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Identification and metabolite profiling of *Sitophilus oryzae* L. by 1D and 2D NMR Spectroscopy

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Abstract

The polyphagous insect *Sitophilus oryzae* L. (Coleoptera:Curculionidae) has a tremendous adaptability in feeding behaviour, making it a serious invasive pest of stored cereals. The present study identifies the metabolite composition of *Sitophilus oryzae* (*S. oryzae*) using Nuclear Magnetic Resonance (NMR) spectroscopy. Assignment of 1D-proton by NMR, ^{1}H-^{1}H COSY, 2D-TOCSY ^{1}H-^{1}H, had been done. Amongst the various biochemically important metabolites isoleucine, valine, leucine, β-hydroxybutyrate, lysine, glutamate, glutamine, proline, lactate, alanine, di-methylamine, α-glucose, β-glucose, choline, glycerophosphorylcholine and tyrosine are present in *S. oryzae*. In wheat-fed *S. oryzae*, the presence of threonine and the absence of lactate is observed. In rice-fed *S. oryzae*, however, the presence of lactate and the absence of threonine were observed. Barley-fed *S. oryzae* shows presence of both tyrosine and lactate. It is concluded that the pest *S. oryzae* has adaptability on different stored cereals and grains, depicting the presence of earlier reported metabolites. The present study aims to identify the key metabolic components and associated enzymes in *Sitophilus oryzae* fed on different cereals.

Keywords: NMR spectroscopy, *Sitophilus oryzae*, stored grain pest, metabolites identification, pest control

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Introduction

Sitophilus oryzae L. (Coleoptera: curculionidae) commonly known as rice weevil is an invasive primary pest of stored food grains (Hill, 1990). It infests the major stored cereals, such as wheat, rice, barley, corn and sorghum, causing high quantitative and qualitative losses. Matthews (1993) reported that stored grain insect pests damage 10–30% of grains produced worldwide due to improper storage. Insecticides and fumigants, widely used to protect stored grains from insect infestations (Slavin, 2004), are toxic to mammals, cause environmental pollution and have lethal effects on non-target organisms. Further, the development of insecticide resistance leads to the 'pesticide treadmill' and necessitates in alternative control measures.

Successful control of insect pest (*S. oryzae*) requires a better understanding of its key metabolic components and associated enzymes (Govindan *et al*., 2000). The traditional bio-analytical procedures to study the biological effects and physiochemical properties are of little use to elucidate the different biochemical composition of the compounds actually occurring in the biomatrix (Dowell *et al*., 1999; Paliwal *et al*., 2004). Such disadvantages have been partially overcome by using NMR spectroscopic techniques in the study of complex bio-mixtures (Nicholson & Wilson, 1989). It is well

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established that insect pests become resistant relatively quickly and develop resistance mechanisms in due course of evolution against various xenobiotics and foreign chemicals. This resistance development mechanism is induced by stimulating the production of enzymes that can increase their tolerance to various insecticides. Insects' enzymes, hormones as well as the presence of cytochrome P-450 monooxygenases, all together are involved in the degradation of insecticides and other xenobiotics (René, 1999). However, various dietary chemicals, both plant based and man-made, based on recognition systems, stimulate the production of enzymes that degrade insecticides and other xenobiotics within the insect's body (Terriere, 1984). Thus, an understanding of correlations among the biochemistry, physiology, endocrinology and nutrition of insects is necessary for the development of novel strategies against insect pests.

It has been observed that the ratios of amylase/proteinase in homogenates of isolated midguts of *S. oryzae* (L.), fed primarily on cereals or cereal products, are extremely high (Baker, 1986, 1988). Whereas, insects that feed and develop on animal products or foods with relatively high protein content have shown higher general proteinase (caseinolytic activity) and amino-peptidase activity and much higher proteinase/amylase ratios than the granivorous coleopterans (Liang *et al.*, 1991). ^{1}H NMR spectroscopic metabolic profile studies of invertebrates, especially in arthropods, have not been researched much, although the possibilities for studying pathological and physiological processes are manifold (Higham *et al.*, 1986; Fan, 1996). Previous ^{1}H NMR spectroscopic studies (Nicholsan & Wilson, 1989; Thompson, 1990), on insects' haemolymph also suggest its feasibility on insect pest management by identifying the major metabolites, including the presence of amino acids (Fields & White, 2002).

Here, we present a 1D-proton NMR spectroscopic assignment study on the rice weevil *S. oryzae* and demonstrate the pest's survival and adaptability to major cereal grains (rice, wheat and barley). In the present study, the key metabolic components and their associated enzymes as metabolic biomarkers have been identified using *S. oryzae* as the target insect to ^{1}H NMR and TOCSY ^{1}H-^{1}H for designing novel strategies for pest control.

Materials and methods

Insects culture and sample processing

Sitophilus oryzae were originally obtained from the Department of Entomology, Chandra Shekhar Azad Agricultural University, Kanpur, India. The insects were cultured on rice, wheat and barley grains respectively; 50–55 live insects (randomly selected adults) were collected from culture from each cereal and frozen in liquid nitrogen for 5 min and then pulverized in a mortar. The addition of 5 ml perchloric acid (14%), was done while stirring continuously. After centrifugation at 10,000 rpm for 15 min, the supernatant was neutralized with 4M KOH between pH 7.2–7.4 and centrifuged once more for 15 min to remove potassium perchlorate. The samples were lyophilized and dissolved in 500 µl of D_2O (Sigma-Aldrich) before NMR analysis. One-dimensional-proton NMR experiments were done on whole insects (mixed males and females). The insects cultured on different cereals were processed thrice and the experiments were done for each individual sample.

