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Synthesis of Haptens and Conjugates for ELISA of Glycitein: Development and Validation of an Immunological Test

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Two carboxylic acid haptens of glycitein were synthesized, with a spacer arm at the C2 position. They differed in the length of the spacer arm, with the length of the spacer arms being three or four carbon atoms, and were named Δ3-glycitein and Δ4-glycitein haptens, respectively. The different haptens were coupled to bovine serum albumin (BSA), and the coupling efficiency was assessed by MALDI mass spectrometry. Polyclonal antibodies were generated against the BSA conjugates. An additional conjugate of Δ4-glycitein hapten was generated with swine thyroglobulin (Thyr). Enzyme-linked immunosorbent assays (ELISAs) based on the competition between free glycitein and Δ4-glycitein−Thyr conjugates for specific antibodies were developed. The IC_{50} of the standard curves was 15.6 ng mL^{-1} with anti-Δ3-glycitein and 62.5 ng mL^{-1} with anti-Δ4-glycitein, that is, 10.9 and 44 pmol/well, respectively. With the Δ3-glycitein antibody, interassay and intra-assay variations were 12.2 and 11.5%, respectively. Specificity tests did not show any significant cross-reaction with any other soy isoflavone. This specificity is not influenced by the length of the spacer arm. The assay was validated by measurements performed on plasma samples as well as on soy-based foodstuffs and on soy-based food supplements.

KEYWORDS: Phytoestrogens; glycitein; haptens; polyclonal antibodies; ELISA; plasma; soy; foodstuff; food supplements

INTRODUCTION

Glycitein (4′,7-dihydroxy-6-methoxyisoflavone) is present in soy and soy-based foods together with genistein and daidzein and their glycosylated forms. All of these substances are known as estrogenic compounds capable of interacting with estradiol receptors (ER) (*1*). Glycitein contents range from 5 to 10% of total isoflavones in soybeans (*2*) and can reach 50% in soy germ (*3*). Both raw materials are increasingly used in human food and food supplements (*4*). Glycitein has been shown to exhibit estrogenic activity comparable to that of other isoflavones in the uterotrophic test in mice (*5*). In competitive binding tests for ERs it was shown to have greater affinity with ERβ than with ERα and displacement activity similar to that of daidzein (*6*). This study also reported that the activation of estrogen-dependent transcription was better via ERβ than via ERα and similar to that of other soy isoflavones. In parallel, glycitein, unlike genistein or daidzein, exhibits no significant effect on MCF-7 cell proliferation (*6*). Although a better knowledge of glycitein bioavailability could help in understanding the health effect of this particular compound, data are still scarce. Setchell et al. (*7*) described plasma parameters obtained on a unique male volunteer. Richelle et al. (*8*) described the plasma and urinary pharmacokinetic data on six European postmenopausal women. Metabolism of glycitein was also compared to that of other isoflavones, daidzein and genistein, on seven women and seven men (*9*). The plasmatic concentration 24 h after dosing revealed that a modest gender difference in glycitein bioavailability was suggested at high doses admin-

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istered with soy germ (*9*). Several studies reported data on glycitein urine excretion (*10*).

However, better knowledge on glycitein plasma availability could help in understanding the health effect of this particular compound. The ability to measure the plasma concentration of glycitein may help in understanding the origin of some of the large variations registered in the health effect of soy in animal or human trials.

In addition, large differences in compositions were already recorded between soy-based food supplements (*7, 11*), but only genistein and daidzein concentrations had been measured in these studies. The availability of an assay for glycitein plasma concentration would help (i) to check for the origin of soy extracts incorporated in soy-based supplements and (ii) to explain at least part of the differences in effects observed from one formula to another.

High-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry had been applied to the quantitative measurement of isoflavones in complex mixtures (*12–17*). This technique is relevant for biological fluids, whereas HPLC with ultraviolet detection is often chosen for routine analysis, especially in vegetable matter (*18*). These approaches often require a preliminary hydrolysis to simplify chromatographic profiles and improve sensitivity. Furthermore, the choice of a universal internal standard is still a challenging process (*16*). Considering the costs and availability of MS/MS instruments as well as the time required for the analysis, the elaboration of new ELISA tests for glycitein appears to be a relevant issue. Such tests can provide fast results and thus be used for large sets of samples, that is, for epidemiological studies with a modest financial input.

In this work, we report the improved synthesis of glycitein used as standard in the assay, as well as the preparation of two new different haptens of glycitein, each bearing a particular spacer arm of specific length, that is, three carbon atoms (Δ3) or four carbon atoms (Δ4). We also present the synthesis of hapten−protein conjugates, their characterization, and the production of the corresponding antibodies as well as the ELISA tests. The cross-reactivity of the antibodies is also discussed. The new technique is tested on soy-based foodstuffs and supplements as well as plasma samples from volunteers challenged with glycitein-containing food supplements.

MATERIALS AND METHODS

Chemicals. 3-Hydroxy-4-methoxybenzaldehyde (99%), hydrogen peroxide solution (30%), selenium dioxide (98%), boron trifluoride diethyl etherate (BF_3-Et_2O), and methyl 4-chloro-4-oxobutyrate (97%) were purchased from Sigma Aldrich Chemical Co. (Saint Quentin Fallavier, France), whereas hexamethyldisilazane (HMDS) (98%), methyl 5-chloro-5-oxovalerate (97%), and methanesulfonyl chloride (98%) were obtained from Lancaster Synthesis Ltd. (Bisheim, France). 4-Hydroxyphenylacetic acid (98%) was purchased from Acros Organics France (Noisy, France).

THF was distilled over sodium wire and benzophenone under argon atmosphere and used immediately. Dimethylformamide (DMF) was dried by distillation over CaO and stored over 4 Å molecular sieves. *n*-BuLi (2.5 M solution in hexane) was purchased from Acros Organics France and used after titration with diphenylacetic acid. All moisture-sensitive reactions were performed under argon atmosphere in oven-dried or flame-dried glassware.

All biological reagents were from Sigma-Aldrich except for the secondary antibody, that is, goat anti-rabbit IgG−horseradish peroxidase purchased from DAKO (Trappes, France).

