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A metabonomic analysis of insect development: ^{1}H-NMR spectroscopic characterization of changes in the composition of the haemolymph of larvae and pupae of the tobacco hornworm, *Manduca sexta*

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ABSTRACT: The analysis of complex biological samples, principally biological fluids, to obtain global metabolite profiles using techniques such as high resolution NMR spectroscopy has been termed metabonomics. This work describes the application of metabonomics to characterize the biochemical changes associated with development during the various instars of the larval stages and in the pupal stage of the tobacco hornworm *Manduca sexta*. The levels of many small molecule metabolites change during development. Thus, alanine, glutamate, lysine, succinate, lactate, and betaine are present at relatively high levels during the feeding period of larval life, but decrease at the onset of wandering. Compounds that increase in concentration as the insects prepare to pupate include citrate, fatty acids, and succinate. The presence of variable levels of putrescine in haemolymph is interesting, given its possible role in juvenile hormone action. The potential of high resolution ^{1}H-NMR spectroscopy for the metabonomic analysis of insect developmental chemistry is discussed.

KEYWORDS: biochemical profile, haemolymph, *Manduca sexta*, NMR

INTRODUCTION

Insect haemolymph is a complex biofluid containing a wide range of substances including proteins, lipids, low molecular mass organic molecules, and inorganic ions, as well as various cell types (plasmatocytes, granular cells, etc.). Haemolymph undergoes significant shifts in composition as insects progress through larval and pupal development. For example, much is known about developmentally related changes in the levels of particular haemolymph proteins[1], especially the hexamerin family of storage proteins[2]. The application of molecular genetic techniques has enabled developmentally regulated changes in the synthesis of even the minor protein components of haemolymph to be examined in great detail[3].

By comparison, surprisingly, much less is known about changes in the levels of most small organic molecule components of haemolymph. Changes in the concentrations of only a few important metabolites have been studied. For example, Siegert[4] examined the concentrations of glucose and trehalose in the haemolymph during the development of the tobacco hornworm, *Manduca sexta,* relating these to changes in the activity of the fat body enzyme glycogen phosphorylase. Subsequently, Siegert et al[5] studied these same metabolites during the period immediately before and after ecdysis, while Siegert[6] extended this to the period surrounding pupal ecdysis.

It is very laborious to study individual small molecule metabolites one at a time in a large number of developmentally timed samples, and under normal circumstances, it is only where there is an *a priori* reason to do so that such studies are performed. The use of powerful multiparametric analytical methods, such as ^{1}H-NMR spectroscopy, used as part of metabonomics studies to provide a "global" view of haemolymph composition may provide a useful means of investigating haemolymph composition during development[7,8]. Although ^{13}C-NMR spectroscopy has

been used to study insect carbohydrate metabolism specifically[9–12], we and others have employed ^{1}H-NMR spectroscopy in a number of preliminary studies to characterize the small metabolite composition of insect haemolymph. We have studied larvae of the holometabolous insects *Bombyx mori*[13] and *M. sexta*[14], whilst Moriwaki's group has studied hemimetabolous insects, namely, aphids[15] and planthoppers[16]. ^{1}H-NMR spectroscopy was also used to study the differences in the haemolymph composition of solitary and gregarious final instar nymphs of the hemimetabolous desert locust *Schistocerca gregaria*[17], revealing clear biochemical differences between the two phases. Here we have extended our previous studies in *M. sexta* to a more detailed ^{1}H-NMR spectroscopy investigation of haemolymph composition during larval development.

MATERIALS AND METHODS

Biological materials

Tobacco hornworms, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) were reared at the University of Bath, as described by Reynolds et al[18]. In brief, insects were reared individually at 25 °C, 50% R.H. under a diapause-averting light regime (17 h light: 7 h dark) on a standard artificial diet containing casein, wheatgerm, sucrose, salts, choline chloride, cholesterol, linseed oil, corn oil, and agar.

Larvae were selected according to the stage of development for each experiment. The first day (i.e. 0–24 h) of each stage is described by convention as "day 0". Third and fourth stage larvae were selected on day 1 of those stages (i.e. during the period 24-48 h after reaching the appropriate stage), while for the fifth stadium and the wandering stage of the fifth instar, insects were selected to represent each day of that stage. Pupae were selected daily during days 0–4 and again on day 10.

