VITAMIN ANALYSIS IN BODY FLUIDS AND FOODSTUFFS WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

accuracy and precision under routine conditions

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Stellingen

1. De precisie van een analysemethode ter bepaling van een (endogene) stof in een lichaamsvloeistof dient te worden vastgesteld voor routine-omstandigheden en voor gehalten die relevant zijn voor de diagnostiek.
   - Mancini G, Carbonara AO & Heremans JF (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry, p. 235-54, Pergamon Press, UK.
   - Speek AJ, Wongkham C, Limratana N, Saowakhonta S, Schreurs WHP (1986) Microdetermination of vitamin A in plasma using high-performance liquid chromatography with fluorescence detection. J Chromatogr 382: 284-9.

2. Een goede "recovery" van de toegevoegde te bepalen verbinding zegt niets omtrent de juistheid van de toegepaste analysemethode.
   - o.a. dit proefschrift

3. Bij veel gepubliceerde methoden voor de bepaling van vitamines in bloed wordt bij gebruik van een interne standaard ten onrechte geen aandacht geschonken aan het mogelijk introduceren van een systematische fout.
   - dit proefschrift.

4. Bij publicatie van onderzoek naar het verlies aan provitamine A-activiteit van groenten, dat optreedt als gevolg van de bereiding, dient de toegepaste bemonsteringsmethode te worden vermeld.
   - Sweeney JP, Marsh AC (1971) Effect of processingon provitamin A in vegetables. J Am Diet Ass 59: 238-43.
   - Ogunlesi AT, Lee CY (1979) Effect of thermal processing on the stereoisomerisation of major carotenoids and vitamin A value of carrots. Food Chem 4: 311-8.
   - Renqvist UH, De Vreeze AC, Evenhuis B (1978) The effect of traditional cooking on carotene content in tropical leafy vegetables. The Ind J Nutr Dietet 15: 154-8.

5. De door Pepping gepresenteerde methode om de juistheid van de door hem toegepaste HPLC-methode voor plasma-retinolbepalingen te testen komt neer op het vergelijken van twee bijna gelijke HPLC-methoden en zegt niets omtrent de juistheid van deze methoden.
   - F. Pepping (1988) Xerophthalmia and post-measles eye lesions in children in Tanzania. Proefschrift, Landbouwuniversiteit Wageningen.

6. De hantering van het begrip "recovery" ter karakterisering van ionenuitwisselende-chelaterende harsen dient te worden ontraden.
   - Beinrohr E, Rojcek J, Garai J (1988) Pre-concentration of trace metals from acidic sample solutions on a thin layer of activated carbon using dithizone as a chelating agent. Analyst 113: 1831-5.

7. De resultaten van het onderzoek van Long en Browner naar de invloed van water op de excitatiecondities van argonplasma stemmen overeen met de bevindingen van Maessen en medewerkers.
   - Long SE, Browner RF (1988) Influence of water on conditions in the inductively coupled argon plasma. Spectrochimica Acta **43B**: 1461.
   - Maessen FJMJ, Kreuning G, Balke J (1986) Experimental control of the solvent load of inductively coupled argon plasmas and effects of the chloroform plasma load on their analytical performance. Spectrochimica Acta **41B**: 3.

8. De door de leiding van wetenschappelijke instellingen op de onderzoekers uitgeoefende druk om regelmatig te publiceren, leidt vaak tot voortijdige publikatie en dientengevolge tot verspilling van wetenschappelijk potentieel.

9. Wetenschappelijk onderzoek verricht aan bevolkingsgroepen in ontwikkelingslanden wordt dikwijls ten onrechte als ontwikkelingshulp gepresenteerd.

10. Het bedrag dat Zwitserland jaarlijks voor ontwikkelingshulp beschikbaar stelt - relatief één van de hoogste bedragen binnen Europa - is een fractie van het bedrag dat dit land aan de ontwikkelingslanden onttrekt door het handhaven van het bankgeheim.

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accuracy and precision under routine conditions

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Chapter 1. GENERAL INTRODUCTION

The vitamins are a group of organic compounds with varying structures and properties. Their classification as a group is based on their common properties: they are all essential to life and well-being of man and animals, they are not or insufficiently produced by the body and they are minor constituents of food. Early in this century, investigations on diseases such as beriberi, scurvy and pellagra have led to the discovery of this class of compounds and to the "deficiency disease" concept.

An essential element of vitamin research is the development of assays. During the 1960s several manual vitamin assays were in use in our department (Department of Clinical Biochemistry, CIVO-TNO Toxicology and Nutrition Institute) for various vitamin research projects. These assays, applicable to certain body fluids and foodstuffs, were based on optical measurements or specific microbiological growth, mostly without or with little pre-purification, so that interferences by sample components were likely to occur. The throughput was low because the assays could not be automated. Furthermore, large day-to-day variations occurred which made the methods unfit for longitudinal studies. In the mid-1960s, the decision to analyse body fluids necessitated methods for the analysis of low vitamin concentrations in small samples with a complex composition. This led to the necessity of separating vitamins from sample components followed by sensitive and specific on-line detection. The impact of the then fairly developed technique of gas chromatography was strong. However, vitamins have no or a low vapour pressure and are heat-labile, and their detection by gas chromatography is far from specific. Therefore, and because of the obvious advent of more precise and sensitive instruments for column liquid chromatography, the separation method of choice became high-performance liquid chromatography (HPLC). The difference between HPLC and classical liquid chromatography is in the use of stationary phases with smaller particle sizes but with larger specific surfaces, which results in a higher resolution and the need to apply a relatively high pressure. Consequently, sophisticated instruments for solvent delivery and sample introduction have to be used.

Then, a start was made to develop reliable and sensitive semi-automated methods for the analysis of vitamins in a large variety of foodstuffs and body fluids. The aim was to achieve a better accuracy and precision and a

higher throughput. Some of these methods were newly developed and published in international journals, others were modifications of existing methods.

All these methods have been implemented in our laboratory for application in large-scale routine analysis of vitamins A, β-carotene, E, C, K_3, B_1, B_2 and B_6 in body fluids such as blood, plasma and urine, as well as in foodstuffs and beverages. Most of the methods concern vitamin analysis on behalf of nutrition research. In Chapter 3, "The HPLC methods for vitamin analysis", all methods discussed in this thesis are presented as abstracts. Section 3.1 lists the bibliographic descriptions and abstracts of our papers on HPLC vitamin analysis. Our non-published methods are given in detail in Appendices 1-6.

Newly developed methods tend to be hastily published. The analyst who adopts the methods in the laboratory may be faced with certain imperfections after some time: accuracy may be impaired by specific conditions of the patient or the sample which were not taken into account when the method was developed. Furthermore, the precision values given for newly published assays are those obtained by the designer of the method who is well trained and eager to present a high precision. Such precision figures should not be taken too literally when judging whether an assay is suitable to be used in specific studies, especially longitudinal ones. Therefore, in this thesis, the analytical quality of data, in terms of accuracy and precision, generated under routine conditions with the HPLC methods mentioned are considered, and discussed in relation to specific characteristics of the vitamin, matrix, method and equipment. In Chapter 4, "Reliability of the HPLC methods for vitamin analysis", the most frequently occurring systematic errors are discussed and the observed "large-scale routine" precision figures are given per assay and compared with literature data. Furthermore, the contribution of some steps in the methods to the imprecision is presented and discussed.

Generally, knowledge concerning the analyte and its matrix is useful for alert control on the reliability of data obtained with an analytical method. Therefore, in Chapter 2, information on the structure and properties of the relevant vitamins and their occurrence, form and level in body compartments and foodstuffs is given.

Chapter 2. THE VITAMINS: CHARACTERISTICS AND ANALYSIS

2.1 Introduction

Procedures for the analysis of vitamins are necessary to establish the vitamin status of humans or animals, to determine the potency of foods and feeds and to control the (long-term) quality of vitamin-containing pharmaceutical preparations.

The challenges in vitamin analysis are three-fold. First, the instability of vitamins implicates some limitations in sample preparation since oxidative degradation is catalysed by oxygen, heat, light, alkali and metal ions, and especially by combinations of these. The sample preparation procedure also should not change the form of the vitamin, i.e. cis-trans isomerization should be avoided. Second, since vitamins are only minor components of complex mixtures such as body fluids or foodstuffs, interferences may readily occur. They should be avoided. Third, most challenging, all positional isomers, which often differ in biological activity, should be distinguished. Furthermore, the methods should be precise, should be easy to operate, should have a high throughput and should not be too tedious and costly. Aqueous and fat-solvent extracts, proceeding from sample pre-treatment, should quantitatively contain all water- and fat-soluble vitamins, respectively. These extracts are the starting point of more or less multi-vitamin analysis by HPLC.

In the past decades many types of separation and/or detection techniques have been applied to vitamin analysis. However, since they all are afflicted with certain drawbacks, most of them have been superseded by HPLC with more or less specific detection. The following analytical methods have been applied for vitamin analysis before HPLC became the technique of choice.

2.1.1 Animal bioassays

Biological tests have a utility that cannot be replaced by specific chemical or physical methods. The physiological response of an animal species to a certain vitamin, derivative or analogue can be assessed by biological procedures only, because the complexities of uptake, absorption, transport and metabolism are integrated into a meaningful whole by this type of test only. Most commonly used is the growth-response test in vitamin-deficient test

animals. Although they can provide useful information, bioassays are too time-consuming and costly for large-scale routine analyses.

2.1.2 Microbiological assays

Many micro-organisms have been shown to require a specific vitamin for growth and reproduction. This finding has been applied in vitamin assays. The growth of the micro-organisms in a liquid extract of a foodstuff or blood sample can be measured and compared to a standard. The methods are based on measurement of turbidity in a colorimeter as a measure of the number of cells of the microorganism. Assays of this type take less time than animal bioassays and are simple, inexpensive and very sensitive. However, the drawbacks, viz. interference by antimicrobial substances such as preservatives and antibiotics and/or their metabolites, long analysis times, high variability, in general lack of differentiation between vitamers, and unsuitability for automation, have made them less popular than the currently applied liquid chromatographic methods. However, they still serve as methods of reference.

2.1.3 Physico-chemical methods

Physico-chemical methods for vitamin analysis are based on measurements of light intensity after little or no sample pre-purification. They are distinguished in spectrophotometric and fluorometric methods.

In spectrophotometry the absorption of light of the relevant wavelength is measured and compared with the extinction coefficient or with the absorption of an external standard. Fluorometric methods are based on the measurement of the intensity of light emitted by the analyte as a result of irradiation with light of higher energy, and comparison with an external standard.

Common critical points with these optical methods are the inpossibility to differentiate between vitamers and the insufficient selectivity, i.e. the lack of a reliable blank. A blank is obtained by decomposition of the vitamin by agents or UV radiation. Interferences may occur when reactions with (unknown) sample components yield compounds that interfere with the optical measurement. A popular method to obtain a reliable blank has been removal of the vitamin by its enzymatic conversion, since enzyme action is generally specific. The selectivity of spectrophotometric assays has been improved by

derivatization or conversion of the analyte by a more or less specific chemical reaction and spectrophotometric measurement of the resulting compound. Fluorometric assays are generally more selective than methods based on measurement of light absorption since only a few compounds exhibit fluorescence, and since such a compound only interferes if both its excitation and emission wavelengths approximate those of the vitamin. Especially methods based on fluorescence of a compound resulting from derivatization or conversion of the vitamin by chemical reaction have been quite popular because of their relatively high selectivity. However, quenching of fluorescence may yield negative aberrant readings, especially when sample dilution is not permitted due to lack of sensitivity.

Another critical point is the small difference between sample and blank value as compared to these values which negatively influences precision.

2.1.4 Chromatographic procedures

The drawbacks mentioned above can be largely avoided by physically separating the analyte and the matrix components. Available techniques are thin-layer chromatography (TLC), paper chromatography (PC), gas chromatography (GC) and column liquid chromatography (LC).

TLC, a convenient and cost-effective technique, is applied mostly for the analysis of vitamin isomers, metabolites and degradation products. A general problem is the standardization of conditions such as temperature, vapour saturation, nitrogen atmosphere and protection from light. Several factors, especially the direct contact of the vitamin on the plate to air, obviously inhibit quantitative analyses. Pre-treatment of the plates by spraying with an antioxidant-containing solution and the use of reversed-phase plates have been applied to avoid degradation, but results were not satisfactory. However, TLC is still the method of choice for a check of radiochemical purity (McKenzie et al. 1977).

Although GC is a powerful separation technique it is rarely applied to vitamin analyses because these compounds have a low heat stability, a high molecular weight and a low vapour pressure. The consequently required high column temperature leads to long analysis times and may cause degradation. These drawbacks can be eliminated by derivatization. However, such procedures are laborious and may become a source of error. Furthermore, unlike in LC,

the usual GC detectors (such as the flame ionization detector) do not contribute to the selectivity of the method so that (multi-step) sample pre-purification is necessary. GC methods for the determination of fat-soluble vitamins have been reviewed by Sheppard et al. (1972), and many GC methods for the analysis of vitamins by Nelis et al. (1985).

The quoted disadvantages of TLC and GC can be largely overcome by separating the analyte and the matrix components by column LC, especially when more or less selective detection is required. Advantages are that the vitamins are dissolved during the entire procedure, and that these methods are quantitative, can be automated, are non-destructive and are carried out at room temperature. This implies that column LC eliminates the risk of thermodegradation, protects the vitamins against conversion and against influence of light during chromatography. Classical column LC (gravity fed) is characterized by a low efficiency, a poor sensitivity and long analysis times because of using large-particle stationary phases. The introduction of microparticulate (3 - 10 µm) stationary phases has led to the development of HPLC which is characterized by a considerably higher efficiency, resolution, speed and sensitivity, while retaining the advantages mentioned.

In the following section characteristics of the vitamins of importance to the analyst are discussed, i.e. definition, structure, properties, and occurrence, form and level in body compartments and foodstuffs. Since for diagnostic purposes vitamins are determined in body fluids, the concentrations and borderline values ($P_{2.5}$ and $P_{97.5}$) in body fluids as determined by HPLC are summarized in Table 1.

Table 1. Mean, median, range, and borderline values of vitamin concentrations in body fluids of normal Dutch adults as determined by HPLC.

Vitamin	Fluid	Unit	Mean	Median	Range	$P_{2.5}$ - $P_{97.5}$	n
A	plasma	µmol/l	2.1	2.0	0.5 - 3.8	0.9 - 3.5	162
Carotenoids	plasma	µmol/l	2.2	1.8	0.9 - 4.5	1.0 - 4.1	162
E	plasma	µmol/l	28.6	27.4	17.4 - 89.8	18.0 - 43.2	161
C	blood	µmol/l	39	41	3 - 86	6 - 72	154
B_1	blood	nmol/l	131	129	72 - 190	98 - 177	154
B_2	blood	nmol/l	327	320	240 - 440	240 - 440	162
B_6	blood	nmol/l	67	65	20 - 122	38 - 101	162

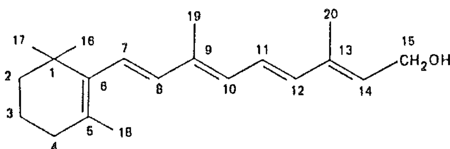
Total carotenoids has been determined by spectrophotometry.

2.2 Vitamin A

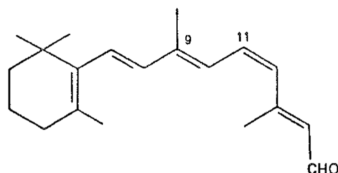
2.2.1 Definition and structure

The name vitamin A is used for all β-ionone derivatives or structurally closely related compounds other than provitamin A carotenoids. Essential for vitamin A-activity is the β-ionone ring. The all-trans form of retinol, a fat-soluble long-chain alcohol of which seven isomeric forms are known, is the representative of the vitamin A group with the highest biological activity (Fig. 1). The characteristics of a large number of vitamin A-related compounds have extensively been described by Hejno (1964) and Schwieter & Isler (1967).

Since in the past the activity of the various vitamin A-active compounds were not yet elucidated, the international unit (IU) was used for quantitation. One IU is equivalent to the activity of 0.30 μg all-trans retinol, of 0.344 μg all-trans retinyl acetate or of 0.55 μg all-trans retinyl palmitate (Roels 1967). Vitamin A concentrations should now preferably be expressed as all-trans retinol in μmol/L body fluid or mg/kg foodstuffs (Int. Union Nutr. Sci. 1978).



retinol: $C_{20}H_{30}O$



11-cis-retinaldehyde: $C_{20}H_{28}O$

Fig. 1. Formulae of all-trans retinol (M = 286.46) and 11-cis retinaldehyde (M = 284.46). Carbons 1 to 6 form the β-ionone ring essential to biological activity.

2.2.2 Properties

Retinol and its esters and analogues are soluble in most organic solvents but not in aqueous solutions. In concentrated solutions they are light yellow to red. The UV absorption of retinol in organic solvents has its maximum in the range 325 - 335 nm. All compounds display natural fluorescence (333-470 nm).

Relevant physical properties of the main representatives of the vitamin A group are given by Hejno (1964) and Olson (1984).

Retinol and, to a lesser extent, its esters are labile when exposed to heat, acid, oxygen, light, and especially to combinations of these factors. The pure vitamins and their solutions in organic solvents are stable for periods of up to several months when stored in a dark and cool place, preferably under a nitrogen atmosphere.

For nutritional and medical use beadlets are produced by coating *all-trans* retinyl esters in the presence of antioxidants with a gelatin-carbohydrate mixture. About 90% of the vitamin A-activity of animal feed or human foodstuffs enriched with these beadlets is retained for at least 6 months if the material is stored under exclusion of too much humidity, oxygen or heat.

2.2.3 Natural sources and occurrence in the human body

Vitamin A, as long-chain fatty acid *all-trans* retinyl esters, is only found in animal tissues. Common dietary sources are dairy products, internal organs such as liver, heart and kidney, and many species of fish such as tuna, sardine and herring. The retinol contents of some important sources are given by Marks (1975). However, only part of the daily supply of retinol comes from animal sources. Plants, especially green leafy vegetables, produce large amounts of provitamin A carotenoids (Isler et al. 1965). Another large proportion of the requirement of vitamin A is met by the conversion in the human body of certain carotenoids into vitamin A (Kläui & Bauernfeind 1981).

Consumed retinyl esters are hydrolysed, absorbed and finally stored in fat-storing cells of the liver as esters of long-chain fatty acids. Under normal conditions, over 90 % of the vitamin A in the human body is stored in the liver. Palmitate ester is the predominant form, and only small amounts of stearate and oleate esters are found (Lindner et al. 1971). Concentrations in the liver can be high, up to 500 mg retinol per kg, but a level of 200 - 300 mg/kg is considered as normal. The liver releases the vitamin via a highly regulated process to the plasma and peripheral tissue. In plasma vitamin A is present as retinol bound to an equimolar complex of a specific retinol-binding protein (RBP) and the tetrameric form of transthyretin (prealbumin). Plasma levels in the range 0.5 - 3.8 μmol/L are considered as normal. In the case of reduced intake, the store in the liver decreases whereas the plasma

level remains normal. When the liver becomes depleted the plasma level drops sharply.

2.2.4 Analytical procedures

The methods used since some decades for the determination of vitamin A contents of body fluids and foodstuffs are bioassays, light absorption methods without and with derivatization reaction, and fluorometric and chromatographic methods. Due to the general drawbacks mentioned in Section 2.1 only methods based on liquid chromatographic separation are currently in use. Some representative HPLC methods for the analysis of vitamin A in body fluids and foodstuffs are discussed below.

Plasma and serum analysis. Currently, HPLC methods have been described for assays of all-trans retinol in plasma or serum samples using UV detection (Nierenberg 1984, Catignani & Bieri 1983, Nelis et al. 1983, Howells et al. 1983, Driskell 1982) or fluorescence detection (Collins & Chow 1984, Mansourian et al. 1982). These methods generally require at least 100 μL of plasma which makes them impractical for field surveys when, for each subject, only a few capillaries filled with whole blood are available for several assays. In addition, many methods require evaporation of the extraction solvent since the extraction and HPLC solvents are not compatible. This step may cause analyte losses and, moreover, makes large-scale routine analysis time-consuming.

Therefore, a micromethod for all-trans retinol assays in 5 μL plasma or serum without the above drawbacks has been developed (Speek et al. 1986a, abstract in Section 3.2.1).

Tear fluid analysis. During the past decade interest in the composition of human tear fluid has grown. Tear fluid is present as the corneal film and in the conjunctival sac. It mainly serves to protect the eye from exogenous antigenic substances and to maintain the structural integrity of the cornea and conjunctival sac. Its composition is complicated (Van Haeringen 1981) and shows much interspecies variation. In patients suffering from severe xerophthalmia (resulting from prolonged vitamin A deficiency) deviations in the corneal and conjunctival mucus layer (Sprague 1978) and decreased tear production (Sommers & Emran 1982) have been reported.

Recently, all-trans retinol was found to be present in human and rabbit tear fluid (Ubels & MacRae 1984). Human tear fluid levels were found in the range <0.4 - 10.6 µg/L by Speek et al. (1986b). It is not yet clear whether retinol analysis in tear fluid may be useful in clinical eye research. Since the HPLC method described by Ubels & MacRae lacks sensitivity and speed, a faster and more sensitive method for retinol assays in human tear fluid has been proposed (Speek et al. 1986b, abstract in Section 3.2.2).

Foodstuff analysis. The HPLC methods applied are methods to determine simultaneously vitamin A derivatives (reviewed by Lambert et al. 1985a) and to determine vitamin A potency as total all-trans retinol content. Spectrophotometric, fluorometric, gravity-fed liquid chromatographic and HPLC methods have been reviewed extensively by Parrish (1977).

Since retinyl esters have the same molar biological activity as retinol, food samples are often saponified to determine the vitamin A potency as total (all-trans) retinol concentration. Saponification, which is actually an alkaline digestion, also serves to free the vitamin from the stabilizing matrix, from lipids and from co-extractable compounds that might interfere with the assay, whereas isomerization is not likely to occur.

The HPLC method given in Appendix 1 (abstract in Section 3.2.3) includes an alkaline saponification procedure. Precautions against oxidative degradation of vitamins are taken during saponification, extraction and chromatography by adding an antioxidant. Metal ions, which may catalyse decomposition of vitamins during saponification, are neutralized by adding sodium sulphide. The extract in diisopropyl ether resulting from the pre-purification step prior to HPLC can be used for HPLC analysis of all fat-soluble vitamins.

2.3 Provitamin A carotenoids

2.3.1 Definition and structure

Provitamin A carotenoids are a class of aliphatic or aliphatic-alicyclic compounds composed of eight isoprene groups, joined such that the arrangement of the isoprenoid units is reversed in the centre of the molecule. This implies that the two central methyl groups are in the 1,6 position and the other non-terminal methyl groups in the 1,5 position (IUPAC-CNOC, CBN 1972). A series

of conjugated carbon-carbon double bonds forms the chromophoric system (Karrer & Zucker 1950). They can be divided into hydrocarbons (carotenes) and their oxygenated derivatives (oxycarotenoids or xanthophylls), β-carotene ($C_{40}H_{56}$; M = 536.89) being the representative with the highest biological vitamin A-activity (Fig. 2).

The structural formulae and the relative provitamin A-activity of various carotenoids are given by Bauernfeind (1972).

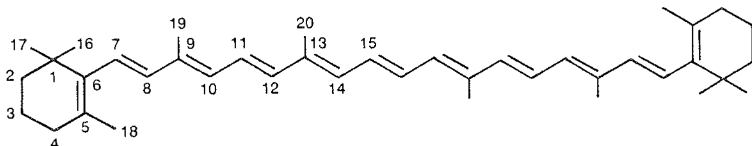


Fig. 2. Structure of β-carotene. Carbon atoms 1-15 form the retinyl structure essential to vitamin A-activity.

2.3.2 Properties

Provitamin A carotenoids are yellow to red compounds soluble in almost all organic solvents, barely soluble in oils and insoluble in aqueous solutions. They absorb light in the yellow region but do not fluoresce. The absorption maxima, ranging from 430 to 455 nm, and the molar absorption coefficients of several representative carotenoids are given by De Ritter & Purcell (1981).

The hydrocarbon carotenes and, to a lesser extent, the oxycarotenoids are labile when exposed to oxygen, direct sunlight or high temperature, and especially to combinations of these factors (Simpson et al. 1976, Simpson & Chicester 1981). Exposure possibly results in oxidative degradation and cis-trans isomerization.

Carotenoids added to foods, pharmaceuticals and food and feed products are oil- and water-dispersible liquid products or dry products with or without antioxidants. The stability after being mixed with foodstuffs is satisfactory. Relevant aspects of the use of carotenoids as food and feed additives are described by Kläui & Bauernfeind (1981).

2.3.3 Natural sources and occurrence in the human body

Carotenoids are synthesized de novo in higher plants and some microorganisms. They occur in the green chloroplasts present in the parenchyma of the leaves and hence contribute considerably to pigmentation. Nature has been estimated to produce about 100 million tonnes of this pigment per year (Isler et al. 1965). Major dietary sources of carotenoids are plants, butter and milk. An extensive list of carotenoids levels in natural products have been given by Bauernfeind (1981).

The main contribution of provitamin A carotenoids to nutrition, especially in developing countries, is the in vivo conversion of some carotenoids into all-trans retinol (Goodman 1969, Simpson 1983). The intake of carotenoids and the conversion into retinol have to supplement the intake of preformed all-trans retinol up to its recommended daily allowance (RDA, 750 µg for normal adults).

Relatively little is known about the metabolism of carotenoids. Dietary carotenoids are partly and at least in man rather unselectively absorbed and stored in most organs and tissues, secreted unchanged in the faeces, and some are converted into retinol. They are found especially in liver, blood, fat tissue and breast milk (Goodman 1969). Normal and borderline plasma levels are listed in Table 1. However, these levels should not be interpreted without taking relationships between plasma values of carotenoids and retinol into account (WHO 1982).

2.3.4 Analytical procedures

Plasma and serum analysis. Although there is still controversy as to the relevance of plasma carotenoid levels as an indicator of vitamin A status (Le Francois et al. 1981, Olson 1981), β-carotene and, to a lesser extent, other provitamin A corotenoids are now routinely determined in human plasma as part of nutritional assessment programmes.

The carotenoid pattern of human plasma is very complex. The predominant carotenoids are β- and α-carotene, β-cryptoxanthin, lycopene and lutein. In recent publications on HPLC for serum carotenoid determination (Broich et al. 1983, Peng et al. 1983, Driskell et al. 1983) most attention has been focused on β- and α-carotene and lycopene. In all methods a deproteinization step is included, viz. addition of (m)ethanol followed by organic extraction of the

carotenoids. Either an aliquot of the organic layer is directly injected onto the HPLC column (Broich et al. 1983, Peng et al. 1983), or the organic layer is evaporated to dryness and the residue redissolved in a solvent compatible with the HPLC solvent (Driskell et al. 1983). Driskell et al. applied an internal standard, such as the synthetic derivative dimethyl-β-carotene. Although all methods are thoroughly checked for good recovery of carotenoids so that no internal standard is needed, the internal standard procedure using dimethyl-β-carotene includes a check for incidental errors during routine analysis.

Detailed serum carotenoid analysis provides relevant information on the provitamin A nutritional status, but is too complicated and too time-consuming for epidemiological studies. The combined determination of total carotenoids (expressed as β-carotene) and specific β-carotene gives all the information needed. Therefore, we propose the method described in Appendix 2 (abstract in Section 3.3.1).

Food analysis. The methods for the determination of carotenoids in foods and feeds used since some decades can be classified into methods to determine the biological activity of certain carotenoids (Gridgeman 1945), methods to determine the composition of (synthetic) mixtures, methods on behalf of carotenogenesis studies, and methods to determine provitamin A-activity. There are only few basic methods available, but modifications adapting analytical methods to facilities present and to individual preference are manifold.

To reveal the composition of complex natural or synthetic mixtures and to study carotenogenesis, many available separation and identification methods are required. A study on the HPLC analysis of many carotenoids from plant, animal, or synthetic origin has been published by Langer (1976). Such methods for complete analysis consist of extraction, gross purification by saponification, separation into classes according to the number of hydroxyl groups, isolation by chromatography and, finally, identification and quantification by measurement of the absorption in the yellow region. Depending on the nature of the sample under investigation one or more steps may be omitted. Many approved methods for detailed carotenoid analysis are reviewed by De Ritter & Purcell (1981) who present a schedule from which, for each type of sample, a method for detailed carotenoid analysis can be selected from literature. Due to the complexity of the analytical problem these methods are rather laborious.

From the viewpoint of nutritional value detailed determination of all carotenoids is useless since there are many uncertainties in the conversion factor. Determination of total carotenoids (expressed as β-carotene) and of specific β-carotene yields sufficiently informative figures. The provitamin A-activity as <u>all-trans</u> retinol equivalents (RE) is calculated using the conversion factors given by the WHO (1982): 1/12 × (total carotenoids minus β-carotene) + 1/6 × β-carotene.

In the generally accepted spectroscopic method as proposed by the Association of Official Analytical Chemists (AOAC 1980) the carotenes are separated from the oxygenated compounds by using gravity-fed chromatography columns, whereafter the fractions are measured by visible spectroscopy and the WHO equation mentioned is applied. However, the individual carotenes and the carotenoid esters are not separated. This may lead to overestimation of the provitamin A-activity, especially when the β-carotene represents only a small fraction of total carotenoids. Many data obtained with this method have been published in the USDA Agriculture Handbook No. 456 (Adams 1975), and Food Composition Tables for East Asia and Africa (Leung et al. 1972 and 1968, respectively). Overestimation has been reported by Gebhardt et al. (1977) for clingstone peaches, by Zakaria et al. (1979) for tomatoes, by Pepping et al. (1988) for East African vegetables and by Speek et al. (1988) for commonly eaten Thai vegetables and fruits. The first two authors conducted more or less detailed carotenoid analyses, whereas the latter two determined only total carotenoids, and α- and β-carotene. Simpson and Chicester (1981) reviewed many HPLC methods for detailed carotenoid analysis. Most methods present a more reliable estimate of the provitamin A value than does the AOAC method, but some of them are rather laborious.

In our laboratory β-carotene and carotenoids are determined to establish both the vitamin A-activity of foodstuffs and the plasma carotenoid status of people. For an easy-to-handle and relatively fast HPLC method it is important that β-carotene is not only separated from the many less active carotenoids but also from the less active and most frequently occurring α- and γ-carotene. Cis-trans isomerization should be avoided. The method described in Appendix 1 (abstract in Section 3.2.3), is proposed for the analysis of total carotenoids and β-carotene in foodstuffs and subsequent calculation of the provitamin A-activity. Although the β-carotene containing extract cannot be directly injected on the C18 column, such a column has been chosen for reasons mentioned in Section 3.1.

2.4 Vitamin E

2.4.1 Definition and structure

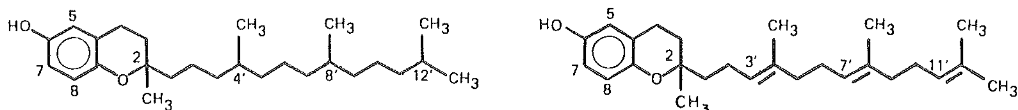
The term vitamin E is be used as the generic description for all tocol and tocotrienol derivatives exhibiting qualitatively the biological activity of α-tocopherol (IUPAC-IUB 1974). The tocol and tocotrienol structures are shown in Fig. 3. Both have a 6-chromanol ring structure and an aliphatic side chain. The tocols have a phytil side chain; the tocotrienols have a similar chain, but with double bonds at the 3′, 7′, and 11′ positions. Both occur as a variety of isomers with different biological activity. These differ by the number and location of methyl groups on the chromanol ring. The epimeric configuration at the 2 position is apparently dominant in determining biological activity. Since there are three asymmetric carbon atoms, at positions 2, 4′ and 8′, there are eight possible optical isomers.

The isomer with the highest biological activity is the only naturally occurring stereoisomer of α-tocopherol, RRR-α-tocopherol or [d]-α-tocopherol, which henceforth will be referred to as α-tocopherol, $C_{29}H_{50}O_2$, M = 430.7. The synthetic vitamin E is all-rac-α-tocopherol (IUPAC nomenclature), formerly designated as [dl]-α-tocopherol, and is used as a standard in analytical procedures. One IU is equivalent to 1 mg all-rac-α-tocopheryl acetate. Vitamin E-activity in food is calculated from the activities of the individual isomers and is expressed as (RRR-)α-tocopherol equivalents (TE). One TE is 1 mg (RRR-)α-tocopherol.

2.4.2 Properties

Tocopherols and tocotrienols are insoluble in water, but they are almost completely soluble in oils, fat and fat solvents. Their UV absorption spectra show maxima between 290 and 298 nm. Furthermore, they display a strong natural fluorescence (296-320 nm).

Tocopherols are stable with regard to heat and alkali in the absence of oxygen and are not affected in acid solution up to 100 °C. However, they are readily oxidized by atmospheric oxygen, which process is accelerated by exposure to light and heat and by the presence of iron and copper ions. The esterified forms are more stable. Since the acetate form shows similar biological activity, tocopherols are usually provided commercially in this form.



tocol structure   trienol structure

Fig. 3. The tocol and tocotrienol structures and the relative biological activity of their derivatives (McLaughlin & Weihrauch 1979).

Position of methyls	Tocol structure	Relative biol. act.	Trienol structure	Relative biol. act.
5, 7, 8	α-tocopherol	100 %	α-tocotrienol	30 %
5, 8	β-tocopherol	25-40 %	β-tocotrienol	5 %
7, 8	γ-tocopherol	1-10 %	γ-tocotrienol	-
8	δ-tocopherol	1 %	δ-tocotrienol	-

2.4.3 Natural sources and occurrence in the human body

All eight RRR isomers of the tocol and tocotrienol series are widely distributed in nature. Vegetable oils, nuts and the embryos of many seeds are rich sources. Seed oils contain the highest levels, up to about 1 g/kg. In lower concentrations, they are also found in plants, milk, milk products and egg yolk. The contents of E vitamers for vegetable oils are given by Bauernfeind (1980) and Speek et al. (1985), and for foods of animal and plant origin by Bauernfeind (1980).

The concentrations of the various E vitamers can vary considerably per source. For seed oils the concentration ranges are characteristic of the type of oil (Speek et al. 1985). Furthermore, for many foods reported α-tocopherol values diverge by a factor of up to 5. Seasonal variations can also be large. Due to these ranges and because natural tocopherols are not very stable, precise values of vitamin E intake cannot be calculated from food composition tables, but should proceed from analysis of the foods prior to consumption.

