HPLC analysis of vitamin B_6 in foods

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ACADEMIC DISSERTATION
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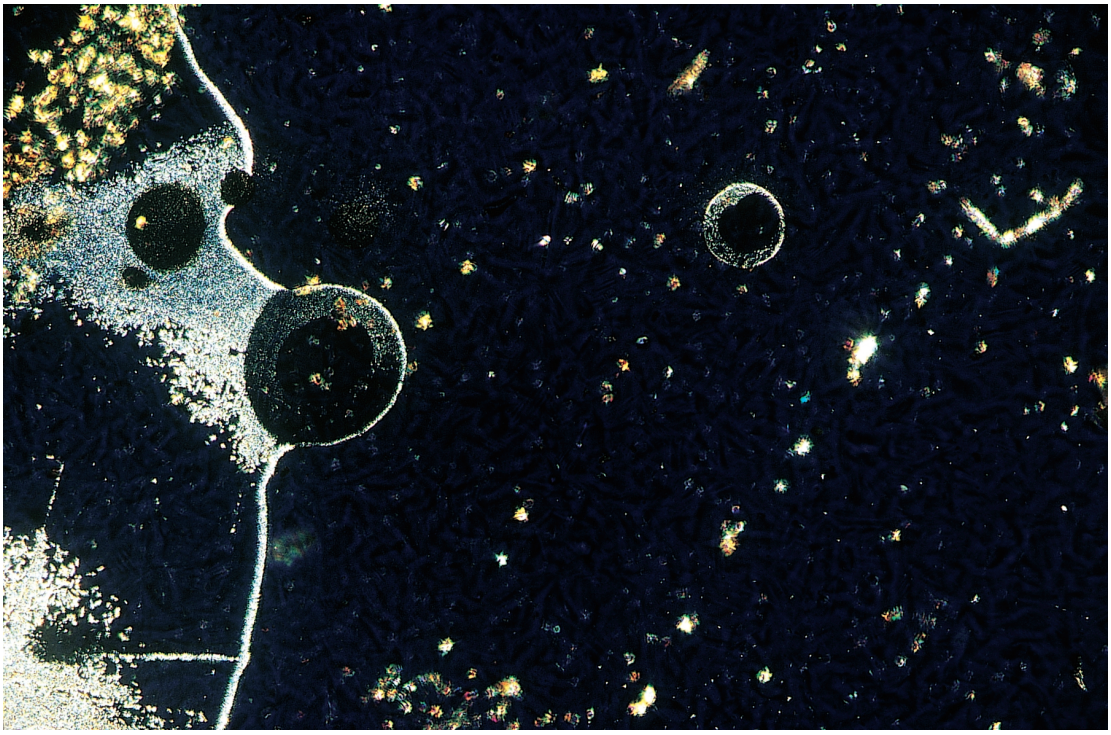

Photo: pyridoxine hydrochloride crystals photographed with polarizing filters (9 x magn.) © Velimatti Ollilainen

"Typically, the more useful a technology is, the more likely people are to adopt it before completely understanding it. Chromatography's tremendous usefulness has ensured that its practice is far ahead of theory."

(Wirth 1994)

Preface

This study on vitamin B_6 was conducted at the Department of Applied Chemistry and Microbiology, University of Helsinki as a part of the project "The Nutrient Content of Finnish Foods - Water-Soluble Vitamins" during the years 1994–1999.

The world of liquid chromatography combined with the analysis of vitamins is an attractive one. I've enjoyed this work which has been both challenging and educational. The development in liquid chromatography has provide sophisticated tools for vitamin analysts. However, there is still a lot of work is to be done. During these years, many people have been involved in this study. Without their support, this work would not have succeeded. I wish to thank them all.

To my supervisor, professor Vieno Piironen, I like to express my deepest gratitude. All her advice and support in the course of time has been vital. I would like to thank her for reading my manuscript and her stimulating criticism.

I wish to thank emeritus professor Pekka Koivistoinen who once made me "an offer I couldn't refuse". His attitude to food chemistry research was most encouraging. His enthusiasm pushed forward my interest in the field of liquid chromatography and vitamin analysis. Professor Pertti Varo is worthy of my sincere thanks for his expertise in the research group, and being an excellent teacher for me.

Dr. Anu Hopia and Dr. Liisa Vahteristo are kindly acknowledged for our continual discussions about food chemistry, liquid chromatography or many other matters. Many new aspects arose during these innovative conversations and arguments. I am indebted to Dr. Anna-Maija Lampi and Dr. Päivi Ekholm for their patience with me with statistics. Antti Uusi-Rauva is acknowledged for all his advice during the years, and especially when I was conducting isotope measurements.

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My special thanks go to Paul Finglas for introducing me to the impressive world of intercomparison studies in the past years. I also want to thank Dr. Henk van den Berg and Dr. Antal Bognar for the most rewarding discussions concerning the vitamin measurements. It has been my pleasure to work with all three of you.

Kirsti Risunen and Jorma Löytynoja are acknowledged for their technical skill and expertise in the course of sampling and maintaining the laboratory facilities. I also wish to thank the other staff and students of our department for the pleasant working atmosphere.

The financial support provided by Ministry of Agriculture and Forestry, Ministry of Social Affairs and Health, and Centre for Metrology and Accreditation is gratefully acknowledged.

Finally, I wish to thank my dear family and as well as my friends for their ever-continuing support. My warmest thanks belong to Ulla who has gave me support during all these years. She had the strength to believe that this work would be finished some day.

Helsinki, January 2000

Velimatti Ollilainen

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List of abbreviations

according to Riekkola 1995, and Huopalahti et al. 1994

α	separation factor, $\alpha=k_2/k_1$
A_s^2	peak symmetry, (peak symmetry)^2, $A_s^2=(b/a)^2$ calculated on the base line
$A_{4.4}$	peak symmetry calculated on the basis of 4.4% peak height, $A_{4.4}=(b/a)$ calculated at 4.4% of peak height
APCI	atmospheric pressure chemical ionization
C_m	concentration in the mobile phase
CAS RN	Chemical Abstract Service Number
CEN	European Commitee for Standardization
CMC	critical micelle concentration
C_s	concentration in the stationary phase
CV_r	coefficient of variation within laboratories
CV_R	coefficient of variation between laboratories
D	minimum detecability
DPN	4-deoxypyridoxine (4-desoxypyridoxine)
DPN•HCl	4-deoxypyridoxine hydrochloride
DPNP	4-deoxypyridoxine-5'-phosphate
d_p	particle diameter (µm)
EI	electron impact ionisation
ESD	error standard deviation
EU-MAT	European Union Measuring and Testing Programme
E%	energy percent
ε	molar absorption coefficient (l mol^{-1} cm^{-1} or l mmol^{-1}cm^{-1})
F_5	width from peak start to total retention time at the 5% of peak height (min)
FAB	fast atom bombardment
GC-MS	gas chromatography – mass spectrometry
Hcy	homocyst(e)ine
HP	hydrophobicity index
HPLC	high-performance liquid chromatograph(y)
HPN	6-hydroxypyridoxine (2-methyl-3,6-dihydroxy-4,5-bis(hydroxymethyl)-pyridine)
i.d.	inner diameter
ISTD	internal standard, internal standard method
J	coupling constant (Hz)
k_e	retention factor (relative retention), $k=(t_R-t_M)/t_M$
l	column length (cm, m)
λ_{max}	wavelength value related to a maximum UV/VIS absorption (nm)
LC-MS	liquid chromatography – mass spectometry
MALDI	matrix associated laser desorption ionization
M_W	molecular weight (g/mol)
MCA	monochloroacetic acid, $ClCH_2COOH$
MPA	metaphosphoric acid, $(HPO_3)_n$
MPCSC	6-methyl-2-pyridone carboxaldehyde
MS	mass spectra, mass spectrometer

MS^n	multiple scan mass spectra
m/z	mass – charge -ratio
N	number of theoretical plate, $N=16(t_R / w_b)2$
NCI	negative-ion chemical ionization
N/l	number of theoretical plates per meter
N_{sc}	surface coverage (μmol/m^2), $N_{sc}= [10^6 P_C/1200n_C-P_C(M_W-I)]$ [1/S]
n_C	number of carbons in the bonded silane molecule
NMR	nuclear magnetic resonance
PA	4-pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-hydroximethylpyridine)
P_C	percent carbon in the bonded phase
PCA	perchloric acid, $HClO_4$
PCI	positive-ion chemical ionization
pK	equilibrium constant
pK_a	dissociation constant
PL	pyridoxal (2-methyl-3-hydroxy-5-hydroxymethyl-4-carboxal-pyridine)
PL•HCl	pyridoxal hydrochloride
PLP	pyridoxal-5'-phosphate (2-methyl-3-hydroxy-5-[(phosphooxy)methyl]-hydroxime-thyl-4-carboxalpyridine)
PM	pyridoxamine (3-hydroxy-2-methyl-5-hydroxymethyl-4-aminomethylpyridine)
PM•2HCl	pyridoxamine dihydrochloride
PMP	pyridoxamine-5'-phosphate (3-hydroxy-2-methyl-5-[(phosphooxy)methyl]-4-ami-nomethylpyridine)
PMP•2HCl	pyridoxamine-5'-phosphate dihydrochloride
PN	pyridoxine (2-methyl-3-hydroxy-4,5-bis(hydroxymethyl)-pyridine)
PN base	pyridoxine cation (Mw 170 g mol^{-1})
PN•HCl	pyridoxine hydrochloride
PNG	pyridoxine glycoside(s), also 5'-*O*- β-D-glucopyranosylpyridoxine
PNG%	pyridoxine glycoside%, PNG%=(PNG/ΣΣ B_6)×100
PNP	pyridoxine-5'-phosphate(2-methyl-3-hydroxy-5-[(phosphooxy)methyl]-4-hy-droxymethylpyridine)
PNX	isolated derivative of pyridoxine
PS-DVB	poly(styrenedivylbenzene)
R_s	peak resolution, $Rs=2(t_{R2}-t_{R1})/(w_{b1}+w_{b2})$
R_r	relative response factor
RSD%	relative standard deviation, percent, (mean/standard deviation)×100
RSD_r	repeatability relative standard deviation
RSD_R	reproducibility relative standard deviation
δ	chemical shift (ppm)
S	specific surface area of the unbonded silica (m^2/g)
SAX	strong anion-exchange
SiOH	silanol index value
SI-MS	secondary-ion mass spectra
SCX	strong cation-exchange(r)
SPE	solid-phase extraction
SSA	5-sulfosalicylic acid, (2-hydroxy-5-sulfobenzoic acid)
t_M	hold-up time, retention time of unretained compound
t_R	total retention time

TCA	trichloroacetic acid, Cl_3CCOOH
TEA	triethylamine, $(CH_3CH_2)_3N$
T	USP tailing factor, $T=w_5/2F_5$
V_0	interparticle volume of the column, void volume
w_b	peak-width at base
w_h	peak-width at half height
w_5	width of the peak at the 5% height
U	unit
ΣB6	sum of PLP, PMP, PNP, PL, PN, and PM
ΣΣB6	sum of ΣB6 and PNG

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The objective of this work was to evaluate the methods for determination of vitamin B_6 in foods. To achieve this, the literature review focused on sample treatment and liquid chromatographic analysis of vitamin B_6 related compounds. In the experimental part, the chosen sample pretreatment and the high-performance liquid chromatographic (HPLC) method were validated, and used to produce vitamin B_6 data on various food items commonly consumed in Finland. The main emphasis of the sample treatment was on the extraction efficiency and the maintenance of the original concentration profile of the vitamers. Several acid extraction procedures were tested for this purpose. Perchloric acid was chosen as the extraction agent. Routine food analysis was then performed using dilute ice-cold perchloric acid extraction followed by an internally standardized ion-paired reversed-phase liquid chromatography. Food samples were hydrolyzed with β-glucosidase and alkaline phosphatase enzymes, phosphorylated and glycosylated vitamers were quantitated before and after the enzymatic digestion. This procedure enabled the extraction of vitamin B_6 compounds in their intact forms, and the measurement of free, phosphorylated and glycosylated forms. The maintenance of the concentration profile of the vitamers was verified by using ^{14}C -labeled pyridoxal-5'-phosphate in the examination of the extraction procedure. The extraction efficiency and laboratory performance were confirmed by interlaboratory studies. Up-to-date data on vitamin B_6 content of about fifty common food items was produced. The data includes the results from meat and poultry, fish and fish product, dairy product, cereal and vegetable, and ready-to-eat food samples. Free and phosphorylated vitamin B_6 compounds were measured in all food groups, and the glycosylated vitamer fraction was analyzed in all plant-derived foods. The results obtained in this work showed that vitamin B_6 content of nearly all foods of plant origin was mainly comprised of glycosidically bound pyridoxine derivatives. These bound analytes are normally not taken into account in traditional analytical methods, and food composition tables lack the data of glycosylated pyridoxine. The role of the glycosylated pyridoxines need to be clarified in terms of their analytical and physiological nature. If, as it is currently assumed, the availability of the bound forms is limited for humans, the role of vegetables, cereals and other foods of plant-origin as a source of vitamin B_6, as well as the analytical methods should be reassessed.

Key words: food, vitamin B complex, pyridoxine, pyridoxal, pyridoxamine, HPLC, liquid chromatography, analytical methods, vitamin analysis

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1 Introduction

The pioneer work of Eijkman in 1906 is considered to be the first recognition of the concept of vitamin as he suggested the presence of an "antipolyneuritis factor" in rice which is "indispensable to health". On the next decade, Funk's theory of the four vitamins opened the new possibilities in nutrition research as the etiology of diseases could be linked to diet and was no longer limited to the "germ theory" only. The subsequent empirical phase combined with the development of the experimental tools yield the discovery of several vitamins in a relatively short time. The substances which are classified as vitamins according to present knowledge were identified within only five decades after Eijkman's work.

The identification of vitamin B_6 compounds was one the most rapid one after the discovery that vitamin B complex includes several, chemically and physiologically dissimilar organic compounds. The vitamin "B_6 family" was surprisingly soon characterized after their discovery , within only one decade. At first the biological methods like microbiological assays enabled their measurement in various biological matrices, and these biological methods were rapidly followed by the intrumental chromatographic procedures. In the late 1970's, Japanese researchers showed that vitamin B_6 in plant-origin materials is mainly constituted of carbohydrate derivatives of pyridoxine and their different vitamin activity from that of the free vitamers was proposed. In addition of traditional "vitamin actions" for vitamin B_6, the new roles for pyridoxine related compounds in human health have also been suggested. These includes their proposed task for instance in hyperhomocyst(e)inaemia and in cancer.

The growing interest in the interactions between diet and health requires a more precise analytical methodology for vitamins. In the food labeling and maintaining the food data bases the appropriate methods are needed. However, the harmonization of analytical procedures is complicated by the fact that term vitamin is a physiological rather than chemical one, expressing a certain physiological activity which is related to the chemical substances responsible for this activity. In a traditional point of view, the vitamin B_6 activity is vested to the free and phosphorylated vitamers but the other derivatives of pyridoxine may not be included to that consideration. In the beginning of the new century, Eijkman's and Funk's question "what is a vitamin" is still of current interest as this must be concluded whenever the analytical procedures are being evaluated in the laboratory performing vitamin analysis.

The first part of the present work includes the literature review mainly focusing on the methodology of vitamin B_6 for the biological materials. In the experimental section, the suitability of the high-performance liquid chromatographic method for food analysis was evaluated, and the chosen method was used for the determination of vitamin B_6 compounds in the most common food items.

2 Literature Review

2.1 Chemical and physical properties of vitamin B_6 compounds

2.1.1 Nomenclature and chemical structures

Free and phosphorylated vitamers

Vitamin B_6 compounds can be classified as derivatives of 2-methyl-3-hydroxypyridine (Fig. 1). Vitamin B_6 is a group name for compounds having vitamin B_6 activity: this group of compounds includes the free vitamers pyridoxine (2-methyl-3-hydroxy-4,5-bis(hydroxymethyl)-pyridine, CAS RN 65-23-6), pyridoxal (2-methyl-3-hydroxy-5-hydroxymethyl-4-carboxalpyridine), CAS RN 66-72-8 and pyridoxamine (3-hydroxy-2-methyl-5-hydroxymethyl-4-aminomethylpyridine), CAS RN 85-87-0, and their phophorylated forms; pyridoxal-5'-phosphate (2-methyl-3-hydroxy-5-[(phosphooxy)methyl]-hydroximethyl-4-carboxalpyridine, CAS RN 54-47-7), pyridoxamine-5'-phosphate (3-hydroxy-2-methyl-5-[(phosphooxy)methyl]-4-aminomethylpyridine), CAS RN 529-96-4 and pyridoxine-5'-phosphate (2-methyl-3-hydroxy-5-[(phosphooxy)-methyl]-4-hydroxymethylpyridine), CAS RN 447-05-2. Free vitamers are commercially available as crystalline hydrochlorides, like pyridoxine•HCl (CAS NR 58-56-0), pyridoxal•HCl (CAS No. 65-22-5), and pyridoxamine•2HCl (CAS RN 524-36-7). Pyridoxine•HCl (Mw 205.64 g mol^{-1}) is the UPS reference standard. Members of "the same vitamin family" are called vitamers (Combs 1992). The terms pyridoxol, PN or vitamin B_6 are also being used as a synonym for pyridoxine.

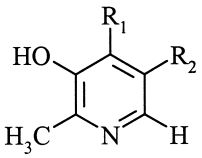

Fig. 1. Structures of vitamin B_6 compounds.

R1	R2	Common name
$-CH_2OH$	$-CH_2OH$	Pyridoxine
$-CHO$	$-CH_2OH$	Pyridoxal
$-CH_2NH_2$	$-CH_2OH$	Pyridoxamine
$-COOH$	$-CH_2OH$	4-pyridoxic acid
$-CH_3$	$-CH_2OH$	4-deoxypyridoxine
$-CH_2OH$	$-CH_2OPO(OH)_2$	Pyridoxine-5'-phosphate
$-CHO$	$-CH_2OPO(OH)_2$	Pyridoxal-5'-phosphate
$-CH_2NH_2$	$-CH_2OPO(OH)_2$	Pyridoxamine-5'-phosphate
$-CH_2OH$	$-CH_2OC_6O_5H_{11}$	Pyridoxine-5'-glucoside

In aqueous solutions vitamin B_6 compounds exist in various ionic forms depending on e.g. pH and temperature (Snell 1963); in a cationic form (I) in acidic environments, as a mixture of zwitterionic (II) and unionized (III) form in neutral solutions, and as an anion (IV) in alkaline solutions (Fig. 2). However, a dipolar ionic form of pyridoxine (II) predominates in neutral media. Pyridoxamine is positively charged in neutral solutions due to its basic 4-aminomethyl group. The aldehyde group of pyridoxal enables also hemiacetal (V) and quinoidic (VI) structures in addition to free aldehyde (VII) and hydrate (VIII) forms (Fig. 3).

Free and phosphorylated B_6 vitamers crystallize as white to off-white platelets or rods, the commercial preparations normally being hydrochlorides. Their melting or decomposition points are generally over 200°C, the melting points of free bases being lower. In general, B_6 vitamers dissolve in water (ca. 0.5–1g/2ml) and in 95% ethanol (0.5–1g/100ml) but are practically insoluble in most organic solvents. Pyridoxic acid is only slightly soluble in water and alcohol. Dilute acidic vitamer solutions are rather stable and tolerate thermal processings (like autoclaving) but pyridoxal degrades in alkaline solutions. Pyridoxine, when autoclaved in a neutral solution, forms a dimer in which the 4'-hydroxymethyl group of one pyridoxine molecule is linked with the nitrogen atom of the other pyridoxine

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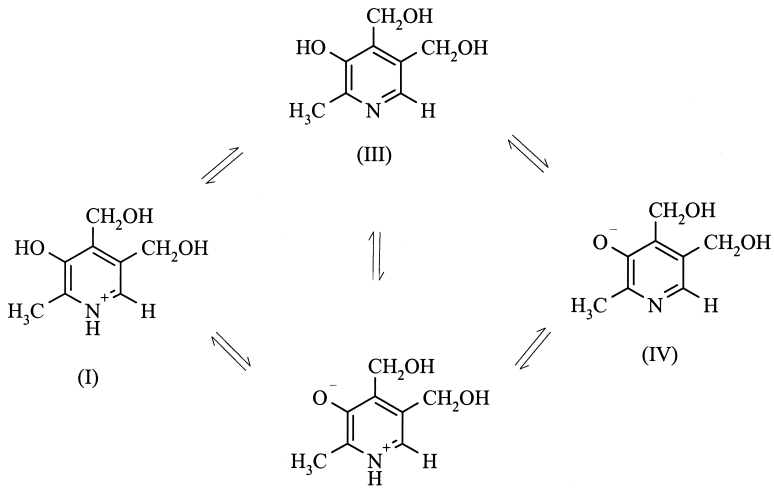

Fig. 2. Ionic forms of pyridoxine.

molecule (Snell 1963). All vitamer forms are relatively light-sensitive and are destroyed by strong oxidazing agents.

Glucosidically bound forms

Nelson and his coworkers (1977) described the bound form of vitamin B_6 present in orange juice as a small nondialyzable (M_w < 3500 daltons) molecule which binds both pyridoxine and pyridoxal. The non-protein character of the vitamin B_6 conjugate present in orange juice was verified by protease digestion as the results derived from the enzymatic hydrolysis indicated no increase in vitamin B_6 activity after protease treatment in contrast to a lyophilized yeast sample similarly treated. Thus, the isolate did not have a protein-binding nature. However, no further structure interpretation for this heat stable non-protein compound was given.

Carbohydrate derivatives of pyridoxine are reported to be present only in plant foods. It is supposed that their utilization by humans as a source of vitamin B_6 is limited. The first eluciated compound, 5'-*O*-(β-D-glukopyranosyl) pyridoxine ($C_{14}H_{21}NO_2$, Mw 331.1g mol^{-1})(Fig. 4), was isolated and characterized from rice bran by Yasumoto et al. (1977). It was proposed that the transglycosylation of pyridoxine enables the formation of both 5'- and 4'-derivatives. However, the 5'-substituted form was considered to be the dominate form. Its white crystals are soluble in water and in boiling 75% ethanol. The

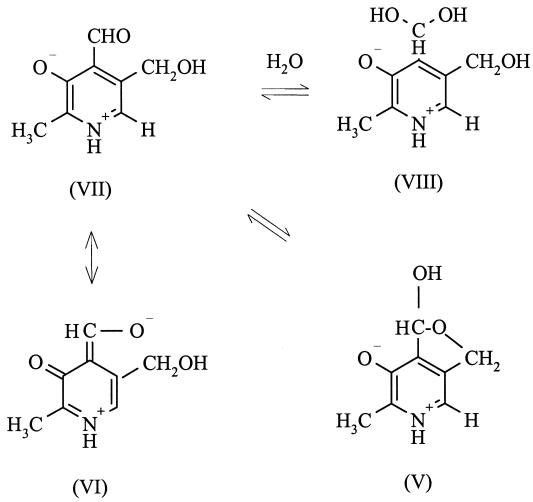

Fig. 3. Free aldehyde (VII), hydrate (VIII), quinoidic (VI) and hemiacetal (V) forms of pyridoxal.

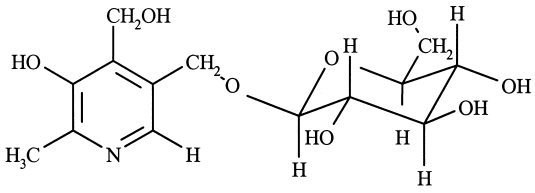

Fig. 4. The structure of 5'-*O*-(β-D-glucopyranosyl) pyridoxine.

β-glycosidic bond is reported to be stable in non-mineral acid solutions, such as in perchloric acid solution (Schramm and Bitsch 1993, Toukairin-Oda et al. 1989), in trichloroacetic acid solution (Gregory and Ink 1987, van Schoonhoven et al. 1994) and in metaphophoric acid solution (Sampson et al. 1995), or in neutral buffer solutions (Kabir et al. 1983a) but it is hydrolyzed in mineral acids especially when acid extraction is combined with a thermal process like autoclaving (Kawai et al. 1971a). Preparation of α-glycosylated pyridoxines (Suzuki et al.1997) and nonlabeled, deuterated as well as tritiated β-glucosidic forms (Gregory and Nakano 1997) has been recently reported.

Other glycosylated vitamers reported in rice are 5'-*O*-(β-cellobiosyl)pyridoxine, 4'-*O*-(β-D-glucosyl)-5'-*O*-(β-cellobiosyl)pyridoxine and 5'-*O*-(β-glucotriosyl)pyridoxine (Tadera et al. 1988). A glycosylated pyridoxine, probably esterified with malonic acid or 3-hydroxy-3-methyl-4-carboxy-butanoic acid, (named B6X) existed in rice and wheat bran, peas and soybeans (Tadera et al. 1983, Tadera et al. 1985 and Tadera et al. 1986a). This compound(s) was considered to be an esterified pyridoxine glucoside as it gives a microbial response after alkaline treatment and hydrolysis of the glycosidic bond. This more precisely unidentified compound was only a minor of B_6 compounds present in those plant foods. A suggestion that PN glucosides exist in potato as a mixture of mono- and diglucosides was presented by Addo and Augustin (1988).

Pyridoxine glucoside-forming activity seems to be distributed particularly to the microbe genera *Sarcina* and *Micrococcus*; pyridoxine 5'-α-glucoside and pyridoxine 4'-α-glucoside were synthesized from pyridoxine and glucosyl donors via transglucosidation (Ogata et al. 1968, Ogata et al. 1969a). Sucrose, maltose and phenyl-α-D-glucoside acted as glucosyl donors. The formation of β-galactosides of pyridoxine, 4'-O-galactopyranosyl-1–4-galactopyranosyl pyridoxine, 4'-O-galactopyranosylpyridoxine, 5'-O-galactopyranosyl-pyridoxine using *Sporobolomyces singularis* has been recently published by Suzuki and Uchida (1997).

Referring to the data published by several research groups, it is obvious that a major portion of vitamin B_6 exists in the form of carbohydrate derivatives of pyridoxine in the plant materials. Their distribution, chemical forms and importance as a source of vitamin B_6 in humans is still not well understood. Traditional analytical methods which are based on the release of free vitamers from the sample matrix do not recognize these bound vitamin forms. For this reason the present food composition data bases do not contain this information. In addition, data concerning the changes in their amount during the maturation of vegetables, fruits, and related plant food materials, as well as their stability in the food processing systems are scarce.

Other forms of vitamin B_6

The study of Bishop and Tryfiates (1989) indicated a novel vitamin B_6 metabolite identified as adenosine-N6-diethylthioether-N1-pyridoximine-5'-phosphate (Fig. 5). Animal and human tumor cells incubated with pyridoxine formed this Schiff's base conjugate of vitamin B_6 with

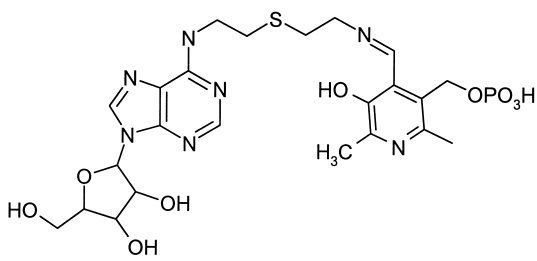

Fig. 5. The structure of adenosine-N6-diethylthioether-N1-pyridoximine-5'-phosphate.

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Table 1. UV-Spectral characteristics of free B6 vitamers and pyridine (Metzler and Snell 1955).

Compound	Solvent	λ_{max}(nm)	ε(1 $mmol^{-1}cm^{-1}$)
Pyridoxine			
– cation	0.1M HCl	291	8.6
– neutral	alcohol	286	5.7
– dipolar ion	pH 6.8	324	7.2
– anion	0.1M NaOH	310	6.8
Pyridoxal			
– cation	0.1M HCl	288	9.0
– neutral	60% dioxane	280	4.1
– dipolar ion	pH 6.9	317	8.9
– anion	pH 10–11	302	5.7
Pyridoxamine			
	0.1 HCl	292	8.2
	98% dioxane	287	3.4
	pH 6.7	326	7.9
	0.1M NaOH	310	7.2
4-Deoxypyridoxine			
– cation	0.1M HCl	282	8.3
– dipolar ion	neutral	313	8.1
– anion	0.1M NaOH	301	7.1
Pyridine			
– cation	0.05M HCl	256	5.7
– uncharged	0.02M NH_3	256	2.8

adenosine diethylthioether, and its formation was highest in the rapidly growing least differentiated cells. The metabolite corresponded to 10–30% of the total vitamin B_6 metabolites and its formation was inversely related to tumor differentiation (Tryfiates et al. 1991). This novel vitamin B_6 compound was assumed to be a minor product of vitamin B_6 metabolism in tumors and cultured tumor cells (Gregory 1992). Based on the findings that cancer patients in the actice disease phase had 3–4 fold higher plasma levels of this B_6 metabolite, a role for it as a circulating marker for human cancer detection was proposed (Tryfiates 1996, Tryfiates et al. 1996).

2.1.2 Spectral characteristics

UV and fluorescence spectra

Due to the ionic nature of B_6 vitamers, their UV absorbance maxima depend on pH and solvent used. Two absorption maxima are normally present in the UV-VIS absorption spectra of the vitamers (Fig. 6). These two maxima are considered to be derived from those of pyridine. The

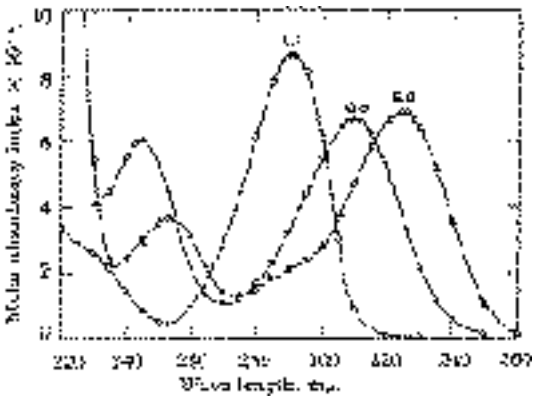

Fig. 6. The UV absorption spectrum of pyridoxine at various pH values. (Reprinted with permission from [Metzler and Snell 1955, *Journal of Americal Chemical Society* 77: 2431–2437].Copyright [1955] American Chemical Society.)

Table 2. Fluorecence characteristics of B_6 vitamers (Bridges et al. 1966).

Vitamer	Excitation/emission wavelenght (nm)	Fluorescence intensity*	pH range of the maximum fluorescence
Pyridoxine	332/400	238	6.5–7.5
	320/380	168	12.0–14.00
Pyridoxal (hemiacetal)	330/382	207	6.0
	310/365	283	12.0
Pyridoxal-5'-phosphate	330/410	11	6.0
	315/370	17.5	12.0–14.0
Pyridoxamine	337/400	370	4.0–5.5
	320/370	410	14.0
4-Pyridoxic acid	320/420	770	1.5–4.0
	315/425	650	6.1–9.4
4-Pyridoxolactone	365/423	32	2.5–4.8
	360/430	2150	8.7–13.0

* expressed as fluorescence intensity relative to the fluorescence intensity of 3-hydroxypyridine anion at pH11.0 considered as 100

main absorbance maximum of pyridoxine in acid and neutral media lies at wavelengths of ca. 290 and 325nm, respectively, compared to the wavelength of 310nm in alkaline solutions. The highest molar absorption coefficient is achieved in acid solutions; the value of coefficient is reduced when the maximum wavelength shifts to higher wavelengths (Metzler and Snell 1955). Table 1 shows the molar extinction coefficients for free B_6 vitamers and pyridine.

Vitamin B_6 compounds possess a native state fluorescence; in acid media the maximum excitation and emission wavelengths are located at ca. 300 nm and 375 nm. In neutral solutions the maximum excitation and emission wavelengths lies at ca. 330–340 nm and 385–400 nm (Table 2). The maximum fluorescence intensity of pyridoxine is found at pH 7 (Duggan et al. 1957). Natural fluorescence of B_6 vitamers is considered to have the characteristics of the hydroxypyridine moiety (Peterson et al. 1955). Pyridoxal-5'-phosphate exhibits a lower fluorescence response than the other vitamers. It is also lower than that of free pyridoxal as the cyclic hemiacetal structure is hindered in the phosphate ester form. The free aldehyde group present in PLP is considered to diminish the fluorecence of the aromatic system by withdrawing the electrons in the ring system. Raising the pH of the measuring solvent to neutral or to weakly alkaline enhanced the relative fluorescence of PLP (Bridges et al. 1966) especially when this procedure is combined with the formation of a bisulphite adduct (Coburn and Mahuren 1983). A comprehensive fluorescence study on hydroxypyrines and vitamin B_6 compounds has been published by Bridges et al. (1966).

UV-absorbance of the isolated 5'-*O*-(β-D-glucopyranosyl)pyridoxine fraction had a maximum at the wavelength of 292 nm in 0.1M hydrochloric acid as well as at 246 nm and 310 nm in 0.1M sodium hydroxide solution (Yasumoto et al. 1977). The molar fluorescence is assumed to be the same as that of pyridoxine (Gregory and Ink 1987).

2.1.3 Chemical reactions

The free vitamers, pyridoxine, pyridoxal and pyridoxamine, exhibit the typical chemical reactions of para unsubstituted aromatic phenols. Colorimetric measurements based on reactions

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with the diazo derivative of sulfanilic acid and with 2,6-dichloroquinone have been used for quantitative measurement of vitamin B_6. In addition, one or more alcoholic hydroxy groups in the molecule enable ester formation with acylating reagents. Many of these reactions, however, have insufficient selectivity and/or sensitivity to be applied in the analysis of food and other biological materials.

2.1.4 Some reactions of pyridoxal and pyridoxal-5'-phosphate

Pyridoxal and its phosphate ester are considered to be the most reactive B_6 vitamers, and many of their reactions take place in sample treatment during the analytical procedure. Some of the reactions related to the aldehyde group of the molecule are utilized to modify the fluorescence detection in liquid chromatography while other reactions are involved with the formation of bounded forms of pyridoxal and its phosphate ester with proteins which may yield reduced vitamin B_6 activity. Transamination, the interconversion of PL to PM and *vice versa*, leads to the changes of the vitamer distribution during sample treatment compared to the original.

Although they are rather stable in pure acidic and neutral solutions, many special reactions are involved with pyridoxal due to its reactive aldehyde group. Pyridoxal is quite stable in acidic solutions in dim environments and the presence of light catalyzes its degradation. Generally, pyridoxal-5'-phosphate reacts more rapidly and more completely than its free vitamer as the stabilizing hemiacetal structure of pyridoxal is disabled in phosphorylated vitamer; PLP has been reported to be 1.5–2 times more reactive than the free pyridoxal under similar conditions (Gregory and Hiner 1983). Phosphorylated pyridoxal reacted with bisulphite forming a hydroxysulfonic derivative which is more fluorescent than native PLP in neutral or slightly alkaline media (Coburn and Mahuren 1983). The reaction with ammonia compounds after elimination of a water molecule yields a carbon-nitrogen double bond in the product molecule. Thus, PLP-semicarbazone is formed after the reaction of PLP with semicarbazide. This acid catalyzed nucleophilic attact reaction had an optimum in a slightly acidic pH, and the formed vitamer-semicarbazone conjugate exhibits strong fluorescence at pH 12 (Gregory 1980a). Pyridoxal was converted to pyridoxine within two hours by reduction by sodium borohydride in alkaline medium (10mM in 0.2M NaOH) (Chaikin and Brown 1949). To decrease the reaction time, a more concentrated borohydride solution (0.1M) was needed (Reitzer-Bergaentzle et al. 1993). This approach has been successfully utilized in an analytical method generally adopted in France.

In aqueous solutions of amino acids the transamination reaction (Fig. 7) occurs slowly at room temperature. This reaction is catalyzed by heat and di- and trivalent metal ions, like copper, iron and aluminium salts (Metzler and Snell 1952). As the imine formation and its breakdown reaction are rapid processes, the rate-limiting step in this non-enzymatic transamination was suggested to be the tautomeric rearrangement of the imine (Metzler 1957). The labilization of bonds in α-carbon (Fig. 7, III) allows for (i) racemization and elimation reactions as well as (ii) the decarboxylation and (iii) the aldolitype reaction. The pH optimum for the reaction between pyridoxal and amino acids was estimated to be 4.5 in a metal ion catalyzed reaction (Metzler and Snell 1952) and in a range of pH 5 to 7 in a non-catalyzed reaction (Cennamo 1964). In a model system described by Metzler and Snell (1952), an equimolar ratio of pyridoxal and pyridoxamine was formed within one hour when 10mM pyridoxal solution was heated (100°C) in the presence of 10mM glutamic acid at pH 5.0, and the same product mixture ratio was obtained from an equal mixture of pyridoxamine and ketoglutaric acid. The use of an enzyme preparation in the sample extraction procedure with a rather long incubation time produced a decreased amount of pyridoxal and the formation of pyri-

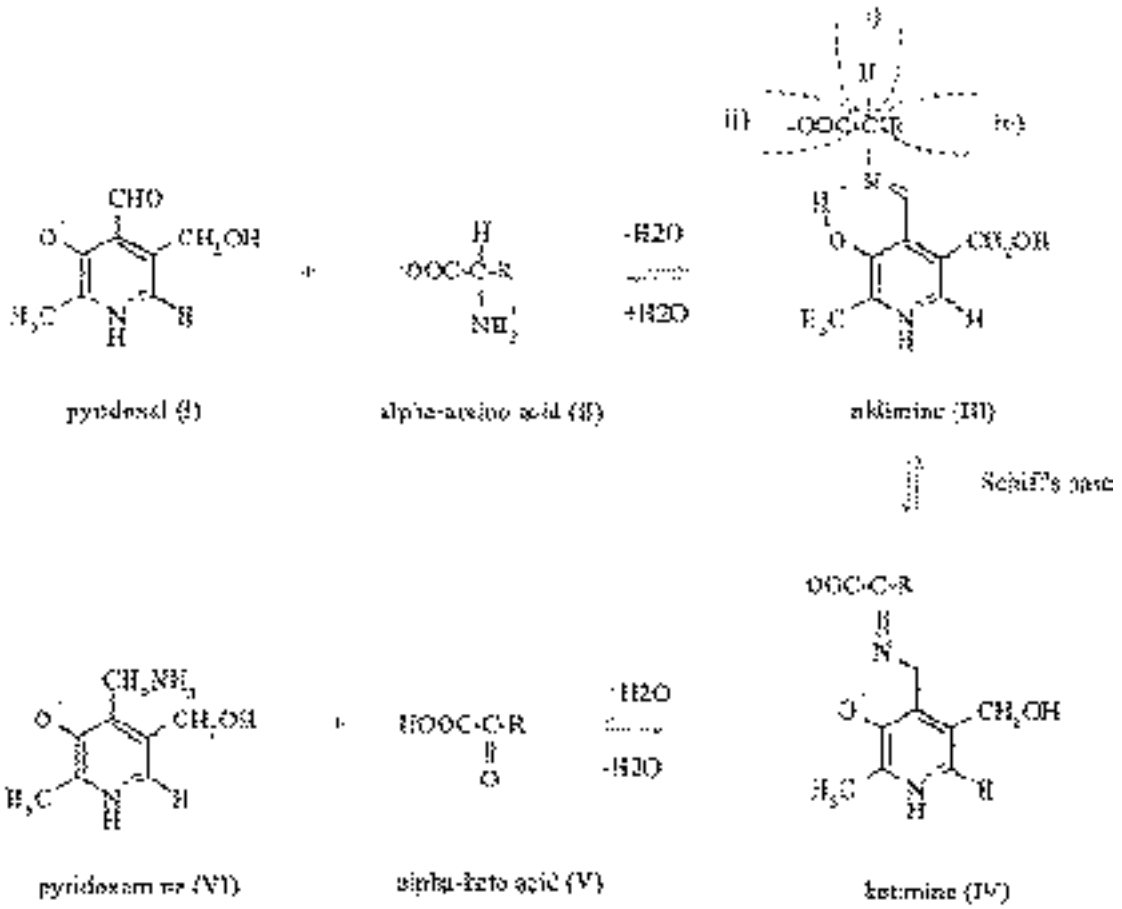

Fig. 7. Non-enzymatic transamination of pyridoxal.

doxamine probably via the transamination route (van den Berg et al. 1996).