Haemolymph samples (400–700 µl) were taken from larvae by cutting the abdominal horn. Prior to sampling, the larvae were anaesthetized by immersion in water until they had ceased to move. The time that this took varied according to the stage. For fifth instar larvae this was about 15 min. In the case of third and fourth stages and smaller fifth stage larvae, it was necessary to pool haemolymph samples for analysis. For pupae, haemolymph was taken from an abdominal incision, taking care to avoid contamination with the fat body. The exuding haemolymph was collected into a 1.5 ml polypropylene microcentrifuge tube. Approximately 30 mg recrystallized phenylthiourea was added to each haemolymph sample to inhibit endogenous phenoloxidase activity that leads to blackening of the haemolymph[19]. Samples were frozen and stored on solid CO_2 and transported to Imperial College, London, for analysis.

NMR spectroscopy

One-dimensional ^{1}H-NMR spectra were obtained with conventional water peak suppression using a Bruker AMX600 NMR spectrometer (Bruker Biospin Ltd., Coventry, UK), operating at the 600.13 MHz ^{1}H observation frequency, and with the samples held at a temperature of 303 K during data acquisition (this was typically ca. 5 min for standard one-dimensional ^{1}H-NMR spectra). Typically, 64 transients were acquired into 32768 data points covering a spectral width of 8,012 Hz. A line-broadening of 0.3 Hz was applied before Fourier transformation and all spectral data sets were processed using standard software supplied by the instrument manufacturer. Five examples of each selected developmental stage were analysed. The spectra shown are representative.

RESULTS

^{1}H-NMR spectroscopy of haemolymph from larval and pupal stages

At all stages of development, the ^{1}H-NMR spectra from the whole haemolymph of developing

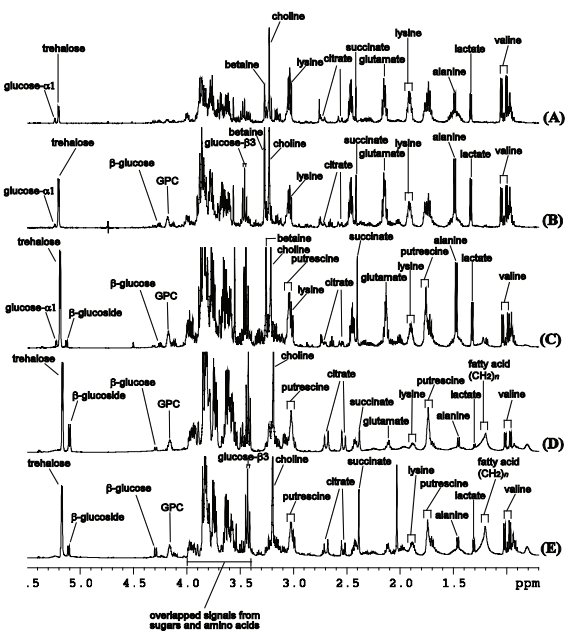

Fig. 1 600 MHz ^{1}H-NMR spectra of the haemolymph of *Manduca sexta* larvae. Haemolymph samples were collected from one day-old third stage larvae (A), fourth stage (B), fifth stage (C), wandering stage larvae (D), and pupae (E).

M. sexta were observed to contain peaks from a large number of low molecular weight metabolites, many of which have been previously identified[14]. Most of these signals were found in the aliphatic portion of the spectrum with the aromatic region showing resonances only from tyrosine, phenylalanine, and phenylthiourea (an added preservative). For this reason, in the figures, only the aliphatic portions of the spectra are shown.

The spectra of 1 day-old insects for each of the larval, wandering, and pupal stages are shown in Fig. 1. As these spectra show, haemolymph composition did not remain constant through development and a wide range of metabolic changes can be observed, particularly at the onset of wandering and the pupal stages. While the concentrations of amino acid such as valine, and other branched chain species, remained essentially constant, that of alanine increased by approximately 300% from the third to the fifth stages before falling by approximately 50% in the wandering and pupal stages. Other compounds such as glutamate and lysine showed a similar behaviour, being present in high concentrations on day 1 of the third, fourth, and fifth stages, but markedly decreased by around 500% on the first day of the wandering and pupal stages. The organic acids acetate, lactate, and succinate also varied in concentrations depending upon the stage of development. Thus, citrate was present, but not prominent, in haemolymph from third, fourth, and fifth stage insects, but was found in increased quantities in the later stages. In contrast, both succinate and lactate were found in large amounts in larval haemolymph until the onset of the wandering stage of the fifth instar, at which point the proportions of both decreased rapidly by approximately 600%. In the pupal stage, succinate concentrations recovered but those of lactate remained low. Glucose showed a general decrease over the progression of the larval stages whilst trehalose markedly increased by more than 5 times to reach a maximum in the fifth instar, and then decreased somewhat.

