Preparing of HPLC method for cholecalciferol determination and fat content assessment in grains

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I. Theoretical part


2. Theoretically treat about vitamin D and its physiological importance.

3. The principal of fat content determination in cereals.

4. Briefly treat about lipids in cereals.

II. Experimental part

1. Characteristics of the samples, chemicals and appliance.

2. Implementation of HPLC methodology for vitamin D determination on liquid chromatograph Dionex Ultimate 3000 using cholecalciferol standard.

3. Fat content determination in cereals.

4. Results, discussion, conclusion.
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ABSTRAKT

Czech abstract

Pšenice je jednou z nejvíce pěstovaných obilnin ve světě a díky vyššímu obsahu lipidů je významným zdrojem energie a obživy pro obyvatelstvo. Prvním cílem diplomové práce bylo nalézt vhodnou metodu pro stanovení celkového obsahu tuku v pěti vybraných družích pšenice. Byly proto vyzkoušeny tři metody vhodné pro stanovení tuků. V první metodě byla použita směs chloroformu a methanolu, druhou metodou byla Soxhletova extrakce a jako třetí byla testována ISO norma 7302. Nejpřesnější výsledky (v porovnání s deklarovanými hodnotami) byly dosaženy postupem podle metody ISO 7302. Druhá část práce byla zaměřena na zavedení HPLC metody pro stanovení cholecalciferolu. Pomocí standardu byly zjištěny optimální podmínky pro jeho stanovení, které byly při vlnové délce 265 nm a průtoku mobilní fáze 0.6 ml.min⁻¹. Retenční čas cholecalciferolu byl za těchto podmínek 6.5 – 6.6 min. Tyto hodnoty byly poté použity při vlastním stanovení cholecalciferolu v mléce.

Klíčová slova: obiloviny, pšenice, tuk, cholecalciferol, HPLC

ABSTRACT

English abstract

The wheat is one of the most cultivated cereal in the world and due to the higher content of lipids, it is important source of energy and living for the population. The primarily aim of the thesis was to find suitable method for total fat content determination in five varieties of the wheat. They were tested three methods. The first one used mixture of chloroform and methanol, the second method was the Soxhlet extraction and in the third case was tested the ISO norm 7302. The most exact results (in comparison with declared values) were achieved with the procedure according to the ISO norm method. The second focus of this thesis was on preparing of HPLC method for cholecalciferol determination. They were established optimal conditions for its determination using the standard which were at the wavelength of 265 nm and at the mobile phase flow rate of 0.6 ml.min⁻¹. The retention time was 6.5 - 6.6 min. These conditions were used for cholecalciferol determination in milk.

Keywords: cereals, wheat, lipids, cholecalciferol, HPLC
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Acknowledgements, motto and a declaration of honour saying that the print version of the Master's thesis and the electronic version of the thesis deposited in the IS/STAG system are identical, worded as follows:

I hereby declare that the print version of my Master's thesis and the electronic version of my thesis deposited in the IS/STAG system are identical.
CONTENTS

INTRODUCTION ........................................................................................................... 10
THEORY ..................................................................................................................... 11
1 CEREALS.................................................................................................................. 12
  1.1 ANATOMY OF CEREAL GRAIN AND ITS CHEMICAL COMPOSITION .......... 13
  1.2 WHEAT AND ITS CLASSIFICATION ................................................. 15
     1.2.1 Kamut (Khorasan wheat) ............................................................. 16
     1.2.2 Green spelt ................................................................................. 16
     1.2.3 Spelt wheat ................................................................................. 17
2 VITAMIN D AND ITS HEALTH EFFECT ................................................. 19
3 METHODS OF VITAMIN D DETERMINATION .................................... 23
  3.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ......................... 24
     3.1.1 INSTRUMENTATION ................................................................ 25
4 LIPIDS IN CEREALS............................................................................................ 30
5 TOTAL FAT CONTENT DETERMINATION ............................................. 31
ANALYSIS ................................................................................................................ 32
6 METHODOLOGY ................................................................................................. 33
  6.1 CHEMICALS ......................................................................................... 33
  6.2 INSTRUMENTS ...................................................................................... 33
  6.3 METHODS OF TOTAL FAT CONTENT DETERMINATION ...................... 34
     6.3.1 SAMPLES .................................................................................. 34
     6.3.2 FAT CONTENT DETERMINATION BY EXTRACTION OF
         CHLOROFORM AND METHANOL MIXTURE .................................. 35
     6.3.3 FAT CONTENT DETERMINATION BY SOXHLET EXTRACTION ....... 36
     6.3.4 TOTAL FAT CONTENT DETERMINATION BY CSN ISO 7302 ............ 36
  6.4 PREPARING OF HPLC METHOD FOR CHOLECALCIFEROL
      DETERMINATION BY HPLC – UV/VIS ............................................. 37
     6.4.1 DETERMINATION OF CHROMATOGRAPHIC CONDITIONS FOR
         CHOLECALCIFEROL DETECTION BY HPLC-UV/VIS ....................... 37
     6.4.2 MEASURING OF THE CHOLECALCIFEROL CALIBRATION CURVE
         BY HPLC – UV/VIS ..................................................................... 38
     6.4.3 SEPARATION OF CHOLECALCIFEROL AND ERGOCALCIFEROL
         STANDARDS BY HPLC – UV/VIS ................................................... 38
  6.5 PREPARING OF HPLC METHOD FOR A-TOCOPHEROL
      DETERMINATION BY HPLC – UV/VIS ............................................. 39
     6.5.1 DETERMINATION OF CHROMATOGRAPHIC CONDITIONS FOR
         A-TOCOPHEROL DETECTION BY HPLC – UV/VIS ......................... 39
     6.5.2 MEASURING OF THE A-TOCOPHEROL CALIBRATION CURVE
         BY HPLC – UV/VIS ..................................................................... 39
     6.5.3 SEPARATION OF CHOLECALCIFEROL AND A-TOCOPHEROL
         STANDARDS BY HPLC – UV/VIS ................................................... 40
INTRODUCTION

Vitamin D belongs to the group of fat soluble vitamins. The main function of this vitamin is to maintain serum calcium and phosphorus levels which are important for bone mineralization. The required intake of vitamin D is between 200 and 600 IU – International Unit (5 – 15 µg) a day, depending on the age. Unfortunately, the common food contain low amount of this vitamin and because of that, most of the advanced countries fortify their food by vitamin D as prevention of rickets. The most important sources of vitamin D are cod liver oil and fish, such as salmon, herring and cod. From the frequently consumed food, the significant sources are butter, margarine, eggs and liver.

Cereals are the most important source of living. People all over the world eat more cereals than any other kind of food. They strongly influence nutritional balance, because they contain all necessary nutrients including fats and vitamins. In many regions, cereals are the main source of saccharides and proteins for inhabitants. Wheat was one of the first cereal grains to be cultivated and it spread to all parts of the world because of its good adaptability to various climates and soils. It grows on every continent except Antarctica.

The increasing interest in level of fats has heightened the need for development of new varieties of cereals, which have constitution that we need, e.g. lower level of fats or more vitamins.

The primarily aim of this thesis is to find an optimal method for total fat content determination in cereals. Afterwards, the method is tested with five varieties of wheat, such as “khorasan wheat”, green spelt, spelt wheat, bread wheat and spelt groats. The second purpose is to set up conditions for vitamin D determination on new HPLC apparatus Dionex Ultimate 3000. The setting of optimal wavelengths and mobile phase flow rate is done by vitamin D\(_2\) and D\(_3\) standards. Consequently the parameters are used for vitamin D determination in milk and margarine.
I. THEORY
Cereals are cultivated grasses which are extended worldwide. They can grow in temperate and tropical regions as well. Cereals were named according to the Roman goddess Ceres, who was the grain giver. They are still very important today because all sorts of cereals are staple food for population. Their world productivity is around three tonnes per hectar in average. People use primarily the grain; the other parts of the plant can be used as animal feed or animal bedding [1, 2].

Cereals belong to the large monocotyledonous plant family called the *Gramineae*. The flowering organs grow on a stem called the rachis, which can be branched, and in turn bears spikelets which may carry more flowers at each node of the rachilla. The spikelets can be arranged in a loose panicle or in a tight spike. The length of the internodes of the rachis and of the rachilla, and the number of flowers at each node of the spikelet design the overall structure. Each spikelet is subtended by two bracts or leaf-like organs named the glumes, and each flower in the spikelet is enclosed in two bract–like organs called the lemma and palea. The lemma may be elongated to form a long awn. In some cereals or cereal varieties the lemma and palea may remain attached to the grain; these are termed hulled or husky grains, such as oats and most barley, as opposed to naked grains such as most wheat and maize [1]. The structure of cereal flowering organs can be seen in figure 1.

Most of the cereals are dioecious. Each flower has male organs which are the three anthers, and female organs, the ovary, which carries two feathery stigmas [1].
1.1 Anatomy of cereal grain and its chemical composition

As mentioned above, the cereal grain is used for human nutrition and has been a staple food for humans for thousands of years. Botanically, the grain is fruit and named *caryopsis*. Morphological grain structure is the same in general, but it can differ in shape, size or share of layers [2, 3, 6].

