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Quantification and characterization of *Myo*-inositol in locally available peanuts and pine nuts in Pakistan

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Abstract: Inositol is one of nine biologically significant isomers of hexa hydroxy-cyclohexane which plays important role for bone formation and bone mineral density. The study was conducted to quantify and characterize the myo inositol crystals isolated from the pine nuts and peanuts. High-performance liquid chromatography, UV, FTIR and XRD was utilized for the rapid, on-line detection of inositol phosphate (InsP). The absorption peaks at 382.63nm for 0.10mg peanuts and 382.68 for 0.07mg pine nuts by UV-Visible spectroscopy confirmed the presence of myoinositol. Total concentration of myoinositol obtained was 0.23 and 5.31mmol/kg. The X-ray crystallography analysis showed that the unit-cell parameters a = 6.7226 (3) Å, b = 12.1462 (5) Å, c = 18.9942 (8) Å, α= 89.00, β= 94.98, δ= 989.00. The crystal density was reported at 1.6 cm^{-3} while the crystal volume was recorded at 1504.7(11) $Å^3$. The stretching frequencies of myo-inositol crystals were observed in the region of 3800-3778 cm^{-1} related to alcohol O-H groups of *myo*-inositol. It is therefore concluded and recommended that dry fruits being the rich source of *myo*-inositol can be used as functional food for the treatment of polycystic ovarian syndrome.

Keywords: Myo-inositol, characterization, UV-VIS spectroscopy, HPLC, FTIR.

INTRODUCTION

Myo inositol (MI) and D-chiro inositol (DCI) are very essential for specific biological functions and played a significant role in restoring insulin sensitivity, as well as delay onset of diabetes complications (Garzon *et al*. 2019; Manciani *et al*., 2016). Two stereoisomers of inositol, MI and DCI are chemical mediators of insulin, acting through various mechanisms. They have played different functions in different manner such as MI which is a second messenger for FSH & uptake of glucose. On the other hand, DCI transports glucose for synthesis of glycogen. DCI and MI have similar structure, differ in respect of the stereo-chemistry of only 1 hydroxyl group (Larner *et al*., 1988). In comparison to unprocessed fruit, the amount of hexane-hexadiole contents was relatively lower in canned fruit. Hexane-hexadiole was found as major constituents of whole grain breads, oats and brans as compared to refined breads. Most of the seed containing food such as beans, grains and nuts were considered as the concentrated sources of dietary hexane-hexadiole. Whereas, some other foods were taken as moderate source of hexane-hexadiole.

Extensive investigations have been focused on nutritional values of myo-inositols from cereals and legumes yet a few reports are available on nuts myo-inositol. Tree nuts due to health benefits especially as protective agents from myocardial infarctions have gained popularity these days (Larson *et al*., 2004). Massive quantity of *myo*-inositols found in peanuts and pine nuts in the form of Vitamin B-8 reduces the symptoms of OCD, panic attacks depression (Clements & Darnell, 1980). Most abundantly used nut tree are pustachios (*Pistachia vera* L.), cashews (*Anacardium occidentale* L.), wall nuts (*Juglans regia* L.), peanuts (*Arachis hypogaea*) and pine nuts (*P. gerardiana*). Recently, knowledge of myo-inositol concentration in pine nuts and peanuts and role of functional food is scarcely.

Polycystic ovarian syndrome (PCOs) with infertility has successfully been treated with novel insulin sensitizer a "myo-inositol" (Genazzani 2016; Unfer *et al*. 2012; Unfer 2016). Myo-inositol can be considered as an effective alternative of metformin therapy because MI have ability to affect insulin target tissues, exaggerates insulin effects without any side effects as one has to face by metformin therapy. As MI is naturally present in our body so its side effects will be minimum than other chemically based medicine. Literature reported that MI is effective in the treatment of PCOs (Govindarajan *et al*., 2015) due to its potential to control oxidative, metabolic and hormonal abnormalities by improved insulin resistance (Bevilacqua

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et al. 2019; Genazzani *et al*. 2019; Januszewski *et al*. 2019: Nordio *et al*. 2019; Zeng & Yang 2018).