The spectra also contained signals from an unidentified carbohydrate species, which was observed in the fifth instar. It was maximal at the wandering stage, and then decreased again in the pupa. For this substance, a doublet signal was observed at 5.11 ppm with a J-coupling typical of an axial-axial C-H bond conformation, suggesting it to be from a H1 proton of a β-hexose. From a 2-dimensional TOCSY spectrum (not shown) this peak is spin-coupled to another at 3.60 ppm, again typical of H2 of a hexose moiety. From a ^{1}H-^{13}C heteronuclear HSQC experiment, the signal at

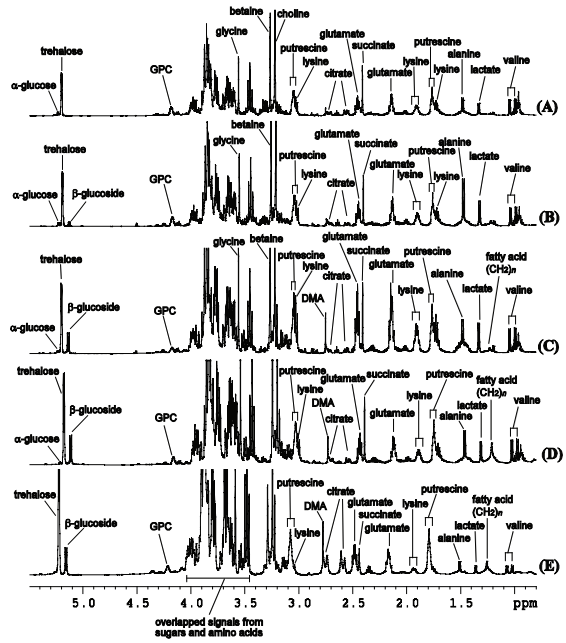

Fig. 2 600 MHz ^{1}H-NMR spectra of the haemolymph of *Manduca sexta* larvae, showing the spectral region δ 0.80–5.50. The haemolymph samples were collected from 0 (A), 1 (B), 2 (C), 3 (D), and 4 (E) day-old larvae from the feeding period of the fifth stage. DMA - dimethylamine.

5.11 ppm has an attached carbon with a chemical shift of ca. 103 ppm (data not shown), all consistent with a β-glucoside residue.

Putrescine was found in significant quantities in the haemolymph from the fifth instar onwards. Betaine, which gave rise to two sharp singlets in the NMR spectrum, was prominent in spectra of haemolymph from the third to the fifth stages but was greatly reduced in the haemolymph of wandering and pupal stages. A sharp singlet, typical of resonances for $^{+}NMe_3$ groups of choline derivatives, was also observed in haemolymph at all stages and, given the characteristic signal seen at 4.15 ppm, was assigned as phosphorylcholine.

Finally, in samples from wandering and pupal stage insects, but not earlier, a number of broader peaks were detected, typical of long chain fatty acid moieties. Given the lack of signals typical of glycerides, these are unlikely to be from triglycerides or phospholipids, and therefore we conclude that these signals are probably due to free fatty acids.

Spectra from fifth stage *M. sexta* during the feeding period

Fifth stage larvae of *M. sexta* feed steadily during the first four days after ecdysis, increasing

