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DEUTERIUM-LABELED BENZYLADENINE: SYNTHESIS AND APPLICATION AS A SURROGATE

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This paper is dedicated to Professor Dr. Albert Padwa on the occasion of his 75th birthday.

Abstract – N^6-Benzyladenine (benzyladenine), a plant growth regulator, was efficiently deuterated by the hydrogen–deuterium (H–D) exchange reaction catalyzed by palladium on carbon-ethylenediamine complex [Pd/C(en)], while use of palladium on carbon (Pd/C) as a catalyst led to a low deuterium incorporation at room temperature or complete removal of the N^6-benzyl group at 110 °C or higher temperature. The obtained benzyladenine-d_5 was used as an internal standard (surrogate) for the quantification of residual benzyladenine in fruits, vegetables, cereals, and beans using LC/MS/MS. Satisfactory recovery of benzyladenine between 94.2 and 105.7% (100.4% on the average) was obtained. The agrochemical could be detected within the concentration range of 0.25–0.50 ng/g in agricultural products using the present quantification method.

INTRODUCTION

N^6-Benzyladenine (benzyladenine) has been used as a plant growth promoter of vegetables, cereals, and fruits, since it was found to be a synthetic cytokinin[1] that activates cell division in plants, leading to the promotion of leaf growth,[2,3] inhibition of leaf senescence and preservation of chlorophyll.[4,5] Although the standard for the residual quantity of benzyladenine in agricultural products was established by Japan's Food Sanitation Law, the quantification method requires a cumbersome sample preparation and is often difficult to reach a high measurement sensitivity.

Recently, deuterium-labeled compounds have been employed as the internal standards, surrogates, for the quantitative analysis of pesticides in crops by gas or liquid chromatography (GC or LC) combined with mass spectrometry (MS).[6–8] Although the ^{3}H-, ^{13}C-, and ^{15}N-labeled benzyladenine derivatives[9–14] were prepared, their use as a surrogate has not been well-studied, and the synthesis of deuterated benzyladenine has not been reported.

Existing methods for the preparation of deuterium labeled compounds are categorized into the following two types: multi-step synthetic methods starting from small deuterium-labeled precursors,[15–17] and the post-synthetic hydrogen–deuterium (H–D) exchange of the unlabeled compounds.[18–23] The latter methods are much more useful in terms of time and cost efficiency, since deuterium atoms could be introduced into the existing compounds. We have recently reported an efficient and chemoselective H–D exchange reaction catalyzed by activated carbon-supported transition-metal catalysts in the presence of deuterium oxide (D_2O) and hydrogen gas (Figure 1). When palladium on carbon (Pd/C) was used as the catalyst, hydrogen atoms on the benzylic carbon of alkylbenzenes was effectively replaced with deuterium atoms at 50 °C.[24,25] Raising the temperature to 110–160 °C could extend the deuterated portions to all the hydrogen atoms on the alkyl chains,[26,27] and heteroaromatic nuclei including the base moieties of the nucleosides were also deuterated.[28,29] The use of platinum on carbon (Pt/C) instead of Pd/C preferentially promoted the H–D exchange reaction on aromatic rings,[30,31] while rhodium on carbon (Rh/C) catalyzed the deuteration of the non-activated alkanes bearing no functionalities.[32] We have also found that ruthenium on carbon (Ru/C) efficiently catalyzed the chemoselective H–D exchange reaction on the carbon atoms adjacent to the hydroxyl groups of primary and secondary alcohols.[33]

