Quantification of Free and Bound Pantothenic Acid in Foods and Blood Plasma by a Stable Isotope Dilution Assay

Michael Rychlik[†]

Institut für Lebensmittelchemie der Technischen Universität München, Lichtenbergstr. 4, D-85748 Garching, Germany

A stable isotope dilution assay for quantification of pantothenic acid in food and blood plasma uses a 4-fold labeled isotopomer of the vitamin as an internal standard. Pantothenic acid and its labeled analogue were detected as trimethylsilyl derivatives by gas chromatography−mass spectrometry, showing a minimized spectral overlap. In starch a detection limit of 44 μg/kg, an intrasample relative standard deviation of 6.7%, and recovery values ranging between 97.5 and 99.4% were determined. Total pantothenic acid content was determined in rice, milk powder, apple juice, and blood plasma after enzymatic hydrolysis of the vitamin's conjugates; free pantothenic acid was quantified prior to enzyme treatment. Almost all results were found to be in good agreement with literature data.

Keywords: *Blood plasma; gas chromatography−mass spectrometry; pantothenic acid; rice; stable isotope dilution assay*

INTRODUCTION

The water-soluble vitamin (*R*)-pantothenic acid (PA) occurs in foods in its free form as well as bound in coenzyme A (CoA) or acyl carrier protein (ACP). The latter conjugates of PA are physiologically active in steroid, fatty acid, and phosphatide metabolism. PA, also called vitamin b_5, cannot be synthesized by animals and therefore has to be assimilated with food.

The most commonly employed method of PA analysis is measuring turbidimetrically the growth of *Lactobacillus plantarum* in a PA-deficient medium (Tanner et al., 1993). Because this procedure requires long incubation times up to 24 h and the maintainance of sterile working conditions, there have been attempts to develop alternative methods.

Chromatographic methods, however, have not been found to be suitable for several reasons. High-performance liquid chromatography, on one hand, suffers with PA's weak UV absorption above 230 nm and with no reproducible formation of fluorescent derivatives (Hudson et al., 1984). On the other hand, gas chromatography requires derivatization of PA, and therefore, the addition of an internal standard (IS) is essential. Banno et al. (1990) used (*R*)-5-[(2,4,-dihydroxy-3,3-dimethyl-1-oxobutyl)amino]pentanoic acid, a homologue of PA, as IS, but the structural difference may cause discrimination between this IS and PA.

By contrast, recently developed enzyme-linked immunosorbent assays (ELISAs) promise to be fast alternatives to the microbiological methods (Finglas et al., 1988; Gonthier et al., 1998). In comparative studies ELISAs revealed results similar to those of the microbiological assays (Finglas et al., 1988). Nevertheless, the major drawbacks of ELISA are its lower sensitivity (Gonthier et al., 1998) and the lack of commercial availability.

As we reported on the analysis of flavor compounds (Rychlik and Grosch, 1996) and of the mycotoxin patulin (Rychlik and Schieberle, 1999), stable isotope dilution assays (SIDA) exhibit excellent sensitivity and reliability and are accurate alternatives to other quantification methods. By use of isotopomers of the analytes as internal standards, losses during cleanup and derivatization can be exactly corrected. Therefore, a SIDA using gas chromatography−mass spectrometry has been developed in the present study, thus introducing this technology into the analysis of PA.

METHODS AND MATERIALS

Chemicals. The following chemicals were obtained commercially from the sources given in parentheses: [^{15}N,$^{13}C_3$]-β-alanine, *N*,*O*-bis(trimethylsilyl)trifluoracetamide (BSTFA), D_2O, 2-methoxyethanol, (*R*)-(−)-pantolactone, (*R*)-pantothenic acid hemicalcium salt (Aldrich, Steinheim, Germany), acetone, ammonium sulfate, calcium oxide, dichloromethane, diethylamine, ethyl acetate, hydrochloric acid, $KHCO_3$, methanol, $NaHCO_3$, pyridine, sodium acetate, Na_2SO_4, toluene (Merck, Darmstadt, Germany), Dowex 1 x 8 (Serva, Heidelberg, Germany) alkaline phosphatase, 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris), heparin lithium salt (170 USP units/mg), pigeon liver acetone powder (Sigma, Steinheim, Germany), sodium 3-(trimethylsilyl)-[2,2,3,3-d_4]-propionate (Isotec, Miamisburg, OH).

Food Samples. Polished and unpolished long-grain rice, skimmed milk powder, apple juices, and edible corn starch (Fixella-Remiga, Krefeld, Germany) were purchased in a local store.

Blood Plasma. Porcine blood was obtained from a local butcher; human blood was withdrawn from the antecubital vein. Clotting of blood (100 mL) was prevented by addition of lithium heparin (8.8 mg, 1500 USP units). Plasma was then obtained by centrifugating the blood at 2000 *g* for 5 min.

Synthesis of Calcium [^{15}N,$^{13}C_3$]-(*R*)-Pantothenate. [^{15}N,$^{13}C_3$]-β-alanine (100 mg, 1.07 mmol) was dissolved in water (2 mL), and calcium oxide (29.1 mg, 0.52 mmol) was added to the solution. The mixture was stirred until the calcium oxide was dissolved, then the water was evaporated in vacuo. Methanol (5 mL), diethylamine (2 μL) and (*R*)-(−)-

[†] Phone: +49-89-289 132 55. Fax: +49-89-289 141 83. E-mail: michaelr@leb.chemie.tu-muenchen.de.

