Determination of B-group Vitamins in Food Using an LC-MS Stable Isotope Dilution Assay

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Declaration:

Hereby I declare that this doctoral thesis, submitted for the doctoral degree at Tallinn University of Technology, is my original investigation and achievement and has not been submitted for any academic degree.

/Kristel Hälvin/

B-grupi vitamiinide määramine toiduainetes kasutades LC-MS isotoop-lahjenduse meetodit

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Contents

Abstract

General consumer awareness of what constitutes a healthy diet has improved. Because of this and the fact that many compounds, including B-group vitamins, are essential nutrients in food, there is a pressing need to develop accurate and high throughput analytical methods to evaluate the nutritional composition of food. Despite the rapid development of analytical techniques, the simultaneous quantification of all B-group vitamins in foods remains challenging. The research presented in this dissertation concerns the development, validation, and testing of an LC-MS - stable isotope dilution assay (SIDA) based method and prototype analytical assay that allows for the simultaneous, high throughput quantification of B-group vitamins in foods. LC-MS(-MS)-SIDA is currently recognized as the most sensitive and reliable method for this purpose and can be conducted using a relatively simple sample preparation procedure, compared with conventional methods. To develop an effective LC-MS-SIDA method and prototype analytical assay to determine vitamins B_1, B_2, B_3, B_5, and B_6 in foods, we optimized LC-MS conditions to separate and detect B-group vitamers, tested various extraction conditions, including enzymes for liberation of free vitamers from phosphorylated forms and cofactors, and validated the method using reference food samples and inter-laboratory comparative tests with other food matrices. Mild acidic extraction with acid phosphatase and β-glucosidase treatment was found to be the most efficient method to determine the total concentration of most B-group vitamins in food. To determine the total concentration of vitamins B_3 and B_5 in foods, additional isotope labelled vitamers (e.g. nicotinamide riboside (NR), nicotinamide mononucleotide (NMN), NAD^+, pantetheine, and acetyl-CoA) should be included in the assay kit because none of the enzymes tested were able to completely liberate simple vitamers from these compounds. Currently, precise quantification of these components is made difficult because isotope-labelled versions of these compounds are not commercially available. Therefore, we tested a method that made

use of *in house* prepared ^{15}N-labelled yeast extract fractions as the source of isotopically labelled internal standards for the determination of some B_3 vitamin derivatives, e.g. NR, NMN, and NAD$^+$. To determine the total concentration of vitamin B_6 the extraction conditions should be further optimized to completely liberate protein bound pyridoxal and pyridoxal phosphate. Our results demonstrate that our LC-MS-SIDA method and corresponding analytical assay are powerful tools to study the stability and distribution of B-group vitamins in foods and microorganisms, and, in the future, should allow one to determine the total concentration of all bioavailable vitamin forms.

Kokkuvõte

Tarbijate teadlikkuse kasv tervislikust toitumisest on loonud vajaduse täpsete ja suure läbilaskevõimega analüüsimeetodite järele hindamaks toiduainete toiteväärtust, sealhulgas B-grupi vitamiinide sisaldust. Vaatamata analüütiliste meetodite kiirele arengule on B-grupi vitamiinide samaaegne määramine jäänud endiselt tõeliseks väljakutseks. Käesolevas doktoritöös töötati välja, valideeriti ja testiti suure läbilaskevõimega vedelikkromatograafia massispektromeetria – isotoop-lahjenduse metoodika (LC-MS-SIDA) koos analüütilise määramiskomplekti prototüübiga B-grupi vitamiinide samaaegseks kvantitatiivseks määramiseks toiduainetes. LC-MS(-MS)-SIDA meetod on hetkel kõige tundlikum ja usaldusväärsem meetod, mis võimaldab kasutada suhteliselt lihtsaid proovi ettevalmistamise protseduure, võrreldes tavapäraste meetoditega. Tõhusa LC-MS-SIDA metoodika ja analüütilise määramiskomplekti väljatöötamiseks vitamiin B_1, B_2, B_3, B_5 ja B_6 määramiseks toiduainetes me optimeerisime LC-MS tingimusi B-grupi vitameeride lahutamiseks ja detekteerimiseks, testisime erinevaid ekstraktsiooni tingimusi, sh ensümaatilist ekstraktsiooni lihtsate vitameeride vabastamiseks fosforüleeritud vormidest ja kofaktoritest, ning valideerisime metoodika, kasutades erinevate toiduainete referentsmaterjale ja laboritevahelisi võrdluskatseid teiste toidumaatriksitega. Kõige tõhusamaks metoodikaks suurema osa B-grupi vitamiinide kogukontsentratsiooni määramiseks toiduainetes osutus ekstraktsioon nõrgalt happelises keskkonnas koos ensümaatilise töötlusega, kasutades happelist fosfataasi ja b-glükosidaasi. Vitamiin B_3 ja B_5 kogukontsentratsiooni määramiseks toiduainetest tuleks määramiskomplekti täiendavalt lisada mitmeid isotoop-märgistatud vitameere (nt nikotiinamiidribosiid (NR), nikotiinamiid mononukleotiid (NMN), NAD^+, panteteiin, atsetüül-CoA), kuna ükski kasutatud ensüümidest ei suutnud lihtsaid vitameere nendest ainetest täielikult vabastada. Hetkel on nende ainete täpne kvantifitseerimine aga keeruline, kuna nende isotoopmärgistatud analoogid ei ole

kommertsiaalselt kättesaadavad. Selle probleemi lahendamiseks katsetasime metoodikat, mis võimaldas kasutada isevalmistatud ^{15}N-märgistatud pärmiekstrakti mõningate isotoop-märgistatud vitamiin B$_3$ vitameeride allikana (nt NR, NMN, NAD$^+$). Vitamiin B$_6$ kogukontsentratsiooni määramiseks tuleks ekstraktsioonitingimusi optimiseerida, et vabastada ka valk-seotud püridoksaal ja püridoksaalfosfaat. Meie tulemused näitavad, et väljatöötatud LC-MS-SIDA metoodika ja vastav analüütiline määramiskomplekt võimaldab uurida B-grupi vitamiinide stabiilsust ja erinevate vitameeride sisaldust toiduainetes ja mikroorganismides ning peaks pikemas perspektiivis võimaldama kõikide biokättesaadavate vitameeride määramist.

List of publications

The following publications form the basis of this dissertation and are reproduced in the appendices with permission from the publishers.

Publication I

Hälvin K, Paalme T, Nisamedtinov I (2013) Comparison of different extraction methods for simultaneous determination of B complex vitamins in nutritional yeast using LC/MS-TOF and stable isotope dilution assay. Anal Bioanal Chem 405(4):1213-22

Publication II

Hälvin K, Paalme T, Nisamedtinov I (2013) Method and Analytical Kit for Simultaneous Quantification of B Complex Vitamin Content in Food. WO/2013/010553

Publication III

Mihhalevski A, Nisamedtinov I, Hälvin K, Ošeka A, Paalme T (2013) Stability of B-complex vitamins and dietary fiber during rye sourdough bread production. J Cereal Sci 57:30-38

Publication IV

Hälvin K, Nisamedtinov I, Paalme T (2014) Comparison of different extraction methods to determine free and bound forms of B-group vitamins in quinoa. Anal Bioanal Chem 406(28):7355-7366

Publication V

Paalme T, Kevvai K, Vilbaste A, Hälvin K, Nisamedtinov I (2014) Uptake and accumulation of B-group vitamers in *Saccharomyces cerevisiae* in ethanol-stat fed-batch culture. World J of Microb Biotech 30(9):2351-9

Publication VI

Hälvin K, Paalme T, Vilbaste A, Nisamedtinov I (2014) LC-MS for quantification of B-group vitamins in yeast. Advances in Science and Industrial Production of Bakers´ Yeast. Proceedings of the 27th VH Yeast Conference. Publication in progress.

The author´s contribution to these publications was as follows

In Publication I, the author designed the study, carried out all experiments, interpreted the data, and was responsible for publication of the manuscript.

In Publication II, the author contributed to designing the study, carried out all experiments, interpreted the data, and was responsible for publication of the manuscript.

In Publication III, the author contributed to designing the study and interpreting the data and was involved in writing the manuscript.

In Publication IV, the author designed the study, carried out all experiments, interpreted the data, and was responsible for publication of the manuscript.

In Publication V, the author contributed to designing and interpreting the analytical part of the study.

In Publication VI, the author designed the study, carried out part of the experiments, interpreted the data, and was responsible for publication of the manuscript.

List of Conference Presentations

Hälvin K, Vilbaste A, Paalme T, Nisamedtinov I. Determination of vitamin B_3 vitamers in milk and milk products by LC-MS SIDA. Poster Presentation at 20th International Mass Spectrometry Conference, 24-29 August, 2014, Geneva, Switzerland

Hälvin K, Paalme T, Vilbaste A, Nisamedtinov I. LC-MS for quantification of B-group vitamins in yeast. Oral Presentation at 27th VH Yeast Conference, 14-15 April, 2014, Istanbul, Turkey

Hälvin K, Nisamedtinov I, Paalme T. Determination of free and bound B-complex vitamins in quinoa by LC-MS-SIDA. Poster Presentation at XVII Euroanalysis 2013, 25-29 August, 2013, Warsaw, Poland

Hälvin K. Application of SIDA for LC-MS quantification of B-complex vitamins in biological samples. Oral Presentation at DSM Nutritional Products Ltd (Analytical Research Center), 3-4 June, 2013, Basel, Switzerland

Hälvin K, Vilbaste A, Paalme T, Nisamedtinov I. A Study on accumulation of B-complex vitamins in Saccharomyces cerevisiae by LC-MS and stable isotope dilution assay. Poster Presentation at 3rd International Conference on FOOD-OMICS, 22-24, May 2013 Cesena, Italy

Hälvin K, Paalme T, Nisamedtinov I. Limitations of LC-MS in B-complex vitamins determination. Poster Presentation at 2nd International Vitamin Conference, 23-25 May, 2012, Copenhagen, Denmark

Hälvin K, Mihhalevski A, Nisamedtinov I, Ošeka A, Paalme T. Determination of the stability of B-complex vitamins during rye sourdough bread production using LC-MS and stable isotope dilution assay (SIDA). Poster Presentation at Advances and Controversies in B-Vitamins and Choline, 5-8 March, 2012, Leipzig, Germany

Hälvin K, Paalme T, Nisamedtinov I. Simultaneous quantification of the B-complex vitamins in dried yeast using UPLC-MS and isotope labeled internal standards. Oral Presentation at FoodBalt 2010, 29-30 October, 2010, Tallinn, Estonia

Hälvin K, Ošeka A, Tammel K, Paalme T, Nisamedtinov I. Effect of hydrolytic enzymes on simultaneous analysis of the B-complex vitamin content in dry yeast using LC-MS and isotope labeled internal standards. Poster Presentation at 1st International Vitamin Conference, 19-21 May, 2010, Copenhagen, Denmark

Hälvin K, Nisamedtinov I, Ošeka A, Paalme T. Quantification of B-group vitamins in food using isotope dilution mass spectrometry (IDMS) and lyophilized internal standards. Poster Presentation at 18th International Mass Spectrometry Conference, 30 August - 3 September, 2009, Bremen, Germany

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ACRONYMS

AOAC   Association of Official Analytical Chemists

ATCC   American Type Culture Collection

AThDP adenosine thiamine diphosphate

AThTP adenosine thiamine triphosphate

BAEE   N-benzoyl-L-arginine ethyl ester

BCR   Community Bureau of Reference

CCFFT Competence Center of Food and Fermentation Technologies

CRM   certified reference material

CoA   coenzyme A

EN   European Standard

ESI   electrospray ionisation

FAD   flavin adenine dinucleotide

FMN   flavin mononucleotide

GIT   gastrointestinal tract

HDL   high-density-lipoprotein

HPLC   high-performance liquid chromatography

HCl   hydrochloric acid

LC   liquid chromatography

LOD   limit of detection

LOQ   limit of quantitation

MS   mass spectrometry

m/z   mass to charge ratio

NA   nicotinic acid

NAD^+ nicotinamide adenine dinucleotide

NADH   nicotinamide adenine dinucleotide reduced

$NADP^+$ nicotinamide adenine dinucleotide phosphate

NADPH nicotinamide adenine dinucleotide phosphate reduced

NAM   nicotinamide

NaR   nicotinic acid riboside

NIST   National Institute of Standards and Technology

NMN   nicotinamide mononucleotide

NR   nicotinamide riboside

PL   pyridoxal

PLP   pyridoxal phosphate

PM   pyridoxamine

PMP   pyridoxamine phosphate

PN   pyridoxine

PNG   pyridoxine glucosides

PNP   pyridoxine phosphate

RP   reversed phase

SIDA   Stable Isotope Dilution Assay

SIL   stable isotope labelled

SRM   standard reference material

TDP   thiamine diphosphate

TMP   thiamine monophosphate

TOF   time-of-flight

TTP   thiamine triphosphate

UV   ultraviolet

WE   Wernicke's encephalopathy

WKS   Wernicke-Korsakoff syndrome

THESIS

1. Introduction

General consumer awareness of what constitutes a healthy diet has improved. Because of this and the fact that many compounds, including B-group vitamins, are essential nutrients in food, there is a pressing need to develop accurate and high throughput analytical methods to evaluate the nutritional composition of food. Despite the rapid development of analytical techniques, the simultaneous quantification of B-group vitamins in foods remains challenging, with major hindrances being their low concentration in the food matrix, relatively low stability, as well as their different structures and chemical properties [Gentili et al 2008]. Liquid chromatography mass spectrometry (LC-MS), or LC-MS-MS, combined with a stable isotope dilution assay (SIDA) is currently the most sensitive and selective method and can be conducted using relatively simple sample preparation procedures, compared with conventional methods. Although there are several reports that have used LC-MS-MS together with isotope labelled internal standards to determine B-group vitamins in tablets and dietary supplements [Chen et al 2007, Goldschmidt & Wolf 2010, Phinney et al 2011], studies involving real food samples are rather limited. Method development for the simultaneous determination of B-group vitamins in natural samples is quite challenging, mainly due to the complexity of the sample preparation processes and unavailability of isotope-labelled internal standards for many naturally occurring vitamers. Thus, to determine the total content of vitamins in food, the extraction of vitamers from the food matrix should be maximized and the vitamers should be converted into the same forms as the internal and external standards used in the assay. Ideally, the method should also allow for high throughput analysis which can be accomplished with a single/few-step procedure for complete extraction and the same chromatographic conditions for all vitamins under study. The standard extraction procedures currently used [EVS-EN 14122:2003 for vitamin B_1, EVS-EN 14152:2003 for vitamin B_2, EVS-EN 15652:2009 for niacin, EVS-EN 14663:2006 for vitamin B_6

(including its glycosylated forms)] are designed for single vitamin analysis and determine the total concentration of the pure vitamin compound, i.e. without providing information on the distribution of individual vitamers within a given group. In addition, these methods use extraction buffers that are unfriendly for MS detection (e.g. hydrochloric acid) or high temperatures which are physiologically unrealistic and are not suitable for the analysis of some B-group vitamers (e.g. pantothenic acid). An alternative would be to use hydrolytic enzymes instead of acid hydrolysis for the liberation of simple vitamers from their bound forms, as proposed by Ndaw and co-workers [2000]. Thus, a suitable enzyme or enzyme mixture with the required activities should be used and an MS compatible extraction buffer should be preferred when determining B-group vitamins in foods.

The aim of this dissertation is to develop an LC-MS-SIDA based method and a suitable analytical assay for the simultaneous, high throughput quantification of B-group vitamins in foods. The resulting LC-MS-SIDA method and analytical assay were validated with standard reference materials and two inter-laboratory comparative tests using different food samples. In addition, testing was carried out using a variety of other food samples.

2. Literature review

Vitamins are a heterogeneous group of organic compounds that are, in trace amounts, essential for the normal functioning of living organisms. They are generally not synthesized in mammals and thus, must be obtained from our daily diet. Vitamins are classified into two groups based on their solubility: fat soluble vitamins (A, D, E, and K) and water soluble vitamins (vitamin C and B-group vitamins).

In the current study we concentrate on B-group vitamins, and specifically on vitamers that contain structural units of thiamine (B_1), riboflavin (B_2), nicotinic acid and nicotinamide (B_3), pantothenic acid (B_5), and pyridoxine, pyridoxal, and pyridoxamine (B_6). Other B-group vitamins that contain structural units of biotin (B_7), tetrahydrofolate (B_9), and cobalamin (B_{12}) were omitted from this study, because their extraction conditions and concentrations in foods are different from other B-group vitamins.

2.1. B-group vitamins

The group of B-vitamins is relatively large and includes vitamins B_1, B_2, B_3, B_5, B_6, B_7, B_9, and B_{12}. As stated above, each of these B-vitamins exist as individual groups of chemically related compounds termed 'vitamers' which contain the same structural unit and have, after digestion and consumption, similar biological activity [Gregory 2012]. B-group vitamins serve various biological functions as coenzymes or precursors of cofactors that function in the metabolism of cells. The term 'bioavailability', as applied to vitamers in human nutrition, refers to the proportion of the quantity of the given vitamer in the food ingested that undergoes intestinal absorption and utilization by the body [Ball 2004].

2.1.1. Vitamin B$_1$

Vitamin B$_1$ exists naturally in a large variety of animal and plant products in a simple form, thiamine, and as one or more of its derivatives: 2-(1-hydroxyethyl)thiamine, thiamine monophosphate (TMP), thiamine diphosphate (TDP), thiamine triphosphate (TTP), adenosine thiamine diphosphate (AThDP), and adenosine thiamine triphosphate (AThTP) (Fig 1). The concentration of the two latter forms, however, is relatively low in food. In addition to these distinct chemical forms, thiamine can be also bound to proteins in microorganisms, animal tissues, and plants [Iwashima & Nishimura 1979, Muniyappa & Adiga 1979, Mitsunaga et al 1986].

The major biologically active form of vitamin B$_1$ is TDP, which serves as a cofactor for several enzymes involved in carbohydrate and amino acid metabolism. TDP works as a coenzyme for three enzymes involved in the oxidative decarboxylation of α-keto acids in the tricarboxylic acid cycle; specifically, the pyruvate dehydrogenase complex (EC 1.2.4.1), the α-ketoglutarate dehydrogenase complex (EC 1.2.4.2), and branched-chain α-keto acid dehydrogenase complex (EC 1.2.4.4). In addition, TDP works as a coenzyme for transketolase (EC 2.2.1.1), which is found in the cytosol and is involved in the pentose phosphate pathway. In higher organisms, thiamine also appears to be related to nerve impulse transmission [Cooper & Pincus 1979].

Thiamine appears in most foods to be either highly or totally available for absorption and utilization in the human gastrointestinal tract (GIT) [Gregory 1997]. Prior to absorption, the phosphorylated forms of thiamine (predominantly as TDP) are hydrolysed into free thiamine in the intestinal lumen by gastrointestinal phosphatases [Rindi & Laforenza 2000]. Absorption of free thiamine takes place primarily in the proximal part of the small intestine via specific carrier-mediated processes [Rindi & Laforenza 2000, Said et al 2004]. Two human thiamine transporters within intestinal epithelial cells (hTHTR-1 and hTHTR-2) have been identified to date [Said 2011]. In addition, thiamine can be obtained via

de novo synthesis by naturally occurring microflora within the large intestine [Wilson 2005, Said 2011]. Thiamine in excess of the body's needs is rapidly excreted in the urine together with small amounts of its metabolites (2-methyl-4-amino-5-pyrimidine carboxylic acid, 4-methyl-thiazole-5-acetic acid, and thiamine acetic acid) [Tanphaichitr 1999].
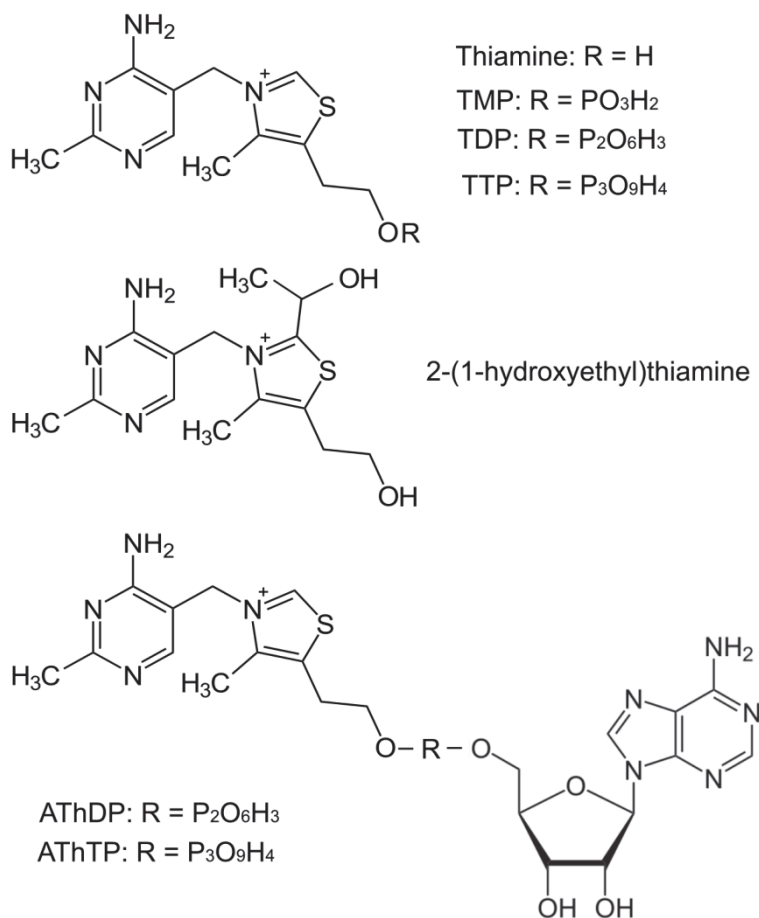
Thiamine: R = H
TMP: R = PO_3H_2
TDP: R = $P_2O_6H_3$
TTP: R = $P_3O_9H_4$

2-(1-hydroxyethyl)thiamine

AThDP: R = $P_2O_6H_3$
AThTP: R = $P_3O_9H_4$

Figure 1. Vitamin B$_1$ vitamers

Medical disorders in humans related to thiamine deficiency are beriberi, Wernicke-Korsakoff syndrome (WKS) (an alcohol–linked neurological disorder) [Butterworth 2003]. Beriberi occurs as dry beriberi, which is characterized by peripheral neuropathy consisting of symmetrical impairment of sensory and motor nerve conduction velocities, and wet beriberi which is characterized by oedema, tachycardia, cardiomegaly, and congestive heart failure, in addition to peripheral neuropathy. WKS typically consists of two components, a short–lived and severe condition termed Wernicke's encephalopathy (WE), and a long–lasting and debilitating condition known as Korsakoff's psychosis. The symptoms of WE include mental confusion, paralysis of the nerves that move the eyes, and an impaired ability to co-ordinate movements. Most alcoholics with WE develop Korsakoff's psychosis, a chronic neuropsychiatric syndrome characterized by behavioural abnormalities and impairment of memory related functions [Butterworth et al 1993, Zubaran et al 1997]. Thiamine deficiency could also be related to Alzheimer's disease and heart failure [Lu'o'ng & Nguyen 2011, DiNicolantonio et al 2013]. Preliminary evidence suggests that thiamine, together with other nutrients, may lower the risk of developing cataracts [Cumming et al 2000, Jacques et al 2005].

2.1.2. Vitamin B_2

Vitamin B_2 exists naturally in a variety of animal and plant products in a simple form, riboflavin, and as one of its two derivatives: flavin cofactors riboflavin-5´-phosphate (flavin mononucleotide, FMN) and flavin adenine dinucleotide (FAD) (Fig 2). Riboflavin and its cofactors, in turn, can be non-covalently bound to proteins resulting in flavoproteins [Adiga et al 1988, Massey 2000]. Some of the flavins can also be covalently bound to proteins but these do not appear to be available for absorption in the human GIT [McCormick 1972]. In addition, some plants may contain glycosylated riboflavin (e.g. riboflavin-5´-α-D-glucoside), however, the nutritional significance of this form appears to be minor [Gregory 1998].

The metabolically active forms of riboflavin are FMN and FAD, which function as cofactors for a variety of oxidative enzymes. FMN and FAD are usually tightly bound to enzymes (flavoproteins), and in some cases even via covalent bonds (e.g. in succinate dehydrogenase) [Walker & Singer 1970]. Flavoproteins are able, in various degrees, to catalyze both one- and two-electron redox reactions and play a wide variety of roles in our intermediary metabolism.

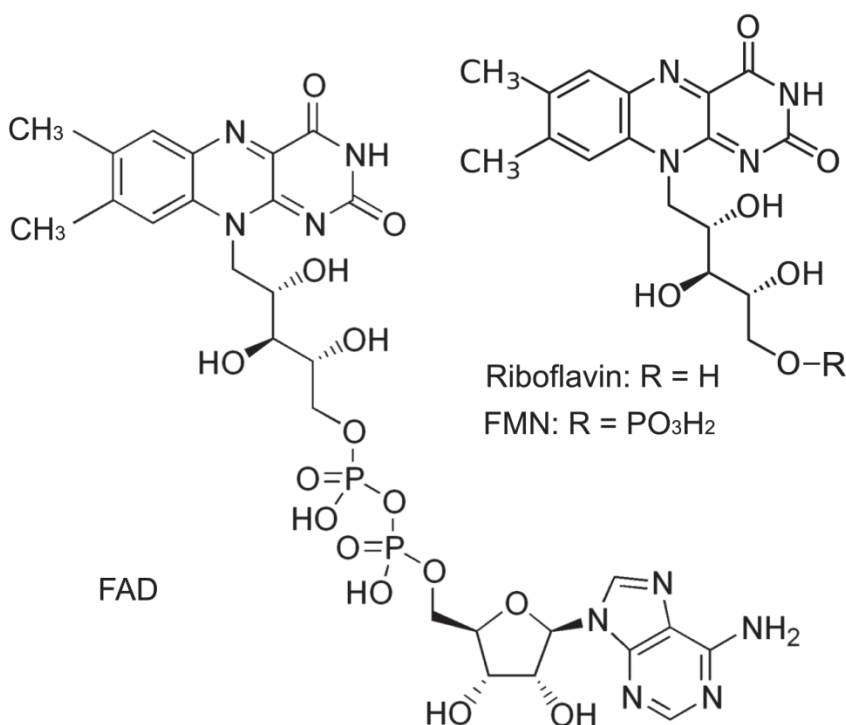Riboflavin: R = H
FMN: R = PO_3H_2

Figure 2. Vitamin B_2 vitamers

The bioavailability of riboflavin containing compounds (flavins) seems not to depend on the form that is digested [Dainty et al 2007]. A prerequisite for absorption is that protein bound riboflavin and flavins

are released in the stomach by gastric acidification [Merrill et al 1981]. The flavins released (FMN and FAD) are then dephosphorylated to riboflavin by nonspecific phosphatases in the brush border membranes of enterocytes in the proximal part of the small intestine [Daniel 1983]. The absorption of free riboflavin in the small intestine occurs on the brush-border membrane vesicle via a carrier-mediated system [Said & Arianas 1991]. Two human riboflavin transporters have been identified in intestinal epithelial cells: hRFT-1 and hRFT-2 [Said 2011]. In addition, colonic microflora is able to synthesize considerable amounts of riboflavin [Wilson 2005]. Riboflavin in excess of the body's needs is excreted in urine and to some extent in sweat, mainly as riboflavin or other related metabolites, e.g. 7-hydroxymethylriboflavin, 8α-sulfonylriboflavin, 8-hydroxymethylriboflavin [Tennent & Silber 1943, Chastain & McCormick 1987, Monteiro & Perrone 2013].

In humans, the clinical features of human riboflavin deficiency do not have absolute specificity. Early symptoms may include weakness, fatigue, mouth pain and tenderness, as well as burning and itching of the eyes. More advanced deficiency may give rise to cheilosis (lesions of the lips), angular stomatitis (angles of the mouth), dermatitis, and vascularization of the cornea. Riboflavin seems to have a putative role in protecting against cancer and cardiovascular disease and there may be implications for the handling of dietary iron [Powers 2003]. Riboflavin deficiency may also be associated with both the development of cataracts and a reduction of the metabolism of other B-group vitamins, notably folates and vitamin B_6 [Leske et al 1995, Powers 2003].

2.1.3. Vitamin B_3

Vitamin B_3 exists naturally in a variety of animal and plant products as one of two simple forms, nicotinamide (NAM) and nicotinic acid (NA), and as their derivatives, such as nicotinamide riboside (NR), nicotinamide mononucleotide (NMN), nicotinamide adenine dinucleotide (NAD^+/NADH), nicotinamide adenine dinucleotide phosphate

(NADP+/NADPH), and their acid analogues, e.g. nicotinic acid riboside (NaR), nicotinic acid adenine dinucleotide [Navazio et al 2000, Matsui et al 2007, Bogan et al 2009] (Fig 3). In addition to these free forms, the coenzymes NAD+ and NADP+ can be also bind to proteins [Bellamacina 1996]. Also, glycosylated forms of vitamin B₃ exist in some grains (e.g. corn and wheat), however, these are thought to exhibit little or no nutritional availability in humans [Carter & Carpenter 1982, Gregory 1998].

NAM, NA, and NR constitute the three salvageable NAD+/NADP+ precursor vitamers. The latter coenzymes function as electron carriers in a wide variety of metabolic oxidation–reduction reactions. Also, there are several types of enzymes that use NAD+ as a co-substrate to modify proteins (e.g. poly-ADP-ribose polymerases, mono-ADP-ribosyl-transferases, NAD+-dependent deacetylases or sirtuins), nucleic acids (e.g. tRNA 2´-phosphotransferases, ADP-ribosylating proteins), and small molecules (e.g. ADP-ribosylcyclases) [Lin 2007]. In addition to salvage, the precursor vitamers NA and NAM can both be synthesized *in vivo* in the human body from the essential amino acid L-tryptophan [Fukuwatari et al 2004].

The bioavailability of B₃ vitamers from animal-derived products is considered higher than from plant-derived foods because the latter often contains NA in chemically bound forms (e.g. covalently bound complexes with small peptides and carbohydrates), which results in decreased bioavailability [Ghosh et al 1963, Mason et al 1973, Carter & Carpenter 1982]. For example, even after conventional cooking, the majority of bound NA forms in mature cereal grains remain biologically unavailable [Ghosh et al 1963, Wall & Carpenter 1988]. Free NA and NAM are rapidly absorbed in both the stomach and the upper small intestine [Bechgaard & Jespersen 1977]. There is also evidence for the absorption of NAM and NA across the human buccal mucosa [Evered et al 1980]. NA uptake is proposed to occur through a specific high-affinity, acidic pH-dependent, Na+-independent carrier-mediated mechanism

[Nabokina et al 2005, Said 2011] while the transport mechanisms of NAM are still not known. There are no mammalian enzymes which can convert NAM to NA, however, it has been suggested that this transformation can occur by the activity of oral and intestinal microflora [Shimoyama et al 1971, Stratford et al 1996]. Thus, in principle, ingested NAM could be deamidated to NA prior to absorption. The reduced coenzymes NADH and NADPH are acid labile and tend to decompose in the gastric juice [Wall & Carpenter 1988]. Ingested NAD^+ (or $NADP^+$) is thought to be metabolized to NMN by a pyrophosphatase present in intestinal secretions and to a much lesser extent in pancreatic juice. For absorption, NMN can be hydrolyzed to NR and eventually to free NAM by three membrane-bound enzymes excreted by intestinal cells, specifically, 5'-nucleotidase (EC 3.1.3.5), purine nucleosidase (EC 3.2.2.1), and uridine nucleosidase (EC 3.2.2.3) [Gross & Henderson 1983]. Nevertheless, only the expression of 5'-nucleotidase (EC 3.1.3.5) has been found in humans, thus, it is possible that NR can also be absorbed directly. NAD^+ can also be hydrolyzed directly to NAM by the membrane bound NAD^+ glycohydrolase (EC 3.2.2.5) [Pfister et al 2001]. In addition to vitamin B_3 uptake from food, natural microflora within the large intestine can also synthesize small amounts of NA [Wilson 2005]. Excess of both NAM and NA is converted by the liver to methylated derivatives (e.g. N'-methyl-nicotinamide, methyl pyridone carboxamides), which are excreted together with urine [Mrochek et al 1976].

The main human disease related to vitamin B_3 deficiency is pellagra (symptoms: dermatitis, inflammation of the mucous membranes, diarrhoea, and psychiatric disturbances). NA is effective in increasing high-density-lipoprotein (HDL) cholesterol and lowering triglycerides, and may also protect against Alzheimer´s disease and age related cognitive decline [Morris et al 2004, Sauve 2008].
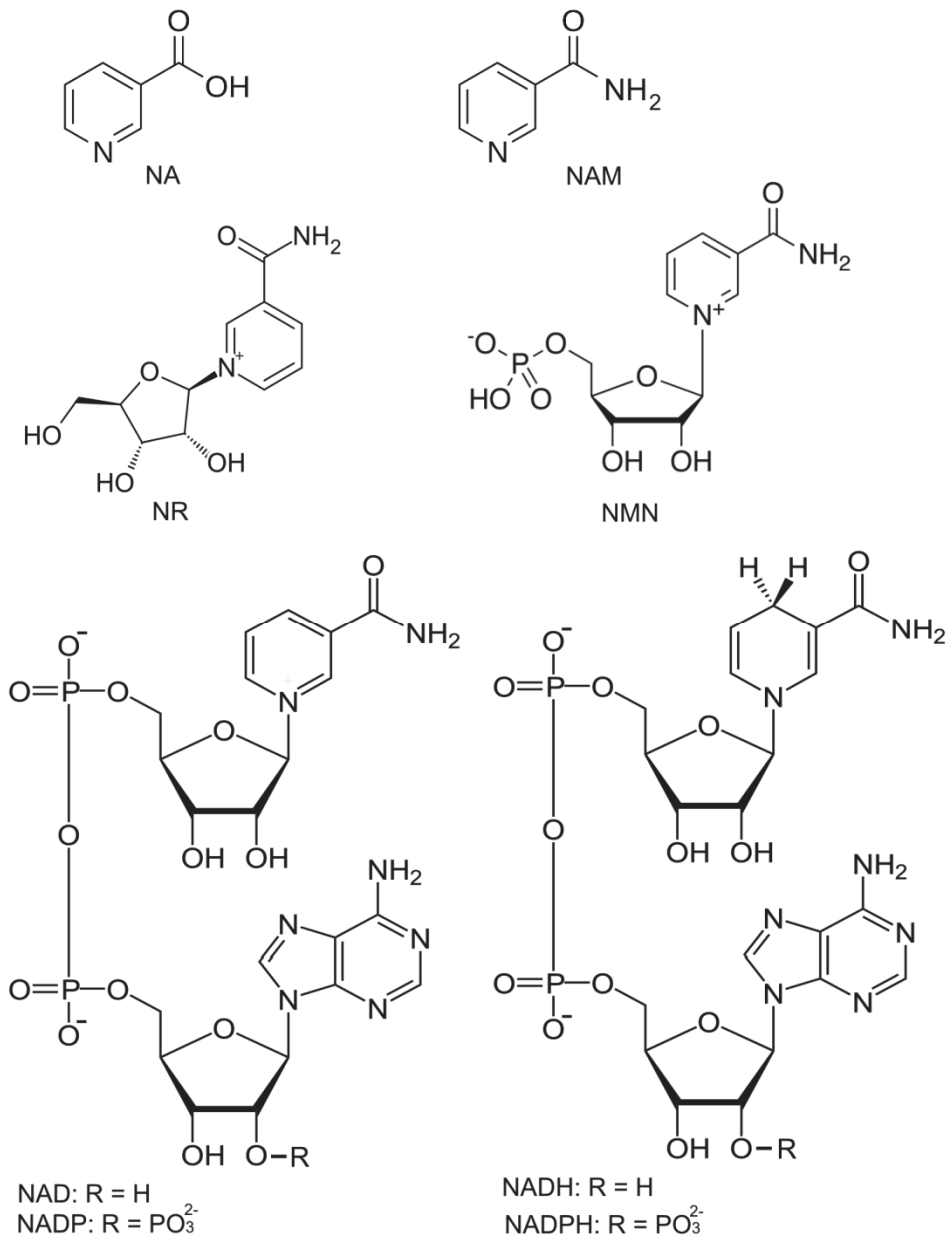
Figure 3. Vitamin B$_3$ vitamers

2.1.4. Vitamin B$_5$

Vitamin B$_5$ exists naturally in foods in the simple form pantothenic acid, and as one or more of its derivatives: coenzyme A (CoA), pantetheine, and 4'-phosphopantetheine (Fig 4). Both 4'-phosphopantetheine and acyl-CoA esters can, in turn, be bound to proteins [Frolov & Schroeder 1998, Branen et al 2001, Byers & Gong 2007, Xiao & Chye 2011]. No evidence exists for the presence of dietary forms of glycosylated pantothenic acid [Gregory 1998].
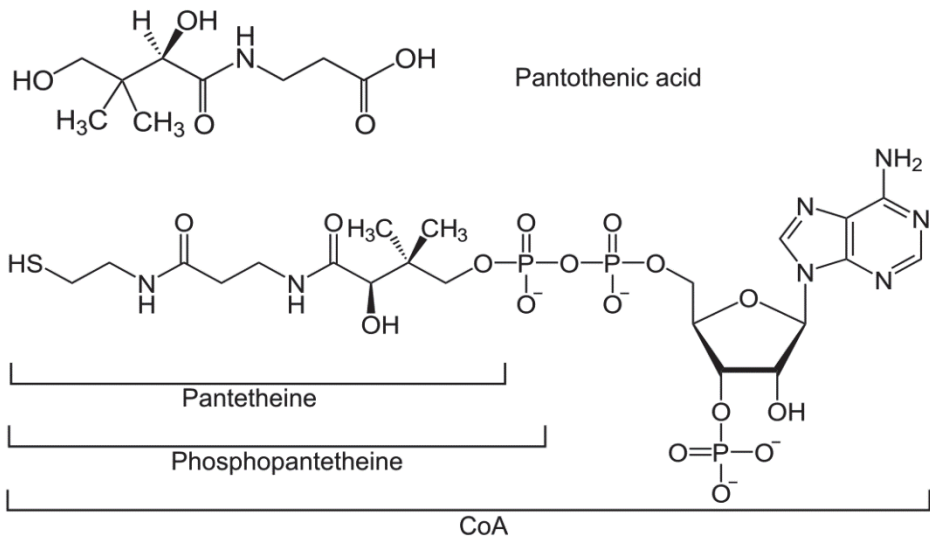
Figure 4. Vitamin B$_5$ vitamers

Pantothenic acid is used as the main precursor in the synthesis of CoA, which plays a central role in the acyl transfer reactions in energy production (via acetyl CoA), fatty acid oxidation, cholesterol synthesis, acetylcholine synthesis, and amino acid catabolism. CoA acts as an acyl carrier to transport carbon atoms within cells.

Little information is available about the bioavailability of pantothenic acid derivatives; however, it is estimated to range from 40 to 60 % of the total pantothenic acid intake in an average diet [Tarr et al 1981]. Ingested vitamin B_5 vitamers are thought to be hydrolysed to free pantothenic acid in the intestinal lumen by the non-specific action of pyrophosphatases, phosphatases, and pantetheinase, secreted from the intestinal mucosa [Shibata et al 1983]. Absorption of liberated pantothenic acid mainly takes place in the jejunum, although, there seems to be no significant difference in the uptake rate in the upper, middle, or lower sections of the small intestine [Shibata et al 1983]. It is known that colonic microflora can also synthesize pantothenic acid [Said et al 1998]. Intestinal absorption of panthothenic acid in the small and large intestine involves a saturable and Na^+-dependent carrier-mediated process that is shared with biotin and lipoate [Said 2009]. Excess pantothenic acid is excreted in an intact form by the urine stream; however, small amounts are also excreted in both faces and sweat [Tennent & Silber 1943, Bender 2003].

Pantothenic acid deficiency in humans is exceptionally rare and has been observed only in cases of severe malnutrition. Symptoms include headache, fatigue, insomnia, intestinal disturbances, numbness, and tingling of hands and feet [Hodges et al 1958, Tanphaichitr 1999]. A dimeric form of pantothenic acid pantethine has a cholesterol-lowering effect [Gaddi et al 1984].

2.1.5. Vitamin B_6

Vitamin B_6 exists naturally in foods in three simple forms: pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), and as phosphorylated forms of these: pyridoxine phosphate (PNP), pyridoxal phosphate (PLP), and pyridoxamine phosphate (PMP) (Fig 5). In addition, some fruits, vegetables, and grains may contain glycosylated PN (e.g. PN-5´-β-D-glucoside, Fig 5) [Kabir et al 1983, Gregory & Sartain 1991]. Both PL and PLP can reversibly bind to proteins through aldimine (Schiff base) and

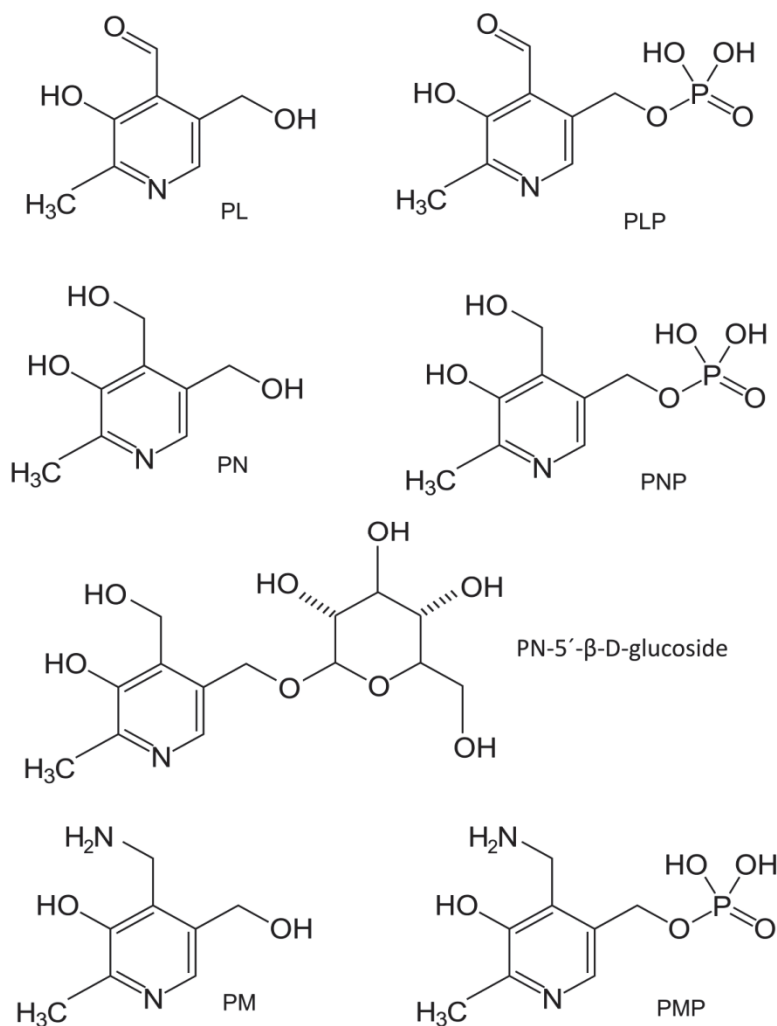substituted aldamine linkages, and are thought to be nutritionally significant [Vacca et al 2008].

Figure 5. Vitamin B$_6$ vitamers

PLP, the main biologically active form of vitamin B$_6$, has multiple roles as a versatile cofactor of enzymes that almost exclusively function in the

metabolism of amino compounds. With regards to amino acid metabolism, the reactions catalysed by PLP include deamination, transamination, decarboxylation, trans-sulphuration, and desulphuration. In certain aminotransferases, PMP also functions as the coenzyme.

Information about the bioavailability of vitamin B_6 derivatives is limited. Previous studies have shown that about 75 % of vitamin B_6 in a mixed diet (the average western diet) is assimilated in the human GIT [Tarr et al 1981]. Prior to absorption, dephosphorylation of the phosphorylated B_6 vitamers is carried out by membrane bound alkaline phosphatase in the intestinal mucosa of the small intestine [Mehansho et al 1979]. The uptake of PN, and presumably PL, and PM occurs by a specific acidic pH (but not Na^+)-dependent carrier-mediated mechanism [Said et al 2008]. The stability of PLP-protein linkages is reversibly dependent on pH and is normally readily dissociated by our gastric fluid [Ball 2004]. The bioavailability of PN glucosides (PNG) (e.g. PN-5´-β-D-glucoside) is thought to be approximately 50 % in humans [Gregory 1998]. PN-5'-β-D-glucoside is hydrolysed to PN by cytosolic PNG hydrolase (EC 3.7.1.4) and brush border lactase-phlorizin hydrolase (EC 3.2.1.108/62) prior to absorption or is absorbed intact by intestinal epithelial cells in a similar manner to that of PN [Mackey et al 2004]. Significant quantities of vitamin B_6 that are readily absorbed are also synthesized by naturally occurring microflora within the large intestine [Wrong et al 1981, Wilson 2005]. The excess vitamin B_6 is excreted in urine mainly as 4-pyridoxic acid, but also as PL, PM, PN, and their phosphates. A small amount of vitamin B_6 is also excreted in our feces [Lui et al 1985].

A primary clinical deficiency of vitamin B_6 in humans is rarely encountered with normal nutrition. According to Hodges et al [1962], vitamin B_6 deficiency may cause scaling of the skin, foul breath, severe gingivitis, soreness and discoloration of the tongue, and dry cracked lips. A severe deficiency of vitamin B_6 may lead to impaired development of brain lipids and incomplete myelination of nerve fibres. Thus, sufficient

availability of B_6 in humans plays a critical role in the structural and functional integrity of the central and peripheral nervous systems [Dakshinamurti et al 1990].

2.2. Analysis methods of B-group vitamins

Despite rapid developments of various analytical techniques, the simultaneous quantification of B-group vitamins in foods remains challenging, with major hindrances being their low concentration in the food matrix, relatively low stability, as well as their different structures and chemical properties [Gentili et al 2008]. The variation in metabolic activity and bioavailability of multiple forms of vitamers adds complexity to an overall assessment of the nutritional value of each vitamin in food. The bioavailability of a particular vitamer depends on the rate or extent of release of the active vitamer moiety from its conjugated forms and on its absorption efficiency in the human GIT [Gregory 2012]. Hence, proper analytical methods should allow one to determine all nutritionally active forms of each respective vitamin in food [Gregory 2012].

The diversity of vitamer forms complicates the reporting of analytical results. The problem becomes even more complex when one considers a huge variety of analytical methods used for vitamin analysis in foods. The standard vitamin analysis methods (e.g. AOAC and EN) usually determine the total concentration of each vitamin without substantial details about the content of individual vitamers. The latter, however, is important in order to properly assess the bioavailability and nutritional value of vitamins within a given food product. Furthermore, determination of the vitamer distribution contributes to the study of vitamin biosynthesis and metabolic processes in living organisms.

Another critical issue concerning the development of vitamin analysis methods is a lack of reliable reference materials to test the method against. These are required to improve both the reliability and accuracy

of methods. Most commercially available reference materials, such as multivitamin tablets (e.g. NIST 3280), fortified nutritional formulas (e.g. NIST 1849a) have certified values for the simple forms of vitamins only. There are also some reference materials (e.g. slurried spinach NIST 2385, pig liver BCR 487, wholemeal flour BCR 121) where the total B vitamin concentration values are provided, however, without indicating the distribution of individual vitamers.

2.2.1. Vitamin extraction

Extraction of B-group vitamins from the food matrix and their quantitative conversion into forms that can be analytically determined is one of the most critical and complicated parts of vitamin determination. The problems mostly arise due to the diverse chemical structures and resulting chemical properties of the vitamers. Thus, it is difficult to find universal extraction conditions for all analytes, particularly in case of those forms which are bound to the food matrix. In general, to extract and liberate vitamins from the food matrix prior to their conversion into forms that can be determined analytically, acid or alkaline hydrolysis or enzymatic treatment is typically applied under conditions that are not always universal for all vitamins.

Conventional methods for the extraction and liberation of thiamine, riboflavin, and PL/PN/PM from their bound forms include acid hydrolysis (usually with 0.1N HCl) at high temperatures (usually 121 ^{o}C for 30 min or 100 ^{o}C for 60 min) followed by enzymatic treatment to completely convert the derived vitamer forms into simple forms (e.g. thiamine, riboflavin) [van den Berg et al 1996, Bognar & Ollilainen 1997, Ndaw et al 2000, EVS-EN 14122:2003, EVS-EN 14152:2003, EVS-EN 14663:2006, EVS-EN 15652:2009]. The high temperature acid pre-treatment step is recommended in order to denature the proteins and to convert starch into soluble sugars. For vitamin B_1 and B_2, takadiastase (from fungi) or another appropriate enzyme preparation containing phosphatase and pyrophosphatase activity is used to hydrolyse the phosphate bonds,

while for vitamin B_6, acid phosphatase and β-glucosidase treatment is used to hydrolyse the phosphorylated and glycosylated forms, respectively.