NMR experiments

1D-proton NMR

For the 1D-proton NMR experiments, insect samples were dissolved in 500 µl of D_2O to provide a field-frequency lock for NMR spectrometer. One and two-dimensional ^{1}H NMR experiments were performed using a Bruker (Fallanden, Switzerland) *Avance* DRX 300 MHz FT-NMR spectrometer (7.2 Tesla, 54 mm vertical-bore magnet) equipped with a 5 mm multinuclear inverse probehead with a Z-shielded gradient, operating at a proton frequency of 300.13 MHz. One-dimensional NMR spectra were obtained using a spin echo experiment (carr-purcell-meiboom-gill) CPMGPR water presaturation to remove resonances from macromolecules and other species with short T2 relaxation times, at 298 K with 32,768 data points, spectral width (SW) of 3591 Hz, 128 scans, with a total relaxation time of 7.68 s per scan; and the binomial 90° pulse was set to 32 µs. FIDs (Free induction decay) were multiplied by an exponential function with a line broadening of 0.01 Hz, prior to Fourier transformation (Nicholson & Wilson, 1989; Phalaraksh *et al.*, 1999).

2D-NMR

^{1}H-^{1}H total correlation spectroscopy (TOCSY) spectra were acquired using transients collected for 256 increments into 1024 data points per increment over a spectral width of 3172.589 Hz. A relaxation delay of two seconds was included. These raw data were multiplied by sine-bell squared apodisation functions prior to zero-filling to 512 points on the spin-coupling axis. The spin lock was achieved using the MLEV-17 sequence for a period of 60 ms, with the spinlock strength of 7 kHz; a relaxation delay of two seconds was included, and the water peak was suppressed as described above. In addition to chemical shifts, two- and three-bond scalar coupling connectivities (spin correlation) were obtained simultaneously for a number of metabolites by detecting off-diagonal cross peaks which are symmetric with respect to the diagonals.

Results

Complete assignments in the 1D-proton NMR spectra of *S. oyzae* infesting wheat, barley and rice are shown in figs 1, 2 and 3 and tables 1 and 2, respectively. Figures 4, 5 and 6, present the 2D-^{1}H-^{1}H COSY NMR spectrum in the range of 0–8 ppm in wheat, 1–5 ppm in barley and 1–5 ppm in rice-fed *S. oryzae*, respectively. Two-dimensional-TOCSY of wheat-fed (in the range of 0–8 ppm), barley-fed (in the range of 1–5 ppm) and rice-fed (in the range of 1–5 ppm) *S. oryzae* are shown in figs 7, 8 and 9, respectively. Amongst the various important metabolites, isoleucine, valine, leucine, β-hydroxybutyrate, lysine, glutamate, glutamine, proline, lactate, alanine, di-methylamine, α-glucose, β-glucose, choline, glycerophosphorylcholine and tyrosine were present in *S. oryzae*. In wheat-fed *S. oryzae*, the presence of threonine and absence of lactate was observed. However, in rice-fed *S. oryzae*, the presence of lactate signal and absence of threonine was evidenced. Barley-fed *S. oryzae* showed presence of tyrosine as well as lactate.

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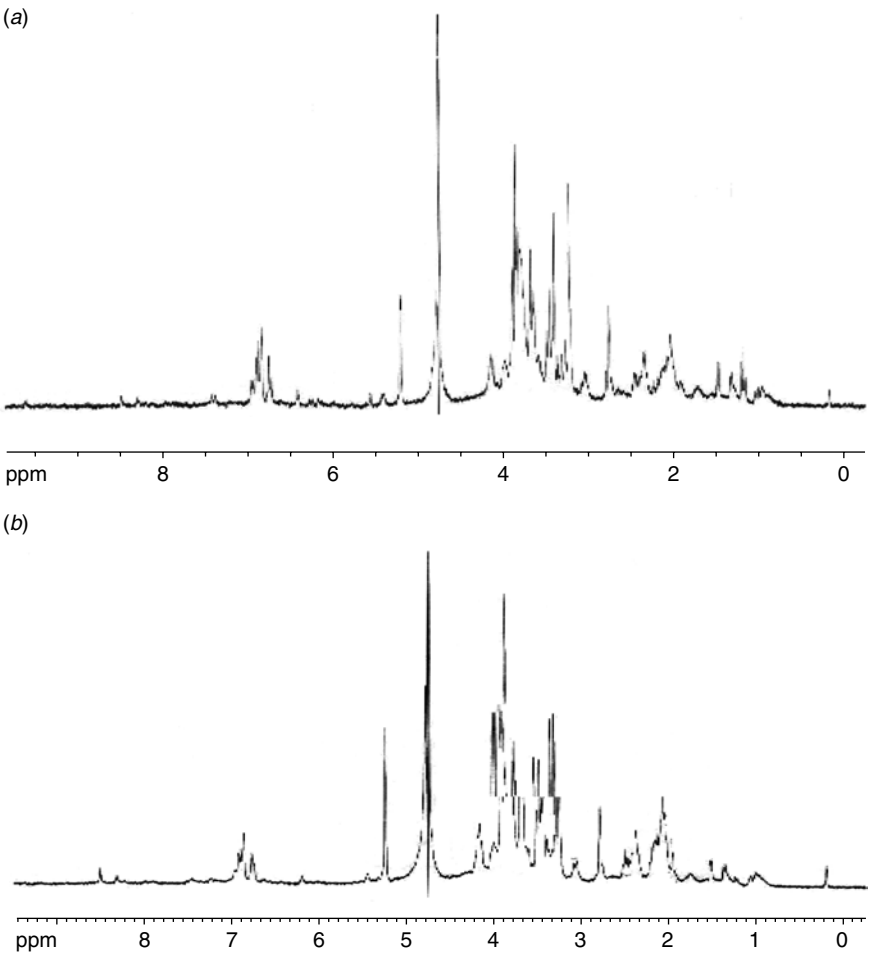

Fig. 1. (a) 1D-NMR of wheat-fed *Sitophilus oryzae*; (b) 1D-NMR of barley-fed *Sitophilus oryzae*.