Each cereal grain is made up of three main sections. The multi-layered outer skin of the kernel is known as bran. It accounts for approximately 14% of the seed and its function is to protect the other two parts of the kernel from sunlight, pests, water or disease. Bran is a fibre-rich part and contains other important nutrients such as vitamin B, minerals (iron, zinc, copper, magnesium) and phytonutrients [4, 6].

The main body of the kernel is the *endosperm*, which forms 84 - 86% of the grain weight and primarily consists of starchy saccharides, proteins and a small amount of vitamins and minerals. The *endosperm* ensures nutrition for the embryo and it is the element of final
products. Surrounding the endosperm is the aleurone layer, which makes up a relatively small part of the seed. The aleurone contains enzymes that can start the reaction which changes starch into sugar (maltose, maltotriose etc.) using grains for specialized uses such as brewing beer [4, 6].

The inner part of the kernel is the germ (2 – 3%). It sprouts into a new plant after fertilization by the pollen. It is rich in proteins, fats, saccharides, dietary fiber, vitamins B and E, antioxidants and phytonutrients. The germ is typically removed during milling of refined flour because it can influence bread making quality, and the oils in the germ can go rancid if the flour is stored for a long time [6, 7].

The chemical composition most of the cereals is very similar. The moisture content of commercial lots of wheat may vary between 12 and 18%, depending on the weather during the harvest. The whole grain consists of saccharides (65 - 70%), proteins (9 - 13%), fats (2%), dietary fibres (11%) and minerals (1.5 - 3%). The composition depends on species, growing conditions or variety [2, 4, 7].

![Cereal grain and its anatomy](image-url)  
*Figure 2: Cereal grain and its anatomy [10]*
1.2 Wheat and its classification

Wheat is the leading cereal grain produced, consumed and traded in the world today. The history of wheat cultivation reaches far back into ancient times and points to the Middle East as a possible geographical region of its origin [1, 3].

The genus *Triticum*, which involves thousands of species and varieties, can be divided into three groups differing in chromosome numbers. *Triticum monococccum* (einkorn) is a diploid species (haploid chromosome number 7) used as a human food in Egypt where it has been found in 4000 year old tombs of the Pharaohs. *Triticum dicocccum* (emmer) is a tetraploid wheat (haploid chromosome number 14) with ancient history of its cultivation, but still grown in some parts of Europe. The most used descend for pasta production is *Triticum turgidum* ssp. *turgidum* conv. *durum* (macaroni wheat). *Triticum aestivum* is a hexaploid and the most commonly grown wheat today [1, 8].

**Table 1**: Classification of the Genus *Triticum* [8]

<table>
<thead>
<tr>
<th>Systematic name (Chromosomes)</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Triticum monococccum</em> L.</td>
<td>einkorn wheat</td>
</tr>
<tr>
<td><em>Triticum monococccum</em> ssp. <em>boeticum</em> (Zoiss.)</td>
<td>wild einkorn wheat</td>
</tr>
<tr>
<td><em>Triticum monococccum</em> ssp. <em>monococccum</em></td>
<td>einkorn wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> L.</td>
<td>emmer wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> ssp. <em>dicocccoides</em> (Körn)</td>
<td>wild emmer wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> ssp. <em>dicocccum</em> (Schrank)</td>
<td>(true) emmer wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> ssp. <em>paleocolchicum</em></td>
<td>kolchis wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> ssp. <em>turgidum</em> conv. <em>turgidum</em></td>
<td>rivet wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> ssp. <em>turgidum</em> conv. <em>durum</em></td>
<td>macaroni wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> ssp. <em>turgidum</em> conv. <em>turanicum</em></td>
<td>khorasan wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> ssp. <em>turgidum</em> conv. <em>polonicum</em></td>
<td>polish wheat</td>
</tr>
<tr>
<td><em>Triticum turgidum</em> ssp. <em>carthlicum</em></td>
<td>dinkel wheat</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L.</td>
<td>spelt wheat</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> ssp. <em>spelta</em></td>
<td>spelt wheat</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> ssp. <em>macha</em></td>
<td>macha wheat</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> ssp. <em>vulgare</em></td>
<td>bread wheat</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> ssp. <em>compactum</em></td>
<td>club wheat</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> ssp. <em>sphaerococccum</em></td>
<td>Indian dwarf wheat</td>
</tr>
</tbody>
</table>
1.2.1 Kamut (Khorasan wheat)

Kamut is a brand name of the ancient wheat called khorasan. It is a relative of modern durum wheat originally grown in the region reaching from Mesopotamia to Egypt. Typical for khorasan wheat is that it has never been hybridized or genetically modified and has high quality standards. It is popular because of its natural sweet and nutty taste, high versatility, great nutrition and smooth texture [8].

It is important from the nutritional point of view as well – rich in proteins and minerals, specially selenium, zinc and magnesium. Due to the high content of selenium (400 – 1000 ppm), khorasan wheat is a very good source of this mineral. It can be also regarded as high-energy source. The high percentage of lipids provides more energy than saccharides in the body. This is the reason why khorasan wheat is suitable for people who are sensitive to modern wheat [8].

Table 2: Nutritional values of Khorasan wheat [8]

<table>
<thead>
<tr>
<th>Composition</th>
<th>Nutritional value [g .100 g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>14.98</td>
</tr>
<tr>
<td>Saccharides</td>
<td>59.30</td>
</tr>
<tr>
<td>Starch</td>
<td>56.97</td>
</tr>
<tr>
<td>Fat</td>
<td>2.21</td>
</tr>
<tr>
<td>Ash</td>
<td>1.68</td>
</tr>
<tr>
<td>Alimentary fibres</td>
<td>12.03</td>
</tr>
</tbody>
</table>

1.2.2 Green spelt

Green spelt is originally a spelt grain, which belongs to the winter crop. In the past, people feared the bad autumn weather thus they started to crop it in July as a green grain. After the harvest, the grain was roasted above fire made from the beech wood. Today, the process of the drainage is going on in the modern hot-air appliance. During the drying, proteins of the grain are destroyed so the flour cannot be used for baking. Otherwise, thanks to this process, grains are stable and get typical nutty taste and aroma [9].
Green spelt used to be grown in a small region in south Germany, called Bauland. The first Green spelt was made around 1660. People used it as trimmings or baked it. Nowadays, it is used all above into the soup [9].

1.2.3 Spelt wheat

Spelt wheat is one of the oldest cereals. It was grown in prehistoric times on the East. It has been grown in Europe for centuries, mainly in Italy, south Germany, Spain, Belgium, Switzerland, England and Poland. In the Czech Republic it was grown in the 18th century and was used as a coffee substitute. Nowadays, the production of spelt wheat increases in Europe, Canada and the USA again [9, 10].

Spelt is a hexaploid hulled wheat with bulky root system. It is resistant to disease of spike and leaves. Spelt has low nutrition requirements and the plant is valued for its high nutritional value of the seed [9, 10].

Spelt wheat contains almost all nutrients which are important for human nutrition. In comparison with Triticum aestivum L., it contains more proteins (16 – 17%) because of the larger aleurone layer. It is rich in gluten (35 - 45%) and has high mineral content (K, Mg, S). It is also a rich source of vitamins B (thiamin and riboflavin), easily digestible, a good source of the fibre and has a positive impact on the immune system [9, 10].

Spelt is used for a wide range of products which are made from it. They are husked grains, groats, green grains, semolina, flour, spelt bulgur, pasta, flakes, müsli, pop spelt, spelt beer or spelt coffee. Its nutty taste, high nutritional value and good digestibility are valuable in bakery and confectionary [9, 10].
Figure 3: 1 – Bread wheat; 2 – Kamut (Khorasan wheat); 3 – Green spelt; 4 – Spelt wheat
2 VITAMIN D AND ITS HEALTH EFFECT

Vitamin D is an essential micronutrient for certain human population. It is fat-soluble vitamin, which plays an important role, along with the essential minerals calcium and phosphorus, in the maintenance of healthy bones and teeth. Vitamin D belongs to the group of lipophilic steroid compounds with antirachitic effect, known as calciferols. The most common forms, having vitamin D activity in foods, are ergocalciferol (vitamin D$_2$) and cholecalciferol (vitamin D$_3$), which differ in their side chain and their appearance. Ergocalciferol and its provitamin D$_2$ form ergosterol is produced in plants, fungi, molds and lichens by the irradiation of ergosterol. Cholecalciferol is widely distributed in animals and is produced by the action of sunlight on 7-dehydrocholesterol in the skin. On irradiation, the provitamins are converted to previtamin D which undergoes thermal transformation to vitamin D [11, 12, 13, 14, 15].

The hormonally active form is 1,25-dihydroxyvitamin D$_3$. Its latter metabolite stimulates the intestine to absorb calcium and phosphate by two independent mechanisms and acts with parathyroid hormone to mobilize calcium, accompanied by phosphate from the bone fluid compartment into the bloodstream. Thus, the most important physiological functions of vitamin D are stimulating the intestinal uptake of Ca$^{2+}$ and phosphate, and promoting the incorporation of these ions into bones [14, 15, 16].