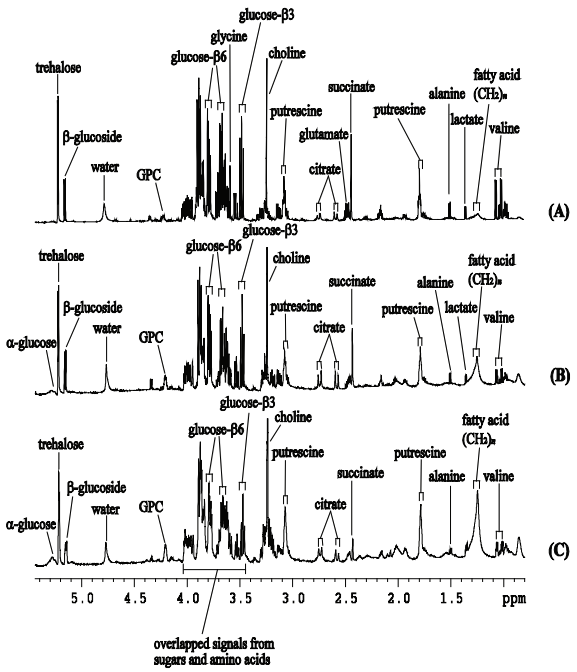

Fig. 3 600 MHz ^{1}H-NMR spectra of the haemolymph of *Manduca sexta* larvae, showing the spectral region δ 0.80–5.50. The haemolymph samples were collected from 0 (A), 2 (B), and 4 (C) day-old wandering stage larvae.

markedly in size from about 1.5g to about 10g. A number of systematic changes were observed to occur in the composition of haemolymph during this time (Fig. 2). These changes include a decrease in glucose, and increases in trehalose (approximately three-fold from the first day to the fourth day of the fifth stage) and the previously mentioned unknown glucose-containing substance. The phosphorylcholine, betaine, and putrescine signals remained essentially constant. Amino acid concentrations were approximately constant over the first three days of the feeding period of the fifth stage but decreased somewhat on day 4. The concentrations of lactate and succinate were variable, and that of citrate decreased sharply on the first day of the fifth stage and then remained at a low level. In these samples, glycine could also be observed, with an increase in the later stages of the instar. Finally, a singlet assigned to dimethylamine was observed, but only on days 2–4 of the feeding period of the fifth stage.

Spectra from fifth stage *M. sexta* during the wandering period

On the fifth day after ecdysis to the fifth stage, *M. sexta* larvae leave their food, empty their gut, and begin to "wander", seeking out a pupation site and preparing to initiate metamorphosis. The first day of this "wandering stage" is designated here as day W0. Actual locomotion occurs mainly in the first day. Subsequently the larvae lose the capacity to move, and prepare for ecdysis to the pupal stage by secreting a new cuticle underneath the old one. Since these larvae are now in effect starving, changes in the metabolic profile would be expected.

Fig. 3 shows ^{1}H-NMR spectra of haemolymph from wandering stage larvae, taken on days W0, W2, and W4. The concentrations of some haemolymph components, e.g. phosphorylcholine, citrate, putrescine, and fatty acid levels, increased with time during the wandering period. Levels of other components, such as trehalose, the unknown glucose-containing compounds, succinate, alanine, and branched chain amino acids were observed to decrease as development progressed.

Spectra from haemolymph of pupal stage of *M. sexta*

The pupal stage of *M. sexta*, under diapause averting conditions, lasts about 21 days. However, it is difficult to obtain samples of haemolymph that are

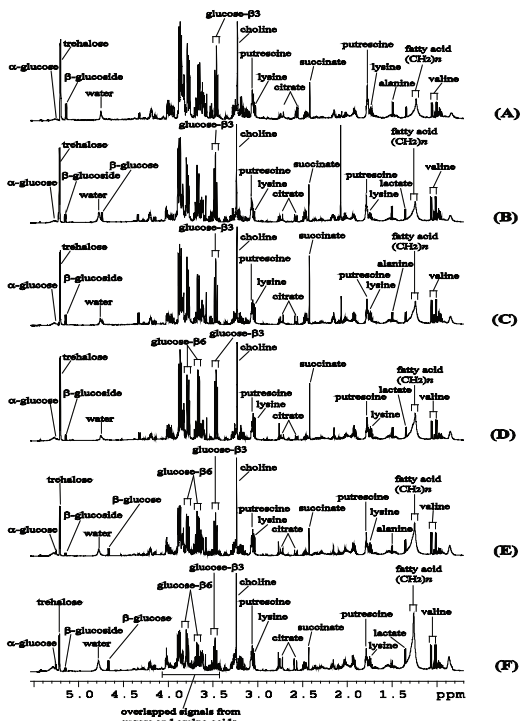

Fig. 4 600 MHz ^{1}H-NMR spectra of the haemolymph of *Manduca sexta* larvae, showing the spectral region δ 0.70–5.50. The haemolymph samples were collected from 0 (A), 1 (B), 2 (C), 3 (D), 4 (E), and 10 (F) day-old pupae.