Wheat fed Sitophilus oryzae *(L.)*

Identification of the resonances resulting from the presence of a number of amino acids can be ambiguously achieved by comparison of 1D and 2D data. An organic acid, i.e. the threonine γ-CH3 doublet, is visible at 1.34 ppm, which is coupled with the α-CH at 3.58 ppm (TOCSY). Citrate gives a distinct doublet pattern in the spectra, which was visible at 2.52 and 2.69 ppm in the COSY spectrum. Also, acetate singlet is visible at 1.92 ppm. The β-CH multiplet of proline at 2.02 ppm was assigned through the coupling to the α-CH at 4.15, δ-CH at 3.75, δ-CH at 3.40, γ-CH at 3.33 and β-CH at 2.33 ppm (TOCSY). The δ-CH_3 doublet of isoleucine was assigned at 0.92 ppm, and this was coupled with the γ-CH_2 at 1.25 ppm (TOCSY). Alanine is assigned by its β-CH_3 doublet at 1.47 ppm, which is coupled with the α-CH quartet at 3.78 ppm (TOCSY). Prominent resonances can be observed from glutamate with the β-CH_2 at 2.08 and γ-CH_2 at 2.34 ppm. The lactate β-CH_3 doublet was visible at 1.33 ppm that was coupled with the α-CH_3 at 4.1 ppm. Tyrosine presence in wheat is shown by the proton NMR signal at 6.7 ppm. Also, there was a presence of phenylalanine (NMR signal at 3.97 and 3.26 ppm), and lysine (NMR signal at 3.01 and 1.91 ppm). The prominent choline singlet was observed at 3.21 ppm from the $-NMe^+{}_3$ groups which were coupled with the O-CH_2 at 4.01 ppm, visible in the TOCSY spectra. The resonance from glycerophosphorylcholine was visible at 3.23 ppm that coupled with the multiplet at 3.59 ppm and triplet at 4.18 ppm, visible in the 1D and TOCSY spectra. Presence of carbohydrates in the insect metabolites was also observed. These included resonance from α- and β-glucose that can be assigned through TOCSY spectra. α-glucose H_4 double-doublet was observed at 3.44 ppm that was coupled with the H_6 multiplet at 3.84 ppm, doublet of H_1 at 5.22 ppm and multiplet on H_6 at 3.70 ppm. Sharp H_1 doublet of disaccharide trehalose was observed at 5.19 ppm that showed connectivity with the H_2 at 3.62 ppm (TOCSY).

Barley fed Sitophilus oryzae

The identification of the resonances resulting from the presence of a number of amino acids can be ambiguously achieved by comparison of 1D and 2D data. These include β-CH_3 multiplet of isoleucine assigned at 1.25 ppm that shows coupling with the δCH_3 at 0.94, β-CH at 1.97 and with γ-CH_2 at 1.01 ppm (TOCSY). The δ-CH_3 doublet of leucine is assigned at 0.97 ppm that shows coupling with the β-CH_2 at 1.72 ppm and with the α-CH at 3.7 ppm (TOCSY). Valine

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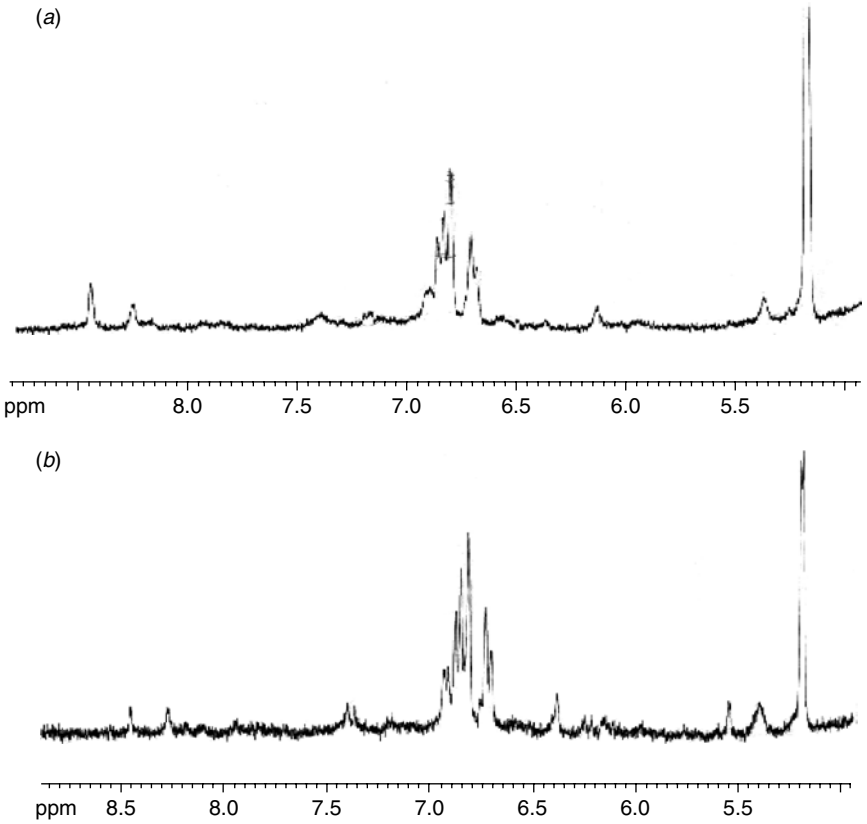

Fig. 2. 1D-NMR of *Sitophilus oryzae* in chemical shift range (5.5–8.5): (a) barley fed and (b) rice fed.