![Ergocalciferol and cholecalciferol](image)

**Figure 4:** Ergocalciferol and cholecalciferol
Table 3: Adequate intakes for vitamin D [17]

<table>
<thead>
<tr>
<th>Age / Sex</th>
<th>Vitamin D [µg]</th>
<th>Vitamin D [IU]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants, Children and Adolescence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 18 years</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 to 50 years</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>51 to 70 years</td>
<td>10</td>
<td>400</td>
</tr>
<tr>
<td>70+</td>
<td>15</td>
<td>600</td>
</tr>
<tr>
<td>Pregnant woman</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>Nursing woman</td>
<td>5</td>
<td>200</td>
</tr>
</tbody>
</table>

Certain human population depends on dietary sources of vitamin D because of insufficient biosynthesis of the vitamin due to the inadequate skin exposure to the sunlight. Most food contain only low amount of vitamin D, so it has become the practice in many countries to fortify frequently consumed foods (e.g. baked goods, grain products, milk and milk products, infant foods) to prevent the rickets. For the fortification of foods are used both vitamin D$_2$ and vitamin D$_3$. Other foods are enriched indirectly as the result of the supplementation of animal feeds with the vitamin [14, 19].

Table 4: Vitamin D activities in food [14]

<table>
<thead>
<tr>
<th>Food</th>
<th>Vitamin D [IU.100g$^{-1}$]</th>
<th>Food</th>
<th>Vitamin D [IU.100g$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine milk</td>
<td>0.3 – 54</td>
<td>Herring</td>
<td>330</td>
</tr>
<tr>
<td>Butter</td>
<td>35</td>
<td>Salmon</td>
<td>220-440</td>
</tr>
<tr>
<td>Eggs</td>
<td>28</td>
<td>Cabbage</td>
<td>0.2</td>
</tr>
<tr>
<td>Cod</td>
<td>85</td>
<td>Corn oil</td>
<td>9</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>10,000</td>
<td>Spinach</td>
<td>0.2</td>
</tr>
</tbody>
</table>

The International Unit (IU) is a unit of measurement for the amount of a substance which is based on measured biological activity or effect. 1 IU of vitamin D activity is equivalent to 25 ng of cholecalciferol and 1µg of cholecalciferol is equivalent to 40 IU [18].
The vitamin D deficiency can result from inadequate irradiation of the skin, from insufficient intake from the food or from impairments in the metabolic activation of the vitamin. Although the sunlight can provide the means of biosynthesis of vitamin D, it is well-documented fact that many people, particularly those in extreme latitudes during the winter months, do not receive sufficient solar irradiation to support adequate vitamin D status. Even people in sunnier climates may not produce adequate vitamin D if their life keeps them indoors [14].

The classic symptoms of the vitamin D deficiency are bone softening and weakening. The skeletal diseases are rickets in children and osteomalacia in adults [14].

Rickets first appears in 6- to 24-month children with the impaired mineralization of the growing bones and accompanying bone pain, muscular tenderness, and hypocalcemic tetany. Tooth eruption can appear later. Older children show deformations of the softened femurs which bend under the weight of the body. Rickets is frequently associated with low dietary intakes of calcium, as in the avoidance of milk products [14].

Osteomalacia signs and symptoms are more generalized than those of rickets, e.g. muscular weakness and bone tenderness, particularly in the spine, shoulder, ribs, or pelvis. Lesions involve the failure to mineralize bone matrix which continues to be synthesized by functional osteoblasts. Patients with osteomalacia are at increased risk of fractures of all types but particularly those of the wrist and pelvis. Deficient intake of vitamin D can be also one of the sources of osteoporosis in old age because the ability to synthesize vitamin D in skin is lower than in young age [11,14].

The abundance (hypervitaminosis) can cause anorexia, vomiting and calcium loosening from bones which can set in kidney or stomach. Also mental expression and cardiac arrhythmia can appear [12].

However, no mutagenic, cancerogenic or teratogenic effects were observed. In the view of fact that vitamin D and its metabolites can go through the placenta to the embryo, it is important to keep in mind teratogenic hazard for women who have high level of vitamin D in blood. It can cause psychical and mental disturbances in newborns [12].

Recent studies also demonstrate that vitamin D may have positive influence on hypertension, colon cancer and autoimmune diseases.
Figure 5: Findings in patients with rickets [20]
3 METHODS OF VITAMIN D DETERMINATION

High performance liquid chromatography (HPLC) has been the method of choice for determining fat-soluble vitamins in food since the middle of 1970s to the present day. It is the only recognized standard method for determining any of the fat-soluble vitamins, which can be applied to all types of food. The advantage of this method is due to no need of derivatization, nondestructive operation, greater separation and detection selectivity. The nondestructive nature of HPLC allows it to be used as a preparative purification method as well as a quantitative technique [15].

Most of the published HPLC methods for determining vitamin D in foods are concerned with estimating the vitamin D content in supplements, such as milk in various forms, infant formulas and margarine. The amount of naturally occurring vitamin D in supplemented foods is usually negligible and it is necessary to determine only the vitamin D that is added. The levels of vitamin D are very low (e.g. 7.5 - 12.5 \(\mu g\cdot 100g^{-1}\) in milk powder) [15].

First of all, the representative sample must be prepared. The amount of the sample material which is used for analyses depends on the material. The vitamin should be quantitatively extracted from the food matrix in a form that can be measured by the HPLC technique. An effective extraction procedure serves to homogenize and concentrate the sample, isolate the vitamin analyte from its association with protein, eliminate known interfering substances and destroy any indigenous enzyme activity. Vitamin D, as well as the other fat-soluble vitamins, is photosensitive. Therefore all operations with vitamin solutions and vitamin-containing materials must be carried out in subdued light or in dark glassware [15].

Methods of extracting the fat-soluble vitamin from food matrices include alkaline hydrolysis, enzymatic hydrolysis, alcohohlysis, direct solvent extraction, and supercritical fluid extraction of the total lipid component. The prepared extracts may require some form of cleanup before the vitamin can be measured. The requirement of cleanup depends upon the ration of analyte to interfering substances and also upon the sensitivity and selectivity of the used HPLC method [15].
3.1 High Performance Liquid Chromatography

High performance liquid chromatography is a method which separates compounds that are dissolved in the sample. The separation of these components depends on different migration. Basically, it is migration in the system of two phases – stationary phase and mobile phase. The sample is applied between these two mutually immiscible phases. The mobile phase is going through the stationary phase and the sample is drifted by the mobile phase. Components of the sample can be caught up by stationary phase. They are hold there and gradually separated [21, 22].

There are many ways to classify liquid column chromatography. If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified [24]:

1. Adsorption chromatography
2. Ion-exchange chromatography
3. Size exclusion chromatography

In adsorption chromatography the stationary phase is an adsorbent (like silica gel or any other silica based packings) and the separation is based on repeated adsorption-desorption steps. Concerning this type of chromatography, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography [24].

In normal-phase chromatography, the stationary bed is strongly polar in nature (e.g. silica gel) and the mobile phase is nonpolar (such as n-hexane or tetrahydrofuran). Polar samples are thus retained on the polar surface of the column packing longer than less polar materials [24].

Reverse-phase chromatography is opposite of normal-phase. The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is polar liquid, such as mixtures of the water and methanol or acetonitrile [24].

Eluent polarity plays the highest role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type, constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run [24].
Adsorption chromatography depends on the chemical interactions between solute molecules and specifically designed ligands chemically grafted to chromatography matrix. Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can be separated by reversed phase chromatography [23].

The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic ligand, i.e. the stationary phase [23].

### 3.1.1 Instrumentation

HPLC instrumentation includes mobile phase reservoir, pump, injector, separation column, detector and data system [24].

The chromatographic process begins by injecting of the solute onto the top of the column. Separation of the components starts when the analytes and the mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (peak) on the recorder. Detection of the eluting components is important and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram [24].

![Figure 6: Scheme of an HPLC instrument](image-url)
**Mobile phase reservoir:** The most common type of the solvent reservoir is a glass bottle. Mostly these bottles are supplied with special caps, teflon tubing and filters to connect to the pump [24].

The HPLC **pump** is considered to be one of the most important components in a liquid chromatography system. Pump provides a continuous constant flow of the eluent through the HPLC injector, column and detector. There are two basic classifications of the pumps: constant-pressure pump which is mainly used for column packing, and constant-flow pump widely used in all common HPLC applications [24].

**Injector:** The samples are injected into the HPLC by an injection port. Usually it consists of an injection valve and a sample loop. The sample is dissolved in the mobile phase before the injection into the sample loop. Then it is drawn into a syringe and injected into the loop by the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 µl to over 500 µl. Today, the sample injection is typically automated in modern HPLC systems [26].

Stopped-flow injection is a method whereby the pump is turned off allowing the injection port to attain atmospheric pressure. The syringe containing the sample is then injected into the valve in the usual manner, and the pump is turned on. For syringe type and reciprocation pumps, the flow in the column can be brought to zero and rapidly resumed by diverting the mobile phase by means of a three-way valve placed in front of the injector. This method can be used up to very high pressures [26].

