

## A common HPLC-PDA method for amino acid analysis in insects and plants

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A common method for analysis of 17 amino acids from various insect species and plant parts was standardized using HPLC-PDA. Prior to hydrolysis, lyophilization of test samples was found indispensable to remove excess moisture, which interferes in hydrolysis and separation of amino acids. After the hydrolysis of plant and insect samples, 500 and 100  $\mu$ L of boiling HCl, respectively for reconstitution, and 20  $\mu$ L of hydrolyzed samples used for derivatization, provided best results. Gradient profile of mobile phase and run time up to 65 min were standardized to (i) overcome the problems related to eluting underivatized sample part, (ii) optimize the use of mobile phase and run time, and (iii) get better separation of different amino acids. Analysis of *Chilo partellus* larvae reared on sorghum seedling powder based artificial diet indicated that arginine and histidine quantities were on par in both samples. However, methionine was higher, and leucine, isoleucine, lysine, phenylalanine, threonine and valine were lower in sorghum seedlings than in *C. partellus* larvae, suggesting compensation of these amino acids by the insect through voracious feeding, as is being expected from artificial diet. This method was found highly sensitive, reproducible and useful for the analysis of amino acids for better understanding of insect-plant interactions.

**Keywords:** Amino acids, Gradient profile, HPLC-PDA, Insect, Plant, Standardization

There are 22 standard amino acids, of which selenocysteine and pyrrolysine are incorporated into proteins by distinctive biosynthetic mechanisms, while remaining 20 are directly encoded by the universal genetic code. The determination of amino acid requirements for 20 different insect species using Rose's deletion method has shown that the L-forms of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are essential, while the L-forms of alanine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, proline, serine and tyrosine are non-essential for majority of the insects<sup>1,2</sup>. Several amino acids that are non-essential for rat (proline, serine, cystine, and glycine) were found essential for some insects<sup>3-5</sup>. Essential amino acids are those which contribute to protein having a carbon skeleton and cannot be synthesized *de novo* by the insects<sup>6</sup>, and their effects on insect growth and development are dose- and species-dependent<sup>2,4,7-9</sup>. Host plant allelochemicals, specific anatomical features and

nutrients have all been invoked as determinants of host plant quality, and the variation in performance and abundance of herbivorous insects is resultant of variation in host plant quality<sup>10,11</sup>.

Most of the research efforts to identify insect-resistant crop genotypes have been on identifying the morphological, anatomical, and anti-nutritional plant characters. However, the manipulation of nutritional composition of host plants could also play an important role in their defense mechanism. Poor understanding of biochemical mechanisms of insect-plant interactions has been the biggest impediment in development and deployment of insect-resistant crop plants. This has been mainly because of major focus of biochemical profiling on host plants, while mapping the changes in biochemical profile/nutritional requirements of the target insects in response to feeding on resistant/susceptible host plants has received little attention. Imbalance in nutritional composition could also be one of the possible means of managing insect pests. In insects, the required balance of nutrients is generally related with natural foods of the species<sup>12</sup>, on which they might respond differently such that they can alter the total amount of ingested food, move from one food to another with a different nutrient

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balance, or can regulate the effectiveness of nutrients. In such a scenario, the knowledge on biochemistry of host plants vis-à-vis target insect species in response to feeding on diverse foods could be helpful in better understanding the insect-plant interactions.

In order to map the biochemical asynchrony in host plants and target insect species; a common, standard and repeatable biochemical protocol/method is the minimum requirement. Efforts are on for determining the role of different amino acids available in host plants and their bioavailability to the target insect species for growth and development, for which accurate and reliable method of quantitative analysis is essential. Amino acid analysis using high performance liquid chromatography (HPLC) is dependent on various steps viz., collection and processing of test samples for hydrolysis, methodology of sample hydrolysis, derivatization procedure of hydrolyzed samples, gradient mixture of different eluents and flow time, and the detector, for better separation of different amino acids. Till date no common suitable HPLC-photodiode array detector (HPLC-PDA) based amino acid analysis method involving all the necessary steps for their separation and applicable to different insect species and the host plant parts, have been reported to pursue the role of different amino acids in plant-insect interactions research. Therefore, present studies have been aimed at standardizing a common method for separating amino acids from different insect groups with varying feeding habits and different plant parts.

