JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

2DLC-UV/MS Assay for the Simultaneous Quantification of Intact Soybean Allergens Gly m 4 and Hydrophobic Protein from Soybean (HPS)

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ABSTRACT: Top-down approaches for quantification of proteins based on separation and mass spectrometric assays hold promise due to their high specificity and avoidance of both proteolytic steps and need for generation of monoclonal antibodies. In this study, a 2DLC-UV/MS assay was developed for the simultaneous quantification of two intact soybean allergens, hydrophobic protein from soybean (HPS) and Gly m 4. Both of these allergens were purified from soybean seeds followed by complete characterization. The method validation consisted of evaluating linearity, precision, and recovery. A linear relationship (R^2 > 0.99) between concentrations of the two proteins and their respective peak areas was observed over the concentration ranges from 6.9 to 355.1 μg/mL and from 11.9 to 599.8 μg/mL for Gly m 4 and HPS, respectively. For the 4 day validation study, precision range (%CV) was observed to be from 4.7 to 9.2% for HPS and from 6.3 to 9.4% for Gly m 4. The assay recovery range (%RE) was observed to be from −1.1 to −13.7% for HPS and from −3.5 to 15.2% for Gly m 4. The assay was applied on 10 non-transgenic commercial lines to quantify the relative levels of the two allergens. The HPS and Gly m 4 levels ranged from 64 to 479 μg/g and from 204 to 637 μg/g, respectively. To the best of the authors' knowledge, this represents the first 2DLC-UV/MS assay for the simultaneous quantitation of selected allergens at the intact level.

KEYWORDS: *soybean allergens, quantification, HPS, Gly m 4*

INTRODUCTION

There is a growing regulatory need to develop analytical methods for the accurate quantification of allergens in food crops for monitoring changes, if any, in their concentrations as a result of genetic modification or due to cross-breeding between different genetic lines.[1−3] Successful implementation of these methods will aid in our understanding of allergen levels within grains from different crop varieties and any contribution from environmental factors among others and will help in determining thresholds for each allergen above which an allergenic response is elicited.[4]

Soybean has been classified as an allergenic food and contains multiple allergenic proteins.[5] Eight of these proteins have been classified as soybean allergens by the International Union of Immunological Societies (IUIS).[6] The most popular methods by far for allergen quantification are immunoassays, using either monoclonal[7] or polyclonal antibodies.[8] These are highly sensitive and relatively easy to use but suffer from limited specificity, and the results depend on the type of immunoassay, the standards used, and the specificity and affinity of the antibodies.[9] Recently, several analytical methods focusing on proteomics have been leveraged for quantitation of different allergens.[10] Approaches based on multiple reaction monitoring (MRM) have been used to target multiple soybean allergens.[11,12] Although very robust, especially for lower abundance proteins, issues related to enzymatic digestion efficiency[10,13] and possible lack of specificity still need to be addressed.[14,15] Some of these issues can be resolved by developing assays that circumvent the digestion step using liquid chromatography with ultraviolet or mass spectrometer based detectors or a combination of both. In the presence of reference standards, this top-down approach can provide a relatively straightforward and specific method for quantification of specific intact proteins. Specific intact proteins have been quantitated previously using LC-UV-,[16] LC-MS-,[17] and 2DLC-UV/MS-based[18] approaches. These assays allow for accurate quantification, as the reference protein itself is being used to quantify the protein of interest. Moreover, this approach also has the potential to quantify levels of individual protein isoforms.

In this study, we have simultaneously quantified two intact allergenic proteins, hydrophobic protein from soybean (HPS) and Gly m 4, using a single 2DLC-UV/MS-based assay.

HPS is an allergen that causes asthma in individuals allergic to soybean dust.[19] HPS is an approximately 8.3 kDa protein with 80 amino acids containing several hydrophobic amino acid moieties.[20] HPS shows a pattern of eight cysteine residues which form four disulfide bridges similar to several nonspecific lipid-transfer proteins.[21] HPS and Gly m 1 soybean allergens share the same primary amino acid structure.[22] The complete functional role of HPS is still not known, but it likely influences physical properties of seed surface by mediating the attachment of endocarp tissues to the seed surface.[23]

Gly m 4 is an approximately 17 kDa protein with 157 amino acids and is classified as a major allergen.[24] Gly m 4 was first

Received: January 7, 2014
Revised: April 7, 2014
Accepted: May 1, 2014
Published: May 1, 2014

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identified at the mRNA level as one of the genes in the family of stress-induced, developmentally regulated soybean genes.[25] The physiological function of Gly m 4 is also still unknown. Gly m 4 is highly homologous with the birch pollen allergen Bet v 1.[24] The three-dimensional structures of recombinant Gly m 4 (rGlym 4) and Bet v 1 are nearly identical, thereby causing high cross-reactivity via the same IgE antibodies.[24] In a recent paper, our laboratory published a quantitative assay for the determination of endogenous intact Gly m 4 protein.[26] The endogenous Gly m 4 protein has 88.7% sequence homology with the theoretically predicted Gly m 4 sequence. The endogenous Gly m 4 protein belongs to the same stress-induced gene family as SAM 22 and H4 proteins.[26]

In this study, both HPS and Gly m 4 were isolated from soybean seeds. Following isolation, purification, and detailed characterization, a multiplexed 2DLC-based approach was developed for simultaneous quantification of the two proteins. The two orthogonal chromatographic dimensions used were strong anion exchange chromatography (SAX) and reversed phase chromatography (RPC) as the first and second dimensions, respectively. The advantage of using the 2DLC-based approach over single-dimension separation assays was the ability to completely resolve the protein of interest from other coeluting components and the ability to multiplex. The assay was evaluated for selectivity, linearity, sensitivity, and recovery and was used to determine the concentrations of Gly m 4 and HPS in different commercial soybean lines.