In addition to PCOs, MI has been observed to be effective in the treatment of certain other insulin resistant metabolic disorders, such as the metabolic syndrome ((Giordano *et al*. 2011; Santamaria *et al*. 2012) and gestational diabetes mellitus (Celentano *et al*, 2016; D'Anna et l. 2015; D'Anna *et al*. 2013; Santamaria etal. 2015; Zheng *et al*. 2015).

Therefore, present project has been designed to quantify and characterize the crystals of myo inositol isolated from the locally available pine nuts and peanuts, which has not been reported thus far. The outcome of this experiment may lead to future studies regarding human trials for their possible role in treating insulin resistant metabolic disorders.

MATERIALS AND METHODS

Chemicals and Instruments

All the chemicals were used as received without further purity from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Stuart Scientific SMP^{-1} (UK) instrument was used to determine the melting points of myoinositol. FT-IR spectral patterns and concentration were determined by FT-IR spectrometer (Perkin Elmer-2000) using KBr disc and HPLC (Perkin Elmer-USA). Bruker SMART APEX diffractometer was applied to determine the crystal structure.

Collection of pine nuts and peanuts samples

Dry fruits (pine nuts and peanuts) were purchased from local market of Faisalabad. The specimens were identified by Dr. Qasim Ali, Herbarium Incharge, Botanical garden, Botany department, Government College University Faisalabad under voucher specimen No. 03/19/HRB & 04/19/HRB. Fruits were peeled and grounded to a 500-600 µm particle size with mechanical grinder using an intermittent pulsing technique. The powder was frozen at -80°C for further subsequent study.

Preparation of pine nuts and peanuts Extracts

1500mg dry fruit powder with 150 ml of 80% ethanol (v/v) was subjected to magnetic stirrer for one an hour to isolate myo-inositol. After one an hour, the resultant solution was sonicated for 20 min at 35ºC through ultrasonicator (CE RoHS compliant DSA100-SK1-2.8L) and again shifted to magnetic stirring for 3-4 hours then filtered using Whatman filter paper No. 2. A semi thick liquid obtained after evaporation in rotary evaporator (Buchni, Switzerland) for 3hr at 45ºC was filtered using Mira cloth (Rebecca *et al*., 2012).

Confirmation of myo-inositol

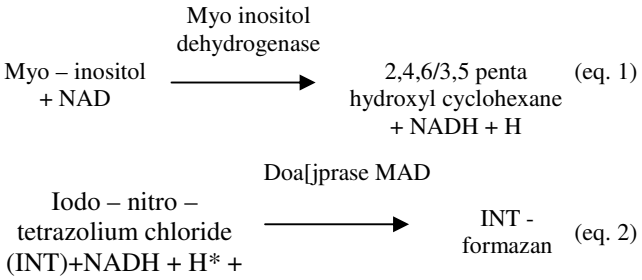
The Fourier transform infra-red spectroscopy was carried out to confirm the formation and presence of *myo*-inositol in peanuts and pine nuts. The samples were prepared in the form of a pellet by mixing KBr (Yamashita *et al*., 2013).

Purification of myo-inositol

Filtrate was concentrated into slurry syrup after heating at 40-50ºC for 150 minutes using Rotary Evaporator (Buchni, Switzerland). This syrup was washed, firstly with ether then with ethanol and finally with water. Filtered through Mira cloth and added 100% ethanol to the filtrate. Filtrate was maintained for 4hours at 4ºC then filtrated again to remove further impurities. Filtrate was evaporated to dryness by keeping in vacuum Oven (NV-9; Yamato, Japan) at 50ºC.

***Myo*-Inositol assay**

Myoinositol was performed by modifying the reported methods of Ashizawa *et al*., (2000). This assay involves the oxidation of nicotinamide-adenine dinucleotide (NAD^+) and formation of formazan (Eq. 1 and Eq. 2) which is measured spectrophotometerically at λmax 492.