Instruments. Melting points were determined with a Stuart Scientific melting point apparatus SMP3 and are uncorrected. ^1H and ^{13}C NMR spectra were recorded with a Bruker AC-300 FT (^1H, 300 MHz; ^{13}C, 75 MHz) or with a Bruker AC 250 (^1H, 250 MHz; ^{13}C, 63 MHz) using TMS as an internal standard. The chemical shifts (δ) and coupling constants (J) are respectively expressed in parts per million and hertz. IR spectra were recorded with a Perkin-Elmer Paragon 1000 FT-IR spectrophotometer. Merck silica gel 60 (70−230 mesh, 0.063−0.200 mm) was used for flash chromatography. Thin-layer chromatography (TLC) was performed using TLC plates (0.25 mm, particle size 15 μm, pore size 60 Å) purchased from SDS (Peypin, France). TLC plates were run in the same solvent system as flash chromatography. The spots were visualized with a UV lamp. Mass spectra (both high and low resolution) were acquired by the CESAMO (Bordeaux, France) on a QStar Elite mass spectrometer (Applied Biosystems). The instrument was equipped with an electrospray ionization (ESI) source and spectra were recorded in the positive mode. The electrospray needle was maintained at 4500 V and operated at room temperature. Samples were introduced by injection through a 10 μL sample loop into a 200 μL min^{-1} flow of methanol from the LC pump.

For the ELISA technique the optical densities (OD) were read on a Dynex MRX II microtitration plate reader at 490 nm. The ELISA technique was carried out on microtitration plates (NUNC maxisorp) with 96 wells. Positive controls were wells receiving coating and a specific antibody without any free phytoestrogen. The standard curves were expressed according to the following formula: $OD_{sample}/OD_{positive\ control} = f$ [log (concentrations)].

Chemical Synthesis. *4-Methoxybenzene-1,3-diol (**3**).* 3-Hydroxy-4-methoxybenzaldehyde (**1**) (6.0 g, 39.4 mmol) was added to a well-stirred mixture of hydrogen peroxide solution (30%, 8.9 mL, 86.7 mmol) and SeO_2 (0.35 g, 3.1 mmol) in CH_2Cl_2 (100 mL) at 0 °C. The mixture was allowed to warm to room temperature overnight, filtered, and washed with water (100 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 40 mL). The combined organic phases were washed with 10% solution of $NaHSO_3$ in water (100 mL) and then with brine (saturated aqueous NaCl solution) (50 mL). The organic layer was then vigorously stirred with saturated aqueous Na_2CO_3 solution (200 mL) for 2 h at room temperature. After separation of the organic phase, the pH of the aqueous phase was adjusted to pH 4−5 with 1 M hydrochloric acid and was extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated under reduced pressure to afford the target compound (**3**) as a brown oil (5.2 g, 95%) used without chromatographic purification in the next step: ^1H NMR (CD_3OD, 300 MHz) δ 3.79 (s, 3H, OCH_3), 6.25 (dd, $J = 3.0$ Hz, 8.6 Hz, 1H, Har, 6), 6.36 (d, $J = 3.0$ Hz, 1H, Har, 2), 6.76 (d, $J = 8.6$ Hz, 1H, Har, 5); ^{13}C NMR (CD_3OD, 75 MHz) δ 153.2 (CQ), 148.7 (CQ), 142.7 (CQ), 114.7 (CHar, 5), 106.6 (CHar, 6), 104.6 (CHar, 2), 57.6 (CH_3); IR (cm^{-1}, KBr) 3384, 2840, 1609, 1513, 1468, 1296, 1227, 1160, 1120, 1028, 969.

*1-(2,4-Dihydroxy-5-methoxyphenyl)-2-(4′-hydroxyphenyl)ethanone (**4**).* BF_3-Et_2O (260 mL, 2.0 mol) was added dropwise to a mixture of **3** (6.88 g, 49.1 mmol) and 4-hydroxyphenylacetic acid (8.96 g, 58.9 mmol) at room temperature under argon atmosphere. The reaction mixture was maintained at 80 °C overnight, allowed to cool to room temperature, and poured onto ice. The aqueous layer was extracted with Et_2O (3 × 100 mL). The combined organic phase was subsequently washed with saturated aqueous KOAc solution (150 mL), saturated aqueous $NaHCO_3$ solution (150 mL), and brine (150 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The brown oil obtained was purified by flash column chromatography (CH_2Cl_2/MeOH, 96:4) to give a brown solid ($R_f = 0.25$), which was purified again by recrystallization from ethanol/water (1:1) to afford the desired compound as orange powder (8.6 g, 64%, mp 150−151 °C): ^1H NMR (CD_3OD, 250 MHz) δ 3.79 (s, 3H, OCH_3), 4.12 (s, 2H, CH_2), 6.30 (s, 1H, Har, 3), 6.74 (d, $J = 8.6$ Hz, 2H, Har, 3′,5′), 7.11 (d, $J = 8.6$ Hz, 2H, Har, 2′,6′), 7.32 (s, 1H, Har, 6); ^{13}C NMR (CD_3OD, 75 MHz) δ 204.0 (C=O), 161.7 (CQ), 157.4 (CQ), 156.8 (CQ), 142.4 (CQ), 131.3 (CHar, 2′,6′), 127.4 (CQ), 116.5 (CHar, 3′,5′), 114.0 (CHar, 6), 111.9 (CQ), 104.5 (CHar, 3), 57.1 (OCH_3), 45.3 (CH_2, 8); IR (cm^{-1}, KBr) 3355, 2977, 1637, 1611, 1513, 1309, 1268, 1232, 1202, 1158, 957.

