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ISOTOPIC AND OTHER TECHNIQUES FOR ORGANIC MICRONUTRIENT ANALYSIS AND DEVELOPMENT OF QUALITY ASSURANCE PROCEDURES

Report of a Consultants Meeting

Vienna, Austria , 4 – 8 December 2000

INTERNATIONAL ATOMIC ENERGY AGENCY

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ISOTOPIC AND OTHER TECHNIQUES FOR ORGANIC MICRONUTRIENT ANALYSIS AND DEVELOPMENT OF ANALYTICAL QUALITY ASSURANCE PROCEDURES

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A report prepared by the
Section of Nutritional and Health-Related Environmental Studies
Division of Human Health
Department of Nuclear Sciences and Applications
International Atomic Energy Agency
P.O. Box 100, A-1400 Vienna, Austria

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SUMMARY

The Nutritional and Health Related Environmental Studies Section (NAHRES), Division of Human Health at the IAEA has been very active in supporting activities related to measurement problems of both organic and inorganic nutrients faced by several developing countries. In preparation to a systematic approach to help the needy developing countries, a new initiative under "Nutritional Metrology in Practice" has been initiated for the years 2002 and 2003.

As a first step, a small group of consultants were sought to advice the Agency on the current methodological status related to nutrient measurements. The consultants who participated in the meeting were: Dr. Katherine E. Sharpless, Dr. Paul M. Finglas, Dr. Clive West and Dr. Tee E. Siong. Recognizing the double burden of under- and over-nutrition in developing countries, the following nutrients were selected as priority under the Nutritional Metrology program: vitamin A and carotenoids, folate, vitamins B_1, B_2, B_6, and B_{12}. The capabilities for the analyses of these nutrients in foods and serum vary extensively among the developing countries. This refers to availability of expertise, equipment, and quality control programs in these countries. Of particular interest to the Agency is the use of isotopic methods to measure these nutrients since several nutritional monitoring programmes are likely to seek this technology for future projects supported by the IAEA. There is therefore an urgent need for enhancing capabilities in these countries.

This group of experts assessed the current status of quality assurance programs, training and quality control materials available for measurement of vitamin A and Beta-carotene, folate, vitamin B1, B2, B6 and vitamin B12 in food/serum/plasma and made their recommendations for future work in these areas: (i) extend/establish quality assurance programs for vitamin A/carotenoids and folate for food and blood analyses in developing countries; (ii) establish training linkages between expert and non-expert laboratories for vitamin A/carotenoids, folate, and other B vitamins; (iii) circulate existing food and biological CRMs and other QC material to existing and new networks of laboratories in developing countries for the analysis of vitamin A/carotenoids, folate, and other B vitamins; (iv) develop accessible/inexpensive control materials for these vitamins; (v) develop isotopically labeled reference materials (isolates and intrinsically labeled foods) for use in bioavailability studies and (vi) develop appropriate LC-MS procedures for vitamin A/carotenoids and folates in regional centers.

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1. INTRODUCTION

Undernutrition and overnutrition are major problems in developing countries. With respect to undernutrition, low intakes of vitamins, trace elements, minerals, protein, and energy are important while overnutrition contributes to chronic diseases including coronary heart disease, diabetes, and cancers at certain sites. The incidence of such chronic diseases appears to be exacerbated by low intakes of various organic micronutrients. The extent of the problem may not be fully recognized because of a lack of analytical expertise in the developing countries.

The Nutritional Metrology activities foreseen for 2002 and 2003 seek to remedy this situation [Iyengar, 2000]. Recognizing the double burden of under- and over-nutrition in developing countries, we have selected a number of nutrients for particular priority as part of the Nutritional Metrology program: vitamin A and carotenoids, folate, and vitamins B_1, B_2, B_6, and B_{12}. The recommendations discussed below should greatly enhance the quality of data output by participants, and permit greater comparability of data between studies, in the Nutritional Metrology program.

2. ORGANIC MICRONUTRIENT METHODOLOGY AND QUALITY ASSURANCE ISSUES

Up until five or ten years ago, programs related to improving nutritional status in developing countries with respect to vitamin A and folic acid were directed towards increasing the intake of pro-vitamin A, folic acid (the form used for food fortification), and naturally occurring folates. However, it is now recognized that bioavailability of these nutrients is important and more information is required on the physiological and dietary factors that affect absorption. In recent years, techniques have been developed for this purpose but as yet the data generated have been limited to a few stable-isotope studies in industrialized countries.

The capabilities for the analyses of these nutrients in foods and serum vary extensively among the developing countries. This refers to availability of expertise, equipment, and quality control programs in these countries. There is therefore an urgent need for enhancing capabilities in these countries. This will greatly help developing countries in their effort to better characterize the extent of the problem as well as in the evaluation of intervention programs.

Liquid chromatographic (LC) methods can be used for the analysis of all of these analytes in foods and blood or serum, although microbiological techniques are frequently used for analysis of the B vitamins in foods (Table 1). LC methods can be automated and run attended, allowing for high sample throughput. Stable isotope techniques are used for assessing the bioavailability and whole body-pool sizes of retinol (and its formation from carotenoids), β-carotene and folate. In addition, stable isotopes are sometimes used as internal standards in LC analysis, as well.

Expertise and analytical performance can be improved by:

- 'twinning' analysts in developing countries with experienced analysts in industrialized countries or from the same geographic region who have demonstrated proficiency; and

- participation in a quality assurance program, as discussed below.

TABLE 1: SUMMARY OF AVAILABLE METHODS FOR THE DETERMINATION OF SELECTED VITAMINS IN FOODS AND BIOLOGICAL MATERIALS

	Vitamin A	Carotenoids	Folate	Vitamin B1	Vitamin B2	Vitamin B6	Vitamin B12
LC-Absorbance Detection	X	X	X	X	X	X	
LC-Fluorescence Detection	X		X	X	X	X	
Microbiological Assay			X	X	X	X	X
Radioassay kit			X			X	X
Enzyme-Protein Binding Assay/ELISA			X			X	X
Gas Chromatography – Mass Spectrometry			X				
LC-Mass Spectrometry	X	X	X				
Accelerated Mass Spectrometry			X				

National Institute of Standards and Technology (NIST) and Wageningen University are currently actively involved in proficiency testing schemes and training for fat-soluble vitamin analyses, including vitamin A and carotenoids. Institute of Food Research (IFR) is one of the EU-designated training centers for micronutrient analyses and bioavailability with particular expertise in carotenoids, folate, and B vitamins.