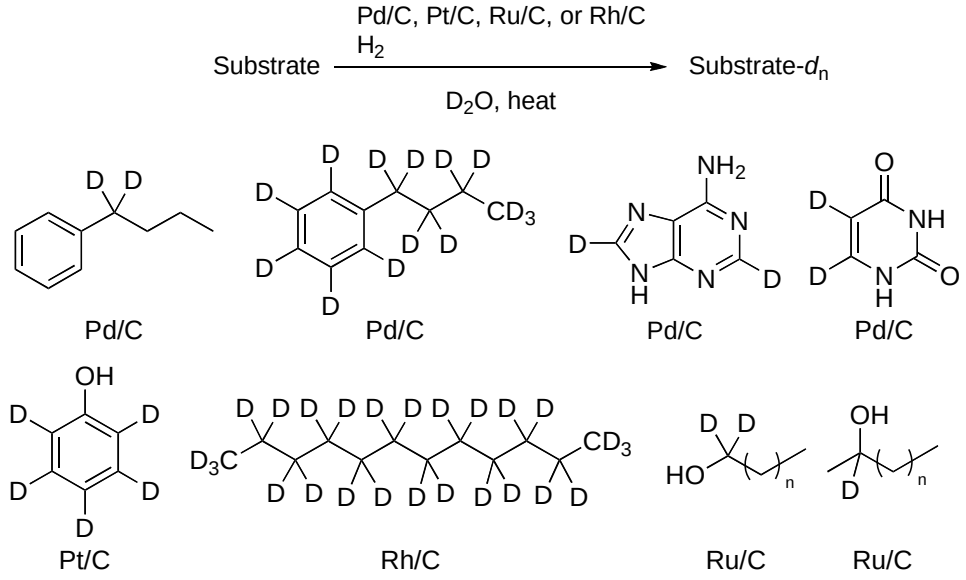


Figure 1. H–D exchange reactions of various organic molecules using Pd/C, Pt/C, Rh/C, or Ru/C in the presence of hydrogen and D_2O

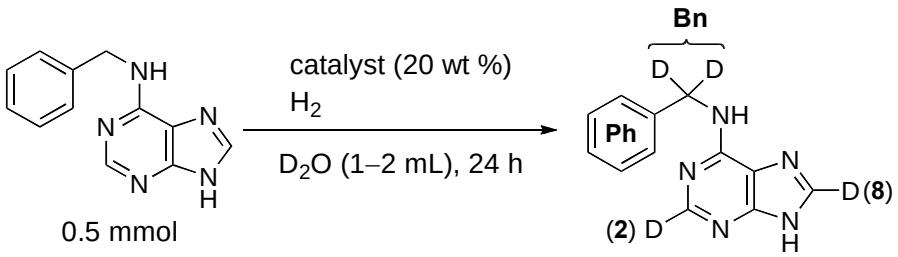
The benzylamino group is quite labile under the hydrogenation conditions catalyzed by transition metals.[34] The deuteration of benzyladenine by such H–D exchange reactions should therefore be challenging from the point of view of organic chemistry. In this paper, we demonstrated the efficient synthesis of deuterated benzyladenine and its application as the surrogate for the quantification of residual benzyladenine in agricultural products.

RESULTS AND DISCUSSION

Synthesis of deuterated benzyladenine

The H–D exchange reaction of benzyladenine using 10% Pd/C as a catalyst at room temperature was first investigated. The deuteration hardly took place on the purine ring at room temperature (Table 1, Entry 1), while raising the temperature to 110 °C led to the complete and undesirable removal of the N^6-benzyl group to give the corresponding adenine as the sole product (Entry 2). The hydrogenolysis of the benzylamino moiety was tolerated at room temperature by the replacement of the catalyst with 5% Pt/C, which is generally effective for the H–D exchange on the aromatic nuclei, but the deuterium efficiency

Table 1. H–D exchange reaction of N^6-benzyladenine



Entry	Catalyst	Temp (°C)	D content (%)[a]			Yield (%)
			Ph	Bn	2 and 8 positions[b]	
1	10% Pd/C	rt	1	27	7	42[c]
2	10% Pd/C	110		debenzylation		0
3	5% Pt/C	rt	0	20	18	86
4	5% Pt/C	110	9	33	37	60[c]
5	5% Pd/C(en)	110	11	77	53	92
6[d]	5% Pt/C	180	10	84	79	82
7	5% Pd/C(en) + 5% Pt/C	110	10	71	75	82
8	5% Pd/C(en) + 5% Pt/C	180	20	96	88	70[c]
9[e]	5% Pd/C(en)	180	17	97	94	47[c]

[a] Determined by ^{1}H NMR. [b] ^{1}H NMR signals for the 2- and 8-positions were not assigned, and the numbers shown were averaged. [c] Adenine was obtained in 24% (Entry 1), 20% (Entry 4), 21% (Entry 8), and 24% (Entry 9), respectively. [d] The product of Entry 5 was used as the starting material. [e] 5% Pd/C(en) (30 wt %) was used.

was only 20% at the benzylic position and purine nucleus (Entry 3). The deuterium efficiency was improved to approximately 35% by increasing the temperature to 110 °C, although the partial debenzylation instead occurred (Entry 4).