7-Hydroxy-3-(4′-hydroxyphenyl)-6-methoxy-4H-chromen-4-one (Glyn). BF_3-Et_2O (14 mL, 111 mmol) was added dropwise to a solution of **4** (6.1 g, 22 mmol) in dry DMF at room temperature under argon atmosphere. The reaction mixture was heated to 50 °C, and methane-

sulfonyl chloride (7.6 g, 5 mL, 66 mmol) was added dropwise. After heating at 70 °C for 4 h, the reaction mixture was allowed to cool to room temperature overnight, poured onto ice, and then extracted with ethyl acetate (3 × 100 mL). The combined organic phase was subsequently washed with saturated aqueous KOAc solution (150 mL), saturated aqueous $NaHCO_3$ solution (150 mL), and brine (150 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The brown solid obtained was purified by recrystallization from ethanol/water (1:1) to afford the desired compound as beige crystals (2 g, 32%, mp 337 °C decomp. [lit. 337−339 °C (*19*)]: ^{1}H NMR (DMSO-d_6, 300 MHz) δ 3.86 (s, 3H, OCH_3), 6.81 (d, J = 8.6 Hz, 2H, Har, 3′,5′), 6.91 (s, 1H, Har, 8), 7.38 (d, J = 8.6 Hz, 2H, Har, 2′,6′), 7.41 (s, 1H, Har, 5), 8.2 (s, 1H, Har, 2), 9.53 (OH), 10.56 (OH); ^{13}C NMR (DMSO-d_6, 75 MHz) 174.8 (C═O), 157.6 (CQ), 153.3 (CQ), 152.8 (CHar, 2), 152.1 (CQ), 147.3 (CQ), 130.5 (CHar, 2′,6′), 123.4 (CQ), 123.2 (CQ), 116.7 (CQ), 115.4 (CHar, 3′,5′), 105.1 (CHar, 5), 103.3 (CHar, 8), 56.2 (OCH_3); IR (cm^{-1}, KBr) 3404, 3071, 2940, 1616, 1578, 1557, 1520, 1480, 1419, 1374, 1277, 1233, 1208, 1178, 841; MS (ESI+) *m/z* 591 $[2M + Na]^+$, 307 $[M + Na]^+$, 285 $[M + H]^+$; HR-MS (ESI+) *m/z* calcd for $C_{16}H_{13}O_5$, 285.0757 $[M + H]^+$; found, 285.0769.

3-(7-Hydroxy-3-(4-hydroxyphenyl)-6-methoxy-4-oxo-4H-chromen-2-yl)propanoic acid (Δ3-Glycitein). A solution of lithium hexamethyldisilazide LiHMDS was prepared by the addition of *n*-BuLi (14 mL, 2.5 M in hexane) to a solution of the freshly distilled HMDS (7 mL, 33.5 mmol) in dry THF (30 mL) at −78 °C under argon atmosphere. The mixture was allowed to warm to −30 °C for 30 min and cooled again to −78 °C before addition via cannula to the stirred solution of **4** (2.0 g, 7.3 mmol) in dry THF (50 mL) at −78 °C. The reaction mixture was stirred for 2 h, and a solution of 4-chloro-4-oxobutyrate (6.62 g, 5.4 mL, 43.7 mmol) in dry THF (30 mL) was slowly added. The reaction mixture was allowed to warm to room temperature overnight, poured onto ice, acidified with 10% HCl to pH 2−3, and stirred for 1 h at room temperature. The solution was extracted with ethyl acetate (3 × 75 mL). The organic phase was washed with brine (50 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was then diluted in THF (15 mL) and cooled to 0 °C. The ice-cooled 1 M LiOH solution (80 mL) was added dropwise, the mixture was stirred for 2 h at room temperature, and the pH was adjusted to pH 2−3 with 10% HCl. The aqueous phase was extracted with ethyl acetate (3 × 40 mL). The combined organic extracts were washed with water (50 mL) and brine (50 mL), dried ($MgSO_4$), and concentrated under reduced pressure. Flash chromatography using CH_2Cl_2/methanol (9:1) as eluent gave the desired compound (Δ3-glycitein) as pale yellow crystals (R_f = 0.28, 1.5 g, 58%, mp 268 °C): ^{1}H NMR (CD_3OD, 300 MHz) δ 2.70 (t, J = 7.5 Hz, 2H, CH_2), 2.92 (t, J = 7.5 Hz, 2H, CH_2), 3.97 (s, 3H, OMe), 6.91 (d, J = 6.4 Hz, 2H, Har, 3′,5′), 6.95 (s, 1H, Har, 5), 7.14 (d, J = 6.4 Hz, 2H, Har, 2′,6′), 7.51 (s, 1H, Har, 8); ^{13}C NMR (CD_3OD, 75 MHz) δ 178.9 (C═O), 176.3 (COOH), 166.3 (CQ), 158.5 (CQ), 154.9 (CQ), 154.1 (CQ), 148.6 (CQ), 132.9 (CHar, 2′,6′), 125.2 (CQ), 123.9 (CQ), 117.0 (CQ), 116.5 (CHar, 3′,5′), 105.7 (CHar, 8), 103.9 (CHar, 5), 56.8 (OCH_3), 32.0 (CH_2), 29.2 (CH_2); IR (cm^{-1}, KBr) 3546, 3213, 1699, 1618, 1575, 1521, 1481, 1279, 1259, 1205, 1183, 1027, 838, 755; MS (ESI+) *m/z* 735 $[2M + Na]^+$, 379 $[M + Na]^+$, 357 $[M + H]^+$; HR-MS (ESI+) *m/z* calcd for $C_{19}H_{17}O_7$, 357.0974 $[M + H]^+$; found, 357.1005.

4-(7-Hydroxy-3-(4-hydroxyphenyl)-6-methoxy-4-oxo-4H-chromen-2-yl)butanoic acid (Δ4-Glycitein). According to the procedure described above for compound Δ3-glycitein), the intermediate **4** (2.0 g, 7.3 mmol) was reacted with methyl 5-chloro-5-oxovalerate (7.24 g, 6 mL, 44 mmol). Flash chromatography using CH_2Cl_2/methanol (9:1) as eluent gave a beige solid (R_f = 0.33), which was purified again by recrystallization from ethanol/water (1:1) to afford the desired compound as white crystals (0.9 g, 33%, mp 162 °C): ^{1}H NMR (CD_3OD, 250 MHz) δ 1.95 (pentuplet, J = 7.3 Hz, 2H, CH_2), 2.27 (t, J = 7.3 Hz, 2H, CH_2), 2.61 (t, J = 7.3 Hz, 2H, CH_2), 3.91 (s, 3H, OMe), 6.85 (d, J = 6.4 Hz, 2H, Har, 3′,5′), 6.87 (s, 1H, Har, 2), 7.07 (d, J = 6.4 Hz, 2H, Har, 2′,6′), 7.42 (s, 1H, Har, 5); ^{13}C NMR (CD_3OD, 63 MHz) δ 178.8 (C═O), 176.7 (COOH), 167.3 (CQ), 158.4 (CQ), 154.8 (CQ), 154.1 (CQ), 148.4 (CQ), 132.9 (CHar, 2′,6′), 125.3 (CQ), 123.9 (CQ), 116.8 (CQ), 116.5 (CHar, 3′,5′), 105.5 (CHar, 8), 103.9 (CHar, 5), 56.7 (OCH_3), 34.2 (CH_2), 32.9 (CH_2), 23.8 (CH_2); IR (cm^{-1}, KBr) 3517, 3208, 1712, 1625, 1591, 1516, 1478, 1440, 1391, 1289, 1227, 1165; MS (ESI+) *m/z* 763 $[2M + Na]^+$, 393 $[M + Na]^+$, 371 $[M + H]^+$; HR-MS (ESI+) *m/z* calcd for $C_{20}H_{19}O_7$, 371.1131 $[M + H]^+$; found, 371.1185.

