_3	8000	40000	10 mL = 0.4 mL stock + 9.600 mL 50% MeOH

8.1.2 Ketoconazole and positive inhibitory control solutions

Table 13: Preparation of ketoconazole working solutions.

No.	Conc. (µM)	Conc. working solutions 1-7 (µM)	Procedure (MeOH content 1%)
Stock			Stock = 1.0 mM
WS1	0.05	0.20	1 mL = 10 µL WS7 + 9.9 µL MeOH + 980.1 µL KPO
WS2	0.10	0.40	1 mL = 20 µL WS7 + 9.8 µL MeOH + 970.2 µL KPO
WS3	0.15	0.60	1 mL = 30 µL WS7 + 9.7 µL MeOH + 960.3 µL KPO
WS4	0.30	1.20	1 mL = 60 µL WS7 + 9.4 µL MeOH + 930.6 µL KPO
WS5	0.50	2.00	1 mL = 100 µL WS7 + 9 µL MeOH + 891 µL KPO
WS6	1.00	4.00	1 ml = 200 µL WS7 + 8 µL MeOH + 792 µL KPO
WS7	5.00	20.00	10 ml = 200 µL stock + 9800 µL KPO

Table 14: Preparation of positive inhibitory control solution for VP3.

No.	Conc. (µM)	Conc. working solutions (µM)	Procedure (EtOH content 3.67%)
WS7			WS7 = 20.00 µM
PIC_{VP3}	0.34	1.36	1 mL = 68 µL WS7 + 36.7 µL EtOH + 895.3 µL KPO

Table 15: Preparation of positive inhibitory control solution for VP4.

No.	Conc. (µM)	Conc. working solutions (µM)	Procedure (EtOH content 3.28%)
WS7			WS7 = 20.00 µM
PIC_{VP4}	0.34	1.36	1 mL = 68 µL WS7 + 32.8 µL EtOH + 899.2 µL KPO

8.1.3 VitaePro solutions

Table 16: Dilution series when making VitaePro working solutions for VP3 and VP4.

No.	Conc. (mg/ml)	Conc. working solutions 1-9 (mg/ml)	Procedure VitaePro 3 (EtOH content 3.67%)
Stock			Stock = 522.94 mg/mL
WS1	0.01	0.04	1 mL = 10 µL WS6 + 36.3 µL EtOH + 953.7 µL KPO
WS2	0.03	0.12	1 mL = 10 µL WS7 + 36.3 µL EtOH + 953.7 µL KPO
WS3	0.10	0.40	1 mL = 10 µL WS9 + 36.3 µL EtOH + 953.7 µL KPO
WS4	0.30	1.20	1 mL = 30 µL WS9 + 35.6 µL EtOH + 934.4 µL KPO
WS5	0.50	2.00	1 mL = 50 µL WS9 + 34.9 µL EtOH + 915.1 µL KPO
WS6	1.00	4.00	2 ml = 200 µL WS9 + 66.1 µL EtOH + 1733.9 µL KPO
WS7	4.00	12.00	2 ml = 600 µL WS9 + 51.4 µL EtOH + 1348.6 µL KPO
WS8	7.00	28.00	1 ml = 700 µL WS9 + 11 µL EtOH + 289 µL KPO
WS9	10.00	40.00	10 ml = 764.9 µL stock + 9235.1 µL KPO
WS0	0.00	0.00	1 ml = 36.7 µL EtOH + 963.3 µL KPO
No.	Conc. (mg/ml)	Conc. working solutions 1-9 (mg/ml)	Procedure VitaePro 4 (EtOH content 3.28%)
Stock			Stock = 585.77 mg/ml
WS1	0.01	0.04	1 mL = 10 µL WS6 + 32.5 µL EtOH + 957.5 µL KPO
WS2	0.03	0.12	1 mL = 10 µL WS7 + 32.5 µL EtOH + 957.5 µL KPO
WS3	0.10	0.40	1 mL = 10 µL WS9 + 32.5 µL EtOH + 957.5 µL KPO
WS4	0.30	1.20	1 mL = 30 µL WS9 + 31.8 µL EtOH + 938.2 µL KPO
WS5	0.50	2.00	1 mL = 50 µL WS9 + 31.2 µL EtOH + 918.8 µL KPO
WS6	1.00	4.00	2 ml = 200 µL WS9 + 59 µL EtOH + 1741 µL KPO
WS7	4.00	12.00	2 ml = 600 µL WS9 + 45.9 µL EtOH + 1354.1 µL KPO
WS8	7.00	28.00	1 ml = 700 µL WS9 + 9.8 µL EtOH + 290.2 µL KPO
WS9	10.00	40.00	10 ml = 682.9 µL stock + 9317.1 µL KPO
WS0	0.00	0.00	1 ml = 32.8 µL EtOH + 967.2 µL KPO