10.1021/jf9913054 CCC: $19.00
Published on Web 03/23/2000

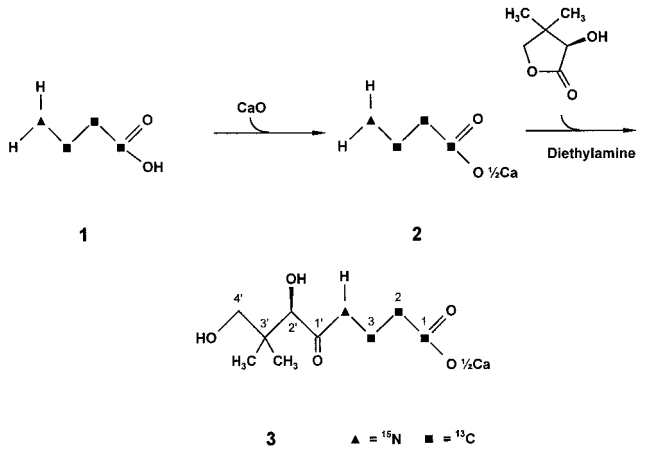

Figure 1. Synthetic route used in the preparation of [^{15}N,$^{13}C_3$]-pantothenic acid.

pantolactone (161.2 mg, 1.24 mmol) were added to the residue, and the mixture was heated in an atmosphere of nitrogen to 70 °C for 8 h. The target compound (180.6 mg) was precipitated by pouring the cold mixture into cold acetone and purified by recrystallization from methanol/acetone.

NMR spectra were recorded with an AM 360 (Bruker, Karlsruhe, Germany). ^{1}H NMR: transmitter frequency 360.13 MHz, spectral width 7246.377 Hz, repetition time 3.2 s, 16 scans, 32K data set. ^{13}C NMR: transmitter frequency 90.56 MHz, spectral width 23 809 Hz, repetition time 2.5 s, 256 scans, 64K data set, 1 Hz line broadening. Processing was performed by multiplication with a Lorentz−Gaussian function prior to transformation. Shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)-[2,2,3,3-d_4]-propionate and J values are in hertz. Numbering of calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate is detailed in Figure 1.

^{1}H NMR (D_2O): δ 0.90 (s, Me), 0.94 (s, Me), 2.43 (dm, $^2J_{HC}$ = 118, H-2), 3.40 (d, $^2J_{HH}$ = 11, H-4′), 3.45 (dm, $^2J_{HC}$ = 138, H-3), 3.52 (d, $^2J_{HH}$ = 11, H-4′), 3.99 (s, H-2′).

^{13}C NMR (D_2O): δ 38.9 (ddd, $^1J_{C2-C3}$ = 36.3, $^1J_{CN}$ = 10.9, $^2J_{CC}$ = 3.6, C-3), 39.4 (dd, $^1J_{CC}$ = 36.3 and 45.1, C-2), 183.2 (dd, $^1J_{C1-C2}$ = 45.1 and $^2J_{CC}$ = 4.4, COOH)

Preparation of Pantetheinase. *Activated Resin*. Anion-exchange resin Dowex 1 x 8 (5 g) was stirred in hydrochloric acid (50 mL, 1 mol/L) for 10 min and filtered with suction. The procedure of stirring followed by filtering was repeated once in hydrochloric acid, then 10 times in water. The residue was then suspended in water (50 mL) and adjusted to pH 8.0 by addition of Tris buffer (12.1 g dissolved in 100 mL of water, adjusted to pH 8.3 with concentrated hydrochloric acid).

Enzyme Preparation. Pigeon liver acetone powder (1 g) was suspended in an aqueous solution of $KHCO_3$ (5 mL, 0.02 mol/L) at 0 °C. The suspension was transferred into centrifuge tubes using a second portion of aqueous $KHCO_3$ (5 mL, 0.02 mol/L) and centrifuged for 20 min at 0 °C at 10000*g*. After the supernatant was filtered through a cheesecloth, the filtrate was added to one-half of the activated resin and the suspension was shaken for 5 min in a shaker box at 4 °C. Later, the mixture was centrifuged for 20 min at 0 °C at 10 000 *g* and the supernatant filtered through a fresh cheesecloth. After addition of the remaining half of activated resin followed by shaking during 5 min at 4 °C, the mixture was centrifuged for 20 min at 0 °C at 10000*g*. The supernatant was finally filtered through another fresh cheesecloth and frozen until required for analysis. Before use, the frozen pantetheinase solution was thawed in an ice/water bath.

Extraction of Rice and Skimmed Milk Powder for SIDA. Rice (3 g) was ground in a grain mill (Bosch, München, Germany) followed by stirring the flour in an aqueous solution of sodium acetate (50 mL, 0.02 mol/L, pH 5.6) for 1 h at 20 °C. Analoguously, skimmed milk powder (0.5 g) was extracted using the same solvent and same conditions.

After addition of an aqueous solution of calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate (3 μg), the extract was filtered. For analysis of total PA an aliquot of the filtrate (3 mL) was subjected to enzyme hydrolysis as outlined below. For analysis of free PA another aliquot of the filtrate (10 mL) was washed with dichloromethane (10 mL) and, after addition of hydrochloric acid (1 mL, 18 mol/L) and ammonium sulfate (4 g), PA was extracted with ethyl acetate (2 × 15 mL).

Extraction of Apple Juice for SIDA. Apple juice (50 mL) was spiked with an aqueous solution of calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate (5 μg) and then extracted with ethyl acetate (2 × 30 mL).

Extraction of Blood Plasma for SIDA. For analysis of total PA, blood plasma (3 mL) was subjected to enzyme hydrolysis as outlined above. For analysis of free PA, an aqueous solution of calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate (1 μg) was added to the plasma (3 mL), which was then was washed with dichloromethane (3 mL). After addition of hydrochloric acid (1 mL, 18 mol/L) and ammonium sulfate (3 g), the plasma was centrifuged at 5000 *g* for 5 min and PA was extracted from the supernatant with ethyl acetate (2 × 15 mL).

Enzyme Hydrolysis for Quantification of Total Pantothenic Acid. For liberation of bound PA, the filtrated extract or the blood plasma was sterilized at 120 °C for 15 min and cooled to 37 °C. Then solutions of pantetheinase (0.4 mL), alkaline phosphatase (0.8 mL, 2%), $NaHCO_3$ (1 mL, 0.85%), Tris buffer (6 mL, pH 8.3, preparation as detailed ahead), toluene (0.1 mL), and an aqueous solution of calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate (3 μg) were added to the sterilized extract (3 mL) and the mixture was incubated for 8 h at 37 °C. Subsequently, the hydrolysate was washed with dichloromethane (3 mL) and acidified by addition of hydrochloric acid (1 mL, 18 mol/L). Then ammonium sulfate (3 g) was added and the solution was extracted with ethyl acetate (2 × 15 mL).