Another quality assurance problem is that related to stable isotope-labeled materials used in nutrition studies such as the measurement of the bioavailability/bioefficacy of carotenoids and folic acid. No reference materials exist in this area for method validation or to verify the quality of the data generated. The application of newly emerging isotopic methods for the determination of vitamin A and folate status and bioavailability would benefit enormously from this initiative. This would also enable better comparability between results of future IAEA Coordinated Research Projects (CRPs) and Technical Cooperation (TC) projects in this area.

3. VITAMIN A AND CAROTENOIDS

3.1. Quality assurance program in developing countries

Deficiencies in vitamin A (and pro-vitamin A compounds such as β-carotene) are associated with an increased risk of morbidity and mortality, nutritional blindness, certain forms of cancer, and macular degeneration. Tuberculosis is one of the major causes of death

in developing countries, and vitamin A and zinc supplementation has resulted in a decrease in various tuberculosis indicators. Programs are aimed at improving the vitamin A status of populations in developing countries, but the effectiveness of such interventions must be monitored through reliable, accurate analysis of vitamin A and carotenoids in serum from representative samples.

The current state of the practice in LC analysis provides within-laboratory reproducibilities of 3 to 5% for retinol (vitamin A) analyses in serum when measured by experienced analysts. In comparison, within-laboratory reproducibilities have been observed to be as poor as 120% for measurements of retinol in serum in less expert laboratories in both developed [Duewer et al., 2000] and developing countries [Brouwer et al., 2000]. Similar poor performance has been reported for the analysis of folic acid (see below) and other micronutrients. Thus, we recommend that laboratories in developing countries that participate in intervention studies also participate in a quality assurance (QA) program for vitamin A and carotenoid analysis. Active participation in such programs has been shown to improve the proficiency of analytical laboratories over time [Duewer et al., 2000]. Four such programs are in existence – two for serum analysis and two for food:

- The Micronutrients Measurement Quality Assurance Program is available from NIST in the United States. For a fee of $2000 (for non-U.S. laboratories), a laboratory receives two sets of five serum samples for analysis each year as well as feedback relating that laboratory's performance to the performance of other participating laboratories. Telephone/e-mail consultations are provided upon request. Occasionally, this program has also included food samples. Because of the cost involved, this program is not readily available to laboratories in developing countries.

- Recently, a proficiency program was coordinated from Wageningen in the Netherlands to assess capabilities of African laboratories for the analysis of retinol and carotenoids in serum [Brouwer et al., 2000]. Funds are now being sought to extend this program to introduce a quality assurance scheme and to provide support to laboratories to improve their performance.

- The Asian Pacific Food Analysis Network (APFAN), coordinated by Pieter Scheelings of Brisbane, Australia, provides food samples to laboratories in the Asian Pacific region in order to assess proficiency.

- The Association of Southeast Asian Nations (ASEAN) Food Data System, another program involving food analysis, is coordinated by Dr. Prapasri Puwastien at the Institute of Nutrition of Mahidol University in Bangkok.

There is a need to extend these programs to laboratories in other developing countries, especially Africa and the Americas. Existing networks such as IAEA laboratories, APFAN, and ASEAN can be used where appropriate, and new ones established in Africa and the Americas, as required.

3.2 Training

After laboratories within a geographic region have demonstrated proficiency as part of one of these QA programs, analysts in these laboratories will be able to provide training to other laboratories within the region. Grants could be provided at the technician level for training at these regional laboratories. Grants would fund travel for trainees as well as expenses incurred by the training itself. Continued demonstration of proficiency and site

visits from the supervisory agency would permit the regional laboratories to continue their training programs. A limited amount of analytical equipment (e.g., analytical columns, spare parts for LC pumps, etc.) could be provided through the program.

3.3. Availability of quality control materials

Certified reference materials (CRMs) are available for vitamin A and carotenoids in both serum and food, but the cost of these materials prohibits their use by developing countries who must first refine their analytical techniques. "Quality control materials" [as opposed to CRMs or reference materials (RMs)] are necessary for development of analytical expertise by laboratories in developing countries. (Frequently, these laboratories have problems with the reproducibility of their methods, and results may differ by orders of magnitude). Such materials would have values (tentatively) assigned in a less rigorous fashion than that typically employed for value assignment of CRMs or RMs, but would be of use for laboratories refining their analytical skills and allow traceability of their data to international standards if value-assigned against CRMs. These control materials may take the form of lyophilized serum, whole blood, or regional foodstuffs, as appropriate. NIST and Institute for Reference Materials and Methods (IRMM) have considerable experience in the production of natural-matrix reference materials such as these.

3.4. Quality assurance program for stable isotopes

IAEA is currently funding several CRPs involving vitamin A interventions and the use of stable isotopes. Stable isotopes of retinol and β-carotene are used to assess bioefficacy, i.e., the conversion rate of β-carotene to retinol. However, no reference materials exist to verify the accuracy of the analytical methods used to measure these isotopes. Thus, a reference material containing labeled retinol and β-carotene could be spiked into serum and made available to researchers in this area. Coordinators of the NIST Micronutrients Measurement QA Program have experience in spiking (unlabelled) retinol into serum.

3.5. Bioavailability of carotenoids

Communities in developing countries rely heavily on plant material as their main source of provitamin A. Limited data are available on the bioavailability of carotenoids from these foods using stable isotopes. Depending on the type of food, method of cooking, etc., bioavailability may vary. Further research needs to be conducted in this area using standardized protocols for stable isotope techniques and evolving LC-MS technologies. In this respect, both the isolated labeled compounds and an intrinsically labeled food material could be used to study the physiological factors influencing absorption and conversion of provitamin A carotenoids to retinol. This would have a major impact on nutrition intervention programs in developing countries.