8.2 Standard curves and quality control validations

8.2.1 First experiment with VP3

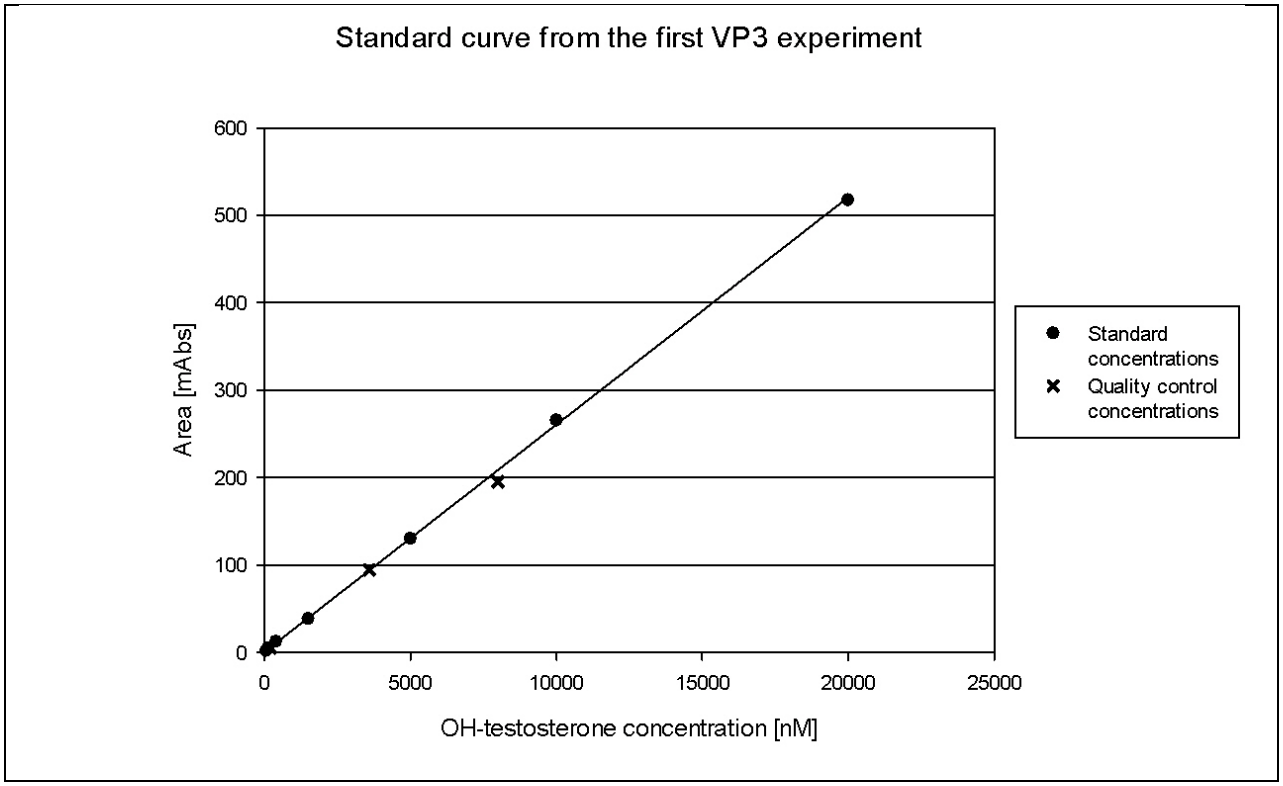

Figure 20: The VP3 standard curve from the first experiment. Regression equation: y = 0.0260x + 0.8622, R^2-value: 0.9996.

Table 17: Data from the first VP3 experiment showing the accuracy for all QCs.

QC	Nominal concentration [nM]	Calculated concentration [nM]	Accuracy (%)
1	200	174.5	87.3
1	200	163.0	81.5
2	3600	3613.0	100.4
2	3600	3613.0	100.4
3	8000	7486.1	93.6
3	8000	7482.2	93.5