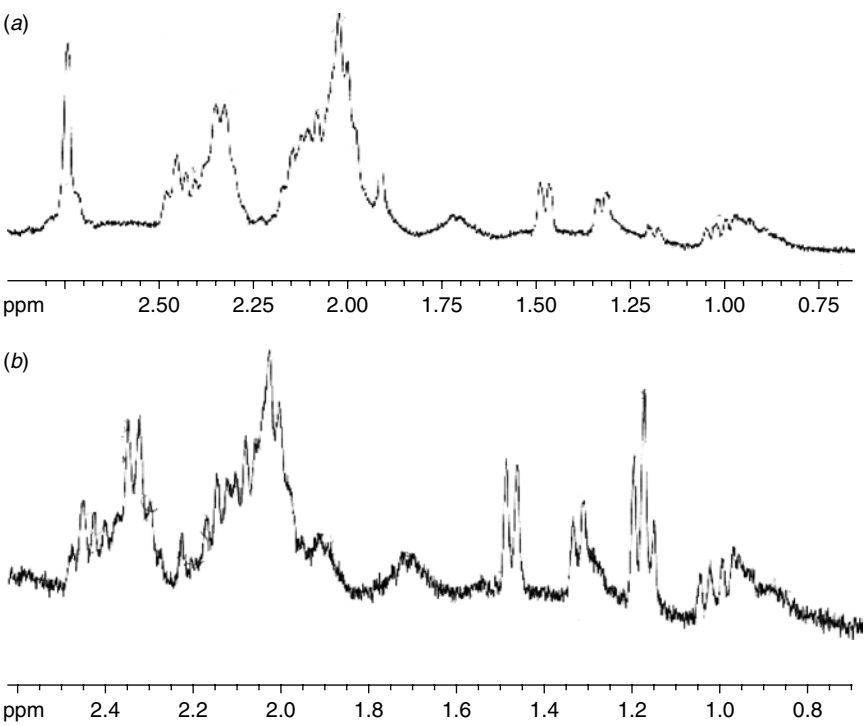

Fig. 3. 1D-NMR of *Sitophilus oryzae* in chemical shift range (0.8–3.0): (a) barley fed and (b) rice fed.

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Table 1. Assignation of different peaks of 1D-NMR on wheat-, barley- and rice-fed *Sitophilus oryzae*.

Peak No.	Chemical shift	Wheat	Barley	Rice	Metabolites
1	8.4	X	X	X	Formate
2	8.25	X	X	X	–
3	7.4	X	X	X	Phenyl Alanine/ thiourea
5	6.9–6.8	X	X	X	Hydroxy phenyl alanine
6	6.7	X	X	X	Tyrosine
8	6.25	X	**	**	–
9	5.55	X	**	X	
10	5.4	X	X	**	–
11	5.2	X	X	X	Trehalose
12	4.2	**	X	X	Lactate
13	4	X	X	X	β-glucose
14	3.9	X	X	X	Phenyl alanine
16	3.82–3.75	X	X	X	α-glucose/ Glutamate
17	3.6	X	X	X	Trehalose
19	3.5–3.35	X	X	X	α/β-glucose
20	3.3	**	**	X	–
21	3.26	X	X	X	Phenylalanne
23	3	X	X	X	Lysine
24	2.75	X	X	X	Di-methyl amine
25	2.46	X	X	X	Glutamate
26	2.41	X	X	X	Succinate
27	2.36	X	X	X	Proline
28	2.29	X	X	X	Valine
29	2.14	X	X	X	Glutamate
30	2.01	X	X	X	Proline
31	1.9	X	X	X	Acetate
32	1.73	X	X	X	Leucine
33	1.47	X	X	X	Alanine
34	1.34	X	X	**	Threonine
35	1.3	**	X	X	Lactate
36	1.2	X	X	X	β-hydroxybutyrate
37	1.02	X	X	X	Isoleucine/Valine

X, present; **, absent.

γ^1-CH_3 is assigned at 1.03 ppm, which was coupled with the β-CH multiplet at 2.29 ppm, γ-CH_3 at 0.99 ppm and α-CH at 3.61 ppm (TOCSY). Alanine is assigned by its β-CH_3 doublet at 1.47 ppm, which was coupled with the α-CH quartet at 3.78 ppm (TOCSY). Threonine γ-CH_3 is visible at 1.34, which was coupled with the β-CH at 3.58 ppm (TOCSY). Tyrosine presence is shown by the proton NMR signal at 6.7 ppm. Phenylalanine dd signal is visible at 3.97 and 3.26 ppm. Prominent resonances of lysine are observed as multiplet of β-CH_2 at 1.91 ppm that was well coupled with the δ-CH_2 at 1.73 ppm, and ε-CH_2 at 3.01 ppm (TOCSY). The resonance of glutamate can be observed as β-CH_2 at 2.07 ppm and the resonances of glutamine can be observed from the β-CH_2 at 2.16 ppm that is coupled with the α-CH at 3.77 ppm (TOCSY). The proline signals were assigned at γ-CH at 3.34 ppm coupled with the α-CH at 4.1 ppm. Among organic acids, β-hydroxybutyrate methyl doublet, visible at 1.20 ppm, was coupled to the signal at 2.59 ppm (TOCSY). The lactate β-CH_3 doublet is visible at 1.33 ppm that was coupled with the α-CH_3 at 4.1 ppm. Sharp H^1 doublet of disaccharide trehalose was observed at 5.19 ppm; this shows connectivity with the H_2 at 3.62 ppm, confirming the presence of carbohydrates.

