Application for the Approval of the Vitamin D2 Yeast Concentrate

Regulation (EC) N° 258/97 of the European Parliament and of the Council of 27th January 1997 concerning Novel Foods and Novel Foods ingredients

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GENERAL INTRODUCTION

Lallemand proposes to market a yeast (*Saccharomyces Cerevisiae*) containing an enhanced level of vitamin D2 (hereafter referred to as "vitamin D2 yeast concentrate") as a nutritional food ingredient and food supplement ingredient in Europe.

Today, vitamin D2 is an authorized form of vitamin D in the EU (Regulation (EC) 1170/2009) and it is extracted (using solvents) from irradiated yeast by companies like Synthesia, a food operator based in the Czech Republic. Lallemand believes that there is a market for a less processed form of vitamin D2 obtained from yeast – as in the way some customers prefer raw sugar to refined sugar or whole grain wheat to white flour.

Therefore, Lallemand proposes to market this less processed form of vitamin D2 (using the same irradiated yeast used by companies such as Synthesia) as an alternative form to the extracted vitamin D2 in all its food applications (current foods and foods supplements) wherever vitamin D2 is authorized.

Yeast and its derivatives have a long history of safe use in the EU (as elsewhere in the world) in foods and in supplements (whether for baking or as sources of vitamin B, minerals, proteins or for its functional and taste properties like flavor enhancers). Vitamin D2 from yeast has a long history of authorized and safe use as a source of vitamin D.

The unprocessed form of vitamin D2 from yeast was widely used in the 1920's and 1930's as a form of vitamin D supplementation in Canada and US without any reported safety concern.

In Canada, Lallemand's vitamin D2 yeast has been confirmed not to be a Novel Food and has been approved by Health Canada as a form of vitamin D supplementation in foods where vitamin D is allowed to be added (Appendices A, B). In the US, Lallemand's vitamin D2 yeast is also approved for food use by the FDA (Appendix C). In the EU, even though this less processed form of vitamin D is used in feed and we strongly suspect it has been used in food prior to the development of the extraction process, we have not been able to document significant consumption in the EU before the cut-off date of 1997.

Therefore, we are seeking its approval under Regulation EC 258/97 of the European Parliament and of the Council of 27 January 1997 concerning Novel Foods and Novel Food ingredients, under category 1(2)(d)., ingredients obtained from microorganisms that have existing food uses.
Accordingly, this dossier has been prepared following the Commission Recommendation of 29th July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of Novel Foods and Novel Foods ingredients (hereafter referred to as the Commission recommendation of 1997).

Section 4 of the Commission recommendation of 1997 identifies six classes of NF, facilitating the safety and nutritional evaluation of a given NF or NF ingredient. Of the six classes identified, vitamin D2 yeast concentrate would be classified in Class 2 as a "Complex Novel Food from non-GM source", since the production of vitamin D yeast has been developed by conventional techniques, with no use of genetic modification. While the yeast that vitamin D2 yeast concentrate is derived from *Saccharomyces cerevisiae*, has a history of food use in the community, vitamin D2 yeast concentrate would be allocated under sub-class 2.1: a complex novel food from a non-GM source with a history of food use of the source material

The essential information requirements corresponding to this classification are developed in this dossier and detailed under the following sections:

I. Specification of the Novel Food
II. Effect of the production process applied to the Novel Food
III. History of the organism used as the source of the Novel Food
IX. Anticipated intake/extended use of the Novel Food
X. Information from previous human exposure to the Novel Food or its source
XI. Nutritional information on the Novel Food
XII. Microbiological information on the Novel Food
XIII. Toxicological information on the Novel Food

For each category (I through XIII), structured schemes have been developed by the Scientific Committee for Food (SCF), which consist of a decision-tree-like set of questions designed to elicit sufficient data for a comprehensive safety and nutritional evaluation of the Novel Food. As outlined below in Sections I through XIII, the required questions are identified and subsequently addressed with the appropriate data.
Sections IV to VIII were omitted, since they are not applicable.

I SPECIFICATIONS OF VITAMIN D2 YEAST CONCENTRATE

Based on the SCF guidelines, the following questions must be answered in the affirmative to ensure sufficient information pertaining to the specifications of the novel food:

- Is appropriate analytical information available on potentially toxic inherent constituents, external contaminants and nutrients?
- Is the information representative of the novel food when produced on a commercial scale?
- Is there an appropriate specification (including species, taxon, etc. for living organisms) to ensure that the novel food marketed is the same as that evaluated?

These questions have been addressed collectively in the section I.

I.1 COMMON NAME OR USUAL NAME

The common name for the product which is the object of this application is: Vitamin D2 yeast concentrate, which contains ergocalciferol (vitamin-D2).

Yeast is a eukaryotic microorganism classified in the kingdom Fungi, and there are about 1,500 species that have been described. Among the most common yeast species, *Saccharomyces cerevisiae* has been used in baking and fermented alcoholic beverages for thousands of years and is well-defined for use in foods. (See part III - History of use for more details).

As described in section II.2 (Production Process), Vitamin D2 yeast concentrate is produced by the use of ultraviolet light (UVB) in a photochemical reaction with the conversion of endogenous ergosterol in yeast to ergocalciferol (vitamin D2). The C9-C10 bond of the ergosterol is broken, followed by a thermal isomerization to form vitamin D2 (see Figure 1).

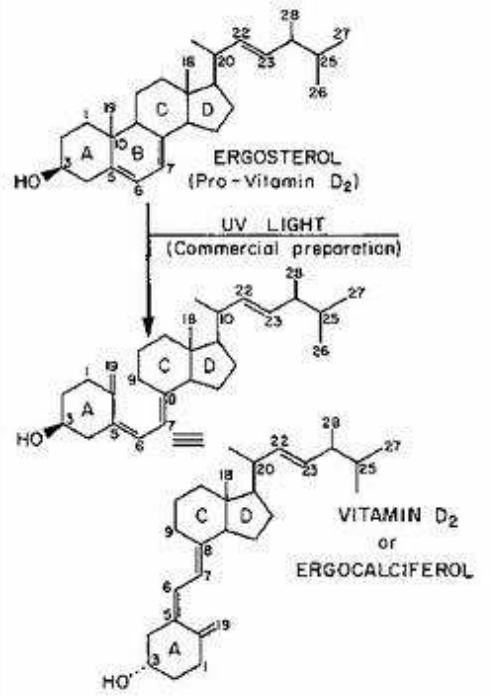


Figure 1: Conversion of ergosterol to vitamin D2 by UV irradiation

It has been noted by the SFC (Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Vitamin D, SCF 2002) that vitamin D2 is formed by UV irradiation of its precursor ergosterol. Ergosterol is found in yeast and the synthesis of vitamin D2 from ergosterol rarely occurs in nature. Synthetic vitamin D2 produced by the irradiation of ergosterol used to be the form added to food or given as supplements.

If isolated from yeast, vitamin D2 appears as white crystals that are insoluble in water but soluble in alcohol, chloroform, ether and fatty oils. However, the vitamin D2 in Vitamin D2 yeast concentrate is not isolated and/or purified from yeast as a chemical entity or resin. Rather, in vitamin D2 yeast concentrate, the vitamin D2 is present as a natural ingredient in yeast after a photoreaction with UVB light.

Ergocalciferol (vitamin D2) is a form of vitamin D which is permitted for addition to foods and to food supplements by the European regulations and is listed in the following:

- Annex II (Vitamin and mineral substances which may be used in the manufacture of food supplements) and,
- Annex III (vitamin formulation and mineral substances which may be added to foods)

of Regulation (EC) 1170/2009 amending Directive 2002/46/EC of the European Parliament and of Council and Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards the lists of vitamin and minerals and their forms that can be added to foods, including food supplements**).**

I.2 CHEMICAL NAME AND CHEMICAL ABSTRACT SERVICE (CAS) NUMBER

These names consist of yeast and Yeast vitamin D2 or ergocalciferol, contained therein (Vitamin D2 yeast).

The chemical name for vitamin D2 is 9,lO-sec0(5Z,7E)-5,7,10(19),22-ergostatetraene-3-01 or (38,5Z,7E,22E)-9,10-secoergosta-5,7,10(19),22-tetraen-3-o1.

The Chemical Abstract Service Registry Number (CAS-RN) for vitamin D2 is 50-14-6.

I.3 EMPIRICAL FORMULA AND MOLECULAR WEIGHT

The empirical formula for vitamin D2 is$C_{28}H_{44}0$ and the molecular weight is 396.66g/mol.

I.4 STRUCTURAL FORMULAE

The chemical structure of vitamin D2 is shown in Figure 2.

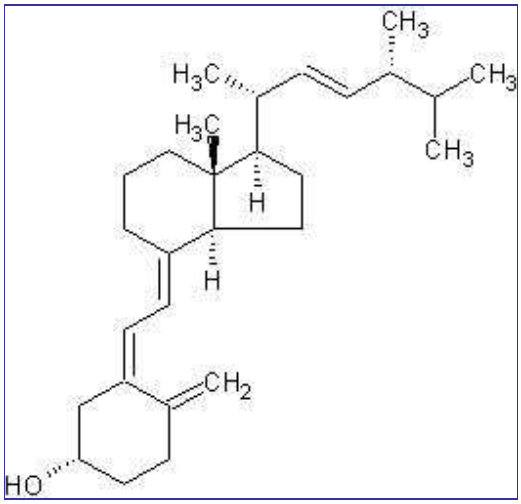


Figure 2: Chemical structure of vitamin D2

I.5 HPLC CHARACTERIZATION

The vitamin D3 produced in the human body is formed in the skin from 7-dehydrocholesterol through a two-step process in which the B ring of the sterol molecule is broken under ultraviolet light (UV) (e.g., sunlight), and the pre-vitamin D3 so formed isomerizes to vitamin D3 in a thermo-sensitive but noncatalytic process. According to the literature, by-products such as lumisterol and tachysterol are potentially produced during the UV photochemical reactions. However, there is no scientific evidence that lumisterol or tachysterol is harmful (Holick 1981, Gilchrest 2006).

Ergosterol, the precursor of vitamin D2 which is endogenous in yeast, can be converted to vitamin D2 via similar UV photochemical reactions. However, the extent to which the photo-conversion of the intracellular ergosterol in yeast cells to vitamin D2 (ergocalciferol) produces any significant amounts of other sterols, and possibly compounds similar to lumisterol and tachysterol is not known.

I.5.1 Product Characterization

To ascertain the potential existence and quantitative distribution of these sterols in regular baker's yeast (control yeast before UV treatment) and vitamin D2 yeast concentrate (after UV photo-reactions), control and vitamin D2 concentrate yeast samples were produced in Lallemand's R&D laboratory and analyzed subsequently using the vitamin D HPLC analysis method. The detailed analytical protocol used is described in Appendix I.5.1A. The sample saponification and extraction procedures used in this method of analysis were adapted from *Dimartino* (2007) and Huang *et al*. (2009). No clean-up steps are involved in this HPLC analytical procedure.

A typical chromatogram for the HPLC analysis of the control yeast samples spiked with internal vitamin D3 standard, which was added before saponification of yeast sample to assess vitamin D recovery efficiency during the sample analysis, is shown in Appendix I.5.1B, which illustrates the HPLC profile of the sterols extracted from 1 g of regular dry baker's yeast not subjected to UVB photo-reactions. There are only two major peaks observed. The first peak is eluted at about 33.94 minutes while the second peak is eluted at 43.75 minutes. Based on comparative elution time and UV spectra analyses between the standards and the sample, the first peak is identified to be vitamin D3 (the internal standard spiked) and the second peak is identified to be ergosterol (the precursor of vitamin D2 in yeast), respectively. Ergocalciferol (vitamin D2) is eluted at about 32.3 minutes and none could be detected in this sample, which is consistent with the fact that it is a control yeast sample before UV photo-treatment.
Quantitatively, the ergosterol content in yeast for this control sample is determined to be 7.05 mg/g or 0.705%. The amount of the vitamin D3 recovered in the analysis is determined to be 1.58 mg, which is very close to the amount of vitamin D3 initially spiked (1.6 mg). As a result, the vitamin D3 recovery efficiency is close to 100% (98.55%), indicating excellent vitamin D recovery of the analysis method.

Appendix I.5.1C shows a typical chromatogram for the HPLC analysis of vitamin D2 yeast concentrate samples spiked with internal vitamin D3 standard. The chromatogram illustrates the HPLC profile of the sterols extracted from 1 g of vitamin D2 concentrate. The same protocol of sample preparation and HPLC parameters for separation used in the analysis of the 1 g of the control baker's yeast sample were employed in the analysis of the Vitamin D2 concentrate sample. As shown in Appendix I.5.1C, there are 4 major peaks in the chromatogram for the vitamin D2 yeast concentrate.

Besides the vitamin D3 and ergosterol peaks, 2 new peaks are observed for the vitamin D2 yeast concentrate sample. Of the 2 new peaks, the large one is identified to be vitamin D2 with an elution time at 32.26 minutes, whose identity is confirmed by UV spectra of the peak. The vitamin D2 peak accounts for 16.03% of the total area and the vitamin D2 content in yeast is determined to be 1.06 mg/g or 4 230 000 IU/100g. The very high vitamin D2 content achieved in yeast is a result of the UV treatment of the yeast.

The second new peak is eluted at 35.63 minutes, which is identified as tachysterol based on comparative elution time and UV spectra analyses between the tachysterol standard and the sample. The tachysterol standard was provided to us by Dr Holicks' laboratory[1] since it is very difficult to obtain from other commercial sources. As shown in Appendix I.5.1C, the tachysterol peak is much smaller than the vitamin D2 peak and the area of the tachysterol peak accounts for only about 4.27% of the total area in the chromatogram. However, no quantitative determination of the tachysterol content in the sample was accomplished.

The ergosterol content in yeast for the vitamin D2 yeast concentrate is determined to be 6.56 mg/g or 0.656%, which is about 10% lower than that of the control yeast sample. This makes sense since part of the ergosterol is converted to vitamin D2 during the UV photochemical reactions. Excellent vitamin D3 recovery efficiency (97.54%) is also achieved during the analysis of the vitamin D2 yeast sample, further confirming the excellent vitamin D recovery of the analysis method.

Overall, the peak eluted at 35.63 minutes, which is identified as tachysterol, appears to be the only chromatographic difference between the control yeast (before UV treatment) and the vitamin D2 yeast concentrate (after UV treatment), and again, the potential level of this compound would be small by its peak area. Furthermore, tachysterol is inert and non-toxic according to literature (Holick 1981; Gilchrest 2006)). There is no difference in the other sterol components between the control yeast and the vitamin D2 yeast. The results of the two comparative chromatograms also indicate that vitamin D2 (ergocalciferol) and ergosterol are the two major sterols in the vitamin D2 yeast concentrate.

Analysis of the commercial vitamin D2 yeast concentrate produced in the plant is also routinely conducted in Lallemand's laboratory using the HPLC analytical procedure described above. This particular commercial sample was analyzed with our HPLC procedure and its chromatogram is shown in Appendix I.5.1D. Appendix I.5.1D appears almost identical to Appendix I.5.1C and both

[1] Dr. Michael Holick, Boston University; Phone: 617-638-4546 and e-mail: mfholick@bu.edu

chromatograms have 4 major peaks, indicating very similar sterol distributions between the lab-prepared and commercially produced vitamin D2 yeast concentrates.

To assess the consistency, reliability and precision of our HPLC analysis procedure, the vitamin D2 yeast concentrate (lot #91620D0K1) was analyzed 6 separate times. The vitamin D2 results for the 6 tests are shown in Table 1. The mean content of the 6 analyses of vitamin D2 in the concentrate is determined to be 3290000 IU/100g with a relative standard deviation (RSD) of 3.59% (See Table 1). The low RSD suggests a small degree of scatter and good precision of the analysis method.