Pyridoxal reacts with the amino group of proteins forming e.g. pyridoxyl-ε-lysine (Fig. 8a). When a pyridoxal solution containing cysteine is heated, a portion of pyridoxal was irreversibely converted to thiazolidine condensation product which still, after hydrolyzation, was reported to be available as a source of vitamin B_6. The heating of condensed milk yielded the mercapto derivative of pyridoxine (Fig. 8b) (Srncova and Davidek 1972).

2.2 Nutrition and physiological functions

2.2.1 Utilization

The bioavailability of a nutrient depends on its extent of intestinal absorption and metabolic utilization. Vitamin B_6 compounds can exist in forms that are readily absorbed but poorly metabolized to forms which act as active coenzymes. On the other hand, intestinal absorption

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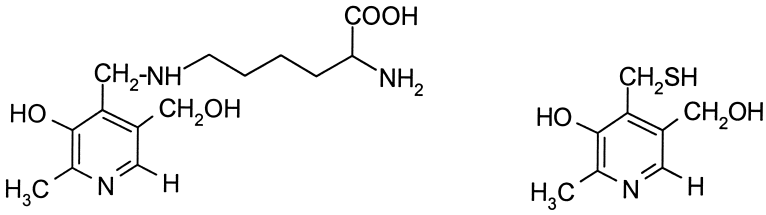

Fig. 8. The structures of a) pyridoxyl-ε-lysine and b) mercaptopyridoxine.

a) b)

may limit a vitamer's utilization (Gregory 1988b). During food processing, like the spray-drying of milk, vitamers can chemically react with other food components to yied products that have decreased vitamin B_6 activity. In addition, the nature of the food matrix (like materials rich in fiber) is suggested to have an influence on vitamin bioavailability.

In general, B_6 vitamers are freely absorbed as nonphosphorylated forms via passive diffusion in the jejunun and ileum. Phosphate esters are dephosphorylated during absorption by the membrane-bound alkaline phosphatase. Free vitamers are then rephosphorylated after crossing the cell membranes and phosphorylated pyridoxine and pyridoxamine are oxidized to the biologically active coenzyme form, pyridoxal-5'-phosphate.

2.2.1.1 Free and phosphorylated forms

As free pyridoxine, pyridoxal, pyridoxamine and their related phosphorylated forms are being converted to each other by numerous enzymes in tissues, it is thought that they possess equal biological vitamin activity in rats and humans. Trials performed on animals (Nguyen et al. 1983) and microbes (Polansky et al.1985), however, it has been shown that individual B_6 vitamers may have different biological activity or growth response depending on the indicator used. The vitamin level in diet supplementation or in the diet may also affect the results (Gregory and Litherland 1986). For instance, the nonphosphorylated vitamers gave a fairly uniform response on plasma pyridoxal-5'-phosphate concentration in rat but a slightly different growth response and feed efficiency was observed (Nguyen et al. 1983). It was shown that the response of the rat to free B_6 vitamers was dependent on the protein level in the diet; the response decreased in low-protein diet compared to that of the normal dietary protein level. The interaction between dietary protein intake and the role of vitamin B_6 in protein metabolism (Nguyen et al. 1983) as well as the gross composition of a diet, and its processing history (Nguyen and Gregory 1983) was established.

2.2.1.2 Glycosidically bound pyridoxines

The main vitamin B_6 fraction in plant-derived foods consists of glycosylated pyridoxine. It is reported that it forms 5–70% of the total vitamin content in plant foods but it is absent in animal products. Several different glycosylated pyridoxine derivatives have been found in nature, however, the bioavailability studies have mainly focused on 5'-*O*-(β-D-glukopyranosyl) pyridoxine, so called pyridoxine-β-glycoside.

An inverse relationship in human between the percentage of glycosylated vitamin B_6 present in food and the extent of availability of the vitamin B_6 was reported by Kabir et al. (1983b). Incomplete utization of glycosidic pyridoxine as vitamin B_6 in the rat was showed by Ink et al. (1986) and the bioavailability of pyridoxine-β-glucoside was estimated to be ca. 40% or less

compared to that of pyridoxine. Trumbo et al. (1988) arrived at the same estimation; the utilization of pyridoxine glucoside relative to the molar response of pyridoxine was only 10–30% in the rat. Seven to nine percent of ingested pyridoxine glycoside was excreted in urine in its intact form at all dosage levels investigated. Their results showed that pyridoxine glucoside is incompletely utilized and the extent of utilization in rat is not influenced by the amount of its intake. Further studies of Trumbo and Gregory (1988) revealed that pyridoxine glucoside and pyridoxine are well absorbed in the intestine but the glycosidic form is then poorly metabolized: ca. 20% of ingested bound form was converted to pyridoxine in rat. Pyridoxine glucoside was absorbed without prior conversion to pyridoxine and the limiting factor seemed to be its hydrolysis to biologically active pyridoxine. As vitamin B_6 deficiency did not enhance the utilization of pyridoxine glucoside, vitamin B_6 status has little or no effect on the utilization. Intact pyridoxine glucoside was not secreted in the milk of lactating rats although pyridoxine derived from the limited hydrolysis of pyridoxine glucoside was delivered to the mammary gland (Trumbo and Gregory 1989). Pyridoxine glucoside comprised ca. 15% of the total vitamin B_6 content in Nepalese human milk (Reynolds 1988). However, this contrasts with other studies where little or no pyridoxine glucoside was found in human milk (Gregory and Ink 1987, Andon et al. 1989). The reason for the higher concentration of pyridoxine glucoside in the breast milk of Nepalese women compared with American women remain unclear.

The utilization of glycosidic bound pyridoxine was later found to be higher in humans than was earlier found in rats (Gregory et al. 1991, Nakano et al. 1997).When pyridoxine and its glucoside were admistrated simultaneously, a greater extent of utilization was measured; the bioavailability of orally administrated deuterated pyridoxine glucoside ranged from 50% to 58% compared to that of pyridoxine. Utilization of glucoside considerably decreased when it was administrated intravenously. These findings supported the suggestion that β-glucosidase activity in the intestinal mucosa or microflora increases the availability of pyridoxine glucoside in human. Animal trials showed that the utilization of pyridoxine glucoside varies in different species. The glucosidic form was effectively absorbed in rats but 80% of it was rapidly excreted in urine. Thus, its bioavailability was estimated to ca. 20% compared to that of pyridoxine (Gregory et al. 1991, Gilpert and Gregory 1992). The bioavailability of pyridoxine glucoside, based on urinary excretion of the administrated tritium labeled pyridoxine glucoside, was only 10–30% in rats, but 70% in mice and hamsters, and 90% in guinea pig. The use of mice or hamsters as model species in utilization trials instead of mice or rats was then suggested (Banks and Gregory 1994). However, the results of their study indicated that pyridoxine glucoside metabolism differs between rat, mice and hamster as well as in human. Overall, the bioassay methods based on animals have been found, in many cases, to be unsuitable for evaluating the bioavailability of vitamin B_6 (Gregory and Litherland 1986). An effect of the physiological condition on utilization was reported by Cheng and Trumbo (1993). During pregnancy the utilization of pyridoxine glucoside in rats was increased and was quite similar to that of pyridoxine. Hormonal differences like changes in the activity of uterine glycosidases may explain these findings.

It is proposed that pyridoxine-β-glucoside nutritionally operates in several ways: it can be utilized as a source of partially available vitamin B_6 but it also acts as a weak antagonist hence preventing the utilization of pyridoxine (Gilpert and Gregory 1992). Pyridoxine glucoside inhibited the uptake of pyridoxine in rats. Utilization of pyridoxine was retarded in isolated rat liver cells incubated in a equimolar mixture of pyridoxine and pyridoxine-β-glucoside. It was concluded that pyridoxine glucoside uses the same transportation system as pyridoxine. As the amount of transported glucoside was only 20% of transported pyridoxine, a permeability barrier due to steric hindrance at the transporter was proposed by Kawai et al. (1972a) and Zhang et

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al. (1993). Unlabeled pyridoxine glucoside administrated simultaneously with ^{14}C-labeled pyridoxine altered the metabolism and *in vivo* retention of pyridoxine in rats. Changes in metabolic patterns as well as possible inhibition of certain enzymes or modulation of the β-glucosidase activity were suggested (Nakano and Gregory 1995). It was proposed that the interaction between pyridoxine-β-glucoside and nonglycosylated B_6 vitamers occur also in human metabolism (Hansen et al. 1996). Their study showed that women consuming a diet rich in PNG exhibited a decrease in vitamin B_6 status indicators. The total loss of the total vitamin B_6 intake was estimated to be 15–18%.

Pyridoxine 5'-α-glucoside and pyridoxine 4'-α-glucoside are formed by certain microbe genera, like *Sarcina* and *Micrococcus* (Ogata et al. 1968, Ogata et al. 1969a) but these α-glucosylated compounds are expected to occur less commonly in nature. Their bioavailability as a source of pyridoxine differs remarkably from those of related β-glucosides. It has been demonstrated that α-glucosides are actively transferred into rabbit erythrocytes (Kawai et al. 1972b) and they are readily utilized by the rat (Joseph et al. 1996, Tsuge et al. 1996). The microbiological activity for *Saccharomyces ovarum* was 20% compared to that of pyridoxine for a 24h incubation. Microbiological activity was increased after a prolonged incubation time and reached 50% of the activity of equivalent of mole amount of pyridoxine. (Kawai et al. 1971a). Synthetized 4'-α- and 5'-α glycosylated derivatives of pyridoxine probably use different transport mechanism than pyridoxine thus having no inhibitory effect on pyridoxine transport. Both forms were readily converted to pyridoxine in rat liver. Pyridoxine-5'-α-glucoside was more rapidly hydrolyzed than the related 4'-form in the experiment of Joseph et al. (1996) and it was concluded that 5'-α-glucoside of pyridoxine, if present in diet, may have some nutritional importance for the intake of vitamin B_6.

It is quite evident that more detailed information of the free, phosphorylated and glycosylated vitamers, and their distribution in food is essential. The vitamin intake and its relevance to vitamin B_6 status can not be completely evaluated on the basis of current food composition data as proper information for different vitamers including glycosylated forms and their distribution is not available.

2.2.1.3 Chemically modified forms

The reaction products of B_6 vitamers are usually incorporated during food processing. More reactive vitamers like pyridoxal, pyridoxamine and their related phosphates, can be bound to an amino or sulfhydryl group of proteins forming Schiff's bases or disulfides. Known products of these reactions are for instance pyridoxyl-ε-lysine and a thiazolidine condensation derivative. The extent to which these modified forms are utilized vary. Generally, pyridoxyl-ε-lysine is poorly utilized (Tsuge et al. 1996) having only 50% activity relative to the free form (Gregory & Kirk 1977, Gregory 1980d). Even an antivitamin B_6 effect of intact ε-pyridoxyllysine has been put forward (Gregory and Kirk 1978a).

Various vitamin activity values for sulfur-containing thiazolidine derivatives formed in evaporated heat-sterilized milk after reaction with cysteine have been reported; thiazolidine forms had no activity in rats, 20% activity in *S. ovarum* (former *carlsbergensis*), and 60–70% activity in *Neurospora sitophila* compared to that of pyridoxal (Bernhart et al. 1960). The reaction with free sulfhydryl groups of milk protein was proposed to yield the formation of bis-4-pyridoxyl disulfide (Wendt and Bernhard 1960). However, the amount of the product formed via this reaction route was estimated to be so small that the decreased vitamin activity found in processed foods could hardly be explained in this way. Thus, the formation of a compound active for microbes but inactive for mammals was proposed (Gregory and Kirk 1977). The formed peptide – pyridoxal phosphate complex gave evidence for an acid stable pyridoxylamino structure.

An inactive mebolite, 6-hydroxypyridoxine HPN (Fig. 9), was found after processing plant foods rich in ascorbic acid (vitamin C). It was

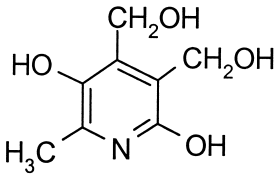

Fig. 9. The structure of 6-hydroxypyridoxine.

suggested that the formation of HPN was the result of the oxidative degradation of ascorbic acid (Tadera et al.1986b) and it was generated by hydroxyl radicals. The pH and temperature optimum for its formation were 4–7 and 30–50°C, respectively. 6-Hydroxypyridoxine showed neither vitamin activity in *S. uvarum* (Tadera et al. 1986b) or in rat (Gregory and Leatham 1990) nor antivitamin activity in *S. uvarum* (Tadera et al. 1986b). It was concluded that the formation of this pyridoxine conjugate causes vitamin losses during food processing, cooking and storage in the presence of ascorbic acid. However, the data of its amount and relevance to the vitamin B_6 losses in processed foods is still scarce.

2.2.1.4 Interaction of other food components – food digestibility

The fiber content of a diet has been proposed to lower vitamin B_6 availability by binding pyridoxine to some extent. This conclusion was based on findings that vitamin B_6 was less effectively utilized from whole-wheat bread than from white bread or from fortified white bread (Leklem et al. 1980). It is was suggested that increased fecal excretion contributed to the lower vitamin availability. However, the difference in utilization efficiencies was small. Lindberg et al. (1983) demonstrated that adding wheat bran to the diet slightly lowered the bioavailability of vitamin B_6 (ca. 17%) in young men but did not adversely affect vitamin B_6 status if the vitamin intake was adequote. The bioavailability of added vitamin also was decreased in rat when vitamin B_6 was added to a rice breakfast cereal (Gregory 1980b). Later studies of Nguyen and associates could not confirm either the inhibitory effect of pectin, cellulose or wheat bran in rat and chicks (1981b) or *in vitro* physical interaction between vitamin B_6 and eight purified polysaccharide fractions (1981a). The same kind of results were obtained with wheat bran and cellulose in rats by Hudson et al. (1988) and in humans by Shultz and Leklem (1987). No effect of dietary fiber on the vitamin B_6 status among vegetarian and nonvegetarian elderly individuals was found in the Netherlands (Löwik et al. 1990). The net bioavailability of vitamin B_6 in an average American diet ranged from 61% to 81% evaluated by plasma PLP data and from 73% to 92% according to urinary data compared to the availability of pure vitamin (Tarr et al. 1981). The availability of vitamin B_6 was reported to be higher in meat and fish foods than in plant-derived foods. It was proposed, however, that dietary fiber has little or no effect on utilization of vitamin B_6 in humans. On the other hand, the slow digestibility of fibrous food may need even more emphasis.

In general, many results of the reported studies can only be interpretated with difficulty as several factors, like interactions or reactions with other compounds present in foods, physiological status and nutrition of an individual as well as different chemical forms of vitamin B_6 compounds, will have an impact on the vitamin utilization.

2.2.2 Coenzyme function

The B_6 vitamers are metabolically interconverted via the dephosphorylation -phosphorylation, oxidation – reduction and deamination – amination reactions by enzymes like pyridoxal kinase, phosphohydrolases, pyridoxal dehydrogenase and transaminases. The metabolically active form of vitamin B_6 is pyridoxal-5'-phosphate (PLP) which serves as a coenzyme in several enzyme systems. Flavin mononucleotide requiring pyridoxal phosphate oxidase (EC 1.4.3.5), regulating the conversion of pyridox-

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ine and pyridoxamine to the active coenzyme has been suggested to be the limiting enzyme in vitamin B_6 metabolism (Kazarinoff and McCormick 1975).

Vitamin B_6 dependent enzymes are mainly involved with amino acid metabolism. The most evident group of enzymes is transaminases, most of which use α-ketoglutaric as an amino group acceptor. The activation coefficient of erythrocytic aspartate aminotransferase (EAST-AC) has been widely used as a parameter to indicate the availability of PLP and thus reflect the vitamin B_6 status of a body over a long period (van den Berg et al. 1978, Löwik et al. 1990, Costa de Carvalho et al. 1996). Other enzymes which are PLP coenzyme dependent include decarboxylases, and amino acid side-chain altering enzymes. Racemaces, involved in the utilization of amino acids, have been found in certain microorganisms. In addition, vitamin B_6 is also required for tryptophan-niacin conversion (kynereninase) and the synthesis of heme (δ-amino levulenic acid synthetase) and glycogen phosphorylase. The role of PLP in lipid metabolism converting linoleic acid to arachidonic acid and in steroid hormone receptors has also been suggested.

Pyridoxal phosphate coenzyme is bound to various apoenzymes by the formation of a Schiff's base between the keto-carbon of the coenzyme and the ε-amino group of a lysyl residue of the apoencyme. Reactions catalyzed by PLP seems to involve the binding of amino acid substrate to the internal aldimine group by the transaldimination reaction to form an external aldimine (Snell 1990). Delocalization of the electrons in the enzyme-substrate complex leads to the formation of a carbanion at the α-carbon of the substrate which weakens its bonds resulting in the heterolytic cleavage of one of the three bonds in the α-carbon. The bond located in α-carbon which is cleaved is determined by the particular PLP-dependent enzyme; in the decarboxylation of amino acids, the formed enzyme-substrate complex weakens the bond of the carboxyl group resulting in the loss of carbon dioxide.

2.2.3 Proposed role(s) in hyperhomocyst(e)inaemia

The increased homocysteine (Hcy) level in plasma seems to be a risk factor for vascular diseases. The concentrations of homocysteine rise in chronic renal failure (Wilcken and Gupta 1979) and elevated levels are associated with atherosclerosis and coronary artery disease. Increased plasma levels are considered to be those over 15μmol/l while normal Hcy level varies from 5 to 15μmol/l depending on age and sex. Certain diseases and medical treatments as well smoking and the excessive consumption of coffee are known to raise Hcy plasma levels. Dietary fats, fruits, vegetables, vitamins and the amount of their consumption are expected to affect the homocysteine levels in the plasma. The role of genotype is also under investigation. As metabolic evidence for low serum folate, vitamin B_{12} and vitamin B_6 concentrations and elevation of serum homocysteine level in elderly people was found in studies carried out in Belgium, Germany and the Netherlands, early detection of tissue deficiency of these vitamins was recommended (Joosten et al. 1993). In humans, dietary supplementation with vitamin B_6 enhances the glutathione activity and the effect is most apparent in elderly people (Grimble 1997). Cravo et al. (1996) suggested that the chronic alcoholism may increase the plasma Hcy level by interferring with the disposal of homocysteine. Chronic alcoholism is known to interfere with one-carbon metabolism which involves folate and vitamin B_6. Significantly lower PLP level in serum and folate level in red blood cells were found among alcoholics than those of controls. Interaction of PLP in the the desulfhydration route of cystein and homocystein to pyruvate and 2-oxobutyrate has been proposed (Harper et al. 1979).

Plasma folate, vitamin B_{12} and pyridoxal phosphate were inversely associated with the plasma homocysteine concentration (Robinson et al. 1995, Selhub et al. 1995). Folate was the most effective agent for reducing total plasma homocysteine levels in cases of a vitamin B_{12}

deficiency in rats and in patients. Vitamin B_6 had no effect on fasting plasma total concentration but did reduce post-methionine load Hcy levels in plasma. Similar results were found in the Framingham Heart Study; homocysteine exhibited a strong inverse association with plasma folates but weaker associations with plasma vitamin B_{12} or vitamin B_6. Verhoef and associates (1996) suggested that the impairment of remethylation of homocysteine to methionine which is dependent on folate and vitamin B_{12} was the predominant cause for high homocysteine levels and not the vitamin B_6 related transsulfuration route. No inverse correlation between plasma levels of homocysteine and vitamin B_6 was found in their study performed in Boston.

In general, plasma folate, cobalamine and pyridoxal phosphate levels are inversely related to homocysteine concentration. More detailed information concerning the factors and metabolic routes effecting homocysteine levels in plasma need further research. For instance, the clinical benefit of vitamin supplementation has not yet been demonstrated.

2.2.4 Recommended dietary intake

The requirement for vitamin B_6 increases as the intake of protein increases. This relationship is connected to the role of pyridoxal-5'-phosphate in amino acid metabolism. The recommended dietary allowance for vitamin B_6 is 2.0mg/day for men and 1.6mg/day for women (Food and Nutrition Board 1989) which is adequate for an avarage protein intake of 100g/day for men and 60g/day for women. An additional allowance of 0.5mg of vitamin B_6 per day is recommended during lactation.

The recommended daily intake for vitamin B_6 in the Nordic Countries is set at 0.015mg of vitamin B_6 per one gram protein when the protein intake counts as 15 E% (NNR 1996). The same intake value is recommended for pregnant and lactating women. This recommended intake corresponds to a daily intake of 1.5mg and 1.2mg for men and women, respectively. The average requirement is set at 0.013mg/d per one gram protein and the highest recommended intake for an individual person is 50mg per day.

The adequate protein intake from natural foods is considered to ensure the recommended vitamin B_6 intake as vitamin B_6 compounds and proteins occur together in natural foods. Vitamin B_6 deficiency is rarely observed and it is assumed that the low vitamin intake is related to the people deficient in other B-complex vitamins too. The risk group includes elderly people whose energy and food intake is generally decreased and chronic alcoholics (Food and Nutrition Board 1989). The amount of vitamin B_6 in food in Nordic countries is estimated to be 1.5–2.2mg of vitamin B_6 per 10MJ (NNR 1996).

Nevertheless, there is still evidence for low vitamin B_6 intake among the population in the industrialized countries. PLP level in plasma samples from ca. 2500 elderly people participating in the Euronut SENECA study (17 towns in 11 European countries) showed that the prevalence of biochemical vitamin B_6 deficiency was widespread (Haller et al. 1991). Later on the marginal vitamin B_6 status of elderly people (546 persons, aged 74–76) in the SENECA study was reported by van der Wielen and coworkers (1996). Approximately 30% of the males and 40% of the females had dietary vitamin B_6 intake below the mean minimum requirements. A French nutrition survey study conducted in Burgundy (337 middle-aged and healthy subjects consisted of 157 males and 180 females) showed the vitamin B_6 intake below the French recommended dietary intake in 11% of the males and 28% of the females (Costa de Carvalho et al. 1996). Bailey and her associates (1997) reported that the risk for biochemical deficiency was greater for vitamin B_6 and B_2 than for folate, thiamine or ascorbic acid in the elderly British population studied (sixty men, eighty-five women). However, it was concluded that the requirements of the elderly for vitamins need review.

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2.3 Occurrence and distribution of vitamin B_6 in foods

Poultry, fish, offals, pork and egg are considered as rich sources of dietary vitamin B_6. Good sources are also unmilled grain and rice, soy beans, oats and nuts whereas dairy products and red meat are assumed to be poor sources. The bioavailability of vitamin B_6 among different foods, however, varies widely. Food groups can be divided into two categories according to bound vitamers as well as the distribution of free and phosphorylated vitamers. The main component(s) in foods of the plant origin, especially in vegetables and cereals, is pyridoxine which is bound to a carbohydrate moiety (Kabir et al. 1983a, Gregory and Ink 1987, van Schoonhoven et al. 1994, Sampson et al. 1995, Sampson et al. 1996). The content of either glycosylated or free pyridoxine may be considered to be characteristic for the plant-derived foods while phosphorylated pyridoxal and pyridoxamine form the main vitamer fraction in flesh and dairy foods. Ca. 80% of the total vitamin B_6 activity in fresh animal tissues is derived mainly from phosphorylated pyridoxal and pyridoxamine and to a smaller extent from free pyridoxal and pyridoxamine (Rabinowizt and Snell 1948, Polansky and Toepher 1969). Pyridoxine phosphate (PNP) is generally assumed to be a minor vitamin B_6 fraction in foods (Vanderslice et al. 1980, Tadera and Naka 1991).

Variations in the sample treatment procedures (e.g. extraction media and conditions) and the later improvements in methodology, especially in modern liquid chromatography, make it difficult and in some cases not even sensible to compare the vitamer distribution results derived from different era and research groups.

2.3.1 Meat, offals and fish

Main B_6 vitamers present in flesh foods were either free or phosphorylated pyridoxal and pyridoxamine, the proportion of free pyridoxine was smaller. Bowers and Craig (1978) found that the the sum of pyridoxamine and pyridoxal formed ca. 80% of the total vitamin B_6 content in chicken meat and Polansky and Toepfer (1969) obtained a figure of 92–95% the major vitamer being pyridoxal except in liver which was rich in pyridoxamine. A similar distribution was reported for beef, lamb, and pork. Phosphorylated forms of pyridoxal (63%) and of pyridoxamine (35%) predominated in raw chicken breasts (Olds et al. 1993), the total vitamin B_6 content being 0.4–0.6mg/100mg (Ang 1980, Olds et al. 1993). Free pyridoxamine counted only for 2% and no pyridoxine was reported by Olds et al. (1993). Almost an equal amount of total pyridoxal and pyridoxamine (40%) was present in turkey meat while free pyridoxine accounted for as much as 20% of the total vitamin B_6 (Bowers and Craig 1978). The total vitamin B_6 content in fresh pork meat varied from 0.46 to 0.57 mg/100g, the major free vitamers being pyridoxamine and pyridoxal (Esteve et al. 1998). The vitamin content of flounder, oyster and shrimp was derived mainly from pyridoxal and pyridoxamine thus resembling meat. The main vitamer in the canned sockeye salmon and tuna was reported to be pyridoxamine (Polansky and Toepfer 1969). This is probably due to the transamination during and after the canning process.

2.3.2 Plant foods

As much as two thirds of the total vitamin activity in some plant food is reported to be derived from bound pyridoxine; such typical materials are carrot, orange (Gregory and Ink 1987, Gregory 1988a) and cereals (Sampson et al. 1995, 1996). Banana was also rich in free pyridoxine. On the other hand, certain nuts and almonds contained only a low level of glycosylated pyridoxine and the main form was free pyridoxine. The native glucosidase activity in almonds (Chiari et al.1997) may explain this phenomenon. The neutral pH optimum β-glucosidases of almonds

are reported to be capable of hydrolyzing a number of plant glucosides (Lai et al. 1992). A smaller amount of free and phosphorylated pyridoxal was found in plant-derived foods. Pyridoxamine content was reported to vary in different materials; e.g. in tomato the content of total pyridoxamine is significant different to that of carrot.

Kabir and his group (1983a) measured the vitamin B_6 content of several food items microbiologically before and after β-glucosidase treatment. The amount of glucosidic bound pyridoxine in plant foods varied from 5 to 82%; in general, high values were found in grains and legumes. Processed broccoli and cauliflower samples gave the higher values compared to those of the raw material. No explanation for the increased values was given. Sampson and coworkers (1995) showed that pyridoxine glucoside and free pyridoxine consisted the main B_6 vitamers in wheat representing ca. 35–81%, and 21–64% of the total vitamin content in three wheat cultivars analyzed. In their study the amount of PLP and PMP accounted for 12% of the total vitamin content in one cultivar and only pyridoxine and its glycoside were present in two other wheat cultivars. A significant variation in the content of pyridoxine and pyridoxine glucoside was found in 22 North American and Canadian wheats (Sampson et al. 1996). American hard wheat contained more vitamin B_6, mostly in the form of pyridoxine glucoside, compared to Canadian hard wheat. The mean values for pyridoxine glucoside (67%), pyridoxine (33%) were given while other vitamers were not reported.

Glycosylated pyridoxine formed the major B_6 vitamer fraction also in legumes. Pyridoxine glucoside accounted for 40%, 63% and 75% of the total vitamin B_6 in haricot beans, lentils and chick beans, respectively (Sierra and Vidal-Valverde 1997). The amount of free vitamers after acid phosphatase digestion varied 5–17% for pyridoxamine, 15–35% for pyridoxal, and 5–8% for pyridoxine in their samples.

2.3.3 Milk, milk products and eggs

The total vitamin B_6 content of fluid milk ranged from 0.03mg to 0.05mg/100g in the studies of Gregory and Mabbit (1959, 1961), Polansky and Toepher (1969), Dong et al. (1980), Coburn and Mahuren (1983), Laukkanen et al. (1988), Toukairin-Oda et al. (1989) and Sieber et al. (1996). Vitamin B_6 activity in fresh fluid milk consisted of both free and phosphorylated vitamers, pyridoxal being the predominant form (Sieber et al. 1996, Argoudelis 1997) whereas pyridoxal phosphate and pyridoxamine phosphate, and free pyridoxamine each formed less than 10% of the total content. A high proportion of PLP (36%) in milk has also been reported by Toukairin-Oda et al (1989). The results of Coburn and Mahuren (1983) showed that PLP was the main vitamer B_6 fraction (51% of the total amount) in fresh bovine milk while increased amount of PMP and pyridoxal were found in pasteurized milk. The amount of free pyridoxine in milk was low (Gregory and Mabbit 1961) or pyridoxine was not found (Rabinowitz and Snell 1948, Sieber et al. 1996, Argoudelis 1997). Lim et al. (1982) reported that pyridoxine formed almost 80% of the total vitamin B_6 content in non-fat dry milk, a result which is contradictory to the results published by other researchers. Neither the processing procedure nor possible enrichment of the material, however, was described in their paper. Practically no change in the vitamer distribution between fresh milk and skimmed milk powder was found in the results of Polansky and Toepher (1969).

The change in the vitamer distribution was observed in "processed" milk products; the amount of pyridoxamine and PMP in the condensed/evaporated or pastorized milk were increased compared to that of fresh milk. The increased proportion of pyridoxamine, probably derived from pyridoxal or its phosphorylated form, was present in cheeses (Polansky and Toepfer 1969, Coburn and Mahuren 1983, Bitsch and Möller 1989b). As only a small amount of PMP was present in fresh milk, it was suggested to be formed during the process (Gregory and

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Mabbit 1961). A short-term microwave heating of fresh milk (83°C for 4min) did not increase the amount of pyridoxamine according to Sieber et al. (1996). A lower total vitamin B_6 content was observed in cultured butter milks than in milks whereas a higher level of vitamin B_6 was present in yoghurts (Polansky and Toepher 1969, Laukkanen et al. 1988).

The vitamin B_6 content of human milk resembled that of cow's milk; the total amount of 0.02mg/100g was reported by Morrison and Driskell (1985) and the proportion of pyridoxal, pyridoxamine, and pyridoxine was 81%, 13%, and 6%, respectively. The lower proportion of phosphorylated PL was present in human milk compared to that of bovine milk; free pyridoxal formed the main portion (65–83%) while PLP (6–28%), pyridoxine (0–4%), and PMP (1–2%) accounted for the rest of the vitamin B_6 activity (Coburn and Mahuren 1983, Hamaker et al. 1985, Bitsch and Möller 1989a). Only traces (less than one percent or not determined) of free pyridoxamine and no PNP was found.

Raw chicken egg yolk contained 0.44mg vitamin B_6 per 100g and the major vitamer was pyridoxal-5'-phosphate (96%). Pyridoxamine (2%) and pyridoxamine-5'-phosphate (2%) were minor components. The amount of free pyridoxine and pyridoxal were traces or not detected (Argoudelis 1996). The same kind of vitamer distribution was published by Toukairin-Oda et al. (1988).

2.4 Analysis of vitamin B_6 compounds

Traditional sample extraction procedures and their modifications for vitamin B_6 analysis are based on mineralic acid hydrolysis combined with a heating step such as boiling in a water bath or an autoclaving process (AOAC 1995a, 1995b, COST91). An enzymatic digestion is commonly added after the heating treatment to disintegrate the sample matrix and to ensure the quantitative hydrolysis of phosphate esters (van den Berg et al. 1996). After these pretreatment steps only pyridoxine, pyridoxal, and pyridoxamine can be measured as phosphorylated forms are hydrolyzed to their corresponding free vitamers. Mineralic acid sample disintegration and extraction combined with an autoclaving process hydrolyses, at least partially, the glycosidic bond present in glycosylated vitamin B_6 forms. The minor glucosidase activity present in mixed commercial enzyme preparations like Takadiastase caused the hydrolysis of glycosidic bond as well (van Schoonhoven et al. 1994). Thus, the traditional extraction procedures with or without mixed enzyme preparations will most likely release the free B_6 vitamers from their corresponding phosphate and glycoside conjugates. Normally, the total vitamin B_6 content as the sum of free vitamers is reported after the microbiological assay. The content of individual free vitamers can be measured if cation-exchange chromatographic separation on an open column is performed prior to the microbial assay. The information on the original distribution of phosphate esters and glycosidic vitamers in the sample, however, is then lost.

The use of the modern liquid chromatography allows the measurement of individual vitamers, and the original vitamer distribution can be achieved. If the original vitamer distribution of the material is needed (free, phosphorylated and glycosylated vitamers), the procedures, conditions and reagents used in the sample pretreatment and chromatographic separation should be carefully chosen to avoid unnecessary hydrolysis and interconversion of the vitamers but still maintaining the quantitativity of the extraction. The use of a heating step in the extraction procedure helps the disintegration of the matrix but samples rich in protein inevitably produce changes in vitamer distribution due to transamination and dephosphorylation reactions. This should be taken into account when the individual vitamer data from studies using different methodology are compared.

If free, phosphorylated as well as glycosidi-

cally bounded B_6 vitamers are to be measured, non-mineral acids or buffer solutions should be used in the sample extraction. All pretreatments and chromatographic separations should be perform at room temperature or lower. Testing of the enzyme activity including also the probable minor side-activities of the preparations should be considered.

2.4.1 Extraction and hydrolysis techniques

Extraction procedure for measurement of vitamin B_6 compounds usually includes hydrolysis in acidic media and is followed by a sample matrix disintegration with enzymatic hydrolysis. The aim of the acid treatment of the sample is to release protein-bound vitamin B_6 forms. Depending on the extraction acid (mineral acids vs. non-mineral acids) and conditions (temperature and time) used, free vitamers are also released from their corresponding phosphate esters or glycosylated derivatives. The release of vitamers is normally completed by performing an enzymatic digestion after acid treatment. Proteases like pancreatin and pepsin have been used to verify the efficiency of the hydrolysis technique or *in vitro* digestion (Ekanayake and Nelson 1988). The hydrolysis of beef or soybean samples was verified by protease treatment; no release of B_6 vitamers was observed after the protease digestion compared to nonenzymatic treatment. Thus, the nonenzymatic sample treatment was considered to be quatitative (Kabir et al. 1983). Extraction procedures for vitamin B_6 compounds are thoroughly reviewed in the papers of Gregory (1988a) and of Rizzolo and Polesello (1992).

2.4.1.1 Mineral acid extraction

Thermal extraction in acidic solution is the most commonly used procedure to release protein-bound vitamers and to hydrolyze the phosphorylated forms. These methods are based on the work of Atkin et al. (1943) in which it was shown that maximal extraction for most biological samples was achieved by autoclaving the samples in 0.055N sulfuric acid at 128°C for 60 minutes. A more concentrated acid solution, 0.44N, was needed for cereal matrix. Sulfuric acid was replaced with hydrochloric acid, and the most suitable condition for the extraction was suggested to range from pH 1.7 to 1.8 (Rubin et al. 1947). Hydrochloric acid extraction was further evaluated by Rabinowitz and Snell (1947); an autoclaving period of one hour with 0.055M hydrochloric acid was adequate for hydrolyzing pyridoxal-5'-phosphate whereas liberation of pyridoxal from its phosphate was slower with more concentrated acid solutions.

Several hours (4–5h) were needed to release pyridoxamine from its phosphate ester. Only 50% of synthetized PMP was hydrolyzed after 10h at 100°C in 6N sulfuric acid (Peterson et al. 1955). Rather vigorous extraction condition, 2M hydrochloric acid, was needed for bound vitamers present in rice bran matrix. Later on these vitamin B_6 compounds were identified as glycosylated derivatives of pyridoxine. On the basis of these studies, the sample pretreatment for the present microbiological assay was developed; extraction with 0.44M hydrochloric acid (two hours at 121°C) for plant foods and with 0.055M hydrochloric acid (five hours at 121°C) for animal products (Toepher and Polansky 1970). The modification of this method is still in use as a procedure recommended by AOAC (1995a, b).

Mineral acid extraction with hydrochloric acid, the pH of the solution adjusted first to 1.7 and then increased to 4.6, without a thermal process was used for determination of vitamin B_6 in fortified infant formulas. High recovery values were obtained with this extraction procedure (Ayi et al. 1986). Autoclaving for 2 hours in 0.5M hydrochloric acid solution was presented as a sample disintegration step for pasta products. Recovery values ranged from 83% to 92%, lowest values achieved with pyridoxal (Tolomelli et al. 1991). A method applying nitric acid as an extractant for vitamin B_6 has also been published; pharmaceutical multi-vitamin preparations were dissolved in dilute nitric acid solution (1mM) and the mixture was heated on a boiling water bath

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for 30 minutes. After filtration the sample was ready-to inject to liquid chromatograph (Callmer and Davies 1974). Relatively low recovery (97%) for pharmaceutical preparation but rather good precision values (as a standard deviation for peak height values of repeated injections) were reported.

At present, mineral acid extraction using either hydrochloric acid (Yasumoto et al. 1977, Addo and Augustin 1988, Ekanayake and Nelson 1988, Steiner et al. 1993, Bognar and Ollilainen 1997) or sulfuric acid (Wehling and Wetzel 1984, Rees 1989, COST 1991, Olleta et al. 1993, Agostini and Godoy 1997, Esteve et al. 1998) are combined with a heating treatment (autoclaving or boiling water-bath) to ensure extraction efficiency. However, interferring compound(s) mainly preceding pyridoxal after mineral acid hydrolysis may complicate the interpretation of the reversed-phase chromatogram (Bognar 1985, COST1991).

2.4.1.2 Chemical deproteinating agents

Many extraction procedures using a non-mineral acid for the release of the protein-bound B_6 vitamers from biological sample matrix have been reported in the literature; sample hydrolysis with metaphosphoric acid, perchloric acid, sulfosalisylic acid, trichloroacetic acid or with certain buffers enables the extraction of phosphoric acid esters and glycosidically bond conjugates of vitamin B_6 in their intact forms. Several sample pretreatment procedures are published and their use is ideal when the "true" vitamer distribution of the sample is needed. However, many of these methods still lack proper validation data on the extraction efficiency.

Trichloroacetic acid (TCA)

Trichloroacetic acid has been largely used for protein denaturation in the measurement of vitamin B_6 compounds. The main advantages for the use of trichloroacetic acid are its extraction efficiency, it has some ionic characteristics which may enhance the resolution of measured analytes in reversed-phase liquid chromatography, and it can be removed from the aqueous phase by organic solvent extraction with diethyl ether.