Preparation of the Tris(trimethylsilyl) Derivative of Pantothenic Acid. Solutions of PA in ethyl acetate were dried over anhydrous Na_2SO_4 and evaporated in a stream of nitrogen to dryness. Then *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (100 μL) and pyridine (100 μL) were added and the mixture was heated for 60 min at 80 °C in a closed vial. After cooling to room temperature, the solution was evaporated to dryness in a stream of nitrogen, and hexane (100 μL) was added.

High-resolution mass spectrometry of Tris(trimethylsilyl)-[^{15}N,$^{13}C_3$]-PA, (relative intensities in parentheses) in the electron impact mode: m/z = 103.0565 (100%), 73.0463 (95%), 295.1363 (80%), 157.1029 (57%), 247.1485 (56%), 117.0720 (51%), 205.0912 (38%), 75.0248 (28%), 147.0649 (26%), 424.2024 (23%), 296.1436 (15%).

Gas Chromatography/Mass Spectrometry (GC−MS). High-resolution gas chromatography (HRGC) was performed by means of a type 5300 gas chromatograph (Carlo Erba, Hofheim, Germany) using capillary DB-5 (30 m × 0.32 mm fused silica capillary, film thickness of the stationary phase d_f = 0.25 μm; Fisons Instruments, Mainz, Germany).

The samples were applied by the cold on-column technique at 60 °C. One minute after injecting the sample, the temperature of the oven was raised to 280 °C at a rate of 15 °C/min. The flow rate of the carrier gas helium was 2 mL/min.

Mass spectra in the electron impact (EI) mode were recorded by means of an MAT 95 S (Finnigan MAT, Bremen, FRG) and in the chemical ionization (CI) mode by means of an ITD 800 (Finnigan MAT, Bremen, FRG) with methanol as the reagent gas. Ionization energy in the EI mode as well as in the CI mode was 70 eV. Mass chromatography was performed by coupling capillary DB-5 to the mass spectrometers.

Determination of Response Factors and Calculation of the Amount of Pantothenic Acid. Solutions of calcium pantothenate and calcium [^{15}N,$^{13}C_3$]-pantothenate were mixed in five mass ratios ranging from 0.15 to 4.5 to give a total volume of 10 mL and a PA content of 100 μg. After addition of hydrochloric acid (1 mL, 18 mol/L) and ammonium sulfate (5 g) the mixtures were extracted with ethyl acetate (2 × 15 mL). Subsequently, the PA mixtures were derivatized and subjected to GC−MS as outlined before. Response factors R_f were calculated according to following equation

$$R_f = \frac{A_{13C,15N-PA} m_{PA}}{A_{PA} m_{13C,15N-PA}}$$

where A_{PA} is the area of unlabeled Tris(TMS)PA in mass trace $m/z = 291$, $A_{13C,15N-PA}$ is the area of labeled Tris(TMS)PA in mass trace $m/z = 295$, $m_{13C,15N-PA}$ is the amount of added labeled PA, and m_{PA} is the amount of added unlabeled PA.

Calculations of the amount of pantothenic acid (C) in foods and plasma by SIDA were made using the following equation

$$C = \frac{A_{PA} m_{13C,15N-PA} R_f}{A_{13C,15N-PA}}$$

where A_{PA} is the area of unlabeled Tris(TMS)PA in mass trace $m/z = 291$, $A_{13C,15N-PA}$ is the area of labeled Tris(TMS)PA in mass trace $m/z = 295$, $m_{13C,15N-PA}$ is the amount of added labeled PA, and R_f is the response factor (=0.99).

Determination of Detection and Quantification Limits. Amounts of 100, 200, 400, and 1000 ng of PA were added to edible corn starch (1 g) and analyzed as detailed before. Each sample was analyzed in triplicate. Detection limits (DL) and quantification limits (QL) were calculated according to Hädrich and Vogelgesang (1999). DL is the concentration calculated from the maximum height of the 95% confidence interval at the zero addition level. QL is the addition level for which the lower 95% confidence limit equals the upper 95% confidence limit of the addition level at the DL.

RESULTS AND DISCUSSION

Synthesis of Calcium [^{15}N,$^{13}C_3$]-Pantothenate. Because of its high separation performance, capillary gas chromatography of trimethylsilyl (TMS) derivatives was chosen to analyze PA. Taking into account the natural isotopic abundance of C-13 and Si-30 in the Tris(TMS)PA molecule, a mass increment of 3 u or more for the labeled isotopomer is necessary to avoid spectral overlap of the analyte at the m/z monitored for the IS. Therefore, the commercially available [^{15}N,$^{13}C_3$]-β-alanine (**1**) was employed for introducing a 4-fold labeling into the pantothenic acid molecule, as can be seen from Figure 1.

By combination of the methods of Wilson et al. (1954) and Williams (1947), **1** was converted to its hemicalcium salt **2** and condensed with (*R*)-(−)-pantolactone in the presence of diethylamine to form the hemicalcium salt of [^{15}N,$^{13}C_3$]-(*R*)-pantothenic acid (calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate) **3** in an overall yield of 72.7%. By contrast, different reaction conditions, like heating (*R*)-(−)-pantolactone with the sodium salt of **1** in a dry mixture according to Williams et al. (1940) or using 2-methoxyethanol as the solvent (Kagan et al., 1957), resulted in lower yields.