![HPLC injector scheme](image)

**Figure 7:** HPLC injector scheme [27]
The **column** is the heart of the system where separation occurs. The columns used for HPLC are generally made of stainless steel and are manufactured. Straight columns of 15 to 50 cm length and 1 to 4 mm diameter are generally used, although smaller microbore or open tubular columns are available. Microbore columns have an internal diameter of 1 to 2 mm and are generally 25 cm long. They can sustain the flow rates of 0.05 to 0.20 cm$^3$.min$^{-1}$ as opposed to 2 cm$^3$.min$^{-1}$ of conventional HPLC columns [29].

The separation effect, time of the analysis and the pressure go up with rising length of the column. Contrariwise they go down with rising particles diameter.

The preparative columns are also available with internal diameter of up to 25 mm. They can sustain flow rates of up to 100 cm$^3$.min$^{-1}$. The most important preparative column feature is that it protects column against impurities and indissoluble materials. Also partial separation can occur here [29].

![HPLC column scheme](image)

**Figure 8:** HPLC column scheme [28].

The **detector** for HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled [30].
There are many types of the detectors that can be used with HPLC. Some of the most common detectors are ultraviolet-visible (UV-VIS), fluorescence, refractive index (RI), radiochemical, electrochemical (EC), infra-red (IR), mass spectral (MS), nuclear magnetic resonance (NMR), and light scattering (LS) [30].

**UV – VIS detector:** In most cases HPLC method development is carried out with ultraviolet detection using either a variable-wavelength or a diode-array detector (DAD). The popularity of this detector is because of its easy using, low purchase prize and broad application area. The detector measures the change in absorbance of light in the 190 - 350 nm region (UV) or the 350 – 700 nm region (visible). A filter or grating is used to select the required wavelength for measurement. Ultraviolet lights sources are either mercury lamps, or deuterium and xenon lamps. Halogen lamps are used in VIS region. The light from the lamp passes through an UV-transmitting flow cell connected to the column and impinges on a diode which measures the light intensity. It is possible to change the detection wavelength during measurements, for the filters can be switched via keyboard, time program, or external analog control. UV-VIS detectors are concentration sensitive. The sensitivity is approximately $10^{-8}$ or $10^{-9}$ mg.ml$^{-1}$ [15, 30, 34].

**The photodiode array detector** (PDA) passes the total light through the flow cell and disperses it with a diffraction grating. The dispersed light is measured by an array of photosensitive diodes. The array of diodes is scanned by the microprocessor. The reading for each diode is summed, and the total is averaged. PDA detectors can simultaneously measure the absorbance at all wavelengths versus time. The amount of data storage is a key feature in the PDA [15].

**Fluorescence detectors** are very sensitive and selective for solutes that fluoresce when are excited by UV radiation. They measure the ability of a compound to absorb then re-emit light at given wavelengths. Each compound has a characteristic fluorescence. The excitation source passes through the flow-cell to a photodetector while a monochromator measures the emission wavelengths. Fluorescence detection is usually more sensitive than absorption detection. It has sensitivity limit of $10^{-9}$ to $10^{-11}$ mg.ml$^{-1}$ [30, 31].

For **refractive index detector** is used mechanism where a light beam is refracted to different extents by different compounds. This detector is not sensitive and the selectivity differences are negligible for homolous compounds [31].
Infrared detector is often used for qualitative analysis of limited range of organic solvents. Every organic molecule absorbs infrared light at one wavelength or another. The mobile phase must not be self-absorbent at the required wavelength. The suitable mobile phases are hexane, dichloromethane or acetonitrile. The sensitivity is not greater than that of refractive index detectors [33, 34].

Electrochemical detectors are based on the electrochemical oxidation or reduction of the analyte and can be applied to the analysis of selected compounds such as phenols. However, the adsorption of reacted molecules on the surface of the electrodes can reduce the conductivity that is why a pulse voltage is applied and it cleans the electrode surface between measurements [31].

The mass detectors are very sensitive and selective instruments. The principle of their function is based on weighting the mass of solute eluted from the column. Mass spectrometers have three distinct features: the source, the analyzer and the detector. Differences in these three components differentiate the types of MS techniques that are useful with HPLC. For all MS techniques, the analyte is first ionized in the source, since the MS can only detect charged species. Ions of discrete mass/charge ratios are then separated and focused in the mass analyzer. The final focused beam impinges on a detector that determines the intensity of the beam [33].

The last part of HPLC apparatus is data system. The visualization of the detector signals helps to clarify the separation conditions. Pen recorders were originally used but now the integrator is popular due to the automatic reporting of both the retention time and the peak area or the height. The use of the integrator makes quantitative analyses easier. Computer-based integrators are powerful for the storage and further arrangement of data and can also be used for the column evaluation and as a system controller [31].
4 LIPIDS IN CEREALS

Cereal grains contain relatively low levels of lipids, usually from 1 to 3% in barley, rice, rye and wheat and from 5 to 9% in corn and oat. These lipids are, nevertheless, important constituents of the grain and grain products. Most of the lipids are present in the germ which, in the case of wheat and corn, serves as a source for oil production. The weight percentage of the germ from the whole grain is only about 2.54% but the amount of lipids there is about 64%. On the other hand, the endosperm which composes 80% of the grain weight, contains only about 3.3% lipids. The germ and the aleurone cells consist of triglycerides while phospholipids and glycolipids (fatty materials containing phosphorus and sugars respectively) predominate in the endosperm [3, 34, 35].

Cereal lipids do not differ significantly in their fatty acid composition. The fatty acids in the cereal lipids are mostly polyunsaturated. The predominant acid is a linoleic acid. The total amount of all unsaturated lipids in cereals is about 75% which is good news in terms of nutrition but a problem during long storage of flour; oxidation of the polyunsaturated acids can lead to off-flavours. Wheat lipids also influence baking quality [34, 35].

The wheat flour contains 1.5 – 2.4% lipids, depending on milling extraction rate. Part of this lipid is nonstarch lipid which can be extracted at room temperature with a polar solvent, water-saturated butanol. This lipid comprises about 75% of the total lipid in flour. The remaining 25% are bound to starch lipids. Nonstarch-bound and starch-bound lipids in wheat differ in their composition. The major constituents in nonstarch-bound lipids are triacylglycerides and digalactosyl diacylglycerides, while in starch-bound lipids the major constituents are lysophosphatides. A decrease in amyllose content is accompanied by a decrease in the lipid content. The ratios of nonstarch-bound lipids classes are dependent on the flour extraction grade. An increase in extraction grade increases the triacylglyceride content, since more of the germ is transferred into the flour [35].
5 TOTAL FAT CONTENT DETERMINATION

Lipids can be generally divided into two groups: simple and complex lipids. Simple lipids include esters of fatty acids and glycerol (triglycerides) and unsaponifiable matter, which are soluble in non-polar organic solvents such as hexane or light petroleum. Complex of polar lipids include phospholipids, glycolipids, lipoproteins, oxidized or polar glycerides, and free fatty acids. Because of these components, complex lipids are extracted in polar solvents, such as methanol [12].

The determination of the total fat content is very common in food analysis. There is need to use standard method for relevant material or there can be used one of the physical method. The method for lipid phase compound determination is more complicated. The lipids must be isolated by milder extractive method and the extract is fractionated by one of the chromatographic methods [36].

The isolation procedure is different and depends on the method. If there is used mild isolation method (e.g. Soxhlet analysis), there are often used hexane, petrolether, diethylether or chloroform as solvents. The lipids from liquid material must be extracted by more polar system, mostly chloroform and methanol but there can be used also ethanol or diethylether and methanol [36].

The choice of the extraction method is very important for the further characterization of the lipid material. The extraction of fat can be performed for quantitative determination of the fat content or to simply obtain lipids for further investigation by chemical or chromatographic methods. Some of the methods are standardized by national and international organization for one type of the product [37].

The very common method for the total fat content determination is a Soxhlet extraction. It is used to extract solutes from solids, using any desired volatile solvent, which can be water-miscible or water-immiscible. The method is suitable for analysis of materials rich in neutral lipids and with low water content. The system of Soxhlet extraction has three components. A solvent vapor reflux condenser is the top part. The middle part involves a thimble holder with a siphon device and a side tube. The thimble holder connects to a round-bottomed flask at the bottom [38, 39].
II. ANALYSIS
6 METHODOLOGY

6.1 Chemicals

Chloroform (supplier Ing. Petr Lukeš, Uh. Brod)
n-Hexan (Ing. Petr Švec, PENTA)
Methanol (Ing. Petr Švec, PENTA)
LC-MS Grade Methanol for HPLC (Fisher Scientific UK Limited, Bishop)
Ethanol 96%
Formic acid 85% (Ing. Petr Švec, PENTA)
Hydrochloric acid 37% (supplier Ing. Petr Lukeš, Uh. Brod)
Potassium hydroxide (Ing. Petr Švec, PENTA)
Redistilled water

6.2 Instruments

Analytical balance (Adam – Alfa 210 LC, Kern)
Preparative balance (Kern, Germany)
Mixer BRAUN
Mixer HEIDOLPH instruments MR 1000
Fritted glass (S1 type)
Water bath MEMMERT
Vacuum evaporator RV-05-ST (IKA WERKE, Germany)
Soxhlet apparatus
Common laboratory glass
HPLC apparatus (Dionex Ultimate 3000)

- Mobile phase reservoir – glass bottle with GL cap
- Degasser
- Binary pump
- Autosampler
- Injector with valve needle for autosampler
- Preparative column MetaGuard 4.6 mm Polaris C18, 5 µm
- Column Acclaim 120 C18 (2.1 x 150 mm, 5 µm)
- UV/VIS detector (possibility of setting of 4 wavelengths from 190 to 650 nm)
- MS detector Amazon X (BRUKER)
- PC with software Chromelone Xpress and HyStar (ver. 3.2)

6.3 Methods of total fat content determination

6.3.1 Samples

For total fat content determination in cereals were chosen samples of five nontraditional species of wheat. They were Kamut (Khorasan wheat), Green spelt, Spelt wheat, Bread wheat and Spelt groats (kernotto). There were used BIO products from the PRO-BIO company, residing in the Czech Republic. The samples were stored in dry and dark space at the room temperature. All samples were pulverized before the analysis.