## Materials and Methods

**Sample collection and processing**—The insect samples from two orders viz., Lepidoptera (*Helicoverpa armigera*, *Chilo partellus*, and *Spodoptera exigua*) and Hemiptera (*Lipaphis erysimi*); mature dry grains and lyophilized seedling power of sorghum were used for the amino acid analysis. The collection of samples and amino acid analysis were carried out at the Division of Entomology, Indian Agricultural Research Institute, New Delhi, India. The *H. armigera* and *C. partellus* larvae were collected from the insects reared on artificial diets, *S. exigua* larvae from pigeonpea crop, and *L. erysimi* nymphs and adults from the rapeseed and mustard crop. The collected insect and plant samples were stored in the deep freeze at -20 °C for further analysis.

**Sample amount**—Various sample quantities viz., 25, 50, 75 and 100 mg of each of the test insects and

plant parts were used initially to determine the appropriate quantity for the detection of different amino acids. The sample quantities of test plant parts and insects were individually collected in 6 × 50 mm sample tubes.

**Lyophilisation of samples**—Before hydrolysis, the samples were lyophilized for 90 min. In case of lyophilized samples this step can be skipped. For example, in this study, the lyophilized sorghum seedling powder was directly used for hydrolysis, whereas the whole grains powder was first lyophilized and then used for further hydrolysis. In case of insects, the samples were first kept in deep freeze for 30 min, weighed, crushed and lyophilized for 90 min.

**Hydrolysis of proteins/peptides into amino acids**—The vacuum-dried samples were hydrolyzed with 200 µL of constant boiling 6N HCl and 40 µL of phenol through vapour-phase hydrolysis. The samples were dried in an oven at 112-116 °C for 20-24 h. After completion of hydrolysis, excess HCl was wiped off and the tubes were vacuum dried for 90 min. The plant and insect samples were reconstituted with 500 and 100 µL of 20 mM boiling HCl, respectively.

**Derivatization of amino acids**—The reconstituted 20 µL samples were derivatized with AccQ-Fluor reagent kit (WAT052880-Waters Corporation, USA). AccQ-Fluor borate buffer (60 µL) was added in the sample tube with micropipette and vortexed. Thereafter, 20 µL of AccQ-Fluor reagent was added and immediately vortexed for 30 sec, and the contents were transferred to maximum recovery vials. The vials were heated for 10 min in a waterbath at 55 °C before separation of amino acids using HPLC.

**Separation of amino acids**—The AccQ-Fluor amino acid derivatives were separated on a Waters 2707 Module HPLC System attached to a PDA (Model PDA 2998). A 10 µL sample was injected into a Waters reversed phase AccQ Tag Silica-bonded Amino Acid Column C<sub>18</sub> (3.9 mm × 150 mm) using auto sampler (Waters 2707). The Waters AccQ Tag Eluent A Concentrate (WAT052890) was diluted to 10% in Milli-Q water and used as eluent A, and 60% acetonitrile as eluent B in a separation gradient with a flow rate of 1.0 mL/min. The separation gradient used was 0-2 min (100% A), 2.0 min (98.0% A), 15.0 min (93.0% A), 19.0 min (90.0% A), 32.0 min (67.0% A), 38.0 min (0.0% A), and 56.0 min (100.0% A). The amino acids were detected using PDA at 254 nm with the column condition set at 37 °C. The amino acid peaks were acquired using Empower Pro Software®

by Waters Corporation (2005-08) and were calculated based on amino acid calibration standard (Thermo Scientific Amino Acid Standard H, Prod # NCI0180) run at five concentrations 10, 20, 30, 40 and 50  $\mu\text{L}$  having 2.5  $\mu\text{moles/mL}$  of L-forms of alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine HCl, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine, and 1.25  $\mu\text{moles/mL}$  of cystine in 0.1N HCl. Amino acid assignments were visually checked to verify the peak assignment. Injections (10  $\mu\text{L}$ ) of 10, 20, 30, 40 and 50  $\mu\text{L}$  of amino acid standard corresponds to 100, 200, 300, 400 and 500 pmol, respectively, of each amino acid, except cystine which had half of their concentrations. The proportional molar concentration for each amino acid was calculated based on the concentration of standard amino acids and expressed as  $\mu\text{g}$  amino acid/mg sample.

