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Pantothenic Acid Quantification: Method Comparison of a Stable Isotope Dilution Assay and a Microbiological Assay

Michael Rychlik[a*] and Dora Roth-Maier[b]

[a] Lehrstuhl für Lebensmittelchemie der Technischen Universität München, Lichtenbergstr. 4, D-85748 Garching, Germany
[b] Fachgebiet Tierernährung des Departments Tierwissenschaften der Technischen Universität München, Hochfeldweg 6, D-85350 Freising-Weihenstephan, Germany

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Abstract: Different foods and feedstuffs were analyzed for pantothenic acid (PA) by the recently developed stable isotope dilution assay (SIDA) and by the standard method, a microbiological assay (MA). The SIDA involved the use of [$^{13}C_3$, ^{15}N]-pantothenic acid as the internal standard and detection by liquid chromatography-tandem mass spectrometry. The analysis of identical extracts minimized systematic bias due to equal extraction yields and enabled an ideal comparison between both methods.

For the samples derived from plants a good accordance between the MA and the SIDA of total PA was found, whereas for the products of animal origin, higher contents were measured by MA than by SIDA. From the results of treatments by pantetheinase and phosphatase on the one hand and papain and diastase on the other, it was concluded that MA is able to measure a significant amount of bound PA. Furthermore, the data imply that microbial enzymes were able to cleave PA conjugates more effectively than pantetheinase and phosphatase treatment.

Key words: Method comparison, microbiological assay, pantothenic acid, stable isotope dilution assay

Introduction

(R)-Pantothenic acid (PA) is a vitamin that occurs in foods and feedstuff in its free form as well as in conjugates such as coenzyme A (CoA) or acyl carrier protein (ACP). As PA is involved in metabolism of steroids, fatty acids, and phosphatides, data on its dietary uptake by humans as well as by cattle are important.

The reference method for quantifying PA is a microbiological assay (MA) measuring turbidimetrically the growth of *Lactobacillus plantarum* in a medium deficient in PA. However, as MA is lengthy and lacks specificity [1], there are increasing efforts to replace it by more ac-

* Corresponding author

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curate methods. Therefore, a stable isotope dilution assay (SIDA) has been developed recently using [$^{13}C_3$, ^{15}N]-PA as the internal standard, detecting the isotopomers by gas chromatography-mass spectrometry [2] or liquid chromatography-tandem mass spectrometry [3]. In the latter reports, the results of PA quantitation in foods compared well with literature data. However, a direct comparison of SIDA with the reference method is still lacking. Therefore, the objective of the present study was to perform a site-by-site comparison of identical samples analyzed by both assays.

Experimental

Chemicals

The following chemicals were obtained commercially from the sources given in parentheses: (R)-pantothenic acid hemicalcium salt, (Aldrich, Steinheim, Germany); acetonitrile, ammonium sulfate, diastase, dichloromethane, ethyl acetate, formic acid, hydrochloric acid, $KHCO_3$, methanol, $NaHCO_3$, papain, sodium acetate, Na_2SO_4 (Merck, Darmstadt, Germany); alkaline phosphatase, coenzyme A from yeast, and liver acetone powder from pigeon (Sigma, Deisenhofen, Germany).

[^{15}N, $^{13}C_3$]-pantothenic acid was synthesized as reported recently [2].

Sample preparation for SIDA and MA

Cereals were ground in a grain mill (Bosch, München, Germany) and hazelnuts were minced in a blender (Privileg, Quelle, Fürth). Porcine liver, whole eggs, and mushrooms were lyophilized and the residue ground in a mortar. The resulting powders (2 g) were stirred for 24 hours at 20°C in an aqueous solution of sodium acetate (160 mL, 0.02 mol/L, pH 4.6) containing papain (80 mg) and diastase (80 mg). To inactivate the enzymes, the extracts were then autoclaved at 121°C for 8 minutes and adjusted to a pH of 6.8 by addition of NaOH. Each extract was then filtered and divided into two halves, one of which was used for SIDA and the other for MA.

SIDA for quantification of free and total pantothenic acid

To an aliquot (10 mL) of each extract, calcium [^{15}N, $^{13}C_3$]-(R)-pantothenate (5 µg) was added and the resulting solution, after passing through a syringe filter (0.4 µm, Millipore, Bedford, MA, USA), was analyzed for free PA by liquid chromatography-tandem mass spectrometry (LC/MS/MS).