The purity and constitution of calcium [^{15}N,$^{13}C_3$]-(*R*)-pantothenate were confirmed by application of ^{1}H NMR and ^{13}C NMR experiments. Comparing ^{1}H NMR spectra of the isotopomeric pantothenic acids revealed that both triplet signals of the protons at C-2 and C-3 in unlabeled PA were split in labeled PA into duplets of multiplets by heteronuclear $^1J_{HC}$ and $^2J_{HC}$ coupling with adjacent ^{13}C atoms. Because $^1J_{HC}$ coupling constants exceed 100 Hz, the resonance signals of the hydrogens at C-3 in labeled PA no longer overlap the pair of duplet signals formed by the coupling ($^2J_{HH}$ = 11 Hz) of geminal hydrogens at C-4′ (Figure 2). In an earlier study Fritz and Löwe (1962) explored this geminal coupling and attributed it to the fixed conformation of the pantoyl moiety in PA. Because the rotation about the bond between C-2′ and C-3′ is sterically hindered, the asymmetric center C-2′ makes the geminal hydrogens nonequivalent.

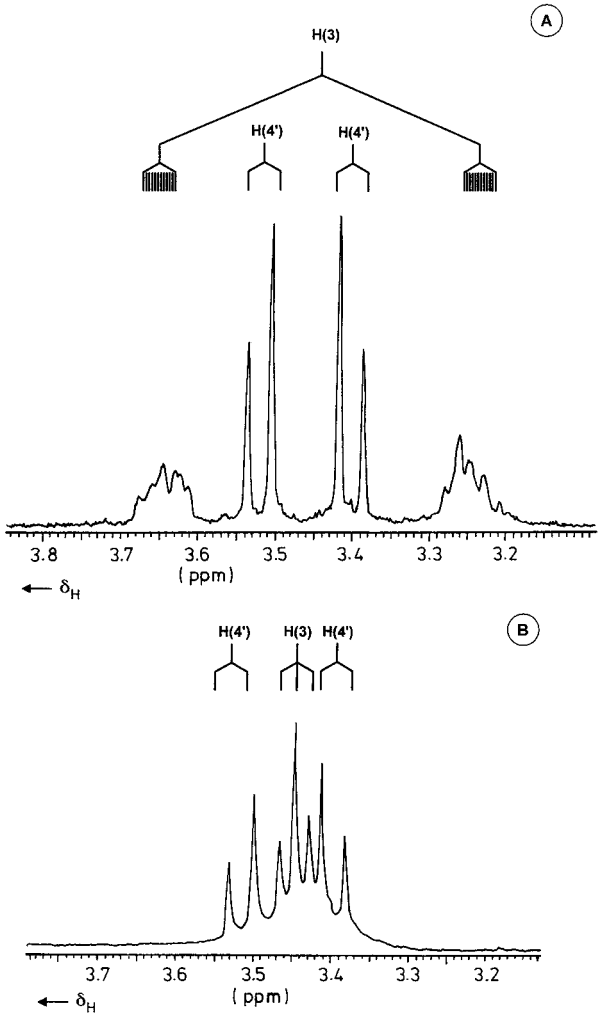

Figure 2. ^{1}H NMR signals of the geminal protons at C-4′ and of the proton at C-3 (for numbering see Figure 1) in labeled (A) and unlabeled (B) pantothenic acid.

The ^{13}C NMR data reveal homonuclear $^1J_{CC}$ and $^2J_{CC}$ coupling of the introduced ^{13}C atoms. In addition, the ^{13}C atom at position 3 shows heteronuclear ^{13}C−^{15}N coupling with a coupling constant of 10.9 Hz.

Gas Chromatography and Mass Spectrometry of Derivatized Pantothenic Acid. Since PA is not volatile enough for gas chromatographic (GC) analysis, it has to be derivatized. In this study, the TMS derivative was generated by reacting PA with a mixture of BSTFA and pyridine according to the method reported by Banno et al. (1990). The mass spectrum of the derivative in CI mode shown in Figure 3 revealed it to contain three TMS groups, which is in compliance with the results of Prosser and Sheppard (1971). According to ^{1}H NMR experiments, the latter authors concluded the TMS moieties to be bound to the two hydroxyls and the carboxyl grouping. The mass spectra in chemical ionization mode as well as in electron impact mode (shown in Figure 4) of labeled Tris(trimethylsilyl)-pantothenic acid revealed a mass shift of 4 u compared to the mass of unlabeled isotopomer. This finding is consistent with the incorporation of the labeled nitrogen and the three labeled carbon atoms into the PA molecule.

Quantification by SIDA Using GC−MS. *Selection of the Most Suitable Ions for Quantification.* Superior

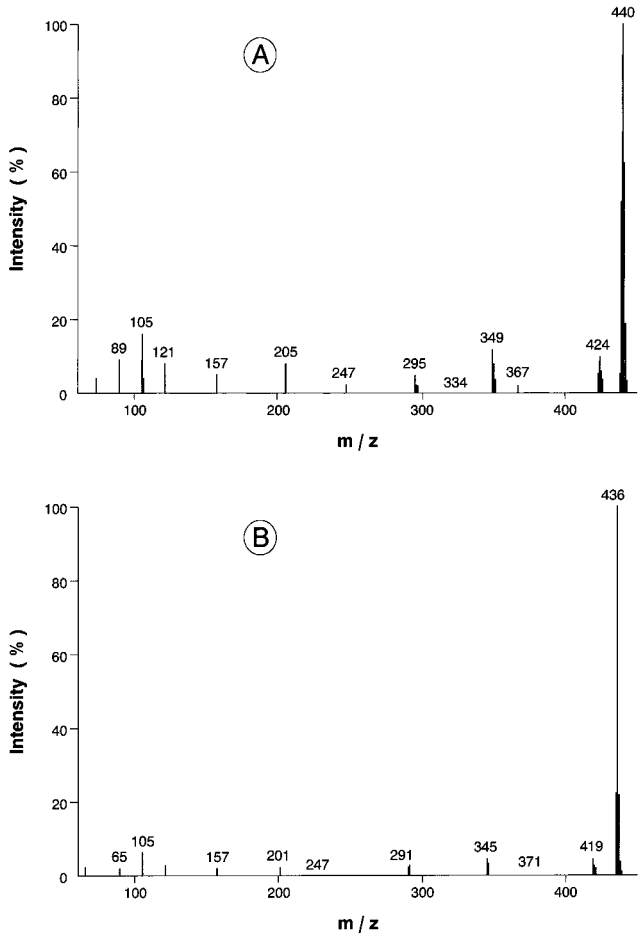

Figure 3. Mass spectrum of labeled (A) and unlabeled (B) Tris(trimethylsilyl)pantothenic acid in chemical ionization mode.

