ANALYTICAL PLATFORM FOR ESTABLISHMENT OF FOOD COMPOSITION DATA FOR VITAMINS – EXEMPLIFIED BY VITAMIN D

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Abstract – The analytical data for food samples are no better than the sampling strategy and the performance of the analytical methods. Furthermore, lack of standardised methods for nutrients in foods can be a source of error. But thorough investigation of the components that need to be quantified and careful selection of the method combined with documentation of especially accuracy will secure reliable results. In this paper the state-of-the art for vitamin D analyses in food is used as an example.

Keywords: analyses, vitamin D, food composition, validation

1. INTRODUCTION

Dietary intake of vitamins comes mainly from food, thus reliable data on food contents is essential. The analytical data are no better than the sampling strategy and the performance of the analytical methods.

The sampling strategy depends on the purpose of the study whether it is for either food composition data or investigating new methodologies.

The need for standardized analytical methods to determine the content of vitamins in food is on the agenda internationally. This is a challenging task as the activity of each of the 13 vitamin is expressed by more than one compound.

It is essential for the analytical method of choice is to have specific information on which criteria should be included. AOAC's Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) has set up Standard Method Performance Requirement (SMPR) for the vitamins of interest [1]. SMPR defines the analytes which shall be included in the quantification, analytical range, limit of quantitation, and repeatability. The SPIFAN only deal with infant formula and adult nutritionals. Hence the scope is not as complicated as for assessing nutrients for food composition databases.

This paper describes general tasks to consider when choosing an analytical method for a specific task. Among the 13 vitamins especially vitamin D has been in focus in nutritional science for the last 15 years which has included. The necessity to include 25-hydroxyvitamin D in food composition tables has received a lot of attention. Therefore the analytical platform used for establishing data on vitamin D for the Danish Food Composition Database [2] and for the analytical platform in the EU 7PP-research project ODIN (Food-based solutions for optimal vitamin D nutrition and health through the life cycle) [3] will be used as an example.

2. VITAMIN D IN FOOD

The vitamin D content of food is composed of different vitamers. The parent vitamin D, cholecalciferol ($vitD_3$) and ergocalciferol ($vitD_2$), and the metabolites, 25-hydroxyvitamin D_3 ($25OHD_3$) and 25-hydroxyvitamin D_2 ($25OHD_2$). Figure 1 shows the structure of different vitamers.

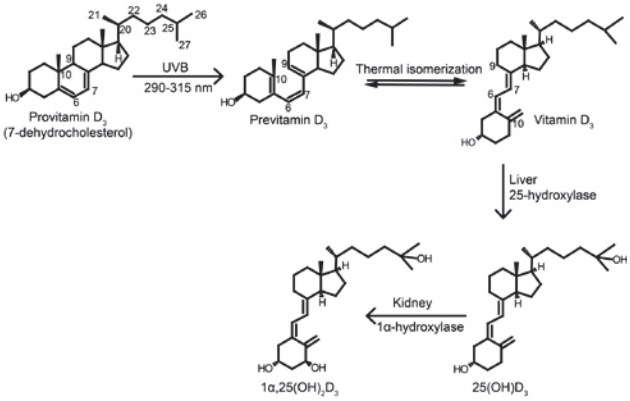

Fig. 1. Structures included in the vitamin D_3 production by UVB-exposure.

$VitD_3$ is the main active compound as only lower amounts of $25OHD_3$ are present in foods of animal origin e.g. fish, meat, eggs and milk. The levels vary from 30 µg $vitD_3$/100 g in salmon to 0.03 µg/100 g in milk [4]. Furthermore, $vitD_2$ may be found in high amounts up to 60 µg/100 g in wild mushrooms and UV-exposed farmed mushrooms, and in low amounts at 0.03 µg/100 g in dairy products, which also show similar low contents of $25OHD_2$ [5][4].

