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Reverse phase HPLC method for detection and quantification of lupin seed γ-conglutin

Sharmilee Mane[a], Scott Bringans[b], Stuart Johnson[c], Vishnu Pareek[a], Ranjeet Utikar[a,*]

[a] Department of Chemical Engineering, Curtin University, Perth, WA, 6845, Australia
[b] Proteomics International, Nedlands, Perth, WA, 6009, Australia
[c] School of Public Health, Curtin Health Innovation Research Institute, Curtin University, Perth, WA, 6845, Australia

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A B S T R A C T

A simple, selective and accurate reverse phase HPLC method was developed for detection and quantitation of γ-conglutin from lupin seed extract. A linear gradient of water and acetonitrile containing trifluoroacetic acid (TFA) on a reverse phase column (Agilent Zorbax 300SB C-18), with a flow rate of 0.8 ml/min was able to produce a sharp and symmetric peak of γ-conglutin with a retention time at 29.16 min. The identity of γ-conglutin in the peak was confirmed by mass spectrometry (MS/MS identification) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The data obtained from MS/MS analysis was matched against the specified database to obtain the exact match for the protein of interest. The proposed method was validated in terms of specificity, linearity, sensitivity, precision, recovery and accuracy. The analytical parameters revealed that the validated method was capable of selectively performing a good chromatographic separation of γ-conglutin from the lupin seed extract with no interference of the matrix. The detection and quantitation limit of γ-conglutin were found to be 2.68 μg/ml and 8.12 μg/ml respectively. The accuracy (precision and recovery) analysis of the method was conducted under repeatable conditions on different days. Intra-day and inter-day precision values less than 0.5% and recovery greater than 97% indicated high precision and accuracy of the method for analysis of γ-conglutin. The method validation findings were reproducible and can be successfully applied for routine analysis of γ-conglutin from lupin seed extract.

1. Introduction

γ-Conglutin, a minor seed globulin (~4-5% w/w of total globulins) of the legume lupin [1], has gained global attention because of its characteristic property in controlling glycemic levels based on reports on cellular and animal models, and human post-prandial studies [2–4]. This paper reports a simple and precise RP-HPLC method for detection and quantitation of γ-conglutin.

γ-Conglutin exists as an oligomer of four subunits at neutral pH [5], which has been shown to undergo pH-dependant association-dissociation between monomeric and oligomeric structures [6]. At pH 5 and lower, the tetramer dissociates into monomers with molecular weights of 48 kDa consisting of disulphide bridged two polypeptide chains of 27–30 kDa and 16–18 kDa respectively [7]. Current methods of quantitation of γ-conglutin include indirect ELISA (enzyme linked immunosorbent assay) [8], and HPLC-chip-multiple reaction monitoring (MRM) label-free method [9]. However, the use of immuno-enzymatic methods such as ELISA have limitations of cross-reactivity or non-specific binding with other food proteins, which can lead to false results [10,11]. While HPLC-chip MRM method is promising for absolute quantitation of γ-conglutin [9], the possibility of adapting this method for routine analysis in the pharmaceutical industry is limited due to the high expenses involved in the mass spectrometry analysis. Therefore, there is a need to develop a sensitive, specific, rapid and easy method for detection and quantitation of γ-conglutin from lupin seed extract.

In recent years, high-performance liquid chromatography (HPLC) with greater sensitivity and faster analysis time has emerged as one of the most important tools of analytical chemistry [12,13]. In particular, reverse phase HPLC (RP-HPLC) has become the technique of choice for

Abbreviations: HPLC, high pressure liquid chromatography; TFA, trifluoroacetic acid; MS/MS, mass spectroscopy; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; MRM, multiple reaction monitoring; RP-HPLC, reverse phase HPLC; FDA, Food and Drug Administration; AOAC, Association of Official Analytical Chemists; ICH, International Conference on Harmonisation; DAFWA, Department of Agriculture and Food Western Australia; BCA, bicinchoninic acid; PVDF, polyvinylidene fluoride; BSA, bovine serum albumin; DAD, diode-array detector; *Rs*, resolution; LOD, limit of detection; LOQ, limit of quantitation; CV, coefficient of variation; RSD, relative standard deviation; RT, retention time; *As*, peak asymmetry; *m/z*, mass to charge ratio; SD, standard deviation

* Corresponding author.
E-mail address: R.utikar@curtin.edu.au (R. Utikar).