Table 5: Fat content in used samples declared by producer

<table>
<thead>
<tr>
<th>Kamut [g.100g(^{-1})]</th>
<th>Green Spelt [g.100g(^{-1})]</th>
<th>Spelt wheat [g.100g(^{-1})]</th>
<th>Bread wheat [g.100g(^{-1})]</th>
<th>Spelt groats [g.100g(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6</td>
<td>3</td>
<td>2.5</td>
<td>1.9</td>
<td>2.5</td>
</tr>
</tbody>
</table>
6.3.2  Fat content determination by extraction of chloroform and methanol mixture

This method was tested for fat content determination in cereals although it is useful all above for food of animal origin. They were done four series of testing. Based on the results, the method was modified in each series.

The first series of the analysis passed according to advised technique. It was weighed 5 g of the sample which was mixed with seventeen-fold volume of the chloroform and methanol mixture (2:1). Afterwards it was homogenized for 5 minutes, filtered by fritted glass and once again was added two-fold volume of the methanol and chloroform (2:1). Separated bottom part was moved into separatory funnel and was added 25 ml of redistilled water. The separatory funnel was shaken for a few minutes and then it was left to separate it. The next bottom part was moved into new separatory funnel, was added mixture of the chloroform – methanol - water in the rate of 3:48:47, was shaken for few minutes and was left to separate it again. The bottom part from this funnel was moved into empty dry and weighed titration flask and the solvent was evaporated on the vacuum evaporator. The temperature of the water was 40°C [36].

In the second analysis, the time of the homogenization was modified. Samples were homogenized for 10, 20, 30 resp. 40 minutes.

In the third analysis, it was poured 15 ml of redistilled water after 30 minutes of homogenization and afterwards it was homogenized again for 10 minutes.

In the fourth analysis was added twenty-fold volume of methanol - chloroform (2:1) instead of seventeen-fold volume as in previous experiments. The homogenization took for 1 hour and then it was added only 25 ml of redistilled water.

The sample was tested five times in each series.

The results were calculated as:

\[
R = \frac{A - B}{C} \cdot 100
\]

A  weight of evaporated flask (g)
B  weight of empty flask (g)
C  weight of the sample (g)
This method was tested only with Kamut. The aim of this experiment was to find out if it is available method for fat content determination in cereals.

### 6.3.3 Fat content determination by Soxhlet extraction

It was weighed 5 g of the sample and it was loaded into a porous cellulosic sample thimble and placed into the thimble holder. 150 ml of the solvent (n-hexane) with couple of boiling chips were added to the flask which was heated on a heating mantle and the cool water was turned on at the same time. The solvent was vaporized. It passed through the side tube and went to the reflux condenser. When it condensed, it dropped on the solid substance contained in a thimble and extract soluble compounds. When the liquid level filled the body of extractor, it automatically siphoned into the flask. This process continued repeatedly at the solvent in the flask was vaporized and condensed. The total time was 4 hours. When the extraction was completed, the heat and cooling water were turned off. The extraction solvent containing the solute was put into a baker. After that, the extracted components were isolated by the evaporation [38, 39].

The sample was tested five times.

Only the Kamut was used for this method. The task of the experiment was to find out if it is appropriate method for total fat content determination in cereals.

### 6.3.4 Total fat content determination by CSN ISO 7302

It was weighed 8 g of the analytical sample. The sample with magnet stirrer was put into the volumetric bottle where was poured 10 ml of ethanol. It was homogenized as long as homogenous mixture appeared. After that, 8 ml of formic acid and 12 ml of hydrochloric acid and water solution (7:3) were added and the homogenization process continued for 5 minutes. Then the bottle was put into the water bath heated at the temperature of 75 °C for 20 minutes. After cooling down of the bottle, the extraction process continued. There were poured 18 ml of ethanol and 50 ml of hexane into the bottle and was homogenized for 5 minutes and consequently there were separated two phases. The hexane phase on the top was shifted through the filter paper into the dry and weighed evaporation flask. Then was added 30 ml of hexane and it was homogenized for next 5 minutes, left it separated again and the hexane phase on the top was removed through the filter paper into the same evaporation flask with first extract. This extraction with 30 ml of hexane was repeated two times.
Finally, the hexane extract was evaporated on the vacuum evaporator with the temperature of 50°C. Cold evaporation flask was weighed [40].

Total fat content expressed as percent from the weight of the sample was calculated as:

\[
R = \frac{m_2 - m_1}{m_0} \cdot 100
\]

\[m_0\] weight of the sample (g)

\[m_1\] weight of the flask (g)

\[m_2\] weight of the flask after extraction (g)

Each sample was tested five times. The purpose of the experiment was to make sure that it is relevant method for total fat content determination in cereals.

### 6.4 Preparing of HPLC method for cholecalciferol determination by HPLC – UV/VIS

For preparing of HPLC method for cholecalciferol determination was used net standard of cholecalciferol (FLUKA, BioChemika).

#### 6.4.1 Determination of chromatographic conditions for cholecalciferol detection by HPLC-UV/VIS

It was prepared the standard solution of cholecalciferol at the concentration of 1 mg.ml\(^{-1}\) (100 mg of standard cholecalciferol was dissolved in 100 ml of methanol). From this solution was provided 10 ml of the new solution at concentration of 50 µg.ml\(^{-1}\) (5 ml of standard solution was mixed with 9.5 ml of methanol).

In the chromatographic separation was used the column Acclaim 120 C18 (2.1 x 150 mm; 5 µm). The elution was isocratic. The composition of the mobile phase was methanol - redistilled water (95:5). They were tested the flow rates of the mobile phase of 0.4, 0.5 and 0.6 ml.min\(^{-1}\). The temperature of the column was 30°C and the stress in the column was 91, 126 resp. 135 bar. The detection signal was scanned by the UV/VIS detector at wavelengths of 254, 265, 274 and 310 nm. It was measured three times at each condition.

The aim of this part was to discover the optimal conditions (wavelength) for cholecalciferol detection and its retention time.

The results of the experiment are in chapter 7.4.
6.4.2 Measuring of the cholecalciferol calibration curve by HPLC – UV/VIS

It was used standard solution of cholecalciferol at the concentration of 1 mg.ml\(^{-1}\). From this solution were prepared new solutions at the concentration of 5, 10, 15, 20, 25 and 50 µg.ml\(^{-1}\).

The column Acclaim 120 C18 (2.1 x 150 mm; 5 µm) was used in the chromatographic separation. The mobile phase (methanol - redistilled water in the rate of 95:5) flow rate was 0.6 ml.min\(^{-1}\). The elution was isocratic, at the temperature of 30°C and the stress in the column was 130 bar. The detection signal was scanned by the UV/VIS detector at wavelengths of 254, 265, 274 and 310 nm. The calibration curve was made as dependence of the peak area [mAV.s] on the cholecalciferol concentration [µg.ml\(^{-1}\)]. Each concentration was measured three times.

The results of cholecalciferol concentration curve determination are in chapter 7.5.

6.4.3 Separation of cholecalciferol and ergocalciferol standards by HPLC – UV/VIS

The cholecalciferol and ergocalciferol standard solutions at the concentration of 50 µg.ml\(^{-1}\) were prepared according to the technique which is described in the chapter 6.4.1.1.

It was used the column Acclaim 120 C18 (2.1 x 150 mm; 5 µm). In the separation, the mobile phase composition was changed. The rate methanol – redistilled water was 95:5, 90:10, 85:15 and 80:20. In the first three cases, the flow rate was 0.6 ml.min\(^{-1}\), in the last case, the flow rate was 0.5 ml.min\(^{-1}\). The elution was isocratic. It was measured at the temperature of 30°C, the stress was 137 bar. The UV/VIS detector scanned the signal at wavelengths of 254, 265, 274 and 310 nm.

The scope of this experiment was to separate cholecalciferol and ergocalciferol.

The results of the separation process are in chapter 7.6.
6.5 **Preparing of HPLC method for α-tocopherol determination by HPLC – UV/VIS**

It was used standard of α-tocopherol for preparing of HPLC method for α-tocopherol determination.