After absorption of dietary vitamin E, tocopherols circulate in the lymph and blood bound to lipoproteins. They are distributed according to the fat composition of each fraction (Gallo-Torres 1980). Tocopherols are also transported in erythrocytes where they appear to be located primarily in the cell membrane. The concentration is about 20 % of that in plasma (Kayden et al.

1973). A relatively rapid exchange between erythrocytes and plasma takes place. After transport vitamin E is deposited in tissues largely unmodified and in its unesterified form. The content of α-tocopherol deposited in human tissue varies from 7 mg/kg for kidney to 150 mg/kg for adipose tissue. Normal human α-tocopherol tissue contents are given by Farrell (1980).

Normal plasma α-tocopherol levels of adults including the borderline values are given in Table 1. The α-tocopherol level is 10 - 100 times the levels of β- and γ-tocopherol. δ-Tocopherol and tocotrienols are usually not present in human plasma. Children (2 - 6 years), neonates and premature infants have lower values, i.e. 9 - 16, 4 - 9, and 4 μmol/L respectively.

2.4.4 Analytical procedures

Due to the drawbacks of methods without physical separation HPLC is the technique of choice for vitamin E analysis. Some representative methods are discussed below.

Plasma and serum analysis. HPLC methods for plasma E vitamers analysis can be divided into straight-phase (SP) and reversed-phase (RP) methods, into methods determining only α-tocopherol and methods determining α- as well as β- and γ-tocopherol, into methods with and without the use of an internal standard and into methods with absorption and with fluorescence measurement as detection. Sample sizes vary from 50 to 500 μL, and analysis times from 4 to 40 min. All methods include a protein precipitation step by addition of (m)ethanol and extraction into an organic solvent followed by HPLC.

Nelis et al. (1985) has mentioned some advantages of RP systems over SP systems, i.e. column stability, better reproducibility of retention times, and better peak shape. However, a strong disadvantage is the incompatibility of the extracting solvent (n-hexane) with the mobile phases usually applied in RP systems. The evaporation and redissolution step needed makes large-scale routine analyses cumbersome. Since n-hexane can be directly injected onto SP columns and since we have observed excellent stability of these systems in our laboratory, we prefer the use of SP-HPLC systems for E vitamer analysis. Furthermore, unlike RP-HPLC, SP-HPLC allows a fast and easy differentiation of positional isomers which is in agreement with the well-known stereochemical selectivity of silicic acid. Van Niekerk (1973) was the first

to report the complete separation of α-, β- and γ-tocopherol on silica columns.

HPLC methods with tocol as internal standard have been described by Abe & Katsui (1975) and by De Leenheer et al. (1978, 1979). Both methods differentiate between the three tocopherol isomers present in plasma. However, if recovery of the analyte(s) is reproducible and close to 100 %, the use of an internal standard in HPLC is only useful to detect incidental errors, for instance in sample handling.

UV detection at the optimum wavelength of 292 nm and fluorescence detection at the wavelength pairs 292-320 or 205-320 nm ensure detectability of subnormal α-tocopherol human plasma levels. However, fluorescence detection is superior to UV detection with respect to sensitivity and selectivity (McMurray & Blanchflower 1979). If attention is also paid to the minor amounts of (less active) β- and γ-tocopherol, fluorescence detection is a necessity. Sensitivity can be enhanced further by excitation of the compounds at 205 nm instead of at > 292 nm (Tangney et al. 1981) which may reduce sample size to 50 μL (Hatam & Kayden 1979).

On the basis of the above characteristics of HPLC and detection systems the method presented in Appendix 3 (abstract in Section 3.4.1) is proposed for the simultaneous determination of α-, β- and γ-tocopherol in human plasma or serum.

<u>Food analysis</u>. The most common E vitamers in foods are the four tocopherols and α-tocotrienol. Many papers on the HPLC analysis of E vitamers have been published. SP-HPLC with fluorescence detection has superseded previously used GC, TLC, chemical methods, and combinations of these. The vitamin E pattern of edible oils, a rich source, can be analysed very easily. The pioneer paper by Van Niekerk (1973), describing the analysis of E vitamers in vegetable oils without pre-purification, is the current basis of analysis. Only dilution of the oils with n-hexane followed by chromatography on a SP column and fluorometric detection permits fast and reliable quantitation of many E vitamers. Many other methods (Fukuba et al. 1979, Tangney et al. 1981, Speek et al. 1985) are based on this method. However, RP systems have been used as well, particularly for the quantitation of α-tocopheryl acetate (Shaikh et al. 1977, Eriksen 1980), for the simultaneous determination of retinol and α-tocopherol (Söderhelm & Andersson 1978) and for simultaneous fat-soluble vitamin assays (Barnett et al. 1980).

More sample clean-up is usually required for analysis in foods and feeds. McMurray et al. (1980) comparing several tocopherol isolation techniques found lipid organic extraction followed by saponification the most effective approach. In view of laboratory efficiency the saponification and extraction procedure as used to isolate vitamin A and carotenoids is also used to isolate E vitamers.

Our method for the simultaneous determination of α-, β-, γ- and δ-tocopherol in foods, feeds, tissues and pharmaceutical preparations, based on the above principles, is described in Appendix 1 (abstract in Section 3.2.3). For the analysis of E vitamers in seed oils the saponification and extraction step, being the sample pre-treatment of the above method, can be omitted. Dilution of the oils with n-hexane permits direct injection and analysis (Speek et al. 1985, abstract in Section 3.4.3).

2.5 Vitamin C

2.5.1 Definition and structure

The term vitamin C is used as the generic descriptor for all compounds exhibiting the biological activity of L-ascorbic acid (AA) (IUNS/AIN 1977, Fig. 4). Strictly speaking, this compound, a carbohydrate derivative, is not a carboxylic acid but a lactone. It owes its acidic properties to the presence of the conjugated enediol system which causes the H atom at the O-3 position to become highly proteolytic. An important member of the vitamin C family is dehydro-L-ascorbic acid (dHAA), the dehydrogenation product of AA (Fig. 4). Both compounds, which have the same biological activity with respect to the prevention or cure of scurvy, form a redox system in the human body. The epimer D-ascorbic acid (Fig. 4), also known as erythorbic acid (EA) or iso-vitamin C, has little or no biological activity (Yourga et al. 1944, Ikeuchi 1955).

One IU Vitamin C is 50 μg AA. Concentrations of vitamin C are expressed as concentrations of AA.

2.5.2 Properties

AA is soluble in water (up to 33 %, w/v), methanol and glycerol, and is insoluble in ether, chloroform, oils, fats and fat solvents. Maximum UV absorption in aqueous solutions is at 245 nm at pH 2 and at 265 nm at pH 6.4. The compound has no fluorescent properties. The redox system AA - dHAA is the most important chemical property of vitamin C and forms the basis for analysis with electrochemical detection and for its physiological properties and stability. Dry crystals of AA are rapidly oxidized when exposed to air and light, which reaction is accelerated by alkali and copper and iron ions and inhibited by reductants such as homocysteine and some other thiols. AA in solutions of metaphosphoric acid containing ligands such as EDTA is fairly stable. Physico-chemical properties have been listed extensively by Jaffe (1984).

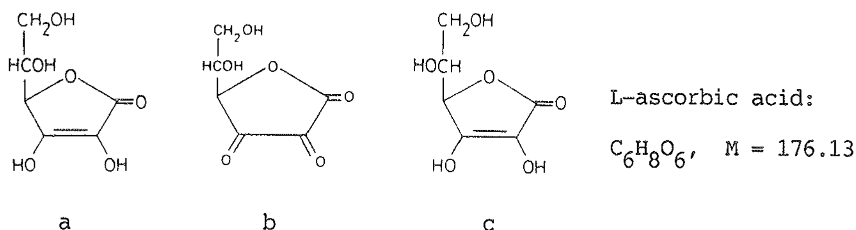


Fig. 4. Structural formulae of L-ascorbic acid (a), dehydro-L-ascorbic acid (b) and erythorbic acid (c).

2.5.3 Natural sources and occurrence in the human body

AA is widely distributed in animals and plants, probably in equilibrium with dHAA. It occurs in high concentrations in vegetables, potatoes and fruits, in animal organs such as liver and kidney, and, to a considerably lesser extent, in meat. The vitamin C content not only varies between foodstuff categories, but also within categories, depending on species, degree of ripeness, origin, storage conditions, and handling before analysis. A comprehensive compilation of concentrations in raw and processed foods has been published by Walt & Merril (1963).

Crystalline AA, protected against oxidation by micro-encapsulation in silicon resin, ethyl cellulose or stearyl alcohol, is commercially available

for the manufacture of tablets. The use of EA as an antioxidant in foodstuffs is prohibited in the Netherlands under the Dutch Food Law.

AA is readily absorbed from the gastro-intestinal tract. Unlike most of the water-soluble vitamins, it appears that limited stores of AA are held in the body. There is no evidence so far of the existence of a specific carrier. For normal health maintenance ascorbate in humans should exist as a pool of 1500 mg distributed through the body with specific tissues having high concentrations (Knox & Goswami 1961, Evans et al. 1982). The clinical signs of vitamin C deficiency (scurvy) are accompanied by reduction of the body ascorbate pool. Lowering of the pool to 600 mg due to low intake triggers physiological changes. Below 300 mg clinical signs of scurvy appear. These changes are reflected by plasma levels (see also Table 1) which fall from the normal level of 15 - 86 μmol/L to 7 - 14 μmol/L at the onset of the clinical signs of scurvy.

2.5.4 Analytical procedures

The methods used since some decades to determine vitamin C are physicochemical methods. Due to the drawbacks mentioned only methods based on HPLC separation are currently in use. Some representative HPLC methods for vitamin C analysis in body fluids and foodstuffs are discussed below.

Blood, plasma and serum analysis. A number of HPLC methods for vitamin C analysis in biological samples with electrochemical (Pachla & Kissinger 1976, Carr & Neff 1980, Tsao & Salimi 1981) and UV detection (Rose & Nahrwold 1981, Liebes et al. 1981, Keating & Haddad 1982) have been described. However, HPLC methods with electrochemical detection allow only the determination of AA, while UV measurement suffers from the very low absorbance of dHAA. Keating & Haddad (1982) described a HPLC method with UV detection for the analysis of AA and dHAA in foodstuffs. They enhanced the absorbance of dHAA by pre-column derivatization with o-phenylene diamine to 3-(1,2-dihydroxyethyl)furo[3,4-b]quinoxaline-1-one. A drawback is that AA is easily oxidized to dHAA during sample handling. This oxidation probably also occurs during deproteinization of blood when oxygen is released from oxyhaemoglobin (Blum & Ling 1959). Due to this and to the possible previous oxidation during blood collection, transport and storage, systematic errors in the determination of AA may arise. To avoid this error we developed a method wherein this oxidation step

is incorporated (Speek et al. 1984a, abstract in Section 3.5.1). The resulting figure, vitamin C as the sum of AA and dHAA, is very informative since AA and dHAA are equally biologically active.

Foodstuff analysis. For vitamin C determinations in foodstuffs not only the presence of both AA and dHAA, and the inactivity of diketogulonic acid have to be taken into account, but also the possible addition of the synthetic and inactive EA which only serves as an antioxidant.

HPLC methods with UV detection (Geigert et al. 1981, Bui-Nguyên 1980, Dennison et al. 1981, Keating & Haddad 1982) and electrochemical detection (Pachla & Kissinger 1976, Rückemann 1980) have been published. Methods with electrochemical detection only permit the assessment of the reduced forms, AA and EA. Dennison et al. (1981) described a HPLC method for the analysis of total vitamin C in beverages by UV measurement of AA after reduction of dHAA with homocystein. Keating & Haddad (1982) and Wimalasiri & Wills (1983) described HPLC methods with UV detection for the simultaneous determination of AA and dHAA. However, they did not consider possible interference by EA. Therefore, and since AA and dHAA have equal biological activity, we developed a HPLC method for the analysis of total vitamin C and total isovitamin C in foodstuffs (Speek et al. 1984b, abstract in Section 3.5.2).

2.6 Vitamin K

2.6.1 Definition and structure

The term vitamin K is used as a generic descriptor for 2-methyl-1,4-naphthoquinone (menadione, vitamin K_3) and its derivatives (IUPAC-IUB 1974). The common physiological characteristic of K vitamins is their anti-haemorrhagic effect. The main naturally occurring members are the 2′,3′-trans form of 2-methyl-3-phytyl-1,4-naphthoquinone (phylloquinone, vitamin K_1) and the class of 2-methyl-3-n-prenyl-1,4-naphthoquinones (menaquinone-n, MK-n, vitamin K_2) which differ from phylloquinone by the unsaturated side-chain consisting of some isoprene units (Fig. 5). Furthermore, the existence of menaquinones with a partly saturated side-chain, hydroquinone-n(Hx), of bacterial origin (Rietz et al. 1970), and of demethylmenaquinones, without the methyl group at the 2 position, has been demonstrated.

The presence of the methyl group at the 2 position of the naphthoquinone structure is essential to biological activity. The active forms in the body seem to be phylloquinone and menaquinone-4. The literature on the relation between biological activity and structure, form and route of administration has been reviewed by Suttie (1978).

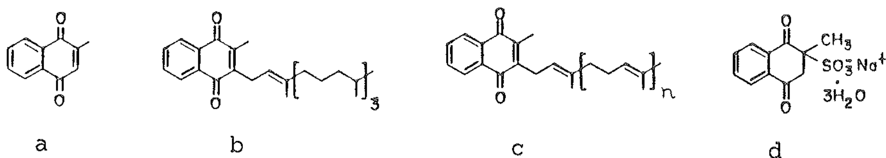


Fig. 5. Structural formulae of some naturally occurring biologically active K vitamers (b,c), the basic compound (a), and a synthetic one (d).

a: menadione, $C_{11}H_8O_2$ b: phylloquinone (K_1), $C_{31}H_{46}O_6$
c: menaquinone-n (K_2) d: menadione sodium bisulphite (K_3)

2.6.2 Properties

The naturally occurring K vitamins have a strong lipophilic nature in common. They are readily soluble in more or less non-polar organic solvents such as acetone, chlorinated hydrocarbons and hexane but, depending on molecular weight, only slightly soluble or even insoluble in polar solvents such as water, alcohol and acetonitril.

The natural as well as the synthetic forms are sensitive to light, alkali and reducing agents. The two synthetic K vitamers are more stable in feed mixtures than the natural forms.

The K vitamins are substituted 1,4-naphthoquinones and, therefore, have the general properties of the quinones. They show characteristic UV absorption with maxima in the range 240 - 280 nm and molar absorption in the order of 20,000. The wavelength of the maxima varies and the molar absorption coefficients decrease with the length of the chain. The compounds do not have natural fluorescence. The reduced forms, the hydroquinones, have quite different UV absorption spectra and do show fluorescence. Many data and spectral and physical characteristics of the phylloquinones and menaquinones have been summarized by Dunphy & Brodie (1971a).

2.6.3 Natural sources and occurrence in the human body

About half the human vitamin K requirement is satisfied by dietary phylloquinone, and about half by absorption of bacterially produced menaquinones (Olson 1980). Phylloquinone is widely distributed in nature, and animal products often contain a mixture of menaquinones.

Tables of the vitamin K contents of common foods (listed by Parrish 1980) are available, but they are not satisfactory. The values published have apparently been recalculated in an unspecified way from data originating from a chick bioassay not intended to yield absolute values (Dam & Schönheyder 1936, Dam & Glavind 1938). Data from chemical assays, GC and HPLC are available as well. As a result of differing assays and of the diversity typical of foodstuffs, only concentration ranges are known. Relatively good vitamin K sources are green and leafy vegetables. Since human requirement is low, most foods contribute markedly to vitamin K requirement.

The metabolism of K vitamins in humans is far from clear. After absorption, dietary vitamin K (menadione, phylloquinone or a mixture of menaquinones) is mainly transported by the lymphatic system. There is no evidence that the vitamin is modified. It has been shown to be associated with chylomicrons (Blomstrand & Forsgren 1968) and with serum lipoproteins (Shearer et al. 1970). There is no evidence of the existence of specific carrier proteins. The K vitamers found in human plasma and tissues are phylloquinone and menaquinone-4 (Taggart & Matschiner 1969, Thierry et al. 1970). Phylloquinone is converted into the inactive 2,3-epoxide after its action as co-factor of carboxylase enzymes (Larson et al. 1981).

Symptoms of serious vitamin K deficiency in man are prolonged blood-clotting times resulting in intramuscular haemorrhages.

Vitamin K deficiency has greater practical significance in poultry, especially in young chickens. Deficiency can be prevented in most poultry by supplementing 2 - 200 μg vitamin K_3 per kg per day (Doisy & Matschiner 1970).

2.6.4 Analytical procedures

Since very little is known about the vitamin, most of the analytical methods developed in the past decades are parts of studies aiming at answering questions on absorption, metabolism and actions of the several K vitamers. General problems in this field are the large variety of natural and synthetic K

vitamers and their metabolites, their marginal presence in body fluids and tissues, their possible interconversion and/or isomerization during analysis, and the possible degradation due to exposure to high temperature and light. Since this thesis refers to vitamin analysis on behalf of nutrition research, only methods referring to this field will be discussed. Methods for separation and detection of natural and synthetic K forms and their metabolites, homologues, derivatives and photodegradation products on behalf of vitamin K research has been reviewed by Lefevere (1985).

Plasma and serum analysis. The K vitamins naturally occurring in human plasma are phylloquinone, menaquinone-4 synthesized by colonic bacteria and the metabolite phylloquinone-2,3-epoxide. It is generally recognized that the concentration of plasma phylloquinone sufficiently reflects the nutritional status of humans.

Literature on the assessment of nutritional status by HPLC is abundant. The HPLC methods used for the determination of physiological levels of phylloquinone can be divided into systems based on adsorption and partition chromatography and systems based on UV, post-column reaction fluorometric and electrochemical detection. Some representative and relevant methods for the analysis of physiological plasma vitamin K levels based on the above detection principles have been described by Hiroshima et al. (1979), Haroon et al. (1984, 1986), Abe et al. (1979), Hart et al. (1985), Lefevere et al. (1979 1982), Hirauchi et al. (1986), Pietersma-de Bruyn & Van Haard (1986), Ueno & Suttie (1982), Lambert et al. (1986), Leclercq et al. (1983), Van Haard et al. (1986), and Langenberg & Tjaden (1984a, 1984b).

The on-line post-column reduction of phylloquinone, electrochemically or chemically by hydrogenation with borohydride derivatives, followed by fluorometric detection of the reaction product, limits the pre-purification procedures to liquid-liquid extraction with or even without an additional column chromatography step. This type of detection is very sensitive. The detection limit and/or the plasma volume used have been markedly reduced. Electrochemical reduction may be an elegant alternative but also a wasteful one if an advanced electrochemical detector is used only as reactor.

Because of their high sensitivity and specificity, the small sample size and relatively easy sample pre-purificaton, the methods with post-column

reduction of phylloquinone followed by fluorometric measurement of the reduced vitamin seems to be most suitable for assessment of the vitamin K status of humans.

Food analysis. Analysis of the naturally occurring vitamins phylloquinone (of plant origin) and menaquinones (of animal origin) provides sufficient information on the nutritional value of foods.

Williams et al. (1972) analysed several fat-soluble vitamins in cod liver oil and pharmaceutical preparations and were the first to report the usefulness of HPLC for vitamin K analysis. More papers have appeared since. The factors underlying this success are the same as described above for plasma and serum analysis (no thermodegradation, interconversion or isomerization, more specific detection, high sensitivity and easy fraction collection).

The methods applicable to the field of nutrition research should be able to detect naturally occurring levels of phylloquinone and menaquinones in foods and feeds. They are mostly applied to infant formulae and mainly differ in sample pre-treatment. Some representative and relevant methods for the analysis of vitamin K in specific foodstuffs have been published by Williams (1972), Thompson (1979), Barnett (1980), Haroon (1982), Bueno (1983), Hwang (1985), Zonta (1985), Sakano (1986), Langenberg (1986) and Lucock (1987). These methods are sufficiently selective and sensitive and are far superior to TLC and GC methods for food analysis.

The method of Thompson (1979) is the only one referring to more than one type of food samples. Because of the rather non-specific detection by UV absorption measurement, a three-step pre-purification is needed. Application of the specific and sensitive detection used for plasma assays, based on post-column reduction of phylloquinone followed by fluorometric detection of the reaction product, may be an improvement.

Since, due to the low requirement, dietary vitamin K deficiency rarely occurs in adults, no method for vitamin K analysis in foods is currently being used in our laboratory.

Feed analysis. Since vitamin K deficiency frequently occurs in young poultry, the feed is enriched with this vitamin. For reasons of absorption and stability the water-soluble menadione sodium bisulphite (MSB, Fig. 5) is often used because the biologically active menaquinone-4 can be formed in vivo from

menadione. The importance of vitamin K in animal nutrition is described by Scott (1966).

Numerous methods for the determination of MSB in feeds have been published: spectrophotometric methods (Koetsveld 1950, European Community 1979, Hassan 1981), GC methods (Libby & Sheppard 1965, Winkler & Yoder 1972) and HPLC methods (Rannft & Rückemann 1978, Manz & Maurer 1982). The drawbacks of the spectrophotometric and GC methods are well-known; they have been superseded by HPLC.

The sensitivity of the HPLC method of Rannft & Rückemann only permits determinations in premixes and supplements. The detection limit of the HPLC method with UV detection of Manz & Maurer is 0.5 mg/kg. However, we observed that UV detection of menadione in complete diet samples at contents below ca. 2 mg/kg is often subject to interference by sample components.

Due to the lack of selectivity of UV detection of menadione we developed a HPLC method with post-column reaction fluorometric detection (Speek et al. 1984c, abstract in Section 3.6.1). In a post-column reaction coil the menadione is hydrogenated by sodium borohydride to 2-methyl-1,4-dihydroxy-naphthalene with is detected fluorometrically. This detection system is a modification of that of Abe et al. (1979), i.e. extended with debubbling of the reagent and air segmentation, which are actually improvements.

2.7 Vitamin B_1

2.7.1 Definition and structure

The names thiamin(e), aneurine (UK) and vitamin B_1, and the obsolete terms polyneuramin, antiberiberi vitamin and antineuric vitamin refer to a single compound with the structural formula given in Fig. 6. Thiamin consists of a substituted pyrimidine and thiazole ring connected by a CH_2 bridge and has the empirical formula $C_{12}H_{17}N_4O_2$. The carbon atom 2 in the thiazole molecule is essential to the catalytic and biological activities of thiamin: any substitution completely eliminates these activities (Barton & Rogers 1950).

Several decades ago, the thiamin deficiency disease beriberi has been highly prevalent in many countries of the world, particularly Asia. It was the first disease recognized as a nutritional deficiency disorder (Grijns 1901, Eijkman 1890). The present vitamin concept as a class of compounds

being trace nutritional factors was first conceived from early studies on beriberi, and the class name "vitamins" has been derived from early work on the chemical nature of the "antiberiberi factor" in the diet (Funk 1911). The fascinating story of the discovery of thiamin and the development of the vitamin concept has been told by a.o. Bicknell & Prescott (1953) and Williams (1961).

2.7.2 Properties

Thiamin is available as its double salt with hydrochloric acid, thiamin chloride hydrochloride. In its dry form the compound is stable up to 100 °C. In an acid medium, below pH 5.5, thiamin is more heat-resistant whereas it is unstable in alkaline and neutral medium. The compound decomposes when exposed to ultraviolet light. Thiamin is highly soluble in water, partly soluble in alcohols and acetone, and insoluble in fat solvents. In solutions with $pH < 5$ thiamin shows two absorption bands with maxima at 235 and 267 nm, but it does not fluoresce.

Thiamin is also available as its mono-, di- and triphosphate (Fig. 6). The active coenzyme form of thiamin is thiamin diphosphate or cocarboxylase, which is stable for several months when stored in a dry state in the dark at 6 °C. In highly alkaline solutions thiamin and its phosphates are oxidized by agents such as $[Fe(CN)_6]^{3-}$ and cyanogen bromide to thiochrome and its phosphates respectively. Alkaline solutions of these compounds are stable for at least three days. The compounds all have almost the same fluorescent characteristics with an excitation maximum at 375 nm and emission maxima at 432 to 435 nm. This property is useful in determinations.

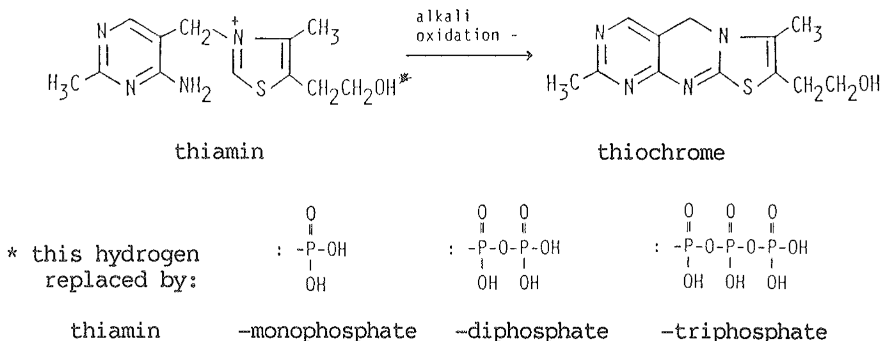


Fig. 6. Structural formulae of thiamin (M = 265.4), thiochrome, and thiamin mono-, di- and triphosphate.

2.7.3 Natural sources and occurrence in the human body

Thiamin occurs in many foods, but in most foods its content is low. The concentrations vary about 25-fold. In most animal products thiamin occurs in two phosphorylated forms and, occasionally, as mono- and disulphide. The predominant form is diphosphate (95 - 98 %). In plant products most of the vitamin occurs in the non-phosphorylated form. Oils and fats and highly refined foods (sugars) do not contain thiamin, and the content in green vegetables, fruits and sea foods is low. Major sources of thiamin are yeast, potatoes and the pericarp and germ of cereals, probably providing 30 to 40 % of the daily intake. Since milling of cereals lowers the thiamin content, in many countries the white flour is fortified with B vitamins. Watt & Merril (1963) and Burton (1965) have recorded the thiamin contents of a broad range of natural and processed foods.

Thiamin is rapidly and actively absorbed from the small intestine whereafter it is phosphorylated in the cytoplasm by thiamin pyrophosphokinase to thiamin diphosphate (ThDP, also named thiamin pyrophosphate, TPP, or cocarboxylase). The body store of total thiamin of a normal adult is about 30 mg of which more than 90 % is present as ThDP. The vitamin has been demonstrated in tissue, in organs (heart, kidney and liver) and in blood in amounts of about 60, 22 and 0.8 % of the body pool respectively. The mono- and triphosphates as well as free thiamin have also been demonstrated. The blood level of total thiamin is about 100 - 180 nmol/L (Schrijver & Speek 1982) of which about 95 % is present as ThDP. Most whole blood thiamin is present in erythrocytes (90 %) and in leucocytes (5 %). Small amounts of free thiamin are found in plasma although the body is incapable of storing the free vitamin. No evidence has been produced of a specific thiamin carrier in the blood.

Total thiamin concentrations in whole blood below 100 nmol/L and an α-ETK (erythrocyte transketolase, see below) value above circa 1.2 are considered as critical values (Sauberlich et al. 1974, Schrijver & Speek 1982).

2.7.4 Analytical procedures

A generally accepted approach to the assessment of nutritional thiamin status has been developed by Brin (1963, 1964). The erythrocyte transketolase (ETK) activity and its ratio to the activity with in vitro stimulation by ThDP (α-ETK or ThDP effect) are measures of the thiamin status. The method, which

strictly is a functional test, is based on the catalytic effect, measured as a reaction rate, of the ThDP-dependent transketolase on the in vitro conversion of ribose 5-phosphate. This method has been described in detail by Smeets & Müller (1971) and by Vuilleumier et al. (1983).

The thiamin status can also be assessed by measuring the concentrations of ThDP or total thiamin in whole blood. All three parameters, ETK, α-ETK and concentrations of total thiamin or of ThDP, are generally considered as useful biochemical indices of the thiamin status. As this thesis focuses on HPLC analysis, only HPLC analysis of total thiamin and thiamin phosphates in blood and foodstuffs are discussed below.

Body fluid analysis. Many papers have recently appeared on HPLC analysis of thiamin in whole blood, serum, erythrocytes, tissue, urine and foods. The procedures can be differentiated into methods measuring free thiamin and its mono-, di-, and triphosphates and methods measuring total thiamin by incorporating an enzymatic hydrolysis step in the sample pre-treatment procedure. Straight-phase (silica) as well as reversed-phase columns (-C18, -NH2 and polystyrene divinyl benzene) are used. The detection in most methods is based on the conversion in an alkaline medium by potassium ferricyanide of thiamin and its phosphates into thiochrome and its phosphates, and fluorescent measurement of the latter compounds. Furthermore, since the thiochrome reaction is instantaneous, pre-column as well as post-column conversion can be applied.

Methods for the determination of total thiamin in whole blood have been described by Schrijver & Speek (1982) who used straight-phase chromatography on silica followed by post-column conversion to thiochrome, and in foods, body fluids, urine and faeces by Böttcher & Böttcher (1986) who used pre-column conversion followed by chromatography on silica-NH2.

Methods for the determination of the coenzyme ThDP in erythrocytes have been described by Warnock (1982), and in erythrocytes and whole blood by Floridi et al. (1984) and Baines (1985). They all more or less improved the pre-column conversion technique and chromatographed the thiochrome diphosphate on silica-NH2.

Methods for the determination of thiamin and its mono-, di-, and triphosphates in tissue have been described by Sanemori et al. (1980) who used pre-column conversion with BrCN followed by chromatography on octadecyl silica; in animal tissue by Kimura et al. (1982) and in human and rat blood by Kimura

& Itokawa (1985) who used chromatography on octadecyl silica followed by post-column conversion; and in urine and cereals by Hilker & Clifford (1982) who used column LC pre-purification on octadecyl silica followed by anion exchange chromatography and UV measurement. Methods for the determination of urinary thiamin and riboflavin without pre-purification have also been described by Mansourian et al. (1982) who used chromatography on a radial compression module (RCM) with a silica column followed by post-column conversion.

We applied the method of Schrijver & Speek (1982) (abstract in Section 3.7.1) for large-scale routine analysis of total thiamin in blood.

Food analysis. Since the biological activity of dietary thiamin and its three phosphates is determined by the thiamin part of the molecule, mostly total thiamin is determined by incorporating an enzymatic hydrolysis step in the sample pre-treatment procedure. Van de Weerdhof et al. (1973) were probably the first to successfully determine total thiamin and riboflavin by HPLC in unfortified foods using chromatography on silica. Thiamin was detected by post-column conversion to thiochrome and fluorometric measurement of the latter while riboflavin was detected using its native fluorescence. Many modifications have been published since. Toma & Tabekhia (1979) determined total thiamin, riboflavin and niacin in rice using a octadecyl silica column and UV measurement. Ohta et al. (1984) determined thiamin in rice in a similar way but with post-column conversion and fluorometry. Ang & Moseley (1980) determined total thiamin and riboflavin in meat and meat products using pre-column conversion of thiamin followed by chromatography on silica and fluorometry. Wimalasiri & Wills (1985) determined thiamin and riboflavin in foods using an octadecyl silica RCM column followed by post-column conversion and fluorometric measurement. Ayi et al. (1985) determined total thiamin in infant formulae by cation exchange LC pre-purification followed by chromatography on silica-CN and UV measurement.

All HPLC methods for thiamin analysis in foods are basically similar. The use of UV measurement for thiamin analysis in samples with more complicated composition necessitates an additional pre-purification step as compared to fluorometric measurement of its conversion product thiochrome.

The methods mentioned are characteristic of thiamin analysis but cover the literature only partly. Kawasaki & Sanemori (1985) have published a very extensive review of the literature giving many methods in detail, but without

discussion. Therefore, we discuss them with respect to specific advantages and disadvantages related to certain essential parts of the method, viz. the choice between post- and pre-column derivatization, between straight- and reversed-phase packing materials and between fluorescence and UV detection.

An advantage of pre- versus post-column derivatization is less peak broadening and hence better resolution. However, in the case of thiochrome formation pre-column derivatization has several disadvantages. First, thiochrome and its phosphates are only stable at pH > 8. This implies that the elution solvent should have a pH of at least 8; the silica(-based) packings are then considerably more soluble than at pH < 6. This phenomenon seriously affects the column's life-time, a characteristic rarely taken into account. Therefore, the paper of Bontempts et al. (1984) is interesting. They separated thiochrome and its mono-, di-, and triphosphate on a polystyrene divinyl benzene packing, which is stable at alkaline pH, using a solvent buffered at pH 8.5. They investigated the capacity factor, plate height and resolution obtained with this material and found it to be very promising for chromatography of thiamin and its phosphates. Nishimune et al. (1972) had already separated thiamin and its phosphates on Sephadex G10, but the last peak (TTP) had an elution volume of as much of 400 mL with a width of about 50 mL. A second disadvantage of the pre-column derivatization is the possible presence in the sample extract of an unknown amount of antioxidant using reagent. Third, the yield of the reaction, depending on several factors, is only about 67 % (Nicholson et al. 1958). Finally, pre-column derivatization implies, in case of large-scale routine analysis, a considerable "waiting time" of the derivatized samples in the injector tray. This is a drawback since thiochrome may be decomposed by (unknown) excess of oxidant (Ziporin et al. 1962).

The disadvantages of pre-column technique can be overcome by applying post-column conversion. Thiamin and its phosphates are then first chromatographed and subsequently converted. Substances interfering with the reaction only hampers accuracy if they co-elutes with thiamin. Furthermore, thiochrome is measured within a minute after being formed so that long-term instability does not play a role. As a result of the mechanical stability of HPLC and the proportioning pump, the reaction circumstances in the post-column coil are well reproducible.

All compounds in question, i.e. thiamin and its three phosphate esters or, in the case of pre-column conversion, thiochrome and its three phosphate esters, can be separated with both types of packing materials. The choice

between straight- and reversed-phase columns therefore should be based on the life-time of the column.

We observed a longer life-time of reversed-phase columns as compared to silica columns when applied in analysis of thiamin in blood extracts. However, the life-time of silica packings for thiamin analyses can be appreciably lengthened by applying the RCM silica column (Waters Assoc., USA). The plastic column, containing spherical 5-μm silica particles, is packed in a rubber sleeve which is radially pressurized in a mechanically pressurized glycerol bath. As a result of suppression of channel formation, the life-time of this column appears to be considerably longer than that of stainless steel columns packed with 5-μm irregularly shaped silica particles (Schrijver & Speek 1982), i.e. 600 vs. 100 whole blood extracts. However, a disadvantage is the fixed length of the RCM column. Since thiamin is strongly retained on this silica column and since the ethanol concentration of the mobile phase should be lower than the concentration at which precipitation in the post-column reaction coil may occur after addition of alkaline potassium ferricyanide, the flow rate has to be rather high (3.2 mL/min).