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cost-effective analysis of bioactive proteins and peptides on account of its selectivity, excellent resolution and high accuracy in quantitation [14]. These features make RP-HPLC an ideal candidate for detection and quantitation of γ-conglutin. At present, only two reports are available on the use of RP-HPLC for analysis γ-conglutin. Duranti et al., have reported the use of RP-HPLC to separate glycosylated and deglycosylated forms of γ-conglutin [15], whereas Garzón-de la Mora et al., used RP-HPLC to purify γ-conglutin and other conglutins from total globulin fraction [16]. As such, there is no report available for quantitation of γ-conglutin by RP-HPLC. The aim of this work was to develop a simple, selective, sensitive, accurate and precise RP-HPLC method using a C-18 column and a linear gradient of water and acetonitrile. The developed method was validated for its analytical characteristics such as specificity, linearity, sensitivity, reproducibility, precision and accuracy in accordance with guidelines provided by various standards.

2. Experimental materials and methods

2.1. Materials

Mature seeds of *Lupinus angustifolius* (Coromup variety) were provided by the Department of Agriculture and Food Western Australia (DAFWA). N-hexane, sodium acetate, glacial acetic acid were used for the extraction process. Sodium chloride, sodium phosphate (monobasic), sodium phosphate (dibasic), tris buffer were used for the cation exchange chromatography process. Acetonitrile and trifluoroacetic acid were used for HPLC (99.9% assay purity). All the chemicals purchased were of analytical grade from Sigma-Aldrich Pty Ltd (Perth, Western Australia). Bicinchoninic acid (BCA) protein assay reagent kit was obtained from ThermoFisher Scientific (Perth, Western Australia). Aqueous solutions were made using ultrapure water (Ariumpro ultrapure water system, Sartorius Australia Pty Ltd, Perth, Western Australia) in the laboratory.

2.2. Purification of γ-conglutin standard

A purified fraction of γ-conglutin was obtained from *L. angustifolius* kernel flour by a combination of acidic extraction and cation exchange chromatography. The purified protein was desalted with ultrapure water through a 10 kDa membrane filter (Minimate tangential flow filtration capsule, Pall Corporations, Perth, Western Australia) and freeze dried (Alpha 1–4 LDplus freeze dryer, John Morris Scientific, Perth, Western Australia). A stock solution of pure γ-conglutin was prepared by dissolving accurately weighed 100 mg of freeze-dried γ-conglutin in 10 ml of ultrapure water (10 mg/ml) slowly under mild stirring conditions at room temperature. The resulting solution was centrifuged at 10,000 rpm for 10 min at room temperature and the supernatant was filtered through a 0.45 μ PVDF (polyvinylidene fluoride) syringe filter. In-house standard solution of pure (97% w/w) γ-conglutin (10 mg/ml) was then stored at −20 °C until further analysis.

2.3. Protein concentration measurement by BCA analysis

The protein concentration of lupin protein extract and standard γ-conglutin solution was determined by bicinchoninic acid (BCA) analysis by using Pierce BCA protein assay kit [17] (ThermoFisher Scientific, Perth, Western Australia) at 562 nm using bovine serum albumin (BSA) as a standard protein

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of γ-conglutin under non-reducing and reducing conditions was performed according to Laemmli [18] using 12% precast polyacrylamide gel (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia). Standard γ-conglutin solution (collected HPLC peak) was diluted with Laemmli sample buffer (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia) for non-reducing conditions, whereas reducing sample was prepared by adding reducing buffer (β-mercaptoethanol to Laemmli sample buffer) followed by heating the resulting sample at 95 °C for 10 min. Electrophoresis was performed with TGX (tris-glycine) running buffer using Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia) at 150 mV for 1 h.

Proteins were fixed and gels were stained using a silver staining protocol. The molecular weights of major peptides in the samples were estimated by comparing their migration with broad range molecular weight SDS-PAGE markers (Bio-Rad Laboratories Pty Ltd, Perth, Western Australia).

2.5. Reverse phase HPLC method

The HPLC system used was an Agilent Technologies 1200 series HPLC model equipped with quaternary pump module, vacuum degasser, autosampler with 100 μl injection loop, thermostatted column compartment and a diode-array detector (DAD). Data acquisition was performed by Agilent's ChemStation software. Reverse phase analysis was carried out with an Agilent Zorbax 300SB C-18 column (4.6 mm ID × 250 mm length, 5 μm particle size) at 25 °C. Mobile phase consisted of 0.1% v/v TFA in ultrapure water (phase A) and 0.1% v/v TFA in acetonitrile (phase B). Before adding TFA, both the phases were degassed using a sonication bath for 10 min.