We previously developed a Pd/C-ethylenediamine complex [Pd/C(en)] on the basis of the catalyst poisoning property of ethylenediamine, which possesses no catalytic activity for the hydrogenation of various reducible functionalities including benzyl ethers and benzyl alcohols.[35,36] The Pd/C(en) was then employed as the catalyst in anticipation of the high deuterium incorporation without removal of the benzyl group.[37] As a result, the N^6-debenzylation was efficiently suppressed during the deuteration of benzyladenine at 110 °C with moderate deuterium contents (Entry 5). The obtained deuterium-labeled benzyladenine was again used as the substrate for the deuteration. Thus, it was stirred in D_2O at 180 °C together with 5% Pt/C under a hydrogen atmosphere. The deuterium incorporation efficiency was slightly improved, but not enough for practical use (Entry 6).

A synergistic effect of the simultaneous use of Pd/C and Pt/C on the H–D exchange reactions was previously observed, which efficiently incorporates deuterium atoms into both aromatic and aliphatic moieties of a variety of substituted arenes.[38] The combined use of 5% Pd/C(en) and 5% Pt/C was then examined (Table 2, Entries 7 and 8). The deuterium efficiency was improved at 110 °C compared to the single use of either 5% Pt/C or 5% Pd/C(en) (Entry 7 vs. Entries 4 and 5, respectively), and the debenzylation, which was observed in the 5% Pt/C-catalyzed reaction (Entry 4), was suppressed probably because of the catalyst poison effect by the coordinated ethylenediamine of 5% Pd/C(en) (Entry 7). The increase in the reaction temperature to 180 °C remarkably promoted the H–D exchange reaction, although the debenzylation was also observed (Entry 8). We finally found that the independent use of an increased amount of 5% Pd/C(en) (30% of the substrate weight) at 180 °C led to an excellent deuterium incorporation, but with the lowest acceptable yield level (Entry 9). The deuterated benzyladenine obtained from Entry 9 in Table 1 was employed for its quantitative analyses in agricultural products.

Quantitative analysis of benzyladenine

Selection of the surrogate

The total ion chromatogram (TIC) of the deuterated benzyladenine showed several ion peaks for *m*/*z* 230.1, 231.1, 232.2, and so on. The compound derived from the highest peak, *m*/*z* 231.1, was selected as the surrogate, which was supposed to be the protonated benzyladenine-d_5. Its MS/MS spectrum indicated the following three ions: m/z 230.9 for $[M+H]^+$ of benzyladenine-d_5, 152.0 for [M–benzene], 94.0 for benzyl-d_3 (Figure 2). The comparison with the MS/MS spectrum of the non-deuterated benzyladenine supported the structure of the surrogate as benzyladenine-d_5 which has a total 5 deuterium atoms, i.e., 1 deuterium on the benzene ring, two on the benzyl site, and two on the adenine nucleus.

When the product ions of the precursor ion for *m*/*z* 225.9 of the protonated benzyladenine were monitored in Q3, the ion for *m*/*z* 91.0 was observed with the highest intensity. Therefore, the ion was assigned as the target ion and the ion for *m*/*z* 226.0 as the qualifier ion. The ion for *m*/*z* 94.0 with the highest sensitivity was determined as the internal standard by the similar monitoring of the product ion of the ion for *m*/*z* 230.9 of the deuterated benzyladenine.