Preparation of Conjugates. Each hapten (Δ3-glycitein or Δ4-glycitein) was coupled either to BSA (for injection into rabbits) or to Thyr (for coating). The conjugation procedure was inspired from refs *20* and *21*. Briefly, tributylamine (17.3 μL, 0.072 mmol), isobutyl chloroformate (4.7 μL, 0.036 mmol), and hapten (compound Δ3-Glycitein or Δ4-Glycitein, 0.036 mmol) were added to 1 mL of DMF at 4 °C and stirred on an ice bath for 30 min. The resulting activated hapten solution in DMF was added dropwise to the protein solution (BSA: 6.1 mg, 0.72 μmol or Thyr: 48.24 mg, 0.072 μmol) in 5 mL of borate buffer (borate−boric buffer 0.2 M, pH 8.7). The mixture was stirred for 6 h at room temperature, dialyzed against phosphate buffer saline (PBS; 0.01 M, pH 7.4, 0.9% NaCl) and then against distilled water. The hapten−protein conjugates were lyophilized and stored at −20 °C.

MALDI-MS. Mass spectra were recorded in the linear mode with a Bruker Reflex III mass spectrometer equipped with a UV laser (337 nm). Samples (10 μM) were mixed (1:1 v/v) with matrix solutions (sinapinic acid or α-cyano-4-hydroxycinnamic acid, 10 mM in water/acetonitrile containing 0.1% trifluoroacetic acid), and 1 μL of the mixture was applied on a stainless steel target and left for drying at room temperature. Spectra were acquired in the external calibration mode using BSA as a reference.

Immunization of the Rabbits. Immunization was achieved on New Zealand sanitary controlled rabbits, which were both pathogen and germ free, from Millegen (Labège, France). Rabbits were first ear-sampled for preimmunserum test. Hapten−BSA conjugates (500 μg) were injected each time. For the first injection the conjugate was dissolved in 1 mL of PBS/complete Freund's adjuvant (50:50). For the subsequent injections the antigen was dissolved in PBS/incomplete Freund's adjuvant (50:50). Injections were performed at multiple points according to the following injection schedule. The first three injections were performed at 1 week intervals. Two additional injections followed thereafter at a 3 week interval. One week after the fifth injection a test was performed on a 5 mL blood sample to check the specificities and titers of antibodies. Fifty milliliters was collected 1 week after the last injection. Serum was obtained after blood clotting at 4 °C for 24 h and centrifuged at 3000*g* for 10 min at 4 °C. Sera were stored at −20 °C in small aliquots. For each hapten−BSA conjugate two rabbits were injected. The efficiency of the immunization was tested in ELISA (as described below) by a direct binding of the antibody onto the coated conjugate. Subsequently, only the best of the two antibodies was used. Titers of all antisera were compared using the same Thyr−Δ4-glycitein conjugate coating antigens (0.1 μg mL^{-1}) and comparing the OD for a 1/40000 antibody dilution at 490 nm.

Validation of the Assay. *Origin of the Samples.* Blood samples of 17 women included in a 4 month clinical study were collected 12 h following the consumption of the last intake of soy-based supplements. The food supplement used was Phytosoya fort, from Arkopharma Laboratories, containing 35 mg of total isoflavones in two intakes a day (i.e., 70 mg total isoflavone and 57 mg in aglycone equivalent). The clinical study was monitored by Arkopharma (*22*).

Isoflavone Extraction from Plasma. Genistein, daidzein, and glycitein contained in 500 μL of plasma were first hydrolyzed using β-glucuronidase aryl-sulfatase from *Helix pomatia* (Roche) according to the protocol described by Bennetau-Pelissero et al. (*23*).

The hydrolysis was monitored using glycitin as external standard. A liquid−liquid extraction was then performed using acidified ethyl acetate (1% HCl). After evaporation to dryness, extracts were stored at −20 °C until assay. Three external standards of extraction containing genistein and daidzein were run in parallel to check for extraction recovery.

Extraction Procedure for Soy-Based Foods and Soy-Based Tablets. The extraction procedure for foodstuffs and food supplements (tablets or capsules) was as described by Bennetau-Pelissero et al. (*23*). The procedure required an enzymatic hydrolysis before ethyl acetate extraction. The recovery was monitored as previously described (*23*).

Table 1. Optimal Conditions for Each Assay Procedure

antisera		anti-Δ3-glycitein	anti-Δ4-glycitein
coating Δ4-glycitein−Thyr (μg mL^{-1})		0.1	0.25
specific antibody dilution		1/40000	1/40000
second antibody dilution		1/10000	1/10000
standard curve limit (ng mL^{-1})		1000−0.49	1000−0.49
IC_{50}[a] (ng mL^{-1})		15.6	62.5
detection limit (ng mL^{-1})		0.98	3.9
slope		0.0805 ± 0.009	0.0778 ± 0.08
interassay variation n = 11 (%)	low concentrations	10.52	ND
	high concentrations	12.50	ND
intra-assay variation n = 12 (of 11 plates) (%)	low concentrations	10.25	ND
	high concentrations	10.73	ND

[a] IC_{50} = concentration of the analyte required for 50% inhibition of the antibody binding to the coating antigen.