8.2.2 Second experiment with VP3

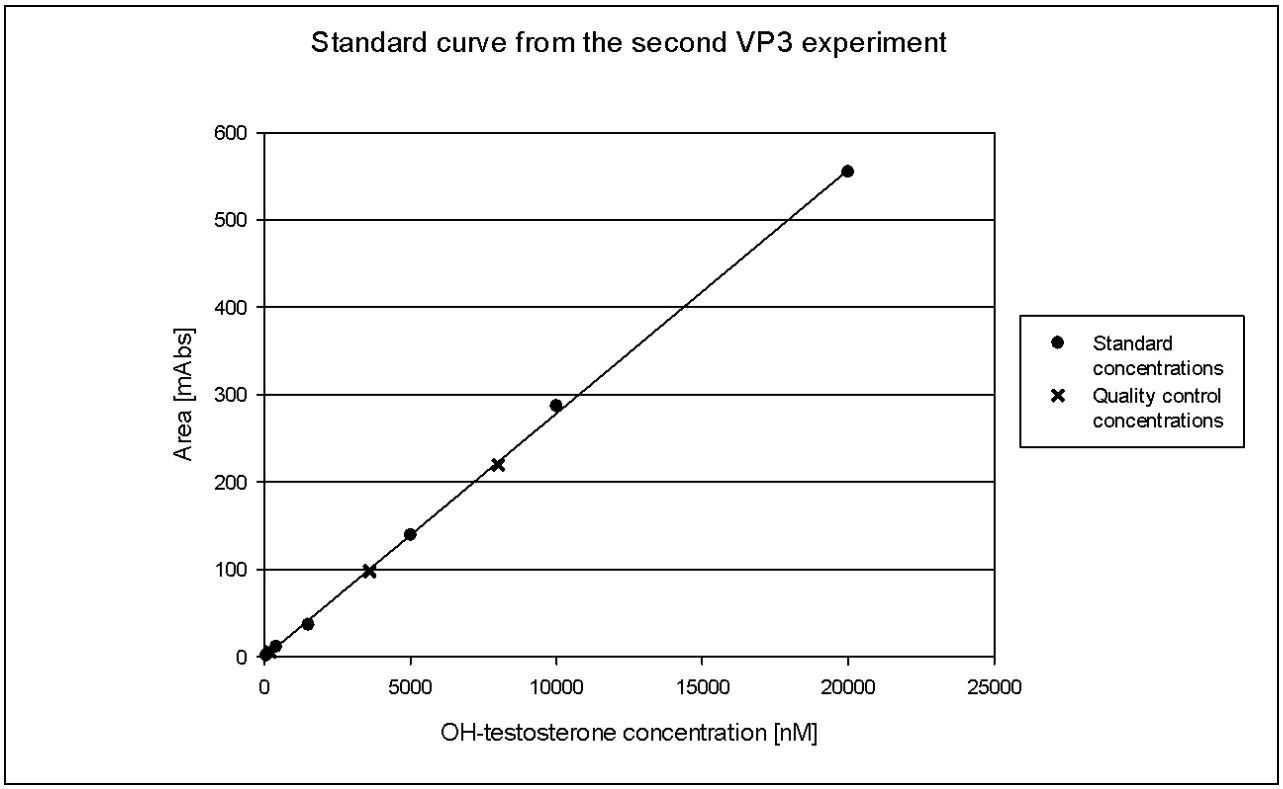

Figure 21: The VP3 standard curve from the second experiment. Regression equation: y = 0.0279x + 0.1807, R^2-value: 0.9988.

Table 18: Data from the second VP3 experiment showing the accuracy for all QCs.

QC	Nominal concentration [nM]	Calculated concentration [nM]	Accuracy (%)
1	200	197.8	98.9
1	200	197.8	98.9
2	3600	3484.6	96.8
2	3600	3534.7	98.2
3	8000	7860.9	98.3
3	8000	7864.5	98.3