MATERIALS AND METHODS

Chemicals. The commercially available, non-transgenic soybean seeds were purchased from the following: H387 (lot JK1700) from Hoffman (Hoffmann, IL, USA); 363 (lot PS9-3633) from Phillips (Hope, KS, USA); William 82 (lot 9S-10-107) from Missouri Seed Foundation (Columbia, MO, USA); 3930 (lot S080184 GR01-596) from Croplan LC (St. Paul, MN, USA); C3884N (lot 1208294) from LG Seeds (Elmwood, IL, USA); pH-4396 (lot 01A (75148)) from Porter Hybrid (Wilmington, OH, USA); HS38C60 (lot SZ090151) from Hi Soy (Bloomington, IL, USA); 029311K (99915) (lot DSR-3590 029301K) from Dairyland/Precision Soya (West Bend, WI, USA); XP843-252 (lot 9E394N) from Brown Seed (Neoga, IL, USA); and Maverick (lot 8S-18-07) from Missouri Seed Foundation. Dithiothrietol (DTT), iodoacetamide (IAM), and E-64 enzyme inhibitor were purchased from Sigma (St. Louis, MO, USA). Sodium chloride, glycerol, LC-MS grade trifluoroacetic acid (TFA), HPLC grade isopropanol (IPA), tris buffer, hexanes, and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). A Steriflip disposable vacuum filtration system with a 0.22 μm membrane filter was purchased from Millipore (Billerica, MA, USA). Trypsin was obtained from Roche Applied Sciences (Indianapolis, IN, USA). For all analyses, Milli-Q (Millipore) deionized water was used.

Generation of HPS and Gly m 4 Reference Standards. Both HPS and Gly m 4 proteins were purified from a non-transgenic (Maverick) soybean line using similar conditions. Soybean kernels were first ground to a fine powder with a Robot Coupe grinder (model RSI 2Y-1, Robot Coupe USA, Inc.) using an equal amount of dry ice. This was followed by defatting the milled soybean seeds with hexanes (milled soybean seeds/*n*-hexanes, 1:0.19 g/mL) for 1 h at room temperature and subsequent removal of hexanes by centrifugation. The procedure was repeated two more times. The dry defatted seed material was distributed between 22 50-mL conical centrifuge tubes, with approximately 7 g of defatted soybean seeds in each tube. Allergens were extracted with 40% v/v IPA/water (seed/extraction buffer 1:5 g/mL) for 1 h at room temperature. Supernatant, containing residual fine flour particles, was filtered using a 0.45 μm filter membrane. The wet seed material was subjected to a second round of extraction with 40% v/v IPA/water, and the extract was subjected to filtration as before. The extracts were subsequently subjected to centrifugal separation at 20000*g* at 4 °C to remove the fine particles. Cold acetone precipitation of the extracted proteins was performed at −20 °C for 2 days (extract/acetone = 1:4). The resulting precipitate was subjected to centrifugal separation at 7200*g* for 15 min, and the supernatant was discarded. The protein precipitate was resolubilized in the solubilization buffer (0.1 M Na_2SO_4/0.2 M NaH_2PO_4/0.05% Tween-20/5% glycerol, pH 8). The purification of HPS and Gly m 4 from the resolubilized crude protein solutions was accomplished using reversed-phase HPLC in two steps: during the first step, HPS and Gly m 4 were crudely separated from each other and the other components in the extract; during the second step, each of the target components (HPS and Gly m 4) was purified to homogeneity. For both steps, reversed phase chromatography was performed using a linear gradient of buffer A (0.1% TFA in water) and buffer B (0.1% TFA in ACN) mobile phases on a Waters XBridge300 C4 250 × 4.6 mm column (Milford, MA, USA). During the first step, a linear gradient from 24 to 29% B was performed to crudely isolate HPS and a linear gradient from 36 to 39% B was performed to isolate Gly m 4. The HPS and Gly m 4 fractions were pooled, lyophilized to dryness, and reconstituted in 5% acetonitrile/0.1% TFA. The HPS-containing fraction was subjected to RP chromatographic separation using a linear gradient between 23 and 31% B over 40 min. HPS-containing fractions were pooled and lyophilized. The Gly m 4-containing fraction was subjected to RP chromatographic separation using a linear gradient between 35.5 and 45.5% B. The Gly m 4 fractions were pooled and lyophilized. The HPS fractions were resolubilized in dissolution buffer containing 100 mM Tris buffer at pH 8.5. For Gly m 4-containing fractions, 10 mM DTT was added to prevent dimerization of Gly m 4. For the generated reference standards, protein concentration was determined by quantitative amino acid analysis. The pooled fractions were aliquoted into vials and stored at −80 °C. Gly m 4 protein has one cysteine residue and, as mentioned, DTT was added to the reference standard to prevent dimerization. In contrast, the presence of DTT in the solvent results in a mixture of variably reduced and nonreduced species for HPS. Consequently, the two reference standards were resolubilized in different storage buffers and also analyzed separately to obtain their respective UV response factors prior to analysis of soybean extracts. The concentrations of HPS and Gly m 4 reference standards were determined by quantitative amino acid analysis based on the amino acid analyses method from Agilent Technologies.