To extract and liberate nicotinamide (NAM) and nicotinic acid (NA) bound to the food matrix together with other vitamin B_3 derivatives, either acid hydrolysis (0.1 M HCl, 100 ^oC for 60 min) or acid hydrolysis followed by alkaline hydrolysis (~1 M NaOH, 120 ^oC for 60 min), or enzymatic treatment with NAD^+ glycohydrolase can be used [EVS-EN 15652:2009]. During acid hydrolysis, NA is liberated from its derivatives, e.g. NAD^+, and NAM is also partially hydrolysed to NA [Elvehjem & Teply 1943]. The following alkaline hydrolysis results in complete hydrolysis of NAM into NA and also liberates the bound forms, e.g. covalently bound complexes of NA and NAM with small peptides and carbohydrates, which are not considered bioavailable [Mason et al 1973, Carter & Carpenter 1982]. Enzymatic treatment with NAD^+ glycohydrolase liberates NAM from NAD^+ and $NADP^+$ but does not liberate NAM from the other bound vitamers of vitamin B_3, e.g. nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN).

To quantify vitamin B_5, pantothenic acid should be liberated from its conjugates, e.g. CoA and pantetheine. Overnight incubation with pigeon liver pantetheinase and alkaline phosphatase has been used [Rychlik 2003]. Notably, in contrast with other B vitamins, hydrochloric acid (HCl) extraction cannot be used for vitamin B_5 because pantothenic acid degrades into β-alanine and pantoic acid under acidic conditions [Velišek & Davidek 2000]. Thus, acid hydrolysis is unsuitable if one wants to simultaneously determine B_5 together with other B vitamins.

For the simultaneous extraction of B-group vitamins other than pantothenic acid, HCl extraction can be combined with enzymatic digestion. For example, Viñas et al [2003] used HCl extraction followed by enzymatic digestion with taka-diastase to determine vitamins B_1, B_2, B_3, PN, and PL in different baby foods, cereals, and fruits. Because HCl can also be detrimental to MS instruments, volatile buffers are preferred

to extract vitamins from food when MS analysis is used. For example, acetic acid and ammonium acetate containing extraction solutions have been used to extract simple vitamers (thiamine, riboflavin, NAM, pantothenic acid, and PN) from foods and nutritional formulations [Aslam et al 2008, Phinney et al 2011, Santos et al 2012, Zand et al 2012].

As an alternative to acid hydrolysis, enzymatic liberation of bound vitamers from the food matrix has been used. Ndaw and co-workers [2000] showed that the treatment of various food samples with a mixture of enzymes containing α-amylase, acid phosphatase, β-glucosidase, and protease activities led, in a single extraction step, to the release of thiamine, riboflavin, and vitamin B_6 vitamers (PN, PL, PM) from phosphorylated, glycosylated, and protein-bound forms, even when acid hydrolysis was not applied. Additional α-amylase was used for starchy foods for better extraction and to resolve possible filtration problems. Jakobsen [2008] further optimized the enzymatic treatment step of this method by introducing ultrasonication to shorten the duration of the enzymatic liberation of protein- and phosphate-bound thiamine and riboflavin. In both studies, the concentration of vitamins B_1 (as thiochrome), B_2, and B_6 was determined using high-performance liquid chromatography (HPLC) coupled with fluorescence detection.

2.2.2. General overview of the methods used to detect B-group vitamins

The most common methods to determine B-group vitamins include microbiological, chemical, and LC based methods. Other methods, such as capillary electrophoresis, gas chromatography, radioimmunoassay, and the use of an enzyme-linked immunosorbent assay are less commonly applied and thus are not reviewed in detail here.

Microbiological assays are based on the absolute requirement of a test microorganism for one or more specific vitamers and thus tend to either under- or overestimate the total content of bioavailable forms. Despite this, microbiological assays are officially recognized methods and are still

commonly used for B-group vitamin analysis: AOAC 940.33 for B_2; 944.13, 985.34 for B_3; 961.15, 985.32 for B6; 945.74, and 992.07 for B_5 determination. The microorganisms used are the bacteria *Lb. rhamnosus* ATCC No. 7469 for vitamin B_2 (riboflavin) and yeast *S. uvarum* ATCC No. 9080 for vitamin B_6 (different response to PN, PL and PM) determination. *Lb*. plantarum ATCC No. 8014 is used for both vitamin B_3 (equal response to NA, NAM and NAD^+) and vitamin B_5 (equal response to pantothenic acid and pantetheine). There is also a microbiological method to determine vitamin B_1, however, it is seldom applied. To ensure complete availability of vitamins under study for test microorganisms, the extraction methods presented above should be applied. While microbiological methods are highly sensitive, they are relatively time-consuming and laborious and require multiple determinations to achieve the required precision when determining mean values [Chen et al 2009]. In addition, they are often imprecise because the vitamers that belonging in the same vitamin group may not be equally assimilable by a given test organism.

Chemical detection methods are usually based on a colorimetric reaction between the structural unit of a given vitamin and a chromogen where the absorbance of the resulting reaction products can be measured photometrically. For example, the concentration of NA and NAM can be determined by the König reaction (AOAC 961.14) in which NA and NAM that has been previously liberated from food matrix and from their derivatives react with cyanogen bromide (CNBr) to yield yellow colored pyridinium compounds. The concentration of thiamine can be measured using the thiochrome reaction (AOAC 942.23, 953.17, 957.17, 986.27) in which free thiamine is converted by reaction with alkaline potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$) into its fluorescent oxidation product thiochrome. The latter is extracted with isobutanol and quantified fluorometrically. The major shortcomings in these chemical detection methods is specificity because several other substances may interfere with the chromogen and commonly only the total concentration of

vitamin is determined without providing sufficient details about the content of individual vitamers.

LC based methods which use fluorescence, ultraviolet (UV), and MS detection are more selective compared with microbiological and chemical methods. As with all LC based methods, separation is based on the different physical and chemical interactions of the compounds in the sample with the mobile and stationary phases. To separate B-group vitamins, a reversed phase (RP) column is commonly used as the stationary phase. In reversed phase columns, the separation of molecules occurs mainly because of differences in the hydrophobicity of the compounds. In recent years, hydrophilic interaction liquid chromatography (HILIC), a variant of normal-phase chromatography, in which polar sorbents are employed with high-organic, low-aqueous eluents, has been used as an alternative to RP-LC to improve separation [Yang et al 2013]. Chromatographic separation is typically followed by UV detection which enables one to simultaneously determine thiamine, riboflavin, NAM, NA, pantothenic acid, PN, PL, and PM, yet has low sensitivity and selectivity compared to fluorescence detection. Fluorescence detection can be used only for vitamers that act as fluorophores such as riboflavin, PN, PL, and PM; thiamine must be converted to thiochrome prior to fluorescence detection. However, despite the increased selectivity compared with microbiological and chemical methods, traditional LC based methods based on UV or flourimetric detection still have several shortcomings. For example, they are commonly designed for single vitamin analysis and often lack sufficient sensitivity and selectivity and furthermore may produce chromatograms with unresolved interferences with other compounds from the sample matrix if proper purification procedures are not carried out. To overcome these problems LC has been coupled with MS which has proven to be a more selective, rapid, and sensitive method to determine B-group vitamins in food and dietary supplements [Leporati et al 2005, Chen & Wolf 2007, Gentili et al 2008].

2.2.3. LC-MS-SIDA

Coupling LC with MS allows one to remarkably increase analysis selectivity and sensitivity, compared with conventional LC based methods. Using LC-MS, the molecules of interest are first separated in a LC, followed by ionization to acquire positive or negative charges which can then be separated in a mass analyser according to their mass to charge ratio (m/z). Because separation is based on the mass to charge ratio (m/z) and the signal is proportional to the concentration of ions, LC-MS can provide both qualitative and quantitative information about the analytes under study. Several ionization techniques exist, e.g. electrospray ionisation (ESI), atmospheric pressure chemical ionisation and atmospheric pressure photo-ionisation. Due to the high-polarity of B-group vitamins, the ESI techniques are commonly used to ionize these compounds. ESI is considered a "soft" ionisation source, meaning that relatively little energy is imparted to the analyte which results in minimal or a complete lack of fragmentation [Pitt 2009]. Different mass analysers exist, e.g. quadrupole, time-of-flight (TOF), and ion trap analysers. For the quantification of vitamins, a triple-quadrupole MS is most commonly used [Leporati 2005, Midttun et al 2005, Chen et al 2007, Gentili et al 2008, Chen et al 2009].

Despite the popularity of LC-ESI-MS in quantitative analysis there are also some shortcomings to this method. A major drawback of LC-ESI-MS is the so-called matrix effect which refers to either the suppression or enhancement of ionization of the analyte of interest by other compounds that co-elute with the analyte from the LC column. This can cause large uncertainties when applying the LC-ESI-MS technique for quantification [Kurve 2011]. The most effective solution to compensate matrix effects is to use stable isotope labelled (SIL) internal standards that co-elute with their unlabelled analogues [Wieling 2002]. This technique, also termed Stable Isotope Dilution Assay (SIDA), is based on adding a known amount of an isotope labelled analogue of the analyte under study to the sample. The ratio of the MS signal response of the

isotope labelled compound to that of the analyte under study is then determined [Chen et al 2009]. The response ratio between the MS signals can then be interpolated onto a standard curve to calculate the absolute concentration of the analyte in the unknown sample. Due to their almost identical chemical and physical properties, the ratio of the MS signals of isotopologues remains stable throughout all analytical steps [Rychlik 2011]. The use of isotope-labelled internal standards with MS techniques increases both their accuracy and precision, and both helps to compensate losses of the analytes during sample preparation and simplify the sample pre-treatment procedures [Vogl & Pritzkow 2010]. In order to obtain very accurate results, particular attention must be given to the choice of calibration procedure. The graphical method of producing a calibration curve is recommended when a number of samples with varying concentrations are to be analysed [Sargent et al 2002]. In order to provide linear calibration curves, an overlap between the ions of the internal standard and analyte (and their natural abundance isotopes) should be avoided. Thus, the mass difference between the SIL internal standard and the analyte should ideally be higher than 3 Da [Fay et al 2000]. If possible, deuterated compounds should be avoided because they cause a slight difference in retention times between the analyte and the SIL internal standard and thus result in a different degree of ion suppression between the two analogues [Wang et al 2007].

Although there are several authors who have used LC-MS(-MS) with SIL internal standards to determine B-group vitamins, these studies have made use of tablets and dietary supplements [Chen et al 2009, Goldschmidt & Wolf 2010, Phinney et al 2011] while data for real food samples is rather limited. Developing a method to simultaneously determine B-group vitamins and their vitamer distributions in natural food samples using LC-MS is quite challenging, mainly due to the complexity of the sample preparation processes and lack of availability of SIL internal standards for many naturally occurring vitamers. The extraction yield of vitamers from the real food matrix should be

maximized, and all vitamers should be converted into the same forms as the internal standards incorporated into the sample. Ideally, the method should also allow for high throughput analysis and thus should consist of a simple, single-step procedure for complete extraction and the same chromatographic conditions for all vitamins under study. Currently applied standard extraction procedures (AOAC and EN standard methods) are designed for single vitamin analysis to determine their total concentrations. These methods are time consuming and do not provide additional information about the distribution of individual vitamers. In addition, these methods use extraction buffers which contain compounds that are unsuitable for MS detection (e.g. HCl) and high temperatures which are not suitable for simultaneous analysis of some B-group vitamers (e.g. pantothenic acid). Finally, they are also uncommon to physiological conditions in the human GIT. An alternative would be to use hydrolytic enzymes instead of high temperature acid treatment for simultaneous liberation of vitamins from their bound forms and conversion of those into simple vitamers, as proposed by Ndaw and co-workers [2000]. Thus, proper enzyme or enzyme mixtures with required activities should be selected and MS compatible extraction buffers should be preferred. Also, care should be taken when measuring raw food samples because they contain endogenous enzymes which may modify the structures of the vitamers during sampling and extraction process and may thus lead to false estimation of the vitamer distribution in foods.

3. The aims of the present dissertation

The work presented in this dissertation aims to:

- develop an LC-MS-SIDA method and respective analytical assay for simultaneous determination of vitamin B_1, B_2, B_3, B_5, and B_6 in food products;

- validate the method and the analytical assay using the reference materials and inter-laboratory comparative tests with food samples;

- test the developed method and the analytical assay on various food samples.

4. Materials and Methods

More detailed descriptions of the materials and methods used are available in the six publications that form the basis of this dissertation. The following sections are provided to make this material more accessible.

4.1. Materials

Reference materials

Standard reference materials (SRM) 1849 Infant/Adult Nutritional Formula and 3234 Soy Flour were purchased from the National Institute of Standards and Technology (NIST) (USA) and certified reference material (CRM) BCR 121 (wholemeal flour) was purchased from the Institute for Reference Materials and Measurements (Belgium). These were used to validate the method and analytical assay developed in Publications I and II.

Food samples

Nutritional inactive yeast powder LBI 2130 (lot no 50-804184E), Engevita Bland (lot no 206477E) and yeast extract FNI100 (lot no 10103308) were kindly provided by Lallemand, Inc. (Canada) and used as model foods to develop, test, and validate the method and the respective analytical assay.

Nutritional inactive yeast powders Engevita Bland, lot no 206477E and LBI 2130, lot no 108706E (Lallemand, Inc., Canada) were used in inter-laboratory comparative tests in co-operation with Aérial (Strasbourg, France) and DSM Nutritional Products, Analytical Research Center (Basel, Switzerland), respectively.

Rye and wheat flours, and steel cut rye grains were obtained from Tartu Grain Mill Ltd. (Estonia), red and white rye malts were obtained from Eesti Leivalinnase Ltd. (Estonia), fresh baker's yeast was kindly provided by AS Salutaguse Pärmitehas (Estonia) and was used to produce rye sourdough bread, commercial rye breads made from these ingredients were obtained from a local bakery (AS Leibur). Laboratory baked rye sourdough bread and commercial rye breads were used to study the stability of B-group vitamins during rye sourdough bread production.

Quinoa seeds were kindly provided by Vis vitalis, LLC (Salzburg, Austria) and were used to study the distribution of B-group vitamers in quinoa seeds.

The pure culture of commercial baker's yeast *Saccharomyces cerevisiae*, strain 210NG was kindly provided by AS Salutaguse Pärmitehas (Estonia) and was used to study the accumulation of B-group vitamins in baker's yeast.

Chemicals and standards

All necessary reagents and unlabelled standards of vitamins and cofactors for analyses were purchased from Sigma-Aldrich (Germany), except nicotinamide riboside which was purchased from LGC Standards AB (Borås, Sweden); isotope-labelled standards of vitamins were obtained from Cambridge Isotope Laboratories, Inc. (USA) and Isosciences, LLC (USA).

Enzymes, enzyme inhibitors and ultrafiltration tools

α-amylase from *Aspergillus oryzae* (crude, A6211), β-glucosidase from almonds (crude, G0395), acid phosphatase from potato (P1146), papain from papaya latex (crude, P3375), and taka-diastase from *A. oryzae* (86247) were all purchased from Sigma-Aldrich. Phosphatase inhibitor (88667SPCL) and protease inhibitor (88666SPCL) were sourced from Thermo Scientific (Rockford, IL, USA). Centrifugal filters Amicon Ultra-

0.5, Ultracel-10 Membrane (UFC501008, cut-off 10 kDa) were purchased from Millipore (Malsheim, France).

The components of the analytical assay

The individual components of the analytical assay are: calibration vials, internal standards vials, and enzyme vials. The calibration vials contain known amounts of the unlabelled (natural abundance) B-group vitamers and stable isotope labelled (SIL) standards. The internal standard (IS) vials contain a known amount of the SIL standards. The enzyme vials contain a mixture of hydrolytic enzymes with phosphatase, pyrophosphatase, and glycolytic activities. (Publication II)

Preparation of internal standards vials

100 μL of SIL internal standard solution (10 - 30 mg/L of $[^{13}C_3]$-thiamine-HCl, $[^{13}C_4{}^{15}N_2]$-riboflavin, $[D_4]$-NAM, $[D_4]$-NA, $[^{13}C_3{}^{15}N]$-Ca-pantothenate, $[^{13}C_4]$-PN HCl, and $[D_3]$-PL HCl) were pipetted into amber 2 mL LC-MS vials and freeze-dried (Heto Powerdry PL 3000, Thermo Scientific, USA). All SIL standard solutions were prepared with 0.1 % formic acid (FA). Thus, the working concentrations of SIL vitamers in the internal standards vials are in the range of 0.1 - 0.3 mg/L. The prepared vials were applicable for 10 mL of sample extract. The vials were freeze-dried, capped under an N_2 environment and stored at -20 ^{o}C until use.

Preparation of calibration vials

100 μl of the external standard mixtures containing thiamine HCl, riboflavin, NAM, NA, pantothenic acid, PL HCl, and PN HCl at varying concentrations (10.0, 5, 2, 1, 0.5, 0.25, and 0.1 mg/L) and 100 μL of SIL internal standard solution (1 - 3 mg/L of $[^{13}C_3]$-thiamine-HCl, $[^{13}C_4{}^{15}N_2]$-riboflavin, $[D_4]$-NAM, $[D_4]$-NA, $[^{13}C_3{}^{15}N]$-Ca-pantothenate, $[^{13}C_4]$-PN HCl, and $[D_3]$-PL HCl) were pipetted into amber 2 mL LC-MS vials and freeze-dried. Thus, the working concentrations of the external standard mixtures are 1.0, 0.5, 0.2, 0.1, 0.05, 0.025, and 0.01 mg/L and SIL internal standards in the calibration vials are in the range of 0.1 - 0.3 mg/L. The

prepared calibration vials were applicable for 1 mL of 0.05 M ammonium formate buffer, pH 4.5 (AF buffer). The vials were freeze-dried, capped under an N_2 environment and stored at -20 ^{o}C until use.

Preparation of enzyme vials

100 µl of acid phosphatase solution (5 U) or ultrafiltered (cut off 10 kDa) β-glucosidase solution (5 U) was pipetted into the 2 mL LC-MS vials and freeze-dried. All enzyme solutions were prepared with AF buffer. The prepared vials were designed for use with 10 ml of sample extract. The vials were freeze-dried, capped under an N_2 environment and stored at -20 ^{o}C until use.

Preparation of vials with ^{15}N-labelled yeast extract fraction

These vials were produced as internal standards vials for quantification of those vitamers whose isotopically labelled analogues are not commercially available (i.e. NAD$^+$, NMN and NR). An *in house* prepared ^{15}N-labelled yeast biomass was extracted with hot AF extraction (described in more detail in Paper V). Endogenous enzymes were separated using size exclusion chromatography. 100 µL of ^{15}N-labelled yeast biomass extract (5 - 50 mg/L of ^{15}N-labelled NAD$^+$, NMN, and NR) was pipetted into amber 2 mL LC-MS vials and freeze-dried. All ^{15}N-labelled standard solutions were prepared with 0.1 % FA. The working concentrations of ^{15}N-labelled internal standards in the vials were in the range of 0.05 - 0.5 mg/L. The prepared vials were applicable for 10 mL of sample extract. The vials were freeze-dried, capped under an N_2 environment and stored at -20 ^{o}C until use.

4.2. Methods

The development of the LC-MS-SIDA analysis method and the prototype of the respective analytical assay are described in details in Publications I and II.

4.2.1. Assay for vitamin determination

A working principle of the developed analytical assay for quantitative analysis of B-group vitamins in foods is illustrated in Figure 6. Simple vitamers are determined by dissolving the content of internal standard vials (IS vials) in 0.05 M ammonium formate buffer, pH 4.5 (AF buffer) and adding it to the homogenized sample. In case of the measurement of total vitamins, the content of enzyme vial(s), dissolved in AF buffer, is added to the sample. In both cases the total volume of sample extract is 10 mL. Incubated samples are centrifuged (14 000 rpm, 5 min at room temperature), filtered through a Millex-LG 13mm Philic PTFE 0.2 μm filter (Millipore, Ireland) and analysed by LC-MS. To calibrate the LC-MS system, 1 ml of AF buffer is added into the calibration vials, mixed, and analysed by LC-MS.

The concentration of vitamers in the sample is calculated using linear regression and a seven-point external standard calibration curve (the concentration of each vitamin is in the range of 0.01 – 1.0 mg/L). The concentration of each SIL vitamer in the internal standard vials can be in the range of 0.1 – 1.0 mg/L, for example 0.4 mg/L, but should be equal to that in the calibration vial. The calibration curves are composed by relating each concentration of unlabelled external standard to its relative response factor, as determined by the ratio of the peak intensity of the respective unlabelled external standard to that of the corresponding SIL internal standard.

The principle of the calculation is described below in equations (1) – (3).

First, it is assumed that the integrated peak areas on the mass spectra of isotope labelled and unlabelled vitamers in the calibration standard and

in the sample are proportional to the concentration of respective vitamers:

$$\frac{i_{NATst}}{i_{SILst}} = \frac{c_{NATst}}{c_{SILst}} \qquad (1)$$

$$\frac{i_{NATsmpl}}{i_{SILsmpl}} = \frac{c_{NATsmpl}}{c_{SILsmpl}} \qquad (2)$$

where i_{NATst}, $i_{NATsmpl}$, i_{SILst} and $i_{SILsmpl}$ are the integrated peak areas of the natural (*NAT*) and stable isotope labelled (*SIL*) vitamins in the calibration standard (*st*) and sample extract (*smpl*), respectively. Similarly, c_{NATst}, $c_{NATsmpl}$, c_{SILst} and $c_{SILsmpl}$ refer to the respective concentrations of the natural and isotope labelled vitamins in the standard and sample extract, respectively.

Considering that $c_{SILsmpl} = c_{SILst}$, if the external standard and sample are both spiked with the same amount of SIL standards, the concentration of vitamins in the sample can be calculated as follows:

$$c_{NATsmpl} = \frac{c_{NATst} * i_{NATsmpl} * i_{SILst}}{i_{SILsmpl} * i_{NATst}} \qquad (3)$$

Stability of the SIL vitamers in the IS vials

To study the stability of the SIL vitamers in the IS vials, their concentration was measured using freshly prepared external standards with UV detection over a period of one year.

Stability of the enzymes in the enzyme vials

To study the stability of the enzymes (acid phosphatase and β-glucosidase) in the enzyme vials, their activity was measured using freshly prepared 25 µM solutions of the following co-factors: TMP, TDP, FMN, FAD, PLP, and mixtures of them (mixture 1 contained TMP, FMN, and PLP and mixture 2 contained TDP and FAD) over a period of three months.
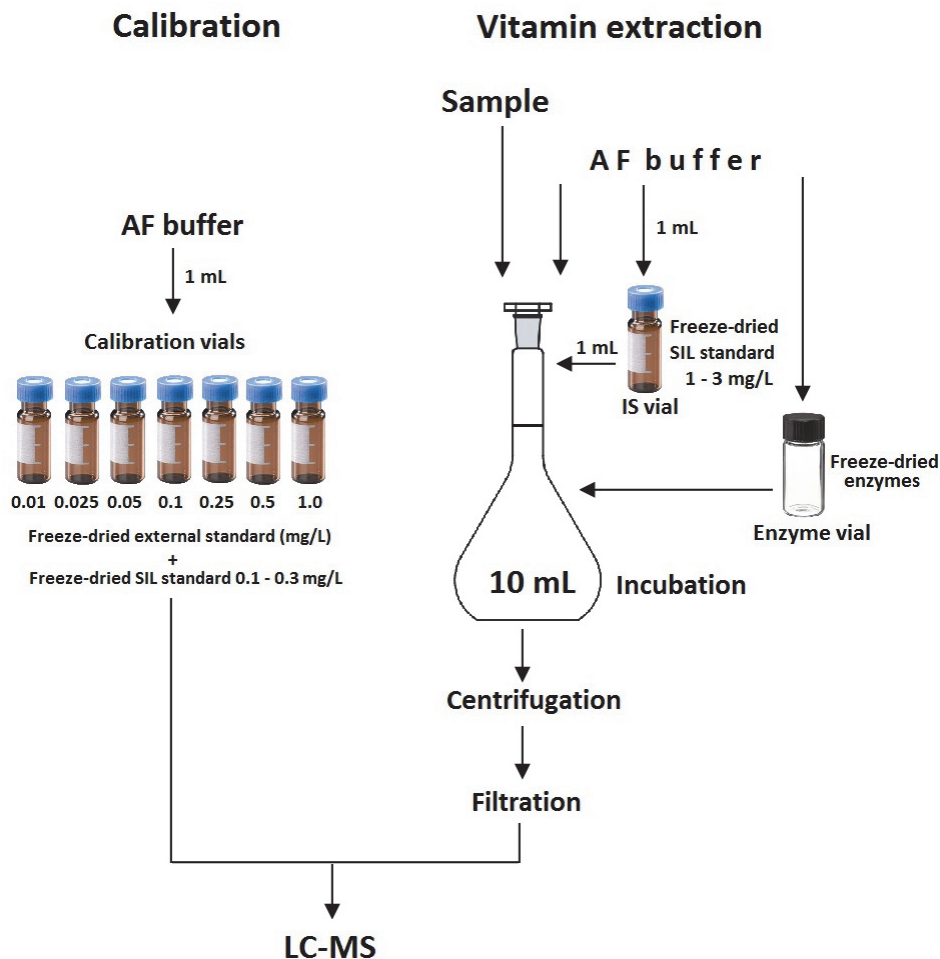
Figure 6. A working principle of the prototype of analytical assay for quantitative analysis of B-group vitamins in foods

4.2.2. Optimization of the analytical assay

Selection of enzymes for the liberation of vitamins from cofactors

The effectiveness of using different enzymes on the liberation of vitamins from their respective cofactors was studied in comparison with

acid hydrolysis. The following enzymes were studied: α-amylase (Cat no A6211, Sigma), β-glucosidase (Cat no G0395, Sigma), acid phosphatase (Cat no P1146, Sigma), papain (Cat no P3375, Sigma), and takadiastase (Cat no 86247, Sigma).

The activities and specificity of the enzymes under study were determined using 25 µM solutions of the following co-factors: TMP, TDP, FMN, FAD, PLP, NAD^+, NADH, and CoA in 0.05 M ammonium formate buffer (pH 4.5). Each solution was incubated with either acid phosphatase (0.12 mg/mL), β-glucosidase (1.2 mg/mL), α-amylase (4.5 mg/mL), takadiastase (5.0 mg/mL), or papain (5.0 mg/mL) for 18 h at 37 °C, followed by determination of liberated thiamine, riboflavin, PL, NAM and pantothenic acid.

Protease side activity in the enzymes was determined using a protease assay kit (Calbiochem, USA). The single enzyme preparation solution (α-amylase 0.225 mg/mL, β-glucosidase 1.2 mg/mL, acid phosphatase 0.12 mg/mL, papain 0.25 mg/mL, takadiastase 5.0 mg/mL) combined with fluorescein thiocarbamoyl-casein and incubation buffer (200 mM Tris-HCl, pH 7.8, 20 mM $CaCl_2$, 0.1 % NaN_3) was incubated at 37 °C for 24 h under subdued lighting conditions using continuous shaking. Protease activity was expressed relative to that of trypsin in N-benzoyl-L-arginine ethyl ester (BAEE) units.

Vitamins were extracted from nutritional yeast powder (LBI 2130, lot no 50-804184E) by an 0.05M ammonium formate buffer, pH 4.5 (AF buffer) with and without adding the enzymes, or mixtures of those. One SIL internal standard solution (the content of IS vial, dissolved in AF buffer) was added to each sample before incubation. The final concentration of SIL internal standards in the extraction mixture was the same as in the calibration vials (i.e. in the range of 0.1 - 0.3 mg/L). Samples were incubated under subdued lighting conditions at 37 °C overnight (18 h). After incubation, the sample extracts were centrifuged (14 000 rpm, 5 min at room temperature), filtered through a Millex-LG 13 mm Philic PTFE 0.2 µm filter (Millipore, Ireland), and injected into LC-MS.

To study the effect of high temperature acid hydrolysis (as described by Ndaw et al 2000) on liberation of B-group vitamins from the cofactors TMP, FMN, TDP, FAD, PLP, NAD⁺, NADH, and CoA, the cofactors were dissolved in 0.1 M HCl and treated in an autoclave at 121 ^{o}C for 30 min, followed by the determination of thiamine, riboflavin, PL, NAM, and pantothenic acid. Similarly, nutritional yeast powder was extracted in an autoclave with 0.1 M HCl, but also with 0.1 M or 0.3 M formic acid as possible alternatives to HCl for MS-TOF analysis. SIL internal standard solution was added to each sample prior to hydrolysis.

Determining the concentration of vitamins by LC-MS

The concentrations of B-group vitamins (thiamine, riboflavin, NAM, NA, pantothenic acid, PL, PN, NR, NMN, NAD⁺) were determined using an ACQUITY UPLC® system coupled with LCT Premier™ XE ESI TOF MS (Waters, USA) with an ACQUITY UPLC HSS C-18 1.8-μm (2.1×150 mm) column. Elution was carried out using water + 0.1 % formic acid and acetonitrile + 0.1 % formic acid gradient: 0–3 min 100 % A; 3–8.5 min 80 % A and 20 % B; 8.5–10 min: 5 % A and 95 % B; 10–15 min 100 % A. We used a flow rate of 0.25 mL/min, an auto-sampler temperature of 4 ^{o}C, a column temperature of 25 ^{o}C and a sample injection of 5 μL. MS analysis was carried out in a positive electrospray ionization mode [M+H]⁺ using a capillary voltage of 2.0 kV, a sample cone voltage of 30 V, a desolvation temperature of 300 ^{o}C and a source temperature of 120 ^{o}C. The following ions were detected (*m/z*): thiamine - [M]⁺ = 265.11, labelled thiamine - [M]⁺ = 268.12, riboflavin - [M + H]⁺ = 377.15, labelled riboflavin - [M + H]⁺ = 383.16, NAM - [M + H]⁺ = 123.06, labelled NAM - [M + H]⁺ = 127.08, NA - [M + H]⁺ = 124.04, labelled NA - [M + H]⁺ = 128.06, pantothenic acid - [M + H]⁺ = 220.12, labelled pantothenic acid - [M + H]⁺ = 224.13, PL - [M + H]⁺ = 168.07, labelled PL - [M + H]⁺ = 171.09, PN - [M + H]⁺ = 170.08, labelled PN - [M + H]⁺ = 174.10, NAD⁺ - [M]⁺ = 664.08, labelled NAD⁺ - [M]⁺ = 671.08, NR - [M]⁺ = 255.09, labelled NR - [M]⁺ = 257.09, NMN - [M+H]⁺ = 335.06, labelled NMN - [M+H]⁺ = 337.06. Full scan mass spectra were acquired in the range of 100 to 500 *m/z*. Data

were collected and reprocessed using Mass Lynx 4.0 software (Waters, Milford, MA, USA).

4.2.3. Method validation

Determination of LOD and LOQ, accuracy and precision

The detection and quantification limits (LOD and LOQ, respectively) as well as the precision and accuracy of the method were determined using nutritional yeast powder (lot no 50-804184E) extracted with only 0.05 M ammonium formate buffer at pH 4.5 (AF buffer). The LODs were determined in six replicates as the amount of vitamer which produced a three times higher signal than the noise of the MS chromatogram baseline of a sample (S/N ratio = 3) after estimating the original amount in a given matrix [Gentili et al 2008]. The LOQs were set at three times the intensity of the LODs of the respective vitamer. The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was carried out by performing three repeated analysis of the same sample on the same day, and the intermediate precision of the method was assessed by the analyses of the same sample on six different days over a period of 1 month under the same experimental conditions. The accuracy of the method was evaluated by determining the recovery after spiking the sample with a known amount of vitamers at three different concentration levels. The recovery was calculated as follows:

$$R, \% = \frac{C_{spiked}}{C_{unspiked} + C_{added}} * 100 \qquad (4)$$

where C_{spiked} is the amount of vitamer determined after spiking the sample, $C_{unspiked}$ is the amount of vitamer determined in the sample before spiking, and C_{added} is the actual amount of vitamer added to the sample. Shortly, a 25-mL aliquot of unlabelled external standard solution at three different concentration levels (0.1, 0.25, and 0.5 mg/L) containing 0.1 - 0.3 mg/L of each SIL internal standard was added to 0.375 g of sample (n = 3). Samples were incubated under subdued

lighting conditions at 37 °C overnight (18 h). After incubation, the sample extracts were centrifuged, filtered and injected into the LC-MS.

The concentration of simple (free) vitamers in reference sample

Infant/Adult Nutritional Formula SRM 1849 was used as a reference sample to validate the method in terms of its ability to determine the concentration of simple vitamers. Vitamins were extracted using AF buffer. SIL internal standard solution (the content of IS vial dissolved in AF buffer) was added to each sample and the samples were mixed thoroughly. The sample extracts were centrifuged, filtered and injected into the LC-MS.

The total concentration of vitamins in the reference samples

Wholemeal flour CRM BCR 121 and Soy Flour SRM 3234 were used as reference samples to validate the method in terms of its ability to determine the total concentration of B-group vitamins. Vitamins were extracted from reference sample CRM BCR 121 using AF buffer with an enzyme mixture containing α-amylase, acid phosphatase, and β-glucosidase while only AF buffer was used in the case of reference sample SRM 3234 to test the effect of endogenous enzymes on the liberation of simple vitamers from their derivatives and the food matrix. The samples were incubated under subdued lighting conditions at 37 °C overnight (18 h). After incubation, sample extracts were centrifuged, filtered and injected into the LC-MS. In case of CRM BCR 121, a SIL internal standard solution (the content of IS vial dissolved in AF buffer) was added to the samples after incubation while the internal standard was added before incubation in the case of SRM 3234.

Inter-laboratory comparative tests with Aérial and DSM

Inter-laboratory comparative tests with nutritional yeast powders were performed in co-operation with external laboratories (Aérial and the Analytical Research Center of DSM Nutritional Products). The samples

analysed were Engevita Bland, lot no 206477E (analysed by Aérial) and LBI 2130, lot no 108706E (analysed by DSM).

Aérial determined all vitamins using individual analysis. Their methods were based on acid or enzymatic extraction, followed by LC with fluorescence detection and are described in more detail in Table 1. In our laboratory, all vitamins were extracted simultaneously from nutritional yeast powder using AF buffer and acid phosphatase solution (the content of enzyme vial). SIL internal standard solution (the content of IS vial dissolved in AF buffer) was added to the samples before incubation. The samples were incubated under subdued lighting conditions at 37 ^{o}C overnight (18 h). After incubation, sample extracts were centrifuged, filtered, and injected into the LC-MS.

Table 1. The methods used by Aérial for B-group vitamin analysis

Vitamin	Extraction method and liberation from the bound forms	Purification and transformation
B$_1$ (thiamine)	α-amylase, acid-phosphatase, papain, acetate buffer (pH 4.5, 37°C, 18h)	Transformation of the vitamer into a fluorescent compound
B$_2$ (riboflavin)	α-amylase, acid-phosphatase, papain, acetate buffer (pH 4.5, 37°C, 18h)	-
B$_3$ (NA, NAM)	0.1M HCl, 1h 100°C	Post column transformation of NA and NAM into fluorescent compounds
B$_5$ (panthotenic acid)	Pepsin, acetate buffer (pH 4.5, 50°C, 18h); alkaline phosphatase, pantetheinase, tris buffer (pH 8, 20°C, 18h)	Purification and post column transformation of pantothenic acid into fluorescent compound
B$_6$ (PN, PL, PM)	Acid-phosphatase, acetate buffer (pH 4.5, 37°C, 18h)	Transformation of PL and PM into PN

DSM conducted their analyses with pre-prepared sample extracts using a HR-Q-Tof (50, 000 FWHM) instrument. The yeast sample extracts were prepared in our laboratory using both diastatic enzymes - α-amylase (250 mg per 1 g of sample) and takadiastase (250 mg per 1 g of sample). Notably, diastatic enzymes were used for purpose of generating interfering peptides in order to demonstrate the efficiency of their higher resolution mass spectrometer. The addition of SIL internal

standard and sample incubation was performed as described above. After incubation, the sample extracts were centrifuged, filtered, and frozen at -80 °C. The frozen samples were sent to DSM on dry ice along with the calibration vials, and were analysed in parallel in our laboratory at the Competence Center of Food and Fermentation Technologies (CCFFT) and in DSM.

5. Results and discussion

The main results of this dissertation are presented in Publications I - VI. The main milestones achieved during method development are discussed below.

5.1. Optimization of LC-MS conditions and enzymatic sample extraction

The LC-MS-SIDA method described in Publication II was developed for the simultaneous determination of vitamins B_1, B_2, B_3, B_5, and B_6 in food products. First, we optimized the LC-MS conditions to separate and detect thiamine, riboflavin, nicotinamide (NAM), nicotinic acid (NA), pantothenic acid, pyridoxal (PL), and pyridoxine (PN) for which the stable isotope labelled (SIL) standards are available. Next, we tested different extraction conditions and studied the effect of enzymes on the liberation of simple vitamers from their derivatives and food matrix using nutritional yeast sample as a model food (Publication I).

5.1.1. Optimization of LC-MS conditions for the separation and detection of B-group vitamers

A fine separation of the peaks of all standard compounds - thiamine, riboflavin, NAM, NA, pantothenic acid, PL, and PN was achieved in an HSS C-18 column with a water + 0.1% formic acid/acetonitrile + 0.1% formic acid gradient (Fig 7); pyridoxamine (PM) had practically no retention in the selected column, and thus, was omitted from this study. Response curves for all the vitamer standards were linear over the concentration range of 0.01 – 1.0 mg/L with correlation coefficients ranging between 0.9995 and 0.9999.
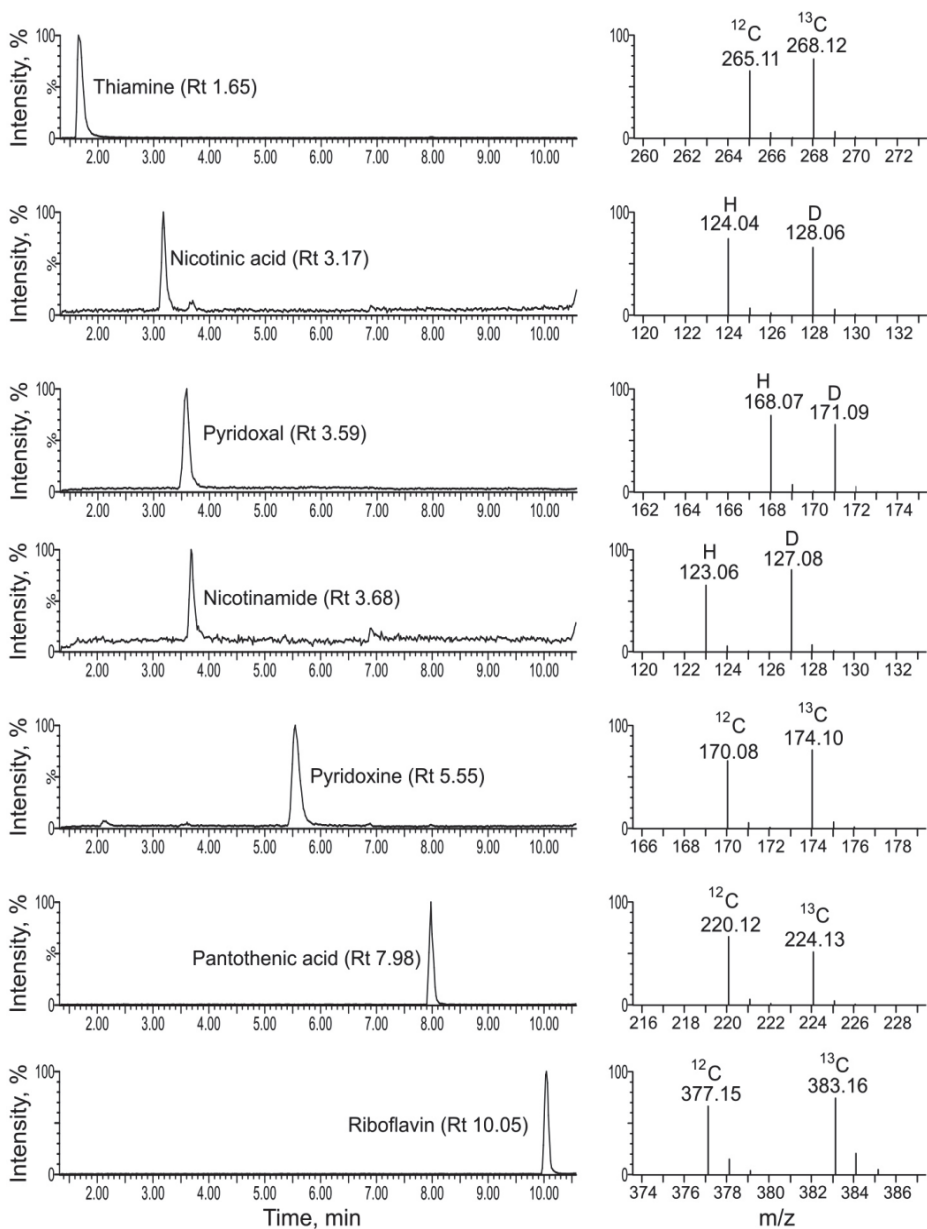
Figure 7. MS chromatograms of unlabelled (^{12}C and ^{1}H) and labelled (^{13}C or D) B-group vitamins (the analytes) (left) and the respective MS spectra (right). MS spectra are illustrative.

5.1.2. The effect of enzymes on the liberation of simple vitamers from their derivatives and the food matrix

In order to liberate matrix bound vitamers and convert them into the same chemical forms as the SIL internal standards used, the effect of different enzymes (α-amylase, β-glucosidase, acid phosphatase, papain, and takadiastase) on pure cofactors was studied (Table 2). A good recovery of thiamine, riboflavin, and PL from their respective cofactors was achieved using all enzymes with the exception of papain. The yields of panthotenic acid, NA, and NAM remained low because complete liberation was not achieved and the respective coenzymes were converted into other compounds such as nicotinamide riboside (NR), nicotinic acid riboside (NaR), and pantetheine.

Each enzyme was then tested individually on a model food sample (natural nutritional yeast powder LBI 2130, lot no 50-804184E) over a range of concentrations in the extraction medium. The results (Publication 1, Table 4) reveal an analytical problem related to interfering effect of compounds that arises due to the side effects of several enzymes used. Thus, the concentration of thiamine increased in an almost linear fashion with increasing dosages of the two diastatic enzymes α-amylase and takadiastase. Similarly, the concentration of riboflavin behaved in an enzyme concentration dependent fashion during α-amylase treatment. Notably, in the case of β-glucosidase and acid phosphatase treatment, the maximum observed thiamine and riboflavin concentrations remained remarkably lower than during treatment with either of the two diastatic enzymes. The effect can be at least partly related to proteolytic side activity, which can produce a number of peptides that interfere with either the vitamers or their isotope labelled variants (Paper 1, Table 5). For example, α-amylase and papain were shown to possess high proteolytic side activity – 2804 and 3304 U/mg, respectively (expressed as BAEE units of trypsin); the other enzymes possessed remarkably lower proteolytic side activity - acid phosphatase 50 U/mg, takadiastase 5.2 U/mg, β-glucosidase 0.12 U/mg.

Table 2. Molar recovery (%) of vitamer subunits from cofactors

	α-amylase (5.0 mg/mL)	β-glucosidase (1.2 mg/mL)	Acid phosphatase (1.2 mg/mL)	Papain (5.0 mg/mL)	Taka-diastase (5.0 mg/mL)	0.1 N HCl 121°C
Thiamine recovery from TMP , %	95	90	100	20	95	10
Thiamine recovery from TDP, %	100	100	95	0	70	10
Riboflavin recovery from FMN, %	90	100	100	70	90	5
Riboflavin recovery from FAD, %	95	100	95	0	50	5
PL recovery from PLP, %	95	100	100	20	100	75
NAM recovery from NAD^+, %	10	10	0	0	5	90
NAM recovery from NADH, %	15	5	5	0	40	5
NR recovery from NAD^+, %	+	+	+ (as NaR)	ND	+	ND
NR recovery from NADH, %	+ (reduced form)	+ (reduced form)	+ (as reduced form of NaR)	ND	+	ND
Pantothenic acid recovery from Co-A, %	0	0	0	0	0	0
Pantetheine recovery from Co-A, %	+	+	+	ND	+	ND

ND – not detected

The concentrations of PN and PL increased only to the extent of 20 - 35 % with the addition of enzyme preparations with high acid phosphatase and pyrophosphatase activity (α-amylase, β-glucosidase, acid phosphatase, and takadiastase). A small effect of enzymatic treatment was also observed for the liberation of NAM and NA, the concentration

of which increased 10 - 30 % with β-glucosidase treatment, while the effect of other enzyme preparations remained negligible. The effect of enzyme treatment on the concentration of pantothenic acid was negligible and remained in the range of statistical experimental error.

In conclusion,

1. For the efficient liberation of thiamine, riboflavin and PN, PM or PL from their corresponding cofactors under the extraction conditions used, the combined effect of two specific enzymatic activities are required: i) acid phosphatase (EC 3.1.3.2) is required to release thiamine from TMP and riboflavin from FMN and PN, PM or PL from PNP, PMP or PLP, respectively, and ii) pyrophosphatase (EC 3.6.1.9) is required to release FMN from FAD, or TMP from TDP and TTP. Both enzymatic activities were sufficiently present in the β-glucosidase and acid phosphatase used.

2. NAM and NA were not released with any of the enzymes studied. This can be explained by the missing purine nucleosidase (EC 3.2.2.1) and NAD^+ glycohydrolase (EC 3.2.2.5) activities which are required to release NAM from nicotinamide riboside (NR) or directly from NAD^+, respectively. Thus, NAD^+ or NR should be quantified directly, using isotope labelled internal standards.

3. The liberation of pantothenic acid from CoA could not be carried out with any of the enzymes studied. This can be explained by the missing pantetheine hydrolase (EC 3.5.1.92) activity, which releases pantothenic acid from CoA-derived pantetheine. Thus, pantetheine should be quantified directly if labelled internal standards are available.

4. Application of relatively impure diastatic enzymes can be complicated because they produce assay interfering compounds.

5.1.3. The effect of acid extraction

The effect of enzymes in liberating vitamins from cofactors was compared to a conventional B-group vitamin extraction method with hot HCl (T = 121 °C, 30 min) (Table 2). The effect of hot formic acid extraction (0.1 M and 0.3 M) was also studied on nutritional yeast sample as a convenient alternative to HCl for LC-MS analysis (Paper 1, Table 3).

The experiments with pure cofactors show that the recovery of thiamine from TMP and TDP, and that of riboflavin from FMN and FAD, were less than 10 % (Table 2) which explains the usage of additional acid phosphatase treatment in approved standard methods (EVS-EN 14122:2003, EVS-EN 14152:2003). On the contrary, the recoveries of PL from PLP and NAM from NAD$^+$ were quite high - 75 and 90 %, respectively.

Similarly, hot acid extraction had no positive effect on the concentration of thiamine and riboflavin in the nutritional yeast sample. Surprisingly, the hot formic acid treatment decreased the thiamine concentration in the yeast sample compared with the HCl treated sample. This effect could be due to the formation of thiamine formic acid conjugates during hot extraction.

A positive effect of hot acid extraction was observed on the concentrations of NAM and NA which increased by about 20 % and 25 %, respectively. The concentration of PL increased by 3 times compared to enzymatic treatment alone (Paper 1, Tables 3 and 4). This can be explained by more efficient hydrolysis of imines by the acid than by the enzymes. Specifically, in living organisms PLP, as an active cofactor, is involved in various reactions with amino acids, including transamination reactions where the aldehyde group of PLP forms a Schiff-base linkage with the ε-amino group of the aminotransferase enzyme [Toney 2005]. Although, it is not known whether such imines would be hydrolyzed in the human gastrointestinal tract, our preliminary experiments with human gastric juice suggest that hydrolysis of PLP covalently bound to

protein is possible and thus imines can contribute to the total vitamin B_6 content in food [Vilbaste et al 2014].

Notably, a detrimental effect on the concentration of pantothenic acid was observed with hot HCl extraction, most likely due to its hydrolysis to β-alanine and pantoic acid [Velišek & Davidek 2000]. The experiments with nutritional yeast sample show that using formic acid extraction the tendency was almost the same as with HCl extraction, excepting the higher recovery of pantothenic acid.

