# Tryptophan-*N*-glucoside in Fruits and Fruit Juices

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In extracts prepared from various fruits as well as in fruit juices a single tryptophan glycoconjugate was detected by HPLC-MS analysis. Product ion spectra demonstrated the *N*-glycosidic linkage of a hexose moiety to the indole nitrogen. For structure elucidation, the novel tryptophan glycoside was isolated from pear juice and identified as $N^1$-($\beta$-D-glucopyranosyl-$^4C_1$)-L-tryptophan by $^1$H, HH−COSY and $^{13}$C NMR spectroscopy. Finally, we disclosed the biosynthetic origin of the novel tryptophan metabolite by demonstrating the enzymatic glycosylation of deuterium-labeled tryptophan, which was applied to pear fruit.

**Keywords:** *Tryptophan-N-glucoside; electrospray ionization; HPLC-MS/MS; biosynthesis*

## INTRODUCTION

In humans, animals and some eubacteria, the essential amino acid L-tryptophan is one limiting factor in protein biosynthesis and is involved in biosynthesis of nicotinic acid derivatives. In plants however, tryptophan serves as precursor for the plant growth hormone indole-3-acetic acid, for phytoalexins, glucosinolates, and alkaloids. Thus, tryptophan plays an important role in the regulation of plant development, in pathogen defense response, and plant−insect interactions (Herderich and Gutsche, 1997). Furthermore, tryptophan-derived alkaloids exhibit a variety of pharmacologically important effects. For example, $\beta$-carbolines such as harman, which has been identified in numerous food samples including soy sauce, vinegar, beer, and wine (Adachi et al., 1991), can act as benzodiazepine receptor antagonists (Cooper, 1987), inhibitors of human monoamine oxidase A (Kim et al., 1997), and comutagens (Meester, 1995). It should be noted that various $\beta$-carbolines occur in edible plants such as cashew nut, walnut, pineapple, and banana (Tsuchiya et al., 1999), while structurally related 1,2,3,4-tetrahydro-$\beta$-carboline-3-carboxylic acids have been detected in various products such as wine, soy sauce (Sen et al., 1995), fruit juices, purees, and jams (Herraiz et al., 1998).

Our interest for the natural occurrence of tryptophan glycoconjugates as a new group of tryptophan derivatives was raised by reports on enzymatically glycosylated tryptophan residues in proteins (Hofsteenge et al., 1994; Hofsteenge et al., 1999). Recently, we demonstrated for the first time the presence of 2-(α-mannopyranosyl)-L-tryptophan, a new metabolite, in human urine. In addition, we identified tryptophan-*N*-glycosides, tryptophan-*C*-glycosyl conjugates, and *glyco*-tetrahydro-$\beta$-carbolines as products derived from chemical condensation of tryptophan and aldohexoses in various food samples (Gutsche et al., 1999). During these studies, observation of one distinct tryptophan-*N*-glycoside in fruit syrup, which was sold as natural sweetener, attracted our attention. Consequently, we studied the occurrence of tryptophan glycoconjugates in plants.

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In this contribution, we report on the detection of a unique tryptophan-*N*-glucoside in numerous fruits and juices, present the isolation and structure elucidation, and demonstrate the biosynthetic formation of this novel tryptophan metabolite.

## MATERIALS AND METHODS

**Apparatus.** HPLC-MS was performed with an Applied Biosystems 140b pump utilizing a TSQ 7000 tandem mass spectrometer system equipped with an ESI interface (Finnigan MAT, Bremen, Germany). Data acquisition and evaluation were conducted on a DEC 5000/33 (Digital Equipment, Unterföhring, Germany) with ICIS 8.1 software (Finnigan MAT, Bremen, Germany). HR-ESI-MS data were recorded on a APEX II Fourier transform mass spectrometer (Bruker Daltonik, Bremen, Germany). HPLC-DAD analysis was performed with a Hewlett-Packard gradient pump (series 1100) and a Hewlett-Packard photodiode array detector (series 1100) including Hewlett-Packard software. For preparative HPLC, a Knauer 64 pump and a Shimadzu SPD-10A UV detector were used. Data acquisition was conducted with Knauer Eurochrom 2000 software. UV−VIS and CD spectra were recorded on a Shimadzu (Duisburg, Germany) UV-2101PC spectrophotometer and a JASCO (Hachioji, Japan) J-600 spectropolarimeter. Optical rotation was determined on a Perkin-Elmer polarimeter 241 MC. NMR spectra were measured in $CD_3OD/CF_3COOD$ with a Bruker 600 spectrometer for $^1$H and HH−COSY spectra and a Bruker 400 spectrometer for $^{13}$C spectra utilizing the chemical shift of the $CD_3OD$ signal for calibration (3.31 ppm for $^1$H NMR, 49.0 ppm for $^{13}$C NMR).