The use of TCA or SSA has been recommended for precipitation of protein and protein hydrolysates. In addition certain enzymes, like α-chymotrypsin (Saidel and Modapallimattam 1970) and pepsin (Alfred and Narasinga Roo 1971), maintained their enzyme activity in dilute TCA solutions. Denaturation and inactivation occurred when 5% or more concentrated acid solution was used. At higher concentrations, TCA is suitable for removing the enzyme protein by precipitation after the enzymatic hydrolysis. So the enzyme activy in the extraction solutions containing trichloroacetic acid should be taken into account. The compatibility of trichloroacetic acid in the microbiological assay should also be verified.

Trichloroacetic acid extraction has been applied to clinical studies (Coburn and Mahuren 1983, Hachey et al. 1985, Shephard et al.1987, Shephard et al. 1989, Mahuren and Coburn 1997), and to human milk (Hamaker et al. 1985) and food and feed analysis (van Schoonhoven et al. 1994) as well as measuring the sugar-derivatives of pyridoxine (Ink et al. 1986, Sierra and Vidal-Valverde 1997). As the phosphate ester and glucosidic bond were not hydrolyzed by TCA the extraction in lowered or ambient temperature, this extraction enabled the determination of free, phosphorylated and glycosylated compounds. In the above mentioned studies, the concentration needed for complete liberation of vitamers normally ranged between 5–10% (w/v). Ubbink and coworkers (1986) showed that albumin bound PLP was quantitatively liberated by a trichloroacetic acid concentration of 3% which is somewhat more dilute than the solutions used by the other researchers. The advantage of using TCA for the separation efficiency of vitamers in the reversed-phase chromatography was stated by Hamaker et al. (1985); the resolution of pyridoxal and its phosphate ester was dependent on the presence of TCA.

The use of trichloroacetic acid as an extracting agent for vitamin B_6 compounds has been evaluated with both individual and collaborative

studies, thus the validity results for this extraction agent are available (Hollman et al. 1993, Berg van den et al.1996). An acceptable repeatability as well as reproducibility value for the total vitamin B_6 content was achieved by using a 5% trichloroacetic acid solution followed by enzymatic hydrolysis for sample matrix disintegration and dephosphorylation. This data was derived from the work of an European collaborative study in which eleven laboratories participated during 1989–1997.

Perchloric acid (PCA)

Perchloric acid has been widely applied as a sample deproteinizing agent. The acid concentration used for the extraction of vitamin B_6 compounds varied considerably; 0.1–0.5M solutions have been used for different foods depending on the protein content (Möller and Bitsch 1988, Bitsch and Möller 1989a, Chase et al. 1992), 0.4M for human plasma (Mascher 1993, 1997), and 1M for biological samples (Pierotti et al. 1984, Tsuge et al.1988, Kimura et al. 1996, Argoudelis 1997, Tsuge 1997). Reynolds and Brain (1992) used PCA only for deproteinizing the sample extract and not for the sample matrix disintegration. Perchloric acid has been used for measurement of both glycosylated derivatives and phosphate esters in their native form (Schramm and Bitsch 1993). Difficulties in the extraction were assumed to be derived from the protein-binding properties of pyridoxal phosphate still present in the dilute perchloric acid solutions (Edwards et al. 1989). An advantage of PCA is that it can be removed by precipitation with alkali, and thus diminishing the risk for the incompatibility of the sample extract/solvent with the chromatographic separation or with the enzymatic hydrolysis. Neutralizing the perchloric acid extraction solvent to pH of 7.5 with dilute potassium hydroxide solution precipitates potassium perchlorate. Even alkali labile pyridoxal is assumed to be stable during this procedure.

Sulfosalicylic acid (SSA)

Some earlier studies applied sulfosalicylic acid as an acid extraction agent in amino acid methodology (Mondino et al. 1972). Later on Vanderslice and his colleagues (1980) published a routine liquid chromatographic method which included a sulfosalicylic acid extraction procedure for vitamin B_6 compounds. A 5% sulfosalicylic acid solution was confirmed to be an effective deproteinizing agent. The prevention of interconversion of B_6 vitamers was also observed. High vitamer recovery values for several different food materials were reported. Since their work sulfosalicylic acid extraction has been used for several food items (Vanderslice et al. 1984, Gregory and Feldstein 1985), for ready-to-eat cereals (Vanderslice et al. 1981b) and for poultry meat (Olds et al. 1993). As sulfosalicylic acid has a native fluorescence in the excitation/emission wavelength related to vitamin B_6 compounds it has to be removed before fluorometric measurement. Sulfosalicylic acid was removed with a purification column containing anion-exchange resin in the work of Vanderslice and coworkers (1981b). However, this purification step diluted the sample extract. This could be a disadvantage when the samples of low vitamin content are to be analyzed.

Metaphosphoric acid (MPA)

One of the first extraction methods for vitamin B_6 using metaphosphoric acid was published by Loo and Badger (1969). Rather low acid concentration, 0.8% (0.17M), was needed for protein denaturation in the assay of vitamin B_6 compounds in brain tissue. It was stated that the removal of lipids prior to metaphosphoric acid extraction effectively released B_6 vitamers. Higher acid concentrations were used in subsequent studies. A 0.5M metaphosphoric acid hydrolysis was applied to chicken meat sample at temperatures below 15°C followed by a heating step in a boiling water bath for 4 minutes (Ang et al. 1988). High recovery values were reported when B_6 vitamers and pyridoxic acid of biological fluid and tissue samples were liberated with 5% to10% metaphosphoric acid solutions by Sharma and Dakshinamurti (1992). The method was suggested to be suitable for analyzing biological liquids with a low B_6 vitamer content. Sampson and

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coworkers (1995, 1996) described a deproteinizing step with cold 5% acid solution for measuring the content of free and glycosylated vitamers of different wheat cultivars. However, a low recovery for added pyridoxal and pyridoxal phosphate was reported

Buffer solutios and water

Dilute buffer solutions have been used for extraction of B_6 vitamers from various foods and other biological materials. Neutral phosphate buffer solution (0.1M, pH7.0) extracted pyridoxal, pyridoxamine and their phosphate esters from rat tissue samples after which the protein precipitation was performed with 0.6M perchloric acid solution (Gregory 1980a, Gregory et al.1981). High recoveries were obtained for all vitamers except for pyridoxal phosphate in certain food matrix. Low recovery for PLP was assumed to be due to the pre-column derivatization with semicarbazide reagent or some impurities quenching the fluorescence intensity in the detection. Extraction with a neutral 0.1M phosphate buffer was applied to the sample preparation for determination of pyridoxine in multivitamin preparations (Razagui and Barlow 1989). This extraction solvent was also suitable for the direct spectrofluorometric measurement. Buffer solutions (pH 6.8) were used in sample extraction for measurement of glycosylated vitamin B_6 in foods; vegetables, fruits, nuts, grains and legumes as well as animal product samples were stirred in 0.1M phosphate buffer solution for two hours at room temperature. The extraction method described was suggested to be usefull for various food matrix (Kabir et al. 1983a). In bioavailability studies of glycosylated pyridoxine Kabir et al. (1983b) used a 0.1M phosphate buffer solution (pH5) for extraction of urine or faces samples.

Extraction with an acetate buffer of pH 4.5 has been utilized for clinical samples (Hefferan et al. 1986, Bötticher and Bötticher 1987) , for fortified breakfast cereals (Gregory 1980c), vitamin preparations (Bühnert 1988) as well as for food samples (Reitzer-Bergaentzle et al. 1993). Acidic acetate solution was easily combined with the followed enzymatic hydrolysis procedure and the method was suitable for the simultaneous measurement of free B_1, B_2 and B_6 vitamers in plasma and serum samples. The validity of the acetate extraction used by Reitzer-Bergaentzle et al. (1993) preceeding the pre-column derivatization of B_6 vitamers into pyridoxine was further evaluated by the collaborative study performed in France (Bergaentzle et al. 1995). A lower pH acetate buffer was used for multivitamin preparate; sample was sonicated with a dilute solution of acetic acid (0.5M) and triethylamine (40mM) at the pH of 3.6 (Lam et al. 1984) or with liquid chromatography's mobile phase (acetonitrile – 10mM phosphate buffer – triethylamine, 8:91.5:0.5, pH 3.8)(Maeda et al. 1989). Good accuracy and recovery values for pyridoxine were obtained in both studies.

Boiling water (Tadera et al. 1986a) or aqueous ethanol (75%)(Tadera et al. 1988) was used for extraction in isolation of glycosylated pyridoxine(s) in cereal samples. Boiling 75% ethanol was considered to possess sufficient enzyme inactivating capability since neither pyridoxine glycoside or pyridoxine was formed from exogenous pyridoxine or glycosylated pyridoxine, respectively. Also transglycosylation to form oligoglucosides caused by β-glucosidase was ruled out. However, the efficiency of ethanol – water -mixture for the extraction of cellobiocyl and glucotriocyl derivatives of pyridoxine in rice bran was not stated. Good recovery and repeatability values for pyridoxine content of multivitamin preparations and elemental diets were reported using a methanol – water -mixture (80:20) (Amin and Reusch 1987), water (Wang and Hou 1988a, Belal 1989) or 2M sodium chloride solution (Iwase 1992) as a dissolving agent. In these studies the extraction procedure could be simplified as the vitamin B_6 content was related only to one vitamer, pyridoxine.

2.4.1.3 Enzymatic hydrolysis

Liberation of B_6 vitamers from their corresponding phosphate esters or glycosides can be performed with acid hydrolysis combined with an autoclaving step or with an enzymatic hydroly-

sis. Rather long autoclaving time, ca. 4–5 hours, in 0.055M hydrochloric acid solution was needed for complete hydrolysis of PMP (Rabinowitz and Snell 1947). Glycosylated pyridoxine is expected to be hydrolyzed more easily in autoclaving with mineralic acid solution than PMP. Dephosphorylation of PLP and PMP using an enzymatic digestion is normally performed overnight and the most commonly used enzymes in vitamin B_6 analysis are phosphatases or the mixed enzyme preparations like Takadiastase and Claradiastase. The time required for releasing the bound pyridoxine with β-glucosidase treatment varied from 30min to several hours depending on digestion conditions. The enzyme treatment, especially with mixed enzyme preparations, also enhance the extraction efficiency due to the sample matrix disintegration by several enzyme activities.

Dephosphorylation

Acid phosphatase preparation has been used for dephosphorylation of PLP and PMP present in food (Addo and Augustin 1988, Reitzer-Bergaentzle et al. 1993) and plasma samples (Guilarte 1983, Hefferan et al. 1986, Mascher 1993, Mascher 1997). The amount of enzyme added to extraction/sample solution ranged from 16–100U/g for food samples and 0.4–2U/g for plasma sample, the pH of the digestion solution being 4.5 to 4.7. Earlier studies of Morrison and Driskell (1985) showed that hydrolysis of human milk samples for 1 h at 37°C resulted in incomplete dephosphorylation. An incubation period of 16h was needed to liberate pyridoxal from its phosphate ester in plasma samples (Mascher 1993) which was a longer period than reported in many other papers. Thus prolonged incubation time, 12h to overnight at 37°C, to ensure the proper hydrolysis was recommended. An acid phosphatase treatment combined with conversion of pyridoxal and pyridoxamine into pyridoxine is well documented including the collaborative study parameters (Reitzer-Bergaentzle et al. 1993, Bergaentzle et al. 1995). Hydrolysis based on alkaline phosphatase has been carried out after perchloric acid extraction. Next to precipitation of perchlorate with alkali, the pH of the sample solution match more easily with alkaline phosphatase hydrolysis omitting/reducing the need for readjustment of pH. Relatively short incubation times were reported for alkaline phosphatase treatment; 30min at 25°C (Möller and Bitsch 1988, Bitsch and Möller 1989a).

Mixed enzyme preparations, like Takadiastase or Clarase, normally contain enough phosphatase activity for dephosphorylation of pyridoxal phosphate and pyridoxamine phosphate. The advantage of the use of a multi-enzyme preparate, Claradiastase, was reported by Bötticher and Bötticher (1987). The enzyme preparate used in their application contained several enzyme activities (diastase, proteinase, phosphatase) making it suitable for simultaneous extraction of B_1, B_2 and B_6 vitamers in blood samples. Esteve and cooperators (1998) applied the AOAC extraction procedure for thiamine and riboflavin to release vitamin B_6 compounds in fresh pork meat; this method included an enzymatic digestion with Takadiastase. A good accuracy estimated through recovery assays was reported. The use of Takadiastase has been proved to yield quantitative hydrolysis of PLP and PMP; the efficiency of dephosphorylation was estimated by measuring the coefficient of variation and the average percentages of recovery/conversion (van Schoonhoven et al. 1994). Takadiastase hydrolysis was further evaluated by the intercalibration and collaborative studies carried out by BCR (van den Berg et al. 1996). It should be noted, however, that prolonged sample digestion with Takadiastase may induce transamination of pyridoxal to pyridoxamine changing the original vitamer distribution. Claradiastase hydrolysis after mineral acid treatment did not yield higher vitamer levels in flour or vegetable samples. On the contrary, a decreased amount of pyridoxal were observed with some samples (Bognar 1985). This was assumed to be a result of transamination or related reactions of pyridoxal. Acid hydrolysis followed by the fungal amylase Clarase digestion was applied to fortified cereal products by Wehling and Wetzel

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(1984). A high reproducibility and accuracy for the analytical method was reported. Nevertheless, pyridoxine was the only B_6 vitamer found in samples analyzed.

Mixed enzyme preparations used for vitamin B_6 analysis, like Takadiastase or Clarase, include several different enzyme activities. Commercial Takadiastase preparation (Serva 35740, Germany) contained α- and β-amylase, adenosine deaminase, nuclease Si, and ribonuclease Ti activity. However, a negligble activity for alkaline phosphatase has been found. Cellulase, α-amylase, invertase, peptidase, phosphatase and sulfatase activity were specified for Claradiastase (Fluka 27540, Switzerland). Enzyme mixture preparations Claradiastase and Takadiastase have resulted in a good extraction efficiency and their use also in multivitamin extraction was proposed (Steiner et al. 1993, van den Berg et al. 1996). A drawback of these mixed preparations can be that the enzyme activity varies from batch to batch and the amount of side-activities is seldom known by the user (Bognar and Ollilainen 1997). Thus, the miscellaneous results in literature may reflect the variability of enzymes used in different studies.

Hydrolysis of glycosylated vitamers

Glucosidase treatment of sample extracts has been carried out in several studies in order to measure the amount of glycosylated B_6 vitamers in samples of plant origin (Kabir et al. 1983a, Kabir et al. 1983b), to test the proposed methodology (e.g. Tadera and Naka 1991) or to confirm the isolated bounded vitamer form (Tadera et al. 1986a). Hydrolysis by β-glucosidase has been performed at pH 5 in phosphate or in acetate buffer for 2–5 hours at 37°C (Kabir et al. 1983a, Kabir et al. 1983b, Sampson et al. 1995, Sierra and Vidal-Valverde 1997). The increase in pyridoxine after this digestion step is mainly considered to be due to hydrolysis of 5'-*O*-(β-D-glucopyranosyl)pyridoxine, the bound B_6 vitamer first isolated from rice bran by Yasumoto et al. (1977). The structural evaluation of 5'-*O*-(β-D-glucopyranosyl)pyridoxine was confirmed by enzyme treatment as this structure was vulnerable to the action of β-glucosidase and not to that of α-glucosidase. Further structural elucidation revealed that also three other pyridoxine glycosides present in rice could be hydrolyzed to free pyridoxine and sugar moieties with β-glucosidase (Tadera et al. 1988). *In vitro* studies of Kawai et al. (1973) clearly showed that both the formation and hydrolysis of pyridoxine-α-glycoside are catalyzed by α-glucosidases. These α-glucosidase enzymes are particularly distributed in the bacteria genera *Sarcina* and *Micrococcus* (Kawai et al. 1972a, b, Kawai et al. 1973) and, the transglycosidation to vitamin B_6 by these microorganisms has been thoroughly studied (Ogata et al. 1969a, 1969b, Kawai et al. 1971b). The synthesis of pyridoxine glucoside by β-glucosidase of rice bran has also been reported (Iwami and Yasumoto 1986).

2.4.2 Chromatographic techniques

Ion-exchange chromatography or ion-pair reversed-phase chromatography are the most commonly used techniques for the separation of vitamin B_6 compounds due to their pH-depended ionic nature. Ionic interaction between the cationic pyridinium or methylamino moiety and the ion-pair reagent or cation-exchange support enables their controlled retention. Partition chromatography of ionic B_6 vitamers using reversed-phase column packings (e.g. octadecyl or octyl phases) usually suffers from the poor retention for PLP and pyridoxamine. The anionic properties of the analytes can only be utilized with the polymer based or related column packings as the dissociation constants of B_6 vitamers lies normally outside the stability range of the normal silica based column packings.

Most of the recently published reversed-phase chromatographic procedures for vitamin B_6 compounds have been performed using chemically bounded phases (CBP), the most prevalent packing being the octadecyl phase (Table 3). The improvements in column packing materials, that is the synthesis of the silica base and the chemically bonded phases, have enabled a

Table 3. Conditions for liquid chromatographic determination of B_6 vitamers.

Sample preparation	Stationary phase	Mobile phase	Detection	Samples/ vitamers	Reference
Extraction with 10%TCA, 2-amino-5-chlorobenzoic acid as internal standard, remove of TCA with diethyl ether	Vydac 401TP-B, 10µm, 300x4.6 mm id	A. 0.02M HCl B. 0.1M sodium phosphate buffer, pH3.3 C. 0.5M sodium phosphate buffer, pH 5.9 gradient elution, 1.5ml/min	Fluorescence, 330/400nm Post-column derivatization with sodium phosphate buffer (1M, pH 7.5) containing 1mg/ml $NaHSO_3$ (4.5ml/h)	Serum, various tissues, milks PLP, PMP, PL, PN, PM, PNG	Cuburn and Mahuren 1983
Homogenization with 5% SSA, DPN as internal standard, remove of lipids with CH_2Cl_2, preparative anion-exhange chromatography to remove SSA β-Glucosidase treatment to verify glycosylated vitamer	Perkin-Elmer ODS, 3µm, 30x4.6mm id	A. 0.033M phosphate buffer, 8mM octanesulfonic acic, pH2.2 B. 0.033M phosphate buffer, pH2.2 – 2-propanol (93:7) gradient elution, 1.8ml/min	Fluorescence, 330/ 400nm Post-column derivatization with potassium phosphate buffer (1M, pH 7.5) containing 1mg/ml $NaHSO_3$ (0.2ml/min)	Plant foods, human milk PLP, PMP, PL, PN, PM, PA	Gregory and Ink 1987
Extraction with 1M PCA, DPN as internal standard, remove of PCA with KOH	TSK Gel ODS-80, 250x4.6mm id	0.05M potassium phosphate, pH 3.5, 0.12M sodium perchlorate, – methanol (99:1) isocratic elution, 0.5ml/min	Fluorescence, 330/ 400nm Post-column derivatization with potassium phosphate buffer (1M, pH 7.5) containing 1mg/ml $NaHSO_3$	Various foods PLP, PMP, PNP, PL, PN, PM, PNG, PA	Tadera and Naka 1991
Extraction with 0.5-1M PCA, DPN as internal standard, remove of PCA with KOH, dephosphorylation with alkaline phosphatase	LiChrospher RP-18, 5µm, 125x4.6mm id	A. methanol B. 0.03M phosphate buffer, pH2.7 – 4mM octanesulfonic acid gradient elution, 1.5ml/min	Fluorescence, 330/ 400nm Post-column derivatization with potassium phosphate buffer (0.5M, pH 7.5) containing 3.7mg/ml $NaHSO_3$ (0.07ml/min)	Liver, milk PLP, PMP, PL, PN, PM, PA	Bitch and Möller 1989
Extraction with 0.05M sodium acetate, deamination of PM to PL with glyoxylic acid, dephosphorylation with phosphatase, reduction of PL to PN with $NaHB_4$	LiChrospher RP Select B, 5µm, 250x5mm id	Acetonitrile – 0.05M potassium phosphate, pH2,5 0.5mM heptanesulfonic acid isocratic elution, 1ml/min	Fluorescence, 290/395nm	Yeast, wheat germ, breakfast cereal PN	Reitzer-Bergaentzle et al. 1993
Extraction with 5%TCA, DPN as internal standard, dephosphorylation with Takadiastase	ODS Hypersil, 3µm, 125x4.6mm id	Methanol – 0.1M phosphate buffer, pH2.15 – 1.25mM octanesulfonic acid isocratic elution, 1.2ml/min	Fluorescence, 333/ 375nm Increase of pH prior detection with 1M K_2HPO_4 (0.3ml/min)	Various plant foods, meats, feeds, yeast PL, PN, PM	van Schoonhoven et al. 1994
Homogenization with 5% TCA, dephospharylation with acid phosphatase	Spherisorb ODS2, 10µm, 300x3.9mm id	Methanol- 0.033M phosphate buffer pH2.2 (2:98)			
β-Glucosidase digestion for glycosylated vitamer(s)		isocratic elution, 1.2ml/min column temperature 17C	Fluorescence, 328/ 390nm Increase of pH prior detection with 0.3M K_2HPO_4 (0.7ml/min)	Lentils, peas, beans PL, PN, PM, PNG	Sierra and Vival-Valverde 1997

HCl; hydrochloric acid, DPN; 4-deoxypyridoxine, KOH; potassium hydroxide, PA; pyridoxic acid, PCA; perchloric acid, PL; pyridoxal, PLP; pyridoxal-5'-phosphate, PN; pyridoxine, PNG; pyridoxine glucoside, PM; pyridoxamine, PMP; pyridoxamine-5'-phosphate, SSA; sulfosalicylic acid, TCA; trichloroacetic acid

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large variety in specificity of the column materials and yielded sophisticated separation procedures. The choice of the composition of the mobile phase, that is organic solvent and buffer, depends on the desired pH and the sample extraction procedure and *vice versa*.

Native state fluorescence, characteristic to vitamin B_6 related compounds, enables their fluorometric detection which is more selective and sensitive compared to that of UV detection. However, improvements in specificity and selectivity in the detection, e.g. use of a mass-selective and other MS applications, are still certainly required to reduce the misinterpretation of the chromatographic data.

2.4.2.1 Open column chromatography

In early studies of vitamin B_6 methodology (e.g. Toepher and Lehman 1961) the separation was based on cation-exchange chromatography in an open column as the cationic characters of pyridinium and aminomethyl group enables their ion-exchange chromatographic separation. Open column chromatography with Dowex AG 50–8X (Toepher and Lehman 1961), sulfonated Amberlite CG120S or related resins (Loo and Badger 1969) for separation of free pyridoxal, pyridoxine and pyridoxamine was described; changing the pH of the elution solvent, three vitamers can be separated according to their pK constants. This procedure combined with microbiological quantitation has been well documented and validation data from the collaborative studies is available (Toepher and Polansky 1970). This approach is still in use in the method recommended by AOAC (1995a, b). A disadvantage of this separation procedure is the limited efficiency and the unwanted dilution of the vitamer fractions. In addition, the method is useful only for free pyridoxine, pyridoxal and pyridoxamine as phosphorylated vitamers need to be hydrolyzed prior to chromatography.

Proper separation efficiency can still be achieved by using an ion-exchange resin with an open column technique despite the higher separation efficiency achieved in the HPLC with modern chemically bounded ion-exchange or partition column packings. Quite recently, a cation-exhange chromatographic separation of vitamers using open-column chromatography was published by Srividya and Balasubramanian (1997). Chromatographic separation was performed prior to the spectrophotometric determination of diazotized 2,4-dinitroaniline derivative of pyridoxine The proposed method was applied for pharmaceutical preparations, biscuit and rice samples.

2.4.2.2 High-performance liquid chromatography

Ion-exchange chromatography

High-performance liquid chromatographic cation-exchange separation has been applied to biological samples (Coburn and Mahuren 1983, Hart and Hayler 1986 , Argoudelis 1988, Mahuren and Coburn 1997) and food materials (Wong 1978). Seven B_6 vitamers: PLP, PNP, PA, PMP, PL, PN and PM, and an internal standard (2-amino-5-chlorobenzoic acid) were separated within 36 minutes using a silica-based ion-exchange column packing (Coburn and Mahuren 1983). The total analysis time varied from 50 to 60 minutes depending on the sample complexity. Baseline chromatographic separation of standards was achieved using a rather long gradient elution with phosphate buffers. Good precision and recovery was obtained with this method. However, to verify the proper peak identity and purity, modifications in solvent gradient were occasionally needed. Shephard and associates (1987) applied a cation-exchange chromatographic method for human plasma samples. Short column life was considered as a drawback of the method as the retention of early eluting compounds, pyridoxal phosphate and pyridoxic acid, decreased rapidly in the course of time. Cation-exhange chromatography was further applied to human tissue samples (Shephard et al. 1989) and other biological samples (Argoudelis 1988) in which good precision and recovery data for six vitamers was reported. Callmer and Davies (1974) published a cation-exhange chromatographic determination of pyridoxine in vitamin preparation using dilute nitric acid as an eluent.

An anion-exhange chromatography of B_6 vitamers and pyridoxic acid was published by Vanderslice et al. (1979); vitamin B_6 compounds were separated as their anion forms at pH 10 with a BioRad A-25 anion exhance packing the eluent being sodium chloride-glycine buffer. This method was applied to food samples in the comprehensive studies of Vanderslice and coworkers; for cereal, milk powder and flesh foods (Vanderslice and Maire 1980, Vanderslice et al. 1980), ready-to-eat cereals (Vanderslice et al. 1981b) and for chicken meat (Olds et al. 1993). If naturally fluorescent sulfosalicylic acid was used as a deproteinating agent in the extraction, it had to be removed by an anion-exchange purification using a Dowex or Aminex A-50 resins prior to the chromatographic separation (Gregory and Ink 1987). The disadvantage of this clean-up procedure was the inevitable dilution of the vitamer fraction.

Partition and ion-pair chromatography

Dilute acidic solutions have been used as a mobile phase with the octadecyl phase; a dilute sulfuric acid (Bognar 1985, COST91 1995, Esteve et al. 1998) or an ammonium sulfate solution (Steiner et al. 1993) were used for the separation of free vitamers in food samples. Despite the polar nature of B_6 vitamers, reversed-phase separation based on partition can yield proper relative retention; only pyridoxamine or pyridoxal-5'-phosphate as such showed an inadequate retention in the partition mode. More commonly the separation of B_6 compounds is established with an ion-pair partition chromatography due to vitamers' ionic nature. The control of retention and selectivity in the ion-pair chromatography have been thoroughly documented by Dong et al. (1988) and Schill (1989). The use of a complexing agent, the ion-pair reagent, enhances the retention of an analyte to the stationary phase or alters the selectivity of the compounds. The retention of an organic ion is affected by the nature of an ion-pairing compound and its concentration, and the presence of other counter ions or components competing for the binding capacity of the stationary phase. By choosing the ion-pair reagent and the separation conditions, the relative retention of vitamin B_6 compounds, especially that of pyridoxamine, can be widely regulated.

The chromatographic separation of free and phosphorylated vitamers as well as pyridoxine-β-glucoside was published by Gregory and Ink (1987). Reversed-phase separation on a 3µm ODS-column was performed with a gradient elution of the mixture of 2-propanol, phosphate buffer and octanesulfonate at pH 2.2 (Gregory and Feldstein 1985) followed by Coburn's and Mahuren's (1983) enhanced fluorerence detection with sodium bisulphite reagent. Good repeatability was achieved and the procedure was applied to a number of plant-derived samples. The method and its modifications have been further applied to food and other biological materials later on by various research groups. The use of an alcyl derivative of sulfonic acid predominates in ion-pair chromatography of B_6 compounds; a number of reports using hexane sulfonic acid (Kawamoto et al. 1983, Wehling and Wetzel 1984, Bötticher and Bötticher 1987, Chase and Soliman 1990, Tolomelli et al. 1991, Reynolds and Brain 1992, Olleta et al. 1993, Blanco et al. 1994, Agostini and Godoy 1997), heptane sulfonic acid (Lam et al. 1984, Hefferan et al. 1986, Bühnert 1988, Rees 1989, Iwase 1992, Reitzer-Bergaentzle et al. 1993) and octane sulfonic acid (Bitsch and Möller 1989, Maeda et al. 1989, van Schoonhoven et al. 1994, Sharma and Daksinamurti 1992, Sampson et al. 1995) or a mixture of alcyl sulphonates (Jaumann and Engelhardt 1985, Addo and Augustin 1988) have been published. The phenolic character of pyridoxine was utilized by Belal (1989); tetrabutyl ammoniumhydroxide enabled the proper separation of pyridoxine and pyrithioxine in a selected pH of 8 in dosage forms.

Enhanced selectivity for the separation of thiamin, riboflavin, pyridoxine and niacin using a reversed-phase column was achieved with a small amount of ammonium hydroxide in the mobile phase (Chase and Soliman 1990). The use of triethylamine did not give the same result in their application. A 16mM concentration of mon-

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ochloroacetic acid (MCA) in the phosphate buffer mobile phase insteadt of trichloroacetic acid improved significantly the resolution of pyridoxamine and its phosphate ester (Ekananayke and Nelson 1988). No explanation of the detailed mechanism for MCA for this particular resolution was given. A similar affect for trichloroacetic acid (TCA) on the separation efficiency has also been suggested (Bognar, personal communication). Japanese researchers used sodium perchlorate (100–120mM) in an isocratic mobile phase to separate six B_6 vitamers, pyridoxine β-glucoside, pyridoxic acid and deoxypyridoxine within a single run. Perchlorate anion was stated to serve as an ion-pair reagent in this application (Tsuge et al. 1986, Tadera and Naka 1991). A small amount of PCA present in the mobile phase consisting of phosphate buffer enabled the separation of seven (Argoudelis 1997) and fourteen (Tsuge 1997) vitamin B_6 related compounds in a single isocratic run. A mobil phase (pH 2.5) containing sodium perchlorate (5mM), alkyl sulphonate (10mM) and methanol separated four free vitamers well in a isocratic run within 14 minutes (Kawamoto et al. 1983). The silanol effect between reversed-phase packings and basic analytes (Stadalius et al. 1988, Li 1992, Sykora et al. 1997) have been diminished by using organic solvent modiers (McCalley 1995) in the mobile phase; free silanols in the packing surface were blocked by adding triethylamine (Mascher1993, Lam et al. 1984, Edwards et al. 1989, Agostini and Godoy 1997) or di-N-butylamine (Bötticher and Bötticher 1987) to the mobile phase used for vitamin B_6 analysis.

The use of a volatile buffer in the mobile phase is favorable for LC-MS applications. Careri et al. (1996) used methanol and ammonium formate in the eluent to apply the mobile phase stream (150µl/min) into a quadrupole mass spectrometer via a particle beam interface. Good repeatability data (RSD% <6%) for pyridoxal, pyridoxine and pyridoxamine was presented.

Detection

Native state fluorescence of vitamin B_6 related compounds enables their selective and sensitive fluorometric detection which is considered to be superior to UV detection. Liquid chromatographic separation of B_6 compounds is mainly done in acid or neutral medium due to the better separation efficiency of ion-pair chromatography in an acidic enviromental (Dong et al. 1988) or to restrictions of silica-based columns in alkaline conditions (Claessens et al. 1996). The maximum fluorescence intensity of pyridoxine is however at pH 7 (Duggan et al. 1957).

The low fluorescence intensity of pyridoxal 5'-phosphate was enhanced with an innovative post-column derivatization by Coburn and Mahuren (1983). After cation-exhange chromatographic separation of PLP, PNP, PA, PMP, PL, PN and PM on a Vydac 401TP-B column in acidic medium, the pH of the mobile phase was raised to neutral by mixing the eluent with a derivatization solution consisting of phosphate buffer (1M, pH7.5) and sodium bisulphite (1mg/ml) prior fluorometric detection. The elevated pH and the formed bisulphite adduct increased the intensity of pyridoxal 5'-phosphate in the FL detection allowing its measurement in the biological samples with a low level of B_6 vitamers. This approach with modifications has been widely accepted later on for the analysis of pyridoxal-5'-phosphate either alone or among other vitamers in food samples (Hamaker et al. 1985, Gregory and Ink 1987, Addo and Augustin 1988, Ekanayake and Nelson 1988, Bitsch and Möller 1989a, Tadera and Naka 1991, Olds et al. 1993) and in other biological materials (Reynolds and Brain 1992, Sharma and Daksinamurti 1992, Sampson et al. 1995, Mahuren and Coburn 1997). Various changes in Coburn's and Mahuren's post-column derivatization have been presented; a pre-column modification for cell-free yeast culture media was published by Argoudelis (1988) whereas Kimura et al. (1996) added bisulphite reagent to the mobile phase. So no further equiment for derivatization in the latter application was needed. In the method reported by Argoudelis (1997), the bisulphite adduct of PLP was formed in acidic pH without raising the pH of eluent with an alkaline solution. On the other hand the elevated pH itself has been

reported to produce a more sensitive and selective detection by reducing the interference found in rice and wheat samples (van Schoonhoven et al. 1994) and legumes (Sierra and Vidal-Valverde 1997). Apparently there was no need for adding bisulphite to the post-column reagent in their applications.

A post-column derivatization using the semicarbazide reaction for increasing the intensity of PLP was first reported by Gregory (1980a). Unfortunately, the fluorescence intensity of formed PLP semicarbazone was not at its maximum in slightly acidic to neutral solutions. The advantage of this derivatization was that the reaction could also be performed in dilute trichloroacetic acid solutions (pH<2) compatible with the sample extraction procedure (Ubbink et al. 1986). The semicarbazide reaction was utilized in the determination of PLP in plasma samples by Vanderslice and Maire (1980) and Mascher (1993, 1997). Gregory and his associates (1981) published a procedure to deaminate pyridoxamine and its 5'-phosphate ester to related aldehyde vitamers by a glyoxylate reagent. The fluorescence of PLP was enhanced by a further semicarbazide reaction. The glyoxylate reaction provided quantitative deamination of PM and PMP, and the formed PLP semicarbazone was stable enough for multiple analysis. The enzymatic hydrolysis of phosphorylated vitamers with an acid phosphatase digestion combined with a glyoxylic acid deamination of pyridoxamine to pyridoxal followed by reduction with sodium borohydride was presented by Reitzer-Bergaentzle and coworkers (1993). The advantage of this excellent pre-column derivatization was that only one vitamer, pyridoxine, needed to be finely measured. The validity of the method was further tested by a collaborative study in which good repeatability and reproducibility was obtained (Bergaentzle et al. 1995). This method has been put forward as an official method in France for vitamin B_6 determination in foods.

Potassium cyanide acted as a catalyst in the oxidation of PL and PLP to 4-pyridoxic acid lactone and 4-pyridoxic acid 5'-phosphate, respectively (Ohishi and Fukui 1968). The formed derivatives possessed enhanced fluorescence intensity compared to the intact aldehydic vitamers. No effect on the fluorescence characteristics of other vitamers was found. This method was succesfully applied to food (Tsuge et al.1988, Toukairin-Oda et al. 1989) and other biological samples (Millart and Lamiable 1989, Tsuge 1997).

UV detection usually lacks the sufficient sensitivity and selectivity for measuring vitamin B_6 compounds in foods and other complex biological materials. The few published papers are mainly limited to the use of UV detection after an ion-exchange purification of extracts (Wong 1978), in the fraction collection step prior to mass spectrometry (Hachey et al. 1985) or to the determination of vitamin B_6 in the vitamin preparations or enriched foods (Agostini and Godoy 1997). The wavelength of a conventional UV detector was set at 254 nm for multivitamin capsule preparations (Amin and Reusch 1987, Maeda et al. 1989) and 280–290 nm for the commercial multivitamin preparations (Callmer and Davies 1974, Lam et al. 1984, Bühnert 1988, Hauenstein 1990, Iwase 1992). An on-line precolumn step was needed as an enrichment and clean-up procedure for measuring vitamin B_6 with UV-detection in the vitamin enriched grape sugar (Jaumann and Engelhardt 1985). The capability of a photodiode array detector for vitamin analysis was evaluated in the study of Arai and Hanai (1988). This system was capable of detecting six vitamins within one chromatographic run. Difficulties in the quantitative and qualitative analysis of a vitamin mixture was supposed to be solved by combining the diode array detection on UV region with fluorometry.

The reaction of pyridoxine with 2,6-dibromoquinone-4-chlorimide in the presence of ammonia yieded a colored compound which exhibits VIS absorption maximum at 650nm (Kawamoto et al. 1983). The proposed method was reported to be approximately seven times more sensitive for measuring pyridoxine in vitamin preparation than the conventional UV detection at 254 nm, and a notable improvement in the selectivity was reported. No further applications of this method have been presented.

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Electrochemical detection for the determination of vitamin B_6 in multivitamin preparations has been proposed by Wang and Hou (1988a, b) and Hou and Wang (1990). The advantage of their application was the improved selectivity and wider linear dynamic range compared to that of UV detection. A subnanogram detection limit (0.5–1ng for PL depending on detection mode, 2ng for PN, and 1ng for PM) was achieved. A mechanism for oxidation of pyridoxal to a dimerisation product was proposed by Hart and Hayler (1986); the approach was suggested for measuring the circulating vitamin B_6 as pyridoxal in plasma.

Internal standard

The accuracy and precision of HPLC analysis can be remarkably improved by using a thoroughly validated internal standard procedure. The choice of an internal standard depends on for instance the chromatographic separation and the commercial availability of the reagent.

4-Deoxypyridoxine has been adopted in routine use for vitamin B_6 analysis for food samples (Gregory and Feldstein 1985, Gregory and Ink 1987 van Schoonhoven 1994, Esteve et al. 1998) and other biological materials (Pierotti et al. 1984, Morrison and Driskell 1985, Sharma and Dakshinamurti 1992). Its chemical characteristics resemble those of other vitamers and it is usually eluted in the middle of the reversed-phase or ion-paired reversed-phase chromatogram. However, the relatively low fluorescence quantum yield of 4-deoxypyridoxine compared to other vitamers may limit its usefulness (Toukairin-Oda et al. 1989). In addition, its stronger retention would increase the total analysis time in the cation-exhange chromatography presented by e.g. Coburn and Mahuren (1983). In this case, deoxypyridoxine as an internal standard was substituted by 2-amino-5-chlorobenzoic acid. Shephard and associates (1987) modified the cation-exchange chromatographic method by using 4'-deoxypyridoxine-5'-phosphate (DPNP) as an internal standard as its chemical structure and fluorescence properties were closer to B_6 vitamers than those of 2-amino-5-chlorobenzoic acid used by Coburn and Mahuren (1983).

3-Hydroxypyridine has also been put forward an internal standard for food samples (Vanderslice et al. 1979, Vanderslice et al. 1980, Olds et al. 1993, Vanderslice et al. 1984). It was stated that this compound was stable, did not coeluate with other vitamers and behaved in the same way as the vitamers throughout the analytical process; hydroxypyridine had fluorescence characteristics similar to other vitamers and it was well separated from the other vitamin B_6 compounds in their ion-exhange chromatogram. However, in the reversed-phase chromatographic method of Ang et al. (1988) neither 3-hydroxypyridine nor 4-pyridoxic acid were suitable for use as internal standards as those compounds were not separated from all the other peaks derived from chicken samples.

Isopyridoxal was used for the analysis of B_6 vitamers in cell-free culture media (Argoudelis 1988) and in milk and egg yolk (Argoudelis 1997). Both deoxypyridoxine and isopyridoxal fullfilled the requirements for an internal standard to quantitate six or seven vitamers in the samples. However, the use of isopyridoxal as an internal standard was recommended due to the better fluorescence response of iso-PL and its more suitable retention characteristics compared to those of DPN. The easy synthesis of isopyridoxal was also put forward as an advantage as this compound is not commercially available at present.