Table 2. Assignments in the ^{1}H NMR spectra of stored grain pest *S. oryzae* infesting different crops.

Compound	1H Shift	Multiplicity	Assignment	13C Shifts
Valine	0.99	d	γ-CH_3	17.4
	1.03	d	γ′-CH_3	18.6
	2.27	m	β-CH	29.7
	3.61	d	α-CH	61.1
Leucine	0.97	d	γ-CH_3	22.7
	1.72	m	β-CH_2	40.5
	3.7	t	α-CH	54.2
B-Hydroxybutyrate	1.2	d	γ-CH_3	–
	2.37	m	α-CH	47.44
Lactate	1.33	d	β-CH_3	20.76
	4.11	q	α-CH	69.33
Alanine	1.47	d	β-CH_3	16.8
	3.78	q	α-CH	51.1
Threonine	1.34	d	γ-CH_3	20
	3.58	d	α-CH	61
Lysine	1.73	m	δ-CH2	26.7
	1.91	m	β-CH_2	30.2
	3.01	t	ε-CH_2	39.5
Proline	2.01	m	γ-CH_2	23.9
	2.34	m	β-CH	29.2
	3.34	t	δ′-CH	–
	4.14	t	α-CH	61.1
Glutamate	2.07	m	β-CH_2	–
Citrate	2.5	dd	α, γ -CH	46.47
	2.65	dd	α′, γ′-CH	46.47
Choline	3.13	s	N-CH_3	54.8
	3.96	t	α-CH_2	68.3
α-Glucose	3.4	d	H_4	70.7
	5.24	d	H_1	93.1
β-Glucose	4.64	d	H_1	97
	3.26	t	H_2	75.2
	3.49	–	H_3	77.0
	3.8	–	H_6	61.8
Trehalose	3.62	m	H_5	72.2
	5.19	d	H_1	94.4
Tyrosine	7.19	d	H_2, H_6	130
Acetate	1.92	s	β-CH_3	24.07
Formate	8.41	s	$HCOO^-$	–
Phenyl Alanine	3.97	dd	α-CH	56.8
	3.26	dd	β-CH	37
Succinate	2.39	s	α, β-CH_2	34.96

s, singlet; d, doublet; t, triplet; dd, double doublet; q, quartet; m, multiplet.

Rice fed Sitophilus oryzae

Amino acids identification and assignments includes the δ-CH_3 of isoleucine, assigned at 0.92 ppm, which is visible in the 1D and TOCSY spectra and was coupled to the signal at 2.29 ppm. The leucine β-CH_2 at 1.72 ppm was coupled to the δ-CH_3 at 0.97 and α-CH at 3.69 ppm (TOCSY). The valine γ^1-CH_3 doublet was visible at 1.04 ppm (TOCSY), and this was coupled to the β-CH at 2.29 ppm. Alanine is assigned by its β-CH_3 doublet at 1.47 ppm, which was coupled with the α-CH quartet at 3.78 ppm (TOCSY). A triplet is present at 3.25–3.50 ppm for β-glucose. The resonance of glutamate can be observed as β-CH_2 at 2.07 ppm, and the resonances of

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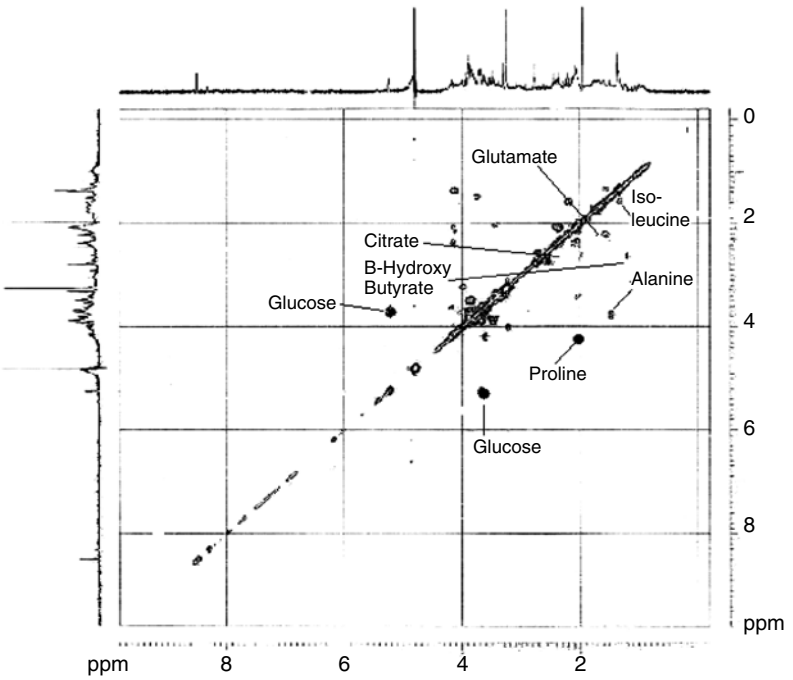

Fig. 4. 2D-^{1}H-^{1}H COSY NMR spectrum in the range of 0–8 ppm in wheat-fed *Sitophilus oryzae*.