Extraction of HPS and Gly m 4 from Soybean Seeds for 2DLC-UV/MS Assay. Milled ground soybean seeds, stored at −20 °C, were thawed at room temperature in a drybox containing Dry-Rite. Approximately 250 mg of milled soybean seeds was weighed, defatted using 1 mL of hexanes, and mixed at 1200 rpm for 1 h at 25 °C on a Thermomixer. The sample was centrifuged at 30130*g* for 5 min at 4 °C. The supernatant was discarded, and the procedure was repeated followed by removal of the residual hexane present in the defatted sample by evaporation for approximately 5 min. The resulting sample was resuspended in 1 mL of protein extraction buffer (40% v/v IPA/water fortified with enzyme inhibitor, E64 at 9.3 μM). This was followed by vortexing and overnight extraction (for 16 h) at 1200 rpm at 25 °C using a Thermomixer. Following extraction, the sample was centrifuged at 30130*g* for 30 min at 4 °C. The supernatant was transferred to a microfuge tube and subjected to additional centrifugation at 30130*g* for 5 min at 4 °C to remove residual particles. The extracts were then aliquoted for subsequent analyses by 2DLC-UV/MS analyses.

Conditions for 2DLC-UV/MS Assay. Two-dimensional liquid chromatography (2DLC) analyses with ultraviolet and mass spectrometric detection was performed in the heart-cutting mode as described previously.[26] The samples were injected as 10 μL injection volumes using a partial loop fill injection mode. A Propac SAX column (Thermo Scientific, Waltham, MA, USA) with dimensions of 250 mm × 4 mm and 5 μm particle size was used as the first-dimension column. A reversed phase Acquity BEH300 C4 column (Waters) at 70 °C with column dimensions of 100 mm × 2.1 mm and 1.7 μm particle size was used in the second dimension. The 2DLC method details are

presented in Table 1. The first-dimension window for elution of Gly m 4 and HPS reference standards was set before the analyses. The first-dimension cuts from respective time windows were diverted toward a 10 port valve containing two 5 mm × 2.1 mm Van Guard BEH300 C4 reversed phase (RP) trap cartridges (Waters). The proteins eluting between 2.2 and 6.0 min were trapped on the first RP trap cartridge, and the proteins eluting between 8.9 and 16.3 min were trapped on the second RP trap cartridge.

Table 1. Gradient Elution Conditions for First and Second Dimensions for Quantitation of HPS and Gly m 4 from Soybean Seeds Extracts

first dimension: SAX				second dimension: reversed phase			
SAX cuts: 2.2−6.0 and 8.9−16.3 min				temperature: 70 °C			
temperature: 30 °C				MPA2: 0.1% TFA in water			
MPA1: 10 mM Tris-HCl, pH 7.6				MPB2: 0.1% TFA in 90/10 2-propanol/acetonitrile			
MPB1: 10 mM Tris-HCl/1 M NaCl, pH 7.6				diode array detector wavelength: 215 nm			
VWD wavelength: 215 nm				bandwidth and slit width: 4 nm			
peak width: 0.2 min				peak width: 0.4 min			
time, min	flow rate, mL/min	% A1	% B1	time, min	flow rate, mL/min	% A2	% B2
initial	0.5	95	5	initial	0.3	86	14
20	0.5	75	25	8	0.3	86	14
21	1	75	25	38	0.3	80	20
26	1	0	100	41	0.3	40	60
31	1	95	5	44	0.3	74	26
36	1	95	5	49	0.3	74	26
37	0.1	95	5	59	0.3	65	35
63	0.1	95	5	62	0.3	40	60
64	0.5	95	5	65	0.3	86	14
				68	0.3	86	14

UV data were acquired using a variable-wavelength detector (Agilent) and a diode array detector (Agilent) for the first and second dimensions, respectively. A solution containing 7:25:68 v/v% of glycerol/acetonitrile/water was teed into the second-dimension effluent before mass spectrometric analysis. Positive-ion electrospray ionization (ESI) was performed on an Agilent 6538 mass spectrometer. The m/z scan range was 500−2500 amu, and the data were acquired at 1.32 spectra/s.

RESULTS AND DISCUSSION

Isolation and Characterization of HPS and Gly m 4 Allergens. HPS was isolated from soybean seeds using a 40% isopropanol solution on the basis of a previously published approach[26] with minor modifications. HPS elutes as two major isoforms; the main isoform (form II) consists of the complete amino acid sequence (1−80), whereas the minor isoform (Form I) is truncated at the N-termini (3−80). Truncation at the N-terminus has been attributed to the proteolytic activity in the soybean hulls and seeds. The reference standard was generated for the main isoform and was denoted as the HPS reference standard for quantification purposes. The HPS reference standard was characterized by reversed phase chromatographic analysis with UV (215 nm) and ESI-MS detection (Figure 1). The reference standard elutes as a single chromatographic peak with a retention time of 15.2 min. The transformed maximum entropy spectrum depicts intact masses for this peak at 8351.3 Da. The observed mass is within 0.006% of its theoretical average molecular mass. Further detailed characterization of the reference standard was carried out using peptide mass fingerprinting with successive in-solution proteolysis with trypsin/endoproteinase Asp-N and an in-solution limited trypsin proteolysis. The sequences were confirmed by ESI/LC/MS and tandem ESI/LC/MS/MS analyses. A summary of mass spectral data and assignments from ESI/LC/MS/MS analyses are presented in Table 2. On the basis of the application of multiple proteases, complete sequence coverage was obtained for the isolated HPS reference standard.