An internally standardized HPLC assay for vitamin B_6 in human serum published by Reynolds and Brain (1992) employed pyridoxamine 5'-phosphate (PMP) as an internal standard for the measurement of pyridoxal 5'-phosphate (PLP). PLP was the only vitamer found in significant quantities in their serum samples. PMP exhibited a more suitable retention than free vitamers in their chromatographic system. Ubbink et al. (1986) applied 6-methyl-2-pyridone carboxaldehyde (MPCSC) as an internal standard for PLP in plasma analysis. A good separation of MPCSC and the semicarbazone derivatives of PL and PLP was achieved using a reversed-

phase chromatography system. Simultaneous determination of pyridoxine, thiamine, and riboflavin in infant formulas was internally standardized by using m-hydroxybenzoic acid as it could be measured both with fluorometric detection (used for vitamin B_6) and UV spectrometric (used for vitamin B_1 and B_2) (Chase et al. 1992). Acetanilide was used in the study of Maeda et al. (1989) for simultaneous determination of nicotinamide, thiamine, riboflavin and its phosphate, pyridoxine, cyanocobalamine, caffeine and sodium benzoate in oral liquid tonics.

2.4.2.3 Gas chromatography and mass spectrometry

Gas chromatography is generally poorly suited for the analysis of vitamin B_6 compounds due to their ionic nature and lack of volatility unless they are dephosphorylated and their polar groups are derivatized. Rigorous pretreatment is also needed to remove nonvolatile compounds and water from the extract. Derivatization procedures include formation of acetyl (Hachey et al. 1985) or trifluoroacetyl (Lim et al. 1982) derivatives. The use of an electron-capture detector enabled a low detection limit (10pg) for all three free vitamer (Lim et al. 1982).

Hachey and coworkers (1985) developed an analysis method for vitamin B_6 forms in biological samples by isotope dilution mass spectrometry. Deuterated vitamers, served as internal standards, were added at the time of trichloroacetic acid extraction and the aliquot of extract was fractionated using cation-exhange HPLC. The fractions were further purified with ion-exhange chromatography, PL and hydrolyzed PLP were reduced to pyridoxine, and the vitamer fractions were acetylated. Mass spectrometry recorded electron impact mass spectra and the quantification was based on the isotopic ratios of deuterated and unlabeled ions. The tediousness and complexity of the method limits its use for routine food analysis. This approach, however, may serve as a accurate reference method for validating other analytical procedures.

Liquid chromatography – mass spectrometry (LC-MS) is more easily applied to vitamin B_6 compounds than GC-MS. Using a semi-microbore column (Ultracarb ODS, Phenomenex, USA) and a binary gradient elution consisting of methanol and ammonium formate (20mM, pH3.57) seven water-soluble vitamins in a standard solution were separated and detected using particle-beam mass spectometry (Careri et al. 1996). For electron impact spectra of the B_6 vitamers, the intensity of the molecule ion was high for all vitamers, and especially for pyridoxal (m/z 167, 67%). The relative abundance of the ions observed was higher in chemical negative (NCI) and positive (PCI) ionization mode compared to that of electron impact ionization. The detection limit values for pyridoxal, pyridoxine and pydoxamine reported were 6ng, 225ng and 400ng, respectively.

2.4.3 Microbiological methods

Microbiological assays for the determination of vitamins are based on "the nutritional requirement of a microorganism for a certain vitamin" (Fawell 1990). Bacteria, molds, protozoa and yeasts can be utilized for the measurement of vitamin B_6. Protozoa, like *Tetrahymena* or *Ochramonas*, have more developed ingestive and digestive system than yeasts or bacteria. Thus, their response to bound vitamers should resemble more closely that of higher animals (Voigt et al. 1979b). On the other hand, protozoa are more sensitive to the growth inhibition caused by salts, and only a few papers concerning their use in vitamin analysis have been published. So yeasts like *Saccharomyces* and *Kloeckera* are most frequently used at present especially as no bacteria utilizes equally all three B_6 vitamers. A comprehensive summary of microbiological methods for vitamin analysis was presented by Bell (1974).

The requirement of vitamin B_6 for the growth of a microorgamism and its use for vitamin determination was published by Atkin et al. (1943). The problem related to a microbiological assay is the variable growth response of an organism to different vitamers. The growth response of *Saccharomyces uvarum* (formerly *S.carlsberg-*

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ensis) for pyridoxamine was 60–80% compared to that of pyridoxal or pyridoxine (Rabinowitz and Snell 1948, Gregory and Mabbit 1959, Polansky and Toepher 1969, Gregory 1982, Gregory 1983). The physiological fundamentals, the influence of pH, ions and temperature on the transport system and metabolism in yeast cells has been examined by Shane and Snell (1976). The extent of utilization of phosphorylated vitamers also varies between microorganisms; the bioavailability of PMP was higher for *Streptococcus faecalis* than for *S. uvarum* or *Str. faecium* when mild acid extraction was performed for milk samples (Gregory and Mabbit 1961). *Str. faecalis* utilized pyridoxamine phosphate as readily as the free vitamer when the mild acid hydrolysis was insufficient to completely hydrolyze pyridoxamine phosphate. Different responses of a microorganism for individual B_6 vitamers inevitability lead to uncertainties in the results. The response differences were later corrected by using an open-column ion-exhange column to separate B_6 vitamers prior microbiologic incubation and an individual calibration was performed for each vitamer (Toepher and Lehmann 1961, Toepher and Polansky 1970, Polansky 1981). This approach is used in the method recommended by the AOAC (1995a, b). However, the chromatographic separation step limits the method's usefulness for routine food analysis (Favell 1990).

The most succesfully used yeasts in the assays are *S. uvarum* (*S. carlsbergensis*) and *Kloeckera brevis* (*K. apiculata*). The former microorganism has been widely recognized for determinations of vitamin B_6 (Toepher and Polansky 1970, Bell 1974, Favell 1990, AOAC 1995a, b). However, an interference factor called "negative drift", especially in cereal sample was reported by Gregory (1980c); an inverse relation between the result and the amount of extract taken into the analysis caused variations in the results. The inhibition effect of neutralization salts (sodium and potassium chlorides) or food preservatives (sodium sorbate, propionate and benzoate) was suggested to explain this annoying phenomenon (Voigt et al. 1979a, b, Guilarte 1984). It was concluded that the standards should be treated as samples to diminish the possible drift. The choice of buffer should also be considered. *Kloeckera brevis* has been reported to show equal responses to three free vitamers (Guilarte 1983, Guilarte and Tsan 1983) which is in contrast to the results of Gregory (1983). Whether this discrepancy was due to the subculturing or some other factors was not resolved. It seems likely that *S. uvarum* and also *K. brevis* only grow on the free vitamers or at least utilize phosphorylated vitamers to a lesser extent (Polansky 1981). If so, the dephosphorylation of related esters either by autoclaving or enzymatically hydrolysing seems to be essential during the course of analysis.

A smart application for the measurement of vitamin B_6 was the use of a microbial biosensor system (Endo et al. 1995). This system used an immobilized *Saccharomyces uvarum* combined to a Clark -type oxygen electrode, and the determination was based on the respiratory activity of the microorganism in the presence of vitamin B_6. Oxygen consumption measured as a current decrease in electrode system was related to the vitamin B_6 amount in the media. A good correlation between proposed method and conventional biological assay was achieved. A high sample input of the biosensor system was reported and one assay could be completed within 15 min.

3 Objectives of the study

This study was a part of the research "Nutrient Content of Finnish Foods; Water-Soluble Vitamins" at the Department of Applied Chemistry and Microbiology/Food Chemistry. The aim of this work was to evaluate and improve the methodology used for the determination of vitamin B_6, to assess the suitability of the developed HPLC method to routine food analysis and to determine the vitamin B_6 content of the most common food items consumed in Finland.

To achieve this

- the extraction procedures compatible for liquid chromatographic determination of free, phosphorylated and glycosylated vitamin B_6 compounds in foods were assessed
- the validity of the method and laboratory's performance was evaluated by intercalibration and collaborative parameters
- up-to-date data on vitamin B_6 content of common food items was produced
- the main glycosylated vitamer fraction of certain plant-derived foods was measured

4 Materials and methods

4.1 Calibrants and enzymes

Vitamin calibrants were purchased from Sigma (MO, USA): 4-deoxypyridoxine hydrochloride (D-0501), 4-pyridoxic acid (P-9630), pyridoxal-5-phosphate (P-9255), pyridoxal hydrochloride (P-9130), pyridoxamine-5-phosphate hydrochloride (P-9505), pyridoxamine dihydrochloride (P-9380) and pyridoxine hydrochloride (P-9755). Labeled pyridoxal phosphate, ([4,5-^{14}C]pyridoxal-5'-phosphate (Fig. 10), measured activity 314kBq (8.5µCi), 71mBq/mmol (1.93mCi/mmol)) was delivered by Amersham International plc, UK. Concentrations of the standard stock solutions were checked spectrophotometrically using molar absorption coefficients (Table 4). Coefficient values obtained from the manufacturer (Claypool 1994) were in accordance to those published by Metzler and Snell (1955). The performance of spectrophotometry was checked against a certified reference holmium oxide kyvette (Milton Roy, USA).

Alkaline phosphatase from calf intestine (EC 3.1.3.1., Boehringen-Mannheim, Germany) and β-glucosidase from almonds (EC 3.2.1.21., Fluka, Switzerland) were used in the enzymatic hydrolysis. The hydrolysis efficiency of phosphatase and of β-glucosidase was tested with sample extracts.

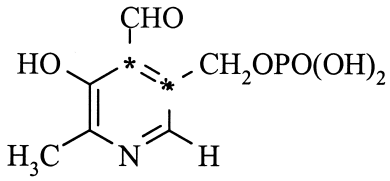

Fig. 10. ^{14}C-labeled pyridoxal-5'-phosphate (^{14}C-carbons marked as *).

4.2 Equipment

An analytical liquid chromatography equiment controlled by a Millennium Chromatography

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Table 4. Molar absorption coefficients (e) of vitamin B_6 compounds (Claypool 1994).

Compound	ε ($l\ mmol^{-1}cm^{-1}$)	λ_{max} (nm)	solution, pH	M_W ($g\ mol^{-1}$)
PLP	5.02	388	0.1M phosp. buffer, pH7	247.1
PMP•HCL	8.37	326	0.1M phosp. buffer, pH7	284.6
PL•HCl	8.96	288	0.1M HCl	203.6
PN•HCL	7.30	323.8	0.1M phosp. buffer, pH7	205.6
DPN•HCl	8.10	314	0.1M phosp. buffer, pH7	189.6
PM•2HCl	4.6	253	0.1M phosp. buffer, pH7	241.1

DPN•HCl; 4-deoxypyridoxine hydrochloride, PL•HCl; pyridoxal hydrochloride, PLP; pyridoxal-5' -phosphate, PN•HCl; pyridoxine hydrochloride, PM•2HCl; pyridoxamine dihydrochloride, PMP•HCl; pyridoxamine-5'-phosphate hydrochloride

Manager software package (v.2.12.x – 2.15.x, Waters, USA) consisted of three chromatography pumps (Waters M510, USA), an autosampler (700 Satellite or 712, Waters, USA), an air-bath column temperature module (Waters, USA) and a fluorescence detector (M470, Waters, USA). A binary gradient elution of the mobile phase was performed with two HPLC pumps while the third pump was used for the post-column derivatization. Data signal was collected via an A/D converter (SAT/IN interface, Waters, USA) and processed with the Millennium Chromatography Manager software package. Calculations in internal standard method were verified using a HP 42S calculator (Hewlett-Packard, USA).

Preparative liquid chromatography was performed using a Delta Prep liquid chromatograph (Waters, USA) with a UV-VIS (Waters 484, USA) and a fluorescence detector (Waters 470, USA). A column packing device manufactured by Shandon (UK) used for packing the in-house made analytical HPLC columns included a single piston pressure intensifier unit and a stainless steel slurry chamber (50ml, Shandon, UK).

A Varian Unity-500 NMR-spektrometer (Varian, USA) was used for measuring the proton NMR spectra. Mass spectra was performed with a Finnigan MAT 90 mass spectrometry (Finnigan, USA). ^{14}C-activity was measured using a Wallac 1411 Liquid scintillation counter (LKB, Sweden).

4.3 Food samples, sample collection and pretreatment

Food samples analyzed were chosen on the basis of their consumption in Finnish diet and their estimated role in vitamin B_6 intake. Sales volumes of individual food items purchased from main wholesale food chains were used for selecting items to be analyzed. The food sample list was prepared in cooperation with the Finnish Food Data Bank (National Institute of Health, Helsinki). Sampling was performed during June 1994 and January 1995. Collected food samples represented common food items in six main food groups; meat and poultry (16 items), dairy products and egg (9 items), vegetables and nuts (8 items), cereals (7 items), fish (6 items), others (5 items), the total number of analyzed food items being 51 (Appendix 1).

Food collection was generally based on the procedure applied in previous vitamin studies performed at our department (Piironen 1986, Heinonen 1990, Mattila 1995, Vahteristo 1998); ten retail shops, located in the Helsinki area and

representing the main wholesale food chains in Finland, were selected based on their market share. If the commodities were not available in these shops, other shops, related to the same wholesale chain if possible, were used. Pork liver, pork kidney, reindeer, lamb and elk samples were purchased from a slaugher house or from a wholesale house. Pike and pikepearch were caught from Baltic Sea, whitefish from Baltic Sea and from Lake Inari. Rainbow trout was cultivated in the sea area. Baker's yeast was from Alko Oy, the Finnish State Alcohol Company. The size of collected subsamples varied from 300g to 1kg depending on the food item and its package size; a minimum subsample size was kept at approx. 300g. Ten subsamples per each food item were collected and the subsamples were pooled to form a composite sample.

Pretreatment was carried out as soon as possible in practice after sample collection. Meat samples were deboned and cut into cubes (ca. $1cm^3$) with a stainless steel knife. Any excess of adipose and connective tissue as well as artifical coverings were removed. Frozen chicken (broiler) and hen, were allowed to thawn, deboned, deskinned, and the meat and fat were cut into cubes with scissors. Frozen peas were mixed as such. Fish samples were obtained as fillets. Egg yolk was separated from raw eggs using normal household methods. Liquids (e.g. milks) and powders (e.g. flours) were mixed and pooled. Subsamples of each food item were mixed in a plastic container and divided into ca. 200g portions. These portions were vacuum-packed into polyethylene-nylon laminate bags, frozen and stored at –20°C until analysed. Pretreament and the sample preparation were done under dimmed light and in cool conditions whenever practically possible.

A commercial infant formula, fortified with pyridoxine, was used as an in-house reference material. The content of three packages (of 400g) were mixed, divided into portions of 10 to 20g, vacuum-packed into aluminium coated polyethylene-nylon laminate bags, frozen and stored at -70°C until analysed.

4.4 Evaluation of liquid chromatographic method and extraction procedure

4.4.1 Analytical liquid chromatography

4.4.1.1 Chromatographic parameters and column testing protocol

Interparticle volume (V_0), retention factor (k_e), separation factor (α), peak resolution (Rs), symmetry (A_s^2) and the plate number of the column (N) calculated according to Snyder and Kirkland (1979) were used to characterize the chromatographic performance. The interparticle volume of the column was measured using either uracil or sodium nitrate detected at a wavelength of 254nm (Wells and Clark 1981). The limit of detection (LOD) was evaluated as three times signal-to-noise ratio. Octadecyl columns were characterized by measuring the column performance (as theoretical plate number, N), the silanol index (SiOH) and the hydrophobicity (HP) values according to Walters (1987). The aminopropyl packing was tested using manufacturer's intructions (Spherical Materials and Columns for HPLC 1976).

4.4.1.2 Tested column packings and mobile phases

Reversed-phase and reversed-phase ion-pair chromatographic techniques were applied to separate vitamin B_6 compounds. Chromatographic separation was performed using octadecyl or aminopropyl phases (Table 5). Novapak C18 (Waters, USA), µBondapak C18 (Waters, USA), Vydac 201HSB (Separations Group, USA), Spherisorb S5ODS2 (formerly PhaseSeparations, UK) and Spherisorb $S5NH_2$ (formerly PhaseSeparations, UK) columns consisted of chemically modified silica. The silica-based column packings chosen were mainly those of low-acidity and/or end-capped materials and thus expected to be suitable for the separation of basic compounds (Walters 1987, Stadalius and

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Table 5. Characteristics of the tested HPLC column packings.

Column packing	Particle diameter (µm)	Pore diameter (nm)	Surface area (m^2/g)	Pore volume (cm^3/g)	Carbon loading (%, w/w)	Type of phase ligand, functionality	End-capped
S5ODS2	5	8	220		12	tri	yes
$S5NH_2$	5	8	220		2	tri	yes
201HSB5	5	8	500		13.5	poly	yes
µC18	10	12.5	330		10	mono	yes
C18	4	6	120	0.3	7	mono	yes
PRP-1	5	7.5	415	0.79	*	*	*
RP-18	9	$30^{5\mu m}$	$350^{5\mu m}$		*	*	*

Packing	Brand name, particle size, column dimensions, manufacturer
S5ODS2	Spherisorb S5ODS2, 5µm, 250x4,6mm i.d., PhaseSep, UK
$S5NH_2$	Spherisorb $S5NH_2$, 5µm, 250x4,6mm i.d., PhaseSep, UK
201HSB5	Vydac 201HSB5, 5µm, 250x4,6mm i.d., Separation Group, USA
µC18	µBondapak C18, 10µm, 300x3,9mm i.d., Waters, USA
C18	Novapak C18, 4µm, 150x3,9mm i.d., Waters, USA
PRP-1	Hamilton PRS-1, 5µm, 250x4,1mm i.d., Hamilton, USA
RP-18	Polyspher RP-18, 9µm, 150x4.6mm id, Merck, Germany

* poly(styrene-divinylbenzene) based

Snyder 1988). Two polymer packings tested, based on the poly(styrene divylbenzene) (PS-DVB) structure, were Polyspher RP-18 (Merck, Germany) and Hamilton PRP-1 (Hamilton, USA).

The columns tested were commercial products except for S5ODS2 and $S5NH_2$ columns which were home-made. Octadecyl or aminopropyl phase (Spherisorb, PhaseSeparations , UK), ca. 3g of packing material per 50ml, was suspended in acetone or in a mixture of methanol: water (90:10), respectively. The slurry was packed downward with a constant pressure (450atm, 46MPa), the packing solvent being methanol (Spherical Materials and Columns for HPLC 1976). All analytical columns apart from the aminopropyl column were connected with a guard column (Novapak C18 cartridge 10x3.9 mm i.d., Waters, USA or Spherisorb S5ODS2 25x2.1 mm i.d., PhaseSeparations, UK).

The mobile phase constituted of buffer (prepared from orthophosphoric acid, 85% Baker 6024 or its potassium salts), dilute acetic acid (Merck 1.00063, >99.8%, Germany) or dilute sulfuric acid. 2-Propanol, methanol, acetonitrile, all of HPLC grade (Baker, USA or Rathburn, UK), were tested as organic solvents in the mobile phase. Peak tailing, especially that of pyridoxamine, was diminished using triethylamine (Fluka 09340, pa., Switzerland) in the mobile phase (Stadalius and Snyder 1988). In order to increase the retention of pyridoxamine, an ion-pair was formed using alkyl sulfonates (1-octanesulfonic acid, sodium salt, Sigma O-8380, appr. 98%, USA). The mobile phase was then a mixture of the organic solvent and the buffer.

4.4.1.3 Post-column derivatization

The detectability of pyridoxal-5'-phosphate was improved by post-column derivatization. The fluorescence response of PLP was enhanced by forming a sulphite adduct in an elevated pH (Coburn and Mahuren 1983); a sodium phos-

phate buffer solution (0.5M, pH7.5) containing sodium bisulphite (36mM) was pumped into the mobile phase through a union tee prior detection. The fluorescence excitation and emission wavelengths were then set at 330nm and 400nm, respectively. The effect of post-column derivatization on the fluorescence intensity of B_6 vitamers was evaluated by comparing the detector signal to that of a non-derivatized detection (emission wavelength 300nm, exitation wavelength 375nm).

4.4.2 Preliminary experiments on extraction procedure

One mineral acid and four non-mineral acids were tested for sample extraction using plant-derived samples. The procedures used were based on literature review. Sulfuric acid extraction (0.1M solution prepared from Merck 731, 95–97%, Germany) was used with or without an autoclaving process (Bognar 1985, COST91 1985). Non-mineral acid extractions were 0.5M perchloric acid (made from Baker 6063, 70–72%, The Netherlands) (Bitsch and Möller 1989a), 5% (w/v) sulfosalicylic acid (Gregory and Ink 1987), 0.5M metaphosphoric acid (prepared from Fluka 79615, ~65% HPO_3, puriss, Switzerland) (Ang et al. 1988) and 10% (w/v) trichloroacetic acid (made from Merck 807, z.A., ACS, Germany) (Coburn and Mahuren 1983). Autoclaving was omitted in non-mineral acid extractions. All analysis were done in duplicate.

The criteria for the chosen extraction procedure were the extraction efficiency, maintaining if possible the B_6 vitamer distribution in its intact form during extraction, and the compability to the reversed-phase liquid chromatographic separation procedure. After this preliminary experiment, the main emphasis was focused on the investigation of the perchloric acid extraction procedure (see 4.5.1).

4.4.3 Solid-phase extraction

Solid-phase extraction (SPE) was tested to find out whether interferring compounds present in the sample extracts could be removed, and vitamin B_6 compounds concentrated as well. Partition chromatography, using octadecyl, octyl, phenyl and cyanopropyl phases (Analytichem International, USA or Baker, the Netherlands), and cation-exchange chromatography, using carboxymethyl, propylsulfonic acid and benzenesulfonic acid phases (Analytichem International, USA or Baker, the Netherlands) were evaluated. The amount of sorbent material in SPE cartridges ranged from 300–500mg. A strong anion-exchange phase, trimethylaminopropyl from Analytichem International (USA), was used to remove sulfosalicylic acid when samples were extracted according to the method of Gregory and Ink (1987).

SPE packings were conditioned according to the manufacturers' guide lines; first with methanol followed by a buffer solution compatible to sample extract. Adequately diluted sample extract solution was applied to the SPE cartridge at a flow rate not exceeding 2ml/min. Total ionic capacity of the phases (meq/g packing material), informed in manufacturer's instructions were used. The flow rate was regulated using a vacuum manifold (SPE21, Baker, the Netherlands) with additional valves, made of stainless steel and teflon, for each SPE cartridge. The SPE cartridge was washed with the solution in which the sample was applied or with the solvent of equal or less solvent/ionic strenght. Analyte(s) was eluted from the SPE cartridge by enhancing the solvent strength of the eluent; increasing the portion of the organic solvent, the ionic strength or ion selectivity, or changing the pH. Washing and elution volumes were maintained ca. two times the interparticle volume of the cartridge (void volume, bed volume) which was estimated to be 1.2µl/mg cartridge packing. Elution was repeated until the analyte was eluted from the phase. All fractions (sample applying solvent, washings as well as elution fractions) were collected and analyzed by HPLC.

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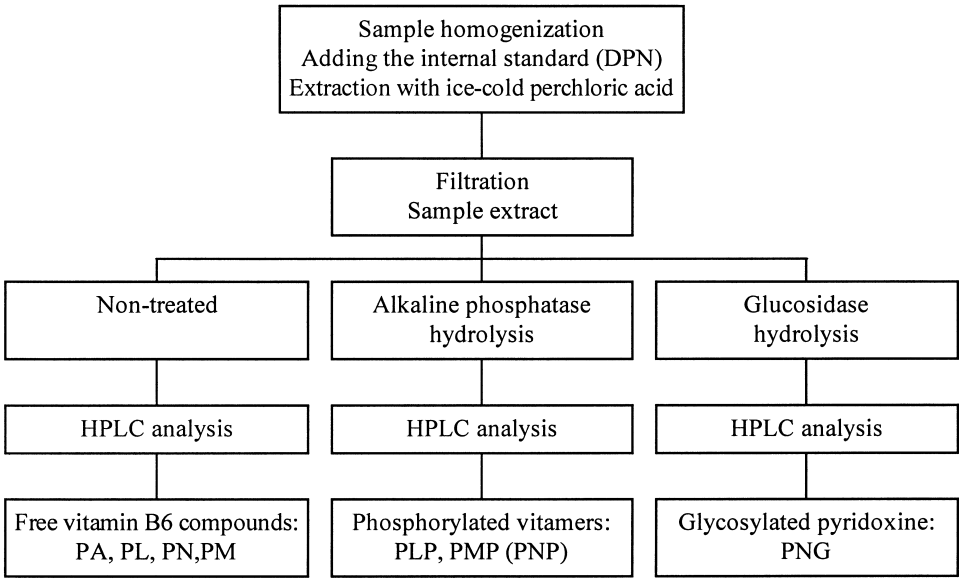

Fig. 11. Schematic presentation of the routine food analysis. (PA = pyridoxic acid, DPN = 4-deoxypyridoxine, PL = pyridoxal, PLP = pyridoxal-5'-phosphate, PN = pyridoxine, PNG = pyridoxine glucoside, PM = pyridoxamine, PMP = pyridoxamine-5'-phosphate).

4.4.4 Statistics

Comparison of means was performed by using an ANOVA or a t-test. When the tests' basic assumptions of equal variances were not matched, the Kruskal-Wallis or the Mann-Whitney test to compare the medians of the samples, or the Kolmogorov-Smirnov test to compare the distributions of the samples was performed. A multiple range test was performed to determine which means were different from the others. Box and whisker plot was used to detect outside and far outside data points (outlier values). All tests were performed at a 95.0% confidence level.

If the amount of repetititon for individual measurements was inadequate for a proper statistical evaluation, the variation was assumed to the same (approximately 12%) for each mean values, and the statistical evaluation was performed using these "recalculated" values. This approach is mentioned in the context of the results when applied. All statistical calculations were performed using Statgraphic Plus for Windows v.3.0 software package (Manugistics, USA).

4.5 Chosen method for routine food analysis

4.5.1 Extraction procedure

A modified perchloric acid extraction of Bitsch and Möller (1989a) combined with an alkaline phosphatase and β-glucosidase digestion was chosen for routine food analysis from the tested extraction procedures (see. 4.4.2). Food samples were extracted with ice-cold dilute perchloric acid followed by enzymatic hydrolysis. All sample extracts were digested with alkaline phosphatase while extracts from plant-derived samples were also treated with β-glucosidase (Fig. 11). All determinations were done in triplicate;

Table 6. Factors for converting vitamin B_6 compounds to pyridoxine base (PN, Mw 170 g mol^{-1}).

Compound	PLP*	PA	PMP•HCl*	PL•HCl	PN•HCl	PM•2HCl	PNG*
Factor	0.836	0.929	0.7059	0.836	0.8274	0.7059	0.8274

* PLP determined as PL, PMP as PM, and PNG as PN
PA; pyridoxic acid, PL•HCl; pyridoxal hydrochloride, PLP; pyridoxal-5'-phosphate, PN•HCl; pyridoxine hydrochloride, PM•2HCl; pyridoxamine dihydrochloride, PMP•HCl; pyridoxamine-5'-phosphate hydrochloride, PNG; pyridoxine glucoside

three separate vacuum-packed sachets of each pooled food item were analyzed.

Homogenized sample (3–10g) was mixed with 50 ml 0.5M perchloric acid, 4-deoxypyridoxine was added as an internal standard, and the mixture was homogenized in an ice-water bath for 60 sec using an Ultra-Turrax blender. The homogenate was made up to a known volume (normally 100ml) and filtrated. A portion was taken (8ml) and the pH was adjusted to 7.5 with 5M and 0.1M potassium hydroxide solution to precipitate perchlorate. After 30 minutes the mixture was filtered or centrifuged and made up to a known volume (10ml).

4.5.2 Enzymatic digestion

In order to analyse free, phosphorylated and glycosylated vitamin B_6 derivatives, the sample extract was divided into three parts. Free forms (PA, PL, PN, and PM) were determined using non-enzymatically treated extract, and two other portions of sample extract were digested with alkaline phosphatase and with β-glucosidase.

Phosphorylated forms (PLP, PMP, and in case of baker's yeast also PNP) were quantitated after alkaline phosphatase treatment (Bitsch and Möller 1989a). An alkaline phosphatase solution (40µl containing 60U) was added to sample extract solution (2ml), and the mixture was incubated for 30 min at room temperature. Sodium acetate solution (0.25M) and dilute hydrochloric acid (0.2M) were used to adjust the pH to 3.8, and the mixture was filtered prior to liquid chromatography. Every sample extract was injected in duplicate. Sample extracts from plant-derived foods containing glycosylated vitamin B_6 derivatives (PNG) were digested also with β-glucosidase (Gregory and Ink 1987). In that case, a 2ml portion of the sample extract solution from which perchlorate has been precipitated was adjusted to the pH 5.0 with sodium acetate solution (0.25M) and dilute hydrochloric acid solution (0.2M). β-Glucosidase solution (20U in 0.2ml) was added, and the incubation at 37°C was stopped after 4h by adding 50µl of trichloroacetic acid (100% w/v). After filtration the mixture was ready to inject into the liquid chromatograph. Enzyme blank samples were treated like food samples. Two HPLC injections were done for each enzyme hydrolyse extract.

The amount of alkaline phosphatase needed to convert phosphate esters into their free forms was tested by monitoring the phosphorylated and their free vitamers using beef steak as a test material. The efficiency of β-glucosidase hydrolysis was evaluated using the extract from raw carrot. The disappearance of the tentatively identified glycosylated pyridoxine, and the simultaneously increased amount of free pyridoxine were assumed to be markers for adequate glucosidase activity during the glucosidase digestion.

4.5.3 Liquid chromatographic separation

An ion-paired reversed-phase chromatography was chosen to separate vitamin B_6 compounds in routine food analyses. The separation of vitamin B_6 forms was performed using a Waters Novapak C18 cartridge column (4µm,

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150x3.9mm i.d., Waters, USA,) connected to a guard column (Novapak C18 cartridge 10x3.9 mm i.d., Waters, USA). The mobile phase, a modification of Gregory and Ink (1978), consisted of 33 mM phosphate buffer and 8 mM 1-octanesulfonate (pH 2.2), and 2-propanol. After a 2 min lag phase (2% 2-propanol), a linear gradient from 2% to 20% of 2-propanol was performed in 10 minutes followed by a 10 minute lag phase (20% 2-propanol). The total analysis time was 37 minutes. Post column reagent, sodium bisulphite (36mM) in phosphate buffer (0.5M, pH 7.5), at a flow rate of 0.1ml/min was used to enhance the fluorescence of pyridoxal-5'-phosphate, the total flow rate of mobile phase being 1.2 ml/min. Column temperature was set at 30°C with an air-bath module (Waters, USA). The injection volume ranged normally 50 to 75µl. Detection was based on the measurement of fluorescence signal (excitation and emission wavelengths set at 330nm and 400nm, respectively).

4.5.4 Calculations and expressing the results

The results were calculated using an internal standard method in which peak area ratios were plotted against concentration ratios at nine concentration levels. 4-Deoxy-pyridoxine was used as the internal standard compound. The fluorescence response against concentration was fitted using a linear equation. The results were enzyme blank corrected. Calculations were performed with a Millennium 2010 Data Manager -software package (v.2.12.x – 2.15.x, Waters, USA) or by using a HP 42S calculator (Hewlett-Packard, USA). Phosphorylated vitamers, PLP and PMP, were determined as their free vitamers before and after alkaline phosphatase hydrolysis:

$$PLP = PL_{\text{amount after phosphatase treatment}} - PL_{\text{amount before phosphatase treatment}}$$

$$PMP = PM_{\text{amount after phosphatase treatment}} - PM_{\text{amount before phosphatase treatment}}$$

In the case of baker's yeast, the amount of pyridoxine phosphate was also determined:

$$PNP = PN_{\text{amount after phosphatase treatment}} - PN_{\text{amount before phosphatase treatment}}$$

Amount of glucosylated pyridoxine was determined as total PNG calculated as PN before and after β-glucosidase digestion:

$$\text{Total PNG} = PN_{\text{amount after glucosidase treatment}} - PN_{\text{amount before glucosidase treatment}}$$

Vitamin B_6 content (ΣB6), expressed as PN, was calculated as the sum of free and phosphorylated forms:

$$\Sigma B6 = PLP + PMP + PNP + PL + PN + PM$$

Total vitamin B_6 content (ΣΣB6) included glucosidic pyridoxine (calculated as PN) as well:

$$\Sigma\Sigma B6 = \Sigma B6 + \text{total PNG}$$

Results of all vitamin B_6 compounds were expressed as pyridoxine, M_w 170 g mol^{-1} (Table 6). Values were given with two or three significant digits based on uncertainty calculations (see 4.5.6.4) and repeatability relative standard deviation (RSD_r). RSD_r of the replicate analysis (n=3) for food analysis normally varied between 1–15%.

4.5.5 Comparison of the results with national food composition tables

In order to compare the results of this study to national food composition tables, some foreign data was recalculated to standardize the vitamin B_6 expression form. A conversion factor of 0.8274 was used to convert the amount of pyridoxine hydrochloride to that of pyridoxine.

4.5.6 Evaluation of the chosen method

Validity of the chosen method for routine food analysis was investigated by evaluating the stability of PLP during the analytical procedure, measuring the chromatographic parameters for

the internal standard, and determining recovery values for added standards, monitoring repeatability by using an in-house reference material, and by participating in both international and national intercalibration studies. Laboratory proficiency for performing vitamin B_6 analysis was tested by taking part in the collaborative and certification studies organized by BCR's EU-MAT -programme.

4.5.6.1 Stability of pyridoxal-5'-phosphate

The stability of phosphorylated pyridoxal in the chosen perchloric acid extraction procedure was further tested using ^{14}C-labeled pyridoxal-5'-phosphate as the aldehydic form is considered to be the most reactive vitamer in aqueous solution containing amino acids and related compounds. The effect of extraction media and the sample matrix on interconversion and hydrolysis of PLP were evaluated by adding labeled pyridoxal phosphate into the extraction procedure with the sample matrix. Tested sample matrices were beef, raw carrot and whole wheat flour. All analyses were done in duplicate.

[4,5-^{14}C]pyridoxal-5'-phosphate in aqueous solution was diluted with water and the activity of 39.3kBq (1.1µCi, corresponding approx. 140µg of labeled PLP) was added to the food sample prior to homogenization. The sample was extracted using the chosen perchloric acid procedure excluding enzyme hydrolysis, and an extract portion of 75 µl was injected into analytical liquid chromatography. Five fractions per minute (ca. 240µl) were collected during the whole chromatographic run. A liquid scintillation cocktail (3ml, Hionic-Fluor scintillation cocktail, Packard Instrument, USA) was added to the collected fraction, and the ^{14}C-activity was measured using a liquid scintillation counter. The quench correction was performed for each material, and the results were calculated as disintegrations per minute (dpm). The purity of the labeled pyridoxal phosphate was measured by injecting diluted ^{14}C-PLP solution into analytical liquid chromatography, collecting fractions over the chromatogram and measuring the activity of the collected fractions.

4.5.6.2 In-house monitoring

Peak identification was based on the retention time in the chromatogram, spiking the sample extract with standard solutions, and on phosphatase and glucosidase treatments. Recovery of an added standard was measured using beef steak and a commercial infant formula as sample matrixes. A commercial product, fortified infant formula, was used as an in-house control sample; the control sample was analyzed every three week. Retention factor (k_e), relative response (peak area /concentration), peak width at half height (w_h), peak symmetry ($A_{4.4}$) and peak tailing (T, according to USP) for the internal standard (4-deoxypyridoxine) were monitored using the Millennium software's system suitability option.

4.5.6.3 Intercalibration and collaborative studies

The validity of the procedure for routine food analysis was verified with intercalibration and collaborative proficiency tests. Laboratory performance was evaluated by taking part in BRC EU-MAT Measurement and Testing Programme and later on by participating the BCR's certification study. Twelve European laboratories participated in the 3rd EU-MAT intercomparison in which the vitamin B_6 results derived both from the in-house methods of individual laboratories and from a common "optimal" extraction protocol were given (van den Berg et al. 1996). Certification data consisted of the results from fourteen laboratories. Sample materials were lyophilized pork liver, mixed vegetable, whole meal flour and milk powder. Both high performance liquid chromatographic and microbiological methods were used in the above mentioned studies.

Chromatographic performance was further evaluated by an interlaboratory study between two German laboratories and our laboratory (Bognar and Ollilainen 1997); standard solutions and sample extracts, prepared by the Federal Research Centre for Nutrition (Stuttgart, Germany) were analyzed in all three laboratories. All analyses were performed with two liquid chro-

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matographic methods (an isocratic reversed-phase chromatography with FL detection and a gradient reversed-phase chromatography with FL detection) and with one microbiolocical assay using *Saccharomyces cereviase* ATCC 9080 as the test organism.

Also a two sample intercomparison (with whole wheat flour and beef steak samples) was performed with VTT Technical Research Centre (Finland) in which rerversed-phase liquid chromatographic results were compared to those derived from a microbiological assay in which *Saccharomyces cereviase (uvarum)* ATCC 9080 was used as the test organism.

4.5.6.4 Estimating the uncertainty of the results, and statistical calculations

The uncertainty of the results was estimated by calculating the expanded uncertainty for pyridoxine results of the in-house control sample. Based on the internal standard standardization of pyridoxine, the 95% confidence interval (CI) of the result was estimated according to Caulcutt and Boddy (1983). Error standard deviation (ESD) values used for the evaluation of uncertainty were produced by the Millennium 2010 Data Manager software package (v. 2.12.X–2.15.X., Waters, USA).

Estimating the expanded uncertainty of the result included balance calibration and performance, repeatability in weighing, error in volumetric glassware and pipettes, and temperature dependense of volumetric glassware, purity and formula weight of weighed reference (standard) materials, and the performance of spectrophotometric measurement. If the limits given in certificates or in equipment specifications were expressed without the confidence level or the shape of the distribution was not known, a rectangular distribution was used (Eurachem 1995). The following assumptions were also made; a random error of the test method was not related to the concentration and the residuals were normally distributed.

Outlying variance, homogeneity of variances, and normality of distribution of mean values were tested in intercalibration and collaborative studies using the Cochran test, the Bartlett test and the Kolmogorov-Smirnov-Lilliefors -test, respectively. A Nalimov or Dixon's test was performed to identify outlying mean values (Caulcutt and Boddy 1983, Sokal and Rohlf 1995). When the homogeneity of variances did not match the Bartlett test, either a non-parametric Kruskal-Wallis or a two-sample comparison, Mann-Wilcoxon test, was applied. The tests were performed at a 95.0% confidence level. The statistical calculations were performed by Statgraphic Plus for Windows 3.0 software package (Manugistics, USA).

4.6 Characterization of bound pyridoxine

Pyridoxine glycoside (PNG) from raw carrot and whole wheat flour was extracted with ice-cold perchloric acid solution and purified using a preparative liquid chromatography. PNG fraction was further purified using reversed-phase and cation-exhange chromatography. The glycosylated pyridoxine fraction was characterized with NMR and mass spectrometry, and with enzymatic hydrolysis (Fig. 12).

4.6.1 Isolation

1 kg of raw carrot was homogenized in 2000ml perchloric acid (0.5M) for three minutes in an ice-water bath. The homogenate was filtered through a nylon cloth to remove excess solid material and the filtrate was then centrifuged. The filtrate was further concentrated prior to preparative chromatography in a vacuum-rotavapor, the temperature of the water bath not exceeding 20°C. The same procedure was also applied to the wheat sample.