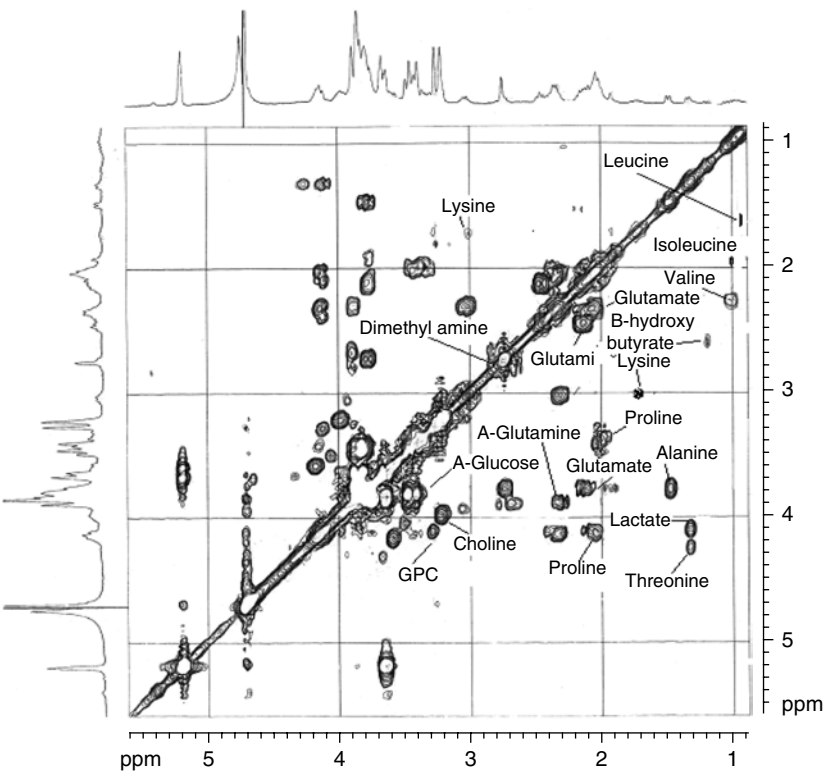

Fig. 5. 2D-^{1}H-^{1}H COSY NMR spectrum in the range of 1–5 ppm in barley-fed *Sitophilus oryzae*.

glutamine can be observed from the β-CH_2 at 2.16 ppm that is coupled with the α-CH at 3.77 ppm (TOCSY). The proline signals were assigned at γ-CH at 3.34 ppm coupled with the α-CH at 4.1 ppm. Tyrosine presence is shown by the proton NMR signal at 6.7 ppm. Phenylalanine dd signal is visible at 3.97 and 3.26 ppm. Prominent resonances of lysine are

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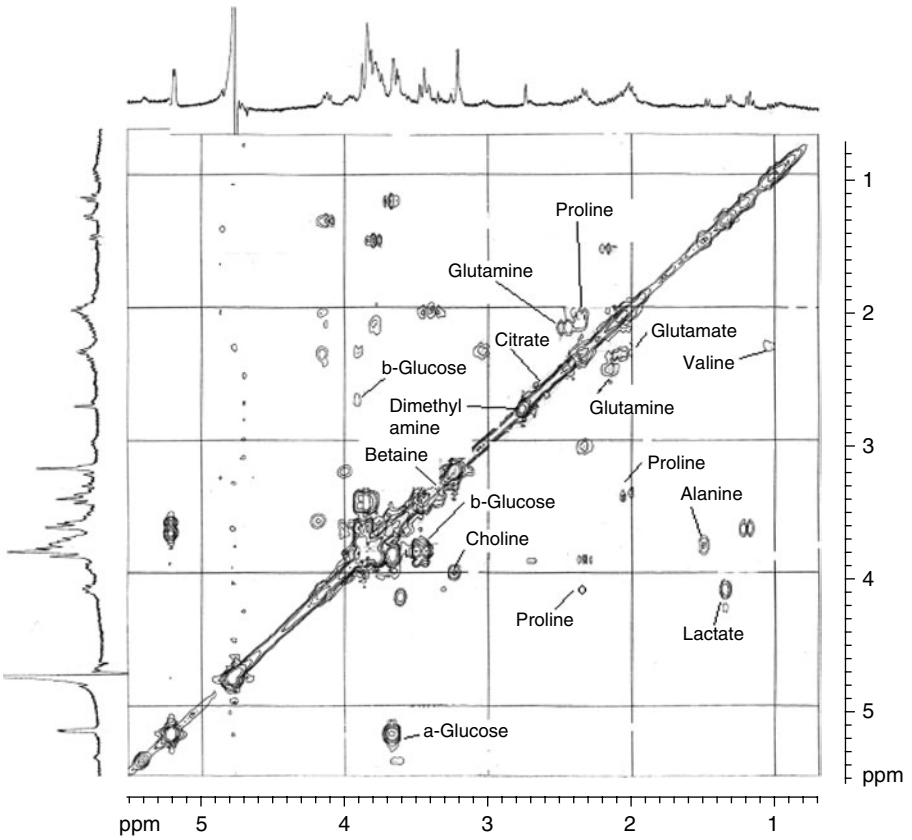

Fig. 6. 2D-^{1}H-^{1}H COSY NMR spectrum in the range of 1–5 ppm in rice-fed *Sitophilus oryzae*.

