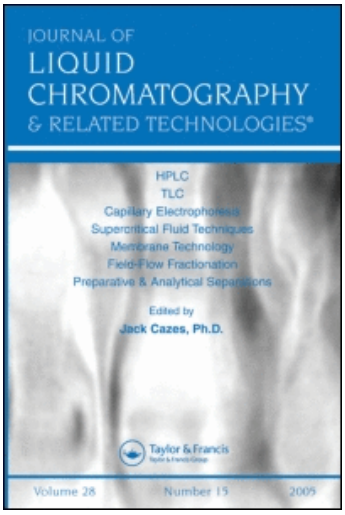

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Sierra, I. and Vidal-Valverde, C.(1997) 'A Simple Method to Determine Free and Glycosylated Vitamin B_6 in Legumes', Journal of Liquid Chromatography & Related Technologies, 20: 6, 957 — 969
To link to this Article: DOI: 10.1080/10826079708013666
URL: http://dx.doi.org/10.1080/10826079708013666

J. LIQ. CHROM. & REL. TECHNOL., 20(6), 957–969 (1997)

A SIMPLE METHOD TO DETERMINE FREE AND GLYCOSYLATED VITAMIN B_6 IN LEGUMES

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ABSTRACT

A simple high performance liquid chromatography (HPLC) method for the determination of pyridoxamine, pyridoxal, pyridoxine and glycosylated pyridoxine in legumes was described. Samples were extracted with trichloroacetic acid and incubated with acid phosphatase and β-glucosidase enzymes. The analytical separation was achieved by isocratic elution (methanol:potassium phosphate buffer) in an octadecylsilica column within 13 min. High recoveries and precision were obtained. This procedure provides a good alternative to previous methods for the determination of vitamin B_6 in legumes.

INTRODUCTION

Vitamin B_6 is the name given to 3-hydroxy-2-methylpyridine derivatives that exhibit biological activity of pyridoxine in rats.[1] It occurs naturally in foods as pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their corresponding 5'-phosphates.

In certain plant foods, besides these biologically active vitamers, a remarkable content of glycosylated forms of PN (G-PN), including 5'-O-(β-D-glucopyranosyl) pyridoxine as the principal fraction, have been found.[2,3] The nutritional significance of these forms is related to their low bioavailability compared to B_6 vitamers.[4,5,6]

The measurement of vitamin B_6 in foods, constitutes a complicated analytical problem due to the existence, at relatively low levels, of multiple forms of this vitamin. The validity of methods depends mostly, on their particular application.[7,8] Some procedures developed for the quantification of B_6 vitamers exhibit several disadvantages for their use in routine work: lengthy procedure time in microbiological methods[9] or sophisticated gradient elution[10] and long retention times [11] in HPLC methods. In the case of quantification of glycosylated forms of PN, some HPLC methods have been reported for the direct analysis of 5'-O-(β-D-glucopyranosyl) pyridoxine,[12,13] however due to the existence of different glycosylated forms and the absence of readily obtainable standards for them, the indirect quantification of these forms as free PN constitute a better approach to measurement of the glycosylated species.[14]

Despite all the existing methods, there is very little information on the quantification of vitamin B_6 in certain complex food matrices such as legumes, which are known to be one of the best sources of this vitamin.[15] Most vitamin B_6 content data of legumes found in the literature, have been obtained by microbiological assay after thermal extraction in acidic media.[16,17] These conditions cause hydrolysis of glycosylated derivates of the vitamin, resulting in an overestimation of the nutritionally available vitamin B_6 content in legumes.[7] The effectiveness of many of the existing HPLC methods for determining the B_6 vitamers,[18,19] when they are applied to complex matrix food like legumes, have not been fully assessed, and many problems can appear, especially due to interferences with the matrix compounds.