6.5.1 **Determination of chromatographic conditions for α-tocopherol detection by HPLC – UV/VIS**

The α-tocopherol standard solution at the concentration of 50 µg.ml\(^{-1}\) was prepared according to the method described in chapter 6.4.1.1.

For the α-tocopherol determination was used the column Acclaim 120 C18 (2.1 x 150 mm; 5 µm). The composition of the mobile phase was methanol – redistilled water (95:5). The elution was isocratic. The flow rate was 0.6 ml.min\(^{-1}\), the temperature was 30°C and the stress in the column was 136 bar. The signal detection was scanned by the UV/VIS detector at wavelengths of 210, 265, 274 and 305 nm.

The purport of the experiment was to design the optimal conditions for α-tocopherol detection and its retention time.

The results are in chapter 7.7.

6.5.2 **Measuring of the α-tocopherol calibration curve by HPLC – UV/VIS**

It was used standard solution of α-tocopherol at the concentration of 1 mg.ml\(^{-1}\). New standard solutions were prepared from this solution at the concentration of 5, 10, 15, 20 and 25 µg.ml\(^{-1}\).

The column Acclaim 120 C18 (2.1 x 150 mm; 5 µm) was used in the chromatographic separation. The mobile phase (methanol - redistilled water in rate 95:5) flow rate was 0.6 ml.min\(^{-1}\), the elution was isocratic, the temperature was 30°C and the stress in the column was 132 bar. The UV/VIS detector scanned the signal at wavelengths of 210, 265, 274 and 305 nm. The calibration curve was made as dependence of the peak area [mAV.s\(^{-1}\)] on the α-tocopherol concentration [µg.ml\(^{-1}\)]. Each concentration was measured three times.

The results are in chapter 7.8.
6.5.3 Separation of cholecalciferol and $\alpha$-tocopherol standards by HPLC – UV/VIS

The cholecalciferol and $\alpha$-tocopherol standard solutions at the concentration of 50 $\mu$g.ml$^{-1}$ were prepared according the technique which is described in the chapter 6.4.1.

It was used the column Acclaim 120 C18 (2.1 x 150 mm; 5 $\mu$m). In the separation, the mobile phase constitution was methanol – redistilled water in the rate of 95:5, the flow rate was 0.6 ml.min$^{-1}$ and the stress was 131 bar. The elution was isocratic. The UV/VIS detector scanned the signal at wavelengths of 210, 265, 274 and 305 nm. The experiment was repeated three times.

The results are in chapter 7.9.

6.6 Cholecalciferol determination in raw milk and margarine by HPLC – UV/VIS

6.6.1 Samples

They were used samples of raw bovine milk and margarine Rama Multivita (Unilever). The content of vitamin D in raw milk is 0.3 – 4 IU.100 ml$^{-1}$(0.0075 – 0.1 $\mu$g.100 ml$^{-1}$) [37] and the producer of margarine denotes 7.5 $\mu$g.100 g$^{-1}$ of vitamin D.

6.6.2 Saponification

The hot saponification consists of treating the sample with ethanolic and aqueous KOH. 10 ml of raw bovine milk was mixed with 30 ml of 1.9 M ethanolic KOH and next 10 ml of raw bovine milk was mixed with 30 ml 3.8 M aqueous KOH. It was also weighed 2 x 10 g of margarine and samples were mixed with 40 ml of 1.9 M ethanolic KOH and 40 ml of 3.8 M aqueous KOH. The flasks with ethanolic KOH were put in for 30 min. to the water bath warmed at the temperature of 60°C. After this time, the samples were put out from the bath and cooled down. The temperature of the bath was increased at 95°C and samples with aqueous KOH were put in for 30 min. and afterwards were cooled down [13].

6.6.3 Extraction

The unsaponifiable fraction is extracted with organic solvents that are not miscible in the water. It was poured 50 ml of n-hexane to all flasks with the samples, they were homogenized on the mixer for 5 min. and then they were separated. The hexane layer was poured
into the evaporation flask and was evaporated on the vacuum evaporator at the temperature of 50°C. After the evaporation it was added 10 ml of methanol [13].

### 6.6.4 Detection

The cholecalciferol was detected by HPLC apparatus Dionex Ultimate 3000. It was used the column Acclaim 120 C18 (2.1 x 150 mm; 5 µm). The mobile phase constitution was methanol – redistilled water (95:5), the flow rate was 0.6 ml.min⁻¹, the temperature was 30°C and the stress was 130 bar. The elution was isocratic. The UV/VIS detector measured wavelengths at 210, 265, 274 and 305 nm. The experiment was repeated five times.
7 RESULTS AND DISCUSSION

7.1 Results of total fat content determination by extraction of chloroform and methanol mixture

The conditions of all experiment series are listed in chapter 6.3.2. The aim of this experiment was to determine total fat content in Kamut and based on the results it was established if this method can be used for total fat content determination in cereals.

Table 6: Total fat content in Kamut after first series of testing

<table>
<thead>
<tr>
<th>Number of experiment</th>
<th>Fat content in Kamut [g.100g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.48</td>
</tr>
<tr>
<td>2</td>
<td>1.48</td>
</tr>
<tr>
<td>3</td>
<td>1.47</td>
</tr>
<tr>
<td>4</td>
<td>1.50</td>
</tr>
<tr>
<td>5</td>
<td>1.49</td>
</tr>
<tr>
<td><strong>Average [g.100 g⁻¹]</strong></td>
<td><strong>1.48</strong></td>
</tr>
<tr>
<td><strong>Standard deviation [g.100g⁻¹]</strong></td>
<td><strong>0.010</strong></td>
</tr>
</tbody>
</table>

As shown in table 6, the average fat content in Kamut after first series was 1.48 ± 0.010 g.100g⁻¹. According to these results and in comparison with the producer statements, the original method is not suitable for total fat content determination in cereals.

In the second series, the conditions were modified as mentioned in chapter 6.3.2.

Table 7: Total fat content in Kamut after the second series of testing

<table>
<thead>
<tr>
<th>Time of homogenization [min.]</th>
<th>Fat content in Kamut [g.100g⁻¹]</th>
<th>Average [g.100g⁻¹]</th>
<th>Standard deviation [g.100g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.73 1.74 1.73 1.69 1.75</td>
<td><strong>1.74</strong></td>
<td><strong>0.008</strong></td>
</tr>
<tr>
<td>20</td>
<td>2.21 2.21 2.22 2.26 2.21</td>
<td><strong>2.22</strong></td>
<td><strong>0.019</strong></td>
</tr>
<tr>
<td>30</td>
<td>1.30 1.56 1.34 1.35 1.29</td>
<td><strong>1.37</strong></td>
<td><strong>0.099</strong></td>
</tr>
<tr>
<td>40</td>
<td>0.37 0.21 0.10 0.36 0.32</td>
<td><strong>0.27</strong></td>
<td><strong>0.103</strong></td>
</tr>
</tbody>
</table>
As summarized in table 7, the time of homogenization strongly influenced the results of the fat content determination. The best results were after 20 min. of the homogenization. The total fat content in Kamut was 2.22 ± 0.019 g.100g\(^{-1}\) which was closest to the statements mentioned on the package of the sample.

The conditions in third series were modified again. The technique is in chapter 6.3.2.

**Table 8:** Total fat content in Kamut after the third series of testing

<table>
<thead>
<tr>
<th>Number of experiment</th>
<th>Fat content in Kamut [g.100g(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.96</td>
</tr>
<tr>
<td>2</td>
<td>1.96</td>
</tr>
<tr>
<td>3</td>
<td>1.97</td>
</tr>
<tr>
<td>4</td>
<td>1.98</td>
</tr>
<tr>
<td>5</td>
<td>1.90</td>
</tr>
</tbody>
</table>

**Average [g.100g\(^{-1}\)]**  1.95  
**Standard deviation [g.100g\(^{-1}\)]**  0.028

The total fat content in Kamut after addition of 15 ml redistilled water and 40 min. of the homogenization was 1.95 ± 0.028 g.100g\(^{-1}\) as table 8 presents. The result is closer to the producer statements than the result after first series.

**Table 9:** Total fat content in Kamut after the fourth series of testing

<table>
<thead>
<tr>
<th>Number of experiment</th>
<th>Fat content in Kamut [g.100g(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.32</td>
</tr>
<tr>
<td>2</td>
<td>5.10</td>
</tr>
<tr>
<td>3</td>
<td>4.46</td>
</tr>
<tr>
<td>4</td>
<td>5.24</td>
</tr>
<tr>
<td>5</td>
<td>4.91</td>
</tr>
</tbody>
</table>

**Average [g.100g\(^{-1}\)]**  5.01  
**Standard deviation [g.100g\(^{-1}\)]**  0.306

The table 9 gives the results after 1 hour of the homogenization. The total fat content in Kamut was 5.01 ± 0.306 g.100g\(^{-1}\). In comparison with statements on the package, the difference in the fat content is wide.
7.2 Results of total fat content determination by Soxhlet extraction

The experiment was made according to the conditions listed in chapter 6.3.3. The aim was to determine the total fat content by Soxhlet extraction, compare the results with statements on the package of the sample and establish if the method is suitable for total fat content determination in cereals.