Test	Date Tested	Lot#	Vitamin D2 (IU/100g)
1	Sept. 28, 2011	91620D0K1	3189000
2	Sept. 28, 2011	91620D0K1	3338000
3	Sept. 30, 2011	91620D0K1	3111000
4	Sept. 30, 2011	91620D0K1	3376000
5	Oct. 21, 2011	91620D0K1	3303000
6	Oct. 21, 2011	91620D0K1	3424000
Average		**91620D0K1**	**3290000**
SD			**118000**
RSD			**3.59%**

Table 1: Vitamin D2 results for the 6 tests of the commercial vitamin D2 yeast concentrate lot #91620D0K1

The vitamin D content of this commercial lot (lot #91620D0K1) had been previously analyzed by an external laboratory (Covance Laboratories) with a modified official method -AOAC Method 982.29 (See Appendix I.5.1E for the AOAC method, and Appendix I.5.1F for the modifications by Covance). The mean vitamin D2 content in yeast from the triplicate analyses of this concentrate sample by Covance is determined to be 3 263 000 IU/100g, which is very close to our average analysis result (3 290 000 IU/100g) as it is shown in certificates of analysis form Covance in Appendix I.5.1G. The excellent agreement of our vitamin D2 results with the results from Covance further cross-validates our analysis method.
Please note that the lot numbers indicated on the certificates of analysis from Covance (Appendix I.5.1G) correspond to sample identification numbers but are related to our commercial lot #91620D0K1.

I.5.2 Quantitative Analysis

To achieve product quantification of the yeast concentrate, two concentrate samples from our production plant were sent to Dr. Holick, who is our scientific partner and a worldwide specialist on vitamin D, for quantitative analysis (production lot #02468D0K1 and lot #02561D0K1). These samples were saponified and analyzed under the same conditions as described above, with the following exceptions:
1) chromatographic peaks were monitored and recorded with a detector setting of 265nm and;
2) standards for ergosterol, tachysterol and vitamin-D2 were used to measure the quantity of these compounds in the two samples (standard for tachysterol, which is hardly commercially available, is synthesized by Holick's laboratory).

The results of Holick's analysis indicate the amount of tachysterol and ergocalciferol (vitamin D2) in these two samples are 140mg/Kg and 672mg/Kg (Lot #02561DOK1) and 145mg/kg and 825mg/Kg (Lot #02468DOK1), respectively (Appendix I.5.2 A).

According to these results and to the intended use for our vitamin D2 yeast concentrate, tachysterol does not present a safety/toxicity risk (see section XIII.6 – Tachysterol toxicity).

I.6 PRODUCT SPECIFICATIONS AND ANALYSES

I.6.1 Product Specifications

The vitamin D2 yeast concentrate is in a form of fine beige granules (e.g when yeast is fluid bed dried) or powder (when yeast is ground or when yeast is spray or roller dried).

The typical range of concentration for vitamin D2 in vitamin D2 yeast concentrate, as specified in product data sheet (see Appendix I.6.1), is from 1 800 000 UI/100g to 3 500 000 UI/100g.

Vitamin D2 yeast concentrate will be commercialized for bakery applications and other food applications, including food supplements. The specifications will depend on the application. Typical microbiological specifications for bakery applications are as follows:

I.6.1.1. Microbiological specifications of Vitamin D2 yeast

Designation	Specification	Reference
E.Coli (/g)	< 10	FDA BAM
Salmonella (/25 g)	Negative	FDA BAM
Coliforms (/g)	< 1000	FDA BAM

I.6.2 Analytical Method for Vitamin D2 content determination

As described in Appendix I.6.2E, a method has been validated for the dosage of vitamin D2 in a range from 0.08 IU/g to 5000 IU/g, in a wide variety of matrices. This includes, but are not limited to, pet foods, cereals, supplements, premixes, infants formulas, and most food products.

In order to demonstrate the validity of the determination of the vitamin D2 content in the vitamin D2 yeast concentrate and in products thereof, Lallemand conducted a validation study. The validation study of the method was accomplished by Covance Laboratories[2]. The method of analysis used to measure vitamin D2 in foods is adapted from AOAC method 982.29 (AOAC, 2000; Appendix I.5.1E and Appendix I.5.1F for the modifications by Covance).

A copy of the validation report is provided in Appendix I.6.2A.

A sample of concentrate (production Lot#02468DOK1) and bread made from this concentrate (as an example of application) was sent to Covance. The concentrate was estimated to have 2,880,000 IU vitamin-D2/100g as this sample had not been previously analyzed by Covance.

The formula for the bread scaled to make four loafs of bread which contain 400 IU vitamin-D2/100g is provided in Appendix I.6.2B. It was calculated that to meet this level of vitamin-D2 in the bread, 0.222 g of concentrate would be mixed with 80g of conventional cream yeast with no vitamin-D2 (Appendix I.6.2B).

A summary of the results provided by Covance is presented along with comments on collaborating analyses.

The mean concentration of triplicate analyses of vitamin-D2 in the concentrate was determined to be 3,440,000 IU vitamin-D2/100g with a relative standard deviation (RSD) of 3.37% (Table 1; Appendix I.6.2A). The concentrate was further analyzed after being spiked with a vitamin-D2 standard at levels of 50, 100 and 250% of the mean value; each spiked sample was analyzed in quadruplicate. The percent recovery of vitamin-D2 in the three-spiked samples is reported in Table 2, Appendix I.6.2A and ranged from 90-119% with a mean recovery of 98.2% and a RSD of 7.8%.

The mean concentration of triplicate analyses of vitamin-D2 in the bread was determined to be 489 IU vitamin-D2/100g with a RSD of 8.03% (Table 3; Appendix I.6.2A). As accomplished with the concentrate, the bread was analyzed after being spiked with a vitamin-D2 standard at levels of 50, 100 and 250% of the mean value; each spiked sample was analyzed in quadruplicate. However as explained in the Covance report (Table 4 and Appendix 3; Appendix I.6.2A), the results of two analyses are not included in the data summary.

[2] Covance laboratories Inc, 3301 Kinsman Blvd Madison, Wisconsin 53704 ; Phone: 608-241-4471

The percent recovery of vitamin-D2 in the three-spiked bread samples, (10-observations) is reported in Table 4, Appendix I.6.2A and ranged from 80.2-109% with a mean recovery of 89.9% and a RSD of 8.9%.

Covance took additional steps to support the results obtained in the validation study of their slightly modified and accepted AOAC Method 982.29 (Appendix I.5.1F and Appendix 2 of the Appendix I.6.2A). This additional validation effort was the analysis of the Concentrate and bread using their LC-MS/MS procedures designated VDMS (Appendix 2, page 18 of Appendix I.6.2A, and Appendices I.6.2 C, D and E). These attachments provide additional information on Covance's VDMS methodology.

The data for the VDMS determination of concentrate and bread are provided in Table 5; Appendix I.6.2A. Covance, using their VDMS methodology, determined the level of vitamin-D2 in the concentrate and bread to be 3 405 000 IU of vitamin D2 and 516 vitamin D2 IU/100g, respectively. These values compare within one-percent (1%) and five-percent (5%) of the values observed in the concentrate (Appendix I.6.2A Table 1) and the bread (Appendix I.6.2A Table 3) as determined in the primary validation study, respectively.

It is interesting to mention that the same concentrate sample has also been analyzed by Holick, and they measured a content of 3 300 000 IU vitamin D2/100g (see Appendix I.5.2A).
This means that a difference of only approximately 4% in the value of vitamin D2 content in the same sample of concentrate was determined using two different methods by the two laboratories.

II EFFECT OF THE PRODUCTION PROCESS APPLIED TO VITAMIN D2 YEAST CONCENTRATE

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient information pertaining to the effect of the production process applied to the novel food:

- Does the novel food undergo a production process?
- Is there a history of use of the production process for the food? If no, does the process result in a significant change in the composition or structure of the novel food compared to its traditional counterpart?
- Is information available to enable identification of the possible toxicological, nutritional and microbiological hazards arising from use of the process?
- Are the means identified for controlling the process to ensure that the novel food complies with its specification?
- Has the process the potential to alter the levels in the novel food of substances with an adverse effect on public health?
- After processing is the novel food likely to contain microorganisms of adverse public health significance?

These questions are addressed in the following sections.

Please note that the Scientific Committee for Food (SCF, 2002) has stated that ''Synthetic vitamin D2 produced by irradiation of ergosterol used to be the form added to food or given as supplements.''.

II.1 RAW MATERIALS USED IN THE PRODUCTION PROCESS

The manufacturing process only uses *S.Cerevisiae* strains that are commonly produced and used in the EU for bakery, distillery, wine, brewer's or as nutritional yeast as raw material.

Strains are kept by Lallemand as a "master cell bank" and a "Working cell bank". The "master cell bank" is issued from the growth of the initial isolate on a yeast culture medium. From this initial isolate, five identical colonies are isolated and submitted to genetic testing, to determine purity and to obtain the genetic profile of the strain (fingerprinting).
The colonies are then used to inoculate a plate to create the "master cell bank" stock: 2 vials in glycerol at -80°C and 2 vials in glycerol in the vapour phase of liquid nitrogen (around -196°C). These vials are stored at the Biotechnology Research Institute (BRI, Montreal, Canada). For safety concerns, another vial in glycerol at -80°C is kept in a different location.

The "Working cell bank" is derived from the "master cell bank". One vial of strain in glycerol at -80°C is used for the inoculation and yield in about 2 vials of "Working cell bank". These vials are kept in glycerol at -80°C.

II.2 PRODUCTION PROCESS

The commercial production yeast- also called commercial fermentation is a multistep process. The production process of the vitamin D2 yeast concentrate is described in the flow chart below.

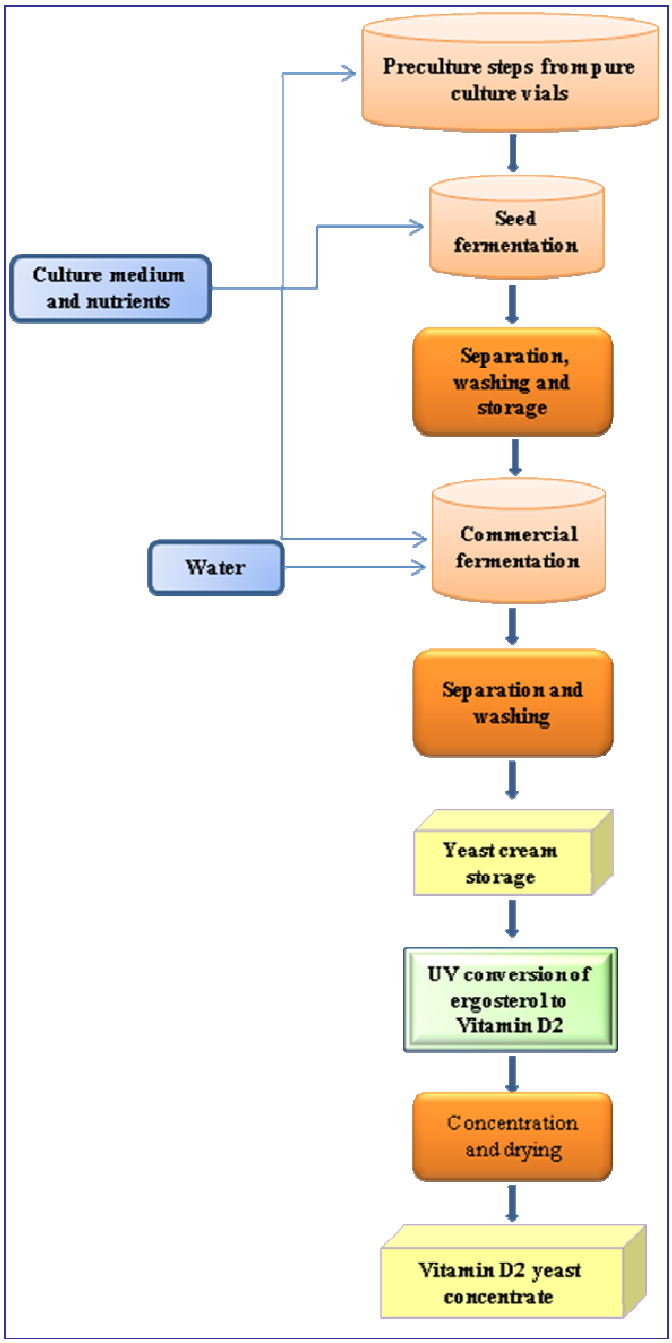


Figure 3: Production process – Vitamin D2 yeast concentrate production

II.2.1 Yeast Commercial Production

The process starts with a pure culture tube of a frozen vial of the appropriate yeast strain. This yeast serves as the inoculum for the pre-pure culture tank, a small pressure vessel where seed is grown in medium under strict sterile conditions. Following growth, the content of this vessel is transferred to a larger pure culture fermentor where propagation is carried out. The grown cells are transferred to a series of progressively larger seed and semi-seed fermentors.

At the end of the semi-seed fermentation, the content of the vessel is pumped into a series of separators that separate the yeast from the spent molasses. The yeast is then washed with water and pumped into a storage tank where the yeast cream is held until it is used to inoculate the commercial fermentation tanks.

The commercial fermentation is the final step in the fermentation process. At the end of fermentation, the fermentor broth is separated, washed and concentrated to yield a yeast cream.

This cream yeast is the starting material of the vitamin D2 yeast concentrate process.

II.2.2 Commercial Production of Vitamin D2 Yeast Concentrate

The obtained conventional yeast cream is continually passed by UV lamps. The obtained vitamin D2 cream is further processed to obtain the highly concentrated vitamin D2 yeast.
A sample of each production lot of concentrate is sent to an outside laboratory, such as Covance, for vitamin D2 determination (see section II.3 – Quality control). Microbiological contaminants are also controlled (see section XII – Microbiological information on vitamin D2 yeast concentrate).

II.2.3 Information on Process Development

This production process is the result of a huge research and development work, from laboratory to pilot scale, including an in-depth study of the UV wavelength effect on the yeast and a complete scale-up work.

Innovation in process development for the yeast production is contributed by Lallemand Research & Development group at the Biotechnology Research Institute in Montreal. A multidisciplinary team works to improve and develop the yeast manufacturing process. Knowledgeable yeast physiologists and engineers work together and undertake experiments in order to have a better understanding of the yeast behavior.

Production process development includes the scale-up and the transfer of laboratory results into production and implementation in the plants. Additionally, technical improvements for the transfer of laboratory results, improvement and rationalization of production processes, optimization of

output and productivity, and implementation of new techniques in the production facilities are performed.

The main focus of production development is the optimization of end-point targets for yeast stability and yield for yeast counts. Examples of production parameters evaluated would include media composition, feeding rates, pH, aeration, addition of emulsifier or drying time. Research and Development evaluates new technologies for the measurement of critical points such as final biomass, activity and shelf-life of the yeast, colour of the yeast, as well as methods for quality control yeast counts and detection of contaminants.

II.3 Quality Control

A sample of every production lot is sent in triplicate to an outside laboratory for the determination of the vitamin D2 content in the vitamin D2 yeast concentrate. Microbiological analyses are achieved in-house following internationally recognized methods.

A copy of the certificates of analysis for 3 batches is given in Appendix II.3. As it can be seen from this Appendix, the process leads to a product which is consistent and in conformity with the product specifications (available in Appendix I.6.1).

As explained in section I.6.1 (Product Specification) the vitamin D2 yeast concentrate will be commercialized for different foods applications, such as bakery, food supplements, etc. Depending on the application, the specifications and related analyses will be adapted according to the specification of the final product where the vitD2yeast concentrate is added.

All production operations in Lallemand plants are International Organization of Standards (ISO) certified and operate under Good Manufacturing Practices (GMP).

II.4 Comparison of Vitamin D2 Yeast Concentrate to Traditional Counterparts

In this section, vitamin D2 yeast concentrate is compared not to a traditional product but to the sum of two products: firstly to yeast and secondly to vitamin D2.

When compared to the yeast, the genetic stability testings (see section II.5.1) have demonstrated that the genetic profile of the yeast is not modified by the production process, so that *Saccharomyces cerevisae* in the vitamin D2 yeast concentrate is equivalent to the *Saccharomyces cerevisae* that is present on the QPS list and is widely used in food and beverages.

When compared to vitamin D2, it is known that vitamin D2 is produced from yeast exposure to UV. This process has been reported by several authors:

Roman and Arima (1957) stated that “irradiated yeast containing vitamin D2 had been widely used in both human and animal nutrition”.
Rose and Harrison (1970) reported the activity of a yeast manufacturer “who employs a strain of saccharomyces with very high ergosterol content in preparing a special food yeast containing 120 000 IU vitamin D2 per gram dry product”.

The production process of vitamin D2 yeast by the exposure to the UV light is also used today on an industrial scale and is in the public domain.