HPS has been interchangeably referred to as Gly m 1, a soybean allergen, in several papers. On the basis of one report, the two proteins are essentially the same protein but post-

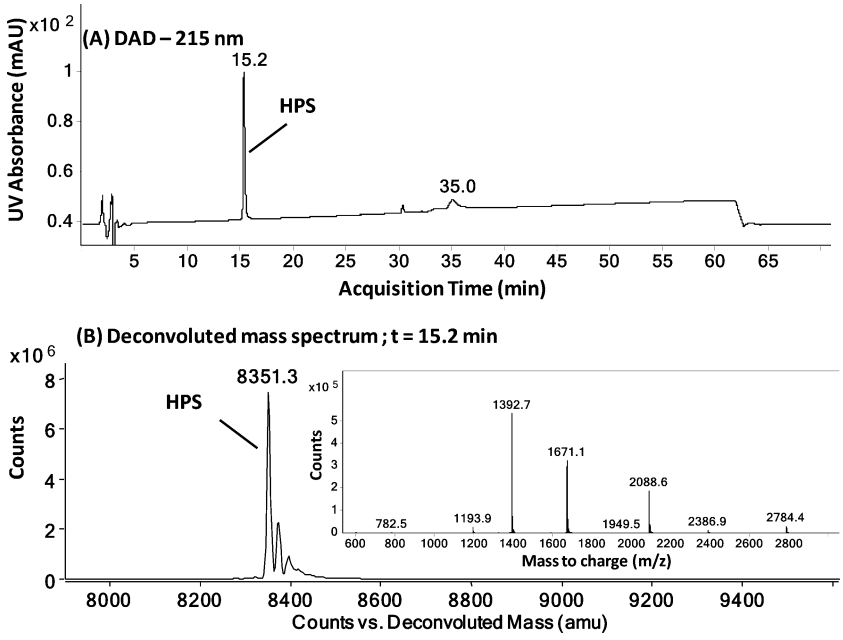

Figure 1. Characterization of reference HPS standard: (A) blank subtracted LC-UV (215 nm) chromatogram of intact reference HPS standard; (B) deconvoluted mass spectrum of reference HPS standard (inset, multiple charge envelope mass spectrum).

dx.doi.org/10.1021/jf500087s | J. Agric. Food Chem. 2014, 62, 4884−4892

Table 2. Summary of Peptides Identified by LC-MS/MS Analysis of HPS Reference Standard Subjected to Successive Proteolytic Cleavage Using Trypsin and Endoproteinase AspN

sequence location	sequence	mass		delta ppm
		theor	exptl	
A(1−9)	ALITRPSCP	1013.5328	1013.5327	−0.1
A(1−25)	ALITRPSCPDLSICLNILGGSLGTV	2626.3823	2626.3807	−0.57
A(1−26)	ALITRPSCPDLSICLNILGGSLGTVD	2741.4092	2741.4068	−0.87
A(10−25)	DLSICLNILGGSLGTV	1630.8600	1630.8589	−0.67
A(10−26)	DLSICLNILGGSLGTVD	1745.8870	1745.8858	−0.66
A(26−36)	DDCCALIGGLG	1149.4794	1149.4791	−0.34
A(27−36)	DCCALIGGLG	1034.4525	1034.4519	−0.62
A(37−49)	DIEAIVCLCIQLR	1601.8269	1601.8252	−1.09
A(37−69)	DIEAIVCLCIQLRALGILNLNRNLQLILNSCGR	3835.0747	3835.0689	−1.51
A(50−58)	ALGILNLNR	982.5924	982.5919	−0.48
A(59−69)	NLQLILNSCGR	1286.6765	1286.6759	−0.45
A(70−79)	SYPSNATCPR	1151.5030	1151.5028	−0.18
A(70−80)	SYPSNATCPRT	1252.5506	1252.5500	−0.5

translationally processed in a different way.[22] Although there is a potential N-glycosylation site near the carboxyl terminus (aa 74−76), similar to other studies,[20] our laboratory did not detect the presence of glycosylation on the asparagine amino acid residue in HPS. The two purified HPS isoforms were subjected to isoelectric focusing. The p*I* of forms I and II were found to be 6.7 and 7.0, respectively (data not shown). No glycosylation was observed for either of the two isoforms of HPS. Further research is needed to unequivocally resolve whether glycosylation is indeed present on the Gly m 1 protein as stated in the literature.

The Gly m 4 reference standard was also generated from soybean seeds. Purification and detailed characterization of the Gly m 4 reference standard has been reported previously.[26] Similar to HPS, complete sequence coverage was also obtained for the Gly m 4 protein.

The concentrations of HPS and Gly m 4 reference standards were determined by quantitative amino acid analysis to be 770 and 402 μg/mL, respectively. Both HPS and Gly m 4 reference standards were diluted to the required concentration for 2DLC-UV/MS assay using 10 mM Tris, pH 8.5, buffer.

Method Development. The 2DLC-UV/MS-based Gly m 4 quantification method[26] was further expanded to accommodate quantitation of HPS in a single analysis. As aforementioned, the method consists of using a two-dimensional liquid chromatographic approach operating in the "heart-cutting" mode with strong anion exchange (SAX) chromatography and reversed phase chromatography in the first and second dimensions, respectively, followed by UV and mass spectrometric detection. The mass spectrometric confirmation for the absence of any coelution with HPS and Gly m 4 peaks during the separation of protein extracts was observed to be critical for ensuring that the required selectivity is achieved.

It was observed that HPS does not bind to the anion exchange column and elutes in the column void volume along with other nonretained proteins. The components eluting between 2.2 and 6.0 min were introduced into the second dimension. Careful method development was required in the second dimension to ensure the absence of any coelution. Figure 2 depicts the chromatographic separation achieved in the second dimension for the two major isoforms along with respective mass spectrometric characterization. As shown in Figure 2A, the optimized method has sufficient resolution to separate HPS isoforms from one another and from other components eluting from the heart-cut SAX chromatographic fraction. The raw mass spectra (Figures 2B,C) and the corresponding deconvoluted mass spectra (Figures 2D,E) further confirm the specificity of the developed 2DLC method.

In contrast, Gly m 4 elutes between 8.9 and 16.0 min during the linear salt gradient. Prior work from our laboratory has shown that the two isoforms of Gly m 4 elute in this window. The two isoforms were observed to have the same sequence but with slightly different anion exchange retention behavior, presumably due to different conformations. In this method, both of these isoforms were quantified as a single peak.