For enzyme hydrolysis of PA conjugates, pantetheinase was prepared from pigeon liver acetone powder as reported recently [2]. Solutions of pantetheinase (0.4 mL), alkaline phosphatase (0.8 mL, 2%) and $NaHCO_3$ (1 mL, 0.85%) were added to the extract (5 mL). The mixture was then alkalized to pH 8 by addition of aqueous sodium hydroxide (1 mol/L) and incubated for 8 hours at 37°C. Subsequently, the hydrolysate was acidified to pH 5.6 by addition of hydrochloric acid (1 mL, 18 mol/L) and submitted to LC/MS/MS.

Efficiency of enzyme hydrolysis was tested by treating coenzyme A (20 µg) as previously detailed and by quantifying PA by SIDA.

Microbiological assay (MA)

The extracts were assayed for pantothenic acid with *Lactobacillus plantarum* (ATTC 8014) in the medium of Strohecker and Henning [4] (Difco™ Pantothenate Assay Medium; Becton, Dickinson and Company Sparks, MD 21152). *Lactobacillus plantarum* growth was measured spectrophotometrically after an 18-hour incubation time at 460 nm (Uvicon 933, Kontron Instruments, Eching, Germany).

Liquid chromatography/tandem mass spectrometry (LC/MS/MS)

LC/MS/MS was performed by means of an LCQ (Finnigan MAT, Bremen, Germany) coupled to a spectra series high-performance liquid chromatograph (Thermo separation products, San Jose, CA, USA) equipped with an Aqua C-18 reversed phase column (250 × 4.6 mm; 5 µm, Phenomenex, Aschaffenburg, Germany). Fifty µL of the sample solutions were chromatographed using gradient elution with variable mixtures of aqueous formic acid (0.1%, solvent A) and acetonitrile (solvent B), at a flow of 0.8 mL/min. After flushing the column for 9 minutes with 7% B, a 13-minute linear gradient was programmed to 17% B followed by a further 3-minute linear gradient to 25% B. Then, the concentration of B was raised immediately to 100%, maintained for 5 minutes and subsequently brought back to the initial mixture for another 5 minutes to allow for column equilibration. During the first 8 minutes of the gradient program, the column effluent was diverted to waste to ensure an adequate spray stability. For [^{15}N, $^{13}C_3$]-pantothenic acid, the mass transitions (m/z precursor ion/m/z product ion) 224/206 and 224/188 and for unlabeled pantothenic acid, the mass transitions 220/202 and 220/184 were chosen. The isolation width of the precursor ion was adjusted to 3 Da and the isolation width of the product ion was set to 1 Da in order to detect the production most selectively. The mass spectrometer operated

in the positive electrospray mode with a spray needle voltage of +5 kV and a spray current of 20 µA. The temperature of the capillary was 200°C and the capillary voltage was +13 V. The sheath and auxiliary gas nitrogen nebulized the effluent with flows of 68 and 19 arbitrary units, respectively. The ion trap was operated at a helium pressure of 10^{-3} Torr.

Results and discussion

Design of the study and performance of the methods

As the purpose of this study was to survey the differences between MA and SIDA, various feedstuffs and foods from plants as well as of animal origin were analyzed. For sample preparation we employed a standard procedure described by Roth-Maier *et al* [5]. This method involved extracting PA with acetate buffer (pH 4.6) and liberating by papain and diastase (PD) treatment those parts of PA that are encapsulated within the food matrix. Following this incubation of 24 hours at 37°C, the extracts were filtered and divided in two halves. The first half was analyzed by monitoring spectrophotometrically the growth of *Lactobacillus plantarum*. To the second part of the extract, the isotopomeric standard [^{15}N, $^{13}C_3$]-PA was added and the solution was analyzed by LC-tandem mass spectrometry as reported recently [3]. This study design made sure that not only identical foods were analyzed but also identical extracts, thus avoiding imprecision due to varying extraction yields. As SIDA without further enzyme treatment cannot measure PA conjugates such as coenzyme A or acyl carrier protein, aliquots of the solutions were incubated additionally with pantetheinase and phosphatase. MA, however, has been shown partly to be able to measure even PA conjugates [6]. Therefore, further enzyme treatment for MA was omitted.

During the present study, both methods appeared to be sufficiently sensitive and precise for PA quantification. Even the orange juice sample, showing very small PA content, could be quantified and the mean CV of multiple measurements was 5.0% for MA and 10.4% for SIDA. However, the requirement on laboratory equipment and skills are rather different as, on the one hand, sterile working conditions and experience in handling microorganisms are necessary for MA. On the other hand, SIDA requires preparation of the isotopomeric standard and the expensive LC/MS/MS equipment. Regarding analysis times, MA took about 24 hours for incubation and turbidimetry of the bacteria whereas for SIDA, the time of a LC/MS/MS run was only about 30 minutes (Fig. 1).