free of fat body contamination in the later part of the stage. Accordingly, we focussed our attention on the early part of pupal-adult development. Fig. 4 shows spectra taken on days 0, 1, 2, 3, 4, and 10 of the pupal stage. Levels of trehalose and the unknown glucose-containing compounds decreased by approximately 100% compared to the late wandering stage, but glucose concentration had increased somewhat. Phosphorylcholine, putrescine, lactate, and the branched chain amino acids were essentially unchanged, whilst the concentration of alanine decreased. Succinate and citrate showed some variability but generally decreased over time. The fatty acid content of the haemolymph had markedly increased by day 10 of pupal development. A major singlet at 2.06 ppm was seen for the first time on day 1 of this instar, was present at a lower level on day 2, but had essentially disappeared in the later stages. This peak is most probably from an N-acetyl group of a small molecule metabolite, such as N-acetylaspartate, N-acetylglutamate, or an N-acetylated carbohydrate, but is as yet unidentified.

DISCUSSION

^{1}H-NMR spectroscopy of insect whole haemolymph

^{1}H-NMR spectroscopy has been extensively used for the study of a wide range of vertebrate biofluids and tissue extracts as part of metabonomic studies, for example to study physiological variation[20], but has been much less widely employed for the study of insect biochemistry and development. However, the limited number of studies that have been performed in the investigation of both holo- and hemimetabolous insects clearly indicate the potential of the method to provide "global" metabolite profiles. Thus, in the present study we have detected and profiled a wide range of endogenous metabolites, many of which are also commonly observed in mammalian biofluids[21]. Whilst haemolymph spectra are complex, this does not preclude analysis, especially as many of these resonances have been assigned previously, particularly with the aid of 2-dimensional NMR spectroscopic methods[14].

An important difference between the results of this study and the previous study of Phalaraksh et al[14] concerns the relative abundance of glucose and trehalose in *M. sexta* haemolymph. Although the levels of these two metabolites vary considerably during development, we have found in the present work that signals from glucose are present at all stages only at very low levels, whereas there is always a much stronger signal from trehalose. This is exactly as expected from a large body of previous work[22].

In our previous paper, the relative abundance of trehalose and glucose was reversed[14]. We speculated that this unexpected finding might have been due to enzymatic degradation of trehalose during sample handling and/or the acquisition of spectra, and indeed Moroiwaki et al[15] found that trehalose was converted to glucose during NMR analysis of aphid haemolymph unless trehalase inhibitor was added. Degradation of trehalose would have been possible during the extended analysis times employed. In the present case, with much shorter NMR runs, we find that glucose levels are low, as expected.

According to a previous study[23], *M. sexta* haemolymph should contain quite large amounts of trehalose 6-phosphate. So far we have not identified a ^{1}H-NMR signal corresponding to this metabolite. Another molecule predicted to be present in *M. sexta* haemolymph, but has never been identified, is α-glycerophosphate. All ^{1}H-NMR peaks from this substance would appear in the region between 3.6–3.9 ppm (http://www.bmrb.wisc.edu/metabolomics) and would be obscured by the complex bands from other carbohydrates such as trehalose and α-CH protons of amino acids. This metabolite should be present in significant amounts[24].

Developmental changes in the metabonomic profile of haemolymph

Obvious differences were observed in the small molecule metabolite composition of haemolymph, both within and between the different stages of larval and the pupal development, not only in the ratios of the metabolite concentrations, but also in the presence or absence of certain metabolites at different stages. An examination of the day 1 spectra for each stage of development shows that each has a characteristic metabolite profile, whilst those obtained during the fifth stage clearly reveal that this profile is dynamic and changes significantly as these stages of development progress.

These results are in general consistent with previously published data from direct chemical or biochemical analyses. For example, it is well known that there is a switch from storage of sugars in haemolymph (and glycogen in fat body) as larval development proceeds, and that lipid stores increase during the pupal stage[25–27]. It has also been demonstrated that haemolymph titres of trehalose, glucose, and to some extent lipids decrease during the last larval moult, followed by an increase of lipid concentrations after pupation[4,5].