In our laboratory we gathered experience with running large-scale routine analyses of total thiamin in foodstuffs and urine for five years. The original method for determinations in whole blood (Schrijver & Speek 1982) (abstract in Section 3.7.1) has been modified to extend column life-time and to reduce the down-time of the system. Based on the arguments mentioned, we have chosen chromatography on a silica RCM column followed by post-column reaction fluorometric detection. The methods for thiamin analysis in foodstuffs and urine are described in Appendix 4 (abstract in Section 3.7.2).

2.8 Vitamin B_2

2.8.1 Definition and structure

Riboflavin is 7,8-dimethyl-10-(1'-D-ribityl)isoalloxazine, an isolaxazine with a ribitol side-chain (Fig. 7). The International Union of Nutrition Sciences Committee on Nomenclature and the Committee on Biochemical Nomenclature of IUPAC-IUB have designated riboflavin as the official name. The name vitamin B_2 is frequently used, and vitamin G, lyochrome, ovoflavin, lactoflavin, uroflavin and hepatoflavin are historical names. The major biological

forms of the vitamin are free riboflavin and two of its derivatives, flavin mononucleotide (FMN, riboflavin 5'-monophosphate) and flavin adenine dinucleotide (FAD)(Fig. 7).

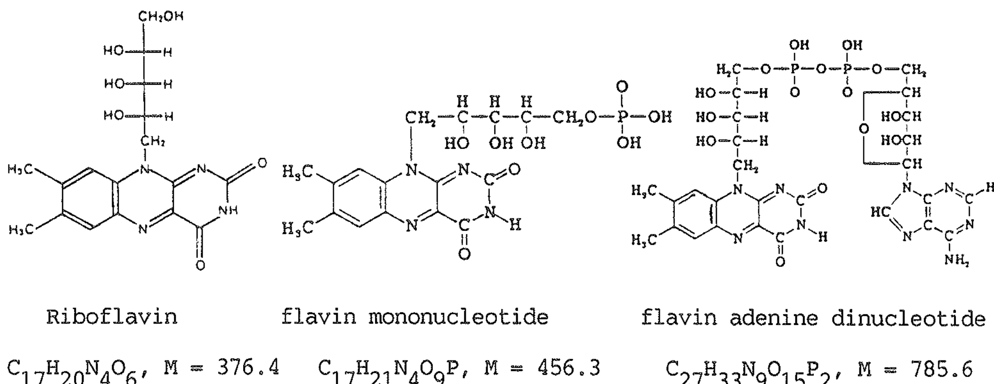


Fig. 7. Structural formulae of the three major riboflavin (vitamin B_2) forms.

2.8.2 Properties

Riboflavin consists of orange-yellow crystals which melt at about 280 °C with decomposition. The vitamin is slightly soluble in water, fairly soluble in acid solutions and insoluble in organic solvents. Neutral aqueous solutions are greenish yellow with absorption maxima at 223, 268, 359-375, 446 and 475 nm. Furthermore, the compound has fluorescent characteristics with excitation and emission maxima at about 470 and 525 nm respectively. The absorption and fluorescence spectra are pH-dependent. The vitamin is relatively heat-stable and is very light-sensitive, especially at a low pH.

2.8.3 Natural sources and occurrence in the human body

The richest natural source of vitamin B_2 is yeast which is not a common component of the human diet. Milk and milk products contribute best to dietary vitamin B_2, accounting for ca. 50 % of daily vitamin B_2 intake in the Western world. Meat, eggs and legumes are important sources, contributing about 25 %, whereas fruits, fresh vegetables and cereal grains contribute about 10 %. Vitamin B_2 occurs in food as free riboflavin, FMN and FAD. Riboflavin contents of many foodstuffs are given by Foy & Mdaya (1975) and Cooperman & Lopez (1984).

After absorption, dietary B_2 vitamers are transported to and stored in the liver and other tissues where they are converted into FAD and bound to specific flavoproteins. Liver, heart and kidney have the highest riboflavin content, of which 70 to 90 % is in the form of FAD. Free riboflavin accounts for less than 5 % of the stored flavins. Liver contains about one third of the total body store. Remarkably, these flavin depots are maintained even in case of severe deficiency.

Blood (mainly the erythrocytes) contains all three forms, with FAD as the predominant form: FAD 240 - 440 nmol/L, FMN about 30 nmol/L, riboflavin about 15 nmol/L (Speek et al. 1982). Levels below 240 nmol FAD per litre blood and an "FAD effect" (α-EGR, see below) over about 1.4 are considered to indicate vitamin B_2 deficiency.

2.8.4 Analytical procedures

A biochemical method to diagnose riboflavin deficiency is analogous to that for thiamin deficiency. The erythrocyte glutathion reductase (EGR) activity and its ratio to the activity with in vitro stimulation by FAD (α-EGR or FAD effect) are measures of the vitamin B_2 status. The method, which strictly is a functional test, is based on the catalytic effect, measured as a reaction rate, of the FAD-dependent EGR on the in vitro reduction of NADH. The technical details of this technique have been described by Tillotson & Baker (1972) and evaluated by Bayoumi & Rosalki (1976). This way of establishing the vitamin B_2 status has pros and cons, just as for vitamin B_1. The main disadvantage is the influence of certain diseases on EGR activity. Another method of evaluating the vitamin B_2 status is the measurement of FAD (the most abundant vitamer) in blood or erythrocytes. Both types of test are considered to evaluate the vitamin B_2 status of humans.

Dietary free riboflavin, FMN and FAD have a similar molar biological activity. Therefore, to establish the vitamin B_2 potency of foodstuffs, mostly total riboflavin is measured by incorporating an enzymatic hydrolysis step in the sample pre-treatment procedure. As this thesis focuses on HPLC analysis, only HPLC analyses of FAD (blood) and total riboflavin (foodstuffs) is discussed below.

<u>High-performance liquid chromatography</u>. Numerous methods for the HPLC determination of flavins in pharmaceutical preparations, foodstuffs, blood (serum)

and urine have been described. They include sample pre-treatment and straight- or reversed-phase chromatography followed by fluorescence or UV detection. Photolysis can readily occur, so that precautions must be taken. The type of sample pre-treatment strongly depends on the type of sample.

Flavins in pharmaceutical preparations (often FMN) are freed by dissolving the sample in proper solvents such as methanol:water (1:1 or 8:2) (Amin & Reusch 1987). The methods used to dissolve the preparations include ultrasonication (Kirchmeier & Upton 1978), shaking at ambient temperature (Ghisla et al. 1980) or at 6 °C (Nuttall & Busch 1971), and heat treatment (Wittmer & Haney 1974). The vitamin is completely dissolved. FMN in the resulting solution is separated on a reversed-phase column and detected by measurement of its UV absorbance. Amin & Reusch (1987) determined seven water-soluble vitamins including riboflavin from vitamin pills in one run by means of ion-pair reversed-phase chromatography with UV detection.

Procedures for the extraction of flavins from foodstuffs mostly include acid extraction in an autoclave at 120 °C followed by cooling to room temperature, pH adjustment and enzymatic hydrolysis to convert all flavins into riboflavin. The clear extract, after filtration, is directly injected onto, mostly, a reversed-phase HPLC column whereafter riboflavin is detected fluorometrically. On such columns, eluted with solvents containing ion-pair reagents (aliphatic sulphonic acids) several water-soluble vitamins can be separated and quantitated, such as riboflavin and thiamin in selected foods (Fellman et al. 1981) and in meat (Ang & Moseley 1980), riboflavin, thiamin and niacin in foods (Skurray 1981), riboflavin and FMN in foods (Lumley & Wiggins 1981), riboflavin, thiamin and pyridoxine in fortified cereal products (Wehling & Wetzel 1984), riboflavin and pyridoxine in infant formulae (Ayi et al. 1986), and riboflavin, thiamin, pyridoxine, ascorbic acid, niacin and folic acid in white sauce (Nandhasri & Suksangpleng 1986).

The flavins in blood and tissue homogenates are mostly freed from their binding proteins by acid treatment (trichloroacetic acid). The supernatant, sometimes after additional purification on a Sep-pak C18 column, is directly injected onto the HPLC column to analyse the various flavins or, after pH adjustment and enzymatic hydrolysis, to analyse total flavins as riboflavin. Typical methods are the fluorometric determination of FAD, FMN and riboflavin in whole blood (Speek et al. 1982; Floridi et al. 1985), the determination of riboflavin in serum (Lambert et al. 1985b), and the simultaneous determination of riboflavin, FMN and FAD in fish serum (Ichinose et al. 1985) and in

plankton body fluid (Ichinose & Adachi 1986). The method of Speek et al. (1982) (abstract in Section 3.8.1) has been applied in our laboratory in large-scale routine.

Urine samples are mostly acidified before storage to stabilize riboflavin. HPLC separation and fluorometric detection permits direct injection of a centrifuged urine aliquot. Typical urinary riboflavin analyses have been described by Smith (1980), Gatautis & Naito (1981) and Mansourian et al. (1982).

Since the flavin-containing extracts can be directly injected onto silica as well as reversed-phase HPLC systems for flavin analysis, and since separation can be achieved with both types of chromatography, column life-time should be the factor determining the choice. The general tendency is the use of reversed-phase columns, mostly silica-C18 but sometimes silica-NH2, for chromatography since these columns are much more stable for this application than silica columns (Lumley & Wiggins 1981).

The methods for vitamin B_2 analysis in foodstuffs and urine as routinely applied in our laboratory are described in Appendix 5 (abstract in Section 3.8.2).

2.9 Vitamin B_6

2.9.1 Definition and structure

Vitamin B_6 (pyridoxine) is the generic term for six vitamers. The B_6 group includes three interconvertible forms: the alcohol form pyridoxine (PN), the aldehyde form pyridoxal (PL), and the amine form pyridoxamine (PM). Their more stable N-hydrochlorides are commercially available. B_6 vitamers occur in body fluids and tissues both in their free forms and phosphorylated at the 5' place. The structural formulae are shown in Fig. 8.

2.9.2 Properties

The various free forms of the vitamin as well as their phosphates and hydrochlorides are readily soluble in water and slightly soluble in ethanol. The important representative PN is fairly stable in dry heat but not in moist heat. All B_6 forms are unstable in intensive visible and UV light, especially in a neutral or alkaline medium. They are fairly stable in acid solutions

stored in the dark. PL hydrochloride decomposes at its melting point of 206 °C; it is fairly stable when used in pharmaceutical preparations. Bauernfeind & Miller (1981) observed a loss of 10 % after storage for one year at 23 °C. The B_6 vitamers have fluorescent characteristics with excitation maxima in the range 295–305 nm and emission maxima in the range 370–380 nm.

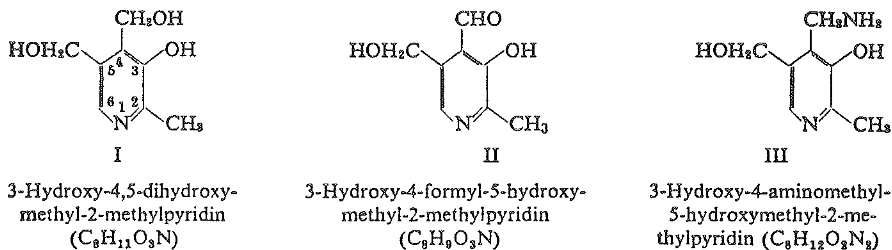


Fig. 8. Structural formulae and IUPAC designations of the B_6 vitamers.
I: pyridoxine (PN), $C_8H_{11}NO_3$, M = 169.1; II: pyridoxal (PL), $C_8H_9NO_3$, M = 167; III: pyridoxamine (PM), $C_8H_{12}N_2O_2$, M = 241.1

2.9.3 Natural sources and occurrence in the human body

PN is widely distributed, albeit in low concentrations, in plant tissue, whereas PL and PM and their 5′-phosphates (PLP, PMP) are the predominant forms found in animal tissue. Relevant to the analyst is the possible binding of the various forms in biological systems. The most common bond for PLP, the biologically most active vitamer, is the Schiff base with the ε-amino group of a lysine residue (Fisher et al. 1958). Schiff bases can be hydrolysed by deproteinizing agents such as trichloroacetic acid (TCA) and perchloric acid. However, PN is sometimes present as 5′-O-(β-D-glucopyranosyl) (Gregory & Ink 1987), and PL can react with amino acids such as histidine, tryptophan, glycine and cysteine (Snell 1981). The three forms are widely distributed at low concentrations in animal and plant tissues. The main sources of vitamin B_6 are given by Marks (1975).

Very little is known about the factors influencing absorption of B_6 vitamers. Dietary vitamin B_6 is absorbed and rapidly converted into the cofactor forms PLP and PMP (McCoy & Colombini 1972). Total body stores of vitamin B_6 have been estimated to be between 20 and 150 mg with a total turnover of 3 % per day (Tillotson et al. 1966, Shane 1978). About half of the vitamin B_6 is

stored in the muscle as phosphorylase-bound PLP. Normal blood levels of PLP are given in Table 1. Levels below 38 nmol PLP per litre blood and a "PLP effect" (α-EGOT, see below) over circa 2.3 are considered to indicate deficiency. Furthermore, PLP and PMP are stored in liver, brain and kidneys. In plasma, both phosphorylated and non-phosphorylated B_6 vitamers and the metabolite pyridoxic acid (PIC) have been demonstrated.

The first vitamin B_6 deficiency manifestations are a reduction of body pool size, followed by lowering of concentrations of the vitamers in blood and tissue and of its metabolite PIC in urine (Sauberlich 1981).

2.9.4 Analytical procedures

A total analysis of vitamin B_6 in foods and body matrices is hampered by the complexicity of the matrix, by the multiple forms in which vitamer B_6 occur, by the different chemical bonds with matrix proteins and, particularly, by their low levels.

A biochemical method of diagnosing vitamin B_6 deficiency is, just as for diagnosing thiamin and riboflavin deficiency, measurement of the PLP-dependent erythrocyte glutamate oxaloacetate transaminase (EGOT) activity with and without stimulation in vitro by PLP, expressed as the ratio, α-EGOT (PLP effect). This functional test is based on the catalytic effect, measured as a reaction rate, of the PLP-dependent EGOT on the transamination of aspartate to oxaloacetate. The technical details of this technique have been described by Stanulovic et al. (1967). This way of establishing the vitamin B_6 status has pros and cons, as in the case of thiamin and riboflavin. The main disadvantage is the influence of certain diseases on EGOT activity.

Another method of evaluating the vitamin B_6 status is the measurement of PLP concentration in blood or erythrocytes.

To establish the vitamin B_6 potency of foodstuffs, one mostly tries to measure the concentrations of the various vitamin B_6 vitamers.

As this thesis focuses on HPLC analysis, only HPLC analyses of PLP (blood) and total PN, PL and PM (foodstuffs) are discussed below.

High-performance liquid chromatography. The literature on HPLC analysis of vitamin B_6 is abundant due to difficulties encountered, especially with regard to the large number of vitamers and the relatively low concentrations. The methods generally include acid extraction with centrifugation, sometimes

pre-column reaction fluorometric labelling, reversed-phase (C18) chromatography with or without ion-pair reagents, followed by on-line detection based on measurement of pre- or post-column formed fluophores, or of native fluorescence. The methods differ in number of vitamers determined simultaneously, which depends on the aim of the investigation. Below some representative methods are grouped according to the aim of the investigation.

Analysis of all vitamers for metabolism and absorption studies. Analytical methods for this purpose include methods for the simultaneous separation and detection of the three free and the three phosphorylated forms as well as methods determining the sum of one of the three free vitamers and its phosphorylated form by incorporation of an enzymatic hydrolysis step.

Some representative methods for the simultaneous determination of the six forms in body fluids and tissues include acid extraction (perchloric acid or TCA), with filtration or centrifugation and sometimes pre-purification of the extract on a cation exchange resin (Tryfiates & Sattsangi 1982). Separation is commonly done on a C18 column eluted with buffer-methanol, and detection by measuring the native fluorescence (300 - 375 nm). Pierotti et al. (1984) and Tryfiates & Sattsangi (1982) used a sulphonate ion pair reagent in the solvent. The UV detection at 313 nm reported by the latter authors only allows analysis of samples with concentrations above the endogenous level. Coburn & Mahuren (1983), Hollins & Henderson (1986) and Sheppard et al. (1987) enhanced the sensitivity for PLP and, to a much lesser extent, for PL by post-column reaction fluorometry by making use of their reaction with $NaHSO_3$. Vanderslice & Maire (1980) analysed all plasma B_6 vitamers and arrived at separation by using a dual-column system in series - both filled with Aminex A-25 resin but eluted at different temperatures - and improved the sensitivity by measuring the native fluorescence of each compound at its excitation and emission maxima. Their chromatographic run, however, takes as much as about 2 h, and resolution is rather poor.

Some representative methods with enzymatic hydrolysis determining the sum of each free vitamer and its phosphorylated form are those of Yoshide et al. (1978) and Hefferan et al. (1986). Yoshide et al. extracted the B_6 vitamers from rat blood. After enzymatic hydrolysis, the free forms, with deoxypyridoxine (DOP) as the internal standard, were separated on an ion-exchange column (Gel LS-160) and detected fluorometrically. Hefferan et al. (1986) analysed blood of deficient and control rats in basically the same way. The

free vitamers were separated by ion pair chromatography on a C18 column and detected by measuring their native fluorescence.

Assessment of nutritional status. The nutritional status parameters mostly used are plasma PLP and, to a lesser extent, plasma PL. Hart & Hayler (1986) determined both levels in plasma. After acid extraction and centrifugation the compounds are separated on a 10-μm C18 column and detected electrochemically. However, although the compounds are separated, they appear on the descending slope of a background peak. A guard cell is necessary to purify the HPLC solvent. The method of Schrijver & Speek (1981) (abstract in Section 3.9.1) for the determination of PLP and PL in whole blood has been used in our laboratory for many years. B_6 vitamers are freed of the Schiff base link with albumin by acid treatment whereafter they are separated on a C18 column. Both vitamers in the HPLC effluent are converted into their semicarbazones by an on-line post-column reaction with semicarbazide. The fluorescence of the semicarbazones is strongly enhanced by adding alkali in a second on-line reaction coil whereafter deficient levels can be detected fluorometrically. Although PLP and PL are differentiated they are not separated from other B_6 vitamers. The fluorescence of the other forms is low and cause only negligible interference. Since in this method an unknown fluophore in blood of patients with renal disease appears to interfere with PLP, another method based on pre-column derivatization using the same reagent and with a modified HPLC system, is used (Ubbink et al. 1985). In both methods the down-time is relatively short, about 120 samples can be run per two days, the columns have a life-time of more than 500 samples and the precisions are reasonable (Chapter 4).

Food analysis. Methods for the HPLC determination of B_6 vitamers in foodstuffs include methods for the simultaneous analysis of all three free vitamers and their phosphorylated forms, and methods analysing the sum of each free vitamer and its phosphorylated form by incorporating an enzymatic hydrolysis step. Some methods - for infant formulae - determine only the most abundant vitamer, PN, simultaneously with thiamin and riboflavin (Ayi et al. 1986). The general schedule is again acid treatment of the sample, filtration or centrifugation, chromatography on a C18 HPLC column, and detection of native fluorescence.

Representative methods for the analysis of all six vitamers in foodstuffs have been described by Vanderslice et al. (1980) and Hamaker et al. (1985).

The method of Vanderslice et al. includes extraction with sulfosalicylic acid, automated LC purification and HPLC separation by means of the dual-column system with Aminex A-25 resin mentioned above for plasma analysis. Sensitivity is improved by measuring the native fluorescence of each compound at its excitation and emission maxima. Hamaker et al. (1985) separated the six forms on a C18 column and enhanced the sensitivity of the nutritional status parameter PLP by post-column $NaHSO_3$ derivatization.

Representative methods for the analysis of the sum of a free vitamer and its phosphorylated form are described by Wong (1978), who uses Taka-Diastase for dephosphorylation and a strong cation exchange column for the analysis of fruits and vegetables, and by Morrison & Driskell (1985), who use ion pair chromatography on a C18 column with DOP as the internal standard for the analysis of human milk. Wang as well as Morisson & Driskell measure native fluorescence for detection. Methods with UV detection are only suitable for the determination of high concentrations in relatively pure samples. Capuano & Daghetta (1980) simultaneously determined retinyl palmitate, α-tocopherol acetate and pyridoxine dipalmitate in edible oils by direct injection of a diluted sample onto a silica column. After separation the vitamins are detected by combined UV absorption and fluorometric measurement.

The references mentioned are just a selection from many papers published recently. Vitamin B_6 analysis is extensively reviewed by Vanderslice et al. (1985).

All methods mentioned have their pros and cons. Our purpose was assessment of the vitamin B_6 potency of foodstuffs, i.e. reevaluation of the Dutch Food Composition Table. The three vitamers pyridoxine, pyridoxal and pyridoxamine have to be separated and quantitated. However, dietary free and phosphorylated forms have similar molar biological activities. Therefore, we designed a method for the determination of the sum of the free and phosphorylated form of each of the three vitamers. The method is based on acid extraction, enzymatic hydrolysis and HPLC separation of the resulting three free vitamers on a C18 column. By means of pH adjustment in a post-column reaction coil the intensities of the native fluorescence of the B_6 vitamers only slightly increase but - which is more important - the excitation and emission wavelength maxima shift in such a way that the method is less susceptible to interferences by co-eluting matrix components. A large variety of foodstuffs can be analysed for the sum of each free vitamer and its phosphorylated form. The method is given in Appendix 6 (abstract in Section 3.9.2).

Chapter 3. THE HPLC METHODS FOR VITAMIN ANALYSIS

3.1 Choice of the HPLC system

Since many types of silica and non-polar chemically bonded phase packing materials and a wide range of solvents and modifiers are available now for HPLC, separation of just a few compounds generally is not really a problem any more. However, some other conditions associated with the HPLC system should be taken into account. These conditions, in order of our priority, are summarized below.

1. The system should be able to completely separate the compound(s) of interest from other compounds present in the sample extract evoking a signal in the detection system. A compromise is possible in the case of analysis of body fluids for diagnostic purposes. If the interference is small and constant for every sample, such an inaccuracy plays a minor role because the same inaccuracy goes for the reference values as well.

   Note. This condition has to be fulfilled for a large variety of samples, such as foodstuffs and body fluids. Our methods for a particular vitamin in different matrices significantly differ in sample pre-treatment. The respective HPLC systems only differ as to the length of the column. This length is so chosen that the chromatographic run time is not the minimum time with sufficient resolution, but a time so much longer that factors such as column alteration, small deviations in composition, in injection volume and/or in detector settings do not immediately cause an unacceptable loss of resolution. In the literature vitamin determinations have been described based on straight-phase (SF), reversed-phase (RP) and ion-exchange systems, operated with a variety of solvent mixtures. However, for only some of these all conditions mentioned under 2 - 7 are met. The choice of a system should be made on the basis of these conditions and the order of priority must be taken into account.

2. If post-column reaction detection is applied the mixture of eluent and reagent solution should be of such a nature that a fairly high - although not necessary quantitative - conversion of the analyte(s) of interest is achieved within the reactor residence time.

Note. This condition limits the choice of HPLC eluent. If more than one derivatization reaction is available, the HPLC system should be chosen such that condition 3 is met, since complete separation can be achieved with different eluent systems.

3. The composition of the HPLC eluent should allow direct injection of the extracts resulting from sample pre-treatment.

Note. The fat or water solubility of the vitamin determines the hydrophilic or lipophilic character of the extraction solvent and, hence, the choice between a SP (fat-soluble vitamins) and RP (water-soluble vitamins) HPLC system. However, there are some exceptions based on the other conditions mentioned.

One exception is the determination of menadione sodium bisulphite (synthetic vitamin K_3) in fodders. This water-soluble vitamin is converted during sample pre-treatment into the fat-soluble menadione, which is extracted in n-hexane. Direct injection of n-hexane requires a lipophilic solvent and hence a SP system. However, derivatization of menadione (chemical reduction to the highly fluorescent diaminonaphthalene) can only be achieved in a hydrophilic solvent. Therefore, an HPLC system was chosen consisting of a RP system (5-μm C18) with a water-methanol mixture. This necessitates the introduction of an evaporation (n-hexane) and redissolution (ethanol) step. A compromise is the injection of a maximum aliquot of not more than 10 μL of the extract in n-hexane. This results in well resolved peaks and good accuracy.

Another exception is the HPLC system for β-carotene determinations, since extracts of plasma/serum and foodstuffs often also contain a variety of more polar carotenoids. Direct injection of the extract on a silica column and elution with 1,2-dichloroethane results in a well separated β-carotene peak. However, the more polar carotenoids are eluted much later than β-carotene or not at all. The retention of these carotenoids causes deactivation of the silica, which results in faster elution of β-carotene with each injection. After injection of about ten samples the β-carotene from a control sample is eluted faster and, hence, shows a sharper and higher peak compared to the former control sample. Therefore, separation on a C18 column is necessary since polar carotenoids elute before β-carotene. Consequently, the extracts have to be evaporated and redissolved in the HPLC

solvent prior to injection. Many efforts have been made to avoid this laborious and tedious step. Until now we have failed to find a solvent that extracts carotenoids from the plasma-ethanol and saponification mixtures and can be injected on an C18 column without interfering with the chromatographic process.

The determination of thiamin is another exception. The extract of a blood or a foodstuff sample is directly injected on a radial compression module with a cartridge containing spherical silica particles. Elution with a buffer-methanol mixture results in a well resolved thiamin peak. However, many alternative methods with RP systems are available (Section 2.7).

4. Detection should be as selective as is possible and the sensitivity should be sufficiently high to detect degrees of deficiency (body fluids) and to detect relevant minimum contents in foodstuffs.

Note. To achieve selectivity, first the possible benefits of selective detection, with or without using derivatization reactions, should be incorporated in the method. This markedly reduces sample pre-treatment which is a requirement for running large-scale routine analyses. However, the effect on accuracy and precision of either a derivatization reaction or the more laborious sample pre-treatment should be taken into account.

5. The system should have sufficient ruggedness. Small deviations in specifications of the stationary phase, in the composition or pH of the HPLC eluent or extraction solvents, in injection volume, in flow rate, etc., should not disturb the chromatographic process.

Note. During long-term routine analyses slight modifications of the original methods have occasionally been introduced by us. It is recommended that the ruggedness test (Fisher 1984) be carried out before a method is introduced in the laboratory for routine assays.

6. The columns should have a reasonable life-time. Since the cost of a HPLC column is a minor fraction of the total cost of an analysis, no compromises should be made.

Note. The life-time of the home-packed silica columns for vitamin A and E determinations is very long, viz. over 500 samples. The C18 columns used for the analyses of vitamins C, K_3, B_2 and β-carotene last sufficiently long as well. The life-time of the radial compression module columns used

for the vitamins B_1 and B_6, which contain spherical 7-μm silica and 7 μm C18-bonded silica, respectively, strongly depends on the amount of proteins injected. Therefore, a proper pH control of the extract is necessary.

7. The use of acute toxic and carcinogenic solvents should be avoided as much as possible.

The methods for vitamin analysis developed with the conditions associated with the HPLC system given above taken into account, are presented below as abstracts. For detailed information the bibliographic descriptions of the relevant paper are given, or, if the method is not published, details are given in the relevant appendix.

Vitamin	Matrix	Abstract	Reference
Vitamin A	plasma	3.2.1	Speek et al. 1986a
	tear fluid	3.2.2	Speek et al. 1986b
	foodstuffs	3.2.3	Appendix 1
β-carotene and total carotenoids	plasma	3.3.1	Appendix 2
	foodstuffs	3.3.2	Appendix 1, Speek et al. 1986c
Vitamin E	plasma	3.4.1	Appendix 3
	seed oils	3.4.2	Speek et al. 1985
	foodstuffs	3.4.3	Appendix 1
Vitamin C	blood	3.5.1	Speek et al. 1984a
	foodstuffs	3.5.2	Speek et al. 1984b
vitamin K_3	feed	3.6.1	Speek et al. 1984c
vitamin B_1	blood	3.7.1	Appendix 4 and Schrijver & Speek 1982
	foodstuffs/urine	3.7.2	Appendix 4
vitamin B_2	blood	3.8.1	Speek et al. 1982
	foodstuffs/urine	3.8.2	Appendix 5
vitamin B_6	blood	3.9.1	Schrijver & Speek 1981
	foodstuffs	3.9.2	Appendix 6

3.2. METHODS OF ANALYSIS OF VITAMIN A

3.2.1 Microdetermination of vitamin A in human plasma using high-performance liquid chromatography with fluorescence detection

A.J. Speek, C. Wongkham, N. Limratana, S. Saowakontha and W.H.P. Schreurs. Journal of Chromatography, Biomedical Applications 382 (1986) 284-289.

Vitamin A has an influence on metabolic processes and plays a specific role in vision. Also, a possible role in the prevention and therapy of cancer is acknowledged. Furthermore, in many developing countries vitamin A deficiency occurs among children during the weaning period, which constitutes a public health problem owing to the possibly resulting xerophthalmia, which may cause blindness.

A fast and sensitive high-performance liquid chromatographic method for the determination of vitamin A (all-trans retinol) in 5 µL human plasma is described. After dilution with 95 µL saline the solution is deproteinized with ethanol and extracted with n-hexane. The retinol in the n-hexane layer is separated on a silica column eluted with a mixture of n-hexane, methylene chloride and n-propanol (90:10:1) and detected fluorometrically (333-470 nm). Vitamin A can be determined in concentrations as low as 40 nmol/L. A single analysis can be completed in 12 min while the analysis of a series of 60 samples takes about 8 h. The within-assay and between-assay coefficients of variation were 3.9 and 5.0 % respectively. The between-assay analytical recovery of retinol added to plasma was 97.0 ± 6.8 % (mean ± SD).

The small volume in assay permits incorporation of vitamin A analyses in field surveys of children of which only a few capillaries filled with whole blood are available. Retinol was determined in plasma of Thai children in the age range 3 - 6 years. Data are given in the legend of Fig. 10.

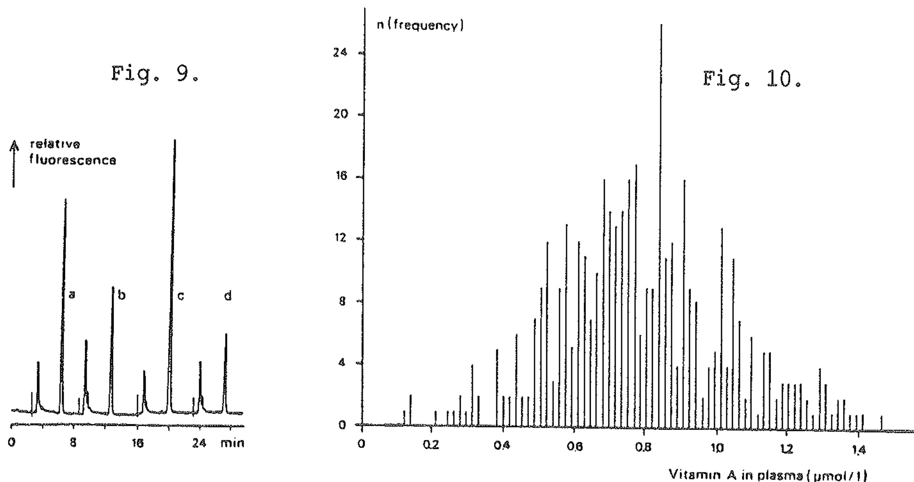


Fig. 9. Typical HPLC elution profiles of the working standard solution (a, 50 nmol/L) and of n-hexane extracts of plasma samples of Thai children (b, c and d: 1.19, 2.55 and 0.74 µmol/L vitamin A in plasma, respectively.

Fig. 10. Frequency distribution of the vitamin A concentration in plasma of Thai children in the age range 3 - 6 years. Total range: 0.12 - 1.47 µmol/L; average, 0.79 µmol/L; n = 429.

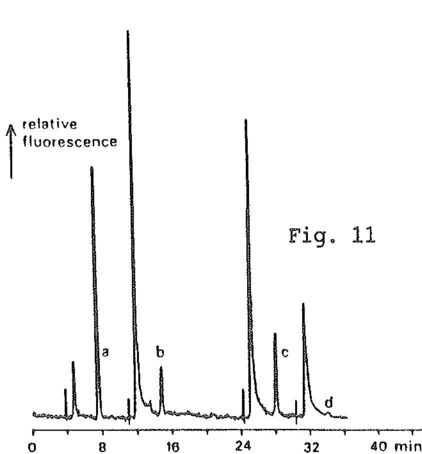
3.2.2 Fluorometric determination of retinol in human tear fluid using high-performance liquid chromatography

A.J. Speek, E.J. van Agtmaal, S. Saowakontha, W.H.P. Schreurs and N.J.van Haeringen. Current Eye Research 5 (1986) 841-845.

Tear fluid refers to the fluid present as the precorneal film and in the conjunctival sac. It mainly serves to protect the eye from exogenous antigenic substances and to maintain the structural integrity of the cornea and conjunctival sac. It has not been elucidated as yet whether tear fluid retinol levels correlate with plasma levels and/or clinical eye examination data.

A fast and sensitive high-performance liquid chromatographic method for the determination of vitamin A (all-trans retinol) in 50 µL human tear fluid is described. After deproteinization with ethanol and extraction with n-hexane retinol in the n-hexane layer is separated on a silica column eluted with a mixture of n-hexane, methylene chloride and n-propanol (90:10:1, v/v/v) and detected fluorometrically (333-470 nm). Retinol can be determined in concentrations as low as 0.4 ng/mL. A single analysis can be completed in 12 min while the analysis of a series of 50 samples takes about 8 h. The within-assay and between-assay coefficients of variation are 3.1 and 4.2 %, respectively. The between-assay analytical recovery of retinol added to tear fluid is 97.4 ± 6.0 % (mean ± SD).

Retinol was determined in tear fluid of nine adult volunteers. The amounts observed ranged from < 0.4 - 10.6 ng/mL.



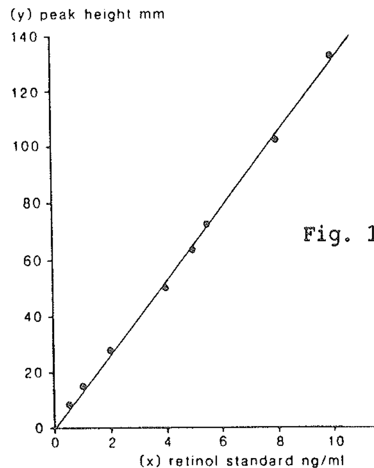


Fig. 11. Typical HPLC elution profiles of the working standard solution (a, 5.5 ng/mL) and of n-hexane extracts of human tear fluid samples (b and c, 3.3 and 5.3 ng/mL respectively). Peak d is all-trans retinol from an extract of a tear fluid sample (1.9 ng/mL) diluted with saline to a concentration of 0.4 ng/mL (detection limit).