The objective of this study was to evaluate each vitamer and the glycosylated forms of vitamin B_6 in legumes, developing a simple method which could be used in routine work. For this purpose the extraction procedure and the HPLC conditions were optimized. The extraction of vitamers B_6 from lentils, chick peas, and haricot beans was carried out using a chemical deproteinating agent, and enzymatic hydrolysis with acid phosphatase enzyme in order to determine the sum of the free and phosphorylated forms of each B_6 vitamer. Glycosylated pyridoxine was quantified indirectly as free PN released by enzymatic treatment of legume extracts with β-glucosidase. The quantification of B_6 vitamers were carried by HPLC in isocratic mode after the adequate pH adjustment of the eluate, in a post-column reaction coil, in order to avoid fluorescence interferences from impurities present in legume extracts.

MATERIAL AND METHODS

Chemicals and Reagents

Methanol HPLC grade was obtained from Lab-Scant Ltd. (Dublin, Ireland). Acid phosphatase from potato (0.4 U/mg specific activity), β-glucosidase from almonds (14 U/mg specific activity), pyridoxamine dihydrochloride (PM-2HCl), pyridoxal hydrochloride (PL-HCl), pyridoxine hydrochloride (PN-HCl) and pyridoxal 5'-phosphate (PLP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pyridoxamine 5'-phosphate hydrochloride (PMP-HCl) were obtained from Merck (Darmstadt, Germany).

Water was purified for chromatographic use with a Milli-Q system (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA) and for other procedures with a Milli-Ro system (Waters Chromatography Division). All other chemicals were of analytical grade.

Samples

Lentils (*Lens culinaris*, c.v. "castellana"), chick peas (*Cicer arietinum*, c.v. "blanco lechoso"), and haricot beans (*Phaseolus vulgaris*, c.v. "riñon") were purchased from a local market. The seeds were ground to pass 0.18 mm sieve and stored in the dark until analysis. Five replicates of each sample were analyzed to provide B_6 vitamer concentration data.

Extraction Procedure

0.4 g of lentils, chick peas, or haricot beans flour were homogenized with 40 mL 5% (w/v) trichloroacetic acid (TCA) for 30 min, filtrated through No. 40 Whatman filter paper and levelled with Milli-Ro water to 50 mL. An aliquot of the filtrate (5 mL) was transferred to a test tube and the pH was adjusted to 4.8 with 4M sodium acetate. 0.4 mL of 6.0 mg/mL aqueous solution of potato acid phosphatase was added and samples were incubated with shaking for 5 h at 37°C in a water bath. The reaction was quenched by the addition of 1.2 mL of 20% (w/v) TCA.

Upon cooling to ambient temperature, an aliquot was filtered through a 0.22 μm pore size nylon filter membrane and analyzed by HPLC. The extract was stored at 6°C for 2 days.

To hydrolyse the glycosylated forms of PN, 1.5 mL of a 1.25 mg/mL aqueous solution of β glucosidase were added to the previously described extract, prior pH adjustment to 5.0. Samples were incubated at 37 °C for 5 h in a shaking water bath. The reaction was quenched with 1.2 mL of 20% (w/v) TCA. An aliquot was filtered through a 0.22 μm pore size nylon filter membrane and analyzed by HPLC. The extract was stored at 6°C for 2 days.

Chromatographic Conditions

Determination of vitamin B_6 by HPLC was carried out using a modular chromatograph (Waters Associates, Milford, MA, USA), equipped with a Model M 510 pump, two Model M 45 pumps, a Rheodyne sample injector with a 50 μL loop and a Waters 470 scanning fluorescence detector (λ_{Ex} 328 nm, λ_{Em} 390 nm). Data were processed on a PC (NEC Corporation, Boxborough, MA, U.S.A).

The analytical column was a ODS2 Spherisorb, 10 μm, 300 mm x 3.9 mm i.d. (Sugelabor S.A, Madrid, Spain) with a guard column containing C_{18} Porasil B, 20 mm x 3.9 mm i.d. (Waters Associates).

