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Single Base Extension and Fourier-Transform Infra-Red Spectroscopy Techniques; Further Approaches in Discriminating Hazelnut-Adulterated Olive Oil

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Background: Confirmation of olive oil authenticity and particularly virgin olive oil has a great importance. Several advanced chemical and genetic analyses have been used to monitor especial components; however, each has its limitations especially when detecting hazelnut-adulterated olive oil.

Objectives: The objective of this research was to assess the presence of trace amount of hazelnut oil in olive oil (less than 10%) by Single Base Extension (SBE) and Fourier Transform InfraRed Spectroscopy (FTIR).

Materials and Methods: The study was based on the analysis of chloroplast DNA sequences using SBE to detect Single Nucleotide Polymorphisms (SNPs) in highly preserved DNA regions among olive and hazelnut species to differentiate pure and adulterated olive oil by means of two parallel tools; ABI PRISM sequencing and AcycloPrime Single Nucleotide Polymorphism Detection. Fourier -Transform InfraRed technique was used for FTIR spectrum comparisons of pure olive oil and hazelnut-adulterated one, as well.

Results: Total DNA was extracted successfully from pure and hazelnut-adulterated olive oil, and it provided properly acceptable amplification with the primers designed on chloroplast region of both species and their admixture oil in different ratios; 50: 50, 70: 30, and vice versa. However, for lesser than 10% hazelnut oil in olive oil only SBE analysis provided recognizable results. FTIR spectra of oil samples were assessed at frequency regions of 4000 - 700 cm^{-1}. Eight wave numbers (3007, 1373, 1237, 1120, 1098, 1032, 965, and 722 cm^{-1}) of eleven differentiating ones were selected as candidate wave-numbers to distinguish pure and adulterated olive oil.

Conclusions: SBE technique proved to be an effective strategy to verify olive oil authenticity, especially from hazelnut-adulterated olive oil. However, FTIR technique provided trustable results only when higher than 10% hazelnut oil is present in olive oil.

Keywords: cpDNA; FTIR; Genetic Analysis; Olive Oil Traceability; *rbcL* Sequence; SNPs

1. Background

Olive oil authentication is performed routinely through chemical analysis by monitoring several components such as sterols, phenols, fatty acids, alcohols, and etc. (1-6). However, several difficulties have been encountered in distinguishing pure olive oil from its admixture with other vegetable oils especially with hazelnut. Hazelnut oil provides characteristics strongly similar to olive oil such as similar fatty acids, triglyceride, sterol, and tocopherol components. These components do not differ sufficiently to readily distinguish olive oil from hazelnut oil even before mixing. On the other hand, the determination of stigmastadiene produced by the dehydration of sterols during the bleaching of oils can enable the detection of refined oils in unrefined olive oil (IOC method), but it is not always successfully assessed. Recently DNA-based techniques have been introduced to overcome these barriers and to promise satisfactory performance of the results for precision and sensitivity (7). Furthermore, the deposition of sequences of olive genome on NCBI database and the application of molecular markers in this discipline offered more benefits, although olive oil usually provides very low yields of DNA and has variable degrees of degradation which may limit the applicability of molecular markers (8, 9). The first documented research on olive oil DNA isolation refers to Muzzalupo and Perri (10). However, other progresses were achieved using SCAR (11), AFLP (9), RAPD, ISSR, and SSR (12), SSRs (13, 14), since they are considered the most interested molecular markers in olive oil varietal identification. Rotondi et al. (14) performed a comparison between genetic results, chemical and sensory properties of monovarietal olive oils and demonstrated a very good correspondence between the clustering obtained by SSR analysis and the clustering based on selected fatty acids composition (15).

Implication for health policy/practice/research/medical education:
Single base extension technique offers a potent tool in olive oil authentication; however, FTIR technique could not be a one hundred percent tool.

As plant cells have one nuclear and two cytoplasmic (chloroplast and mitochondrial) genomes, the nuclear genome undergoes recombination during sexual reproduction, the other two do not, and therefore chloroplast and mitochondrial DNAs are more useful for taxonomic studies. Chloroplast DNA (cpDNA) has been extensively used in phylogenetic reconstructions and a number of potentially useful regions are easily amplified using universal primers (16), even more it is used for cultivar identification in olive oil (17). It is supposed that PCR analysis on cpDNA/mtDNA with further molecular analysis such as SNP detection would provide trustable results in olive oil authentication.

The chemometrics techniques play an important role in the study of edible fats and oils, especially for the authentication study (18). Among them, FTIR received more attention as assesses the association between the concentration of analyte and its spectra (19) by fast acquisition of a great numbers of spectral data (20). Furthermore it is considered as a valid tool in the study of edible oils and fats that could serve as a "fingerprint technique". This analytical approach presents good sensitivity and a great simplicity in sample preparation and data elaboration and is considered as a valid tool to authenticate extra virgin olive oil (21). Some attempts in using FTIR to distinguish olive oils from different geographical origin (22, 23) and different genetic varieties (24) have been proposed, as well (25).