A Delta Prep liquid chromatograph (Waters, USA) was connected to a UV-VIS -detector (Waters 484, USA) and a fluorescence detector (Waters 470, USA). The preparative liquid chro-

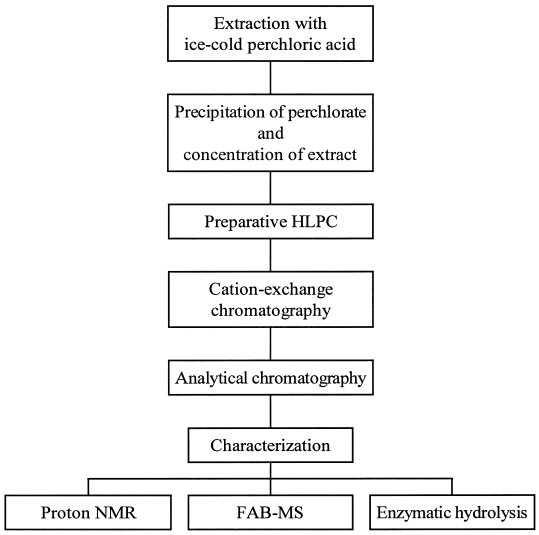

Fig. 12. Isolation and characterization of glycosylated pyridoxine. (NMR = nuclear magnetic resonance spectroscopy, FAB-MS = fast atom bombardment mass spectroscopy).

magraphic method, except for the flow rate of the mobile phase, was scaled up on the basis of the analytical liquid chromatographic method. Separation of the glucosidic fraction of PN was performed using a µBondapak C18 column (15–20µm, 100x25mm i.d., radial compression column, Waters, USA) connected to a guard column (µBondapak C18, 10µm, 10x25mm i.d., Waters, USA). The flow rate of the gradient elution, a mixture of 2-propanol, phosphate buffer, and ion-pair reagent, was set at 10ml/min. Post-column derivatization was omitted in the preparative chromatography. Fractions were manually collected, and the individual fractions containing bound pyridoxine were pooled.

Pooled fractions were concentrated in a vacuum-rotavapor and the residues were redissolved into 9 ml of water. The fractions were injected into the preparative chromatograph in order to remove buffer salts and ion-pair reagent. The mobile phase was then a mixture of methanol and water (85:15) the flow rate being 10ml/min. The fractions were manually recollected, and checked by analytical liquid chromatography. Desalted, bound pyridoxine containing fractions were pooled and lyophilized.

Proposed bound pyridoxine fractions were further purified with ion-exchange open-column chromatography based on the method of Gregory and Ink (1978). An open-column (32x20mm) was packed with a cation-exhange resin (Dowex AG 50W-X8, 200–400 mesh), regenerated with 1M ammonium hydroxide solution and equilibrated with 33mM ammonium hydrogenphosphate solution (pH 2.2). The lyophilized extract, dissolved into a 30ml portion of water, was applied to column, and the column was washed with 50mM ammonium acetate solution (pH4.0, 25ml) and with 250mM ammonium acetate solution (pH4.0, 65ml). The analyte was eluted from the resin with 250mM ammonium acetate solution (pH7.0, 65ml). Fractions of 10ml were collected and checked using analytical liquid chromatography.

The ammonium acetate buffer used in the ion-exhange chromatography was removed by analytical liquid chromatography using 10% (v/v) methanol as a mobile phase, the flow rate being 1.2ml/min. Lyophilized analyte extract was dissolved in 10% methanol (1ml) , injected and the fractions containing bound pyridoxine were collected, pooled, lyophilized and dissolved in deuterium oxide (99.8%, Merck, Germany). The sample was relyophilized and redissolved in deuterium oxide (99.95%, Merck, Germany) prior NMR measurement.

4.6.2 β-glucosidase digestion

As the reversed-phase chromatogram normally contains three peaks, named as X_1 (k_e~8.3), X_2 (k_e ~10.0), and X_3 (k_e ~11.8) which disappear after the digestion with β-glucosidase, the fraction that gives an increased fluorescence response in analytical liquid chromatography at the k-value of pyridoxine (k_e ~13.3–13.6) being taken for further testing. The amount of this fraction was then measured as pyridoxine using analytical HPLC. Quantitation with an external standard method was based on the assumption

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that the molar fluorescence responses of pyridoxine and its glycosylated derivative are equal (Gregory and Ink 1987).

4.6.3 Structural evaluation

Proton-NMR of the isolate was measured in deuterium oxide using a Varian Unity-500 NMR-spectrometer at the Institute of Biotechnology (Helsinki). Proton-NMR-spectra and COSY and TOCSY correlation spectrum from wheat were recorded. The carrot fraction contained impurities in a such amount that the evaluation of its NMR spectrum was rejected. Proton spectra of D(+)-glucose (Merck 8337, Germany) and pyridoxine hydrochloride (Sigma P-9755, USA) dissolved in D_2O were measured in a same manner as for bound pyridoxine fractions.

The FAB-ionization technique was used to perform mass spectrum of the analyte. The sample was applied to the mass spectrometry via a directly coupled sample probe. Glycerol and nitrobenzyl amine (for positive ion measurement) and triethanol amine (for negative ion measurement) were used as matrix modificators. Measurements were done in low resolution mode with Finnigan MAT 90 mass spectrometry equipment at the Environment laboratory of Helsinki.

5 Results

5.1 Liquid chromatography

5.1.1 Choice of the column and the mobile phase

Peak symmetry of pyridoxal, pyridoxine, deoxypyridoxine, and pyridoxamine in reversed-phase chromatography was measured using different column packings and mobile phases (Table 7). Excellent peak shape for free B_6 vitamers was achieved with the µBondapak packing in spite of a relative high silanol index value (SiOH) for that particular packing material measured by Walters' test (Table 8). The characteristics of the column packings were needed because the separation efficiency is greatly influenced by the cationic nature of vitamin B_6 compounds. Peak shape of pyridoxamine, e.g. using 201HSB5 packing, could be improved by adding triethylamine as a modifier to the mobile phase but this reduced peak shape for the other free vitamers.

Vitamin B_6 compounds possess both hydrophobic and ionic properties which were utilized in liquid chromatographic separation. Chemically bonded reversed-phases were chosen to be tested for separation of B_6 vitamers since adequate retention for free vitamers is generally achieved with these packings. However, the use of an ion-pair reagent was needed to ensure the adequate retention in liquid chromatography for phosphorylated pyridoxamine (k_e ~ 20 in the chosen method for routine food analysis). Rather modest column efficiency (as N/m) in Walters' test was achieved with poly(styrene-divinylbenzene)(PS-DVB) based "reversed-phase like" polymer packing compared to silica based column packings (Table 8). The aminopropyl phase gave an insufficient retention of vitamin B_6 compounds, and its further testing was discontinued.

A good peak separation was achieved using 2-propanol as a organic modifier in the gradient elution (Fig. 13). Selectivity of isopropanol was found to be superior to acetonitrile or methanol (data not shown). By lowering the pH of the mobile phase to approx. 2–3, the baseline disturbances could also be diminished which resulted in increased selectivity of fluorescense detection. The low pH of the mobile phase also

Table 7. Peak symmetry values for B_6 vitamers in reversed-phase columns tested.

Column	Mobile phase	Symmetry (A_s^2)			
		PL	PN	DPN	PM
S5ODS2	40mM H_2SO_4,isoc.	1.8	5.4		1.0
	33mM phosphate buffer, pH2.2, isoc.	3.1	5.2		1.0
	170mM HOAc, MeOH, IP, grad.	11.1	7.1	16.0	3.2
201HSB5	170mM HAc, MeOH, IP, grad.	2.1	2.0	1.3	2.5
	170mM HAc, MeOH, IP, grad., 1%TEA	3.2	3.2	3.1	1.0
µC18	170mM HAc, MeOH, IP, grad.	1.0	1.0	1.0	1.0
C18	33mM phosphate buffer, pH2.2, IPA, grad. IP	2.1	2.1		2.0

Columns:

S5ODS2 Spherisorb S5ODS2, 5µm, 250x4.6mm i.d., PhaseSeparation, UK
201HSB5 Vydac 201HSB5, 5µm, 250x4.6mm i.d., Separation Group, USA
µC18 µBondapak C18, 10µm, 300x3.9mm i.d., Waters, USA
C18 Novapak C18, 4µm, 150x3.9mm i.d., Waters, USA

HAc; acetic acid, IP; ion-pair, IPA; 2-propanol, MeOH; methanol, TEA; triethylamine

favoured the conditions required for the formation of an ion-pair between alkyl sulfonates and cationic analytes like pyridoxamine.

Table 8. Characterization of octadecyl columns tested according to Walters method (1987).

Packing	SiOH	HP	Efficiency (N/m)
S5ODS2	0.51	5.14	52 000
201HSB5	0.64	3.96	11 000
µC18	1.71	3.18	14 000
C18	0.59	4.56	84 000
RP-18	0.20	nd*	4 400

SiOH; silanol index, HP; hydrophobicity index
nd* antracene not eluted in a proper retention time

Packings:

S5ODS2 Spherisorb S5ODS2, 5µm, 250x4,6mm i.d., PhaseSep, UK
201HSB5 Vydac 201HSB5, 5µm, 250x4,6mm i.d., Separation Group, USA
µC18 µBondapak C18, 10µm, 300x3,9mm i.d., Waters, USA
C18 Novapak C18, 4µm, 150x3,9mm i.d., Waters, USA
RP-18 Polyspher RP-18, 9µm, 150x4.6mm id, Merck, Germany

On this basis, the selection of the chromatography mode for separation of vitamin B_6 compounds was later focused on ion-paired reversed-phase chromatography. Chromatographic parameters (Tables 7 and 8) favoured the use of Waters Novapak C18 cartridge column (4µm, 150x3.9mm i.d., Waters, USA) which was later on connected with a guard column (Waters Novapak C18 cartridge) in routine food analysis. Retention of pyridoxamine was adjusted to a value of ca. k_e 20 by choosing 1-octanesulfonate to serve as an ion-pair reagent. Gradient elution then consisted of 33 mM phosphate buffer and 8 mM 1-octanesulfonic acid (pH 2.2), and 2-propanol column temperature being set at 30°C. Retention factor for PLP, PA, PMP, PL, PN, DPN, and PM were then 1.4, 2.1, 5.6, 12.2, 13.6, 14.6 and 21.3, respectively. Tentatively identified 5'-*O*-β-D-glucopyranosyl pyridoxine was eluted preceeding PL at k_e value of 11.6–11.8 in this chromatographic system.

5.1.2 Post-column derivatization

The relative fluorescence responses of B_6 vitamers differs noticeable, pyridoxal-5'-phophate and 4-deoxypyridoxine (internal standard) hav-

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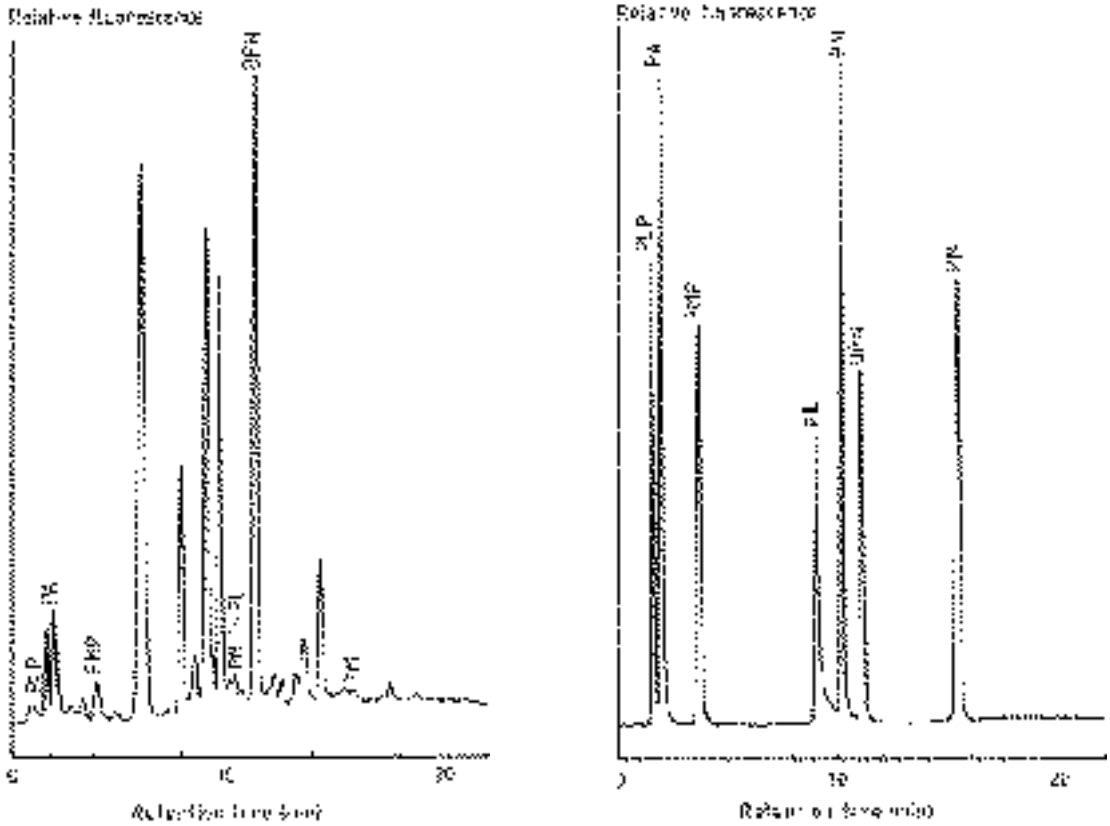

Fig. 13. Ion-paired reversed-phase chromatogram of a) a carrot sample and b) standards. (PA = pyridoxic acid, DPN = 4-deoxypyridoxine, PL = pyridoxal, PLP = pyridoxal-5'-phosphate, PN = pyridoxine, PM = pyridoxamine, PMP = pyridoxamine-5'-phosphate).

ing the lowest responses (Fig. 14). The response of PLP was increased five-fold after the formation of a sulphite adduct at elevated pH. The relative effect of derivatization on the responses of other vitamers was smaller. Post-column derivatization reagent, 35mM sodium hydrogensulphite in 0.5M phosphate buffer (pH7.5) mixed with the mobile phase yielded the highest response for PLP. Optimum condition for PLP was thus obtained by pumping a derivatization reagent through a union tee into the mobile phase with a flow rate of 0.1ml/min which corresponded approximately 3mM hydrogen sulphite concentration in the mobile phase prior to detection (Fig. 15). In post-column derivatized LC system, the fluorescence excitation and emission wavelengths were set at 330nm and 400nm, respectively.

5.2 Sample extraction

5.2.1 Preliminary experiments for the extraction procedure

The result of the total B_6 vitamin content of a broccoli sample seemed to differ using four different non-mineral acids as the extracting solution. Tested non-mineral acids, sulfosalicylic acid, metaphosphoric acid, trichloroacetic acid,

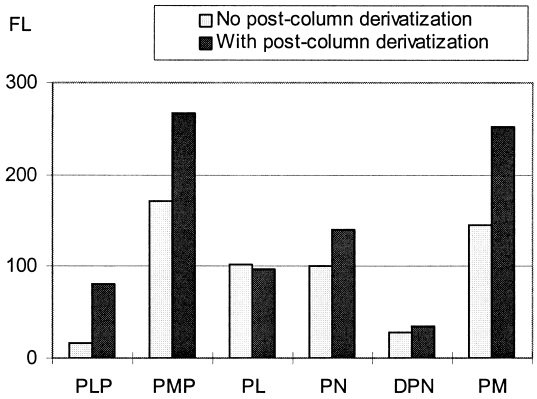

Fig. 14. Relative fluorescence responce of vitamin B_6 compounds with and without post-column derivatization. The relative fluorescence responce of PN without post-column derivatization set at 100. (DPN = 4-deoxypyridoxine, FL = relative fluorescence, PL = pyridoxal, PLP = pyridoxal-5'-phosphate, PN = pyridoxine, PM = pyridoxamine, PMP = pyridoxamine-5'-phosphate).

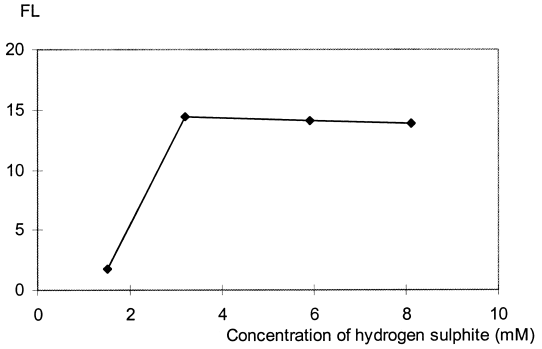

Fig. 15. The effect of the amount hydrogensulfite in the mobile phase on the fluorescence responce of PLP. (FL = relative fluorescence, PLP = pyridoxal-5'-phosphate).

and perchloric acid showed also varieties in the vitamer distribution (Fig. 16a). The statistical difference in the results could not be directly evaluated due to an insufficient number of repeated measurements. If the variation in the individual mean values of the total results for each acid treatment was assumed to be the same (and of magnitude 12%), a statistical difference within the results was found; the results derived from the sulfosalicylic acid and perchloric acid extractions were consistent, but they were different compared to those of metaphosphoric acid and trichloroacetic acid extractions, and *vice versa*. However, this was not the case with the banana matrix; in the total vitamin results of banana sample, the sulfosalicylic acid and trichloroacetic acid hydrolysis gave the same result (Fig. 16b).

Sulfuric acid digestion without an autoclaving process extracted bounded pyridoxine but it hydrolyzed partially the glycosidic bond (Fig. 17). When sulfuric acid extraction was

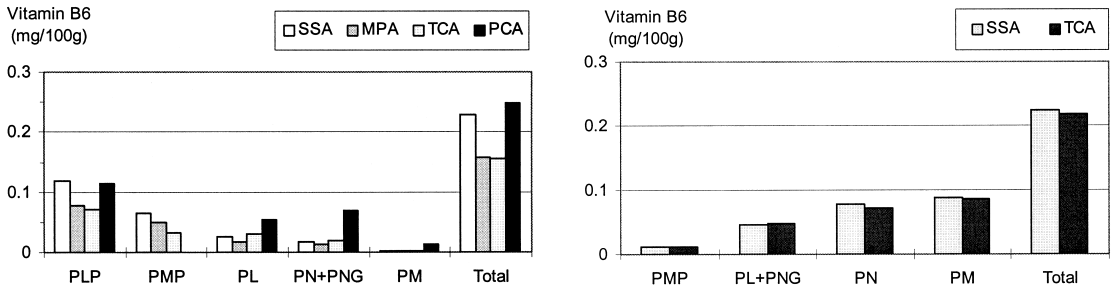

Fig. 16. Effect of sample extraction on vitamin B_6 content of a) a raw broccoli sample extracted with non-mineral acids combined with a β-glucosidase treatment (replicate analyses) and, b) a banana sample (replicate analyses), no enzyme treatment, PLP not determined. (SSA; sulfosalicylic acid hydrolysis, MPA; metaphosphoric acid hydrolysis, TCA; trichloroasetic acid hydrolysis, PCA; perchloric acid hydrolysis, PL = pyridoxal, PLP = pyridoxal-5'-phosphate, PN = pyridoxine, PNG = pyridoxine glucoside, PM = pyridoxamine, PMP = pyridoxamine-5'-phosphate).

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combined with an autoclaving treament (121°C, 30min), glycosidic pyridoxine in carrot extract was almost completely hydrolysed to free pyridoxine (data not shown). Homogenizing the sample with an ice-cold perchloric acid and omitting the autoclaving process extracted glycosylated pyridoxine in its intact form; practically no pyridoxine was measured after plain acid treatment while this was not the case with sulfuric acid. The explanation for an increased amount of PM after perchloric acid/glucosidase digestion remained unclear as the phosphatase activity of the used enzyme preparate was not measured. The sample treatment procedure in which the samples were extracted with dilute ice-cold perchloric acid (and excluding the autoclaving step) was chosen for a more detailed examination.

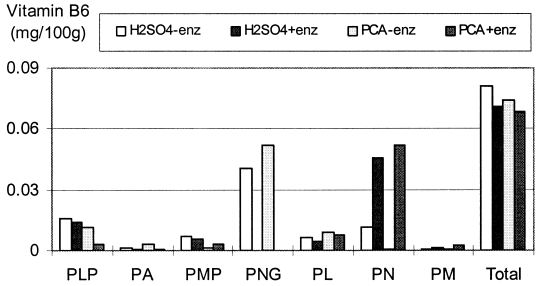


Fig. 17. Effect of sample extraction on vitamin B_6 content of a carrot sample extracted with sulfuric acid and with perchloric acid (replicate analysis). (H_2SO_4; sulfuric acid hydrolysis with and without β-glucosidase treatment, PCA; perchloric acid hydrolysis with and without β-glucosidase treatment, PA = pyridoxic acid, PL = pyridoxal, PLP = pyridoxal-5'-phosphate, PN = pyridoxine, PNG = pyridoxine glucoside, PM = pyridoxamine, PMP = pyridoxamine-5'-phosphate).

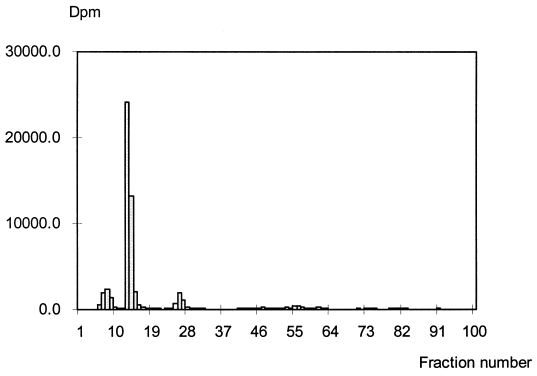
5.2.2 Evaluation of the chosen perchloric acid extraction

Perchloric acid extraction was further tested using ^{14}C-labeled pyridoxal-5'-phosphate. The measured total ^{14}C-activity of purchased standard compound was 314 kBq and the purity was approximately 76% calculated on the basis of distribution of percentage ^{14}C-activity of untreated standard in the reversed-phase chromatogram (Fig. 18a). 12% and 8% of the total ^{14}C-activity was located in the chromatogram at retention factor values of k_e=0.5 and k_e=5.6. The former ^{14}C-active fraction eluted at near interparticle volume (V_0) was considered as an unknown compound (named unknown 1) whereas the latter active fraction eluted at the same retention factor value as PMP.

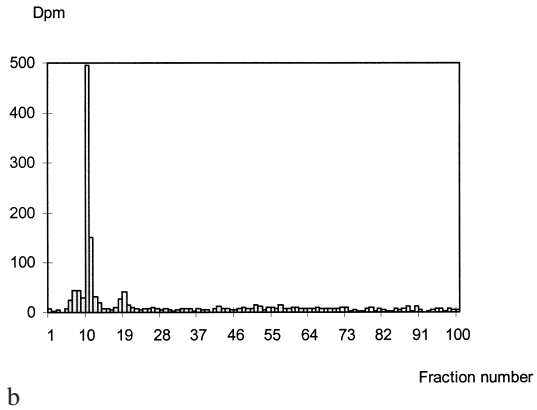
A small change in ^{14}C-activity distribution was found after the labeled pyridoxal phosphate standard was extracted with the routine perchloric acid extraction procedure (Fig. 18b). The activity in PLP fraction decreased by approximately 3% after perchloric acid extraction, and increment of activity was found in the PMP fraction, in unknown 3 fraction and in PL fraction representing the change of 0.8, 1.6 and 0.8% in the total activity, respectively.

The sample matrix effect on the stability of PLP during the acid extraction was evaluated by adding labeled PLP standard to beef steak, carrot and whole wheat flour samples and measuring the activity distribution in the reversed-phase chromatograms (Fig. 18c-e). Approximately, 10% of ^{14}C-activity of labeled PLP added to beef steak and carrot samples was lost during the extraction procedure and the main increased activity was relocated in fractions of PL and of unknown 2 (Fig. 18c, d). The chemical structure of the supposed break-down product of PLP, referred to as unknown compound 2, is not known. The percentage activity related to the released free PL due to hydrolysis of phosphate ester lingage of PLP was 7% and 14% in beef and carrot sample matrices, respectively (Fig. 19). For the whole wheat flour matrix, the ^{14}C-activity in pyridoxal fraction was increased by approximately 26%.

When labeled PLP standard was extracted with any of three tested sample matrices, part of the ^{14}C-activity was relocated in a unknown compound 2 fraction (k_e~3.9) instead of unknown compound 3 fraction (k_e~11). Formation of the



a



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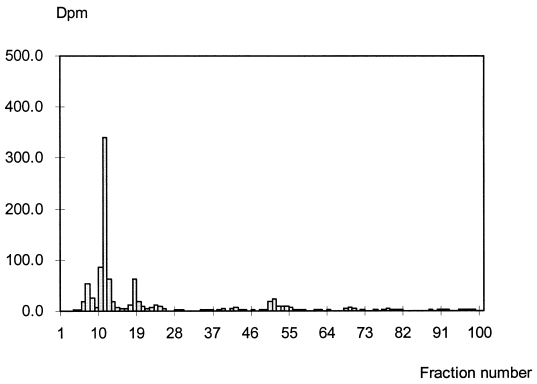

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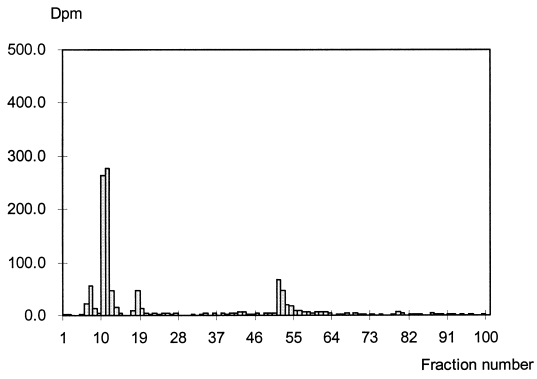

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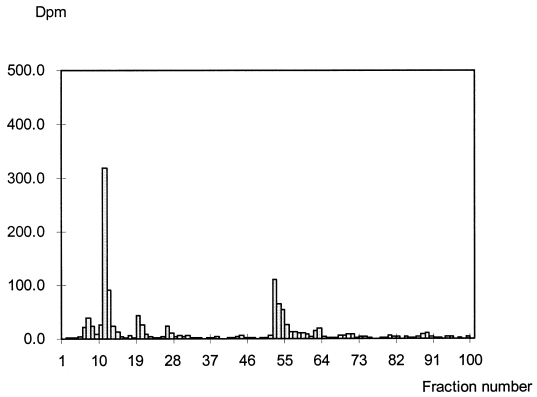

e

Fig. 18. Distribution of ^{14}C-activity in a reversed-phase chromatogram of

a) a labeled PLP standard sample directly injected into HPLC omitting perchloric acid extraction procedure,
b) a labeled PLP standard sample measured by HPLC after the perchloric acid procedure,
c) a labeled PLP standard added to beef steak sample, extracted with perchloric acid solution and measured by HPLC
d) a labeled PLP standard added to carrot sample, extracted with perchloric acid solution and measured by HPLC and
e) a labeled PLP standard added to whole wheat sample, extracted with perchloric acid solution and measured by HPLC.

Five fractions (each of ca. 240µl) per minute collected during the whole chromatographic run (20min). Dpm = disintegrations per minute, PLP = pyridoxal-5'-phosphate.

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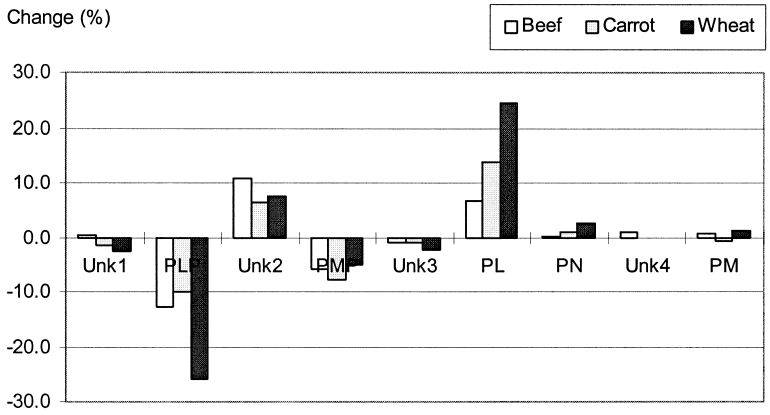

Fig. 19. Changes in the vitamer distribution of ^{14}C-labeled pyridoxal-5'-phosphate added into three food matrices (beef, carrot and wheat), and compared to perchloric acid extracted ^{14}C-PLP standard calculated on the basis of ^{14}C -activities. (PL = pyridoxal, PLP = pyridoxal-5'-phosphate, PN = pyridoxine, PM = pyridoxamine, PMP = pyridoxamine-5'-phosphate, Unk 1,2,3,4 = Unknown compounds).

latter fraction was found during the extraction procedure without any food matrix.

5.2.3 Enzymatic digestion

Phosphorylated pyridoxal and pyridoxamine were converted into their free forms using an alkaline phosphatase preparate from calf intestine. The minimum amount of alkaline phosphatase needed to ensure adequate dephosphorylation was estimated to be 0.4–0.8U enzyme per mg sample in this sample extraction procedure (Fig. 20).The amount of alkaline phosphatase needed to release free vitamers from their phosphate esters was tested using beef steak sample which is rich both in pyridoxamine phosphate and pyridoxal phosphate; the amount of phosphorylated pyridoxamine and pyridoxal in beef covered approximately 44% and 46% of the vitamin B_6 activity, respectively.

The carrot matrix where pyridoxine glucoside accounts for the major portion of vitamin B_6 compounds was used for testing glucosidase

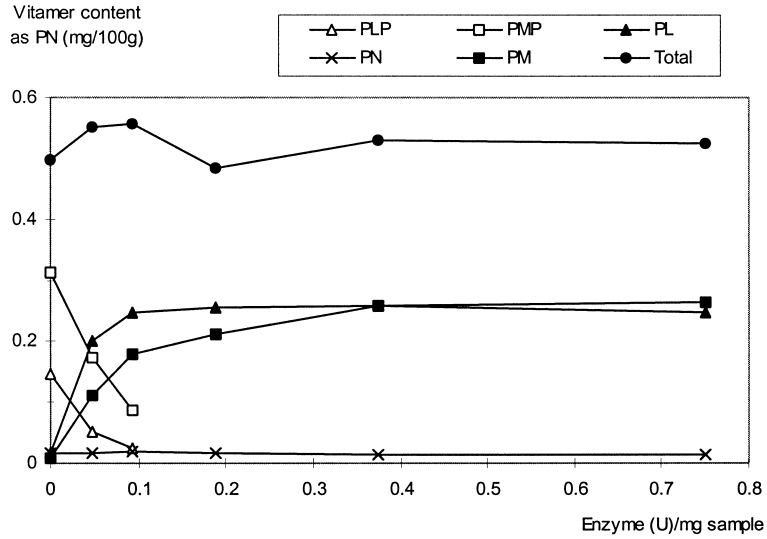

Fig. 20. Effect of the amount of alkaline phosphatase on the hydrolysis of phosphorylated pyridoxal and pyridoxamine in beef steak matrix. (PL = pyridoxal, PLP = pyridoxal-5'-phosphate, PN = pyridoxine, PM = pyridoxamine, PMP = pyridoxamine-5'-phosphate, Total = sum of PLP, PL, PM, PMP and PN).

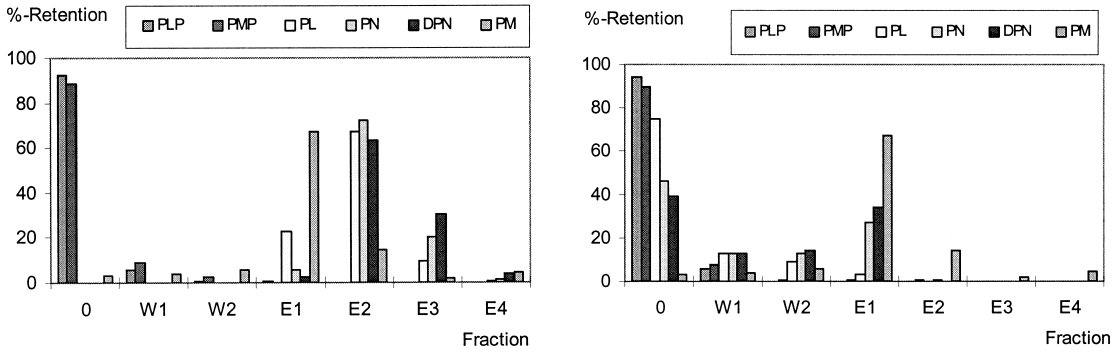

Fig. 21. Retention of vitamin B_6 compounds on a) a benzene sulfonic acid SPE phase (replicate analysis) and b) a propyl sulfonic acid SPE phase (replicate analysis). (0 = fraction through the cartridge,W1-2 = washings, E1-E4 = elutions, DPN = 4-deoxypyridoxine, PL = pyridoxal, PLP = pyridoxal-5'-phosphate, PN = pyridoxine, PM = pyridoxamine, PMP = pyridoxamine-5'-phosphate).

hydrolysis. A completely disappeared PNG peak (k_e=11.6, t_R=9.8 min) in the reversed-phase chromatogram was considered to show an adequate enzymatic hydrolysis. No fluorescence signal at a k_e value of 11.6 in the chromatogram was measured after hydrolysis procedure using ca. 0.13U of β-glucosidase per mg sample.

5.2.4 Solid-phase extraction

Purification of the sample extract and concentration of vitamin B_6 compounds using a solid-phase extraction (SPE) technique was tested. As the adequate retention on reversed-phase SPE materials was not achieved due to a large variation in polarity of B_6 compounds (data not shown), the separation of vitamin B_6 compounds based on the cationic nature of either pyridinium ion or aminomethyl group was evaluated. Weak cation-exchange functional group such as carboxymethyl (pK~4.8) retained vitamers from standard solutions but it was not effective enough with sample extracts (data not shown). Free B_6 derivatives, PL, PN, DPN and PM, could be isolated with a stronger cation-exhange phase by using the benzene sulfonic acid phase (Fig. 21a). Propyl sulfonic acid phase did not retain free vitamers sufficiently (Fig. 21b.). Also phosphorylated vitamers (PLP and PMP) were lost using both phases. The most cationic compound, PM, retained strongly in both phases (Fig. 22).

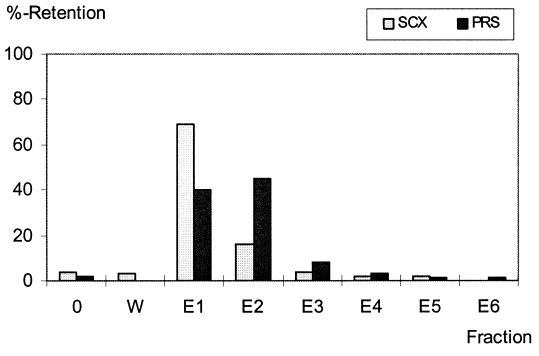

Fig. 22. Retention of pyridoxamine on a propyl sulfonic acid (PRS) and benzene sulfonic acid (SCX) SPE-phases (replicate analysis). (0 = fraction through the cartridge, W = washing, E1-E4 = elutions).

When the extraction method of Gregory and Ink (1987) was evaluated, sulfosalicylic acid (SSA) has to be removed by an anion-exhange SPE because of its natural fluorescence properties. Removal of SSA by solid-phase extraction led to an unwanted dilution of the analyte extract. As the same extraction efficiency was achieved with the more easily removable perchloric acid (PCA), extraction with sulfosalicylic acid was excluded.

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Table 9. Repeatability parameters for 4-deoxypyridoxine (internal standard).

Sample group		Repeatability relative standard deviation (RSD_r) for				
	n	k'	$w_{\_}$	R_r	$A_{4.4}$	T
Standards	178	3.0	9.9	14.0	9.4	5.9
Meat and meat products	174	2.3	10.0	14.3	7.7	5.0
Milk and milk products	90	1.7	6.6	11.4	9.5	6.1
Cereals and vegetables	359	1.8	21.4	14.5	7.7	3.7
Fish and fish products	68	0.8	5.8	7.9	7.1	4.8
Miscellanous	13	0.1	3.6	10.6	n.d	n.d
Sum	882					
Weighed mean		2.0	13.9	12.9	8.1	4.7

n; number of chromatographic runs, k_e; retention factor, $w_{h;}$ peak-width of at half height, R_r; relative responce (peak area/concentration injected), $A_{4.4}$; peak asymmetry calculated as width at the 4.4% peak height, T; tailing factor according to USP, n.d.; not determined

5.3 Validity of the method chosen for routine food analysis

5.3.1 General parameters

Retention factor (k_e) values for PLP, PA, PMP, PL, PN, DPN, and PM in the chosen ion-paired reversed-phase chromatographic separation were 1.4, 2.1, 5.6, 12.2, 13.6, 14.6 and 21.3, respectively. The retention factor, chromatographic peak shape (peak width at half height, asymmetry, USP tailing) and relative response (peak area/concentration) for 4-deoxypyridoxine (DPN) in calibration and quantitation runs were monitored during the routine food analysis (Table 9). The retention of 4-deoxypyridoxine on the chromatographic system was reproducible, the relative standard deviation of retention factor being ca. 2%. A higher variation was found in the peak width at half height and in the relative responce values. However, the relative standard deviation of peak symmetry or USP tailing were both under 10%.

The limit of detection (LOD) was estimated to be 50–150pg pyridoxine per injection which corresponded approximately to 1.4–4.2µg pyridoxine per 100g food sample using the presented routine food analysis procedure. The recovery of an added analyte to beef steak and in-house reference material (commercial infant formula, fortified with pyridoxine) ranged normally from 72 to 107% (Table 10). The highest variation (150%) was measured for PLP. The repeatability relative standard deviation (RSD_r) for the vitamin B_6 content of an in-house reference material (a commercial fortified infant formula powder) was 5.2% (n=6, triplicate analysis), mean and standard deviation being 0.44 and 0.023 mg PN/100g fresh weight (Fig. 23).

Table 10. Recovery (%) of added B_6 vitamers (mean±SD, n=4).

Vitamer	Beef steak	Infant formula
PLP	77±16.5	150±50
PA	77±14.2	83±5.2
PMP	90±4.8	82±16.8
PL	83±0.7	72±9.2
PN	98±2.3	95±7.3
DPN	94±3.8	107±9.4
PM	95±2.2	107±10.4

DPN; 4-deoxypyridoxine, PA; pyridoxic acid, PL; pyridoxal, PLP; pyridoxal-5'-phosphate, PN; pyridoxine, PM; pyridoxamine, PMP; pyridoxamine-5'-phosphate

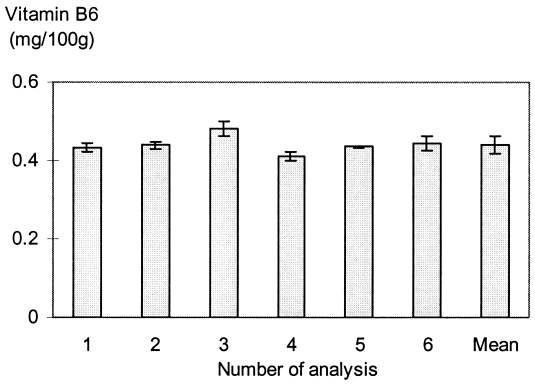


Fig. 23. Repeatability of determination of the in-house reference material (a commercial fortified infant formula powder); mean value of means, standard deviation, and repeatability relative standard deviation 0.44 mg PN/100g FW, 0.023 mg PN/100g FW and 5.2%, respectively.