Fig. 12. Calculated regression curve for the peak height [y] and the retinol concentration in n-hexane [x] for the concentration range 0.17 - 10 ng/mL. The regression equation was [y] = 12.99 [x] (SD = 0.121; $p < 0.0001$). This corresponds to a tear fluid all-trans retinol concentration range of 0.51 - 30 ng/mL.

3.2.3 Determination of total vitamin A, total carotenoids and β-carotene and E vitamers in foodstuffs, pharmaceutical preparations and body tissues by high-performance liquid chromatography

Laboratory method of TNO-CIVO Institute Toxicology and Nutrition, Department of Clinical Biochemistry

After sample digestion and hydrolysis of their esters by alkaline saponification the vitamins are extracted in diisopropyl ether. This extract quantitatively contains the fat-soluble vitamins A, E, and the carotenoids.

All-trans retinol in the extract is separated on a silica column eluted with a mixture of n-hexane, methylene chloride and n-propanol (90:10:1) and detected fluorometrically (333-470 nm). Vitamin A can be determined in concentrations as low as 0.2 μg/g. The between-assay coefficient of variation is 4.1 %. The between-assay analytical recovery of retinol added to foodstuffs is 98.2 % ± 7.2 (mean ± SD).

Total carotenoids in the extract is determined spectrophotometrically by measurement of the absorbance at 445 nm and expressed as β-carotene. Total carotenoids can be determined in concentrations as low as 0.2 μg/g. The between-assay coefficient of variation is 8.1 %. The between-assay analytical recovery of β-carotene added to foodstuffs, measured by spectrophotometry prior to HPLC, ranged from 95 to 99 %.

β-Carotene in the extract is separated on a C18 column eluted with a mixture of methanol, acetonitril, chloroform and water (200:250:90:11, v/v) and determined by measurement of its absorbance at 445 nm. β-carotene can be determined in concentrations as low as 0.2 μg/g. The between-assay coefficient of variation is 8.6 %. The between-assay analytical recovery of β-carotene added to foodstuffs measured by HPLC ranged from 94 to 98 %.

E vitamers are separated on a silica column eluted with a mixture of n-hexane and diisopropyl ether (95:5, v/v) containing 1 mg BHT per liter and detected fluorometrically (296-320 nm). E vitamers can be determined in concentrations as low as 0.5 μg/g per vitamer. The between-assay coefficients of variation for the E vitamers ranges from 3.1 % to 9.4 %. The between-assay analytical recovery of α-tocopherol added to foodstuffs is 96.6 % ± 6.2 (mean ± SD).

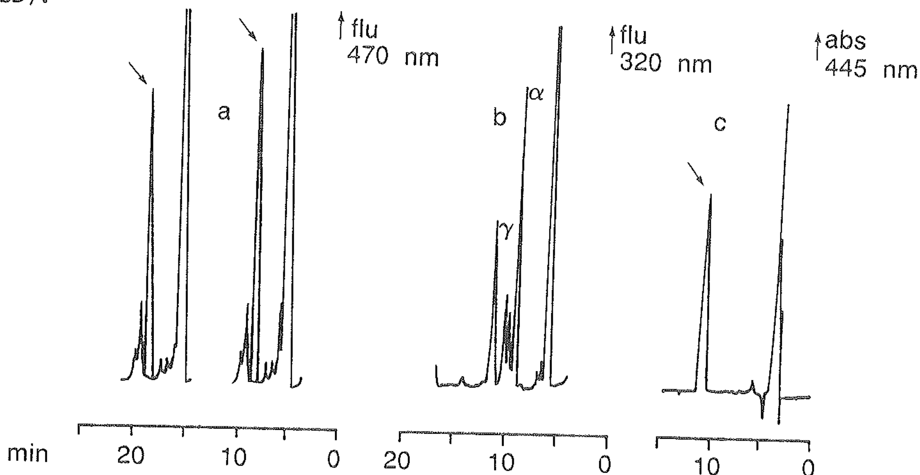


Fig. 13. Typical HPLC elution profiles of foodstuff extracts for analysis of a: all-trans retinol (arrow) in a complete meal, b: α- and γ-tocopherol in a biscuit and c: β-carotene (arrow) in a dog food.

3.3. METHODS OF ANALYSIS OF TOTAL CAROTENOIDS AND β-CAROTENE

3.3.1 Determination of total carotenoids and β-carotene in human plasma using spectrophotometry and high-performance liquid chromatography.

Laboratory method of TNO-CIVO Institute Toxicology and Nutrition, Department of Clinical Biochemistry

A variety of provitamin A carotenoids, of which β-carotene is a main constituent, occurs in human tissue and plasma. The intestinal cleavage of these compounds to form vitamin A-active retinoids represents a major contribution to nutrition.

A fast and sensitive method for the determination of total carotenoids and β-carotene in human plasma is described. After deproteinization with ethanol the carotenoids are extracted with n-hexane.

Total carotenoids in the extract is determined spectrophotometrically by measurement of the absorbance at 445 nm and expressed as β-carotene. Total carotenoids can be determined in concentrations as low as 0.5 μmol/L. The between-assay analytical coefficient of variation is 9.8 %. The between-assay analytical recovery of β-carotene added to plasma, measured by spectrophotometry prior to HPLC, ranged from 94 to 102 %.

β-carotene in the n-hexane layer is separated on a C18 column eluted with a mixture of methanol, acetonitril, chloroform and water (200:250:90:11, v/v) and determined by measurements of its absorbance at 445 nm. β-carotene can be determined in concentrations as low as 0.5 μmol/L. The between-assay analytical coefficient of variation is 10.3 %. The between-assay recovery of β-carotene added to plasma measured by HPLC ranged from 95 to 99 %.

A single analysis of total carotenoids and β-carotene can be completed in 30 min, while the analysis of a series of 15 samples takes about 8 h.

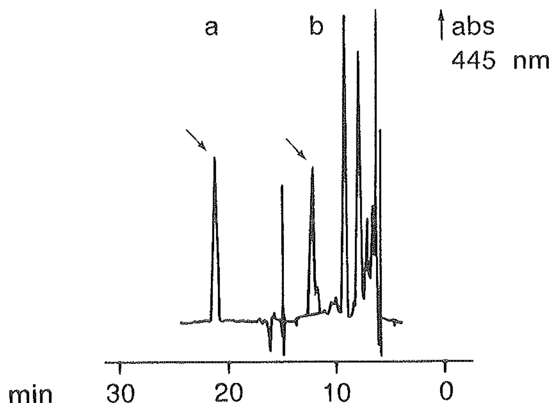


Fig. 14. Typical HPLC elution profiles of the working standard solution (a, 2 μmol/L) and (b) of an n-hexane extract of a human plasma sample. The arrow indicates β-carotene.

3.3.2 Determination of total carotenoids and β-carotene in foodstuffs, pharmaceutical preparations and body tissues by high-performance liquid chromatography

Laboratory method of TNO-CIVO Institute Toxicology and Nutrition, Department of Clinical Biochemistry

This method is given in Appendix 1 (abstract in Section 3.2.3).

3.3.3 Determination of β-carotene content and vitamin A-activity of vegetables by high-performance liquid chromatography and spectrophotometry

A.J. Speek, C.R. Temalilwa and J. Schrijver. Food Chemistry 19 (1986) 65-74.

Carotenoids, several of which has vitamin A-activity, occur invariably in photosynthetic tissues and are responsible for the colour of many vegetables and fruits. The intestinal cleavage of these compounds to form vitamin A-active retinoids represents their major contribution to nutrition.

A fast and sensitive high-performance liquid chromatographic method for the determination of the provitamin A, β-carotene, in vegetables is described. After alkaline saponification and organic extraction β-carotene is separated from other carotenoids on a C18 column eluted with a mixture of methanol, acetonitril, chloroform and water (200:250:90:11, v/v) and determined by measurement of its absorbance at 445 nm. The total amount of carotenoids is determined by measurements of the absorbance at 445 nm of the extract prior to HPLC. β-carotene can be determined in concentrations as low as 0.2 µg/g. The between-assay coefficient of variation is circa 8.6 %. The between-assay analytical recovery of β-carotene added to vegetables ranged from 95 to 100 %.

Results are presented of the analysis of β-carotene and total carotenoids in some vegetables bought at a local market in Dar es Salam. The percentage β-carotene, of the total carotenoid content, showed a great variation. The vitamin A-activity of the vegetables was calculated from the analytical results using the in vivo conversion factors given by the WHO (1982).

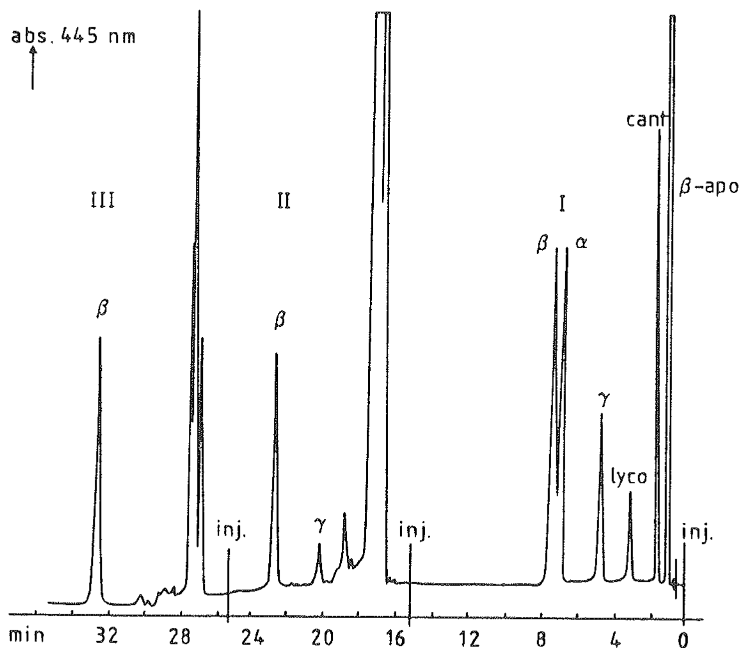


Fig. 15. Typical HPLC elution profiles of the working standard solution (I) spiked with α- and γ-carotene, canthaxanthin, β-apo-8'-carotenal and lycopene, and of extracts of pumpkin (II) and dried potato leaves (III).

3.4 METHODS OF ANALYSIS OF E VITAMERS

3.4.1 Fluorometric determination of α-, β- and γ-tocopherol in human plasma or serum by high-performance liquid chromatography

Laboratory method of TNO-CIVO Institute Toxicology and Nutrition, Department of Clinical Biochemistry

The several E vitamers differ in biological activity. α-Tocopherol, and in a lesser extent γ-tocopherol, occur in human plasma. The mechanism of action of E vitamers is not yet fully understood. Actually known is the antioxidant effect both in the body and in edible oils.

A fast and sensitive method for the determination of α- and γ-tocopherol in human plasma is described. After deproteinization with ethanol the E vitamers are extracted with n-hexane. α-, β- and γ-Tocopherol in the n-hexane layer are separated on a silica column eluted with a mixture of n-hexane and diisopropyl ether (10:1, v/v) containing 1 mg BHT per liter and detected fluorometrically (296-320 nm). α-Tocopherol can be determined in concentrations as low as 1 μmol/L. A single analysis can be completed in 15 min while the analysis of a series of 40 samples takes about 8 h. The between-assay coefficient of variation of α-tocopherol analysis is 6.1 %. The between-assay analytical recovery of α-tocopherol added to plasma ranged from 96 to 101 %.

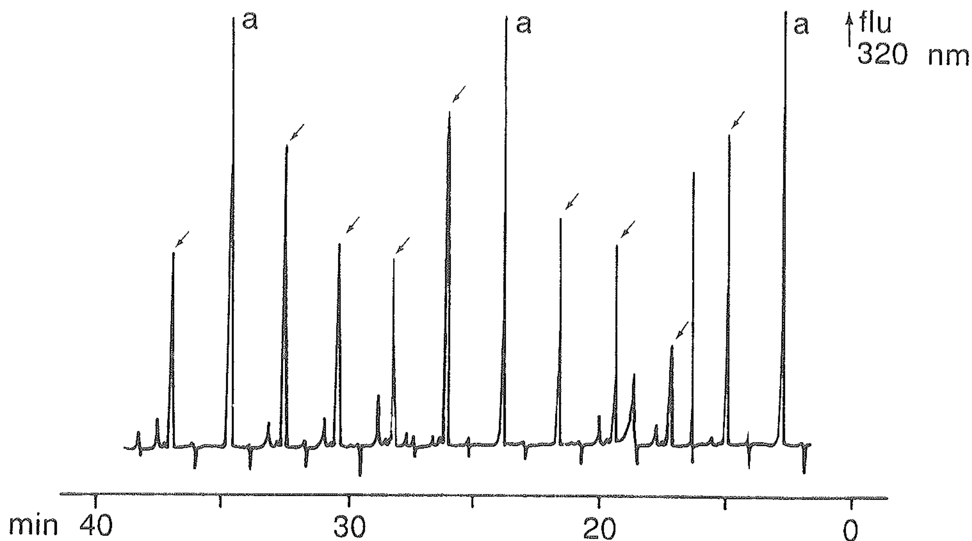


Fig. 16. Typical HPLC elution profiles of the working standard solution (a) and of n-hexane extracts of human plasma samples. The arrow indicates α-tocopherol.

3.4.2 Determination of E vitamers in foodstuffs, pharmaceutical preparations and body tissues by high-performance liquid chromatography

Laboratory method of TNO-CIVO Institute Toxicology and Nutrition, Department of Clinical Biochemistry

This method is given in Appendix 1 (abstract in Section 3.2.3).

3.4.3 Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorescence detection

A.J. Speek, J. Schrijver and W.H.P. Schreurs. Journal of Food Science 50 (1985) 121-124.

The vitamin E group includes several tocopherols, tocotrienols, isomers and derivatives that differ in biological activity. The biological activities of α-, β-, γ- and δ-tocopherol and α-tocotrienol are in the ratio 10:4:1:0.1:3. The mechanism of action of E vitamers is not yet fully understood. Actually known is the antioxidant effect both in the body and in oils.

A high-performance liquid chromatographic method is described for the simultaneous analysis of the E vitamers α-, β-, γ- and δ-tocopherol and α-tocotrienol in seed oils. After diluting the oils with n-hexane, E vitamers are separated on a silica column eluted with a mixture of n-hexane and diisopropyl ether (10:1, v/v) containing 1 mg BHT per liter and detected fluorometrically (296-320 nm). Standardization is achieved with the aid of electron-impact mass spectrometry and HPLC. E vitamers can be determined in concentrations as low as 0.5 μg/g per vitamer. The within-assay and between-assay coefficients of variation for the 4 vitamers ranged from 2.9 % to 9.4 %. The between-assay analytical recoveries of α- and γ-tocopherol and α-tocotrienol added to maize germ oil were 93, 94 and 95 %, respectively.

Vitamin E composition of several hot and cold pressed seed oils, originating from maize germs, olives, soy beans and sesame, safflower and sunflower seeds, was investigated. No clear differences were observed between E vitamer concentrations of hot and cold pressed oils of the same origin. On the other hand, vitamin E composition of oils of different origin varied widely. Of the oils examined, only maize germ oil contained α-tocotrienol in detectable amounts (about 2 %). Esterified vitamers were not detected.

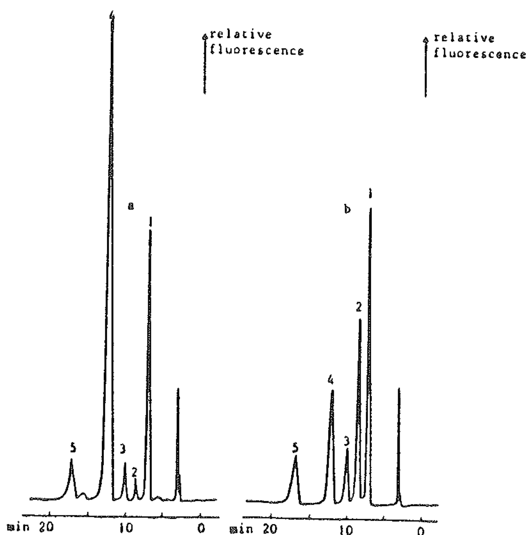


Fig. 17. Typical HPLC elution profiles of a diluted maize germ oil sample (a) and of the working standard solution (b): 1, α-tocopherol; 2, α-tocotrienol; 3, β-tocopherol; 4, γ-tocopherol; 5, δ-tocopherol.

3.5 METHODS OF ANALYSIS OF VITAMIN C

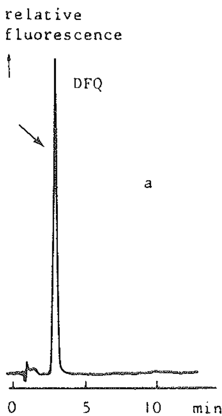
3.5.1 Fluorometric determination of total vitamin C in whole blood by high-performance liquid chromatography with pre-column derivatization

A.J. Speek, J. Schrijver and W.H.P. Schreurs. Journal of Chromatography, Biomedical Applications 305 (1984) 53-60.

Vitamin C in human blood consists of L-ascorbic acid and its oxidized form dehydro-L-ascorbic acid. Both forms are equally biological active.

A semi-automated high-performance liquid chromatographic method is described for the determination of total vitamin C in whole blood. After deproteinization of whole blood and enzymatic oxidation of L-ascorbic acid to dehydro-L-ascorbic acid, the latter is condensed with o-phenylene diamine to its quinoxaline derivative. This derivative is separated on a C18 column eluted with a mixture of 0.08 M phosphate buffer and methanol at pH = 7.8 and detected fluorometrically (355-425 nm). Routine vitamin C determinations can be carried out in a series of 100 samples within 48 h. Total vitamin C in whole blood can be determined in concentrations as low as 0.2 µmol/L. The within-assay and between-assay coefficients of variation were 3.7 % and 4-.6 %, respectively. The between-assay analytical recovery of L-ascorbic acid added to whole blood samples was 97.0 ± 7.0 % (mean ± SD). References values of vitamin C in whole blood of normal healthy Dutch adults were found in the range 20 - 80 µmol/L.

Special attention was paid to the stability of vitamin C in whole blood and of its quinoxaline derivative in the extract. Result showed that total vitamin C in whole blood is stable for eight days at -20 °C, provided ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid and glutathione are immediately added to the blood sample. The quinoxaline derivative of vitamin C in blood extracts is stable for at least 24 h if stored in the dark at 4 °C.



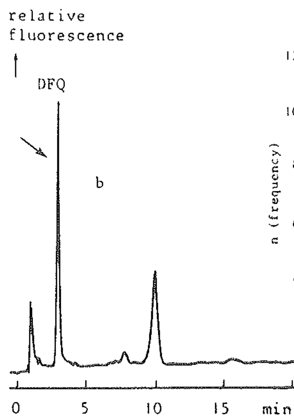


Fig. 18. Typical HPLC elution profile of an extract of a whole blood sample, arrow: vitamin C quinoxaline derivative.

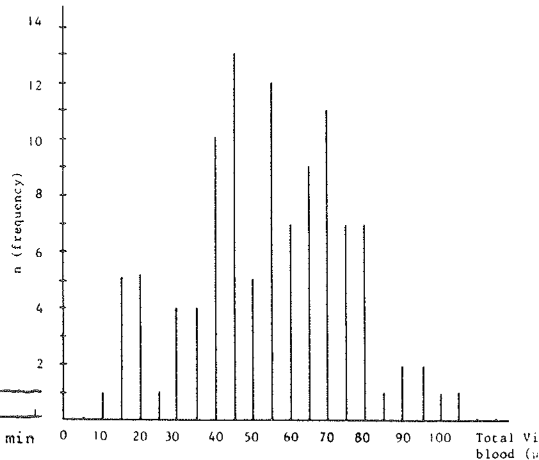


Fig. 19. Frequency distribution of the concentration of total vitamin C in whole blood of healthy Dutch adults, mean = 54 µmol/L (n = 108).

3.5.2 Fluorometric determination of total vitamin C and total isovitamin C in foodstuffs and beverages by high-performance liquid chromatography with pre-column derivatization

A.J. Speek, J. Schrijver and W.H.P. Schreurs. Journal of Agricultural and Food Chemistry **32** (1984) 352-355.

Ascorbic acid (AA) is widely distributed in high concentrations, particularly in citrus fruits and green vegetables. The C-5 epimer of AA, isovitamin C or erythorbic acid (EA), which is much less biological active, may be used as an antioxidant in foods and beverages.

A sensitive high-performance liquid chromatographic method is described for the simultaneous determination of total vitamin C (L-ascorbic acid plus dehydro-L-ascorbic acid, dHAA) and its C-5 epimer, total isovitamin C (erythorbic acid plus dehydro-erythorbic acid, dHEA), in foodstuffs and beverages. After extraction AA and EA are oxidized enzymatically to dHAA and dHEA with the aid of ascorbate oxidase (EC 1.10.3.3). The latter compounds are condensed with o-phenylene diamine to their highly fluorescent quinoxaline derivatives. These derivatives are separated on a C18 column eluted with a mixture of 0.08 M phosphate buffer and methanol at pH = 7.8 and detected fluorometrically (355-425 nm). The total vitamin C and isovitamin C can be determined in concentrations as low as 0.2 μg/g per vitamin. The within-assay coefficient of variation of the analysis of total vitamin C in a complete meal was 2.1 %. The between-assay analytical recovery of AA and EA added to a complete meal were 97.1 and 99.3 % respectively. The amounts of dHAA and dHEA present in foodstuffs and beverages can be determined separately by the same procedure with omission of the enzymatic oxidation.

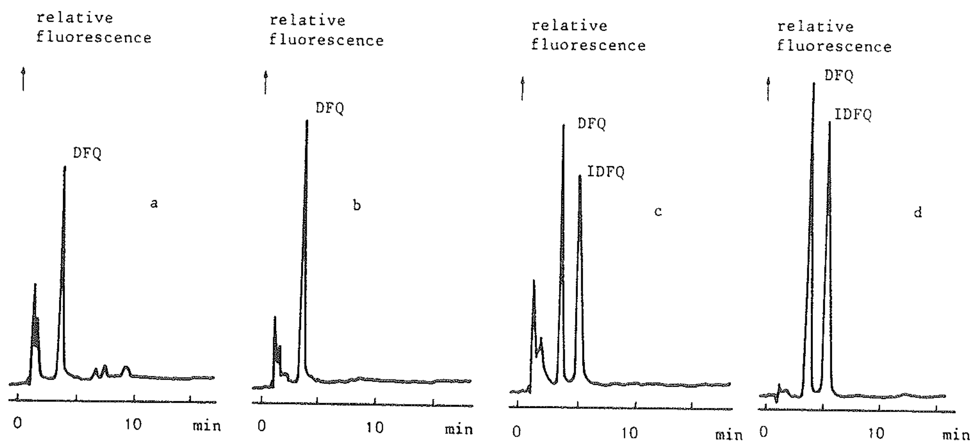


Fig. 20. Typical HPLC elution profiles of a derivatized fresh green paprica extract (a), of a diluted orange juice (b), a diluted orange juice with added erythorbic acid (c) and of the working standard solution (d). DFQ and IDFQ: quinoxaline derivatives of dHAA and dHEA, respectively.

3.6 METHODS OF ANALYSIS OF VITAMIN K_3

3.6.1 Fluorometric determination of menadione sodium bisulphite (vitamin K_3) in animal feed and premixes by high-performance liquid chromatography with post-column derivatization

A.J. Speek, J. Schrijver and W.H.P. Schreurs. Journal of Chromatography 301 (1984) 441-447.

The biologically active vitamin K forms phylloquinone (vitamin K_1) and the menaquinones (vitamin K_2 series) play an important role in blood coagulation and bone mineralization. Since vitamin K deficiency frequently occurs in young poultry, the feed is enriched with menadione sodium bisulphite (vitamin K_3) which is a precursor of the biologically active menaquinone-4.

A high-performance liquid chromatographic method for the determination of menadione sodium bisulphite (vitamin K_3) in animal feed and premixes is described. After aqueous extraction, the vitamin is converted into menadione, which is extracted and separated on a C18 column eluted with a mixture of water and ethanol (4:6, v/v). After menadione has been reduced in a post-column reaction coil, fluorometric measurement (325-425 nm) of the reduced vitamin permits determination of menadione sodium bisulphite at concentrations as low as 0.02 μg/g. The within-coefficient of variation of the method applied to feeds is 6.5 %. The within-assay analytical recovery of menadione sodium bisulphite added to feeds is 94.4 ± 6.8 % (mean ± SD).

Several types of animal feed has been analysed according to the method described and also with the European Community colorimetric method. From high concentration levels down to circa 15 μg/g the results obtained with the two methods are in good agreement. For concentrations in the range 1-3 μg/g (complete diets) a tendency to lower results by the HPLC method is observed. The detection limit of the HPLC method is considerably lower than that of the European Community colorimetric method.

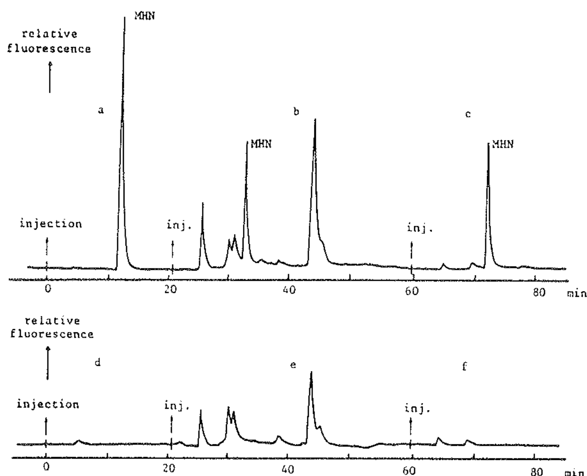


Fig. 21. Typical HPLC elution profiles of (a) the working standard solution (1 μg/mL), (b) of an extract of a cat food (0.1 μg/g) and (c) of a premix (180 μg/g); (d), (e) and (f) are elution profiles of the samples (a), (b) and (c), respectively, with the derivatization reagent replaced by HPLC mobile. MHN = 2-methyl-1,4-dihydroxy-naphthalene.

3.7 METHODS OF ANALYSIS OF VITAMIN B_1

3.7.1 Reliable semi-automated method for the determination of total thiamin in whole blood by the thiochrome method with high-performance liquid chromatography

J. Schrijver, A.J. Speek and W.H.P. Schreurs. Annals Clinical Biochemistry 19 (1982) 52-56.

Vitamin B_1 (thiamin) is mainly present in blood as its biological active form thiamin diphosphate. This compound takes part as a coenzyme in a number of reactions catalysed by enzyme systems in the metabolism of carbohydrates.

A sensitive method for the determination of total thiamin in whole blood has been developed which is suited for routine analysis. After extraction, and enzymatic hydrolysis of thiamin phosphate esters, thiamin is separated on a silica column eluted with a mixture of sodium and potassium phosphate and ethanol. Thiamin is converted to thiochrome in a post-column reaction coil. The latter compound is detected fluorometrically (367 - >430 nm). By calculating the concentration of thiamin on-line with the aid of a computer, it is possible to complete one analysis within 4 h. Routine thiamin determinations can be carried out in a series of 120 samples within 48 h. The within-assay and between-assay coefficients of variation of the analysis of total thiamin in whole blood are 4.2 and 4.4 %, respectively. The between-assay analytical recovery of thiamin diphosphate added to whole blood samples is 99.9 $\pm$ 1.7 % (mean $\pm$ SD).

The HPLC method described has been applied to the analysis of thiamin in plasma and erythrocytes. In agreement with other reports, it was found that about 90 % of total thiamin is present in the erythrocytes. Reference values of thiamin in human blood were found to be in the range 95 - 155 nmol/L with a mean value of 115 nmol/L.

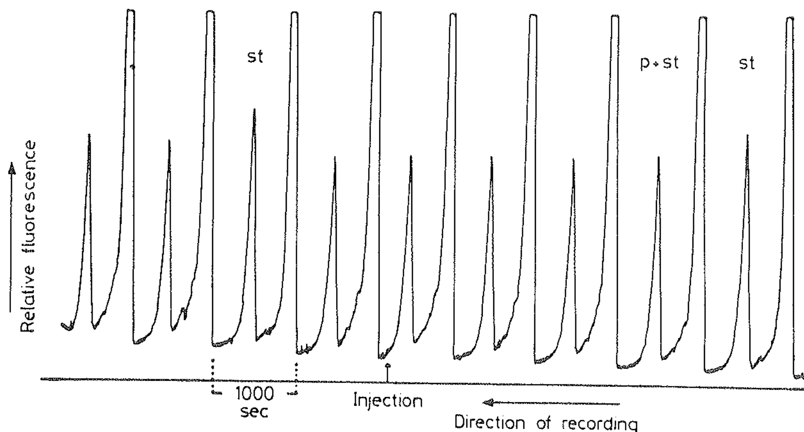


Fig. 22. Typical HPLC elution profiles of the working standard solution and of whole blood extracts. Concentrations of total thiamin are given in nmol/L. st: standard solution; p + st: blood sample with added thiamin diphosphate.

3.7.2 Determination of total thiamin in foodstuffs and urine by high-performance liquid chromatography with post-column reaction fluorometric detection

Laboratory method of TNO-CIVO Institute Toxicology and Nutrition, Department of Clinical Biochemistry

Foodstuff samples are extracted with hydrochloric acid. After adjustment of the pH and enzymatic hydrolysis of thiamin phosphate esters, thiamin is separated on a silica column eluted with a mixture of sodium and potassium phosphate and ethanol. Thiamin is converted to thiochrome in a post-column reaction coil. The latter compound is detected fluorometrically (367- >430 nm). A single analysis can be completed within 4 h. Routine thiamin determinations can be carried out in a series of 20 samples within 24 h. The within-assay coefficient of variation of the analysis of total thiamin in foodstuffs is 3.4 %. The between-assay analytical recovery of thiamin diphosphate added to foodstuffs samples is 96.9 ± 6.8% (mean ± SD).

Acidified urine samples are buffered and centrifuged. The thiamin in the resulting solutions is separated and detected as described above for foodstuff samples. A single analysis can be completed within 20 min. Routine thiamin determinations can be carried out in a series of 50 samples within 24 h. The within-assay coefficient of variation of the analysis of thiamin in urine is 5.4 %. The between-assay analytical recovery of thiamin added to urine samples is 94.6 ± 7.4% (mean ± SD).

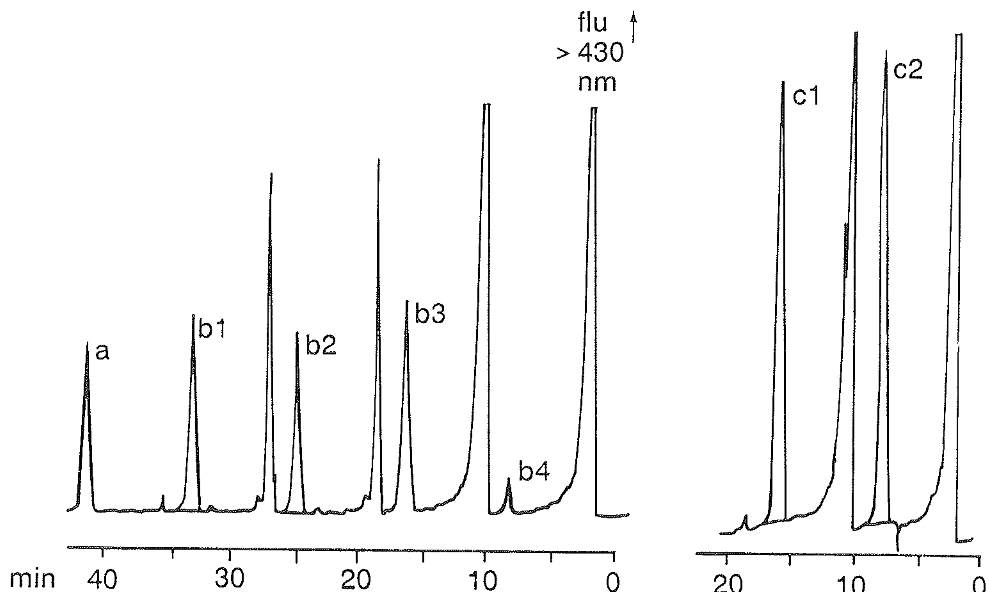


Fig. 23. Typical HPLC elution profiles of (a) a thiamin working standard solution, (b1-b4) of extracts of foodstuff samples (complete meals) and (c1-c2) of buffered acidified urine samples.

3.8 METHODS OF ANALYSIS OF VITAMIN B_2

3.8.1 Determination of the B_2 vitamer flavin adenine dinucleotide in whole blood by high-performance liquid chromatography with fluorometric detection

A.J. Speek, F. van Schaik, J. Schrijver and W.H.P. Schreurs. Journal of Chromatography, Biomedical Applications **228** (1982) 311-316.

In the gut riboflavin (Rb) taken up with food is phosphorylated to flavin mononucleotide (FMN) by the intestinal mucosa during absorption. In tissue cells FMN can be converted to the biologically active B_2 vitamer flavin adenine dinucleotide (FAD). FMN and FAD serve as cofactor in most of the oxidation-reduction reactions catalysed by the flavin enzymes.

A high-performance liquid chromatographic method for the determination of FAD in whole blood is described. Blood is deproteinized by addition of trichloroacetic acid. After adjustment of the pH and centrifugation, FAD in the supernatant is separated from FMN, Rb and interfering compounds on a C18 column eluted with a mixture of 0.3 M phosphate buffer and 16.7 % (v/v) methanol and detected fluorometrically (470-525 nm). FAD can be determined in concentrations as low as 20 nmol/L. Also FMN and Rb are detected although the sensitivity is not sufficient to analyse accurately the low endogenous levels. The analysis of a series of 40 samples takes about 24 h. The within-assay and between-assay coefficients of variation are 2.9 and 4.4 % respectively. The between-assay analytical recovery of FAD added to blood is 97.6 % $\pm$ 3.8 (mean $\pm$ SD).

FAD has been determined in the blood of healthy Dutch volunteers. A total range of 260 - 390 nmol/L was found with a mean value of 310 nmol/L (n = 70).