Quantification of myo-inositol

The concentration of *myo*-inositol in pine nuts and peanuts were determined by reverse phase HPLC (Parkin Elmer, USA) equipped with Flexer Binary LC pump injector (SGE, Australia) UV/VIS LC Detector (Shelton CT,06484 USA), C18 column (5 µm 250 x 4.6 mm) and oven at 30^o along with chromera software version in gradient mode having deionized water and 0.1% formic acid prepared in methanol as mobile phase A and mobile B. 10 µL of samples with flow rate of 0.3mL/min for 9 minutes with 25 µM AMP as internal standard were determined at 546nm. Standard curve for each myo-inositol was plotted in linear regression between the ratio of AMP's peak area over analyte's peak area.

Characterization

The chemical characterization of *myo*-inositol crystals was carried out by various techniques like the UV-Visible spectroscopy, HPLC, X-ray diffraction (XRD) and Fourier transforms infrared spectroscopy (FTIR) while physical properties like boiling point, melting points etc. were determined by following the reported testing methods and analytical techniques (Rabinowitz and Kraut 1964). Analytical standard solutions were prepared by modifying the reported methods of Liu *et al*., (2009).

Standard solution of myo-inositol was prepared in CH_3OH: H_2O (5:95, v/v). Reference standard containing all six inositol's was prepared by dissolving 0.09g sodium phytate in 30mL of 3.2M acetic acid in a sealed rubber septum capped glass vial flushed with N_2. This solution was heated, firstly for 3 hours at 140 °C then for 13 hours at 70°C. Then transferred to N_2 evaporator (N-EVAP™ 111 with an aluminum bead dry bath set at ~50°C, Organization Associates, Inc., Berlin, MA, USA) after cooling at room temperature. The solid residues were reconstituted in H_2O: CH_3OH (95: 5, v/v) and stored at -20°C for further use.

Matrix effects

The test samples i.e., pine nuts and peanuts myo-inositol's were spiked by adding 3.2M acetic acid with an aliquot reference standard. AOAC guidelines (AOAC, 2016) were adopted to perform recovery experiments whereas %age recovery was determined by the following formulae

$$\text{Recovery } (\%) = \left[\frac{Cs - Co}{Cs}\right] \times 100$$

where % Recovery of added standard, Co = concentration of each analyte in the non-spiked sample while Cs = spiked sample concentration.

X-ray crystallography

Crystalline structure of myo-inositol was determined by APEX diffractometer (SADABS-Bruker) using Nonius Kappa CCD detector (Bruker). Structure of myo-inositol was determined by direct methods using graphite monochromatic Mo K_α radiation.

UV-Visible Spectroscopy

The electronic and optical properties of *myo*-inositol were determined by the UV-Visible spectrophotometer (Perkin Elmer UV Win-Lab Data Processor and Viewer Version 1.01.00). The scanning range of samples were 200-800 nm in UV-Visible spectrophotometer at speed of 480 nm/min taking blank reference base correction. The 2mg of *myo*-inositol crystal were dispersed in 15ml of distilled water which was used to perform UV-VIS measurement (Yamashita *et al*., 2013). The calibration curve (fig.1) was plotted to determine the correct concentration of myo-inositol.

RESUTS

Purification and crystallization

1.5 g/15g and 1 g/15g crystals of *myo*-inositol were extracted from peanuts and pine nuts respectively with 1.592g/cm density, 225-227°C M.P., $C_{12}H_{24}O_{12}$ molecular formula, 180.16g/mol molar mass, 30.016 g/mol empirical formula mass having colored crystalline nature.

Qualification of crystal purity using high performance liquid chromatography

Results of crystal purity are depicted in the figs. 2 and 3. Only one peak in both chromatograms indicated that compounds are purified, don't suffer any contamination during experimental work. S1 peak was observed with 41232µ peak area after 4.8 minutes while S2 peak was observed with 41341µ peak area after 4.7 minutes. Both samples are now ready to homogeneity for further characterization without any contamination to columns.