Examples:

- For many years, Synthesia, a company based in Czech Republic, has been using this process. Vitamin D2 is produced from yeast and using irradiation followed by other purtification steps using solvents. Ergocalciferol has been manufactured at Synthesia since 1962. Other producers use the same process as a means of making large quantities of vitamin D2 for the supplement industry. A large portion of the supplements available on the market are vitamin D2. It has been a continuous source since the early 1940's for the supplement industry.

- In *MedPedia* (November 3, 2011) under the definition of "vitamins" we can read: "in supplements and fortified foods, vitamin D is available in two forms, D2 (ergocalciferol) and D3 (cholecalciferol). Vitamin D2 is manufactured by the UV irradiation of ergosterol in yeast".

- In *ArticlesBase* web site, an article by Ante posted on July 31, 2009 states that "vitamin D2 ….derived by irradiating yeast…" and "vegans use this form of vitamin D because it is not derived from animals in any way".

Several products containing vitamin D2 are available on the European market:

- In the UK, there are several products using vitamin D2.
  - "Pure" (www.puredairyfree.co.uk) from Kerry.
  - "Suma Soy Spread" (www.sumawholesale.com) from Suma.
- In France, the company Triballat has been marketing a soya drink that contains vitamin D2 from yeast. http://www.sojasun.com/produits/gamme.html.
- Vitablend, a company based in the Netherlands, also markets a vitamin originating from yeast for food applications in France and Europe.

As it has been mentioned above, the food company based in Czech Republic (Synthesia) certified in writing that vitamin D2 has been manufactured by UV irradiation since 1962. Although in the Synthesia process, the exposure of the ergosterol to the UV light takes place only when ergosterol has been isolated from yeast, the process of UV irradiation is the same and used in the production of both vitamin D2 as a mineral substance and of our product vitamin D2 yeast concentrate to convert ergosterol to ergocalciferol.

II.5 STABILITY OF VITAMIN D2 YEAST

II.5.1 Genetic Stability of Vitamin D2 Yeast

Ultraviolet (UV) photoreactions can potentially cause mutations in micro-organisms. Under intensive doses, UV light can be effective in inactivating viruses, bacteria, yeast and moulds; a means of purification, sterilization and/or sanitation. These mutations could be generated as a result of the reactions of UV photons with cellular DNA, damaging the nucleic-acids. The most potent UV wavelength for micro-organism mutation and inactivation is 254 nm, and is the wavelength to be used in the commercial production of Vitamin D2 yeast concentrate. The formation of pyrimidine dimers and other photo-products of nucleic acids inhibit DNA replication and transcription and hence, prevent the cell or virus from multiplying. Because of the extended hours that the yeast is undergoing the photochemical reaction, genetic changes of the vitamin D enriched yeast might occur.

Nevertheless, our vitamin D yeast is produced in a submerged UV reactor system, in which a large amount of liquid yeast with high dry matter content is continuously treated via fast circulation. Due to poor transmission and penetration of UV rays in the concentrated liquid yeast and the large amount of yeast in the UV reactor, the UV dosage received by yeast cells is low, which would have almost no detrimental effect on yeast, as evidenced by the observation that the baking performance of the treated yeast is not significantly affected. Therefore, the UV intensity and dosage acting on yeast cells in our UV reactor system are much lower, compared to the UV rays used in mutagenesis (usually direct UV irradiation with high intensity). No significant mutations would be expected in our vitamin D yeast products, which is supported by our genetic analysis described below which show that no genetic variations are observed between the treated and un-treated yeasts.

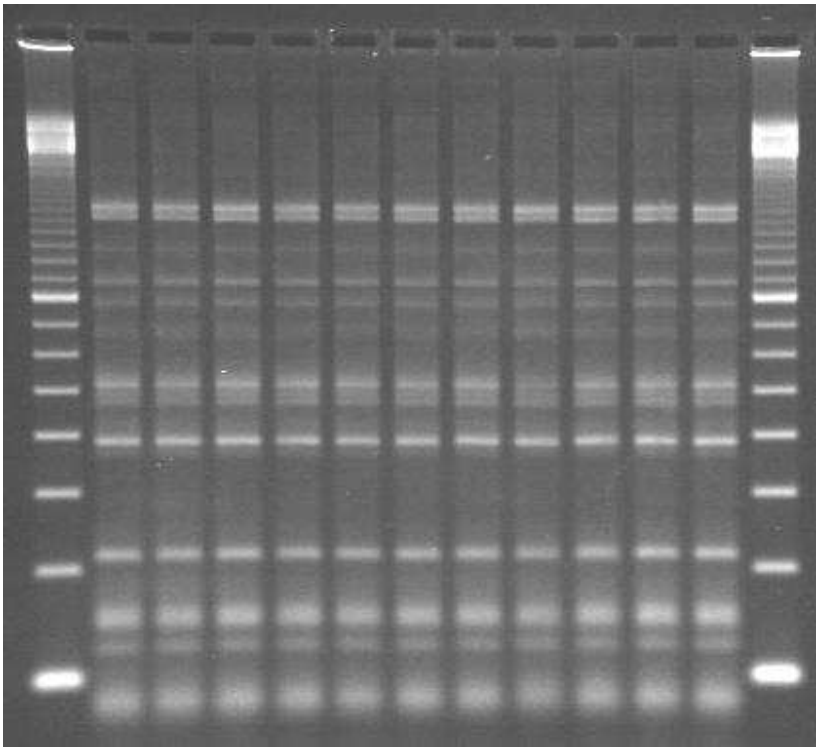
A study was conducted to evaluate the genetic stability of the vitamin D2 yeast during the photoreaction at 254 nm (Bertrand 2010). All analytical data observed, reported and discussed in the original file/report are summarized below.

We believe we have reliable genetic analysis methods in place to detect potential genetic variations and mutations in our commercial products. For UV mutagenesis in yeast, some references say that no gross chromosomal rearrangements are induced by UV mutagenesis (James and Kilbey 1977) while others observed an increase of the mobile element Ty transposition (Morawetz and Hagen 1990). Therefore, we believe that although cyclobutane pyrimidine dimers and pyrimidine-pyramidone or photoproducts are the most important DNA lesions induced by UV radiation, mobile elements will also transpose, and therefore both our RAPD-PCR and RFLP techniques should enable to detect it. In organisms other than yeast, other RAPD techniques and RFLP have been used to detect mutants from wild-type (Levall *et al*. 1994, Shafique *et al*. 2009, 2011). Our RADP-PCR will be the preferred technique for chromosomal rearrangements while RFLP will definitively better detect point mutations.

Materials and Methods

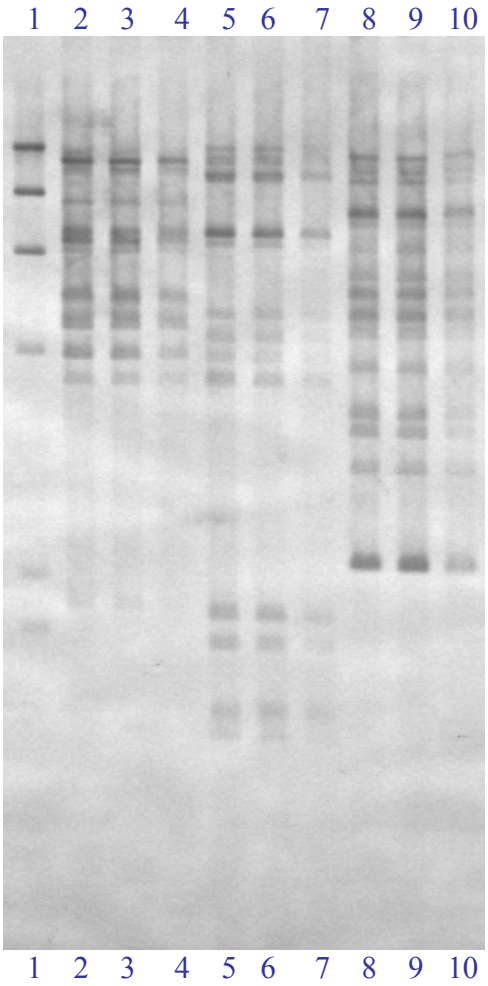
Genetic analysis was achieved on ten colonies of a vitamin D2 concentrate yeast sample from production after irradiation with U.V. light. It is important to notice that the irradiation time was exceptionally long compared with classical irradiation time for commercial production.
Isolated colonies were compared to control strain 256ng #256 from Lallemand Yeast Culture Collection. Samples were analyzed by a rapid polymerase chain reaction (PCR) with primers targeting inter-delta sequences and restriction length fragment polymorphism (RLFP) with enzymes *Pst*I, *EcoR*I and *Sal*I and probe TY.

Results and Discussions



1) Ladder 100 bp
2) Cream Yeast Sample Colony #1
3) Cream Yeast Sample Colony #2
4) Cream Yeast Sample Colony #3
5) Cream Yeast Sample Colony #4
6) Cream Yeast Sample Colony #5
7) Cream Yeast Sample Colony #6
8) Cream Yeast Sample Colony #7
9) Cream Yeast Sample Colony #8
10) Cream Yeast Sample Colony #9
11) Cream Yeast Sample Colony #10
12) 256ng #256
13) Ladder 100 bp

Figure 4: PCR (RAPD) of UV irradiated yeast with primers targeting inter-delta sequences



1) DNA Ladder
2) Cream Yeast Sample Colony #1 with *EcoR* I
3) Cream Yeast Sample Colony #2 with *EcoR* I
4) 256ng #256 with *EcoR* I
5) Cream Yeast Sample Colony #1 with *Pst* I
6) Cream Yeast Sample Colony #2 with *Pst* I
7) 256ng #256 with *Pst* I
8) Cream Yeast Sample Colony #1 with *Sal* I
9) Cream Yeast Sample Colony #2 with *Sal* I
10) 256ng #256 with *Sal* I

Figure 5: RFLPS of UV irradiated yeast with restriction enzymes *EcoR*I *Pst*I, *Sal*I and TY probe

Conclusions

The genetic profile of each colony analysed after UV irradiation demonstrated an identical genetic profile to the microbank control strain 256ng #256 by PCR (Figure 4) and RFLP (Figure 5).

II.5.2 Stability of Vitamin D2 Yeast Concentrate

Lallemand has conducted studies to assess the stability of vitamin D2 yeast concentrate. Three lots samples from commercial productions in the Lallemand Montreal Canada plant of Vitamin D2- were stored for this stability study. These samples were vacuum-packed in aluminum foil immediately after production and stored at room temperature. This information is summarized in Table 2. All samples, before and after storage, were forwarded to Covance Laboratories for vitamin D analysis in accordance with AOAC method 982.29 (AOAC, 2000).

Lot#	Production date	Initial vitamin D2 content (IU/100g)	Duration of storage (months)	Final vitamin D2 content (IU/100g)	% difference relative to initial result
82911D0K1	Dec-2008	2 400 000	34	2 350 000	- 2.08%
92995D0K1	Jan-2009	2 030 000	33	2 120 000	4.43%
91035D0K1	Feb-2009	2 160 000	32	2 390 000	10.65%

Table 2: Stability data for vitamin D2 yeast concentrate

These results do not indicate any significant loss of vitamin D2 in vitamin D2 yeast concentrate stored at room temperature for periods ranging from 32 to 34 months, proving that the product is stable throughout its three years shelf-life.

III HISTORY OF THE ORGANISM USED AS THE SOURCE

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient information pertaining to the history of the source organism:

- Is the novel food obtained from a biological source, *i.e.*, a plant, animal or microorganism?
- Has the organism used as the source of the novel food been derived using GM?
- Is the source organism characterized?
- Is there information to show that the source organism and/or foods obtained from it are not detrimental to human health?

These questions have been addressed collectively in the following section.

III.1 HISTORY OF USE OF YEAST

Saccharomyces cerevisiae is a non-pathogenic yeast that has been used in several countries in food such as bakery, winemaking, brewery, distillery, etc.

In the US, the Food and Drug Administration (FDA) listed *S. cerevisiae* as generally recognised as safe (GRAS):

- http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?filter=cerevisiae&sortColumn=&rpt=grasListing
- http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.896

In the EU, *S. cerevisiae* is also under the list of taxonomic units proposed for QPS status (Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA, the EFSA Journal, 2007).

- www.**efsa**.europa.eu/de/scdocs/doc/587.pdf

In Australia, *S. cerevisiae* is included in the Australian Register of Therapeutic Goods Ingredients https://www.ebs.tga.gov.au/ebs/ANZTPAR/PublicWeb.nsf/IngredientsPublic?OpenView&Start=7401&Count=20 under the "ingredients, all" page 371.

At the international level, the IDF (The International Dairy Federation) in collaboration with the European Food and Feed Cultures Association (EFFCA) assembled a list of microorganisms with a documented history of safe use in food. *Saccharomyces cerevisiae* is listed in this inventory (Mogensen *et al*., Bulletin of the International Dairy Federation, N°377/2002).

As demonstrated in section II.5.1 (genetic stability), the genetic profile of the yeast strain is not modified by UV irradiation. Therefore, we consider that our irradiated yeast is equivalent to the non-irradiated *Saccharomyces cerevisiae* and therefore is safe.

III.2 HISTORY OF USE OF VITAMIN D2 YEAST

In the 1920's, rickets was a major public health problem in the United States, and the discovery that the irradiation of food could render various foods antirachitic constituted an important milestone in the history of rickets. In 1927, it was determined that ergosterol was convertible to vitamin D_2 by ultra-violet irradiation (Rosenheim and Webster 1927). This process was patented and licensed to pharmaceutical companies, which led to the development of a medicinal preparation of vitamin D_2 and caused a rapid decline in the prevalence of rickets in children (WARF; Wolf 2004). In addition, irradiated yeast or activated ergosterol became the major vitamin D source for food fortification, mainly cereals, leading to a public health campaign to eradicate rickets by the 1930's (Rajakumar 2007).

The first evidence of the efficacy of irradiated yeast as a source of vitamin D was reported by Hess, one of the greatest nutritional clinical researchers of the early 20th century.

Hess and his colleagues reported as early as 1924 that various foods could develop antirachitic properties by means of ultra-violet irradiation with a quartz mercury vapour lamp (Hess and Weinstock 1925). They conducted investigations with several irradiated foods (fluid milk, dry milk, wheat flour, spinach, etc.) in order to determine their potential benefits for combating infantile rickets.

In 1925, the potential benefits of irradiated milk were reported (Hess and Weinstock 1925). In further communications, the authors considered the technical aspects of milk irradiation and reported that the process could be performed under fully controlled conditions resulting in a product which was reliable and constant, without any disagreable taste or odour and not deprived of its essential vitamins (Supplee *et al*. 1932). Hess reported in 1932 that irradiated milk had been used on a considerable scale in Germany and recommended the use of such milk for infants and children, especially in large communities (Hess 1932).

Hess and his colleagues also studied the therapeutic effects of viosterol (irradiated ergosterol) (Hess *et al.* 1930) and irradiated yeast. The high ergosterol content of yeast prompted the adoption of ultraviolet rays by which ergosterol is converted into vitamin D_2. They used irradiated brewers' or bakers' yeast. The dried yeast was spread in a thin layer and irradiated at a distance of 1 foot for a period of one-half hour. A daily dose of irradiated yeast suspended in milk was found highly effective in animals and in infants (Hess 1927).

In 1932, at the symposium of the Food and Nutrition Section of the American Public Health Association, Hess pointed out that, instead of producing antirachitic milk by means of direct irradiation, this end might be accomplished by means of feeding cows activated yeast (Hess 1932).

The use of irradiated yeast as feeding material to increase the vitamin D content in cow's milk was studied by other researchers. Thomas and MacLeod (1931) reported that the vitamin D activity of cow's milk was increased several times by supplementing feed rations with irradiated yeast and irradiated ergosterol. Similar results were obtained by Schmidt (Schmidt, 1953).

The concept of "yeast milk" to designate milk from cows fed irradiated yeast was used by Jeans in his report to the Committee on Foods on the value of different varieties of vitamin D in milk. The author reviewed the results obtained in animals (chickens and cows) fed with different sources of vitamin D formed either in nature or by the action of ultra-violet radiation (cod liver oil, irradiated milk, irradiated cholesterol, irradiated yeast and irradiated ergosterol) (Jeans 1936).

