

NIR SPECTROSCOPY BASED METABOLIC PROFILING OF EARTHWORMS FROM WESTERN GHATS, ONE OF THE BIODIVERSITY HOTSPOTS OF THE WORLD

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ABSTRACT

The NIR spectroscopic analysis of the coelomic fluid of nine different species of earthworms (*Lampito mauritii*, *Branchiura sowerbyi*, *Dashiella* sp., *Megascolex cochinchensis*, *Megascolex travancorensis*, *Perionyx excavatus*, *Progizzardus varadiamensis*, *Megascolex sylvicola* and *Sparganophilus* sp.) belonging to five different families have been used. This technique provides a family level discrimination of earthworms. The potential use of this information as base line data for future taxonomical, physiological or toxicological studies is emphasized. Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) and Partial Least Square Discriminant Analysis (PLSDA) of the near infra-red spectral data of the coelomic fluid show that the families Megascolecidae and Octochaetidae may have different ancestry. Both PCA and PLSDA provide 100 % calibration of the five different families. The dendrogram of HCA shows that the four families (Almidae, Sparganophilidae, Megascolecidae and Octochaetidae) of Oligochaetes are well separated with no overlapping trends between them. The morphometric classification shows perfect corroboration with metabolomic profiling using coelomic fluid.

Key Words: Near Infra Red Spectroscopy, Coelomic Fluid, Earthworms, Chemometrics, Morphometric Classification

INTRODUCTION

1 Evolution of Near Infra Red Spectra

Historically, the discovery of NIR energy is ascribed to Herschel in 1800. The large credit has to be given to researchers in the field of agricultural science, foremost Norris, who have recognized the potential of this technique already in the early fifties. It was noted that the photographic plate, invented in 1829 by Niepce and Daguerre, had some NIR sensitivity. This enabled Abney and Festing to record the spectra of organic liquids in 1880s. This work was of great significance; not only did it represent the first serious NIR measurements but also the first interpretations, because Abney and Festing recognized both atomic grouping and the importance of the hydrogen bond in the NIR spectrum. Stimulated by their work, Coblentz (1905) constructed a spectrometer using a rock salt prism and a sensitive thermopile connected to a mirror galvanometer. This instrument was highly susceptible to both vibration and thermal disturbances. Around 1905 he produced a series of papers and ultimately recorded the spectra of several compounds. Coblentz discovered that no two compounds had the same spectrum, even when they had the same complement of elements. That is each compound had a unique finger print. The first quantitative measurement was the determination of atmospheric moisture at the Mount Wilson observatory by Fowle (1912). Work on the diffuse scattering of light in both transmission and reflection, by Kubelka and Munk in 1931, opened the door to NIR measurements on solids. Hotelling (1933) wrote a classical paper on principal components analysis (PCA) and formulated a mathematical approach for representing. In 1968a, Ben-Gera and Norris published their initial work on applying Multiple Linear Regression (MLR) to the problem of calibration relating to agricultural products. It is interesting to note that up to 1970; only about 50 papers had been written on work concerning NIR. NIR technology has evolved rapidly since 1970 and has now gained wide acceptance. In many sectors, it is now the measurement of choice. The modern NIR

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technology relies heavily on computer not only for its ability to control and acquire the data from the instrument, but to facilitate calibration and multivariate data analysis (Hindle, 2008). The personnel computer became the driving force behind NIR instrumentation.

2 Advantages of NIR over other Vibrational Spectroscopy

As already mentioned that the vibrational frequencies are very sensitive to the structure of investigated compound, and so there is the widespread application of infra-red spectroscopy for structure elucidation. NIR spectrum is not only a finger print of the chemical composition but also a signature of the physical state of order of the material under investigation. Although the three techniques are very different in several aspects, their basic physical origin is the same: signals in the Near Infra Red (NIR), Mid Infra Red (MIR) and Raman spectra of chemical compounds can be observed as a consequence of molecular vibrations. However, while Raman spectroscopy is a scattering technique, mid infrared and NIR spectroscopy are based on the absorption of radiation.

The NIR region contains almost exclusively absorption bands that can be assigned to overtone and combination vibrations. Unfortunately, the overlap of these overtone and combination bands strongly decreases the specificity of NIR spectroscopy. However, the availability of chemometric evaluation procedures for qualitative discrimination and quantitative determination and also the perception that the low band intensities can be advantageously exploited in terms of larger sample thickness and therefore much easier sample handling has eventually led to the breakthrough of the NIR technique (Martens, 1989; Massart *et al.*, 1997; Kramer, 1998; Naes *et al.*, 2001). Whereas scanning near infra red (NIR) and mid infra red spectrometers operate with a polychromatic source for individual frequency range from which the sample absorbs specific frequencies corresponds to its molecular vibrational transitions (mostly overtone and combinational vibrations for near infra red and fundamental vibrations for mid infra red), in Raman spectroscopy the sample is irradiated with monochromatic laser light whose frequency may vary from visible to the NIR region.

NIR spectroscopy covers the wave number range adjacent to the mid infra red and extends to up to the visible region (4000-12,500 cm^{-1}). NIR absorptions are based on the overtones and combinational vibrations of the investigated molecule, and owing to their lower transition probabilities, the intensities usually decrease by a factor of 10 to 100 for each step from the fundamental to the next overtone (Groh, 1988; Siesler, 1991; Siesler *et al.*, 2002). As far as the quantitative evaluation of the vibrational spectra is concerned, mid infra red and near infra red spectroscopy follow Beer's law, whereas in Raman spectroscopy, intensity is directly proportional to the concentration of the compound to be determined. It is also noted that, the NIR spectroscopy is the method of choice in view of the comparatively large sample volume or thickness.

The expenditure of running a near-infrared analysis, in comparison with morphologic studies, would be outweighed by (1) higher through-put of data, (2) ease of identification, and (3) the time and cost of maintaining animals would be avoided. Finally, near-infrared spectrometry instrumentation currently available on the market is rugged and is able to withstand field use (Cole *et al.*, 2003). The absorption is affected by the internal and external biochemical composition of the organism, and different organisms can have unique absorption spectra. Generally, the constituents must be present at the parts-per-thousand level or greater to be detected by NIR spectroscopy (Mayagaya *et al.*, 2009). It should be emphasized, however, that NIR spectroscopy is not only a routine tool but has also a tremendous research potential, which can provide unique information not accessible by any other technique.

3 Application of NIR in Biological Sciences

Peuchant *et al.*, (1987) reported use of NIR reflectance spectroscopy and multiple linear regressions for measuring cholesterol in the human serum samples. From 1992 to 1995, Hall and Pollard studied the determination of proteins, triglycerides and glucose in human sera using NIR spectroscopy with chemometrics such as least squares regression and Partial least squares (PLS) regression (Du *et al.*, 2008). Several research groups reported their trials for the in vivo NIR determination of blood glucose in human blood. Important application of NIR analysis are found in the determination of the constituents in the

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alcoholic beverages like beer, wines etc. and other nutritional drinks like fruit juices, coffees, teas etc. (Kingtonn and Jones, 2008); wool textile manufacturing industries (Hammersley and Townsend, 2008); textile industry (Ghosh and Rodgers, 2008); petrochemical industry (Buchanan, 2008); pharmaceutical industry (Ciurczak, 2008). It is also noted that the development of NIR for routine quality control testing in agricultural products by Karl Norris and co-workers in 1968 (BenGera and Norris, 1968 a and b) and Shenk and Hoover (1976) paved the way for successful NIR method development based on computer applications (Kradjel, 2008). The most publicized and pursued use of NIR in the life science is for in situ glucose measurements (Ciurczak, 2008) and also studying the cardio vascular diseases (Urbas and Lodder, 2008).