The mobile phase consisted in a mixture of methanol and 0.033M potassium phosphate buffer pH 2.2 (2:98, v/v). The flow rate was 1.2 mL/min and the column temperature was 17 °C.

The acidic pH of the eluate was adjusted to pH 7 ± 0.5 in a post-column reaction coil with 0.3 M dipotassium hydrogen phosphate solution pumped at a flow rate of 0.7 mL/min from a second pump.

A mixture methanol:water (30:70), delivered from a third pump, was used to clean retained substances in the chromatographic column between injections. The cleaning step lasted 5 min and the conditioning of the column with mobile phase another 5 min.

Standard Solutions

Individual stock standard solutions were prepared by dissolving 4.2 mg of PM-2HCl, 14.1 mg of PL-HCl, 9.7 mg of PN-HCl, 5.0 mg of PMP-HCl and 7.0 mg of PLP in 100mL Milli-Q water and adding 0.5 mL of orthophosphoric acid. These stock solutions were stable for 2 months stored in the dark at 4°C.

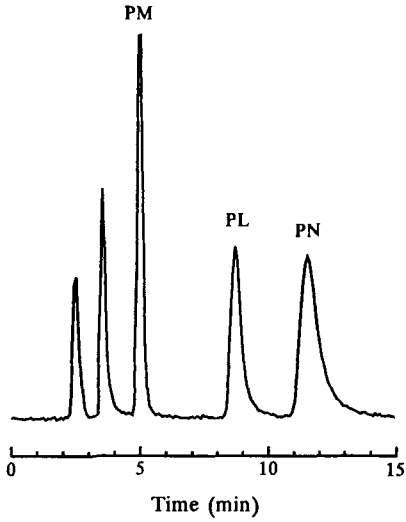

Figure 1. HPLC chromatogram of processed working standard mixture containing pyridoxamine (PM = 12 ng/ml), pyridoxal (PL = 24 ng/ml) and pyridoxine (PN = 34 ng/ml).

Mixed working standard solutions were prepared daily by transferring appropriate volumes of PM, PL and PN stock solutions to 100 mL conical flasks, diluting with 40 mL 5% (w/v) trichloroacetic acid in Milli-Ro water and subjected to the same extraction procedure used with samples.

The overall concentrations ranges for each vitamer were 0.8-12 ng/mL for PM, 0.8-24 ng/mL for PL, and 1-34 ng/mL for PN. Calibration curves were obtained by plotting areas versus concentration of processed working standard solutions.

Recovery Experiments

Vitamer recoveries were determined by spiking the legume samples with standard vitamers (0.69 μg/g PM, 1.35μg/g PL, 0.59 μg/g PN, 0.52 μg/g PMP, 0.82 μg/g PLP) before extraction. Recoveries were calculated by comparing the difference between the measured concentration in the spiked and non spiked samples respect to amount of standard B_6 vitamers added. Phosphorylated vitamers were recovered as their respective unphosphorylated forms.

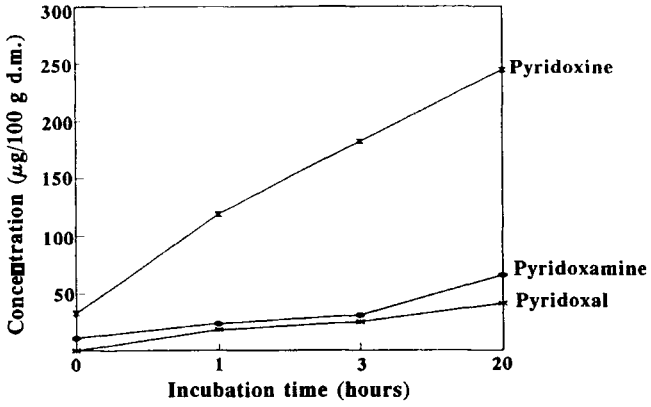

Figure 2. Effect of Takadiastase enzyme on B_6 vitamers content of lentils.