**Reagents.** Water, acetonitrile, and methanol (all of HPLC grade), trifluoroacetic acid (TFA) (spectroscopic grade), and Lichroprep C18 (40−63 $\mu$m particle size) were obtained from Merck (Darmstadt, Germany). L-[Indole-$d_5$]-tryptophan was from Cambridge Isotope Laboratories (Andover, MA). All chemicals were of analytical purity. Membrane filters with pore size 0.2 $\mu$m were from Ziemer (Mannheim, Germany). Fruits and fruit juices were purchased at local markets.

**Sample Preparation.** Juice samples were filtered through membrane filters of pore size 0.2 $\mu$m. The fresh and ripe fruits were intensively crushed for several minutes with a kitchen blender and centrifuged at 8000 g for 10 min. The supernatant was filtered through membrane filters of pore size 0.2 $\mu$m. The resulting solutions were directly subjected to HPLC-ESI-MS/MS analysis. We analyzed three pear juices (two from concentrate, one not from concentrate) and two apple juices (not from concentrate). For quantitative analysis of fruits, we used samples of two pear cultivars (Packhams, Williams Christ;

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weight 120−140 g) and of three apple cultivars (Elstar, Jonagold, Gala Royal; weight 150−250 g). Furthermore, we analyzed one sample each of apricots (ca. 220 g), kiwi (ca. 70 g), and plums (ca. 130 g) and two samples each of raspberries (70−90 g) and peaches (170−200 g). The amount of juice obtained after centrifugation was about two-third of the fresh weight of the fruit sample.

**Identification of Tryptophan-*N*-glucoside and Quantitative Determination in Fruits and Fruit Juices by HPLC-MS/MS.** Chromatographic separation was performed on a Symmetry C18 column (150 mm × 2.1 mm i.d., 5 μm) (Waters, Milford, MA) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was acetonitrile. HPLC was programmed as follows: pressurizing with 50% B, 10 min equilibration time at 5% B, and linear gradient elution (0 min, 5% B; 30 min, 30% B). The flow rate was 200 μL/min and the injection volume was 5 μL for all analyses. For pneumatically assisted ESI, the spray voltage was set to 3.5 kV; the temperature of the heated inlet capillary was 220 °C. Nitrogen served both as sheath (60 psi) and auxiliary gas (10 units). Product ion scanning was performed at a collision gas pressure of 267 mPa of Ar and a collision energy of 20 eV with a total scan duration of 1.0 s for a single spectrum. The most abundant product ions were chosen for the selected reaction monitoring (SRM) experiments. Selected ion pairs were as follows: SRM I, *m/z* 367/247, 367/229 for detection of *C*-glycosyl conjugates and *glyco*-tetrahydro-β-carbolines with a scan duration of 0.5 s for each experiment; SRM II, *m/z* 367/205, 367/188 for detection of *N*-glycosides with a scan duration of 0.5 s for each experiment; SRM III, *m/z* 367/188 with a scan duration of 1.0 s. SRM I and II were used for the initial screening; SRM III was chosen for quantitative determinations. Quantitative evaluations were performed with standard solutions (0, 0.1, 0.5, 1.0, 5.0, 10.0, 20.0 μg/mL) of tryptophan-*N*-glucoside in water for external calibration (calibration function $y = 1.5955x - 0.0952$, $r = 0.9997$). Values were calculated from peak areas of the product ion *m/z* 188 as obtained by HPLC-ESI-MS/MS analysis with SRM III.