Assay Procedure. Coating of the wells was performed overnight with the Δ4-glycitein−Thyr conjugates (200 μL/well) in solution in a carbonate buffer (0.05 M, pH 9.6) at 4 °C. The wells were then saturated with PBS−Tween−PS−DMSO (PBS containing 1 mg mL^{-1} BSA, 0.05% Tween 20, and 1% DMSO) at 37 °C for 30 min. Plates were washed three times with PBS−Tween−DMSO (PBS, 0.05% Tween 20, 1% DMSO). Serial dilutions of the analyte in PBS−Tween−PS−DMSO were prepared as standard curves, and 100 μL/well was added to the plate. Specific antibodies in PBS−Tween−PS−DMSO were then added (100 μL/well). The incubation lasted for 2 h at 37 °C. The plates were washed three times with PBS−Tween−DMSO. Then 200 μL/well of the second antibody was added in PBS−Tween−PS−DMSO. The incubation was performed at 37 °C for 30 min. To measure peroxidase activity, 200 μL/well of substrate solution containing 0.005 M *o*-phenylenediamine and 0.00025% H_2O_2 in citrate−phosphate buffer (0.15 M, pH 5.0) was added. The reaction took place for 30 min at room temperature and was stopped with 4 M H_2SO_4 (50 μL/well). ODs were read at 490 nm. The standard curves were expressed as $\log(\text{glycitein concentrations}) = f[(B_i - NC)/(B_0 - NC)] \times 100$, where B_i is the OD of sample/standard wells. B_0 (maximum of binding) is obtained by the antibody reacting on coating without any competition. NC (negative control) is obtained without adding primary antibody. The standard curves counted 12 points in duplicates with a 2-fold increase between concentrations. From all of the experimental tests achieved, the best conditions retained are presented in **Table 1**. From this table it appears that the assay setup with the anti-Δ3-glycitein is more sensitive than that using the anti-Δ4-glycitein.

Cross-Reactivity Tests. The cross-reactivities of the antibodies were tested using a competitive procedure, according to the optimized conditions summarized in **Table 1**. Besides a standard curve obtained with the analyte, the antibody was exposed to serial dilutions of other compounds in PBS−Tween−PS−DMSO. The concentrations of the competitors varied from 50 to 0.003 μg mL^{-1} with a 4-fold decrease between concentrations.

Validation Tests. The assay was undertaken on seven different soy-based foodstuffs and on nine soy-based supplements. For these samples, dilutions had to be performed prior to analysis. In most cases the final dilution ranged between 1/5000 and 1/20000. Each value is the mean of three determinations. The variation coefficient of all these measures never exceeded 11%. In parallel, in each sample genistein and daidzein assays were performed in triplicates using the corresponding ELISAs as described by Bennetau-Pelissero et al. (*23*). Again, the variation coefficients never exceeded 10%.

RESULTS AND DISCUSSION

Immunogen Design. The crucial step in the development of an immunological assay is the production of antibodies with high specificity and selectivity, capable of recognizing the target compound from parent structures and metabolites in complex biological matrices. Glycitein is a small molecule and, thus, cannot act as an immunogen. The design and synthesis of corresponding haptens and hapten−protein conjugates are essential for successful antibody generation. Previous studies on the design and synthesis of different haptens of the isoflavonoids, such as formononetin, daidzein, biochanin A, genistein, and equol, demonstrated a significant difference in immunogenic activity in relation to the linker position on the isoflavone skeleton (*24, 25*). The linkers bearing a carboxylic function required for covalent coupling to carrier proteins were introduced at C7-OH, C8, and C2 positions. In previous works it was shown that the C2 position was the best choice for linker anchorage because the discriminating functions are preserved and favorably exposed for specific antibody recognition.

Therefore, the C2 site in glycitein was targeted for the synthesis of corresponding haptens, and the length of the alkyl chain was varied to study its influence on the specificity and sensitivity of the raised antibodies.

Synthetic Work. In an attempt to shorten the synthetic work, the preparation of glycitein used as standard during immunoassay development, as well as the preparation of two different haptens, was performed via the deoxybenzoin route (**Figure 1**). The key intermediate deoxybenzoin **4** was prepared from commercial 3-hydroxy-4-methoxybenzaldehyde **1**, which was transformed by oxidative rearrangement to corresponding formate **2** (not isolated), which was directly hydrolyzed to 4-methoxybenzene-1,3-diol **3**. Different experimental conditions and different protections of functional groups were reported in the Dakin oxidation reaction to afford compound **3** yield at 68−80% (*26–28*). In our optimized procedure based on the conditions reported by Lang'at-Thoruwa et al. (*19*) the yield of **3** was increased to 95%. The deoxybenzoin was prepared via the condensation of compound **3** and 4-hydroxyphenylacetic acid, followed by cyclization (*29*) to give an overall glycitein yield of 20%. The enolate of **3** generated by treatment with lithium hexamethyldisilazane (LiHMDS) was quenched with an appropriate acyl chloride derivative (*25*). The basic hydrolysis of the cyclized intermediates afforded yields of the targeted haptens Δ3-glycitein and Δ4-glycitein of 58 and 33%, respectively. The haptens were coupled to BSA for antibody production.

Analysis of BSA Conjugates. Considering the three-dimensional structure of BSA, only 26 ϵ-NH_2 groups of surface lysines (of 59 lysine residues of BSA) are theoretically available for coupling with haptens (*30*). A high ratio of hapten per molecule of BSA ensures the efficiency of the coupling reaction. Therefore, it is very important to quantify the hapten/BSA ratio before the administration of BSA conjugates to rabbits for antibody generation.

The conjugates were analyzed by matrix-assisted laser desorption−ionization mass spectrometry (MALDI-MS). The average molecular weight of each conjugate was calculated from both singly $(M + H)^+$ and doubly $(M + 2H)^{2+}$ charged peaks using two different matrices (**Table 2**). This method allowed the average number of bound molecules to be determined with