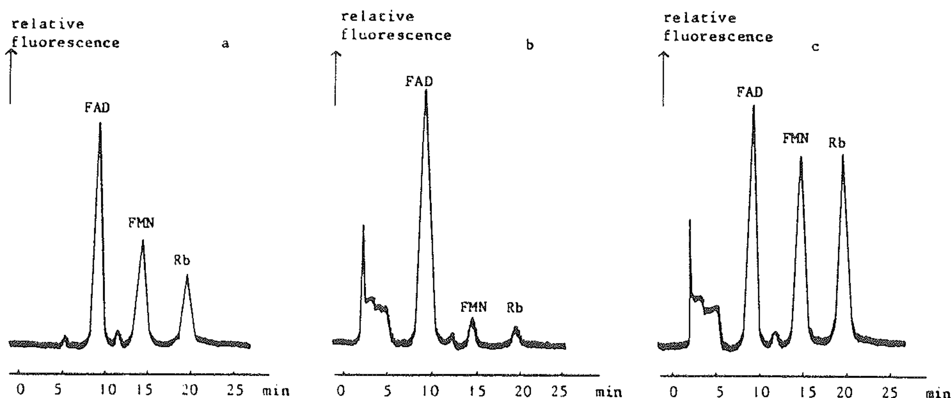


Fig. 24. Typical HPLC elution profiles of the B_2 vitamers from (a) the working standard solution, (b) a whole blood sample and (c) a whole blood sample obtained from a healthy adult volunteer 12 h after supplementation with 60 mg of FMN.

3.8.2 Determination of total riboflavin in foodstuffs and urine by high-performance liquid chromatography with fluorometric detection

Laboratory method of TNO-CIVO Institute Toxicology and Nutrition, Department of Clinical Biochemistry

Foodstuff samples are extracted with hydrochloric acid. After adjustment of the pH and enzymatic hydrolysis of flavins, riboflavin is separated on a C18 column eluted with a mobile phase consisting of 20 % (v/v) methanol in water and detected fluorometrically (462-520 nm). A single analysis can be completed within 4 h. Routine riboflavin determinations can be carried out in a series of 20 samples within 24 h. The within-assay coefficient of variation of the analysis of riboflavin in foodstuffs is 2.6 %. The between-assay analytical recovery of riboflavin added to foodstuffs samples is 93.7 ± 5.9 % (mean ± SD).

Acidified urine samples are diluted with hydrochloric acid, buffered and centrifuged. The riboflavin in the resulting solutions is separated and detected as described above for foodstuff samples but with a reduced flow-rate. A single analysis can be completed within 20 min. Routine riboflavin determinations can be carried out in a series of 40 samples within 24 h. The within-assay coefficient of variation of the analysis of riboflavin in urine is 6.3%. The between-assay analytical recovery of riboflavin added to urine samples is 96.6± 8.1% (mean ± SD).

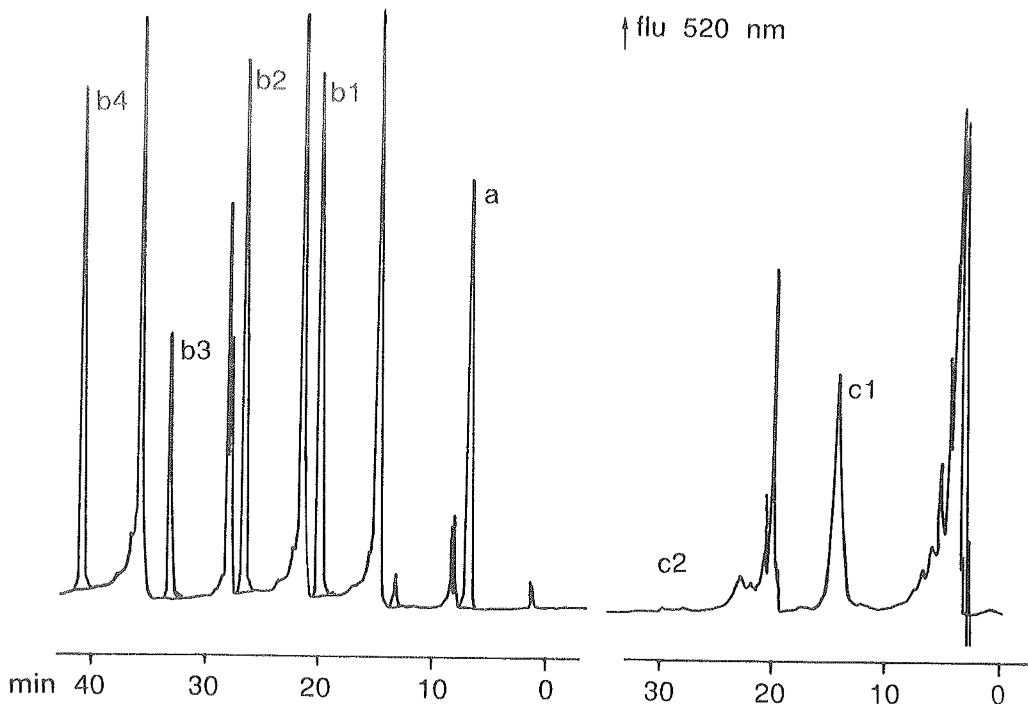


Fig. 25. Typical HPLC elution profiles of (a) a riboflavin working standard solution, (b1-b4) of extracts of foodstuff samples (complete meals) and (c1-c2) of two diluted urine sample.

3.9 METHODS OF ANALYSIS OF VITAMIN B_6

3.9.1 Semi-automated fluorometric determination of pyridoxal 5′-phosphate (vitamin B_6) in whole blood by high-performance liquid chromatography

J. Schrijver, A.J. Speek and W.H.P. Schreurs. International Journal of Vitamin and Nutrition Research 51 (1981) 216-222.

Vitamin B_6 is the generic term for the three naturally occurring nonphosphorylated (pyridoxine, pyridoxal and pyridoxamine) and the 5′-phosphorylated forms of the vitamin B_6 family. The biologically most active member, pyridoxal 5′-phosphate (PLP), is the cofactor directly involved in the cellular amino acid metabolism.

A sensitive method for the determination of PLP in blood is described. After acid extraction PLP is separated on a C18 column eluted with 0.05 M phosphate buffer at pH = 2.9. After reaction of PLP in a post-column reaction coil to its semicarbazone, the latter compound is measured fluorometrically (367-478 nm). By calculating the concentration of PLP on-line with the aid of a computer, it is possible to run 125 samples within 48 h in routine analysis. The within-assay and the between-assay coefficients of variation of the analysis of PLP in whole blood were 3.3 and 4.7 % respectively. The between-assay analytical recovery of PLP added to whole blood was 100.0 ± 7.1 % (mean ± SD).

Reference values of PLP in human whole blood were found to be in the range 55 - 120 nmol/L with an mean value of 80 nmol/L.

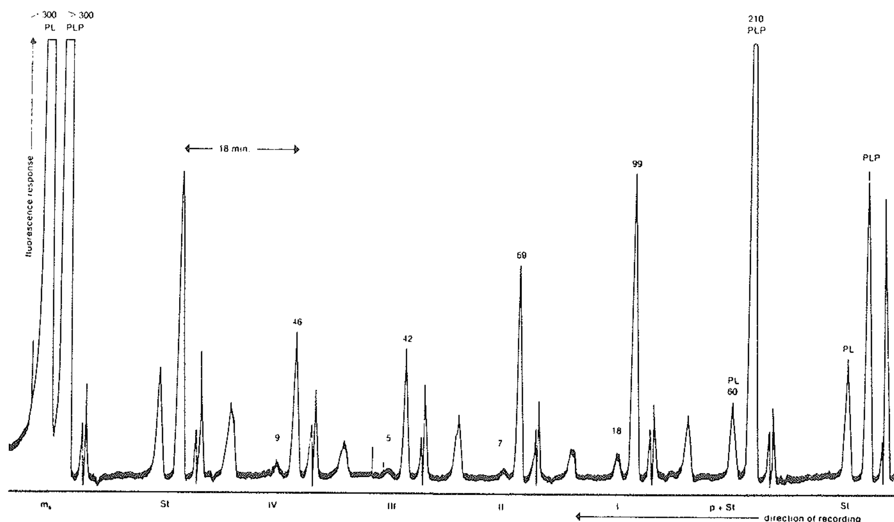


Fig. 26. Typical HPLC elution profiles of (a) the working standard solution and (b) of pyridoxal 5′-phosphate (PLP) and pyridoxal (PL) from human whole blood samples. Concentrations are given in nmol PLP per litre of blood.

3.9.2 Determination of total pyridoxal, pyridoxine and pyridoxamine (vitamin B_6) in foodstuffs by high-performance liquid chromatography with fluorometric detection

Laboratory method of TNO-CIVO Institute Toxicology and Nutrition, Department of Clinical Biochemistry

After addition of internal standard solution, extraction with trichloroacetic acid and adjustment of the pH, phosphorylated B_6 vitamers are hydrolysed enzymatically. The resulting three free forms in the extract, pyridoxine, pyridoxal and pyridoxamine, are separated on a C18 column eluted with a mobile phase consisting of 3 % (v/v) methanol in a mixture of 5 mL Pic-B8 modifier in 0.1 M phosphate buffer. After addition of a phosphate buffer in a post-column reaction coil the enhanced fluorescence is measured (333-375 nm). A single analysis can be completed within 5 h. Routine total vitamin B_6 determinations can be carried out in a series of 20 samples within 24 h. The within-assay coefficients of variation of the analysis of B_6 vitamers in foodstuffs ranges from 5.5 to 7.8 %. The between-assay analytical recoveries of pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate added to foodstuffs samples ranges from 94 to 103 %.

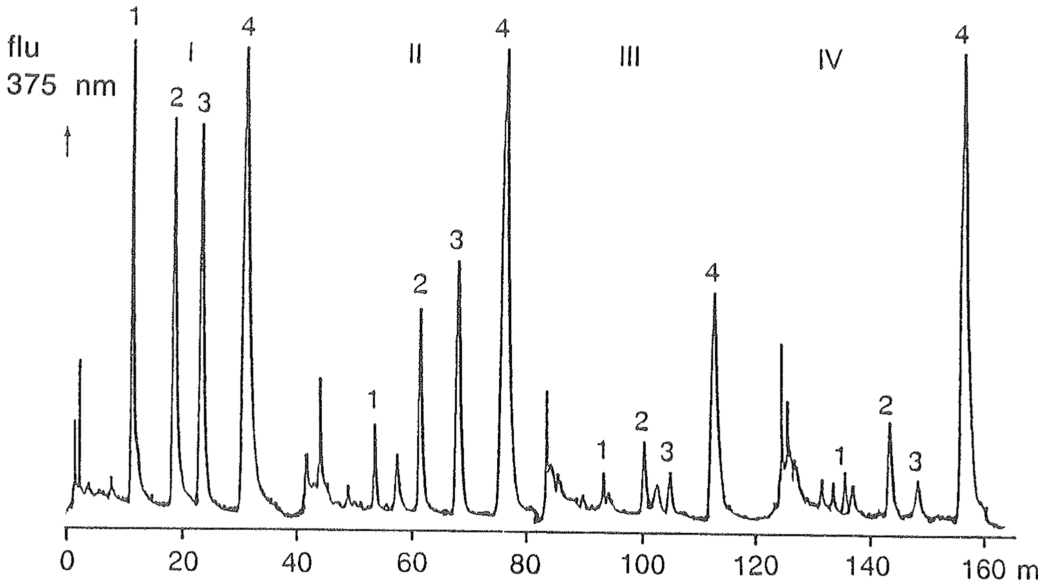


Fig. 27. Typical HPLC elution profiles of the working standard solution (I) with pyridoxal (1), pyridoxine (2), pyridoxamine (3) and 4-deoxypyridoxine (4) and of an extract of banana (II), of potato chips (III) and of corn-flour (IV).

Chapter 4. RELIABILITY OF THE HPLC METHODS FOR VITAMIN ANALYSIS

4.1 Introduction and terminology

Analytical methods are never perfect with regard to reproducibly producing true values. The usefulness of figures resulting from analytical determinations depends on the size of the error in the figures projected on the intended use. Therefore, in this chapter, the reliability (or performance characteristics) of the HPLC methods for vitamin analysis is discussed, i.e. the extent and - if possible - the source of the components forming the total error. Since the nomenclature concerning these performance characteristics is not yet definitively established, the terms used in this chapter are explained below.

The true value is the correct concentration of the analyte in the sample under investigation. By application of the analytical method an analytical figure (data) is obtained. If the analytical method is error-free this analytical figure is equal to the true value. Since it is not, the analytical figure deviates from the true value; the difference is the error. When more than one determination is done the mean value (mean) is obtained. When the number of determinations approximates infinity, the calculated mean value approximates the true mean value, which is the asymptotic value of the distribution function. The bias is the difference between the true mean value and the true value and is a characteristic of the analytical method.

The error is the cumulative effect of several more specified errors, i.e. the random error, the systematic error and possible incidental error(s) (blunder). The random error is the difference between the observed value and the true mean value and is a characteristic of the analytical figure (data). It is governed by the distribution function. If this function is normal (Gaussian) the extent of the random error is reflected by the calculated standard deviation or its relative value, i.e. the coefficient of variation which is the standard deviation divided by the mean. The variance is the dispersion parameter of the normal distribution function and is simply the square of the standard deviation. The systematic error is the non-random part of the error and is a characteristic of the analytical method. When the number of determinations approximates infinity, the systematic error approaches the bias. Incidental errors are outright human mistakes.

An analytical method is regarded as accurate if it is not afflicted with a systematic error or if this error is negligible with regard to the intended use of the analytical figure. The term (in)accuracy quantitatively describes the (lack of) accuracy (systematic error) in an analytical method, it comprises the bias and the imprecision. An analytical method is regarded as being precise if the standard deviation is small with regard to the intended use of the analytical figure. The term (im)precision quantitatively describes the (lack of) precision of an analytical method and is identical to the standard deviation. The repeatability, as measured by the within-assay standard deviation (Section 4.3.2.1) is a measure of the internal variance. An assay shows good repeatability if the data obtained in the same laboratory with the same equipment by the same operator within a short interval of time are comparable, i.e. are within an acceptable small range. The reproducibility as measured by the between-assay standard deviation (Section 4.3.2.1) is a measure of the external variance. An assay shows good reproducibility if comparable results are obtained with the same method but in different laboratories with different equipment by different operators. Since the experiments underlying this thesis are obtained with the same equipment in the same laboratory, our between-assay standard deviations concern the same equipment in the same laboratory used by different operators but within a large time interval (approximately 3 months).

The term reliability (or performance characteristics) quantitatively describes the extent of the error and comprises the systematic error, the random error and possible incidental errors.

In this chapter the reliability of the methods for vitamin analysis by HPLC are discussed with emphasis on accuracy and precision.

Section 4.2 describes and - if possible - estimates some vitamin-, sample- and method-specific factors influencing the accuracy of the HPLC methods for vitamin analysis. For reasons mentioned below, inaccuracies in the methods cannot be estimated unequivocally.

Section 4.3 deals with the observed precisions. The precision data have been calculated on the basis of data resulting from the analysis of quality control samples incorporated in the series of routine analyses of body fluids and foodstuffs carried out lately in our laboratory. A comparison with literature values is made. For certain parts of the methods for body fluids attention is paid to their contribution to imprecision: the extraction step,

the column chromatographic step, the sample volume in assay, the level of the vitamin and the detection using pre- or post-column derivatization. Furthermore, the effect of technicians working in shifts on precision has been investigated. For the methods for foodstuff analysis the contribution of the type of foodstuff, of the moisture content, of the sampling, of the evaporation and dissolution step and of the particle size to the imprecision have been investigated.

In Section 4.4 some frequently occurring incidental errors are mentioned as well as some ways of detecting them.

4.1.1 Use of analytical results of vitamin analysis

Body fluids. Concentrations of vitamins in body fluids are mostly used for diagnosis. Therefore, the value obtained from a patient has to be compared with the so-called reference values (EPTRV 1982). These values are obtained from a large group of healthy persons comparable to the patient. The vitamin values indicative for diagnosis (below normal, borderline, normal and above normal) are calculated from the frequency distribution. A large group of persons is needed since intraspecific variation is large. The method of calculation of the reference values from normal and non-normal distributions has been described by Wayne Bruce (1984), while a distribution-free method is given by Rümke & Bezemer (1972). The reliability of a diagnosis derived from a laboratory result, irrespective of the diagnostic sensitivity of the test, depends on the error in the result.

Foodstuffs. Vitamin concentrations of foodstuff samples are mostly determined on behalf of food consumption inquiries as part of an investigation on deficiency diseases, to establish food composition tables and to determine the stability of the vitamin during processing and/or storage. If the analysis is performed on behalf of a food consumption inquiry, accuracy is essential since the observed values have to be correlated with other observations of the target group. If the analysis serves for establishing food composition tables, reasonable accuracy is required but the precision may be of minor importance since intrafoodstuff variations are large compared to the method's precision.

4.2 Accuracy - systematic error

4.2.1 Introduction

The systematic error is the positive or negative deviation of the calculated mean value from the true value due to an imperfection of the method. In practice the observed difference also comprises a random error.

In clinical analysis the true value is commonly unknown, since a matrix such as a body fluid without the analyte is hard to obtain and since it is usually impossible to incorporate a known amount of the compound of interest in the sample in its natural surroundings, i.e. in the living cell and/or bound to its specific carrier protein. Since foodstuffs mainly consist of materials of plant and animal origin, accuracy tests for vitamin analysis in foods are also afflicted by the lack of samples with known concentration (reference samples). Therefore, the occurrence of a systematic error is difficult to recognize. Often clinically inexplicable results (body fluids) or a large deviation from the expected value (foodstuffs) are indications. Although the analytical chemist, when developing a method, tries hard to detect and eliminate systematic errors, most of the methods suffer from an unknown small systematic error in practice. However, in the analysis of body fluids for diagnostic purposes this error does not matter if it is small and constant for every sample from every patient analysed, and if it is incorporated in the reference values. Therefore, reference values are related to the method applied and sometimes even to the equipment used.

The occurrence of a systematic error also can be detected by means of a comparison study using different analytical techniques.

Due to the lack of reference samples for vitamin analysis in foodstuff as well as in body fluids possible inaccuracies in the HPLC methods for vitamin determinations cannot be established unequivocally. However, some vitamin-, sample- and method-specific factors influencing the accuracy of the HPLC methods have been detected and are discussed below. Some instrument-related factors are discussed as well. Although errors caused by these factors, strictly speaking, do not fit into the definition of systematic error, they are included because they may seriously hamper accuracy in the practice of large-scale routine analysis. Also temporarily occurring systematic errors have been classified as factors afflicting accuracy.

These vitamin-, sample- and method-specific factors influencing accuracy are differences in particle size of analyte (carrier) and matrix, carry-over, isomerization, pellet adsorption, partial extraction, incomplete dephosphorylation, presence of additives, degradation, ghost peaks, interferences and temperature effects. They are described and, as far as possible, quantitated below in order of possible occurrence in the analytical procedure.

4.2.2 Sampling

Sampling of a large amount of material to obtain a representative aliquot for laboratory analysis may cause an inaccuracy as a result of difference in size of the sample and the added vitamin carrier particles.

A large variation, up to a factor of 2, has been observed in the results of total retinol analyses in synthetic dry fodders. The cause appeared to be the difference in size between fodder and added vitamin carrier particles. This synthetic preparation consists of retinyl acetate-coated bran with a particle size of 1 - 1.5 mm, whereas the fodder particle size is below 0.5 mm. The preparation is mixed with the fodder in a concentration of about 3 g/kg. Gentle mechanical shaking makes the smaller fodder particles sink in the container which renders the sample inhomogeneous. Although erroneous results due to inhomogeneity of the sample cannot be considered as a systematic error, we mention this problem since it seriously hampers accuracy (and precision, see 4.3.4.2) of vitamin A determinations in synthetic fodders. Therefore, we recommend to roughly mix a large amount of sample with a spoon and to grind in a Cyclotec type 1093 sample mill at least 30 g of the sample to a particle size below 0.5 mm. An amount of at least a 10 g has to be taken for the assay directly after grinding. In this way the accuracy can be improved (< 8 % of the calculated value) while the within-assay coefficient of variation will be within the range 3.2 - 6.3 % (Table 7a).

4.2.3 Sample pre-treatment

Proportioning 1-mL whole blood aliquots. Carry-over is the measurement of analyte present in the analytical system as a result of analysis of the previous sample. Carry-over via the pipette can be avoided by the use calibrated pipettes with disposable tips. However, the use of such pipettes, operated on air displacement, affects reliability since the amount of blood left on the

inner wall is only constant within a certain range. Since whole blood is a rather viscous liquid, we have tested the use of capillettors equipped with a cylinder with piston (Boehringer, No 286419) for whole blood proportioning. They are advantageous in that, after addition of the 1-mL aliquot to the test tube, virtually no blood remains in the tip. Furthermore, the tip can be used for at least one series of samples per day. We observed in blanks run directly after a blood sample from a person supplemented with thiamin (> 400 nmol/L blood) thiamin peaks corresponding with a whole blood concentration of up to 8 nmol/L. This concentration may be the difference between low/normal and deficient levels. Further investigations revealed the cause to be carry-over via the capillettor, and not via the automatic injector. Therefore, in our laboratory analyses are now routinely repeated for samples analysed directly after a sample with a concentration far above normal has been handled.

Cis/trans isomerization. This phenomenon may occur during sample pretreatment and plays a role in the determinations of the vitamins A, K_1 and β-carotene. Since certain vitamins have a number of double bonds in their structure, several stereo-isomers exist. They often differ in biological activity. A systematic error may occur as a result of cis/trans isomerization during the analytical procedure, i.e. if the isomer formed is separated from the original form on the HPLC column and/or if the detector responds differently. This phenomenon has been observed in the determination of the biologically most active and most abundant vitamin A isomer in foodstuffs, all-trans retinol, since silica is capable of separating geometric isomers on the basis of their difference in polarity. Injection of an aliquot of the all-trans retinol standard solution results in an all-trans retinol peak preceded by a 13-cis retinol peak with a height of less than 1 % of the all-trans peak. Saponification and extraction of the standard solution yield a chromatogram with a 13-cis and an all-trans peak too, but 13-cis is raised to a few per cent of the all-trans peak. Most HPLC analyses of foodstuffs for all-trans retinol produce chromatograms showing a 13-cis retinol peak with a height of a few per cent of the all-trans peak. Since the saponification step is necessary it cannot be established whether the biologically less active 13-cis retinol originates from the sample or from all-trans retinol conversion during the saponification procedure. From the saponification experiment with the standard solution mentioned one may assume that the small amount of 13-cis retinol appearing in the foodstuff chromatograms originates, at least

largely, from originally present <u>all-trans</u> retinol. This means that cis/trans isomerization causes a small (< 2 %) negative systematic error in HPLC analysis of <u>all-trans</u> retinol in foodstuffs. This small and probably constant systematic error can be avoided by using a saponified standard solution as working standard. However, uncertainties remain, such as the influence of sample components on the rate of isomerization. Recently numerous food samples have been analysed for <u>all-trans</u> retinol in our laboratory. We have never observed a 13-cis peak higher than 3 % of the <u>all-trans</u> retinol peak, so we consider our saponification procedure reliable.

The cis/trans isomerization of <u>all-trans</u> retinol during saponification has been quantitated by Steuerle (1985), who determined the degree of isomerization by adding <u>all-trans</u> retinyl acetate (in powder form) to 8 vitamin A-free blends, followed by saponification, extraction and HPLC. All 5 known <u>cis</u>-isomers (13-, 11- and 9-cis, and 9,13- and 11,13-dicis) and 5 different saponification procedures were taken into account. Steuerle concluded that the degree of isomerization, depending on the type of feed and the conditions of saponification, ranges from 4 to 40 %. Schwartz (1987) investigated the isomerization of <u>all-trans</u> palmitate. The ratio 13-cis/all-trans increased as a result of heat treatment, whereas direct sunlight induced primarily the formation of 9-<u>cis</u> retinyl palmitate. Mulry et al. (1983) observed that the use of chlorinated solvents in the presence of light promotes cis/trans isomerization.

A possible inaccuracy in the determination of <u>trans</u>-phylloquinone (vitamin K_1) in human plasma due to cis/trans isomerization has been observed by Pietersma-de Bruyn & Van Haard (1985). Human plasma contains the biologically active trans form and not the inactive cis form. These forms are not separated on the analytical C18 column and respond similarly to the detection system (electrochemical reduction followed by fluorometric detection of the reduced forms). A systematic error may occur since the standard (Sigma, No V 3501) occasionally contains <u>cis</u>-K_1 in a concentration of up to 15 % (w/w). Since both forms equally respond to the detection system the cis/trans ratio in the standard can be determined by isoretention HPLC on a silica column. The error can also be avoided by pre-purifying the standard by HPLC followed by spectroscopic measurement of <u>trans</u>-K_1. An incidental inaccuracy may occur by the presence of light during sample pre-treatment causing considerable conversion into the cis form. This compound is removed during sample pre-treatment which causes a negative error.

Carotenoids also readily undergo stereoisomerism in solution. This phenomenon is catalysed by light and heat, and is dependent on time (Zechmeister 1962). The most common induced stereoisomers are central cis-isomers. Measurements of β-carotene added to foodstuffs and plasma in our method (Speek et al. 1986c) show recoveries in the range 94 - 100 %. From this finding it may be concluded that stereoisomerism does not effect analysis of this nutritionally important member of the carotenoid family.

Adsorption to precipitates. Vitamins are compounds that are not simply mixed in the matrix but rather present in specific compartments of the (on a micro scale) inhomogeneous sample and/or bound to its specific carrier protein. For example, plasma retinol is bound to retinol-binding protein which forms a 1:1 molar complex with the tetrameric form of transthyretin (prealbumin), whereas vitamin E is found within the cell membrane of blood cell erythrocytes and bound to lipoproteins in plasma and plasma PLP forms a Schiff's base with ε-amino groups of lysine residues. The sample extraction procedure should completely free the vitamins from their binding sites without deterioration. If a vitamin is not completely freed or, after being freed as a first step in sample pre-treatment, is adsorbed to the denaturated proteins, the analytical data bear a negative systematic error. Since the macrocomposition of body fluids is constant within certain ranges, this systematic error is a constant one. Application of the standard addition method, the usual method to avoid such an error, is not possible since one cannot add a known amount of vitamin in its natural surrounding.

We have tried to estimate the extraction efficiency of the methods for analysis of water-soluble vitamins in whole blood by measuring the adsorption of vitamin to the precipitate after extraction. A series of whole blood samples of normal adults were extracted with trichloroacetic acid (TCA) solution. After centrifugation and removal of the supernatant, the precipitate was extracted twice more. The three portions of supernatant were analysed for the vitamin by HPLC. The sum was regarded to be the total amount of vitamin in the blood sample. The percentage of vitamin left in the precipitate after the first extraction was calculated by subtracting the amounts of vitamin found in the supernatants after the second and third extraction from the total amount of vitamin. This amount includes vitamin dissolved in occluded supernatant and vitamin adsorbed to the precipitate. The percentage of occluded supernatant was determined by extracting the same blood samples with

TCA solution but in the presence of added ^3H_2O (about 15 × 10^4 dpm). After centrifugation and removal of the supernatant, the precipitate was analysed for the percentage of occluded supernatant by counting ^3H_2O in both precipitate and supernatant. The extraction efficiency was calculated by subtracting the percentage of vitamin present in the precipitate after the first extraction, corrected for the percentage of vitamin dissolved in occluded supernatant, from 100 percent. For the analysis of the water-soluble vitamins in blood we obtained the results given in Table 2.

Table 2. Retention of vitamins and supernatant in the precipitate after treatment of 1 mL of blood with trichloroacetic acid solution (TCA).

vitamin	TCA solution (mL)	% of total remaining in the precipitate			calculated extraction efficiency (%)
		of vitamin (mean ± SD)	of supernatant (mean ± SD)	n	
B_1 (thiamin)	1.5	22.4 ± 1.5	22.9 ± 1.0	10	100.5
B_2 (FAD)	3	17.0 ± 2.1	11.0 ± 0.9	10	94.0
B_6 (PLP)	5	23.4 ± 2.9	11.3 ± 0.9	10	87.9
C (AA + dHAA)	4	10.3 ± 1.3	9.9 ± 0.4	5	99.6

From table 2 we conclude that the vitamins B_2 and B_6 are not completely released from their (chemical) bond to the protein precipitate of whole blood using the sungle TCA extraction step. A correction for this negative systematic error is necessary. Assuming an equal adsorption behaviour of different blood precipitates, this correction can be constant for samples with a more or less constant macrocomposition such as body fluids. However, this method of correction is rather risky since uncertainties remain, e.g. possible differences in adsorption behaviour resulting from differences in blood protein composition caused by disease. Recovery experiments cannot reveal such errors because the added vitamin is not brought in its natural surrounding.

The percentage of vitamin left in the precipitate has been determined also for the fat-soluble vitamins. Since it appeared to be less than a few per cent of the total, it has not been corrected for in the calculation.

Since foodstuffs mainly contain proteins of animal and plant origin, the same type of vitamin bonding as for body fluids plays a role. PL and PLP in foodstuffs are bound as the Schiff's base to ε-amino groups of lysine residues. Furthermore, PN is sometimes present as 5'-O-(β-D-glucopyranosyl)

(Gregory & Ink 1987) whereas PL can react with amino acids such as histidine, tryptophan, glycine and cysteine (Snell 1981). The extraction efficiencies have not been determined because, as a result of the large variation in composition, it is not feasible to establish them for any type of foodstuff. Furthermore, extraction procedures for foodstuffs - saponification for the fat-soluble vitamins and acid extraction at 120 °C for the water-soluble ones - are more harsh than the pre-treatment procedures of body fluids. The possible error in foodstuff analysis may be small and irregular. Since the amounts of water-soluble vitamins (Appendices 4 - 6) found in the second extract of several foodstuff samples appeared to be a few percent of the total, it has not been corrected for in the calculation.

Volume correction for precipitates. Whole blood and plasma are relatively concentrated solutions. As a result of pre-treatment the sample is finally split into a precipitate and a liquid. Therefore, it has to be taken into account that, for analysis of water-soluble vitamins, a 1-mL sample contributes less than 1 mL to the final volume. Since whole blood contains 85 % water (Bergmeyer 1975) the contribution is 0.85 mL when 1-mL aliquots of whole blood are analysed for water-soluble vitamins.

Sample pre-treatment of foodstuffs for the analysis of water-soluble vitamins includes heating with acid. The volume of the extracting acid increases slightly owing to breaking cell walls. Since foodstuffs vary widely in composition, no correction factor has been established. Wettish foodstuffs may be dried before analysis by lyophilization.

Since fat-soluble vitamins in biological fluids as well as in foodstuffs are completely extracted by phase transfer, no volume corrections are needed.

Incomplete extraction. During development of methods for vitamin analysis in foodstuffs attention has been focused on complete extraction of the vitamin from all types of foodstuff. However, a systematic error due to incomplete extraction may occur in the determination of the menadione content of animal feed to which menadione sodium bisulphite has been added. Alteration of the sample, to a small extent, decomposes the latter water-soluble compound to fat-soluble menadione. This form of the vitamin is co-extracted with the sodium bisulphite form (ultrasonication with 40 % ethanol in water). The latter compound is then converted into menadione whereafter the total amount of

menadione in the extract is measured by HPLC. However, the extraction efficiency of menadione appeared to be dependent on the fat content of the sample (Speek 1985, non-published results). Aliquots of a rather old sample (thus containing menadione) with a known amount of added menadione sodium bisulphite have been analysed after the addition of increasing amounts (ranging from 3 to 20 %, w/w) of edible oil. The difference between added amount and measured amount of menadione appeared to be related to the amount of added oil due to a less effective extraction of the menadione form of the vitamin. This negative systematic error, ranging from 5 to 15 % of the known value (added oil ranging from 3 to 20 %), can be avoided by extraction of the sample with 90 % ethanol in water. However, since other fat-soluble compounds may be co-extracted, interferences are then likely to occur.

Solubility of extraction solvent. During pre-treatment of plasma and foodstuff samples fat-soluble vitamins are extracted from an aqueous phase into an organic phase. Dissolution of the extraction solvent in the aqueous phase yields a positive systematic error. This phenomenon has been investigated for foodstuff and plasma analysis.

Pre-treatment of foodstuffs for the analysis of fat-soluble vitamins results in a solution of all fat-soluble vitamins in diisopropyl ether. This solution has to be washed up to three times with 100-mL portions of doubly distilled water. Since diisopropyl ether is slightly soluble in water, the vitamins in the resulting solution are slightly concentrated. Aliquots of a retinol solution in diisopropyl ether have been analysed for retinol before and after washing three times with 100-mL portions of water. The retinol concentration in the solution after washing appeared to be higher - range 3 to 5 %, n = 12 - than the original concentration. We eliminated this positive systematic error by washing with water saturated with diisopropyl ether. Van der Schalm (personal communication 1986) eliminated this error by chromatography of the saponification mixture on Extrelut 20 columns (Merck, no. 11737). A 30-mL aliquot of this mixture is added to 20 mL 10 % (w/v) sodium ascorbate solution whereafter an aliquot of 20 mL of the resulting solution is brought on the Extralut 20 column. Elution with 45 mL of 2.5 % (v/v) ethanol in cyclohexane yields a solution quantitatively containing the vitamins A and E.

Extraction experiments have also been carried out with the all-trans retinol standard solution in n-hexane for determinations in plasma or serum. A number of 2-mL aliquots of this standard solution were extracted with 1 mL of

a 1:1 water:ethanol mixture. HPLC analysis for retinol in the standard solution before and after extraction did not show any change in retinol concentration.

Incomplete dephosphorylation in extracts of body fluids. An enzyme is a protein that serves as a specific catalyst of biochemical reactions. By formation of hydrogen and disulphide bridges and by action of certain co-factors the polypeptide chains are folded to a specific tertiary structure. The catalytic action of an enzyme is determined by its specific surface structure.

Pre-treatment including a reaction catalysed by enzymes comprises a certain risk since enzymes are vulnerable compounds. The reaction circumstances have to be defined very well to maintain the surface structure. Minor changes of the reaction circumstances, i.e. of pH, temperature and ionic strength, caused by matrix components may change this structure, which consequently causes inactivation of the enzyme. The presence of inhibitors such as substrate analogues, certain heavy metal ions and ligands in the mixture also may cause inactivation. Substrate analogues are recognized by the enzymes surface and compete with the analyte. Enzymes often contain a certain metal ion in their active center. The inhibitory action of heavy metal ions in the reaction mixture is based on replacement of the enzyme's metal ion. The inhibitory action of ligands like EDTA is complexation of the enzyme's metal ion. Other types of inhibitor are compounds like cyanide and hydrogen sulphide. They inactivate metal catalysts of the enzyme by forming very stable complexes with the metal. Therefore, recovery experiments with conjugated (mostly phosphorylated) vitamins are recommended.