RESULTS AND DISCUSSION

In Figure 1, a chromatogram of processed PM, PL and PN standards is shown. Good resolution of the three B_6 vitamers in a relatively short time (13 min) was obtained. The retention times (min) were 4.98 ± 0.01 for PM, 8.69 ± 0.01 for PL and 11.52 ± 0.02 for PN. The limit detection of vitamers ranges between 0.2-0.4 ng/mL.

Peak areas were linearly related to the amount injected over the range investigated for each vitamer (0.04-0.60 ng/injection for PM, 0.04-1.20 ng/injection for PL and 0.06-1.70 ng/injection for PN). Correlation coefficients obtained for these calibration curves were always > 0.990.

Preliminary studies to hydrolyze the phosphorylated B_6 vitamers in legumes were carried out using Takadiastase enzyme.[20] As indicated in Figure 2, very high increases of PN was obtained after the enzymatic procedure (212 μg/100g), and since phosphorylated PN in foods occurs in very low amounts,[20] it was clear that in legumes some others forms of PN were hydrolyzed by the Takadiastase enzyme.

Figure 3 shows the results obtained with variable amounts of acid phosphatase enzyme to hydrolyse the phosphorylated B_6 vitamers of legumes and the plateau that was reached with 2.4 mg of enzyme. The increases of PN after the enzymatic hydrolysis ranged between 0-27 μg/100g.

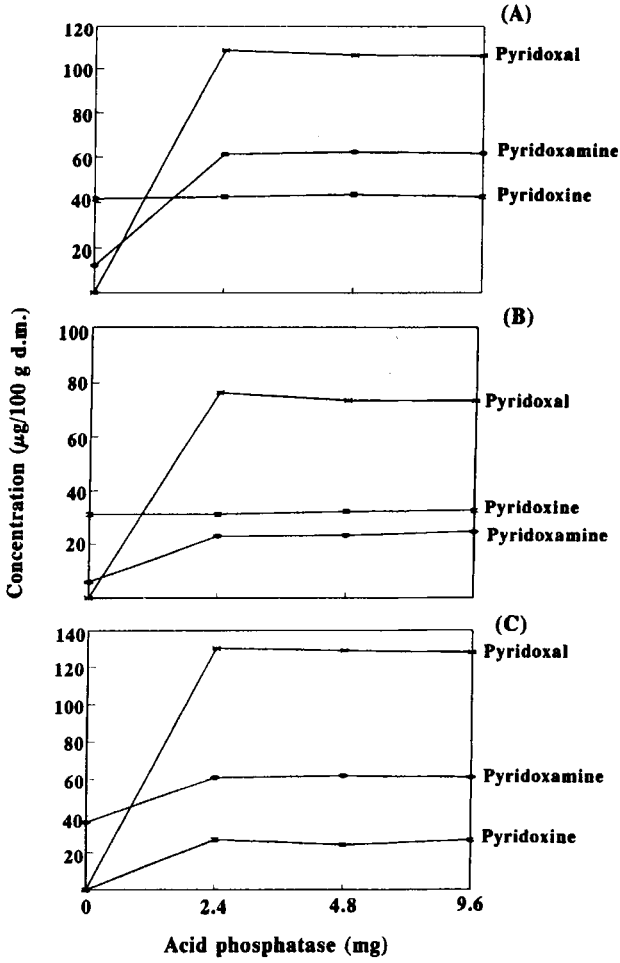

Figure 3. Content of B_6 vitamers in legumes, extracted with variable amounts of acid phosphatase enzyme. a) lentils, b) chick peas, c) haricot beans.