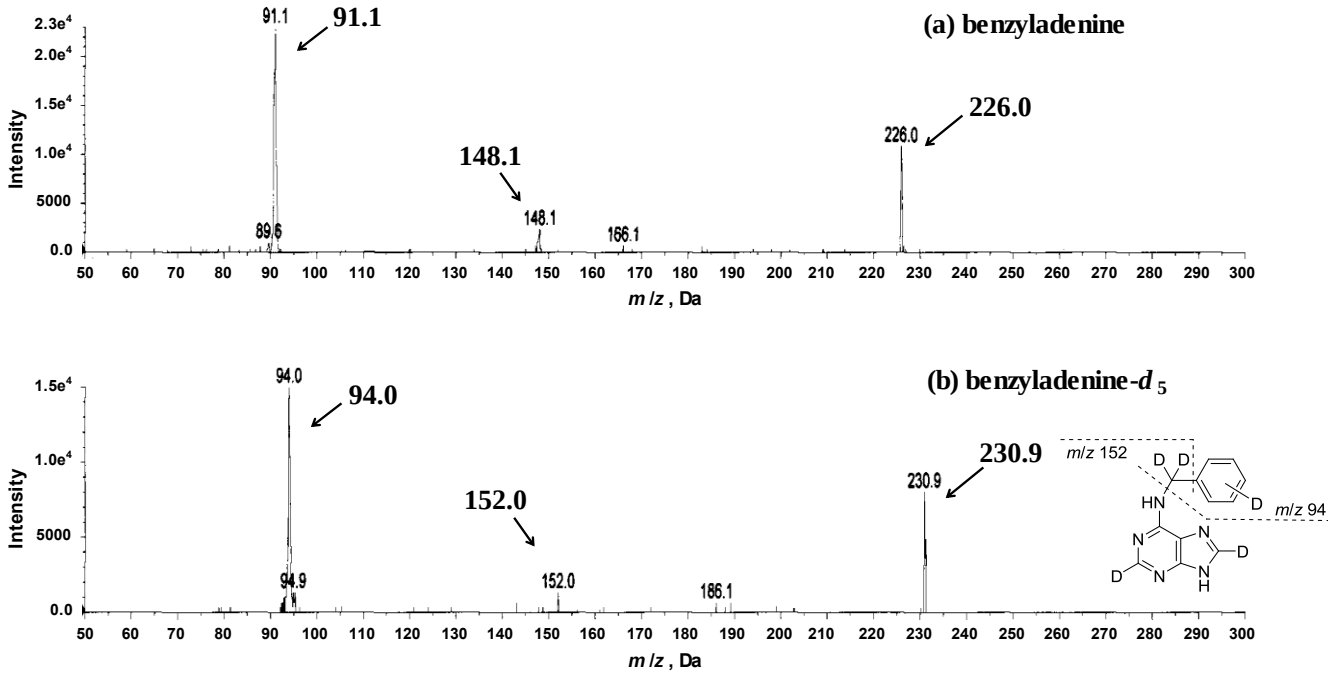


Figure 2. MS/MS spectra of benzyladenine (a) and benzyladenine-d_5 (b)

Recovery of the benzyladenine from agricultural products

Ion suppression effects of the acetonitrile extracts from agricultural products by mass spectrometry were first investigated (Table 2).[39] The ratio of the MS peak area of the matrix standards extracted from grapefruit and lemon to that of the solvent standards were 0.84 and 0.83, respectively. Benzyladenine added on these products was not well recovered (83.4 and 75.7%, respectively) by the external standard method. The good correlation between the ratio of the matrix standards to solvent standards and the recovery ratio of grapefruit and lemon suggests that the low recovery should be attributed to the ion suppression effect. On the other hand, the use of benzyladenine-d_5 as the internal standard improved the recovery from grapefruit and lemon up to 98.7 and 94.2%, respectively. Furthermore, the surrogate method was applicable for the recovery tests of all 15 products without any loss in sensitivity. The limit of detection (S/N = 3) of benzyladenine in fruits and vegetables was 0.25 ng/g, and 0.50 ng/g in cereals and beans.

Table 2. Recovery of benzyladenine from agricultural products

sample	matrix STD/solvent STD	Recovery (n = 5)			
	peak area ratio (%)	external standard method		surrogate method	
		recovery ratio (%)	RSD (%)	recovery ratio (%)	RSD (%)
asparagus	0.97	92.9	0.94	101	0.86
pumpkin	0.96	93.8	3.0	104	2.1
cabbage	0.94	91.8	1.2	101	0.86
radish	0.99	98.0	3.2	103	1.7
onion	0.95	89.9	2.7	97.0	1.3
spinach	0.96	87.1	2.1	99.2	0.89
potato	1.0	96.1	1.3	106	1.5
rice	0.93	98.6	1.5	98.7	1.9
soybean	0.98	91.2	1.3	98.4	1.0
almond	0.93	92.9	2.4	98.6	1.1
banana	0.95	96.3	3.1	101	0.97
apple	1.0	96.6	3.1	101	0.97
orange	1.1	92.6	6.7	104	0.81
grapefruit	0.84	83.4	1.7	98.7	0.96
lemon	0.83	75.7	2.8	94.2	2.2
average	0.96	91.8	2.5	100	1.3

RSD: relative standard deviation.

CONCLUSIONS

Deuterium-labeled benzyladenine was successfully prepared by the H–D exchange reaction of benzyladenine using the Pd/C(en)–D_2O–H_2 system. The conditions achieved a high deuterium efficiency on the purine nuclei and benzylic position with minimal removal of the N^6-benzyl group. The ^{1}H NMR spectrum and LC/MS/MS analysis demonstrated that five hydrogen atoms of benzyladenine were replaced with deuterium atoms. The deuterium-labeled benzyladenine was successfully employed as the surrogate to quantify the residual benzyladenine in agricultural products using LC/MS/MS with a high sensitivity and reproducibility.