Another source of systematic error is the occasional blank response of certain enzymes. A blank test with every batch is necessary.

Several trademarks of hydrolysing enzymes and many batches have been tested in our laboratory for blank response and for complete dephosphorylation of endogenous and added thiamin diphosphate (ThDP) in extracts of whole blood. The test material used was whole blood collected in tubes containing heparin, the most commonly used anti-coagulant, citrate and EDTA being used less frequently. The hydrolysing enzymes investigated, Taka-Diastase (Pfalz and Bauer, New York, USA, No. T00040), Clara-Diastase (Fluka, Buchs, Switzerland, No. 27540), Mylase-100 (US Biochemical Corporation, Cleveland, USA, No. 19299) and α-amylase (Sigma, No. A-0273), showed complete hydrolysis of endogenous and added ThDP. Our attention had been drawn to this method after a

group of patients screened for thiamin status appeared to be severely deficient, which was clinically unexplicable. A study of sampling and handling revealed a strong correlation between the measured thiamin deficiency and the presence of the anticoagulant EDTA in the blood. Recovery experiments with ThDP added to blood showed effective dephosphorylation by all four enzymes in extracts from heparin- and citrate-supplemented blood, whereas ThDP in extracts of EDTA-containing blood could not be dephosphorylated completely (for circa 30 %) by Mylase-100. Since EDTA is known to inhibit certain enzymes, we tried to mask EDTA. Addition of an excess of calcium and magnesium ions, both separately and combined, did not enhance dephosphorylation. Finally, addition of a small amount of iron(III) chloride to the deproteinizing solvent was found to yield complete dephosphorylation by Mylase-100 (Appendix 4). This phenomenon may be explained by the diverging stability constants of the EDTA metal complexes.

Another source of systematic error is the occasional blank response of the dephosphorylation enzymes used in thiamin analysis. This blank response, varying from 0 to 17 nmol thiamin per litre blood, is eliminated by dialysis of the enzyme.

Other vitamin- and sample-specific factors influencing pre-treatment. Other minor sample-related systematic errors in methods for body fluids are caused by small differences in whole blood or plasma handling and can only partly be explained. Examples are small differences in the rate of freezing and thawing of whole blood samples to break cell walls, the difference in homogenization by using the vortex or roller just before pipetting 1-mL aliquots and the presence of small amounts of clotted blood or plasma. Furthermore, the rate of pipetting of the 1-mL blood or plasma aliquots into the tube containing deproteinizing agent should not be higher than dropping, faster addition causes slightly lower measured concentrations due to occlusion of the vitamin.

Another sample-related systematic error may occur when the macro composition of foodstuffs or premixes deviates from more or less usual compositions which were taken into account during method development. Premixes are synthetic compositions of which a small amount is mixed with feed to obtain the desired concentration of micronutrients. This implies that certain additives which do not afflict accuracy at their levels in feed may afflict accuracy at their much higher levels in premixes.

Feed often contains copper salt since this metal is essential for production of red blood cells. Metal ions, which may catalyse decomposition of vitamins during sample pre-treatment, are masked by addition of sodium sulphite (Appendix 1). Correct levels of the vitamins A, E and β-carotene have been measured for feed containing up to 20 mg $CuSO_4$ per kg. However, we measured too low vitamin A values (circa 80 % of the calculated value) in premixes containing 15 g $CuSO_4$ per kg of premix. Although there is an excess of sodium sulphide in the saponification flask (circa 8.5 mmol) as compared to the amount of $CuSO_4$ (0.5 mmol when 5 g of premix is taken in the assay), this excess seems too small to sufficiently mask all copper ions. When measured vitamin A concentrations in premixes are below the calculated concentrations we recommend to repeate analysis with less amount of sample in the assay and with extra addition of sodium sulphide.

Analysis of premixes containing high amounts of chalk (calcium carbonate, up to 25 %, w/w) resulted in remarkedly lower values than expected for added thiamin chloride (10 - 50 %). Extraction appeared to be incomplete since the usual amount of hydrochloric acid (4 mL of 1 M HCl, Appendix 4) added to 2 g of premix resulted in a pH of the supernatant of circa 4 instead of in the desired range of 1 - 2. Complete extraction is achieved by addition of 6 M HCl (instead of 4 mL 1 M HCl) up to a pH of the supernatant of 1.5 whereafter resumption of the normal procedure results in correct data for vitamin B_1.

4.2.5 Calibration

All vitamin standard solutions are quantitated by measuring their absorbance at the absorption maximum to avoid errors due to decomposition and/or hygroscopic action of the vitamin. The measured concentration should be equal to or somewhat lower than the concentration calculated from the weight of the standard used to make the solution. An additional quantitation (control) is obtained by comparison of a newly made standard solution to the former standard solution using HPLC. Slight decomposition of the vitamin by degradation or by the influence of light does not produce a systematic error if standard solutions are regularly measured as described.

However, decomposition of the standard to a compound absorbing at the absorption maximum of the vitamin yields a positive systematic error. This phenomenon has been observed for β-carotene standard solutions. A β-carotene standard solution, stored in a normal glass flask, was injected onto a C18

column and eluted. During the run the flask was exposed to direct sunlight for 30 s. The resulting solution, which was obviously more yellow, was directly analysed for β-carotene by HPLC and by spectroscopy at 440 nm (Appendix 1). The resulting peak height represented a β-carotene concentration of 52 % of the original value whereas the measured extinction corresponded with a concentration of about 125 % of the original one. The chromatogram showed some peaks resulting from the exposure to light, probably representing more polar carotenoids with a higher absorption at 440 nm than β-carotene (Fig. 28). The total peak area of the chromatogram after exposure to light was larger than before. Since β-carotene is very light-sensitive, calibration of a standard solution by measuring the absorption may be biased by the presence of more strongly absorbing carotenoids. Therefore, the chromatogram of the standard solution should be checked for fast-eluting carotenoids more polar than β-carotene.

A systematic error as a result of cis/trans isomerization of the vitamin K_1 standard is described above (4.2.3, cis/trans isomerization).

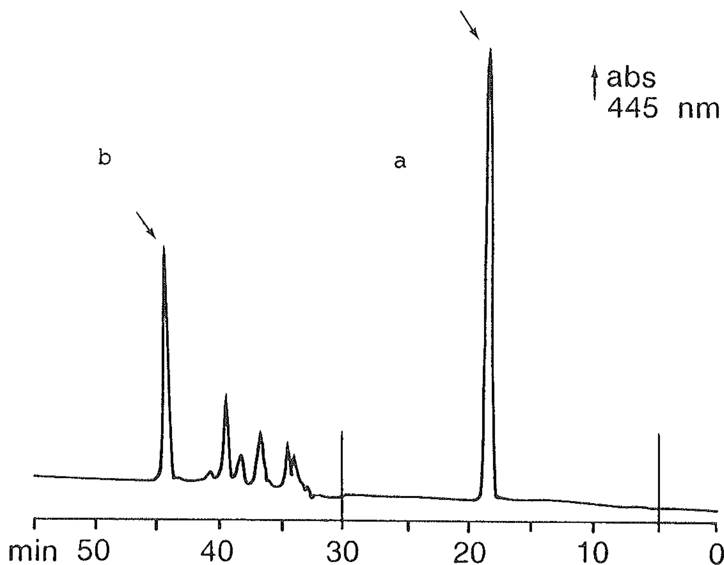


Fig. 28. HPLC elution profile of (a) a working standard solution of β-carotene stored in the dark at -20 °C and (b) of the same solution, exposed for 30 seconds to direct sunlight. The arrow indicates β-carotene.

4.2.5 Injection

A systematic or incidental error may be introduced by improper injection of an extract onto the HPLC column. Some examples observed in our laboratory are given below.

Carry-over. The usual procedure for manual injection includes connection of the steel needle of the syringe with the steel capillary tube being the sample loop entrance using a plastic tube with a length of about 10 cm. The connecting tube may cause a systematic error in the analysis of fat-soluble vitamins. The extent of the error may vary. After analysis of concentrated yellow coloured foodstuff extracts for retinol, the HPLC system was modified for the analysis of β-carotene. Injection of blanks showed a considerable β-carotene peak obviously resulting from extraction of the connecting tube by the injected solvent. We did not observe this effect when analysing vitamins A and E after analysis of foodstuff extracts for fat-soluble vitamins.

The carry-over (cross contamination) of automated injectors as given by manufacturers in their specifications is mostly $\leq$ 0.02 %. We observed carry-over figures even better.

Partial needle obstruction. Aqueous extracts of whole blood containing water-soluble vitamins sometimes contain small protein particles, even after centrifugation. These particles may partly obstruct the injection needle of the automatic injector. We observed an irregular negative error as a result of this partial blockage when using an injector based on sucking a certain volume from the sample vial into a calibrated syringe (Perkin Elmer type ISS 100). Obviously the induced vacuum in the needle causes leakage of air. The use of an automated injector based on mechanically pressing an aliquot of the sample extract through the sample loop (whole-loop injection, Micromeritics type 725) did not yield this error.

Vacuum in injection vial. If volumes larger than about 100 μL are injected with an automated injector designed to suck the extract into a calibrated syringe from a small vial, the use of certain septa allows a reduced pressure in the vial during sampling. The competition between the low pressure in the sucking syringe and in the vial causes the volume injected to be lower than adjusted. This may cause an irregular negative systematic error. The use of

an internal standard solves this problem as well as the problem mentioned above (Partial needle obstruction). Since internal standards fulfilling all requirements are not easy to find, in our laboratory we check for these errors by comparing peak heights (or areas) of the standard of every subsequently injected series of five samples and one standard.

Contaminated short circuit in automated injectors. Certain types of automated injectors use a 4-port injection valve (as with e.g. Micromeritics type 725) to inject the extract. During rotation of the rotor seal the increasing pressure in the system is released through a by-pass valve. The pressure limit of the by-pass valve is regulated by an adjustable spring pressing a steel ball on its seat. This ball-and-seat system may be contaminated and/or corrode if an HPLC line is operated for a long period with aqueous buffers as part of the solvent. Two types of "incidental" systematic errors may occur.

- If the solvent is delivered to the column via two routes, via the sample loop of the injection valve and by leakage via the by-pass valve, the same volume of sample extract is injected over a longer period as without leakage. This results in a loss of resolution and smaller peakheights. Since this phenomenon is irregular, accuracy is impaired.
- If such an injector is placed in another HPLC line operated with a different solvent, this solvent passes the ball and seat of the by-pass valve whenever the injection valve turns and may dissolve a small portion of the contaminating material. This may result in a ghost peak interfering with the analyte. An injector that is long being used for injecting acidified urine samples showed, when used for the E vitamer line, a chromatogram of a blank with an unidentified compound co-eluting with δ-tocopherol.

Therefore, it is now routine in our laboratory to subsequently flush a newly installed injector with several solvents, i.e. subsequently 25 mL-portions of 6 % HNO_3, 0.1 M HCl, acetone and ethanol, with 10-mL portions of water in between. The by-pass valve is flushed by switching off the main power while the injection valve is half-way between inject and load position. After flushing, a blank is injected to confirm the absence of ghost peaks.

4.2.6 Chromatographic step

Interferences. A frequently occurring problem is interference caused by overlap of the analyte peak by a compound originating from the matrix or added

during sample pre-treatment. A compound interferes with the analyte if it is not quantitatively separated from the analyte during pre-treatment or HPLC and is detected.

Systematic errors due to interference can be detected by analysing the sample with substantially different techniques or - which is not customary in the vitamin world because of many practical problems (insufficient amount of analyte, presence of salts and/or contaminants in the eluent or lack of equipment) - by checking the "peak purity" by means of mass spectrometry, nuclear magnetic resonance or infrared spectroscopy. Sometimes peak collection and re-chromatography with a different HPLC system may detect interferences. Speek et al. (1986b) confirmed the peak purity of retinol in human tear fluid by peak collection and re-chromatography.

Another method to detect interferences is application of multi-wavelength detection. If the observed ratios of one or more absorbances at different wavelengths of a sample extract deviates from these ratios as determined for the standard solution, interference is obvious. The diode-array detector, a convenient and highly selective alternative becoming available the last years, has so far not been applied in our laboratory because of the large investment involved.

The absence of an interference can be more likely established when the analyte is converted to a fluorescent derivative to improve selectivity and/or sensitivity. HPLC analysis of menadione in feed extracts with and without the reducing agent sodium borohydride in the reagent solution showed the menadione determination free from interferences (Speek et al 1984c, abstract in 3.6.1).

A sample-specific systematic error occurs when the method involving post-column derivatization (Schrijver & Speek 1981, abstract in 3.9.1) is used for the analysis of PLP in blood of patients with renal disease. The PLP levels of these patients have been observed to be inexplicably high. Modification of the HPLC solvent revealed that the PLP peak is overlapped by the peak of a matrix component occurring at very low concentrations in blood of healthy humans. A modification of the method of Ubbink et al. (1986), based on pre-column derivatization with post-column addition of NaOH solution, is recommended to solve this problem. Although the elution profile resulting from blood of a renal patient is not ideal, PLP can be measured. Chromatography of the same extract but without addition of derivatization reagent results in

approximately the same peak pattern. We regard this as indicative for the "peak purity" of the PLP derivative (Fig. 29). This method yields an about 10 % lower reference range for PLP in blood than the method with post-column derivatization. The latter method probably always includes a small positive systematic error resulting from the presence of a compound the concentration of which is higher than normal in the blood of patients with renal disease.

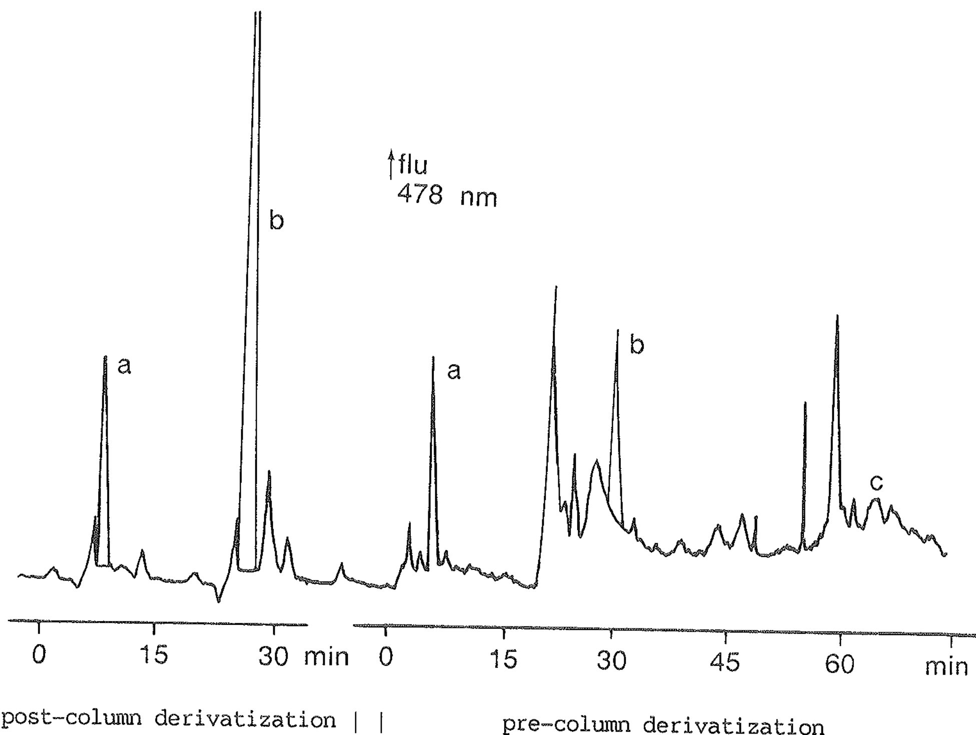


Fig. 29. HPLC elution profiles of the biologically active B_6 vitamer pyridoxal 5'-phosphate in extracts of (a) blood of a healthy adult and (b) of blood of a renal patient, both analysed according the methods with post- and pre-column derivatization. Profile c represents the blood of the same renal patient analysed with the pre-column method but without addition of derivatization reagent.

Analyses of certain foodstuffs for tocopherols have yielded unexplicably high concentrations of β- or γ-tocopherol, dependent on the exact percentage of modifier in the hexane-based HPLC solvent. Peak collection and re-chromatography on the same column but with a modified solvent (Tangney et al. 1981) revealed that the γ-tocopherol peak is interfered with by butylated hydroxyanisol (BHA), an antioxidant sometimes used to stabilize feed. We have

also occasionally observed BHA in edible oils. This interference, which may also lead to faulty identification of the type of oil, has been confirmed by chromatography on the same HPLC system but with absorption detection at 296 nm. The peak height ratios of E vitamers detected by fluorometry and UV-296 absorbance did not match this ratios as determined for the standards.

Sinclair & Slattery (1978) have observed interferences by compounds extracted from stoppers of blood collection tubes with vitamin E. However, Davidson (1979), who extracted several types of stoppers with serum, could not observe any interference with retinol and α-tocopherol as determined by HPLC.

Differences in quality between batches of column packing material. Although manufacturers, generally in vague terms, claim that their batches of the same material are uniform, we have observed considerable differences. These differences affect one compound more than another. Using different batches of the same packing material, we observed changes in the peak pattern (ratios of the relative retentions) of extracts of blood also when the number of plates as determined with the vitamin standard is approximately equal. Since blood has a fairly constant composition, chromatogrammes resulting from the determination of pyridoxal 5'-phosphate (PLP) by post-column derivatization using the same batch of packing material (ODS-Hypersil 3 μm, Shandon) show similar peak patterns for healthy humans. The resolution between PLP and the unknown compound eluting just before PLP slowly decreases with an increasing number of sample extracts being chromatographed. However, the resolution mostly suffices until the end of the column's life-time (approximately 600 samples). For certain batches we have observed the small peak to move under the PLP peak during chromatography of the first 50 samples, causing a positive systematic error. Therefore, control on sufficient high number of plates, and computerized peak area measurement and calculation of the concentration should go with visual control of the peak pattern.

Decomposition of β-carotene during column chromatography. Although β-carotene is protected during HPLC analysis against decomposition by heat and light, we have sometimes observed far too low β-carotene peaks or no peaks at all. This occasionally happened when either a newly packed column or new mobile phase had been put into use. Perhaps the explanation is given by Nierenberg &

Lester (1986) who analysed β-carotene on identical C18 HPLC columns, subsequently incorporated in the same system, but with frits made of different alloys. Their mobile phase consisted of a mixture of acetonitril, methanol, tetrahydrofuran and ammonium acetate. They conclude that, in this solvent mixture, frits made of No 316 stainless steel cause decomposition of β-carotene, whereas frits made of Hastelroy C, Hastelroy C-276 and titanium do not. They hypothesize that the iron content of the No 316 frits and the very large surface area of the frits relative to the column wall and tubing generate surface conditions catalysing the oxidation of β-carotene, possibly to endoperoxides.

First injections. When a HPLC line for the analysis of fat-soluble vitamins including a silica column is started for chromatography of sample extracts, the first injection of a standard solution of a fat-soluble vitamin results in a peak that is about 10 % higher than that of subsequent standards but the peak area is the same. The columns seem to need a few injections to become fully stable even after solvent has been pumped for a considerable time. We cannot explain this phenomenon.

4.2.7 Detection

Specific detection. Very large systematic errors may occur as a result of mistakes in the interpretation of the results of selective detection during method development. The huge advantage of a selective detector, i.e. high sensitivity for the analyte and disregard of even large concentrations of other (co-)eluting compounds, may turn into a disadvantage if no or insufficient peak identification has been done. Although this type of error should be regarded as a blunder it is included here because it causes a systematic error.

On behalf of a vitamin research project a method for the determination of urinary xanthurenic acid (XA) had to be developed. Since preliminary experiments had shown an intense fluorescence of aqueous XA (Sigma, No X-3250) solutions, method development was based on HPLC with fluorometric detection. Finally, a chromatogram of pre-purified urine was obtained showing a well separated XA peak. However, cumbersome field desorption mass spectrometry (presence of phosphate buffer) revealed that the peak resulting from standard

as well as urine samples was the 8-methyl ether of xanthurenic acid (8-O-methyl XA). This compound is present in human urine as well as in the standard (about 0.5 %; Schrijver et al. 1984). An aliquot of the standard solution was repeatedly chromatographed and the eluted compounds were monitored by either a refractometer or a fluorometer or an electro-chemical detector (ECD) in the amperometric mode (650 mV). The results are given in Table 3.

Development of an HPLC method including specific detection should include peak confirmation by substantially different techniques or by the use of a detector with identification options.

Table 3. Detector response of XA and its 8-methyl ether

Compound	Retention time (min)	Detector response (%)*		
		refractometer	fluorometer	ECD
XA	10	30	0	100
8-O-methyl XA	25	20	100	0

*The highest response has been set at 100 %.

Day-and-night temperature rhythm. The temperature in the laboratory usually decreases slowly at the end of the working day and returns to the original temperature in the next morning. When automated HPLC analyses are run, this decrease of temperature results in a shift downwards of the base-line, in lower peaks and in smaller peak areas. The extent of change in peak areas depends on the type of detection: for UV and fluorometric detection this change is small and thus is the effect on the accuracy negligible, but for electrochemical detection the effect may amount to a factor of two. If certain vitamin analysis methods are based on HPLC with electrochemical detection (vitamin C: Pachla & Kissinger 1976, Carr & Neff 1980, Tsao & Salimi 1981; vitamin K: Hart & Hayler 1986, Hart et al. 1985) this may lead to errors. Although the ratio between sample and standard peak is constant for a certain temperature, a considerable "occasional" systematic error occurs during the temperature drop or raise since the injection sequence is five samples followed by a standard. Concentrations are then calculated from standard peak heights corrected by interpolation over the temperature range. This correction is not necessary when the base-line is horizontal again. Electrochemical detectors with thermostated cells are now commercially available.

4.3 Precision - random error

4.3.1 Introduction

The random error (or statistical error) is the difference between the observed value and the true mean value and is a characteristic of the analytical figure (data). If the distribution function is normal (Gaussian) the extent of the random error is reflected by the calculated standard deviation (SD) or by its relative value, the coefficient of variation (CV). More terminology and definitions concerning precision and its extent are given in 4.1. The word "error" is delusive in that it suggests a faulty performance of the procedure. However, the results of repeated analyses of a homogeneous sample fluctuate as well if sample handling and method are flawless. The random error is an accumulation of many small inevitable errors, viz. small variations in proportioning the volumes of sample and reagent, in adjusting to a certain volume, in chemical reaction, in measuring a peak height, in the electronic processing of the detector signal, etc., so that resulting figures are slightly larger, or smaller.

A factor highly influencing the extent of the random error, expressed as the SD or CV, is the extent of the signal-to-noise ratio, i.e. the concentration measured. These concentrations, in case of vitamin analysis, may vary from circa 100 % (purity control of additives) via circa 20 % (premixes) to the relevant detection limits (as low as 0.4 ng/mL). We consider the detection limit as being the vitamin concentration resulting in a signal-to-noise ratio of three. In practise a measured signal comprises a contribution of noise. When low concentrations are measured the signal is low and the contribution of noise to the signal is relatively high. Consequently, a small (inevitable) absolute error in measurement of signal and noise (peak height or area) results in lesser precision at low as compared to high concentrations. It is generally acknowledged that an analytical method, from the viewpoint of precision, has an <u>optimal</u> concentration range, viz. the range wherein the SD is comparable with the expected SD for such a type of determination, and if the SD increases linearly with the concentration, so that the CV remains more or less constant. At a lower concentration range the SD remains constant, so that the CV increases as the concentration decreases. At even lower concentrations (nearby the detection limit) both SD and CV increase with decreasing concentrations.

The precisions of the HPLC methods for analysis of body fluids as well as of foodstuffs when applied under "routine" conditions are given below as standard deviations and/or coefficients of variation. They have been calculated from the set of quality control samples proceeding from one year of large-scale routine analyses. Results are compared with literature values.

For methods for analyses of body fluids, the effect of some methodical characteristics such as the extraction step, the column chromatographic step, the sample volume in the assay, the level of the vitamin, the derivatization step (post- vs pre-column) on the precision have been investigated as well as the effect of changing technicians working in shifts.

For the methods for analyses of foodstuffs the within-assay precision, i.e. the precision combined for a number of different samples, has been calculated from the duplicate values. Furthermore, the contribution of the type of foodstuff, of the moisture content, of the sampling, of the evaporation and dissolution step and of the particle size to the imprecision have been investigated.

4.3.2 Methods

4.3.2.1 Data collection and analysis

Preparation of control samples of body fluids. The quality of methods for analysis of fat-soluble vitamins is controled by analysing aliquots of the same pooled plasma sample, and for water-soluble vitamins by analysing aliquots of the same pooled whole blood sample. Control pooled body fluid samples are prepared from a portion of a few litres of blood obtained from healthy donors. A portion of blood or plasma is split under stirring into three equal aliquots. These aliquots are used to prepare three types of pooled body fluid, i.e. one without vitamin addition (P), one with a low addition (P+) and (only for water-soluble vitamins) with a high addition (P++). The vitamin addition consists of a few millilitres of a rather concentrated solution of the vitamin in dimethylacetamide (fat-soluble vitamins) or in saline (0.9 % NaCl, w/v) (water-soluble vitamins). The vitamin concentrations are raised to about 1.5 and 2 times the endogenous level (P+ and P++, respectively). Portions of 4-mL each of the three aliquots are pipetted into plastic test tubes

which are capped and stored in the dark at -20 °C. Per series of samples one tube of each type of pooled sample is used for quality control.

Routine analysis and incorporation of control body fluids. The vitamin concentrations in body fluids such as whole blood, plasma, serum, cerebrospinal fluid and urine are mostly singularly determined in a series of about 30 to 90 samples per day. Sample pre-treatment is carried out in the morning, and the extracts are chromatographed during the subsequent afternoon and night. Sample pre-treatment and chromatography are carried out by six technicians. All technicians involved work in shifts.

During a certain period of about three months quality control is achieved by incorporating aliquots of pooled body fluid prepared as described above. A series of analyses carried out in one day includes triplicate analyses of P, P+ and P++. Two weeks before a new batch is taken into use the pools are incorporated in routine analysis to obtain reliable values.

Furthermore, five randomly chosen samples from the former series are analysed. The quality control samples are placed at begin, middle and end of the series.

Quality control of analysis of foodstuffs. An essential difference between body fluids and foodstuff samples is the more or less constant macro composition of body fluids. Since a foodstuff sample with a macro composition representative of many types is hard to obtain, we decided to achieve quality control for vitamin analyses in foodstuffs by carrying out duplicate determinations and recovery experiments per sample. If the series consists of samples of the same type recovery experiments are carried out on one out of four samples.

4.3.2.2 Calculation of precision parameters from a set of data

The precision parameters SD_w, SD_b and SD_o have been calculated from the set of quality control data with the following formulae:

$$SD_w^2 = \frac{\Sigma_k \Sigma_j \Sigma_i \ (x_{ijk} - \bar{x}_{jk})^2}{(n_w - 1) * n_b * n_q}$$

$$SD_b^2 = \frac{\Sigma_k \Sigma_j \ (\bar{x}_{jk} - \bar{x}_k)^2}{(n_b - 1) * n_q} - \frac{SD_w^2}{n_w}$$

$$SD_o^2 = SD_w^2 + SD_b^2$$

$$CV_o = \frac{SD_o}{\bar{x}} * 100 \ \%$$

where:

$\bar{x}$: grand mean

SD_w : standard deviation within series

SD_b : standard deviation between series adjusted for SD_w

SD_o : overall standard deviation, the standard deviation when a sample is singularly analysed in an arbitrary series

i,j,k : indices for within series, between series and between periods resp.

n_w : number of replicates within one series (one series per day, $n_w = 3$)

n_b : number of series within one period

n_q : number of periods

All calculations have been carried out with the aid of the Genstat V statistical program (Payne et al. 1987) run on a VAX 11/750 computer (Digital Equipment Corporation).

4.3.3 Results

4.3.3.1 Precision of the methods for vitamin analysis in body fluids.

General precision parameters. The precision parameters of the HPLC methods for vitamin analysis in body fluids are given in Table 4. Since vitamin C in blood is unstable during the storage conditions applied only within-assay data for this vitamin are available.

Table 4. Precision of the HPLC methods for vitamin analysis in pooled body fluids with and without additions.

vitamin	unit	mean	SD_w	SD_b	SD_o	CV_o(%)	n_b	n_q
fat-soluble vitamins in plasma:								
vitamin A	µmol/L	1.48	0.097	0.092	0.134	9.1	43	4
(all-trans retinol)		3.10	0.181	0.171	0.249	8.0	43	4
total carotenoids	µmol/L	1.54	0.101	0.112	0.151	9.8	36	3
		3.37	0.125	0.250	0.280	8.3	36	3
β-carotene	µmol/L	0.31	0.025	0.022	0.032	10.3	20	2
		1.67	0.123	0.063	0.138	8.2	20	2
vitamin E	µmol/L	21.0	1.04	0.73	1.27	6.1	57	4
(α-tocopherol)		40.1	1.57	1.59	2.23	5.6	57	4
water-soluble vitamins in blood:								
vitamin C (AA + dHAA)	µmol/L	62.4	1.58	3.52	–	–	66	–
vitamin B_1	nmol/L	112	3.54	3.98	5.33	4.8	173	4
(total thiamin)		160	5.22	5.50	7.58	4.7	173	4
		206	6.84	6.39	9.36	4.5	173	4
vitamin B_2	nmol/L	226	10.5	5.8	12.0	5.3	50	3
(FAD)		300	9.4	11.3	14.6	4.9	50	3
		371	14.3	16.2	21.6	5.8	50	3
vitamin B_6*	nmol/L	48	1.71	1.00	1.98	4.1	52	4
(PLP)		85	2.14	2.03	2.95	3.5	52	4
		118	3.44	1.83	3.90	3.3	52	4
vitamin B_6#	nmol/L	45	1.62	1.77	2.40	5.4	107	4
(PLP)		75	2.57	2.29	3.44	4.6	107	4
		105	3.89	3.14	5.00	4.8	107	4

*: With post-column derivatization according to Schrijver & Speek (1981).
#: With pre-column derivatization according to Ubbink et al. (1985).

Comparison to literature data. The reported SDs and VCs, as far as available, together with the observed data are given in Table 5. In the literature "routine" precision data are rarely given. The reported "optimal" values result from analyses on a pooled sample carefully carried out by experienced workers (usually the designers of the methods), who are eagerly to show high precision, directly after the method has been designed and tested.

Table 5. Precision of HPLC methods for routine analysis of vitamins in pooled body fluids (this thesis) in comparison with reported ("optimal") precision data.

vitamin	mean value, µmol/L		SD_o, µmol/L		CV_o, %		source*
	routine	optimal	routine	optimal	routine	optimal	
A (1 mL)	1.48	2.9	0.134	0.108	9.1	3.7	1
A (5 µL)	1.55	1.8	0.206	0.120	13.0	6.7	2
β-carotene	0.31	0.14	0.036	0.015	10.3	10.4	3
E	20.9	24.5	1.27	1.00	6.1	4.0	4
B_1	112	110	5.33	7.1	4.8	6.4	5
B_2	226	308	12.0	15.8	5.3	5.2	6
B_6	48	75	1.98	3.98	4.1	5.3	7

*: Sources of reported ("optimal") precision data are: 1: Nelis et al. 1983; 2: Speek et al. 1986a; 3: Thurnham et al. 1988; 4: De Leenheer et al. 1978; 5: Schrijver & Speek 1982; 6: Speek et al. 1982; 7: Schrijver & Speek 1981.

Influence of sample volume on precision of plasma vitamin A analysis. The micro method for plasma retinol (Speek et al. 1986a) been applied for about 4000 plasma samples of Thai children subjected to a controlled nutritional intervention study (Egger et al. 1985). Mostly, single analyses were done on 5-µL aliquots of plasma diluted with 95 µL saline, but for certain series, when more plasma was available, 10-µL or 20-µL aliquots were used. The volumes of saline used were then adapted to 90 and 80 µL, respectively. Per series of about 40 singular analyses per day five control plasma samples were taken from a 400-µL vial (stored in the dark at -20 °C). To estimate whether the use of plasma volumes smaller than 1 mL for HPLC assays lowers precision, the SDs of the method routinely used in our laboratory (1 mL) and of the micro HPLC method have been calculated (Table 6).

Table 6. Influence of sample volume on precision of plasma vitamin A analysis.

assay volume µL	mean µmol/L	SD_o µmol/L	CV_o %
5	1.55	0.202	13.0
10	1.58	0.143	9.0
20	1.56	0.139	8.9
1000	1.48	0.134	9.1

4.3.3.2 Precision of methods for vitamin analysis in foodstuffs

To trace possible effects of the type of foodstuff on precision, the samples have been classified according to type. Most foodstuffs could be assigned to one of the following groups:
code 1: milk powders
code 2: foodstuffs based on liquid milk (infant food and parenteral food)
code 3: premixes (with vitamin addition)
code 4: dry foodstuffs (moisture content < 12 %)
code 5: wet foodstuffs (moisture content > 12 %)
code 6: fruit juices

Quality control of determinations of a vitamin in foodstuff is achieved by carrying out duplicate determinations and recovery tests because a representative pooled sample was not available (4.3.2.1). The SD has been calculated from the two values obtained from each sample analysed for the same vitamin. The calculated variances (SD^2) have a χ_1^2 distribution with a decreasing frequency of the SD^2 with increasing SD (Fig. 30). Since a χ_1^2 distribution can not be transformed to a normal one, nor calculation of the overall SDs and CVs neither analysis of variance to demonstrate significance of observed differences of precision parameters, was possible. Therefore, the precision parameters of the HPLC methods for vitamin analysis in foodstuffs, grouped according to vitamin and type of foodstuff, are given as median and mean CVs in Tables 7a and 7b.