The reverse phase HPLC method used for analysis of standard γ-conglutin solution (prepared as described in section 2.2) is presented in Table 1. A linear gradient of phase B was employed in 36 min, followed by regeneration and re-equilibration of the column in the next 14 min before further injections. The injection volume for all samples was set to 50 μl and the flow rate of the method was maintained at 0.8 ml/min. The detection of protein was carried out at 280 nm.

2.6. Validation of reverse phase HPLC method

The performance of the RP-HPLC method for detection and quantitation analysis of γ-conglutin was validated as per Food and Drug Administration (FDA) programs for analytical methods [19], Association of Official Analytical Chemists (AOAC) International [20] and International Conference on Harmonisation (ICH) guidelines [21]. The analytical characteristics validated were selectivity, linearity, quantitation and detection limits, precision, recovery and accuracy as stated further in this section.

2.6.1. Selectivity

Selectivity is the ability of a method to accurately assess the analyte in presence of other interfering components (matrices or potential impurities) that may be expected to be present in the sample [22]. Specificity of a method is determined by adding specific interference (lupin protein extract) in the presence of target analyte (γ-conglutin) and evaluating the resolution of analyte peak from the nearest eluting peak

Table 1
Reverse phase HPLC method for analysis of γ-conglutin.

Time (min)	Mobile phase (%)	
	Phase A	Phase B
0	100	0
16	100	0
26	50	50
36	0	100
41	0	100
45	100	0
50	100	0

in the matrix [23]. Resolution (*Rs*) is expressed as the ratio of the difference in retention time (t_1 and t_2) between the two peaks to the mean of their base widths (W_1 and W_2) as given in equation (1),

$$Rs = 2(t_2 - t_1)/(W_1 + W_2) \tag{1}$$

2.6.2. Linearity and range

Linearity study verifies the range of concentration where a response of the analyte is directly proportional to its concentration [13]. Linearity of a method was evaluated by plotting a calibration curve of peak area (Y) as a function of γ-conglutin concentration (X) using linear regression analysis. The calibration solutions were prepared in ultrapure water with ten different concentrations of γ-conglutin starting from quantitation limit, 8, 15, 25, 50, 100, 250, 500 and 1000 μg/ml. Each concentration was analysed in five independent replicates for validation of the analyte response [24]. The slope, s, intercept, c, correlation coefficient, r^2, and the respective variances were determined using the Excel linest function on peak area ratio at different calibration levels.

2.6.3. Sensitivity, detection limit and quantification limit

Sensitivity is the change in the analyte response divided by the corresponding change in the analyte concentration [22]. Sensitivity of the analytical method was estimated in terms of the limit of detection (LOD) and limit of quantitation (LOQ). LOD is the lowest concentration of the analyte that can be detected and distinguished from the noise level of the system but not necessarily quantified as an exact value [24]. LOQ is the lowest concentration of the analyte which can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were determined by the Eq. (2) and (3) respectively as suggested by ICH [21].

$$LOD = 3.3 \times \sigma/S \tag{2}$$

$$LOQ = 10 \times \sigma/S \tag{3}$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.6.4. Precision

Precision is a measure of reproducibility of the analytical method and is expressed as the degree of agreement between a series of measurements obtained from the multiple sampling of the same homogeneous sample [21]. Precision of the analytical method was determined by repeatability (intra-day) and intermediate precision (inter-day) [25] by adding three different concentrations (0.08, 0.10, 0.12 mg/ml) of analyte (γ-conglutin) to the sample matrix (lupin protein extract). Each concentration was analysed in five independent replicates. Repeatability of the method was determined by analysing the sample five times on the same day, whereas intermediate precision was assessed by analysing the sample on five different days over a period of one week [13]. The overall repeatability mean and standard deviation were calculated to determine the absolute value of the coefficient of variation (CV) which is the ratio of the repeatability, i.e. standard deviation, to its mean [23]. The precision was expressed as the percentage of the relative standard deviation, % RSD, of responses at respective concentrations [26].

2.6.5. Accuracy and recovery

The accuracy of the analytical method is the degree of closeness between the true value of an analyte in the sample and the value determined by the method [21]. It is sometimes referred to as trueness [26]. The accuracy of the method was determined by conducting a recovery experiment. Recovery was expressed as the percentage that was determined by comparing the responses of sample matrix in its original state (lupin protein extract) to the response after addition (spiking) of a known mass of analyte (γ-conglutin) [23,26,27]. To the pre-analysed sample matrix (lupin protein extract), a known amount of analyte (γ-conglutin) was added at 80, 100 and 120% level of the original amount and analysed by the proposed HPLC method [27].