5.3.2 Uncertainty of results

The total uncertainty of results was divided between errors caused by calibration and those due to preparation of analysis sample (Table 11). Uncertainly related to internal standard standardization, calculated as RSD%, was double that for preparation of the analysis sample. The total uncertainy for pyridoxine was estimated to be ca. 12%. Based on this calculation, the results derived from the routine food analysis were expressed using two or three significant numbers. Uncertainty related to the spectrophotometric measurement of the concentration of the stock standard solutions was approx. 1%. The 95% confidental interval ($CI_{95\%}$) for concentration, slope, and intercept for in-house reference material were calculated on the basis of four randomly chosen calibration data sets of pyridoxine, pyridoxal, and pyridoxamine (Table 12). The origin value was located in the calculated $CI_{95\%}$ of the intercept in all twelve calibration sets except in two cases (pyridoxine; calibration no 2, pyridoxal; calibration no 3).

5.3.3 Interlaboratory and laboratory proficiency tests

Validity of the analytical method for routine food analysis and the performance of the laboratory were evaluated by taking part in EU Measurement and Testing (MAT) Programme's 3rd intercomparison. Each laboratory reported its results for a tested ("optimal") method and for the laboratory's own ("in-house") method. Results from twelve laboratories were submitted (Fig. 24 a-c). Repeatability relative standard deviation (RSD_r) for a particular laboratory (variation within a laboratory) varied from 5% to 13% being much lower than the reproducibility relative standard deviation RSD_R (variation between laboratories) (Table 13). Our laboratory's results (laboratory no. 2 in Figures 24a-c) were located within the 95% confidental interval calculated for either all data or outlier excluded data. Furthermore each laboratory's performance could

Table 11. Total uncertainty of pyridoxine result derived from an in-house reference material (infant formula) (mean value 0.324mg PN/100g fresh weight).

Procedure	mean±SD	uncertainty as %	range
Calibration	0.324 ± 0.03708	11.4	0.287 – 0.361
Sample preparation	0.324 ± 0.01591	4.9	0.308 – 0.340
Total uncertainty (u_{Tot})	0.324 ± 0.04035	12.4	0.284 – 0.364
Result	0.32 ± 0.040		

Table 12. Uncertainty associated with the calibration of a) pyridoxine, b) pyridoxal and c) pyridoxamine; 95% confident interval ($CI_{95\%}$) for concentration of an in-house reference material (infant formula), for slope, and for intercept (4-deoxypyridoxine as an internal standard, nine concentration levels, two injections at each level).

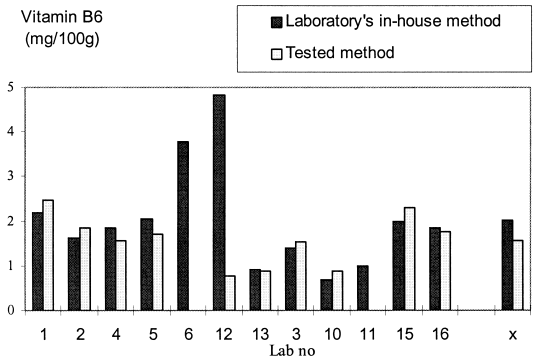
Calibration	Concentration				Slope				Intercept			
	lower	mean	upper	range	lower	mean	upper	range	lower	mean	upper	range
a) pyridoxine												
1	0.286926	0.324008	0.361089	0.074	5.245390	5.363290	5.481190	0.24	-2.663737	1.241910	5.147557	7.8
2	0.322839	0.330329	0.337819	0.015	4.504873	4.523283	4.541693	0.037	0.136360	0.801412	1.466464	1.3
3	0.276147	0.296115	0.316084	0.040	5.015526	5.036334	5.134714	0.12	-1.435908	0.550152	2.536212	4.0
4	0.315917	0.347536	0.379155	0.063	5.163761	5.075120	5.361081	0.20	-0.320164	2.437870	5.195904	5.5
Mean				0.05				0.2				5
SD				0.026				0.09				2.7
b) pyridoxal												
1	0.059081	0.082763	0.106444	0.047	4.146367	4.203542	4.260717	0.11	-3.423120	-1.490640	0.441845	3.86
2	0.120235	0.127733	0.135231	0.015	3.668108	3.689492	3.710876	0.043	-0.966250	-0.425840	0.114583	1.08
3	0.089894	0.109092	0.128290	0.038	4.122300	4.165300	4.208300	0.086	-3.117330	-1.562720	-0.008120	3.11
4	0.029069	0.056734	0.084398	0.055	3.690527	3.749976	3.809425	0.12	-3.354470	-1.658680	0.037122	3.39
Mean				0.04				0.09				3
SD				0.017				0.034				1.2
c) pyridoxamine												
1	0.037805	0.053577	0.069348	0.032	11.890760	12.165780	12.440790	0.55	-3.861200	-0.121580	3.618034	7.5
2	0.052238	0.056151	0.060064	0.0078	8.910810	8.949223	8.987636	0.077	-0.496500	0.250033	0.996564	1.5
3	0.030368	0.038484	0.046600	0.016	14.538650	14.312890	14.538650	0.45	-4.392560	-1.656350	1.079870	5.5
4	0.049424	0.056779	0.064134	0.015	11.831120	11.707610	11.584100	0.25	-1.852450	-0.435110	0.982224	2.8
Mean				0.02				0.3				4
SD				0.010				0.21				2.7

Table 13. Statistics of EU-MAT interlaboratory study on vitamin B_6 (Berg van den et al. 1996).

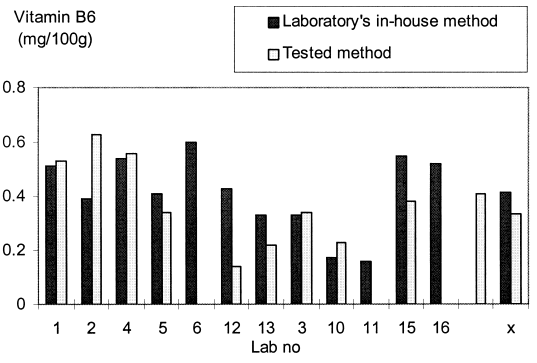
	Pig's liver		Mixed vegetables		Wholemeal flour	
	In-house	Optimal	In-house	Optimal	In-house	Optimal
Number of laboratories	12	10	12	10	12	11
Mean of means*	2.01	1.57	0.41	0.38	0.27	0.26
RSD_r (%)	6	13	12	5	11	7
RSD_R (%)	63	36	38	40	51	43
Number of outliers**	–	1	–	–	–	–

* as PN (mg/100g)
** according to Nalimov test
RSD_r; repeatability relative standard deviation, RSD_R; reproducibility relative standard deviation

be assessed by its results for the BCR's certification study for reference materials; our laboratory's vitamin B_6 results were included within the 95% confidental interval calculated from the mean values derived from eleven European laboratories.



a



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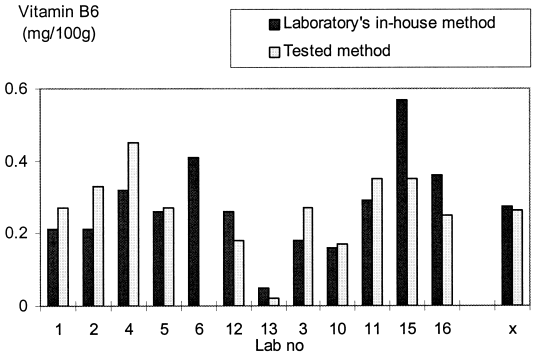

c

Fig. 24. Intercomparison results of EU-MAT study on vitamin B_6 x = mean value, laboratory 2 = University of Helsinki. Samples: a) pig's liver, b) mixed vegetable and c) wholemeal flour. (Redrawn from the results of van den Berg et al. (1996) with permission from Dr. van den Berg).

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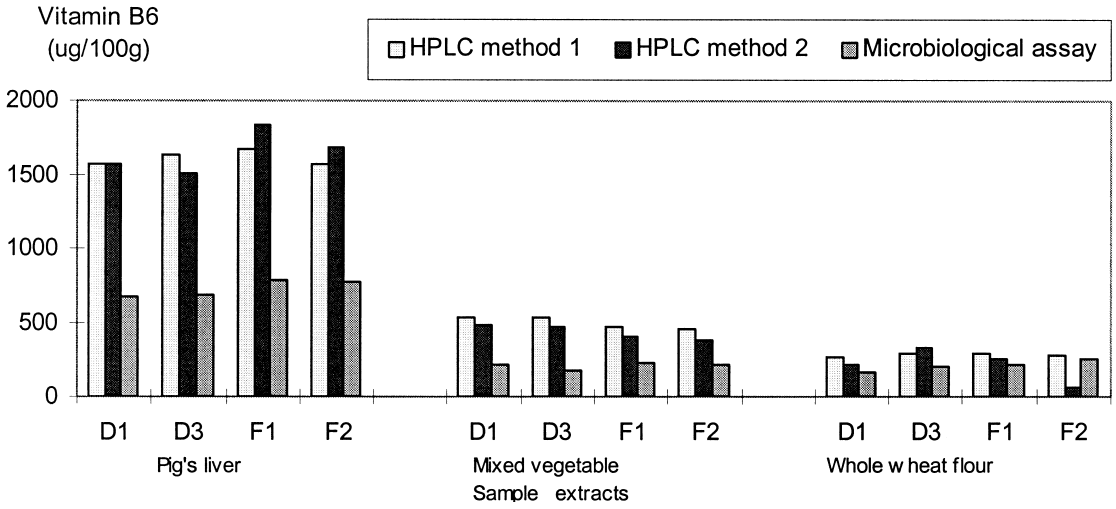

Fig. 25. Interlaboratory results of the circulated sample extracts. Redrawn from the results of Bognar and Ollilainen (1997). Sample treatment: D1 = 5% trichloroacetic acid, 20°C, 30min, b-glucosidase, D3 = 0,1 M hydrochloric acid, 120°C, 30min, β-glucosidase, F1 = 5% trichloroacetic acid, 20°C, 30min, Takadiastase, F2 = 0,1 M hydrochloric acid, 120°C, 30min, Takadiastase. Methods: HPLC1 = isocratic elution (BFE, Germany), HPLC2 = gradient elution (University of Helsinki), MA = microbiological assay (LUF, Germany). (Redrawn from the results of Bognar and Ollilainen (1997) with permission from Dr. Bognar).

Moreover, the sample extracts of pig's liver, mixed vegetables, and whole wheat flour prepared by the Federal Research Centre for Nutrition (Stuttgart, Germany) were analyzed in three laboratories. Two liquid chromatographic procedures (HPLC1; isocratic method, HPLC2; gradient method) and one microbiological assay were compared. The results derived from two chromatographic methods were correlative to each other (Fig. 25). There was a statistically significant difference in the results of pigs' liver sample extract between liquid chromatographic and microbiological assay; the medians at the 95% confidence level indicated that a lower result was obtained using a microbiological assay. This was not the case with the two other samples.

Whole wheat flour and beef steak samples were also analyzed in two laboratories in Finland (Fig. 26). In this comparison, the microbiological assay seemed to give the same (for beef sample) or a little higher value (for whole wheat four) compared to those of the liquid chromatographic method when the variation in the mean values for both methods is assumed to be the same (and of the magnitude of 12%).

5.4 Routine food analysis

5.4.1 Flesh foods: meat, fish and poultry

The most abundant B_6 vitamers in beef, pork, lamb, reindeer, elk, and poultry meat were pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate representing ca. 88.3–94.4 % of the vitamin B_6 content (Table 14). A significant amount of free pyridoxine was present only in beef shoulder and kidney sample. Offals like pork liver, beef liver, broiler liver, beef kindey, and pork kidney also contained free vitamers, especially pyridoxamine. Poultry meat was rich in pyridoxal phosphate. Inactive metabolite, 4-pyridoxic acid, was the main vitamin B_6 compound in pork and beef liver but not in kidney or in poultry liver. A low percentage of PLP was found in processed food items; sausages and meat balls

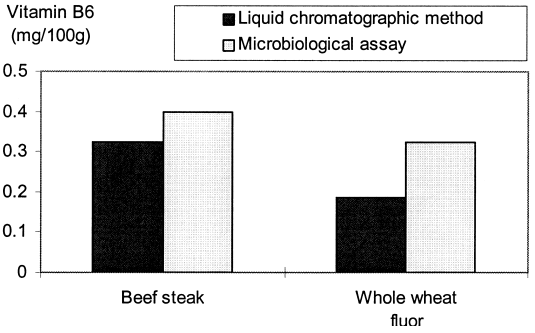

Fig. 26. A two-sample (beef steak and wheat flour) comparison, HPLC (Univ of Helsinki), microbiological assay (VTT Technical Research Centre, Finland).

which contained mainly PMP. The highest vitamin contents were found in liver; 0.85, 0.81, and 0.66mg/100g for beef liver, broiler liver, and pork liver, respectively.

Distribution of B_6 vitamer in fish resembled that of meat; the amount of vitamin B_6 consisted mainly of phosphorylated pyridoxal and pyridoxamine. However, free vitamers and especially pyridoxic acid were also present (Table 15).

5.4.2 Dairy products and egg

Free and phosphorylated pyridoxal, and pyridoxamine phosphate but also to some extent free pyridoxamine, were typical for liquid milk products whereas free and phosphorylated pyridoxamine formed the major vitamin fraction in cheese (Table 16). Pyridoxal phosphate was the dominating vitamer in egg yolk. Infant formula based on cow milk was fortified with pyridoxine. Only a small amount of pyridoxine was found in baker's yeast and egg yolk. In addition baker's yeast contained a minor fraction of pyridoxine phosphate. This vitamer was not found in any other food item in this study.

Table 14. Vitamer distribution and vitamin B_6 content of meat and meat products (mean±SD, n=3, as PN mg/100g fresh weight).

Food item	Vitamer						Sum
	PLP	PA	PMP	PL	PN	PM	
Minced meat, beef	0.17±0.004	0.006±0.0033	0.16±0.003	0.024±0.0014	0.0047±0.0006	tr	0.36
Minced meat, beef and pork	0.21±0.019	0.018±0.0026	0.075±0.0022	0.017±0.0001	0.014±0.0001	0.0053±0.00002	0.32
Beef shoulder	0.14±0.014	0.0043±0.00097	0.09±0.014	0.0061±0.00055	0.06±0.082	0.006±0.0011	0.30
Pork shoulder	0.22±0.014	0.020±0.0043	0.087±0.0094	0.010±0.0018	0.0159±0.00067	0.013±0.0015	0.35
Elk, steak	0.352±0.0089	n.d.	0.129±0.0038	0.022±0.0014	0.0062±0.00020	0.0061±0.00012	0.52
Lamb, steak	0.112±0.0044	0.0065±0.00097	0.060±0.0019	0.0106±0.00059	0.0071±0.00039	0.0047±0	0.19
Reindeer, steak	0.20±0.036	n.d.	0.187±0.0091	0.0093±0.00087	0.0057±0.00064	0.0086±0.00072	0.41
Beef liver	0.17±0.013	0.55±0.069	0.23±0.005	0.026±0.001	0.069±0.003	0.36±0.005	0.85
Beef kidney	0.019±0.0009	0.016±0.0011	0.070±0.0046	0.041±0.0029	0.073±0.0028	0.171±0.0042	0.37
Pork liver	0.135±0.0013	1.07±0.078	0.30±0.011	0.037±0.0056	0.0694±0.00053	0.116±0.0019	0.66
Pork kidney	0.108±0.0065	0.035±0.0083	0.269±0.0047	0.065±0.0049	0.0212±0.00027	0.073±0.0017	0.54
Broiler liver	0.093±0.0071	0.0076±0.00070	0.179±0.0043	0.11±0.011	0.021±0.0015	0.40±0.019	0.81
Broiler without skin	0.33±0.022	0.019±0.0011	0.054±0.0013	0.022±0.0030	0.0072±0.00093	0.0062±0.00014	0.42
Hen without skin	0.313±0.0079	0.023±0.0012	0.081±0.0015	0.015±0.0018	0.0100±0.00047	0.0061±0.00001	0.43
Dry sausage, salami type	0.0016±0.00025	0.0066±0.00058	0.095±0.0063	0.0110±0.00063	0.0147±0.00067	0.134±0.0062	0.26
Sausage, "lenkki"	0.0033±0.00093	0.0013±0.00080	0.042±0.0024	0.0187±0.00094	0.0073±0.00064	0.0058±0.00020	0.077
Meatballs	0.0159±0.00096	0.006±0.0013	0.0414±0.00043	0.0088±0.00070	0.017±0.0022	0.0090±0.00070	0.092
Broiler meatballs	0.029±0.0051	0.0124±0.00082	0.048±0.0025	0.0097±0.00056	0.0174±0.00020	0.0110±0.00036	0.11

PA; 4-pyridoxic acid, PL; pyridoxal, PLP; pyridoxal-5'-phosphate, PM; pyridoxamine, PMP; pyridoxamine-5'-phosphate, PN; pyridoxine, Sum; PLP+PMP+PL+PN+PM

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Table 15. Vitamer distribution and vitamin B_6 content of fish (mean±SD, n=3, as PN mg/100g fresh weight).

Food item	Vitamer						Sum
	PLP	PA	PMP	PL	PN	PM	
Baltic herring, fillets	0.061±0.0077	0.006±0.0010	0.08±0.014	0.0099±0.00084	0.00626±0.000096	0.021±0.0033	0.18
Pike, sea, fillets	0.13±0.012	0.0360±0.00076	0.039±0.0023	0.0069±0.00014	n.d.	0.00043±0.000056	0.17
Pikepearch, sea, fillets	0.11±0.024	0.036±0.0087	0.044±0.0072	0.0081±0.00033	0.0031±0.00020	0.005±0.0015	0.17
Rainbow trout, cultivated, fillets	0.301±0.0097	0.013±0.0010	0.058±0.0036	0.0093±0.00095	0.0147±0.00067	0.0055±0.00053	0.39
Whitefish, sea, fillets	0.29±0.010	1.9±0.33	0.046±0.0021	0.0219±0.00077	0.0056±0.00062	0.0049±0.00017	0.37
Roe paste, smoked, salted	0.0008±0.00069	n.d.	0.0005±0.0004	0.0014±0.00048	0.00289±0.00047	0.038±0.0010	0.0432

PA; 4-pyridoxic acid, PL; pyridoxal, PLP; pyridoxal-5'-phosphate, PM; pyridoxamine, PMP; pyridoxamine-5'-phosphate, PN; pyridoxine, Sum; PLP+PMP+PL+PN+PM

5.4.3 Plant-derived foods

The presence of bound pyridoxine and several vitamers was characteristic for plant derived foods: six vitamer and glycosylated pyridoxine were present in almost every food item (Table 17a and 18a).

Generally, pyridoxine and its bound form (pyridoxine glycoside) as well as free and phosphorylated pyridoxamine form a significant portion of B_6 vitamer content in cereals. Typically, pyridoxal phosphate and pyridoxic acid were only minor components except in rolled oats in which a relatively large portion of pyridoxic acid

Table 16. Vitamer distribution and vitamin B_6 content of dairy products, egg, and bakers yeast (mean±SD, n=3, as PN mg/100g fresh weight).

Food item	Vitamer						Sum
	PLP	PA	PMP	PL	PN	PM	
Milk, 1.9% fat	0.010±0.0011	0.022±0.0026	0.009±0.0013	0.020±0.0026	n.d.	0.0045±0.00056	0.044
Milk, 3.8% fat	0.0069±0.00048	0.0196±0.00068	0.006±0.0017	0.0200±0.00035	n.d.	0.0047±0.00014	0.038
Whipping cream	0.0035±0.00028	0.024±0.0011	0.005±0.0016	0.0054±0.00024	n.d.	0.0027±0.00014	0.017
Cheese, Edam 40	0.0004±0.00035	n.d.	0.030±0.0035	0.0016±0.00100	n.d.	0.015±0.0016	0.047
Cheese, Edam 20	n.d.	0.0048±0.00081	0.049±0.0028	0.0015±0.00047	n.d.	0.018±0.0027	0.068
Cream cheese	n.d.	0.0019±0.00069	0.0229±0.00064	n.d.	n.d.	0.007±0.0013	0.030
Skim milk powder	0.090±0.0013	0.101±0.0021	0.085±0.0086	0.168±0.0037	n.d.	0.042±0.0025	0.39
Infant formula*	0.006±0.0013	0.040±0.0080	0.050±0.0014	0.0499±0.00074	0.35±0.0126	0.050±0.0012	0.52
Egg yolk	0.303±0.0031	0.025±0.0014	0.0039±0.00064	0.0055±0.00029	0.0066±0.00064	0.0105±0.00044	0.33
Baker's yeast**	0.04±0.015	n.d.	0.19±0.037	0.099±0.0049	0.017±0.0028	0.0471±0.00035	0.40

* fortified with pyridoxine

**PNP: 0.019±0.0049

PA; 4-pyridoxic acid, PL; pyridoxal, PLP; pyridoxal-5'-phosphate, PM; pyridoxamine, PMP; pyridoxamine-5'-phosphate, PN; pyridoxine, Sum; PLP+PMP+PL+PN+PM

Table 17a. B_6 vitamer distribution in cereal and rice (mean±SD, n=3, as PN mg/100g fresh weight).

Food item	Vitamer						
	PLP	PA	PMP	PL	PN	PM	PNG
Barley flour	0.007±0.0020	0.0254±0.00089	0.0081±0.00046	0.009±0.0011	0.028±0.0010	0.033±0.0012	0.077±0.0076
Oats, rolled	n.d.	0.108±0.0051	0.032±0.0020	0.013±0.0014	0.033±0.0030	0.0272±0.00078	0.03±0.016
Rye flour	0.0109±0.00032	n.d.	0.024±0.0013	0.0144±0.00072	0.062±0.0016	0.02866±0.000064	0.119±0.0044
Wheat flour, graham	0.0074±0.00074	n.d.	0.016±0.0016	0.012±0.0012	0.040±0.0040	0.016±0.0016	0.036±0.0036
Mixed wheat and rye flour	0.0079±0.00080	0.0072±0.00021	0.0099±0.00086	0.0100±0.00036	0.031±0.0014	0.0138±0.00020	0.071±0.0019
Wheat germ	0.083±0.0014	n.d.	0.152±0.0032	0.037±0.0037	0.156±0.0059	0.108±0.0036	0.56±0.012
Rice. polished	0.0038±0.00031	0.0088±0.00072	0.0087±0.00061	0.0129±0.00095	0.021±0.0013	0.0175±0.00082	0.020±0.0028

PA; 4-pyridoxic acid, PL; pyridoxal, PLP; pyridoxal-5'-phosphate, PM; pyridoxamine, PMP; pyridoxamine-5'-phosphate, PN; pyridoxine, PNG; pyridoxine glucoside (calculated as PN)

Table 17b. Vitamin B_6 and total vitamin B_6 contents and PNG percentage of cereal and rice (mean±SD, n=3, as PN mg/100g fresh weight).

Food item	ΣB6	ΣΣB6	PNG%
Barley flour	0.084	0.16	47.7
Oats, rolled	0.11	0.14	23.4
Rye flour	0.14	0.26	45.8
Wheat flour, graham	0.091	0.13	28.5
Mixed wheat and rye flour	0.073	0.14	49.1
Wheat germ	0.54	1.1	51.0
Rice. polished	0.064	0.084	23.5

ΣB6; PLP + PMP + PL + PN + PM, ΣΣB6; ΣB6 + PNG, PNG%; PNG/ΣΣB6 × 100

was found. The highest vitamer content in flour samples was found in rye, 0.26mg/100g, although the difference between analyzed flour samples was rather small. Barley flour, mixed wheat and rye flour, and polished rice contained all six vitamers, even though pyridoxic acid was not measured or found in whole wheat or rye flour. Glycosidic derivative(s) of pyridoxine was found in all cereal samples including rice, and its portion varied from 23% (rolled oats and polished rice) to 51% (wheat germ), an average being ca. 40% of the total vitamin B_6 content (Table 17b).

Several vitamers including pyridoxine glycoside(s) were present in vegetables, roots and nuts and the ratio of glycosylated pyridoxine to free and phosphorylated vitamers had a large variation; PNG % was ca. 8, 40 and 77 % for frozen peas, broccoli and carrot, respectively (Tables 18a-b). Nearly half of the total amount of vitamin B_6 in peanut was in glycosylated form(s) whereas only a low concentration of bound pyridoxine, 0.0021mg/100g representing less than 1% of total vitamer content, was found in hazelnut. The PNG portion in baby-foods was higher in foods based on vegetable ingredients than those based on mixed vegetable and meat ingredients: ready-to-eat potato-carrot puree baby-food contained almost two third of its total vitamer as glycosidic pyridoxine.

5.4.4 Comparison with national food composition tables

The present data was generally in accordance to the results published in national food composi-

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Table 18a. B_6 vitamer distribution in plant-derived foods (mean±SD, n=3, as PN mg/100g fresh weight).

Food item	Vitamer						
	PLP	PA	PMP	PL	PN	PM	PNG
Broccoli	0.032±0.0024	0.106±0.0073	0.0149±0.000810	0.0214±0.00009	0.021±0.0016	0.0021±0.0002	0.060±0.0050
Carrot	0.009±0.0026	0.0044±0.00089	0.0019±0.00016	0.011±0.0012	n.d.	0.00057±0.00053	0.073±0.0047
Potato	0.028±0.0055	0.030±0.0033	0.007±0.0042	0.018±0.0026	0.020±0.0017	0.024±0.0013	0.17±0.032
Tomato	0.0093±0.00060	0.020±0.0036	0.0030±0.00070	0.012±0.0015	0.023±0.0050	0.023±0.0015	0.017±0.0038
Peas, frozen	0.023±0.0026	n.d.	0.0395±0.00083	0.0109±0.00026	0.0080±0.00049	0.0136±0.00054	0.0087±0.00071
Hazelnuts	0.014±0.0066	0.067±0.0027	0.0042±0.00079	0.30±0.038	0.0032±0.00048	0.00396±0.000056	0.0021±0.00056
Peanuts	n.d.	0.061±0.0042	0.0107±0.00043	0.050±0.0099	0.036±0.0032	0.0127±0.00030	0.082±0.0051
Baby food, veal and vegetables	n.d.	0.016±0.0023	0.0151±0.00054	0.022±0.0017	0.0013±0.00018	0.0120±0.00026	0.0210±0.00064
Baby food, potato and carrot puree	n.d.	n.d.	0.0025±0.00013	0.0143±0.00045	n.d.	0.0037±0.00020	0.0381±0.00088

PA; 4-pyridoxic acid, PL; pyridoxal, PLP; pyridoxal-5'-phosphate, PM; pyridoxamine, PMP; pyridoxamine-5'-phosphate, PN; pyridoxine, PNG; pyridoxine glucoside (calculated as PN), ΣB6; PLP + PMP + PL + PN + PM, ΣΣB6; ΣB6 + PNG, PNG% ; PNG/ΣΣB6 × 100

tion tables (Tables 19–23). However, meat sorting or labeling was found to hamper this comparison. One of the main differences was found in the values for baker's yeast; relatively high value was reported in Danish food composition table compared to other food composition tables. This difference can not only be explained by the variation in dry matter content.

Table 18b. Vitamin B_6 and total vitamin B_6 contents and PNG percentage of plant-derived foods (as PN mg/100g fresh weight).

Food item	ΣB6	ΣΣB6	PNG%
Broccoli	0.091	0.15	39.9
Carrot	0.022	0.096	76.7
Potato	0.098	0.27	63.6
Tomato	0.070	0.087	19.7
Peas, frozen	0.095	0.10	8.4
Hazelnuts	0.33	0.33	0.6
Peanuts	0.11	0.19	42.7
Baby food, veal and vegetables	0.051	0.072	29.3
Baby food, potato and carrot puree	0.021	0.059	64.9

ΣB6; PLP + PMP + PL + PN + PM, ΣΣB6; ΣB6 + PNG, PNG% ; PNG/ΣΣB6 × 100

Table 19. Comparison of national food data bases; vitamin B_6 content of meat and meat products (as PN mg/100g fresh weight).

	Den[1)]	Fin	Ger	Swe	UK[1)]	US[1)]	This study
Minced meat, beef	–	0.27	..	0.44	0.22	0.22	0.36
Minced meat, beef and pork	–	–	–	–	–	–	0.32
Beef shoulder	0.360	0.32	..	0.50	0.19	–	0.30
Pork shoulder	0.26	0.31	..	0.36	0.37	0.29	0.35
Elk, steak	–	..	–	0.44	–	..	0.52
Lamb, steak	0.17	0.20	0.13	0.20	0.17	0.14	0.19
Reindeer, steak	–	..	–	0.44	–	..	0.41
Beef liver	0.83	0.83	0.71	1.00	0.69	0.78	0.85
Beef kidney	0.36	0.32	0.39	0.43	0.26	0.42	0.37
Pork liver	0.70	0.68	0.59	0.85	0.56	0.57	0.66
Pork kidney	0.370	0.25	0.55	0.44	0.21	0.36	0.54
Broiler liver	0.66	0.80	0.80	0.80	0.33	0.63	0.81
					–		
Broiler without skin	0.36	0.43	0.50	0.43	0.35	0.36	0.42
Hen without skin	0.50	0.6	..	–	–	0.23	0.43
Dry sausage, salami type	0.12	0.15	..	0.15	0.12	0.09	0.26
Sausage, lenkki	–	0.15	0.14	0.15	–	0.11	0.077
Meatballs	–	–	–	0.20	–	–	0.092
Broiler meatballs	–	–	–	0.20	–	–	0.11

[1)] values recalculated to pyridoxine base
.. value missing
– food item not comparable or missing

Den; Møller 1985, Fin; Rastas et al. 1993, Ger; Souci et al. 1994, Swe; SLV 1993, UK; Holland et al 1991, US; USDA 1999

Table 20. Comparison of national food data bases; vitamin B_6 content of fish (as PN mg/100g fresh weight).

	Den[1)]	Fin	Ger	Swe	UK[1)]	US[1)]	This study
Baltic herring, fillets	–	0.37	..	0.37	–	–	0.18
Pike, sea, fillets	0.12	0.12	0.15	0.12	–	0.097	0.17
Pikepearch, sea, fillets	–	0.3	..	0.12	–	–	0.17
Rainbow trout, cultivated, fillets	–	0.98	..	0.69	–	0.512	0.39
Whitefish, sea, fillets	–	0.3	–	0.12	–	0.248	0.37
Roe paste, smoked, salted	0.17	..	–	0.2	–	–	0.043

[1)] values recalculated to pyridoxine base
.. value missing
– food item not comparable or missing

Den; Møller 1985, Fin; Rastas et al. 1993, Ger; Souci et al. 1994, Swe; SLV 1993, UK; Holland et al 1991, US; USDA 1999

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Table 21. Comparison of national food data bases; vitamin B_6 content of dairy products and egg (as PN mg/100g fresh weight).

	Den[1)]	Fin	Ger	Swe	UK[1)]	US[1)]	This study
Milk, 1.9% fat	0.040	0.04	0.046	0.05	0.05	0.036	0.044
Milk, 3.9% fat	0.039	0.04	0.036	0.04	0.05	0.035	0.038
Whipping cream	0.016	0.02	0.036	0.03	0.03	0.022	0.017
Cheese, 20 Edam type	0.066	0.07	–	–	–	–	0.068
Cheese, 40 Edam type	0.07	0.06	0.073	0.09	0.07	0.063	0.047
Cream cheese	0.046	0.04	0.056	0.06	0.03	0.039	0.030
Infant formula, fortified	–	–			–	–	0.52
Skim milk powder	0.40	0.6	0.280	0.49	0.50	0.286	0.39
Egg yolk	0.25	0.3	0.300	0.3	0.25	0.324	0.33
Baker's yeast	0.91	–	0.684	0.6	0.50	0.356	0.40

[1)] values recalculated to pyridoxine base
– food item not comparable or missing

Den; Møller 1985, Fin; Rastas et al. 1993, Ger; Souci et al. 1994, Swe; SLV 1993, UK; Holland et al 1991, US; USDA 1999

Table 22. Comparison of national food data bases; vitamin B_6 content of cereal (as PN mg/100g fresh weight).

	Den[1)]	Fin	Ger	Swe	UK[1)]	US[1)]	This study[2)]
Barley flour	0.2[3)]	0.33	0.560[3)]	0.33	–	0.263	0.16
Wheat flour, graham	0.41	0.35	0.46	0.35	0.41	0.282	0.13
Mixed wheat and rye	0.09	–	–	0.30	–	–	0.14
Oats, rolled	0.17	0.18	0.160	0.18	0.27	..	0.14
Rye flour	0.29	0.35	0.233	0.35	0.29	0.222	0.26
Wheat germ	0.79		0.492	1.82	2.73	1.08	1.1
Rice, polished	0.12	0.30	0.150	0.15	0.26	0.290	0.084

[1)] values recalculated to pyridoxine base
[2)] glycosylated forms included
[3)] whole grain
.. value missing
– food item not comparable or missing

Den; Møller 1985, Fin; Rastas et al. 1993, Ger; Souci et al. 1994, Swe; SLV 1993, UK; Holland et al 1991, US; USDA 1999

Table 23. Comparison of national food data bases; vitamin B_6 content of plant-derived foods (as PN mg/100g fresh weight).

	Den[1)]	Fin	Ger	Swe	UK[1)]	US[1)]	This study[2)]
Broccoli	0.17	0.4	0.280	0.16	0.11	0.132	0.15
Carrot	0.054	0.12	0.270	0.07	0.11	0.122	0.096
Potato	0.12	0.44	0.307	0.26	0.36	0.215	0.27
Tomato	0.083	0.10	0.100	0.10	0.11	0.066	0.087
Peas, frozen	0.108	0.12	0.046[3)]	0.12	0.07	0.101	0.10
Hazelnuts	–	0.61	0.313	0.61	0.49	0.506	0.33
Peanuts	0.4	0.59	0.440	0.30	0.49	0.288	0.19
Baby food, veal and vegetables	–	–		–	–	0.098	0.072
Baby food, potato and carrot puree	–	–		–	–	0.067	0.059

[1)] values recalculated to pyridoxine base
[2)] glycosylated forms included
[3)] canned
– food item not comparable or missing

Den; Møller 1985, Fin; Rastas et al. 1993, Ger; Souci et al. 1994, Swe; SLV 1993, UK; Holland et al 1991, US; USDA 1999

5.5 Characterization of bound pyridoxine

The reversed-phase chromatogram of whole wheat extract contained three main unknown peaks, named X_1 (k_e~8.3), X_2 (k_e~10.0), and X_3 (k_e~11.8) which disappeared during the sample extract digestion with β-glucosidase (Fig. 27). Further emphasis was focused on the X_3 fraction which eluted before pyridoxal (k_e~12.2). The chemical structure of isolated X_1 or X_2 fractions remains unclear. The X_3 fraction was collected and repurified by open-column ion-exchange chromatography. The amount of isolated and purified X_3 analyte (later called PNX), quantitated as pyridoxine using an external standard method, was approximately 0.075µmol (13µg) and 0.1µmol (17µg) starting from 1000g of raw carrot and 750g of wholemeal flour. The recovery after ion-exchange purification was estimated to be ca. 65%.

5.5.1 Enzymatic hydrolysis

The collected PNX fractions were combined and a part of the pooled fraction was enzymatically hydrolyzed using a β-glucosidase preparate; isolated PNX yielded the increased fluorescence signal at a k_e value of 13.6 in reversed-phase chromatogram. Thus, the enzymatic hydrolysis of the PNG fraction increased the size of the chromatographic peak at the region of PN. Spiking of the enzymatically treated PNX extract with pyridoxine standard solution confirmed this. The amount of pyridoxine derived from bound form was almost equimolar to PNX calculated as free pyridoxine.

5.5.2 Proton NMR spectroscopy

The isolated and purified PNX fraction was dissolved in deuterium oxide (99.8%), lyophilized and redissolved in deuterium oxide (99.95%). The proton spectrum of the isolated PNX frac-

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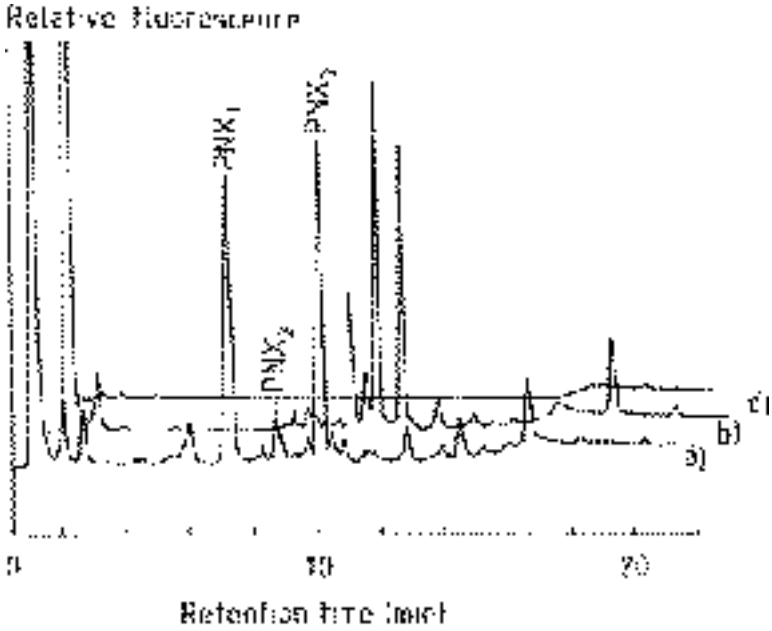

Fig. 27. A reversed-phase chromatogram of carrot sample: a) before b-glucosidase hydrolysis, b) after β-glucosidase hydrolysis and, c) enzyme blank.

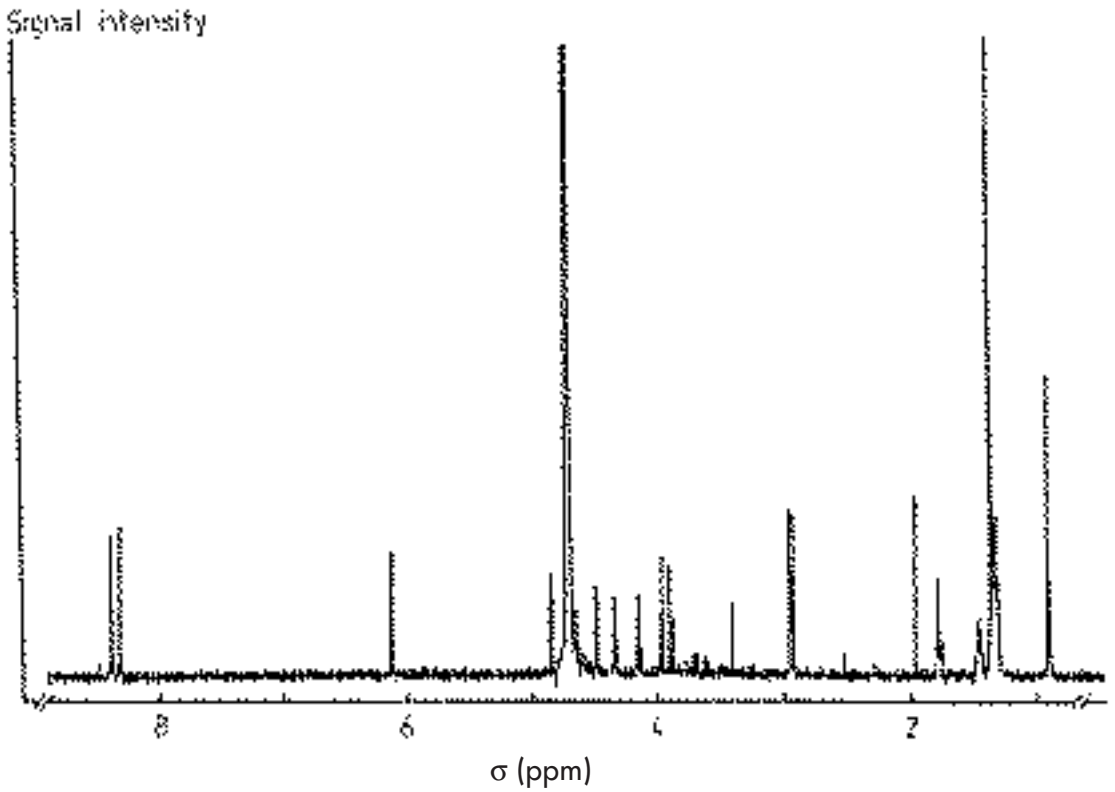

Fig. 28. Proton spectrum of PNX_3 fraction isolated from wholemeal flour.