8.2.3 First experiment with VP4

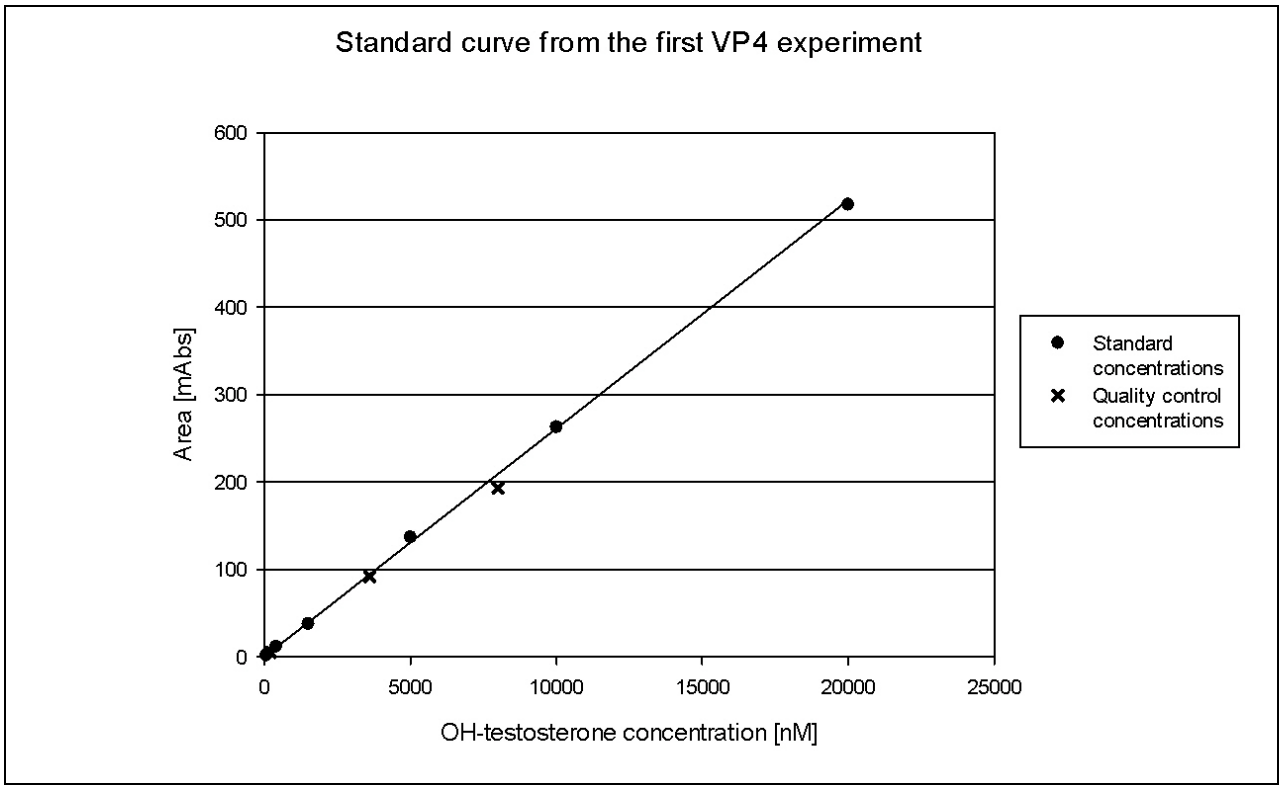

Figure 22: The VP4 standard curve from the first experiment. Regression equation: y = 0.0261x + 0.5069, R^2-value: 0.9994.

Table 19: Data from the first VP4 experiment showing the accuracy for all QCs.

QC	Nominal concentration [nM]	Calculated concentration [nM]	Accuracy (%)
1	200	179.8	89.9
1	200	176.0	88.0
2	3600	3513.1	97.6
2	3600	3490.2	97.0
3	8000	7386.7	92.3
3	8000	7386.7	92.3