Coelomic fluid of earthworms contains the coelomocytes and many molecular components involved in innate immunity apart from numerous metabolites. The consistency of the coelomic fluid differs between different species of earthworms, and also depends upon the humidity of the air in which the worms' live (Aja *et al.*, 2010). Roch *et al.*, (1986) studied the amino acid composition of the coelomic fluid of the earthworm, *Eisenia fetida andrei* and compared the relationships with other animal groups. Several functions have been attributed to coelomic fluid. These include hemolysis, haemagglutination (Mohrig *et al.*, 1996) and antibacterial property (Roch *et al.*, 1986). Coelomic fluid protease patterns can be seen as promising biomarker candidates in environmental monitoring studies and can be considered as nearly species-specific in earthworms (Kauschke *et al.*, 1997).

It is now known that Infra-red Spectroscopy of biological materials provides chemical and structural information for identifying and characterizing biological molecules. Channa *et al.*, (2007) analysed human gallstones using FT-IR and Paradkar *et al.*, (2002), made a comparison of FT-IR, FT-Raman, and NIR Spectroscopy in a maple syrup adulteration study. A wide range of minute organic molecules can be characterized and quantified using NIR analysis of biofluids, particularly urine and blood plasma. It was found to be successful in detecting metabolic changes induced by xenobiotics in mammalian systems (Ciurczak and Drennen, 2002).

Williams and Norris (2001) reported that the near-infrared spectroscopy was used for protein and moisture analysis in hard and soft wheat flour and Lewis *et al.*, (2005) utilized it to locate and identify contaminants in single or multiple samples.

Petibois *et al.*, (2001) pointed out a limitation of FTIR when it is used for the study of complex biological samples like plasma. When FT-IR is used in such cases, the absorption spectra of some biomolecules obstruct the other absorption patterns. Other disadvantages are associated with solvent interference and sample cell assembly. Over other vibration techniques, the advantages of NIR measurements depend upon high precision output and the provision of flexibility built into it. It requires only minimal sample preparation without further sample separation.

The principle of this technique is based on the spectra-structure correlations existing between a measured spectral responses caused by harmonic vibrations occurring at infra-red frequencies. The harmonic vibrations depends upon the several factors like sample thickness, type of absorbing molecules present within the sample and the quantity of absorber. Quantitative methods are possible where changes in the response of the near infrared spectrometer are proportional to changes in the concentration of chemical components, or in the physical characteristics of samples undergoing analysis. Taking the multifarious potentialities of NIR, it can be considered as successful technique for the species discrimination from bacteria to chicken (Baker *et al.*, 1999; Dowell *et al.*, 1999; McElhinney *et al.*, 1999 Sikulu *et al.*, 2000; Rodriguez-Saona *et al.*, 2004). Similarly, Bergholt (2011) studied the importance of spectral variations by analysing gastric malignancies based on PCA and linear discriminant analysis by using both NIR excited auto- fluorescence and Raman spectra.

The present attempt is to study whether there is significant difference in the chemical composition of the coelomic fluid of earthworms collected from different families by exploring the near infrared spectral data and whether this can be used as a biomarker in future studies.

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MATERIALS AND METHODS

I Collection of Earthworms and Extraction of Coelomic Fluid

The coelomic fluid was recovered from earthworms procured from four major regions of Kerala state in India belonging to 9 different species and five different families (Table 1). The study areas fall within the Western Ghats region, one of the ecological hotspots of the world and known for the rich biodiversity and high endemicy (Praveen and Nameer, 1999). The Taxonomic description of nine species of earthworms is represented in Appendix. Earthworm coelomic fluid was retrieved from the coelomic cavity by electric shock treatment (5v) to obviate any damage to the organism. The coelomic fluid was then centrifuged at 10000 rpm for 10 minutes to remove all the coelomocytes and other particular materials. The supernatant was then collected for spectrometric analysis. The samples are designated as S1, S2, S3, S4, S5, S6, S7, S8 and S9.

Table 1: Species of earthworms collected from various localities in Kerala

Family	assigned code	species	habitat	locality	soil type	pH of CF
Megascolicidae	S1	<i>L. mauritii</i>	Banana plantation	Thiruvananthapuram, Vanchiyoor	upland laterite	7.47
Tubificidae	S2	<i>B.sowerbyi</i>	Near canal	Kottayam, Pala	clay loam	7
Octochaetidae	S3	<i>Dashiella</i> sp.	Paddy field	Palakkad, Chittoor	clay loam	7.2
Megascolicidae	S4	<i>M. cochinensis</i>	Paddy field	Palakkad, Chittoor	clay loam	7.2
Megascolicidae	S5	<i>M. travancorensis</i>	Muddy area	Thiruvananthapuram, Nalanchira	low land laterite	7.62
Megascolicidae	S6	<i>Perionyx excavatus</i>	Coconut trees	Thiruvananthpuram, Karumam	sandy clay loam	7.51
Almidae	S7	<i>P. varadiamensis</i>	Bamboo trees	Trissur, Varadium	mid land laterite	7.56
Megascolicidae	S8	<i>M. sylvicola</i>	Near lake area	Thiruvananthpuram, Karumam	sandy clay loam	7.54
Sparagnophilidae	S9	<i>Sparagnophilus</i> sp.	Near lake area	Thiruvananthpuram, Karumam	sandy clay loam	7.53

Foot note: CF: Coelomic Fluid

Appendix

***Lampito mauritii* Kinberg, 1867**

Collectors' name: Jaya M, Aja M and Vijayakumaran Nair K

General habitat: banana plantation, vegetation sites, coconut plantation

Soil texture: gravelly sandy clay, clay, sand, gravelly clay, loamy sand, gravelly loam, caly loam, gravelly clay loam, sandy clay loam, sandy clay, clay loam, sandy loam

Physiography: mid land, low land, high land, mountaneus region

[Diagnostic Features from: descriptions of Stephenson, 1923; Gates, 1972; Blakemore, 2006 and also present observation]

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External Features

Size: 80-210 mm × 2.5 - 5 mm

Number of segments: 166 – 190.

Pigmentation: dark yellow with purplish tinge at anterior end.

Prostomium: prolobous or epilobous ½, Segments 5 and 6 biannulate, the rest of those in front of the clitellum triannulate

First dorsal pore: in 10/11

Setae: perichaetine, numbers 38/vi, 44/x, 34/xxi, and 33 in the middle of the body.

Clitellum: 14– 17 (=4), annular with transparent clitellum

Male pores: in 18 with large round papillae

Female pores: double, but very near each other, anteriorly on 14.

Spermathecal pores: three pairs, in 6/7, 7/8 and 8/9

Genital Marking: none

Internal Anatomy

Septa: 7/8-12/13 thickened.

Gizzard: in 6

Calciferous glands: absent; oesophagus in 10-13 with internal lamellae

Last heart: 13

Ovaries: fan-shaped with several egg-strings in 13; ovaries small, acinous in 14.

Nephridia: meroic

Testes and funnels: free in 10 and 11.

Seminal vesicles: in 9 and 12, irregularly cut up into small lobes.