Concerning clinical studies in humans, considerable work was done in the 1930's to determine the antirachitic efficacy of various irradiated substances. Several clinical studies were conducted involving children. Drake *et al*. (1934) conducted a study on 529 infants, aged 3 weeks to 8 months and largely of British and northern European descent over a period of 5 winter months. The subjects were given vitamin D from several different sources: cod liver oil, viosterol and irradiated milk for comparison of their antirachitic effectiveness.

In another study, Drake, Tisdall and Brown reported on the effect of daily administration of vitamin D as irradiated yeast in the prevention and cure of rickets during 5 winter months. For ease of administration the irradiated yeast was mixed with farina and given to 69 infants (Drake et *al*. 1936).

Observational studies were conducted on 1228 infants during the winter months of 3 consecutive years (1933 to 1936) with the same objective (Drake 1937). No differences were observed in terms of antirachitic effectiveness of vitamin D administered in the form of cod liver oil, a mixture of fish liver oils of high potency, irradiated cholesterol, or irradiated milk. The effectiveness of irradiated yeast (which was administered mixed with cereals and thus found to be more effective in the prevention of rickets) was also commented upon.

In Europe, Kon and Mayzer (1930) conducted investigations in Poland with pure baker's yeast. For activation, the yeast was spread by means of a sieve in thin layers on metal trays and exposed to the radiation of a Hanan quartz mercury-vapour lamp for 30 minutes. The study involved 12 rachitic children who received irradiated yeast suspended in a milk mixture once daily. The outcome of this study was the disappearance of the symptoms in the course of six to eight weeks. In addition to demonstrating the effectiveness of the irradiated yeast, the interest and application of this study involved not only its therapeutic use but also other considerations. For instance, the authors drew attention to a possible indiscriminate use of irradiated preparations by the general population and recommended that antirachitic prophylaxis was left in the hands of physicians. However, they recognized that in the context of a large-scale antirachitic action in Poland, irradiated yeast seemed to fulfil the requirements of reliability, low price and ease of preparation.

Other investigators used irradiated yeast in clinical studies to evaluate the antiirachitic value of different vitamin D sources (Compere et *al*., 1935).
Despite the effectiveness in healing rickets, no safety issues were noticed in any of the reported clinical investigations.

Many scientific articles and other general publications reporting on the health benefits of vitamin D2 yeast can be found. Those articles support the suitability of vitamin D2 yeast for different consumer groups such as organic foods for vegans (vegetarian) and Kosher foods for Jewish persons.

Finally, our vitamin D2 yeast has been approved by the FDA in the USA, including its use in bakery (Appendix C letter from FDA August 2007).

It has also recently been approved by Health Canada. This decision has been recently published in the Canada Gazette (part I, February 19 2011): “Health Canada has received a submission to permit the optional addition of vitamin D2 yeast to yeast-leavened bakery products at level of 90 IU per 100g. Health Canada has completed the safety assessment of the proposal (…) Evaluation of available data has demonstrated that the addition of vitamin D to the foods described above at a level of up to 90 UI per 100g of product, as consumed, is safe.(see Appendix B).

IX ANTICIPATED INTAKE/EXTENT OF USE OF VITAMIN D2 YEAST CONCENTRATE

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient information pertaining to the intake/extent of use of the novel food:

- Is there information on the anticipated uses of the novel food based on its properties?
- Is there information to show anticipated intakes for groups predicted to be at risk?
- Will introduction of the novel food be restricted geographically?
- Will the novel food replace other foods in the diet?

These questions have been addressed collectively in the following section.

IX.1 CONDITIONS OF INTENDED FOOD USE

Vitamin D2 yeast concentrate is intended to be used as an alternative form of vitamin D2 and therefore its intended uses are the same as for vitamin D2. We anticipate that it will appeal mainly to those food and supplement producers aiming at the vegan or vegetarian consumers, which is a small, yet growing segment.

Vitamin D2 is permitted for addition to foods and to food supplements in the EU and is listed in Regulation (EC) 1170/2009, Annex II (Vitamin and mineral substances which may be used in the manufacture of food supplements) and in Annex III (vitamin formulation and mineral substances which may be added to foods).

Estimated consumption of vitamin D2 (Section IX.3) is based on bread based products and supplements which are the major categories that we envisage will contain vitamin D2 yeast concentrate.

The individual proposed food uses and use-levels in the EU are summarized in Table 3.

Proposed food use	Proposed use levels (µg vitD/100g)
White bread	5
Wholemeal bread	5
Brown, granary and wheatgerm bread	5
Other breads	5
Food supplements	5

Table 3: Proposed food uses for vitamin D2 and proposed levels of vitamin D

Please, note that not all the types of breads listed above will always contain vitamin D2 yeast concentrate.

The level of use of vitamin D2 yeast concentrate will contribute to a maximum of 100% of RDA of vitamin D. In practice, the food operators' use of vitamin D in food products is generally 15 % or 30% of RDA per serving dose. As an example, if we take into account a product containing 30% of RDA in vitamin D (i.e. 1.5 µg of vitamin D) per serving, 3 mg of vitamin D2 yeast concentrate (of 2 000 000 UI/100g) will have to be incorporated. However, for the estimation of consumption, as a worst case scenario, we have taken the 100% of RDA in vitamin D.

IX.2 FOOD LABELING INSTRUCTIONS/FOOD INGREDIENTS

Lallemand proposes that food products or supplements containing vitamin D2 yeast concentrate use on their ingredient list the words "vitamin D yeast," or "vitamin D2 yeast".

IX.3 ESTIMATED CONSUMPTION OF VITAMIN D2 YEAST

IX.3.1 Estimated Consumption from Proposed Food Uses

Lallemand considers vitamin D2 yeast concentrate as an alternative source of vitamin D2 and its uses should be the same as the forms of vitamin D listed in Annex III of Regulation (EC) 1170/2009 amending Directive 2002/46/EC of the European Parliament and of Council and Regulation (EC) No 1925/2006 of the European Parliament and of the Council with regard to the lists of vitamin and minerals and their forms that can be added to foods, including food supplements.

However, in practice, Lallemand's intention to market vitD2 yeast concentrate is for bread products and food supplements, and thus the estimates of potential maximum dietary intake of vitamin D coming from this source is based on the proposed usage levels summarized in Tables 4, 5 and 6 respectively for adults, children 11 to 18, and children 4 to 10.

The estimates represent potential maximum intake levels based on the proposed uses of vitamin D2 yeast concentrate (Cf. Table 3 in section IX.1). The estimates are maximum theoretical intakes that provide a conservative basis for evaluation of safety.

Dietary consumption of vitamin D was based on food consumption data as part of the United Kingdom (UK) survey of food intake, nutrient intake and nutritional status of the British population (adults aged 19 to 64, children 11 to 18, and children 4 to 10): National Diet and Nutrition Surveys (NDNS). In 2010, the results from the first year of the National Diet Nutrition Survey (NDNS) 2008/2009 were published (Bates *et al*. 2010).

Adults

Proposed food use	Proposed use levels (µg/100g)	Mean daily adult consumption of bread (g) [1]		Estimated mean daily consumption of Vitamin D (µg) [2]		Estimated high level daily consumption of Vitamin D (µg) [3]	
		Male	Female	Male	Female	Male	Female
White bread	5	77	51	3.85	2.55	11.55	7.65
Wholemeal bread	5	60	36	3	1.8	9	5.4
Brown, granary and wheatgerm bread	5	42	36	2.1	1.8	6.3	5.4
Other breads	5	31	22	1.55	1.1	4.65	3.3

Table 4: Estimated daily intake of vitamin D for adults from proposed food uses

[1] 19-64 years food consumption (only food consumers) (NSDS data; Bates 2010)
[2] Assuming all products contain vitamin D from vitamin D yeast at the indicated level ("worst case" scenario)
[3] Calculated as 3times mean value

Children 11 to 18

Proposed food use	Proposed use levels (µg/100g)	Mean daily children consumption of bread (g) [1]		Estimated mean daily consumption of Vitamin D (µg) [2]		Estimated high level daily consumption of Vitamin D (µg) [3]	
		Male	Female	Male	Female	Male	Female
White bread	5	75	55	3.75	2.75	11.25	8.25
Wholemeal bread	5	51	31	2.55	1.55	7.65	4.65
Brown, granary and wheatgerm bread	5	38	33	1.9	1.65	5.7	4.95
Other breads	5	35	28	1.75	1.4	5.25	4.2

Table 5: Estimated daily intake of vitamin D for children 11 to 18 from proposed food uses

[1] 11-18 years food consumption (only food consumers) (NSDS data; Bates 2010)
[2] Assuming all products contain vitamin D from vitamin D yeast at the indicated level ("worst case" scenario)
[3] Calculated as 3times mean value

Children 4 to 10

Proposed food use	Proposed use levels (µg/100g)	Mean daily children consumption of bread (g) [1]		Estimated mean daily consumption of Vitamin D (µg) [2]		Estimated high level daily consumption of Vitamin D (µg) [3]	
		Male	Female	Male	Female	Male	Female
White bread	5	48	45	2.4	2.25	7.2	6.75
Wholemeal bread	5	41	25	2.05	1.25	6.15	3.75
Brown, granary and wheatgerm bread	5	51	29	2.55	1.45	7.65	4.35
Other breads	5	30	21	1.5	1.05	4.5	3.15

Table 6: Estimated daily intake of vitamin D for children 4 to 10 from proposed food uses

[1] 4-10 years food consumption (only food consumers) (NSDS data; Bates 2010)
[2] Assuming all products contain vitamin D from vitamin D yeast at the indicated level ("worst case" scenario)
[3] Calculated as 3times mean value

In order to provide a conservative estimate (worst case scenario) all categories of bread that may contain vitamin D2 yeast concentrate were assumed to contain the maximum allowable levels of vitamin D (100% of the RDA, i.e. 5 µg/100g).

High level consumption is based on the 97.5th percentile and we have assumed this can be as high as 3 times the mean. Following this assumption, as a worst-case scenario, for a consumer who would exclusively choose bread containing vitamin D2 yeast concentrate and consume high amounts of white bread, the estimate of intake of the 97.5 percentile will be around 11 µg of vitamin D per day. In making this assumption we note that it is extremely unlikely that the same consumers would simultaneously be high level consumers of all bread types.

While this is above of the recommended daily intake in the EU for vitamin D (which is 5 µg) it is, nonetheless, significantly less than the RDA issued by the Institute of Medicine (IOM, 2010) that is, for adults, 600 UI/Day= 15 µg (the conversion being 1µg= 40 UI) and the Upper safe limit set by EFSA of 50 µg/day for adults, EFSA 2006) and IOM (100 µg/day, IOM 2010).

By the same token, for children, in the worst case scenario and on the basis that the 97.5th high percentile is calculated as 3 times the mean, if we consider the highest level of consumption of white bread, the estimate of intake of the 97.5 percentile will be respectively around 11 µg and 7 µg of vitamin D per day for children from 11 to 18 and from 4 to 10 years. These values are lower than the upper safe limit defined by EFSA in 006 (50 µg/day for children from 11 to 17 and 25 µg/day for children from 3 to 10) and IOM in 2010 (100 µg/day for children from 9 to 17, 75 µg/day for children from 4 to 8 and 62.5 µg/day for children aged of 3).

As it will be shown in Section X.1.1 (Intake of vitamin D in Europe), despite the fact that the fortification regulation allows the addition of vitamin D to all foods, the intake of vitamin D by the European population is far below the recommendations. Therefore, the use of vitamin D2 yeast concentrate will not lead to an overconsumption of vitamin D2.

IX.3.2 Estimated Consumption from Proposed Food Supplements Uses

In addition to the use of vitamin D2 as a source of vitamin D in food, Lallemand intends to commercialize the vitamin D2 yeast concentrate as a source of vitamin D to be added to capsules, tablets and other food supplements.

The intake of vitamin D coming from food supplements is limited compared to food sources. According to the U.K National Diet and Nutrition Survey (NSDS) program (Henderson *et al*. 2003), the intake of vitamin D coming from food supplement sources has been estimated to be only 12% of the global intake in vitamin D for men and 24% for women.

Moreover, the figures of the sales of vitamin D in some European countries show that the consumption of vitamin D as a food supplement is limited (see Table 7).

Geographies	2005	2006	2007	2008	2009	2010
Belgium	4,009.3	4,548.9	4,991.4	5,353.7	7,398.3	10,772.0
Denmark	3,629.3	3,579.4	3,483.4	3,685.1	4,083.1	4,397.2
Finland	10,273.3	10,088.4	9,937.0	11,167.9	11,547.6	12,069.5
Germany	81,803.2	81,999.6	81,753.6	80,944.2	79,891.9	78,677.6
Greece	5,364.1	5,665.6	6,667.8	7,210.6	7,876.1	8,506.2
Ireland	6,156.1	5,719.0	5,507.4	5,665.4	5,772.5	5,636.4
Norway	40,687.4	41,480.9	42,206.8	43,050.9	43,782.8	44,654.0
Spain	5,656.3	5,571.5	5,515.8	5,466.1	5,430.0	5,400.7
Sweden	6,576.6	6,622.7	6,662.4	7,262.0	7,988.2	8,247.8
United Kingdom	121,319.4	144,271.8	157,775.6	160,666.3	163,092.3	168,311.3

Table 7: Vitamin D sales retail volume (modelled, in units*1000)

Research sources: Consumer health: Euromonitor from trade sources / national statistics

Lallemand has estimated the market of vitamin D2 yeast concentrate to be around 2% (at the maximum) of the global market of vitamin D food supplement, therefore, exposure from food supplement uses, from a population standpoint, will be a small part of the total exposure for adults (cf section IX.3.1).

IX.4 CONCLUSION

In this section, we have described the intended uses of vitamin D2 yeast concentrate, which are the same as vitamin D2 (authorized today as a source of vitamin D in the EU in Annex III of Regulation 1170/2009). According to Regulation 1170/2009, vitamin D2 can be added to all foods; however, in practice, not many foods are supplemented with vitamin D and according to the data from food consumption databases, as it will be shown in section X, the intake of vitamin D by the European population is far below recommended levels.

For instance, in the UK, the NSDS 2003 report (Henderson *et al*. 2003) concludes that mean daily intake of vitamin D from all sources was 4.2µg for men and 3.7µg for women (less than the recommended daily intake of 5 µg). In the EVM (Expert group on Vitamins and Minerals) report (2003) this figure is 3µg for men and women

Moreover, vitamin D2 yeast concentrate is estimated to replace only a part of the vitamin D uses and contribute to a small part of the overall vitamin D consumption (not all the foods are enriched with vitamin D and not all the enriched vitamin D foods will use the vitamin D2 yeast concentrate).

In the food supplement market, Lallemand estimates that the market share for vitamin D2 concentrate will be around 2% maximum of the vitamin D supplement market.

This data taken together show that the consumption of vitamin D2 yeast concentrate in food and in food supplements uses will be limited and will not lead to any risk of vitamin D overconsumption by the European population.

X INFORMATION FROM PREVIOUS HUMAN EXPOSURE TO VITAMIN D2 YEAST

Based on the SCF guidelines, the following questions must be answered in the affirmative to ensure sufficient information pertaining to previous human exposure to the novel food:

- Is there information from previous direct, indirect, intended or unintended human exposure to the novel food or its source which is relevant to the EU situation with respect to production, preparation, population, lifestyles and intakes?
- Is there information to demonstrate that exposure to the novel food is unlikely to give rise to mitochondrial, toxicological and/or allergenicity problems?

These questions have been addressed collectively in the following sections.

As it has already been stated, vitamin D2 yeast concentrate consist of vitamin D2 from one side, and yeast from the other side. This section has been addressed by taking into account:

- Previous human exposure to vitamin D on one side and
- Previous exposure to yeast (*Saccharomyces cerevisiae*) on the other side

X.1 NATURAL OCCURRENCE OF VITAMIN D IN THE DIET

In its opinion on the tolerable upper intake level of vitamin D in September 2002 (also expressed in Tolerable upper intake levels for vitamins and minerals, EFSA 2006), the Scientific Committee on Food (SCF 2002) has described the occurrence of vitamin D in food as follows:

Vitamin D forms in food

Vitamin D comprises two closely related substances of nutritional importance: vitamin D3 (cholecalciferol), which is the physiological form, and the synthetic analogue vitamin D2 (ergocalciferol). The two forms only differ by the side chain to the sterol skeleton (Holick 1999). It has been assumed, based on studies in the 1930's showing no conclusive difference between vitamin D3 (from cod liver oil) and D2 in their preventing effect against infantile rickets, that vitamin D2 for practical purposes could be regarded as equal to vitamin D from cod liver oil. There is no contemporary evidence showing that vitamin D3 and D2 are equally efficient in increasing the circulating metabolite proximate to the active form. Indeed, later studies have shown important biological differences in this respect between these forms (Trang *et al*. 1998).