## Results

**Standardization of sample amount and preparation protocols**—Determination of sample quantity prior to separation of amino acids is highly desirable as the protein content is variable across sample types, and other compounds present in the test samples which also interfere in their separation. Based on the sample

quantities used for standardization (25, 50, 75 and 100 mg), 50 mg of the plant parts was found optimum for better separation of amino acids, whereas in case of insects, whole insect(s) was used as sample and later on amino acid quantity calculated for 50 mg sample weight, and the data reported herein is based on these standardized sample weights (Table 1). It was also observed that the vacuum drying of samples before and after hydrolysis resulted in better separation of amino acids. Amount of HCl used in making up of final volume of the test samples after hydrolysis depends on the composition of test sample, for example 500  $\mu\text{L}$  of boiling HCl was required to dissolve 50 mg plant part samples, while 100  $\mu\text{L}$  of boiling HCl was found enough for insect samples, which could be because of cellular composition and contents of the test samples. In derivatization, 20  $\mu\text{L}$  from the final volume resulted in better quantifiable results for all amino acids tested, as some of the amino acids were present in very low concentration in the test samples.

**Standardization of gradient run time**—Gradient method was optimized for better separation of different peaks, wherein all the test amino acids eluted within 35 min without interfering with the next run of standards. However, the insect or plant sample run with the same gradient method eluted underivatized

Table 1— Amount of amino acids ( $\mu\text{g}/\text{mg}$ ) in different insects and plant parts using HPLC-PDA method

|                     | Sorghum plant parts<br>(Dry weight basis) |              | Insects (Fresh weight basis) |                        |                                 |                              |
|---------------------|-------------------------------------------|--------------|------------------------------|------------------------|---------------------------------|------------------------------|
|                     | Seedlings                                 | Mature seeds | <i>Lipaphis<br/>erysimi</i>  | <i>Chilo partellus</i> | <i>Helicoverpa<br/>armigera</i> | <i>Spodoptera<br/>exigua</i> |
| Aspartic acid (Asp) | 24.95                                     | 14.54        | 7.74                         | 4.48                   | 3.16                            | 6.34                         |
| Serine (Ser)        | 15.56                                     | 10.87        | 9.11                         | 5.71                   | 4.85                            | 11.97                        |
| Glutamic acid (Glu) | 22.05                                     | 28.29        | 8.38                         | 4.57                   | 4.81                            | 8.74                         |
| Glycine (Gly)       | 46.81                                     | 17.40        | 12.91                        | 19.09                  | 12.39                           | 24.85                        |
| Histidine (His)     | 6.10                                      | 4.01         | 3.18                         | 6.31                   | 5.21                            | 8.87                         |
| Arginine (Arg)      | 12.43                                     | 4.81         | 5.84                         | 10.33                  | 8.36                            | 12.11                        |
| Threonine (Thr)     | 13.58                                     | 5.50         | 6.90                         | 6.45                   | 5.54                            | 11.11                        |
| Alanine (Ala)       | 56.42                                     | 35.72        | 10.95                        | 14.94                  | 8.57                            | 15.45                        |
| Proline (Pro)       | 19.69                                     | 18.86        | 7.90                         | 9.42                   | 6.94                            | 13.18                        |
| Cystine (Cys)       | 0.93                                      | 0.28         | 0.05                         | 0.12                   | 0.08                            | 0.14                         |
| Tyrosine (Tyr)      | 2.03                                      | 1.64         | 6.90                         | 8.52                   | 7.61                            | 9.97                         |
| Valine (Val)        | 18.26                                     | 7.69         | 5.55                         | 5.85                   | 5.38                            | 10.09                        |
| Methionine (Met)    | 15.09                                     | 9.21         | 31.59                        | 30.30                  | 28.35                           | 53.47                        |
| Lysine HCl (Lys)    | 12.61                                     | 5.56         | 5.44                         | 4.44                   | 2.75                            | 7.62                         |
| Isoleucine (Ile)    | 9.47                                      | 3.19         | 3.46                         | 4.23                   | 3.68                            | 6.63                         |
| Leucine (Leu)       | 22.62                                     | 15.68        | 6.93                         | 8.99                   | 7.16                            | 13.61                        |
| Phenylalanine (Phe) | 12.20                                     | 3.28         | 5.04                         | 5.70                   | 6.41                            | 9.42                         |

part after 35 min of sample run. When sample run period was increased up to 65 min, the extended 30 min run time was found useful for eluting the underivatized sample parts to get rid off the interference in separation of different amino acids during the next sample run.