Table 7a. Precision parameters of the HPLC methods for analysis of fat-soluble vitamins in foodstuffs.

vitamin	group#	conc., mg/kg		SD, mg/kg		CV, %		n
		median	mean	median	mean	median	mean	
vitamin A (all-trans retinol)	1	25	47	0.69	1.2	1.9	3.7	52
	2	4	9	0.07	0.43	1.5	3.2	47
	3	99	107	4.8	6.6	6.2	6.3	24
	4	16	22	0.29	0.86	2.7	3.4	88
	5	10	16	0.42	0.71	3.6	6.2	37
	overall	19	32	0.35	1.4	2.6	4.1	248
β-carotene	1	0.3	0.9	0.01	0.06	5.7	8.7	37
	2	0.2	0.7	0.01	0.05	4.9	11.0	36
	3	34	43	1.6	1.6	3.1	3.9	24
	4	0.4	2.2	0.01	0.06	3.7	6.2	63
	5	0.3	2.5	0.02	0.10	4.7	8.6	31
	overall	0.5	6.8	0.03	0.27	4.8	8.6	191
vitamin E (mg/g, α-tocopherol)	1	11	13	2.3	3.4	2.6	2.7	14
	2	34	35	0.28	0.51	1.2	1.6	29
	3	64	88	1.6	2.4	2.4	2.6	24
	4	46	54	0.74	1.2	1.8	2.7	42
	5	34	48	1.9	2.7	5.8	6.6	24
	overall	29	51	1.0	1.8	2.2	3.1	133

#: food groups codes are:
code 1: milk powders
code 2: foodstuffs based on liquid milk (infant food and parenteral food)
code 3: premixes (with vitamin addition)
code 4: dry foodstuffs (moisture content < 12 %)
code 5: wet foodstuffs (moisture content > 12 %)
code 6: fruit juices

Table 7b. Precision parameters of the HPLC methods for analysis of water-soluble vitamins in foodstuffs.

vitamin	group#	conc., mg/kg		SD, mg/kg		CV, %		n
		median	mean	median	mean	median	mean	
vitamin C (AA + dHAA)	2	22	39	0.9	1.4	2.8	3.3	70
	4	92	104	0.8	1.1	2.9	3.4	43
	5	86	112	2.4	3.2	5.7	7.6	36
	6	120	284	2.7	3.2	2.7	3.1	35
	overall	45	115	1.9	2.4	3.2	4.0	184
vitamin K_3 (menadion)	3	105	208	3.6	4.1	4.6	5.3	27
	4	0.3	2.9	0.03	0.09	3.7	7.6	36
	5	0.4	2.7	0.04	0.13	4.8	8.6	40
	overall	15	57	2.0	2.7	4.3	6.5	103
vitamin B_1 (total thiamin)	1	4.2	5.0	0.07	0.15	2.9	3.3	74
	2	3.2	3.7	0.09	0.11	2.7	3.0	29
	3	103	108	2.8	3.2	2.8	3.8	12
	4	5.5	6.6	0.08	0.15	1.4	1.8	35
	5	3.4	7.4	0.28	0.58	7.8	9.5	15
	6	1.3	1.2	0.01	0.01	0.3	1.2	5
	overall	4.5	12.5	0.08	0.39	2.7	3.4	170
vitamin B_2 (total riboflavin)	1	6.8	6.6	0.07	0.14	1.9	2.5	41
	2	3.3	4.9	0.05	0.10	1.5	2.2	25
	3	120	129	2.7	3.1	2.6	3.1	16
	4	4.5	6.1	0.04	0.09	0.9	1.3	21
	5	5.9	6.8	0.37	0.56	7.9	8.2	17
	6	0.9	1.3	0.01	0.01	0.8	1.7	7
	overall	6.0	21.3	0.07	0.54	1.8	2.6	127

#: See Table 7a for food group codes.

4.3.4 Discussion and conclusions

4.3.4.1 Precision of the methods for vitamin analysis in body fluids

General precision parameters.

Almost all methods show a somewhat higher SD_o and lower CV_o when applied to samples with the vitamin added than when applied to samples without addition (Table 4).

The higher concentrations of the pooled body fluids we used as control samples are not wholly comparable with the lower ones since the added vitamin is not present in its natural surrounding, i.e. bound to its carrier (lipo)-protein in the living cell or in the plasma. Nevertheless, we conclude from the higher SD_o and the slightly lower or equal CV_o for the samples with vitamin added that all of the methods are, with regard to their precision, optimal for the normal concentration range of the vitamins. The term optimal is defined in 4.3.1.

Comparison with literature. From Table 5 it may be concluded that the precision obtained for large-scale routine analyses of fat-soluble vitamins is generally less than reported "optimal" precisions, whereas "optimal" precision obviously can be obtained for large-scale routine analysis of water-soluble vitamins. This may be explained by the absence of a liquid-liquid extraction step in methods for analysis of water-soluble vitamins.

Extraction step

It is concluded from the CV_os given in Table 4 that the methods for analysis of fat-soluble vitamins in plasma are less precise than those for analysis of water-soluble vitamins in blood.

Since the concentrations of both the fat- and the water-soluble vitamins in the pooled samples are considerably higher than the detection limits this phenomenon may be explained from a major difference between methods for fat-soluble vitamins and those for water-soluble vitamins, viz. the extra extraction step. However, there are more differences: straight-phase (vitamins A, E, B_1) versus reversed-phase chromatography (β-carotene, vitamins C, B_2 and B_6); an extra enzymatic dephosphorylation step (vitamin B_1); detection by measurement of absorption (β-carotene), native (vitamins A, B_2 and E) or induced fluorescence (vitamins C, B_1 and B_6). Due to these differences we cannot unequivocally establish the origin of the observed difference in precision.

Column chromatographic step

The concentration of total carotenoids in the extract of plasma is determined through direct spectrophotometry (445 nm). Thereafter the extract is reconstituted into n-hexane. The β-carotene in this extract is separated by HPLC and detected by measurement of its absorbance at 445 nm. The CV_os are 9.8 % and 10.8 %, respectively.

From the small difference between these precision figures we conclude that the column chromatographic step does not markedly contribute to the overall coefficient of variation. Evidently, for this plasma carotene determination, the protein precipitation and vitamin extraction steps contribute most to imprecision. This finding is more or less confirmed by the good precision figures (CV_ws of less than 0.05 % for almost all automatic samplers as well as for manual whole-loop injectors) obtained for repeatedly injected aliquots of an extract. Unfortunately, we could not investigate the contribution of the column chromatography and (reaction-) detection step to the imprecision for the other vitamins since direct fluorometric measurements are interfered by co-extracted plasma components.

Influence of sample volume on precision of plasma vitamin A analysis. The precision data (SDs and CVs) for the several volumes in the retinol assay are given in Table 6. To estimate whether the use of plasma volumes smaller than 1 mL for HPLC assays results in a lower precision, the precisions of the method routinely used in our laboratory (with 1 mL) and of the micro HPLC method have been compared. The sign test has been applied on the CV_ws obtained from analysis using 1-mL, and 5-, 10- or 20-μL aliquots of the pooled sample per series per volume used. The following three conclusions can be drawn.

- There is no difference between the precisions of the methods with 10, 20 and 1000 μL.

The figures obtained are fairly comparable with the figures reported by Van Haard et al. (1987) whose method for analysis of retinol in 200 μL of plasma yielded a CV_w, CV_b and CV_o of 5.0, 9.0 and 11.0 %, respectively.

The similar precision of the method applied to 10 and 20 μL of plasma may be explained from the type of capillary pipette used. For quantitation of 5-μL and 20-μL aliquots the same 25-μL adjustable capillary pipette was used. A 10-μL aliquot of plasma was taken with a fixed-volume 10-μL pipette with a

capillary diameter half as large as that of the adjustable 25-μL pipette. With regard to these precision data, the major point arising from the study of the precision and accuracy of mechanical-action micropipettes of Kratochvil & Motkosky (1987) should be taken into account. They observed that the precision for a variety of micropipettes is rather good, i.e. CV of the order of 1% or less. However, the accuracy varies considerably from tip to tip and depends to a large extent on the skill of the operator.

- The precision of the method using 5 μL (CV_o = 13.0 %) is markedly lower as compared to those obtained with 10, 20 or 1000 μL in the assay (CV_o = 9.0, 8.9 and 9.1 %, respectively).

- The precision of the micromethod with 5 μL applied to large-scale routine analyses by more than one technician (CV_o = 13.0 %) is considerably lower than the "optimal" precision (CV_o = 6.7 %). It may be assumed that the imprecision is markedly enlarged by the large sample load and by the fact that the technicians are working in shifts. This aspect is of sufficient importance to discuss it more extended.

Effect on precision of working in shifts. An effort has been made to calculate the contribution of handling by different technicians working in shifts to the imprecision. However, to obtain a balanced design with the same number of analyses for every technician for every batch of pooled body fluid, the data sets had to be reduced so much that no reliable conclusions could be drawn. Therefore, the averages of P, P+ and P++ of all series of the same batch of control sample have been compared by analysis of variance with the technician involved as variable. For all six technicians we observed a small but significant occasional difference between two averages. Since these differences were found only occasionally and were of a similar order of magnitude for all the technicians, we conclude that there was no difference in performance between the technicians but that all of them occasionally produced slightly aberrant values.

Relation between precision and endogenous vitamin concentration. The methods for analysis of vitamins A and E in 1 mL of plasma only differ in composition of the hexane-based mobile phase and in the settings of the fluorometer. After analyses of vitamin A, vitamin E is subsequently analysed in the same

n-hexane extract after changing the solvent and the detector settings, and after equilibration of the system. There is a large difference in endogenous concentrations (A: 0.2 - 4 µmol/L, average 1.5 µmol/L; E: 4 - 96 µmol/L, average 21 µmol/L). To establish a possible difference in precision, we compared both precisions by using the sign test. In 40 days of analyses we obtained 40 CV_ws both for vitamin A and vitamin E analysis in the same extract. In 38 cases CV_w was larger for vitamin A than for vitamin E. From this result it may be concluded that the method for analysis of vitamin E is significantly more precise than that for analysis of vitamin A. This finding cannot be explained from the higher endogenous vitamin E concentration since the fluorescence yield of vitamin E is markedly lower than that of vitamin A. This results in detection limits for these similar assays of 0.05 and 1 µmol/L for vitamin A and E, respectively. This means for both methods that application to the normal vitamin concentration range results in approximately the same signal to noise ratio. The finding of Van Haard et al. (1987) is conflicting with our observation. They obtained CV_os of 10.2 % and 14.1 % respectively for their determinations of vitamin A and E. Their method, with 200 µL of plasma, is based on protein precipitation, extraction in dichloromethane and reconstitution of this solvent into n-hexane. After separation of the vitamins on a straight-phase column they are detected by measurement of absorption. The extra evaporation and redissolution step may be responsible for the high CV_os as compared to the CV_os we observed for methods with direct injection of the extract (9.1 % and 6.1 % for vitamin A and E, respectively).

<u>Precision of vitamin B_6 analyses with post- or pre-column derivatization.</u>
A disadvantage of post-column derivatization is the peak broadening in the reaction coil and hence loss of resolution, while time-consuming reactions cannot easily be applied. An advantage is the well defined reaction conditions for all samples. Disadvantages of pre-column derivatization are the exposure of the total extract to the reagent (side reactions) and the difference in reaction time as a result of sequential injection. An advantage is the lack of peak broadening as mentioned above.

The determination in blood of pyridoxal 5′-phosphate (PLP), the biologically most active B_6 vitamer, has been carried out by applying two methods both based on the formation of the derivative of PLP with semicarbazide hydrochloride in acid medium to form the fairly stable PLP-semicarbazone which is highly fluorescent in alkaline medium. For many years, up to the end

of 1985, the method of Schrijver & Speek (1981) has been used. This method is based on protein precipitation, chromatography of the released PLP on a C-18 column followed by semicarbazone formation in a post-column reaction coil, addition of alkaline in a second coil and, finally, measurement of fluorescence. Since we found evidence that blood of patients with renal disease contains a substance interfering with PLP, we adopted the method of Ubbink et al. (1986) which is based on pre-column semicarbazone formation. The semicarbazone is chromatographed on a C-18 column and, after alkaline was added in a post-column reaction coil, measured fluorometrically. Of both methods four data-sets of P, P+ and P++ were available. The precision parameters are given in Table 4. To investigate whether the observed precisions for both methods differ, the sign test was applied to the differences between two CV_ws with a corresponding order in the series of two data sets, one obtained with post-, the other with pre-column derivatization. To exclude any effect of different technicians we selected four pairs of such data sets, each analysed by the same pair of technicians (one doing the extraction and the other the HPLC analyses of the extract). Other factors such as different dates of analysis, alteration of equipment, and differences in batches of column pack material could not be excluded. For P, P+ and P++ we obtained a few more positive than negative values. From these results we conclude that, irrespective of effect on accuracy, the post-column method is somewhat more precise than the pre-column method, although the difference is not significant.

4.3.4.2 Precision of the methods for vitamin analysis in foodstuffs

Tables 7a and 7b show that, generally, the within-assay precision of HPLC methods for the determination of water- and fat-soluble vitamins in foodstuffs are approximately equal which contrasts with the finding for the overall assay precisions for determinations in body fluids (4.3.4.1). This difference may be explained from the more favourable solid/liquid ratio in foodstuff analyses so that less reproducible adsorption effects play just a minor role, if any, as compared to analyses of body fluids. The standard deviations are sufficiently low for application of the methods to foodstuffs.
Tables 7a and 7b also show that the type of foodstuff usually does not strongly affect the within-assay precision of the particular HPLC method. However, there are some striking differences in precision which are discussed below.

Tables 7a and 7b show for all vitamins a markedly higher median and mean within-assay CV for analysis of wet foods than for dry foods (moisture contents > 12 % and < 12 %, respectively). Consequently, we conclude that vitamin analysis is more precise in dry foods than in wet foods. This difference seems to be stronger for analysis of water-soluble vitamins. We conclude that these effects are caused by the inhomogeneity of samples resulting from partial separation of water.

For all types of foods the mean within-assay CV is markedly higher for the determinations of β-carotene and vitamin K_3 (menadione) than for the other methods. A difference between the β-carotene and menadione method and the other methods is that both extracts containing β-carotene or menadione cannot be directly injected onto the HPLC column. From this fact, and from the well-known instability of both vitamins (2.3.2 and 2.6.2), we assume that precision is affected negatively by the necessary extra evaporation and redissolution step. Experiments revealed that, dependent on the type of foodstuff, a too long time period between evaporation and redissolution (varying from 2 to 20 min) impairs accuracy. Addition of a small amount of antioxidant (BHT) to the extract to protect the vitamin did not bring any improvement.

Vitamins A and E in a foodstuff sample are analysed in the same extract in diisopropyl ether. Nevertheless, if vitamins A and E are analysed in the extract of a fortified feed (classified under code 3), the within-assay precision is generally less for vitamin A analysis (mean CV = 6.3 %) than for vitamin E analysis (mean CV = 2.6 %) and for the analysis of endogenous vitamin A. This difference may be explained by the composition of many fortified feeds which consist of a basic feed enriched with a number of additives. The vitamins are added by mixing the feed with vitamin carriers. In the case of vitamin A bran coated with retinyl acetate is commonly used whereas fortification with vitamin E is often achieved by adding a silica carrier coated with α-tocopheryl acetate. However, there is a large difference in particle size between the silica and bran carriers. The silica particles have a size that is generally comparable to the size of the feed particles (< 0.5 mm) and are not separated from the feed when the sample is manipulated. The larger bran particles (1 - 1.5 mm) have been observed to be separated from the feed particles when the sample is gently shaken. These inhomogeneity effects obviously hamper the within-assay precision if a limited amount is sampled for analysis. Thorough mechanical mixing improves the results. These inhomogeneity effects can be reduced to variation coefficients below about 5 %

(n = 3) by thoroughly grinding an aliquot of at least 30 g of feed to particles < 0.5 mm.

The effect of a high CV could be especially expected for feeds with a low (added) vitamin content since the influence of a few more vitamin carrier particles in the assay on the analytical result is larger as the carrier density of the feed is lower. However, since the data obtained for samples of this type are 'diluted' with many data obtained from samples with low endogenous concentration we could not observe this effect.

4.4 Incidental errors

Incidental errors can be very large; they result from incidental avoidable mistakes such as incorrect labelling of samples, exchange of reagents, measuring the height of the wrong peak, transposing two digits of a display or calculation errors. This type of errors may yield strongly aberrant results. Due to the diversity in types of incidental error, this phenomenon is not liable to a systematic investigation.

When a series of samples of body fluids or foodstuffs are analysed, every separate operation within a method is completed for all consecutive samples of the series before the next step in the procedure is started. This means that handling can be checked for correctness by incorporating control samples and recovery experiments in the series. Recoveries in the range of 90 - 110 % imply that no measurable incidental errors have been made in the procedure for the whole series.

Occasional incidental errors may be revealed by differences in duplicate analyses or, in the case of single analyses, by differences in repeated analyses. An experimentally obtained value is suspect in the case of body fluids:

- if the value is not compatible with former values of the patient in case of a certain treatment;
- if the value is far beyond the reference range;

and in the case of foodstuffs:

- if the value considerably differs from the concentration claimed by the manufacturer;
- if the value is not compatible with former values of the sample in case of a storage experiment.

Chapter 5. GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Accuracy

5.1.1 Body fluids

The analytical chemist, when developing a method, tries to achieve optimal accuracy by combining the benefits of more or less selective detection, high column resolution and sample pre-purification. The accuracy of the methods described in this thesis is acceptable. Nevertheless, a number of factors impairing accuracy (Section 4.2.2 - 4.2.7) could be established only because results appeared to be conflicting with other observations of the patient.

Since blood samples offered for analysis are, by definition, drawn from patients, it is recommended to test HPLC methods for vitamin analysis in body fluids for interferences and for changes of the adsorption characteristics of the blood precipitate (Section 4.2.3) possibly resulting from a disease (such as renal disease, Section 4.2.6) and/or from medication.

Apart from these investigations into interferences or incomplete extraction, the selectivity of the method (the 'peak purity') in cases of doubt should preferably be checked by using a detector with identification options such as the diode-array detector (multi-wavelength detection) or, if no unequivocal confirmation is obtained, by peak collection followed by re-chromatography on a different HPLC system or by mass spectrometry.

Since vitamins are labile when exposed to light - especially direct sunlight - it is recommended to analyse vitamins in a laboratory screened from natural light and equipped with special ultraviolet-free lights. Attention should also be paid to another aspect of exposure to light, cis/trans isomerization (Section 4.2.3). Therefore, even when only one form occurs naturally, we recommend the use of HPLC systems capable of separating the cis/trans isomers, and the incorporation of recovery tests with the natural form of the vitamin.

A source of aberrant results appeared to be the variability in properties of different batches of the same reversed-phase column packing material, probably resulting from a relatively poor reproducibility of the chemical reaction between the basic silica material and the modifying agent. Since, for practical reasons, we try to achieve a high selectivity by combining highly

selective detection with minimum sample pre-treatment, the extract contains many compounds at relatively large concentrations but with a low response to the detection system. The obtained peak pattern is usually similar for fresh blood samples of different persons, but it is often different for control samples due to a difference in storage time of these samples as compared to fresh patient's samples. When a column is replaced by another one filled with the same type of packing material but from another batch, the chromatogrammes obtained from fresh patient's samples as well as from 'aged' control samples should be checked in order to find out whether they show the correct peak pattern.

Certain vitamin determinations are sum determinations: the sum of the parent compound and its phosphorylated forms are determined as the total amount of the parent compound. We recommend to test new batches of dephosphorylating enzymes for complete dephosphorylation for the several types of body fluids with different anticoagulants (Section 4.2.3).

5.1.2 Foodstuffs

In the case of foodstuff analysis possible inaccuracies caused by new additives or by the addition of more than the usual amount of additive should be investigated (Section 4.2.3, other vitamin- and sample-specific factors). The efficiency of the extraction procedure should also be tested for large changes in macro composition as compared to the composition taken into account during method development (Section 4.2.3, solubility of vitamins in sample components). Furthermore, attention should be paid, as with the analysis of body fluids, to factors causing cis/trans isomerization and decomposition of vitamins.

A serious problem is the influence of the particle size of the vitamin carriers added to premixes on accuracy (Section 4.2.2, Sampling). Since the precision is also strongly affected, this topic is discussed below (Section 5.2.2).

5.2 Precision

5.2.1 Body fluids

The precision figures for the methods of vitamin analysis in body fluids are satisfactory (Table 4). For most of the methods it has been established that the overall SD increases with increasing concentration, whereas the overall CV remains constant or decreases only slightly. This implies that the precisions of the methods are optimal for the normal vitamin concentration ranges.

The protein precipitation and organic extraction step (part of the methods for the analysis of fat-soluble vitamins only) contribute most to imprecision, and the column chromatographic step least (4.3.4.1). The methods for analysis of water-soluble vitamins generally show a better large-scale routine precision than those for the analysis of fat-soluble vitamins. Comparison with "optimal" precision data from the literature (Table 5) yielded surprising results. Large-scale routine analyses of fat-soluble vitamins are less than "optimally" precise, but "optimal" precision appears to be attainable for water-soluble vitamins. The latter may be explained by the additional organic solvent extraction step included in the methods for fat-soluble vitamins.

The volume of body fluid in the assay seems to afflict precision only when extremely small volumes are used, such as with the 5-μL aliquots used for vitamin A analyses in plasma (Speek et al., 1986a; abstract in 4.3.4.1).

When a certain vitamin determination is carried out by several technicians working in shifts, the precision is not lower than for one technician handling this determination. However, it could be established that all of them occasionally produce slightly aberrant data.

It was established that vitamin E analysis in plasma, with an average endogenous concentration of 21 μmol/L, is more precise than vitamin A analysis in the same plasma samples, for which case the average endogenous vitamin concentration is 1.5 μmol/L (overall CVs of 6.1 and 9.1 %, respectively). The methods only differ in detector settings and in additions to the hexane-based solvent. However, it cannot be unequivocally concluded that, at these vitamin levels, concentration affects precision since the fluorescence yield of vitamin E is much lower than that of vitamin A, which results in a different detection limit (1.0 and 0.05 μmol/L for vitamin E and A, respec-

tively) to about the same extent as the concentration difference. The precisions of the method are independent of concentration, provided the concentration lies within the normal range for the vitamin in question.

Vitamin B_6 in blood has been determined by applying two similar HPLC methods, one with post-column and the other with pre-column derivatization, both with semicarbazide as reagent. From the viewpoint of precision, application of post-column derivatization is advantageous as the reaction circumstances are similar for all samples. Application of pre-column derivatization implies large differences in reaction time as a result of subsequent injection. From precision data (Table 4) one can conclude that, under large-scale routine conditions, the post-column method is somewhat more, but not significantly more, precise. (4.3.4.1).

5.2.2 Foodstuffs

The within-assay precisions of the HPLC methods for the determination of water- and fat-soluble vitamins in foodstuffs are approximately equal (Tables 7a and 7b). This contrasts with the finding for the overall precisions of analyses in body fluids (Table 4). This difference may be explained by the more favourable solid/liquid ratio in foodstuff analyses, so that less reproducible adsorption effects play no or just a minor role compared to analyses of body fluids. Since the solubility of the extractant diisopropyl ether in the washing water cannot be neglected, we recommend to wash with 100 mL of water saturated with diisopropyl ether.

The type of foodstuff to be analysed generally does not strongly influence the within-assay precision of a HPLC method. However, some exceptions - which are mentioned below - should be noted.

Vitamin analyses in dry foods (moisture content < 12 %) appear to be more precise than analyses in wet foods (moisture content > 12 %), particularly for the analysis of water-soluble vitamins. We recommend to eliminate or minimize these inhomogeneity effects by thoroughly mixing a sufficient amount of sample directly before taking an aliquot for analysis. Grinding samples frozen in liquid nitrogen is an effective method, but it is rather tedious for routine application.

Apart from the factors afflicting precision and accuracy mentioned above, attention has to be paid to the evaporation and redissolution step as needed for β-carotene and vitamin K_3 determinations. This step cannot be avoided

since we are not able to fulfil all conditions mentioned in 3.1. When the vitamin is not protected by solvent it is more vulnerable to the influence of oxygen. Therefore, we recommend to evaporate at 37 °C under a gentle stream of oxygen-free and dry nitrogen and to add the new solvent directly after the evaporation flask has run dry. Experiments have revealed that, dependent on the type of foodstuff, too long intervals between evaporation and redissolution (from 2 till 20 min) hamper accuracy and repeatability. Addition of a small amount of the antioxidant butylated hydroxytoluene to the extract to protect the vitamin fails to improve the situation.

A premix is a feed supplemented with vitamins and other nutrients. Feeds with appropriate concentrations are obtained by mixing small amounts of premix with feed containing a low vitamin concentration. We observed that the particle size of the vitamin carrier causes inhomogeneity which influences accuracy and precision. Bran particles (carrier of vitamin A esters) larger than the feed particles concentrate in the upper layer of the sample as a result of mechanical action. Therefore, we recommend to roughly mix and subsequently grind a large amount of sample as described under 4.2.1 (Sampling). In this way the accuracy can be improved (< 8 % of the calculated value), and the within-assay precision will be within the range mentioned in Table 7a.

5.3 General conclusion

Finally, we conclude that the combination of simple sample pre-purification, separation and on-line selective detection makes high-performance liquid chromatography, in terms of accuracy, precision, sensitivity and capacity, the best available analytical tool for vitamin analysis in body fluids and in foodstuffs. In contrast with other separation techniques such as gas and thin-layer chromatography, HPLC is harmless to labile vitamins during analysis. A striking difference with formerly applied methods such as bioassays, microbiological and physicochemical methods is the capability of HPLC to differentiate between vitamers varying in biological activity. Consequently, the impact of HPLC on biochemical, epidemiological and medical research is large.

SUMMARY

HPLC methods for the analysis of the vitamins A, β-carotene, E, C, K_3, B_1, B_2 and B_6 in body fluids and/or in foodstuffs have recently been developed and been routinely applied in our laboratory for large-scale analyses. In this thesis the various HPLC methods are described and are discussed in terms of reliability, i.e. accuracy and precision.

Chapter 1 (General introduction) describes the rationale underlying the development of HPLC methods: basically a need for more accuracy (especially differentiation between vitamers with different biological activity), precision, sensitivity and capacity, as required for advanced vitamin research.

In Chapter 2 (The vitamins: characteristics and analysis) many disadvantages and some advantages of several types of formerly applied vitamin assays are summarized: bio- and microbiological assays, direct optical assays (spectrophotometry and fluorometry) and chromatographic assays (classical liquid chromatography and gas and thin-layer chromatography). Furthermore, the chapter provides general information on several vitamins relevant to the analyst, viz. information on structure, chemical and physical properties, and occurrence, form and level in body compartments and foodstuffs. For each vitamin some representative HPLC methods described in the literature are discussed.

In Chapter 3 (The HPLC methods for vitamin analysis) the conditions pertaining to particular HPLC systems are given, which are determined by the characteristics of the analyte and its matrix and by certain requirements of the methods themselves. The HPLC methods for vitamin analysis discussed in this thesis have been developed with most of these conditions taken into account and are presented as abstracts. For detailed information reference is made to the relevant paper or, if the method is not published, the detailed method is given in the appendix.

In the introduction to Chapter 4 (Reliability of the HPLC methods for vitamin analysis) the relevance of good accuracy and precision is discussed against the background of application, i.e. clinical diagnosis, assessment of nutritional status or evaluation of the contribution of foodstuffs to the recommended daily allowance of a particular vitamin.

Section 4.2 (Accuracy - systematic error) describes factors influencing accuracy related to vitamin, matrix, method, equipment and combinations of these.

Section 4.3 (Precision - random error) presents the precisions of analytical methods for plasma or blood as well as for foodstuffs when applied under routine conditions, as standard deviations and/or coefficients of variation. Furthermore, this section describes how, for the determinations in body fluids, the precision is affected by certain sample- and method-related factors. Subsequently the extraction step, the separation on the HPLC column, the vitamin concentration, the plasma volume in assay, differences in achievement between individual technicians working in shifts, and the application of post- or pre-column derivatization are discussed. Data from the literature are discussed. The precisions of the determinations in foodstuffs, including some relevant conclusions, are presented as median and average standard deviations and coefficients of variation.

In Section 4.4 (Incidental errors) some frequently occurring incidental errors are mentioned as well as some ways of detecting them.

In Chapter 5 (General discussion, conclusions and recommendations) the conclusions are summarized and recommendations are given.

Finally, in Appendices 1 - 6, the non-published HPLC methods discussed in this thesis are described.

SAMENVATTING

De laatste jaren zijn een aantal HPLC-methoden voor de bepaling van vitamines in lichaamsvloeistoffen en voedingsmiddelen ontwikkeld en als routine-bepalingen toegepast, met name methoden voor de bepaling van de vitamines A, β-caroteen, E, C, K_3, B_1, B_2 en B_6. In dit proefschrift wordt, op basis van de opgedane ervaring, de betrouwbaarheid van de methoden besproken, met het accent op de juistheid en precisie.

In hoofdstuk 1 (General introduction) worden de redenen gegeven om de bovengenoemde HPLC-methoden te ontwikkelen. Deze redenen betroffen vooral de voor vitamine-onderzoek vereiste verbetering van de juistheid (vooral het onderscheid tussen vitameren met verschillende biologische activiteit), de precisie, gevoeligheid en capaciteit.

In hoofdstuk 2 (The vitamins: characteristics and analysis) worden tal van nadelen en enige voordelen van verschillende typen eerder toegepaste analyse-methoden voor vitamines samengevat: dierproeven, microbiologische methoden, directe optische methoden (spectrofotometrie en fluorimetrie) en gas en dunne-laag chromatografische methoden. Tevens verschaft dit hoofdstuk de analytisch chemicus informatie over structuur, chemische en fysische eigenschappen, en voorkomen, vorm en concentratie van deze vitamines in lichaamscompartimenten en voedingsmiddelen.

In hoofdstuk 3 (The HPLC methods for vitamin analysis) wordt, uitgaande van enige eigenschappen van vitamine en matrix en van enige aan de methode te stellen eisen, de keuze van het te ontwikkelen HPLC-systeem besproken. De HPLC-methoden behandeld in dit proefschrift zijn ontwikkeld rekening houdend met genoemde eigenschappen en eisen en zijn gepresenteerd in samengevatte vorm. Voor gedetailleerde informatie wordt verwezen naar verschenen publicaties. Niet-gepubliceerde methoden zijn volledig beschreven in de appendices.

In hoofdstuk 4 (Reliability of the HPLC methods for vitamin analysis) wordt de betrouwbaarheid van de HPLC-methoden voor vitamine-analyses besproken. In de inleiding (4.1) wordt de belangrijkheid van juistheid en precisie behandeld tegen de achtergrond van de toepassing, met name klinische diagnostiek, de bepaling van de vitaminestatus en de voedingsmiddelenanalyse.

In paragraaf 4.2 (Accuracy - systematic error) worden een aantal aan vitamine, matrix, methode en apparatuur (en combinaties daarvan) gerelateerde factoren behandeld die de juistheid beïnvloeden.

In paragraaf 4.3 (Precision - random error) worden de precisies van de methoden voor zowel plasma of bloed als voedingsmiddelen vermeld als standaarddeviaties en variatiecoëfficiënten. Tevens wordt voor de bepalingen in plasma of bloed de invloed van een aantal aan monster en methode gerelateerde factoren op de precisie besproken, met name de invloed van de extractiestap, de scheiding op de HPLC-kolom, de concentratie van het vitamine, het plasmavolume, individuele verschillen tussen laboratoriummedewerkers die in wisseldienst werken, en het toepassen van voor- of na-kolom derivatisering. Gegevens uit de literatuur worden ter vergelijking eveneens vermeld. Voor de methoden voor voedingsmiddelenanalyse worden de precisies gegeven als mediane en gemiddelde standaardafwijkingen en variatiecoëfficiënten, en worden enige conclusies getrokken.

In paragraaf 4.4 (Incidental errors) worden een aantal regelmatig voorkomende "blunders" gegeven alsmede een aantal aanknopingspunten om dit type fouten te op te sporen.

In hoofdstuk 5 (General discussion, conclusions and recommendation) worden de conclusies samengevat en worden aanbevelingen gedaan.

APPENDICES: THE NON-PUBLISHED HPLC METHODS FOR VITAMIN ANALYSIS

All HPLC methods for vitamin analysis discussed in this thesis are presented as abstracts in Chapter 3, Sections 3.2 - 3.9. For detailed information the bibliographic descriptions of the relevant paper are given, or, if the method is not published, details are given in the relevant appendix.

Vitamin	Matrix	Abstract	Reference
Vitamin A	plasma	3.2.1	Speek et al. 1986a
	tear fluid	3.2.2	Speek et al. 1986b
	foodstuffs	3.2.3	Appendix 1
β-carotene and	plasma	3.3.1	Appendix 2
total carotenoids	foodstuffs	3.3.2	Appendix 1, Speek et al. 1986c
Vitamin E	plasma	3.4.1	Appendix 3
	seed oils	3.4.2	Speek et al. 1985
	foodstuffs	3.4.3	Appendix 1
Vitamin C	blood	3.5.1	Speek et al. 1984a
	foodstuffs	3.5.2	Speek et al. 1984b
vitamin K_3	feed	3.6.1	Speek et al. 1984c
vitamin B_1	blood	3.7.1	Appendix 4 and Schrijver & Speek 1982
	foodstuffs/urine	3.7.2	Appendix 4
vitamin B_2	blood	3.8.1	Speek et al. 1982
	foodstuffs/urine	3.8.2	Appendix 5
vitamin B_6	blood	3.9.1	Schrijver & Speek 1981
	foodstuffs	3.9.2	Appendix 6

APPENDIX 1.

Determination of total vitamin A, total carotenoids and β-carotene, and E vitamers in foodstuffs, pharmaceutical preparations and body tissues by high-performance liquid chromatography

PRINCIPLE

Vitamin esters in the sample are hydrolysed by alkaline saponification (Mulder 1957) whereafter the vitamins are extracted in diisopropyl ether. All-trans retinol is separated on a silica column and determined fluorometrically. E vitamers are separated on another silica column and determined fluorometrically. Total carotenoids in the extract are determined spectrophotometrically. β-carotene is separated on a C18 column and detected by measurement of the absorbance at 445 nm.

EXPERIMENTAL

Apparatus

All-trans retinol

HPLC is performed with a system incorporating a Gilson Model 302 constant-flow pump (Gilson, Villiers-le-Bel, France), a Rheodyne injection valve type 7010 and a Kratos type FS 950 Fluoromat flow-through fluorometer equipped with a mercury light source (type FSA 110), an excitation interference filter of 365 nm (type FSA 401) and an emission cut-off filter of 440 nm (type FSA 427). A Knauer stainless steel column (250 x 4.6 mm ID) is laboratory-packed with Polygosil 60-5, 5 μm (Macherey-Nagel, Düren, FRG, No. 8283) by the balanced-density slurry technique on a column packing apparatus designed at our institute, using a Haskel pump type DSTV 150 (Ammann Technik, Stuttgart, FRG). The slurry and packing solvents are methylene chloride and n-hexane respectively (Merck, Darmstadt, FRG, Nos 6050 and 4368, respectively). Elution profiles are displayed on a Kipp BD-8 recorder (Kipp & Zonen, Delft, The Netherlands).