Spermathecae: present, two diverticula

Prostates: tubuloracemose in 18-19 with muscular ducts

Penial setae: 1½ - 2 mm. long, with a single curve, tip hoursehoe-shaped with semicircular concavity, flattened; numerous rings of large slender spines standing off somewhat from the shaft.

Remarks: Gates (1972) comments on the regenerative capacity of this species and Ismail (1997) discusses the ability of this species to recycle organic matter and to ameliorate poor soils, including highly alkaline sodic soils.

***Dashiella* sp.**

Collectors' name: Jaya M and Aja M

Date of collection: 18-9-2009 to 4- 11- 2010

General habitat: banana plantation, paddy field, coconut plantation, cultivated area, residential area

Soil texture: sandy clay loam

Physiography: mid up land

[Diagnostic Features from: present observation]

External Features

Size: 13 mm × 0.2 mm, pre clitellar and post clitellar; 0.1 clitellar region

Prostomium: prolobous, setae: perichaetine; 17 setae per segment

Shape: towards the posterior end tapering

First dorsal pore: 9/10, lightly seen; 12/13 distinct

Clitellum: 13-16

Number of segments: 308; annulation beginning from segment 12 onwards

Female Pore: in 14 as transverse slit

Male Pore: a pair in 17 as transverse slit

Genital Marking: in 15 and 16

Tubercula pubertatis: in 18

Internal Anatomy

Septa: 5/6, 6/7 and 7/8 thick

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Gizzard: double, in 5 and 6

Nephridia: meroic

Last heart: in 14, Calciferous gland: extramural; 3 pair, in 11-13

Seminal vesicle: in 11 and 12

Ovary: in 11 and 12

Ovisac: in 12

Spermathecae: 2 pair, 7/8 and 8/9 with monodiverticula

Testis: in 9 and 10

Prostate: confined to 18

Remarks: The species possesses distinct features, which are different from the *Dashiella khandalaensis*. These include two pair of monodiverticulate spermathecae; the genital marking in 15 and 16 and the nature of the prostate.

Megascolex cochiniensis Stephenson, 1915

Collectors' name: Jaya M, Aja M and Vijayakumaran Nair K

Date of collection: 1 -3-2006 to 15-1-2008

General habitat: coconut plantation, residential area

Soil texture: clay loam and gravelly clay

Physiography: mid land and low land

[Diagnostic Features from: descriptions of Stephenson, 1923 and also present observation]

External Features

Size: 90-220 mm× 2-4 mm, (2mm preclitellar, 3mm clitellar and 4mm post clitellar)

Number of segments: 224- 288, up to 14- no annulations, 15-29- triannular, 30-32- tetraannular, 33-50- again triannular, 50 onwards- no annulations, posterior most segments- bi annulated.

Pigmentation: grey, no special pigmentation.

Prostomium: closed epilobous

First dorsal pore: 5/6.

Setae: Perichaetine, *aa* = *2ab* in front of and *3ab* behind clitellum, *zz* = *2yz*; numbers 12/3, 41/5, 54/9, 57/12, 48/19, 36-38 in middle of body.

Clitellum: 14 – 2/3 17(= 3 2/3), however indistinct in the present specimen.

Male pores: as oblique wavy slits on segment 18.

Female pore: 13, median as transverse slit.

Spermathecal pores: in 7/8 and 8/9, in line with *a*.

Genital marking: in line with male pore, comma like oblique slits

Internal Anatomy

Septa: 5/6/7/8- highly thick, 9/10- 11/12 moderately thickened

Gizzard: large and barrel-shaped, in 5.

Calciferous gland: absent, oesophagus swollen and vascular in 12-15

Intestine: begins in 19.

Last heart: in 13.

Nephridia: In front of clitellum nephridia only as tufts by the side of oesophagus; behind clitellum, they form a band (but not a single line) in the anterior half of each segment.

Ovary: 13, one pair

Testis and funnels: free in 10 and 11.

Seminal vesicles: grape - like, in 11 and 12.

Spermathecae: 7/8 and 8/9, 2 pairs; monodiverticulate, spermathecal ampulla ovoid; duct as long as ampulla and less than half as wide; diverticulum arising from ectal end of duct, club-shaped, reaching about to middle of ampulla

Sperm groove: 11/12

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Prostates: limited to 18, each a mass of small rounded lobules; duct passing straight inwards, wider at its termination; accessory prostate near the main glands one on each side situated in front of the main gland; each with a short stalk and same texture as the prostate

Penial setae: absent

Remarks: The presence of accessory gland along with prostate is not mentioned before.

Megascolex travancorensis Michaelsen, 1910

Collectors' name: Jaya M, Aja M, Vijayakumaran Nair K and Surendran

Date of collection: Date of collection: 9-9-2007 to 4- 11- 2010

General habitat: coconut plantation, cultivated area

Soil texture: gravelly sandy clay, sandy clay loam, clay loam, gravelly clay and sand

Physiography: low land, mid upland, mid land, moutaneous region

[Diagnostic Features from: descriptions of Stephenson, 1923 and also present observation]

External Features

Size: 80- 140 mm× Preclitellar region: 2 mm; Clitellar region: 3mm; Post clitellar region: (1-2mm).

Number of segments: 120-280, segment 2-5 biannular; 5-13 triannular

Pigmentation: dark grey before preservation, unpigmented.

Prostomium: epi tanylobous, Clitellum: in 13-16 (=6)

First dorsal pore: 4/5 (3/4 in one specimen).

Setae: Perichaetine, 16 setae per segment in the middle of the body

Male pores: in 19 on slightly raised cushions, which are egg-shaped, their inner borders approximated and parallel, both cushions together almost fill up a somewhat depressed median area, which is bounded laterally and in front by a slight wall.

Female pores: paired.

Spermathecal pores: two pairs, between *a* and *b*, in 7/8 and 8/9, about ½ of the circumference apart.

Genital marking: pair of raised markings in segments 17, 18 and 19 in which it is highly ornamented with folding and wrinkles forming the shape of an eye at the base (=19).

Internal Anatomy

Septa: 6/7 – 12/13 highly thickened, 13/14 and 14/15 moderately thickened

Gizzard: large in 6

Calciferous glands: absent

Testis and funnels: free in 10 and 11

Seminal vesicle: compactly racemose in 11 and 12.

Spermathecae: spermathecal ampulla large, pear-shaped and usually much bent at its ectal end; duct still thinner, very short, mostly concealed in the body-wall; diverticulum enters the ectal end of ampulla; is narrowly club-shaped and somewhat bent at its ectal end; a mass at the ectal end of ampulla seems to represent an incompletely formed spermatophore.

Ovary: 2 pair in 13 and 14

Prostates: racemose in the 17 and part of the following segments with three to five incisions to form lobes

Penial setae: absent

Remarks: The form of the spermathecae relates this form to *M. konkanensis*. The form of TP is also a notable feature to the type *Megascolex travancorensis*. In the present collection, fingerlike projection appears in the segments 15/16/17/18 (in two specimens). The actual function of these structures is not known- may be genital marking or an artifact! It may be mentioned that Aiyer (1929) described a new subspecies *M. travancorensis* var. *proboscidea* of the type *M. travancorensis* from Tenmalai.