EXPERIMENTAL

Synthesis of deuterated benzyladenine

General. The ^{1}H and ^{2}H NMR spectra were recorded by a JEOL AL-400 or EX-400 spectrometer (400 MHz for ^{1}H NMR, 61 MHz for ^{2}H NMR). The chemical shifts (δ) are given in parts per million referenced to tetramethylsilane or residual solvent (2.49 ppm/DMSO). The deuterium content was determined using the internal standard 1,4-dimethoxybenzene. The EI mass spectra were taken by a JEOL JMS-SX 102A spectrometer. The analytical thin-layer chromatography (TLC) was carried out on pre-coated Silica Gel 60 F_{254} plates (Merck, Art 5715) and visualized with UV light. Column chromatography was performed using Merck silica gel 60 (230–400 mesh). 10% Pd/C, 5% Pd/C(en), and 5% Pt/C were obtained from the N.E. Chemcat Corporation, Wako Pure Chemical Industries, Ltd., and

Sigma-Aldrich Corporation, respectively. Deuterium oxide (99.9% isotopic purity) was purchased from Cambridge Isotope Laboratories, Inc., or Division of Spectra Gases, Inc. Benzyladenine (99.9% purity) was obtained from Hayashi Pure Chemical Industries, Ltd. All other reagents were obtained from commercial sources and used without further purification.

General procedures for the H–D exchange reaction (Table 1)

Method A: A mixture of benzyladenine (113 mg, 0.500 mmol) and catalyst (23.0 mg, 20 wt % of benzyladenine) in D_2O (1–2 mL) under a hydrogen atmosphere in a stainless sealed tube was stirred at 110–180 °C (oil bath). After 24 h, the mixture was diluted with MeOH (5 mL) at room temperature and passed through a membrane filter (Millipore Millex-LG, 0.45 μm) to remove the catalyst. The catalyst collected on the filter was washed with MeOH (2 × 5 mL), and the filtrate was concentrated *in vacuo*. The residue was purified by silica-gel column chromatography ($CHCl_3$:MeOH = 10:1).

Method B: A mixture of benzyladenine (113 mg, 0.500 mmol) and catalyst (23.0 mg, 20 wt % of benzyladenine) in D_2O (1–2 mL) under a hydrogen atmosphere in a 15 mL-test tube was stirred at room temperature. After 24 h, the mixture was diluted with MeOH (5 mL) and passed through a membrane filter (Millipore Millex-LG, 0.45 μm) to remove the catalyst. The catalyst collected on the filter was washed with MeOH (2 × 5 mL), and the filtrate was concentrated *in vacuo*. The residue was then purified by silica-gel column chromatography ($CHCl_3$:MeOH = 10:1).

Entry 1. Method B. 10% Pd/C (23.0 mg, 20% of substrate weight) was used as the catalyst instead of 5% Pt/C. Yield: deuterated benzyladenine, 47.5 mg (42%); adenine, 26.9 mg (24%, D efficiency was not determined).

Entry 2. Method A. 10% Pd/C (24.0 mg, 20% of substrate weight) was used instead of 5% Pt/C. The reaction was carried out at 110 °C. Debenzylation took place.

Entry 3. Method B. Yield: deuterated benzyladenine, 97.5 mg (86%).

Entry 4. Method A. The reaction was carried out at 110 °C. Yield: deuterated benzyladenine, 68.0 mg (60%); adenine, 22.8 mg (20%, D efficiency was not determined).

Entry 5. Method A. 5% Pd/C(en) [23.0 mg, 20% of substrate weight] was used as the catalyst. The reaction was carried out at 110 °C. Yield: deuterated benzyladenine, 104 mg (92%).

Entry 6. Method A. The reaction was carried out at 180 °C. Yield: deuterated benzyladenine, 92.3 mg (82%).

Entry 7. Method A. 5% Pd/C(en) [23.0 mg, 20% of substrate weight] and 5% Pt/C (23.0 mg, 20% of substrate weight) were used. The reaction was carried out at 110 °C. Yield: deuterated benzyladenine, 92.9 mg (82%).

Entry 8. Method A. 5% Pd/C(en) [23.0 mg, 20% of substrate weight] and 5% Pt/C (23.0 mg, 20% of substrate weight) were used. The reaction was carried out at 180 °C. Yield: deuterated benzyladenine,

79.1 mg (70%); deuterated adenine, 23.7 mg [21%, 94% deuterium efficiency (average of the 2 and 8 positions)].