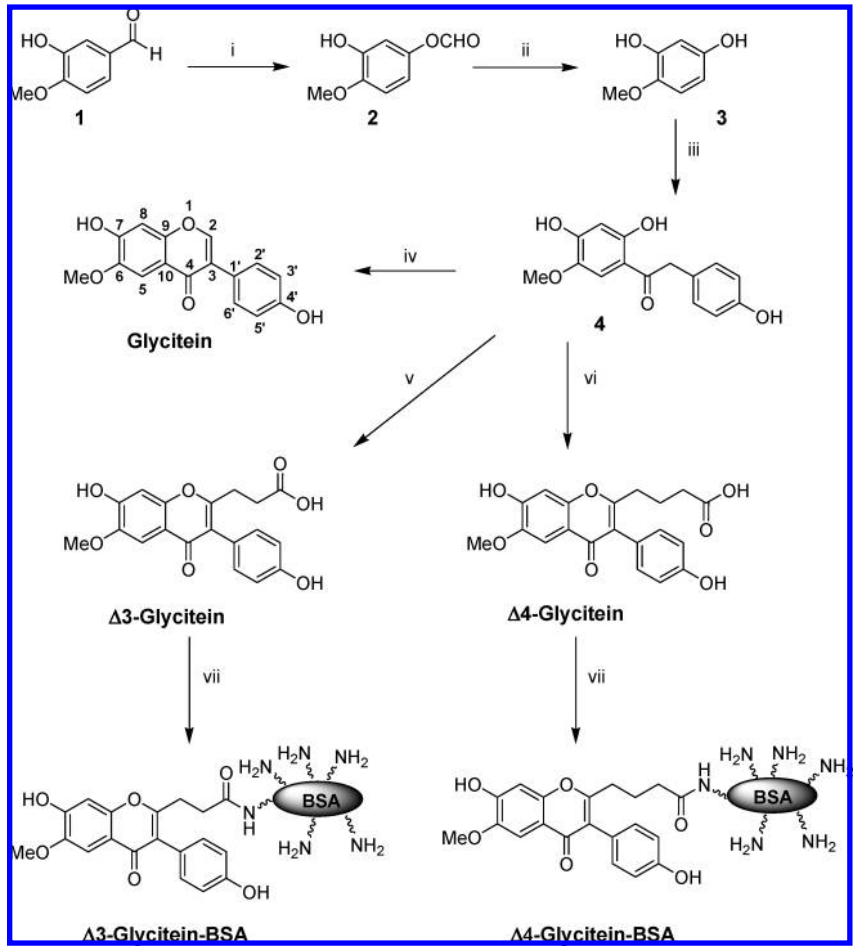

Figure 1. Synthesis of glycitein, C2-haptens, Δ3-glycitein, and Δ4-glycitein, with two lengths of linker, and corresponding BSA conjugates: (i) H_2O_2, 30%, CH_2Cl_2, SeO_2 cat.; (ii) Na_2CO_3 sat.; (iii) BF_3-Et_2O, 4-hydroxyphenylacetic acid, 80 °C; (iv) BF_3-Et_2O, $MeSO_2Cl$, DMF, 70 °C; (v) (1) LiHMDS, $ClC(O)(CH_2)_2CO_2Me$, THF, −78 °C, (2) 10% HCl, (3) 1 M LiOH, 0 °C; (vi) (1) LiHMDS, $ClC(O)(CH_2)_3CO_2Me$, THF, −78 °C, (2) 10% HCl, (3) 1 M LiOH, 0 °C; (vii) tributylamine, isobutyl chloroformate, DMF, 4 °C, then BSA in borate−boric buffer 0.2 M. pH 8.7.

Table 2. Comparison of Hapten Incorporation on BSA Conjugates Determined by MALDI-MS Analysis

	calcd hapten no. per molecule of BSA $(MW_{conjugate} - MW_{BSA})/MW_{hapten}$				
	matrix 1[a]		matrix 2[b]		
conjugate	ion 2^+	ion 1^+	ion 2^+	ion 1^+	av
Δ3-glycitein−BSA	18.4	17.9	21.3	21.0	19.9 ± 1.4
Δ4-glycitein−BSA	22.3	22.1	23.1	22.3	22.5 ± 0.5

[a] α-Cyano-4-hydroxycinnamic acid. [b] Sinapinic acid.

a high level of reproducibility and accuracy. A high degree of modification of available sites on the protein surface was achieved, because about 20 haptens per molecule of BSA were found for each conjugate.

Immunization Tests. As shown in **Table 1**, titers of the antibodies are similar, because for both antibodies the dilution required for ELISA tests was the same (1/40000). This means that when using the same immunization protocol, the arm length did not affect the immunogenic properties of BSA conjugates.

ELISA Tests. The optimal conditions of the ELISAs are shown in **Table 1**. The standard sigmoid curves obtained for each of the antibodies are presented in **Figure 2**. Anti-Δ3-glycitein gives better results because the linear portion of the curve is larger and the sensitivity obtained for 50% binding is lower. This antibody will be used routinely hereafter. Note that the best sensitivity may be due not only to the antibody but also to the heterologous coating conditions (*31*). Indeed, as mentioned earlier, in this assay coating was performed using Thyr−Δ4-glycitein conjugate. The glycitein detection limits reached here were 0.98 ng mL^{-1}, that is, 0.69 pmol/well, with anti-Δ3-glycitein, and 3.9 ng mL^{-1}, that is, 2.7 pmol/well, with anti-Δ4-glycitein. This sensitivity is higher than that described by Aguiar et al. (*17*) and of the same order of magnitude of that recently obtained by several other authors (*10, 32*).

Recently, Grace et al. (*12*) also presented a sensitive and reliable method for phytoestrogen measurement in biological fluids using liquid chromatography−tandem mass spectrometry. This technique is as applicable to a large number of samples as ELISA is, but its drawbacks lie in the running costs and availability of equipment. Intra- and interassay variation coefficients were determined for the anti-Δ3-glycitein antibody. Interplate variations were calculated as variation coefficient of measurements carried out on 11 different microtitration plates. Intra-assay variations were calculated as the mean of 11 variation coefficients. Each coefficient was obtained using 12 measures of the same glycitein samples and on the same plate. The tests were performed for either low (75 ng mL^{-1}) or high (15 μg mL^{-1}) glycitein concentrations. These variations were always below 15% (**Table 1**). Therefore, this assay appeared to be as reliable as other physicochemical techniques.