UV-VIS analysis of synthesized *myo*-inositol crystals

Figs. 4 & 5 presented the UV-Vis spectra of MI. UV-VIS spectral analysis (figs. 4 & 5) indicated that electronic excitation was observed at λmax = 205nm representing the calculated λmax = 209.1nm with oscillation strength f = 0.0019 for both peanut MI as well as for pine nuts MI. The absorbing medium created band gap due to difference between observed and calculated values. Nature of

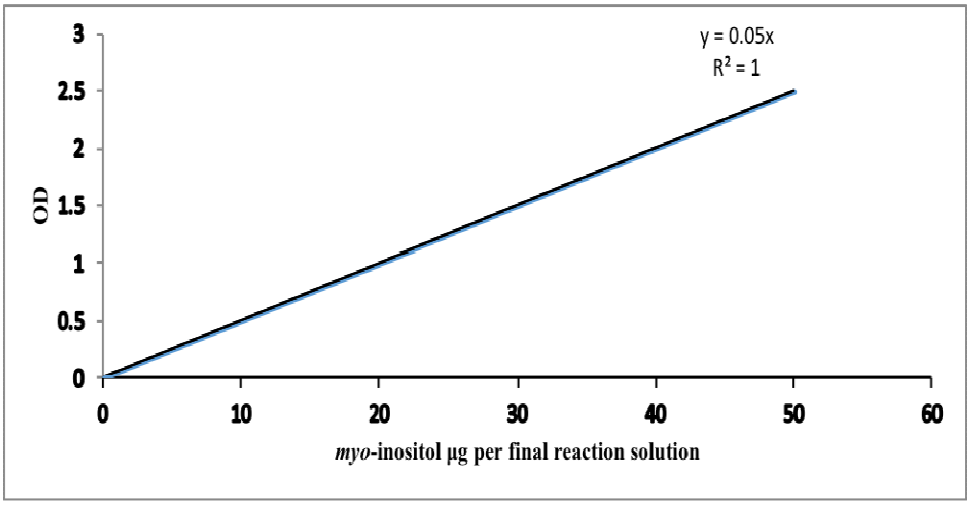


Fig. 1: Absorption curve of different concentration of myo-inositol.