5.2. Development of the assay

The main goal while developing the analytical assay was to utilize universal extraction and chromatographic conditions for the simultaneous quantification of all vitamins under study. In the present study we focused on the determination of vitamers that had commercially available stable isotope labelled (SIL) isotopomers. Due to the high price of many of these isotopomers, the first goal was to optimize their cost per assay. This problem was partially addressed by freeze-drying mixtures of SIL vitamers in chromatography vials. The amount of each vitamer to be freeze-dried in the vials was selected based on their respective LOQs that were previously determined (at least 10 times above the LOQs in yeast). Because the required concentration of each SIL vitamer was too small to precisely weigh, the quantification of the vitamers in the sample was carried out using external standards. Thus, the calibration vials were prepared by adding a solution with known concentration of unlabelled vitamers into the vials containing the same amount of SIL vitamers as in the internal standard vials. Provided that the ratios of MS signal intensities of the labelled and unlabelled vitamers is the same in case of the sample and the calibration solution, this method allows us to determine the concentrations of all vitamers in the sample without knowing the exact concentrations of their internal standards (see Formula 3).

5.2.1. The stability of internal standard (IS) vials

Then the stability of freeze-dried SIL internal standard preparations was also studied (Publication II). The stability of the SIL vitamers in the analytical assay was tested every 1 - 5 months over a period of one year using freshly prepared external standards. All SIL vitamers were found to be stable during the testing period (Fig 8). This knowledge enabled us to prepare calibration vials containing both internal and external standards that accompanied the internal standard (IS) vials in the prototype assay kit.
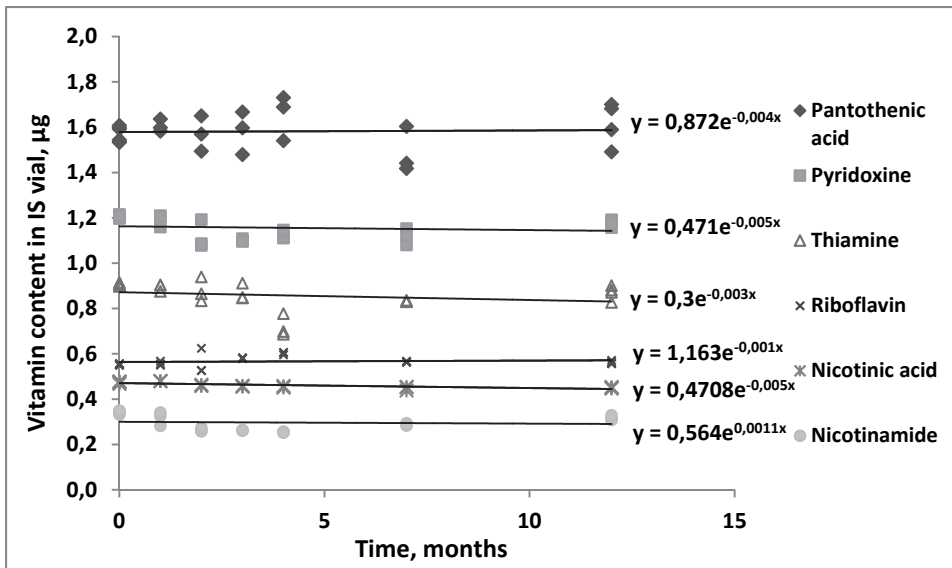

Figure 8. Stability of the freeze-dried isotope labelled vitamers in the sample vials of the analytical assay

5.2.2. Selection of enzymes and the stability of enzyme vials

The enzymes for the prototype of analytical assay kit were selected based on experiments with pure cofactors and nutritional yeast. The results of these experiments demonstrate that the most efficient

enzyme preparations for the liberation of free vitamers from cofactors were those that possessed both acid phosphatase and pyrophosphatase activity with low protease side activity. Thus, acid phosphatase (Cat no P1146, Sigma) and β-glucosidase (Cat no G0395, Sigma) were selected as potential enzymes for the prototype analytical assay kit. β-glucosidase was also chosen due to its glycolytic activity which appears necessary to liberate glycosylated forms of vitamers when present in the food matrix. However, it has to be noted that for some food matrices, proteolytic (side) activity might be necessary to liberate protein bound forms of vitamers. However, in those cases a higher resolution MS should be used to resolve the compounds that interfere with each specific vitamer. Alternatively, conditions that simulate acidic extraction in the human GI tract should be used.

The stability of freeze-dried enzymes was tested over a period of three months and all enzymes were found to be stable over this testing period (not illustrated), the recovery of thiamine from TMP and TDP was in the range of 90-110%, while riboflavin from FMN and FAD was in the range of 85-90% and PL from PLP was in the range of 65-75% using a single cofactor or a mixture of cofactors.

5.3. Validation of the LC-MS-SIDA method and analytical assay

5.3.1. Validation of the LC-MS-SIDA method

The validation of the LC-MS-SIDA method was performed while analysing the extraction of simple vitamers from nutritional yeast powder (LBI 2130, lot no 50-804184E) using 0.05M ammonium formate buffer (pH 4.5) (Publication I). The method validation parameters LOD (μg/g) and LOQ (μg/g), recovery (%) and intra-day and inter-day precision values of the vitamers determined in this study are presented in Table 3.

Table 3. Method validation parameters

Vitamer	LOD (µg/g) (n = 6)	LOQ (µg/g) (n = 6)	Recovery (%) (0.5 mg/L) (n = 3)	Recovery (%) (0.25 mg/L) (n = 3)	Recovery (%) (0.1 mg/L) (n = 3)	Intra-day precision[a] (%) (n = 3)	Inter-day precision[a] (%) (n = 6)
Thiamine	0.11	0.34	105 ± 1	95 ± 7	97 ± 6	2.1	5.8
Riboflavin	0.04	0.12	104 ± 1	113 ± 4	110 ± 4	3.5	4.9
NAM	0.14	0.43	96 ± 2	106 ± 5	104 ± 7	1.7	6.4
NA	0.17	0.51	95 ± 4	100 ± 7	96 ± 3	1.3	3.8
Pantothenic acid	0.08	0.24	93 ± 8	98 ± 2	103 ± 2	2.9	6.6
PL	0.05	0.14	104 ± 2	90 ± 6	95 ± 2	6.5	5.5
PN	0.03	0.09	97 ± 1	105 ± 7	102 ± 6	2.1	1.2

[a] Expressed as coefficient of variance

5.3.2. Validation of the assay performance

The performance of the given analytical assay was validated with three reference materials: SRM 1849 (Infant/Adult Nutritional Formula, Publication I) for the quantification of free vitamins, CRM BCR 121 (wholemeal flour, Publication III) for total concentration of vitamins B_1 and B_6 (the content of the other vitamins are not provided for this sample) and SRM 3234 (soy flour, Publication IV) for total concentration of thiamine, riboflavin, nicotinamide (NAM), nicotinic acid (NA), pantothenic acid, pyridoxal (PL), and pyridoxine (PN). In addition, inter-laboratory comparative tests with nutritional yeasts Engevita Bland, lot no 206477E and LBI 2130, lot no 108706E were carried out together with external food analysis laboratories (Aérial and DSM Nutritional Products, Analytical Research Center).

The concentration of free vitamins determined in SRM 1849 using the given analytical method correlates well with the certified data (Table 4). As for the total vitamin concentration values, the vitamin B_1 concentration determined in CRM BCR 121 correlates well with the certified data, while the concentration of vitamin B_6 is significantly lower than that of the reference value (Table 5). This cannot solely be

explained by the fact that pyridoxamine (PM) remained unquantified with the chromatography method used because the content of PM in flour has been reported to be only 10 – 12 % of the total B_6 content [Polansky & Toepfer 1971]. A more plausible explanation for the lower B_6 content would be incomplete liberation of bound pyridoxal (PL). As proposed in Publication I, some of the PL and PLP might be bound to proteins (e.g. in the form of imines), which could not be completely liberated by the enzymes.

Table 4. The certified concentrations of free B-group vitamers in the SRM 1849 and those determined by us using LC-MS-SIDA method

Vitamer, mg/kg	Certified value	LC-MS-SIDA [a] (n = 30)
Thiamine Cl HCl	15.8 ±1.3	15.5 ± 0.3
Riboflavin	17.4 ± 1.0	17.2 ± 0.3
NAM	97.5 ± 2.3	99.7 ± 1.9
Pantothenic acid	64.8 ± 2.2	63.9 ± 0.9
PN HCl	14.2 ± 1.5	14.2 ± 0.2

[a]Results are expressed as a mass fraction for the material as received and as the mean of at least 30 measurements on five different days during one month. The uncertainties are expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U = k*u_c$, where u_c is uncertainty of the measurement results and k is a coverage factor.

In case of SRM 3234 (soy flour, Publication IV) the total vitamin concentrations correlate well with the certified data (Table 6). However, the distribution of the content of NAM and NA in soy flour was slightly different compared with the certified data while simultaneously correlating well with the certified total content of vitamin B_3. The difference in the distribution of NAM and NA is probably related to the different sample extraction techniques applied, however, because the NIST certificate of analysis only indicates a dilute acid extraction, fundamental conclusions cannot be drawn regarding the differences in extraction methods, e.g. influence of endogenous enzymes, temperature, and pH. In addition, also other vitamin B_3 derivatives,

nicotinic acid riboside (NaR) and nicotinamide mononucleotide (NMN) were observed in the mass spectra of soy flour, after overnight incubation with endogenous enzymes, thus, the total concentration of vitamin B_3 in soy flour can be even higher than reported by NIST.

Table 5. The certified concentrations of B-group vitamers in CRM BCR 121 (wholemeal flour) and those determined by us using LC-MS-SIDA method

Vitamin, µg/100 g of DM	Certified value	LC-MS-SIDA (AVG ± SD, n = 3)
B_1	364 ± 31	394 ± 31
B_2	–	325 ± 135
NA	–	391 ± 28
NAM	–	58 ± 2
B_5	–	658 ± 47
PL	–	28 ± 4
PN	–	189 ± 16
B_6	377 ± 84	217 ± 19

Table 6. The certified concentrations of B-group vitamers in the SRM 3234 and those determined by us using LC-MS-SIDA method

Vitamer, mg/kg dwb	Certified value	LC-MS-SIDA (AVG ± SD, n = 5)
Thiamine	8.60 ± 0.20	8.74 ± 0.26
Riboflavin	3.36 ± 0.04	3.35 ± 0.06
NAM	11.49 ± 0.13	8.62 ± 1.06
NA	4.59 ± 0.17	6.90 ± 1.35
Vitamin B_3 as NAM	16.04 ± 0.24	15.46 ± 2.03
Pantothenic acid	11.45 ± 0.12	11.48 ± 0.62
PL HCl	1.72 ± 0.10	1.82 ± 0.07
PN HCl	0.73 ± 0.09	0.83 ± 0.05

The results of the inter-laboratory comparative test with Aérial using nutritional yeast (Engevita Bland, lot no 206477E) are presented in Table 7 while the methods used by Aérial are described in Table 1 in the Materials and Methods section. A good correlation was seen in the case of vitamin B_1 and vitamin B_3. With vitamin B_2, our method provided higher concentrations and yet with both vitamin B_5, and, in particular B_6, our results were lower than those obtained by Aérial.

Table 7. The results of the inter-laboratory comparative test with Aérial using nutritional yeast as a model food

Vitamin	Aérial	CCFFT
	mg/100g (AVG ± SD, n = 3)	
B_1	0.84 ± 0.21	1.06 ± 0.10
B_2	4.05 ± 0.04	5.66 ± 0.18
NA	9.73 ± 0.35	9.05 ± 0.90
NAM	15.4 ± 0.28	16.11 ± 1.47
B_3 (NA+NAM)	25.13 ± 0.63	25.16 ± 2.37
B_5	15.90 ± 0.82	11.01 ± 0.68
B_6	1.50 ± 0.03	0.18 ± 0.01

It is difficult to explain why the concentration of vitamin B_2 was found to be different because Aérial used a very similar extraction method, i.e. an overnight mild acid extraction (acetate buffer, pH 4.5, 37°C, 18h) with α-amylase, acid-phosphatase, and papain.

The considerably lower result determined by us for vitamin B_6 can be partially explained by PM which was not determined with our chromatography system. Notably, PM can constitute up to a half of the total vitamin B_6 content in Baker´s yeast [Ollilainen 2000]. On the other hand, even when including the PM content, the total vitamin B_6 concentration would still be remarkably lower than that determined by Aérial. Because Aérial used a similar mild extraction (acetate buffer, pH

4.5, 37°C, 18h) with incubation with acid phosphatase, the difference cannot also be explained by the poor liberation of bound pyridoxal. Thus, it could be possible that the method used to convert PM and PL into PN also resulted in the conversion of other compounds that contain the structural units of those vitamers.

The lower result for vitamin B_5 was most likely because our method does not determine CoA and related compounds, e.g. pantetheine and 4´phosphopantetheine. In contrast, Aérial's method includes pantetheinase (EC 3.5.1.92, purified from liver acetone powder from pigeon) treatment in combination with the alkaline phosphatase treatment. Thus, adding pantetheinase to the enzyme mixture of our assay should be considered in the future. Because pantheteinase has an optimum pH between five and seven, the pH of the extraction medium should be slightly increased.

Another inter-laboratory comparative test was carried out using nutritional yeast (LBI 2130, lot no 108706E) in co-operation with DSM Nutritional Products, Analytical Research Center. The aim was to test whether a higher resolution MS HR-Q-Tof (50, 000 FWHM) can resolve overlapping peaks which are a potential cause of the false estimation of the vitamin concentration.

The sample extracts prepared with our assay were analysed in parallel at CCFFT and DSM with the results presented in Table 8. These results suggest that some overlap with peptides or other interfering compounds lead to either an over- or under-estimation of the vitamin concentration in our MS-TOF that has five times lower resolution compared with the machine at DSM.

In case of thiamine our results were 25 % lower than those reported by DSM. This is likely due to the peptide isotopomer $[M + H]^+ + 2 = 268.13$ (Paper 1, Table 5) which interfered with labelled thiamine. Also, another peptide isotopomer $[M + H]^+ + 1 = 265.11$ interfered with unlabelled thiamine. Based on the isotopomer distribution of the given interfering

compound its overlapping effect on the intensity of the peak of interest can be predicted using an isotope distribution calculator (http://www.sisweb.com/mstools/isotope.htm) and corrections can thus be made for unlabelled and labelled thiamine peak intensities. Interestingly, after this correction the thiamine concentration was well in accordance with the DSM results.

Table 8. The results of an inter-laboratory comparative test with nutritional yeast treated with enzyme preparations

Vitamer, mg/100g	DSM	CCFFT
B_1	6.18	6.61* (4.52)
B_2	2.22	2.81
NA	6.90	5.17
Pantothenic acid	10.26	7.58
PL	0.54	0.43
PN	ND	0.19

ND- not detected
* corrected according to isotopomer distribution

With regards to riboflavin, our results were 20 % higher than those reported by DSM. It can be speculated, that there was overlapping of unlabelled riboflavin with interfering peptides (Paper 1, Table 5). NA, PL, and pantothenic acid concentrations were 25, 20, and 25 % lower, respectively, than the concentrations reported by DSM. This suggests a possible overlap of their labelled internal standards with some unknown compounds. Nevertheless, this possibility is rather speculative. Finally, it should also be mentioned that substantial conclusions on this inter-laboratory comparative test cannot be drawn at this stage because it was conducted without replicates. Thus, in the cases when proteolytic (side) activity is necessary to liberate protein bound forms of vitamers, a higher resolution MS should be used to resolve interfering compounds from the vitamers.

5.4. Applications of the assay

The assay developed in Publications I and II was applied for the analysis of B-group vitamins in various food matrices to study the stability of B-group vitamins during rye sourdough bread production (Publication III), the distribution of B-group vitamers in quinoa seeds (Publication IV), the uptake and accumulation of the B-group vitamers in the yeast *Saccharomyces cerevisiae* (Publication V) and to determine the B-group vitamers and their derivatives, including NAD^+, NMN, NR, in nutritional yeast and yeast extract (Publication VI).

5.4.1. The stability of B-group vitamins during rye sourdough bread production (Publication III)

To study both the stability of B-group vitamins during rye sourdough bread production and the distribution of free and bound forms of the vitamins, we performed extractions on both raw materials and baked rye breads (rye bread and fine rye bread) using an ammonium formate buffer (pH 4.5) with and without an enzyme mixture containing α-amylase, β-glucosidase, and acid phosphatase. Notably, additional α-amylase was used to hydrolyse starch which may be intertwined with vitamins and hinder their release [Hucker et al 2014]. The results are presented in Table 3 in Paper III. It was found that enzyme treatment increased the thiamine yield in both the raw materials and in the breads. The bound vitamer forms comprised up to 70 % of the total thiamine, 60 % of the total riboflavin, 60 % of the total pyridoxine (PN), and 50 % of the total pyridoxal (PL). Nevertheless, it is important to mention that the content of bound forms in these raw materials which were not previously thermally treated (e.g. flours), could have been even higher because the extraction method used does not inactivate endogenous enzymes. The distribution of free and bound forms of vitamin B_3 and B_5 could not be determined because the enzymes used do not liberate nicotinic acid (NA), nicotinamide (NAM), and pantothenic acid from their derivatives (i.e. NR, NMN, NAD^+, CoA) (Publication I).

During bread making, the decrease in the concentration of vitamers in rye bread and fine rye bread were in the range of 20 – 40 % for thiamine, 25 – 50 % for NA and 45 – 65 % for PL, and in fine rye bread 50 % for riboflavin and 15 % for PN, as calculated according to the mass-balance of the enzyme extracted samples from both flours and breads. There was, however, a significant increase in the NAM concentration (by tenfold) in both breads during processing. The increase in NAM was probably related to the conversion of NAD^+ into NAM during the lactic acid fermentation of sourdough. The concentration of pantothenic acid remained the same which indicates that this compound is stable during the baking conditions of rye breads. The loss of thiamine, riboflavin, and PN during baking was similar to that reported in earlier studies [Batifoulier et al 2005, 2006].

Our results reveal that the nutritional databases report significantly higher vitamin B_3, B_5, and B_6 content in cereals than were determined in our work (Paper III, Tables 3 and 4). This was expected because the acid phosphatase, β-glucosidase, and α-amylase enzymes that we used lack side activities that completely liberate NA, NAM, and pantothenic acid from their bound forms. In the case of vitamin B_6, PL, and PLP bound into imines is the most likely cause of under-determination considering the mild extraction conditions used. In addition, other factors can also cause such differences. For example, grain from different geographic locations may vary considerably in its vitamins content. Also, information in the nutritional databases is derived from several sources and is usually analysed using different analytical methods. For example, Kall [2003] showed that the vitamin B_6 content in foods determined using a microbiological assay and HPLC was systematically different and the variance of the results depended mainly on the extraction method and the sample matrix. In most cases, vitamin determination methods are optimized separately for the maximal recovery of each vitamin and do not take into account the bioavailability of the compounds containing the structural unit of the vitamin. This often results in an overestimation of the vitamin content, particularly for those which are based on

microbial assays or involve high temperature acid extraction that is very different compared with the conditions in the human GIT. This means that for vitamin analysis in foods, the extraction conditions and enzymes common within the human GIT should ideally be used.

5.4.2. Comparison of different extraction methods to determine the distribution of free and bound forms of B-group vitamins in quinoa (Publication IV)

Determining the distribution of different forms of vitamers in foods is important with respect to understanding the metabolism of vitamins and cofactors in the food chain. The efficiency of various sample pre-treatment processes was studied to determine the vitamer distribution in quinoa (Paper IV, Fig 1). Under mild extraction conditions (e.g. 0.05M ammonium formate buffer, pH 4.5) the activity of endogenous enzymes is not inhibited in food and, thus, may change the distribution of different vitamin forms. For this reason, both enzyme inhibitors and ultrafiltration were evaluated for their ability to eliminate the activity of endogenous enzymes (Paper IV, Fig 2).

To determine the concentration of free vitamers in quinoa seeds, we found that the most efficient method was short cold extraction of finely ground seeds together with protease and phosphatase inhibitors. These inhibitors, which were initially designed to protect cell lysates and other biological samples from dephosphorylation and proteolytic degradation, work by inhibiting the activity of a wide range of phosphatases and proteases. These were also found to be effective in inhibiting the endogenous liberation of some of the free vitamers from their bound forms. Thus, the addition of phosphatase and protease inhibitors to the sample extract reduced the concentration of both free riboflavin and PL. In contrast, the concentration of free thiamine and PN did not decrease with the addition of enzyme inhibitors. It can be speculated that the endogenous phosphatases responsible for thiamine and PN liberation from their bound forms are insensitive to sodium fluoride, which was the

active compound in the phosphatase inhibitor preparation used. Surprisingly, this treatment gave the highest result for NA, probably by preventing the conversion of NA into nicotinic acid mononucleotide by nicotinate phosphoribosyltransferase (EC 2.4.2.11) [Ashihara et al 2005]. Notably, none of the extraction methods used had a significant influence on the recovery of free pantothenic acid.

Ultrafiltration was found to be efficient in removing endogenous enzymes. Another benefit of ultrafiltration is that compounds that interfere with the mass spectra of the compounds of interest are reduced considerably in the sample extract. This effect is probably related to the removal of soluble proteins and other high molecular weight compounds, the hydrolysis products of which could interfere with the spectra of vitamers during LC-MS determination, as shown in the case of thiamine in Figure 9. Thus, ultrafiltration is recommended as both an effective sample purification method and sample fixation step.

The importance of eliminating the activity of endogenous enzymes is well illustrated with an example where overnight incubation at 37 ^{o}C, both with and without additional enzymes (phosphatase and β-glucosidase) were almost equally efficient in releasing the bound forms of vitamin B_1, B_2, and B_3 vitamers. Despite some activity of endogenous enzymes in liberating the bound forms of PL, the most efficient method for the complete release of its bound forms was thermal pre-treatment (30 min at 100 ^{o}C) followed by overnight incubation with an enzyme that displays acid phosphatase activity. This suggests that some PL or PLP in quinoa might be also bound into imines and is not liberated by endogenous enzymes (Publication I). The most efficient pre-treatment for releasing the bound forms of PN was overnight incubation with β-glucosidase. This indicates the existence of pyridoxine glucosides (PNG) in quinoa. This result was expected because in plants PN can exist in the form of PNG [Gregory 1998]. Interestingly, various sample pre-treatment steps had no effect on the concentration of vitamin B_5. This is probably due to the fact that neither the addition of enzymes nor the activity of

endogenous enzymes within quinoa liberated pantothenic acid from its bound forms (CoA, pantetheine etc). Considering that some of these bound forms may have nutritional value, their quantification remains important. To liberate the bound forms of pantothenic acid, additional pantetheinase and alkaline phosphatase treatment has been suggested by Rychlik [2000].
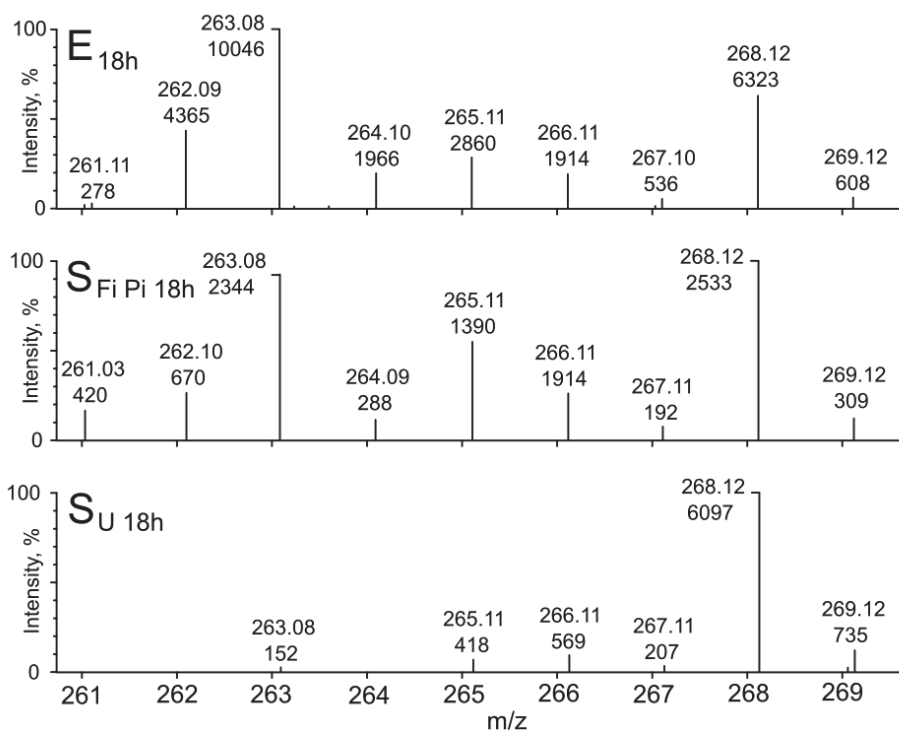

Figure 9. Interfering compounds (m/z 262.10 and 263.08) in the mass spectrum of thiamine (m/z 265.10). E_{18h} - overnight incubation with endogenous enzymes, $S_{Fi\ Pi\ 18h}$ - overnight incubation with protease and phosphatase inhibitors, $S_{U\ 18h}$ - overnight incubation of ultrafiltered sample

5.4.3. The uptake and accumulation of B-group vitamers in *Saccharomyces cerevisiae* (Publication V)

In this work we studied the accumulation of thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine in the baker's yeast *Saccharomyces cerevisiae*. Biomass sample fixation in cold methanol was used to measure the intracellular concentrations of vitamins and cofactors at different specific dosage rates.

An increase in the specific dosage of B-group vitamins clearly increased the total intracellular concentration of B_1, B_3, and B_5 vitamers and cofactors. However, no additional accumulation of B_2 or B_6 vitamers was observed when additional vitamins were added to the growth medium (Paper V, Table 1). A common feature of all vitamins studied in the present work, with the exception of riboflavin, was that their specific accumulation rates were lower than their respective apparent uptake rates. This could either be due to an underestimation of vitamin and cofactor accumulation or vitamin degradation. Underestimation of vitamin and cofactor accumulation can occur due to losses during sample processing or due to vitamers that remain undetermined for technical reasons. The latter was clearly the case for vitamin B_5, and most likely also B_6, where CoA and protein-bound PLP were not determined. Although the sample was fixed in cold methanol, which denaturates the cell membranes and inhibits endogenous enzymatic activity in the cell, the activity of endogenous enzymes could partly resume during the subsequent extraction with ammonium formate. Therefore, it is possible that the intracellular concentrations of free vitamers may be even lower than than those reported and thus the concentration of cofactors and other more complex vitamer forms in yeast cells is probably higher. Also, losses of vitamers during sample preparation cannot be excluded, because the internal standards were added to the sample extracts only before LC-MS analysis to prevent absorption of internal vitamer standards by the yeast cells. Thus, in order to determine the exact distribution of the vitamers in the yeast cells,

sample preparation techniques, e.g. endogenous enzymes inactivation, should be further optimised.

5.4.4. LC-MS for quantification of B-group vitamins in nutritional yeast and yeast extract (Publication VI)

The LC-MS-SIDA based method and a prototype analytical assay developed in Publication II, was used together with *in house* prepared ^{15}N-labelled yeast extract fractions to evaluate the content of B-group vitamins in both nutritional yeast powder Engevita Bland (lot no 206477E) and yeast extract FNI 100 (lot no 10103308). In addition to mild acid extraction combined with enzyme treatment, hot HCl (0.1 N) extraction was also used to assess the total amount of pyridoxal in the samples. The results are presented in Figures 2 – 5 within Paper VI.

The LC-MS-SIDA method with respective analytical assay was found to be suitable for the determination of both the free forms of all vitamers under study (thiamine, riboflavin, PL, PN, NA, NAM, and pantothenic acid) and the total concentration of vitamin B_1, vitamin B_2 in nutritional yeast and yeast extract. It was impossible to determine the total concentration of vitamin B_3 and vitamin B_5 because the enzymes used lack proper side activities to completely liberate NA, NAM, and pantothenic acid from their derivatives. Also, it was impossible to determine the total concentration of vitamin B_6 because one of the vitamin B_6 vitamers, PM, remained unquantified with the chromatography method used. The efficiency of the hot HCl extraction in liberating PL and PLP from imines was also determined in this study. The concentration of pyridoxal in nutritional yeast sample increased 1.7 times compared to enzymatic extraction. Also, after hot HCl extraction pyridoxal was found in yeast extract (0.15 ± 0.02 mg/100g). *In house* prepared ^{15}N-labelled yeast extract fraction was found to be suitable for the determination of NAD$^+$, NMN and nicotinamide riboside (NR) and thus can be used to determine the derivatives of NAM.

6. Conclusions

The LC-MS-SIDA method employing enzyme assisted extraction in mild acidic conditions and the developed prototype assay consisting of internal standard, enzyme, and calibration vials provides a good basis for the further development of commercial high throughput assays for quantitative analysis of B-group vitamins in foods.

At the present stage of the assay development, additional conclusions can be made:

I   The LC-MS-SIDA method with respective analytical assay was found to be suitable for the determination of the free forms of all vitamers studied (thiamine, riboflavin, PL, PN, NA, NAM, and pantothenic acid) and the total concentration of vitamin B_1 and B_2.

II   Acid phosphatase and β-glucosidase are currently the most efficient commercially available enzymes for the liberation of simple vitamers from more complex bound forms considering the lack of undesirable side activities.

III   For the complete determination of the total concentrations of vitamin B_3 and B_5 in foods, several isotope labelled vitamers (e.g. NR, NaR, NMN, NAD^+, pantetheine, and acetyl-CoA) should be included with the assay kit because none of the commercial enzymes tested were able to completely liberate simple vitamers from these compounds.

IV   To determine the total concentration of vitamin B_6, the extraction conditions should be further optimized to liberate bioavailable protein bound PL and PLP. For this, conditions simulating the gastric part of the human GI tract could be used.

V   The intrinsic activity of endogenous enzymes should be avoided whenever the distribution of free and bound vitamins is to be studied, i.e. proper sample fixation and extraction methods should be developed.

VI   To reduce the errors caused by matrix effects during quantification in the MS, either ultrafiltration (cut off 10 kDa) or use of high resolution MS spectrometers (50, 000 FWHM) is recommended.

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APPENDICES

PUBLICATION I

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Comparison of different extraction methods for simultaneous determination of B complex vitamins in nutritional yeast using LC/MS-TOF and stable isotope dilution assay

Comparison of different extraction methods for simultaneous determination of B complex vitamins in nutritional yeast using LC/MS-TOF and stable isotope dilution assay

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Abstract The application of LC/MS-TOF method combined with stable isotope dilution assay was studied for determination of thiamine, riboflavin, nicotinamide, nicotinic acid, pantothenic acid, pyridoxal, and pyridoxine in food. Nutritional yeast powder was used as a model food matrix. Acid extraction was compared with various enzymatic treatments in ammonium formate buffer to find a suitable method for the conversion of more complex vitamers into the same forms as the used isotope-labeled internal standards. The enzyme preparations α-amylase, takadiastase, β-glucosidase, and acid phosphatase were all able to liberate thiamine and riboflavin. The diastatic enzyme preparations α-amylase and takadiastase also expressed proteolytic side activities resulting in the formation of small peptides which interfered with the mass spectra of thiamine and riboflavin. Liberation of nicotinamide and pantothenic acid from NAD^+ and CoA, respectively, could not be achieved with any of the studied enzyme preparations. Hydrochloric acid extraction at 121 °C for 30 min was found to be destructive to pantothenic acid, but increased the liberation of pyridoxal.

Introduction

The group of B complex vitamins is diverse with respect to structure, molecular weight, chemical properties, and physiological activity. In spite of the rapid development of analytical techniques, simultaneous quantification of these water-soluble vitamins in foodstuffs remains challenging, with major hindrances being their low concentration in the food matrix, relatively low stability, as well as their different chemical structures [1]. Liquid chromatography (LC)-based methods using conventional UV or fluorescence detection often lack sufficient sensitivity and selectivity and may produce chromatograms with unresolved interferences with other compounds from the sample matrix if proper cleanup procedures are not carried out. To overcome these problems, modern methods couple LC with mass spectrometry (LC/MS and LC/MS/MS) which have proven to be rapid and sensitive methods for the determination of B complex vitamins in food and dietary supplements [1–3]. However, isotope-labeled internal standards should be applied to compensate for losses of the analytes during sample preparation and for matrix effects during MS and MS/MS analysis [4]. The use of isotope-labeled internal standards with MS techniques increases accuracy, precision, and helps to simplify considerably the sample pretreatment procedures [5]. Although there are several authors who have used isotope-labeled internal standards for determination of B complex vitamins in tablets and dietary supplements, the data for real food samples are limited [6–9]. The main hindrances, which need to be overcome when real food samples are analyzed, involve complex sample preparation processes and

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unavailability of isotope-labeled internal standards of naturally occurring vitamers. Thus, the extraction of vitamers from the food matrix should be maximized, and all vitamers should be converted into the same forms as the used internal and external standards. The latter is important because, in addition to the simple chemical forms of vitamers which are used in the composition of food or feed supplements, they can also be chemically modified (e.g., phosphorylated, glycosylated, glutamylated, or bound to proteins) or exist in more complex natural forms (e.g., cofactors) [10, 11]. Several authors have suggested a combination of acid hydrolysis and enzyme treatment to extract and dephosphorylate vitamers or liberate them from protein-containing matrices [12, 13]. Acid hydrolysis, often carried out at elevated temperatures, can be detrimental to the stability of several vitamins in the sample and thus may not be appropriate if their simultaneous determination is to be carried out. It has also limitations when mass spectrometry detection is used. Ndaw and co-workers [14] showed that the treatment of various food samples with a mixture of enzyme preparations containing α-amylase, acid phosphatase, β-glucosidase, and papain led, in a single extraction step, to the release of thiamine, riboflavin, and vitamin B_6 from phosphorylated and protein-bound forms, even if the acid hydrolysis was not used. Jakobsen [15] further optimized the method by introducing ultrasonication in order to shorten the duration of enzymatic treatment for liberation of protein- and phosphate-bound thiamine and riboflavin in food. In both of these studies, the concentration of up to three selected B complex vitamins was determined using high-performance liquid chromatography (HPLC) and fluorescence detection.

Consumer demand for reliable nutrition labeling has created the need for a high throughput method for quantitative B complex vitamins determination. This method should consist of a simple, single-step procedure for complete extraction of water-soluble vitamins and the same chromatographic conditions for analysis. In the present work, we used nutritional yeast as a model to study the effect of different enzymatic extraction methods for simultaneous quantification of thiamine, riboflavin, nicotinamide, nicotinic acid, pantothenic acid, pyridoxal, and pyridoxine using LC/MS-time of flight (TOF) and stable isotope dilution assay.

Materials and methods

Chemicals

Unlabeled standards of nicotinamide (99.9 %), nicotinic acid (99 %), thiamine HCl (99 %), pyridoxine HCl (99.9 %), pyridoxal HCl (99 %), Ca pantothenate (99 %), and riboflavin (99 %) were purchased from Sigma-Aldrich (Steinheim, Germany). The isotope-labeled standards of

[D$_4$]-nicotinamide (98 %), [D$_4$]-nicotinic acid (98 %), [^{13}C$_3$]-thiamine HCl (99 %), and [^{13}C$_4$]-pyridoxine HCl (99 %) originated from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA); [^{13}C$_3$^{15}N]-Ca-pantothenate (98 %), [^{13}C$_4$,^{15}N$_2$]-riboflavin (98 %), and [D$_3$]-pyridoxal HCl (99 %) were sourced from Isosciences, LLC (King of Prussia, PA, USA). Thiamine monophosphate chloride dihydrate (99 %), thiamine pyrophosphate chloride ($\geq$97 %), riboflavin 5′-monophosphate sodium salt dihydrate (75.9 %), flavin adenine dinucleotide disodium salt hydrate (97 %), β-nicotinamide adenine dinucleotide hydrate (98 %), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (99 %), and Coenzyme A hydrate (99 %)—used for the determination of phosphatase and/or pyrophosphatase and/or purine nucleosidase and/or pantetheine hydrolase activities of the enzyme preparations as well as for testing the efficacy of hydrochloric acid treatment were all purchased from Sigma-Aldrich. Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals Ltd. (Walkeburn, Scotland, UK), formic acid (LC/MS grade), ammonium formate (puriss. p.a., for HPLC), and hydrochloric acid (puriss. p.a., ACS) were all purchased from Sigma-Aldrich. High purity water was produced by a Millipore water purification system (Millipore S.A.S., Malsheim, France).

Enzymes

α-amylase from *Aspergillus oryzae* (crude, A6211), β-glucosidase from almonds (crude, G0395), acid phosphatase from potato (P1146), papain from papaya latex (crude, P3375), and taka-diastase from *A. oryzae* (86247) were all purchased from Sigma-Aldrich.

Samples

Commercial nutritional yeast powder Engevita Bland (lot. 50-804184E) was obtained from Lallemand, Inc. (Montreal, Canada). The performance of the used LC/MS method was assessed by analyzing a standard reference material (SRM) 1849—Infant/Adult Nutritional Formula which was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

Standard preparation and calibration

External standard stock solutions were prepared fresh daily as follows: 25 mg of nicotinamide, nicotinic acid, pantothenic acid, pyridoxal HCl, pyridoxine HCl, and thiamine HCl was weighed separately into 100-ml volumetric flasks and dissolved to the marked volume with 0.05 M ammonium formate buffer (pH 4.5). For preparation of riboflavin stock, 10 mg was weighed into a 500-ml volumetric flask

and dissolved to the marked volume with 0.05 M ammonium formate buffer. A working external standard mixture was prepared by transferring the aliquots of each stock solution into a 100-ml volumetric flask and diluting to the volume with 0.05 M ammonium formate buffer to produce a standard solution with a final concentration of 1.0 mg/L. Further serial dilutions were then made to prepare standard solutions with a concentration of 0.5, 0.2, 0.1, 0.05, 0.025, and 0.01 mg/L.

Internal standard stock solutions were prepared by dissolving separately 1 mg of each isotope-labeled vitamer in 5 mL 0.05 M ammonium formate (pH 4.5) buffer. Internal standard solution (20 mg/L) was prepared by combining aliquots of the stock solutions and stored at +4 °C for up to 1 month.

Concentration of each vitamin in the sample was calculated using seven-point calibration curves. Calibration curve standards were prepared by adding 980-μL aliquots of external standards' mixture with varying concentrations (1.0, 0.5, 0.2, 0.1, 0.05, 0.025, and 0.01 mg/L) into the amber LC-MS vials containing 20 μL of internal standard solution (20 mg/L). Thus, the final concentration of each isotope-labeled internal standard in the vials was 0.4 mg/L. The calibration curves were composed by relating the varying concentrations of unlabeled external standards to their relative response factors as determined by the ratio of the peak intensity of the unlabeled external standards to that of the corresponding labeled internal standards.

Sample preparation

Fine ground samples (0.375 g) of nutritional yeast powder were weighed into 25-mL volumetric flasks, 0.5 mL of internal standard solution (containing 20 mg/L of $[^{13}C_3]$-thiamine HCl, $[^{13}C_4,^{15}N_2]$-riboflavin, $[D_4]$-nicotinamide, $[D_4]$-nicotinic acid, $[^{13}C_3{}^{15}N]$-Ca pantothenate, $[^{13}C_4]$-pyridoxine HCl, and $[D_3]$-pyridoxal HCl) was added, filled to the volume with 0.05 M ammonium formate buffer (pH 4.5), mixed thoroughly and transferred to 50-mL centrifuge tubes. To study the effect of enzymatic treatment on the extraction and liberation of B complex vitamers, one of the following enzyme preparations or the mixture of the enzymes was added to the sample before incubation: α-amylase (0.5–4.5 mg/mL), β-glucosidase (0.2–1.6 mg/mL), acid phosphatase (0.04–0.2 mg/mL), papain (1.0–10.0 mg/mL), and takadiastase (1.0–10.0 mg/mL). The mixture of enzymes contained α-amylase (4.5 mg/mL), β-glucosidase (1.2 mg/mL), acid phosphatase (0.12 mg/mL), and takadiastase (5.0 mg/mL). The concentration of enzyme preparations in the mixture was chosen based on previously published works by Ndaw et al. [14] and Jakobsen [15]. Incubation was followed at 37 °C under subdued lighting conditions for

16 h using continual shaking, followed by the analysis of sample extracts as described below.

For acid extraction, 0.375 g of sample was weighed into a 25-mL volumetric flask, 0.5 mL of internal standard solution was added, filled to the volume with 0.1 M HCl, 0.1 M formic acid, or 0.3 M formic acid, mixed thoroughly, and transferred into a 100-mL flask. Hydrolysis was carried out in the autoclave at 121 °C for 30 min as described in Ndaw et al. [14], followed by the analysis of sample extracts as described below.

The reference sample SRM 1849 (0.5 g) was weighed into 100-mL volumetric flasks, 2 mL of internal standard solution was added, filled to the volume with 0.05 M ammonium formate buffer (pH 4.5), mixed thoroughly, and analyzed as described below.

Concentration of vitamins or the interfering compounds in the enzymes was analyzed as follows: 112.5 mg of α-amylase, 40 mg of β-glucosidase, 5 mg of acid phosphatase, 250 mg of papain, and 250 mg of takadiastase were weighed separately into 25-mL volumetric flasks, 0.5 mL of internal standard solution was added, filled to the volume with 0.05 M ammonium formate buffer (pH 4.5), mixed thoroughly, incubated, and analyzed as described below. The compounds which interfere with internal standards were determined by incubating enzymes without internal standard solution.

Two milliliters of each sample extract was centrifuged (14,000 rpm, 5 min at room temperature) to remove insoluble matter and filtered through a Millex-LG 13 mm Philic PTFE 0.2 μm filter (Millipore, Ireland). One milliliter aliquots of this filtrate were then transferred to the amber LC/MS vials and injected into the LC/MS-TOF.

Determination of enzyme activities and efficiency of acid hydrolysis

The phosphatase, pyrophosphatase, purine nucleosidase, and pantetheine hydrolase activities of the used enzymes were determined by preparing 25-μM solutions of the following vitamer precursors: thiamine mono- and diphosphate (TMP and TDP), flavin mononucleotide, and flavin adenine dinucleotide (FMN and FAD), NAD^+ and reduced nicotinamide adenine dinucleotide (NADH) and Coenzyme A (CoA) in 0.05 M ammonium formate buffer (pH 4.5). Each solution was incubated with 25 ml of acid phosphatase (0.12 mg/mL), β-glucosidase (1.2 mg/mL), α-amylase (4.5 mg/mL), takadiastase (5.0 mg/mL), and papain (5.0 mg/mL) for 18 h at 37 °C, followed by determination of liberated thiamine, riboflavin, nicotinamide, and pantothenic acid.

Protease activity was determined using a protease assay kit (Calbiochem, USA): 100 μL of the single enzyme preparation solution was combined with 50 μL of fluorescein

thiocarbamoyl-casein (FTC-casein) and 50 µL of incubation buffer (200 mM Tris–HCl, pH 7.8, 20 mM CaCl$_2$, 0.1 % NaN$_3$). Concentration of the enzyme solutions was as follows: α-amylase 0.225 mg/mL, β-glucosidase 1.2 mg/mL, acid phosphatase 0.12 mg/mL, papain 0.25 mg/mL, and takadiastase 5.0 mg/mL. Incubation was carried out at 37 °C for 24 h under subdued lighting conditions using continuous shaking. The reaction was stopped by the addition of 500 µL of 5 % trichloroacetic acid. Denatured FTC-casein was separated from the reaction mixture by centrifugation (12,000 rpm for 5 min at room temperature), and the concentration of FTC-peptides in the supernatant was determined by absorbance at 492 nm. Protease activity was expressed relative to that of trypsin in N-benzoyl-L-arginine ethyl ester (BAEE) units.

To study the effect of acid hydrolysis, 25 mL of TMP (25 µM), FMN (25 µM), TDP (25 µM), FAD (25 µM), NAD$^+$ (25 µM), or NADH (25 µM) in 0.1 M HCl were treated in autoclave at 121 °C for 30 min, followed by the determination of thiamine, riboflavin, and nicotinamide.

LC/MS-TOF analysis

All analyses were performed using an ACQUITY UPLC® system coupled with LCT Premier™ XE ESI TOF MS (Waters, Milford, MA, USA). For LC separation, an ACQUITY UPLC HSS C-18 1.8-µm (2.1×150 mm) column was used. Elution was carried out using two eluents (A: water+ 0.1 % formic acid and B: acetonitrile+0.1 % formic acid) applied in the following gradient mode: 0–3 min 100 % A; 3–8.5 min 80 % A and 20 % B; 8.5–10 min: 5 % A and 95 % B; 10–15 min 100 % A. A flow rate of 0.25 mL/min, an autosampler temperature of 4 °C, a column temperature of 25 °C, and a sample injection of 5 µL were used. Mass spectrometry analysis was carried out in a positive electrospray ionization mode [M+H]$^+$ using a capillary voltage of 2.0 kV, a sample cone voltage of 30 V, a desolvation temperature of 300 °C, and a source temperature of 120 °C. The following ions were detected (m/z): thiamine—[M+H]$^+$=265.11, labeled thiamine—[M+H]$^+$=268.12, riboflavin—[M+H]$^+$=377.15, labeled riboflavin—[M+H]$^+$=383.16, nicotinamide—[M+H]$^+$=123.06, labeled nicotinamide—[M+H]$^+$=127.08, nicotinic acid—[M+H]$^+$=124.04, labeled nicotinic acid—[M+H]$^+$=128.06, pantothenic acid—[M+H]$^+$=220.12, labeled pantothenic acid—[M+H]$^+$=224.13, pyridoxal—[M+H]$^+$=168.07, labeled pyridoxal—[M+H]$^+$=171.09, pyridoxine—[M+H]$^+$=170.08, labeled pyridoxine—[M+H]$^+$=174.10. Full scan mass spectra were acquired in the range of 100 to 500 m/z. Data were collected and reprocessed using MassLynx 4.0 software (Waters, Milford, MA, USA).

Method validation

The detection and quantification limits (LOD and LOQ, respectively) as well as the precision and accuracy of the method were determined by using a nutritional yeast powder extracted only with 0.05 M ammonium formate buffer (pH 4.5). The LODs were determined in six replicates as the amount of vitamer which produced a three times higher signal than the noise of the MS chromatogram baseline of a sample (S/N ratio=3), after estimating the original amount in a given matrix [1]. The LOQs were set at three times of the LODs of the respective vitamer.

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was carried out by performing three repeated analysis of the sample on the same day, and the intermediate precision of the method was assessed by the analyses of the sample which were carried out on six different days over a period of 1 month under the same experimental conditions.

Accuracy was evaluated by determining the recovery of the method by spiking the sample with a known amount of vitamers at three different concentration levels. The recovery was calculated as follows:

$$R, \% = 100 \left(C_{\text{spiked}} / \left(C_{\text{unspiked}} + C_{\text{added}} \right) \right)$$

where C_{spiked} is the amount of vitamer determined after spiking the sample, C_{unspiked} is the amount of vitamer in the sample before spiking, and C_{added} is the amount of vitamer added to the sample. Shortly, a 25-mL aliquot of vitamers' standard solution at three different concentrations (0.1, 0.25, and 0.5 mg/L) containing approximately 0.4 mg/L of each internal standard was added to 0.375 g of sample (n=3) and subjected to the sample treatment procedure as described above.

The performance of the used method was assessed by analyzing the standard reference material (SRM 1849). This reference material was selected because it was the only commercial reference material that contained most of the vitamers determined in this study.

Results and discussion

Fine separation of the peaks of all studied vitamers was achieved in an HSS C-18 column using a gradient system (Fig. 1). The retention times of isotope-labeled vitamins coincided with those of natural abundance, except for nicotinic acid and nicotinamide, the isotope-labeled internal standards of which had a shorter retention time (3.10 instead of 3.17 and 3.55 instead of 3.68, respectively; not illustrated). This was most likely caused by the deuterium label, which changed the compounds less lipophilic than their unlabeled forms and reduced their retention time on a

Fig. 1 MS chromatograms of unlabeled (^{12}C and ^{1}H) and labeled (^{13}C or D) B complex vitamins (*left*) and the respective MS spectra (*right*)
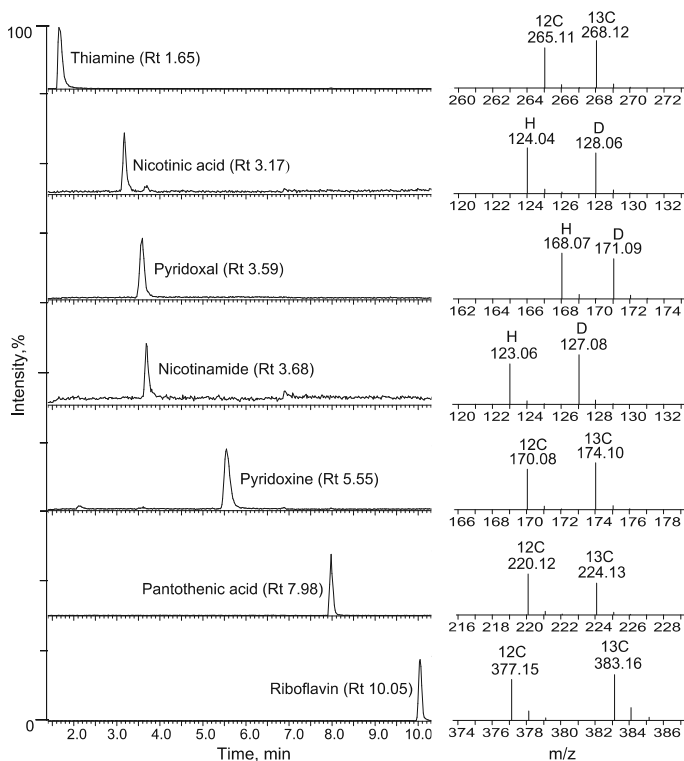
reversed phase chromatography system [16, 17]. This difference of the retention times was taken into account when choosing the retention time windows for the quantification of nicotinic acid and nicotinamide by MS-TOF. The vitamer standards remained stable during 18 h incubation at 37 °C in 0.05 M ammonium formate buffer (pH=4.5).