2.7. Mass spectrometry (MS/MS analysis)

The HPLC peak of the standard γ-conglutin sample was collected and dried in a SpeedVac (Thermofisher Scientific, Perth, Western Australia). Dried γ-conglutin was digested with trypsin and generated peptides were extracted according to standard techniques [28]. Peptides were analysed by electrospray ionisation mass spectrometry using the Agilent 1260 Infinity HPLC system coupled to an Agilent 1260 Chipcube Nanospray interface on an Agilent 6540 mass spectrometer. Peptides were loaded onto a ProtIDChip-150 Agilent C-18 column and separated with a 10 min linear gradient of 5–40% acetonitrile and 0.1% (v/v) formic acid in water at a constant flow rate of 600 nL/min. A voltage of 2050 V at a temperature of 350 °C was applied between the needle and the source. An acquisition scan rate of 5 spectra/s with a collection rate of 200 ms/spectra was used for MS, whilst a scan rate of 1 spectra/s with a collection rate of 1000 ms/spectra was used for MS/MS respectively. MS data were collected in the range of 290–1700 *m/z* for MS and 60–1700 for MS/MS. Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with MSPnr100 database [29] and viridiplantae (Green plants) selected as the taxonomy. The MSPnr100 is a fasta file compiled from all known reference protein sequences including NCBI, Refseq, UniProt, EuPathDB and Ensembl.

3. Results and discussion

3.1. Reverse phase HPLC method

Pure γ-conglutin is freely soluble in ultrapure water at room temperature, therefore a simple linear gradient of water and acetonitrile was used to develop the method for its analysis. A C-18 column was selected for analysis to obtain optimal resolution of peaks in the sample [12]. Initially different trials with mobile phase gradients were performed, which resulted in an asymmetric peak with tailing effect. Therefore, an ion pairing agent (TFA) was added to the mobile phase as it is known to reduce tailing effects [12]. While developing a method, care was taken to provide sufficient equilibration and regeneration to avoid noise generated by the system. The run time of the developed method was 50 min, in which last 14 min were for regeneration and re-equilibration before further injections.

The developed method (Table 1) resulted in a sharp peak with a retention time (RT) at 29.16 min, as represented in Fig. 1. γ-Conglutin was seen to elute from the reverse phase column approximately between 60 and 70% acetonitrile. The percentage area of the peak (Fig. 1) was observed to be 97%, indicating the purity of the analyte (γ-conglutin) loaded on the column [12]. While developing method, the shape of the peak is considered as a performance characteristic for good quantitative results and is represented as peak asymmetry factor, *As*. Peak asymmetry factor is calculated as $^{b}/_{a}$ where b is the distance from the peak midpoint to the trailing edge of the peak measured at 10% of peak height and a is the distance from the leading edge of the peak to the peak midpoint measured at 10% of peak height [12]. Asymmetry factor of 0.95-1.10 is considered to be a symmetric peak with no tailing and fronting [12]. The asymmetric factor for the peak with RT of 29.16 min (Fig. 1a) was calculated to be 1.04, which represents an acceptable symmetric nature of peak for accurate quantitation.

The peak (Fig. 1a) was collected post HPLC detection and analysed by SDS-PAGE (Fig. 2) and mass spectrometric analysis (Fig. 3) to confirm its identity as γ-conglutin before validating the method. Non-reducing SDS-PAGE of the peak fraction (lane 2, Fig. 2) showed the presence of γ-conglutin at 48 kDa in its monomeric form. The same fraction under reducing conditions was seen to be reduced into its individual polypeptides at ∼30 kDa (large chain) and ∼18 kDa (small

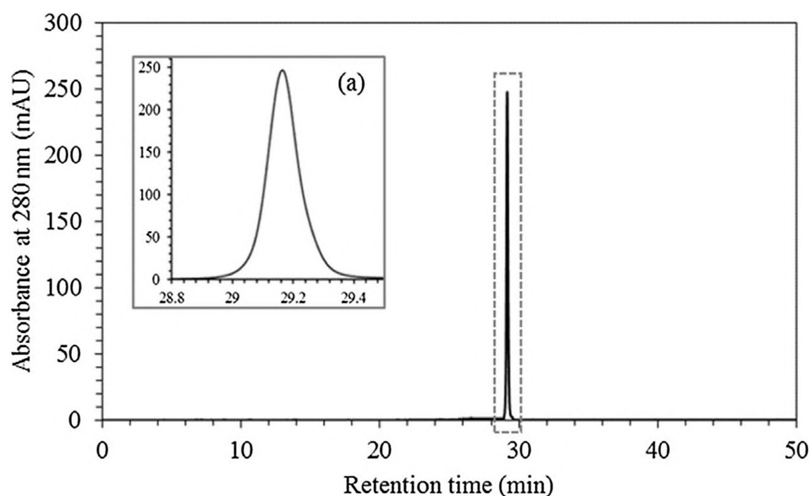

Fig. 1. Spectral chromatogram of pure γ-conglutin (1 mg/ml).