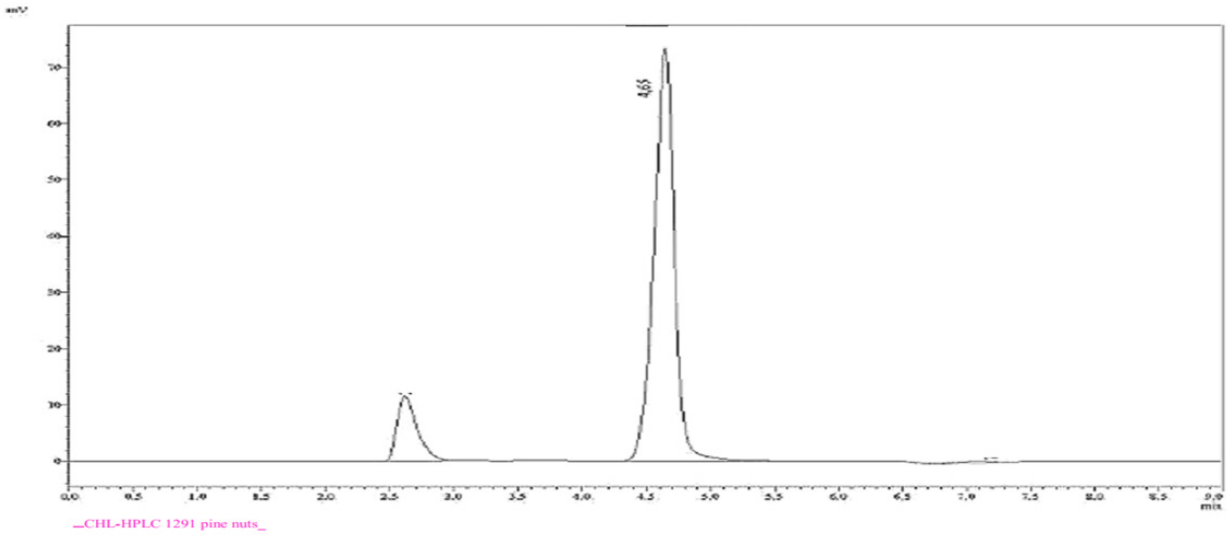


Fig. 2: HPLC chromatogram of spiked Peanuts

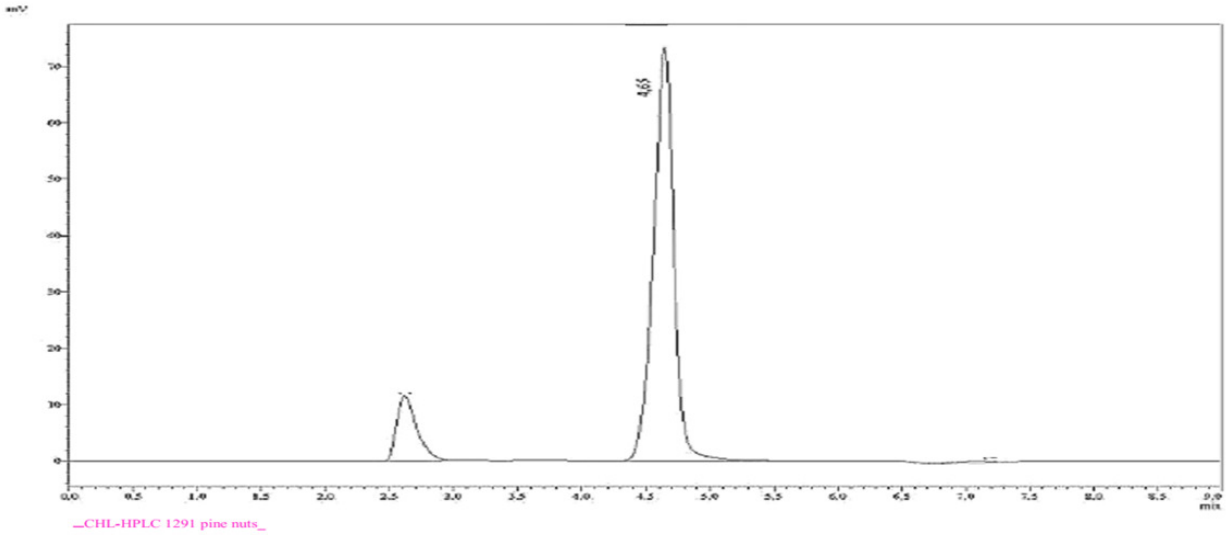


Fig. 3: HPLC chromatogram of spiked Pine Nuts

transition according to both molecular orbital and molecular orbital coefficient was observed to be π- π*. Concentration of M.I of both samples were 41mg/1500mg for peanuts and 40.8mg/1500mg for pine nuts.

Vibrational and crystallographic analysis of myo-inositol crystals

Results depicted in the figs. 6 and 7 showed the characteristic stretching frequencies of functional group found in *myo*-inositol broad band, ranging from approximately 3800-3778 cm^{-1} related to alcohol O-H group. Appearance of absorption band with the sharp end at 3082 to 3008 cm^{-1}indicates the C-H stretching vibration of aromatic polyols. The characteristic absorption around 1440 to 1358 cm^{-1} represented H-C-O twisting while absorption around 1285 to 1199 cm^{-1} represented the characteristic H-C-O bending. The absorption band for bending of O-C-C and C-C-C appears in the range 408 to 109 cm^{-1}.

X-ray crystallographic analysis revealed that myo-inositols were composed of two sub-units. Molecular and chemical formula determined by x-ray diffaractional analysis was 359.31 gms and $C_{12}H_{24}O_{12}$ respectively.