Several conditions were evaluated to optimize simultaneous extraction of the two allergenic proteins from soybean seeds. These included (i) selection of appropriate solvent, (ii) extraction time, and (iii) use of enzyme inhibitors. Once optimized for solvent, two different seed to solvent (w/v) ratios (1:4 and 1:7) were also evaluated.

The different solvents that were evaluated were 60% ethanol, 25% 2-propanol, 40% 2-propanol, and 55% 2-propanol. On the basis of the analyses, 40% 2-propanol extraction solvent was selected because of the maximum recovery obtained for both of the proteins. Of the two solvent ratios evaluated, the 1:4 ratio provided maximum extraction of the two proteins. Similarly, extraction times (2 h, 4 h, and overnight (16 h)) were compared to understand the level of recovery for the two proteins. On the basis of the analyses, overnight extraction (16 h) was selected. As reported previously, HPS elutes as two main isoforms during reversed phase chromatography along with other minor truncations. A number of enzyme inhibitors were evaluated to limit the truncations occurring during the extraction process. Of the different enzyme inhibitors evaluated, E-64, a cysteine protease inhibitor, was found to be critical in preventing further N-terminal truncation of HPS. Because the truncated version of HPS differs in only two neutral amino acids, an identical chromatographic response factor was assumed for the two isoforms, and quantitation was carried out using purified reference standard for HPS (form II). No truncations were observed for Gly m 4 in the presence or absence of E-64. A representative chromatographic and mass spectrometric profile obtained for the separation of soybean extracts is shown in Figure 3. The "heart-cuts" employed in the SAX dimension and the SAX chromatography profile are depicted in Figure 3A. The reversed-phase chromatography profiles for the first and second cuts are shown in Figure 3B.

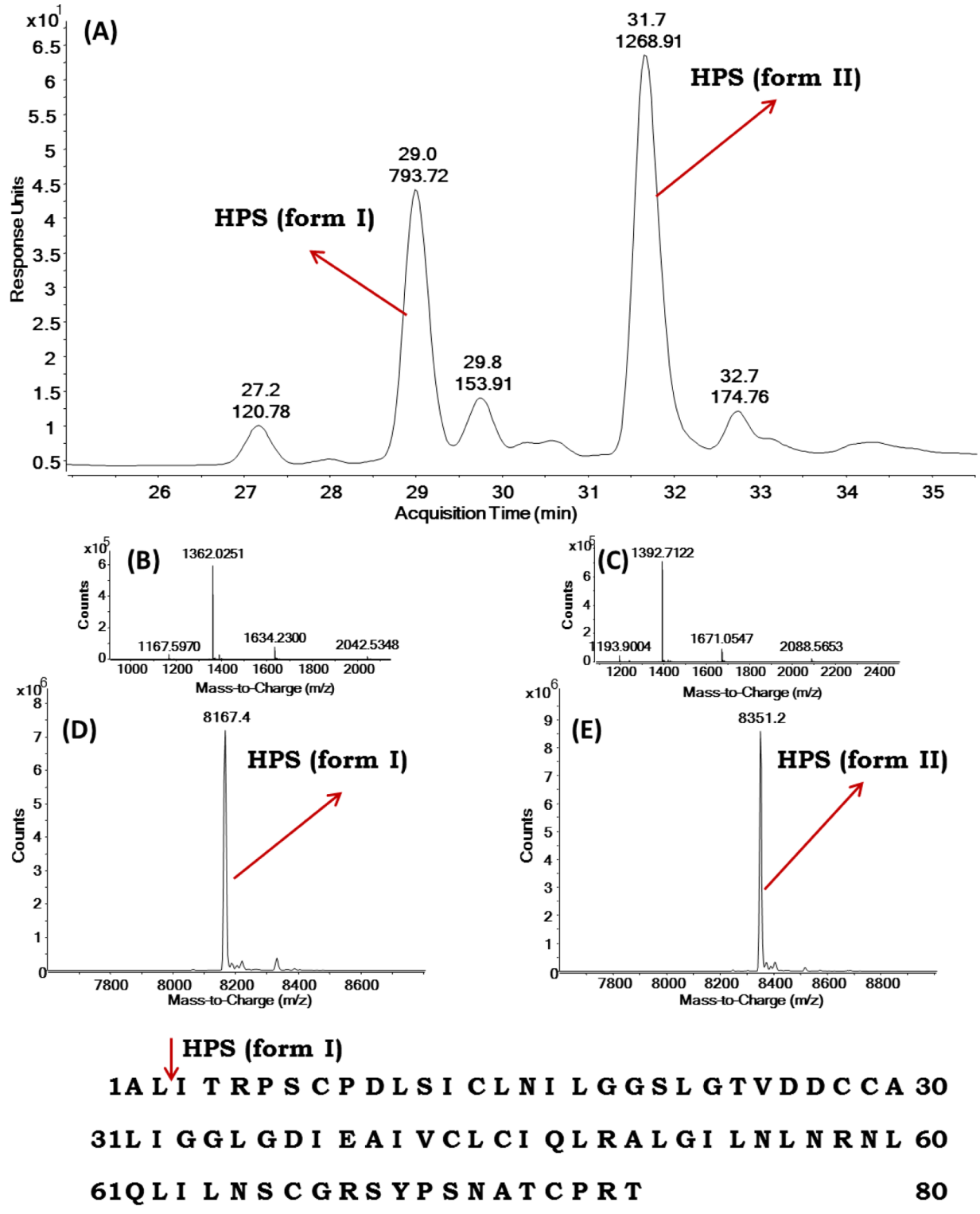

Figure 2. Representative (A) UV (215 nm) chromatogram from reversed-phased (second dimension) chromatography of soybean seed extract, (B) raw mass spectrum of HPS (form 1), (C) raw mass spectrum of HPS (form II), (D) deconvoluted mass spectrum of HPS (form I), and (E) deconvoluted mass spectrum of HPS (form II).