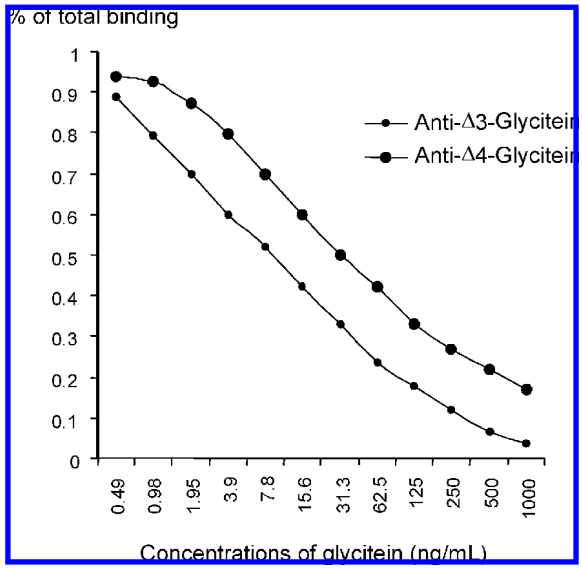

Figure 2. Standard curves obtained with the two antibodies. Points are the mean of duplicates. Equation of the competitive assay is log[glycitein concentrations] = $f(B_i - NC)/(B_0 - NC) \times 100$, where B_i is the optical density of the sample or standard, NC is the negative control obtained without antibody, and B_0 is total binding without any competitor. Analyte−antibody combinations are listed in **Table 1**.

Table 3. Cross-Reactions (Percent) with Each Antibody

	anti-Δ3-glycitein	anti-Δ4-glycitein
genistein	0.32	0.25
daidzein	1.28	1
equol	0.02	<0.1
biochanin A	0.12	0.25
formononetin	0.96	0.5
naphthoflavone	0.48	0.125
naringenin	<0.05	<0.05
chrysin	<0.05	<0.05
quercetin	<0.05	<0.05
flavone	0.027	<0.125
17β-estradiol	<0.02	<0.125
luteolin	0.02	<0.125
eriodyctiol	0.032	NS
hesperitin	<0.024	NS
O-DMA	0.024	NS

Cross-Reactivity of the Antibodies. Two antisera, anti-Δ3-glycitein and anti-Δ4-glycitein, were tested in detail for cross-reactivities (**Table 3**). The cross-reactivity of an antibody is defined to be its ability to react with a molecule distinct from that for which it was initially raised. None of the structurally related substances tested was responsible for a cross-reaction of >1.3%. These data indicate a high specificity of both antisera. It is important to note that there is no significant difference in specificity between antibodies raised against Δ3-glycitein− and Δ4-glycitein−BSA conjugates, which differ in linker length. Thus, glycitein is well-discriminated, and the cross-reactions with the parent compounds likely to be present in food matrices or in biological samples at the same time as glycitein are very low.

These results allow using this ELISA assay for soy, soy-based products, and biological fluids from persons fed soy-based food items. Indeed, similar specificity was previously obtained with antibodies raised against isoflavone compounds also coupled to carrier proteins via a carboxylic linker at C2 position (*25*). This specificity can be explained by the favorable exposure of discriminating functions, such as the methoxy group on C6 present only on glycitein and not on other isoflavonoids. In previous works (*24, 25*), different haptens of isoflavones were synthesized with a linker on the hydroxyl group in C7 or on the C8 position. These haptens generated antibodies with high cross-reactivity between daidzein and genistein and between biochanin A and formononetin. It was explained through the fact that protein linkage of these haptens masked the C5 position and did not allow discriminating between molecules having or not a hydroxyl group at the C5 position. Among the different haptens synthesized, those with the linker in the C2 position were shown to generate the antibodies with the best specificity allowing discrimination between the compounds with a OH group at C5. In addition, choosing the linker in the C2 position is likely not to interfere with other functional groups branched on the A ring such as the MeO function present on the C6 position in glycitein. It is also important to emphasize that the variation of the alkyl chain length of the linker with the same position of anchorage has no influence on the efficiency of the coupling reaction with the carrier protein and on the cross-reactivity of the antibodies raised. On the other hand, the slight lengthening of the linker (from three to four carbons) caused a 4-fold decrease of the detection limit and IC_{50} (**Table 1**). The loss of the sensitivity can be explained by the lower covering of protein surface by the antigen with the longer linker and is in agreement with the previous study on 8-prenylnaringenin (*33*). It can also be due to heterologous competitive conditions because anti-Δ3-glycitein is challenged in the assay with free glycitein and Thyr−Δ4-glycitein, which can favor the binding to free compound.

Validation of the Assay. The assay was undertaken on seven different soy-based foodstuffs (**Table 4**) as well as on nine soy-based supplements (**Table 5**). Generally, foodstuffs are prepared from soybean, which is known to contain only a small percentage of glycitein (*3*). This was confirmed by our ELISA assay. In all foodstuffs, glycitein appeared to account for <10% of total isoflavone except in the soy sauce, where it accounted for 25%. In soy-based food supplements, there was a greater variability, showing that the origin of the raw material used in the preparation of isoflavone extracts was more variable (**Table 5**).

Indeed, some preparations were based on soybean (*34*), some were based on soy germ known to contain large proportions of glycitein (*24*), and some were prepared from synthetic isoflavones (*35*). In the last case, glycitein was absent. As already mentioned (*7, 23, 36*), the claimed dose and the dose measured in our laboratory did not always correspond. This was explained by the presentation of our data in equivalent aglycone, whereas manufacturers gave their data in glycosylated forms, which included different proportions of glycosyl, acetyl, and malonyl forms. In France, the health authorities recently recommended citing isoflavone content in aglycone equivalents, but this recommendation has not been followed by manufacturers so far (*37*).

The assay was also tested on plasma samples collected from women taking Phytosoya fort supplements for 4 months. Plasma glycitein concentrations were found to be in the 40.14−369.59 ng mL^{-1} range (**Table 6**). Phytosoya fort is classically prepared from soy germ and contains 45% of daidzein, 44% of glycitein, and 11% of genistein (**Table 5**). On the basis of what is known from Richelle's work (*8*) on the bioavailability of these isoflavones, it was expected to find rather modified percentages of each isoflavone in plasmas. Indeed, following chronic intake, as mentioned previously by Mathey et al. (*38*), a dynamic equilibrium is classically reached, and plasma and urinary levels do not always reflect the ingested proportions of isoflavones.