4. FOLATE

4.1. Quality assurance program in developing countries

Deficiencies in folate are associated with an increased risk of neural tube defects, coronary heart disease, stroke, and certain cancers. This is an emerging area and there are few quality assurance schemes in operation within industrialized countries and none in developing regions. Thus we recommend that a quality assurance (QA) program for folate analysis be established. Active participation in such QA programs has been shown to improve the proficiency of analytical laboratories over time [Duewer et al., 2000].

Inter-laboratory variation in results for serum and red cell folate have been assessed by both European and international working groups. Considerably variation (18 to 41%) was observed both between different methods (microbiological, radioassay, and HPLC), as well as between-laboratories using similar assay method (van den Berg et al., 1994). In a similar study, involving 20 international laboratories experienced in folate analyses in blood, overall CVs of 25 to 40% were reported for serum and red cell folate with 2- to 9-fold differences in concentrations between methods, with the greatest variation occurring at critical low folate concentrations (Gunter et al, 1996).

4.2. Training

After laboratories within a geographic region have demonstrated proficiency as part of a QA program, analysts in these laboratories will be able to provide training to other laboratories within the region. Grants could be provided at the technician level for training at these regional laboratories. Grants would fund travel for trainees as well as expenses incurred by the training itself. Continued demonstration of proficiency and site visits from the supervisory agency would permit the regional laboratories to continue their training programs. A limited amount of analytical equipment (e.g., analytical columns, spare parts for LC pumps) could be provided through the program.

4.3. Availability of quality control materials

Four CRMs for folate in food are available from the EU's IRMM [Finglas et al., 1999]. These materials are certified for total folate and have information values for 5-methyltetrahydrafolic acid (5-MTHF). The cost of these materials renders them inappropriate for use by developing countries who must first develop accurate and reproducible analytical techniques. "Quality control materials" (as opposed to CRMs or RMs) are necessary for development of analytical expertise by laboratories in developing countries. (Frequently, these laboratories have problems with the reproducibility of their methods, and results may differ by orders of magnitude.) Such materials would have values (tentatively) assigned in a less rigorous fashion than that typically employed for value assignment of CRMs or RMs, but would be of use for refining their analytical skills and expertise. These control materials may take the form of regional foodstuffs, as appropriate. This has been started by networks such as APFAN and ASEAN but lack of funds and international guidance has restricted progress.

Quality control materials are available for folate in serum and whole blood from various commercial suppliers, and the cost of these is not prohibitive. These are currently being used by a group of European laboratories as part of an EU FP5 project on folate functionality and bioavailability ("Folates: From Food to Functionality in Optimal Health;" see http://ifr.bbsrc.ac.uk/folate). This could be extended to include developing countries.

4.4. Quality assurance program for stable isotopes

Isotopically labeled folates are now commercially available for folic acid and 5-MTHF (6S-isomer) and could be used as QA material to assist the development of combined liquid chromatographic - mass spectrometric (LC-MS) procedures in regional laboratories in developing countries. This technique has been established in the Queensland Health laboratory in Brisbane and has the potential for setting up in the Institute of Nutrition, Mahidol University in Thailand.

4.5. Bioavailability and functionality of folate

IFR has developed intrinsic labeling techniques for producing ^{13}C- and ^{15}N- folates in spinach, a rich natural source of the vitamin. Both the isolated labeled compounds and the intrinsically labeled spinach could be used to study the physiological factors influencing absorption and cleavage in vivo, and the release and absorption of folates from foods as consumed (Wolfe et al., 2000).

4.6. Additional folate studies

Folate and vitamins B_6 and B_{12} may lower plasma levels of homocysteine, which is an emerging risk factor for coronary heart disease and stroke. Various dietary intervention studies are currently being conducted in industrialized countries, and these could be further extended to developing regions. Methods for plasma homocysteine based on LC or immunoassay techniques need to be established in regional centers in these areas. Relatively inexpensive control materials for plasma homocysteine analysis are available.

5. OTHER B VITAMINS (B_1, B_2, B_6, AND B_{12})

There is also continuing interest and need for determining the prevalence of vitamins B_1 and B_2 deficiency among communities in developing countries. Similarly, vitamin B_{12} deficiency plays a significant role in anemia among specific groups of the population, notably the elderly, in these countries. The increased use of food folic acid fortification strategies in industrialized countries has added to potential problem of the masking of vitamin B_{12} deficiency in the population. Less is known about the performance of methodologies for the determination of these vitamins in food and blood. There is therefore a need to assess this for laboratories in developing countries. Thus a QA program similar to that mentioned above for retinol and folate analyses in food and blood should be established using unlabeled quality control material. Food CRMs are available from IRMM for all of these B vitamins [Finglas et al., 1999], but the cost of these can be prohibitive for use in developing countries.

6. SUMMARY RECOMMENDATIONS

The following initiatives are recommended:

- Extend/establish quality assurance programs for vitamin A/carotenoids and folate for food and blood analyses in developing countries.
- Establish training linkages between expert and non-expert laboratories for vitamin A/carotenoids, folate, and other B vitamins.
- Circulate existing food and biological CRMs and other QC material to existing and new networks of laboratories in developing countries for the analysis of vitamin A/carotenoids, folate, and other B vitamins.
- Develop accessible/inexpensive control materials for these vitamins.
- Develop isotopically labeled reference materials (isolates and intrinsically labeled foods) for use in bioavailability studies.
- Develop appropriate LC-MS procedures for vitamin A/carotenoids and folates in regional centers.