Perionyx excavatus Perrier, 1872

Collectors' name: Aja. M., Jaya M and K. Vijayakumaran Nair

Date of collection: 9 -7-2007 to 15-2-2009

Habitat: Coconut plantation, Banana Plantation

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Soil texture: Sand, gravelly clay, gravelly loam, gravelly sandy clay, sandy clay loam, gravelly sandy loam, sand

Physiography: low land, upland, mid land

[Diagnostic Features from: descriptions of Stephenson, 1923; Gates, 1972; Julka, 1981; Blakemore, 2006 and also present observation]

External Features

Size: 23-180 mm×2-5 mm

Shape: body dorsoventrally flattened

Number of segments: 75-165.

Pigmentation: deep purple to reddish-brown dorsally, pale ventrally.

Prostomium: open epilobous, Clitellum: annular, 12-17 (=5).

First dorsal pore: in 4/5 or 5/6.

Seta: perichaetine: small black and numerous per segment from 2: 44 on 12, 40-54 on 20; No setae between confluent male pores but tips of black penial setae present around male pores

Male pores: in 18, closely-paired in deep wedge-shaped clefts in a common depressed but tumid field.

Female pores: in 14

Spermathecal pores: two pairs, in 7/8 and 8/9

Internal Anatomy

Septa: 7/8 and 8/9 have some thickening others weak, 9/10-11/12 appear to incorporate seminal vesicles.

Gizzard: vestigial, in 6

Last heart: in 12.

Calciferous glands: in 10-12; the oesophagus is swollen and vascularised, while in 13 it is especially swollen to almost form lateral pouches, the internal surfaces have ridges

Nephridia: holoic

Testes and funnels: free in 10 and 11.

Seminal vesicles: in 9-12.

Ovaries: in 13 as large pair of palmate to saccular glands with numerous egg strings.

Spermathecae: with large ovoid ampulla; duct short and narrow; in 8 and 9 paired with large tapering or bilobed ampullae each with various closely-attached flat or lobed iridescent diverticula; duct short and stout.

Prostates: large almost spherical racemose glands with central U-bent duct joined entally by vas deferens, or conical by folding around thick duct, confined to 18.

Penial setae: may be in a group of 4-6 on each side, medial from the male pores; 0.6 mm. long, with indistinctly quadrangular smooth tip and many rings of long thin teeth.

Remarks: Several authors stated the variability in the genus is noted, especially in terms of size (Michaelsen, 1909). Stephenson (1923) mentioned that the spermathecal diverticula and the male field area may be quite indistinct. Beddard (1886) has found very large variations in the numbers and position of the genital apertures.

***Progizzardus varadiamensis* Nair 2010**

Collectors' name: Jaya. M, Aja M, Vijayakumaran Nair K

Date of Collection: 2-7-07 to 13- 8 2009

General Habitat: banana plantation, Bamboo trees

Soil texture: gravelly clay, gravelly clay loam

Physiography: low land, mid land

2010. *Progizzardus varadiamensis* Nair, Megadrilogica, 14, (3), pp.53-58.

[Diagnostic Features from: descriptions of Nair *et al.*, 2010]

External Features

Size: 55-100 mm ×2-4 mm

Shape: quadrangular in cross section.

Number of segments: 200-320

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Pigmentation: Pre-clitellar region is pink.

Prostomium: prolobous, Clitellum: in 4-10, annular

Dorsal pore: absent.

Setae: lumbricine, 8 setae per segment, $aa:ab:bc:cd:dd=0.39:1:0.51:1:1.6$ ($dd>aa$); in between the ventral setae is a light shady area longitudinally, with an external longitudinal folding present in segment 11 onwards

Genital markings: 21/22/23/24/25/26, seen as 5 patches

Genital setae: 3 pairs in 7, 8 and 9.

Internal Anatomy

Septa: 6/7, 7/8, 8/9, 9/10 and 10/11 highly thick.

Gizzard: in 4th segment.

Last heart: in 11, calciferous gland: in 6-11

Female pore: in 9/10.

Ovary: with ovisac in segment 10 (rarely 13).

Spermathecae: multiple, 4 pairs in (holotype) and 5 pairs in (paratypes) in segments 5/6/7/8/9, which is always in association with a constricted blood vessel and adiverticulate.

Seminal vesicles: 4 pairs in 9,11,12,13

Male pore: in 13.

Nephridia: holoic, one pair in each segment, 12th segment onwards. **Calciferous glands:** usually in segments 6-11 (in holotype) or sometimes more extensive (in paratypes), extending up to the segment 33

Sperm grooves: present anteriorly

Prostate: absent.

Remarks: The species usually lives in wet or marshy areas where the pH ranges from 6-7, with 8% moisture. The worms are seen at an average depth of 10 to 30 cm. These are usually seen to coexist with black ants. The casts are granular and the cocoon has both ends tapering. The cocoon is greyish in colour and the mean incubation time under laboratory condition was 17 days. The mean length and diameter of the cocoons are 2.65 ± 0.36 and 2.10 ± 0.26 , respectively.

Megascolex sylvicola Michaelsen, 1907

Collectors' name: Jaya M, Aja M and Vijayakumaran Nair K

Date of collection: 5 -7-2007

General habitat: near lake area, banana plantation

Soil texture and Physiography: gravelly sandy loam and mid land

[Diagnostic Features from: descriptions of Stephenson, 1923 and also present observation]

External Features

Size: 97- 185 mm \times 2½ - 3½ mm

Shape: towards the posterior end tapering.

Number of segments: 200.

Pigmentation: light grey

Prostomium: epi-tanylobous, Clitellum: post clitellated adult, clitellum not distinct

First dorsal pore: in 6/7

Setae: small, rather enlarged in the anterior half of the anteclitellar region; rings irregularly but broadly interrupted dorsally, especially at the anterior end; regularly broken ventrally, $aa = ca. 2ab$; setae a and b regularly placed throughout the body; numbers 10/3, 12/4, 11/5, 15/13, 21/17, 27/24, and 30 at the hinder end.

Male pores: in lines a and b , on minute papillae, the papillae surrounded by a common whitish wall of dumbbell shape.

Spermathecal pores: in line of a , in 7/8 and 8/9.

Genital Marking: 17, as paired transverse slits

Genital pappillae: a pair in each segment as pappillae, 18, 26, 47 and 52

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Internal Anatomy

Septa: 6/7-13/14 thickened, 7/8-9/10 highly thickened.

Gizzard: large, in 5

Calciferous gland: absent, oesophagus simple and swollen in 13

Last heart: in 13

Nephridia: Behind clitellum in each segment a pair of meganephridia as well as a number of micronephridia; in front of this only micronephridia.

Phagocytic organ: 59 onwards

Testis and funnels: free in 11.

Seminal vesicles: racemose, in 12.

Spermathecae: 2 pair in 7/8 and 8/9, spermathecal ampulla pear-shaped, passing without break into the duct with two diverticula, club-shaped or nearly cylindrical each with a single seminal chamber

Prostates: reniform prostate in 17; A pair of accessory gland in 19 seen as small glandular swollen tubes.

Penial setae: absent

Remarks: The shape of the prostate is compared to that of *Woodwardiella uzeli*. As it is a post clitellated adult, the clitellum is not well marked. Michaelsen (1907b) also did not record the position of the clitellum. The presence of accessory gland in the 19th segment is not reported in the original description.