**Separation of amino acid standards**—The separation and resolution of peaks of all the 17 amino acids present in the standard was found satisfactory using this method. The first amino acid was eluted at 13.59 min and the last amino acid was separated at 34.26 min run time (Fig. 1). The peak pairs such as arginine/threonine, valine/methionine and isoleucine/leucine, which are not completely separated by the UV detector, the current method using the PDA detector resulted in clear separation of peaks of these amino acid pairs.

**Separation of amino acids in different plant parts and insect species**—The current HPLC-PDA based amino acid analysis method gave clear separation of all the 17 amino acids from the seedlings (Fig. 2A) and mature seeds (Fig. 2B) of sorghum, and the test insect species viz., mustard aphid (Fig. 2C), spotted stem borer (Fig. 2D), pod borer/cotton bollworm (Fig. 2E), and beet armyworm (Fig. 2F) fed on different food sources. The sequence of elution of all the test amino acids in different plant parts and insect species was similar to that of standards. Elution of some unknown amino acids was also observed within the elution time range of the test amino acids in case of sorghum seedlings and the insect species.

**Comparative analysis of amino acid composition in test samples**—This method could analyze very low to very high concentration of different amino acids across different plant parts and the insect species tested (Table 1). The amount of all the test amino acids (except glutamic acid) was higher in sorghum seedlings than in the mature seeds, indicating direct relationship between activities/functions being performed by various plant parts and the transmission/accumulation of different amino acids in different plant parts. Like large animals, insects need a dietary source of arginine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (all in the L-form), and are considered as essential amino acids. This method successfully analyzed all the essential amino acids (except tryptophan), along with other non-essential amino acids from different insect species. The amount of essential amino acid - methionine and the non-essential amino acid - tyrosine in different insect species was 3 to 5 times higher than in different plant parts (Table 1).

Amount of the essential amino acids valine, methionine and lysine HCl recorded from *S. litura* larvae was almost double the amount present in other test insects. The amount of essential amino acids viz., histidine, arginine, isoleucine, leucine and phenylalanine recorded in the sucking insect, *L. erysimi* was lower than that in biting and chewing insects viz., *C. partellus*, *H. armigera* and *S. litura*. In case of non-essential amino acids, the amounts of aspartic acid, glutamic acid, alanine, proline and

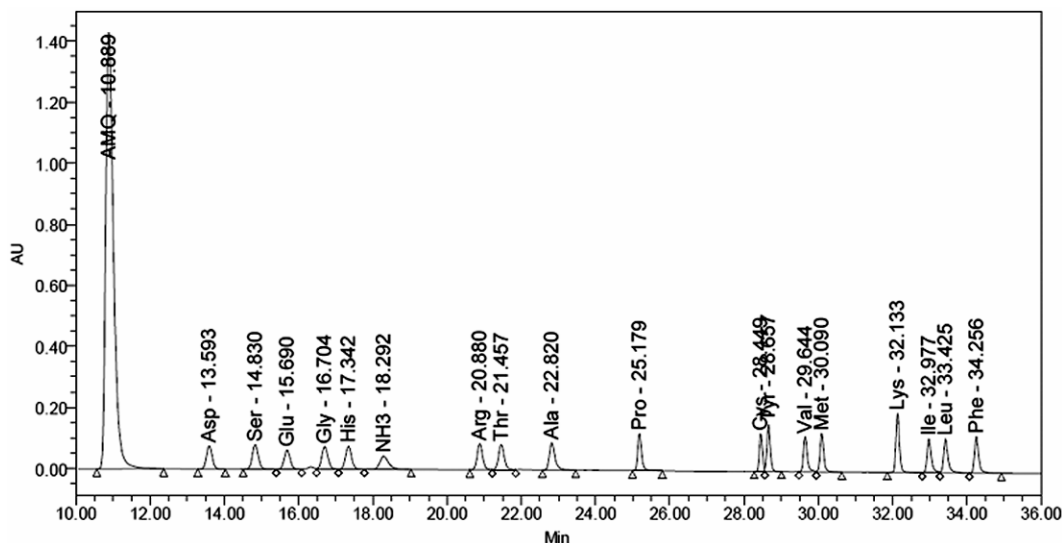


Fig. 1—Chromatogram of amino acid standards separated using HPLC-PDA.