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CONSULTANTS' MEETING

ISOTOPIC AND OTHER TECHNIQUES FOR ORGANIC MICRONUTRIENT ANALYSIS AND DEVELOPMENT OF ANALYTICAL QUALITY ASSURANCE PROCEDURES

INFORMATION SHEET

1. Background

The International Atomic Energy Agency (IAEA) has been supporting activities in the field of nutrition since the 1970s. In 1987, it established a sub-programme on nutrition related research, with a focus on applications of isotopic techniques for measuring nutrients in foods and in the human body. In 1992, the IAEA increased its efforts to bridge the gap between industrialized and developing countries' access to isotopic techniques for monitoring the nutritional status, thereby arousing interest among the UN, national and bilateral organizations in the use of isotopic techniques in food and nutrition. In recent years, new strategies are being sought to address micronutrient malnutrition that is in epidemic proportions in developing societies today. Women and children representing the largest segment of society are in need of protection because of their increased vulnerability to malnutrition. Multi-factorial interventions to prevent malnutrition, infectious disease and environmental pollution are priority issues that need to be addressed as urgent public health problems. The IAEA is contributing to these efforts by facilitating the development of a variety of isotope based techniques to improve monitoring techniques for both nutrients and pollutants and to identify effective strategies in nutrition intervention schemes particularly among vulnerable groups in developing regions around the world.

Recently, the Nutritional and Health Related Environmental Studies Section (NAHRES), Division of Human Health of the IAEA has been very active in supporting activities related to measurement problems of both organic and inorganic nutrients faced by several developing countries. In preparation to a systematic approach to help the needy developing countries, a new initiative under "Nutritional Metrology in Practice" has been initiated in the year 2002 and 2003 budget cycle. As a first step, it is planned to assemble a small group of consultants to seek their advice on the current methodological status related to nutrient measurements. Of particular interest to the Agency is the use of isotopic methods since several nutritional monitoring programmes are likely to seek this technology for future projects supported by the IAEA.

2. IAEA's involvement with stable isotopes and organic nutrient measurements

In recent years, NAHRES has turned more toward the use of stable isotopes. Work with stable isotopes involves nuclear techniques, which are also exploited by the joint FAO/IAEA Division and the Section of Isotope Hydrology and Geochemistry, at the IAEA. For the light isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O), largely as a consequence of the activities of physiologists and nutritionists using IRMS, this type of analysis is now available with a high degree of automation with no significant reduction in performance. GC/MS (gas chromatography/mass spectrometry) and LC/MS (liquid chromatography/mass spectrometry) are never likely to be tools of choice for stable isotope ratio measurements but GC/C/IRMS (gas chromatography, combustion, IRMS) developments will be significant and will reduce isotope costs in many applications. For the heavy elements lack of automation inhibits the development of good models for absorption, storage and turnover but magnetic sector ICPMS with multiple collectors is probably the way forward.

The IAEA is involved in a range of nutritional topics covering quantitative measurements of vitamins in foods and biological fluids, assessment of absorption of nutrients, nutrient supplementation, measurement of body nutrient status and energy metabolism, among others. However, the IAEA recognises that the most serious gaps, and thus the greatest difficulties in long-term inter-laboratory harmonisation, lie with the vitamins, partly because they tend to be less stable in comparison with minerals, and partly because there are no "gold standard" assay methodologies, for the relevant biological materials (However, there are some radioimmuno-assay methods for several of them).
For these and other reasons, the IAEA closely follows the progress in application of stable isotope and other methods in human nutrition research and recognises the need to periodically assess the state of the practice by bringing together experts for consultants' meetings. It is anticipated that this group of experts will be able to assess the state of practice for vitamin A and Beta-carotene in foods, folate in serum and whole blood, vitamin B6 in plasma, vitamin B12 in serum/plasma, vitamin C in plasma, 25(OH) vitamin D, vitamin k in plasma, red cell B-vitamin status indices for B1, B2, B6 and possibly biotin.

3. Objectives

- To assess the state of practice of stable isotope and other methodologies used for vitamin assays
- To identify new strategies for improving sensitivity of organic nutrient monitoring techniques for use in Co-ordinated Research Projects and Technical Co-operation (TC) Projects in nutrition
- To obtain an objective assessment of *"the state of practice"* comparative measurement methods for organic nutrients in foods, tissues and body fluids and their applicability to specific matrices.
- To assess the availability of natural matrix reference materials certified for specific vitamins and to identify areas where new analytical quality assurance initiatives are needed

4. Expected outcomes

- Working material addressing the above-mentioned issues, describing on-going or recent work carried out by each participating scientist, to be presented during the meeting and made available to the IAEA.
- Strategy to approach analytical quality control issues of vitamin measurements in support of the "Nutritional Metrology" activities foreseen for Programme and Budget for 2002 and 2003 cycle.
- Recommendations on use of improved comparative methodologies based on stable isotopes and other methods for strategic health applications for IAEA's Co-ordinated Research Projects and field applications in TC projects.
- Technical guidance for initiating preparation of natural matrix reference materials for vitamins presently not certified.
- Short report (not more than 20 pages) with recommendations, to be drafted and adopted by participants during the meeting. It is anticipated that the discussions during this consultants' meeting will shed light on the problems outlined above and facilitate formulating future projects to resolve the issues using nuclear and isotope based techniques.

AGENDA

MONDAY, 4 DECEMBER 2000 JOINT SESSION

09:00 – 09:30	**Registration/Opening Session**
09.30 – 10.00	Gathering at the Meeting Room/coffee
10:00 – 10:15	Welcome and Introduction of the participants
10.15 - 10.45	NAHU perspectives (Dr. S. Groth, DIR-NAHU)
10.45 – 11.00	Aim of the meeting (s) (Dr. G.V. Iyengar, SH, NAHRES)
11.00 - 11.20	The world of Stable Isotope Measurements (Dr. A. Coward, U.K.)
11.20 - 11.40	The world of Organic Nutrient Measurements (Dr. C. West, Netherlands)
11.40 - 12.00	Stable Isotope Activities at the IAEA (Dr. M. Groening , NAAL-NAPC)
12.00 - 12:30	Discussions/selection of general chairpersons
12:30 - 14:30	Lunch/Administrative matters
14:30 – 17:30	**SESSION 1, Presentations by the consultants and discussions**
14:30 – 15:30	Dr. C. West, Netherlands.
15.15 - 16.00	Dr. Tee E Siong, Malaysia
16.00 - 16.30	Coffee break
16:30 – 17:30	General discussion/Administrative matters