Table 24. Signals in ^{1}HNMR spectrum of PNX isolated from wholemeal flour measured in deuterium oxide (t=35°C).

Chemical shift δ_H (ppm)	Signal form (intensities)	Coupling constant J (Hz)	Tentative characterization	Analyte
0.9				Unknown
1.3				Unknown
1.35	multiple		methyl protons in CH_3CH_2OH, impurity	unknown (impurity)**
1.6				Unknown
1.8				Unknown
2.8				Unknown
3.2				Unknown
3.85	douple douplet (1:3)	Ja,b ~ 3.4		carbohydrate moiety*
3.95	douple douplet (3:1)	Ja,b ~ 3.8		carbohydrate moiety*
4.15	quartet (1:3:3:1)	Ja,b ~ 7,2 Ja,c ~ 13.9 Ja,d ~ 20.7 Jb,c ~ 6.8 Jb,d ~ 13.5 Jc,d ~ 6.8	methylene protons in CH_3CH_2OH, impurity	unknown (impurity)**
4.35	quartet (?) (1:3:3:1)	Ja,b ~ 3.4 Ja,c ~ 3.0 Ja,d ~ 9.4 Jb,c ~ 3.0 Jb,d ~ 6.0 Jc,d ~ 3.0		carbohydrate moiety*
4.45	douple douplet (1:1:1:1)	Ja,b ~ 3.0 Ja,c ~ 4.9 Ja,d ~ 8.3 Jb,c ~ 1.9 Jb,d ~ 5.3 Jc,d ~ 3.4	anomeric H in C1-glu	carbohydrate moiety*
4.6–4.8				Water
4.85	douplet			carbohydrate moiety*, signal disappeared at 23°C
6.15	douplet	Ja,b ~ 6.4		carbohydrate moiety*
8.30	two singlets	(J ~ 42.5)	aromatic H in C6-pyr	pyridoxine moiety
8.40				

tion of wholemeal flour showed that the analyte was pure enough for proper NMR measurement (Fig. 28) whereas the fraction isolated from carrot still contained impurities. Therefore, the proton spectrum of PNX fraction (Table 24) and COSY and TOCSY spectra (Fig. 29) of only whole wheat flour were measured. The NMR spectra of D-glucose and pyridoxine hydrochloride in deuterated water were also recorded (Table 25).

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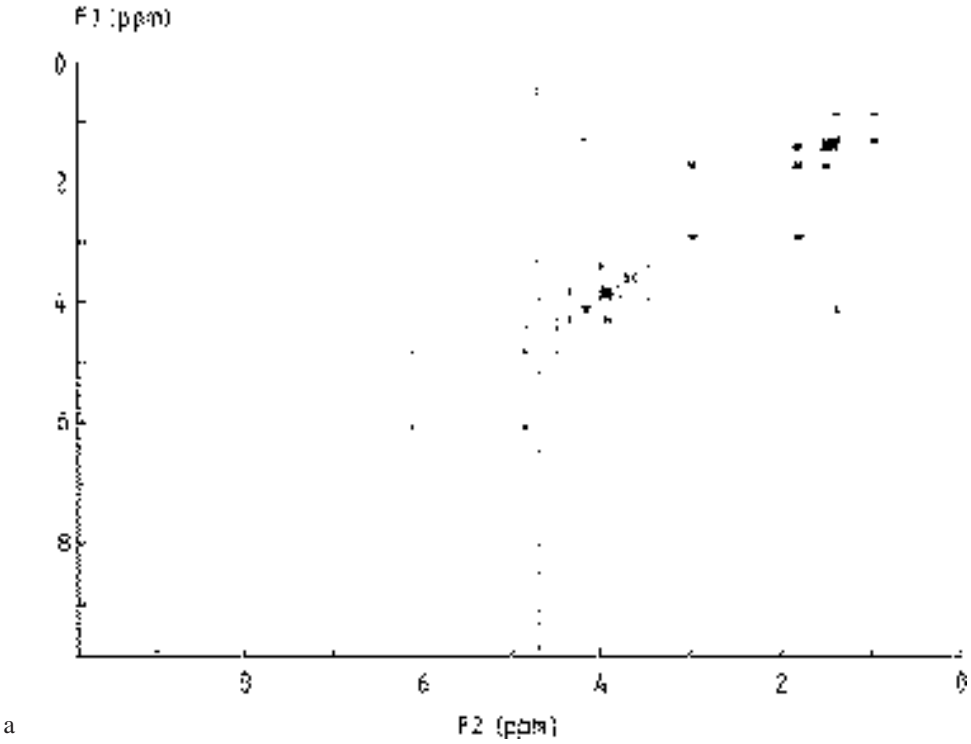
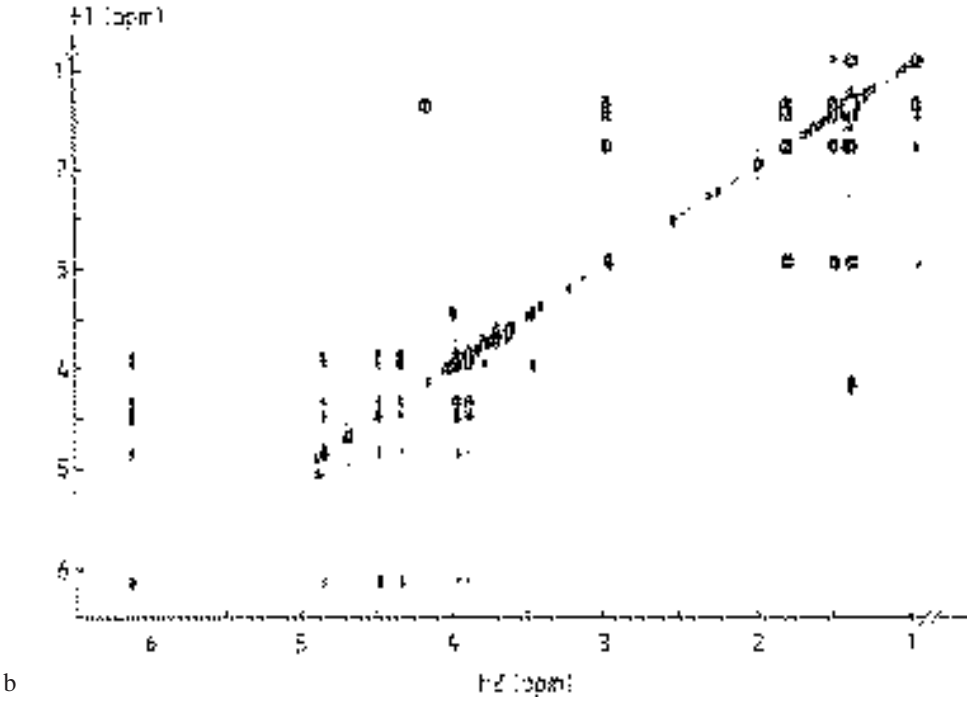


a



b

Fig. 29. COSY- (a) and TOCSY-spectra (b) of PNX fraction of wholemeal flour.

Table 25. Signals in ^{1}HNMR spectrum of D-glucose and pyridoxine hydrochloride measured separately in deuterium oxide (t=35°C).

Chemical shift δ_H (ppm)	Signal form (intensities)	Coupling constant J (Hz)	Tentative characterization	Analyte
4.57	douplet (1:1)	Ja,b ~ 8.1	axial anomeric proton in β-anomer	D-glucose
4.7	singlet		impurity	water
5.16	douplet (1:1)	Ja,b ~ 3.9	equatorial anomeric proton in α-anomer	D-glucose
Intensities of δ_H 4.57 and δ_H 5.16 signals: 1:2			α- and β-anomer in equilibrium stage in deuterium oxide	D-glucose
2.5	singlet		methyl protons	pyridoxine; 2C-CH_3
4.7	singlet		impurity	water
4.9	singlet (?)		4'- methylene protons (-CH_2-O-)	pyridoxine; 5C-5'CH_2-O-
8.0	singlet		aromatic proton	pyridoxine; 6C-H
Intensities of δ_H 2.5, δ_H 4.9 and δ_H 8.0 signals: 1:4.5:4.9				

5.5.3 FAB mass spectrometry

FAB mass spectra of PNX fraction isolated from whole meal flour and carrot samples were measured in glycerol, nitrobenzylalcohol and triethanolamine matrices (Appendix 2).

6 Discussion

6.1 Liquid chromatography

6.1.1 Column packings

The chromatographic retention of B_6 vitamers based on partition chromatography is mainly affected by the substitution of the pyridinium ring while the peak symmetry is dependent on the cationic nature of the analyte. The cationic nature of B_6 vitamers, due to the pK-values of pyridinium nitrogen group and functional groups (4-carboxal, 4-hydroxymethyl, and 4-aminomethyl) is characteristic for these compounds. This ionic nature of vitamers enables the use of ion-exchange- or ion-pair-chromatography in their separation. It was therefore expected that the proper resolution and separation efficiency achieved with octadecyl packings is a result of both ionic and partition characteristics of the analytes. An aminopropyl phase possessed only a limited retention for vitamin B_6 compounds, as can be also seen in the report of Belal (1989), and its use was thus rejected.

Adsorption activity

Peak tailing (poor peak symmetry), caused by the coulombic interaction between cationic vitamers (pK-value for pyridinium ring 7.9–9.0 according to Snell 1963) and especially that of in PM (aminomethyl group; pK 10.5) and either residual silanols and/or metal activity of the base

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silica was found at some extent with all column packings. This characteristic should be especially true for non-end capped bounded phases (Stadalius and Snyder 1988). The effect of this unfavorable phenomenon on separation efficiency can be deminished by choosing a fully endcapped, low-acidic, and low-metal activity silica material. The concentration of silanol groups in silica packing was presumed to be ca 8µmol/m^2 (Unger et al. 1976, Köhler and Kirkland 1987). Lower silanol surface concentration values expressed as cation-exchange capacity, 0.7–0.8 meq/g for plain silica , were obtained when the surface silanol was measured by direct titration with alkali in the presence of aqueous salts (Khurana and Ho 1988). It should be noted that in the latter study only one commercial silica gel packing was tested. It has been estimated that due to steric hindrance in the silica surface geometry only half of the silanol surface can be bound and the rest of the surface, ca. 4.2 µmol/g (Nasuto and Rózylo 1995, Bereznitski et al. 1996, Buszewski et al. 1997), must be derivatized by using an additional process. The desired silica packing for basic compounds should only contain a few unbonded groups for adsorption interaction. Various methods, such as secondary silanization (Nasuto and Rozylo 1995), douple endcapping (Fiorianti et al. 1995), the use of a high density coverage (Buszewski 1991) or mobile phase additives (Musch and Massert 1988, Li 1992), and various silica treatment procedures (Köhler and Kirkland 1987) have been applied to reduce the unwanted residual silanol activity.

An excellent peak shape for the B_6 vitamers in the present study was achieved with a µBondapak packing (Table 7) even thought a relative high silanol index value was measured for that particular packing material by Walters' test (Table 8). Similar results are reported by Wehling and Wetzel (1984) and Bötticher and Bötticher (1987). The efficiency of this column material (expressed as plate numbers) was lower than those of other octadecyl phases (except for polymer phases) due to larger particle size (10µm), and contrastingly the silanol index value was clearly higher than in other ODS phases. Thus, silanol activity alone did not explain this result. Asymmetry values for B_6 vitamers were generally higher using Spherisorb ODS2 and Vydac HS packings. The difference of a µBondapak or Novapak packing compared to other octadecyl phases is propably due to silica synthesis chemistry but its details were not available. With other column packings, peak shapes, especially for pyridoxamine, could be improved by adding triethylamine to the mobile phase as a modifier (Table 7). However, the asymmetry values should be considered only as a guideline since the measurement of column efficiency is affected by many factors. Even the slightest use of amine modifiers, ion-pair reagents or buffer salts in the mobile phase can change the efficiency, and once the column has been exposed to these compounds, the native selectivity/efficiency may be permanently changed. The use of basic modifiers in the mobile phase may also shorten the column's life. In addition, the variation in pH of the mobile phase after adding triethylamine or ammonia into the mobile phase was considered to be harmful to the succesful use of amine modifiers.

Traces of various metals at some extent are always incorporated in silica matrix (van den Driest et al. 1988); concentration levels in commercial parent silica can vary remarkably: e.g. 10–350 µg/kg of aluminium, 40–420µg/kg iron, and 10–5600µg/kg sodium. Their concentration should be under 50 ppm level which is suggested to be an accepted upper limit for ideal support (Buszewski et al. 1997). The interaction between metal impurities in the silica support and ionic analyte may be seen as tailing peaks and as reduced column efficiency. The deterioration of column efficiency for ionic compounds is reported to be quite similar to residual silanol effect. Accurate data, however, on metal impurity concentrations in commercial silica supports is not readily available. This makes it difficult to evaluate what is the primary reason for the poor peak shape of ionic analytes in certain packing materials.

Unwanted adsorption interactions were expected to be diminished by choosing a high hy-

drophobicity, and a low silanol activity column packing material such as Waters Novapak C18 support (Waters, USA).

Phase type and pore size

The octadecyl column packings in this comparison were of the monofunctional (both Waters packings) and tri- or polyfunctional type (Spherisorb and Vydac packings). Monomeric chemically bounded phases in liquid chromatography are probably more preferred to polymeric phases; the advantages of a monomeric phase are faster mass transfer, increased stability in aqueous solvents, and more homogenous organic coating with decreased amounts of residual silanol groups. Thus, many of the current commercial packings are synthesized using the monosilane process. The monomeric chemically bounded silica phase combined with a high surface coverage was the most apolar stationary phase and possessed the maximal selectivity for aromatic test substances (Buszewski and Galushko 1995). It was also proposed that the chemically bound ligands of the phases with dense CBP coverage existed in a more ordered configuration owing to stronger chain-to-chain interactions.

However, certain compounds with some degree of planarity in their molecule shape are more effectively separated using polymeric phases; better resolution of polycyclic aromatic hydrocarbons (Sander and Wise 1984) and carotenoids (Jinno and Lin 1995) have been achieved using polymeric coatings instead of monomeric phases. By decreasing the temperature, an enhanced selectivity for separation of α- and β-carotenes was obtained, the optimum temperature being at 20°C. Phase transitions in chemically bonded phase were then assumed to cause this selectivity – temperature -dependence. In addition, column selectivity was assumed to be more directly related to bonded phase surface coverage values (N as μmol/m^2) whereas the absolute retention was more dependent on carbon loading. The morphology of the polymeric phases and its relevance to specificity of separation, however, is still poorly understood.

Column separation efficiency using two poly(styrene-divinylbenzene)(PS-DVB) based polymeric reversed-phase packings, Polyspher RP-18 and Hamilton PRP-1, was remarkably lower than that of silica based materials. However, better column efficiency values for an anion-exchange polydivinylbenzene resin have been reported (Nair et al. 1996). The decreased efficiency could not be explained by the particle size as d_p of the polymeric supports was almost equal to those of silica based particles. Generally speaking, retention factor values were much higher than those of silica based supports showing an increased hydrophobicity of the polymeric packing. Hydrophobicity value for the polymeric packing could not be determined by using the test procedure developed for chemically modified octadecyl silica packings. Thus, the column chemistry related to polymeric support and its functionality differs notably from ordinary, silica based chemically bound octadecyl phases. This was also shown e.g. by Pastores et al. (1995) in their work with a non-porous PS-DVB packing which was developed to eliminate all pore diffusion effects.

The pore size of the parent silica material is recommended to have a sufficient large diameter and pore system to ensure the fast mass transfer of solutes and adsorption-desorption kinetics. For low molecular weight analytes (M_w <3kD), pore sizes of 6–15nm is recommended (Regnier 1987). The columns tested in the present work belong to this category. Larger pore size was needed for efficient separation of compounds with higher molecular weight (Pearson et al. 1982). The effect of pore structure changes during the surface modification on mass transfer of the solutes is still somewhat unclear (Unger et al. 1976).

Several chemically bounded octadecyl phases were tested to separate B_6 vitamers in various food matrices. Reversed-phase columns tested were based on the literature review; the separation efficiency achieved by small mesopore reversed-phase particles(d_p ~3–5μm) is normally superior to e.g. ion-exchange chromatography. The sufficient retention factor of even phospho-

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rylated compounds (namely PLP and PMP) can be achieved by using octadecyl phases. The retention factor values (k_e) for PLP, PA, PMP, PL, PL, PN, DPN, and PM were 1.4, 2.1, 5.6, 12.2, 13.6, 14.6 and 21.3, respectively. The relatively high retention factor for pyridoxamine ($k_e > 20$) was needed to avoid coelution of pyridoxamine with two baseline disturbance "peaks" in retention factor values between 15 and 17. System peaks were assumed to be the source of these baseline disturbances. Tentatively identified 5'-*O*-β-D-glucopyranosyl pyridoxine was eluted at the k_e-value of 11.8, just before PL. Even adequate relative retention was obtained for the first eluted B_6 vitamer, pyridoxal-5'-phosphate. However, early eluting substances may time to time, depending on the sample matrix, disturb PLP's proper baseline measurement.

This column packing comparison showed the importance of column testing or their evaluation by other means to make sure that column packing is suitable for a particular analytical purpose, and this is especially true for basic analytes. The development of column packings and their chemistry may be considered to be the main driving force in liquid chromatrography today, and an ever-increasing number of different products are launched every year. This means that any column test, such as ours, should be considered as a guideline as it represents the situation at the time it was done.

6.1.2 Mobile phase and column temperature

The mobile phase used for routine food analysis was a modification of Gregory and Ink's (1987) method consisting of a gradient elution with a mixture of 2-propanol and phosphate buffer. A better column efficiency for separating B_6 vitamers was achieved using 2-propanol as the organic modifier than using methanol or acetonitrile. Methanol has been reported to yield a better peak shape of basic compounds compared to acetonitrile when the influence of the organic solvent modifier in reversed-phase chromatography was studied by McCalley (1995). In our study a more stable retention for B_6 vitamers was achieved by using a rather low pH (2.2) of 50mM phosphate buffer in the mobile phase. The retention of pyridoxamine was adjusted with octanesulfonic acid as ion-pair reagent in the mobile phase. The retention of pyridoxamine can be readjusted whenever needed by changing the length of alkyl chain of the ion-pair reagent (hydrophobicity/size of the ion-pair moiety). The use of octanesulfonate (sodium salt) produced an adequate retention as well as improved peak resolution and peak shape for pyridoxamine in our chromatographic system. Concentration of the ion-pair reagent in the mobile phase (C_m) was adjusted to 8mM in the present work in line with the findings of Dong et al. (1988) and Knox and Hartwick (1981). The latter group measured the maximum concentration of alkyl sulfates located in the octadecyl bonded silica surface. This reflected the maximum surface concentration (C_s) of ca. 2 μmol/m^2 for octyl sulphonate and pointing to a nearly monomolecular layer of adsorbed counter ion. The critical micelle concentration (CMC), the concentration after which the relative retention would sharply decrease, was still significantly higher for the alkyl sulphonates studied. The same kind of findings were also earlier reported by Giles and coworkers (1974). In the work of Knox and Hartwick it was assumed that the degree of retention of a positively charged analyte was directly based on the charge formed on the surface of stationary phase, proving that the formation of the ion-paired complex (hetaeron – cationic compound) in the mobile phase plays a minor role, if any.

Column stability is affected by the type and the concentration of the organic mobile phase modifiers. A decreased stability in basic eluents containing methanol was reported using phosphate or carbonate buffer compared to borate or glycine buffers (Claessens et al. 1996). However, the usefulness of borate or glycine buffer in vitamin B_6 analysis is somewhat limited due to its restricted buffer capacity in the mobile phases at low pH ranges. The acidic pH of the mo-

bile phase in the reversed-phase chromatography was needed to achieve the proper selectivity and retention for the vitamers. Phosphate buffers were also considered to be free from some interacting or unwanted reactions; borate buffers form complexes with B_6 vitamers while glycine buffers may interact with pyridoxal (Metzler and Snell 1955).

As the column temperature was set at 30°C without thermostating the mobile phase, the formation of a temperature gradient in the column was evident. It was estimated that the ambient room temperature in the laboratory normally varied between 20 and 25°C. Thus, the radial temperature profile caused by the viscous heat dissipation of the eluent was opposed to that formed by the unthermostatted mobile phase. The temperature difference in our chromatographic system is in accordance with the results of Welsch et al. (1996) which showed that the optimum precooling of the eluent was achieved when the temperature of the eluent was ca. 8°C below the column temperature. This temperature difference was estimated to be enough to compensate for the viscous heat dissipation, and its advantages for the separation efficiency and reproducibility were stated in their report.

6.1.3 Post-column derivatization

The molar absorptivities as well as relative fluorescence responses of B_6 vitamers differ noticeable from each other and are influenced by the selected pH value (Metzler and Snell 1955). The lowest response (expressed as a ratio of fluorescence intensity and concentration) was measured with pyridoxal and pyridoxal-5'-phosphate, and with 4-deoxypyridoxine (internal standard). The fluorescence response for PLP using bisulphite adduct reagent as a post-column reagent was ca. five-fold compared to that of an underivatized compound (Fig. 14). The effect on other vitamers was weaker. Only the response of pyridoxal decreased with post-column derivatization, and the difference was considered to be insignificant in practical laboratory work. The use of pre-column derivatization with sodium hydrogensulphite in an ion-exchange chromatography of B_6 vitamers reduced the fluorescence intensity of other vitamers (Argoudelis 1988). This contrasts with our results but may be explained by the different pH-values of the mobile phase used in their ion-exchange and in our present partition chromatography. Optimum conditions in our study were achieved by pumping the derivatization reagent, 35mM sodium hydrogensulphite in 0.5M phosphate buffer (pH 7.5) into the mobile phase (Fig.15). Increasing either the amount of hydrogen sulphite in the derivatization reagent or the flow rate of the derivatization solvent did not yield a higher response.

6.2 Evaluation of the chosen method for routine food analysis

6.2.1 Acid hydrolysis and the stability of PLP

Perchloric acid extraction procedure was tested using ^{14}C-labeled pyridoxine-5'-phosphate. The measured total ^{14}C-activity of purchased standard was 314 kBq the purity being 76.0% (Fig. 18a). 12% and 8% of the total ^{14}C-activity was eluated in the chromatogram at the same retention as unknown 1 (k_e 0.5) and PMP (k_e 4.5), respectively. Unknown 1 represented a breakdown product derived from PLP; as 14C-carbon atoms were located in a rather stable pyridinium ring in positions 4 and 5, this breakdown product probably still consisted of a pyridinium ring. The second minor activity fraction was eluted at a retention factor of 4.5, which is the same as that of PMP. When different sample extraction procedures for lyophilized pork liver samples were tested, a portion of pyridoxal was assumed to have been converted into pyridoxamine (Bognar and Ollilainen 1997). This transamination phenomenon was then considered to be the result of a long incubation time in takadiastase

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hydrolysis, and was caused by the sample matrix as well. Whether this conversion occurs similarly in different buffer solutions remains unclear.

Only a minor change in the activity distribution occured when labeled pyridoxal phosphate standard was extracted with perchloric acid. The activity of PLP decreased during perchloric acid extraction, and an increase in activity was found at the retention factor values of pyridoxamine phosphate, unknown 3 and pyridoxal. The total change approx. 3% was measured. Thus, the most labile B_6 vitamer in this study, pyridoxal 5'-phosphate, can be quantitatively extracted using an ice-cold perchloric acid precedure and then separated with ion-paired reversed-phase chromatography in its nearly intact form when the sample matrix effect is excluded.

The sample matrix affected on the stability of PLP during the extraction. When labeled phosphorylated pyridoxal was exposed to beef, carrot and whole wheat flour matrices, and then extracted and measured via HPLC, the distribution of ^{14}C activity was changed. ^{14}C-activity of labeled PLP added to beef steak and carrot samples was partially lost during the extraction procedure. The main activity increase was located in the fractions of pyridoxal and unknown 2 (Fig. 19). The increased amount of free pyridoxal due to hydrolysis of phosphate ester lingage of PLP was, however, only 7% and 14% in beef and carrot sample matrices, respectively. Added ^{14}C-labeled PLP consisted ca. 8% of the total activity as an "impurity" of which the main part was located after extraction procedure either in PL or in unknown 2 fraction. The results showed that the conversion of one B_6 vitamer to another vitamer form in these conditions includes both interconvertion of a functional group and ester linkage hydrolysis. This was also found in the work of Bognar and Ollilainen (1997). The main B_6 vitamers naturally present in beef are PLP, PMP and PL representing 65%, 23% and 5% of the vitamin content making it a suitable matrix for stability testing. In the case of whole wheat flour, 26% of originally added ^{14}C-activity was found either in pyridoxal or in PNG fraction since these derivatives were eluted very near each other and could not be exactly separated using this fraction collection. In carrot, ca. 70% of total vitamin fraction is made up of glucosylated pyridoxine, while PLP (9%) and pyridoxal (11%) formed the minor vitamer fractions. The chemical structure of the suggested breakdown product of PLP, referred as unknown compound 2, was not solved. When labeled PLP was extracted without any sample matrix, the main fraction containing ^{14}C-activity was unknown compound 3 instead of unknown 2 in the food matrices tested.

Changes in these ^{14}C-activity patterns were considered to be reflected in the matrix effect on the vitamin B_6 compound distribution. In wheat matrix the main change, probably the dephosphorylation of PLP into free pyridoxal, took place to a greater extent than in other two matrices. It is noteworthy that the interconversion of pyridoxal to pyridoxamine was quite minimal, and a small increase of pyridoxamine was observed showing that vitamin B_6 compounds in beef and carrot matrices can be measured nearly in their unaltered forms using perchloric acid as an extracting agent.

In spite of the rather gentle extraction procedure, that is perchloric acid hydrolysis performed on an ice-water bath protected from light, the degradation, interconversion and dephosphorylation of pyridoxal-5'-phosphate could not be completely avoided in extraction and the preceding sample preparation. This was especially true for the wheat matrix. Dephosphorylation of B_6 vitamers has been reported to be associated with the unit operations used such as acid hydrolysis (Peterson et al. 1955) or exposure to salt and alcohol solutions (Shimada et al. 1993), and the rapid dephosphorylation step was followed by a slow transamination phase (Shimada et al. 1993). The expected reactivity of pyridoxal's aldehyde group with amino compounds during applied extraction with ice-cold perchloric acid could not be observed. So the transamination of pyridoxal or pyridoxal-5'-phosphate to related amino forms reported by many reseachers (Metzler and Snell 1952, Gregory and Kirk 1977) was not

found when the extraction procedure with ice-cold perchloric acid was used. In practice no difference in the distribution of ^{14}C-activity in beef sample compared to that of carrot or wheat sample was recorded in the reversed-phase chromatogram. This was probably due to some sort of inactivation, and also a less suitable pH-value of acid extractant for that particular reaction(s). In a citrate-phosphate buffer solution model, the maximum degradation rate for pyridoxal was reported at pH 5, pyridoxamine loss increased in higher pH values while pyridoxine was considered to be the most stable vitamer (Saidi and Warthesen 1983). A factor that possibly also diminished the transamination reaction rate in the present study was the lower extraction temperature compared to traditional sample treatments. Thermal destruction of B_6 vitamers at elevated temperatures has been reported by many research groups (Srncova and Davidek 1972, Evans et al. 1981, Navankasattusas and Lund 1982, Gregory and Hiner 1983). The catalytic nature of transition metal and aluminium ions in transamination (Snell 1954, Cennamo 1964) could also be reduced by the suggested ion-pairing character of perchlorate anion. Formation of ε-pyridoxyllysine via a non-enzymatic browning reaction as demonstrated by Gregory and Kirk (1978a) was not considered to occur to any great degree in our procedure as this reaction is generally related to long-term storage of dehydrated materials.

6.2.2 Enzymatic hydrolysis

Alkaline phosphatase treatment

Pyridoxal and pyridoxamine were released from their phosphorylated forms by an alkaline phosphatase enzyme prepared from calf intestine. Beef steak matrix which is rich in both pyridoxamine phosphate and pyridoxal phosphate was used as a test matrix since phosphate ester linkage in pyridoxamine phosphate has been reported to be more stable e.g. during acidic thermal extraction procedures than that of pyridoxal phosphate (Gregory and Mabbit 1961).

The adequate amount of alkaline phosphatase was ca. 0.4–0.8U enzyme /mg sample in this sample extraction procedure (Fig. 20). Increasing the amount of enzyme did not yield a higher vitamin B_6 content. The enzyme preparation used was free of B_6 vitamers; no measurable amount of any vitamer was found in the enzyme's blank analysis, thus higher enzyme concentrations can be used if needed. Removing enzyme protein by precipitation can be performed after hydrolysis with trichloroacetic acid, but this was not found to be necessary; no deterioration of column performance was observed when this step was omitted from the routine procedure.

The limited availability of pyridoxamine-5'-phosphate reference standard may complicate method development work in the future. Many reagent suppliers have informed us that this reference material is no longer being commercially produced (personal communications), and it may not be obtainable in future. As the phosphate ester linkage in PMP is considered to be more permanent than that of PLP during the dephosphorylation process, the hydrolysis efficiency of pyridoxamine phosphate should somehow be confirmed.

β-Glucosidase treatment

Carrot matrix was chosen for testing glucosidase hydrolysis. Glucosically bounded pyridoxine forms ca. 50–70% of the total B_6 vitamer content in carrot (Gregory and Ink 1987). Its fraction can be clearly separated from other peaks using e.g. the ion-paired reversed-phase chromatography. However, several peaks (normally two to three) in our chromatogram disappeared after β-glucosidase incubation. The fraction (k_e=11.6, t_R=9.8 min) eluting right before pyridoxal (k_e=12.2, t_R =10.2 min) in the reversed-phase chromatogram was assumed to be 5'-*O*-β-D-glucopyranosyl pyridoxine (PNG). No measurable signal at k_e of 11.6 in the chromatogram was observed after hydrolysing the sample extract (0.13U of β-glucosidase per mg sample). The chemical nature of those two other peaks remained unknown. The tentative peak identification based on the glucosidase treat-

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ment only can be complicated by the fact that a minor amount of side-activities, such as phosphatase activity, were unfortunately present in this enzyme preparation. Thus, the disappearance of some of the unknown compounds in the chromatogram after the enzymatic hydrolysis with glucosidase can partly be as a result of hydrolysis of the possibly occuring phosphate ester linkages. As the measurement of glycosylated pyridoxine was based on the difference in the pyridoxine amount before and after the β-glucosidase treatment, the presence of minor but unwanted phosphatase activity in enzyme preparate may lead to misinterpretations for samples which contain phosphorylated pyridoxine as well. Pyridoxine phosphate, however, was only found in baker's yeast and not at all in plant-derived samples.

6.2.3 Solid-phase extraction

Partition chromatography based on chemically bonded reversed-phases, namely octadecyl, octyl, phenyl, and cyanopropyl sorbents, was first evaluated. The results of those tests revealed that the polarity characteristics of B_6 vitamers differs to such an extent that proper retention and elution conditions with one sorbent material suitable for all the vitamers was quite unlikely to be found. The most polar vitamers, phosphate esters of pyridoxal and pyridoxamine, and pyridoxic acid, did not retain enough in reversed-phase packings from aqueous sample extract matrices. Free vitamers being less polar analytes retained in the octadecyl and the octyl phases but not in a more polar cyanopropyl phase. Nor did phenyl sorbent which based on π-electron interactions work sufficiently well. When the analytical procedure was considered as a whole, it was thought to be useless to apply the same separation phenomenon, partition chromatography both in the purification step and in the analytical separation.

Cation-exchange chromatographic purification/concentration proved to be more effective than partition in sample extract purification as all B_6 vitamers show more or less cationic features due to their cationic pyridinium or aminomethyl chromophore. A weak cation-exchange sorbent containing the carboxymethyl group ($-CH_2COOH$) retained free PL, PN, DPN, and PM in the standard solutions. The pK value for carboxymethyl group is ca. 4.8 being suitable for strong cations also as the negative charge of the sorbent can be neutralized by adjusting pH. However, this ionic interaction was greatly decreased when the added standards were coeluted with the sample matrix. Because the weak cation-exchange sorbent failed to retain free vitamers in the sample extracts, two strong cation-exchange phases were then tested. Most commercial sorbents have either the propylsulfonate or propylbenzenesulfonate group chemically bonded to the parent silica. Due to the low pK value of the sulfonate ion, cationic analytes had be eluted using high ionic strengths and/or by neutralizing the positive charge of the analyte. Strong cation-exchange phases are thus generally suitable for weak cations, such as the pyridinium ion, only. Modifying the elution solvent system, pyridoxamine was succesfully retained and eluted using aromatic sulfonic acid as cation exhange material. The elution efficiency, that is the release of PM, was then improved by using an elution solvent of high pH value combined with a high solvent strength because of pyridoxamine's basic nature (pK ~10.5). However, this yielded to alkaline elution solvents which, in general, are incompatible with the traditional HPLC columns due to the lability of silica phase in alkaline media. This limiting factor has been resolved by using poly(styrenedivylbenzene) or alumina based column packings in liquid chromatography which are more stable in large pH areas like pH 1–13 (Mao and Fung 1997). These chemically resistant polymer phases enable the use alkaline mobile phases and/or injection solvents. The separation efficiency of polymer phase columns tested in our work was, however, insufficient for adequate separation of vitamin B_6 compounds and unknown substances in food samples.

The removal of sulfosalicylic acid prior to liquid chromatography was necessary when the extraction method of Gregory and Ink (1987) was applied as sulfosalicylic acid possesses a natural fluorescence very similar to B_6 compounds. Anion-exchange solid-phase extraction with trimethylaminopropyl as a functional group trapped sulfosalicylic acid effectively from the sample extract. The selecticity of the proposed clean-up procedure is complicated by the dissociation constant (pK <2.5)) value of the phosphate residue in pyridoxal-5'-phosphate and in pyridoxamine-5'-phosphate. For the quantitative elution of phosphorylated vitamers from the anion-exchange sorbent, the elution solvent volume of several cartridge void volumes was required. Thus, an unwanted dillution of the analyte solution during this clean-up procedure was inevitable. In practical term, this would have caused an additional concentration step.

In summary, one solid-phase extraction material suitable for quantitative analysis of both more polar (PLP, PA, and PMP) and less polar (PL, PN and PM) vitamin B_6 compounds was not found as B_6 vitamers represent a wide range of polarities due to both cationic and anionic functional groups being present. Secondly, the fluorescence characteristics of vitamin B_6 compounds are related to the 3-hydroxypyridinium structure which lacks the selectivity for vitamin B_6 compounds if the selective retention is based on the pyridinium ion. At the pH range of 1–2, 3-hydroxypyridine occurs as a cation and a dipolar ion, and their derivatives are fluorescent (Bridges et al. 1966). Any unknown compound present in the chromatogram derived from the fluorescence detector's signal may have a similar chemical hydroxypyridine structure. Thus, purification based on the ion-exhange properties of pyridinium ion may not yield proper selectivity in solid-phase extraction followed by fluorescence detection. Evidence of this phenomenon can be found in the results of Wong (1978). When cation-exchange liquid chromatography was applied to the separation of B_6 vitamers, a strong unknown peak preceding the pyridoxal peak interferred with the baseline separation and there was still a need for cleaning procedure for the sample extracts.

For the above mentioned reasons, the ion-exchange solid-phase extraction in purification and in routine food analyses was omitted.

6.2.4 Validity of the routine food analysis method

The retention factor values (k_e) for B_6 vitamers ranged from 1.4 to 21.3 which was considered to be adequate for quantitative work. As a general rule, retention factors ranging from 2 to 6 are recommended (Snyder and Kirkland 1979), the upper limit is, however, a guideline. Thus vitamin B_6 compounds except for PLP fulfill the minimum conditions in this work. In the routine food analysis method chosen, the amount of PLP was calculated on the basis of pyridoxal as the difference between the enzyme treated and non-treated sample extract. Using this method, the early elution of PLP in ion-paired reversed-phase chromatography was circumvented. Chromatographic peak performance (peak symmetry or peak tailing and relative response) as well as the repeatability of retention for the internal standard, 4-deoxypyridoxine, were good (Table 9). The variations in the above mentioned values are derived from both sample matrix effect and the decreased column performance during routine analysis period. Thus the continuous documentation of these parameters was necessary. The accepted performance limits should ultimately match the quality requirements of an individual laboratory, and only general recommendations are given in international directions like ISO/IEC Guide 25 (1990).

The sensitivity of the method was generally sufficient, and the main difficulties were related to the proper identification of the analyte's signal. This task occasionally required laborious standard spiking procedures for the sample extracts. The recovery results of an added standard were satisfactory for individual vitamers ranging from 72% to 107%. A similar total vita-

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min B_6 recovery values was reported by Gregory (1980c) for their liquid chromatographic method. A recovery value of 150% for PLP was achieved in our work for fortified infant formula sample thus showing there were problems with this most labile vitamin B_6 compond in that particular sample matrix. This phenomenon was not found in beef sample matrix. In addition, the early elution of PLP in our ion-paired reversed-phase chromatographic system made the reliable measurement of PLP more difficult. Phosphorylated pyridoxal was then measured in the routine food analyses as the difference between the PL amount of phosphatase hydrolysed extract and of nontreated sample extract.

The greatest source of uncertainty inherent to the results was the standardization even though nine concentration levels were used in the standardization procedure. It was estimated that the uncertainty related the to internal standard standardization was two times that for the preparation of analysis sample. The total uncertainty for pyridoxine was approximately 12%. Thus, the main efforts to reduce the uncertainty in the results should be further apparently focused on improvement of the standardization procedure.

Intercalibration studies showed good agreement between 12 European laboratories for pig's liver, mixed vegetable, and wholemeal flour samples (van den Berg et al. 1996). Our laboratory's results in that intercalibration study were in accordance with the mean values calculated from the results derived from the participating laboratories. Generally, the results derived from each laboratories' own in-house methods revealed some inconsistencies between liquid chromatographic and microbiological assays. The coefficient of variation within laboratory (CV_r) was considered to be acceptable (ranging from 5% to 13%) whereas the variation between laboratories (CV_R) was higher than expected.