Vitamin D3 and vitamin D2, together with the provitamins they are made from, are all derivatives of sterols, their chemical structure resembles cholesterol, bile acids and the sex hormones. Vitamin

D2 is formed by UV radiation from its precursor ergosterol. Ergosterol is found in plants, especially yeast and fungi. The synthesis of ergocalciferol from ergosterol hardly takes place in nature. Plants are thus a poor source of vitamin D2. Synthetic vitamin D2 produced by irradiation of ergosterol was the form formerly added to food or given as supplements. During the past two decades, vitamin D3 has also been used to fortify milk, margarine and other foods worldwide, and although the use of vitamin D2 in food and supplements still is widely used, its use is less than before. Vitamin D3 is formed from its precursor 7-dehydrocholesterol, which is found in ample amounts in the skin and fat depots in animals and man. Vitamin D is relatively stable in fat solutions, e.g. is not inactivated by pasteurisation or sterilisation. It oxidises in contact with air and in acid solutions and is inactivated when exposed to sunlight.

Vitamin D from breast milk

The British Food composition tables (Holland *et al*. 1991) use the value 0.4 µg/L vitamin D in human breast milk. The same value is used in the Norwegian food composition tables. However, the literature reports a quite large range of concentrations, varying from 0.1 to 1.2 µg/L. A variety of compounds with vitamin D activity (metabolites) are present in human milk, but 25(OH)D accounts for the majority of the antirachitic activity (Reeve *et al*. 1982; Weisman *et al*. 1982; Ala-Houhala *et al*. 1988; Hillman 1990). Human milk even from a vitamin D-sufficient mother provides a marginal amount of total vitamin D activity. The 25(OH)D level was higher in hind- than in foremilk (Ala-Houhala *et al.* 1988). Vitamin D activity in human milk of unsupplemented mothers was lower in the winter than in the summer. The influence of supplementation with 25 µg ergocalciferol or cholecalciferol or 50 µg cholecalciferol on vitamin D activity in human milk in summer and winter was investigated by Ala-Houhala *et al*. (1988). They found that supplementation with 50 µg of vitamin D could increase vitamin D activity of milk in the winter to that of unsupplemented mothers in the summer, but the responses varied widely among individuals. Markestad (1983) found a strong correlation between infant and maternal plasma 25(OH)D concentrations both at birth and after 6 weeks in unsupplemented infants born in the winter in the northern areas. The 25(OH)D concentrations in the infants were considerably reduced and reached levels associated with rickets during this period. It appears that sun exposure of the infant is a very important determinant for vitamin D status. Although a study in Caucasians from central USA showed that bone mineralisation was normal in unsupplemented and exclusively breast-fed infants up to 16 weeks (Roberts *et al*. 1981), most studies agree that fully breast-fed infants have a reduced vitamin D status after 6 weeks of age if no supplemental D is given. The general recommendation therefore is that infants should be supplemented with vitamin D.

Vitamin D intake from food

Only a few foods contain vitamin D, i.e. vitamin D3, naturally in quantities that have an impact on the dietary intake: fish liver, fish liver oils, fatty fish and egg yolks. Thus, some countries practice fortification of certain foods with vitamin D, most often milk, margarine and/or butter. The mean intakes in different studies vary with age group, food and supplementation habits and gender. Recent publications from various parts of Europe all show that a substantial part of the population including pre-school children has a vitamin D intake below the recommended dietary intakes

(Davies *et al*. 1999; de Jong *et al*. 1999; Koenig and Elmadfa 2000; Lehtonen-Veromaa *et al*. 1999; Ortega et al, 1995; van der Wielen et al, 1995). The low intake is confirmed by results from the SENECA study, an investigation of the diet and health of 824 elderly people from 19 towns in 11 countries (Greece, Portugal, Italy, Spain, France, Switzerland, Hungary, Belgium, Netherlands, Denmark and Norway). Thirty-six per cent of the men and 47% of the women had 25(OH)D concentrations below 30 nmol (van der Wielen *et al*. 1995).

Surprisingly, lowest mean 25(OD)D concentrations were found in southern European countries; more than 80% of Italian and Greek women had values below 30 nmol compared with 18% in Norway. One factor associated with better vitamin D status was increased fish consumption, but the main reasons for the relatively good vitamin D status in the Scandinavian countries are probably fortification of food and a higher percentage of people taking vitamin D supplements. Cod liver oil was taken regularly by 35% of all men and 34% of all women in Norway in 1997, and the percentage was higher among the elderly (Norkost 1997). A much lower prevalence of vitamin D deficiency was found in the French general adult population; of 1191 adults 11% was below 30 nmol 25(OH)D in serum (Chapuy *et al*. 1997; Guinot *et al*. 2000). They found a correlation to latitude and skin exposure, as 24% of those with low exposure was deficient.

The intake of vitamin D in Europe can be illustrated by the following table, which gives the mean dietary vitamin D intakes in several European countries (EFSA report on tolerable upper intake levels for vitamin D, EFSA, 2006).

Country	Type of survey	n	Method	Supplements*	Mean	97.5%
Austria[a]	Individual	2488	24h recall	Not defined	4.0	22.2
Germany[b]	Individual (M) Individual (F)	854 1134	7-day dietary record	- -	4.0 3.1	16.8 11.9
UK[c]	Individual (M) Individual (F) Individual (M) Individual (F)	1087 1110 1087 1110	7-day weighed inventory	- - + +	3.4 (2.9) 2.5 (2.2) 3.8 (3.0) 3.1 (2.3)	9.9 6.9 12.7 12.6
Italy[d]	Household	2734	7-day record	+	3.0	8.4
Netherlands[e]	Household	5958	2-day record	-	3.7	8.9
Norway[f]	Individual (M) Individual (M) Individual (F) Individual (F)	1298 - 1374 -	Semiquantitative FFQ last year, 180 food items	- + - +	5.8 11.2 4.0 10.3	13.0 37.6 10.3 33.3
Ireland[g]	Individual (M) Individual (F)	662 717	7-day estimated food record	+ +	3.7 3.7	13.5 14.9

* + data included supplements; - data excluded supplements.
[a] Elmadfa *et al* (1998). [b] Heseker *et al* (1994) - values are the median.
[c] Gregory *et al* (1990) - values are the mean with the median in parentheses.
[d] Turrini (1996). [e] Hulshof and Kruizinga (1999). [f] Norkost (1997).
[g] IUNA (2001).

Table 8: The daily intakes of vitamin D (µg/day)

The Recommended Daily Allowance (RDA) for vitamin D in Europe is 5 µg/day (Commission Directive 2008/100/EC amending Council Directive 90/496/EEC on nutrition labeling for foodstuffs).

However, the intake of vitamin D by the European population is far below RDA, as it was reported in 2002 by the SCF (Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Vitamin D, 2002) and also recently, in 2010, as mentioned in the Parliament magazine, (http://www.theparliament.com/digimag/vitamindsupplement), which report indicates that about 50% of Europe's population is deficient in vitamin D.

Moreover, this fact can be illustrated by two important surveys recently achieved in Germany and UK.

Since there is no food consumption database at European level, we have used national surveys. Some of them do not contain information on vitamin D and/or don't use standardized analytical methods, which make difficult the calculation of vitamin D intake. However, there are 2 countries where national data exist on vitamin D consumption, Germany and the UK. Both reports are commented below:

X.1.1 Germany: Nationale Verzehrsstudie II

In 2007, the Nationale Verzehrsstudie II (NVS II) provided information on the nutrient and energy intake of more than 15 000 Germans (from 14 to 80 year-old), their current food consumption, and on lifestyle and eating behavior (what, when, where and why do Germans eat?). This survey contains information on the vitamin D consumption.

The median vitamin D intake for men is 2.9 µg / day and in women at 2.2 µg / day (Table XXX).

For men and women from 14 to 64 years old, the median vitamin D intake is well below the 5 µg/day Recommended Daily Allowance (RDA) for vitamin D in Europe (Table 9 and Figure 6).

Moreover, for people older than 65 years, The Population Reference Intake (PRI) recommended by the Committee is 10 µg/day (SCF, 1993).
The intake of vitamin D is also not attained in the senior population. For persons aged 65-80 the median vitamin D intake is only about a quarter of the recommendation.

In all age groups, 82% of men and 91% of women do not meet the recommendations for vitamin D consumption. This is particularly true among young people and young adults (> 86% for men, > 96% for women) and seniors (94% for men, 97% for women).

Vitamin D intake (µg/day)	n	Mean	Median	Standard error	Upper 5 percentile	Lower 5 percentile	Reference values	Median intake as % of reference values	People (%) under the reference values
Men	7093	3.8	2.9	0.04	9.6	0.9			82
14-18	712	2.7	2.2	0.08	6.9	0.8	5	44	91
19-24	510	3.0	2.3	0.11	7.0	0.8	5	46	86
25-34	690	3.5	2.8	0.11	8.0	0.9	5	56	81
35-50	2079	3.8	2.9	0.07	9.7	0.9	5	58	78
51-64	1633	4.2	3.3	0.09	10.9	1.0	5	66	74
65-80	1469	4.4	3.3	0.11	10.6	1.0	10	33	94
Women	8278	2.9	2.2	0.03	7.0	0.7	5		91
14-18	700	2.0	1.6	0.06	4.4	0.5	5	32	97
19-24	510	2.0	1.6	0.07	4.8	0.6	5	32	96
25-34	972	2.6	2.0	0.07	6.4	0.7	5	40	91
35-50	2694	2.7	2.2	0.04	6.3	0.8	5	44	90
51-64	1840	3.4	2.6	0.07	8.8	0.8	5	52	83
65-80	1562	3.4	2.6	0.07	8.2	0.8	10	26	97

Table 9: Intake of vitamin D from food sources in Germany (µg / day) and comparison with the reference values in different age groups for men and women

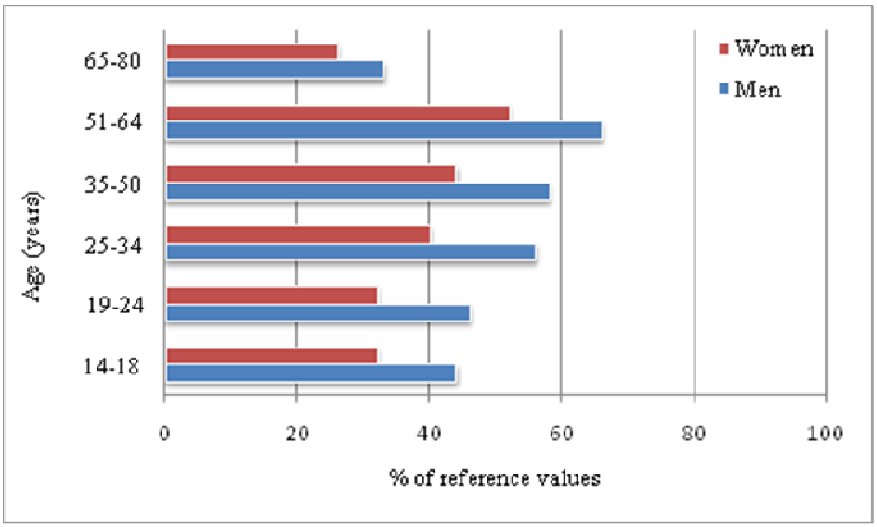


Figure 6: Median vitamin D intake (Germany) as % of reference values

X.1.2 U.K.: The National Diet and Nutrition Survey

The National Diet and Nutrition Surveys (NDNS) are a series of government-funded surveys of food intake, nutrient intake and nutritional status of the British population (adults aged 16 to 64), undertaken to support nutritional policy and risk assessment.

In 2010, the results from the first year of the National Diet Nutrition Survey (NDNS) 2008/2009 were published (Bates *et al*. 2010). The NDNS rolling programme aims to provide quantitative data on the food and nutrient intakes, sources of nutrients and nutritional status. The programme is carried out in all four countries of the United Kingdom (UK) and is designed to be representative of the UK population.

As shown in Table 10 and Figure 7: Median vitamin D intake (UK) as % of reference values, the median daily intake of vitamin D from food sources in all the age group is lower than the European RDA. We can see that this intake is lower than 50% of the RDA for all groups except for the 19-64 men group where it is 56%.

	Age	n	Mean	Median	Standard error	Upper 2.5 percentile	Lower 2.5 percentile	RDA*	Median intake as % of reference values
Men	4-10	119	1.9	1.8	1.0	4.1	0.5	5	36
	11-18	114	2.5	2.3	1.4	6.5	0.8	5	46
	19-64	181	3.1	2.8	1.8	8.0	0.6	5	56
Women	4-10	119	2.0	1.9	1.0	4.0	0.4	5	38
	11-18	110	2.1	1.8	1.3	5.6	0.6	5	36
	19-64	253	2.7	2.3	1.8	7.4	0.4	5	46

*RDA: Recommended Daily Allowance (Directive 2008/100/EC)

Table 10: Intake of vitamin D from food sources in UK (µg / day) and comparison with RDA in different age groups for men and women

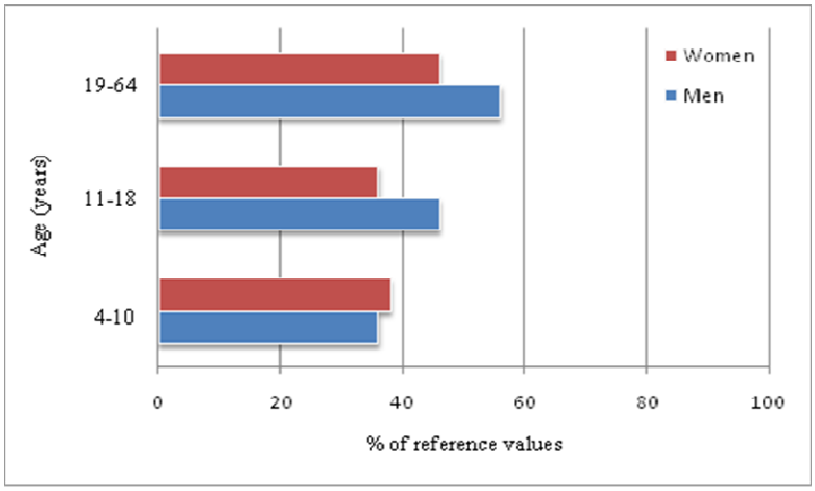


Figure 7: Median vitamin D intake (UK) as % of reference values

A comparison with the past surveys is also available in the 2010 NSDS report, showing that the lack of vitamin D intake from food sources is a recurrent problem in UK. For all age groups, the median intake of vitamin D from food sources is almost the same or lower between the last survey (1997 for young people and 2001 for adults) and the 2008-2009 one year program.

		Age	n	Mean	Median	Standard error	Upper 2.5 percentile	Lower 2.5 percentile	RDA*	Median intake as % of reference values
Men	1997 NSDS young people	4-10	410	2.3	2.0	1.3	5.4	0.5	5	40
		11-18	416	2.9	2.5	1.8	7.0	0.6	5	50
	NSDS 1 year programme 2008/09	4-10	119	1.9	1.8	1.0	4.1	0.5	5	36
		11-18	114	2.5	2.3	1.4	6.5	0.8	5	46
	2000/01 NSDS adults	19-64	833	3.8	3.2	2.7	10.0	0.8	5	64
	NSDS 1 year programme 2008/09	19-64	181	3.1	2.8	1.8	8.0	0.6	5	56
Women	1997 NSDS young people	4-10	397	2.0	1.8	1.2	4.5	0.5	5	36
		11-18	448	2.2	1.9	1.4	5.4	0.5	5	38
	NSDS 1 year programme 2008/09	4-10	119	2.0	1.9	1.0	4.0	0.4	5	38
		11-18	110	2.1	1.8	1.3	5.6	0.6	5	36
	2000/01 NSDS adults	19-64	891	2.9	2.3	2.4	9.0	0.4	5	46
	NSDS 1 year programme 2008/09	19-64	253	2.7	2.3	1.8	7.4	0.4	5	46

Table 11: Comparison of vitamin D intakes from food sources (µg / day) with past NSDS surveys.