TUESDAY, 5 DECEMBER 2000

09:00 – 12:30	**SESSION 2, Presentations by the consultants and discussions**
09.00 - 10.00	Dr. P. Finglas, U.K.
10.00 - 11.00	Dr. K. Sharpless, U.S.A.
11.00 - 11.30	Coffee Break
11.30 - 12.30	Discussion to identify areas needing attention
12.30–14:30	**Lunch and administrative arrangements, if any**
14:30 – 15.30	**SESSION 3, Selected topics for in-depth discussions**
	Moderator Dr. C. West
	Input: all participants

Suggested topics:

- To assess the state of practice of stable isotope and other methodologies used for vitamin assays
- To identify new strategies for improving sensitivity of organic nutrient monitoring techniques for use in Co-ordinated Research Projects and Technical Co-operation (TC) Projects in nutrition
- To obtain an objective assessment of *"the state of practice"* comparative measurement methods for organic nutrients in foods, tissues and body fluids and their applicability to specific matrices.
- To assess the availability of natural matrix reference materials certified for specific vitamins and to identify areas where new analytical quality assurance initiatives are needed

15:30 – 16:00	**Coffee break**	
16.00– 17.30	**Framework discussion on contributions to ICN-17 in Vienna in August 2001:**	JOINT SESSION
	Stable Isotope User's Group	
	Moderator: Dr. T. Walczyk	
	Introduction by Dr. G.V. Iyengar	
	Input by stable isotope CS group	
	Comments/contributions by Vitamin CS group	

WEDNESDAY, 6 DECEMBER 2000

09:00 – 12:30 SESSION 4: Selected topics for in-depth discussions (continued):
Moderator: Dr. C. West
Contributors: All participants

Suggested topics

- To assess the state of practice of stable isotope and other methodologies used for vitamin assays
- To identify new strategies for improving sensitivity of organic nutrient monitoring techniques for use in Co-ordinated Research Projects and Technical Co-operation (TC) Projects in nutrition
- To obtain an objective assessment of *"the state of practice"* comparative measurement methods for organic nutrients in foods, tissues and body fluids and their applicability to specific matrices.
- To assess the availability of natural matrix reference materials certified for specific vitamins and to identify areas where new analytical quality assurance initiatives are needed

- **Other topics to be brought for discussion**

Proposed actions and identifying authors for writing specific segments
Coffee break as desired

Lunch

14.30 - 17.00 SESSION 5, Combined session with both CS groups JOINT SESSION

- Short reports by both groups by respective chairpersons and moderators.
- Comparative assessment: isotopic vs non-isotopic methods

Approaches for certification of organic nutrients in natural matrix materials: specific examples
Other topics to be brought for discussion

THURSDAY, 7 DECEMBER 2000

09:00 – 12:30 **SESSION 6, Preparation of draft report**
Completion of individual contributions as assigned
Lunch

14.30 - 17.30 **SESSION 7, Preparation of draft report (continued)**
Coffee break as needed

FRIDAY, 8 DECEMBER 2000

09:00 – 12.00 SESSION 8, Finalising the Report

Timetable for further action needed
Conclusions of the meeting
Closing of the meeting

LIST OF PARTICIPANTS

Dr. Tee E. SIONG
Head, Human Nutrition Division
Institute for Medical Research (IMR)
Jalan Pahang
50588 Kuala Lumpur
Malaysia
Tel.: + 60 3 298 6704
Fax: + 60 3 294 3575
E-mail: teees@imr.gov.my

Dr. Clive WEST
Wageningen Agricultural University
Human Nutrition
P.O. Box 8129
6700 EV Wageningen
The Netherlands
Tel.: +31 317 482589
Fax: +31 317 483342
E-mail: clive.west@staff.nutepi.wau.nl

Dr. Paul M. FINGLAS
Diet Health and Consumer Science Division
Institute of Food Research
Colney Lane
Norwich, NR4 7UA
Norfolk, GB
Tel.: + 44 1603 255318
Fax: + 44 1603 507723
E-mail: paul.finglas@bbsrc.ac.uk

Dr. Katherine E. SHARPLESS
Research Chemist
National Institute of Standards and Technology
100 Bureau Drive Stop 8392
Gaithersburg, MD 20899-8392
USA
Tel.: + 1 301 975 3121
Fax: + 1 301 977 0685
E-mail: katherine.sharpless@nist.gov

IAEA SECRETARIAT :

Dr. Manfred GROENING
Isotope Hydrology Section
Agency's Laboratories Seibersdorf
Department of Nuclear Sciences and Applications

Tel: + 43 1 2600 21740
Fax: + 43 1 26007
E-mail: M.Groening@iaea.org

Dr. S. GROTH
Director
Division of Human Health
Department of Nuclear Sciences and Applications

Tel: + 43 1 2600 21650
Fax: + 43 1 26007
E-mail: S.Groth@iaea.org

Dr.G.V. IYENGAR
Head, Section of Nutritional and Health-Related Environmental Studies
Division of Human Health
Department of Nuclear Sciences and Applications

Tel: + 43 1 2600 21657
Fax: + 43 1 26007
E-mail: V.Iyengar@iaea.org

Institute of Food Research

ISOTOPIC AND OTHER TECHNIQUES FOR ORGANIC MICRONUTRIENT ANALYSIS

**Paul M. Finglas, Organic Micronutrient Group,
Nutrition Health & Consumer Science Division,
Institute of Food Research, Norwich, UK**

OUTLINE

- General overview of vitamin methods
- Current status of CEN/TC275/WG9
- Recommended Status Methods for B-vitamins (EU FLAIR CA No 10)
- Individual B-Vitamins (isotopic/other)
- QA & Reference Materials

OVERVIEW OF METHODS FOR B-GROUP VITAMINS

- Microbiological assays
- Chemical (thiochrome, niacin)
- HPLC (uv, diode-array, F & EC), LC-MA
- Radio-protein bindings assays (biotin, B_{12}, folate & B_6) (radioassay kits)
- Enzyme-protein binding assays/ELISAs (folate, biotin, B_{12}, B_6 (IMx system, Biacore)
- GC-MS, LC-MS & AMS (folates)

CURRENT STATUS OF CEN TC275/WG9 (Food)

- CEN Published Methods:
  - Vitamin A (Parts 1&2; LC-UV)
  - Vitamin E (LC-F)
  - Vitamin D_3 & D_2 (LC-UV)
- Methods under CEN Enquiry:
  - Vitamins B_1 & B_2 (LC-F)
  - Vitamin C (LC-UV)
  - Vitamin K_1 (LC-F)
  - Vitamin B_6 (MA & LC-F) (pre-standards)
  - Total Folate (MA) (pre-standard)

ON-GOING & FUTURE CEN WORK

- Method intercomparison study for vitamin B_6 using LC-F as PN
- Draft Standard Method for vitamin B_6 using above
- Draft Standard Method for folic acid by LC-UV (?)
- Draft Standard Method for carotenoids by LC-UV (?)