***Sparganophilus* sp.**

Collectors' name: Jaya M and Aja M

Date of collection: 6 -2-2009

General habitat: banana plantation

Soil texture: gravelly sandy loam

Physiography: mid land

Type species: *Sparganophilus tamesis*

[Diagnostic Features from: present observation]

External Features

Size: 8.8 mm× (0.2 mm- pre clitellar; 0.2 mm- clitellar; 0.3 mm post clitellar and 0.2 mm towards the posterior)

Setae: perichaetine, 12 setae per segment

Shape: anterior end is blunt and the posterior end is tapering

Number of segments: 280, tetra annulations (four annulations) from segment 2 onwards

First dorsal pore: 2/3

Spermathecal pore: absent

Clitellum: absent, some segments are bulging in 43, 44 and 45

Tubercula Pubertatis: absent

Internal Anatomy

Prostate: a pair, rudimentary on 28 the segment

Gizzard: absent

Nephridia: from 2 onwards, holoic

Phagocytic organ: 180th segment onwards, towards the posterior side

Last heart: in 9

Remarks: In one specimen the spermathecae are not found. This may be an abnormality or parthenogenetic morph of the type.

2 Near Infrared Spectra Acquisition

The scanning NIR spectrometers operate with a polychromatic source for the individual frequency range from which the sample absorbs specific frequencies corresponding to its molecular vibrational transitions that is overtone or combinational vibrations. Owing to their lower transition probabilities the intensities usually decrease by a factor of 10 – 100 for each step from fundamental to the next overtone (Wenlandt

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and Hecht, 1966). NIR spectra of 9 samples over 700 nm – 1400 nm spectral regions at 1 nm interval were recorded.

3 Chemometrics and Data Analysis

Qualitative and quantitative near infrared (NIR) spectroscopic methods typically require the application of multivariate calibration algorithms and statistical methods (i.e. chemometrics) to model NIR spectral responses to chemical or physical properties of the samples used for calibration (Aaltonen, 2007).

Three pattern recognition techniques (both supervised and unsupervised) namely Principal component analysis, Hierarchical cluster analysis and Partial least square discriminant analysis were performed using algorithms from PLS Toolbox 5.8.2 supported by Matlab environment and the results and outcomes were compared. A Proper preprocessing technique namely smoothing (Savitzky - Golay filters; window-15 point, order-2) coupled with auto-scale was used to remove background noise and to increase spectral resolution. (In this study, auto- scaling was done such that variables (columns) with large standard deviation are down-weighted relative to variables with small standard deviation. This changes the relative sum-of-squares for the preprocessed data and the first principal component will now capture the largest sum-of-squares relative to the mean of the weighted matrix after preprocessing).

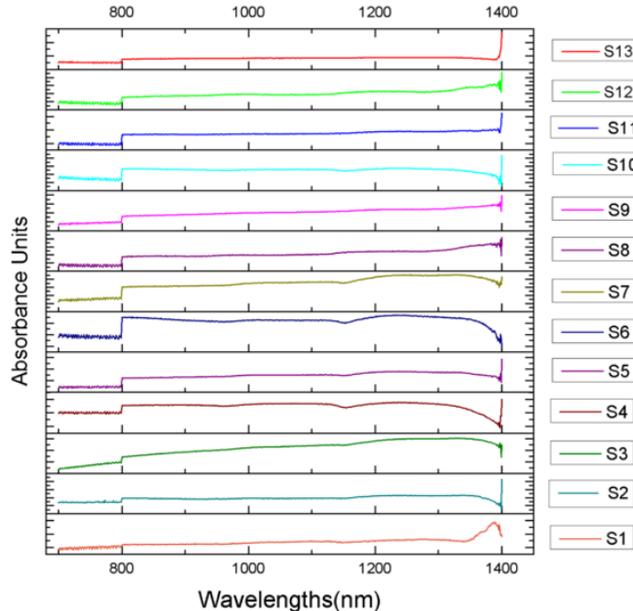
RESULTS AND DISCUSSION

Results

The present approach is to provide a metabolic fingerprint based on the biochemical profile of the oligochaetes.

1 NIR Spectra Collection

Near infrared spectra of 13 samples were recorded for 700 nm – 1400 nm spectral region, at 1 nm spacing with a NIR spectrophotometer Cary 5000 (SI No. EL 03127331) with a resolution of 0.01 nm. The spectra were collected in 1 nm data intervals.



Foot note: 13 sample spectra are shown which contains a training set of 9 samples and a test set comprising 4 independent samples.

Figures 1- 4: *Lampito mauritii* - S1, *Branchiura sowerbyi* - S2, *Dashiella* sp. - S3, *Megascolex cochinchensis* – S4, *Megascolex travancorensis* – S5, *Perionyx excavatus* - S6, *Progizzardus varadiamensis* - S7, *Megascolex sylvicola* - S8 and *Sparganophilus* sp. - S9.

Figure 1: NIR spectra of coelomic fluid of earthworm samples

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An average spectrum of four scans for each sample was obtained. All the spectra were recorded in absorbance units (Figure 1). The sample spectra were divided into training set comprising of 9 samples (n=9) and test set comprising of 4 independent samples (n =4), one from each group.

2 Principal Component Analysis (PCA)

Prior to PCA application, the data were preprocessed using Smoothing (order: 2, window: 15 point) combined with auto-scale. The first two Principal components (PCs) were found to carry the maximum variation among the data matrix (Table 2). The optimum number of components (2) was determined by the root mean square error of calibration (RMSEC: 0.00400684). Leave-one-out cross-validation was used for calibrating the model. In order to show the differences among the earthworm NIR spectra, the scores plot using the first and the second PCs were used (Figure 2). Four clusters based on PC1 were also identified. Each cluster represents one type. The farther the distance between the clusters, the more different the spectra are. The results show the ability of NIR technique to discriminate earthworms at their family level in the present study. This model can be used as a calibration model for identifying test samples by applying the model on other new spectral data, which belong to test samples (Obeidat *et al.*, 2008).

Table 2: Percent Variance Captured by PCA Model

Principal Component Number	Eigen value of Covariance (X)	% Variance Captured of This PC	% Variance Captured Total
1	6.71e+002	95.92	95.92
2	2.08e+001	2.97	98.89
3	5.34e+000	0.76	99.66
4	1.28e+000	0.18	99.84
5	7.41e-001	0.11	99.94
6	3.13e-001	0.04	99.99
7	6.36e-002	0.01	100.00