The deconvoluted mass spectra of HPS forms I and II and Gly m 4, shown in Figures 3C−3E, confirm the specificity of the 2DLC method.

Method Validation. Basal level of endogenous Gly m 4 and HPS was first determined in a control non-transgenic soybean sample for evaluating linearity and recovery of the 2DLC-UV/MS assay. The basal levels of endogenous HPS and Gly m 4 were observed to be 337.3 and 185.7 μg/g, respectively. Representative chromatograms from the second-dimension separation of protein extract at different HPS/Gly m 4 levels are presented in Figures 4A,B. The assay was observed to be linear over the evaluated range with R^2 values of 0.996 and 0.997 for HPS and Gly m 4, respectively. The evaluated linear ranges for HPS and Gly m 4 were from 11.9 to 599.8 μg/

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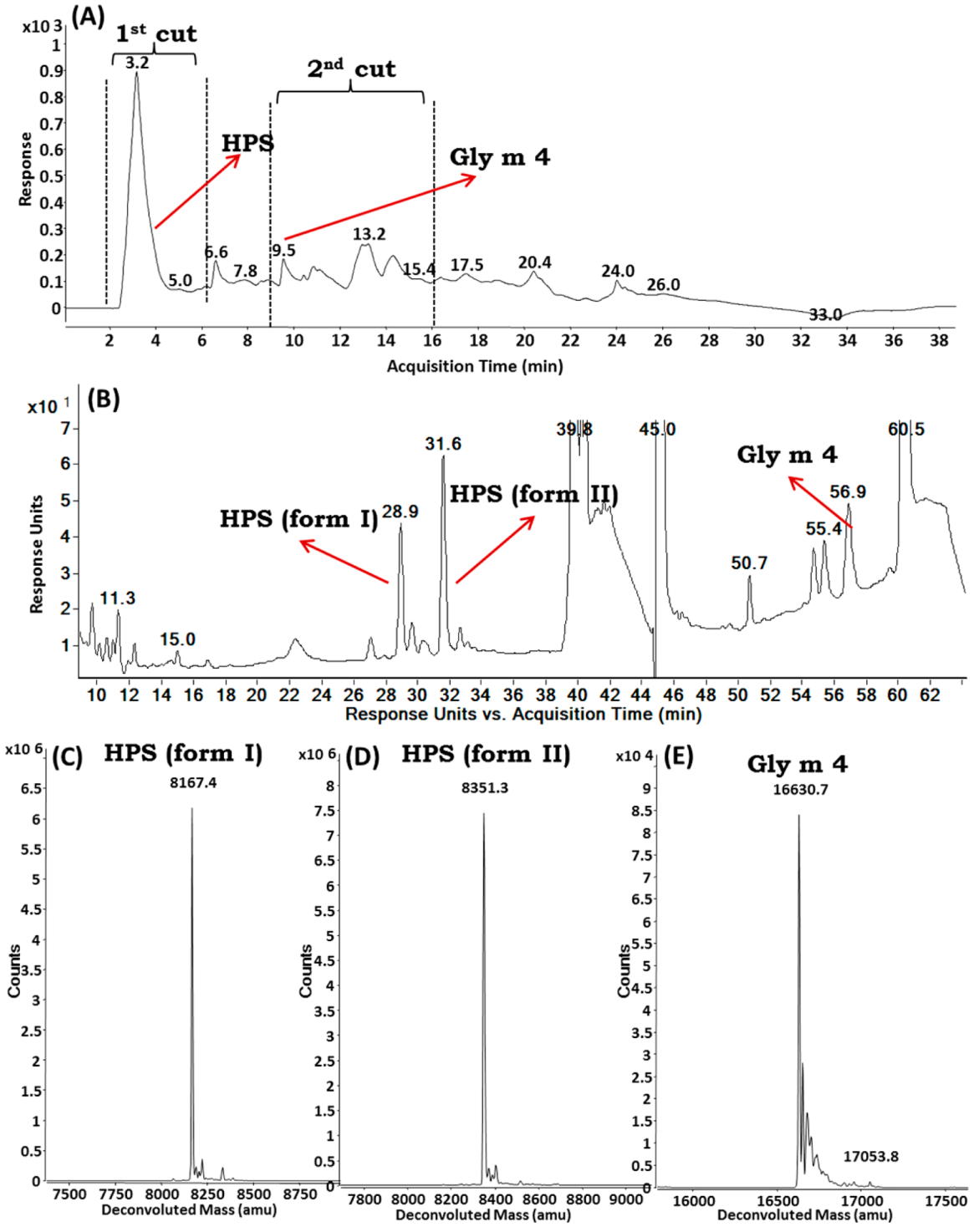

Figure 3. Representative 2DLC-UV/MS analyses of soybean extract from control soybean line: UV chromatograms (215 nm) from (A) first- and (B) second-dimension separations; (C) deconvoluted mass spectrum of component at retention time 28.9 min; (D) deconvoluted mass spectrum of component at retention time 31.6 min; (E) deconvoluted mass spectrum of component at retention time 56.9 min.

mL and from 6.9 to 355.1 μg/mL, respectively (Figure 4C,4D). The evaluated range was in the physiologically relevant range as also observed by the levels of these two allergens in the non-transgenic commercial lines.