The significance of the observed developmental

changes in metabolite levels is as yet uncertain but a few points may be made. Elevated levels of alanine may reflect enhanced gluconeogenesis. Pyruvate cycling of this type was detected in *M. sexta*[12] using ^{13}C-NMR. Gluconeogenesis would be expected to occur where some amino acids were available in excess. It seems quite likely that diet-fed insects do indeed have an excess of amino acids in their diet, since artificial diets are usually designed to allow growth at the maximum possible rate, which means avoiding N-limitation.

An alternative hypothesis is that the presence of alanine in the haemolymph may reflect the use of proline as a circulating energy source. In some insects (e.g. flying beetles[28]), proline is produced from fat catabolism in the fat body, and then exported to the haemolymph. It is then taken up by muscles and converted to alpha-ketoglutarate by transamination, producing alanine which is then recycled to the fat body. This occurs as a way of fuelling flight, and has not as far as we are aware been reported to occur in lepidopteran larvae. But if this cycle did occur in *M. sexta* larvae, then we might expect high levels of alanine in haemolymph to accompany the use of proline as an energy source. This would presumably only occur when the insects were active. If this were the case we would expect alanine levels to be negatively correlated with proline levels, but there is no evidence for this in the spectra. We discuss the point here mainly to point out the power of the NMR technique to generate testable hypotheses.

The presence of lactate in haemolymph may be artifactual. The insects were immobilized prior to sampling by immersion under water (the anaesthesia is due to a combination of anoxia and CO_2 accumulation), and it is possible that under these conditions anaerobic respiration may occur, producing lactate. Insects are often said not to respire anaerobically, but the process has been observed to occur under experimentally anoxic conditions[29].

Betaine (trimethylglycine) is a prominent component of larval haemolymph, but is absent from non-feeding stages of the insect. Betaine is a metabolite of choline and its presence in larval haemolymph may reflect the inclusion of choline at high concentration in the artificial diet on which the larvae feed. The signal for choline itself, however, remains prominent in wandering and pupal haemolymph, after feeding has ceased.

The presence in haemolymph of significant concentrations of the polyamine putrescine is interesting. Polyamines have been shown to regulate neural development in crickets by modulating the actions of juvenile hormone[30,31]. It is not clear from our data, however, that the level of putrescine in haemolymph is correlated with changing levels of juvenile hormone.

The potential of ^{1}H-NMR in insect physiology

The picture revealed here by the use of high field ^{1}H-NMR to produce metabolite profiles of haemolymph through the various stages of larval and pupal development graphically shows, if further illustration were needed, that haemolymph is a highly complex and dynamic biofluid whose composition changes systematically during development. The advantage of using a "global" metabolite profiling technique such as ^{1}H-NMR, which does not specifcally target any specific class of biomolecules, is that there is no requirement to preselect analytes, allowing the detection and further investigation of unexpected compounds, together with a facile means of observing the way in which lipids, Krebs cycle intermediates, polyamine, and amino acids vary in relation to each other in a single analysis. An example of such an unexpected finding is the unknown glucose-containing molecule visible at most stages of development which is an obvious target for isolation and identification.

This study represents the first detailed investigation of insect development using this method. We believe that this "metabonomics" approach (which is not limited to biofluids, but which is equally amenable to tissue extracts, etc.) provides an ideal framework for the chemical study of insect development. The combination of this type of metabonomics profiling with proteomic and transcriptomic data to provide a "systems biology" approach to this area is an obvious next stage, which may provide insight into previously unexplored fundamental problems.

^{1}H-NMR spectroscopy has been used in the present study to provide information about haemolymph composition and how this changes with time in relation to development. We note, however, that spatial information on particular metabolites may also prove to be of interest in studying the developmental chemistry of insects. For example, it would be extremely interesting to localize the tissue source of the putrescine that we detected in the *M. sexta* haemolymph samples. ^{1}H-NMR spectroscopy is also capable of providing images of developing insects that contain chemical information. The first attempt was made some years ago by Goodman et al[32] who used ^{1}H-NMR imaging to localize lipid storage sites in lepidopteran pupae. Since then, the resolving power of ^{1}H-NMR spectroscopic imaging has increased greatly in terms of both chemistry and

spatial resolution. For example, a recent study of tumour pH had a resolving power of ca. 32 microns[33]. Hence such techniques might provide an attractive experimental approach.

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