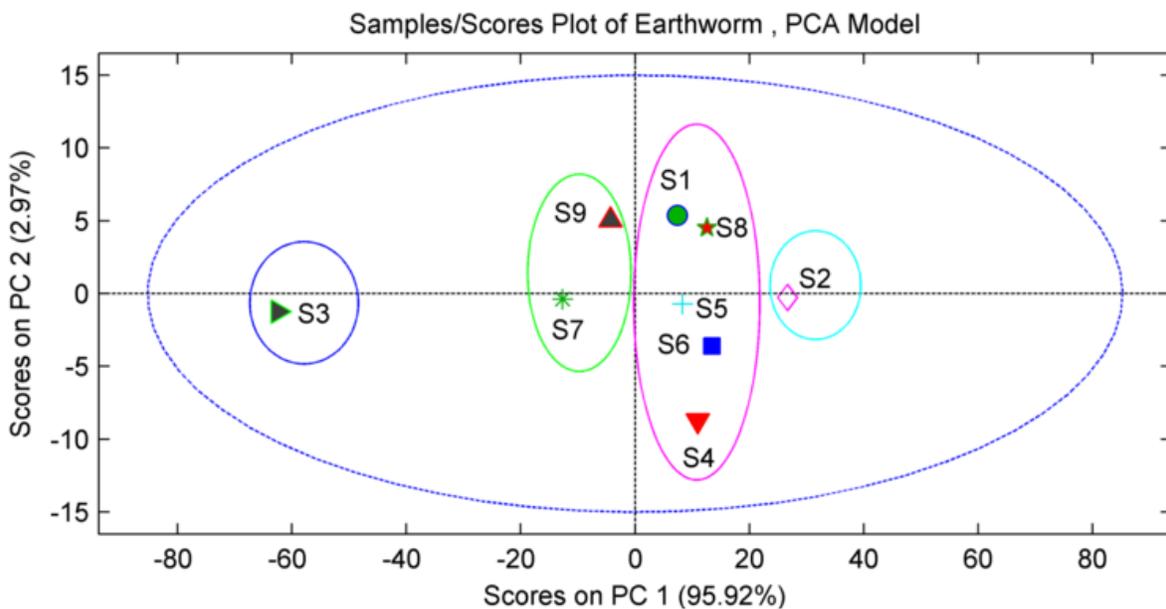


Figure 2: Score plot of PCA model of earthworms

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3 Hierarchical Cluster Analysis (HCA)

HCA is an unsupervised technique that uses the information obtained from measured variables to reveal the natural clusters existing between the studied samples (Peterson, 2002; Skrobot *et al.*, 2005). The dendrogram obtained from HCA, where four clusters or classes can be identified are recorded (Figure 3).

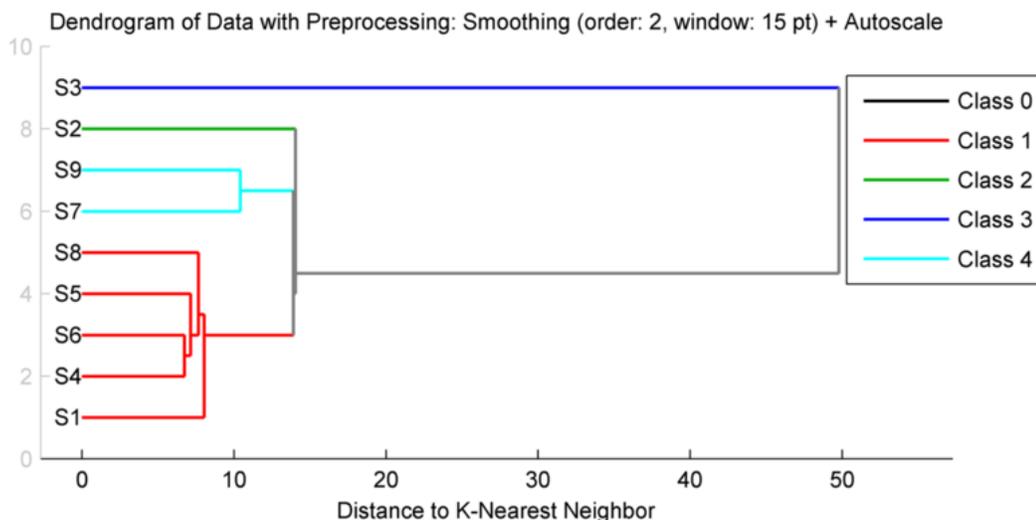


Figure 3: HCA dendrogram of earthworm samples using K- Nearest Neighbor

4 Partial Least Square Discriminant Analysis (PLSDA)

The earthworm coelomic fluid samples were investigated by another chemometric tool, PLSDA, which is a supervised pattern recognition technique. The results were compared with those from PCA. The two Latent Variables (LVs) were found to carry the maximum variation among the dataset. Like PCA, in PLSDA model, four separate clusters (each cluster representing one type) were identified clearly using the first and the second latent variables (Figure 4). For further inspection, the prediction of each worm sample is explored within the two latent variables -- PLSDA model. The samples of each worm type cluster independently of other samples within the estimated threshold calculated from Baye’s Theorem. Table 3 gives the statistics for each class of earthworms for calibration of PLSDA model for pre-processed NIR data.

Table 3: Statistics for each y-block column PLSDA Model

Statistical	class 1	class 2	class 3	class 4
Parameters				
Modeled Class:	9.507712e+001	9.775008e+001	9.873996e+001	1.015316e+002
Sensitivity (Cal):	1.000	1.000	1.000	1.000
Specificity (Cal):	1.000	1.000	1.000	1.000
Class. Err (Cal):	0	0	0	0
RMSEC:	0.0211209	0.128526	0.117245	0.0255265
Bias:	-1.19349e-015	2.19269e-015	7.21645e-016	-1.23512e-015
R ² Cal:	0.997419	0.904426	0.944327	0.993403

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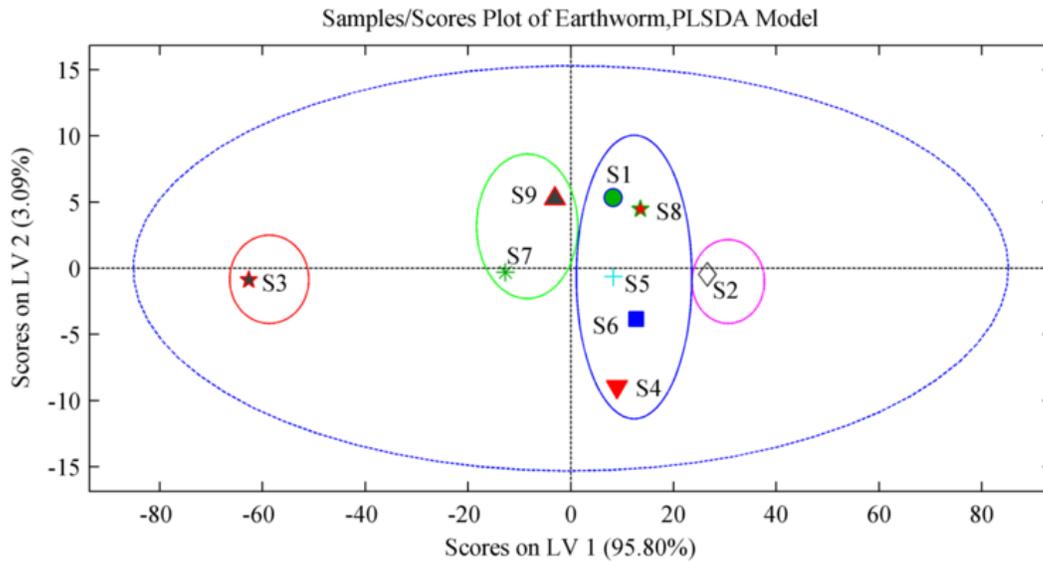