Responses for all the vitamins were linear over the working range (0.01–0.9 mg/L) with correlation coefficients ranging between 0.9995 and 0.9999. Prior to comparison of different extraction procedures, the used method was validated by using the sample extracted only with ammonium formate. The method validation parameters LOD (micrograms per liter) and LOQ (micrograms per liter), recovery (percentage), and intra-day and inter-day precision values of the vitamers determined in this study are shown in Table 1.

The performance of the used method was assessed by analyzing the standard reference material (SRM 1849) which contained most of the vitamers determined in this study. Our results correlated well with the certified data (Table 2).

Effect of different extraction methods

The concentration of seven B complex vitamers in the yeast sample, extracted with ammonium formate and the enzyme mixture, is given in Table 3. In addition, hot acid extraction was conducted using hydrochloric acid (0.1 M) and formic acid (0.1 and 0.3 M). The effect of formic acid extraction was studied additionally as a convenient alternative for hydrochloric acid for MS-TOF analysis.

Hydrochloric acid extraction at 121 °C in the autoclave had no effect on the concentration of thiamine and riboflavin, while the concentration of nicotinamide and nicotinic acid increased by about 20 and 25 %, respectively, and that of pyridoxal nearly three times (Table 3). In contrast, the concentration of pantothenic acid decreased 25 times. Using formic acid extraction, the tendency was almost the same, except for the higher recovery of pantothenic acid and an adverse effect on the thiamine concentration, which decreased about 30 %.

Using pure standard compounds, the recovery of thiamine from TMP and TDP, and that of riboflavin from FMN and FAD was less than 10 % during hydrochloric acid extraction. This explains why similar thiamine and riboflavin concentrations were observed with and without the acid extraction. On the contrary, extraction in 0.05 M ammonium formate in the presence of a mixture of α-amylase, β-glucosidase, acid phosphatase, and takadiastase increased the values of thiamine and riboflavin concentration

Table 1 Method validation parameters

Vitamer	LOD (μg/g) (n=6)	LOQ (μg/g) (n=6)	Recovery (%) (0.5 mg/L) (n=3)	Recovery (%) (0.25 mg/L) (n=3)	Recovery (%) (0.1 mg/L) (n=3)	Intra-day precision[a] (%) (n=3)	Inter-day precision[a] (%) (n=6)
Thiamine	0.11	0.34	105±1	95±7	97±6	2.1	5.8
Riboflavin	0.04	0.12	104±1	113±4	110±4	3.5	4.9
Nicotinamide	0.14	0.43	96±2	106±5	104±7	1.7	6.4
Nicotinic acid	0.17	0.51	95±4	100±7	96±3	1.3	3.8
Pantothenic acid	0.08	0.24	93±8	98±2	103±2	2.9	6.6
Pyridoxal	0.05	0.14	104±2	90±6	95±2	6.5	5.5
Pyridoxine	0.03	0.09	97±1	105±7	102±6	2.1	1.2

[a] Expressed as coefficient of variance

significantly (Table 3). The reasons for this increase are discussed in next sections.

Besides the free forms, nicotinamide and nicotinic acid occur naturally in the composition of pyridine coenzymes NAD^+, nicotinamide adenine dinucleotide phosphate, and their reduced analogues, but also in the form of nicotinic acid adenine dinucleotide [18] and nicotinic acid adenine dinucleotide phosphate [19]. To assess the effect of acid extraction in nicotinamide and nicotinic acid liberation, the pure compounds NAD^+ and NADH were used as substrates. Extraction with 0.1 M HCl resulted in approximately 90 % of NAD^+ being hydrolyzed to nicotinamide which can explain the 20–40 % increase in nicotinamide concentration, observed with the acid extraction (Table 3). Notably, the recovery of nicotinamide during acid hydrolysis of NADH was not observed. According to Lin et al. [20], up to 40 % of nicotinamide in yeast is in the form of NADH. This suggests that the concentration of nicotinamide, determined in our work by acid extraction, could still be an underestimate. On the other hand, the reduced coenzymes NADH and NADPH are acid-labile and are not thought to be bioavailable as they tend to break down in the gastric juice [21].

Table 2 Vitamer concentrations in the SRM 1849

Vitamin, mg/kg	Our results[a] (n=30)	Certified concentration
Thiamine Cl HCl	15.5±0.3	15.8±1.3
Riboflavin	17.2±0.3	17.4±1.0
Nicotinamide	99.7±1.9	97.5±2.3
Pantothenic acid	63.9±0.9	64.8±2.2
Pyridoxine HCl	14.2±0.2	14.2±1.5

[a] Results are expressed as a mass fraction for the material as received and as the mean of at least 30 measurements on five different days during 1 month. The uncertainties are expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U=k×u_c$, where u_c is uncertainty of the measurement results and k is a coverage factor

The most common chemical forms of vitamin B_6 in biological systems are the 5′-phosphate esters of pyridoxine, pyridoxal, and pyridoxamine. In this study, pyridoxamine could not be adequately determined due to its very low retention under the chromatographic conditions used. In the case of pyridoxine, all extraction methods tested provided similar results. On the contrary, liberation of pyridoxal with acid extraction was shown to be much more efficient than enzymatic pretreatment. In living organisms, pyridoxal phosphate is an active cofactor and is involved in various reactions with amino acids, including transamination reactions where the aldehyde group of pyridoxal phosphate forms a Schiff-base linkage with the ε-amino group of the aminotransferase enzyme [22]. At high temperatures, pyridoxal or pyridoxal phosphate may yield the formation of such imines in the presence of amino acids [23]. Under acidic conditions, imines become hydrolyzed [24]. Thus, it may be possible that the heat inactivation and high drying temperatures (150 °C) applied during the production of inactive dry yeast powder may have caused the formation of the respective imines, which could not be hydrolyzed by the enzymes but were hydrolyzed at lower pH values during acid extraction. It is not known whether such imines would be hydrolyzed in the human gastrointestinal tract, and thus, their contribution to total vitamin B_6 content in food remains to be elucidated.

While a significant decrease in pantothenic acid concentration was observed with acid treatment, the effect of enzyme treatment was rather small. The significant decrease in the concentration of pantothenic acid can be explained by its hydrolysis to β-alanine and pantoic acid under acidic conditions [25]. This was confirmed in our study by the low recovery observed after treatment of pantothenic acid standard with 0.1 M HCl.

Effect of individual enzymes on liberation of B complex vitamers

We evaluated the effect of different commercial enzyme preparations on conversion of natural B complex vitamins

Table 3 The effect of extraction with acids and with the enzyme mixture (α-amylase 4.5 mg/mL, β-glucosidase 1.2 mg/mL, acid phosphatase 0.12 mg/mL, and takadiastase 5.0 mg/mL) on the liberation of B complex vitamins in yeast into the seven vitamers quantified in this work

Vitamer	No treatment	0.1 M HCl	0.1 M formic acid	0.3 M formic acid	Enzyme mixture
Thiamine	34±2	36±1	23±1	24±1	104±6
Riboflavin	12±0	13±1	12±0	12±1	75±1
Nicotinamide	130±2	155±6	186±8	181±6	136±6
Nicotinic acid	111±1	141±2	133±8	131±6	142±1
Pantothenic acid	140±4	6±0	70±2	54±3	153±5
Pyridoxal	2.00±0.26	8.48±0.33	7.69±0.34	8.64±0.39	2.41±0.12
Pyridoxine	1.23±0.03	1.03±0.06	1.19±0.03	1.21±0.08	1.47±0.05

Results are expressed as micrograms per gram of sample dry weight [means±SD ($n=2$–5)]. The concentration of vitamers in enzyme mixture was taken into account in the calculations

into the seven vitamers quantified in this work. Each enzyme preparation was tested individually over a range of concentrations in the extraction medium with the results presented in Table 4.

The concentration of thiamine increased in an almost linear fashion with increasing dosages of the two diastatic enzyme preparations (α-amylase and takadiastase) without signs of cessation even at very high concentrations (4.5 and 10 mg/mL in the case of α-amylase and takadiastase, respectively). Similarly, the concentration of riboflavin behaved in enzyme concentration-dependent fashion during α-amylase treatment. In the case of β-glucosidase, acid phosphatase, and papain treatment, the maximum observed thiamine and riboflavin concentrations remained remarkably lower than with the diastatic enzyme preparations −48±1 and 41±3 mg/g dry weight, respectively.

This was surprising because the recovery of thiamine from TMP and TDP was almost 100 % using the extraction media which contained either β-glucosidase, acid phosphatase, or α-amylase. Also, FMN and FAD were converted into riboflavin to the extent of 100 % by these three enzyme preparations. For the efficient liberation of thiamine and riboflavin from the corresponding cofactors under enzymatic extraction conditions, the combined effect of two specific enzymatic activities is required: (1) acid phosphatase (phosphate-monoester phosphohydrolase, EC 3.1.3.2) is required to release thiamine from TMP and riboflavin from FMN and (2) organic pyrophosphatase (ectonucleotide pyrophosphatase, EC 3.6.1.9) is required to release FMN from FAD or TMP from TDP and TTP (thiamine triphosphate). Our results indicate that both enzymatic activities were sufficiently present in β-glucosidase (1.2 mg/mL), acid phosphatase (0.12 mg/mL), and α-amylase (4.5 mg/mL), while in the case of takadiastase (5.0 mg/mL), the recoveries were somewhat lower (68 and 49 % for thiamine and riboflavin, respectively). Almost no ectonucleotide pyrophosphatase+ phosphate-monoester phosphohydrolase activity was determined in papain.

Despite the high enzymatic potential of β-glucosidase and acid phosphatase to liberate chemically bound forms of thiamine and riboflavin, the concentrations measured were remarkably higher when the diastatic enzyme preparations α-amylase and takadiastase were used. Both enzyme preparations have previously been shown to possess proteolytic side activity [14], an effect confirmed also in this study. Thus, protease activity in the enzyme preparations used (expressed as BAEE units of trypsin) was determined as follows: papain 3304 U/mg, α-amylase 2804 U/mg, acid phosphatase 50 U/mg, takadiastase 5.2 U/mg, and β-glucosidase 0.12 U/mg. While the amylolytic activity is unlikely to be necessary for liberation of vitamins from the yeast matrix, it is not clear whether the proteolytic side activity of α-amylase and takadiastase really supported the liberation of thiamine and riboflavin from the yeast matrix or was the observed increase in the vitamin concentration an artifact of the detection method. To distinguish between these two possibilities, we studied the interfering background in the LC/MS spectra, caused by α-amylase and takadiastase. The results suggest that the extensive increase of thiamine and riboflavin peaks in the mass spectra, observed with the treatment of the two diastatic enzymes, was likely caused by protease activity which released di- and tripeptides with a similar retention time and m/z ratio (see next section).

The concentrations of pyridoxine and pyridoxal increased only to the extent of 20–35 % with the addition of most enzyme preparations, including the ones with high acid phosphatase+pyrophosphatase activity. The lower efficiency of enzymatic liberation, compared to that of acid extraction, can be explained by the resistance of imines to enzymatic hydrolysis, as described in the previous section.

A small effect of enzyme treatment was also observed for the liberation of nicotinamide and nicotinic acid, the concentration of which increased about 10–20 %. More specifically, the concentration of nicotinic acid increased up to 30 % when the sample was treated with β-glucosidase or an

Table 4 Effect of enzymatic pretreatment of yeast to convert B complex vitamins into the seven vitamers quantified in this work

Vitamers						
	Acid phosphatase					
	0 mg/mL	0.04 mg/mL	0.08 mg/mL	0.12 mg/mL	0.16 mg/mL	0.20 mg/mL
Thiamine	34±2	42±1	43±1	47±2	46±1	48±2
Riboflavin	12±0	40±3	39±1	40±1	40±3	38±2
Nicotinamide	130±2	156±12	149±6	150±11	139±1	155±11
Nicotinic acid	111±1	127±3	114±0	117±3	115±7	110±4
Pantothenic acid	140±4	134±11	136±5	130±7	128±11	130±0
Pyridoxal	2.00±0.26	3.18±0.22	2.87±0.00	3.07±0.17	2.80±0.11	2.94±0.11
Pyridoxine	1.23±0.03	1.36±0.10	1.35±0.02	1.33±0.01	1.42±0.08	1.42±0.08
	β-glucosidase					
	0 mg/mL	0.2 mg/mL	0.4 mg/mL	0.8 mg/mL	1.2 mg/mL	1.6 mg/mL
Thiamine	34±2	42±1	42±1	42±2	50±1	50±2
Riboflavin	12±0	24±1	38±1	38±2	38±2	38±2
Nicotinamide	130±2	144±5	149±8	142±4	150±1	134±1
Nicotinic acid	111±1	121±6	121±8	126±1	146±3	141±14
Pantothenic acid	140±4	129±4	132±12	135±7	142±8	152±7
Pyridoxal	2.00±0.26	2.49±0.06	2.75±0.09	2.54±0.17	2.51±0.32	2.80±0.24
Pyridoxine	1.23±0.03	1.21±0.04	1.18±0.17	1.17±0.05	1.41±0.12	1.26±0.07
	α-amylase					
	0 mg/mL	0.5 mg/mL	1.0 mg/mL	1.5 mg/mL	3.0 mg/mL	4.5 mg/mL
Thiamine	34±2	50±2	51±2	66±1	67±0	79±1
Riboflavin	12±0	32±2	48±3	56±5	64±3	64±4
Nicotinamide	130±2	152±2	145±7	145±4	118±4	147±3
Nicotinic acid	111±1	114±2	103±3	98±1	87±4	101±4
Pantothenic acid	140±4	149±7	155±8	157±1	147±2	158±9
Pyridoxal	2.00±0.26	2.06±0.58	1.97±0.09	2.13±0.43	2.36±0.01	2.58±0.45
Pyridoxine	1.23±0.03	1.16±0.05	1.34±0.10	1.30±0.06	1.16±0.00	1.45±0.06
	Takadiastase					
	0 mg/mL	1.0 mg/mL	2.5 mg/mL	5.0 mg/mL	7.5 mg/mL	10.0 mg/mL
Thiamine	34±2	43±0	45±3	62±1	67±2	75±5
Riboflavin	12±0	33±2	41±0	42±1	39±1	41±1
Nicotinamide	130±2	171±22	156±3	173±1	156±5	157±9
Nicotinic acid	111±1	115±6	105±0	112±8	98±1	101±7
Pantothenic acid	140±4	141±8	150±0	150±8	145±2	143±5
Pyridoxal	2.00±0.26	2.69±0.12	2.98±0.29	3.32±0.14	3.12±0.14	2.82±0.08
Pyridoxine	1.23±0.03	1.27±0.05	1.49±0.04	1.59±0.07	1.58±0.01	1.52±0.06
	Papain					
	0 mg/mL	1.0 mg/mL	2.5 mg/mL	5.0 mg/mL	7.5 mg/mL	10.0 mg/mL
Thiamine	34±2	42±1	45±0	45±0	46±1	47±1
Riboflavin	12±0	20±1	25±0	44±0	45±6	42±1
Nicotinamide	130±2	124±6	147±5	169±2	167±9	178±20
Nicotinic acid	111±1	105±7	93±2	101±0	93±5	104±17
Pantothenic acid	140±4	155±2	154±4	147±6	141±3	143±9
Pyridoxal	2.00±0.26	0.84±0.03	0.90±0.04	1.22±0.03	1.20±0.01	1.08±0.24
Pyridoxine	1.23±0.03	0.38±0.04	0.42±0.07	0.54±0.01	0.68±0.03	0.70±0.02

Results are expressed as micrograms per gram of sample dry weight [means±SD ($n=2–5$)]. The concentration of vitamers in the enzyme preparations was taken into account in the calculations

enzyme preparation mixture containing β-glucosidase, while the individual effect of other enzyme preparations remained largely ineffective. Experiments with pure NAD$^+$ showed that in the case of all enzyme preparations, the nicotinamide recovery remained below 10 %, which can be explained by the absence of purine nucleosidase activities in the used enzyme preparations. This was also strongly supported by the increased intensities of N-ribosyl nicotinamide peaks on the mass spectra of β-glucosidase, takadiastase and, in particular, α-amylase-treated NAD$^+$ (not illustrated). Interestingly, instead of N-ribosyl nicotinamide, N-ribosyl nicotinic acid was observed on the mass spectra of acid phosphatase-treated NAD$^+$ (not illustrated). This can be explained by potato nicotinamide riboside deaminase activity, which catalyses the conversion of N-ribosyl nicotinamide into N-ribosyl nicotinic acid [26].

The effect of enzyme treatment on the concentration of pantothenic acid was negligible and remained in the range of statistical experimental error (Table 4). CoA is the principal biologically active form of pantothenic acid which can be hydrolyzed to pantothenic acid by the combined action of ectonucleotide pyrophosphatase, phosphate-monoester phosphohydrolase, and pantetheine hydrolase (EC 3.5.1.92). Our experiments with pure CoA showed that the activities of first two enzymes were sufficiently high in the enzyme preparations used, except for papain and β-glucosidase. Pantetheine hydrolase activity was most likely absent in all enzyme preparations, as was suggested by the increased intensities of pantetheine lines on the mass spectra and the absence of free pantothenic acid in CoA hydrolysates (not illustrated). Consequently, for quantification of pantothenic acid, the pantetheine concentration should be measured (or alternatively CoA in an enzymatically untreated sample).

Limitations of LC/MS-TOF quantification of B complex vitamins

In spite of several advantages that LC/MS-TOF quantification offers, there are also limitations which have to be considered when using this technique. One of the greatest challenges with the quantification of B complex vitamins using LC/MS-TOF is the interference by other compounds in the sample extract. Thus, overlap of the MS-TOF spectra of a vitamin or its isotope-labeled internal standard with any other compound of similar m/z value may easily result in over- or underestimation of the vitamin concentration in the sample. Our study revealed that most of the used enzyme preparations contain B complex vitamins or compounds with similar retention time and m/z value. For example, search in the METLIN Metabolite Database [27] for compounds with similar masses revealed various di- and tripeptides, which could interfere with the mass spectra of thiamine, riboflavin, and pantothenic acid (Table 5).

Table 5 Compounds interfering with the quantification of B complex vitamins in the enzymes (micrograms per gram of enzyme, means±SD (n=3), expressed as concentration of the respective vitamin)

Enzymes and interfering compounds	Thiamine [M+H$^+$]=265.11	Riboflavin [M+H$^+$]=377.14	Nicotinic acid [M+H$^+$]=124.04	Pantothenic acid [M+H$^+$]=220.12	Pyridoxal [M+H$^+$]=168.06	Pyridoxine [M+H$^+$]=170.08
α-amylase	–	43.9±3.9	2.7±0.1	–	–	–
β-glucosidase	–	43.7±1.7	–	–	–	–
Papain	–	17.6±6.6	5.4±0.1	2.6±0.2	–	–
Takadiastase	0.5±0.0	4.9±0.2	1.0±0.1	2.3±0.1	0.6±0.0	1.7±0.1
Peptides interfering with vitamin[a]	–	Ala Thr Trp, Pro Phe Asn [M+H$^+$]=377.17	–	–	–	–
Peptide isotopomers interfering with vitamin[a]	Thr Gly Ser, Ser Ser Ala [M+H$^+$]+1=265.11	–	–	Ile Ser, Leu Ser, Thr Val [M+H$^+$]+1=220.13	–	–
Peptide isotopomers interfering with internal standard[a]	Ser Gly Cys [M+H$^+$]+2=266.13	–	–	–	–	–

[a] Peptide sequences are not defined

Also, interfering isotopomers of di- and tripeptides were found in the enzyme preparations. Thus, papain, takadiastase and, in particular α-amylase, contained compound(s) with a mass $[M+H^+]=264.12$, corresponding to nine-carbon tripeptides consisting of either Thr, Gly, Ser or Ser, Ser, Ala. The MS peaks of the ^{13}C isotopomers ($[M+H^+]+1$) of these unknown compounds overlapped with the thiamine peak ($[M+H^+]=265.11$) on the mass spectra, thus increasing its intensity. According to isotope distribution calculations (http://www.sisweb.com/mstools/isotope.htm), the intensities of ^{13}C ($[M+H^+]+1$) isotopomers of nine-carbon compounds would constitute 11 % of that of ^{12}C ($[M+H^+]$) peak of the respective compound. The calculated ratio of $[M+H^+]=265.11$ peak intensity to that of $[M+H^+]=264.12$ was approximately 10 % in papain and α-amylase and 40 % in takadiastase, suggesting that thiamine was absent in papain and α-amylase.

Similarly, an interfering compound with a mass of $[M+H^+]=219.13$ was determined in takadiastase, α-amylase, and papain (Table 5). The peak of ^{13}C isotopomer ($[M+H^+]+1$) of this compound overlapped with that of pantothenic acid ($[M+H^+]=220.12$). Search for the compounds with a similar mass provided the closest match with six candidate molecules, all nine-carbon dipeptides, consist of either Ile, Ser or Leu, Ser or Thr, Val. The ratio of $[M+H^+]+1=220.12$ peak intensity to that of $[M+H^+]=219.13$ was 25 % in papain, 18 % in takadiastase, and 10 % in α-amylase, suggesting that pantothenic acid was absent in α-amylase.

In addition to the impurities in the enzyme preparations, the formation of assay-interfering compounds, occurring with incubation with crude enzyme preparations, can cause false quantification of B complex vitamins in biological samples. A good example is the observed extensive increase in the concentration of thiamine and riboflavin when incubated with diastatic enzymes (Table 4). Fungi express a wide variety of proteolytic enzymes, including dipeptidyl and tripeptidyl peptidases [28]. These enzyme preparations could be responsible for the formation of small MW peptides, in particular di- and tripeptides. These low MW peptides may have similar masses to those of B complex vitamins or their isotope-labeled counterparts and thus, interfere directly with the quantification even if the exact mass MS-TOF spectrometer with a resolving power of 10,000 full width at half maximum is used. Using enzyme preparations that are free of proteolytic side activity, protease inhibitors, or hydrochloric acid for extraction and liberation of vitamers the formation of peptides can be avoided. The effect of proteolytic treatment during vitamin extraction from different food matrices needs a separate study.

In conclusion, LC/MS-TOF with stable isotope dilution assay can be used without a pre-concentration step for simultaneous determination of B complex vitamins in yeast. However, several isotope-labeled vitamers (e.g., pantetheine, N-ribosyl nicotinamide, and N-ribosyl nicotinic acid) should be additionally synthesized for complete determination of B complex vitamin activities in foods. Another challenge of this approach is the selection of enzyme preparations with sufficient and highly specific activities for the conversion of natural vitamers to analytically suitable forms while minimizing side activities that cause the formation of compounds that interfere with the MS-TOF analysis.

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PUBLICATION II

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Method and Analytical Kit for Simultaneous Quantification of B Complex Vitamin Content in Food

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(54) Title: METHOD AND ANALYTICAL KIT FOR SIMULTANEOUS QUANTIFICATION OF B-COMPLEX VITAMIN
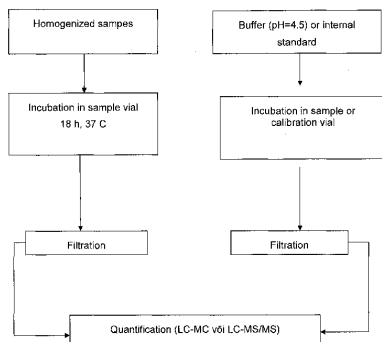CONTENT IN FOOD

(57) **Abstract**: The present invention relates to an analytical vitamin
analysis kit, the method of its preparation, and the method of its ap-
plication. The given analytical kit can be used for the simultaneous de-
termination of the quantity of five B-complex vitamins (B 1, B2, B3,
B5 and B6) and individual vitamers in drugs, food, food- and bio-sup-
plements, feed, and other biological samples using LC-MS isotope in-
ternal standard assay. The analytical kit is based on the use of sample
vials containing lyophilized or dried isotope labelled internal standards
of the B-complex vitamers with a mix of hydrolytic enzymes, used to
convert vitamers and cofactors into the same chemical forms as the
used isotope labelled internal standards, and for their following quanti-
fication by means of LC-MS or LC-MS/MS.

FIG 1

WO 2013/010553 A1

METHOD AND ANALYTICAL KIT FOR SIMULTANEOUS QUANTIFICATION OF B-COMPLEX VITAMIN CONTENT IN FOOD

5 **Field of the invention**

The present invention concerns the method and the respective analytical kit for quantitative nutritional composition analysis, and more specifically, for the simultaneous determination of the content of B-complex vitamins B1, B2, B3, B5 and B6 in food, food- and bio-supplements and feed.

10 **Background of the invention**

Simultaneous determination of B-complex vitamins in food and other biological samples is challenging due to their low concentration in the sample, relatively low stability as well as diverse chemical structure and physical properties of the individual vitamers. Liquid chromatography combined with mass or tandem mass spectrometry (LC-MS or LC-MS/MS)

15 are widely used analysis techniques for the determination of water soluble vitamins. The vitamins are extracted from the sample along with other low molecular weight compounds and separated by LC according to their physico-chemical properties, followed by detection with MS or MS/MS using the mass to charge ratios of ionized vitamers. Each vitamer is quantified using its respective MS peak intensity.

20 Leporati *et al.* (2005) discloses the use of LC-MS/MS to determine the simplest vitamers of six B-complex vitamins (thiamine, riboflavin, pyridoxine, nicotinamide, nicotinic acid, pantothenic acid, and folic acid) in pasta products by using various extraction methods: hydrochoric acid extraction for thiamine, riboflavin, pyridoxine, nicotinamide, nicotinic acid, and acetic acid extraction for pantothenic acid, and sodium phosphate-sodium citrate/ascorbate

25 extraction (pH 8) for folic acid.

Quantitative mass spectrometry is complicated by matrix-associated ionization effects caused by compounds that co-elute from the LC at the same retention time as the compound under study and may significantly affect the intensity of its MS peaks. To compensate the matrix-associated ionization effects, stable isotope labelled (SIL) vitamers can be used as internal

30 standards due to their nearly identical chemical and physical properties. Thus, SIL internal

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standards can be added to the sample and the concentration of the vitamer under study can be calculated based on the ratio of MS peak intensities of natural and SIL forms.

Hachey *et al.* (1985) and Midttun *et al.* (2005) disclose the quantification of vitamin B2 and B6 related vitamers (riboflavin, pyridoxal, pyridoxine, pyridoxamine, pyridoxal phosphate, pyridoxine phosphate, and pyridoxamine phosphate) by LC-MS/MS and SIL standards in liver and urine and in human blood plasma, respectively. Sample extraction was performed with trichloroacetic acid that contained a known amount of SIL internal standards of these vitamers and the concentration was calculated based on the ratio of MS peak intensities of natural and SIL forms.

The activity of each B-complex vitamin is defined as a sum of concentration of different chemical substances (vitamers), which all have a similar biological activity of that particular vitamin. For example, bioactive forms of vitamin B1 and B6 in food are their phosphorylated forms, thiamine mono-, di- and triphosphates and pyridoxal, pyridoxine and pyridoxamine phosphates, respectively. Ndaw et al. (2000) and Jakobsen (2008) used enzymatic extraction to liberate vitamers from a food matrix and to dephosphorylate them. USA patent application No. US20090093009 discloses an application which limits the use of acid phosphatase to liberate different phosphorylated forms of thiamine into free (i.e. non-phosphorylated) thiamine.

Besides the simplest chemical forms of the B-complex vitamins B1 (thiamine), B2 (riboflavin), B3 (nicotinic acid and nicotinamide), B5 (pantothenic acid) and B6 (pyridoxine and pyridoxal) and their phosphorylated and glycosylated forms there are several cofactors and associated metabolites in food which also may possess the activity of the given vitamins. In the human gastrointestinal tract (GIT) these cofactors are converted into the simplest forms of vitamers and absorbed in the small intestine. For example, potential vitamin B2 sources (riboflavin precursors) in food are flavin coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which are hydrolyzed in the small intestine by alkaline phosphatase (EC 3.1.3.1.) into riboflavin and then absorbed (Daniel *et al.* 1983).

Similarly, potential vitamin B3 (nicotinic acid and nicotinamide) sources in food are coenzymes nicotinamide adenine dinucleotides (NAD, NADH, NADP, and NADPH), which are converted to nicotinamide mononucleotide and adenosine monophosphate by NAD pyrophosphatase (EC 3.6.1.22) found in small intestine juice secretes (and also, to a small

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extent, in pancreatic juice). Nicotinamide mononucleotide is further hydrolyzed to nicotinamide riboside and then further to free nicotinamide by enzymes associated with the epithelial cells of the small intestine (Gross and Henderson, 1983).

An additional source of vitamin B5 (pantothenic acid) in food is Coenzyme A (CoA) which is
5   hydrolyzed in the small intestine to pantetheine by non-specific pyrophophatase and phosphatase reactions. Pantetheine is hydrolyzed to free pantothenic acid by pantetheinase, an enzyme secreted by the gastrointestinal mucosa, and then absorbed in the jejunum part of the small intestine (Shibata et al. 1983).

Additionally, B-complex vitamers may be glycosylated (e.g., vitamers which possess vitamin
10   B6 activity) or bound to proteins (e.g. vitamers which possess vitamin B2 activity) in food (Gregory 3rd, 1998; White III *et al.*, 1986). Protein bound vitamers dissociate in the stomach due to low pH caused by hydrochloric acid.

In order to express total B-complex vitamin content in food it is important to quantitatively determine all respective B-complex vitamers and their precursors which are relevant from a
15   nutritional point of view. The individual determination of each and every compound with B-complex vitamin activity is tedious and time-consuming. To resolve this issue the relevant vitamers could be converted into the simplest chemical forms of their respective vitamins (e.g., thiamine in case of vitamin B1, riboflavin in case of vitamin B2, nicotinamide and nicotinic acid in case of vitamin B3, pantothenic acid in case of vitamin B5 and pyridoxal or pyridoxine
20   in case of vitamin B6) using enzymes with appropriate specificity and activity. The resulting primary forms of these B-complex vitamins can be simultaneously quantified using LC-MS/(MS). The method described in the present invention, where quantitative determination of B-complex vitamins is based on the sum of molar concentrations of their respective vitamers is unique and has not been previously disclosed in the research and patent literature. This method
25   has significant advantages over conventional methods (e.g. HPLC or microbiological methods) that are currently applied for quantification of B-complex vitamins. For example, physiological differences between bacteria used in the microbiological assay and the human organism can cause over- or underestimation of some vitamin activities (Bender, 2003).

In the analysis of the nutritional value of food, the availability of user-friendly and high-
30   throughput analytical methods is becoming increasingly important. Hence, there is an obvious

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need for method and supporting analytical kit allowing one to simultaneously (i.e. in the same extraction and chromatographic run) determine the content of different B-complex vitamins.

The proposed analytical kit which supports such simultaneous quantification of B-complex vitamins contains: i) sample vials containing known quantities of lyophilized or dried isotope labelled vitamers and/or enzyme preparations, ii) calibration vials, containing, in addition to the extraction vial content, known amounts of the same unlabeled vitamers at different concentrations or alternatively iii) calibration mixture vials containing known amounts of unlabeled vitamers.

UK patent application GB1411381 discloses a method of making an assay kit comprising vials arranged for quantitative determination of a compound in a sample to be added to a vial wherein each vial contains a standard amount of a lyophilized labelled (radioactively or stable isotope labelled) version of the compound to be determined and a standard amount of a lyophilized specific reagent to react (for example to form a complex) with the compound to be determined. An assay kit may also comprise vials which are intended for use as standards, which contain, in addition to the lyophilized specific reagents and labelled standards, also a known amount of the lyophilized unlabelled version of the compound to be determined. At the same time, the amount of a specific reagent in the vial is set to be insufficient to react with all labelled and unlabelled compound. Assuming that a specific reagent reacts with the labelled and unlabelled (natural) compound in stoichiometrically equivalent amounts, it is possible to determine the concentration of unreacted labelled compound in the sample, and, in addition, calculate the concentration of unlabeled (natural) compound in the sample. This invention discloses an example which is based on immunoanalysis, wherein lyophilized reagents in the vial are antibodies which form complexes with compounds of interest.

Summary of the invention

The present invention relates to the method and analytical kit, which can be used for the simultaneous determination of B-complex vitamin (B1, B2, B3, B5 and B6) content in food, food- and bio-supplements, and feed. For simultaneous quantitative determination of the given five B-complex vitamins, LC-MS (or LC-MS/MS) with stable isotope internal standard assay kit is used. In contrast to the conventional methods (microbiological or HPLC-based methods) the present invention considerably simplifies the determination procedure, increases the accuracy of the analysis, and allows one to reduce the analysis time and labour.

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The analytical kit described in the present invention consists of one or more sample vials and one or more calibration vials. The sample vials contain a known amount of appropriate lyophilized stable isotope labelled B-complex vitamers with and without lyophilized mixtures of hydrolytic enzymes or enzyme preparations with specific activities. The calibration vials

5   contain a known amount of the same lyophilized stable isotope labelled B-complex vitamers with or without a mixture of lyophilized hydrolytic enzymes or enzyme preparations with specific activities and a known amount of the same unlabelled (natural abundance) B-complex vitamers.

Sample vials, which contain lyophilized stable isotope labelled internal standards of the B-

10   complex vitamers with hydrolytic enzymes or enzyme preparations with specific hydrolytic activities, are used to convert the B-complex vitamers and their respective cofactors into the same chemical forms as the stable isotope labelled internal standards used. Sample vials, which do not contain an enzyme mixture, are used for the quantification of vitamers, which are already in the same chemical forms as the used internal standards, for example for fortified

15   food analysis.

Calibration vials, which contain a mixture of known amounts of isotope labelled B-complex vitamers with and without enzymes, and known amounts of unlabeled (natural abundance) B-complex vitamers at different concentrations, and/or a mixture of hydrolytic enzymes or enzyme preparations with a specific activities, are used to generate a calibration curve, when

20   an extraction buffer of an equal volume to that of the sample extract is added to the calibration vials.

Alternatively, the calibration mixture that contains only unlabelled B-complex vitamers can be used for calibration. In this case, their contents are dissolved in an extraction buffer of an equal volume to that of the sample extract and added to the sample vials instead of the sample

25   extract. The content of B-complex vitamins (B1, B2, B3, B5 and B6) in the sample is determined as the sum of the molar concentration of the respective individual vitamers.

Sample vials of the present analytical kit are prepared by dispensing a fixed volume of a solution of appropriate isotope labelled B-complex vitamers of a known concentration with and without a mixture of hydrolytic enzymes or enzyme preparations with specific activities

30   under oxygen-deprived conditions (i.e., under an inert gas atmosphere) into appropriate vials and by lyophilizing the contents of the vial.

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Calibration vials of the present analytical kit are prepared by dispensing a fixed volume of a solution of the same isotope labelled B-complex vitamers and the same unlabeled B-complex vitamers at different concentrations and/or a mixture of hydrolytic enzymes or enzyme preparations with specific activities under oxygen-deprived conditions into appropriate vials and by lyophilizing the contents of the vial.

5

After capping the vials under an inert gas atmosphere, the sample and calibration vials, described above, are usable for at least one year for quantitative analysis of five B-complex vitamins (B1, B2, B3, B5 and B6) in food, food- and biosupplements, or in feed using LC-MS (or LC-MS/MS).

10   The present invention (the method and analytical kit) allows one to reduce the analysis time and labour used for sample preparation and analysis, allows one to conduct high throughput quantitative analysis of at least five B-complex vitamins (B1, B2, B3, B5 and B6) as well as folic acid and vitamin C in drugs, food, food- and bio-supplements, feed, and other biological samples.

15   The main differences between the inventions described in the "background of the invention" section are:

- the sample and calibration vials contain solid substances with a long shelf life;

- the sample vials of the present analytical kit contain isotope labelled internal standards of B-complex vitamers and/or hydrolytic enzymes or enzyme preparations, which do not chemically

20   change themselves during the analysis process and act as catalysts (in case of enzymes) by liberating B-complex vitamers contain in the sample from phosphorylated, glycolylated and protein bound complexes and by converting them into the same chemical forms as the internal standards used.

- the mixture of enzymes or enzyme preparations possesses protease and/or α-amylase and/or

25   acid phosphatase and/or acid pyrophosphatase and/or β-glucosidase activity and does not possess any activities, which may decrease B-complex vitamers activity and/or interfere with LC-MS (or LC-MS/MS) determination.

- for quantification, a homogenised sample is added into a sample vial and for calibration the same amount of extraction buffer is added to the calibration vials, or alternatively, a

30   calibration mixture containing unlabeled vitamer standards at different concentrations is added

to another sample vial, samples and calibration solutions are incubated on a shaker, filtered, and the concentration of vitamers is determined by the LC-MS isotope internal standard method;

- absolute activity of five B-complex vitamins (B1, B2, B3, B5 and B6) is calculated by summarizing the molar concentrations of the respective vitamers and recalculated into respective vitamin activity units (e.g. μmol in 1 g of sample, μg in 1 gram of sample etc).

Brief description of the drawings

The present invention is illustrated with drawings, where:

Fig. 1 shows a principal scheme of the method of the present invention;

Fig. 2 – 6 show enzymatic conversion reactions of vitamers with B1, B2, B3, B5, and B6 activities;

Fig. 7 shows an example of MS chromatograms of unlabelled (^{12}C and ^{1}H) and labelled (^{13}C or D) B-complex vitamers (left) and the respective MS spectra (right);

Fig. 8 shows the stability of isotope labelled internal standards in the sample vials over a period of one year of storage under subdued lighting conditions at -20 ^{0}C.

Detailed description of the invention

The present invention relates to the method and to an appropriate analytical kit used for the simultaneous determination of the content of five B-complex vitamins (B1, B2, B3, B5 and B6) in food, food- and bio-supplements, and feed by LC-MS (or LC-MS/MS) isotope internal standard assay. The method is comprised of the following steps:

- a fixed amount of the selected isotope labelled internal standards of B-complex vitamers with and without a mixture of hydrolytic enzymes or enzyme preparations with specific activities is dried or lyophilized into sample vials;

- in the sample vial with enzyme mixture, the vitamers and cofactors in the sample, which possess the B-complex vitamins activity, are converted into the same chemical forms as the respective stable isotope labelled internal standards during a sample incubation of between 2 – 24 h, preferably 18 h at a temperature of between 20 - 50 ^{0}C, preferably 37 ^{0}C, under acidic conditions where the pH is between 2.0 – 5.0, preferably 4.5, to prevent microbial growth;

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- in addition to the sample vials described above, calibration vials containing the same dried or lyophilized unlabelled vitamers standard mixture at different concentrations is included in the analysis kit;

- to determine the concentration of vitamers in the sample a calibration curve is generated by
5   using calibration vials. Alternatively, sample vials can be used, wherein only unlabelled (natural abundance) B-complex vitamers (meaning calibration mixture) at different concentrations are added.

- the content of B-complex vitamins is calculated as the sum of the molar concentrations of the vitamers, which possess the respective vitamin activity, and is expressed in appropriate
10   concentration units (for example μg of thiamine in 1 g of sample etc.);

- alternatively, the content of one sample vial can be used for more than one sample analysis, by adding a known amount of the vial's content, which is previously dissolved with an extraction buffer, to several samples. The contents of calibration vials can be used in the same way.

15   EXAMPLE 1: Determination of vitamins B1, B2, B3, B5 and B6 content in inactive dried yeast.

0.15 g of inactive dried yeast is weighed into a 10 ml sample vial with enzymes, 0.15 g of inactive dried yeast is weighted into a 10 ml sample vial without enzymes, 10 ml of 0.05 M ammonium formate buffer (pH 4.5) is added into the vials, the contents of the vials are mixed
20   thoroughly, and incubated with frequent shaking at 37 ^{0}C for 18 h in the dark. Following this, 2 ml from each of the incubation mixtures is centrifuged (14000 rpm/min) for 5 minutes at 10 ^{0}C and filtered (Millex-LG 13mm Philic PTFE 0.2 μm, Millipore, Carrigtwohill, Ireland). 1 ml of this filtered extract is added into an empty 2 ml LC-MS vial and injected into the LC-MS system.

25   To generate a calibration curve, 1 ml of 0.05 M ammonium formate buffer (pH 4.5) is added into each vial in the set of 2 ml calibration vials (containing different amounts of unlabelled vitamers), the contents of the vials are mixed thoroughly, and incubated with frequent shaking at 37 ^{0}C for 18 h in the dark and analysed by LC/MS. Vitamers are determined using ACQUITY UPLC® liquid chromatograph (Waters, Milford, MA, USA), which consists of an
30   autosampler and gradient pumps. The liquid chromatograph instrument is coupled with an

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LCT Premier™ XE ESI TOF MS System (Waters, Milford, MA, USA), which consists of an electrospray ionization (ESI) source, a time-of-flight (TOF) mass analyzer, and a dual microchannel plate detector assembly detector.

Vitamers are chromatographically separated using a reversed phase chromatography column
5   ACQUITY UPLC HSS C-18 1.8 μm (2.1 x 150 mm) (Waters, Milford, MA, USA) and two eluents (A: water + 0.1 % formic acid and B: acetonitrile + 0.1 % formic acid) applied in the gradient mode as follows: 0 - 3 min 100% A; 3 - 8.5 min 80% A and 20% B; 8.5 – 10 min: 5% A and 95% B; 10 – 15 min 100% A. One analysis run time is 15 minutes, with an applied flow rate of 0.25 ml/min, a sample injection of 5 μl and a column temperature of 25 ^{0}C. Mass
10   spectrometry is carried out in a positive ionization mode using a capillary voltage of 2000 V, a cone voltage of 30 V, a temperature source of 120 °C, and a desolvation temperature of 300^{0}C.

Natural vitamers and their labelled forms are identified by retention times and mass to charge ratios (m/z) (Tabel 1, Fig 7). Data are collected and processed using Mass Lynx 4.0 software (Waters, Milford, MA, USA).

15   **Table 1.** Retention times (Rt) and mass to charge ratios (m/z) of isotope labelled (^{13}C, ^{2}H and ^{15}N) and unlabeled (natural abundance) of B-complex vitamers being identified.

Vitamin	Rt, min	m/z
Thiamine	1.57	265.11
Thiamine [^{13}C$_3$]	1.61	268.12
Riboflavin	9.95	377.15
Riboflavin [^{13}C$_4$,^{15}N$_2$]	9.95	383.16
Nicotinamide	3.60	123.06
Nicotinamide [^{2}H$_4$]	3.47	127.08
Nicotinic acid	3.09	124.04
Nicotinic acid [^{2}H$_4$]	3.00	128.06
Pantothenic acid	7.82	220.12
Pantothenic acid [^{13}C$_3$, ^{15}N]	7.82	224.13

Pyridoxal	3.56	168.07
Pyridoxal [^{2}H$_3$]	3.55	171.09
Pyridoxine	5.61	170.08
Pyridoxine [^{13}C$_4$]	5.60	174.10

The concentration of each vitamin in the sample is calculated using a seven-point external calibration curve (0.05 – 3.75 µM in the case of thiamine, 0.03 - 2.48 µM in the case of riboflavin, 0.10 - 7.66 µM in the case of nicotinamide, 0.10 -7.63 µM in the case of nicotinic acid, 0.05 - 4.16 µM in the case of pantothenic acid, 0.07 - 5.68 µM in the case of pyridoxal, and 0.07 - 5.40 µM in the case of pyridoxine). The concentration of labelled internal standard is 1.13 µM in the case of thiamine, 0.53 µM in the case of riboflavin, 4.10 µM in the case of nicotinamide, 3.28 µM in the case of nicotinic acid, 0.91 µM in the case of pantothenic acid, 1.20 µM in the case of pyridoxal, and 1.18 µM in the case of pyridoxine. The calibration curves are composed by relating each concentration of unlabelled external standard to its relative response factor as determined by the ratio of the peak intensity of the respective unlabelled external standard to that of the corresponding labelled internal standard. The coefficients of linear regression are as follows: r^2 = 0.9999 (y = 0.7545x + 0.0105) for thiamine, r^2 = 0.9999 (y = 2.3417x + 0.0123) for riboflavin, r^2 = 0.9995 (y = 0.2068x + 0.0106) for nicotinamide, r^2 = 0.9998 (y = 0.3568x – 0.0082) for nicotinic acid, r^2 = 0.9999 (y = 1.1862x + 0.0047) for pantothenic acid, r^2 = 0.9993 (y = 0.9202x + 0.033) for pyridoxal, r^2 = 0.9999 (y = 1.0826x – 0.007) for pyridoxine.

The concentration of vitamers is calculated by the ratio of corresponding spectral lines in the sample and calibration vial using formula 1

$$^{12}C_{smpl} = \frac{^{12}C_{std} \times {}^{13}i_{std}}{^{12}i_{std}} \times \frac{^{12}i_{smpl}}{^{13}i_{smpl}}$$ (1), where,

$^{12}C_{smpl}$ – concentration of vitamer in sample vial

$^{12}C_{std}$ - concentration of unlabeled vitamer standard in the calibration vial

$^{12}i_{std}$ – signal intensity of unlabelled vitamer standard in the calibration vial;

$^{13}i_{std}$ – signal intensity of isotope labelled vitamer standard in the calibration vial

$^{12}i_{smpl}$ – signal intensity of vitamer in the sample vial;

$^{13}i_{smpl}$ – signal intensity of isotope labelled internal standard in the extraction vial.

To calculate the content (B) of a specific vitamin, for example vitamin B1 activity in the sample (µg of thiamine in 1 g of sample), the following equation is used:

$$B = \frac{\sum {}^{12}C_{Bi\_smpl}}{m_{smpl}} \times V \times M_B \qquad (2),$$

where,

$^{12}C_{Bi\_smpl}$ is the concentration of respective vitamer i possessing vitamin B1 activity in the extraction solution calculated from calibration curve, µM;

m_{smpl} is the sample weight in the sample vial, g;

V is the volume of extraction solution in the sample vial, L;

M_B is the molecular weight of the vitamer used to express the results (for example in case of vitamin B1, molecular weight of thiamine is used), Da.

Results obtained by using extraction vials with and without enzyme preparations are shown in Table 2.

Table 2. Vitamer concentrations and the respective B-complex vitamin content in inactive dried yeast (mean ± S.D., n = 3), by using extraction vials without enzyme preparation (A) and with enzyme preparation (B).

Vitamin	A, µg g^{-1}	B, µg g^{-1}
Thiamine	34.2 ± 1.7	47.2 ± 1.7
B1 (as thiamine)		47.2 ± 1.7
Riboflavin	12.3 ± 0.4	40.4 ± 0.5
B2 (as riboflavin)		40.4 ± 0.5
Nicotinamide	130.1 ± 2.3	149.7 ± 11.2
Nicotinic acid	110.9 ± 1.4	117.2 ± 3.3

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B3 (as nicotinic acid)		265.7 ± 14.5
Pantothenic acid	140.3 ± 4.1	130.1 ± 7.3
B5 (as pantothenic acid)	140.3 ± 4.1	130.1 ± 7.3
Pyridoxal	2.00 ± 0.26	3.07 ± 0.17
Pyridoxine	1.23 ± 0.03	1.33 ± 0.01
B6 (as pyridoxine)		4.36 ± 0.18

EXAMPLE 2: Determination of thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine content in the reference sample SRM 1849 (Infant/Adult Nutritional Formula).

0.1 g of reference sample SRM 1849 is weighed into a 20 ml sample vial without enzymes, 20 ml of 0.05 M ammonium formate buffer (pH 4.5) is added, the content of the vial is mixed thoroughly, 2 ml of the incubation mixture is centrifuged (14000 rpm/min) for 5 minutes at 10 ^{0}C and filtered (Millex-LG 13mm Philic PTFE 0.2 µm, Millipore, Carrigtwohill, Ireland). 1 ml of this filtered extract is added into an empty 2 ml LC-MS vial and injected into the LC-MS system.