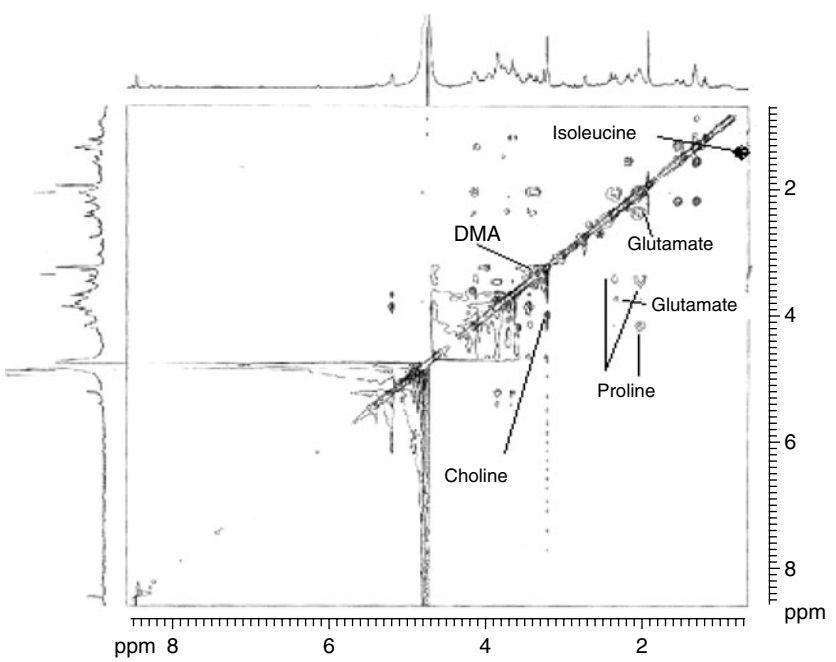

Fig. 7. 2D-TOCSY in the range of 0–8 ppm in wheat-fed *Sitophilus oryzae*.

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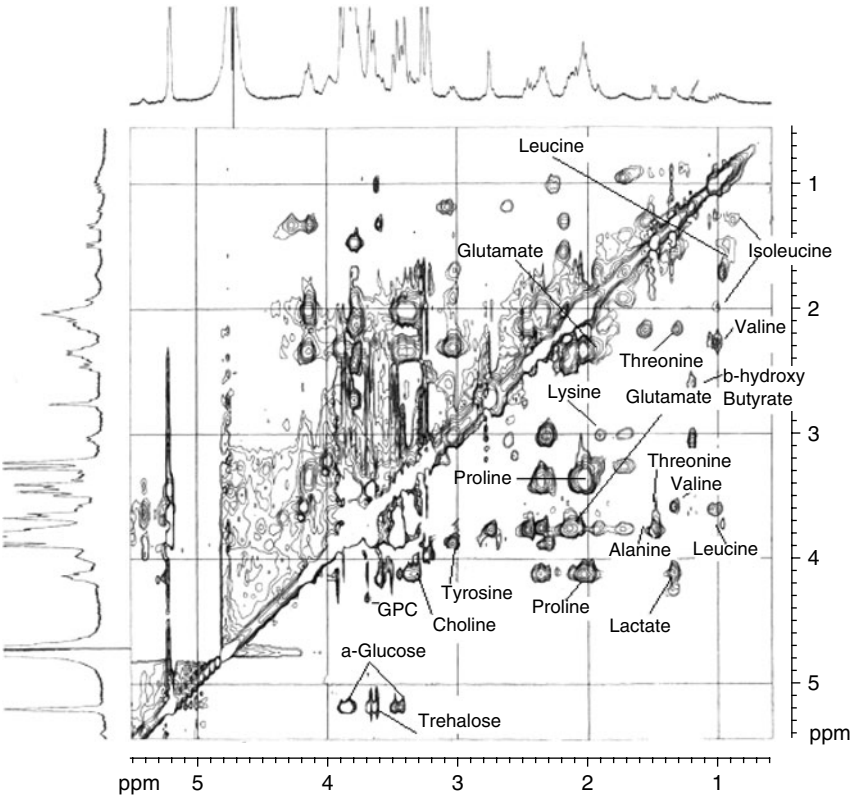

Fig. 8. 2D-TOCSY in the range of 1–5 ppm in barley-fed *Sitophilus oryzae.*

observed as multiplet of β-CH_2 at 1.91 ppm that was well coupled with the δ-CH_2 at 1.73 ppm and ε-CH_2 at 3.01 ppm (TOCSY). The nitrogen containing molecules were also observed in the NMR spectra, with dimethylamine giving a characteristic singlet at 2.74 ppm, visible in the 1D and TOCSY spectra. The prominent choline signal's singlet was observed at 3.22 ppm from the $-NMe^{+}{}_3$ group, which was coupled with the O-CH_2 at 4.05 ppm, visible in the TOCSY spectra. The resonance from glycerophosphorylcholine was visible at 3.23 ppm that was coupled with the double-doublet at 3.52 ppm visible in the 1D and TOCSY spectra. Carbohydrates were also observed. These include resonances from α- and β-glucose that can be assigned through TOCSY spectra. α-glucose H_4 double-doublet was observed at 3.45 ppm that was coupled with the H_6 multiplet at 3.85 ppm and doublet of H_1 at 5.24 ppm. β-glucose H_3 multiplet was visible at 3.49 ppm that was coupled with the H_1 doublet at 4.67 ppm. Sharp H_1 doublet of disaccharide trehalose was observed at 5.19 ppm that shows connectivity with the H_2 at 3.62 ppm. Among organic acids, lactate β-CH_3 doublet is visible at 1.33 ppm, which was coupled with the α-CH_3 at 4.1 ppm. A characteristic AB doublet pattern of citrate was visible at 2.69 and 2.80 ppm in the TOCSY spectrum.