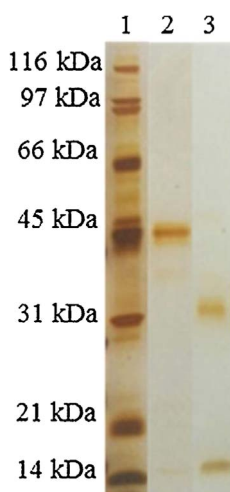

Fig. 2. SDS-PAGE of γ-conglutin peak (RT- 29.16 min).
(L1- molecular weight marker, L2- non-reducing peak fraction and L3- reducing peak fraction)

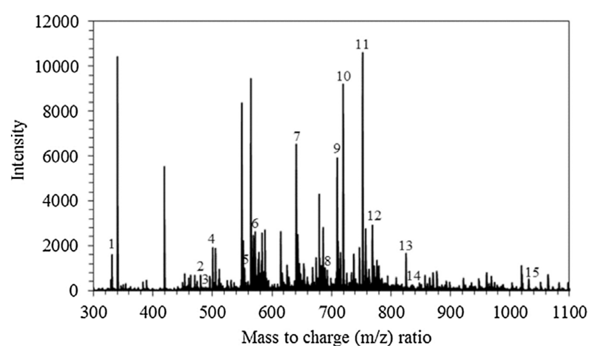

Fig. 3. Mass spectrum of tryptic digest peptides of γ-conglutin HPLC peak.
(The numbers on peaks are peptides matched with those from γ-conglutin database, Q42369)

chain) through breakage of disulphide linkage [5,30].

The same peak fraction (Fig. 1a) was analysed by mass spectrometry (MS/MS) to confirm its identity as γ-conglutin. The sample for MS/MS analysis was prepared and digested with trypsin as mentioned in section 2.7. The obtained MS/MS spectrum (Fig. 3) was analysed using Mascot sequence matching software (Matrix Science) with MSPnr100 database to confirm the protein of interest. The database (MSPnr100) was a Viridiplantae (Green Plants) database downloaded in January 2017 with 5, 315, 975 sequences. A peptide tolerance of 0.2 and MS/MS tolerance of 0.2 with 1 missed cleavage was used as the default setting for protein identification. Individual ions scores > 48 indicate identity or extensive homology ($p < 0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

The protein hit with the maximum score of 540 was observed for γ-conglutin from *L. angustifolius* (tr, Q42369) (Fig. 4). In this case, 126 out of 449 amino acid residues from γ-conglutin database were observed to match with peptides generated from γ-conglutin peak, showing 28% coverage of peptide sequences. The second hit score of 42 was found for basic 7S globulin-like protein from *Cicer arietinum* (NCBI, XP_004494958.1), which is reported to be homologous with γ-conglutin from *L. albus* [31], with 10% coverage.

The characteristic details of the peptides (generated from γ-conglutin peak) confirming the identity of the protein and matching specific peptides from the database of γ-conglutin (Q42369) are represented in Table 2. This coverage coupled with the protein score substantiates the identity of γ-conglutin obtained by the proposed RP-HPLC method.

Thus the proposed method (Table 1) successfully produced a sharp and symmetric peak of γ-conglutin with RT at 29.16 min, with a simple mobile phase gradient. The developed method was then validated in accordance with FDA, AOAC International and ICH guidelines for its intended use as an analytical technique for detection and quantitation of γ-conglutin in lupin samples.

3.2. RP-HPLC method validation

3.2.1. Selectivity

The selectivity of RP-HPLC method for analysis of γ-conglutin was determined by observing the retention time of γ-conglutin after spiking with specific interference (lupin protein extract) and by calculating resolution between γ-conglutin peak and the nearest eluted peak in the extract. The original concentration of γ-conglutin in lupin protein extract was 0.1 mg/ml as calculated with the developed RP-HPLC method by comparing it with γ-conglutin in-house standard. Therefore, 1 mg/ml of lupin protein extract was added as interference to the standard γ-conglutin solution (0.1 mg/ml). The overlay of spectral chromatograms of standard γ-conglutin solution (0.1 mg/ml), original lupin protein extract and γ-conglutin (0.1 mg/ml) spiked with 1 ml of lupin protein extract is represented in Fig. 5.