Entry 9. Method A. 5% Pd/C(en) [34.0 mg, 30% of substrate weight] was used as the catalyst. The reaction was carried out at 180 °C. Yield: deuterium benzyladenine, 53.0 mg (47%); deuterated adenine, 27.1 mg [24%, 96% deuterium efficiency (average of the 2 and 8 positions)]. ^{1}H NMR (DMSO-d_6, 1,4 dimethoxybenzene as an internal standard) δ 8.16–8.09 (s, 0.12H), 7.35–7.19 (m, 4.17H), 4.68 (s, 0.06H). ^{2}H NMR (DMSO) δ 8.16 (br s), 4.71 (br s).

Quantification of benzyladenine

Materials

Acetonitrile, MeOH, and NH_4OAc were purchased from Kanto Chemical Co., Inc. The membrane and glass filters were obtained from Millipore (Millipore Millex-LG SLLG H13 NL, 0.20 μm) and Advantec (GA-100, 1.0 μm), respectively. The deuterated benzyladenine was prepared by the method described in the general procedure for the H–D exchange reaction (Table 1), Entry 9.

Apparatus and conditions

HPLC apparatus and conditions

An Agilent 1200 LC (SL) system with an Agilent ZORBAX Eclipse Plus C18 column (2.1 mm i.d. × 150 mm, 1.8 μm particle size) was used. The HPLC conditions were as follows: Flow rate, 0.2 mL/min; Column temperature, 40 °C; Injection volume, 5 μL; Mobile phase, Solvent A: 5 mmol/L aqueous NH_4OAc solution, Solvent B: 5 mmol/L NH_4OAc solution in MeOH, Gradient profile, 0 min, A:B = 85:15; 1 min, A:B = 60:40, 3.5 min, A:B = 60:40; 6 min, A:B = 50:50, 8 min, A:B = 45:55; 17.5 min, A:B = 5:95, 30 min, A:B = 5:95.

Mass spectrometry (MS) analysis

Quantification was performed using an Applied Biosystems API4000 Q TRAP^{TM} MS/MS system. Multiple reaction monitoring (MRM) analyses were conducted in the positive electrospray ionization (ESI) mode. The ESI conditions were as follows: curtain gas, 30 psi; ion source gas 1, 80 psi; ion source gas 2, 30 psi; ion spray voltage, 4500 V; temperature, 700 °C. MRM parameters are indicated in Table 3.

Table 3. MRM parameters

	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	DP (V)	CE (eV)	CXP (V)
benzyladenine	225.9	226.0	100	71	9	14
	225.9	91.0	100	71	33	14
benzyladenine-d_5	230.9	230.9	100	76	9	44
	230.9	94.0	100	76	35	16

Preparation of standard solutions

Solvent standard (Solvent STD)

A 5.0 ng/mL benzyladenine solution in a mixed solvent of acetonitrile and H_2O (1:1).

Matrix standard (Matrix STD)

Acetonitrile (1 mL) was added on the surface of each vegetable (20 g), fruit (20 g), cereal (10 g), or beans (10 g). To the cereal and beans was also added H_2O (20 mL). After 30 min, acetonitrile (approximately 130 mL) was added, and the agricultural product was homogenized for over 3 min. The mixture was then filtered using a glass filter (1.0 μm), and the filtrate was diluted with acetonitrile to 200 mL. The solution (2 mL) was concentrated *in vacuo* to dryness, and a 10 ng/mL benzyladenine solution (2.0 mL) in a mixed solvent of acetonitrile and H_2O (1:1) to the residue. The resulting solution was diluted with acetonitrile–H_2O (1:1) to 4.0 mL.

Preparation of sample solution

The acetonitrile solutions of benzyladenine-d_5 (4.0 mg/L, 1 mL) and of benzyladenine (2.0 mg/L, 1 mL) were added on the surface of each vegetable (20 g), fruit (20 g), cereal (10 g), or beans (10 g). To the cereal and beans was also added H_2O (20 mL). After 30 min, acetonitrile (approximately 130 mL) was added, and the agricultural product was homogenized for over 3 min. The mixture was then filtered using a glass filter (1.0 μm), and the filtrate was diluted with acetonitrile to 200 mL. The solution (1 mL) was diluted with H_2O to 2 mL, and passed through a membrane filter (0.20 μm). The filtrate was used for the MRM analysis using LC/MS/MS.

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