To generate a calibration curve, 1 ml of 0.05 M ammonium formate buffer (pH 4.5) is added into each vial in the set of 2 ml calibration vials (containing different concentrations of labelled and unlabelled vitamers), the contents of the vials are mixed thoroughly, and incubated with frequent shaking at 37 ^{0}C for 18 h in the dark and analysed by LC/MS. Vitamers are analysed and calculated as in Example 1.

Results are shown in Table 3.

Table 3. Vitamin concentrations in the SRM 1849.

Vitamin, mg/kg	Our results[a]	Certified Concentration
Thiamine (expressed as thiamine Cl HCl)	15.5 ± 0.3	15.8 ± 1.3
Riboflavin	17.2 ± 0.3	17.4 ± 1.0
Nicotinamide	99.7 ± 1.9	97.5 ± 2.3

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Pantothenic acid	63.9 ± 0.9	64.8 ± 2.2
Pyridoxine (expressed as pyridoxine HCl)	14.2 ± 0.2	14.2 ± 1.5

[a] Results are expressed as a mass fraction for the material as received and as the mean of at least 30 measurements on five different days over one month. The uncertainties are expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U=k*u_c$, where u_c is the uncertainty of the measurement results and k is a coverage factor (k=2).

The present invention relates to the method and analytical kit, which is comprised of both calibration and sample vials containing a known amount of lyophilized isotope labelled and unlabelled vitamer standards.

As used herein, a "sample vial" refers to a vial, in a size of 0.5 - 50 ml, made from glass or plastic or any other material, impermeable to UV-light and oxygen, which is hermetically capped in an inert gas atmosphere, and which contains at least one dried or lyophilized isotope labelled vitamer with a vitamin B1, B2, B3, B5, and B6 activity, respectively, and/or a lyophilized or dried enzyme or enzyme preparation or mixture of enzymes with specific hydrolytic activities. The sample vials may be used as extraction vials (preferable size 20 ml) in which extraction of sample is carried out or as vials (preferably vials of an LC autosampler) into which clear pre-extracted and filtrated sample is added.

As used herein, a "calibration vial" refers to a vial, in a size of 0.5 - 50 ml, made from class or plastic, with UV- and oxygen protection, which is hermetically closed in the N_2 or some other inert gas atmosphere, and which contains the same lyophilized or dried isotope labelled vitamers as are containing in the sample vial and also lyophilized or dried unlabelled vitamers and/or a lyophilized or dried enzyme or enzyme preparation or enzyme (enzyme preparations) mixture with specific hydrolytic activities. Preferably, the quantity and number of enzyme or enzyme preparation or enzyme (enzyme preparations) mixture and isotope labelled vitamers are identical in sample and calibration vials.

As used herein, a "calibration mixture" refers to a vial, in a size of 0.5 - 50 ml, made from class or plastic, with UV- and oxygen protection, which is hermetically closed in the N_2 or

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some other inert gas atmosphere, and which contains the same lyophilized or dried unlabelled vitamers with a vitamin B1, B2, B3, B5, and B6 activity as are in the sample vial.

- thiamine, TMP, TDP, TTP and ATDP 0.002 - 200 nmol;

- riboflavin, FMN, and FAD 0.01 – 150 nmol;

5 • nicotinamide, nicotinic acid, NAD, NADH, NADP, NADPH, N-ribosyl nicotinamide, NAAD, NAADP, and N-ribosyl nicotinic acid 0.04 – 400 nmol;

- pantothenic acid, CoA, 4-phosphopantetheine, and pantetheine 0.01 - 200 nmol;

- pyridoxine, pyridoxal, pyridoxamine, PNP, PLP, and PMP 0.01 – 300 nmol.

As used herein, an "isotope labelled B-complex vitamer standards" refer to the following
10 isotope labelled compounds (Fig 2 – 6): B1: thiamine and/or thiamine monophosphate (TMP) and/or thiamine diphosphate (TDP) and/or thiamine triphosphate (TTP) and/or adenosine thiamine diphosphate (ATDP); B2: riboflavin and/or flavin mononucleotide (FMN) and/or flavin adenine dinucleotide (FAD); B3: nicotinamide and/or nicotinic acid and/or nicotinamide adenine dinucleotide (NAD) and/or nicotinamide adenine dinucleotide reduced (NADH)
15 and/or nicotinamide adenine dinucleotide phosphate (NADP) and/or nicotinamide adenine dinucleotide phosphate reduced (NADPH) and/or N-ribosyl nicotinamide and/or nicotinic acid adenine dinucleotide (NAAD) and/or nicotinic acid adenine dinucleotide phosphate (NAADP) and/or N-ribosyl nicotinic acid; B5: pantothenic acid and/or Coenzyme A (CoA) and/or 4-phosphopantetheine and/or pantetheine; B6: pyridoxine and/or pyridoxal and/or pyridoxamine
20 and/or pyridoxine phosphate (PNP) and/or pyridoxal phosphate (PLP) and/or pyridoxamine phosphate (PMP).

As used herein, an "isotope label" refers generally to ^{13}C, ^{15}N, ^{2}H and other stable isotopes that contain at least two atomic positions in the respective isotope labelled B-complex vitamer standard. The amount of internal standard in the sample vial depends on the sample of interest
25 and the size of the vial, but remains within the following range:

- thiamine, TMP, TDP, TTP and ATDP 0.002 - 200 nmol;

- riboflavin, FMN, and FAD 0.01 – 150 nmol;

- nicotinamide, nicotinic acid, NAD, NADH, NADP, NADPH, N-ribosyl nicotinamide, NAAD, NAADP, and N-ribosyl nicotinic acid 0.04 – 400 nmol;

- pantothenic acid, CoA, 4-phosphopantetheine, and pantetheine 0.01 - 200 nmol;

- pyridoxine, pyridoxal, pyridoxamine, PNP, PLP, and PMP 0.01 – 300 nmol.

As used herein, "enzymes with hydrolytic activities" refer to enzymes or enzyme preparations that possess any combination of acid phosphatase (0.1 – 2 000 U/vial), acid pyrophosphatase

5   (0.1 – 2000 U/vial), α-amylase (0.1– 7 500 U/vial), and β-glucosidase (0.1 – 2 000 U/vial) activities. Selection of enzymes can depend on the sample.

As used herein, the "activity of 1 U of acid phosphatase" refers to the activity that is required to liberate 1 µmole of phosphate from p-nitrophenyl phosphate at pH 4.8 at 37 °C per 1 min.

As used herein, the "activity of 1 U of acid pyrophosphatase" refers to the activity that is

10 required to liberate 1 µmole of phosphate from p-nitrophenyl phosphate at pH 4.8 at 37 °C per 1 min.

As used herein, the "activity of 1 U of α-amylase" refers to the activity that is required to liberate 1 µmole of maltose from starch at pH 6.0 at 25 °C per 1 min.

As used herein, the "activity of 1 U of β-glucosidase" refers to the activity that is required to

15 liberate 1 µmole of glucose from salicin at pH 4.0 at 37 °C per 1 min.

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CLAIMS

1. A method for simultaneous quantification of B-complex vitamins in drugs, food, food- and bio-supplements, feed, and other biological samples using LC-MS or LC-MS/MS and isotope labelled internal standard method, comprising:

5   - simultaneous bottling of a known amount of isotope labelled B-complex vitamers possessing vitamin B1, B2, B3, B5 and B6 activity into sample and calibration vials;

- bottling of a known concentration of the same unlabelled vitamers into the calibration vials;

- simultaneous bottling of a mixture of enzyme preparation, which may possess either acid phosphatase, pyrophosphatase, α-amylase, and β-glucosidase activity or all these activities into

10   the sample and calibration vials, lyophilisation and hermetic enclosure of sample vials and calibration vials under oxygen deprived conditions;

- incubation of acidic extract of a sample under study in a sample vial to convert all the containing vitamers into the same forms as the isotope labelled internal standards used;

- calibrating the LC-MS system with calibration vials or a calibration mixture, which are

15   incubated under the same conditions as the sample extract;

- determination of the content of B-complex vitamins as the sum of the molar concentrations of their respective vitamers.

2. The method of claim 1, wherein said the content of at least five B-complex vitamins is determined simultaneously.

20   3. The method of claim 2, wherein said the content of vitamins B1, B2, B3 B5, B6 is determined.

4. The method of claim 1, wherein said the mixture of isotope labelled vitamers, internal standards, and/or a mixture of enzymes or enzyme preparations with specific hydrolytic activities, are lyophilized or dried simultaneously into the sample vials.

25   5. The method of claim 1, wherein said the enzyme preparations with hydrolytic activities is lyophilized or dried into the sample vials.

6. The method of claim 1, wherein said the pH of the said sample is in the range of 2.0 – 5.0.

7. The method of claim 6, wherein said the pH of the said sample is preferably pH 4.5.

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8. The method of claim 1, wherein said the sample extract is incubated for 2 – 24 h within the temperature range of 20 – 50 $^{\circ}$C.

9. The method of claim 8, wherein said the sample extract is incubated, preferably for 18 h at 37 $^{\circ}$C.

10. The method of claim 1, wherein said the concentration of vitamers in the said sample is determined by LC-MS using a calibration mixture, which is added to the sample vials instead of a sample with an equal volume of the sample extract.

11. The method of claim 1, wherein said the mixture of isotope labelled vitamers as internal standards, and unlabelled vitamers, and a mixture of enzymes or enzyme preparations with specific hydrolytic activities, are lyophilized or dried simultaneously into the calibration vials.

12. The method of claim 1, wherein said a mixture of enzymes or enzyme preparations with specific hydrolytic activities in the calibration vials are lyophilized or dried simultaneously.

13. The method of claim 1, wherein said the concentration of vitamers in the internal standard is brought to the same value in the sample and in the calibration vials.

14. The method of claim 1, wherein said a known amounts of isotope labelled vitamers is bottled into sample and calibration vials of the analysis kit and lyophilized or dried.

15. The method of claim 1, wherein, instead of vitamers, said a known amounts of isotope labelled biomass or its extract can be lyophilized or dried into the sample and calibration vials.

16. An analytical kit for simultaneous quantification of the content of five B-complex vitamins (B$_1$, B$_2$, B$_3$, B$_5$ and B$_6$) in vitamin containing drugs, food, food- and bio-supplements, feed, and other biological samples comprising of a pair of calibration and sample vials, which contain an equal amount of isotope labelled vitamers.

FIG 1
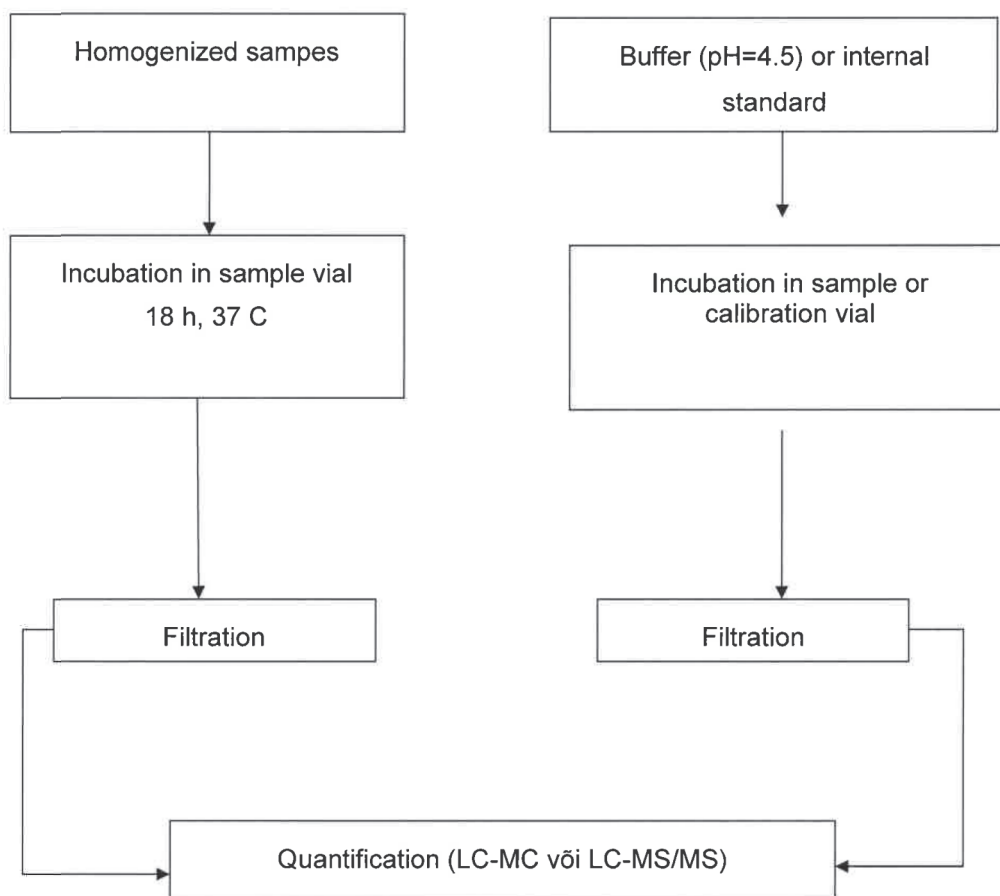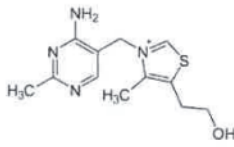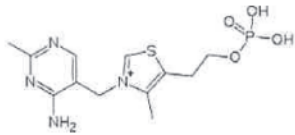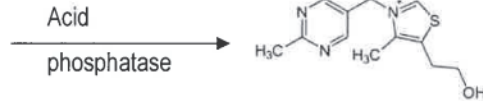
Thiamine

Thiamine monophosphate (TMP)

Acid
phosphatase

Thiamine

Thiamine diphosphate (TDP)
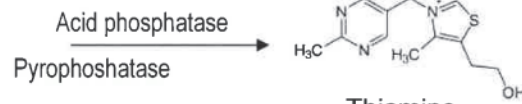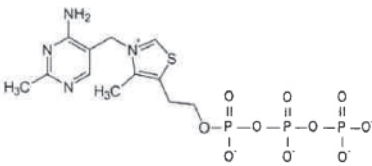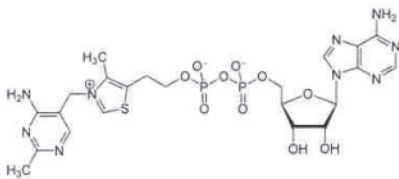
Acid phosphatase
Pyrophoshatase

Thiamine

Thiamin triphosphate (TTP)

Acid phosphatase
pyrophosphatase

Thiamine

Adenosine thiamine diphosphate (ATDP)

Acid phosphatase
pyrophosphatase

Thiamine

Adenosine thiamine triphosphate (ATTP)

Acid phosphatase
pyrophosphatase

Thiamine

FIG 2

Flavine mononucleotide (FMN) → Acid phosphatase / pyrophosphatase → Riboflavin

Flavine adenine dinucleotide (FAD) → Acid phosphatase / pyrophosphatase → Riboflavin
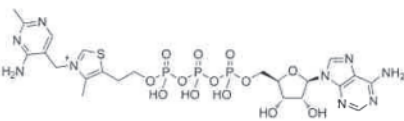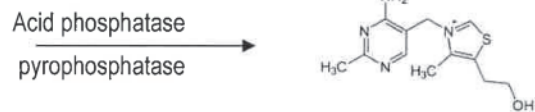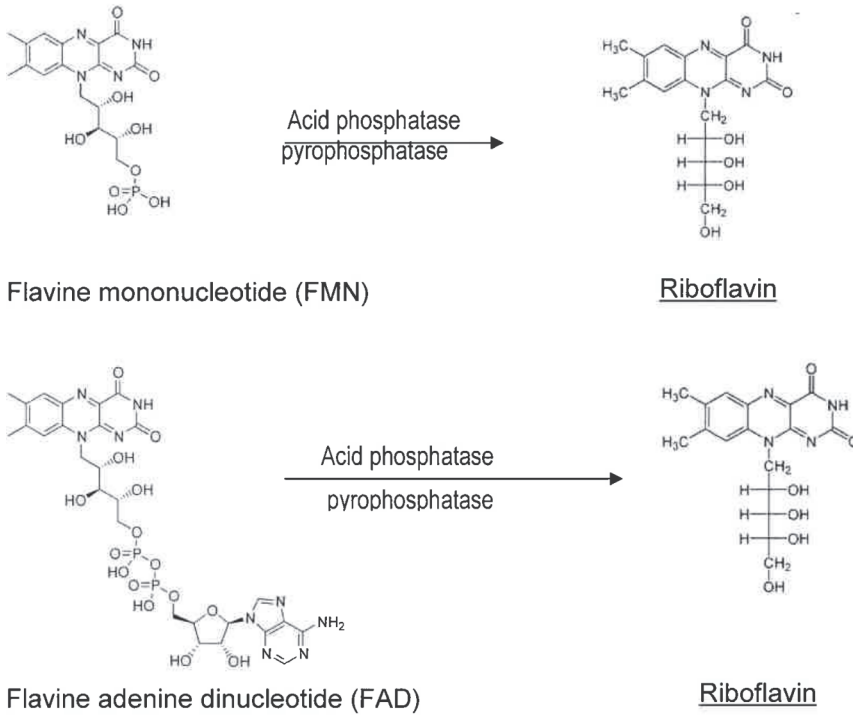
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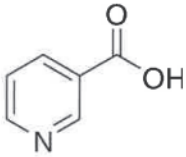
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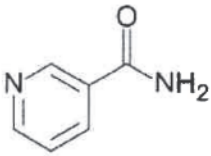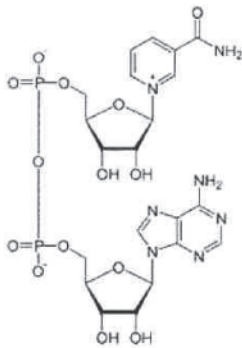
Nicotinamide

Nicotinamide adenine dinucleotide (NADH)

Acid phosphatase
pyrophosphatase
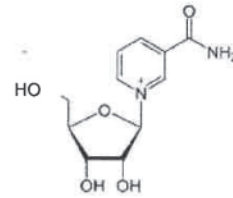
N-ribosyl nucleotide amine

FIG 4

Pantothenic acid

Co-enzyme A   pyrophosphatase   4-phosphopantotheine

Acid phoshatase

4-phosphopantotheine   Pantotheine

FIG 5

Pyrodoxine

Pyridoxal

Pyridoxamine

Pyridoxalphosphate

Acid
phosphatase

Pyridoxal

Pyridoxal phosphate

Acid
phosphatase

Pyridoxine

Pyridoxamine phosphate

Acid
phosphatase

Pyridoxamine
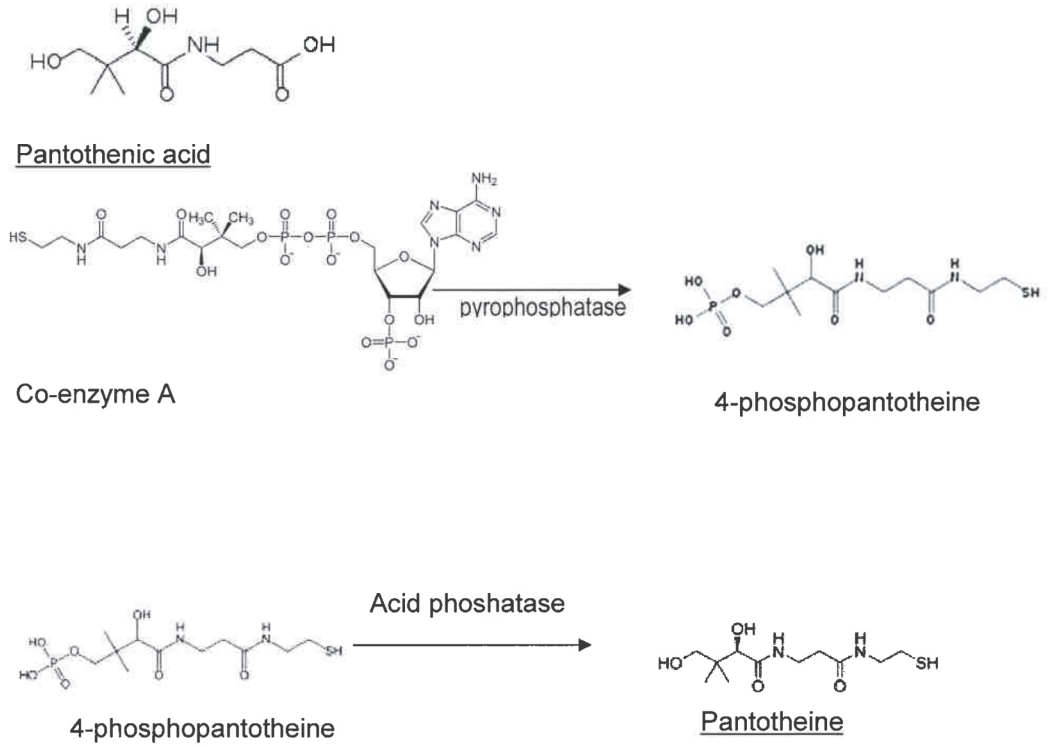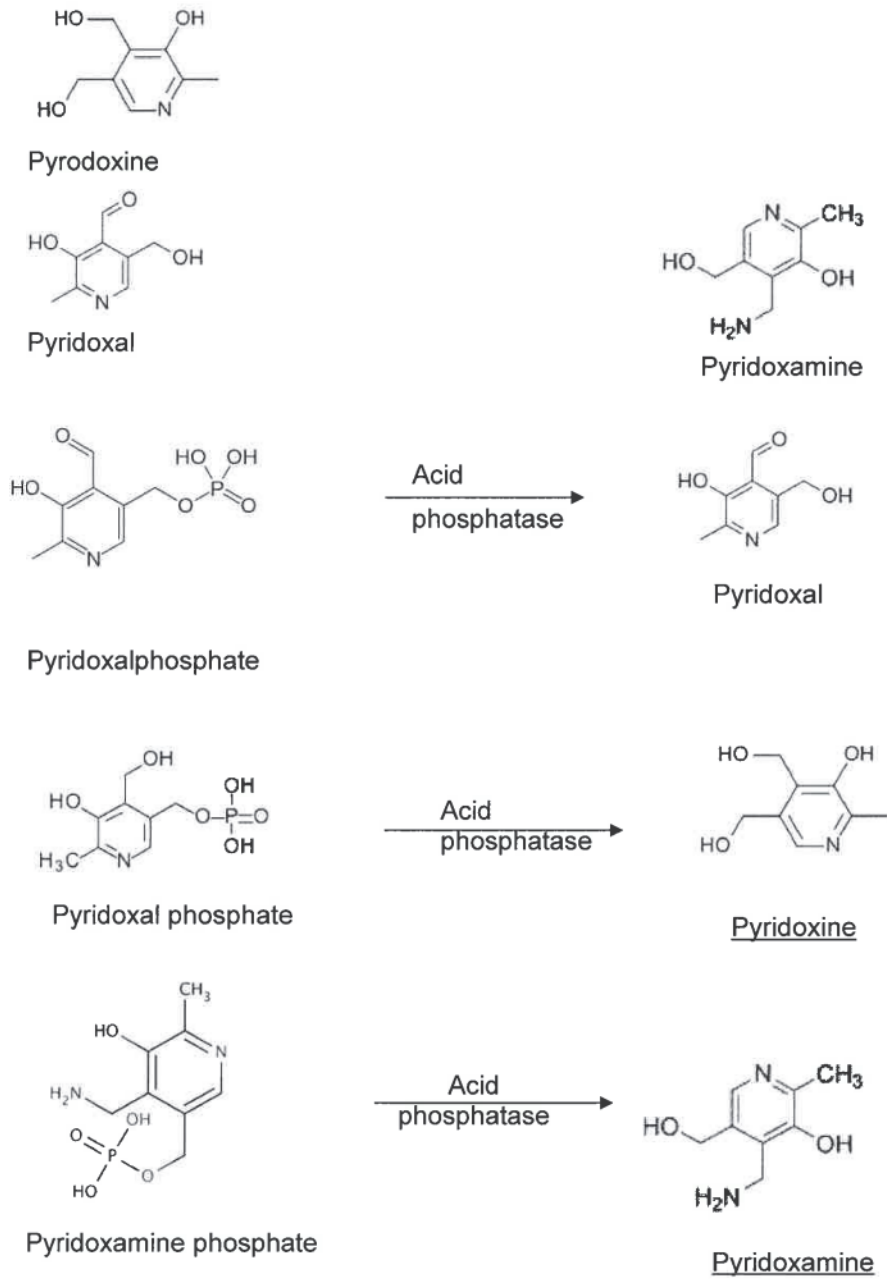
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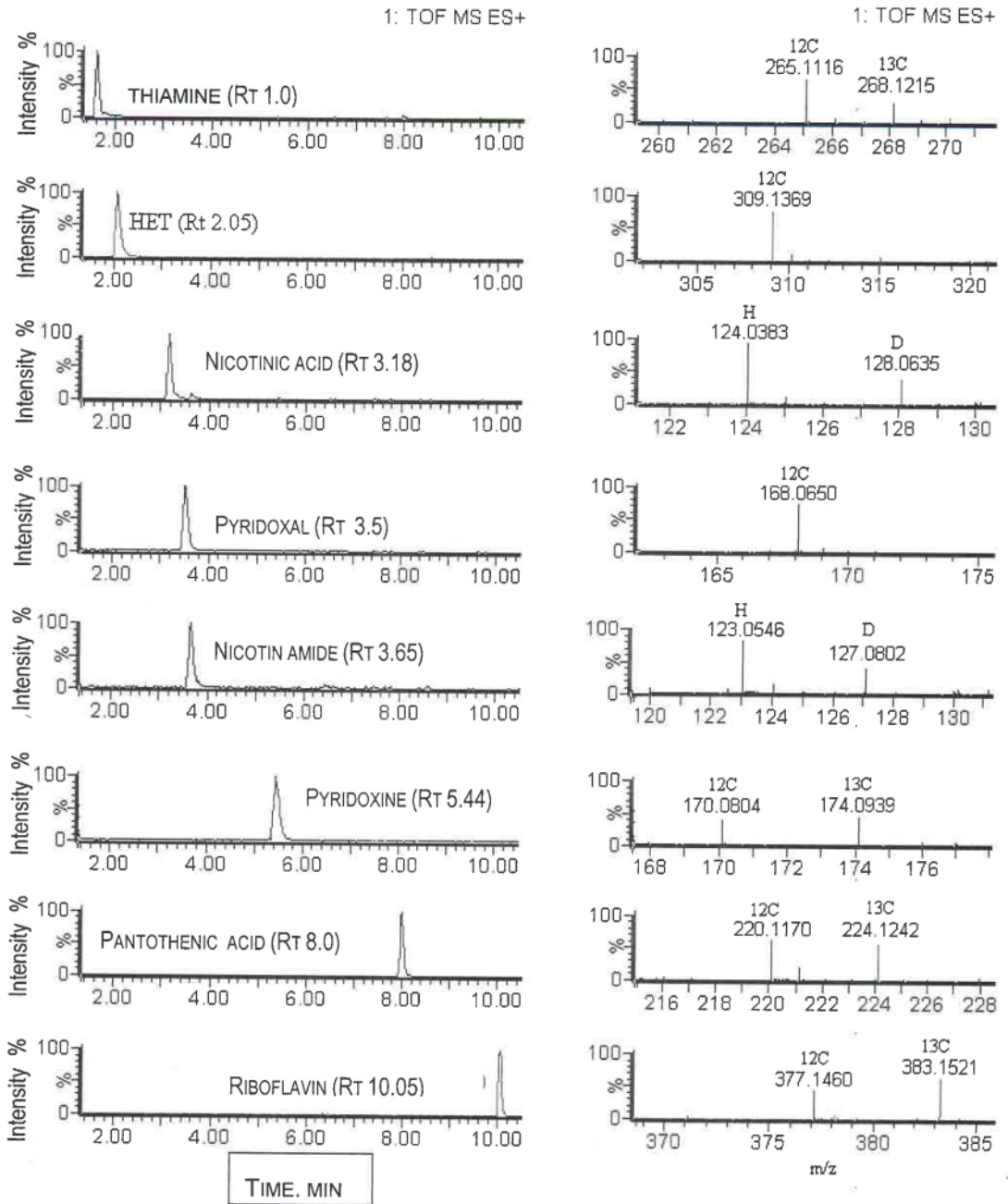
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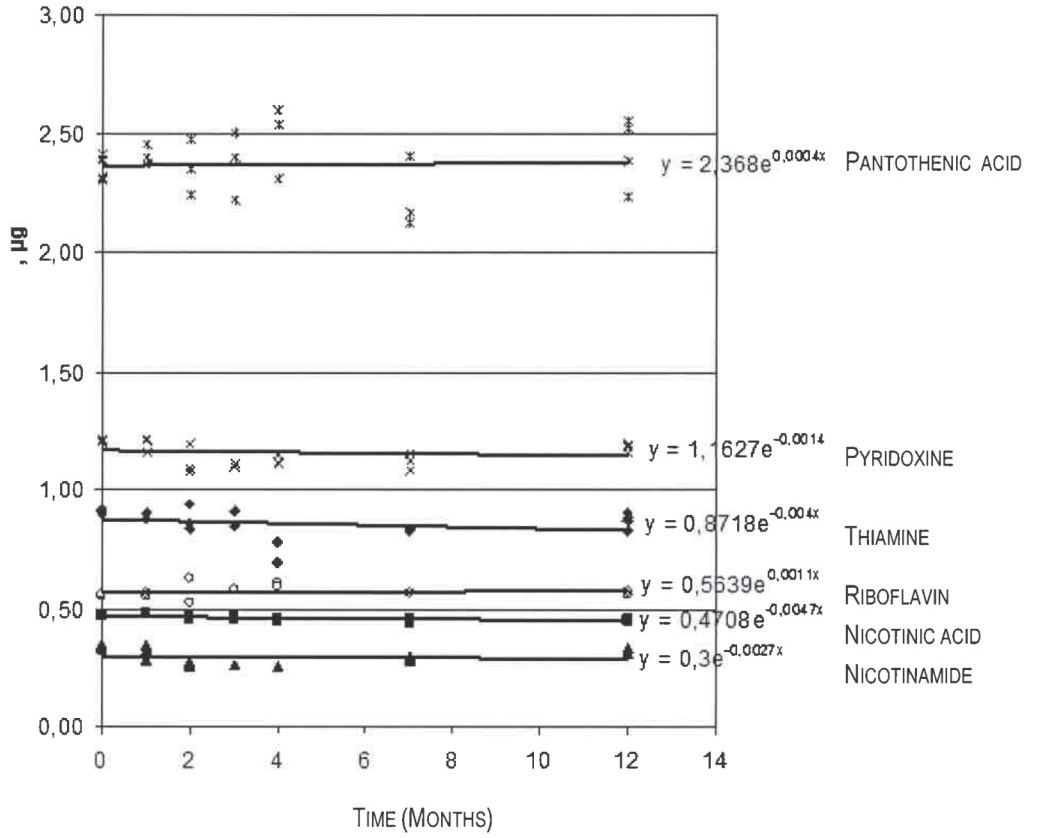
FIG 8

Abstract

The present invention relates to an analytical vitamin analysis kit, the method of its preparation, and the method of its application. The given analytical kit can be used for the simultaneous determination of the quantity of five B-complex vitamins (B1, B2, B3, B5 and

5   B6) and individual vitamers in drugs, food, food- and bio-supplements, feed, and other biological samples using LC-MS isotope internal standard assay. The analytical kit is based on the use of sample vials containing lyophilized or dried isotope labelled internal standards of the B-complex vitamers with a mix of hydrolytic enzymes, used to convert vitamers and cofactors into the same chemical forms as the used isotope labelled internal standards, and for

10 their following quantification by means of LC-MS or LC-MS/MS.

PUBLICATION III

Mihhalevski A, Nisamedtinov I, Hälvin K, Ošeka A, Paalme T

Stability of B-complex vitamins and dietary fiber during rye sourdough bread production

Stability of B-complex vitamins and dietary fiber during rye sourdough bread production

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ABSTRACT

The stability of vitamers: thiamine, riboflavin, nicotinic acid, nicotinamide, pantothenic acid, pyridoxine and pyridoxal, as well as soluble and insoluble dietary fiber was studied in a rye sourdough bread process. The vitamer concentrations were measured in raw materials (rye flours, white and red rye malt, yeast) and the rye sourdough breads made from them by means of LC–MS and stable isotope dilution assay. The content of dietary fiber was determined using a standard enzymatic-gravimetric method. During baking, the concentration of vitamins decreased by 20–45% in the case of thiamine, 25–50% in the case of nicotinic acid, 45–65% in the case of pyridoxal in both breads, 50% in the case of riboflavin and 15% in the case of pyridoxine only in fine rye bread. In contrast, the content of nicotinamide increased during processing by ten fold, presumably due to microbial activity during sourdough fermentation. The ratio of soluble to insoluble dietary fiber increased during rye sourdough processing.

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1. Introduction

Rye sourdough bread is a traditional and popular bread in North–West Russia, the Baltic states, Finland, Denmark, and the North of Germany. Rye sourdough bread is industrially produced by mixing sourdough with flour, followed by fermentation with yeast (leavening), and baking. Rye bread is a recommended part of the diet because it is a good source of various bioactive substances, including B-complex vitamins and dietary fiber.

In industrial product labeling practice, the calculation of the content and composition of vitamins and fiber is based on the composition of their ingredients. However, this may result in incorrect estimations, mostly due to variations in the vitamins and fiber content of the particular ingredients used, but also due to chemical changes that occur during processing.

Dietary fiber is an indigestible complex of carbohydrates and lignin. It is an important functional component, the content of which can change during rye bread processing. Insoluble dietary fiber compounds in rye include cellulose (1–3 %), lignin (1–2 %) (Liukkonen et al., 2006) and the insoluble part of arabinoxylans. Rye grain contains 8–12 % of arabinoxylans, fructan (4.5–6.4%) and

β-glucans (1–3%) (Hansen et al., 2003; Karppinen et al., 2003; Liukkonen et al., 2006). The soluble fibers are fructo-oligosacchrides 1-kestose (0.6%), 1,1-kestotetraose (0.3%), and 1,1,1-kestopentaose (0.3%) (Karppinen et al., 2003). Amount of water-extratable β-glucans is 1.2% in rye (Aura et al., 2007).

Enzymatic as well as thermal treatment can modify the ratio between soluble and insoluble fibers during bread making (Elleuch et al., 2011). During flour hydration, several intrinsic hydrolytic enzymes of cereals (α-amylase, β-xylosidase, α-L-arabinofuranosidase, cinnamoyl esterases, D-xylosidase, β-glucanases and endo-D-xylenase) are activated (Boskov Hansen et al., 2002). Also the enzymes with β-glycolytic activity from lactic acid bacteria (Axelsson, 2007) and yeast (Hauf et al., 2000) can affect the fiber concentration during rye bread processing. The most commonly used methods for the determination of dietary fiber in cereals are enzymatic-gravimetric methods (Lee et al., 1992).

The term *vitamin* refers to a number of *vitamer* compounds with the same biological activity as the respective vitamin (Table 1). Unfortunately, the complete list of vitamers with respective vitamin activity for mammals is still not clear. Several vitamins during food processing, storage and digestion are derived from cofactors as the result of enzymatic activities in both the food matrix and the digestive tract. For example, 2–10% of the vitamin B1 pool in cereals is present as its phosphorylated form thiamine diphosphate (TDP) (Buchholz et al., 2011), which is hydrolyzed by different phosphatases in the human gastrointestinal tract into

Table 1
Vitamins and corresponding vitamers.

Vitamin	Vitamers
B1, thiamine	Thiamine
	Thiamine monophosphate (TMP)
	Thiamine diphosphate (TDP)
	Thiamine triphosphate (TTP)
	2-(1-hydroxyethyl)thiamin (HET)
B2, riboflavin	Riboflavin
	Flavin mononucleotide (FMN)
	Flavin adenine dinucleotide (FAD)
B3, niacin	Nicotinic acid
	Nicotinamide
	Nicotinamide adenine dinucleotide (NAD)
	Nicotinamide adenine dinucleotide phosphate (NADP)
	Nicotinic acid adenine dinucleotide (NAAD)
	Nicotinic acid adenine dinucleotide phosphate (NAADP)
	N-ribosyl nicotinamide
	N-ribosyl nicotinic acid
B5, panthothenic acid	Pantothenic acid
	Coenzyme A (CoA)
	Phosphopantothenic acid
	Pantetheine
	Phosphopantetheine
B6, pyridoxine	Pyridoxine
	Pyridoxal
	Pyridoxamine
	Pyridoxine phosphate (PNP)
	Pyridoxal phosphate (PLP)
	Pyridoxamine phosphate (PMP)
	5′-O-β-ᴅ-glucopyranosyl pyridoxine

thiamine (Rindi and Laforenza, 2000). Similarly, the phosphorylated forms of riboflavin and vitamin B6 can also be found in nature (Cataldi et al., 2002; Roth-Maier et al., 2002).

In practice, the content of B-complex vitamins is expressed as the concentrations of their most simple commercially available chemical forms (e.g. B1 = thiamine chloride hydrochloride, B2 = riboflavin, niacin = nicotinic acid + nicotinamide, expressed as nicotinic acid, B5 = calcium pantothenate, B6 = pyridoxine + pyridoxal + pyridoxamine, expressed as pyridoxine hydrochloride) which often serve as a starting point for the synthesis of respective cofactor(s) in cells. Thus, in most of the food databases, the B-complex vitamin contents (μg per 100 g of food) are expressed as thiamine, riboflavin, niacin, pantothenic acid and pyridoxine hydrochloride equivalents.

Information regarding the stability of B-complex vitamers during the course of rye sourdough bread-making processes is scarce. Thiamine is relatively stable at pH 2.0—4.0, which is a common pH range of the sourdough. In slightly acidic solutions it is stable up to the boiling point. However, during baking, 20—50 % losses of thiamine have been reported (Martinez-Villaluenga et al., 2009). Similarly, riboflavin is relatively heat-stable (Batifoulier et al., 2005). It has been reported that whole wheat bread made with yeast results in 30% enrichment in riboflavin due to the contribution of endogenous yeast riboflavin, while sourdough lactic acid fermentation without yeast did not result in any increase in the bread's riboflavin content (Batifoulier et al., 2005).

Nicotinamide and nicotinic acid are relatively stable during baking (Ottaway, 2002). A small part of chemically bound nicotinic acid (i.e. NAD) can be hydrolyzed by gastric juice and is thus converted into bioavailable forms (Wall and Carpenter, 1988). Pantothenic acid has good stability during most food processing operations, including baking (Ottaway, 2002). Pyridoxine, pyridoxal and pyridoxamine are relatively heat-stable under acidic

conditions and very heat-labile under alkaline conditions (Leklem, 2001). The baking of bread can induce losses of up to 17% for vitamin B6 (Ottaway, 2002).

The current study was carried out to analyze the stability of soluble and insoluble dietary fiber and B-complex vitamers during rye sourdough bread making. To determine vitamer concentrations we used a high throughput LC—MS and stable isotope dilution assay.

2. Materials and methods

2.1. Materials and reagents

Whole grain rye flour R1800, dark rye flour R1370, fine rye flour R705, wheat flour W700, inactive (red) rye malt, active (white) rye malt and steel cut rye grains were obtained from Tartu Grain Mill Ltd. (Tartu, Estonia). The same lots of flour were used for the production of all bread recipes. The commercial rye bread containing dark rye flour (55%), active rye malt (1%) and inactive rye malt (1%), yeast (0.9%), and fine rye bread containing fine rye flour (42%), wheat flour (10%), dark rye flour (7%), active rye malt (1%), and yeast (1%) were obtained from a local bakery.

Dark rye flour (R1370) used for incubation and fermentation experiments was sterilized by γ-irradiation at 10 kGy using a dosimetric system GEX WinDose (Centennial, CO, USA). Bacterial strain *Lactobacillus panis* N915 was isolated from the rye sourdough (Mihhalevski et al., 2011). Fresh baker's yeast was kindly provided by AS Salutaguse Pärmitehas (Kohila vald, Estonia).

Certified reference material BCR 121 (wholemeal flour) was obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). The certified values of B-complex vitamers with uncertainties (μg/100 g dry matter) for BCR 121 were as follows: 364 ± 31 for vitamin B1 (expressed as thiamine equivalents) and 377 ± 84 for B6 (expressed as pyridoxine equivalents).

Acid washed Celite was obtained from Megazyme International Ireland Ltd. (Bray, Ireland). Acetone and acetonitrile (both HPLC grade) were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland, UK), MES (2-(N-morpholino) ethanesulfonic acid), ammonium formate (puriss.p.a., for HPLC), HCl (37% puriss.p.a.) and TRIS (tris(hydroxymethyl) aminomethane) were obtained from Sigma—Aldrich Inc (St.Louis, MO, USA), NaOH was obtained from Lach-Ner (Neratovice, Czech Republic) and Sigma—Aldrich. High purity water was produced by a Millipore water purification system (Millipore S.A.S., Malsheim, France).

Nicotinamide (99.9%), nicotinic acid (99%), thiamine chloride hydrochloride (99%), pyridoxine-HCl (99.9%), pyridoxal-HCl (99%), Ca-pantothenate (99%) and riboflavin (99%), used as external standards were obtained from Sigma—Aldrich. The stable isotope labeled internal standards nicotinamide − [D$_4$, 98%], nicotinic acid − [D$_4$, 98%], thiamine-Cl − [^{13}C$_3$, 99%], pyridoxine-HCl − [^{13}C$_4$, 99%], were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Ca-pantothenate − [^{13}C$_3$,^{15}N, 97.1%], riboflavin − [^{13}C$_4$,^{15}N$_2$, 97.9%] and pyridoxal HCl − [D$_3$, 99%] were from Iso-sciences, LLC (King of Prussia, PA, USA).

α-amylase from *Aspergillus oryzae* (crude, Cat. No. A6211), β-glucosidase from almonds (crude, Cat. No. G0395), and potato acid phosphatase (Cat. No. P1146) were obtained from Sigma—Aldrich.

Thiamine monophosphate chloride dehydrate (99%), thiamine pyrophosphate chloride (≥97%), riboflavin 5′-monophosphate sodium salt dehydrate (75.9%), flavin adenine dinucleotide disodium salt hydrate (97%), β-nicotinamide adenine dinucleotide hydrate (98%), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (99%) and coenzyme A hydrate (99%), were obtained from Sigma—Aldrich.

2.2. Sample preparation

After slicing, the breads were frozen in liquid nitrogen, freeze-dried, ground in a laboratory Waring blender HGBTWTS3 (Waring, Torrington, CT, USA) (particle size from 0.10 to 0.25 mm), packed into plastic bags, and stored at −20 °C until further analysis. Flours, malts and cut rye grains were stored at −20 °C. Steel cut rye grains were ground before analysis (particle size from 0.10 to 0.25 mm).

To study the effect of fermentation on the content of B-complex vitamers the 10 kGy irradiated rye flour was mixed in a ratio of 1:1 with a suspension of *L. panis* N915 in 0.5% NaCl (5×10^6–5×10^7 cfu/mL) using a Stomacher 400 Circulator (Seward Ltd., Worthing, UK) for 15 min at 100 RPM and incubated at 30 °C for 24 h. Control sourdough was made without lactic acid bacteria. After sourdough fermentation, the samples were freeze-dried and ground until further analysis.

2.3. Analytical methods

All samples were analyzed at least in three replicates.

2.3.1. Dry matter

The dry matter content of each sample was measured using a Halogen Moisture Analyser HR83 (Mettler Toledo, USA) and the ash content was determined according to AOAC method (923.03). Samples were treated in a muffle furnace at 550 °C for 10 h.

2.3.2. Total, soluble and insoluble dietary fiber

Total, soluble and insoluble dietary fiber content was measured according to the enzymatic-gravimetric method using an assay kit from Megazyme International Ltd. (Bary, Ireland). Samples were subjected to sequential enzymatic digestion by heat-stable α-amylase, protease, and amyloglucosidase. Insoluble dietary fiber was filtered from the hydrolyzate, washed, dried and weighed. Soluble dietary fiber was precipitated from the filtrate with four volumes of 95% ethanol, filtered and dried. For determination of total dietary fiber, the samples after enzymatic treatment were treated with four volumes of ethanol to precipitate soluble fiber. The residue was then washed, filtered, dried and weighed. Total, soluble and insoluble dietary fiber contents were corrected, based on the protein and ash residues.

2.3.3. Determination of B-complex vitamer concentrations

LC–MS combined with a stable isotope dilution assay was used for the analysis of B-complex vitamer concentrations in the samples. Freeze dried flour and bread samples were weighed (approx 1.0 g) into a 10 mL volumetric flask, filled up with a 0.05 M ammonium formate buffer (pH 4.5), mixed thoroughly, transferred to a 50 mL centrifuge tube and incubated with frequent shaking at 37 °C for 18 h. After incubation, each sample extract was centrifuged at 14 000 RPM for 5 min at 22 °C by Micro CL21R centrifuge (Thermo Scientific, Osterode, Germany) to remove insoluble debris and filtered through a Millex-LG 13 mm Philic PTFE 0.2 μm filter (Millipore, Carrigtwohill, Ireland). 1000 μL aliquots of this filtrate were then transferred to LC-MS sample vials containing 20 μL of internal standard solution containing approximately 20 mg/L of each labeled vitamer.

Fresh baker's yeast samples (approx 0.5 g, DM 21%, in four replicates) were weighted into 2 mL eppendorf tubes, 0.5 mL of 0.05 M ammonium formate buffer (pH 4.5) was added, vortexed, and held in the water bath at 70 °C for 30 min using frequent shaking. After heating, approximately 1.0 g of glass beads (425–600 μm, acid washed, G8772, Sigma–Aldrich) were added to the sample and yeast cells were disrupted using a Genie cell disrupter

(Scientific Industries Inc., New York, USA) for 30 min. The disrupted yeast samples were transferred quantitatively into a 10 mL volumetric flask, filled up with 0.05 M ammonium formate buffer (pH 4.5), mixed thoroughly, incubated, centrifuged, filtered and prepared for LC–MS analysis as described above.

All vitamer analyses were performed using an ACQUITY UPLC® system, equipped with an ACQUITY UPLC HSS C-18 1.8 μm (2.1 × 150 mm) column and coupled with LCT Premier™ XE ESI TOF MS System (Waters, Milford, MA, USA). Elution was carried out using two eluents (A: water + 0.1% formic acid and B: acetonitrile + 0.1% formic acid) applied in the following gradient mode: 0–3 min 100% A; 3–8.5 min 80% A and 20% B; 8.5–10 min: 5% A and 95% B; 10–15 min 100% A. A flow rate of 0.25 mL/min and a sample injection of 5 μL were used. Mass spectrometry was carried out in positive ionization mode $[M + H]^+$ using a capillary voltage of 2000 V, sample cone voltage of 30 V, desolvation temperature of 300 °C and source temperature of 120 °C. Full scan mass spectra were acquired from m/z 100 to 500. Data were collected and processed using Mass Lynx 4.0 software (Waters, Milford, MA, USA).

The m/z ratios of 265.11 and 268.12 for thiamine, 377.15 and 383.16 for riboflavin, 123.06 and 127.08 for nicotinamide, 124.04 and 128.06 for nicotinic acid, 220.12 and 224.13 for pantothenic acid, 168.07 and 171.09 for pyridoxal, 170.08 and 174.10 for pyridoxine were used for the determination of unlabeled and labeled forms of vitamins, respectively.

Internal standard stock solutions were prepared by dissolving separately about 1 mg of each isotope labeled vitamer in 5 mL 0.05 M ammonium formate (pH 4.5) buffer. Internal standard solution (~20 mg/L) was prepared by combining aliquots of the stock solutions. Concentration of each vitamin in the sample was calculated using 7-point calibration curves. Calibration curve standards were prepared by adding 1000 μL aliquots of external standards solution with varying concentrations (0.01–0.9 mg/L) into the LC–MS vial containing 20 μL of internal standard solution. The calibration curves were constructed by relating the varying concentrations of unlabeled external standards to their relative response factors as determined by the ratio of the peak intensity of the unlabeled external standards to that of the corresponding labeled internal standard. Responses for all the vitamins were linear over the working range with correlation coefficients ranging between 0.9995 and 0.9999. The vitamers were separated by LC and the intensities of respective lines of natural abundance and isotope labeled internal standards in the MS spectra were measured (Fig. 1).

2.3.4. The effect of enzymatic treatment and acid hydrolysis on liberation of simple vitamers

To study the effect of enzymatic treatment on the extraction and liberation of B-complex vitamers, 10 mL of a mixture of enzyme solution in 0.05 M ammonium formate (pH 4.5) containing α-amylase (4.50 mg/mL), β-glucosidase (1.60 mg/mL) and acid phosphatase (0.16 mg/mL) was added to 1 g of sample before incubation described in Section 2.3.3. All results were enzyme blank corrected.