Assay recovery (percent relative error, %RE) was also calculated for the two allergens in the respective linear range and is presented in Table 3. The recovery range (%RE) for HPS was observed to be from −1.1 to −13.7% with an average recovery of −4.8%. The recovery range (%RE) for Gly m 4 was observed to be from −3.5% to 15.2% with an average recovery of 5.6%. The validation results for assay intraday and interday precision (percent coefficient of variation, %CV) were calculated for the two allergens. The summarized results are presented in Table 4. Precision in the simultaneous measurement of two allergens at their intact level was measured by use of four different seed lines encompassing low, medium, and

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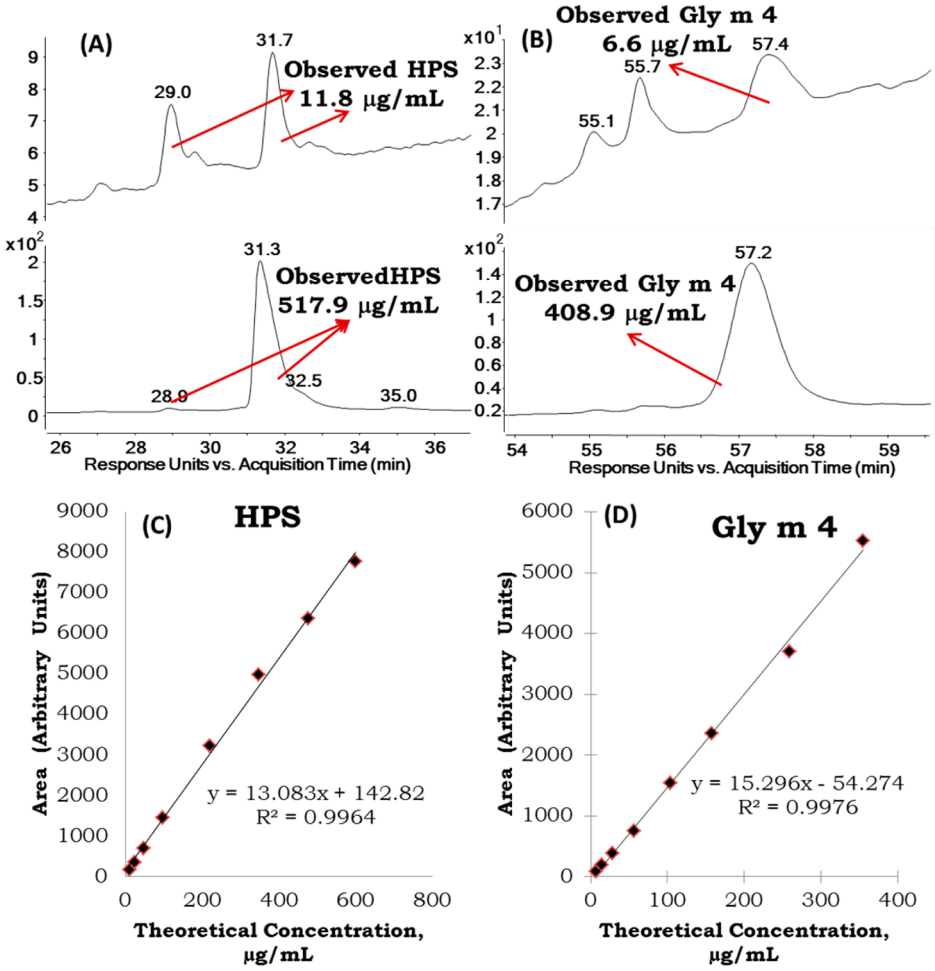

Figure 4. Second-dimension UV (215 nm) chromatograms obtained from the soybean seed kernel extracts spiked with HPS or Gly m 4 reference standards to obtain (A) HPS concentrations of 11.8 and 517.9 μg/mL and (B) Gly m 4 concentrations of 6.6 and 408.9 μg/mL; standard linearity curves for (C) HPS and (D) Gly m 4 over the ranges from 11.9 to 599.8 μg/mL and from 6.9 to 355.1 μg/mL, respectively.

Table 3. Multipoint Recovery of the 2DLC-UV Method for Quantification of HPS and Gly m 4 at Various Concentrations

HPS (μg/mL)			Gly m 4 (μg/mL)		
expected	observed	recovery (% RE)	expected	observed	recovery (% RE)
11.90	11.76	−1.14	6.9	6.6	−3.5
24.14	23.45	−2.84	13.9	14.3	3.1
48.50	46.83	−3.44	27.9	28.9	3.6
97.16	97.16	0.00	56.0	56.0	0.0
220.08	215.79	−1.95	103.4	113.8	10.1
347.59	332.10	−4.46	158.2	174.7	10.4
477.91	424.30	−11.22	258.6	274.7	6.2
599.87	517.88	−13.67	355.1	408.9	15.2
	av	−4.8		av	5.6

high levels of HPS and Gly m 4. For the 4 day validation study, the precision ranges (%CV) were observed to be 4.7−9.2% for HPS and 6.3−9.4% for Gly m 4 at the various allergen concentrations tested. The average precision over all the samples was found to be less than the average target precision of 15.0%.

The stability of the two allergens in seed extracts was evaluated at the autosampler temperature (5 °C) at different time intervals: 24, 47, and 69 h. For the three time intervals studied, compared with the initial time (~9 h), losses of 8.8, 3.6, and 17.1% were observed for HPS and losses of 12.4, 2.4, and 16.7% were observed for Gly m4. On the basis of the above data, the assay was determined to be stable for at least for 47 h when performed using an autosampler maintained at 5 °C.