**Isolation of Tryptophan-*N*-glucoside from Pear Juice.** Pear juice (1 L, adjusted to pH 7) was applied to a Lichroprep C18 column (32 cm × 3.4 cm, 40−63 μm particle size). Before sample application, C18 material was equilibrated with 1 L of water/TFA (100:0.05, v/v). After the column was loaded with sample, it was washed with 350 mL of water/TFA (100:0.05, v/v), and then eluted with 350 mL of water/acetonitrile/TFA (95:5:0.05, v/v/v) and 1 L of water/acetonitrile/TFA (90:10:0.05, v/v/v). Fractions (10 mL each) were analyzed by HPLC with photodiode array detection (200−600 nm) with an Eurospher 100 C18 column (250 × 4 mm i.d., 5 μm) (Knauer, Berlin, Germany) using the same binary gradient as that described for HPLC-MS/MS analysis. Fractions 77−81 containing tryptophan-*N*-glucoside were pooled, freeze-dried, and further purified by semipreparative HPLC on an Eurospher 100 $C_{18}$ column (250 × 16 mm i.d., 5 μm) (Knauer, Berlin, Germany) with water/methanol/TFA (90:10:0.05, v/v/v) as solvent and UV detection at 262 nm. The flow rate was 7.5 mL/min; the retention time of tryptophan-*N*-glucosid was 40 min. The separation procedure yielded 10 mg of a pale yellow amorphous powder: ESI-MS, $[M+H]^+$ *m/z* 367; ESI-MS/MS (20 eV, 267 mPa Ar), *m/z* 350, 331, 247, 230, 205, 188; UV $\lambda_{max}^{H_2O}$, 221 nm (28 000 $M^{-1}$ $cm^{-1}$), 270 nm (6000 $M^{-1}cm^{-1}$); CD ($H_2O$), 224 nm ($\epsilon$ + 1.7), 203 nm ($\epsilon$ −5.8); optical rotation, $\alpha^{25}_D = -20.3$ (0.22, $H_2O$). HR-ESI-MS calcd for $C_{17}H_{23}N_2O_7$ $[M+H]^+$: 367.14998. Found: 367.15001.

**Model Reaction of L-[Indole-$d_5$]-tryptophan with Glucose.** L-[Indole-$d_5$]-tryptophan (10 mg) and 18 mg of D-glucose were dissolved in 320 μL of water, adjusted to pH 1 with 2 M HCl and left at 80 °C for 12 days.

**Application of L-[Indole-$d_5$]-tryptophan to Pear Fruits and Incubation of L-[Indole-$d_5$]-tryptophan in Model Juice.** A 200 μL aqueous solution of L-[indole-$d_5$]-tryptophan (10 mg/mL) was injected subepidermally with a syringe into three pears each (Durondeau, Belgium, weight 120−140 g). After storage at room temperature for 2, 13, and 15 days, the fruit juice samples were prepared as described above and analyzed by HPLC-MS/MS. We used as a model juice an aqueous solution containing 2.2% glucose, 6% fructose, and 1.1% sucrose with a pH of 3.8 (adjusted with malic acid). L-[Indole-$d_5$]-tryptophan (1.5 mg) was dissolved in 100 mL of model juice, the solution (containing 0.02% $NaN_3$ to prevent growth of microorganisms) was stored at room temperature, and aliquots for HPLC-MS/MS analysis were taken after 6 and 23 days. Chromatographic separation was performed as described previously. HPLC was programmed as follows: pressurizing with 50% B, 10 min equilibration time at 5% B, and linear gradient elution (0 min, 5% B; 18 min, 20% B). Conditions for pneumatically assisted ESI were the same as those described previously. The most abundant fragment ions in the product ion spectra of $d_0$-tryptophan-*N*-glucoside (as obtained from MS/MS analysis of tryptophan-*N*-glucoside in pear juice, Figure 1), of $d_5$-tryptophan-*N*-glucoside and of $d_4$-tryptophan-*C*-glucosyl derivative and $d_4$-*gluco*-tetrahydro-β-carbolines (as obtained from MS/MS analysis of a model reaction of $d_5$-tryptophan with glucose, data not shown), respectively, were chosen for the selected reaction monitoring (SRM) experiments. Selected ion pairs were *m/z* 367/188 for $d_0$-tryptophan-*N*-glucoside, *m/z* 372/192 for $d_5$-tryptophan-*N*-glucoside and *m/z* 371/251 for the $d_4$-tryptophan-*C*-glucosyl derivative and $d_4$-*gluco*-tetrahydro-β-carbolines with a scan duration of 0.3 s for each experiment.