X.2 PREVIOUS EXPOSURE TO *SACCHAROMYCES CEREVISAE* IN THE DIET

The vitamin D2 yeast concentrate consists of a yeast from the species *Saccharomyces cerevisiae*. It is amply documented that *S.cerevisiae* has a long history of use for human consumption, via bread, beer, etc... for decades. Such "long use" has further been recognized by EFSA as being a history of "safe use".
In fact, in its Opinion of the Scientific Committte on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganism referred to EFSA (EFSA Journal 2007, 587:1-16), EFSA has noted that "yeast used in food production, particularly brewers/bakers yeast, are considered among the safest of microorganisms (…) Today, the impact of yeast on food and beverage production extends beyond the original and popular notions of bread, beer and wine fermentations by *Saccharomyces cerevisiae* (Querol, Belloch *et al.* 2003; Fleet 2006)".

Therefore, from the point of view of the exposure to yeast, the exposure of the European population to *Saccharomyces cerevisiae* has been widely documented and reported as safe.

X.3 POTENTIAL ALLERGENICITY CONCERNS

The vitamin D2 yeast concentrate is not produced using any allergen so there is no allergenicity concern.

X.4 CONCLUSION

The intended use of the vitamin D2 yeast concentrate is the same as for the vitamin D2 that is currently added to foods and food supplements.
We have seen, from the data on the exposure to vitamin D presented in this section, that the European population does not attain the recommended daily values of vitamin D, and thus the replacement of vitamin D2 by vitamin D2 yeast concentrate is not likely to lead to an overconsumption of vitamin D.

Moreover, the exposure to vitamin D2yeast concentrate will be as the exposure to yeast, which has been widely reported to be safe.

The only difference between the exposure of a mixture of vitamin D2 and yeast from one side, and to vitamin D2 yeast concentrate on the other side, would be the compounds that may originate from the exposure to the UV light.
As it has been shown in Section I.5.1 (Products characterization) and in related HPLC chromatograms (see Appendix I.5.1C and I.5.1D) the only other sterol that is found in samples of

yeast exposed to the UV light is tachysterol. As described by Holick (1981) and Gilchrest 2006), tachysterol is naturally present in the human body and is formed from previtamin D3 to limit the levels of vitamin D during exposure to UV. Both articles mention the fact that tachysterol is biologically inert, thus non toxic. Moreover, tachysterol is formed in very small amounts (see section XII.3 Tachysterol Toxicity and Appendix I.5.2A).

Thus, we believe that the consumption of the vitamin D2 yeast concentrate, based on previous consumption of vitamin D2 on one side, and yeast on the other side, is safe for the European population.

XI NUTRITIONAL INFORMATION ON VITAMIN D2 YEAST CONCENTRATE

Based on the SCF guidelines, the following question must be answered in the affirmative to ensure sufficient nutritional information pertaining to the novel food:

- Is there information to show that the novel food is nutritionally equivalent to existing foods that it might replace in the diet?"

This question has been addressed in the following section.

XI.1 NUTRITIONAL EQUIVALENCE TO EXISTING FOODS

The equivalence of vitamin D2 yeast concentrate to the sum of vitamin D2 and yeast has been discussed in Section II.4 (Comparison of Vitamin D2 Yeast Concentrate to Traditional Counterparts). Therefore, it is expected that vitamin D2 yeast concentrate is nutritionally equivalent to the sum of vitamin D2 and yeast.

XI.2 NUTRITIONAL BENEFITS OF VITAMIN D2 YEAST

Vitamin D2 yeast benefits are the same as the well known benefits of vitamin D, which deficiency is becoming a huge public health issue.

Worldwide it has been reported that children and adults in India, China, Europe, Canada, Japan, Australia, New Zealand and South America are all at high risk for vitamin D deficiency / insufficiency (Romagnoli *et al*. 1999, McGrath *et al*. 2001, Marwaha *et al*. 2005, Markestad *et al*. 1991, Lips *et al*. 2001, Glerup *et al*. 2000, Brunvand *et al*. 1993, Brot *et al*. 2001), and more than 1 billion children and adults are considered at risk (Holick 1989, Holick and Chen 2008a).

Recent reviews present the role and benefits of vitamin D in humans (Holick 2011, Boucher 2011).

Vitamin D deficiency in young children is responsible for a wide variety of skeletal deformities associated with rickets (Holick 2006, Brown *et al*. 1995, Rajakumar *et al*. 2007, Hess 1936).
In adults, vitamin D deficiency increases osteoporosis-related risks, including fragility fractures and reduction in muscle strength, which increases the risk of falls and resultant fractures. It is also responsible for osteomalacia, which can cause throbbing, aching bone pain and muscle weakness (Plotnikoff *et al*. 2003, Holick 2003).

Moreover, epidemiologic evidence and prospective studies have linked vitamin D deficiency with the increased risk of many chronic diseases including:

- Autoimmune diseases (Mathieu *et al*. 1995, Hyppönen et *al*. 2001),

- Cardiovascular diseases (Zittermann et *al*. 2009, Autier et *al*. 2007, Parker *et al*. 2010, Wang *et al*. 2008, Li *et al*. 2004, Maiya *et al*. 2008),
- Cancers (Ahonen *et al.* 2000, Bischoff-Ferrari et *al.* 2006, Bertone-Johnson et *al.* 2005, Boscoe *et al*. 2006, Freedman *et al*. 2010, Gandini *et al*. 2011, Garland *et al*. 1991, 2006 and 2007, Giovanucci *et al*. 2006, Grant *et al.* 2006 and 2009, Knight *et al*. 2007, Lappe *et al.* 2007, Luscombe *et al*. 2001, Moan *et al*. 2008),
- Type II diabetes (Lee et *al*. 2008, Autier et *al*. 2007, Chiu et *al*. 2004, Pittas et *al*. 2006),
- Infectious diseases, like chronic periodontis (Garcia *et al*. 2011), respiratory infections (De Luca et *al*. 2010, Ginde *et al*. 2009, Litonjua et *al*. 2007, Martineau et *al*. 2011, Tolppanen et *al*. 2011) or influenza (Urashima *et al*. 2010).

Today, vitamin D deficiency is being recognized as the most common medical problem worldwide. As expressed in 2002 by the SCF (Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Vitamin D, 2002) the intake of vitamin D by the European population is far below RDA. In fact, recent publications from various parts of Europe all show that a substantial part of the population, including pre-school children, has a vitamin D intake below the recommended dietary intakes (Davies *et al*, 1999; de Jong *et al*,1999; Koenig and Elmadfa, 2000; Lehtonen-Veromaa *et al*, 1999; Ortega *et al*, 1995; van der Wielen *et al*, 1995).

As very few foods naturally contain vitamin D, our vitamin D2 yeast concentrate represents an alternative to maintain sufficient levels of vitamin D intake in the population.

XII MICROBIOLOGICAL INFORMATION ON VITAMIN D2 YEAST CONCENTRATE

Based on the SCF guidelines, the following question must be addressed to ensure sufficient microbiological information on the novel food:

- Is the presence of any microorganisms or their metabolites due to the novelty of the product/process?

This question has been addressed below.

Vitamin D2 yeast concentrate is monitored to meet microbiological standards. As a food product manufacturer, Lallemand recognizes the importance and need for food safety through the microbiological testing of its products.

Every lot of yeast produced and prepared/packaged is analyzed prior to shipment to meet the following microbiological standards:

1. Coliforms/g: less than 1000 / g
2. E. coli: less than 10 / g
3. Salmonella: negative / 25 g

These specifications are the minimum specifications which are applied to the vitamin D2 yeast concentrate for bread and baked goods applications and are cited, together with reference methods, in Section I.6.1 (Product Specification) and are also available in product specifications sheets (Appendix I.6.1).
For other food applications, additional analysis will be achieved according to the specifications of the final product where the vitD2 yeast concentrate is added.

XIII SAFETY AND TOXICOLOGICAL INFORMATION ON VITAMIN D2 YEAST CONCENTRATE

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient toxicological information pertaining to the novel food:

- Is there a traditional counterpart to the novel food that can be used as a baseline to facilitate the toxicological assessment?
- Compared to the traditional counterpart, does the novel food contain any new toxicants or changed levels of existing toxicants?

OR

- Is there information from a range of toxicological studies appropriate to the novel food to show that the novel food is safe under anticipated conditions of preparation and use?
- Is there information which suggests that the novel food might pose an allergenic risk to humans?

These questions have been addressed collectively in the following sections.

XIII.1 BIOAVAILABILITY

The bioavailability of a nutrient or drug is primarily used to describe that fraction of an administered dose that remains unchanged or activated as is the case for vitamin D and maintained in the systemic system. When vitamins D3 and D2 enter the circulatory system they are changed to 25-hydroxyvitamin D3 (25(OH)D3) and 25-hydroxyvitamin D2 (25 (OH)D2), respectively. While serum levels of 25(OH)D are the standard for assessing both vitamin D bioavailability and status, the measurement of this pro-hormone does not differentiate between 25(OH)D3 and 25(OH)D2; the serum value is the sum of both.

There has been some controversy about whether vitamin D2 is as effective as vitamin D3 in maintaining 25(OH)D levels. There are two proposed explanations for these lower levels of serum 25(OH)D2. The first is that 25(OD)D2 is cleared faster from the blood (IOM, 1997), and alternatively the rate of hydroxylation of ergocalciferol to 25(OH)D2 is slower than the rate of hydroxylation cholecalciferol to 25(OH)D3 (Guo e*t al*., 1993).

Two reports (Trang *et al*., 1998; Armas *et al*., 2004) and one review (Heaney, 2008) are frequently cited that indicate vitamin D2 is not as potent as vitamin D3. The recent AHRQ (Chung *et al*. 2009) report and 2 studies by Heaney (2011) and Binkley *et al*. (2011) make reference to the higher potency of vitamin D3.

However, Holick *et al*. (2008b) directly addressed this issue of non-equal potency between vitamin D2 and vitamin D3. In a randomized, placebo-controlled, double-blinded study, 68 healthy adults between the ages of 18 and 84 years received placebo, 1000 IU vitamin D3, 1000 IU vitamin D2, or 500 IU vitamin D2 plus 500 IU vitamin D3 daily for 11 weeks at the end of the winter. The circulating levels of 25(OH)D (mean ± SD) increased to the same extent in all 3 groups. Among groups fed vitamin D2, there was no negative effect on serum 25(OH)D3 levels.

A similar study was conducted in children demonstrating that ingestion of vitamin D2 was equally as effective as vitamin D3 in maintaining their circulating level of 25(OH)D (Gordon *et al*. 2008).

Moreover Biancuzzo *et al*. (2010a and b) showed that vitamin D2 was equally as effective as vitamin D3 in raising and maintaining total serum 25(OH)D when absorbed via 2 different ways (an orange juice and a capsule). They conclude that vitamin D2 is equally bioavailable as vitamin D3.

Moreover, a recent bioavailability study by Hohman *et al.* (2011) show that bread made with vitamin D2 yeast can improve trabecular and cortical bone health as well as a vitamin D3 supplement.
During the 8 week study, 80 four-week old male rats were randomly assigned diets containing 25 IU, 100 IU, 200 IU, or 1000 IU/ kg of vitamin D sourced from either vitamin D3 supplement or bread made with vitamin D2 yeast. The rats plasma 25(OH)D status and bone health markers were monitored The results show that, even if 25(OH)D2 levels are lower than 25(OH) d3 levels, a dose-dependent rise in serum 25(OH)D level is observed with the bread made with vitamin D2 yeast Furthermore, when the vitamin D2 content of the treatment diets was increased from 25 to 1000 IU/ kg, results showed an improvement in bone health markers -- mineral content and density, geometry, volume and connectivity density.

Finally, Lamberg-Allardt *et al*. (2010) have compared the effect on 25(OH)D status of vitamin D2 from bread baked with vitamin D2 yeast and from vitamin D2 supplement. Thirty-eight women from 19 to 41 years were divided in 3 groups. During 4 weeks, Group 1 (n=13) received bread baked with vitamin D2 yeast (corresponding to 1000 UI/day) and a placebo supplement, group 2 received placebo bread and a vitamin D supplement (1000 UI/day) and group 3 received placebo bread and placebo supplement (no vitamin D).
There were no differences in serum 25(OH)D levels between the 3 groups at baseline. Results have shown a significant difference in the variation of 25(OH)D levels between groups 1 and 3, and no significant difference between groups 1 and 2.
These results prove that bread baked with vitamin D2 yeast increases serum 25(OH)D levels has an equal effect on serum 25(OH)D levels as a vitaminD2-containing supplement. This is an evidence that vitamin D2 from bread baked with vitamin D2 yeast is equally bioavailable as vitamin D2 from currently accepted sources.

While there will remain some controversy regarding the difference in potency of vitamin D2 compared to vitamin D3 in maintaining serum 25(OH)D levels, our recent results show that

vitamin D2 from bread baked with vitamin D2 yeast is bioavailable. But it can be stated that no study has ever proved that the bioavailability of vitamin D2 was higher than the one of vitamin D3.

Moreover, it is important to notice that vitamin D2 has been the primary source for the prevention and treatment of vitamin D deficiency in children and adults over the past 50 years (Eliot and Park, 1938; Holick 2007). Is has been demonstrated that as little as 100 IU vitamin D2 is effective in the prevention of rickets (Eliot and Park 1938; Jeans 1950; Holick 2006) The number of studies reporting on the potency and effectiveness of vitamin D2 for a number of health issues is numerous (AHRQ, Chung *et al*. 2009). Furthermore, vitamin D2 supplementation is most commonly used in randomized controlled trials among infants and pregnant or lactating women, compared to vitamin D3 supplementation. Vitamin D2 significantly increased 25(OH)D concentrations in infants, lactating mothers and in cord blood (AHRQ, Chung *et al*.2009).

XIII.2 SAFETY AND TOXICOLOGY OF VITAMIN D2 YEAST

Concerning the safety of the yeast, as already assessed in section III:

In the US, the Food and Drug Administration (FDA) listed *S. cerevisiae* as generally recognised as safe (GRAS):

- http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?filter=cerevisiae&sortColumn=&rpt=grasListing
- http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.896

In the EU, *S. cerevisiae* is also included in the list of taxonomic units proposed for QPS status (Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA, the EFSA Journal, 2007).

- www.**efsa**.europa.eu/de/scdocs/doc/587.pdf

In Australia, *S. cerevisiae* is included in the Australian Register of Therapeutic Goods Ingredients https://www.ebs.tga.gov.au/ebs/ANZTPAR/PublicWeb.nsf/IngredientsPublic?OpenView&Start=7401&Count=20 under the “ingredients, all” page 371.

At the international level, the IDF (The International Dairy Federation), in collaboration with the European Food and Feed Cultures Association (EFFCA), has assembled a list of microorganisms with a documented history of safe use in food. *Saccharomyces cerevisiae* is listed in this inventory (Mogensen *et al*., Bulletin of the International Dairy Federation, Nº377/2002).

As we have demonstrated that the manufacturing process has no impact on the yeast genetic profile (see section II.5.1), we consider that *Saccharomyces cerevisiae* in vitamin D2 yeast is safe for the intended use.

As far as vitamin D safety is concerned, the current situation regarding the upper safe limits in the Europe (EFSA, 2006) and in the US (IOM, 2010) is summarized below:

Table 12: Summary of upper levels(µg/day) for vitamin D

	Age of children	UL[1] (EFSA, 2006)	UL[2] (IOM, 2010)
Adults		50	100 (4000 UI)
Children	14-18	-	100
	11-17	50	-
	9-13	-	100
	3-10	25	-
	4-8	-	75

[1]EFSA (European Food Safety Agency, 2006). Tolerable upper intake levels for vitamins and minerals.
[2] IOM (Institute of Medicine of the National Academies, 2010). Dietary Reference Intakes for calcium and Vitamin D.

It is also important to note that, in its opinion on the tolerable upper intake level of vitamin D, the SCF (2002) has expressed that "The observed reduced ability of vitamin D2 to raise plasma 25(OH)D and the low calcaemic effect of 1α,24(OH)D2 formed at high doses of vitamin D2 including suppressing effect on 25(OH)D3 and 1α,25(OH)2D3 synthesis are most probably the reasons for a lower toxicity of vitamin D2 than of vitamin D3 as the toxic effects are mainly related to the distorted calcium metabolism".