Feeds and foods of plant origin

The results of plant-derived samples are listed and compared to the data of databases [7, 8] in Table I.

For cereals and potatoes, the results of MA and SIDA of total PA were in quite good accordance, with a maximum deviation of 21% for wheat. Moreover, our results were quite well in line with the two databases considered. In the case of orange juice, which contains relatively small amounts of PA, the results of the both methods were also consistent and matched the entrances of the databases.

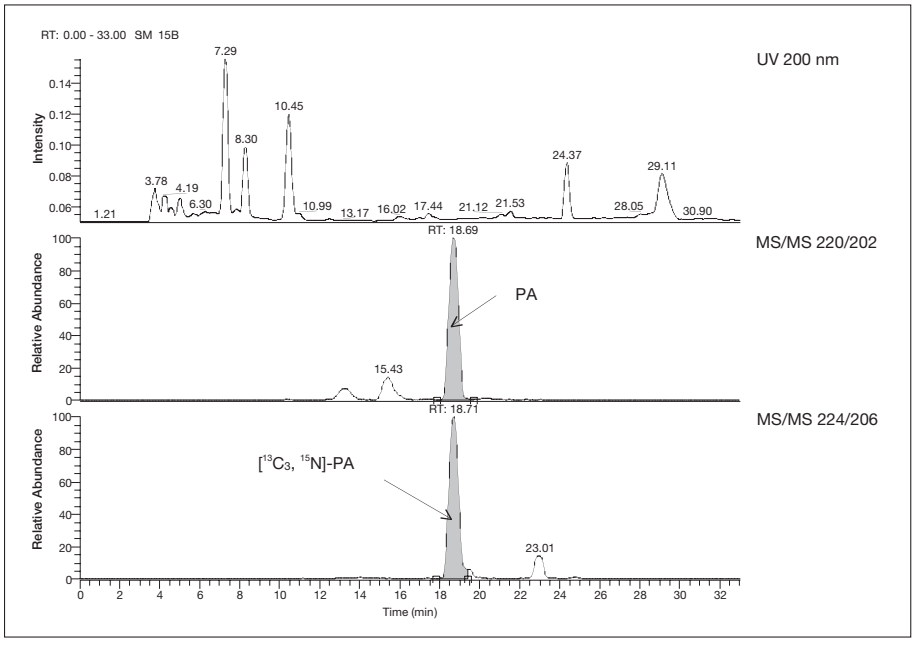

Figure 1: LC/MS/MS chromatograms of mushroom powder. The internal standard [$^{13}C_3$, ^{15}N]-pantothenic acid ([$^{13}C_1$, ^{15}N]-PA) is detected in the trace MS/MS 224/206, unlabeled pantothenic acid (PA) in trace MS/MS 220/202. UV:UV absorption.

Table I: Comparison of pantothenic acid (PA) data obtained by stable isotope dilution assay (SIDA) and by microbiological assay (MA) with those reported in the literature

mg/100 g	SIDA of		MA	Literature data [7, 8]
sample	free PA	total PA		
millet	0.37	0.61	0.76	0.35–1.20
wheat	0.64	0.84	1.07	0.85–1.75
corn	0.22	0.65	0.65	0.48–0.76
potato	0.25	0.25	0.28	0.26–0.50
cocoa	0.67	1.39	1.36	0.2–1.1
hazelnuts	0.85	1.22	1.64	0.918–1.18
soy beans	1.45	1.76	1.91	0.80–1.95
orange juice	0.08	0.08	0.18	0.15–0.17
mushroom	1.17	1.29	1.23	1.5–2.7
whole egg pwd.	7.78	8.09	9.53	5.905–10.0
skim. milk pwd.	5.23	5.61	6.33	3.39–3.50
porcine liver	4.35	5.23	6.59	6.4–7.0

However, the situation was quite different for the remaining samples derived from plants. For cocoa, mushrooms, and soy beans the two methods were also in good agreement, but the data were very different from those in the databases. As the ranges of contents have been reported to be very broad for soy and mushrooms [7], or there were only very few data entrances for cocoa [8], the literature data appear to be inconsistent or incomplete. A possible explanation for this uncertainty is the discrepancy in microbiological results when different microorganisms are employed [9].

For hazelnuts, a significant difference of 25% between MA and SIDA was observed. The reasons for this discrepancy remain open, but the data imply that nuts contain substances that stimulate the growth of the microorganisms without being measured by SIDA, even after all enzyme treatments. These compounds might be yet unknown PA forms or substances that the microorganisms can utilize for their growth and that are structurally different from PA conjugates. This might also hold true of millet, wheat, and orange juice, for which the MA gave significantly higher PA figures than SIDA. In case of cocoa, corn, and mushrooms, the slightly higher figures of SIDA compared to those of MA were not significantly different and were obviously due to random dispersion.