Table 10: Total fat content in Kamut after the Soxhlet extraction

<table>
<thead>
<tr>
<th>Number of experiment</th>
<th>Fat content in Kamut [g.100g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.50</td>
</tr>
<tr>
<td>2</td>
<td>1.61</td>
</tr>
<tr>
<td>3</td>
<td>1.57</td>
</tr>
<tr>
<td>4</td>
<td>1.70</td>
</tr>
<tr>
<td>5</td>
<td>1.71</td>
</tr>
<tr>
<td><strong>Average [g.100g⁻¹]</strong></td>
<td><strong>1.62</strong></td>
</tr>
<tr>
<td><strong>Standard deviation [g.100g⁻¹]</strong></td>
<td><strong>0.079</strong></td>
</tr>
</tbody>
</table>

As shown in table 10, the average total fat content in Kamut was 1.62 ± 0.079 g.100g⁻¹.
7.3 Results of total fat content determination by ČSN ISO 7302

The total fat content determination was made by technique mentioned in chapter 6.3.4. The goal was to find out if the international norm for total fat content determination in cereals is relevant.

Table 11: Total fat content in all samples after the determination by ČSN ISO 7302

<table>
<thead>
<tr>
<th>Number of experiment</th>
<th>Kamut [g.100g⁻¹]</th>
<th>Green Spelt [g.100g⁻¹]</th>
<th>Spelt wheat [g.100g⁻¹]</th>
<th>Bread wheat [g.100g⁻¹]</th>
<th>Spelt groats [g.100g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.72</td>
<td>3.53</td>
<td>3.15</td>
<td>2.28</td>
<td>2.13</td>
</tr>
<tr>
<td>2</td>
<td>2.95</td>
<td>3.34</td>
<td>3.09</td>
<td>2.48</td>
<td>2.22</td>
</tr>
<tr>
<td>3</td>
<td>2.59</td>
<td>3.41</td>
<td>2.84</td>
<td>2.30</td>
<td>2.83</td>
</tr>
<tr>
<td>4</td>
<td>2.71</td>
<td>3.36</td>
<td>2.74</td>
<td>2.28</td>
<td>3.01</td>
</tr>
<tr>
<td>5</td>
<td>2.86</td>
<td>3.52</td>
<td>2.79</td>
<td>2.20</td>
<td>2.62</td>
</tr>
<tr>
<td>Average</td>
<td>2.78</td>
<td>3.43</td>
<td>2.92</td>
<td>2.31</td>
<td>2.56</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.126</td>
<td>0.079</td>
<td>0.166</td>
<td>0.093</td>
<td>0.340</td>
</tr>
</tbody>
</table>

As can be seen in table 11, the average fat content in Kamut was 2.78 ± 0.126 g.100g⁻¹, in Green Spelt was 3.43 ± 0.079 g.100 g⁻¹, in Spelt wheat was 2.92 ± 0.166 g.100g⁻¹, in Bread wheat was 2.31 ± 0.093 g.100g⁻¹ and in Spelt groats was 2.56 ± 0.340 g.100g⁻¹. The results of the fat content determination by the norm ISO 7302 are very close to statements mentioned on the packages of the samples.

Based on these results, the best method for total fat content determination in cereals is international norm ISO 7302.
7.4 Results of determination of chromatographic conditions for cholecalciferol detection by HPLC-UV/VIS

The experiment conditions are listed in chapter 6.4.1. The scope was to prepare suitable conditions for cholecalciferol determination. It was analyzed at wavelengths of 254, 265, 274 and 310 nm. The best detection was at 265 nm.

Table 12: Measuring of retention time at different flow rates of the mobile phase

<table>
<thead>
<tr>
<th>Flow rate [ml.min(^{-1})]</th>
<th>Retention time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>11.3 – 11.4</td>
</tr>
<tr>
<td>0.5</td>
<td>7.7 – 7.8</td>
</tr>
<tr>
<td>0.6</td>
<td>6.5 – 6.6</td>
</tr>
</tbody>
</table>

As summarized in the table 12, there were tested flow rates of 0.4, 0.5 and 0.6 ml.min\(^{-1}\). The shortest retention time was at the flow rate of 0.6 ml.min\(^{-1}\) consequently this flow rate was chosen for the next experiments. The chromatogram in appendix I is the example of this measuring. The flow rate was 0.6 ml.min\(^{-1}\) and the retention time was 6.5 – 6.6 min.
7.5 Results of the cholecalciferol calibration curve measuring by HPLC – UV/VIS

The calibration curve for cholecalciferol determination was measured according to technique mentioned in chapter 6.4.2. The goal was to find the peak areas for each cholecalciferol concentration. The average values of the peak areas were calculated and are presented in table 13. The graph 1 reveals the regression equation.

Table 13: The results of cholecalciferol calibration curve measuring at different concentrations by HPLC – UV/VIS

<table>
<thead>
<tr>
<th>Cholecalciferol concentration [µg.ml⁻¹]</th>
<th>Peak area [mA.V.s]</th>
<th>Average peak area [mA.V.s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2143</td>
<td>2142</td>
</tr>
<tr>
<td>10</td>
<td>4289</td>
<td>4249</td>
</tr>
<tr>
<td>15</td>
<td>6968</td>
<td>6333</td>
</tr>
<tr>
<td>20</td>
<td>8963</td>
<td>8908</td>
</tr>
<tr>
<td>25</td>
<td>13773</td>
<td>13832</td>
</tr>
<tr>
<td>50</td>
<td>23913</td>
<td>23901</td>
</tr>
</tbody>
</table>

Graph 1: The calibration curve with the regression equation for cholecalciferol determination

It was established the regression equation with correlation coefficient of 0.9951.
7.6 Results of cholecalciferol and ergocalciferol separation by HPLC – UV/VIS

The conditions of the experiments are mentioned in chapter 6.4.3. The aim of this experiment was to separate cholecalciferol and ergocalciferol. The resulting chromatograms of the separation are in appendix II. The retention times both of the vitamins were same and that is why it was difficult to separate these vitamins. In the separation, it was modified mobile phase constitution. As can be seen from chromatogram 1 in appendix II, the vitamins were best separated by the mobile phase in the rate of 95:5.

7.7 Results of determination of chromatographic conditions for \( \alpha \)-tocopherol detection by HPLC-UV/VIS

The experiment conditions are in chapter 6.5.1. The scope was to find suitable conditions for \( \alpha \)-tocopherol determination. It was analyzed at wavelengths of 210, 265, 274 and 305 nm. The best detection was at 210 nm. The retention time was 9.4 – 9.5 min. The resulting chromatogram is in appendix III.

7.8 Results of the \( \alpha \)-tocopherol calibration curve measuring by HPLC – UV/VIS

The calibration curve for \( \alpha \)-tocopherol determination was measured according to technique in chapter 6.5.2. The goal of the experiment was to find the peak areas for each \( \alpha \)-tocopherol concentration. Consequently the average values of the peak areas were calculated and are presented in the table 14. The graph 2 reveals the regression equation.
Table 14: The results of $\alpha$-tocopherol calibration curve measuring at different concentrations by HPLC – UV/VIS

<table>
<thead>
<tr>
<th>Cholecalciferol concentration [µg.ml$^{-1}$]</th>
<th>Peak area [mAV.s]</th>
<th>Average peak area [mAV.s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2502</td>
<td>2432</td>
</tr>
<tr>
<td>10</td>
<td>3808</td>
<td>3836</td>
</tr>
<tr>
<td>15</td>
<td>5948</td>
<td>5973</td>
</tr>
<tr>
<td>20</td>
<td>7840</td>
<td>7714</td>
</tr>
<tr>
<td>25</td>
<td>9681</td>
<td>9651</td>
</tr>
</tbody>
</table>

Graph 2: The calibration curve with the regression equation for $\alpha$-tocopherol determination

It was established the regression equation with correlation coefficient of 0.9966.
7.9 Results of cholecalciferol and $\alpha$-tocopherol separation by HPLC – UV/VIS

The aim was to separate cholecalciferol and $\alpha$-tocopherol according to the conditions listed in chapter 6.5.3. As can be seen from the chromatogram in appendix IV, the vitamins were separated. The retention time for cholecalciferol was 6.5 – 6.6 min. and the retention time of $\alpha$-tocopherol was 9.4 – 9.5 min.

7.10 Results of cholecalciferol determination in raw milk and margarine by HPLC – UV/VIS

The experiment was made according to the technique mentioned in chapter 6.6. The aim was to find out if the conditions of measuring set in chapter 6.4.1. were applicable for self cholecalciferol determination in food.

The saponification both of the samples by ethanolic KOH and saponification of margarine by aqueous KOH failed. It is certain that the conditions of the saponification, like the time and the temperature, were set incorrectly. It also proved that the method is appropriate only for the milk products and the margarine is not the milk product. The extraction of these samples could not be done and so they could not be applied to HPLC.