As seen from Fig. 5, no change in the chromatogram pattern of original extract and the spiked extract was observed, except an increase in peak area at a retention time of 29.16 min (Fig. 5a). The area of γ-conglutin peak in the spiked extract was doubled as the concentration of γ-conglutin was doubled in the spiked extract. It was also observed

1	MARNMAHILH	ILVISLSYSF	LFVSSSSQDS	QSLYHNSQPT	SSKPNLLVLP
51	VQEDASTGLH	WANIHKR**TPL**	**MQVPLLLDLN**	**GK**HLWVTCSQ	HYSSSTYQAP
101	FCHSTQCSRA	NTHQCFTCTD	STTTRPGCHN	NTCGLLSSNP	VTQESGLGEL
151	AQDVLAIHST	HGSK**LGPMVK**	VPQFLFSCAP	SFLAQK**GLPN**	**NVQGALGLGQ**
201	**APISLQNQLF**	**SHFGLK**RQFS	VCLSRYSTSN	GAILFGDIND	PNNNNYIHNS
251	LDVLHDLVYT	PLTISK**QGEY**	**FIQVNAIR**VN	KHLVIPTKNP	FISPSSTSYH
301	GSGEIGGALI	TTTHPYTVLS	HSIFEVFTQV	FANNMPKQAQ	VK**AVGPFGLC**
351	**YDSRKISGGA**	**PSVDLILDKN**	**DAVWR**ISSEN	FMVQAQDGVS	CLGFVDGGVH
401	AR**AGIALGAH**	**HLEENLVVFD**	**LER**SR**VGFNS**	**NSLK**SYGKTC	SNLFDLNNP

Fig. 4. Protein view showing 28% peptide sequence coverage to Q42369. (matched peptides are shown in bold letters)

Table 2
Matching of masses of peptides generated from γ-conglutin peak (Fig. 3) with the peptides available in the database of Q42369.

Peak number	Peptide (*m/z*)	Peptide (Z)	Peptide score[a]	Peptide sequence	Start-end peptide sequence coverage
1	322.69	2	23	LGPMVK	165–170
2	479.92	3	19	QGEYFIQVNAIR	267–278
3	483.25	2	35	VGFNSNSLK	426–434
4	505.01	3	36	KISGGAPSVDLILDK	355–369
5	556.70	3	27	TPLMQVPLLLDLNGK	68–82
6	576.56	4	22	AGIALGAHHLEENLVVFDLER	403–423
7	642.87	2	28	AVGPFGLCYDSR	343–354
8	692.89	2	49	ISGGAPSVDLILDK	356–369
9	709.38	3	65	ISGGAPSVDLILDKNDAVWR	356–375
10	719.38	2	79	QGEYFIQVNAIR	267–278
11	757.02	2	9	KISGGAPSVDLILDK	355–369
12	768.41	3	61	AGIALGAHHLEENLVVFDLER	403–423
13	826.54	2	68	TPLMQVPLLLDLNGK	68–82
14	834.43	2	49	TPLMQVPLLLDLNGK	68–82
15	1040.25	3	60	GLPNNVQGALGLGQAPISLQNQLFSHFGLK	187–216

[a] Peptides with a score < 48 have a < 95% confidence for identity but are useful for confirming the overall protein identity and add to the protein score of 540.

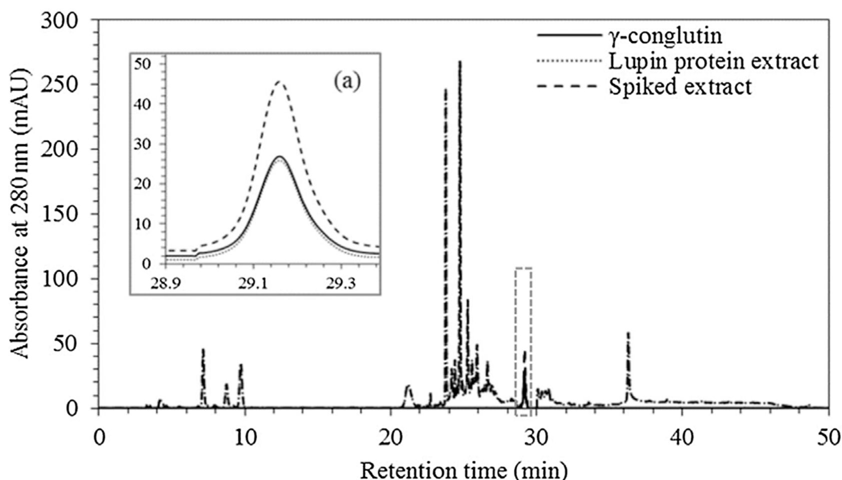

Fig. 5. Overlay of spectral chromatograms of pure γ-conglutin (0.1 mg/ml), lupin protein extract and extract spiked with 0.1 mg/ml γ-conglutin.

from Fig. 5 that the addition of lupin protein extract to standard γ-conglutin solution did not affect the retention time of γ-conglutin peak, which indicates the specificity of the developed RP-HPLC method for analysis of γ-conglutin.