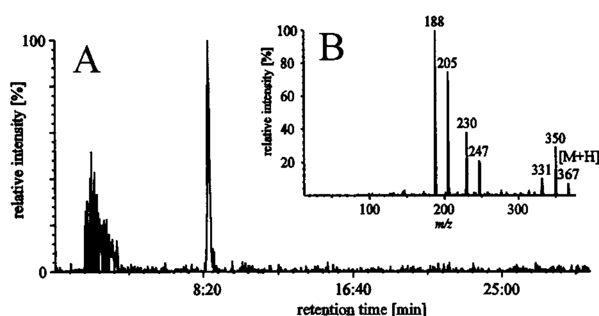


**Figure 1.** HPLC-MS/MS analysis of pear juice. (A) Mass chromatogram of *m/z* 367 ($[M+H]^+$ of tryptophan−hexosides). (B) Product ion spectrum of tryptophan-*N*-glucoside in pear juice (precursor ion *m/z* 367, 20 eV, 267 mPa Ar).

To determine molecular ions of $d_4$/$d_5$-tryptophan-*N*-glucoside obtained from model reactions as well as $d_5$-tryptophan-*N*-glucoside from pear conditions were as follows: Chromatographic separation was performed as described previously. HPLC was programmed as follows: pressurizing with 50% B, 10 min equilibration time at 5% B, and linear gradient elution (0 min, 5% B; 18 min, 20% B). The injection volume was 27 μL for analysis of pear extract and 3 μL for analysis of the model reactions. Conditions for pneumatically assisted ESI were the same as those described previously. Spectra were acquired scanning from *m/z* 369 to *m/z* 379 with a scan duration of 0.5 s.

## RESULTS AND DISCUSSION

By HPLC-ESI-MS/MS analysis, one distinct tryptophan glycoconjugate was detected in numerous fruit extracts and commercially available fruit juices (Figure 1) in concentrations between 0.1 mg/L and > 10 mg/L (Table 1). From retention time and product ion spectra, the compound most likely was a tryptophan-*N*-hexoside. The product ion spectrum obtained by low-energy CID of the molecular ion *m/z* 367 $[M+H]^+$ is dominated by the fragment ions *m/z* 205 and *m/z* 188 corresponding to loss of an intact anhydro-sugar moiety $C_6H_{10}O_5$ and subsequent loss of $NH_3$. This fragmentation pattern is characteristic for *N*-glycosidically linked sugars (Frear et al., 1989), whereas in product ion spectra of *C*-glycosyl derivatives and *glyco*-tetrahydro-β-carbolines, the prominent ions *m/z* 247 and *m/z* 229 result from loss of a

**Table 1. Tryptophan-*N*-glucoside in Fruits and Fruit Juices**

| $n^a$ | sample | tryptophan-*N*-glucoside [mg/L] |
|---|---|---|
| 3 | pear juice | 13.5 ± 1.7 |
| 2 | pear fruit | 7.3 ± 1.4 |
| 2 | apple juice | 0.7 ± 0.2 |
| 1 | apricot fruit | 0.3 ± 0.0 |
| 3 | apple fruit | 0.2 ± 0.1 |
| 2 | peach fruit | 0.2 ± 0.0 |
| 2 | raspberry fruit | 0.2 ± 0.0 |
| 1 | kiwi fruit | ≥0.1 |
| 1 | plum fruit | ≥0.1 |

[a] number of samples analyzed.