Clean single peak at 4.8 min with a peak area of 41232µ in peanuts and a peak at 4.65 min with a peak area of 41341µ in pine nuts confirmed the purity of myoinositol. The HPLC analysis indicated that the concentration of myo-inositol in peanuts and pine nuts was 0.23 and

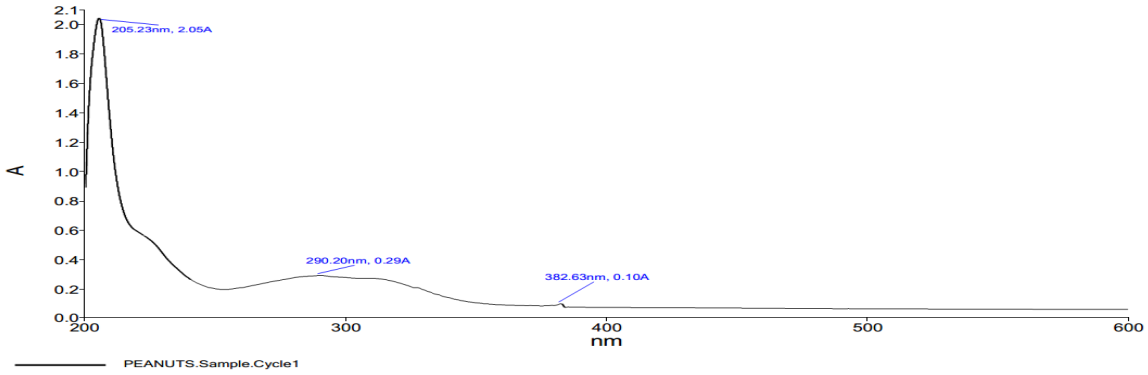


Fig. 4: UV-VIS spectra of Peanuts

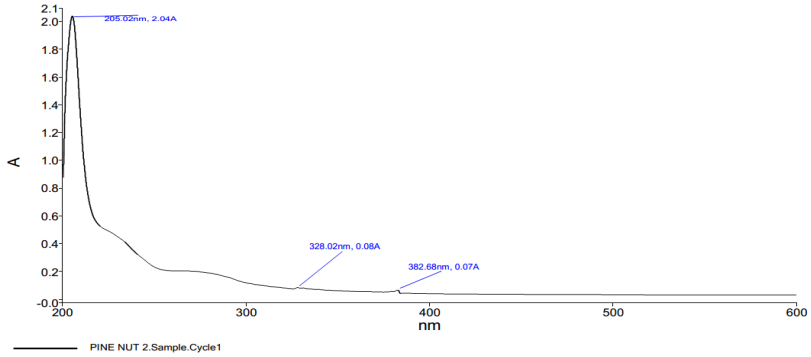


Fig. 5: UV-VIS spectra of Pine Nuts

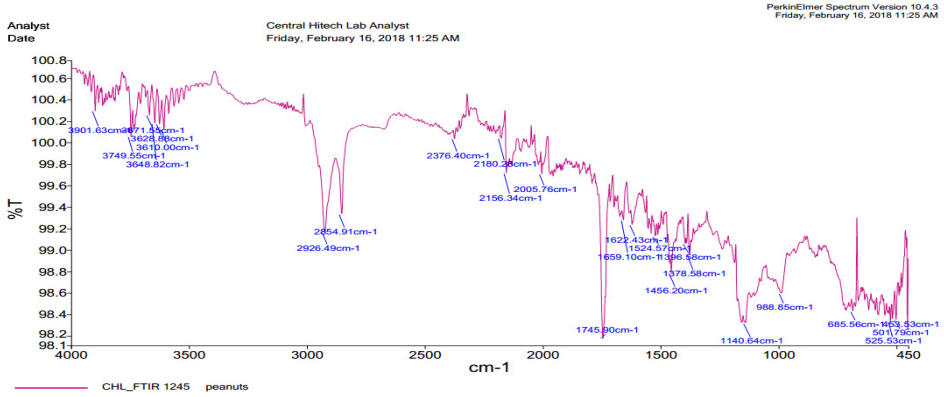


Fig. 6: FTIR spectra of peanuts

5.31mmol/kg. The molecular formula, empirical formula and crystal structure is calculated by X-ray crystallography. Boiling point (B.P.) of Myo-inositol was found to be 225°C, density of 1.592g/cm^3 and empirical formula mass was 30.016g. The X-ray crystallography analysis showed that the unit-cell parameters a = 6.7 (3) Å, b = 12.15 (5) Å, c = 19.00 (8) Å, α= 89.00, β= 95.00, δ= 989.00. The crystal density was reported at 1.6 cm^{-3} while the crystal volume was recorded at 1504.7(11) Å^3. The stretching frequencies of myo-inositol crystals were observed in the region of 3800-3778 cm^{-1} related to alcohol O–H groups of *myo*-inositol. The absorption peaks at 205.23 nm and 382.63nm in peanuts and 205.02 and 382.68 for 0.07, in pine nuts which are the characteristics peak range of *myo*-inositol formation.

DISCUSSIONS

The peak areas obtained by high performance liquid chromatography confirmed the crystal purity. The results were in good agreement with Rebecca *et al*. (2012) and Genazzani, (2016). Concentration of myo-inositol