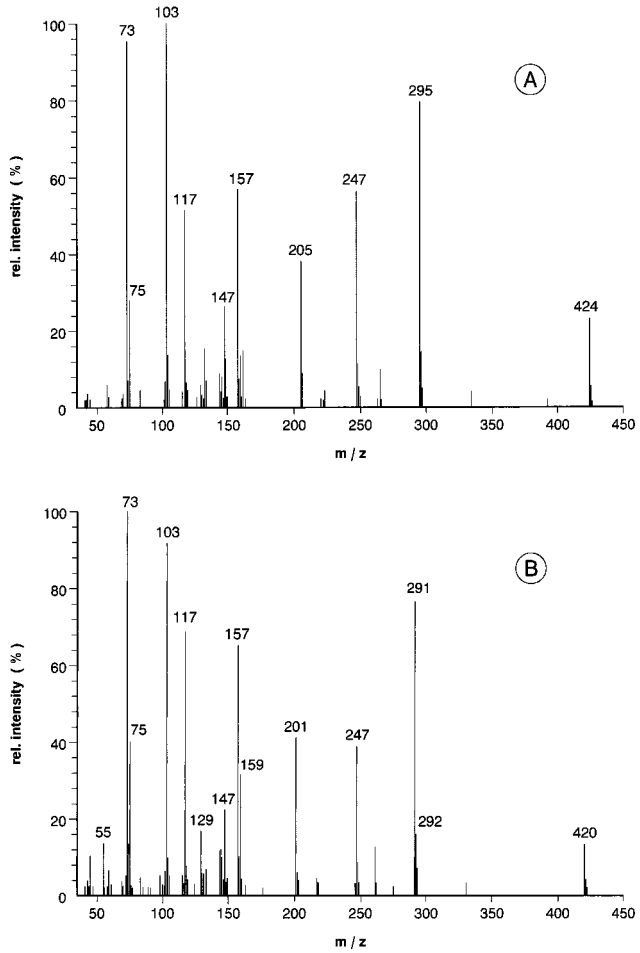

Figure 4. Mass spectrum of labeled (A) and unlabeled (B) Tris(trimethylsilyl)pantothenic acid in electron impact mode.

labeled internal standards have to fulfill several requirements. First, the stability of labeling is imperative. Second, the IS material has to be completely labeled, and third, interferences from naturally occurring isotopes in the analyte at the *m/z* used for measurement of the IS have to be omitted. Noncompliance with the last two prerequisites will result in mass spectral overlap between the unlabeled analyte and the labeled internal standard at the *m/z* chosen for measurement. Consequently, the calibration graph relating measured isotope ratios to mass ratios of the analyte and the IS becomes a hyperbola instead of a straight line. The slope of this ideal straight calibration line represents the response factor R_f, which will be variable in the nonlinear case. This case would stringently require extended approximation methods for quantification (Thienpont et al., 1996). Because the employed [^{15}N,$^{13}C_3$]-β-alanine provides stability and completeness of labeling, the invariability of R_f depends solely on the contribution of the unlabeled analyte at the *m/z* value chosen for monitoring the labeled IS. Regarding mass spectra of the TMS derivatives, in the electron impact mode the fragment ions $[M - 2TMS + 2H]^+$ (*m/z* 295 and 291, respectively) and $[M - CH_3]^+$ (*m/z* 424 and 420), and in the chemical ionization mode the quasimolecular ions (*m/z* 440 and 436), are conceivable mass traces for quantification. To select the most suitable ion species, abundance ratios of the possible ion pairs of IS to analyte were measured for mass ratios ranging from 0.15 to 4.5 and R_f values were calculated (Table 1). For the quasimolecular ion pair *m/z* 440 and 436 in CI mode, the relative standard deviation (RSD) of R_f exceeded 13%, which might be attributed to the lower sensitivity of the employed instrumentation. In EI mode, monitoring the ion pair 424/420 resulted likewise in a high RSD of R_f. By contrast, ion pair 295/291 revealed an R_f closest to 1.0 with the lowest RSD being 4.1%. These findings can undoubtedly be attributed to the elemental composition of the ions *m/z* 295/291 containing only one silicon atom in contrast to the other pairs. Therefore, the fragment $[M - 2TMS + 2H]^+$ of unlabeled Tris(TMS)-PA shows only negligible ion intensity from naturally occurring Si-30 isotopes falling at *m/z* 295 monitored for the respective fragment of labeled Tris(TMS)PA. These results demonstrate that the useful range of mass ratios of analyte to IS covers values between 0.3 and 4.5 for this ion pair. The calibration graph relating intensity ratios to mass ratios is drawn in Figure 5.

Table 1. Response Factors R_f and Mean Relative Standard Deviation (RSD) of R_f for the *m/z* Fragments Chosen for Quantification

	response factor R_f							
	mass ratio of unlabeled to labeled PA						mean	mean RSD[a]
m/z	0.15	0.3	0.5	1.0	2.2	4.5	value[a]	(%)
291/295[b]	0.89	1.02	0.99	0.93	0.96	1.05	0.99	4.1
420/424[b]	0.85	1.37	1.00	0.92	1.05	1.13	1.07	14.2
436/440[c]	0.93	0.97	1.22	1.18	1.05	1.36	1.16	13.1

[a] For R_f for mass ratios ranging from 0.3 to 4.5. Each mass ratio was analyzed in triplicate. [b] In electron impact mode. [c] In chemical ionization mode.