The phosphatase, pyrophosphatase, purine nucleosidase, pantetheine hydrolase activities of the enzymes in used enzyme preparations were determined by preparing the 25 μM solutions of following vitamer precursors: thiamine mono- and diphosphate (TMP, TDP), flavin mononucleotide and flavin adenine dinucleotide (FMN, FAD), NAD$^+$ and reduced nicotinamide adenine dinucleotide (NADH) and Coenzyme A (CoA). Each solution was incubated with 25 mL of acid phosphatase (0.12 mg/mL), β-glucosidase (1.2 mg/mL) or α-amylase (5.0 mg/mL) during 18 h at 37 °C followed by determination of liberated thiamine, riboflavin, nicotinamide, and pantothenic acid by LC–MS.
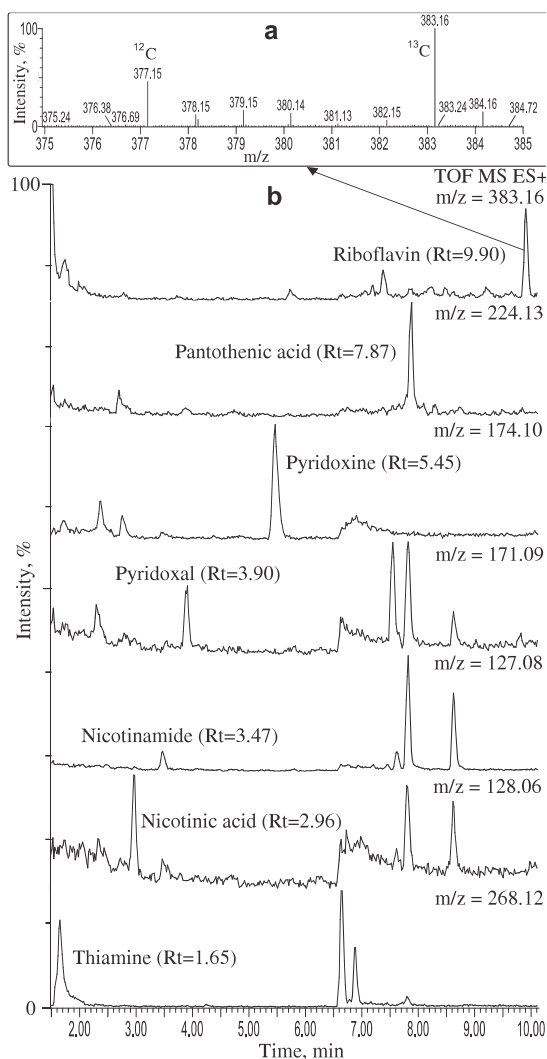
Fig. 1. TOF MS ES + spectrum of riboflavin in rye sourdough bread (a) and UPLC TOF MS ES + chromatograms of B-complex vitamers in rye sourdough bread (b).

Protease activity was determined using a protease assay kit (Calbiochem, USA): 100 μL of the single enzyme solution was combined with 50 μL of fluorescein thiocarbamoyl-casein (FTC-casein) and 50 μL of incubation buffer (200 mM Tris–HCl, pH 7.8, 20 mM $CaCl_2$, 0.1% NaN_3). Concentration of the enzyme solutions were as follows: α-amylase 0.225 mg/mL, β-glucosidase 1.2 mg/mL, acid phosphatase 0.12 mg/mL. Incubation was carried out at 37 °C for 24 h under subdued lighting conditions using continuous shaking. The reaction was stopped by the addition of 500 μL of 5% trichloroacetic acid. Denatured FTC-casein was separated from the reaction mixture by centrifugation (12 000 RPM for 5 min at room temperature) and the concentration of FTC-peptides in the supernatant was determined by absorbance at 492 nm. Protease activity was expressed relative to that of trypsin in BAEE (N-benzoyl-ʟ-arginine ethyl ester) units.

The effect of acid hydrolysis on the liberation of vitamers from biologically active forms was studied as follows: 25 mL of TMP (25 μM), FMN (25 μM), TDP (25 μM), FAD (25 μM), NAD^+ (25 μM) or NADH (25 μM) in 0.1 M HCl were hydrolyzed in an autoclave at 121 °C for 30 min, followed by the determination of thiamine, riboflavin and nicotinamide.

2.4. Calculation of stability of nutrients during processing

The expected concentration of each nutrient in the bread was calculated on a dry mass basis by summing up the contributions from the individual ingredients. This involved measuring the amount of each nutrient found in each ingredient on a dry mass basis.

The recovery of B-complex vitamins after processing was calculated as the percentage of concentration measured in the bread sample divided by the value calculated according to the recipe.

3. Results

All results are presented on a dry matter (DM) basis.

3.1. Stability of dietary fiber during rye sourdough bread processing

The concentrations of soluble, insoluble and total dietary fiber were determined in all ingredients used for rye sourdough bread making as well as in the baked breads (Table 2). Similar values for total dietary fiber (15–17% DM) and for soluble fiber (2–3% DM) were found in whole grain rye flour, white rye malt, red rye malt and steel cut rye. In wheat flour and fine rye flour, the total dietary fiber was found to be only about 3% DM and 7% DM, respectively (Table 2). The soluble fiber content in fine rye flour (1.9% DM) was

Table 2
Total, soluble and insoluble dietary fibre in flours and breads.

Sample	Moisture %	Ash % DM	Total dietary fiber % DM	Soluble dietary fiber % DM	Insoluble dietary fiber % DM
Whole grain rye flour (R1800)	11.3 ± 0.2	1.4 ± 0.1	15.0 ± 0.5	2.34 ± 0.1	12.0 ± 0.8
Dark rye flour (R1370)	11.4 ± 0.2	1.1 ± 0.1	11.0 ± 0.6	2.3 ± 0.4	7.8 ± 0.8
Fine rye flour (R705)	11.8 ± 0.2	0.7 ± 0.03	6.6 ± 0.9	1.9 ± 0.2	3.6 ± 0.2
Wheat flour (W700)	12.6 ± 0.2	0.7 ± 0.04	2.7 ± 0.4	0.7 ± 0.2	1.2 ± 0.3
Non fermented rye malt	6.7 ± 0.2	1.5 ± 0.02	15.2 ± 0.8	2.2 ± 0.2	11.9 ± 1.3
Fermented rye malt	3.5 ± 0.2	1.6 ± 0.03	17.0 ± 1.8	3.1 ± 0.5	11.4 ± 2.0
Cut rye	11.1 ± 0.2	1.5 ± 0.1	16.3 ± 1.3	NT	12.8 ± 1.7
Rye bread					
Experimentally measured	42.4 ± 0.5	2.7 ± 0.01	11.1 ± 1.1	3.4 ± 0.2*	6.2 ± 0.3
Calculated			9.73 ± 0.5	2.02 ± 0.3	6.91 ± 0.7
Fine rye bread					
Experimentally measured	41.8 ± 0.2	2.3 ± 0.03	10.0 ± 0.7*	2.6 ± 0.4*	6.0 ± 0.5*
Calculated			6.02 ± 0.1	1.54 ± 0.1	3.42 ± 0.2

NT – not tested, * - experimental value is different from calculated value ($p < 0.05$).

relatively high compared to that of wheat flour (0.7% DM). The highest concentration of soluble fiber in the raw materials studied was observed in fermented rye malt (3.1% DM).

The concentration of soluble fiber in the rye bread (2.0% DM) and in fine rye bread (1.5% DM), calculated on the basis of their recipes and on the fiber content in flours used were lower than the measured values (3.4 and 2.6% DM, respectively). This also applies to the experimentally determined total dietary fiber concentration of fine rye bread.

3.2. B-complex vitamers in cereal materials and rye bread

The concentrations of seven vitamers of B-complex vitamins: thiamine, riboflavin, nicotinic acid, nicotinamide, pantothenic acid, pyridoxine and pyridoxal were analyzed in reference wholemeal flour, different breads, as well as the raw materials used to make them using extraction with ammonium formate buffer and an LC–MS isotope dilution assay method. These vitamers were well resolved in the reversed phase column under the chromatographic conditions specified in Methods (Fig. 1). The MS spectrum of riboflavin in the internal standard is given as an example.

The concentration of B-complex vitamers in the reference material, raw materials, and breads prepared from them is given in Table 3.

The concentration of thiamine in the reference wholemeal flour, determined without enzymatic treatment, was 376 ± 39 µg/100 g DM (Table 3), which corresponds to the certified value (364 ± 31 µg/100 g DM) for vitamin B1, expressed as thiamine. Using enzymatic extraction the concentration of thiamine increased up to 394 ± 31 µg/100 g DM, which is not significantly higher ($p > 0.05$) than the reference value. On the contrary, the sum of pyridoxal and pyridoxine concentration (expressed as pyridoxine) was 226 ± 15 µg/100 g DM, which was significantly lower than that of the reference value 377 ± 84 µg/100 g DM. Enzymatic

Table 3
Content of B-complex vitamers in reference material, flours, sterile sourdoughs, and breads obtained using the extraction with and without enzyme mix. The recipe based values of vitamers in breads (calculated).

Samples	Thiamine	Riboflavin	Nicotinic acid	Nicotinamide	Pantothenic acid	Pyridoxal	Pyridoxine
	µg/100 g of DM						
Reference material BCR 121 (wholemeal flour)							
Certified value	364 ± 31	–	–	–	–	–	–
Free vitamins	376 ± 39^a	129 ± 25^a	615 ± 45^a	32 ± 1^a	765 ± 54^a	30 ± 2^a	196 ± 13^a
Enzymatic extraction	394 ± 31^a	325 ± 135^b	391 ± 28^b	58 ± 2^b	658 ± 47^a	28 ± 4^a	189 ± 16^a
Steel cut rye							
Free vitamins	230 ± 11^a	96 ± 4^a	257 ± 9^a	17 ± 3^a	304 ± 8^a	31 ± 4^a	115 ± 2^a
Enzymatic extraction	295 ± 20^b	116 ± 10^b	164 ± 26^b	44 ± 8^b	308 ± 15^a	47 ± 6^b	121 ± 5^a
Whole grain rye flour							
Free vitamins	246 ± 6^a	82 ± 6^a	289 ± 33^a	50 ± 1^a	241 ± 10^a	32 ± 7^a	92 ± 2^a
Enzymatic extraction	246 ± 13^a	91 ± 5^a	191 ± 14^a	65 ± 4^b	234 ± 11^a	41 ± 4^a	105 ± 2^b
Dark rye flour							
Free vitamins	236 ± 8^a	67 ± 3^a	185 ± 26^a	31 ± 3^a	174 ± 5^a	26 ± 4^a	75 ± 2^a
Enzymatic extraction	261 ± 4^b	90 ± 3^b	136 ± 11^b	39 ± 6^a	165 ± 16^a	35 ± 3^b	83 ± 3^b
Fine rye flour							
Free vitamins	185 ± 5^a	50 ± 2^a	144 ± 6^a	26 ± 2^a	122 ± 2^a	17 ± 4^a	49 ± 2^a
Enzymatic extraction	186 ± 5^a	58 ± 1^b	98 ± 15^b	40 ± 8^b	114 ± 4^b	23 ± 5^b	49 ± 4^a
Wheat flour							
Free vitamins	185 ± 2^a	37 ± 3^a	140 ± 16^a	18 ± 1^a	198 ± 3^a	19 ± 4^a	53 ± 3^a
Enzymatic extraction	185 ± 6^a	NA	101 ± 26^b	29 ± 3^b	196 ± 12^a	30 ± 1^b	57 ± 5^a
White rye malt							
Free vitamins	48 ± 2^a	147 ± 5^a	649 ± 146^a	0^a	241 ± 29^a	31 ± 6^a	80 ± 3^a
Enzymatic extraction	177 ± 7^b	193 ± 8^b	460 ± 25^b	64 ± 5^b	276 ± 10^a	62 ± 6^b	120 ± 5^b
Red rye malt							
Free vitamins	86 ± 27^a	112 ± 4^a	1138 ± 61^a	175 ± 7^a	NA	ND	49 ± 2^a
Enzymatic extraction	94 ± 9^a	110 ± 1^a	1176 ± 109^a	146 ± 21^a	NA	ND	117 ± 7^b
Yeast							
Free vitamins	2869 ± 80^a	1334 ± 27^a	4855 ± 76^a	1718 ± 21^a	5942 ± 135^a	198 ± 25^a	25 ± 1^a
Enzymatic extraction	4455 ± 719^b	2991 ± 218^b	9003 ± 113^b	3326 ± 11^b	8765 ± 91^b	214 ± 41^a	15 ± 1^b
Rye bread							
Free vitamins	$129 \pm 6^{a\,*}$	$50 \pm 2^{a\,*}$	210 ± 16^a	$431 \pm 31^{a\,*}$	151 ± 9^a	$10 \pm 1^{a\,*}$	$42 \pm 1^{a\,*}$
Enzymatic extraction	$139 \pm 5^{a*}$	89 ± 2^b	117 ± 24^b	$438 \pm 31^{a*}$	168 ± 13^a	$11 \pm 2^{a*}$	73 ± 2^b
Free vitamins, calculated	209 ± 7	65 ± 3	200 ± 23	35 ± 2	168 ± 4	23 ± 3	66 ± 2
Enzymatic extraction calculated	233 ± 5	85 ± 3	156 ± 10	41 ± 5	162 ± 13	31 ± 3	74 ± 2
Fine rye bread							
Free vitamins	$98 \pm 3^{a*}$	$30 \pm 1^{a*}$	$88 \pm 10^{a*}$	$502 \pm 9^{a*}$	$132 \pm 4^{a*}$	$8 \pm 1^{a*}$	$24 \pm 2^{a*}$
Enzymatic extraction	$140 \pm 6^{b*}$	$82 \pm 5^{b*}$	57 ± 5^b	$535 \pm 48^{a*}$	135 ± 8^a	$14 \pm 2^{b*}$	43 ± 3^b
Free vitamins, calculated	176 ± 2	49 ± 1	151 ± 15	26 ± 1	142 ± 3	17 ± 1	48 ± 1
Enzymatic extraction calculated	181 ± 2	165 ± 1	108 ± 4	39 ± 1	136 ± 3	24 ± 1	50 ± 1
Irradiated rye flour without L. panis N915, 0 h							
Free vitamins	199 ± 54^a	50 ± 4^a	259 ± 16^a	9 ± 2^a	396 ± 78^a	38 ± 9^a	100 ± 14^a
Enzymatic extraction	218 ± 7^a	50 ± 3^a	164 ± 6^b	0^b	349 ± 37^a	55 ± 4^a	94 ± 4^a
Irradiated rye flour without L. panis N915, 24 h							
Free vitamins	259 ± 17^a	45 ± 2^a	479 ± 55^a	19 ± 3^a	559 ± 40^a	32 ± 4^a	97 ± 8^a
Enzymatic extraction	246 ± 5^a	45 ± 2^a	344 ± 23^b	11 ± 3^b	520 ± 34^a	40 ± 8^a	101 ± 3^a
Irradiated rye flour with L. panis N915, 24 h							
Free vitamins	242 ± 5^a	46 ± 5^a	80 ± 1^a	613 ± 39^a	223 ± 6^a	31 ± 6^a	88 ± 4^a
Enzymatic extraction	225 ± 6^b	134 ± 35^b	56 ± 16^a	386 ± 13^b	215 ± 13^a	31 ± 3^a	96 ± 5^a

NA – Not available ND – Not detected.
Mean values of the vitamins in flours or rye bread with and without enzymatic treatment followed by a different superscript letter are significantly different at $p < 0.05$.
* – experimental value is different from calculated value ($p < 0.05$).

treatment with an enzyme mixture containing β-glucosidase from almonds did not increase pyridoxal and pyridoxine concentration in the reference flour. The concentration of pyridoxamine could not be adequately determined due to its very low retention under the chromatography conditions used and low concentration.

For vitamins B2, B3 and B5 the certificated values for standard material BCR 121 were not available and thus could not be compared with our results.

The concentration of thiamine was the highest in steel cut rye, whole grain rye flour and dark rye flour, remaining in the range of 235–246 µg/100 g DM. Fine rye flour and wheat flour contained less free thiamine (185 µg/100 g DM). In red rye malt and white rye malt, the concentration of thiamine (85 µg/100 g DM and 47 µg/100 g DM, respectively) was significantly lower than in whole grain rye flour.

Adding the enzymes to the extraction medium increased the concentration of thiamine up to 30%. The effect of enzymes was statistically significant for steel cut rye, dark rye flour, white rye malt, fine rye bread; however, it was not statistically significant for whole grain rye flour, fine rye flour, red rye malt, and rye bread.

Notably, the expected concentration of thiamine in rye bread (calculated based on the recipe and the respective thiamine content in the ingredients used) was 60% higher than that determined experimentally.

The concentration of riboflavin in whole grain rye flour and steel cut rye remained 82–96 µg/100 g DM, 67 µg/100 g DM in dark rye flour, 50 µg/100 g DM in fine rye flour and 40 µg/100 g DM in wheat flour. Enzymatic extraction increased the concentration of riboflavin significantly (10–35%) in both rye flour and rye bread samples. Notably, in wheat flour it was not possible to accurately measure the concentration of riboflavin using enzymatic extraction because of interfering peptides. The concentration of riboflavin in the rye bread was 50 µg/100 g without and 89 µg/100 g with enzymatic extraction. The theoretical riboflavin concentration,

predicted on the basis of recipe, in rye bread was 30% higher than the experimentally measured concentration.

The concentration of pantothenic acid in steel cut rye (about 300 µg/100 g DM) was higher than in rye flours (122–241 µg/100 g DM). The enzyme extraction of samples had no effect on pantothenic acid concentration. The concentration of pantothenic acid calculated from the recipe was similar to that determined experimentally.

Similar to the results obtained on the reference sample, the sum of pyridoxal and pyridoxine in the flours and breads was lower than has been reported in the literature (Table 4). The concentration of pyridoxal in flours and rye breads was 8–31 µg/100 g DM and that of pyridoxine was 24–115 µg/100 g DM. Applying enzymatic treatment increased the concentration of pyridoxal and pyridoxine in the flour samples to some extent, most likely due to the dephosphorylation of corresponding phosphates. For malt samples, the enzymatic treatment had a more pronounced effect (up to 50–60%). Recipe based contents of pyridoxine and pyridoxal in rye bread were 36–59% higher than those determined experimentally, suggesting that both vitamers were unstable during processing.

The concentration of nicotinic acid and nicotinamide in whole grain rye flour and steel cut rye were about 250–300 and 15–50 in µg/100 g DM, respectively. Notably, while the concentration of nicotinamide increased with enzyme treatment, the concentration of nicotinic acid decreased by a considerable extent. The concentration of nicotinic acid in white and red rye malt was significantly higher than that in flours: 650 and 1140 µg/100 g DM, respectively. This can be explained by the increase of nicotinic acid concentration during malting. Interestingly, after rye bread processing the nicotinamide concentration (430 µg/100 g DM) was significantly higher than the theoretically calculated value (34 µg/100 g DM), while the concentration of nicotinic acid did not change significantly. This increase in the concentration of nicotinamide observed

Table 4
The concentration of B-complex vitamins and dietary fiber in flours and breads from different data sources.

Name	Reference	B1	B2	B3	B5	B6	Insoluble DF	Soluble DF	Total DF
		µg/100 g of edible portion					g/100 g of edible portion		
Rye									
Crushed rye grain	Finnish food composition database FINELI	280	100	ND	ND	280	18.1	2.2	18.1
Rye kernels	Danish Food Composition Databank	359	152	1680	1340	234	–	–	14.8
Rye	USDA Nutrient Database for Standard Reference	316	251	4270	1456	294	–	–	15.1
Dark rye flour							–	–	–
Dark rye flour	Finnish food composition database FINELI	300	200	400	–	160	10.8	2.1	9.4
85% extraction	Danish Food Composition Databank	330	82	–	430	–			10.5
Dark rye flour	USDA Nutrient Database for Standard Reference	316	251	4270	1456	443	–	–	23.8
Medium rye flour		287	114	1727	492	268	–	–	11.8
Fine rye flour									
70% extraction	Danish Food Composition Databank	234	49	–	370	–			4.4
Light rye flour	USDA Nutrient Database for Standard Reference	287	90	800	492	234	–	–	8
Rye bread									
Rye bread, 51% rye	Finnish food composition database FINELI	150	120	600	–	–	6.5	1.4	8.1
Rye bread		190	150	400	–	–	8.5	1.6	11.0
Rye dark bread	Danish Food Composition Databank	191	69	850	600	161	–	–	8.6
Rye dark, wholemeal bread		110	54	470	600	130	–	–	6.1
Rye bread	USDA Nutrient Database for Standard Reference	434	335	3805	440	75	–	–	5.8

during rye bread processing can be attributed to lactic acid fermentation.

Notably, compared to the 10 kGy irradiated control sample without LAB the concentration of nicotinamide increased during the fermentation of the flour by lactic acid bacteria (*L. panis* N915) nearly 60 times and that of nicotinic acid and pantothenic acid decreased about 3 and 1.7 times, respectively (Table 3). Such changes were not observed during lactic acid fermentation for the other vitamers.

4. Discussion

In sourdough fine rye breads, the experimentally measured total dietary fiber was higher than that calculated on the basis of the raw materials and the bread recipe. The increase in total dietary fiber during bread processing has been observed previously. Johansson et al. (1984) observed the increase of total dietary fiber content in wheat and rye breads made from low extraction flour and found that this was caused by the formation of resistant starch during baking. The increase in soluble dietary fiber content during bread processing observed in this work can be explained by the partial conversion of insoluble fiber into soluble during rye flour fermentation, scalding, as well as during leavening with yeast. Such redistribution of dietary fiber forms by the activities of intrinsic enzymes in rye flour (α − amylase, β − xylosidase, α − arabino-furanosidase, β-glucanase, endo-xylanase and cinnamoyl esterase) was also suggested by Boskov Hansen et al. (2002).

The decrease in the concentration of specific vitamers during rye sourdough bread processing was significant for thiamine, riboflavin and pyridoxine. In general, the loss of those vitamers during processing, much like degradation at higher temperatures applied during baking, was similar to that observed earlier (Batifoulier et al., 2005, 2006; Martinez-Villaluenga et al., 2009). The lower concentration of thiamine in the red and white rye malt than in grain can be explained by losses of thiamine during the malt production processes, where kilning at high temperatures with air-flow and removal of dried sprouts reduce its content remarkably.

The nutritional databases report significantly higher vitamin B3, B5 and B6 content in cereals than were determined in this work (Tables 3 and 4). This could be due to various reasons. First, the data in the databases derives from several sources and is usually analyzed by different extraction and analysis methods. For example, Kall (2003) showed that vitamin B6 content in foods

obtained with a microbiological assay and HPLC was systematically different and the variance of the results depended mainly on the extraction method and the sample matrix. Second, grains from different geographic locations may vary in vitamer content.

In order to determine whether vitamin content determined in the present work for the cereal products was affected by insufficient or unsuitable enzymatic activities of the enzymes in the extraction solution, we studied the effect of enzyme preparations: α-amylase, β-glucosidase, and acid phosphatase on the recovery of thiamine, riboflavin, nicotinamide and pantothenic acid from their different cofactors: TMP, TDP, FMN, FAD, NAD$^+$, NADH, and CoA (Table 5). More than 90% recovery of thiamine (from TMP and TDP) and riboflavin (from FMN) was observed in case of all enzyme preparations, suggesting their sufficient acid phosphatase and acid pyrophosphatase activities. The α-amylase also had significant proteolytic activity which could cause overestimation of riboflavin in the case of flour samples (including the reference sample) due to formation of peptides with similar m/z to that of riboflavin. This was, in particular, the case for wheat flour riboflavin analysis where the α-amylase treatment increased the riboflavin content by 20 fold. We have modeled the formation of possible peptide fragments with [M + H]$^+$ = 377.15 +/− 0.5 Da from various wheat proteins (gliadins, glutenins, avenin-like proteins) using the UniProt program (www.uniprot.org) and ExPASy Bioinformatics Resources Portal (www.expasy.com) and analyzed their hydrophobicity and retention time in a C18 reversed phase column. As a result, several tetrapeptides, such as TSAV ([M + H]$^+$ 377.203), LSAS ([M + H]$^+$ 377.203), ISGT ([M + H]$^+$ 377.203), VCGV ([M + H]$^+$ 377.18), STGL ([M + H]$^+$ 377.203), and tripeptide QVM ([M + H]$^+$ 377.185) were shown to be possible interfering compounds for riboflavin analysis.

The recovery of nicotinic acid and nicotinamide from NAD$^+$ and NADH was less than 10%, even with treatment by enzyme preparations. This can be explained by the absence of purine nucleosidase activities in the enzyme preparations used. This was also strongly supported by the increased intensities of N-ribosyl nicotinamide peaks, observed in the mass spectrum of β-glucosidase and α-amylase treated NAD$^+$. Interestingly, instead of N-ribosyl nicotinamide, N-ribosyl nicotinic acid was observed in the mass spectra of NAD$^+$ treated with acid phosphatase (Table 5). N-ribosylnicotinic acid was also found in enzymatic extracts of the cereal samples. This observation could be evidence that our enzyme preparations exhibit potato nicotinamide riboside deaminase activity, which catalyzes the conversion of nicotinamide riboside

Table 5
Enzymatic activities and molar recovery (%) of vitamer subunits from Co-factors.

	Acid phosphatase (0.12 mg/mL)	β-Glucosidase (1.2 mg/mL)	α-Amylase (5.0 mg/mL)	0.1 N HCl 121 °C
Proteolytic activity[a]	50 U/mg	0.12 U/mg	2804 U/mg	−
Thiamine recovery, %				
From TMP	101	88	95	10
From TPP	96	105	105	9
Riboflavin recovery, %				
From FMN	98	102	92	6
From FAD	93	106	95	7
Nicotinamide recovery, %				
From NAD	0	9	10	91
From NADH	6	4	13	6
Nicotinamide riboside recovery, %				
From NAD	+(Nicotinic acid riboside)	+	+	ND
From NADH	+(Reduced form of nicotinic acid riboside)	+(Reduced form)	+(Reduced form)	ND
Pantothenic acid recovery, %				
From Coenzyme-A	0	0	0	0
Pantetheine recovery, %				
From Coenzyme-A	+	+	+	NT

+ − identified in the mass spectra by the m/z ratio.
ND − not detected; NT − not tested.
[a] Protease (side) activity in the enzyme preparations used (expressed as BAEE units of trypsin).

into nicotinic acid riboside (Katahira and Ashihara, 2009). Unfortunately, due to the unavailability of nicotinamide and nicotinic acid riboside standards, the recovery values of this vitamer from NAD$^+$ could not be determined. Acid treatment (0.1 M HCl, 121 °C, 30 min) liberated 90% of nicotinamide from NAD$^+$ by cleavage of the N-glycosidic bond. This can explain the higher values for niacin reported in the literature since the standard method usually involves the extraction in 0.1 M HCl at 100 °C for 60 min (EN 14663:2006). On the other hand, the ability of human GI to utilize NAD$^+$ as vitamin B3 is unclear.

The concentration of nicotinamide increased with lactic acid fermentation of the rye dough while that of nicotinic acid decreased. This process cannot be related to direct conversion of nicotinic acid into nicotinamide because this reaction is thermodynamically irreversible under physiological conditions. According to the KEGG metabolic pathways (www.kegg.com), nicotinic acid (and NAD$^+$) can be converted into nicotinamide by lactobacilli over a series of enzymatic reactions. This microbial conversion, as well as the acidic conversion of NAD$^+$ into B3 vitamers in the gastrointestinal (GIT) requires a separate study.

Pantothenic acid as a chemical compound could not be recovered by enzymatic treatment from Coenzyme A. However, all enzyme preparations resulted in liberation of pantetheine, which has been shown to have vitamin B5 activity (Aguilar et al., 2008). Again, due to the unavailability of isotope labeled pantetheine, the vitamer recovery could not be taken into account which, in turn, may have caused the underestimation of vitamin B5 (pantothenic acid) content in our study.

The concentration of pyridoxine + pyridoxal determined in our work was more than 40% lower than the B6 content reported in databases as well as for the reference material. The effect cannot be explained by the fact that pyridoxamine could not be quantified under the chromatographic conditions used because the content of pyridoxamine in flour has been reported to be only 10—12% of the total B6 content (Polansky and Toepfer, 1971). A more plausible explanation for lower B6 content is poor hydrolysis of glycosylated pyridoxine. The latter is common in grains and may account for as much as 56% of total B6 in wheat cultivars (Sampson et al., 1995). It is possible that, in contrast to the standard methods (EN 14663:2006) which use the HCl extraction at 121 °C, the enzyme mixture used in this work was unable to convert 5′-O-β-D-glucopyranosyl pyridoxine into pyridoxine. Notably, the pyridoxine glucoside serves as a source of nutritionally available vitamin B6, with ~50% bioavailability relative to pyridoxine (Nakano et al., 1997).

5. Conclusion

Our results demonstrate that theoretical concentrations of dietary fiber and vitamers calculated on the basis of raw ingredients can differ significantly from concentrations measured in baked rye bread. We attribute this difference to biochemical and microbiological processes that occur during bread making.

The LC—MS method we employ, combined with the stable isotope dilution assay, is a promising method both for the simultaneous analysis of B-complex vitamins, and for studying their stability during food processing. However, for a complete analysis of the B-group vitamins in food using this method, additional labeled vitamers or their precursors should be incorporated in the internal standard solutions and/or extraction steps should be further optimized.

Acknowledgments

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PUBLICATION IV

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Comparison of different extraction methods to determine free and bound forms of B-group vitamins in quinoa

RESEARCH PAPER

Comparison of different extraction methods to determine free and bound forms of B-group vitamins in quinoa

Kristel Hälvin · Ildar Nisamedtinov · Toomas Paalme

Abstract The distribution of free and bound forms of B-group vitamins (B_1, B_2, B_3, B_5, and B_6) was quantified in quinoa seeds using LC-MS-TOF combined with a stable isotope dilution assay. The effectiveness of liberating thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxal, and pyridoxine from the food matrix and cofactors was evaluated for a variety of extraction conditions, including the addition of enzymes. Phosphatase and protease inhibitors, as well as ultrafiltration, were evaluated for their ability to suppress vitamer liberation via enzymes endogenous to quinoa. Cold extraction, together with a mixture of phosphatase and protease inhibitors, is identified as the most efficient treatment to prevent the conversion of cofactors into simple vitamers. Overnight incubation at 37 °C both with and without additional hydrolytic enzyme preparations containing phosphatase and β-glucosidase activity was almost equally effective in releasing the bound forms of the vitamers. This indicates that the endogenous enzymes within quinoa seeds have high activity. β-Glucosidase should be used when the total pyridoxine content is to be determined, and thermal treatment followed by enzymatic treatment with phosphatase activity is recommended to liberate the bound forms of pyridoxal prior to quantification.

Keywords B-group vitamins · Isotope dilution mass spectrometry · Liquid chromatography · MS-TOF · Quinoa

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Introduction

Vitamins are a heterogeneous group of organic compounds that are essential for the normal functioning of living organisms. The B-group vitamins typically function as either a reserve pool or direct precursor for the synthesis of coenzymes. They are not synthesized in mammals and, thus, must be obtained from our daily diet. Each particular group of B vitamins consists of chemically different compounds with similar biological activity and are often called vitamers. Vitamers can exist either in free forms as simple vitamers, e.g., thiamine, and cofactors, e.g., thiamine diphosphate, or bound forms, e.g., bound chemically to proteins or carbohydrates. The bioavailability of the bound forms and cofactors in food strictly depends on how well they are liberated by digestive enzymes into forms that can be absorbed in the gastrointestinal tract. Because different B-group vitamers may vary in both their bioactivity and bioavailability, accurate quantification of individual vitamers should be carried out in order to evaluate their overall nutritional value in food [1].

Selection of effective extraction and determination methods are the key factors that enable accurate and precise vitamin analysis in foods. B-group vitamins are commonly extracted from food by acid and enzymatic hydrolysis, followed by liquid chromatographic (LC) separation with ultraviolet (UV) absorbance or fluorescence detection [2–7] and are optimized to determine the "total" content of vitamins. Coupling LC with mass spectrometry (MS) and stable isotope dilution assay (SIDA) approach allows one to determine the individual forms of vitamers and cofactors from the same sample in a single chromatographic run [8]. Nevertheless, despite the high sensitivity and selectivity achieved with LC-MS-SIDA, accurately determining the distribution of B-group vitamers, i.e., simple vitamers, cofactors, and their precursors, in foods can be quite challenging due to the presence of active endogenous enzymes which may degrade the structures of the

vitamers and their complexes during sampling and extraction. False estimation of the actual vitamer distribution can be avoided by suppressing or inhibiting the activities of endogenous enzymes. Various methods have been used for sample fixation and extraction, e.g., using enzyme inhibitors [9], thermal treatment [10], ultrasonication [11], and microwave heating [12].

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal that has been cultivated in the Andean region of South America for more than 7,000 years. In Northern Europe, interest in cultivating quinoa has grown in recent years due to its high nutritional value, including the abundance of B-group vitamins [13]. Quinoa is either used in a similar manner as cereal crops or as a food additive to increase the nutritional value of different foods [14]. Due to its high B-group vitamin content and a wide range of endogenous enzymes (e.g., amylase and both alkaline and acid phosphatases) [15], quinoa is a perfect model food matrix to study and optimize methods to determine the B-group vitamer distribution in food. The aim of the present work is to evaluate the effect of different sample pre-treatment processes while determining the distribution of free and bound forms of five B-group vitamins (B$_1$, B$_2$, B$_3$, B$_5$, and B$_6$) in quinoa using LC-MS-SIDA.

Materials and methods

Chemicals

Unlabelled standards of nicotinamide (99.9 %), nicotinic acid (99 %), thiamine-HCl (99 %), pyridoxine-HCl (99.9 %), pyridoxal-HCl (99 %), Ca-pantothenate (99 %), and riboflavin (99 %) were purchased from Sigma-Aldrich (Steinheim, Germany). The stable isotope-labelled standards of nicotinamide-[D$_4$, 98 %], nicotinic acid-[D$_4$, 98 %], thiamine-HCl-[^{13}C$_3$, 99 %], and pyridoxine-HCl-[^{13}C$_4$, 99 %] were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA); Ca-pantothenate-[^{13}C$_3$^{15}N, 98 %], riboflavin-[^{13}C$_4$^{15}N$_2$, 98 %], and pyridoxal-HCl-[D$_3$, 99 %] were obtained from Isosciences, LLC (King of Prussia, PA, USA). Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals Ltd. (Walkeburn, Scotland, UK), while formic acid (LC-MS grade), ammonium formate (puriss. p.a., for HPLC), and hydrochloric acid (puriss. p.a., ACS) were all purchased from Sigma-Aldrich. High purity water was produced by a Millipore water purification system (Millipore S.A.S., Malsheim, France).

Enzymes, enzyme inhibitors, and ultrafiltration tools

β-Glucosidase from almonds (crude, G0395) and acid phosphatase from potato (P1146) were purchased from Sigma-Aldrich. Phosphatase inhibitor (88667SPCL) and protease inhibitor (88666SPCL) were sourced from Thermo Scientific (Rockford, IL, USA). Centrifugal filters Amicon Ultra-0.5 and Ultracel-10 Membrane (UFC501008, cutoff 10 kDa) were purchased from Millipore (Malsheim, France).

Samples

Dried quinoa seeds were kindly provided by Vis vitalis, LLC (Salzburg, Austria). Standard reference material (SRM) 3234 Soy Flour was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

Standard preparation and calibration

Standard solutions were prepared as described previously by Hälvin et al. [16] with some modifications. A working external standard mixture containing 1.0 mg/L of nicotinamide, nicotinic acid, pantothenic acid, pyridoxal-HCl, pyridoxine-HCl, thiamine-HCl, and riboflavin was prepared and further serial dilutions were made (0.5, 0.2, 0.1, 0.05, 0.025, and 0.01 mg/L). An internal standard solution mixture containing the respective isotope-labelled vitamers 20 mg/L of [^{13}C$_3$] thiamine-HCl, [^{13}C$_4$^{15}N$_2$] riboflavin, [D$_4$] nicotinamide, [D$_4$] nicotinic acid, [^{13}C$_3$^{15}N] Ca-pantothenate, [^{13}C$_4$] pyridoxine-HCl, and [D$_3$] pyridoxal-HCl was prepared. All standard solutions were prepared with extraction buffer (0.05 M ammonium formate buffer, pH 4.5).

The concentration of each vitamer in the sample was calculated using seven-point calibration curves. Calibration curve standards were prepared by adding 980 µL aliquots of each external standard mixture with varying concentrations (1.0, 0.5, 0.2, 0.1, 0.05, 0.025, and 0.01 mg/L) into amber LC-MS vials containing 20 µL of stable isotope-labelled internal standard solution (20 mg/L). Thus, the final concentration of each isotope-labelled vitamer in the vials was 0.4 mg/L. The calibration curves were composed by relating the concentrations of unlabelled external standards to their relative response factors, as determined by the ratio of the peak intensity of the unlabelled external standards to that of the corresponding labelled internal standards.

Sample preparation

Sample preparation was carried out as described by Hälvin et al. [16] using various modifications described below.

Fine ground samples (0.2 g) of quinoa seeds were weighed into 10-mL volumetric flasks; 0.2 mL of the internal standard solution mixture containing the respective isotope-labelled vitamers (20 mg/L of each vitamer) was added, filled to the volume with extraction buffer, and mixed thoroughly. The various sample pre-treatment processes are described below and summarized in Table 1.

Table 1 Sample pre-treatment processes

Treatment regime	Protease inhibitor	Phosphatase inhibitor	Extraction temp, °C	Sonication (5 min)	Extraction time, min	Ultrafiltration, 10 kDa	Filtration (0.2 μm)	Acid phosphatase (0.1 mg/mL)	β-glucosidase (1 mg/mL)	Incubation 37 °C, 18 h
Effect of extraction conditions										
C	−	−	4	−	<10	−	−	−	−	−
E	−	−	20	−	<10	−	−	−	−	−
S	−	−	20	+	<10	−	−	−	−	−
E_{1h}	−	−	20	−	60	−	−	−	−	−
T^{70}	−	−	70	−	30	−	−	−	−	−
E_{18h}	−	−	20	−	<10	−	−	−	−	+
T^{70}_{18h}	−	−	70	−	30	−	−	−	−	+
$E_{F\ 18h}$	−	−	20	−	<10	−	−	+	−	+
$E_{\beta\ 18h}$	−	−	20	−	<10	−	−	−	+	+
T^{100}_{18h}	−	−	100	−	30	−	−	−	−	+
$T^{100}_{\beta\ 18h}$	−	−	100	−	30	−	−	−	+	+
Effect of inhibitors										
$C_{Fi\ Pi}$	+	+	4	−	<10	−	−	−	−	−
S_{Fi}	−	+	20	+	<10	−	−	−	−	−
S_{Pi}	+	−	20	+	<10	−	−	−	−	−
$S_{Fi\ Pi}$	+	+	20	+	<10	−	−	−	−	−
Effect of filtration										
S_U	−	−	20	+	<10	+	−	−	−	−
$S_{Fi\ Pi\ U}$	+	+	20	+	<10	+	−	−	−	−
$C_{M\ 18h}$	−	−	20	−	<10	−	+	−	−	+
$S_{M\ 18h}$	−	−	20	+	<10	−	+	−	−	+
$S_{U\ 18h}$	−	−	20	+	<10	+	−	−	−	+
$S_{U\ F\ 18h}$	−	−	20	+	<10	+	−	+	−	+
$S_{Fi\ Pi\ U\ 18h}$	+	+	20	+	<10	+	−	−	−	+

The following extraction conditions were studied to determine their effectiveness: cold extraction at 4 °C for 5 min (C), extraction at 20 °C for 5 min (E) and 1 h (E_{1h}), extraction at 20 °C for 5 min with sonication (S), thermal extraction at 70 °C (T^{70}) and at 100 °C (T^{100}). Cold extraction (C) was performed in an ice bath using 3×10 s vortexing cycles, followed by immediate filtration and analysis (total contact time, 5 min). Samples E, T^{70}, and T^{100} were additionally incubated overnight (18 h) at 37 °C under subdued lighting conditions using continual shaking to form sample extracts E_{18h}, T^{70}_{18h}, and T^{100}_{18h}, respectively. The effect of enzyme addition on the liberation of vitamers from their bound forms and cofactors was studied by adding 2 mL of acid phosphatase (0.5 mg/mL in an extraction buffer) or β-glucosidase (5 mg/mL in an extraction buffer) to finely ground samples before incubation (samples $E_{F\ 18h}$ and $E_{\beta\ 18h}$, respectively). In addition, β-glucosidase was also added to high temperature extracted sample ($T^{100}_{\beta\ 18h}$).

The effect of inhibiting enzymes endogenous to quinoa on the distribution of vitamers in the sample was studied by adding phosphatase or protease inhibitors (one tablet of inhibitor dissolved in 2 mL extraction buffer) or both to the finely ground samples followed by extraction using sonication at room temperature for 5 min (samples S_{Fi}, S_{Pi}, and S_{FiPi}, respectively). Phosphatase and protease inhibitors were also added to the cold extracted sample ($C_{Fi\ Pi}$).

The effect of ultrafiltration (cutoff 10 kDa) on removal of endogenous enzymes was studied on the sample extracted using sonication at room temperature for 5 min or the sample containing phosphatase and protease inhibitors (samples S_U and $S_{Fi\ Pi\ U}$, respectively). Ultrafiltration was performed using $14,000 \times g$ for 5 min at room temperature. Sample extracts S_U and $S_{Fi\ Pi\ U}$ were additionally incubated overnight (18 h) at 37 °C under subdued lighting conditions using continual shaking to produce extracts $S_{U\ 18h}$ and $S_{Fi\ Pi\ U\ 18h}$, respectively.

We also studied the effect of microfiltration to remove the bound forms of vitamers. The sample extracted at room temperature was centrifuged ($18,000 \times g$ for 5 min at room temperature) and then microfiltered through a Millex-LG 13 mm Philic PTFE 0.2 μm filter (Millipore, Ireland). Aliquots of this filtrate were then incubated overnight (18 h) at 37 °C under

subdued lighting conditions using continual shaking (sample $S_{M\ 18h}$). A similar procedure was also carried out with the cold extracted and microfiltered sample extract (sample $C_{M\ 18h}$).

Determination of the phosphate-bound forms of vitamers in the ultrafiltered sample extracts was carried out by adding 200 μL of acid phosphatase solution (0.1 mg/mL of acid phosphatase in an extraction buffer) to 200 μL of ultrafiltered sample extract followed by overnight incubation (18 h) at 37 °C under subdued lighting conditions using continual shaking (sample $S_{U\ F\ 18h}$).

LC-MS-TOF analysis

Two milliliters of each sample extract was centrifuged (18,000×g for 5 min at room temperature) and microfiltered (0.2 μm) to remove the insoluble matter. One-milliliter aliquots of this filtrate were then transferred into amber LC-MS vials and injected into the LC-MS-TOF instrument.

The LC-MS-TOF analyses were performed as described by Hälvin et al. [16]. An ACQUITY UPLC® system coupled with LCT Premier™ XE ESI TOF MS (Waters, Milford, MA, USA) with an ACQUITY UPLC HSS C-18 1.8-μm (2.1× 150 mm) column was used. Elution was carried out using water+0.1 % formic acid and acetonitrile+0.1 % formic acid gradient: 0–3 min 100 % A, 3–8.5 min 80 % A and 20 % B, 8.5–10 min 5 % A, and 95 % B, 10–15 min 100 % A at a flow rate of 0.25 mL/min. Mass spectrometry analysis was carried out in a positive electrospray ionization mode.

The following ions were detected: thiamine $[M]^+$=265.11, labelled thiamine $[M]^+$=268.12, riboflavin $[M+H]^+$=377.15, labelled riboflavin $[M+H]^+$=383.16, nicotinamide $[M+H]^+$= 123.06, labelled nicotinamide $[M+H]^+$=127.08, nicotinic acid $[M+H]^+$=124.04, labelled nicotinic acid $[M+H]^+$=128.06, pantothenic acid $[M+H]^+$=220.12, labelled pantothenic acid $[M+H]^+$=224.13, pyridoxal $[M+H]^+$=168.07, labelled pyridoxal $[M+H]^+$=171.09, pyridoxine $[M+H]^+$=170.08, labelled pyridoxine $[M+H]^+$=174.10 m/z. Full scan mass spectra were acquired in the range of 100 to 1,000 m/z. Data were collected and reprocessed using Mass Lynx 4.0 software (Waters, Milford, MA, USA).

Method validation

The method was validated by analyzing SRM 3234—soy flour with the two most efficient extraction methods—cold extraction with inhibitors and overnight incubation with endogenous enzymes. Soy flour 0.5 g (n=5) was weighed into 10-mL volumetric flasks and analyzed as described above.

The method was also validated for quinoa matrix by determining the detection and quantification limits (LOD and LOQ, respectively) as well as the precision and accuracy of the method using the two most efficient extraction methods. The LOD values for both samples were determined in three

replicates and were defined as the amount of a given vitamer which produced an intensity signal three times higher than the noise of the MS chromatogram baseline of a respective sample (S/N ratio=3) after estimating the natural amount of vitamer in a given matrix [17]. The LOQ values were defined as concentrations which were three times higher than their respective LOD values.

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was carried out by performing six repeated analyses of the samples on the same day, while the intermediate precision of the method was assessed using samples that were analyzed on four different days over a period of 2 months under the same experimental conditions.

Accuracy was evaluated by determining the recovery of the method by spiking the samples with a known amount of vitamers at three different concentration levels. The recovery was calculated as follows:

$$R, \% = \frac{C_{\text{spiked}}}{C_{\text{unspiked}} + C_{\text{added}}} \times 100$$

where C_{spiked} is the amount of vitamer determined after spiking the sample, C_{unspiked} is the amount of vitamer in the sample before spiking, and C_{added} is the amount of vitamer added to the sample. Shortly, 1 mL aliquots of vitamers standard solution at three different concentrations (1, 2.5, and 5 mg/L) were added to 0.2 g of sample (n=3) already containing 0.2 mL of internal standard solution and, in the case of $C_{Fi\ Pi}$, protease and phosphatase inhibitors filled to the volume (10 mL) with extraction buffer, mixed thoroughly, and subjected to the sample treatment procedures as described above for samples $C_{Fi\ Pi}$ and E_{18h}.

Results

The effect of extraction conditions

In order to study the extraction efficiency of B-group vitamers from finely ground quinoa seeds, the following extraction conditions were tested: cold extraction at 4 °C for 5 min (C), extraction at 20 °C for 5 min (E) and 1 h (E_{1h}) at 20 °C for 5 min with sonication (S), and thermal extraction for 30 min at either 70 or 100 °C (T^{70} and T^{100}, respectively). Each sample was injected into the LC-MS-TOF immediately after either extraction (C, E, E_{1h}, S, T^{70}) or after overnight incubation with acid phosphatase ($E_{F\ 18h}$) or β-glucosidase ($E_{\beta\ 18h}$, $T^{100}_{\beta\ 18h}$) or without additional enzymes (E_{18h}, T^{70}_{18h}, T^{100}_{18h}).

The concentrations of B-group vitamers were determined after each extraction method and summarized in Fig. 1. It is
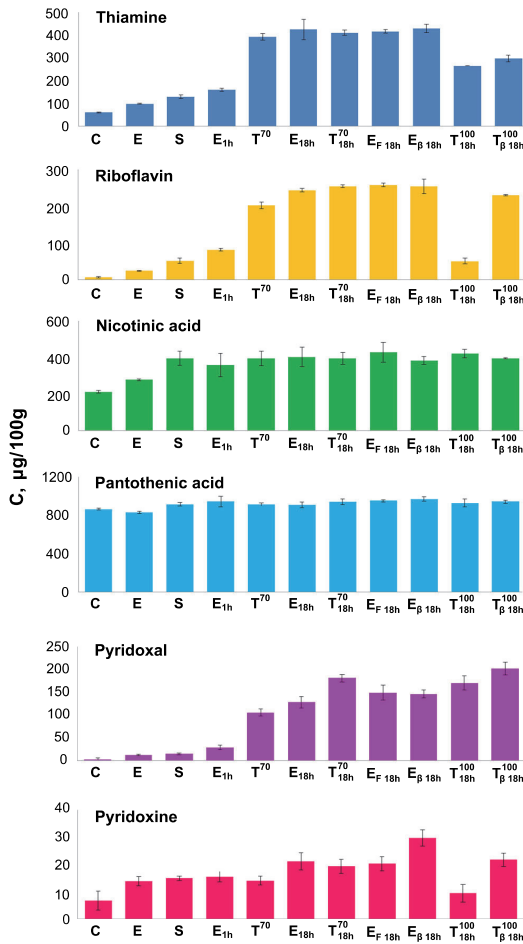
Fig. 1 The effect of extraction conditions on the determined concentration of B-group vitamers in quinoa seeds ($n=2$–4) (see Table 1 for abbreviations describing sample pre-treatments)

clear that the extraction yield of thiamine, riboflavin, nicotinic acid, pyridoxal, and pyridoxine increased with contact time, temperature, and shear stress. The lowest concentrations were obtained with cold extraction (C). All extraction methods carried out at room temperature (samples E, S, and E_{h1}) provided higher concentrations of simple vitamers compared with the cold extraction method. Using these three methods, the concentration of thiamine increased by 1.6, 2.1, and 2.6; riboflavin by 3.8, 7.9, and 12.5; nicotinic acid by 1.3, 1.9, and 1.7; pyridoxal by 5.4, 6.5, and 13.1; and pyridoxine by 2.1, 2.2, and 2.3 times respectively. Thermal treatment at 70 °C (T^{70}) facilitated even more efficient extraction of simple vitamers. Relative to cold extraction, the concentration of pyridoxal, riboflavin, thiamine, nicotinic acid, and pyridoxine increased by 48, 31, 6, 2, and 2 times, respectively. The only exception was pantothenic acid where differences in the simple vitamer concentrations with used extraction methods were practically not seen. This indicates that the observed concentration differences between treatment methods are not related to the rate of solubilization of simple vitamers, and all free forms of vitamers are extracted during 5 min of cold extraction.