SUMMARY OF FLAIR RECOMMENDED STATUS METHODS (1995)

Vitamin	Recommended	Best Available	Promising
A		serum retinol	RDR test
Carotenoids		serum profile	
E	serum α-tocopherol		
D_3	25-OH-D_3		
B_1		ETK test	RBC TPP
B_2	EGR test		
B_6		plasma PLP	
B_{12}	serum cobalamins		s-MMA
Folate	RBC folate		plasma Hcy

FOLATE

- Serum/plasma folate
  - MA, LC-MA
  - RPBA (kits), IMx (chemilumescence ELISA)
  - HPLC (5-MeTHF)
  - GC-MS, LC-MS
- RBC Folate
  - MA (whole blood), LC-MA
  - RPBA (MTHFR genetic errors)
  - GC-MS, LC-MS, AMS

Vitamin B_6

- Plasma PLP
  - HPLC-F with cyanide derivatisation
  - Separates 4-PA
  - Bailey et al. (1999)
- Plasma Aminotransferase Activity
- Urinary excretion of 4-PA
- RBC PLP – better index?

Vitamin B_1 (thiamin)

- Whole Blood
  - ETK stimulation tests
  - Total thiamin by HPLC-F (as thiochrome)
  - RBC Thiamin pyrophosphate by HPLC-F
- Urinary thiamin excretion (HPLC)

Riboflavin (B_2)

- Whole Blood
  - EGR – widely used
  - B_2 levels of little value
- Urinary excretion – early NHANES
  - MA or HPLC-F

Vitamin B_{12}

- Serum/plasma
  - MA
  - RPBA (IMx)/RIA (^{57}Co- & ^{58}Co-B_{12})
- Urinary
  - MMA excretion
  - Schilling Test (RIA)
- RBC – limited value

Biotin

- Nutritional need ?
- MA or RIA (^{14}C- & ^{3}H-biotin)
- Mainly food/plasma

Vitamin C

- Serum/Plasma/Urinary AA
  - HPLC with UV, F or EC detection
- Leukocyte AA
  - Reliable index of tissue stores
- Body Pool Sizes
  - Isotope dilution (^{14}C-label)
  - Stable isotopes?

QA MATERIAL & PROFICIENCY SCHEMES

- BCR/NIST CRMs for vitamins in food
- NIST serum for fat-soluble vitamins
- WHO Whole blood folate standard
- Commercial QC samples
  - Bio-Rad serum/whole blood samples for folate & B_{12}
- Proficiency Schemes
  - NIST for C & fat-soluble vitamins
  - UK EQUAS for folate & B_{12}

Use of isotopes in human nutrition

Clive E. West, *Wageningen & Nijmegen Universities with*
Machteld van Lieshout: Wageningen University

Dewi Permaesih & Muhilal: *Nutrition Research & Development Center, Bogor, Indonesia*

Yan Wang, Xiaoying Xu & Richard van Breemen: *University of Illinois at Chicago*

Michiel Verhoeven, Alain Creemers & Johan Lugtenburg: *Leiden Institute of Chemistry*

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We study this because,

- vitamin A deficiency serious problem in developing countries;

- VAD can be prevented by adequate intake of vitamin A from pre-formed retinol or from provitamin A carotenoids;

- Provit A carotenoids, such as β-carotene, major source of vitamin A in diet of large proportion of world's population

Aims of studies

- Measuring bioefficacy
- Measuring nutrient concentrations (static measurements)
- Measuring nutrient status/body stores
- Measuring nutrient requirements (?)
- Meauring metabolic rates/energy expenditure
- Elucidating metabolic pathways

Definition of bioefficacy and its components

- **Bioavailability**: Fraction of ingested nutrient available for metabolic processes and storage
- **Bioconversion**: Fraction of bioavailable nutrient (absorbed provitamin A carotenoids) converted to active form of nutrient (retinol)
- **Bioefficacy**: Fraction of ingested nutrient (dietary provitamin A carotenoids) absorbed and converted to active form of nutrient (retinol)

Stable versus radioactive isotopes 1

Stable isotopes:

- Are more acceptable for human studies (dangers of radioactive isotopes are usually overstated)
- Generally have less isotope effects
- Their measurement is less sensitive
- Less suitable for tracing metabolic fates
- Generally unsuitable for analytical purposes

Stable versus radioactive isotopes 2

	Stable	Radioactive
Nutrient concentrations (radioimmunoassay)	--	++ (man/animals)
Bioefficacy	++ (man)	++ (animals)
Nutrient status/body stores	as for bioefficacy	
Nutrient requirements	as for bioefficacy	
Metabolic rates/energy expenditure	as for bioefficacy	
Body composition	as for bioefficacy	
Metabolic pathways	+/-	++

Choice of stable isotope for retinol/ carotenoid studies in humans

^{13}C-labelled compounds vs ^{2}H-labelled compounds:

- Less isotopic effect
- Less chance of migration

Intrinsic versus extrinsic labelling

Advantages of intrinsic labelling:

- Labelling in same metabolic pool

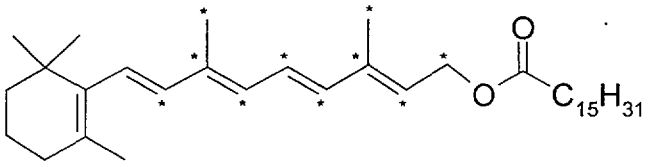
Advantages of extrinsic labelling:

- Labelling in specific positions
- High isotopic purity with no scrambling
- Enables examination of various matrices
- Enables conversion to other compounds to be more readily traced