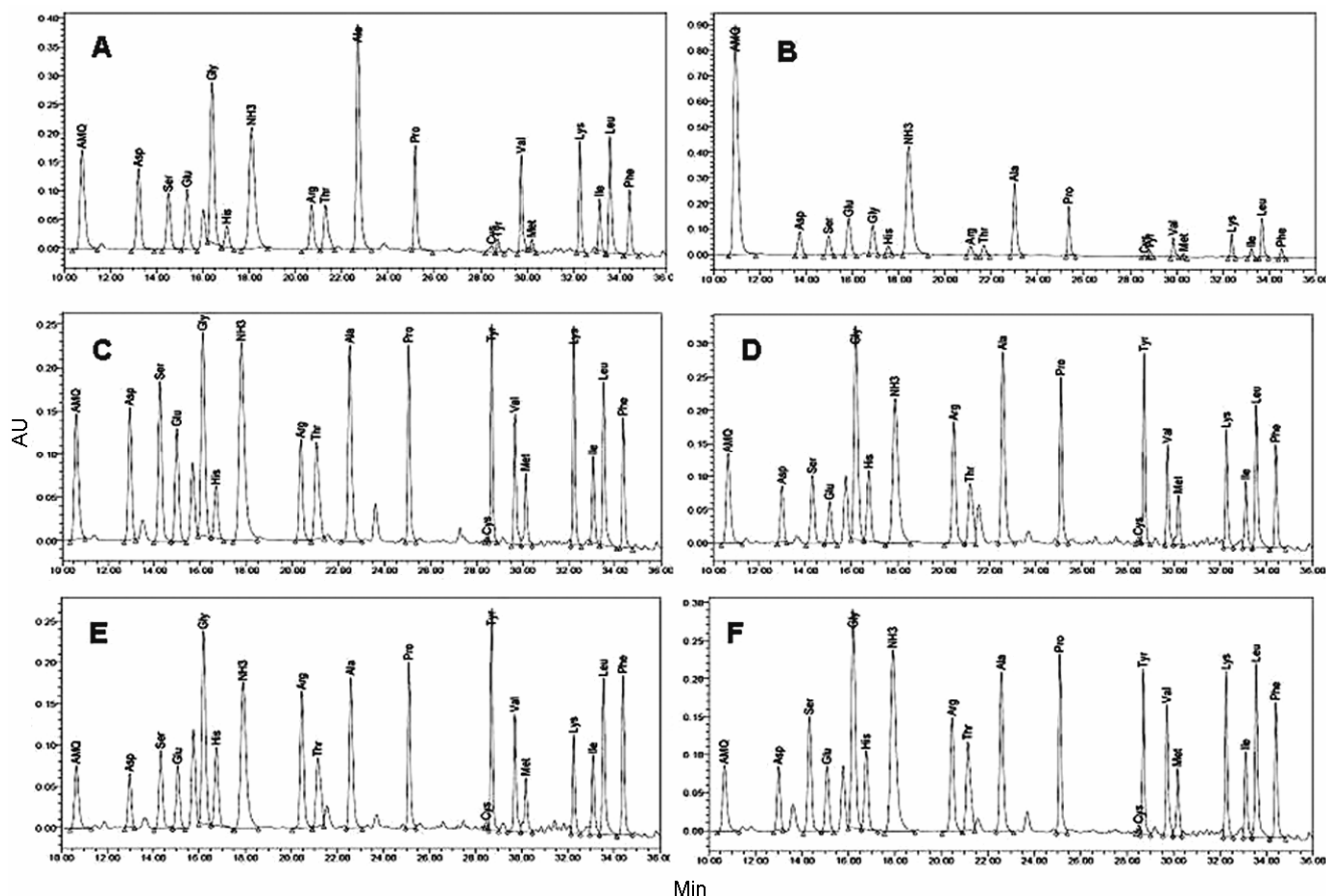


Fig. 2—Separation of different amino acids from: A. sorghum seedlings; B. sorghum seeds; C. mustard aphid, *Lipaphis erysimi*; D. spotted stem borer, *Chilo partellus*; E. pod borer/cotton bollworm, *Helicoverpa armigera*; F. beet armyworm, *Spodoptera exigua* using HPLC-PDA.

tyrosine were 3 to 7 times higher in different plant parts as compared to the insect species tested (Table 1), indicating that the insects too require these amino acids but their functions are not known. The glycine content was found higher in *C. partellus* and *S. exigua* as compared to other insects.