The highest concentration of vitamers was determined in samples which were extracted either at room temperature or at 70 °C and additionally incubated overnight at 37 °C (E_{18h} and T^{70}_{18h}, respectively). With the exception of pyridoxal, the vitamer concentrations determined did not differ between the two extraction methods used (one-way ANOVA, $p=0.05$). The pyridoxal concentration, however, increased by 40 % when thermal treatment was used before overnight incubation, thus indicating that some of pyridoxal or pyridoxal phosphate (PLP) may be bound in forms that are not liberated by endogenous enzymes but rather as a result of thermal treatment in an acidic environment.

Of those samples that were extracted overnight in the presence of acid phosphatase ($E_{F\ 18h}$) or β-glucosidase ($E_{β\ 18h}$), we observed no additional liberation of thiamine, riboflavin, nicotinic acid, and pantothenic acid compared with incubation in the absence of enzymes (one-way ANOVA, $p=0.05$). By contrast, the concentration of pyridoxal increased by 15 % when either acid phosphatase or β-glucosidase was used and the concentration of pyridoxine increased by 40 % when β-glucosidase was used.

Of those samples subjected to thermal extraction at 100 °C (T^{100}_{18h}) and overnight incubation, the concentration of thiamine, riboflavin, and pyridoxine decreased compared with samples extracted at a lower temperature (E_{18h}, T^{70}_{18h}, $E_{β\ 18h}$, $E_{F\ 18h}$). Even when β-glucosidase with acid phosphatase and pyrophosphatase side activity [18] was added after temperature treatment (sample $T^{100}_{β\ 18h}$), the concentrations determined were lower than the maximum concentrations observed in samples E_{18h}, T^{70}_{18h}, $E_{β\ 18h}$, $E_{F\ 18h}$. By contrast, the concentration of both nicotinic acid and pantothenic acid remained unchanged and the concentration of pyridoxal increased when subjected to high temperature extraction. The maximum concentration of pyridoxal was observed when β-glucosidase was added after temperature treatment.

The effect of enzyme inhibitors and ultrafiltration

The results of these extraction experiments suggested that continuous liberation of thiamine, riboflavin, pyridoxal, and pyridoxine from the quinoa matrix occurs as a result of the activity of its endogenous enzymes. To suppress the activity of endogenous enzymes, we studied the influence of a phosphatase inhibitor (S_{Fi}), protease inhibitor (S_{Pi}), both inhibitors ($S_{Fi\ Pi}$), or both inhibitors combined with cold extraction ($C_{Fi\ Pi}$). In another attempt to remove the activity of

endogenous enzymes, ultrafiltration was applied with and without phosphatase and protease inhibitors. The results of these experiments are shown in Fig. 2.

With exception of nicotinic acid, the addition of enzyme inhibitors had no effect on the concentration of vitamers subjected to cold extraction; the values for C and C_{FiPi} were practically the same for thiamine, riboflavin, pantothenic acid, pyridoxal, and pyridoxine. Comparing samples subjected to cold extraction, the concentration of nicotinic acid increased by 2.4 times using a combination of phosphatase and protease inhibitors.

Extraction methods that utilized 5 min of sonication at room temperature (S_{Fi}, S_{Pi}, and $S_{Fi\ Pi}$) displayed no change in the concentrations of thiamine, pantothenic acid, and pyridoxine with the addition of inhibitors (one-way ANOVA, $p=0.05$). Nicotinic acid, however, showed a 15 % lower concentration with the addition of inhibitors compared with the same extraction in the absence of inhibitors. Riboflavin and pyridoxal both displayed a more pronounced effect when

inhibitors were added. The most efficient treatment to suppress riboflavin and pyridoxal liberation by endogenous enzymes used a combination of phosphatase and protease inhibitors ($S_{Fi\ Pi}$). In this case, the concentrations of riboflavin and pyridoxal were six and two times lower, respectively, compared with the same treatment in the absence of inhibitors. The addition of phosphatase inhibitor alone (sample S_{Fi}) decreased the liberation of riboflavin while the protease inhibitor (sample S_{Pi}) acted to limit the liberation of both riboflavin and pyridoxal.

The ultrafiltration of samples S and $S_{Fi\ Pi}$ together with overnight incubation did not influence the concentration of thiamine, riboflavin, nicotinic acid, and pantothenic acid (Fig. 2) (samples S_U and $S_{U\ 18h}$; $S_{Fi\ Pi\ U}$ and $S_{Fi\ Pi\ U\ 18h}$). Surprisingly, the concentration of pyridoxal and pyridoxine increased to some extent during overnight incubation (comparing samples S_U and $S_{Fi\ Pi\ U}$ with samples $S_{U\ 18h}$ and $S_{Fi\ Pi\ U\ 18h}$). This suggests that the liberation of pyridoxal and pyridoxine

Fig. 2 The effect of enzyme inhibitors and the removal of endogenous enzymes on the determined concentration of B-group vitamers in quinoa seeds ($n=2$–4) (see Table 1 for abbreviations describing sample pre-treatments)
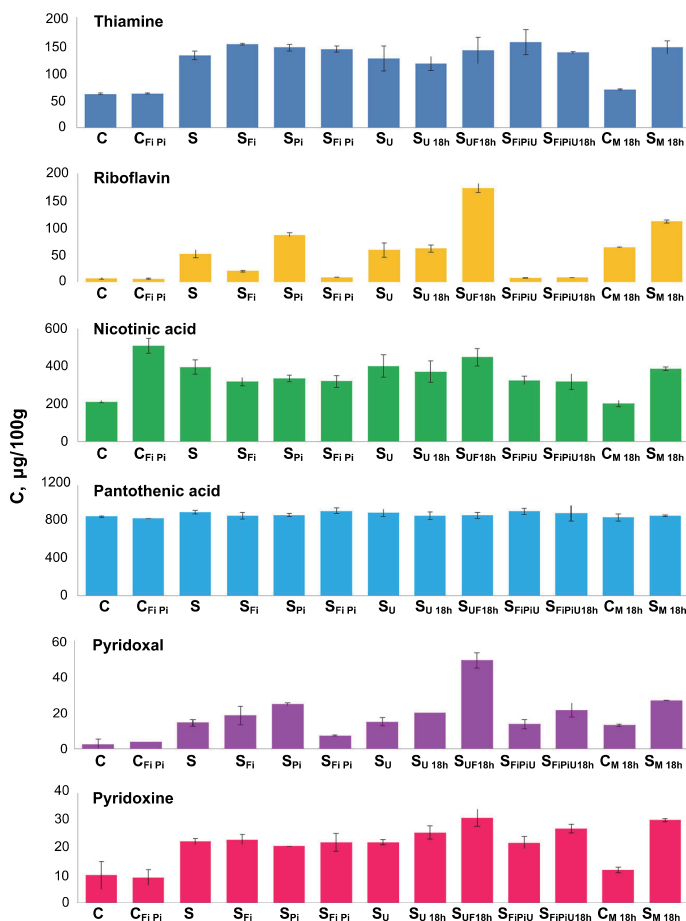
occurs during sample incubation via non-enzymatic reactions. Adding acid phosphatase to the ultrafiltered sample S_U increased the concentration of these simple vitamers. A significant increase in the concentrations of both riboflavin (3 times) and pyridoxal (2.5 times) was observed. This indicates that the endogenous phosphatase activity of quinoa seeds is insufficient to liberate riboflavin and pyridoxal from its phosphorylated forms during 5 min of sonic extraction.

Surprisingly, applying microfiltration before overnight incubation ($S_{M\ 18h}$) induced similar change in concentration compared with ultrafiltration ($S_{U\ 18h}$) for some vitamers. Comparing the overnight incubation of microfiltered and ultrafiltered samples (samples $S_{M\ 18h}$ and $S_{U\ 18h}$) with overnight incubation in the presence of endogenous enzymes (E_{18h}), the concentrations of thiamine, riboflavin, and pyridoxal decreased by 3 and 3.5, 2 and 4, and 5 and 6.5, respectively. Even lower concentrations were measured in sample subjected to cold extraction and microfiltration before overnight incubation ($C_{M\ 18h}$). The concentrations of pyridoxine, thiamine, riboflavin, pyridoxal, and nicotinic acid were 10, 6, 4, 2.5, and 2 times lower, respectively, compared with overnight incubation without filtration (E_{18h}). These results suggest that either enzymes or vitamers were bound to insoluble food matrices and were removed by microfiltration.

Validation

The performance of the LC-MS-TOF method (e.g., separation of the peaks, linear working range, and correlation coefficients of the standards) is described, in detail, by Hälvin et al. [16]. In this study, pyridoxamine could not be adequately determined due to its very low retention under the chromatographic conditions used.

The method of quantification was validated with SRM 3234 (soy flour) using the most efficient method of extracting free vitamers ($C_{Fi\ Pi}$—cold extraction with phosphatase and protease inhibitors) and total content of vitamers (E_{18h}—overnight incubation with endogenous enzymes). The results of the total vitamin concentrations correlate well with the certified data (Table 2). However, the distribution of the content of

nicotinamide and nicotinic acid in soy flour was slightly different compared with the certified data while simultaneously correlating well with the certified total content of vitamin B_3. The difference in the distribution of nicotinamide and nicotinic acid is probably related to the different sample extraction techniques applied; however, because the NIST certificate of analysis only indicates a dilute acid extraction, fundamental conclusions cannot be drawn. In addition, also, other vitamin B_3 derivatives, nicotinic acid riboside, and nicotinamide mononucleotide were observed in the mass spectra of soy flour after overnight incubation with endogenous enzymes; thus, the total concentration of vitamin B_3 in soy flour can be even higher than reported by NIST.

Validation was also performed for quinoa matrix; the method validation parameters LOD and LOQ ($\mu g/g$), recovery (%), and values for both intra-day and inter-day precision are provided in Table 3 (free vitamers) and Table 4 (total concentration of vitamers). The MS chromatograms of the studied vitamers and their respective MS spectra determined in the sample E_{18h} are shown in Fig. 3.

Discussion

Efficacy of extraction methods

In the present work, we study the effect of different sample pre-treatment processes on the concentration of free and bound forms of five B-group vitamins (B_1, B_2, B_3, B_5, and B_6) in quinoa. The lowest concentrations of thiamine, riboflavin, nicotinic acid, pyridoxine, and pyridoxal were measured in the sample subjected to short cold extraction (C). It can be speculated that those vitamers may not have been completely released from the sample matrix with cold extraction and perhaps longer extraction time, sonication, or a higher solvent temperature would result in further extraction. The addition of phosphatase and protease inhibitors to quinoa powder ($S_{Fi\ Pi}$) inhibited the extraction of both riboflavin and pyridoxal which suggests that both are liberated by endogenous enzymes

Vitamer, mg/kg dwb	Free vitamers ($n=5$)	Total content of vitamers ($n=5$)	Certified value
Thiamine	0.33±0.05	8.74±0.26	8.60±0.20
Riboflavin	0.07±0.00	3.35±0.06	3.363±0.041
Nicotinamide (NAM)	8.16±0.69	8.62±1.06	11.49±0.13
Nicotinic acid (NA)	4.13±0.54	6.90±1.35	4.59±0.17
Vitamin B_3 as NAM	12.31±0.30	15.46±2.03	16.04±0.24
Pantothenic acid	11.62±0.12	11.48±0.62	11.45±0.12
Pyridoxal (PL) HCl	0.04±0.01	1.82±0.07	1.72±0.10
Pyridoxine (PN) HCl	0.24±0.03	0.83±0.05	0.734±0.089

Table 2 The certified concentrations of B-group vitamers in the SRM 3234 and those determined by our method (free vitamers and total content of vitamers)

Table 3 Method validation parameters for free forms of vitamers

Free vitamers	LOD (µg/g) (n=6)	LOQ (µg/g) (n=6)	Recovery (%) (0.5 mg/L) (n=3)	Recovery (%) (0.25 mg/L) (n=3)	Recovery (%) (0.1 mg/L) (n=3)	Intra-day precision[a] (%) (n=6)	Inter-day precision[a] (%) (n=4)
Thiamine	0.04	0.11	102±1	106±2	104±3	5.7	8.8
Riboflavin	0.02	0.06	91±1	95±4	95±2	4.2	9.2
Nicotinamide	1.09	3.28	–	–	–	–	–
Nicotinic acid	0.69	2.08	96±9	99±14	89±6	14.4	23.4
Pantothenic acid	0.04	0.11	98±1	100±1	99±1	1.5	3.3
Pyridoxal	0.06	0.18	87±4	97±4	98±7	11.6	13.2
Pyridoxine	0.04	0.13	84±0	85±2	88±4	10.2	15.3

[a] Expressed as coefficient of variance

during extraction at room temperatures. Thus, inhibitors ought to be used when the concentration of riboflavin and pyridoxal is to be determined. By contrast, thiamine extraction was not reduced with the addition of phosphatase inhibitor. It is likely that the enzymes responsible for thiamine liberation from thiamine phosphates are insensitive to this inhibitor. This could be related to the fact that the protein responsible for thiamine phosphate hydrolysis in plants and animals is, according to the KEGG database, unknown and thus could have only slight similarity with conventional phosphatases. In case of pyridoxine, the inhibitory effect of phosphatase inhibitor was also not observed. Thus, it can be assumed that the enzymes responsible for pyridoxine liberation from pyridoxine phosphate are also insensitive to this inhibitor. In contrast with thiamine, riboflavin, pyridoxine, and pyridoxal, the most efficient method for the determination of the free form of nicotinic acid is cold extraction together with inhibitors which gave the highest result. The phosphatase inhibitors probably inhibited the activity of nicotinate phosphoribosyltransferase [EC:2.4.2.11] and, thus, prevented the conversion of NA into nicotinic acid mononucleotide [19]. Thus, cold extraction with phosphatase inhibitors ought to be used when the concentration of free nicotinic acid is to be determined. With regard to pantothenic acid, it was not possible to ascertain a method which provided the lowest concentration because neither cold extraction nor the use of inhibitors had a significant influence on its concentration.
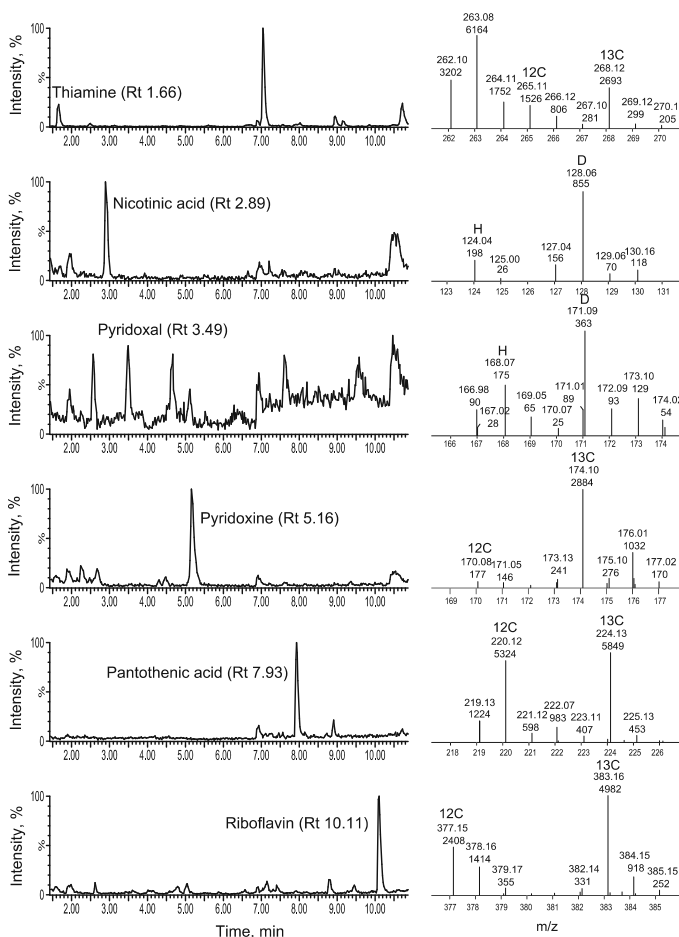
The liberation of free vitamers via enzymatic activity can be efficiently eliminated using ultrafiltration by removing endogenous enzymes, as seen by examining experiment $S_{U\ 18h}$. However, sample pre-treatment procedures should be optimized in order to suppress enzymatic processes prior to ultrafiltration while determining the distribution of vitamers in foods. Another benefit of ultrafiltration, in addition to removing endogenous enzyme activities, is that the content of interfering compounds in the sample extract can be considerably reduced in the mass spectra of vitamers (e.g., thiamine) (Fig. 4). This effect is probably related to the removal of soluble proteins and other high molecular weight compounds; the hydrolysis products of which (e.g., peptides) could interfere with the spectra of vitamers during LC-MS determination [16]. Thus, ultrafiltration is recommended as an additional sample purification as well as fixation step. In addition, our results demonstrate that microfiltration is also able to remove some insoluble matrix-bound enzymatic activity; however, because this also probably affects the results by removing the matrix-bound vitamers, we do not recommend this as a pre-treatment step.

Table 4 Method validation parameters for total content of vitamers

Total vitamers	LOD (µg/g) (n=6)	LOQ (µg/g) (n=6)	Recovery (%) (0.5 mg/L) (n=3)	Recovery (%) (0.25 mg/L) (n=3)	Recovery (%) (0.1 mg/L) (n=3)	Intra-day precision[a] (%) (n=6)	Inter-day precision[a] (%) (n=4)
Thiamine	0.03	0.08	105±1	104±2	106±1	4.2	3.5
Riboflavin	0.03	0.08	96±1	93±2	96±4	3.7	10.1
Nicotinamide	0.80	2.39	–	–	–	–	–
Nicotinic acid	0.67	2.00	87±5	89±5	94±2	15.2	18.5
Pantothenic acid	0.05	0.16	101±3	100±2	102±1	5.2	7.4
Pyridoxal	0.06	0.18	86±8	86±6	85±8	9.8	10.5
Pyridoxine	0.08	0.24	88±5	90±4	88±2	8.6	14.5

[a] Expressed as coefficient of variance

For the sake of simplicity, we seek a "universal" extraction procedure for all water-soluble vitamers to measure the total concentration of vitamins. To determine the total concentration of thiamine and riboflavin, overnight incubation in the presence of endogenous enzymes (E_{18h}) was sufficient while pyridoxal required additional thermal treatment for 30 min at 70 °C (T^{70}_{18h}) prior to overnight incubation to obtain the highest extraction. This suggests that some pyridoxal might be bound into forms that are not liberated by the endogenous enzymes, but can be liberated by thermal treatment under acidic conditions. Those bound forms could be imines, which are hydrolyzed under acidic conditions, seen in our previous study [16]. Moreover, quinoa contains a considerable amount of protein, especially lysine-rich proteins (15 %), which form Schiff base linkages during the formation of these imines [20, 21]. The concentration of pyridoxal increased even further when applying β-glucosidase treatment in combination with

high temperature extraction ($T^{100}_{β\ 18h}$). This increase is probably due to the phosphatase side activity of the β-glucosidase enzyme preparation [18]. Thus, to determine the total concentration of pyridoxal, additional phosphatase activity is required even after thermal treatment. However, thermal extraction at 100 °C negatively affected the concentration of thiamine, riboflavin, and pyridoxine, and further treatment with β-glucosidase, containing acid phosphatase and pyrophosphatase side activities, did not help to reach the maximal extraction observed in samples E_{18h}, T^{70}_{18h}, $E_{β\ 18h}$, and $E_{F\ 18h}$. Thus, high temperature treatment at 100 °C probably inactivated some of the endogenous enzymes responsible for liberation of free vitamers from bound forms. The most efficient extraction method for pyridoxine was β-glucosidase treatment ($E_{β\ 18h}$), which indicates the existence of pyridoxine glucosides in quinoa. This result was expected because in plants, pyridoxine can exist in the form of pyridoxine
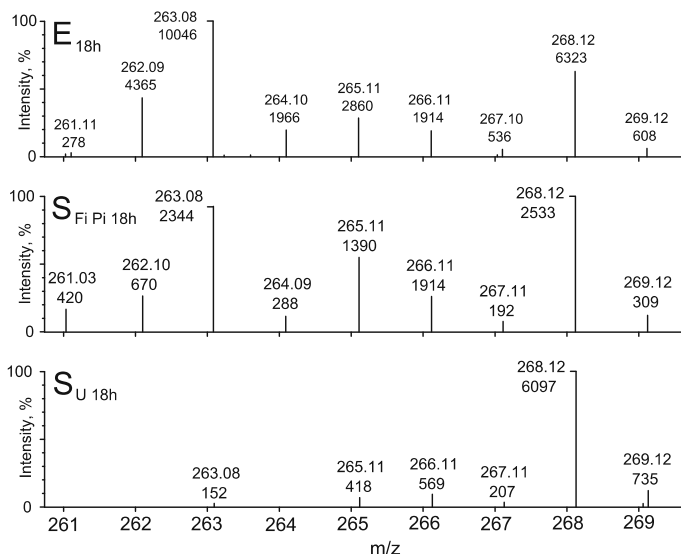
glucosides (e.g., pyridoxine-5′-β-D-glucoside) [22]. Although pyridoxine glucosides are thought to be approximately 50 % bioavailable as a source of vitamin B_6 in humans, their determination ought to be important because glycosylated forms of pyridoxine could form up to 75 % of the total vitamin B_6 pool in fruits, vegetables, and grains [23].

The efficacy of the extraction methods for determination of the total concentration of vitamins in quinoa was evaluated by searching the residual phosphorylated and cofactor forms of thiamine, riboflavin, and pyridoxal (samples E_{18h}, T^{70}_{18h}, $E_{\beta\ 18h}$, $E_{F\ 18h}$, and $T^{100}_{\beta\ 18h}$); external standards were used to identify the compounds of interest: thiamine monophosphate (TMP, $[M]^+=345.06$) and thiamine diphosphate (TDP, $[M]^+=425.03$) in case of vitamin B_1, riboflavin-5′-phosphate (FMN, $[M+H]^+=457.10$) and riboflavin flavin adenine dinucleotide (FAD, $[M+H]^+=786.18$) in case of vitamin B_2, and pyridoxal phosphate (PLP, $[M+H]^+=248.03$) in case of vitamin B_6. No traces of TMP, TDP, FMN, FAD, and PLP were found from the respective samples.

Unfortunately, it was not possible to determine the total concentration of either vitamin B_3 or B_5. With regard to vitamin B_5, the addition of enzymes used in the present study may not have liberated pantothenic acid from its bound forms (CoA, pantetheine, etc.). To liberate bound forms of pantothenic acid, additional pantetheinase and alkaline phosphatase treatment should be used, as suggested by Rychlik [24]. With regard to vitamin B_3, it was not possible to determine the concentration of nicotinamide because the intensity in the MS-TOF was very low, which is probably caused by matrix effects. Although the presence of other bound forms of nicotinic acid and nicotinamide–nicotinamide riboside (NR,

$[M]^+=255.09$), NaR ($[M]^+=256.08$), and nicotinamide mononucleotide (NMN, $[M+H]^+=335.06$) were observed in the MS spectra of quinoa samples (Fig. 5), their exact concentration could not be determined due to a lack of labelled internal standards.

Distribution of free and bound vitamers in quinoa

To determine the concentration of free thiamine, riboflavin, pyridoxine, and pyridoxal, we consider short time cold extraction with enzyme inhibitors to provide the most reliable results. Thus, the concentration of free thiamine in quinoa seeds was found to be 60 μg/100 g (sample $C_{Fi\ Pi}$) which is about 15 % of the total B_1 concentration (400 μg/100 g, samples E_{18h}, T^{70}_{18h}, $E_{\beta\ 18h}$, $E_{F\ 18h}$). In the sample utilizing cold extraction incubated overnight for 18 h after microfiltration ($C_{M\ 18h}$), the respective concentration for thiamine was 70 μg/100 g which indicates that the concentration of thiamine phosphates (e.g., TDP) unbound to a matrix in quinoa seeds is less than a few percent. This suggests that up to 85 % of the total thiamine is bound to the food matrix, most probably to membrane proteins such as pyruvate and α-ketoglutarate dehydrogenase thiamine-diphosphate complexes [25].

The concentration of free riboflavin in quinoa seeds was even smaller than that of thiamine, with about 10 μg/100 g in the cold extracted sample ($C_{Fi\ Pi}$) and 65 μg/100 g in the cold extracted and microfiltered sample incubated overnight ($C_{M\ 18h}$), while the concentration of total vitamin B_2 was found to be 250 μg/100 g (samples E_{18h}, T^{70}_{18h}, $E_{\beta\ 18h}$, $E_{F\ 18h}$). This indicates that about 5 % of the B_2 pool is in the
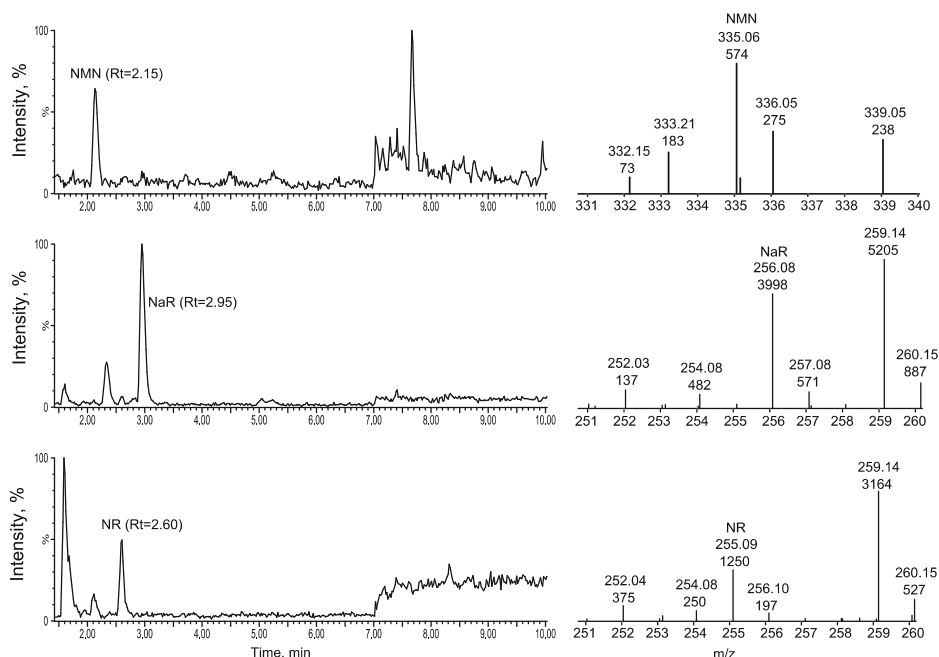
Fig. 5 MS chromatograms of NR, NaR, and NMN (*left*) and the respective MS spectra (*right*) in the sample C

form of free riboflavin and only about 20 % is in the form of the cofactors FMN and FAD unbound to the matrix. These results suggest that vitamin B_2 is mostly found in the insoluble matrix of quinoa seeds (75 %), consisting of membrane-bound FAD binding proteins, for example, succinate dehydrogenase and acyl-CoA dehydrogenase [26, 27].

Of the vitamin B_6 vitamers in quinoa, we measured the concentrations of both pyridoxal and pyridoxine. The concentration of free pyridoxal in cold-extracted quinoa seeds was negligible (4 μg/100 g, sample $C_{Fi\ Pi}$) while the concentration of PLP unbound to the matrix was rather low (10 μg/100 g, sample $C_{M\ 18h}$). In contrast, the total concentration of pyridoxal extracted with overnight incubation in the presence of phosphatase or β-glucosidase was 150 μg/100 g (samples $E_{β\ 18h}$, $E_{F\ 18h}$) and reached up to 200 μg/100 g when applying thermal treatment before incubation with β-glucosidase (sample $T^{100}{}_{β\ 18h}$). This suggests that, practically, all pyridoxal and PLP in quinoa seeds are bound to the insoluble matrix (95 %), of which, approximately 25 % is probably bound to proteins through aldimine (Schiff base) and substituted aldamine linkages and only 5 % is found as soluble PLP. The concentration of free pyridoxine in quinoa seeds was 10 μg/100 g in the cold extracted sample ($C_{Fi\ Pi}$) and practically the same in the sample incubated overnight ($C_{M\ 18h}$) while the concentration of pyridoxine determined after incubation was 30 μg/100 g (samples E_{18h}, $T^{70}{}_{18h}$, and $E_{F\ 18h}$) and reached up to 45 μg/ 100 g when β-glucosidase was used (sample $E_{β\ 18h}$). Thus,

most of the pyridoxine is found in bound forms—approximately 40 % is bound to the insoluble matrix as pyridoxine and pyridoxine phosphate, 35 % is glycosylated, and the rest is found in a free form (25 %).

With regard to vitamins B_3 and B_5, it is impossible to draw conclusions concerning their distribution of different vitamers in quinoa because we only determined the concentration of nicotinic acid and pantothenic acid.

The obtained results were compared with the literature data (Table 5). As there was no comparative literature data concerning distribution of different forms of vitamins in quinoa, only the "total" content of vitamins was compared. In the case of vitamin B_1 and vitamin B_2, our results correlated well with the literature data; in the case of vitamins B_3 and B_6, our

Table 5 Comparison of "total" B-group vitamin content in quinoa seeds as determined in this work and in previous studies

Vitamin (mg/100 g grain)	Ruales and Nair [30]	Ranhotra et al. [31]	USDA [32]	Our results
Vitamin B_1	0.4	0.29–0.36	0.36	0.40
Vitamin B_2	0.2	0.30–0.32	0.32	0.25
Vitamin B_3	No data	1.24–1.52	1.52	0.40[a]
Vitamin B_6	–	–	0.49	0.25[b]

[a] Free nicotinic acid

[b] Total content of pyridoxal and pyridoxine

results were over three and two times lower, respectively; and in case of vitamin B_5, there was no literature data available. The lower result for vitamin B_3 was rather expected as only nicotinic acid was determined in this study. The lower result for vitamin B_6 could partially be explained by the fact that pyridoxamine was not determined. However, it is unlikely that pyridoxamine constitutes half of the total vitamin B_6 content as its content in foods is rather negligible compared to other vitamin B_6 vitamers. For example, the content of pyridoxamine in flour has been reported to be only 10–12 % of the total B_6 content [28]. Also, other factors can affect the results, e.g., origin of the sample as well as different analytical methods.

The most similar food matrix to quinoa seeds for which the distribution of different vitamin forms has been studied is wheat flour. In earlier studies, the distribution of vitamins B_1, B_2, and B_6 in wheat flour has been determined as follows: 50, 30, and 85 % of free forms; 5, 5, and 15 % of free cofactor forms; and 45, 65, and 0 % of protein-bound forms of thiamine, riboflavin, and vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine) [29]. The present work indicates that the proportion of vitamers bound to the food matrix in quinoa is even more significant reaching levels of 85, 65, and up to 90 % for B_1, B_2, and B_6, respectively. In case of soy flour (SRM 3234), the tendency is the same—vitamins B_1, B_2, and B_6 are mostly in bound forms, probably bound to food matrix, as the concentration of free cofactors in foods seems to be low. During food processing: hydration, mechanical, and thermal treatment (samples S and T^{70}), the amount of free thiamine, riboflavin, and pyridoxal significantly increases reaching over 50 % of the total concentration of those vitamers. Thus, we suppose that main mechanism of delivering free vitamers from the food matrix is enzymatic hydrolysis of protein-bound cofactors.

In conclusion, the LC-MS-TOF quantification method employed, combined with SIDA, has proven to be an efficient method to study the distribution of free and bound forms of B-group vitamins in quinoa. However, attention should be paid to developing effective sample pre-treatment procedures. The most suitable method to determine simple vitamers is cold extraction in the presence of phosphatase and protease inhibitors followed by ultrafiltration to prevent enzymatic conversion of cofactors into simple vitamers before injection. To determine the total concentration of vitamers, overnight incubation with endogenous enzymes is recommended. With regard to pyridoxal, thermal treatment followed by enzymatic treatment with phosphatase activity containing enzyme is recommended while pyridoxine is best liberated using β-glucosidase treatment. To determine the total content and distribution of the various forms of vitamins B_3 and B_5 in foods, several isotope-labelled vitamers (e.g., pantetheine, NR, NaR, and NMN) should be synthesized to supplement the isotope-labelled vitamers utilized in this study.

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Uptake and accumulation of B-group vitamers in *Saccharomyces cerevisiae* in ethanol-stat fed-batch culture

ORIGINAL PAPER

Uptake and accumulation of B-group vitamers in *Saccharomyces cerevisiae* in ethanol-stat fed-batch culture

T. Paalme · K. Kevvai · A. Vilbaste · K. Hälvin · I. Nisamedtinov

Abstract The uptake and accumulation of the B-group vitamins thiamine, riboflavin, nicotinamide, pantothenic acid and pyridoxine in *Saccharomyces cerevisiae* was studied by gradually increasing the specific dosage of vitamins in an ethanol-stat fed-batch culture. Thiamine, nicotinamide, pantothenic acid, and pyridoxine were almost completely taken up at low vitamin dosages. Thiamine was determined to be the major accumulating form of vitamin B_1 while most of the assimilated nicotinamide and pantothenic acid accumulated in cofactor forms. Despite the obvious uptake of pyridoxine, accumulation of B_6 vitamers was not observed. In contrast with the other vitamins studied, riboflavin began accumulating in the culture medium immediately after vitamin addition was initiated. By the end of the experiment, the apparent uptake of all vitamins exceeded their accumulation in the cells. Variations in the growth rate of yeast at different vitamin dosages demonstrate the importance of balancing the vitamins in the media during cultivation.

Introduction

Baker's and brewer's yeasts are well known for their high content of B-group vitamins, which also justifies their use as nutritional supplements. Most wild-type strains of *Saccharomyces cerevisiae* are prototrophic for all B-group vitamins. The exception is biotin, the synthesis of which is a variable trait (Hall and Dietrich 2007). In addition to de novo synthesis, a number of vitamers can also be taken up from the growth environment. Considering the complexity of the vitamin synthesis pathways, the uptake of vitamins from the extracellular medium would allow cells to save intracellular resources for other biosynthetic processes.

Vitamin B_1 occurs in yeast as free thiamine and its phosphoesters thiamine phosphate, thiamine pyrophosphate, and thiamine triphosphate, with thiamine pyrophosphate being the main bioactive form. Thiamine is synthesized from pyridoxine and histidine, the precursors of its pyrimidine unit (Tazuya et al. 1989, 1993), and glycine and ribulose 5-phosphate, the precursors of its thiazole unit (Linnett and Walker 1968; White and Spenser 1979, 1982). In addition to de novo synthesis, yeasts can readily take up thiamine from the extracellular environment by the plasma membrane transporter Thi7p ($K_m = 0.18$ μM) (Enjo et al. 1997). In addition, low-affinity transport of thiamine via the nicotinamide riboside tranporter Nrt1p (Thi71p) (Enjo et al. 1997; Belenky et al. 2008; Li et al. 2010) as well as via Thi72p (Mojzita and Hohmann 2006) has been reported. The expression of genes encoding the enzymes involved in the metabolism of

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thiamine (*THI* genes), including *THI7*, are coordinately repressed by exogenous thiamine and induced in its absence, whereas thiamine pyrophosphate seems to serve as a corepressor (Nishimura et al. 1991, 1992; Nosaka et al. 2005).

The de novo synthesis of riboflavin (vitamin B_2) and the cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) occurs from guanosine 5′-triphosphate and ribulose 5-phosphate over a number of consecutive reactions catalysed by Rib1-7p, ATP-dependent riboflavin kinase (Fmn1p), and FAD synthase (Fad1p) (Oltmanns and Bacher 1972; Santos et al. 1995; Reihl and Stolz 2005). The uptake of riboflavin from the growth medium can occur by facilitated diffusion via the plasma membrane transporter Mch5p ($K_m = 17$ μM). In contrast with riboflavin auxotrophic strains, the uptake of riboflavin by commercial baker's yeast strains has been shown to be relatively poor, even under conditions where its extracellular concentration exceeded the K_m value by several times (Perl et al. 1976). Years later, it was suggested that the expression of *MCH5* is regulated by the intracellular flavin content (Reihl and Stolz 2005).

Nicotinamide adenine dinucleotides NAD^+ and $NADP^+$ are essential cofactors for cellular redox reactions and energy metabolism, the de novo synthesis of which can occur from tryptophan via kynurenine (Bedalov et al. 2003) or via the NAD^+ salvage pathway from the B_3 vitamers nicotinic acid and nicotinamide. In *S. cerevisiae,* the uptake of nicotinic acid from the culture medium is mediated by the high affinity permease Tna1p ($K_m = 1.7$ μM), the expression of which is inversely related to the extracellular concentration of nicotinic acid (Llorente and Dujon 2000). No transporters with nicotinamide affinity have been identified (Belenky et al. 2011).

Pantothenic acid (B_5) serves as a precursor for the synthesis of coenzyme A (CoA) in a five-step pathway with some organism-specific variations. The respective enzymes in yeast (Cab1-5p) have been identified (Olzhausen et al. 2009; Ruiz et al. 2009). *S. cerevisiae* is able to perform de novo synthesis of pantothenic acid from β-alanine and pantoate, which are derived from methionine and valine, respectively (White et al. 2001). *S. cerevisiae* can also acquire pantothenic acid from the surrounding medium by the proton symporter Fen2p ($K_m = 3.5$ μM). The transport rate has been shown to be modulated by the content of pantothenate in the growth medium, with maximal rate occurring at low concentrations (Stolz and Sauer 1999).

Pyridoxal 5-phosphate (PLP), the bioactive form of vitamin B_6, has multiple roles as a versatile cofactor of enzymes that almost exclusively function in the metabolism of amino compounds (Stolz and Vielreicher 2003). The B_6 vitamers pyridoxine, pyridoxine 5-phosphate, pyridoxamine, pyridoxamine 5-phosphate, and pyridoxal can

all be converted to PLP via the PLP salvage pathway. The only known carrier of pyridoxine, pyridoxal, and pyridoxamine in *S. cerevisiae* is the proton symporter Tpn1p, which has high affinity for pyridoxine ($K_m = 0.55$ μM) (Stolz and Vielreicher 2003). Expression of *TPN1* is transcriptionally regulated, and is highly induced when either the pyridoxine concentration in the growth medium drops below the threshold concentration needed to support maximal growth, or in response to nitrogen depletion (Gasch et al. 2000).

Among other environmental factors, the assimilation of vitamins may also depend on the growth rate, the sources of carbon and nitrogen, and the state of metabolism (i.e., fermentative or respiratory growth), as occurs with the assimilation of riboflavin (Perl et al. 1976; Reihl and Stolz 2005). The majority of studies that measure B-group vitamin accumulation in yeast have focused on the uptake of one or two particular vitamins in shake flask cultures; studies which utilize industrially relevant fed-batch conditions are scarce. In this work we study the simultaneous assimilation of five B-group vitamins (thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine) by *S. cerevisiae* in an ethanol-stat cultivation using gradually increasing specific dosage of vitamins.

Materials and methods

Fed-batch cultivation with continuous addition of B-complex vitamins

This study utilizes commercial baker's yeast strain *S. cerevisiae* 210NG, kindly provided by AS Salutaguse Pärmitehas (Estonia). A 7 L "BioBench" fermenter (Applikon, The Netherlands), equipped with pH, pO_2, O_2, CO_2, and temperature sensors was used for cultivation. The ethanol concentration was measured online using a "Chemgard" infrared gas monitor (Mine Safety Appliances, USA) and cultivation conditions were controlled using the cultivation control software "BioXpert" (Applikon). Two variable speed pumps (Cole Parmer, USA) were used to pump the feeding media with and without the mixture of B-group vitamins. The feeding media vessels were placed on digital scales (Sartorius, Germany) to precisely determine the pumping rates. Cultivation was carried out at 30 °C and pH 4.75, maintained by titration with 1 M NH_4OH. The dissolved oxygen concentration (pO_2) was controlled at min 20 % of air saturation by adjusting the ratio of air and oxygen in the aeration gas mixture while the reactor was continuously stirred at 800 rpm.

The compositions of the cultivation media were based on those described previously by Nisamedtinov et al. (2010),
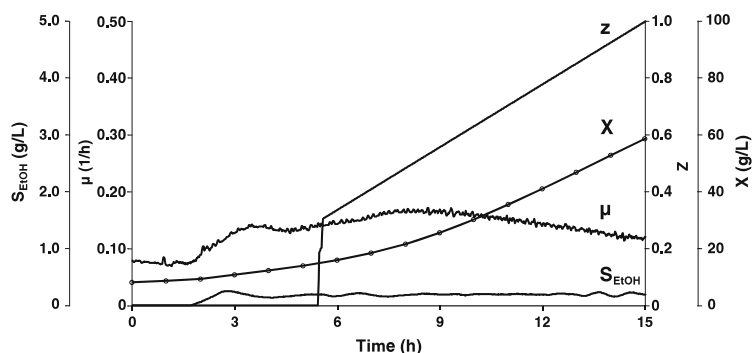
Fig. 1 Ethanol-stat cultivation of *S. cerevisiae* 210NG with gradual increase of B-group vitamins concentration in the feeding medium. *z*—the fraction of vitamin supplemented medium (*Medium 2*) in the feeding medium (in relative units); *X*—biomass concentration (g_{DW}/L), μ—specific growth rate (1/h); S_{EtOH}—ethanol concentration in the cultivation medium (g/L)

and modified in terms of vitamin content. The *Batch medium* contained 50 g/L glucose, 0.5 g/L NaCl, 0.5 g/L CaCl$_2$, 3 g/L KH$_2$PO$_4$, 2.5 g/L NH$_4$Cl, 0.8 g/L MgSO$_4$·7H$_2$O, 1.9 mg/L ZnSO$_4$·7H2O, 1.9 mg/L FeSO$_4$·7H$_2$O, 0.6 mg/L CuSO$_4$·5H$_2$O, 0.5 mg/L MnSO$_4$·H$_2$O, 0.4 mg/L CaCl$_2$·H$_2$O, 0.1 mg/L CoSO$_4$·7H$_2$O, 25 μg/L (NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O, and vitamins (μM): biotin 2, myo-inositol 165. Upon completion of the batch phase and before the start of the fed-batch the following mineral salts were additionally added into the cultivation medium: 6 g NH$_4$Cl, 34 g KH$_2$PO$_4$ and 12 g MgSO$_4$ × 7H$_2$O. Two feeding media (*Medium 1* and *Medium 2*) were used in the fed-batch phase of the experiment. Both media contained 400 g/L glucose, 30 mg/L CaCl$_2$·2H$_2$O, 15 mg/L ZnSO$_4$·7H$_2$O, 15 mg/L FeSO$_4$·7H$_2$O, 5 mg/L CuSO$_4$·5H$_2$O, 4 mg/L MnSO$_4$·H$_2$O, 3 mg/L CaCl$_2$·H$_2$O, 1 mg/L CoSO$_4$·7H$_2$O, 0.2 mg/L (NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O. *Medium 1* contained (μM): biotin 2, myo-inositol 1400; *Medium 2* contained, in addition to biotin and myo-inositol, also (μM): thiamine 180, riboflavin 50, nicotinamide 2750, pantothenic acid, and pyridoxine 120. The vessel of *Medium 2* was covered with aluminium foil in order to prevent light-induced degradation of riboflavin.

The experiment was started by inoculating the *Batch medium* (2 L) with 20 mL of culture grown without vitamins (except biotin and myo-inositol). The yeast was then grown in pH- and pO$_2$-controlled batch culture until nearly all ethanol was consumed. Subsequently, the fed-batch phase was initiated using *Medium 1* in ethanol-stat mode (ethanol set-point, S_{EtOH}^{set} = 0.2 g/L) as described by Nisamedtinov et al. (2010). These conditions allow for cultivation near the maximum respiratory growth rate ($\mu \approx \mu_{crit}$). After stabilization of the culture (as indicated by constant μ), a gradual increase in the specific dosage of vitamins was applied by increasing the fraction of *Medium 2* in the feed from 30 to 100 % over 10 h (Fig. 1).

Sampling routines and determination of vitamer concentrations

The concentrations of vitamers in the biomass and culture medium were determined by LC–MS combined with a stable isotope dilution assay method (Hälvin et al. 2013; Mihhalevski et al. 2013). When available, commercial isotope labelled vitamers where used. For quantification of a number of vitamers (nicotinamide riboside, nicotinamide mononucleotide, and NAD$^+$) we used ^{15}N-labelled yeast hydrolysate, prepared as described by Kevvai et al. (2014). The vitamers were extracted from the freeze-dried hydrolysate with hot ammonium formate (0.05 M, 70 °C, pH 4.5) followed by separation in a Superdex^{TM} Peptide 10/300 GL size-exclusion column (GE Healthcare, USA). The concentration of labelled vitamers was determined by LC–MS using unlabelled internal standards.

Culture samples were withdrawn from the fermenter at 1 h intervals. To determine the extracellular vitamin concentration, 1 mL of sample was immediately centrifuged at 18,000×*g* for 3 min, the supernatant was filtered through 0.2 μm filter and stored at −40 °C until analysis. For analysis of intracellular vitamer concentrations, the biomass samples (10 mL) were promptly quenched in 40 mL of 60 % MeOH-MilliQ solution (−40 °C) as described by Villas-Boas et al. (2005). The quenched samples were centrifuged at 18,000×*g* for 3 min. The yeast biomass pellets were washed twice with 60 % MeOH-MilliQ solution (−40 °C). The washed pellets were freeze-dried and stored at −40 °C until analysis. Vitamin extraction from lyophilized yeast samples (50 mg in five replicates) was carried out in 1 mL of 70 °C 0.05 M ammonium formate buffer (pH 4.5) for 30 min. The sample extracts were centrifuged at 18,000×*g* for 5 min at room temperature and the supernatants were filtered through 0.2 μm filters (Millipore, USA).

To determine the concentrations of free B-group vitamins, 1 mL aliquots of diluted sample extracts (dilution factor 5) were added into LC–MS vials containing isotope labelled internal standards and injected into LCT Premier LC–MS (Waters Corp., Milford, MA, USA). To determine nicotinamide riboside, nicotinamide mononucleotide, and NAD$^+$, 1 mL aliquots of diluted sample extracts (dilution factor 25) were added into LC–MS vials containing purified ^{15}N-labelled yeast hydrolysate and injected into the LC–MS. To determine the total concentration of vitamins, 2 mL aliquots of diluted sample extracts (dilution factor 5) were treated with 0.17 mg of potato acid phosphatase (Sigma, P1146) at 37 °C for 18 h. After incubation, the samples were centrifuged at 18,000×g for 5 min at room temperature, filtered through 0.2 μm filters, and analysed using LC–MS–TOF as described in Hälvin et al. (2013). The following ions were detected (m/z): thiamine—[M]$^+$ = 265.11, labelled thiamine—[M]$^+$ = 268.12, riboflavin—[M + H]$^+$ = 377.15, labelled riboflavin—[M + H]$^+$ = 383.16, nicotinamide—[M + H]$^+$ = 123.06, labelled nicotinamide—[M + H]$^+$ = 127.08, nicotinic acid—[M + H]$^+$ = 124.04, labelled nicotinic acid—[M + H]$^+$ = 128.06, pantothenic acid—[M + H]$^+$ = 220.12, labelled pantothenic acid—[M + H]$^+$ = 224.13, pyridoxal—[M + H]$^+$ = 168.07, labelled pyridoxal—[M + H]$^+$ = 171.09, pyridoxine—[M + H]$^+$ = 170.08, labelled pyridoxine—[M + H]$^+$ = 174.10, NAD$^+$—[M]$^+$ = 664.08, labelled NAD$^+$—[M]$^+$ = 671.08, NR$^+$—[M]$^+$ = 255.09, labelled NR$^+$—[M]$^+$ = 257.09, nicotinamide mononucleotide—[M + H]$^+$ = 335.06, labelled nicotinamide mononucleotide—[M + H]$^+$ = 337.06.