3. SAMPLING

In sampling for food composition data, it is essential e.g. to take into account the variation of vitamin D within a fish from head to tail [5]. Milk from cows having access to outdoor areas in the summer will have a higher vitD level than milk in the winter, due to the production of vitamin D in the skin by exposure to sun [4].

Sampling for research projects e.g. when comparing different feeding strategies one should sample meat cuts with similar levels of fat, as contents of vitamin D and fat are associated [6].

Essential for the analytical results are the condition during sampling and until arrival at the laboratory.

4. STORAGE

The stability of vitamin D is associated with the stability of fats and oils. Vitamin D in food is relatively stable in raw food. Vitamin D_3 in milk powder stored at -20°C are stable for at least 7 years [4], while storage of serum at -20°C resulted in an interference for $25OHD_3$ in serum [7]. Hence, storage at -80°C is recommended for the samples to be analysed.

Once freed from the protection of the food matrix, vitamin D is susceptible to decomposition by oxygen and light, but also suspectible to isomerizations catalyzed by acids and temperature. Therefore the analytical method has to include protection against especially oxidation.

5. HOMOGENISATION AND TEST SAMPLE

The amount of sample taken for homogenisation should be representative i.e. for fish one whole fish filet is required, while for eggs a separation into egg yolk and egg white seems optimal due to the challenging mixing of whole eggs. If whole eggs are used the mixing requires longer time, and precaution to avoid oxidation.

The homogenised sample should be tested for sufficient homogeneity for the amount of test sample acquired by the method. This is especially to check if the test sample is below 1 g.

6. ANALYTICAL METHODS

6.1. Which method to choose?

The isomerisation of vitD to the pre-vitD form during saponification necessitates the use of an internal standard with the same property. Quantification of $vitD_3$ with $vitD_2$ as internal standard (and vice versa) is already well described in a standardised method by use of HPLC-UV [8][9]. The methods are applicable for a wide range of foods, while a recent single laboratory LC-MS/MS based method from AOAC is validated on specific fortified food i.e. infant formula and adult nutritional [10].

No standard method is available for 25OHD, $25OHD_2$ nor $25OHD_3$, but numerous papers in reviewed journals describe the possibility to utilise a similar procedure as for vitD. In 1995, reference [11] was the first to validate a method which used $25OHD_2$ as internal standard for $25OHD_3$ in a HPLC-UV based method. While reference [12], was the first to publish a LC-MS/MS method for the four metabolites in meat and organs.

In our lab we have run quantification of vitD accredited according to EN45001 since 1994 by HPLC-UV/PDA, extended with 25OHD and run accredited according to ISO17025 since 2001, and from 2014 supplemented with a LC-MS/MS method. The experiences obtained are used for the description development of the techniques.

6.2. HPLC-UV/DAD

The extraction principle: saponification and several clean-up steps have been used throughout the years, and the conditions have gradually been optimised especially regarding working environment for technicians. The first step is alkaline hydrolysation at room temperature overnight or at 80°C for 45min, followed by liquid-liquid extraction by either ethyl ether:petroleumether (50:50) og ethylacetate:*n*-heptane (20:80). Further clean-up steps were silica solid phase extraction, and 1-2 semi-preparative steps. Final quantification was assessed on a

separate HPLC-systems for vitD and 25OHD, as separation of the $vitD_2$/$vitD_3$ and $25OHD_2$/$25OHD_3$ in one run in a sufficient manner for a variety of foods is a challenge not yet solved for a baseline solution of each of the D_2- and D_3-metabolites. A DAD-detector (220-320 nm) was used for detection, and 265 nm used for quantification.

6.3.LC-MS/MS

Use of MS/MS-detection depends on the availability of reliable internal standards appropriate for the different vitamin D metabolites. Due to the temperature dependent equilibrium during saponification of pre-vitD/vitD labelled compounds of the metabolites of interest are essential. The huge amount of research and the need to understand vitamin D metabolism have provided a demand for labelled standards, therefore labelled metabolites have become commercially available for $vitD_3$/$vitD_2$ and the hydroxylated metabolites during the last few years.