Total carotenoids and β-carotene

HPLC is performed with a system incorporating a Gilson Model 302 constant-flow pump, a Rheodyne injection valve type 7010 and a Perkin Elmer variable-wavelength detector type LC 75. A Knauer stainless steel column (250 x 4.6 mm ID) is laboratory-packed with ODS-Hypersil 3 μm (Shandon Southern Products, Astmoor, UK, No. 580 x 18) by the balanced-density slurry technique on a column packing apparatus designed at our institute, using a Haskel pump type DSTV 150. The slurry and packing solvents are iso-propanol and methanol respectively (Merck, Nos 9634 and 6009, respectively). Elution profiles are displayed on a Kipp BD-8 recorder.

Spectrophotometry is performed using a Vitraton spectrophotometer type MPS equipped with a tungsten light source and a 445 nm interference filter (Meyvis, Bergen op Zoom, The Netherlands).

E vitamers

HPLC is performed with a system incorporating a Gilson Model 302 constant-flow pump, a Micromeritic type 725 automatic injector and a Perkin Elmer fluorescence detector type 650-10. A Knauer stainless steel column (250 x 4.6 mm) is laboratory-packed with Polygosil 60-5 (Macherey-Nagel, No. 71101) by

the balanced-density slurry technique on a column packing apparatus designed at our institute, using a Haskel pump type DSTV 150. The slurry and packing solvents are methylene chloride and n-hexane respectively (Merck, Nos 6050 and 4368, respectively). Elution profiles are displayed on a Kipp BD-8 recorder.

Reagents

Sodium ascorbate and sodium sulphide are obtained from Merck. A 12 % (w/v) sodium sulphide solution is prepared by dissolving 120 g sodium sulphide in 200 mL distilled water, whereafter 700 mL glycerol (density 1.23) is added. Diisopropyl ether (Merck, No. 800866) is stabilized by adding 10 mg butylated hydroxytoluene (BHT) per litre.

The HPLC mobile phase consists of,

vitamin A determination : n-hexane : methylene chloride : isopropanol = 900 : 90 : 12 (v/v).

β-carotene determination : methanol : acetonitril : chloroform : water = 200 : 250 : 90 : 11 (v/v).

E vitamers determination : n-hexane : diisopropyl ether (containing 10 mg BHT per litre) = 95 : 5 (v/v).

All mixtures are flushed with a gentle stream of helium gas for 10 min before use.

Standardization

Retinol

A stock standard solution of vitamin A (all-trans retinol, Fluka, Buchs, Switzerland, No. 95144) is prepared by dissolving ca. 50 mg in 100 mL absolute ethanol. The retinol concentration of this solution is determined by measuring the absorbance at 325 nm of a 100-fold dilution in a suitable spectrophotometer using E (1 cm) = 1832 at 325 nm for a solution of 1% (w/v) of all- trans retinol in absolute ethanol (Dawson et al. 1986). A working standard solution is obtained by diluting the stock standard solution with diisopropyl ether to ca. 0.5 mg/L. A stock solution of all-trans retinyl acetate (Fluka, No. 95140) in absolute ethanol for recovery experiments is obtained by dissolving ca. 40 mg in 100 mL absolute ethanol. The retinyl acetate concentration of this solution is determined by measuring the absorbance at 325 nm of a 100-fold dilution in a suitable spectrophotometer using E (1 cm) = 1561 at 325 nm for a 1% (w/v) solution of all-trans retinyl acetate in absolute ethanol (Hejno 1964).

Total carotenoids and β-carotene

A β-carotene stock standard solution is obtained by dissolving approximately 20 mg of β-carotene in 10 mL of methylene chloride (Merck, No. 6050) in a 100-mL brown glass flask followed by dilution with n-hexane (Merck, No. 4368) to 100 mL. The concentration of β-carotene in the stock standard solution is determined by spectrophotometry. Of this solution 1 mL is diluted with cyclohexane (Merck, No. 2822) to 200 mL, whereafter the concentration of β-carotene is determined by measuring the absorbance of the diluted solution at 457 nm in a suitable spectrophotometer, using E (1 cm) = 2505 at 457 nm for a solution of 1 % (v/v) β-carotene in cyclohexane (Hejno 1964). A working standard solution is prepared daily by diluting an aliquot of the stock solution with mobile phase to a concentration of ca. 1 mg/L.

E vitamers

A standard solution containing all four tocopherols is made by dissolving the content of an ampoule containing 10 mg each of α-, β-, γ- and δ- tocopherol (Merck, No. 15496) in n-hexane and subsequently diluting it to about 15 mg/L. A stock solution of α-tocopheryl acetate (Sigma, No. T-3376) in absolute ethanol for recovery experiments is obtained by dissolving ca. 60 mg in 100 mL absolute ethanol. The α-tocopheryl acetate concentration of this solution is determined by measuring the absorbance at 284 nm of a 10-fold dilution in a suitable spectrophotometer using E (1 cm) = 43.36 at 284 nm for a 1% (w/v) solution of α-tocopheryl acetate in absolute ethanol (Dawson et al. 1986). All stock and working solutions are stored in the dark at -20 °C and are stable for at least two weeks.

Saponification and extraction

A portion of 1 - 10 g of foodstuff or tissue, or a number of pulverized pharmaceutical pills are brought into a 250-mL brown glass saponification flask. After adding 10 mL of a freshly prepared aqueous solution of 10 % (w/v) sodium ascorbate, 5 mL sodium sulphide solution and 50 mL of a freshly prepared 2 mol/L ethanolic KOH solution, the resulting mixture is refluxed on a boiling water bath for 30 min. The flask is then cooled to room temperature, and after addition of 100 mL diisopropyl ether the contents are mixed by shaking. After separation of the two layers, the upper layer is transferred to a 250-mL brown glass separation funnel containing 100 mL of a 5 % (w/v) KOH solution. After shaking, the lower aqueous layer is discarded and the diisopropyl ether is washed further with consecutive 100-mL portions of water saturated with diisopropyl ether until the reaction to phenolphthalein paper is no longer alkaline. Usually three portions will suffice. The diisopropyl ether, containing the fat-soluble vitamins, is dried with strips of blue-ribbon filter paper. The extract can be directly used for HPLC analysis or stored in the dark at -20 °C for analysis within a week.

Spectrophotometry

The total concentration of carotenoids is determined by measuring the absorbance at 445 nm of the extract using a quartz cuvette in the spectrophotometer with the working standard solution as reference.

High-performance liquid chromatography

Retinol

HPLC is carried out by injecting 100 μL of the extract in diisopropyl ether or the working standard solution onto the Polygosil 60-5 column. The column is eluted isocratically with the mobile phase set at a flow-rate of 1.2 mL/min. The effluent is monitored with the fluorometer set at range 0.1, suppression low, sensitivity 0.76 kV and T/C damping 1 s. The recorder is set at 10 mV full scale. The chromatographic run takes about 12 min per sample.

β-carotene

An aliquot of the extract is evaporated at 37 °C under a gentle stream of nitrogen and redissolved in mobile phase. A 100-μL aliquot of this solution is injected onto the HPLC column. The column is eluted isocratically with the mobile phase set at 1.5 mL/min. The effluent is monitored with the detector set at 445 nm. The recorder is set at 10 mV. The chromatographic run takes about 15 min per sample.

E vitamers

HPLC is carried out by injecting 100 µL of the extract onto the Polygosil column. The column is eluted isocratically with the mobile phase set at a flow-rate of 1 mL/min. The effluent is monitored using the fluorescence spectrophotometer set at a wavelength pair of 296 - 320 nm. The recorder is set at 10 mV full scale. The chromatographic run takes about 18 min per sample.

Recovery experiments

These experiments are carried out by adding a few millilitres of the retinyl or α-tocopheryl acetate or β-carotene stock solution in ethanol to the saponification flask prior to the saponification procedure. The amount of vitamin added should approximately double the vitamin content of the sample aliquot in the flask.

CALCULATION

The vitamin concentrations of the original foodstuff sample are calculated from peak heights with the relevant working standard solution as reference. In routine analysis the working standard solution is run before each series of five samples. The recovery is calculated by comparing the difference between the measured concentrations in the spiked and not spiked sample to the calculated amount of vitamin added per g of sample. The provitamin A-activity as all-trans retinol equivalents (RE) is calculated using the conversion factors given by the WHO (1982): 1/12 × (total carotenoids minus β-carotene) + 1/6 × β-carotene.

APPENDIX 2.

Determination of total carotenoids and β-carotene in human plasma or serum by high-performance liquid chromatography

PRINCIPLE

After precipitation of proteins by addition of ethanol, the carotenoids are extracted. β-Carotene in the extract is separated on a reversed-phase HPLC column and detected by measuring the absorbance at 445 nm. Total carotenoids are determined by measuring the absorbance at 445 nm of the extract prior to HPLC.

EXPERIMENTAL

Apparatus

HPLC is performed with a system incorporating a Gilson Model 302 constant-flow pump, a Rheodyne injection valve type 7010 and a Perkin Elmer variable-wavelength detector type LC 75. A Knauer stainless steel column (250 × 4.6 mm ID) is laboratory-packed with ODS-Hypersil 3 μm (Shandon, No. 580 x 18) by the balanced-density slurry technique on a column packing apparatus designed at our institute, using a Haskel pump type DSTV 150. The slurry and packing solvents are iso-propanol and methanol, respectively (Merck, Nos 9634 and 6009, respectively). Elution profiles are displayed on a Kipp BD-8 recorder. Spectrophotometry is performed using a Vitraton spectrophotometer type MPS equipped with a tungsten light source and a 445 nm interference filter (Meyvis).

Sample extraction

The extraction of plasma is carried out in a room screened from direct sun light. Via a pipette, 1 mL plasma is brought into a 10-mL brown glass test tube; next 1 mL ethanol containing 1.5 g/L BHT is added and the content is mixed. Using a pipette 2 mL n-hexane containing 0.25 g/L BHT is added and the tube content is thoroughly vortexed for 2 min and subsequently centrifuged at 2000 **g** and 4 °C for 5 min. A 1.5-mL aliquot of the n-hexane is pipetted into a brown glass tube whereafter the n-hexane is evaporated to dryness under a gentle stream of nitrogen. The residue is redissolved in 1.5 mL HPLC mobile phase. This solution can either be directly used for spectrophotometry of total carotenoids or for HPLC analysis of β-carotene, or be stored in the dark at -20 °C for analysis within a week.

Standardization

A β-carotene stock standard solution is obtained by dissolving ca. 20 mg β-carotene in 10 mL of methylene chloride (Merck, No. 6050) in a 100-mL brown glass flask followed by dilution with n-hexane (Merck, No. 4368) to 100 mL. The concentration of β-carotene in the stock standard solution is determined by spectrophotometry. A 1-mL aliquot of this solution is diluted with cyclo-hexane (Merck, No. 2822) to 200 mL, whereafter the concentration of β-carotene is determined by measuring the absorbance of the diluted solution at 457 nm in a suitable spectrophotometer, using E (1 cm) = 2505 at 457 nm for a

solution of 1 % (v/v) β-carotene in cyclohexane (Hejno 1964). A working standard solution is prepared daily by diluting an aliquot of the stock solution with a mobile phase to a concentration of 1 mg/L. The solutions are stored in the dark at -20 °C and are stable for at least two weeks.

Spectrophotometry

The total concentration of carotenoids is determined by measuring the absorbance at 445 nm of the extract in the mobile phase using a semi-micro quartz cuvette in the Vitatron spectrophotometer type MPS with the working standard solution as reference.

High-performance liquid chromatography

After spectrophotometry HPLC is carried out by injecting 100 μL of the extract in the mobile phase onto the HPLC column. The column is eluted isocratically with a mobile phase consisting of methanol : acetonitril : chloroform : water = 200 : 250 : 90 : 11 (v/v), set at 1.5 mL/min. The effluent is monitored with the detector set at 445 nm, this being the absorption maximum of β-carotene. The recorder is set at 10 mV. The chromatographic run takes about 15 min per sample.

Recovery experiments

A recovery solution containing ca. 50 mg/L of β-carotene is prepared as described under standardization but with dilution with dimethylacetamide (Merck, No. 803235) in stead of n-hexane. The recovery of β-carotene added to plasma is determined by analysing aliquots of plasma to which 10 μL of the recovery solution per mL of plasma had been added. The original β-carotene concentration is thus increased by 0.5 mg per L of plasma.

CALCULATION

The contents of total carotenoids and of β-carotene in the plasma sample are calculated from absorbances and peak heights respectively with the working standard solution as reference. In routine analysis, this solution is run before each series of five samples. The recovery is calculated by comparing the difference between the measured concentrations in the spiked and not spiked sample with the calculated amount of β-carotene (in g) added per mL of sample.

APPENDIX 3.

Fluorometric determination of α-, β- and γ-tocopherol in human plasma or serum by high-performance liquid chromatography

PRINCIPLE

After precipitation of proteins by addition of ethanol, the E vitamers are extracted, and α-tocopherol (and β- and γ-tocopherol) in the extract is separated on a straight-phase HPLC column and detected fluorometrically.

EXPERIMENTAL

Apparatus

HPLC is performed with a system incorporating a Gilson Model 302 constant-flow pump, a Micromeritic type 725 automatic injector and a Perkin Elmer fluorescence detector type 650-10. A Knauer stainless steel column (125 x 4.6 mm ID) is laboratory-packed with Polygosil 60-5 (Macherey-Nagel, No. 71101) by the balanced-density slurry technique on a column packing apparatus designed at our institute, using a Haskel pump type DSTV 150. The slurry and packing solvents are methylene chloride and n-hexane respectively (Merck, Nos 6050 and 4368, respectively). Elution profiles are displayed on a Kipp BD-8 recorder.

Sample extraction

Plasma is extracted as described for carotenoids (Appendix 2).

Standardization

A stock standard solution of vitamin E (all-rac-α-tocopherol; Sigma No. T-3251) is prepared by dissolving ca. 100 mg in 100 mL absolute ethanol. The vitamin E concentration of this solution is determined by measuring the absorbance at 292 nm of a 10-fold dilution in a suitable spectrophotometer using E (1 cm) = 75.69 at 292 nm for a solution of 1% (w/v) of α-tocopherol in absolute ethanol (Dawson et al. 1986). A working standard solution is obtained by diluting the stock standard solution with n-hexane to ca. 10 μmol/L. This solution and the stock standard solution are stored in the dark at -20 °C and are stable for at least one month.

High-performance liquid chromatography

HPLC is carried out by injecting 50 - 100 μL of the n-hexane layer onto the Polygosil column. The column is eluted isocratically with a mobile phase consisting of n-hexane : diisopropyl ether (containing 10 mg BHT per litre) = 9 : 1 (v/v; Merck, Nos 4368 and 800866 respectively) at a flow-rate of 1 mL/min. The effluent is monitored with the fluorescence spectrophotometer set at a wavelength pair of 296 - 320 nm. The recorder is set at 10 mV full scale. The chromatographic run takes ca. 6 min per sample.

Recovery experiments

A recovery solution containing ca. 2 mmol/L α-tocopherol is prepared by evaporating to dryness a few millilitres of the stock standard solution under a gentle stream of nitrogen and redissolving the residue in dimethylacetamide (Merck, No. 803235). The recovery of vitamin E added to plasma is determined by analysing aliquots of plasma to which 10 μL of the recovery solution per mL of plasma had been added. The original vitamin E concentration is thus increased by 20 μmol/L of plasma.

CALCULATION

The concentration of α-tocopherol (and/or of other tocopherols) of the plasma sample is calculated from peak heights with the working standard solution as reference. In routine analysis, this solution is run before each series of five samples. The recovery is calculated by comparing the difference between the measured concentrations in the spiked and not spiked sample with the calculated amount of α-tocopherol added per mL of sample.

REMARKS

For assessment of the vitamin E status the determination of α-tocopherol suffices since the concentration of this compound in plasma is 10 - 100 times higher than that of the two other E vitamers, i.e. β- and γ-tocopherol. Furthermore, the latter compounds are less active (2.4). A standard solution containing all four tocopherols is made by dissolving the content of an ampoule containing 10 mg each of α-, β-, γ- and δ-tocopherol (Merck, No. 15496) in n-hexane and subsequent dilution. The four vitamers are separated at a flow-rate of not more than 1.2 mL/min. The injection volume can be increased to 250 μL without loss of resolution. Concentrations are calculated as for α-tocopherol.

APPENDIX 4.

Determination of total thiamin in whole blood, foodstuffs and urine by high-performance liquid chromatography with post-column reaction fluorometric detection

PRINCIPLE

Blood is deproteinized by addition of trichloroacetic acid. Food samples are extracted with hydrochloric acid. The pH of both mixtures is adapted whereafter the thiamin phosphates are hydrolysed enzymatically. Acidified urine samples are buffered. The thiamin in the resulting solutions is separated on a HPLC column and oxidized in an on-line post-column reaction coil to thiochrome which is detected fluorometrically.

EXPERIMENTAL

Apparatus

HPLC is performed with a system incorporating a Knauer HPLC constant-flow pump type FR30, a Micromeritics type 725 automatic injector, a radial compression module equipped with a silica cartridge type 8 SI 10 (Waters Assoc., Milford, USA), a Kratos FS 950 Fluoromat fluorescence spectrophotometer equipped with a mercury light source (type FSA 110), an excitation band filter of 365 nm (type FSA 403) and an emission cut-off filter of 418 nm (type FSA 426), and a proportioning pump type Minipuls II (Gilson). Elution profiles are displayed on a Kipp BD-8 recorder and processed with the LIMS 2000 system of Perkin Elmer.

Reagents

Ferric chloride, BDH, No. 6498; sodium ascorbate, Merck, No. 500076; Mylase-100, United States Biochemicals, No. 19299; Taka-Diastase, Pfalz and Bauer, No. T00040. A 20% (w/v) Mylase-100 or Taka-Diastase solution is freshly prepared every day. The mobile phase consists of 250 mL 0.13 mol/L Na_2HPO_4, 250 mL 0.13 mol/L KH_2PO_4, 500 mL water and 367 mL ethanol. The thiochrome reagent consists of 1.5 mmol/L $K_3Fe(CN)_6$ (Merck, No. 4973) in 1.8 mol/L NaOH and is freshly prepared every day. The latter two solvents are flushed with a stream of helium gas 10 min before use. All other chemical are of analytical grade.

Standardization

Stock solutions of thiamin and thiamin diphosphate

Thiamin and thiamin diphosphate stock solutions are prepared by dissolving thiamin chloride.HCl (Sigma, No. T-4625) and thiamin diphosphate (Sigma, No. C-8754) in 0.15 mol/L sulphuric acid to about 2 mmol/L. The concentrations of these stock solutions are checked every two weeks as follows. A 5-mL aliquot of each stock solution is diluted with 0.1 mol/L HCl to 50 mL. Aliquots of 2, 3 and 4 mL of the HCl-diluted thiamin and thiamin diphosphate stock solutions are diluted to 20 mL with 0.1 mol/L HCl. The absorbances at 248 nm of these dilutions are measured in a suitable spectrophotometer. The concentrations of the thiamin and thiamin diphosphate stock solutions are calculated using

E (1 cm) = 13400 at 248 nm for 1.0-mol/L solutions of thiamin as well as of each of its three phosphates in 0.1 mol/L HCl (Penttinen 1976).

Thiamin working standard solutions

The thiamin stock solution is diluted with water to 150 nmol/L, and 0.25 and 0.1 mg/L. The 150-nmol/L solution is treated as a blood sample and serves as the working standard solution for determinations in blood. Aliquots of 5 mL of the 0.1 and 0.25 mg/L solution are treated as a food sample to obtain the working standard solutions for determinations in foodstuffs. Aliquots of 5 mL of the 0.25 and 0.1 mg/L solution are diluted with 1.5 mL 0.15 mol/L H_2SO_4 and 3.5 mL 4.5 mol/L sodium acetate buffer pH 6.2 to obtain the working standard solutions for determinations in urine.

Recovery solutions

The solution for recovery experiments in blood is obtained by diluting the thiamin diphosphate stock solution with physiological saline to ca. 8 µmol/L. A recovery solution for determinations in foodstuffs is obtained by diluting the thiamin stock solution with water to a concentration dependent on the expected concentrations of the samples investigated. A recovery solution for determinations in urine is obtained by diluting 0.1 mL of the thiamin stock solution to 20 mL with 6 mL 0.15 mol/L H_2SO_4 and 13.9 mL 4.5 mol/L sodium acetate buffer pH 6.2. All solution are stored in the dark at 4 °C.

Sample pre-treatment

Blood

Blood samples are frozen at -20 °C and thawed three times before analysis is done. A 1-mL aliquot of a just vortexed whole blood sample is slowly transferred during vortex-mixing into a reagent tube containing 1.5 mL of a solution containing 0.4 mol/L trichloroacetic acid (TCA) and 1.44 g/L ferric chloride. The tube is then allowed to stand for about 60 min at ambient temperature and the suspension is shaken vigorously half-way for 10 s. Then 0.4 mL 4.5 mol/L sodium acetate buffer pH 6.2 is added and the content is mixed (final pH 4.5). After addition of 0.1 mL of the Mylase-100 solution the solution is incubated in a water bath at 45 °C for 2 h. After cooling to room temperature the tube is centrifuged for 15 min at 2000 g. The clear supernatant is directly used for HPLC analysis or stored in the dark at 4 °C for analysis within 40 h.

Foodstuffs

Dependent on the expected content, 0.1 - 5 g finely ground sample is brought into a calibrated 50-mL septum bottle. Subsequently 25 mL water, 4 mL 1 mol/L HCl and about 100 mg sodium ascorbate are added. The content is incubated for 15 min in a pressure cooker at 120 °C. After cooling to room temperature, 6 mL 4.5 M sodium acetate buffer pH 6.2 and 1 mL enzyme solution are added whereafter the solution is incubated for 1 h at 45 °C. During incubation the contents are mixed a few times. The extracts are cooled to room temperature and the volume is adjusted to 50 mL with water. The samples are then filtrated through normal filter paper. The extracts are analysed by HPLC directly or stored at 4 °C for analysis within 40 h.

Urine

In a 10-mL plastic test tube 4.5 mL urine is mixed with 0.15 mL 1.5 mol/L sulphuric acid and 0.35 mL 4.5 mol/L sodium acetate buffer pH 6.2. The tube is centrifuged for 10 min at 2000 g and 4 °C. The extracts are analysed by HPLC directly or stored at 4 °C for analysis within 40 h.

Recovery experiments

Blood

Two pooled blood samples for recovery experiments are obtained by adding 3.75 and 7.5 mL, respectively of the recovery solution of thiamin diphosphate to two portions of 500 mL pooled blood. This blood is frozen at −20 °C and thawed three times before analysis. This way the original total thiamin concentration of the blood increases by 60 and 120 nmol/L, respectively.

Foodstuffs

The recovery of thiamin added to foods is determined by analysing a sample aliquot to which a few millilitres of recovery solution have been added to approximately twice the expected concentration.

Urine

The recovery of thiamin added to urine is determined by analysing 4 mL urine to which 0.5 mL of the 2.5 mg/L thiamin solution have been added. The concentration of the urine sample is raised by 0.31 mg/L.

High-performance liquid chromatography

HPLC analysis is carried out by injecting a 100-μL aliquot onto the HPLC column. The column is eluted isocratically with the mobile phase set at 3.2 mL/min. The effluent of the column is mixed with 0.3 mL/min thiochrome reagent in a 1200 × 1 mm stainless steel reaction coil. The effluent is monitored with the fluorometer. The recorder is set at 10 mV. The chromatographic run takes about 12 min per sample.

CALCULATION

Blood

A Perkin Elmer LIMS 2000 system is connected with the fluorometer. The concentration of total thiamin in the original whole blood sample is calculated from the peak area (mV.s), read in a tangential mode with base-line correction, in which the thiamin working standard solution, which is run before each series of five samples, is used as reference.

Foodstuffs and urine

The thiamin concentrations of the original foodstuff and urine samples are calculated from peak heights with the relevant working standard solutions as references. In routine analyses, the thiamin standard is run before each series of five samples. The recovery is calculated by comparing the difference between the measured concentrations of the spiked and not spiked sample to the calculated amount of thiamin added.

APPENDIX 5.

Determination of total riboflavin in foodstuffs and urine by high-performance liquid chromatography with fluorometric detection

PRINCIPLE

Foodstuffs are extracted with acid. The pH is adjusted whereafter the flavins are hydrolysed enzymatically. The resulting riboflavin in the extract is separated on a C18 column and detected fluorometrically. Urine is diluted with hydrochloric acid and buffer and then injected onto a C18 column.

EXPERIMENTAL

Apparatus

HPLC is performed with a Knauer FR30 constant-flow pump, a Micromeritics type 725 autoinjector and a Shimadzu type RF-530 fluorescence spectrofluorometer. A Knauer stainless steel column (125 x 4.6 mm ID) is laboratory-packed with ODS-Hypersil 5 μm (Shandon, No. 58105010) by the balanced-density slurry technique on a column packing apparatus designed at our institute, using a Haskel pump type DSTV 150. The slurry and packing solvents are 2-propanol and methanol (Merck, Nos 9634 and 6009, respectively). Elution profiles are displayed on a Kipp BD-8 recorder.

Sample pre-treatment

Foodstuffs

An aliquot of 0.1 - 5 g of foodstuff is brought into a septum bottle whereafter 25 mL water, 4 mL 1 mol/L HCl and about 100 mg sodium ascorbate is added. The mixture is incubated in a pressure cooker for 15 min at 120 °C whereafter it is allowed to cool to ambient temperature and 6 mL of a 4.5 mol/L sodium acetate buffer pH 6.2 is added. The flavins are hydrolysed by incubating the mixture for 60 min at 45 °C with 1 mL of a solution containing 20 % (w/v) Taka-Diastase (Pfaltz and Bauer, No. T00040). The septum bottle is shaken a few times during the incubation period. The mixture is allowed to cool to ambient temperature whereafter the volume is adjusted to 50 mL with water. The supernatant is filtrated through normal filter paper and, if still turbid, subsequently through a disposable 0.45-μm filter. The clear filtrate is either used for direct HPLC analysis or stored in the dark at 4 °C for analysis within one week.

Urine

A 2-mL aliquot of urine is brought into a 5-mL plastic reagent tube and mixed with 0.2 mL 1 mol/L HCl and 0.3 mL 4.5 mol/L sodium acetate buffer pH 6.2. After centrifugation for 10 min at 2000 g and 4 °C the resulting liquid is directly used for HPLC analysis.

Standardization

Foodstuffs

A riboflavin stock standard solution is obtained by dissolving about 25 mg of riboflavin (E. Hoffmann-La Roche, Basel, Switzerland) in 300 mL water of 50 °C whereafter 40 mL 0.15 mol/L H_2SO_4 and 18 mL 10 % (w/v) TCA solution are

added. After the vitamin has dissolved, the volume is adjusted to 500 mL with water. Of this solution 1 mL is diluted with water to 50 mL. Two working standard solutions are obtained by treating a 1-mL and a 2-mL aliquot of the diluted stock standard solution as samples. The working standard solutions are freshly prepared for every series of analyses.

Urine

The stock standard solution of riboflavin is the working standard solution for riboflavin determinations in urine and is freshly prepared for every series of analyses.

Recovery experiments

Foodstuffs

The recovery of riboflavin added to foodstuffs is determined by analysing portions of foodstuff spiked with a few millilitres of stock standard solution transferred to the septum bottle prior to extraction in the pressure cooker.

Urine

The recovery of riboflavin added to urine is determined by analysing portions of urine spiked with 0.5 mL stock standard solution added to the tube instead of 0.2 mL 1 mol/L HCl and 0.3 mL sodium acetate buffer.

High-performance liquid chromatography

HPLC is carried out by injecting 100 μL foodstuff extract or diluted urine onto the ODS-Hypersil column. The column is eluted isocratically with a mobile phase consisting of 20 % (v/v) methanol in water. The flow-rate is 1.2 mL/min. The effluent is monitored with the fluorescence spectrophotometer set at a wavelength pair of 462 - 520 nm. The recorder is set at 10 mV full scale. The chromatographic run takes about 15 min per sample.

CALCULATION

The riboflavin concentration of the foodstuff or urine sample is calculated from peak heights with the relevant working standard solution as reference. In routine analysis the working standard solution is run before each series of five samples. The recovery is calculated by comparing the difference between the measured concentrations in the spiked and not spiked sample to the calculated amount of riboflavin added.

APPENDIX 6.

Determination of total pyridoxal, pyridoxine and pyridoxamine (vitamin B_6) in foodstuffs by high-performance liquid chromatography with fluorometric detection

PRINCIPLE

Foodstuffs are extracted with trichloroacetic acid (TCA). The pH is adjusted whereafter the B_6 vitamers are hydrolysed enzymatically. The resulting three free forms in the extract are separated on a reversed-phase column. After addition of a phosphate buffer in a post-column reaction coil the enhanced fluorescence is measured.

EXPERIMENTAL

Apparatus

HPLC is performed with a Gilson Model 302 constant-flow pump, a Perkin Elmer type ISS-100 autoinjector and a Shimadzu type RF-530 fluorescence spectrofluorometer. A Knauer stainless steel column (125 x 4.6 mm ID) is laboratory-packed with ODS-Hypersil 3 μm (Shandon, No. 580 x 18) by the balanced-density slurry technique on a column packing apparatus designed at our institute, using a Haskel pump type DSTV 150. The slurry and packing solvents are 2-propanol and methanol, respectively (Merck, Nos 9634 and 6009, respectively). A Watson and Matlow 4-channel peristaltic pump is used for adding reagent to the post-column coil. Elution profiles are displayed on a Kipp BD-8 recorder.

Standardization

Stock standard solutions of pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), pyridoxal 5'-phosphate (PLP), pyridoxamine 5'-phosphate (PMP) and 4-deoxypyridoxine (DOP, internal standard) from Sigma (Nos P-9130, P-9755, P-9380, P-9255, P-9505 and D-0501, respectively) are made by dissolving 10 mg compound in 100 mL water and adding 3 drops of phosphoric acid. Working standard solution of PL, PN, PM and DOP are made by diluting 100 μL - PL, PN and PM - or 1 mL - DOP - of the stock solutions to 50 mL with 5 % (w/v) TCA solution whereafter these solutions are treated as foodstuffs. The stock solutions are stable for 2 months if stored in the dark at 4 °C.

Sample pre-treatment

An aliquot of 0.1 - 5 g of foodstuff is brought into a septum bottle whereafter 1 mL DOP solution and a few millilitres of PLP and/or PMP solution (to approximately twice the content for recovery experiments) are added. The volume is adjusted to 50 mL with 5 % (w/v) TCA. The mixture is shaken for 30 min, centrifuged for 10 min at 2000 g and filtrated. A 3-mL aliquot of filtrate is brought into a test tube and 0.4 mL 4.5 mol/L sodium acetate buffer pH 6.2 is added. After vortexing 0.1 mL of a solution containing 10% (w/v) Taka-Diastase (Pfaltz and Bauer, No. 00040) is added. The extract is incubated for 3 h on a water bath at 45 °C and mixed regularly. During vortexing 1.5 mL 16.7 % (w/v) TCA is added, and the extract is centrifuged for 10 min at 2000 g. The extract is stored at 6 °C for HPLC within 2 days.

High-performance liquid chromatography

HPLC is carried out by injecting 100 μL of the foodstuff extract onto the ODS-Hypersil column. The column is eluted isocratically with a mobile phase consisting of 3 % (v/v) methanol in a mixture of 5 mL Pic-B8 reagent (Waters Ass., No. 85142) in 0.1 mol/L potassium dihydrogen phosphate and with the pH adjusted to 2.15. The flow-rate is set at 1.0 mL/min. The reagent, 1 mol/L dipotassium hydrogen phosphate, is added to the post-column coil at a flow-rate of 0.33 mL/min. The effluent is monitored with the fluorescence spectrophotometer set at a wavelength pair of 333 - 375 nm. The recorder is set at 10 mV full scale. The chromatographic run takes about 25 min per sample.

Recovery experiments

Recovery experiments are carried out by dissolving relevant amounts of PLP and PMP in the internal standard solution. The concentrations of PLP and PMP have to be approximately doubled.

CALCULATION

The B_6 vitamer concentrations of the foodstuff are calculated from peak heights with the internal standard and the relevant working standard solution as reference. In routine analysis the working standard solution is run before each series of five samples. The recovery is calculated by comparing the difference between the measured concentrations in the spiked and not spiked sample to the calculated amount of B_6 vitamers added.

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CURRICULUM VITAE

Andries Speek werd op 28 februari 1943 te Amsterdam geboren. Na het behalen van het diploma HBS-B aan de Dr.Ir. Lely HBS te Amsterdam begon hij in 1960 met de studie scheikunde aan de toenmalige "Gemeente Universiteit van Amsterdam" (thans Universiteit van Amsterdam). In november 1968 behaalde hij het doctoraaldiploma (hoofdvak emissie spectroscopie, bijvak elektronica), waarna hij zijn militaire dienstplicht vervulde. In september 1970 trad hij in dienst van het Centraal Instituut voor Voedingsonderzoek TNO te Zeist, sectie Verpakkingen en Gebruiksartikelen, voor analytisch-chemisch onderzoek naar de migratie van additieven. In maart 1977 begon hij, als werkgroepleider HPLC, voor de afdeling Klinische Biochemie van hetzelfde instituut, met het ontwikkelen van HPLC-methoden voor de analyse van vitamines in lichaamsvloeistoffen en voedingsmiddelen en met het uitbouwen van het routinelaboratorium voor vitamine-analyses. Tussen 1984 tot 1986 werden zijn werkzaamheden voor TNO onderbroken toen hij ten behoeve van een ontwikkelingsproject als "consultant for analytical biochemistry" was verbonden aan de Khon Kaen Universiteit te Khon Kaen, Thailand.

Andries Speek was born on February 28, 1943 in Amsterdam, The Netherlands. He graduated from the Dr Ir Lely High School at Amsterdam (HBS-B) in 1960 and entered the at that time Municipal University of Amsterdam (now University of Amsterdam). In November 1968 he fulfilled the requirements for the Doctoral Degree in Analytical Chemistry with Emission Spectroscopy and Electronics as major subjects, whereafter he served in the army. In September 1970 he affiliated with the Central Institute for Food and Nutrition Research TNO in Zeist, Section Food Packaging and Utensils where he conducted analytical investigations on the migration of additives. In March 1977 he became groupleader "High-Performance Liquid Chromatography" of the Department of Clinical Biochemistry. HPLC methods for vitamin analysis were developed and the routine laboratory was extended with HPLC methods for vitamin analyses in body fluids and foodstuffs. From 1984 to 1986 he was affiliated as "consultant for analytical biochemistry" with the Khon Kaen University in Khon Kaen, Thailand on behalf of a co-operation project with the Department of Clinical Biochemistry.