Calculation of culture parameters

The specific dosage of vitamer i [$F_i(t)$], mol/g$_{DW}$ which shows the amount of a given vitamer added per unit of dry biomass in the fermenter was calculated as follows:

$$F_i(t) = \frac{\int_{t_0}^{t} S_i^F \cdot z(t) \cdot \mathrm{pump}(t)dt}{X(t) \cdot V(t)}, \tag{1}$$

where S_i^F (M) is the concentration of vitamer i in the vitamin-supplemented feeding medium (Medium 2), $z(t)$ is the fraction of Medium 2 in the feeding medium, pump(t) (L/h) is the total flow rate of the feeding medium into the reactor, $V(t)$ (L) is the current volume of the culture medium, and $X(t)$ (g/L) is the dry biomass concentration in the culture medium, t_0(h) is the fermentation time corresponding to the start of vitamin addition, t(h) is the current fermentation time.

The apparent specific uptake rate of vitamer i [$U_i(t)$, mol/(g$_{DW}$h)] shows the amount of vitamer i assimilated by the cells per unit dry biomass per unit time and was calculated as follows:
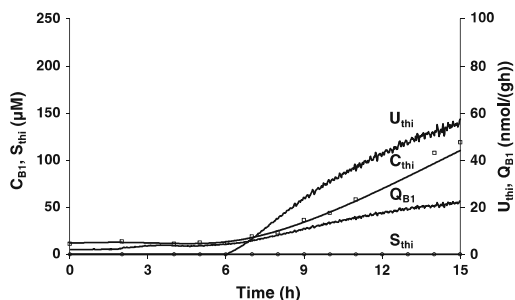

Fig. 2 Accumulation of vitamin B$_1$ in S. cerevisiae 210NG as the concentration of thiamine in the feeding medium was gradually increased. C_{thi}—concentration of thiamine in the biomass (μM); U_{thi}—apparent specific uptake rate of thiamine [nmol/(g$_{DW}$h)]; S_{thi}—concentration of thiamine in the culture medium (μM); Q_{B1}—specific accumulation rate of vitamin B$_1$ in the biomass [nmol/(g$_{DW}$h)]

$$U_i(t) = \mu(t) \cdot \left(F_i(t) - \frac{S_i(t)}{X(t)} \right), \tag{2}$$

where $\mu(t)$ (1/h) is the specific growth rate,

$$\mu = \frac{d(X \cdot V)}{X \cdot Vdt}. \tag{3}$$

The intracellular molar concentration of vitamer i (C_i, M) was calculated with the assumption that the intracellular water space in the yeast cells is constant at v = 2.1 mL/g$_{DW}$ (Okada and Halvarson 1964).

The specific accumulation rate of vitamer i in the dry biomass [Q_i, mol/(g$_{DW}$h)] was calculated as follows:

$$Q_i = \mu \cdot C_i \cdot v. \tag{4}$$

Results

The experiment presented herein used ethanol-stat fed-batch culture which simulates the industrial production conditions of yeast for the purpose of studying the effect of increasing the specific dosage of B-group vitamins (Eq. 1) on the intracellular accumulation of each respective vitamer (Eq. 4). After all ethanol was consumed in the batch phase, the culture was switched to ethanol-stat fed-batch mode. The specific growth rate ($\mu \approx \mu_{crit}$) in the fed-batch after stabilization on a vitamin-free feeding medium (Medium 1) was 0.14 1/h and increased to 0.17 1/h with the gradual increase in the concentration of thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine in the feeding medium (Fig. 1). At higher specific dosages of vitamins and at higher biomass concentration, a slow decrease in the specific growth rate was observed.

The intracellular concentration of thiamine (C_{thi}) during growth on the vitamin-free medium (henceforth referred to as the 'de novo concentration') was 12.1 μM (Fig. 2;

Table 1 Intracellular concentrations of vitamers in an ethanol-stat culture on vitamin free medium and their maximum intracellular concentrations observed at the highest vitamer dosage at 15 h ($C_i^{\text{de novo}}$ and $C_i^{15\text{h}}$, respectively), the concentration of vitamers in the culture medium at the end of the experiment ($S_i^{15\text{h}}$), the maximum apparent uptake rate of vitamers ($U_i^{15\text{h}}$), the specific vitamin accumulation rate during growth without vitamins and at the highest vitamer dosage ($Q_i^{\text{de novo}}$, $Q_i^{15\text{h}}$, respectively)

Vitamer/vitamin	$C_i^{\text{de novo}}$ (μM)	C^{15h} (μM)	S^{15h} (μM)	U^{15h} nmol/ (g_{DW}h)	$Q_i^{\text{de novo}}$ nmol/ (g_{DW}h)	Q_i^{15h} nmol/ (g_{DW}h)
Thiamine	12.1 ± 0.5	105 ± 18	<0.1	57	4	23
Total B$_1$ (Thi + ThiP + ThiPP)	13.0 ± 1.3	113 ± 21	<0.1	57	4	26
Riboflavin	5.9 ± 0.8	7.6 ± 1.8	4.0 ± 0.1	17	3	4
Total B$_2$ (Riboflavin + FMN + FAD)	40.8 ± 3.3	45.4 ± 2.6	4.0 ± 0.1	17	10	13
Nicotinamide (NAM)	59.6 ± 3.4	161 ± 17	0.5 ± 0.1	905	17	37
Nicotinic acid (NA)	9.7 ± 0.9	70.3 ± 8.6	8.4 ± 0.6	0	3	16
Nicotinamide riboside (NR)	8.3 ± 1.2	12.5 ± 3.6	N/A	0	2	3
Nicotinamide mononucleotide (NMN)	70.7 ± 4.8	106 ± 14	N/A	0	20	24
NAD	922 ± 53	2,152 ± 52	N/A	0	265	493
Total B$_3$ (NAM + NA + NR + NMN + NAD)	1,080 ± 63	2,497 ± 96	8.9 ± 0.6	905	307	573
Pantothenic acid	5.8 ± 0.6	62.7 ± 16.6	5.5 ± 0.1	101	2	13
Total B$_5$	N/A	N/A	N/A	101	N/A	N/A
Pyridoxine (PN)	0[a]	0[a]	10.1 ± 0.1	35	0	0
Pyridoxal (PL)	9.3 ± 1.2	16.5 ± 3.5	0[a]	0	3	4
PL + PLP	18.2 ± 1.6	25.1 ± 3.3	0[a]	0	5	7
Total B$_6$	N/A	N/A	N/A	35	N/A	N/A

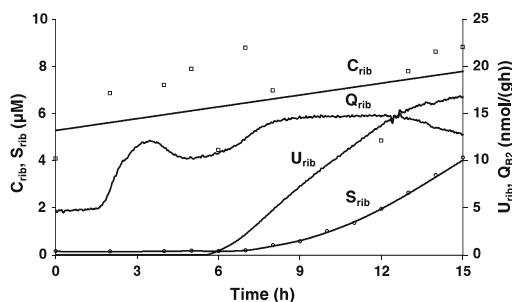*N/A* not analysed

[a] Below detection limit

Fig. 3 Accumulation of vitamin B$_2$ in *S. cerevisiae* 210NG as the concentration of riboflavin in the feeding medium was gradually increased. C_{rib}—concentration of riboflavin in the biomass (μM); U_{rib}—apparent specific uptake rate of riboflavin [nmol/(g_{DW}h)]; S_{rib}—concentration of riboflavin in the culture medium (μM); Q_{B2}—specific accumulation rate of vitamin B$_2$ in the biomass [nmol/(g_{DW}h)]

Table 1), and accounted for over 90 % of the total de novo synthesized B$_1$ vitamers. This ratio remained largely unchanged over the course of the entire experiment. With an increase in the specific thiamine dosage, the intracellular vitamin B$_1$ concentration increased proportionally. The extracellular concentration of thiamine remained negligible ($S_{\text{thi}} < 0.1$ μM) throughout the experiment, which indicates its active uptake by the Thi7p transporter ($K_m = 0.18$ μM; Iwashima et al. 1973; Enjo et al. 1997). The apparent specific uptake rate of thiamine (U_{thi}) was over two times higher than the calculated accumulation rate of vitamin B$_1$ (Q_{B1}) in the cells.

The de novo concentration of riboflavin in the cells (C_{rib}) was around 5.9 μM (Fig. 3; Table 1) and approximately 20 % of the total B$_2$ vitamers determined. In contrast to thiamine, we did not observe an accumulation of riboflavin or the other B$_2$ vitamers in the cells while the riboflavin concentration in the feed was increased, even though the apparent uptake rate (U_{rib}) increased.

The de novo concentration of B$_3$ vitamers and cofactors was found to be two orders of magnitude higher than the other vitamins studied (1,080 μM; Table 1). The majority of the total B$_3$ pool existed as cofactor NAD$^+$ (922 μM). The concentrations of other B$_3$ vitamers were significantly lower: 9.7 μM for nicotinic acid (C_{NA}), 59.6 μM for nicotinamide (C_{NAM}), 8.3 μM for nicotinamide riboside (C_{NR}) and 70.7 μM for nicotinamide mononucleotide (C_{NMN}). With a gradual increase in the concentration of nicotinamide in the feed, the total intracellular concentration of B$_3$ vitamers and cofactors increased to 2,497 μM, mainly due to an increase in the concentration of NAD$^+$ (not illustrated). The extracellular concentration of nicotinamide
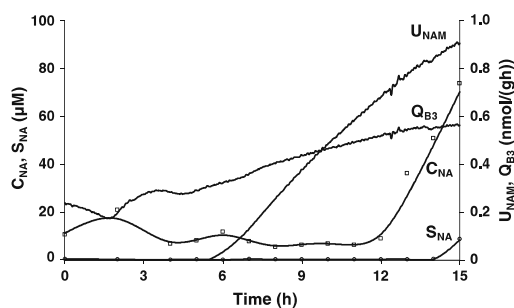
Fig. 4 Accumulation of vitamin B_3 in *S. cerevisiae* 210NG as the concentration of nicotinamide in the feeding medium was gradually increased. C_{NA}—concentration of nicotinic acid in the biomass (μM); U_{NAM}—apparent specific uptake rate of nicotinamide [nmol/(g_{DW}h)]; S_{NA}—concentration of nicotinic acid in the culture medium (μM); Q_{B3}—specific accumulation rate of vitamin B_3 in the biomass [nmol/(g_{DW}h)]
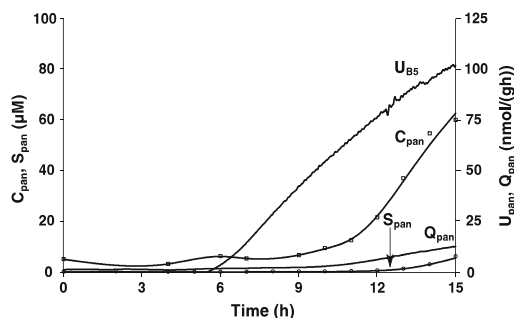

Fig. 5 Accumulation of pantothenic acid in *S. cerevisiae* 210NG as its concentration in the feeding medium was gradually increased. C_{pan}—concentration of pantothenic acid in the biomass (μM); U_{pan}—apparent specific uptake rate of pantothenic acid [nmol/(g_{DW}h)]; S_{pan}—concentration of pantothenic acid in the culture medium (μM); Q_{pan}—specific accumulation rate of pantothenic acid in the biomass [nmol/(g_{DW}h)]
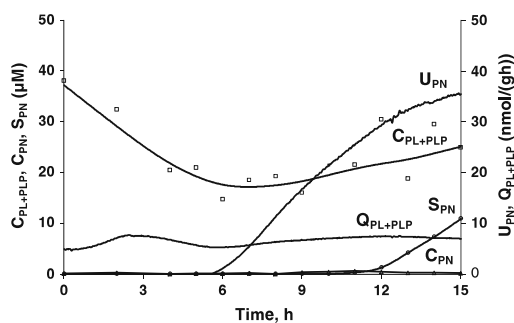

Fig. 6 Accumulation of vitamin B_6 (pyridoxal and pyridoxal phosphate) in *S. cerevisiae* 210NG as the pyridoxine concentration in the feeding medium was gradually increased. C_{PL+PLP}—concentration of pyridoxal and pyridoxal phosphate in the biomass (μM); C_{PN}—concentration of pyridoxine in the biomass (μM); U_{PN}—apparent specific uptake rate of pyridoxine [nmol/(g_{DW}h)]; S_{PN}—concentration of pyridoxine in the culture medium (μM); Q_{PL+PLP}—specific accumulation rate of pyridoxal and pyridoxal phosphate in the biomass [nmol/(g_{DW}h)]

remained close to zero over the course of the entire experiment (S_{NAM}, Fig. 4), suggesting that it was actively transported into the cells. Notably, we observed that nicotinic acid accumulated in the culture medium (S_{NA}) at high specific nicotinamide dosages. The specific uptake rate of nicotinamide (U_{NAM}) was higher than the accumulation rate of total vitamin B_3 (Q_{B3}).

The de novo concentration of pantothenic acid (C_{pan}) was 5.8 μM (Fig. 5; Table 1). The concentrations of other B_5 vitamers and cofactors (pantetheine and coenzyme A) were not determined in the present study, which at least

partly explains why its apparent uptake rate (U_{pan}) was an order of magnitude higher than the accumulation rate of vitamin B_5 (Q_{B5}). As additional pantothenic acid was added in the feed, its extracellular concentration remained low ($S_{pan} = 5.5$ μM), suggesting near complete uptake. Despite this, its accumulation over the de novo concentration in the cells was only observed 3 h after the start of vitamin addition. At high specific dosages of pantothenic acid, we observed its accumulation in the culture medium, which suggests either saturation or feed-back repression of its transport system.

From the B_6 vitamers only pyridoxal and its phosphorylated form were determined to be above the detection limit in the biomass. Of these, pyridoxal phosphate accounted for nearly 50 % of the B_6 vitamer pool; the intracellular concentration of pyridoxine remained below the detection limit over the course of the entire experiment. The concentration of pyridoxal and pyridoxal phosphate (C_{PL+PLP}) at the end of the batch phase was over 35 μM (Fig. 6; Table 1). Notably, with the switch to the ethanol-stat culture without vitamins, the concentration of this pool decreased by approximately twofold to 18.2 μM. During the first stages (6–10 h) of vitamin feeding, the pyridoxine concentration in the culture medium was low, which suggests near-complete uptake. At higher specific dosages, it was found to accumulate in the medium. The specific uptake rate of pyridoxine (U_{PN}) was approximately five times higher than the specific accumulation rate of pyridoxal and pyridoxal phosphate (Q_{PL+PLP}).

Discussion

In the present work the effect of the gradual addition of B-group vitamins (thiamine, riboflavin, nicotinamide, pantothenic acid, and pyridoxine) on their rate of assimilation in *S. cerevisiae* was studied. An ethanol-stat fed-batch culture was used, simulating the conditions often applied in industrial baker's yeast production. The results suggest that while supplementation of the culture with these vitamins is not strictly required, adding them in optimal concentrations can increase the productivity of the process by increasing the specific growth rate. This positive effect can be related to the reduced amount of cellular resources required for de novo synthesis of vitamins and cofactors, as well as the biosynthetic enzymes. Although we only observed a modest increase in the maximum respiratory growth rate (0.03 1/h), it is possible that a more profound effect could be observed while increasing the dosage of vitamins at a reduced rate due to better adaptation. In addition, the relative concentration of some vitamins in the feeding medium may not have been optimal. For example, the de novo concentration of vitamin B_3 in the cells was approximately 100 times higher than that of vitamin B_1, however, the concentration of nicotinamide in the feed was only ten times higher than that of thiamine. It is possible that such imbalances in vitamin concentrations may lead to specific limitations or inhibitory effects.

At high specific vitamin dosages, the specific growth rate started to decrease slowly. This decrease could have been caused by over-accumulation of vitamins and cofactors. However, no growth inhibition could be demonstrated using shake flask experiments with the same vitamin concentrations as used in *Medium 2* (results not illustrated). Thus, the observed decreasing growth rate at high vitamin concentrations was most likely due to the inhibitory effects related to high biomass concentration. We have previously shown that at biomass concentrations higher than 50 g_{DW}/L, a slow and consistent decrease in the specific growth rate occurred (Nisamedtinov et al. 2010).

The gradual increase in the specific dosage of B-group vitamins clearly increased the total intracellular concentration of B_1, B_3, and B_5 vitamers and cofactors, whereas no remarkable accumulation of B_2 and B_6 vitamers over de novo values were observed (Table 1). During vitamin addition, a marked increase in the specific accumulation rates (Eq. 4) relative to the respective de novo values was observed for both B_1 and B_3 vitamers as well as for pantothenic acid (approximately six, two and sixfold, respectively). These observed differences in vitamin and cofactor accumulation rates could be even greater because the maximum values of intracellular vitamin and cofactor concentrations were not reached in the present study.

Although no nicotinamide transporters in *S. cerevisiae* have been identified, its low extracellular concentration ($S_{NAM}^{15h} = 0.5$ µM) compared to its concentration in the cells ($C_{NAM}^{15h} = 160$ µM) suggests that an active transporter should be involved. Interestingly, the intracellular concentration of nicotinamide exceeded that of nicotinic acid by nearly seven times during growth without vitamins, whereas the concentrations of these two vitamers became very similar during vitamin addition (Table 1; Fig. 4). This indicates that following its uptake, nicotinamide was converted into nicotinic acid in the nicotinamidase reaction and then used for NAD^+ synthesis. The observed increase in both the intra- and extracellular nicotinic acid concentrations indicates possible feed-back inhibition or saturation of the NAD^+ synthesis pathway. These results also fit with the findings of Belenky et al. (2011) who found that nicotinamide taken up by cells was readily converted to nicotinic acid and the excess of the latter was excreted from the cells in a Tna1p independent manner. Nicotinic acid may be excreted to mitigate toxic effects caused by its overaccumulation.

Saccharomyces cerevisiae was long considered a pantothenate auxotroph (Stolz and Sauer 1999; Leonian and Lilly 1942). However, White et al. (2001) showed its ability to synthesize it via a pathway involving methionine-derived spermine. The growth of yeast observed in this experiment with a vitamin-free feeding medium supports this fact. Uptake of pantothenic acid occurred immediately after its concentration in the feeding medium was increased; its intracellular concentration, however, started to increase only several hours later. This could be explained by the prompt conversion of imported pantothenic acid into coenzyme A, the concentrations of which in yeast, according to data of Seifar et al. (2013), are in the range of 200 µM, which is several times higher than the intracellular concentrations of pantothenic acid measured in our study (~ 6–60 µM). It is likely that the intracellular pantothenic acid concentration started to increase only after the coenzyme A pools were saturated.

Intracellular pyridoxine levels remained below detection limit throughout the experiment, suggesting its prompt conversion into other B_6 vitamers. However, we did not observe a remarkable increase in the intracellular concentrations of either pyridoxal or pyridoxal phosphate with an increase in pyridoxine dosage. This could result from partial condensation of pyridoxal phosphate with the amino acid substrate to form a Schiff base, a uniform component of all pyridoxal phosphate catalysed enzyme reactions (Eliot and Kirsch 2004). It is possible that pyridoxal from such aldimines may not have been released with the acid phosphatase treatment used in the present study. At higher specific dosages, we observed an accumulation of pyridoxine in the culture medium. It can be speculated that this

was due to saturation or down-regulation of Tpn1p. Stolz and Vielreicher (2003) showed a 15-fold difference in the expression of *TPN1* in pyridoxine free medium compared to a medium containing 2 μM of pyridoxine. Interestingly, we observed a gradual decrease in the intracellular concentration of total pyridoxal (C_{PL+PLP}) after the switch from batch to ethanol-stat fed-batch. The reasons behind this phenomenon remain unclear. It is possible that the decrease was induced by the switch from relatively slow growth on ethanol in the final stages of batch to higher growth rates in the ethanol-stat culture.

The intracellular concentration of riboflavin and related cofactors did not increase significantly with an increase in the specific dosage of riboflavin even though its apparent uptake rate reached a value close to that of its de novo accumulation rate (Fig. 6). By the end of the experiment the extracellular concentration of riboflavin (S_{rib}^{15h}) reached 4.0 μM which was close to the intracellular concentration (7.6 μM). Considering that the riboflavin translocation into the cells is believed to occur only by facilitated diffusion via Mch5p ($K_m = 17$ μM) (Reihl and Stolz 2005) its poor uptake into the cells under these experimental conditions could be expected. It is also possible that the concentration of free riboflavin was overestimated due to endogenous pyrohosphatase activitiy which could have released it from flavin nucleotides during sample preparation. Because *S. cerevisiae* wild type strains are invariably riboflavin prototrophs, our findings, together with previous studies, demonstrate the poor ability of *S. cerevisiae* to transport and accumulate riboflavin (Perl et al. 1976). This raises a general question regarding the physiological roles of plasma membrane transporter Mch5p.

A common feature of all vitamins studied in the present work, with the exception of riboflavin, was that their specific accumulation rates were lower than their respective apparent uptake rates. This was most probably due to an underestimation of vitamin and cofactor accumulation. Underestimation of vitamin and cofactor accumulation can occur due to losses during sample processing or due to vitamers that remain undetermined due to technical reasons. The latter was clearly the case for vitamin B_5, and most likely also B_6, where coenzyme A and protein-bound pyridoxal phosphate were not determined. However, underestimation of the accumulation of the other vitamins was probably not significant. Although the catabolic pathways of vitamins and cofactors are not described in yeast, vitamin turnover may also be a partial reason for the low recovery in some cases. Overestimation of vitamin uptake could result from the instability of vitamins in the culture medium, e.g. in case of light-induced degradation of riboflavin. Nevertheless, according to the information available about the stability of vitamins used in the present study (Troy and Beringer 2006), their degradation in the cultivation medium was most likely negligible.

The present study revealed significant differences in the uptake and accumulation between the B-group vitamins studied. The reasons underlying these differences remain to be elucidated. While in some cases this can be explained by the fact that some related compounds were simply not measured in the present work, in other cases it raises important questions regarding the fate of vitamers in *S. cerevisiae*. New (enzymatic) techniques probably need to be developed to determine all the vitamers in each group. Alternatively, vitamin catabolism may play a much more significant role than has previously been assumed. While our approach does not necessarily reveal the role of individual vitamins with regards to the observed changes in specific growth rate, the smooth increase in the dosage of specific vitamins is, nevertheless, a useful tool to study the accumulation of vitamers under industrially relevant conditions.

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PUBLICATION VI

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LC-MS for quantification of B-group vitamins in yeast

LC-MS for quantification of B-group vitamins in yeast

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Abstract

Consumer demand for reliable nutrition labelling has created the need for high throughput methods for simultaneous quantitative determination of B-group vitamins in foods. In spite of the rapid development of analytical techniques, the simultaneous quantification of water soluble vitamins in vitamin rich foodstuffs, including yeast, remains challenging. The major hindrances include a relatively low concentration and stability of vitamins, as well as their different chemical structures. LC-MS combined with a stable isotope dilution assay (SIDA) is at the moment the most sensitive and selective method, which can be conducted using relatively simple sample pre-treatment procedure compared to conventional methods. Although there are several authors who have used LC-MS-MS with isotope labelled internal standards for determination of B-group vitamins in tablets and dietary supplements the data for real food samples is rather limited. In the present work we demonstrate a rapid, convenient and cost-efficient method for simultaneous determination of vitamins B_1, B_2, B_3, B_5, and B_6 in nutritional yeast and yeast extracts using LC-MS.

Introduction

In spite of the rapid development of analytical techniques, the simultaneous quantification of B-group vitamins in foods, including yeast, remains challenging, with major hindrances being their low concentration in the food matrix, relatively low stability, as well as their different structures and chemical properties [Gentili et al 2008]. LC-MS combined with a stable isotope dilution assay (SIDA) is at the moment the most sensitive and selective method, which can be conducted using relatively simple sample pre-treatment procedure compared to conventional methods. Although there are several authors who have used LC-MS-MS with isotope labelled internal standards for determination of B-group vitamins in tablets and dietary supplements [Chen et al 2007; Goldschmidt and Wolf 2010; Phinney et al 2011] the data for real food samples is rather limited. The method development for simultaneous determination of B-group vitamins in natural samples is quite challenging, mainly due to complexity of sample preparation processes and unavailability of isotope-labelled internal standards of many naturally occurring vitamers. Thus, the extraction of vitamers from the real food matrix should be maximized, and all vitamers should be converted into the same forms as the used internal and external standards. Ideally, the method should also allow high throughput analysis and should, thus, consist of a simple, single-step procedure for complete extraction and the same chromatographic conditions for all vitamins under study. The currently used standard extraction procedures [EVS-EN 14122:2003; EVS-EN 14152:2003; EVS-EN 15652:2009; EVS-EN 14663:2006] are designed for single vitamin analysis and determine the total concentration of vitamin. They are time consuming and usually do not provide additional information about distribution of individual vitamers in food matrix. In addition, these methods use extraction buffers which are unsuitable for MS detection (e.g. hydrochloric acid) and high temperatures which are not suitable for simultaneous analysis of some B-group vitamers (e.g. pantothenic acid) and are also uncommon to physiological conditions in the human GIT. An alternative would be to use hydrolytic enzymes instead of acid hydrolysis for liberation of vitamins from their bound forms, as proposed by Ndaw and co-workers [2000]. Thus, proper enzyme or enzyme mixtures with required activities should be used and MS compatible extraction buffers should be preferred.

Baker's and brewer's yeasts belonging to the genus *Saccharomyces* are well known for their high content of B-group vitamins, which also justifies their use as nutritional supplements, e.g. nutritional yeast, yeast extract. Thus, they are excellent model food matrices for vitamin B-group method development and testing. In the present work we demonstrate a rapid, convenient and cost-efficient method and a respective analytical kit for simultaneous determination of vitamins B_1, B_2, B_3, B_5, and B_6 in nutritional yeast, yeast extracts and for studying the biological enrichment of yeast with B-group vitamins.

Materials and Methods

Chemicals

Unlabelled standards of nicotinamide (99.9 %), nicotinic acid (99 %), thiamine-HCl (99 %), pyridoxine-HCl (99.9 %), pyridoxal-HCl (99 %), Ca-pantothenate (99 %), riboflavin (99 %), β-nicotinamide mononucleotide (95 %) and β-nicotinamide adenine dinucleotide (99 %) were purchased from Sigma-Aldrich (Steinheim, Germany). Nicotinamide riboside was purchased from LGC Standards AB (Borås,

Sweden). The stable isotope labelled standards of nicotinamide-[D_4, 98 %], nicotinic acid-[D_4, 98%], thiamine-HCl-[$^{13}C_3$, 99 %], and pyridoxine-HCl-[$^{13}C_4$, 99 %] were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA); Ca-pantothenate-[$^{13}C_3^{15}N$, 98 %], riboflavin-[$^{13}C_4^{15}N_2$, 98 %] and pyridoxal-HCl-[D_3, 99 %] were obtained from Isosciences, LLC (King of Prussia, PA, USA). Acetonitrile (HPLC grade) was obtained from Rathburn Chemicals Ltd. (Walkeburn, Scotland, UK), while formic acid (LC-MS grade), ammonium formate (puriss. p.a., for HPLC) and hydrochloric acid (puriss. p.a., ACS) were all purchased from Sigma-Aldrich. High purity water was produced by a Millipore water purification system (Millipore S.A.S., Malsheim, France).

Enzyme

Acid phosphatase from potato (P1146) was purchased from Sigma-Aldrich.

Samples

Commercial nutritional yeast powder Engevita Bland (lot no 206477E) and yeast extract FNI 100 (lot. 10103308) were kindly provided by Lallemand, Inc. (Canada). The performance of the used LC-MS method was assessed by analyzing a standard reference material (SRM) 1849 - Infant/Adult Nutritional Formula which was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

Standard preparation and calibration

The concentration of each vitamer in the sample was calculated using seven-point calibration curves. Calibration curve standards were prepared by adding 1 ml of extraction buffer (0.05 M ammonium formate buffer, pH 4.5) was added into calibration vials. Calibration vials A contained external standards' mixtures of thiamine-HCl, riboflavin, nicotinamide, nicotinic acid, pantothenic acid, pyridoxal-HCl, and pyridoxine-HCl with varying concentrations (1.0, 0.5, 0.2, 0.1, 0.05, 0.025, and 0.01 μg) and an internal standard mixture containing the respective isotope labelled vitamers 0.4 μg of [$^{13}C_3$] thiamine-HCl, [$^{13}C_4^{15}N_2$] riboflavin, [D_4] nicotinamide, [D_4] nicotinic acid, [$^{13}C_3^{15}N$] Ca-pantothenate, [$^{13}C_4$] pyridoxine-HCl, and [D_3] pyridoxal-HCl. Calibration vials B contained external standards' mixtures of nicotinamide riboside (NR), nicotinamide mononucleotide (NMN), and nicotinamide adenine dinucleotide (NAD^+) with varying concentrations (1.0, 0.5, 0.2, 0.1, 0.05, 0.025, and 0.01 μg) and an internal standard mixture containing ^{15}N-labelled yeast hydrolysate as a source of labelled NR^+, NMN, and NAD^+.

Thus, the final concentration of each vitamer in the external standards´ mixtures vials were 1.0, 0.5, 0.2, 0.1, 0.05, 0.025, and 0.01 mg/L and internal standard concentration in the vials was 0.4 mg/L. The calibration curves were composed by relating the concentrations of unlabelled external standards to their relative response factors, as determined by the ratio of the peak intensity of the unlabelled external standards to that of the corresponding labelled internal standards.

Sample preparation

The reference sample SRM 1849 (0.5 g) was weighed into 100 mL volumetric flask, internal standard solution (dissolved content of sample vial A, containing the respective isotope labelled vitamers 0.4 μg of [$^{13}C_3$] thiamine-HCl, [$^{13}C_4^{15}N_2$] riboflavin, [D_4] nicotinamide, [D_4] nicotinic acid, [$^{13}C_3^{15}N$] Ca-pantothenate, [$^{13}C_4$] pyridoxine-HCl, and [D_3] pyridoxal-HCl was added, filled to the volume with 0.05

M ammonium formate buffer (pH 4.5), mixed thoroughly. 2 mL of each sample extract was centrifuged (14 000 rpm, 5 min at room temperature) to remove insoluble matter and filtered through a Millex-LG 13mm Philic PTFE 0.2 μm filter (Millipore, Ireland). 1000 μL aliquots of this filtrate were then transferred to the amber LC-MS vials and injected into the LC-MS-TOF.

Samples of nutritional yeast powder and yeast extract (0.2 g) were weighed into 10 mL volumetric flasks in three parallels, internal standard solution (dissolved content of sample vial A or B (containing[15]N-labelled yeast hydrolysate)) was added, filled to the volume with 0.05 M ammonium formate buffer (pH 4.5) and mixed thoroughly. The final concentration of each internal standard in the vials was 0.4 mg/L. To determine the total concentration of vitamins in the samples 1 ml of acid phosphatase solution (10 U/ml) was added to the samples which contained dissolved content of sample vial A. Samples with and without acid phosphatase were incubated at 37 °C under subdued lighting conditions for 16 h using continual shaking. After incubation the sample extracts were centrifuged, filtered, and analysed using LC-MS-TOF.

In comparison to enzymatic extraction hot acid extraction was also tested on nutritional yeast powder and yeast extract using 0.1 M hydrochloric acid. For acid extraction, 0.5 g of sample was weighed into a 50 mL volumetric flask, internal standard solution (dissolved content of sample vial A) was added, filled to the volume with 0.1 M HCl mixed thoroughly, and transferred into a 100 mL flask. Acid extraction was carried out in the autoclave at 121 °C for 30 min as described in Ndaw et al. [2000], followed by the analysis of sample extracts as described below.

LC-MS-TOF analysis

The concentration of B-group vitamins (thiamine, riboflavin, nicotinamide, nicotinic acid, pantothenic acid, pyridoxine, pyridoxal, NR, NMN, and NAD^+) was determined using an ACQUITY UPLC® system coupled with LCT Premier™ XE ESI TOF MS (Waters, USA) with an ACQUITY UPLC HSS C-18 1.8-μm (2.1×150 mm) column. Elution was carried out using water + 0.1 % formic acid and acetonitrile + 0.1 % formic acid gradient: 0–3 min 100 % A; 3–8.5 min 80 % A and 20 % B; 8.5–10 min: 5 % A and 95 % B; 10–15 min 100 % A. A flow rate of 0.25 mL/min, an auto sampler temperature of 4 °C, a column temperature of 25 °C and a sample injection of 5 μL were used. MS analysis was carried out in a positive electrospray ionization mode $[M+H]^+$ using a capillary voltage of 2.0 kV, a sample cone voltage of 30 V, a desolvation temperature of 300 °C and a source temperature of 120 °C. The following ions were detected (*m/z*): thiamine - $[M]^+$=265.11, labelled thiamine - $[M]^+$=268.12, riboflavin - $[M+H]^+$=377.15, labelled riboflavin - $[M+H]^+$=383.16, NAM - $[M+H]^+$=123.06, labelled NAM - $[M+H]^+$=127.08, NA - $[M+H]^+$=124.04, labelled NA - $[M+H]^+$=128.06, pantothenic acid - $[M+H]^+$=220.12, labelled pantothenic acid - $[M+H]^+$=224.13, PL - $[M+H]^+$=168.07, labelled PL - $[M+H]^+$=171.09, PN - $[M+H]^+$=170.08, labelled PN - $[M+H]^+$=174.10, NAD^+ - $[M]^+$=664.08, labelled NAD^+ - $[M]^+$=671.08, NR^+ - $[M]^+$=255.09, labelled NR^+ - $[M]^+$=257.09, NMN - $[M+H]^+$=335.06, labelled NMN- $[M+H]^+$=337.06. Full scan mass spectra were acquired in the range of 100 to 1000 *m/z*. Data were collected and reprocessed using Mass Lynx 4.0 software (Waters, Milford, MA, USA).

Results and Discussion

Fine separation of the peaks of all studied vitamers was achieved in an HSS C-18 column using a gradient system (Figures 1A and B). Responses for all the vitamins were linear over the working range (0.01 – 0.9 mg/L) with correlation coefficients ranging between 0.9995 and 0.9999.

The performance of the used LC-MS method was assessed by analyzing the standard reference material (SRM 1849) which contained most of the vitamers determined in this study. Our results correlated well with the certified data (Table 1).

Table 1. Vitamer concentrations in the SRM 1849.

Vitamin, mg/kg	Our results[a] (n=30)	Certified Concentration
Thiamine Cl HCl	15.5 ± 0.3	15.8 ±1.3
Riboflavin	17.2 ± 0.3	17.4 ± 1.0
Nicotinamide	99.7± 1.9	97.5 ± 2.3
Pantothenic acid	63.9 ± 0.9	64.8 ± 2.2
Pyridoxine HCl	14.2 ± 0.2	14.2 ± 1.5

[a] Results are expressed as a mass fraction for the material as received and as the mean of at least 30 measurements on five different days during one month. The uncertainties are expressed as an expanded uncertainty, U. The expanded uncertainty is calculated as $U=k*u_c$, where u_c is uncertainty of the measurement results and k is a coverage factor.

The concentrations of B-group vitamers in nutritional yeast and yeast extract with and without enzymatic treatment (total content and free forms, respectively) and by using hot HCl extraction are shown in Figures 2, 3, and 4. The concentration of NAD, NMN, and NR in nutritional yeast is shown in Figure 5.
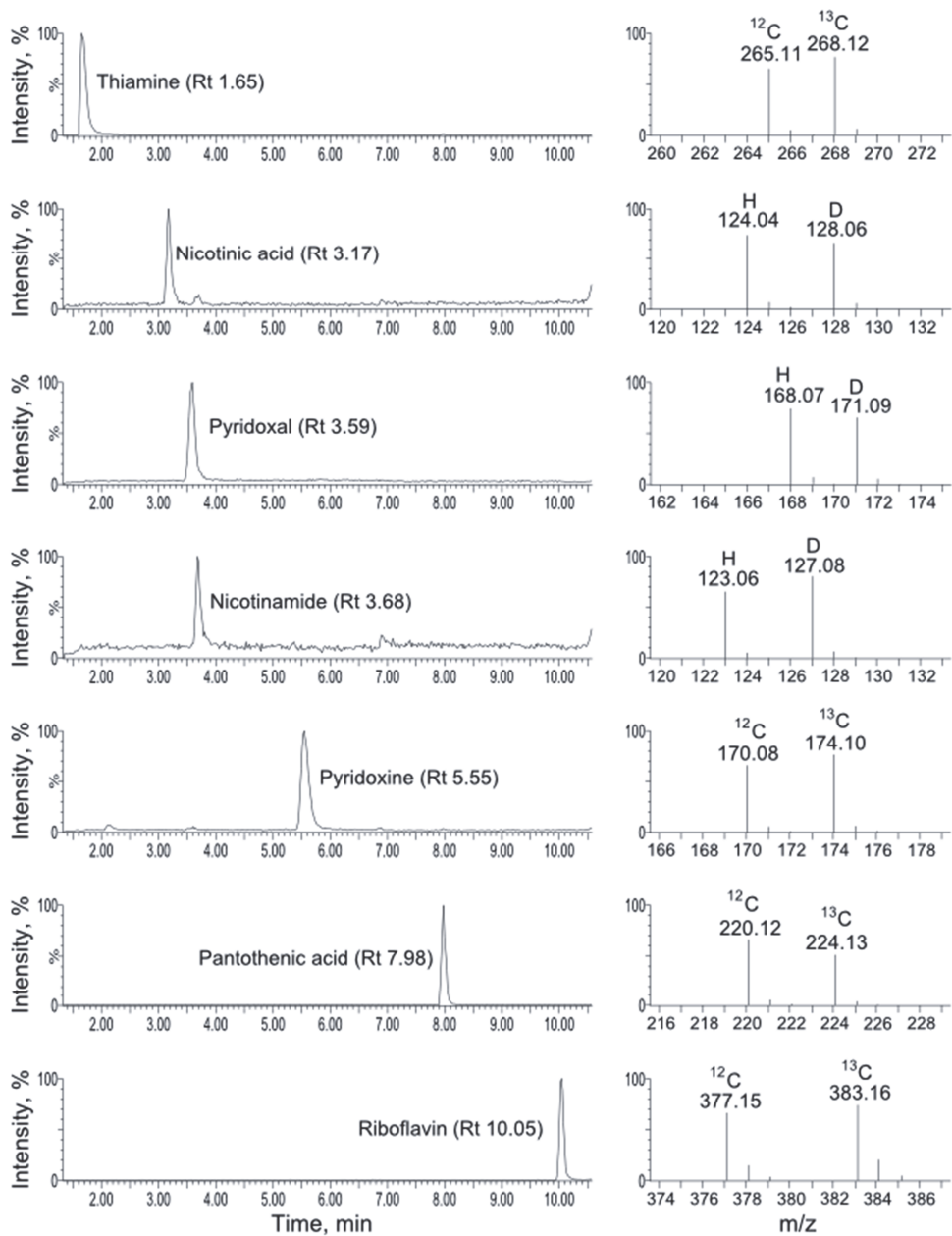
Figure 1A. MS chromatograms of unlabelled (^{12}C and ^{1}H) and labelled (^{13}C or D) B-complex vitamins (left) and the respective MS spectra (right)
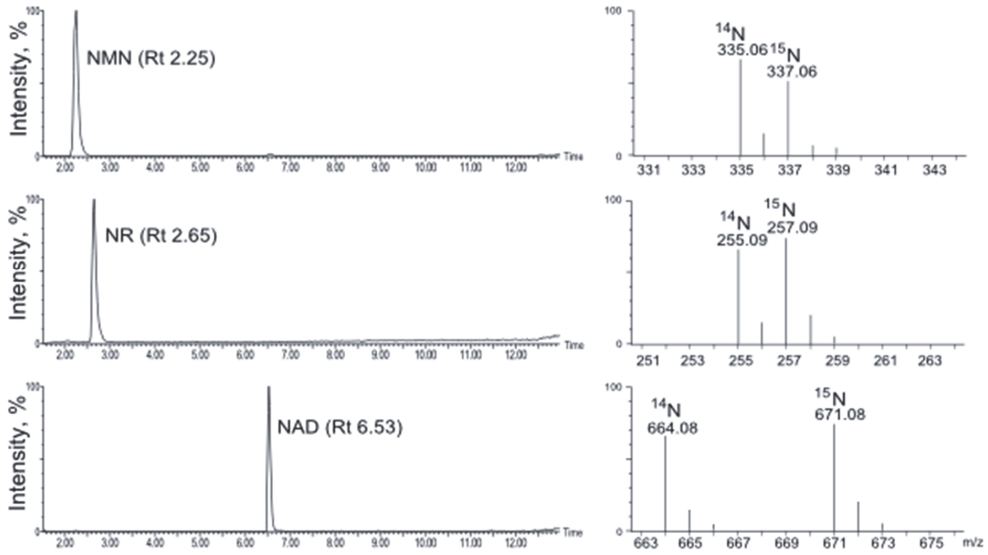
Figure 1B. MS chromatograms of unlabelled (^{14}N) and labelled (^{15}N) NAD, NMN, NR (left) and the respective MS spectra (right)
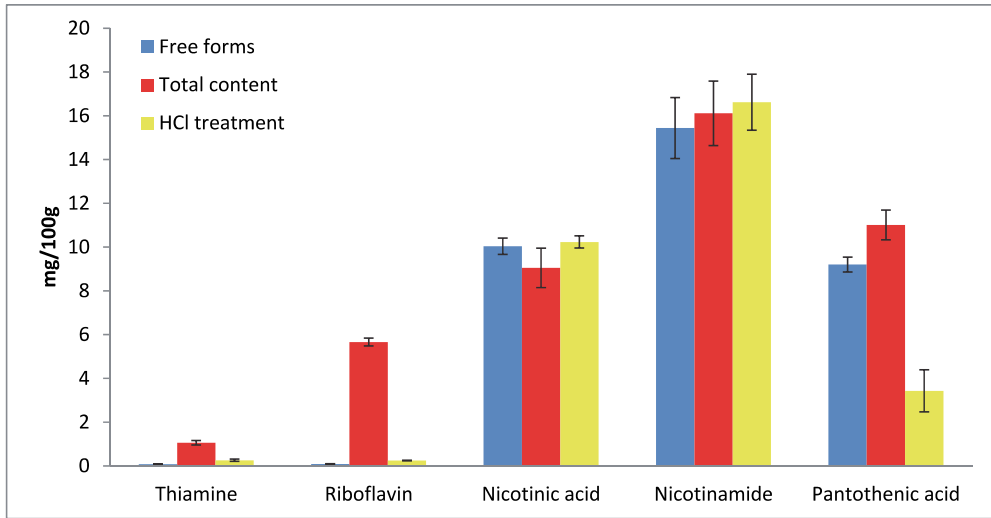

Figure 2. The concentration of thiamine, riboflavin, nicotinic acid, nicotinamide, and pantothenic acid in nutritional yeast with and without enzymatic treatment (total content and free forms, respectively) and by using hot HCl extraction
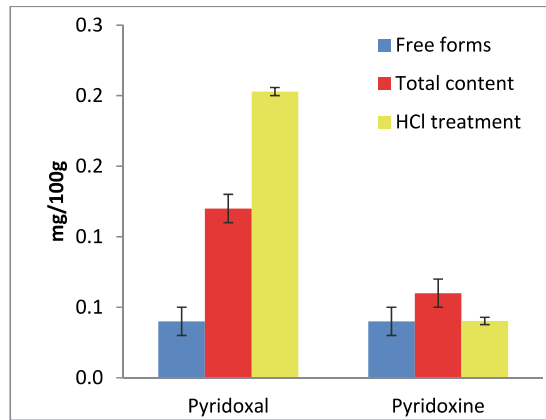
Figure 3. The concentration of pyridoxal and pyridoxine in nutritional yeast with and without enzymatic treatment (total content and free forms, respectively) and by using hot HCl extraction
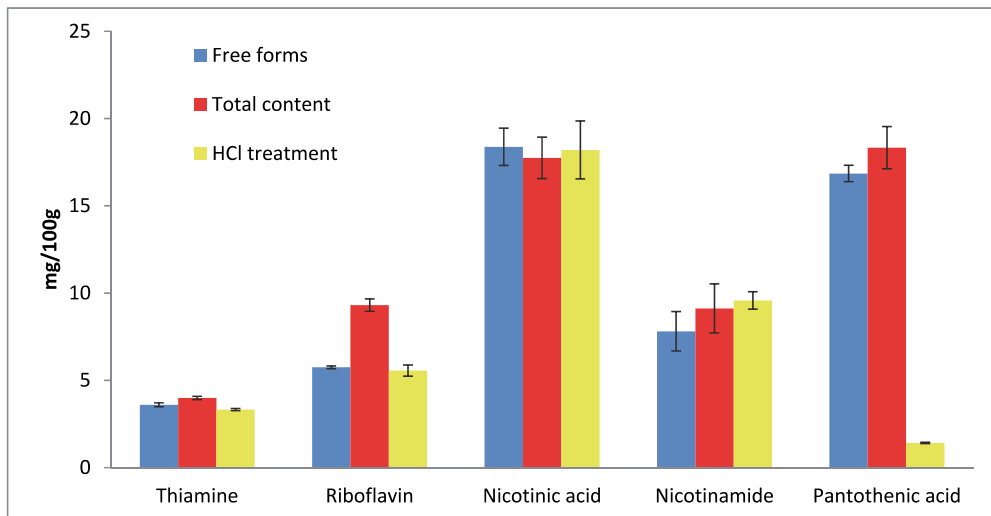

Figure 4. The concentration of thiamine, riboflavin, nicotinic acid, nicotinamide, and pantothenic acid in yeast extract with and without enzymatic treatment (total content and free forms, respectively) and by using hot HCl extraction
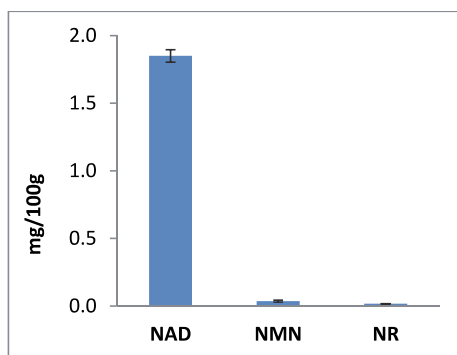
Figure 5. The concentration of NAD, NMN, and NR in nutritional yeast

Enzymatic extraction increased the concentration of thiamine, riboflavin and pyridoxal in nutritional yeast indicating the existence of phosphorylated forms and cofactors of those vitamins. In yeast extract the enzymatic treatment with acid phosphatase increased only the concentration of riboflavin, thus all the other phosphorylated forms of vitamins are probably already hydrolysed into simple forms of vitamins during yeast extract production. The concentration of nicotinamide, nicotinic acid, and pantothenic acid remained the same in the both samples, which is rather expected as the enzyme preparation used does not liberate the bound forms of those vitamers. In yeast extract no pyridoxal and pyridoxine were detected. Thus, those vitamers are probably degraded during yeast extract production.

Nutritional yeast also contained some small quantities of NAD, NMN and NR. As yeast cells usually contain quite considerable amount of NAD [Sporty et al. 2008] most of it is probably degraded into nicotinamide due to high temperatures during nutritional yeast production. Yeast extract contained no traces of NAD, NMN, and NR. Thus, those vitamers are probably degraded into nicotinamide during yeast extract production.

In comparison to enzymatic treatment hot hydrochloric acid extraction was tested on nutritional yeast and yeast extract samples. Hot hydrochloric acid extraction had no effect on the concentration of thiamine, riboflavin, pyridoxine, nicotinamide and nicotinic acid while the concentration of pyridoxal in nutritional yeast increased 1.7 times compared to enzymatic extraction. Surprisingly, pyridoxal was also found in yeast extract (0.15 ± 0.02 mg/100g). The concentration of pyridoxal increased during hot hydrochloric acid extraction probably due to the hydrolysis of imines, which can be formed at high temperatures in the presence of amino acids PL or PLP during the production of nutritional yeast or yeast extract and which could not be hydrolyzed by the enzymes but were hydrolyzed at lower pH values during acid extraction. As it is not known whether such imines would be hydrolyzed in the human gastrointestinal tract their contribution to total vitamin B_6 content in food remains to be elucidated. In addition, a significant decrease in pantothenic acid concentration was observed with a hot hydrochloric treatment in both samples, which is explained by its hydrolysis to β-alanine and pantoic acid under acidic conditions [Velišek and Davidek 2000].

Conclusions

The developed LC-MS-TOF method with soft acid extraction can be applied for simultaneous quantification of respective B-group vitamins in nutritional yeast and yeast extracts.

Acknowledgements

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