Feeds and foods of animal origin

For comparing the PA data in animal-derived products, whole-egg powder, skimmed milk powder, and porcine liver were analyzed by the two methods. As expected, these samples contained much more PA than the foods and feeds of plant origin and appear to be a much better source of dietary PA. In analogy with the latter products, PA data of SIDA were lower than those of MA, but the differences were much more pronounced and amounted up to 1.5 mg/100 g in the case of whole-egg powder. This observation corresponded to that of Walsh *et al* [10], who performed a method comparison between radioimmunoassay and MA for PA analysis. The latter authors found, consistent with our results, a good agreement of the data from plant-derived foods and significantly higher data of the MA for meat.

Regarding the contents of free and total PA, the part of bound PA for these products was below 10% of the total PA content, which is in compliance with our earlier reports [2, 3] and those of other authors [11, 12]. Furthermore, our data for whole-egg powder and liver matched those of the databases, whereas for skimmed milk powder both methods gave significantly higher PA contents than reported. As only one sample was analyzed in the present study, we assume that the discrepancies might be due to different drying technologies used (spray- vs. roller-drying) and the sample analyzed is obviously not representative for these kinds of products.

Effects of the enzyme treatments

To get a more detailed insight into the actions of the enzymes used, extracts of millet, mushrooms, and skimmed milk powder were also analyzed without treatment by papain and diastase (PD). As displayed in Figure 2, for millet and milk powder the PA content of MA and SIDA were found to be lower when omitting PD incubation. In the case of mushrooms, however, no significant discrepancies were observed. Comparing the two methods, the differences between the millet data of MA, SIDA of total PA, and SIDA of free PA were very similar for the quantification with and without PD treatment. Therefore it may be concluded that these enzymes in millet do not alter the ratio between the different PA active substances but only help in liberating these compounds from the matrix. However, for milk powder the difference between MA and SIDA of total PA with PD treatment was significantly higher than without PD incubation, whereas the difference between SIDA of free and total PA remained constant. As SIDA detects PA very specifically, it can be concluded that PD treatment not only liberated encapsulated PA-active compounds in milk powder, but also cleaved conjugates to substances that promote growth of the bacteria without being free PA itself. It might therefore be speculated that these compounds are bound to carbohydrates or peptides.

Determination of coenzyme A

A survey of the literature indicates that CoA is considered the most abundant PA conjugate in organisms [6]. To confirm that this compound was responsible for the differences between SIDA of total and free PA, we additional-

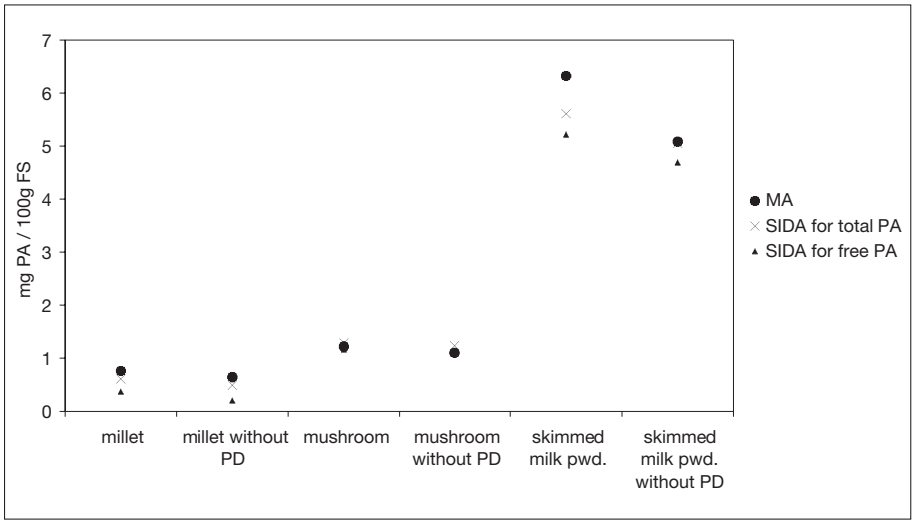

Figure 2: Pantothenic acid (PA) content of different foods measured by microbiological assay (MA), stable isotope dilution assay (SIDA) for free PA and for total PA with and without papain and diastase (PD) treatment.