A peak at a retention time of 14.5 min present at the chromatogram of an non-enzymatic extract (Figure 4) could be related with G-PN forms. This chromatographic peak still remained when the acid phosphatase enzyme was used to hydrolyse the phosphorylated B_6 vitamers, and disappeared when extracts where incubated with β-glucosidase. However, when the enzymatic procedure was carried out with Takadiastase enzyme, this chromatographic peak did not appear, and a very

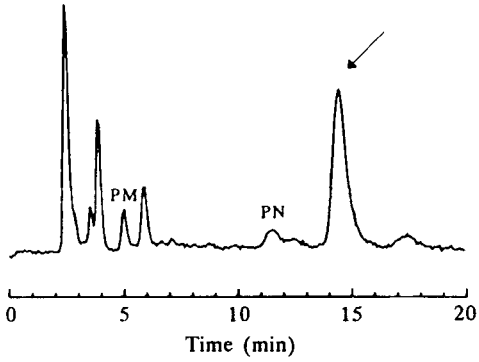

Figure 4. HPLC chromatogram of non-enzymatic lentil extract of B_6 vitamers.

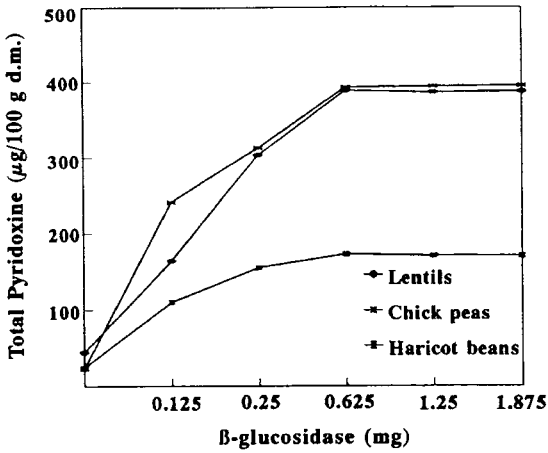

Figure 5. Total pyridoxine content in legumes obtained with variable amounts of β-glucosidase enzyme.

high amount of PN content was obtained. These results suggested that the peak with retention time of 14.5 min corresponds to glycosylated forms of pyridoxine and that the use of Takadiastase enzyme can produce an overestimation of the PN (sum of free and phosphorilated forms) of legumes including the G-PN as PN, while these

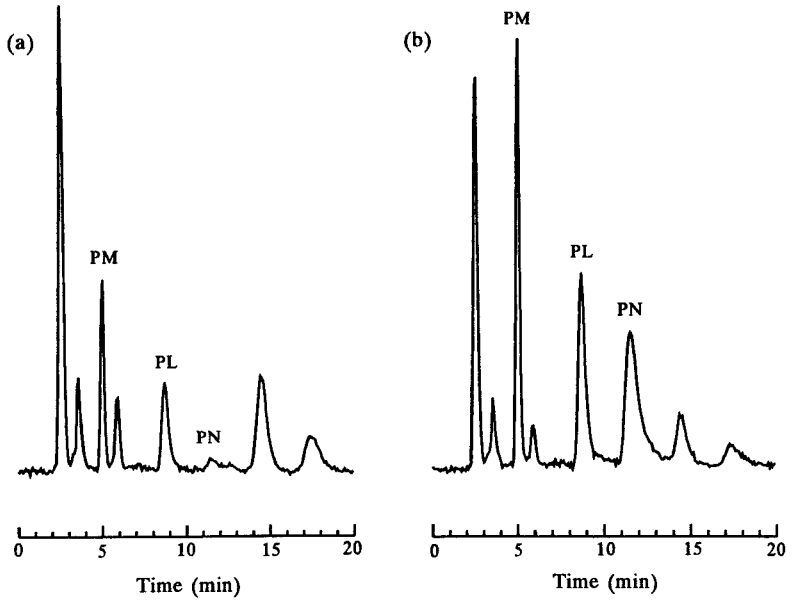

Figure 6. (a) HPLC chromatogram of pyridoxamine (PM), pyridoxal (PL) and pyridoxine (PN) in a haricot bean sample, (b) HPLC chromatogram of a haricot bean extract enriched with PM, PL and PN.

forms have lower biological activity. The G-PN in legumes was quantified indirectly as free PN. The legume extracts, obtained after acid extraction and enzymatic hydrolysis with acid phosphatase, were submitted to the action of β-glucosidase enzyme. Figure 5 indicates the results obtained with different amounts of β-glucosidase enzyme to hydrolyse the G-PN of legumes and it can be observed that a plateau was reached with 0.625 mg of enzyme. The difference between the PN data obtained with and without β-glucosidase enzyme (Figures 3 and 4) gave the G-PN content of legumes.