Moreover, based on several studies, it is now evident that vitamin D is one of the least toxic fat-soluble vitamins and that vitamin D intoxication is one of the rarest medical conditions that is caused by inadvertent or intentional ingestion of excessively high quantities of vitamin D for prolonged periods of time (Holick 1989, Koukia *et al*. 2001, Vieth 2004).

For example, in adults 10 000 IU of vitamin D a day for five months did not alter serum calcium or increase risk of kidney stones (Heaney 2003). Infants in Europe routinely received 200,000 IU of vitamin D intramuscularly for the prevention of rickets (Holick 2006, Rajakumar *et al*. 2007). Young girls in Lebanon (9-17 years) who received 2000 IU of vitamin D a day for one year maintained their blood level of 25(OH)D above 30 ng/ml without any toxicity (Holick 2007).

In a study on efficacy and safety of vitamin D3 intake, Vieth *et al.* (2001) reported that the upper reference serum concentration of 25(OH)D was not exceeded upon daily supplementation with 4000UI of vitamin D3.

Reviews exist on vitamin D safety. In 2007, Vieth concluded that the clinical trial evidence shows that a prolonged intake of 10 000 IU per day of vitamin D3 is likely to pose no risk of adverse effects in almost all individuals in the general population.
Hathcock *et al.* (2007) also concluded that the absence of toxicity in trials conducted in healthy adults that used vitamin D3 at a daily dose of 10 000 IU supports the confident selection of this value as the upper limit.

A complete review of the literature where cases of vitamin D safety / toxicity were reported is available in section XIII.5.

Finally, the toxicity of vitamin D is completely assessed in the SCF opinion (2002). Resulting from this toxicity study, the SCF has defined upper limits for the consumption of vitamin D, that have been fixed at 1000 UI for children from 0 to 10 years, 2000 UI for children to 11 to 17 years, and 2000 UI for adults of more than 17 years.

Lallemand is confident that the proposed use of the vitamin D2 yeast concentrate is safe and there is no risk of exceeding the upper limits defined by the SCF with this intended use.

XIII.3 TACHYSTEROL TOXICITY

As explained in sections I.5.1 (Product Characterization) and II.4 (Comparison of vitamin D2 yeast concentrate to traditional counterparts), the only difference between our vitamin D2 yeast concentrate and existing vitamin D2 is the presence of very limited amounts of tachysterol.

The following two sentences appear in the abstract of Dr. Michael Holick's paper published in 1981.

"When human skin was exposed to stimulated solar ultraviolet radiation epidermal 7-dehydrocholesterol was converted to previtamin D3. During prolonged exposure to stimulated solar ultraviolet radiation, the synthesis of previtamin D reached a plateau at about 10 to 15 percent of the original 7-dehydrocholesterol content, and previtamin D3 was photoisomerized to two biologically inert isomers, lumisterol$_3$ and tachysterol$_3$"

The keys words in this paper are "two biologically inert isomers, lumisterol$_3$ and tachysterol$_3$".

The work of Dr. Holick in this seminal experiment was primarily directed at understanding the mechanism and metabolism of vitamin-D3 production in the human skin. His studies have helped elucidate many aspects of vitamin-D metabolism and nutrition. It is fortuitous that his scientific work helped to verify the safety of tachysterol.

The conclusion of the authors are that since vitamin-D binding protein (DBP) has no affinity for lumisterol-3 and minimum affinity for tachysterol-3, the translocation of these photoisomers into the circulation is negligible, and thus these products are probably sloughed off during the natural turnover of the skin. Both lumisterol and tachysterol are biologically inert.

The non-toxicity of tachysterol is also confirmed by Dr Gilchrest in a book published in 1986:
"Cutaneaous production of vitamin D3 is absolutely dependent on UV radiation present in sunlight in the UVB spectrum (290-320 nm). Continued UVB exposure is not a limitless source of vitamin D as additional UVB further transforms previtamin D3 into biologically inactive metabolites, tachysterol and lumisterol".

Acceptable Daily Intake (ADI)

The ADI is expressed as either a numerical value, (mg/kg bw/d), or as Not Determined (ND). Not Determined (ND) signifies a number of situations where a numerical ADI has not been determined. For example, if exposure to the substance is less than 50 ppb and of no concern at this time, a numerical ADI would not be calculated.

Based on the data from Dr. Michael Holick's laboratory (see Appendix I.5.2A), the amount of vitamin D and tachysterol in one-gram of vitamin D2 yeast concentrate is 750 µg/g (30 000 IU) and 140 µg/g, respectively (rounded values).

There is no data to help establish an ADI for tachysterol. Since a review of the literature provided no data and/or indication of any safety concern regarding tachysterol, as it is considered an inert photoisomer in the UV conversion of ergosterol to ergocalciferol (Holick 1981; Gilchrest 2006), we assume that the ADI for tachysterol is classified as Not Determined (ND).

In summary, with the knowledge that an extremely small amount of tachysterol will be in products made with vitamin D2 yeast concentrate and the available scientific evidence indicates tachysterol has no biological activity, the issue of tachysterol safety/toxicity is considered to be irrelevant.

XIII.4 ALLERGENS

There is no allergen used in the production of the vitamin D2 yeast concentrate.

XIII.5 RECENT SAFETY LITERATURE SEARCH AND REVIEW (JAN 1, 2007 – FEBRUARY 28, 2011)

A literature search of scientific studies was conducted using PubMed for the period January 1, 2007 through November 2, 2011. The primary keywords used in this search were "vitamin-D, "safety" and "toxicity". While numerous reviews articles were found, these are not cited. Among all the scanned and reviewed articles, only a few articles (see summaries below) reporting any adverse effects among humans given vitamin D in clinical intervention studies or self administered high doses of vitamin D were found. These adverse effects are limited and are generally linked with the absorption of very high doses of vitamin D.
Only 2 studies (McKiernan and Wiley2008; Zhu *et al*. 2008) describe side effects following the intake of vitamin D2, the others concern vitamin D3. For one study (Lappe *et al*. 2008) the source of vitamin D is not described.

1. Jackson *et al*., 2006

The Women Health Initiative (WHI) is a 7-year trial that was designed to compare the effects of combined vitamin D3 (400 IU) and calcium (1 000 mg as calcium carbonate) daily intakes versus placebo on bone health in 36,282 postmenopausal women (age 50-79 y). The study provided data for numerous health outcomes of interest dealing with bone health and the risk of fractures. The analysis of the data from the WHI trial by Jackson *et al*. (2006) showed among women taking both calcium and vitamin D supplement there was a small but significant increase in the proportion of women with renal calculi.

Significant limitations have been noted regarding the results observed in the WHI, particularly, compliance issues. It was found that subjects in both supplement and placebo groups were allowed to take additional vitamin D supplements up to 600 IU and later 1000 IU per day and calcium supplements up to 1000 mg per day. At baseline, about one-third of the subject in both supplement and placebo groups were taking vitamin D supplements of at least 400 IU/day and 29% were taking at least 500 mg/day of supplemental calcium. By the end of the trial, 69% of subjects were taking additional supplemental calcium. During the 7 years of the study, only about 60% of subjects (in any given year) were taking at least 80% of the study supplements; at the end of the trial, only 76% were still taking any of the study supplements. It should also be noted that 51% of the participants, following protocol, received estrogen and approximately 11% received other antiresorptive drugs during the study and follow up, both of these treatments might have influenced bone health results and incidents of renal stones (Diaz-Lopez and Cannata-Andia, 2006).

Overall, the validity of the findings of an elevated risk of kidney stones in women subjects from the WHI trial is questionable given the study limitations and the fact that no other longer term intervention studies involving Vitamin D and calcium supplementation since the WHI reported any significant increase in kidney stones. See Hathcock *et al.* (2007) for further comment on the finding of this study.

2. Hathcock *et al*., 2007

Hathcock *et al*. (2007) reviewed 21 clinical studies in which subjects were fed vitamin-D supplements substantially above 1800 IU of vitamin D per day. Of these, 13 studies reported no significant adverse effects and 8 studies between the period of 1985-2005 reported adverse effects. Their review of these studies showed that that the serum 25(OH)D concentrations associated with hypercalcemia were almost exclusively the result of very large doses of vitamin D, and in all instances serum 25(OH)D concentrations reached concentrations well into the hundreds and even thousands of nmol/L. Hathcock *et al*. (2007) and others (Mason and Posen 1979; Morris 2005) concluded that a serum 25(OH)D concentration of ≥700 nmol/L may be needed to evoke hypercalcemia in normal adults. Hathcock *et al*. (2007) could not find any consistent and reproducible adverse effect caused by vitamin D in well-conducted clinical trials with intakes up to 50 000 IU.

The authors also commented on the continued controversy regarding the WHI involving calcium and vitamin D3 supplementation in which there appeared to be an increase risk of renal stones (Jackson *et al*., 2006). With respect to safety, results showed a significant 17% increased risk of renal stone formation in the supplement group (449 cases) compared with the placebo group (381 cases). The high use of self-selected supplements indicates that calcium intake in the experimental group was upwards of 2000 mg. In view of the vitamin D supplement levels of several hundred micrograms that have been administered experimentally without any hypercalcemia, it seems unlikely that the vitamin D treatment contributed to the excess risk of renal stones.

Hathcock *et al.* (2007) estimated that sunshine can provide an adult with vitamin D in an amount equivalent to daily oral consumption of 10 000 IU/day and considered this a safe dose. The incremental consumption of 40 IU/day of vitamin D3 raises serum 25(OH)D by approximately 1 nmol/L (0.4 ng/ml). Therefore, if sun-deprived adults are to maintain serum 25(OH)D concentrations, >75 nmol/L (30 ng/ml), they will require an intake of more than the current UL for vitamin D. The mechanisms that limit vitamin D safety are the capacity of circulating vitamin D-binding protein and the ability to suppress 25(OH)D-1-alpha-hydroxylase. Vitamin D causes hypercalcemia when the "free" concentration of 1,25-dihydroxyvitamin D is inappropriately high. This displacement of 1,25(OH)(2)D becomes excessive as plasma 25(OH)D concentrations become higher than at least 600 nmol/L (240 ng/ml). Plasma concentrations of unmetabolized vitamin D during the first days after an acute, large dose of vitamin D can reach the micromolar range and cause acute symptoms.

The clinical trial evidence showed that a prolonged intake of 10 000 IU/day of vitamin D3 is likely to pose no risk of adverse effects in almost all individuals in the general population; this meets the criteria for a tolerable upper intake level.

3 Burleigh *et al*., 2007

This is a randomized double-blind, controlled study involving 205 acute admissions patients more than 65 years of age to a geriatric medical unit. The objective of the study was to determine whether routine supplementation with vitamin D plus calcium reduces numbers of fallers and falls in a cohort of hospital admissions while they are inpatients. Patients were randomized to intervention of daily vitamin D 800 IU plus calcium 1,200 mg or control group of daily calcium 1,200 mg, until discharge or death.
The baseline characteristics were similar in both groups with a median age 84 years and a median length of stay = 30 days. The study findings showed that in a population of geriatric hospital inpatients, vitamin D did not reduce the number of fallers. Routine supplementation cannot be recommended to reduce falls in this group.

Four cases of asymptomatic hypercalcemia (as defined by serum adjusted calcium >2.60 mmol/L) were observed at time of discharge among three individuals in the intervention arm of this study and one person who died in the control group. In two cases, the hypercalcemia had been present on

admission but the laboratory result was delayed and neither patient received more than 4 days treatment prior to the study drug being discontinued. The other two individuals developed hypercalcemia during the study and were both acutely medically unwell. The few incidents of adverse effects observed in this study did not appear to be associated with the design of the study or vitamin D. While the mean median vitamin D status of the participants upon admission to the study was 22 nmol/L, there was no significant change with vitamin D intervention. The study does report sufficient data to totally evaluate the asymptomatic hypercalcemia in these few elderly subjects.

4 Lappe et *al.*, 2007 and 2008

The study by Lappe *et al*. (2007) was a 4 year, population-based, double-blind, randomized placebo-controlled trial. The purpose of this analysis was to determine the efficacy of calcium alone and calcium plus vitamin D in reducing incident cancer risk of all types. The primary outcome was fracture incidence, and the principal secondary outcome was cancer incidence. The subjects were 1179 community-dwelling women randomly selected from the population of healthy postmenopausal women aged 55 years in a 9-county rural area of Nebraska. Subjects were randomly assigned to receive 1400–1500 mg supplemental calcium/day alone (Ca-only), supplemental calcium plus 1100 IU vitamin D3/day (Ca- D), or placebo.

When analyzed by intention to treat, cancer incidence was lower in the Ca-D women than in the placebo control subjects ($P \leq 0.03$). With the use of logistic regression, the unadjusted relative risks (RR) of incident cancer in the Ca-D and Ca-only groups were 0.402 ($P \leq 0.01$) and 0.532 ($P \leq 0.06$), respectively. When analysis was confined to cancers diagnosed after the first 12 mo, RR for the Ca-D group fell to 0.232 (CI: 0.09, 0.60; $P \leq 0.005$) but did not change significantly for the Ca-only group. In multiple logistic regression models, both treatment and serum 25-hydroxyvitamin D concentrations were significant, independent predictors of cancer risk. Based on these results, it was concluded that improving calcium and vitamin D nutritional status substantially reduces all-cancer risk in postmenopausal women.

During the course of study, there were no serious supplemented adverse events. But, five subjects were diagnosed with renal calculi: 1 subject in the placebo group, 1 subject in the Ca-D group, and 3 subjects in the Ca-only group. These incidences did not differ significantly by group. No patterns of adverse events were seen among the 3 groups. The few incidents of adverse effects observed in this study do not appear to be associated with the design of the study or vitamin D. No adverse events in similar study by Lappe *et al.* (2008) were reported.

5 Kimball and Vieth, 2008

This is a case study report of self prescribed high dose of vitamin D3 over 5–6 years by two men: Subject 1 had been taking 4 000 IU for 3 years followed by 3 years of 8 000 IU. Serum 25(OH)D concentrations averaged 130 nmol/L while taking 4 000 IU /day of vitamin D3. While taking 8 000

IU /day of vitamin D3, mean serum 25(OH)D concentrations were 260 nmol/L with no hypercalcemia or hypercalciuria over the 6 years of vitamin D3 intake.

Subject 2 was a 39-year-old man diagnosed with multiple sclerosis. He initiated his own dose-escalation schedule. His vitamin D3 intake increased from 8 000 to 88 000 IU/day over 4 years. The first evidence of a potential adverse effect was that urinary calcium/creatinine ratios showed an increasing trend, which preceded serum calcium concentrations above the reference range (2.2–2.6 nmol/L). His serum 25(OH)D concentration was 1 126 nmol/L when total serum calcium reached 2.63 mmol/L, and this was only on one occasion. He stopped vitamin D3 supplementation at this point. Two months later, all biochemistry values were within reference ranges; serum 25(OH)D concentrations fell by about one-half, to 656 nmol/L.

These results help to clarify the human response to higher intakes of vitamin D3. Close monitoring of biochemical responses confirmed that an increase in urinary calcium/creatinine ratio precedes hypercalcemia as serum 25(OH)D concentrations rise. The results observed in these two men again demonstrate that serum 25(OH)D concentrations > 250 nmol/L can be obtained and tolerated without causing hypercalcemia.

The lowest observed adverse effect level for vitamin D (for hypercalcemia in normal adults) is 3 800 IU/day. Serum 25-hydroxyvitamin D (25[OH]D) concentrations associated with hypervitaminosis remain undefined. Reported 25(OH) D concentrations resulting from prolonged excessive vitamin D3 intakes have exceeded 700 nmol/L.

6 McKiernan and Wiley, 2008

This Letter to the Editor reports on two elderly women (81 and 75 years of age) who ingested 50,000 IU of vitamin D2 everyday for nine months as prescribed by their physicians. Upon initial examination, one woman had a serum 25(OH)D level of 241 ng/ml, a serum 1,25(OH)D level of 70 pg/ml, and a serum calcium level of 10.0 mg/dl; the second women had a serum 25(OH)D level of 200 ng/mL, a serum 1,25(OH)D level of 38 pg/ml, and a serum calcium level of 9.5 mg/dl.