The cholecalciferol sign appeared only in the milk saponified by aqueous KOH. The resulting chromatogram is in appendix V. The sample was measured twice. The peak areas were 1957 resp. 1974 mAV.s and these values were substituted for “y” to the regression equation (graph 1). From this equation it was calculated the concentration of cholecalciferol in the milk. The result was 4.91 resp. 4.95 $\mu$g.ml$^{-1}$ and consequently it was converted to $\mu$g.100ml$^{-1}$. After that, the cholecalciferol content in 100 ml of raw milk was 0.0491 resp. 0.0495 $\mu$g. According to the Rucker [17], the cholecalciferol content in 100 ml of milk is 0.0075 – 0.1 $\mu$g. It is obvious that cholecalciferol content determined in milk is consistent with set values.
CONCLUSION

The primarily aim of the master thesis has concentrated on finding the optimal method for total fat content determination in cereals. They were chosen five nontraditional species of wheat, such as khorasan wheat, green spelt, spelt wheat, bread wheat and spelt groats. The second purport was to prepare the optimal conditions for cholecalciferol determination by HPLC – UV/VIS.

In the first part of the study, there were tested three methods for total fat content determination. The first one used the mixture of chloroform and methanol. It should be noted that this method was tested only with Kamut. The aim was to find how much fat can be extracted by this method and compare it with statement on the package. Based on the results, it was decided not to continue with this method. It was confirmed that it is not appropriate technique for the cereals. The method of Soxhlet extraction showed similar results as the first experiment. As the most exact method for total fat content determination turned out the ISO norm. The results of this technique were very close to the declared statement and thereby this method was used for total fat content determination in all wheat samples.

The second part of the master thesis has concentrated on vitamin D, all above on cholecalciferol. It was used pure standard of cholecalciferol and a brand-new HPLC apparatus Dionex Ultimate 3000 for preparing the method. Firstly they were found optimal conditions, such as wavelength, mobile phase flow rate and retention time for cholecalciferol detection, and consequently it was measured its calibration curve which was important for further cholecalciferol determination in the food. It was also tried to separate ergocalciferol and cholecalciferol standards on this apparatus but it failed unfortunately. It seems that the problem may be the same retention time both of the vitamins so they cannot be detected. The determination of α-tocopherol and measurement of its calibration curve was done extra. The main purpose was to try the separation of cholecalciferol and α-tocopherol standards. The aim of the latest experiment was to check out if the conditions for cholecalciferol determination are applicable.

Vitamin D is very important for optimal metabolism of the body and for good bone and teeth condition. Unfortunately, its content in common food is very low. The experiment is one of the evidence. It was found that in 100 ml of bovine milk is approximately only
0.05 µg (2 IU) of cholecalciferol. The adequate intake of cholecalciferol for most of the population is 200 IU a day. It follows that we should drink 10 l of the raw bovine milk a day to implement this requirement. However, it is obvious that it is not possible for people to fulfill it and that is why some of the frequently consumed foods are fortified by vitamin D these days. The fortification guarantee the vitamin D intake also for people who cannot expose themselves to the sun.
BIBLIOGRAPHY


LIST OF ABBREVIATIONS

IU International Unit
HPLC High Performance Liquid Chromatography
UV-VIS Ultraviolet-visible
PDA Photodiode Array
RI Refractive Index
EC Electrochemical
IR Infra-Red
MS Mass Spectral
NMR Nuclear magnetic resonance
LS Light Scattering
ISO International Organization for Standardization
ČSN České technické normy,

Czech technical norms
LIST OF FIGURES

**Figure 1:** Generalized structure of cereal flowering organs .............................................. 13

**Figure 2:** Cereal grain and its anatomy ................................................................................. 14

**Figure 3:** 1 – Bread wheat; 2 – Kamut (Khorasan wheat); 3 – Green spelt; 4 – Spelt wheat ........................................................................................................................................... 18

**Figure 4:** Ergocalciferol and cholecalciferol ........................................................................ 19

**Figure 5:** Findings in patients with rickets ........................................................................... 22

**Figure 6:** Scheme of an HPLC instrument ............................................................................ 25

**Figure 7:** HPLC injector scheme ......................................................................................... 26

**Figure 8:** HPLC column scheme ......................................................................................... 27
LIST OF TABLES

Table 1: Classification of the Genus *Triticum* [8] ................................................................. 15
Table 2: Nutritional values of Khorasan wheat [8] ................................................................. 16
Table 3: Adequate intakes for vitamin D [36] ........................................................................... 20
Table 4: Vitamin D activities in food [14] ................................................................................ 20
Table 5: Fat content in used samples declared by producer ................................................... 34
Table 6: Total fat content in Kamut after first series of testing ........................................... 42
Table 7: Total fat content in Kamut after the second series of testing ................................... 42
Table 8: Total fat content in Kamut after the third series of testing ...................................... 43
Table 9: Total fat content in Kamut after the fourth series of testing ................................... 43
Table 10: Total fat content in Kamut after the Soxhlet extraction ......................................... 44
Table 11: Total fat content in all samples after the determination by ČSN ISO 7302 .......... 45
Table 12: Measuring of retention time at different flow rates of the mobile phase ............ 46
Table 13: The results of cholecalciferol calibration curve measuring at different concentrations by HPLC – UV/VIS ................................................................. 47
Table 14: The results of α-tocopherol calibration curve measuring at different concentrations by HPLC – UV/VIS .................................................................................... 49
APPENDICES

Appendix P I: Cholecalciferol determination by HPLC – UV/VIS

Appendix P II: Cholecalciferol and ergocalciferol separation by HPLC – UV/VIS

Appendix P III: α-tocopherol determination by HPLC – UV/VIS

Appendix P IV: Cholecalciferol and α-tocopherol separation by HPLC – UV/VIS

Appendix P V: Cholecalciferol determination in raw milk and margarine by HPLC – UV/VIS
APPENDIX P1: CHOLECALCIFEROL DETERMINATION
BY HPLC-UV/VIS

Chromatogram

Report for "vitamin D3_RA6_03_36"
Printed: 16:56:48  03/16/10  Computer: XM4600-ESI User: mif

Report for "vitamin D3_RA6_03_36"

Chromatogram File:
D:\Data\MIP\Vitamin\vitamin D3_RA6_03_36.d\vitamin D3_RA6_03_36.unt

Created on: 3/16/2010 at: 03:08:18 pm
on Windows system: XM4600-ESI by user: Administrator
with HyStar Version 3.2.44.0
Operator: mif, Laboratory:

Total Chromatogram Runtime: 10.02 min
Flow Rate: 0.600 ml/min
Injection Volume: 5.00 µl
Sample ID: vitamin D3

Signal 1: UV (254.0nm)
Signal 2: UV (265.0nm)
Signal 3: UV (274.0nm)
Signal 4: UV (310.0nm)
APPENDIX P II: CHOLECALCIFEROL AND ERGOCALCIFEROL SEPARATION BY HPLC – UV/VIS

Chromatogram 1: Cholecalciferol and ergocalciferol separation (mobile phase 95:5)
Chromatogram 2: Cholecalciferol and ergocalciferol separation (mobile phase 90:10)

Peak height [mAV]

Time [min.]
Chromatogram 3: Cholecalciferol and ergocalciferol separation (mobile phase 85:15)

Peak height [mAV]

Time [min.]
Chromatogram 4: Cholecalciferol and ergocalciferol separation (mobile phase 80:20)
APPENDIX P III: α-TOCOPHEROL DETERMINATION BY HPLC – UV/VIS

Chromatogram
Report for "vitamin E_RA5_03_67"
Printed: 10/29/13 03/11/10 Computer:NM600-ESI User:mif

Report for "vitamin E_RA5_03_67"

Chromatogram File:
D:\Data\MIP\Vitamin\vitamin E_RA5_03_67\vitamin E_RA5_03_67.unm

Created on: 3/31/2010 at: 05:29:23 pm
on Windows system: WN600-ESI by user: Administrator
with HyStar Version 3.2.44.0
Operator: mif, Laboratory:

Total Chromatogram Run Time: 15.02 min
Flow Rate: 0.600 ml/min
Injection Volume: 5.00 µl
Sample ID: vitamin E

Signal 1: UV (210.0nm)
Signal 2: UV (265.0nm)
Signal 3: UV (274.0nm)
Signal 4: UV (305.0nm)
APPENDIX P IV: CHOLECALCIFEROL AND α-TOCOPHEROL SEPARATION BY HPLC – UV/VIS

Peak height [mAV]

cholecalciferol

α - tocopherol

Time [min.]
APPENDIX P V: CHOLECALCIFEROL DETERMINATION IN RAW MILK BY HPLC – UV/VIS

Chromatogram Report for "vitamin L19_RC3_01_94"
Printed: 17:01:58 04/09/10 Computer: XM4600-BEI User: mlf

Chromatogram File: D:\Data\RF\Vitamins\vitamin L19_RC3_01_94.d\vitamin L19_RC3_01_94.unr

Created on: 4/7/2010 at: 08:09:29 pm on Windows system: XM4600-BEI by user: Administrator with HyStar Version 3.2.44.0
Operator: mlf, Laboratory:

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Total Chromatogram Runtime: 20.02 min
Flow Rate: 0.600 ml/min
Injection Volume: 5.00 µl
Sample ID: vitamin L39
Signal 1: UV (210.0nm)
Signal 2: UV (265.0nm)
Signal 3: UV (274.0nm)
Signal 4: UV (305.0nm)

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[Graph showing chromatogram with peak labeled as cholecalciferol]