Quantitative analysis of vitamin B_6 in legumes by HPLC requires effective separations because of the complex legume matrix. The separation of non-phosphorilated B_6 vitamers with reverse phase on octadecylsilica columns was first reported by other authors.[18,19,20] The method of Gregory and Kirk[18] gives rapid separations of the three vitamers, but their application to complex samples is not sucessfull. A very similar system was used by Lim et al.[19] to separate PM, PL and PN, but when was applied to milk samples good results were not obtained.

Table 1

Recovery of Pyridoxamine (PM), Pyridoxal (PL), Pyridoxine (PN), Pyridoxamine Phosphate (PMP), and Pyridoxal Phosphate (PMP) Added to Legume Samples[a]

B_6Vitamers	Lentils	Chick Peas	Haricot Beans
PM	96 ± 0	96 ± 4	93 ± 3
PL	98 ± 2	98 ± 1	95 ± 2
PN	96 ± 1	95 ± 2	93 ± 1
PMP	106 ± 0	107 ± 2	100 ± 6
PLP	96 ± 1	98 ± 2	94 ± 4

[a] (%) Mean values ± SD of three replicates.

Table 2

Pyridoxamine (PM), Pyridoxal (PL), Pyridoxine (PN), and Glycosylated PN (G-PN) Content in Legumes (μg/100 g d.m.)[a]

Legumes	PM	PL	PN	G-PN[b]	Total B_6[c]
Lentils	63.3 ± 2.6	94.2 ± 2.8	44.2 ± 2.5	343.3 ± 3.4	546.5
Chick Peas	26.9 ± 0.8	74.8 ± 2.3	23.5 ± 1.3	371.5 ± 3.8	496.7
Haricot Beans	64.5 ± 1.2	128.8 ± 1.6	24.0 ± 0.2	146.2 ± 3.0	365.0

[a] Mean values ± SD of five replicates.
[b] G-PN = Difference of PN before and after hydrolysis with β-glucosidase.
[c] Total B_6 = Sum of all vitamers and G-PN calculated as PN.

Speek[20] obtains a good resolution of the three vitamers in some foods (banana, potato chips and corn-flour) however, long analysis time is required (40 min). Figure 6a shows a typical chromatogram of legume sample (haricot bean). The chromatographic procedure used enabled good separations of the three vitamers in a short time (23 min including the cleaning step).

PM, PL and PN peaks of extracts were identified by spiking and comparing their retention times with standards (Figure 6b). Recoveries for exogenous vitamers added to the samples are reported in Table 1. The results obtained provide evidence of a reasonable extraction efficiency comparable to other extraction methods.

Table 2 shows the concentration of PM, PL, PN and glycosylated PN in selected legumes. Regarding the total content of vitamin B_6, it appears that our own results, calculated as PN from the sum of all these forms, are in agreement with the total value of B_6 reported in Food Composition Tables.[21,22]

In conclusion, we propose a simple method that provides a valid approach to the measurement of free and glycosylated forms of vitamin B_6 from legume foods. Differentiation between total and glycosylated vitamin B_6 is of value for an accurate assessment of bioavailable vitamin B_6 from plant foods.

ACKNOWLEDGEMENTS

This work was supported by Comision Interministerial de Ciencia y Tecnologia ALI 96-0480.

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Received August 5, 1996
Accepted August 26, 1996
Manuscript 4221