Figure 4: Score plot of earthworms, PLSDA model

Discussion

The nine different species taken for the study include *Lampito mauritii* (S1), *Branchiura sowerbyi* (S2), *Dashiella* sp. (S3), *Megascolex cochinensis* (S4), *Megascolex travancorensis* (S5), *Perionyx excavatus* (S6), *Progizzardus varadiamensis* (S7), *Megascolex sylvicola* (S8) and *Sparganophilus* sp. (S9). They are belonging to the families Megascolecidae (S1, S4, S5, S6 and S8), Tubificidae (S2), Octochaetidae (S3), Almidae (S7) and Sparganophilidae (S9). From the spectral analysis it was found that the three species of worms belonging to two classes, Class 2 and 4 coming from the same lineage show more or less similar characteristics. Supporting this view and using conventional taxonomic analysis it follows that absence of prostate is a systemic character of these three species. From the NIR spectral analysis we could identify 4 different classes. Class 1 represents five earthworms belonging to the single family Megascolecidae having a pair of prostates with branched system of canals and single gizzard and with perichaetine or lumbricine setal arrangement. Class 3 contains only one sample belonging to the family Octochaetidae having a pair of tubular prostates and meroic nephridia. Octochaetidae as currently defined is debatably polyphyletic requiring restriction. The lineage of Octochaetidae can be traced back to Acanthodrilidae while Megascolecidae may have arisen from the Acanthodrilidae or developed independently (Blakemore, 2006). In consonance with this, the present study shows that Megascolecidae and Octochaetidae might have arisen independently. Eventhough the two samples from class 4 represent two different families (S7 representing Almidae and S9 representing Sparganophilidae); they may have a common ancestral origin. Both families have freshwater and muddy forms with holoic nephridia and no prostates. This is a point to be taken for further investigation. It may be recalled that Jamieson (1988) reviewed the overall phylogeny and higher classification of the oligochaeta based on the cladistic analysis. He placed both Almidae and Sparganophilidae in the single ancestral group Aquamegadriili. The dendrogram shows that these four families (Almidae, Sparganophilidae, Megascolecidae and Octochaetidae) of Oligochaetes are well separated with no overlapping trends between them. The one major advantage of using this technique that is worth mentioning is that the classification at the family level can be done without killing the animals. In addition to this, the morphometric classification is aligning with spectra obtained from the coelomic fluid recovered from different species.

Bundy *et al.*, (2001) made an attempt to characterize the coelomic fluid of the earthworm *Eesnia veneta* by one and two dimensional HNMR spectroscopy with the aim of providing fundamental knowledge about the composition of coelomic fluid. However, the modern NIR technology relies heavily on

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computer not only for its ability to control and acquire the data from the instrument, but to facilitate calibration and multivariate data analysis (Burns and Ciurczak, 2008). This chapter introduces the use of near infra-red spectroscopy as a technique for identification and quantitative analysis for the coelomic fluid of nine earthworm species belonging to five different families. Setting apart the question of the ancestral origin of Almidae and Sparganophilidae, the results in this part of study are consistent with the previous reports. Many reports are available for the use of factorial discriminant analysis or partial least squares regression discrimination along with the NIR spectrum generally produced the best accuracy rates and enabled classification of the spectra up to individual species. It could be also used for the rapid and automated identification of many organisms such as chicken, insects and bacteria even up to the species level (McElhinney *et al.*, 1999; Dowell *et al.*, 2000). Dowell *et al.*, (1999) had used absorption characteristics of cuticular lipids for the classification of insects up to species level. We are interested to use the potential of near infrared (NIR) spectroscopy to compare the chemical signature of multiple samples rather than focusing on characterizing the heterogeneity of the coelomic fluid of single species. Accordingly, NIR is ideal for taxonomists who have to investigate quickly large samples obtained from different populations.

REFERENCES

- Aaltonen J, Strachan CJ, Pollanen K, Yliruusi J and Rantanen J (2007).** Hyphenated spectroscopy as a polymorph screening tool. *Journal of Pharmaceutical and Biomedical Analysis* **44**(2) 477-483.
- Aja M, Nair KV, Jaya M and Reynolds JW (2010).** A comparative study on the morphology of coelomocytes in four species of megascolecid earthworms (annelida: oligochaeta). *Megadrilologica* **13**(10) 1-8.
- Baker JE, Dowell FE and Throne JE (1999).** Detection of parasitized rice weevils in wheat kernels with near infrared spectroscopy. *Biological Control* **16** 88-90.
- Ben-Gera I and Norris KH (1968a).** Direct spectrophotometric determination of fat and moisture in meat products. *Journal of Food Science* **33** 64–67.
- Ben-Gera I and Norris KH (1968b).** Determination of moisture content in soybeans by direct spectrophotometry, *Israel Journal of Agricultural Research* **18** 124-132.
- Bergholt MS, Zheng W, Lin K, Ho KY, The M, Yeoh KG, So JBY and Huang Z (2011).** Molecules combining near-infrared-excited auto fluorescence and Raman spectroscopy improves in vivo diagnosis of gastric cancer. *Molecules* **16**(5) 3740- 3760.
- Buchanan B (2008).** Recent advances in the use of Near IR spectroscopy in the petro chemical industry. In: edited by Burns AD and Ciurczak WE, Hand book of Near Infra-red analysis, CRC press, Taylor and Francis group, New York 521- 527.
- Bundy JG, Osborn D, Weeks JM, Lindon JC and Nicholson JK (2001).** An NMR-based metabonomic approach to the investigation of coelomic fluid biochemistry in earthworms under toxic stress. *FEBS Letters* **500** 31- 35.
- Burns AD and Ciurczak WE (2008).** Hand book of Near Infra-red analysis. CRC press, Taylor and Francis group, New York.
- Channa NA, Khand FD, Khand TU, Lehari M and Memon NA (2007).** Analysis of human gall stone by FT-IR. *Pakistan Journal of Medical Science* **23**(4) 546-550.
- Ciurczak EW (2008).** Biomedical applications of near infra red spectroscopy. In: edited by Burns AD and Ciurczak WE, Hand book of Near Infra-red analysis, CRC press, Taylor and Francis group, New York 647-655.
- Ciurczak EW (2008).** “Process analytical technologies (PAT) in the pharmaceutical industry”. In: edited by Burns AD and Ciurczak WE, Hand book of Near Infra-red analysis, CRC press, Taylor and Francis group, New York 581- 584.
- Ciurczak EW and Drennen JK III (2002).** Pharmaceutical and Medical Applications of Near-Infrared Spectroscopy. Practical spectroscopy, Marcel Dekker, New York 192.