The assay was also evaluated for its robustness with changes in column temperatures. The column temperature was systematically changed in each of the two dimensions, and the levels of Gly m 4 and HPS were compared with their levels observed with first- and second-dimension temperatures of 30 and 70 °C, respectively. Initially, the first-dimension column temperature was fixed at 30 °C, and the second-dimension column temperature was varied at two selected temperatures, 65 and 75 °C. The relative levels of HPS were observed to be 84 and 99.1%, respectively. Similarly, the relative levels of Gly m 4 were observed to be 96.1 and 106.2%, respectively. This was followed by maintaining the second-dimension temperature at 70 °C and setting the first-dimension temperatures of 27 and 33 °C. The relative levels of HPS were observed to be 103.8 and 98.7%, respectively, whereas the relative levels of Gly m 4 were observed to be 106.2 and 106.0%. The method was observed to be more sensitive to temperature changes in the second dimension. It is recommended to precisely control the assay temperatures in both dimensions.

dx.doi.org/10.1021/jf500087s | J. Agric. Food Chem. 2014, 62, 4884−4892

Table 4. Precision of the 2DLC-UV Method for Quantification of HPS and Gly m 4 from Soybean Extracts

		HPS			Gly m 4		
day		low	medium	high	low	medium	high
1	av μg/mL	51.0	74.4	124.1	53.3	92.1	150.6
	av μg/g	198.5	291.6	483.3	206.5	358.8	590.0
	SD	4.4	13.3	16.9	5.5	5.4	7.8
	%CV	2.2	4.6	3.5	2.7	1.5	1.3
2	av μg/mL	51.0	76.8	127.2	55.0	91.8	155.4
	av μg/g	188.6	284.2	470.1	204.3	339.5	574.8
	SD	1.8	5.5	5.0	6.6	4.1	9.9
	%CV	1.0	1.9	1.1	3.2	1.2	1.7
3	av μg/mL	47.1	69.9	119.6	59.0	98.5	148.1
	av μg/g	176.1	261.8	442.4	217.9	364.2	554.7
	SD	3.7	16.1	5.6	7.4	15.1	27.9
	%CV	2.1	6.2	1.3	3.4	4.2	5.0
4	av μg/mL	45.2	65.9	119.6	47.3	86.0	140.8
	av μg/g	168.0	243.1	439.2	173.9	315.9	519.4
	SD	3.7	8.7	5.7	9.1	5.0	7.9
	%CV	2.2	3.6	1.3	5.2	1.6	1.5
4 day av	av μg/mL	47.8	70.1	122.6	53.7	92.1	146.5
	av μg/g	180.8	265.5	458.8	200.7	344.6	554.7
	SD	15.8	24.5	21.4	18.8	21.9	35.3
	%CV	8.7	9.2	4.7	9.4	6.3	6.4

Carry-over present in samples that followed the highest respective HPS and Gly m 4 levels was observed to be 0.1 and 0.6%, respectively. This corresponded to 4.3 and 37.9% of the area of the lowest concentration of HPS and Gly m 4, respectively.

To evaluate extraction efficiency of Gly m 4 and HPS, two successive overnight extractions were carried out. The successive extraction did not yield additional Gly m 4 and HPS protein, suggesting complete extraction of the two proteins in the first extraction.

Simultaneous Analyses of HPS and Gly m 4 in Commercial Soybean Lines. The levels of HPS and Gly m 4 were quantified using this assay in 10 selected non-transgenic commercial lines. The concentrations of the two allergens were observed to be within respective validated linear ranges. The levels of HPS and Gly m 4 in each line are presented in Figure 5. On the basis of the analyses, various levels of HPS and Gly m 4 were observed in different commercial lines with no apparent trend. In most of the samples (7 of 11), Gly m 4 levels were observed to be higher than HPS levels. Significantly greater variability (648%) was observed in expression levels of HPS in the 10 commercial lines compared with variability in expression levels for Gly m 4 (212%). Further statistically designed and replicated studies need to be carried out to determine the source of this variation.

In conclusion, this study demonstrates the use of a 2DLC-UV/MS-based assay for the simultaneous quantification of two allergens of interest at their intact level. The ability to quantify more than one allergen in a single analysis expands the applicability of these assays. Throughput of the assay can be further improved by intelligent method development in either dimension and by using novel trapping approaches. The assay was successfully applied to understand levels of HPS and Gly m 4 in 10 non-transgenic commercial soybean lines. Further research is needed to understand the biovariability of these allergens, especially as a result of growing environment. Another important aspect to understand is the level at which each of these allergen elicits an allergenic response. Moreover, several methods are currently available to quantify proteins of interest. It will be critical to compare these methods to get consistency and understand the biases, if any, inherent within each of these quantitative methods.

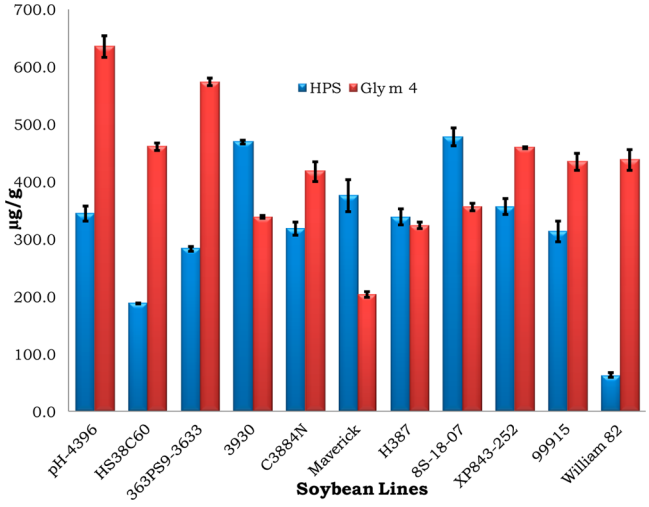

Figure 5. Determination of HPS and Gly m 4 levels in different commercially available soybean lines.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Rod Herman and Paul O'Connor for excellent technical review. We also thank Margaret Covington, Linda Singletary, and Shawna Embrey for their technical assistance and Michelle Mayes for sourcing and preparation of the ground soybean seeds.

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