There are a few important and positive observations that were gained from these prescribed high doses of vitamin D2. Although having high vitamin D intakes for nine months, neither subject had clinical evidence of vitamin D intoxication or hypercalcemia despite the fact their serum 25(OH)D levels were greater than 150 ng/ml, a level suggested to indicate hypervitaminosis D (Holick, 2007). These observations strongly suggest that the upper limit is higher than the current level of 2,000 IU/day and supports the conclusions of Hathcock *et al*. (2007) that hypercalcemia is not normally seen with serum 25(OH)D levels < 250 ng/ml.

In another informative aspect of this letter, the authors followed the levels of total serum 25(OH)D and 25(OH)D2 for 40 days after the women stopped taking the vitamin D supplements. It took about 20 to 30 days for the subjects to achieve the upper limits of normal 25(OH)D levels, 80 ng/ml. In accomplishing these measurements by combined high performance liquid

chromatography-mass spectroscopy (HPLC-MS) methodology, the authors confirmed the half-life of the total serum 25(OH)D (mixture of 25(OH)D3 and 25(OH)D2) and 25(OH)D2 to be approximately 29 and 21 days, respectively.
These observations helped confirm similar values for the clearance of all forms of 25(OH)D from the serum, but perhaps more importantly added further proof that while vitamin D2 has equal equivalent potency to vitamin D3 (Holick *et al*., 2008b), 25(OH)D2 is cleared faster from the blood.

7 Sneve *et al*., 2008

This is a randomized double blind clinical trial with 20 000 IU cholecalciferol twice a week, or 20 000 IU once a week plus placebo, or placebo twice a week, for 12 months. All subjects were given 500 mg calcium supplementation. The objective of the study is to investigate whether cholecalciferol supplementation leads to weight loss in overweight and obese adults. Four hundred and forty five healthy, overweight, and obese men and women age 21–70 years, body mass index (BMI) 28.0–47.0 kg/m^2). Body weight, fatness, and fat distribution parameters were measured by dual-energy X-ray absorptiometry and anthropometry, blood samples and 24-h urinary samples were collected.

At baseline, there were no significant differences between the groups, but there was a significant inverse relation between serum 25-hydroxyvitamin D (25(OH)D) levels and BMI, and a significant positive association between calorie intake and BMI. Three hundred and thirty four subjects completed the study. During the study, there was no significant change in weight, waist-to-hip ratio (WHR) or percentage body fat in any of the groups, nor between them. Parathyroid hormone decreased and 25(OH)D increased significantly in both groups receiving cholecalciferol, and serum levels of 25(OH)D stabilized after 3 months. Serum calcium was unchanged in all groups. Urinary calcium excretion increased in all groups, but there was no significant difference between the groups. Weekly dosage of 20 000–40 000 IU cholecalciferol for 12 months was associated with a low risk of adverse effects, at least in overweight and obese adults living at latitude 708 N. Based on the study findings, it was concluded that significant weight reduction in overweight and obese subjects is unlikely to occur with cholecalciferol supplementation.

During the study, only seven subjects reached a serum calcium value above 2.59 mmol/L, which was the predefined hypercalcemia threshold. Two subjects, one given placebo, developed PHPT and, in retrospect, their baseline serum calcium and PTH levels, although not outside the preset limits for inclusion, indicated a disturbed calcium metabolism. Among the remainder, only one subject (in the 20 000 IU/week group) had to be excluded due to serum calcium of 2.59 mmol/L at the re-examination. Most of the other adverse events were related to gastro-intestinal discomfort, most likely due to the calcium supplementation. Although a group of 449 subjects is small when considering the safety of the applied cholecalciferol doses, it does at least indicate that serious hypercalcemia is not a frequent consequence of administrating cholecalciferol doses in the range 20 000–40 000 IU weekly, at least not in overweight and obese subjects.

8 Zhu *et al*., 2008

The aim of this study is to evaluate the relative importance of vitamin D and calcium treatment on BMD and bone-related chemistry in elderly women with vitamin D insufficiency. Three hundred and two elderly women (age, 77.2 ± 4.6 yr) with serum 25(OH)D concentrations <60 nM participated in a 1 year randomized, double-blind, placebo-controlled trial. All subjects received 1000 mg calcium citrate per day with either 1000 IU ergocalciferol (vitamin D2) or identical placebo (control). The effects of time and time treatment interactions were evaluated by repeated-measures.

At baseline, calcium intake was 1100 mg/d, and 25(OH)D was 44.3 ± 12.9 nM; this increased in the vitamin D group by 34% but not the control group after 1 year (59.8 ± 13.8 versus 45.0 ± 13.3 nM, $p < 0.001$). Total hip and total body BMD increased significantly, and procollagen type I intact N-terminal propeptide (PINP) decreased during the study with no difference between the treatment groups (hip BMD change: vitamin D, +0.5%; control, +0.2%; total body BMD change: vitamin D, +0.4%; control, +0.4%; PINP change: vitamin D, −3.9%; placebo, −2.8%). Although the fasting plasma and urine calcium increased in both groups equally, there was no detectable change in serum PTH. The increase in 25(OH)D achieved with vitamin D supplementation had no extra effect on active fractional intestinal calcium absorption, which fell equally in both groups (vitamin D, −17.4%; control, −14.8%). In patients with a baseline calcium intake of 1100 mg/d and vitamin D insufficiency, vitamin D2 1000 IU for 1 year has no extra beneficial effect on bone structure, bone formation markers, or intestinal calcium absorption over an additional 1000 mg of calcium. Vitamin D supplementation adds no extra short-term skeletal benefit to calcium citrate supplementation even in women with vitamin D insufficiency.

During the study period, there were no significant differences between the vitamin D and the control groups in the rate of incident cancer and vascular disease (ischemic heart disease and stroke). One participant in the vitamin D group had mild asymptomatic hypercalcemia on one occasion. No case of renal calculus was reported.

9 Bäck *et al*., 2009

In a prospective birth cohort study, 123 six-year-old children were investigated for the cumulative incidence of atopic dermatitis, allergic rhinitis or asthma by means of a postal questionnaire. The study objective was to assess the relationship between intake of vitamin D3 during infancy and the development of atopic allergy later in childhood. Based on information received in the questionnaires, the children were divided into two groups based on the arithmetic mean of their vitamin D intakes for the 5th, 7th and 10th months of the study. The 2 groups were divided into those having < 13.0 µg/day of vitamin D per day (low, n=61) and those receiving > 13.1 µg/day of vitamin D (high, n=62). The estimated vitamin D intake values among these children also included the 400 IU from the prescribed supplement. The end result of this study was to determine the

number of children who had some type of manifestations of atopic illnesses during the period of infancy to 6 years of age.

Among the 61 children from the low dose group, 26 (43 %) were reported to have had an atopic illness during this period. Among the children in the high vitamin D intake group, all 62 (100 %) of these children were reported to have had an atopic illness during this period. The primary atopic illnesses reported were atopic dermatitis, allergic rhinitis and allergic asthma. Atopic manifestations appeared to be more prevalent in the group with higher intake of vitamin D3.

However, there were several limitations associated with this study, including:

- Effect of vitamin A: It is not clear why vitamin A was also given to the subjects. The potential interaction between vitamins A and D and effect modifier cannot be eliminated.
- Clinical and laboratory confirmation of self reported allergic illness was not done.
- The estimates of vitamin D intake were based on responses to an infant feeding questionnaire (which was not identified as a validated questionnaire) and vitamin D concentration data from food manufacturers or reference sources. Given the potential for inaccuracies in responses to the dietary questionnaire and differences between the vitamin D concentration data in the reference data and the actual foods consumed, actual vitamin D intakes may differ from the estimated intakes. Additionally, no additional information is provided regarding the dietary intake of the infants to determine if intakes of all nutrients and foods (e.g., milk, peanuts, and other common allergens) were comparable between the low and high vitamin D groups.
- There was no control group of children not receiving vitamin A and vitamin D supplements.

The findings from this study are tentative, and verification with controlled intervention trials would be needed to support a definitive relationship between vitamin D and atopic manifestations (as indicated by the authors).

10 Kaptein *et al.*, 2010

The authors review two case studies of woman that were suffering from life threatening hypercalcemia due to taking concentrated sources of vitamin-D. Their supplements, while labeled to contain 3.75 µg (150 IU), were found to contain 100-1,000 times higher amounts. Laboratory tests on these two women found ionized blood calcium levels of 4.00 mmol/L (16.00 mg/dL) and 4.56 mol/L (18.24 mg/dL), and 25(OH)D levels of 1 372 nmol/L and 644 nmol/L. The conclusion of the authors was as to be expected. Manufacturers must be more prudent in the analysis of their products and consumers must use caution in the use of supplements.

11 Hackman *et al*., 2010

In this prospective randomized trial, 2 regimes of vitamin-D were evaluated to test their efficacy and safety. In one regime (high dose), patients received vitamin D3, 50 000 IU/day for 10 days; in the second regime (low dose), patients received 3 000 IU/day vitamin D3 for 30 days followed by 1 000 IU/day for 60 days. Both groups received 500g of calcium citrate daily. The starting serum 25(OH)D levels for both groups were about 27 nmol/L.

In both groups, the mean increase in serum 25(OH)D levels was similar (55 nmol/L for high dose and 51 nmol/L for low dose). Three individuals displayed hypercalciuria (urine calcium > 7.5 mmol/day), one in the high dose group and two in the low dose group. One patient in the low dose group had a pre-existing mild renal condition and this condition worsened during the trial. The authors concluded that no patient developed hypercalcemia (corrected calcium> 2.6 mmol/L), vitamin-D toxicity (25(OH)D > 200 nmol/L), or nephrolithiasis during the study.

12 Sage *et al*., 2011

In a short letter to the editor, Sage *et al*. (2011) explain that patients with sarcoidosis at Henry Ford hospital (Detroit, USA) with low 25(OH)D3 levels have become hypercalcemic after receiving pharmacologic vitamin D supplementation (50 000 IU/week). Development of hypercalcemia appears to be especially problematic in patients with chronic kidney injury.
They state that due to the possible development of hypercalcemia, vitamin D supplementation should be given with caution, ideally, after the determination of $1,25(OH)_2D3$ levels. For dark-skinned patients with sarcoidosis with low $1,25(OH)_2D3$ and normal parathyroid hormone they recommend 400-800 IU of oral vitamin D per day for 30 days and 50 000 IU per week for 8-12 weeks respectively.

13 Koul *et al*., 2011

Koul *et al*. report 10 cases of hypercalcemia in India due to vitamin D intoxication are presented with features of vomiting, polyuria, polydipsia, encephalopathy and renal dysfunction. All the patients had demonstrable hypercalcemia and vitamin D levels were high in nine of the 10 cases. The patients had received high doses of vitamin D (from 3.6 million IU to 210 million IU over periods ranging from 1 to 4 months, in the form of multiple parenteral injections or weekly oral sachets of vitamin D for various indications) and no other cause of hypercalcemia was identified.
Treatment of hypercalcemia resulted in clinical recovery in nine cases. The authors conclude that hypervitaminosis D must be considered in the differential diagnosis of patients with hypercalcemia in endemically vitamin D deficient areas.

CONCLUSION

This dossier supports the position that Lallemand vitamin D2 yeast concentrate, produced by UV exposure of the yeast *Saccharomyces cerevisae,* should be approved as a source of vitamin D for the addition to food in the EU (current foods and foods supplements). Approval is sought under Regulation EC 258/97 of the European Parliament and of the Council of 27 January 1997 concerning Novel Foods and Novel Food ingredients, under category 1(2)(d), ingredients obtained from microorganisms that have existing food uses.

In this dossier, we have presented the product- Lallemand's vitamin D2 yeast concentrate- as being equivalent to two traditional products: firstly to yeast *Saccharomyces cerevisae* and secondly to vitamin D2. We have made the case that vitamin D2 yeast concentrate is safe to be consumed by the EU population; its consumption does not raise any safety concerns but rather it can bring benefits to consumers since many countries have very low vitamin D intake levels.

Saccharomyces cerevisae has a long history of safe use in the EU in applications, such as bakery, winemaking, brewery, distillery, etc. as well as in supplements. Moreover, it is included in the list of taxonomic units proposed by EFSA for QPS status, it is considered as GRAS in the US and it is on the list of microorganisms with a documented history of safe use in food by (the so-called "IDF" list). As shown in Section II.5.1 (Genetic Stability of Vitamin D2 Yeast) the genetic profile of *Saccharomyces cerevisae* is not modified by the UV irradiation and therefore the irradiated yeast (vitamin D2 yeast concentrate) is equivalent to the non-irradiated *Saccharomyces cerevisiae* and therefore, safe.

Vitamin D2 is an authorized form of vitamin D in the EU (Regulation (EC) 1170/2009) and it is extracted (using solvents) from irradiated yeast by companies like Synthesia, a food operator based in the Czech Republic. Several products containing vitamin D2 are available on the European market, and they are produced by means of the same production process as that used for the production of the vitD2 yeast concentrate.

The production process used by Lallemand for the production of the vitamin D2 yeast concentrate uses UV light and has been reported as a current process for the manufacture of vitamin D2. The Scientific Committee for Food, in its opinion expressed on 4 December 2002 on the Tolerable Upper Intake Level of vitamin D (SCF, 2002), explicitly recognized that the process employing UV light is used for the production of vitamin D2: "*vitamin D2 is formed by UV radiation from its precursor ergosterol.* (…) S*ynthetic vitamin D2 produced by irradiation of ergosterol used to be the form added to food or given as supplements.* Therefore, it can be concluded from that SCF opinion, that UV irradiation has been used for more than 2 decades for the production of vitamin D2 for use in fortified foods and food supplements, thus providing evidence of the use of that process since at least 1982.

Additionally, the food company mentioned above (Synthesia) certified in writing that vitamin D2 has been manufactured by UV irradiation since 1962. Although in the Synthesia process, the

exposure of the ergosterol to UV light takes place only when the ergosterol has been isolated from yeast, the process of UV irradiation is the same. The primary difference is the way that the Synthesia process employs several steps of purification (distillation and extraction with solvents) after irradiation whereas Lallemand's production of vitamin D2 yeast concentrate does not employ an additional step of purification. This makes it a less processed form of vitamin D2 and thereby represents an alternative form to the purified vitamin D2. The only resulting difference is the presence of a small amount of tachysterol as a byproduct in vitamin D2 yeast concentrate, as demonstrated by the product characterization achieved by Lallemand (see Section I.5.1).

Nevertheless, tachysterol is naturally formed in the human body to limit the levels of vitamin D during the exposure to UV (sunlight) and it has been described as a biologically inert substance. Moreover, in the vitamin D2 yeast concentrate, it is present in very small quantities and thus the intake by the population will be very limited according to the intended use described for our vitamin D2 yeast concentrate.
Therefore, we can conclude from the available scientific evidence and intended use that the tachysterol safety/toxicity can be considered as irrelevant.

Vitamin D2 yeast concentrate is produced under rigorously controlled conditions and each production lot is analyzed before release for vitamin D2 content in an accredited laboratory using a validated method adapted from a recognized analytical method (AOAC official method 982.29). Control of microbiological contaminants is conducted in our own quality control laboratory using internationally recognized methods.
The stability of vitamin D2 yeast concentrate is also controlled and the product has been demonstrated to be stable during its 3 year shelf-life.

Regarding bioavailability, a recent study has demonstrated that vitamin D2 from vitamin D2 yeast is bioavailable, and equivalence in bioavailability with vitamin D2 existing sources has been demonstrated (Lamberg-Allardt *et al*. 2010). A literature review on vitamin D safety has shown that only a few articles reported adverse effects among humans given vitamin D in clinical intervention studies or self administered high doses of vitamin D were found. These adverse effects are very limited and are generally linked with the absorption of very high doses of vitamin D.

Finally, it is important to note that Lallemand's vitamin D2 yeast has been approved as a form of vitamin D supplementation in the US and in Canada.
In the US, the Lallemand vitamin D2 yeast is approved by the FDA since 2007. More recently, Health Canada completed the safety assessment of the proposal to fortity some food products using the Lallemand vitamin D2 yeast. The evaluation of the data provided by Lallemand has lead them to conclude that the use of that product as source of vitamin D to fortity certain foods in Canada is safe.

The evidence presented in this dossier substantiates the case for vitamin D2 yeast concentrate as an alternative to vitamin D2 for use in foods and food supplements in the EU, when produced according to the procedures and characteristics outlined. We consider that we have made a clear case for the vitamin D2 yeast concentrate, as described in this dossier, to be approved as a novel food according to Regulation EC 258/97.

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