**Table 2. $^1$H and $^{13}$C NMR Data of the Isolated Tryptophan-*N*-glucoside**

| atom | δ $^1$H [ppm] | δ $^{13}$C [ppm] |
|---|---|---|
| tryptophan | | |
| COOH | | 171.6 |
| 2 | 7.37 (s, 1 H) | 126.0 |
| 3 | | 109.6 |
| 3a | | 129.2 |
| 4 | 7.62 (d, 1 H) | 119.5 |
| 5 | 7.14 (dd, 1 H) | 121.5 |
| 6 | 7.23 (dd, 1 H) | 123.7 |
| 7 | 7.57 (d, 1 H) | 111.7 |
| 7a | | 138.8 |
| 8a | 3.48−3.55 (m, 1 H)[a] | 27.4 |
| 8b | 3.28−3.34 (overlapped by $CD_3OD$ signal)[b] | |
| 9 | 4.28 (dd, 1 H) | 54.4 |
| sugar moiety | | |
| 1' | 5.50 (d, 1 H) | 86.3 |
| 2' | 3.85−3.93 (m, 1 H)[c] | 74.0 |
| 3' | 3.64 (dd, 1 H) | 78.9 |
| 4' | 3.48−3.55 (m, 1 H)[a] | 71.4 |
| 5' | 3.61 (ddd, 1 H) | 80.6 |
| 6'a | 3.85−3.93 (m, 1 H)[c] | 62.6 |
| 6'b | 3.72 (dd, 1H) | |

*J* [Hz]: H-4, H-5 = 7.9; H-5, H-6 = 6.9; H-6, H-7 = 8.3; H-8a, H-9 = 4.0; H-8b, H-9 = 7.6; H-1', H-2' = 9.1, H-2', H-3' = 8.9; H-3',H-4' = 9.1; H-4', H-5' = 9.7; H-5', H-6'a = 2.2; H-5', H-6'b = 5.5; H-6'a, H-6'b = 12.0. [a,c] Overlapping signals, two protons by integration of signal. [b] Signal assignment was confirmed by HH−COSY.

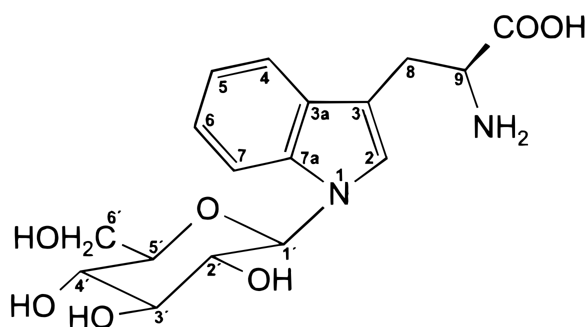


**Figure 2.** $N^1$-(β-D-glucopyranosyl-$^4C_1$)-L-tryptophan.

$C_4H_8O_4$ moiety and subsequent loss of $H_2O$ (Gutsche et al., 1999). Yet information about configuration and conformation of the sugar residue is not readily available from the product ion spectra. Motivated by the adequate concentration of tryptophan-*N*-glycoside in pear juice, we started the isolation from this material. HR-ESI-MS analysis confirmed the molecular formula $C_{17}H_{23}N_2O_7$ for the molecular ion *m/z* 367 $[M+H]^+$ of the compound under study. The structure of the purified compound was deduced by $^1$H, HH−COSY, and $^{13}$C NMR spectroscopy (Table 2, Figure 2). The results are in good accordance with those of the *N*-glucoside isolated from model reactions of tryptophan with glucose (Gutsche et al., 1999). The $^1$H chemical shifts and coupling constants are characteristic for β-D-glucopyranose-$^4C_1$ (Collins and Ferrier, 1996). Both, the chemical shift and the vicinal coupling constant of H-1′ ($\delta_{H-1'}$ = 5.50 ppm, $J_{H-1',H-2'}$ = 9.1 Hz) confirm the *N*-β-glucosidic linkage of the sugar moiety (Avalos et al., 1992). This is further substantiated by the $^{13}$C chemical shift of C-1′ ($\delta_{C-1'}$ = 86.3 ppm), which is comparable to that of the anomeric carbon ($\delta_{C-1'}$ = 85.6 ppm) of the synthetic reference compound $N^\alpha$-acetyl-1-(β-D-glucopyranosyl)-D,L-tryptophan amide (Nyhammar and Pernemalm, 1985). Thus, the identity of the isolated compound was unambiguously established as $N^1$-(β-D-glucopyranosyl-$^4C_1$)-L-tryptophan.