Discussion

The success of *Sitophilus* spp. as a group is their capability to survive a wide range of environmental conditions, high fecundity, dietary habits and adaptability to thrive on different cereals. Their adaptation to different feed materials depends upon the digestive enzymes present in insects. Various components in the meal are hydrolyzed by enzymes belonging to different mechanistic classes, i.e. cathepsin-like (B-type, cistein-proteinase, or D-type, aspartic proteinases). Grain-feeding insects rely on amylases to hydrolyze starch and other sugars. To understand the metabolic profile and identification of key metabolites of the *S. oryzae* fed on different cereal crops, high-resolution 1D-proton NMR spectroscopy has been performed. High resolution NMR spectroscopy has shown that the various metabolites of differently fed *S. oryzae* contain a highly complex mixture of various metabolites as shown in figs 1–9. Despite of high degree of signal overlapping, many resonances could be assigned with the help of 2D-^{1}H-^{1}H COSY methods. Haemolymph of the tobacco hornworm, *Manduca sexta*, was evaluated for understanding the biochemical status in relation to physiological or environmental stress by NMR by Phalaraksh *et al*. (1999).

The structure and interaction of dietary components at the molecular or macromolecular stage characterize the dynamics of diet function (Cohen, 2003). NMR spectroscopy has shown marked differences in metabolites in insects infesting cereal grains of differing chemical constitutions. In other studies, the most abundant disaccharide trehalose concentration in the haemolymph was found to be higher than that of glucose (Thompson, 1990, 1998; Thompson & Borchardt, 1996), showing the highly active biochemical pest status of *Manduca sexta*. This relative stability shows the balance between synthesis and utilization; as trehalose is

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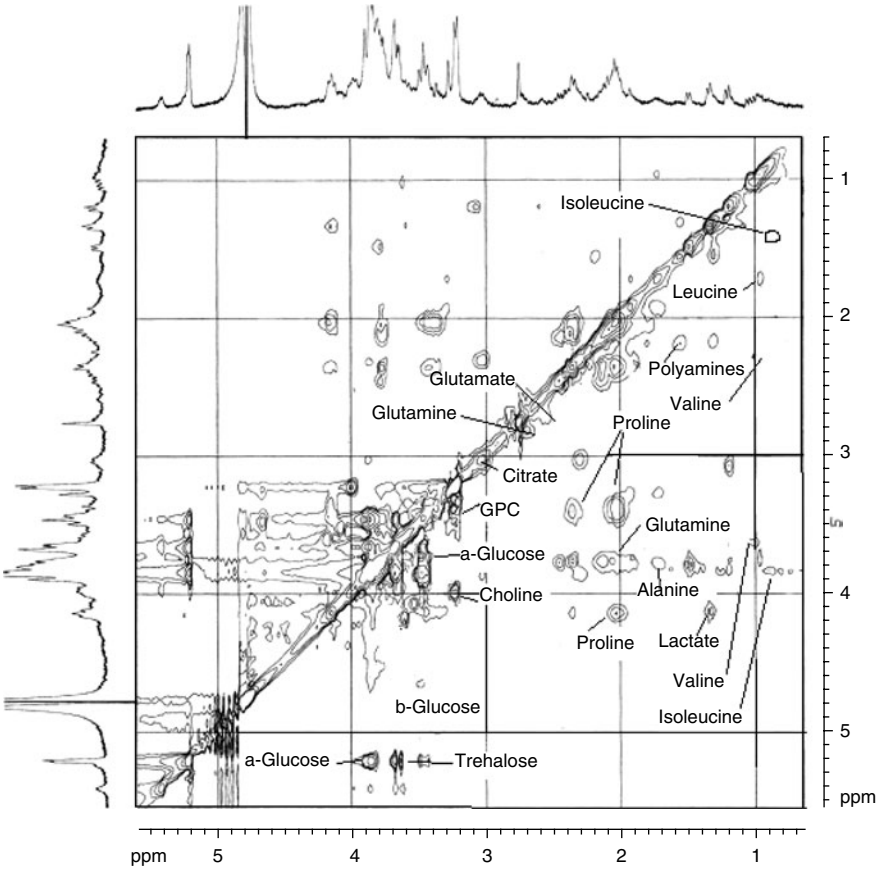

Fig. 9. 2D-TOCSY in the range of 1–5 ppm in rice-fed *Sitophilus oryzae*.

metabolized, more will be produced from glycogen (Gilmour, 1965; Wigglesworth, 1965; Chapman, 1982). The trehalose is derived from glucose via glucose-6-phosphate in the fat body. Since α-amylase is a predominant digestive hydrolase in *S. oryzae*, a major enzyme for hydrolyzing starch, the degree to which cereal diets affect amylase levels may indicate their suitability as potential hosts (Baker, 1988; Anonymous, 1991). *S. granarius* (L.) showed higher growth rates and development of insects on wheat and barley compared to corn, oats or rice examined at 27.5°C and 75% RH (Schwartz & Burkholder, 1991).

Conclusions

An understanding of the key insect metabolites, for the development of newer strategies of pest control is important. In this regard, advances in NMR spectroscopy has opened a new gateway of identifying metabolites in complex mixtures of biological substances. Analysis of crude *S. oryzae* metabolites, fed on different diets (e.g. wheat, barley and rice), through NMR revealed the presence of isoleucine, valine, leucine, β-hydroxybutyrate, lysine, glutamate, glutamine, proline, lactate, alanine, di-methylamine, α-glucose, β-glucose, choline, glycerophosphorylcholine and tyrosine. Wheat-fed *S. oryzae* showed the presence of threonine and absence of lactate. In rice-fed *S. oryzae*, the presence of lactate signal and absence of threonine was evidenced. Barley-fed *S. oryzae* showed the presence of both tyrosine and lactate. These differences in metabolites demonstrate the adaptability of the test species to various types of stored cereals. An understanding of *S. oryzae*'s metabolism of different cereals may assist in designing molecules tailored to disrupt the enzymes systems leading to lethal effects.

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