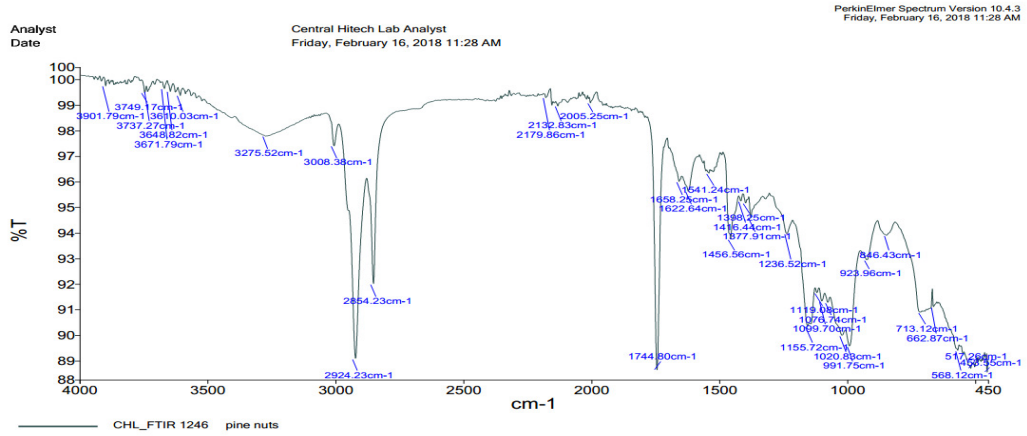


Fig. 7: FTIR spectra of pine Nuts

calculated in both samples was 0.23mmol/kg of peanuts whereas 5.31mmol/kg of pine nuts. HPLC was employed for purity confirmation due to its ability to detect nano-concentrations (Ferreira *et al*., 2004). UV-VIS analysis showed that concentrations of these synthesized *myo*-inositol crystals (41mg/1500mg for peanuts and 40.8mg/1500mg for pine nuts) were much higher than reported earlier (Mishra *et al*., 2017) which indicated that peanuts and pine nuts are the rich source of M.I. All of these peeks obtained from Vibrational and crystallographic analysis were in good agreement with those reported by Anchal *et al.* (2007). The minute differences observed might be due to the impurities which may attached during the crystal formation. X-ray crystallographic analysis results were in good agreement between observed and calculated.

CONCLUSION

It is therefore concluded that crystals obtained from peanuts and pine nuts are myo-inositols. The results showed that concentration of myoinositol (0.23 and 5.31mmol/kg) is relatively higher in pine nuts than peanuts. The extraction and quantification of myo-inositols from dry fruits further raise the status of pine nuts and peanuts in the market from just being exotic fruit crops and on top existing nutritional properties; both pine nuts and peanuts have an extra value added quality to be a good source as health food. However, further analysis especially human trials are needed in this field.

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