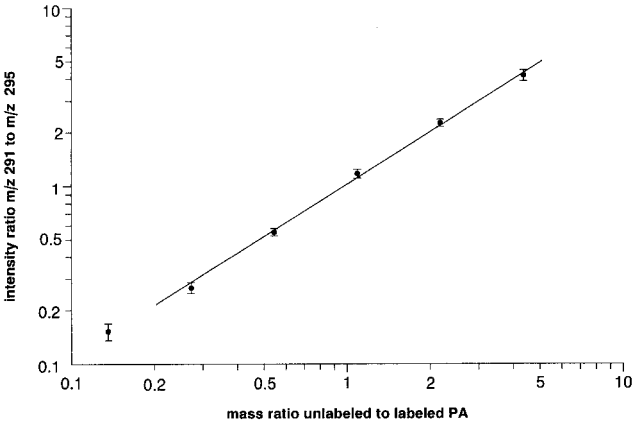

Figure 5. Calibration graph relating measured ion intensity ratios to mass ratios of isotopomeric pantothenic acids on a logarithmic scale.

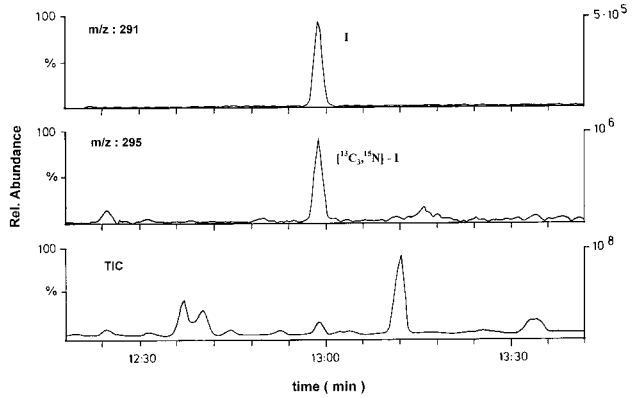

Figure 6. GC−mass chromatogram of an unpolished rice containing 5.56 mg/kg of free pantothenic acid. The internal standard Tris(trimethylsilyl)-[^{15}N,$^{13}C_3$]-pantothenic acid ([^{15}N,$^{13}C_3$]-I) is detected at *m*/*z* 295, unlabeled Tris(trimethylsilyl)pantothenic acid (I) at *m*/*z* 291. TIC is the total ion current.

Route of Analysis. For quantification of PA, foods or blood plasma was extracted in sodium acetate buffer (pH 5.65) according to official method 945.74 (AOAC, 1995). After addition of labeled PA, analyte and IS were extracted into ethyl acetate, converted into the TMS derivatives, and analyzed by GC−MS. Mass chromatography of *m*/*z* 291 and 295 (Figure 6) enables us to compute the ion abundance ratios, mass ratios of PA to the IS, and the PA content in the analyzed sample, successively.

Performance Criteria of the Stable Isotope Dilution Assay. For determining a new method's detection limit, two alternative procedures may be applied. The first of them defines DL as that concentration for which the signal-to-noise ratio (SNR) equals 3 (Williams and Salin, 1988). Although the simplicity of this concept made it popular among analysts, it has been criticized for several reasons (Williams, 1991). First, this is because of the restricted comparability of SNR to that from other laboratories. Second, it is because losses and interferences caused by the sample-preparing procedure are not taken into consideration, and third, it is because SNR is not related to the quantitative information provided by a calibration curve (Meier and Zünd, 1993). Therefore, in the present study the second procedure was chosen. It deduces DL from the confidence interval of a calibration line prepared by spiking a suitable matrix with the analyte (Hädrich and Vogelgesang, 1999).

Table 2. Comparison of Performance Data of the Stable Isotope Dilution Assay (SIDA) to an Enzyme-Linked Immunosorbent Assay (ELISA) Reported by Song et al. (1990) for Quantifying Pantothenic Acid in Starch-Containing Foods

performance criterion	SIDA	ELISA
detection limit	44 μg/kg	*a*
quantification limit	131 μg/kg	400 μg/kg
intrasample RSD in polished rice		
free pantothenic acid	6.7% ($n = 5$)	6% ($n = 10$)
total pantothenic acid	10.5% ($n = 5$)	*a*
recovery of pantothenic acid		95%[b]
addition level: 6 mg/kg	99.4% ($n = 3$)	*a*
addition level: 200 μg/kg	97.5% ($n = 3$)	*a*

[a] Not determined. [b] Mean value.

Because it is necessary to use a matrix that does not contain the analyte, edible corn starch with a PA content below the DL was chosen as a model matrix for starch-containing foods such as cereals. Four standard addition levels of PA in starch were analyzed in triplicate in a manner similar to the determination of the DL in a SIDA for the mycotoxin patulin (Rychlik and Schieberle, 1999). After calculation of the confidence interval of the calibration line, a DL of 44 μg/kg and a QL of 131 μg/kg was computed.

To examine repeatability, free PA was quantified within 5 days in duplicate in one polished rice sample, showing an intrasample RSD of 6.7% ($n = 5$). Because of enzyme treatment prior to quantification, the intrasample RSD of total PA amounted to 10.5% ($n = 5$).

Recovery of PA added to polished rice and starch at different levels was between 97.5 and 99.4% (Table 2). Each addition level was analyzed in triplicate.

A comparison of the newly developed SIDA with a recently reported ELISA (Song et al., 1990) revealed similar performance data regarding repeatability and recovery (Table 2). In view of sensitivity, SIDA shows a lower QL than ELISA in food samples on the assumption of identical extraction procedures.

By contrast, for PA analysis in blood plasma ELISA may be more favorable because of its low sample volumes of 50 μL, whereas for the SIDA sample volumes of 1 mL or more are necessary.

Enzyme Hydrolysis of Bound Pantothenic Acid. Liberation of bound PA was achieved by adding pigeon liver pantetheinase (PLP) and alkaline phosphatase (AP) to the aqueous extracts according to Gonthier et al. (1998). PLP was prepared from pigeon liver acetone powder, which had to be treated with an anion-exchange resin to remove PA from the enzyme solution. In preliminary experiments, PLP and AP were tested for PA content and the degree of liberation of PA from CoA. Eight hours of incubation time at 37 °C hydrolyzed 85% of coenzyme A (70 μg) to PA (17 μg), thus revealing sufficient activity of the enzyme mixture. Quantification of the PA content in the mixture of PLP (0.4 mL) and AP (0.8 mL) showed levels as low as 20 ng, whereas the respective amount of pigeon liver acetone powder (50 mg) contained 1.7 μg prior to ion exchange. Nevertheless, all total PA data were corrected for the small exogenous addition of the vitamin.