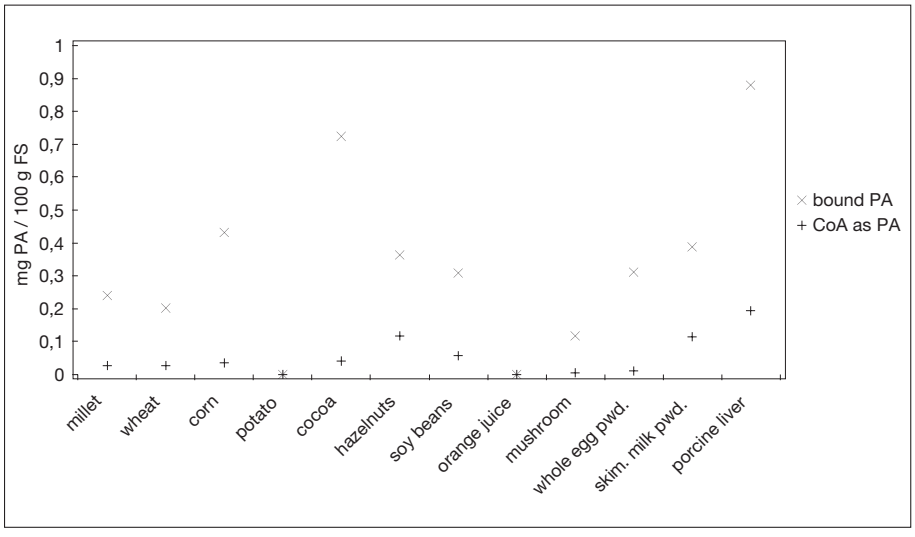

Figure 3: Content of bound pantothenic acid (PA) and Coenzyme A (CoA) in different foods.

ly analyzed the samples for their content of CoA. This was achieved by using the enzymatic recycling procedure reported by Gibon *et al* [13]. This method was sensitive enough to measure the contents of CoA in all samples. However, in nuts and soy beans, an enzyme inhibition of up to 50% of their normal activity was observed. Therefore in additional assays, known amounts of CoA were added to the extracts of these samples to determine the recovery of CoA. Thereafter, this figure was used to compensate for inhibition. Unexpectedly, the CoA contents in the samples shown in Figure 3 appeared to be too low to explain the differences between the SIDA of total and free PA. Therefore, other conjugates of PA are supposed to play a more important role than expected. For example, glucosylated PA has been reported in tomato juices and was found to stimulate bacterial growth much more effectively than PA [14]. If this compound was present in the samples, it may be not reacted by PD and might be partially responsible for increased bacterial growth during MA.

Total PA measurement by MA and SIDA

As noted above, MA for nearly all foods and feedstuffs gives somewhat higher data than SIDA of total PA. This result indicates that MA data using diastase and papain include conjugated PA without the need for further enzymatic treatment. Therefore, our suggestions are contradictory to those of Gonthier *et al* [6], who found PD treatment of minor effectiveness in total PA analysis as it liberated in model assays only 22% of PA from CoA. However, as our results indicate that CoA is not as abundant as expected, PD incubation may be very effective in cleaving PA conjugates for microbial growth. These findings

are in compliance with those of Gonthier *et al* [6], who reported diastase and papain to give for several composite foods the highest PA results in MA measurement compared to other enzyme treatments.

Conclusions

The method comparison presented here revealed for a number of foods and feedstuffs a quite good accordance between the MA and the SIDA of total PA. From this finding and from the results of the different enzyme treatments it can be concluded that MA is able to measure a significant amount of bound PA. Moreover, MA showed significantly higher data than SIDA of total PA, which could only be explained by the presence of compounds that show microbiological PA activity, but do not liberate PA when treated with common deconjugating enzymes. Another possible explanation has been given by Walsh *et al* [10], who suggested that microbial enzymes were able to cleave PA conjugates more effectively than pantetheinase and phosphatase treatment. The structures of these conjugates or PA-active compounds, however, are still unknown and must be elucidated in future studies.

With regard to human and animal nutrition it has yet to be considered which method gives a realistic content of bioavailable PA. Although the action of pantetheinase in intestinal lumen has been detected [15], and absorption of PA has been reported to run via an active transport mechanism[16], PA from foods has been found to show an availability of only 30 to 81% compared to synthetic PA, depending on the food source [5, 17]. As the bioavailability of the single PA forms is still unknown, the question of the best method to measure bioavailable PA remains open.

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Michael Rychlik

Lehrstuhl für Lebensmittelchemie
der Technischen Universität München,
Lichtenbergstr. 4, D-Garching
Phone +49-89-289 132 55
Fax +49-89-289 141 83
E-mail: michael.rychlik@ch.tum.de