The resolution between γ-conglutin and nearest eluting peak decides the degree at which the area of an analyte peak can be accurately measured for a perfectly symmetric Gaussian peak [23]. For minimum usable separation, $Rs > 1$ is recommended by AOAC International [23,32]. The developed method demonstrated the chromatographic separation between γ-conglutin and the nearest eluting peak (Fig. 5) with $Rs = 1.39$. Thus the mobile phase composition and its gradient flow enabled separation of γ-conglutin in the presence of matrix/interference solution.

3.2.2. Linearity and range

Linearity of a method should be evaluated in the concentration span of 80–120% of the expected concentration range [26], starting from the lower limit of quantitation [22]. Therefore, ten different concentrations of γ-conglutin ranging from 8, 15, 25, 50, 100, 250, 500 and 1000 μg/ml were analysed in five independent replicates. The overlay of spectral chromatograms of γ-conglutin at different concentrations is as represented in Fig. 6. The height and area of the peak were observed to increase with an increase in the concentration of γ-conglutin (Fig. 6a). To determine the relation between peak area and concentration of γ-conglutin, the calibration curve was constructed by plotting the mean response factor (peak area) against the respective concentration of γ-conglutin.

Linearity of the method was determined by correlation coefficient and intercept value of the calibration curve. A linear regression curve with a correlation coefficient of greater than 0.95 and an intercept not significantly different from zero is accepted as a standard curve [26]. In the case of γ-conglutin, the linear relation with a good correlation

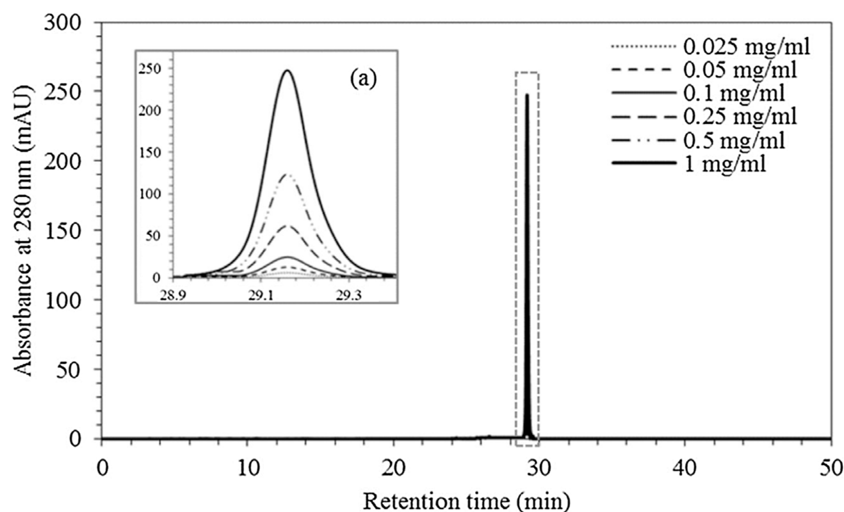

Fig. 6. Overlay of spectral chromatograms of γ-conglutin at different concentrations.

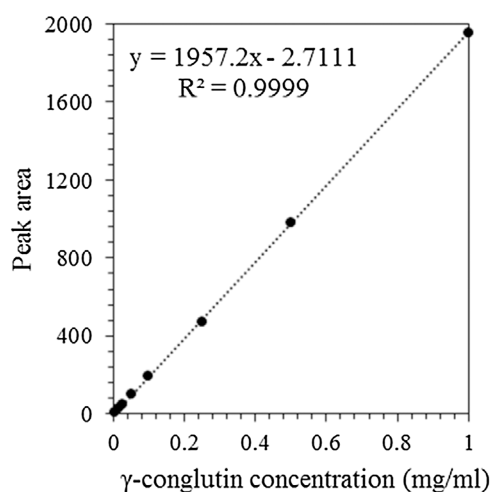

Fig. 7. Calibration curve of γ-conglutin at different concentrations.

coefficient of 0.999 was obtained (Fig. 7), which shows the acceptable fit of the data to the regression line. From the standard equation of the calibration curve, values of slope and intercept were found to be 1957.2 and −2.7111 respectively.