Table 4. Isoflavone Content in Several Foodstuffs Based on Soy

liquid foodstuff	trademark	manufacturer	daidzein (μg mL^{-1})	genistein (μg mL^{-1})	glycitein (μg mL^{-1})	total per mL (mg mL^{-1})	serving size (mL)	total per serving (mg/serving)
soy-based infant formula[a]	Gallia soja	Danone	11.20	22.50	2.63	36.33	900	32.70
soy-based infant formula[a]	Modilac soja	Sodilac	13.10	17.40	3.28	33.78	900	30.40
soy milk	Lait de soja nature	Gerblé	120.08	149.50	15.50	285.08	350	99.78
soy sauce	Bio Shoyu	Lima	11.27	5.55	5.65	22.47	6	0.13

solid foodstuff	trademark	manufacturer	daidzein (μg g^{-1})	genistein (μg g^{-1})	glycitein (μg g^{-1})	total per mL (μg g^{-1})	serving size (g)	total per serving (mg/serving)
soy yogurt	Senja au soja	Danone	48.48	108.10	8.85	165.43	120	19.85
powder for instant milk	Bio Soja Cao	Heuprophax	50.34	67.29	9.89	127.52	3	0.38
soy dessert cream	Dessert soja	Gayelord Hauser	32.46	66.07	1.55	100.08	120	12.01

[a] Servings are those proposed for 4-month-old babies.

Table 5. Isoflavone Concentrations in Various Soy-Based Food Supplements Sold in France

trademark	laboratory	claimed dose (mg/capsule or tablet)	form	daidzein (mg/capsule or tablet)	genistein (mg/capsule or tablet)	glycitein (mg/capsule or tablet)	% of glycitein	total (mg/capsule or tablet)
Bioptimum Soja	Boiron	25	tablet	9.00	3.10	8.68	41.7	20.78
Compléal soja NG	Besins Inc. Nutraceutique	45	capsule	14.35	25.90	0.00	0	40.25
Equilibre feminin	Vitarmonyl	17.5	capsule	5.00	10.70	1.32	7.7	17.02
Evestrel	Theramex	37.5	tablet	9.19	11.61	1.26	5.7	22.06
Féminité Soja 3D	Oenobiol	20	tablet	5.80	4.20	1.97	16.4	11.97
Ménopause	Juvamine	5	capsule	1.20	2.70	0.55	12.3	4.45
Phytosoya	Arkopharma	17.5	capsule	6.69	1.58	6.59	44.3	14.86
Soja Cal	Novagyn	30	capsule	6.30	9.50	1.20	7.0	17.00
Sojyam	Tonipharm	15	capsule	2.33	4.98	0.71	8.8	8.02

Table 6. Concentrations of Isoflavones in the Plasma of Postmenopausal Women Taking a Food Supplement Based on Soy Germ

	concentrations (ng mL^{-1})						
volunteer	daidzein	genistein	glycitein	total	% daidzein	% genistein	% glycitein
PIAN	238.11	70.08	204.52	512.71	46.44	13.67	39.89
FRBE	361.82	126.92	175.38	664.12	54.48	19.11	26.41
PIGE	84.34	55.64	40.14	180.12	46.82	30.89	22.28
DURE	302.73	80.11	171.49	554.33	54.61	14.45	30.94
SIMI	164.68	51.92	172.87	389.47	42.28	13.33	44.39
DACH	245.76	91.18	14.80	482.74	50.91	18.89	30.20
BEEL	115.24	110.09	83.54	308.87	37.31	35.64	27.05
JINE	195.39	79.53	170.99	445.91	43.82	17.84	38.35
BENA	564.64	193.89	353.83	1112.36	50.76	17.43	31.81
LAAN	169.08	159.32	158.21	486.61	34.75	32.74	32.51
ANAN	515.84	182.02	369.59	1067.45	48.32	17.05	34.62
BAHU	126.81	49.56	152.01	328.38	38.62	15.09	46.29
BRDO	343.26	103.23	253.97	700.46	49.00	14.74	36.26
DEMA	202.34	66.19	199.16	467.69	43.26	14.15	42.58
JOHE	308.73	133.14	197.45	639.32	48.29	20.83	30.88
CAMO	111.42	70.92	152.26	334.60	33.30	21.20	45.51
JACH	843.94	29.,80	317.22	1457.96	57.88	20.36	21.76
mean	287.89	112.97	195.20	596.07	45.93	19.85	34.22
SEM	197.59	64.94	86.64	331.64	7.07	6.85	7.73
min	84.34	49.56	40.14	180.12	33.30	13.33	21.76
max	843.94	296.80	369.59	1457.96	57.88	35.64	46.29

Richelle et al. (*8*) reported that the bioavailability of isoflavones ranged as follows: genistein > daidzein > glycitein. Indeed, glycitein seemed to be absorbed less than the other two, and genistein has a longer residence time in plasma due to its slower elimination. Therefore, if the ingested percentages were taken into account, it was expected that the percentage of glycitein in plasma should be lower than in the supplement and the percentage of genistein should be higher. Our results confirm our expectations because the mean percentages in plasma were 46% for daidzein, 20% for genistein, and 34% for glycitein (for further information on daidzein and genistein see ref *11*). Note that, as already mentioned in Mathey et al. (*38*), there was a great variability in plasma levels. Indeed, when total isoflavones were considered, the minimum value was 180.12 ng mL^{-1} and the maximum value was 1457.96 ng mL^{-1}.

In conclusion, it appears in this work that whatever the linker length (three or four carbons) the specificity of the assay is not markedly modified. Therefore, the ELISA presented here is a

useful new method for the rapid and low-cost measurement of glycitein content in foodstuffs, in food supplements based on soy extracts, and in human plasmas, although quantification by liquid chromatography coupled to tandem mass spectrometry may be more sensitive. However, ELISAs are easier to use than chromatographic techniques, especially those coupled to tandem mass spectrometry. They are also more convenient than RIA because they do not need radioisotopes. Finally, compared to other methods, our ELISA is suitable for high-throughput screening of glycitein levels in epidemiological studies. It can also be used by food manufacturers to check their materials before and after processing.

ABBREVIATIONS USED

B_i, OD of sample/standard wells; B_0, OD of positive control; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, estradiol receptor; ESI, electrospray ionization; IC_{50}, concentration for 50% inhibition; HR-MS, high-resolution mass spectrometry; IR, infrared spectroscopy; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; NBS, *N*-bromosuccinimide; NC, negative control, NMR, nuclear magnetic resonance; OD, optical density; PBS, phosphate buffer saline; ppb, parts per billion; ppm, parts per million; PS, pork serum; RIA, radioimmunoassay; TLC, thin-layer chromatography; Thyr, thyroglobulin.

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