Similar results for vegetable and wheat sample extracts were achieved with two different HPLC methods and one microbiological assay when standard solutions and sample extracts were circulated between three laboratories. The results of pig's liver sample derived from a microbiological assay were lower than those of liquid chromatography. The disparity between these results may partly be explanied by the different growth responses of the microorganism *Saccharomyces uvarum* for individual B_6 vitamers. It is generally recommended that microbiological assays should be standardized with each individual vitamer if possible to reduce possible growth response errors. However, vitamer B_6 distribution of pig's liver did not markedly differ from that of vegetable sample. Both liver and vegetable matrices were rich in pyridoxamine (and/or phosphate), and their portion covers approximately 70% of the total vitamin B_6 content while in flour sample more than two thirds are derived from pyridoxine and its glucosylated form(s). If the result disagreement is only caused by the lower growth response of pyridoxamine for the microorganism used, this same phenomenon should have been found in vegetable sample extracts as well which was not the case. Moreover, an intercalibration between two laboratories in Finland showed that the results of the microbiological assay were the same or slightly higher than results derived from a liquid chromatographic method. The factor(s) which caused the disagreement in the results between the liquid chromatographic method and the microbiological assay remained unresolved.

6.2.5 Laboratory proficiency

The performance of the laboratory was tested by participating in the intercomparison and certification studies. These results showed that both the analytical procedure and laboratory work corresponded to the general status between European laboratories involved in these studies. The results calculated on the basis of both external standard and internal standard standardization were in agreement showing that the chosen internal standard, 4-deoxypyridoxine, and its use fulfill the requirements for quantitative work. Our success in the interlaboratory studies and in a certification study (Ollilainen et al., manuscript) we participated in was considered to show

our laboratory's proficiency to carry out vitamin B_6 analyses.

The sets of interlaboratory studies clearly showed that further harmonizing work in the field of vitamin B_6 analysis is still needed. This task should include evaluation of the chromatographic separation and its parameters, the sample preteatments (enzymatic and/or acid hydrolysis), and the standardization procedures for chromatographic and microbiological assays as well.

6.3 Food analysis

In order to produce up-to date food composition data, a considerable amount of food items need to be analysed, and it can be considered that the value of the data obtained is due to the fact that the same validated analytical procedure is applied to various samples. Sample pooling procedure, as performed in this study, limits the obtainable information as the variation between subsamples is lost (Stewart 1995). In practice, in order to handle this number of food items using the described, quite laborious analytical methodology, this compromise had to be made. If information of the variation between subsamples is needed, it will inevitable multiply the number of samples resulting in a need for increased economical resources. Further studies related to food composition work in future should be focused on producing reliable data on the uncertainty of sampling procedures as well. This data would be of considerable help in the accreditation of laboratories.

6.3.1 Flesh foods; meat, poultry, and fish

Phosphorylated vitamers, pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate formed the major fraction of vitamin B_6 content in meat samples (Table 14) and pyridoxine, more characteristic for plant-derived foods, was present only in small amounts. In this respect, beef kidney and shoulder samples were exceptional; free pyridoxine covered one fifth of the total vitamin content in both samples. No detectable free pyridoxine in pork meat or pork meat products has been reported by Esteve et al. (1998); however, small amounts of free PN could be detected in all our meat samples. Free vitamers in our work, however, were generally present in offals. Livers were rich in vitamin B_6 containing ca. 0.85, 0.81, and 0.66mg/100g in beef liver, broiler liver, and pork liver, respectively. Poultry meat contained almost solely pyridoxal phosphate; PLP covered more than 70% of the total vitamin content. Vitamin B_6 levels in kidney samples were much lower. 4-pyridoxic acid, the metabolic end-product of pyridoxine compounds was the main vitamin B_6 compound in livers. It was noteworthy that no PNP could be detected in the liver samples of our study. In some rat liver samples but not in all samples, minor amounts of pyridoxine phosphate were found according to Vanderslice and coworkers (1981a). The majority of the vitamin B_6 activity in fish was composed of phosphorylated pyridoxamine and pyridoxal as the total sum of PLP and PMP ranged between 80–90% of the total vitamin B_6 amount. Free vitamers were only minor constituents except in Baltic herring.

Processing seems somewhat to alter the vitamer distribution in meat samples; a lower percentage of PLP was present in processed foods (sausages and meatball food items) while this was not the case with PMP. This might reflect the lability of phosphate ester linkages of pyridoxal phosphate present in beef and pork.

Pyridoxal and pyridoxamine are reported to be the predominant vitamers in flesh foods (Polansky and Toepher 1969). Due to heating processes like cooking or canning, at least 70% of the total vitamin content was in the form of pyridoxamine whereas the original, non-processed material contained more pyridoxal and less pyridoxamine. These different levels of pyridoxamine in raw and processed products were caused by the transamination of pyridoxal. The formation of pyridoxamine as a result of heating pyridoxal together with glutamic acid

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reached a maximum value at pH 6 at 100°C (Sigg 1985). Conversion of pyridoxamine in part to pyridoxine was observed when the temperature was further raised at 140°C (Sigg 1985). Temperature dependence of transamination toward the formation of pyridoxamine has also been reported in steamed sea urchin gonads (Shimada et al. 1993), in cooked meats (Bowers and Craig 1978) and in cereal based food model systems (Gregory and Kirk 1978b).

It is quite obvious that the extent of transamination varies markedly depending on unit operations to which the food material has been exposed. Furthermore, the interconversion of B_6 vitamers inevitably also occurs during the laboratory operations including sample storage and pretreatment, extraction, digestion and purification steps prior to analytical measurement. This complicates the evaluation of the vitamer distribution data of individual studies, and the earlier published data is not always comparable to present results.

In the analytical sense, meat samples were considered as "easy" samples in terms of sample pretreatment and enzymatic hydrolysis step even thought a more vigorous hydrolysis is needed due to more stable phosphorylated pyridoxamine (Peterson et al. 1955, Toepher and Polansky 1970). The strong hydrolysis of muscle and related tissues, especially if acid treatment is combined with a heating procedure, may yield breakdown products of pyrimidine-like and similar chemical structures. As the fluorescence characteristics of B_6 vitamers is based on the structure of hydroxypyridine moiety (Peterson et al. 1955), the formation of unknown or interferring components which are seen in the chromatogram will be hardly avoided. This was clearly shown in the analytical procedure applied by COST91 (1985) when sulfuric acid extraction followed by an autoclaving step was performed. An unknown, and in many cases also interference, fraction was eluted near to the pyridoxal in the reversed-phase chromatogram. Even if B_6 vitamers can be separated from these compounds, difficulties in the accurate baseline measuring of vitamin B_6 analytes should be expected. In addition, as many of these interferring compounds shows the same polarity nature in partition chromatography and the same ionic characters in ion-exchange chromatography, their specific removal during the sample treatment prior to analytical liquid chromatography will be a challenging task in vitamin B_6 analysis. Thus, a selective sample clean-up step with solid-phase extraction or related techniques would be of great help in the analytical procedure.

6.3.2 Dairy products and egg

A different distribution of B_6 vitamers was found in liquid milks and egg yolk compared to other food groups. The predominant vitamer in light-processed items such as liquid milks was free and phosphorylated pyridoxal covering together approximately 50 to 70% of the total vitamin B_6 activity. The highest portion of PLP, over 90%, was found in egg yolk, a result which is in line with Argoudelis (1997) and Toukairin-Oda et al. (1989).

Changes in the vitamer distribution between fresh and condensed or dried milk presented by Gregory and Mabbit (1961) were not seen in our skim milk powder sample (Table 16) as its vitamer distribution resembled that of milk or cream. In their early studies it was suggested that part of phosphorylated pyridoxamine found in freeze-dried milk was derived from pyridoxal during the processing steps. However, the presence of pyridoxal phosphate in fresh milk could not be measured due to the limitations in their analytical procedure. Thus, a possible substrate for transamination, free or phosphorylated pyridoxal, remained unclear in their results.

The total amount of vitamin B_6 in cheese samples, as expected, correlated in reverse order to the fat content; a lower vitamin amount was found in cheese with a higher fat content. The vitamer distribution in cheese was also remarkably different than that in pasteurized milk; both free and phosphorylated pyridoxal almost completely disappeared while the relative amount of

pyridoxamine and its phosphate in cheese were 2–3 fold compared to that of milk. Whether this was caused by processing or microbiological growth in the cheese maturation process (Gregory and Mabbit 1961) was beyond the scope of this study.

Pyridoxine phosphate, most likely pyridoxine-5'-phosphate, was found in baker's yeast and it was the only food sample in our study in which the pyridoxine content increased after phosphatase digestion. This increase in free pyridoxine was considered to indicate the occurrence of phosphorylated pyridoxine (PNP) in the sample. However, the effect of other unknown enzyme side activities or vitamer interconversion cannot be totally excluded. Results reported by Toukairin-Oda and coworkers (1989) showed that approx. 13% of the total vitamin B_6 content of their baker's yeast sample was derived from phosphorylated pyridoxine. Taking this into consideration, the PNP results of our study for yeast showed lower proportion of PNP than that of the Japanese group. The portion of PNP in other food groups was lower according to above mentioned study.

6.3.3 Plant-derived foods

The primary vitamin B_6 fraction of plant derived foods in our study was glucosylated pyridoxine. In this aspect foods of plant origin differ remarkably from other food groups. Only some plant foods like almonds and hazel nuts lack bound derivative(s) of pyridoxine; it has been proposed that this was due to the natural β-glucosidase activity present in these materials (Chiari et al. 1997, He and Withers 1997, Lai et al. 1992). Total vitamin B_6 activity in cereals is derived from pyridoxine or its bound form (pyridoxine glycoside). Free and phosphorylated pyridoxamine and pyridoxal and free pyridoxic acid form only a minor part of vitamin B_6 compounds. All six vitamers were present in barley flour, mixed wheat and rye flour samples, and also polished rice contained all six vitamers. This is partially contradicts to the results of Sampson et al. (1995) as they did not detect any free pyridoxamine in three wheat cultivars studied. Our results for flour samples were somewhat higher than those reported by Michela and Lorenz (1976); the total vitamin B_6 content for wheat and rye flour were 0.04mg/100g FM and 0.04mg/100g whereas our results for wheat and rye were 0.13mg/100g FM and 0.26mg/100g FM. No vitamer distribution was given in their results. Differencies in the milling process make it difficult to compare these results as the ash content was not given. The effect of milling on the vitamin B_6 content in wheat, triticale and rye was clearly seen in their study; bran was rich in total vitamin B_6 (1.1mg/100g FM) and the milling reduced their vitamin content to one tenth part that of grain. Glycosidic derivative(s) of pyridoxine was determined in all cereal samples; the average portion of total PNG was ca. 40% of the total vitamin B_6 content. These results are in agreement with earlier studies (Kabir et al. 1983a, Gregory and Ink 1987, Sampson et al. 1995). In babyfoods based on vegetable ingredients this bound vitamer fraction constituted about two thirds of the total vitamin content.

Most commonly used analytical procedures include either sample extraction with mineral acids or enzymatic digestion of food matrix prior to quantitative measurement of B_6 vitamers. Many enzyme preparations, like takadiastase, contain normally enough glucosidase side-activity to hydrolyze the glucosidic linkage present in bound vitamin forms. In an analytical sense this simplifies the determination of the total vitamin B_6 content and it is often a desired characteristics in an enzyme preparation. It has also been considered as a marker of the usefulness of an enzyme preparation. Hydrolysis of bound vitamers and phosphorylated forms enables the direct measurement of the total vitamin B_6 content without the need for determination of each vitamin form present. However, after these sample pretreatment steps the information of original vitamer distribution in food is lost.

Plant foods are considered to be the major dietary contributor of vitamin B_6; the portion of the intake derived from cereals and grain, from fruits and vegetables, and from legumes and nuts

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were 21%, 43% and 8%, respectively. Their added up vitamin B_6 content represented almost 70% of daily intake for elderly persons (Manore et al. 1990). The average PNG content of plant-derived foods was estimated to be 40% in our study. In some food items glucosylated pyridoxine may form as much as 60–70 percent of the total vitamin B_6 activity. Thus, the role of glycosidically bond pyridoxine in plant-derived foods cannot be overlooked. More detailed data on the vitamer distribution and occurrence in biological samples like food, and the role of glycosidic forms of pyridoxine in human nutrition is clearly needed. This requires new physiological and analytical/ methodological studies. The data for availability in different animal species and in humans is inadequate but also the effect of processing (Kabir et al. 1983b) and food matrix on utilization should be evaluated. It seems that the availability of B_6 vitamers from different food matrices varies (Gregory 1980c).

6.3.4 Comparison to national food composition tables

The total vitamin B_6 content of dairy products in the present work were in accordance to those values in national food composition tables when the amount of bound pyridoxine was summed together with other B_6 vitamers (Table 21). Some differences were still found, for example in the values for baker's yeast; a relatively high value was reported in the Danish food composition table compared to other food composition tables.

6.4 Characterization of isolated pyridoxine derivative

The amount of isolated PNX fraction (k_e~11.8), quantitated as pyridoxine, was approximately 1.3µg/100g and 2.03µg/100g in carrot and wholemeal flour, respectively. This calculation is based on the assumption that the molar fluorescence response of glycosylated pyridoxine is equal to that of pyridoxine (Gregory and Ink 1987). The recovery after ion-exchange purification was estimated to be ca. 65%. The content of glycosylated pyridoxine varied from 100 to 900 µg/100g in carrot and wheat flour according to Gregory and Ink (1987, Schramm and Bitsch (1991), Bitsch and Schramm (1992), Schramm and Bitsch (1993), and Sampson et al. (1995). Considering those literature values, a high percentage of analyte was lost during the isolation and fractionating process.

6.4.1 β-glucosidase hydrolysis

The reversed-phase chromatogram of plant-origin sample extracts contained several (normally three) peaks, (k_e~8.3, ~10.0, ~11.8) which disappeared during the β-glucosidase digestion. When the proposed main bound vitamin fraction (PNX at k_e ~11.6–11.8) was enzymatically hydrolyzed with β-glucosidase, it yielded an increased pyridoxine peak in the chromatogram. The retention of two other fractions in cation-exchange resin was also different than that of PNX, thus showing unequal cationic features for these two unknown analytes compared to that of PNX. No data on their structures or their chemical natures have been presented in the literature. The most retained unknown fraction (PNX) was considered to include the main glycosylated pyridoxine fraction, and this analyte fraction was taken into NMR spectroscopy and FAB mass spectrometry for further characterization.

6.4.2 Proton NMR spectroscopy

The ^{1}H spectrum of the PNX fraction from wholemeal flour (k_e~11.8) was measured as the isolated analyte was considered to be pure enough for NMR measurement (Fig. 28) whereas the carrot fraction still contained impurity/

unknown compound(s). The anomeric proton of glucose unit was tentatively characterized according to the signal at the δ-value of 4.45 ($J_{a,d}$ ~ 8Hz)(Table 24). The signal of an anomeric proton is expected to be located in a low field due to the attachment of two oxygens atoms to C-1, and should therefore be distinguishable from the other signals (Hall 1964). The measured chemical shift as well as coupling constant values for anomeric proton in the sugar moitety corresponded to those found in literature for β-D-glucopyranose (Lemieux et al. 1958, Rudrum and Shaw 1965). Pure D-glucose, dissolved in D_2O, at equilibrium showed two douplet signals at δ-values 4.57 ($J_{a,b}$ ~ 8.1) and 5.16 ($J_{a,b}$ ~ 3.9) apart from the other carbohydrate moiety signals (Table 25). The intensities of these douplets (1:2) corresponded to tautomeric equilibria of glucose in water; 36% of α-D-glucose and 64% β-D-glucose (Rudrum and Shaw 1965, Lemieux and Stewens 1966).

These findings were also in accordance with the results for the axial H-1 proton in β-D-glucopyranose (δ~4.3–4.4 ppm, J~8Hz). Conformation of C1 structure has been confirmed by free energy calculation of Angyal (1968). NMR measurements of an anomeric proton in D-glucopyranose (Lemieux and Stevens 1966) or D-glucopyranose pentaacetates (Lemieux and Stevens 1965), revealed that equatorial protons give their signal to a lower field than chemically similar but axially orientated protons. The anomeric proton in β-glucopyranosyl moiety of a phenyl propanoid glycoside in lemon was found at δ4.47 (J~8) (Matsubara et al. 1991). The chemical shift of the douplet signal for anomeric proton in 2-phenyl ethyl-β-glucoside located at δ-value of 4.82 ppm (J~7Hz) was reported by Umehara and coworkers (1988). The corresponding signal of the equatorially orientated proton in α-glucoside was found at a δ-value of 4.80 ppm (J~3.8Hz)(Shu and Lawrence 1994).

The anomeric proton of the glucose moiety in glycosylated pyridoxine located in the high field area (δ~4.44 ppm, J~6Hz) was reported by Gregory and Ink (1987); these values were considered to show the β-glycosidic linkage in glucosylated pyridoxine. A douplet at δ 4.22 (J~7), measured in CD_3OD, was ascribed to the β-anomeric proton in bound glucose of 5'-*O*-(β-D-glucopyranosyl) pyridoxine isolated from rice bran (Yasumoto et al. 1977). A singlet (at δ~7.78) was suggested as a signal derived from the aromatic proton of pyridoxine. Tadera et al. (1988) reported NMR data for 5'-*O*-(β-cellobiosyl) pyridoxine; two singlets at δ 4.25 and 4.27 indicated β-glycosidic linkage in the analyte. The anomeric proton of 5'-*O*-[6–*O*-(3-hydroxy-3-methyl-4-carboxy-butanoyl)-β-D-glucopyranosyl] pyridoxine was characterized according to signal at δ 4.21(J=7.0) by Tadera and associates (1983). An anomeric proton resonance of pyridoxine 4'- and 5'-α-D-glucosides measured in deuterated dimethyl sulfoxide has been found at δ 4.89 (1H, d, J=3.5) and δ 4.68(1H, d, J=3.0), respectively (Suzuki et al. 1997).

A low field signal (δ8.3–8.4, J ~ 42.5) as a douplet or two individual singlets in our results was/were assumed to be derived from the aromatic C-6 proton in pyridoxine. The value for the coupling constant was extraordinarily wide. On the other hand, two singlet interpretation did not correlate with the proposed pyridoxine moitety in 5'-*O*- β-D-glucopyranosylpyridoxine. No low field area of the NMR spectrum was presented by Gregory and Ink (1987). Evaluation of the aromatic proton region in the NMR spectra of synthetized and isolated pyridoxine-5'-α-D-glucopyranoside were also missing in the results of Ogata et al. (1969a, b). The low field area of spectrum in our study representing the area for aromatic protons was also different than that of 5'-*O*-(β-cellobiosyl) pyridoxine in rice bran reported by Tadera et al. (1988) as only one singlet (δ~7.92) was present in their spectrum. Suzuki and coworkers (1997) reported that aromatic protons in 4'- and 5'-O- (α-D-glucopyranosyl) pyridoxines gave resonance signals at δ 7.91 (1H, s, PN, 6-H) and at δ 7.86 (1H, s, PN, 6-H). Thus, aromatic proton in pyridoxine was seen in both above mentioned studies as a singlet which was in accordance with expectations.

Pyridoxine hydrochloride gives a four signal NMR spectrum in deuterium oxide: δ~2.68,

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δ~4.84, δ~5.04 and δ~8.21 for methyl protons (2'C), methylene protons in 4'CH_2-, methylene protons in 5'CH_2-, and aromatic proton in 6C, respectively (Handbook of Proton-NMR Spectra and Data 1985). Our results for the pyridoxine moiety of the analyte is generally in accordance with that data, except for the signal of methylene protons in the 4'-carbon as it was overlapped by the water signal. Overall, the NMR signal for hydroxyl protons could not be recorded since the measurements were performed in deuterium oxide. A high field signal in our work at δ~2.8 was characterized as an impurity.

Spin-spin coupling and total spin correlation of the molecule were measured using COSY- and TOCSY-spectra (Fig. 29), respectively. Signals at δ-values of 3.85, 3.95, 4.35, 4.45, 4.85 and 6.15 in whole wheat flour spectrum were assumed to be sign of a spin-spin coupling of the adjacent atoms. Signal intensities of the former coupling system also shoved that these signals were assigned to one coupling system. This result agreed with the proposed structure of glycosylated pyridoxine. Signals at 1.35 and at 4.15 belong to another coupling system, representing an impurity present in the NMR sample. The intensity of the proposed system (δ 3.85–3.95–4.35–4.45–4.85– 6.15) did not correlate with the calculated amount of the isolated fraction when the concentration of the glycosylated fraction was measured using HPLC data. Overall NMR-signal level of that coupling system was estimated to be higher than the result derived from the chromatographic data.

Event though the resolution in the proton NMR spectrum was good, the exact chemical structure of the isolated pyridoxine derivate could not be elucidated using this proton NMR data. Most of the signals can be explained by the proposed molecule structure (5'-*O*- β-D-glucopyranosyl pyridoxine) but too many uncertainties were still present in the interpretation of the proton spectra. Furthermore, the absolute amount of the isolate did not match the intensity of the proton NMR signal; the amount of isolated compound was considered to be higher than expected when the intensity of the NMR signal was taken into account. Thus, it should be critically evaluated whether this discrepancy was due to different molecule stucture of the isolate than expected and/or to the presence of impurity (or impurities) in the isolated PNG fraction.

6.4.3 FAB mass spectrometry

FAB induced mass spectra of the PNX fraction (k_e~11.8) isolated from wholemeal flour and carrot samples were measured using either glycerol, nitrobenzylamine or triethanolamine as a matrix (Appendix 2). The highest m/z-value (621.4, 60%) as a molecule ion using a nitrobenzylamine matrix (in the positive mode) was considered to be too high to show the proposed glycosylated pyridoxine structure when m/z 171 formed the base peak (100%). In addition, if molecular weights are normally given by abundant $[M+H]^+$ ions in positive-ion FAB spectra, the above mentioned m/z-value match with difficulty. Odd-numbered m/z-value for molecule ion, if a truly nonprotonated analyte molecule ion is present in the mass spectra, requires normally an odd number of nitrogen atoms in the proposed structure, which is the case in proposed structure of glycosidically bound pyridoxine. Two triglucosides of pyridoxine identified from rice bran, 4'-*O*-(β-D-glucosyl)-5'-*O*-(β-cellobiosyl)pyridoxine and 5'-*O*-(β-glucotriosyl)pyridoxine, gave both $[M+H]^+$ ions at an m/z-value of 656 in the secondary ion mass spectrometry (SI-MS). The $[M+H]^+$ ion for a diglucoside of PN, 4'-*O*-(β-cellobiosyl) pyridoxine, was detected at an m/z value of 494 (Tadera et al. 1988).

Unexpectedly high m/z-values were also found using a glycerol matrix as the observed m/z-values neither correlated with the suggested protonated oligomers of glycerol adducts ([glycerol]nH^+, m/z (92n+1)) nor with other proposed adducts ($[M+NH4]^+$, $[M+Na]^+$). The mass fraction m/z 171 present in positive-mode CI with glycerol and nitrobenzylalcohol matrices in our work was found in the EI mass spectra of 5'-*O*- β-D-glucopyranosyl pyridoxine by Bitsch

and Schramm (1992). No mass spectrometric interpretation, however, was given in their report.

Careri and her coworkers (1996) have reported electron impact (EI) and chemical ionization (CI) mass spetrometric data for water-soluble vitamin standards produced by a particle beam (PB) mass spectrometry. For the electron impact spectra of free pyridoxine, the intensity of the molecule ion was relatively high 169 (50%), other major fragments being m/z 94 (M-75, 100%), 106 (M-63, 77%) and 151 (M-18, 74%). Unbound pyridoxine using a chemical ionization in negative-ion mode (NCI) formed the major ions as m/z=167 (100%), 149 (74%), 168 (55%), and 151 (35%), and in positive-ion mode (PCI) as m/z= 152 (100%), 170 (76%), 136 (19%). PCI proved to be the most suitable ionization mode for pyridoxine and pyridoxamine. None of above mentioned molecule ions or fragmentation ions could be clearly recognized in our mass spectra.

FAB-MS gave some evidence that both isolates (from carrot and wheat flour) contained the same analyte but the exact interpretation of the mass spectra was interfered from the impurities, probably traces derived from the mobile phase and ion-pair reagents. This being the case, the structure for the proposed 5'-*O*-(β-D-glucopyranosyl) pyridoxine could not be confirmed on the basis of our mass spectra results. Whether this discrepancy is mainly caused by impure analyte or a less suitable ionization technique in mass spectrometric measurements remains unclear. In order to continue this work, further purification of the analyte as well as the use of other MS techniques, like MALDI or APCI-MS^n, is probably needed.

7 Conclusions

In summary, the liquid chromatographic measurement of vitamin B_6 compounds in a complex matrix, like foods and other biological materials, has proved to be a challenge for an analytical laboratory. Depending on the data needed, different analytical approaches can be chosen. Sample treatment, including extraction and detection procedures, chromatographic separation and the quantitation of the analytes will have an effect on the data and its accuracy which are obtained using a particular method.

The present data shows that vitamin B_6 compounds can be determined in their intact forms, and the native distribution of vitamers is maintained during the analytical procedure. Cold perchloric acid extraction followed by enzymatic digestions prior to liquid chromatographic analysis produces data on free, phosphorylated and glycosylated vitamin B_6 compounds. In this approach plant-derived samples were treated with two enzymatic digestions in addition to a non-treated sample which need, however, resources in a laboratory.

At present, the vitamin B_6 method chosen in a laboratory depends on how detailed data is required and for what purpose. The European Committee for Standardization (CEN) has begun to harmonize the methodology for vitamin B_6. In that work a method will be found which will serve as an European standard for quantitative measurement of vitamin B_6 in foods. Further work will be then focused on the sample preparation technique and on the method proficiency testing data available. In addition, further progress in the improvements in identification in the present techniques would be of help. The general selectivity limitations in the present liquid chromatographic methods may be solved to some extent by the development of LC-MS or CE-MS methodology. Reassessment of the biological methods, like microbiological assays, is also needed since these methods are still in gen-

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eral utilization, and their use is expected to continue in the near future in spite of the liquid chromatography's suitability.

Secondly, the latest Finnish Food Composition Table completely lacks data for vitamin B_6, and the data users are involved in consulting the foreign food data bases. In general, the analytical results of the amount of glycosylated pyridoxine in foods are scarce too. The strength of the present data is that it is produced by one method including the interlaboratory data, and the results are thus comparable with each other within the food samples or food groups analyzed. It is therefore to be hoped that the results of the present study will meet those needs.

Thirdly, in the present work it was clearly seen that glycosidically bound pyridoxine derivative(s) accounted for the main portion of vitamin B_6 in nearly all foods of plant origin, and this bound analyte(s) is not taken into account in the traditional methods. The role of the glycosylated pyridoxine(s), needs to be clarified both in analytical and physiological aspects. The utilization of the glycosidically bound forms has remained somewhat unclear despite of the intensive study performed in this field. If the availability of the bound forms is limited for human as it is more or less assumed at present, it means that the role of vegetables, cereals and other plant-derived foods as a source of vitamin B_6 should be reassessed.

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SELOSTUS

B_6-vitamiinin määrittäminen elintarvikkeista suuren erotuskyvyn nestekromatografisella (HPLC) menetelmällä

Velimatti Ollilainen
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B_6-vitamiini (pyridoksiini) on vesiliukoinen vitamiini, joka esiintyy erilaisissa kemiallisissa muodoissa biologisissa materiaaleissa kuten eläinkudoksissa, kasvisolukoissa ja niitä sisältävissä elintarvikkeissa. Elimistössä B_6-vitamiini edesauttaa monia biokemiallisia reaktioita mm. valkuaisaineiden ja glykogeenin aineenvaihdunnassa. Perinteisen vitamiinivaikutuksen lisäksi pyridoksiini saattaa liittyä mm. altistumiseen sydän- ja verisuonitaudeille. Tieteellistä näyttöä näistä uusista yhteyksistä ei kuitenkaan vielä ole saatu.

Perinteisesti B_6-vitamiini on määritetty elintarvikkeista mikrobiologisesti, jolloin pyridoksiini on vapautettu ns. sidotuista muodoista. Tässä työssä selvitettiin nestekromatografian (HPLC:n) soveltuvuutta B_6-vitamiinin vapaiden ja sidottujen muotojen määrittämiseen elintarvikkeista. Kehitetyn menetelmän luotettavuus varmistettiin mm. Euroopan unionin järjestämissä laboratorioiden välisissä vertailututkimuksissa. Menetelmällä määritettiin 50 elintarvikenimikkeen eri B_6-vitamiinimuotojen pitoisuudet. Runsaasti pyridoksiinia tai sen johdannaisia sisälsivät mm. liha ja sisäelimet (maksa ja munuaiset), kananmunan keltuainen ja hasselpähkinä. Kasviperäisten elintarvikkeiden B_6-vitamiini koostui suurelta osin sidotuista glykosyloiduista vitamiinimuodoista. Niiden hyväksikäytettävyydestä elimistössä on varsin ristiriitaisia tietoja. Työssä saadut elintarvikkeiden B_6-vitamiinitulokset liitetään Kansanterveyslaitoksen elintarvikkeiden koostumustietopankkiin ja niitä hyödynnetään mm. ravitsemussuunnittelussa. Työ osoitti olevan tarpeellista yhdenmukaistaa käytettäviä määritysmenetelmiä, jotta eri laboratorioissa saadut tulokset olisivat paremmin vertailukelpoisia. Tätä menetelmien yhtenäistämistä jatketaan mm. Euroopan Standardoimisliiton (CEN) työryhmissä.

Appendix 1

FOOD SAMPLES FOR VITAMIN B_6 ANALYSIS

MEAT AND POULTRY

PY-1	Minced beef (steak)
PY-2	Minced meat, beef and pork
PY-5	Pork shoulder, deboned
PY-6	Beef, shoulder chuck, deboned
PY-3	Beef liver
PY-10	Pork liver
PY-11	Pork, kidney
PY-4	Beef, kidney
PY-7	Broiler, liver
(PY-28	-"-)
PY-12	Dry sausage, salami type
PY-13	Sausage, "lenkki"
PY-15	Lamb, leg
PY-16	Elk, steak
PY-14	Reindeer, steak
PY-9	Broiler, boneless, skinless
PY-8	Hen, boneless, skinless

CEREALS

PY-74	Whole wheat flour (graham)
PY-41	Wheat germ
PY-40	Rye flour
PY-42	Barley flour
PY-44	Oats, rolled
PY-43	Mixed wheat and rye flour, "sämpyläjauho"
PY-45	Rice

FISH AND FISH PRODUCTS

PY-75	Rainbow trout, fillets
PY-96	Pikeperch, sea, fillets
PY-70	Baltic herring, fillets
PY-97	Whitefish, sea, fillets
PY-95	Pike, sea, fillets
PY-76	Roe paste, salted, smoked

READY-TO-EAT FOODS

PY-30	Meatballs
PY-31	Broiler meat balls
PY-32	Veal-vegetable stew (baby food)
PY-33	Potato-carrot puree (baby food)

DAIRY PRODUCTS AND EGGS

PY-20	Whole milk, standardized 3.9 % fat
PY-21	Milk, standardized 1.9 % fat
PY-22	Whipping cream
PY-25	Cream cheese, "Turunmaa"
PY-23	Cheese, Edam type, 40%
PY-24	Cheese, Edam type, 20%
PY-26	Skimmed milk powder
PY-27	Infant formula powder, based on cow's milk
PY-71	Egg yolk

VEGETABLES, POTATO AND NUTS

PY-80	Tomato
PY-81	Broccoli
PY-82	Carrot
PY-83	Potato
PY-84	Peas, frozen
PY-91	Peanuts
PY-92	Hazelnuts
PY-93	Almonds
(PY-94	Almonds)

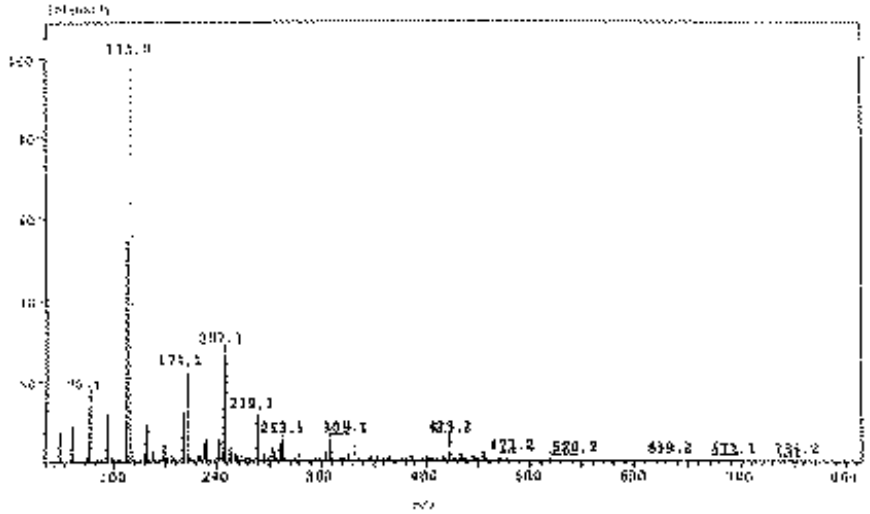
MISCELLANEOUS

PY-90	Yeast, bakers

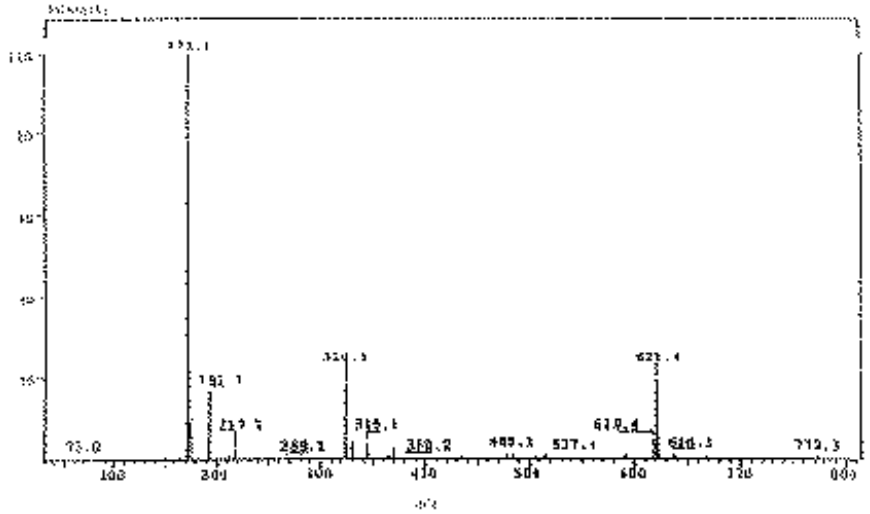
Appendix 2

FAB-MS spectra of PNX from carrot using a) glyserol, b) nitrobenzylamine and c) triethanolamine as a matrix.

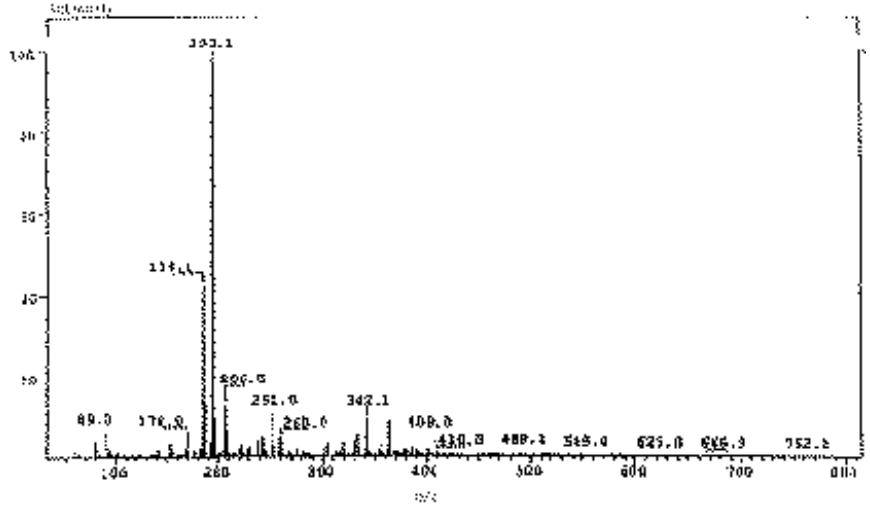
a)



b)



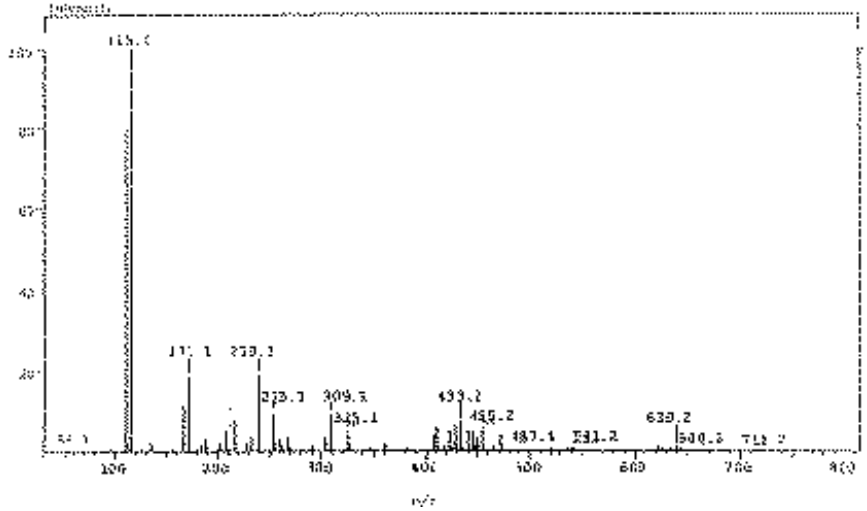
c)



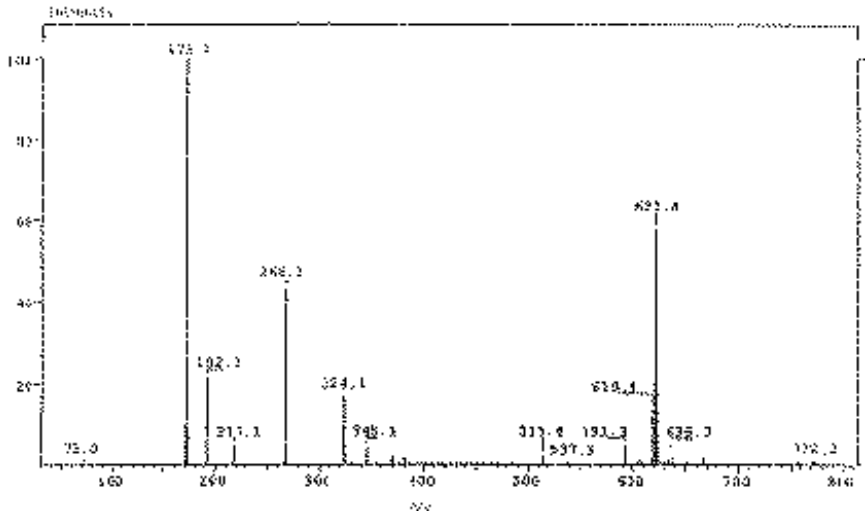
Appendix 2

FAB-MS spectra of PNX from wholemeal flour using a) glyserol, b) nitrobenzylamine and c) triethanolamine as a matrix.

a)



b)



c)