Research Article

- Coblentz WW (1905)**. Investigations of infra-red spectra”, part I. Publication No. 35. Octavo, Carnegie Institute of Washington **6** 1-285.
- Cole TJ, Ram MS, Dowell FE, Omwega CO, Overholt WA and Ramaswamy SB (2003)**. Near-infrared spectroscopic method to identify *Cotesia flavipes* and *Cotesia sesamiae* (Hymenoptera: Braconidae)”. *Annals of the Entomological Society of America* **96** 865-869.
- Dowell FE, Throne JE, Wang D and Baker JE (1999)**. Identifying stored grain insects using near-infrared spectroscopy. *Journal of Economic Entomology* **92**(1) 165-169.
- Dowell FE, Broce AB, Xie F, Throne JE and Baker JE (2000)**. Detection of parasitized fly puparia using near infrared spectroscopy. *Journal of Near Infrared Spectroscopy* **8** 259-265.
- Du YP, Kasemsumra S, Jiang JH and Ozaki Y (2008)**. In vivo and in vitro Near infra red spectroscopic determination of blood glucose and other biomedical components with chemometrics. In: edited by Burns AD and Ciurczak, Hand book of Near Infra-red analysis, W. E., CRC press, Taylor and Francis group, New York 673-698.
- Fowle FE (1912)**. The spectroscopic determination of aqueous vapour, *Astrophysical Journal* **35** 149-162.
- Ghosh S and Rodgers J (2008)**. NIR analysis of Textiles. In: edited by Burns AD and Ciurczak, Hand book of Near Infra-red analysis, W. E., CRC press, Taylor and Francis group, New York 485-520.
- Groh W (1988)**. Overtone absorption in macromolecules for polymer optical fibers, *Makromol. Chemistry* **189** 2861- 2874.
- Hall JW and Pollard A (1993)**. A near infra red spectroscopic determination of serum total proteins, albumins, globulins and urea, *Clinical Biochemistry* **26** 483 – 490.
- Hall JW and Pollard A (1995)**. In Leaping ahead with Near infra red soectroscopy, In: edited by Bhaten GD, Flinn PC, Welsh LA and Blakeney AB, Royal Australian Chemical Institute, Melbourne 421.
- Hammersley MJ and Townsend PE (2008)**. NIR analysis of Wool. In: edited by Burns AD and Ciurczak WE, Hand book of Near Infra-red analysis, CRC press, Taylor and Francis group, New York 465-478.
- Hindle PH (2008)**. Historical Development. In: edited by Burns AD and Ciurczak WE, Hand book of Near Infra-red analysis, CRC press, Taylor and Francis group, New York 3-6.
- Hotelling H (1933)**. Analysis of a complex of statistical variables in to principal components. *Journal of Educational Psychology* **24** 417-520.
- Jamieson BGM (1988)**. On the phylogeny and higher classification of the Oligochaeta. *Cladistics* **4**(4) 367-401.
- Kauschke E, Pagliara P, Stabili L and Cooper EL (1997)**. Characterization of proteolytic activity in coelomic fluid of *Lumbricus terrestris* L. *Comparative Biochemical Physiology* **118B**(2) 235- 242.
- Kingtonn LR and Jones TM (2008)**. Application of NIR Ananalysis of Beverages. In: edited by Burns A D and Ciurczak WE, Hand book of Near Infra-red analysis, CRC press, Taylor and Francis group, New York 457- 463.
- Kradjel C (2008)**. NIR in the dietary supplement industry: Qualitative and quantitative analysis of ingredients, process blends, and final products, In: edited by Burns AD and Ciurczak WE, Hand book of Near Infra-red analysis, CRC press, Taylor and Francis group, New York 613-630.
- Kramer R (1998)**. Chemometric Techniques for quantitative analysis, Marcel Dekker, New York 203.
- Kubelka P and Munk E (1931)**. Ein Beitrag zur Optik der Farbanstriche, *Zeits.Tech.Physik.* **12** 593–601.
- Lewis EN, Kidder LH and Lee E (2005)**. NIR chemical imaging—near infrared spectroscopy on steroids. *NIR news* **16**(5) 1-4.
- Martens H and Naes T (1989)**. Multivariate calibration, Wiley, Chichester and New York 419.
- Massart DL, Vandeginste BGM, Buydens LMC, de Jong S, Lewi PJ and Smeyers- Verbeke J (1997)**. In: edited by Vandeginste BGM and Rutan SC, Hand book of chemometrics and qualimetrics, Part 20A. Elsevier, Amsterdam.

Research Article

- Mayagaya VS, Michel K, Benedict MQ, Killeen GF, Wirtz RA, Ferguson HM and Dowell FE (2009).** Non-destructive Determination of Age and Species of *Anopheles gambiae* s.l. using Near-infrared Spectroscopy. *American Journal of Tropical Medicine and Hygiene* **81**(4) 622–630.
- McElhinney J, Downey G and Fearn T (1999).** Chemometric processing of visible and near infrared reflectance spectra for species identification in selected raw homogenised meats. *Journal of Near Infrared Spectroscopy* **7**(3) 145 - 154.
- Mohrig W, Eue I, Kuuschke E and Hennicke F (1996).** Cross reactivity of Hemolytic and Hemagglutinating Proteins in the Coelomic Fluid of Lumbricidae (Annelida). *Comparative Biochemistry and Physiology* **115A**(1) 19-30.
- Naes T, Isaksson T, Fearn T and Davies T (2001).** A user friendly guide to Multivariate calibration and classification, NIR publications, Chichester, UK 420.
- Obeidat S, Bai BG, Rayson D, Anderson DM, Puscheck AD, Landau SY and Glasser T (2008).** A Multi-Source Portable Light Emitting Diode Spectrofluorometer. *Applied Spectroscopy* **62**(3) 327-332.
- Paradkar MM, Sakhamuri S and Irudayaraj J (2002).** Comparison of FTIR, FT-Raman, and NIR Spectroscopy in a Maple Syrup Adulteration Study. *Journal of Food Science* **67**(6) 2009–2015.
- Peterson LE (2002).** Software Report: CLUSFAVOR 5.0: Hierarchical Cluster And Principal Component Analysis of Microarray-Based Transcriptional Profiles. *Genome Biology* **3**(7) 1-8.
- Petibois C, Cazorla G, Cassaigne A and De le'ris G (2001).** Plasma Protein Contents Determined by Fourier-Transform Infrared Spectrometry. *Clinical Chemistry* **47**(4) 730–738.
- Peuchant E, Salles C and Jensen R (1987).** Determination of serum cholesterol by near-infrared reflectance spectrometry. *Analytical Chemistry* **59** 1816- 1819.
- Praveen J and Nameer PO (1999).** Monitoring bird diversity in Western Ghats of Kerala. *Current Science* **96**(10) 1390-1395.
- Roch PH, Valembos P and Vaillier J (1986).** Aminoacid compositions and relationships of five earthworm defense proteins. *Comparative Biochemical Physiology* **85B**(4) 747-751.
- Rodriguez-Saona LE, Khambaty FM, Fry FS, Dubois J and Calve EM (2004).** Detection and Identification of Bacteria in a Juice Matrix with Fourier Transform–Near Infrared Spectroscopy and Multivariate Analysis. *Journal of Food Protection* **67**(11) 2555-2559.
- Shenk JS and Hoover MR (1976).** Infrared reflectance spectro computer design and application, *Proceedings of the 7th Technicon International Congress* **2**, Tarrytown, NY 122.
- Siesler HW (1991).** Near infra red spectroscopy of polymers. *Makromolekulare Chemie, Macromolecular Symposia* **52** 113 -129.
- Siesler HW, Ozaki Y, Kawata S and Heise HM (2002).** Near-infrared spectroscopy, Principles, instruments, applications, Wiley-VCH, Weinheim 348.
- Sikulu M, Killeen GF, Hugo LE, Ryan PA, Dowell KM, Wirtz RA, Moore SJ and Dowell FE (2010).** Near-infrared spectroscopy as a complementary age grading and species identification tool for african malaria vectors, *Parasites and Vectors* **3** 49.
- Skrobot VL, Castro EVR, Pereira RC, Pasa VD and Fortes IP (2005).** Identification of Adulteration of Gasoline Applying Multivariate Data Techniques HCA and KNN in Cromatographic Data. *Energy and Fuel* **19**(6) 2350-2356.
- Urbas AA and Lodder RA (2008).** Near Infrared Spectroscopy in Cardiovascular Disease. In: edited by Burns AD, Ciurczak WE, Hand book of Near Infra-red analysis, CRC press, Taylor and Francis group, New York 657-671.
- Wenlandt WW and Hecht HG (1966).** Reflectance spectroscopy, In: edited by Elving PJ, Kolthoff IM, Interscience, John Wiley and sons, NewYork.
- Williams P and Norris K (2001).** Near-Infrared Technology in the Agricultural and Food Industries. 2nd Edition, American Association of Cereal Chemists, St. Paul, Minnesota, USA.