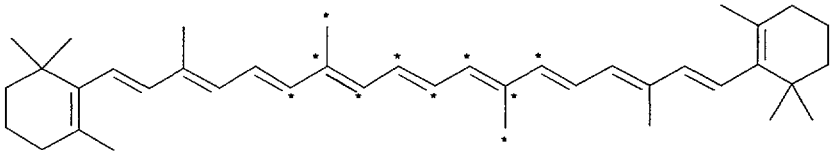
Specifically-labelled compounds

Food-grade; > 95% *all-trans* form

High isotopic incorporation (99%) and no scrambling



[$^{13}C_{10}$]Retinyl palmitate



[$^{13}C_{10}$]-β-Carotene

The capsules contained labelled β-carotene and retinol synthesized by Professor Lugtenburg and colleagues. This synthesized β-carotene and retinol, are each labelled with 10 Carbon-thirteen-atoms at predetermined positions. These compounds are dissolved in oil.

Retinol formed in the body will contain 5 ^{13}C-atoms.

In these figures the asterix indicate ^{13}C-atoms.

Need to use physiological doses

- Problem of lack of sensitivity of analytical methods
- Can be overcome by use of continuous/ periodic dosing instead of single doses, the two approaches
  - based on different assumptions
  - requiring different models
  - providing different information

Choice of analytical method for measuring isotopic enrichment

- NMR: lacks sensitivity
- Combustion followed by GLC-MS: time-consuming and loses information
- HPLC-thermospray MS: lacks sensitivity
- HPLC-electrospray MS: lacks dynamic range
- HPLC-APCI MS: gives good data

Reproducibility of APCI LC-MS measurements for retinol and β-carotene

Reproducibility of duplicate analyses (SD/mean):

- $M_{274,sR}$: 3.3% (n=163)
- $M_{279,sR}$: 4.2% (n=163)
- $M_{547,sC}$: 3.8% (n=147)

Van Breemen et al J Chrom A 1998;794:245-251
Wang et al Anal Chem 2000;72:4999-5003

The LC-MS-method has been developed by Dr van Breemen and colleagues from University of Illinois at Chicago. They developed an LC-MS method to determine the degree of isotopic enrichment of retinol and β-carotene in serum.

This method is based on separation of compounds by HPLC, after hexane extraction.

Then during atmospheric pressure chemical ionisation, β-carotene has base peaks with a mass of m or m+10.

For retinol, there are base peaks with a mass of m, m+10 and also m+5 which comes from the m+10 β-carotene.

Applicability to other vitamins

Radioactive isotopes used up until now for:

- Radioimmunoassay of vitamin B_{12}
- Metabolic/nutrition studies in animals and, to a limited extent, in humans

Stable isotopes used up until now for metabolic/nutrition studies in humans for:

- Vitamin A/β-carotene
- Folate
- Tocopherols
- Vitamin K

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Wageningen University
Biotechnion

Annex 3

Bioefficacy of β-carotene in oil, pumpkin and spinach consumed by children in Indonesia

Machteld van Lieshout, Clive West, Joseph Hautvast. *Division of Human Nutrition & Epidemiology, Wageningen University, The Netherlands*

Dewi Permaesih, Muhilal. *Nutrition Research & Development Center, Bogor, Indonesia*

Yan Wang, Xiaoying Xu, Richard van Breemen. *University of Illinois at Chicago, USA*

Michiel Verhoeven, Alain Creemers, Johan Lugtenburg. *Leiden Institute of Chemistry, The Netherlands*

WOTRO grant: WV 93-271

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Background

Amount of β-carotene required to form 1 µg retinol:

- Sheffield experiment, 1949 (n=2):
  - **3.3 µg** β-carotene in oil
  - **6.0 µg** β-carotene in fruits and vegetables
- De Pee et al, 1998 (Indonesia) (n=238) and Khan et al (Vietnam):
  - **26 µg** β-carotene in dark green leafy vegetables
  - **12 µg** β-carotene in orange fruit
- Tang et al, 1999 (n=41):
  - **27 µg** β-carotene in green and yellow vegetables

Djoko Suharno and Saskia de Pee showed:

women consumed twice the recommended daily allowance of vitamin A from carotenoids but were vitamin A deficient.

Subsequent intervention studies showed:

bioavailability of carotenoids and bioconversion to retinol is much lower than previously thought.

Sheffield Experiments (World War Two) in 2 subjects:3.3 µg β-carotene in oil required to form 1 µg retinol. 6 µg β-carotene in fruits and vegetables required to form 1 µg retinol.

De Pee and colleagues showed in a study in Indonesia: 26 µg β-carotene in dark green leafy vegetables required to form 1 µg retinol. And 12 µg β-carotene in orange fruits required to form 1 µg retinol.

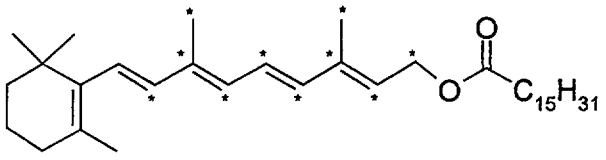
Tang and colleagues showed in a study in China: 27 µg β-carotene in green and yellow vegetables required to form 1 µg retinol.

We have developed a stable isotope method to study bioefficacy of β-carotene in different matrices.