Previous investigation of reactions of tryptophan with aldohexoses had revealed that temperatures above 50 °C and acidic pH are a prerequisite for formation of tryptophan-*N*-glycosides. Furthermore, by the chemical condensation reaction, *C*-glycosyl conjugates and *glyco*-tetrahydro-β-carbolines were always generated together with *N*-glycosides (Gutsche et al., 1999). Therefore, occurrence of a single *N*-glucoside in fruits lacking any heat treatment strongly suggested the specific enzymatic biosynthesis in the plant. Formation of the compound during preparation of the fruit samples could be further excluded by homogenization of the fruits in methanol (data not shown): Thus, largely reduced browning of the homogenate demonstrated that enzymatic activities triggered by the maceration process were prevented by treatment with organic solvent, whereas the concentration of the *N*-glucoside remained unchanged.

To obtain deeper insight into the origin of the novel glycoconjugate, we injected deuterium-labeled tryptophan subepidermally into pear fruits and studied formation of deuterium-labeled tryptophan glycoconjugates. After storage at room temperature for 2−15 days, the fruit extracts were prepared as usual and analyzed by HPLC-MS/MS. To select appropriate ions for these SRM experiments, we performed model reactions, heating L-[indole-$d_5$]-tryptophan with D-glucose to 80 °C at pH 1 for 12 days. As expected, MS analysis of the model reactions yielded the $d_4$-*C*-glucosyl conjugate and $d_4$-*gluco*-tetrahydro-β-carbolines detected by their molecular ions *m/z* 371 $[M+H]^+$ as result of the loss of the deuterium at indole C-2 during the condensation reaction. The product ion spectra of the $d_4$-*C*-glucosyl conjugate and $d_4$-*gluco*-tetrahydro-β-carbolines (data not shown) are dominated by the fragment ions *m/z* 251 and *m/z* 233 corresponding to loss of a $C_4H_8O_4$ moiety and subsequent loss of $H_2O$. However, for the chemically prepared *N*-glucoside molecular ions *m/z* 371 $[M+H]^+$ ($d_4$-tryptophan-*N*-glucoside) besides *m/z* 372 $[M+H]^+$ ($d_5$-tryptophan-*N*-glucoside) were found. This can be explained by deuterium-exchange at C-2: Following reversible protonation of C-3 in strongly acidic medium, H/D-exchange can occur by migration of deuterium from C-2 to C-3 and subsequent loss of deuterium during deprotonation (Gilchchrist, 1995; Jackson and Smith, 1968). In the product ion spectrum of the $d_5$-*N*-glucoside, the prominent ions *m/z* 210 and *m/z* 192 result from loss of an intact anhydro-sugar moiety $C_6H_{10}O_5$ and subsequent loss of $NH_2D$. On the basis of the results of the model experiments with $d_5$-tryptophan, the following ions were selected for SRM experiments: *m/z* 367/188 for $d_0$-tryptophan-*N*-glucoside, *m/z* 372/192 for $d_5$-tryp-

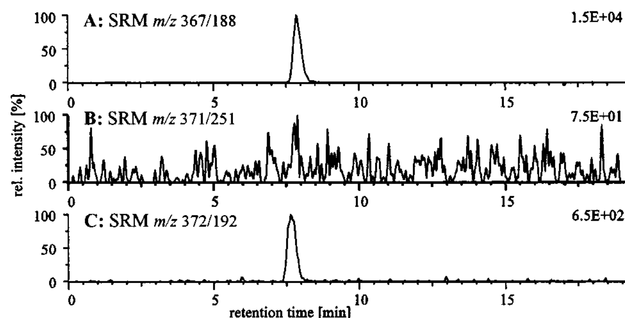


**Figure 3.** HPLC-MS/MS analysis of pear juice obtained after application of $d_5$-tryptophan. (A) SRM for $d_0$-tryptophan-*N*-glycoside. (B) SRM for $d_4$-*C*-glycosyl conjugate and $d_4$-*glyco*-tetrahydro-$\beta$-carbolines. (C) SRM for $d_5$-tryptophan-*N*-glycoside.