Although Gonthier et al. (1998) does not recommend destroying endogenous PA liberating enzymes by heating, in the present examination the extracts were sterilized to prevent microbiological growth.

Pantothenic Acid Concentration in Foods. To test the performance of the new method, free and total

Table 3. Free and Total Content of Pantothenic Acid (PA) in Foods and Blood Plasma

sample	free PA (mg/kg)	total PA (mg/kg)	total PA range of literature data (mg/kg)
apple juice	0.23	0.23	0.2−1.0[a,b]
skimmed milk powder	30.3	31.2	32.8−36.0[b,c,d,e]
polished rice	3.93	4.45	6.3−13.4[b,e]
unpolished rice	5.56	20.7	11−17[b,e]
human blood plasma	152[f]	160[f]	104−197[f,g]
porcine blood plasma	337[f]	404[f]	74.8[f,h]

[a] Haenel (1956). [b] USDA (1999). [c] McCance and Widdowson (1992). [d] Møller (1996). [e] Souci et al. (1994). [f] In ng/mL. [g] Song et al. (1985) and Srinivasan and Belavady (1976). [h] Banno et al. (1990)

PA content was determined in three examplary foods. Apple juice, rice, and skimmed milk powder were chosen as representatives for beverages and for starch- and protein-containing foods, respectively. The measured PA concentrations are presented in Table 3.

Apple juice contained only free PA at levels as low as 230 μg/kg, which is well in line with the range of microbiological data covering 200−1000 μg/kg (Haenel, 1956; USDA, 1999).

The measured total PA content in skimmed milk powder of 31.2 mg/kg is in fair agreement with the microbiological data reported in different food composition tables (McCance and Widdowson, 1992; Møller, 1996; Souci et al., 1994; USDA, 1999) ranging from 32.8 to 36.0 mg/kg. Furthermore, skimmed milk powder does not contain substantial amounts of bound PA, which confirms the findings of Song et al. (1984).

In polished rice a lower PA content was found than reported in the literature. SIDA revealed one sample to contain 3.93 mg/kg of free and 4.45 mg/kg of total PA, whereas food composition tables (Souci et al., 1994; USDA, 1999) report total PA content ranging between 6.3 and 13.4 mg/kg. By contrast, the measured total PA concentration of 20.7 mg/kg in unpolished rice was higher than the data of the composition tables mentioned before as ranging from 11 to 17 mg/kg.

In this context, it is good to regard the difference between free and total PA content in the two rice brands. Whereas the small difference in polished rice is consistent with data published by Gonthier et al. (1998), unpolished rice contains more than twice as much bound PA than free PA.

These discrepancies relative to the literature data may be attributed to the wide variation in PA content of different varieties of rice as revealed by the USDA composition tables (1999). Therefore, the results point to the need for further investigation involving a method comparison between SIDA and the microbiological assay. This study is presently in progress.

Pantothenic Acid Concentration in Blood Plasma. Because PA is widely distributed in foods, there is general consensus that a common diet prevents PA deficiency. However, the dietary intake of some population groups showing deviating food habits or increased PA needs, such as adolescents (Eissenstat et al., 1986), is frequently below the estimated safe level set by the food and nutrition board (1989). To discern potential deficiencies, there is increasing emphasis on the surveillance of the PA blood level. Therefore, the newly developed SIDA was applied to PA analysis in blood plasma. Porcine blood plasma (one sample) and human blood plasma (one sample) were found to contain 337 and 152 ng/mL of free PA, respectively (Table 3). In agreement with the report of Song et al. (1985) the vitamin occurs almost exclusively in the free form. The measured difference between the total and free PA content in porcine blood plasma is not statistically significant ($p = 0.05$; Meier and Zünd, 1993). The human plasma PA level of the measured sample (152 ng/mL) was in compliance with the literature data (Song et al., 1985; Srinivasan and Belavady, 1976) ranging between 104 and 197 ng/mL. The data obtained for porcine blood plasma, however, were nearly 5 times as high as the content reported by Banno et al. (1990). But the results of the latter authors have to be questioned because they found concentrations in human plasma that are considerably lower than the concentrations determined by other investigators (Song et al., 1985; Srinivasan and Belavady, 1976).

CONCLUSIONS

In the present study a SIDA for quantifying total and free PA in foods and blood plasma was developed. The method exhibits excellent recovery, repeatability, and a detection limit that is low enough to measure the vitamin even in foods with small PA content. The sample preparation and GC−MS analysis takes only 5 h in contrast to more than 24 h necessary for the microbiological analysis. Compared to the latter method, the separation performance of high-resolution gas chromatography, the specificity of mass spectrometry, and the use of an isotopomeric internal standard make SIDA highly accurate and suitable for food and clinical analyses as well. Recently developed ELISAs appear to be equally convenient techniques (Song et al., 1990; Gonthier, 1998), but they lack commercial availability. In contrast to the preparation of a serum against PA, the presented isotopomeric standard can easily be generated even by chemists having only little synthetic experience.

Therefore, SIDA should be particularly suitable as a reference method in PA analysis. For clinical analyses a reduction of sample volume would enable us to test the PA status in blood by finger puncture. This goal can be accomplished when sensitivity is enhanced by working in the multiple ion detection mode of a high-resolution mass spectrometer. These studies are presently underway.

ACKNOWLEDGMENT

I am grateful to Mrs. D. Fottner for her excellent technical assistance.

LITERATURE CITED

AOAC. Method 945.74. In *Official Methods of Analysis of AOAC International*, 16th ed.; AOAC International: Gaithersburg, MD, 1997a.