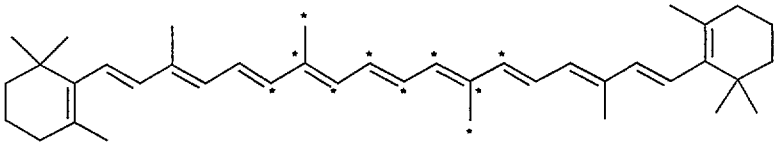
Specifically-labelled compounds

Food-grade; > 95% *all-trans* form

High isotopic incorporation (99%) and no scrambling



[$^{13}C_{10}$]-Retinyl palmitate



[$^{13}C_{10}$]-β-Carotene

Analysis

Measurements in serum:

- Retinol and carotenoid concentration by HPLC
- Isotopic enrichment of retinol and β-carotene by APCI LC-MS

Calculation of carotenoid bioefficacy

- Using *CarRet PIE* (β-***car***otene and ***ret***inol at ***P***lateau ***I***sotopic ***E***nrichment) model

APCI LC-MS analysis of retinol and β-carotene

Sample preparation: As for HPLC-analysis

Separation and detection of compounds:
HPLC coupled to **a**tmospheric **p**ressure **c**hemical **i**onization **m**ass **s**pectrometer

Advantages:
- Sufficient sensitivity, dynamic range and accuracy
- No saponification or pre-column derivatization required
- Isotopic enrichment of retinol in serum with both $^{13}C_5$-retinol and $^{13}C_{10}$-retinol
- Isotopic enrichment of β–carotene in serum with $^{13}C_{10}$-β-carotene

CarRet PIE model I: Intake of retinol and β-carotene

Description	Variable	Formula
Dietary retinol intake, µg	a	
Retinol intake capsules, µg	b	
Total retinol intake, µg	r	a + b
Dietary β-carotene intake, µg	d	
β-Carotene intake capsules, µg	e	
Total β-carotene intake, µg	c	d + e
Enrichment of dietary retinol with $^{13}C_{10}$-retinol, %	$E_{10,dR}$	b / r
Enrichment of dietary β-carotene with $^{13}C_{10}$-β-carotene, %	$E_{10,dC}$	e / c

CarRet PIE model II: Serum retinol and β-carotene

Description	Variable	Formula
Enrichment serum retinol with $^{13}C_{10}$-retinol, %*	$E_{10,sR}$	$M_{279,sR} / (M_{269,sR} + M_{274,sR} + M_{279,sR})$
Enrichment serum retinol with $^{13}C_5$-retinol, %*	$E_{5,sR}$	$M_{274,sR} / (M_{269,sR} + M_{274,sR} + M_{279,sR})$
Enrichment serum β-carotene with $^{13}C_{10}$-β-carotene, %*	$E_{10,sC}$	$M_{547,sC} / (M_{537,sC} + M_{547,sC})$
Proportion serum retinol from dietary retinol	$P_{sR/dR}$	$E_{10,sR} / E_{10,dR}$
Proportion serum retinol from dietary β-carotene	$P_{sR/dC}$	$E_{5,sR} / E_{10,dC}$
Proportion serum β-carotene from dietary β-carotene	$P_{sC/dC}$	$E_{10,sC} / E_{10,dC}$

* Corrected for average enrichment at baseline

Results of 'OIL' study (n=35, all sampled 3x)

- Plateau of isotopic enrichment of retinol and β-carotene in serum reached by Day 21
- 2.4 (95%-CI: 2.3;2.7) μg β-carotene dissolved in oil forms 1 μg retinol in the body
- Coefficient of intra-individual variation: 27%

Van Lieshout et al. Am J Clin Nutr 2000: in press

A plateau of isotopic enrichment was reached by Day 21, therefore, results are presented from data at this time point.

These data show that the relative provitamin A activity of β-carotene compared with that of dietary retinol is 36%, when not taking into account a lower absorption of cis-β-carotene than trans-β-carotene.

When taking into account a lower absorption of cis-β-carotene than trans-β-carotene the relative provitamin A activity of β-carotene compared with that of dietary retinol is 57%.

These data are independent of the absorption of β-carotene from other foods and also from the level of β-carotene intake from other foods.

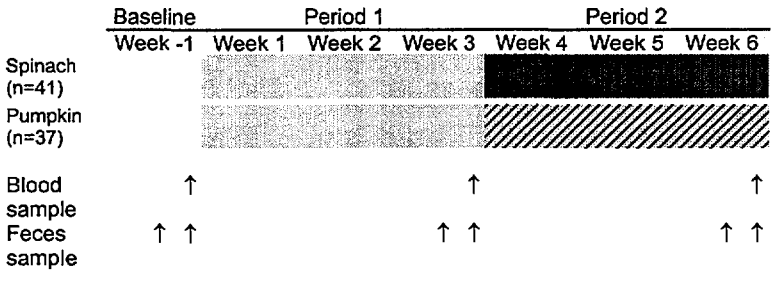
As the lower absorption of cis-β-carotene is well established the data result in a conversion factor of 1.7 ug β-carotene required to form 1 ug retinol in the body

Bioefficacy of β-carotene in oil, pumpkin and spinach consumed by children in Indonesia

Aim: To assess bioefficacy of β-carotene in:

- Oil
- Pumpkin
- Spinach

Study design



Both groups, period 1 + 2: 3 times per day:
- 21 μg $^{13}C_{10}$-retinyl palmitate; 31 μg $^{13}C_{10}$-β-carotene
- Low-retinol, low-carotene basic diet

Supplements: 2 portions per day
Yard long beans
Spinach
Pumpkin

Results of period 1: Bioefficacy of β-carotene in oil

Description	Variable	Total group (n=55)
Relative provitamin A activity of β-carotene in		

Conclusions: Amount of β-carotene in oil that forms 1 µg retinol

Sheffield experiment, 1949 (n=2):
- 3.3 µg β-carotene in oil (bioefficacy: 28.5%)

'OIL' study (n=35; all sampled 3x):
- 2.4 µg β-carotene in oil (bioefficacy: 41.0%)

Period 1 of 'Pumpkin/ Spinach' study (n=55):
- 2.6 µg β-carotene in oil (bioefficacy: 36.2%)

Thus, β-carotene dissolved in oil is **21%** more effective than previously thought

Preliminary conclusions of period 2

- Calculation of amount of β-carotene in pumpkin and spinach required to form 1 μg retinol requires further development of *CarRet Pie* mathematical model
- Ratio spinach/pumpkin (n=51): 2.15
  (will not change after further development of *CarRet PIE* model)
- De Pee et al, 1998 (n=238):
  - 26 μg β-carotene in dark green leafy vegetables (DGLV)
  - 12 μg β-carotene in orange fruit
  - Ratio DGLV/fruit: 2.16

Further information coming from second study

Comparison of bioavailability of β-carotene and lutein in pumpkin and spinach (after analysis of isotopic enrichment of β-carotene and retinol in feces)