8.2.4 Second experiment with VP4

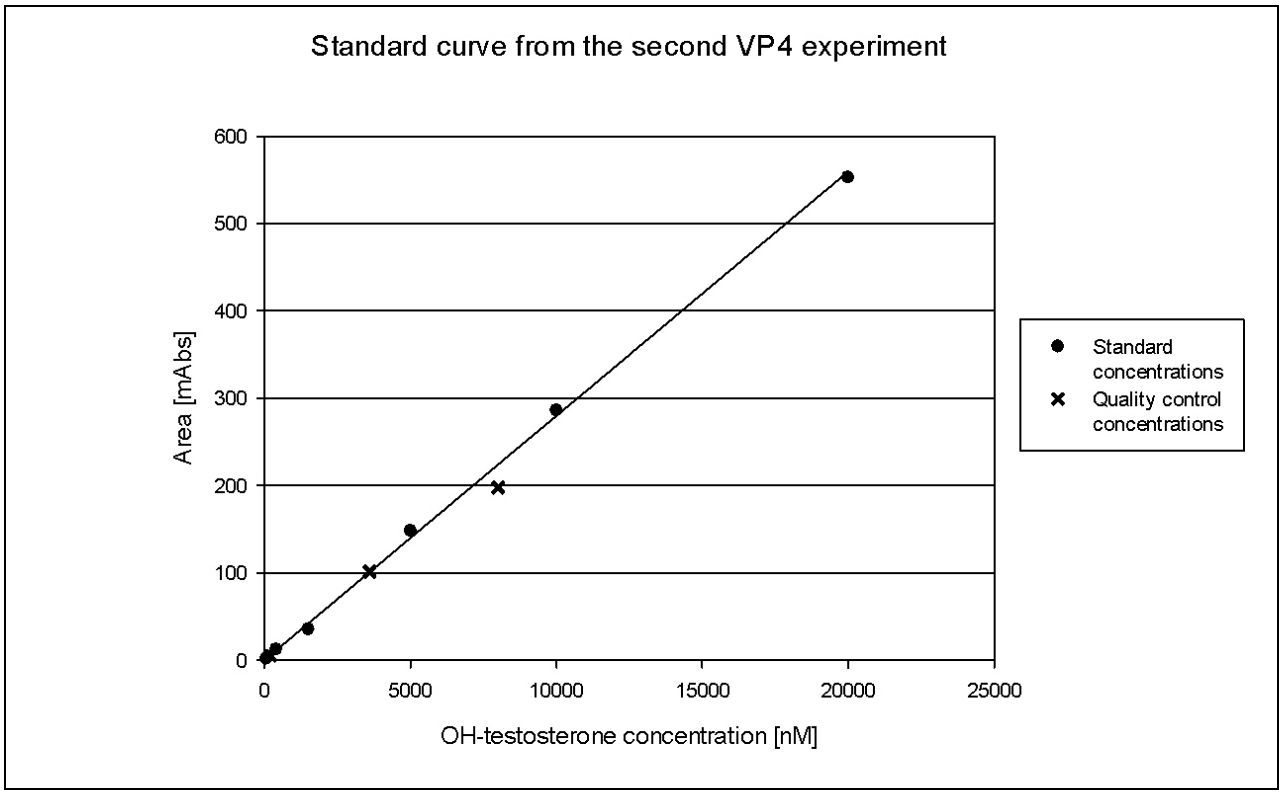

Figure 23: The VP4 standard curve from the second experiment. Regression equation: y = 0.0280x + 0.4617, R^2-value: 0.9979.

Table 20: Data from the second VP4 experiment showing the accuracy for all QCs.

QC	Nominal concentration [nM]	Calculated concentration [nM]	Accuracy (%)
1	200	172.8	86.4
1	200	169.2	84.6
2	3600	3629.9	100.8
2	3600	3590.7	99.7
3	8000	7065.7	88.3
3	8000	7040.7	88.0

8.3 Adverse drug reactions and interactions with VitaePro reported to RELIS

Table 21: An overview of all suspected ADRs and interactions with VitaePro reported to RELIS per December 17th 2015. (S) = suspected drug, (C) = concomitant drug, (I) = interacting drug.

Date	Involved drugs or substances	Clinical or biochemical manifestations
04/22/2008	ADALAT OROS (C) VitaePro (S)	Papular rash Urticaria generalized
08/20/2008	AMARYL (C) CALCIGRAN FORTE (C) DIGITOXIN (C) DIURAL (C) MAREVAN (C) TRIOBE (C) Vitaepro (S)	Obstipation
12/03/2008	VitaePro (S)	Insomnia Irritable excitation Restless
12/05/2008	DIGITOXIN (C) MAREVAN (I) TRIOBE (C) Astaxanthincomplex (I) Luteincomplex (I)	INR increased