3.2.3. Sensitivity, detection limit and quantitation limit

Sensitivity of a method for analysis of γ-conglutin was indicated in terms of detection and quantitation limits. LOD and LOQ were calculated based on 3 times and 10 times the standard deviation of the response factor (peak area) over the slope, respectively [23,26]. LOD and LOQ of γ-conglutin were estimated at 2.68 μg/ml and 8.12 μg/ml respectively.

3.2.4. Precision

Precision of the analytical method was expressed as the percentage of the relative standard deviation, % RSD, of responses at respective concentrations [26]. The precision values are expected to yield the smallest variations among responses, generally, RSD ≤ 2% at each concentration is required for developing highly precise analytical method [26]. Intra-day and inter-day precision of the developed method for analysis of γ-conglutin were found to be in the range of 0.38-0.49% (Table 3). Precision values at each concentration were within the acceptance criteria. The results indicated the high precision of the developed analytical method, with very less variability at different concentrations measured on different days.

3.2.5. Accuracy and recovery

Accuracy of the proposed RP-HPLC method for analysis of γ-conglutin was expressed in terms of recovery at three different levels (80, 100 and 120%) in the range of expected concentration (three independent replicates). Recovery is calculated as the ratio of experimental concentration (concentration extrapolated from calibration curve) to theoretical concentration (added concentration for spiking) of the analyte (γ-conglutin) in the spiked extract. The average percentage recovery for γ-conglutin was found to be 97.77%, 97.01% and 98.63% for 0.08 mg/ml, 0.10 mg/ml and 0.12 mg/ml respectively (Table 4). The results indicated acceptable values of recovery at different concentration levels as recommended by AOAC Guidelines, which defines the high accuracy of the proposed RP-HPLC method for detection and quantitation of γ-conglutin [20].

4. Conclusion

A reverse phase (C-18) HPLC method for detection and

Table 3
Precision analysis (intra-day and inter-day) of γ-conglutin at three different concentrations.

γ-Conglutin added in extract (mg/ml)	Intra-day studies*			Inter-day studies*		
	Response[a] ± SD[b]	RT[c] ± SD[b]	% RSD[d]	Response[a] ± SD[b]	RT[c] ± SD[b]	% RSD[d]
0.08	330 ± 1.62	29.16 ± 0.07	0.49	333 ± 1.58	29.16 ± 0.08	0.47
0.10	378 ± 1.59	29.16 ± 0.08	0.42	382 ± 1.56	29.16 ± 0.09	0.41
0.12	417 ± 1.57	29.16 ± 0.06	0.38	423 ± 1.61	29.16 ± 0.07	0.38

* Average of five determinations.
[a] Response (peak area).
[b] Standard deviation of three readings.
[c] Retention time (min).
[d] Relative standard deviation.

Table 4
Recovery and accuracy studies of γ-conglutin at three different concentrations.

γ-Conglutin in extract	R[a] ± SD[b]	RT[c] ± SD[b]	γ-Conglutin added in extract	R[a] ± SD[b]	RT[c] ± SD[b]	γ-Conglutin recovered	Recovery
(mg/ml)			(mg)			(mg/ml)	%
0.1	150 ± 1.52	29.16 ± 0.05	0.08	330 ± 1.52	29.16 ± 0.03	0.17 ± 0.006	97.77
0.1	150 ± 1.52	29.16 ± 0.06	0.10	378 ± 1.50	29.16 ± 0.02	0.19 ± 0.004	97.01
0.1	150 ± 1.52	29.16 ± 0.04	0.12	417 ± 1.48	29.16 ± 0.03	0.21 ± 0.007	98.63

[a] Response (peak area).
[b] Standard deviation of five readings.
[c] Retention time (min).

quantification of γ-conglutin from lupin seed extract was developed with a simple linear gradient of water and acetonitrile in the presence of TFA. The mobile phase gradient and the flow rate of 0.8 ml/min were found to be appropriate to provide a sharp and symmetric peak ($As = 1.04$) of γ-conglutin with retention time at 29.16 min. The identity of γ-conglutin in a peak was confirmed by SDS-PAGE and mass spectrometry (MS/MS) analysis, which showed 28% peptide sequence coverage of the γ-conglutin precursor (Q42369). The performance characteristics of the developed RP-HPLC method were validated based on guidelines of FDA, AOAC International and ICH. The method was found to be specific, reproducible and accurate for detection and quantitation of γ-conglutin for lupin seed extracts. The validated method can be suitably used for an effective and precise analysis of γ-conglutin in lupin extracts.

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