Shifting to MS/MS-detection may be run with the similar clean-up step as for UV-detection, but without separate injection of the fractions of vitD and 25OHD. However, semi-preparative clean-up step may be replaced by a derivatisation step with 4-phenyl-1,2,4-triazoline-3,5-dione. The PTAD-derivatisation improves the ionization efficiency by electrospray ionization and limits interferences.

6.4. Method performance

The validation of the methods followed the procedure according to ref. [13] for specificity, sensitivity, accuracy (precision and trueness), measuring range, sensitivity (limit of quantification), and ruggedness.

Table 1 summarizes amount of testsample, LOQ, and precision for the HPLC-UV and LC-MS/MS methods.

Table 1. Method characteristics for HPLC-UV and LC-MS/MS.

	Test sample	LOQ, µg/100 g	Precision, %
HPLC-UV	10-300 g	0.004-0.02	5-8
LC-MS/MS	0.2-1 g	0.002	8-10

Especially trueness has been a challenge, as limited laboratories have had methods for vitamin D metabolites.

For $vitD_3$ a collaborative study and an inter-laboratory study based on HPLC-UV methods on 8 different fortified samples, 0.4-14 µg $vitD_3$/100 g showed a reproducibility 6-24% [8], [9].

Similarly, only certified reference materials of margarine and milk powder/infant formula with added $vitD_3$ was available until last year.

In 2016, reference [14] reported the results of a inter-laboratory study on natural level of $vitD_3$, $25OHD_3$ and $vitD_2$ in meat, organs and eggs. Five laboratories participated using their own in-house method. We contributed and applied our LC-MS/MS-method [15]. The samples contained 0.2-4.5 µg $vitD_3$/100 g and 0.1–1.5 µg $25OHD_3$/100g. The repeatability was <10% and <16%, respectively. The low level at 0.04 µg/vit D_3/100 g was assessed based on a reproducibility of 58%. The levels of $vitD_2$ and $25OHD_2$ was <0.1 µg/100 g (n=3), but despite the low level the reproducibility were below 23%. Reproducibility for the MS/MS-based method as presented is satisfactory.

6.5. Routine quality control

Once validated and accredited by the national accreditation body, the routine quality control will include analyses of available certified reference materials and participation in proficiency tests.

Only proficiency test have been available for $vitD_3$ mainly from ref. [16].

Therefore we have relied on house-reference materials in our documentation for a constant level in our laboratory through the years of $vitD_2$, $25OHD_3$ and $25OHD_2$, but also level of $vitD_3$ has been checked in these samples of natural food. We seek to have house-reference materials similar to the test-samples i.e. fat, meat, egg, butter, milk, fish, but also fortified wheat flour and infant formula. An example of comparison between UV- and MS/MS-based method are given in Table 2.

Table 2. Vitamin D_3 quantified by HPLC-UV and LC-MS/MS-method in house-reference materials of salmon and egg yolk, µg/100 g.

	Salmon	Egg yolk
HPLC-UV	15.4 (n=4)	2.66 (n=10)
LC-MS/MS	15.2 (n=18)	2.69 (n=5)

7. DISCUSSION AND CONCLUSION

Apart from the above mentioned elements, it is crucial to know that the main contributor to the precision in the analyses of food derives from the food matrix from the sampling for the homogenised sample.

An essential part is also the practical performance in the laboratory. The technician should be sufficiently trained in the practical part especially the part of an analytical method where the main analytical error or mistake may occur.

The analyses of vitamin D with use of internal standard, makes it easy to train new people in the laboratory. However, the crucial part becomes the preparation of the calibration curve.

The set-up of the samples in analytical series for a study depends on the aim of the study, whether to run all samples at random or pairwise.

A new analytical method shall be compared with the former used methodology before introduced for routine analyses. For vitamin D, HPLC-UV with the necessary clean-up step i.e. SPE and 1-2 semi-preparative HPLC shows no difference with LC-MS/MS based method.

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