01/26/2009	IMIGRAN (C) PARACETAMOL (C) TEGRETOL RETARD (I) TRAMADOL (C) VitaePro (I)	Drug interaction Drug level in blood decreased Epilepsy aggravated

02/09/2009	ACETYLSALICYLIC ACID (C) NAPROXEN (C) VITAEPRO (S) VOLTAREN (C)	Arthritis rheumatoid aggravated
03/31/2009	VitaePro (S)	Dizziness Nausea Syncopal attack
04/16/2009	AMLODIPINE (C) Omega-3 fatty acid (S) SIMVASTATIN (C) VOLTAREN (S) VitaePro (S)	Bleeding gastric ulcer
06/08/2009	MAREVAN (I) Vitaepro (I)	INR increased
08/21/2009	AMLODIPIN ACTAVIS (C) FINASTERID ACTAVIS (C) GLUKOSAMIN COPYFARM (C) LANSOPRAZOLE (C) OMEGA-3-TRIGLYCERIDES INCL. OTHER ESTERS AND ACIDS (C) PARALGIN FORTE (C) VENTOLIN (C) VitaePro (S)	Discomfort Itching Rash NOS
12/18/2009	VitaePro (S)	Elevated liver enzymes

09/09/2010	MAREVAN (I)	INR decreased
	VitaePro (I)	
03/14/2013	AMLODIPINE (C)	Hyperkalemia
	ARANESP (C)	
	ATACAND (C)	
	BURINEX (C)	
	CARDURAN CR (C)	
	NATRIUMBIKARBONAT (C)	
	PARACETAMOL (C)	
	RENAVIT (C)	
	RENVELA (C)	
	ROCALTROL (C)	
	SELO-ZOK (C)	
	SIMVASTATIN (C)	
	TRAMADOL (C)	
	VITAEPRO (S)	
06/22/2015	IMOVANE (C)	Feeling cold
	LIVIAL (C)	Sweating attack
	SPERSADEX MED	
	KLORAMFENIKOL (C)	
	VITAEPRO (S)	