Banno, K.; Matsuoka, M.; Horimoto, S.; Kato, J. Simultaneous determination of pantothenic acid and hopantenic acid in biological samples and natural products by gas chromatography−mass fragmentography. *J. Chromatogr.* **1990**, *525*, 255−264.

Eissenstat, B. R.; Wyse, B. W.; Hansen, R. G. Pantothenic acid status of adolescents. *Am. J. Clin. Nutr.* **1986**, *44*, 931−937.

Finglas, P. M.; Faulks, R. M.; Morris, H. C.; Scott, K. J.; Morgan, M. R. A. The development of an enzyme-linked immunosorbent assay (ELISA) for the analysis of pantothenic acid and analogues. II. Determination of pantothenic acid in foods. *J. Micronutr. Anal.* **1988**, *4*, 47−59.

Food and Nutrition Board. In *Recommended Dietary Allowances*, 10th ed.; National Academy Sciences: Washington, DC, 1989.

Fritz, H.; Löwe, W. Determination of the conformation of pantothenic acid and of pantoyl amide by proton resonance spectroscopy (in German). *Angew. Chem.* **1962**, *74*, 751−752.

Gonthier, A.; Fayol, V.; Viollet, J.; Hartmann, D. J. Determination of pantothenic acid in foods: influence of the extraction method. *Food Chem.* **1998**, *63*, 287−294.

Hädrich, J.; Vogelgesang, J. Concept 2000—a statistical approach for analytical practice—Part 1: Limits of detection, identification and determination (in German). *Dtsch. Lebensm. Rundsch.* **1999**, *95*, 428−436.

Haenel, H. Pantothenic acid in foods. *Ernährungsforschung* **1956**, *1*, 533.

Hudson, T. S.; Subramanian, S.; Allen, R. J. Determination of pantothenic acid, biotin, and vitamin B_{12} in nutritional products. *J. AOAC Int.* **1984**, *67*, 994−998.

Kagan, F.; Heinzelman, R. V.; Weisblat, D. I.; Greiner, W. A novel resolution of pantolactone-the synthesis of D-(+)-calcium pantothenate. *J. Am. Chem. Soc.* **1957**, *79*, 3545−3549.

Meier, P. C., Zünd, R. E. In *Statistical Methods in Analytical Chemistry*; John Wiley: New York, 1993.

McCance, R. A.; Widdowson, E. M. In *The Composition of Foods*; Royal Society of Chemistry: Cambridge, U.K., 1992.

Møller, A. In *Levnedsmiddeltabeller*; Levnedsmiddelstyrelsen: Søborg, Denmark, 1996.

Prosser, A. R.; Sheppart, A. J. GLC of trimethylsilyl derivatives of pantothenyl alcohol and pantothenates. *J. Pharm. Sci.* **1971**, *60*, 909−911.

Rychlik, M.; Grosch, W. Identification and quantification of potent odorants formed by toasting of wheat bread. *Lebensm.−Wiss. Technol.* **1996**, *29*, 515−525.

Rychlik, M.; Schieberle, P. Quantification of the mycotoxin patulin by a stable isotope dilution assay. *J. Agric. Food Chem.* **1999**, *47*, 3749−3755.

Song, W. O.; Chan, G. M.; Wyse, B. W.; Hansen, R. G. Effect of pantothenic acid status on the content of the vitamin in human milk. *Am. J. Clin. Nutr.* **1984**, *40*, 317−324.

Song, W. O.; Wyse, B. W.; Hansen, R. G. Pantothenic acid status of pregnant and lactating women. *J. Am. Diet. Assoc.* **1985**, *85*, 192−198.

Song, W. O.; Smith, A.; Wittwer, C.; Wyse, B. W.; Hansen, R. G. Determination of plasma pantothenic acid by indirect enzyme linked immunosorbent assay. *Nutr. Res.* **1990**, *10*, 439−448.

Souci, S. W.; Fachmann, W.; Kraut, H. In *Food Composition and Nutrition Tables*; Medpharm Scientific Publishers: Stuttgart, Germany, 1994.

Srinivasan, V.; Belavady, B. Nutritional status of pantothenic acid in Indian pregnant and nursing women. *Int. J. Vitam. Nutr. Res.* **1976**, *46*, 433−438.

Tanner, J. T.; Barnett, S. A.; Mountford, M. K. Analysis of milk-based infant formula. Phase V. Vitamins A and E, folic acid, and pantothenic acid: food and drug administration-infant formula council: collaborative study. *J. AOAC Int.* **1993**, *76*, 399−413.

Thienpont, L. M.; Nieuwenhove, B. van; Stöckl, D.; de Leenheer, A. P. Calibration for isotope dilution mass spectrometry—description of an alternative to the bracketing procedure. *J. Mass Spectrom.* **1996**, *31*, 1119−1125.

United States Department of Agriculture (USDA), Agricultural Research Service. In *USDA Nutrient Database for Standard Reference*, release 13; Nutrient Data Laboratory home page http://www.nal.usda.gov/fnic/foodcomp, 1999.

Williams, R. R. Fundamental limitations on the use and comparison of signal-to-noise ratios. *Anal. Chem.* **1991**, *63*, 1638−1643.

Williams, R. J. Pantothenic acid and related growth-promoting compounds. U.S. Patent 2,414,682, 1947; *Chem. Abstr.* **1947**, *41*, 3118.

Williams, R. J.; Mitchell. H. K.; Weinstock, H. H.; Snell, E. E. Pantothenic acid. VII. Partial and total synthesis studies. *J. Am. Chem. Soc.* **1940**, *62*, 1784−1785.

Williams, T. W.; Salin, E. D. Hazards of a naive approach to detection limits with transient signals. *Anal. Chem.* **1988**, *60*, 726−727.

Wilson, E. H.; Weijlard, J.; Tishler, M. Pantothenic acid salts. *J. Am. Chem. Soc.* **1954**, *76*, 5177−5178.

Received for review December 1, 1999. Revised manuscript received February 1, 2000. Accepted February 2, 2000.

JF9913054