## Discussion

Amino acids play central role both as building blocks of proteins and as intermediates in metabolism, and absolutely essential for every metabolic process. Development of a common method for the analysis of amino acids from host plants vis-a-vis insect pests is a paramount to better understand the plant-insect interactions and engineer nutritional manipulation based strategy for the management of insect pests. Several modern instruments notably HPLC and UPLC (ultra performance liquid chromatography) have been deployed for the identification and quantification of different amino acids, wherein several methods have

been reported<sup>13-19</sup>, but these methods require specific and unique methods/protocols for the preparation of samples prior to amino acid analysis and deem cumbersome for insect-plant interaction studies. The hydrolysis, derivatization, gradient mixture and flow time are one of the most important and critical components of sample preparation and separation of different amino acids on the HPLC-PDA.

The sample amount required for the analysis of amino acids is inversely proportional to protein content of the samples. Present studies suggested that around 50 mg sample weight can be kept as a standard weight of the test samples for amino acid analysis, irrespective of the amount of protein present in the test insect and plant samples. Further, in case of insects, whole insect body is highly desirable as dissection of the insect for weight quantitation might result in disproportionate distribution of amino acids in different pieces of the dissected insect body. Taking this fact in to consideration and to make

comparative analysis in different insects, present studies suggested to use whole insect body as sample, and later on amino acid concentration can be calculated as desired. Furthermore, during the present studies it was observed that the sample moisture interferes in the hydrolysis process, and lyophilization step was found indispensable to remove the excess moisture before hydrolysis. Hydrolysis plays a crucial role in the conversion of peptides into free amino acids, and if this step is improperly carried out the separation or intensity of peaks will not be appropriate. During the derivatization process, the hydrolysed part of the test sample needs to be taken out with great care otherwise the presence of solid particles could choke the column and reduce the column performance. All the amino acids were eluted within 35 min using the amino acid standards without influencing the next run of standard. However, insect or plant samples run with the same protocol resulted in appearance of underivatized part in the next sample run. This particular problem could be resolved by little change in the mobile phase gradient and extending the run period up to 65 min. The gradient of mobile phase used in the study provided good separation of various peaks resulting in their better identification and quantitation. Furthermore, the high sensitivity of this HPLC-PDA method is evident from the intensity of peaks of different amino acids as shown in the figures, and even very less quantities of cystine from the test samples were successfully determined (Table 1).

Insects and the host plants need desired amount of amino acids to synthesize various proteins required for structural purposes, as enzymes, receptors, and for transport and storage. The variation in amino acid concentrations in different plant and insect species depends on numerous factors such as nutritional requirement, source of nutrition, biotic and abiotic environmental factors, etc. Essential amino acids are those which cannot be synthesized *de novo* by the insects, and their effects on insect development are dose- and species-dependent<sup>6</sup>. For example, during the present studies, essential amino acid analysis of *C. partellus* larvae reared on the artificial diet containing leaf powder of the susceptible sorghum genotype indicated that the amount of arginine and histidine were on par in host plant seedling and the insect larvae. However, the amount of methionine was higher, while leucine, isoleucine, lysine, phenylalanine, threonine and valine were lower in the sorghum seedlings as compared to the

*C. partellus* larvae, suggesting that the requirement for these amino acids by the insect larvae might have been compensated by increased feeding rate as is being expected while rearing the insects on the suitable artificial diet. Earlier studies have also reported that the insects compensate nutritional requirement by increasing the rate and quantity of food intake<sup>20</sup>, and the amount of amino acids required for different growth, development and life process is highly variable across insect species<sup>2,4,7,8,9,21-25</sup>. Similarly in case of plants, the species/genotypes conferring resistance to insect pests and diseases generally contain higher levels of phenolic compounds and low levels of free amino acids<sup>26-28</sup>. However, specific amino acids have also been reported to be correlated with resistance to aphids in cereals and legumes<sup>29-32</sup>.

In conclusion, reproducibility of the method applied across different sample types is one of the key indicators of a successful method. The present studies resulted in highly sensitive, user friendly, and more importantly a common method using HPLC-PDA for the analysis of different amino acids from various insects and the plant parts for better understanding of insect-plant interactions.

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