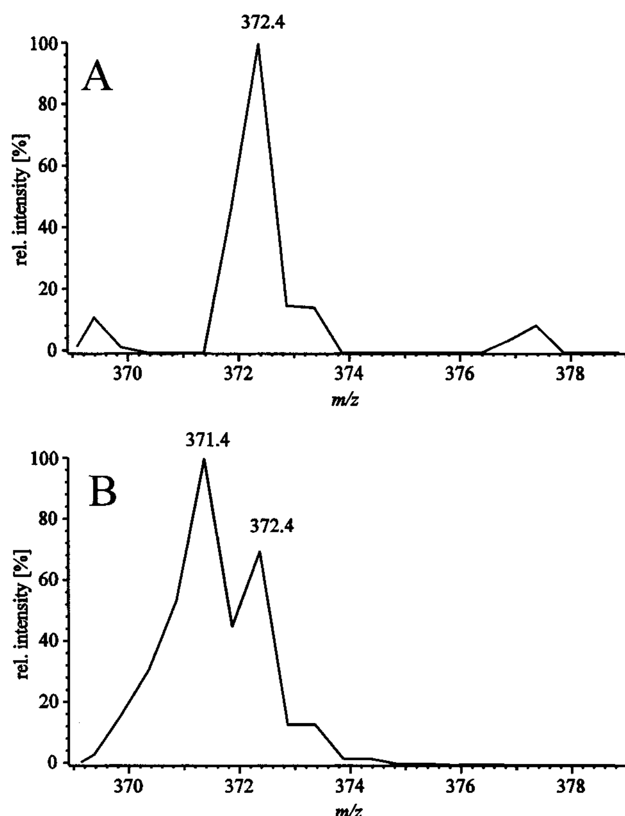


**Figure 4.** ESI-MS spectra showing molecular ions of tryptophan-*N*-glucosides. (A) $d_5$-Tryptophan-*N*-glucoside formed enzymatically in pears after application of $d_5$-tryptophan. (B) $d_4$-/$d_5$-Tryptophan-*N*-glucoside formed chemically.

tophan-*N*-glucoside, and *m*/*z* 371/251 for the $d_4$-tryptophan-*C*-glucosyl derivative and $d_4$-*gluco*-tetrahydro-$\beta$-carbolines.

Studying formation of deuterium-labeled tryptophan glycoconjugates in pear fruits HPLC-MS experiments exclusively proved formation of the corresponding $d_5$-tryptophan-*N*-glucoside, whereas the presence of any chemically formed $d_4$-*C*-glucosyl conjugate and $d_4$-*gluco*-tetrahydro-$\beta$-carboline could be excluded conclusively (Figure 3). To rule-out nonenzymatic condensation of tryptophan and glucose during storage of fruits, we also incubated $d_5$-tryptophan in a model juice containing 2.2% glucose, 6% fructose, and 1.1% sucrose with a pH of 3.8 (adjusted with malic acid). The sugar content and pH value were adjusted according to data published for pear fruits (Belitz and Grosch, 1992). Again, in the "pear model juice", no formation of deuterium-labeled tryptophan glycoconjugates was observed. Finally, we compared the mass spectra of deuterated *N*-glucoside obtained from chemical condensation with that of the deuterated *N*-glucoside found in pear juice after application of $d_5$-tryptophan (Figure 4). As mentioned above, the chemically formed *N*-glucoside yields the molecular ions *m*/*z* 371 $[M+H]^+$ and *m*/*z* 372 $[M+H]^+$ due to deuterium exchange. In contrast, the *N*-glucoside in pear juice exclusively shows the molecular ion *m*/*z* 372 $[M+H]^+$ as result of enzymatic biosynthesis which yields $d_5$-tryptophan-*N*-glucoside from the labeled precursor in a highly specific manner. Taken together, our data convincingly demonstrate the enzymatic formation of the *N*-glucoside from the amino acid tryptophan in pear fruits.

In conclusion, this is the first report on naturally occurring $N^1$-($\beta$-D-glucopyranosyl-$^4C_1$)-L-tryptophan representing a novel intermediate of tryptophan metabolism in plants. Future research will establish the potential of this compound to serve as a marker substance for authenticity control and will provide a reliable analytical basis for the detection of adulteration and processing of fruit products.

## ACKNOWLEDGMENT

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CID, collision-induced dissociation; ESI, electrospray ionization; HH−COSY, homonuclear correlation spectroscopy; HR-ESI-MS, high-resolution electrospray mass spectrometry; MS/MS, tandem mass spectrometry.

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