2. Objectives

The present research was performed with the aim of offering powerful molecular/chemical tools suitable to prove olive oil authenticity and to prevent its adulteration. SBE and FTIR techniques were compared when olive oil was mixed with different ratios of hazelnut oil.

3. Materials and Methods

3.1. Sample Preparation and DNA Extraction

Cold pressed unfiltered virgin olive oil and hazelnut oil were prepared at Core facilities lab, NIGEB, Tehran, Iran and stored at 4°C until worked out. Commercial kit Qiagen QIAamp DNA stool (cod. 51504) was used for DNA extraction. DNA was extracted from 250 μg oil samples (13). This kit is based on resin tablets that absorb PCR inhibitors and silica-gel columns which allow separation of nucleic acids. To test the quality of DNA extracted, two microsatellite markers of the literature (DCA17 and DCA9) were tested on DNA isolated from olive oil by means of PCR (PTC-200 machine, MJ research, USA) amplification and agarose gel electrophoresis separation (Figure 1) (13). From the young leaves of the same olive cultivar which olive oil was extracted, DNA was extracted by commercial DNA extraction kit (DNeasy Plant Mini Kit (cod. 69104)) to compare the reliability of the results when compared with DNA extracted from oil.

3.2. Analysis of Chloroplast DNA

3.2.1. Design of Universal and Specific Primers

Upon a survey on NCBI GenBank, *rbcL*(ribulose-bisphosphate carboxylase large unit) gene was nominated for primer design. Alignment did on the sequence of *rbcL* which used as a template for the design of flanking primers with the software PRIMER3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/) for two species of hazelnut (*Corylus avellana L.* access number: 33113306) and olive (*Olea europaea L.* access number: 2598012). The main concept to select this region was being well-conserved and highly polymorphic, which led to design two primer pairs of common and specific amplification, *rbcL*1 and *rbcL*2, 143 bp and 118 bp, respectively (Figure 2).

It is suggested to provide more copy number of the target zone before performing Single Base Extension (SBE) analysis. SBE analysis requires the use of a single primer that extends one of the strands. Therefore two one-handed primers were designed on *rbcL* sequence and it was decided to perform the analysis on both strands using separately either forward or reverse primer. In Figure 4, the two designed SBE primers are shown. Forward primer is in dark box and it exactly ends before a G, so the addition of a G fluorescent dye-labeled terminator means the presence of hazelnut in the oil sample. In case of Rev-primer (clear box), the addition of a dye-labelled C, should confirm the presence of hazelnut in the sample. The primer pairs' information is addressed in Table 1.

3.2.2. Single Base Extension Approach

Two simple analyses of the *rbcL* polymorphism were designed, both based on the SBE: one makes use of the ABI PRISM automatic sequencer and the Snapshot kit (APPLERA); the other one makes use of the VICTOR machine (Perkin Elmer, USA) and the modified TDI-FP kit (Template-directed Dye-terminator Incorporation with Fluorescence Polarization detection technique) (26).

3.2.2.1. AcycloPrime Single Nucleotide Polymorphism Detection

The AcycloPrime TM II SNP Detection kit was used to determine the base present at a specific location in an amplified DNA by a modification of TDI-FP. This approach is more robust and accurate even in the presence of few copies of DNA (27).

A preliminary PCR with a primer pair designed to amplify the zone including SBE was performed (*rbcL*3 primer pairs, Figure 3). Then a clean-up pre-SBE step operates to

degrade and remove extra dNTPs, inorganic pyrophosphate (PPi) and the rest of primers from the amplification steps to prevent interference with primer extension, by means of enzymatic purification, pyrophosphatase (PPase). By incubating at 80°C for 15 min the enzyme deactivates. AcycloPrime reaction by SNP primer to extend the primer only with one base during the thermal cycles between 10 to 30 cycles was performed. In the TDI-FP, the combination of R110 and Tamra terminators was used because their spectral wavelengths do not overlap. As the final step, FP values read by using VICTOR machine and the allele-calling software, calculates the results (SNPscorer software cat no: ASP001 from PerkinElmer).

3.2.2.2. ABI PRISM Snapshot

ABI PRISM Snapshot is based on the dideoxy single base extension of an unlabeled oligonucleotide primer that was performed by ABI PRISM Snapshot Multiplex Kit (Applied Biosystems).

DNA amplification was performed with the aim of amplifying a region including SBE (*rbcL*3 primer) followed by an enzymatic purification (*SAP*, Shrimp Alkaline Phosphatase and *Exo I*, Exonuclease I) to obtain purified template. The second purification or post-SBE clean-up (*SAP* and *Exo I*) was performed to remove unincorporated ddNTPs. Then the samples were run on ABI PRISM 3700 DNA Analyzer instrument while Hi-Di formamide was already added to each of them, and samples were denatured.

3.3. Spectrum Wavelength Evaluation by Fourier-Transform InfraRed Spectroscopy

FTIR spectra were recorded on FT-IR spectrophotometer (BRUKER, Germany) using KBr discs which were approximately 5 mm in diameter and approximately 1 mm thickness. IR spectra were recorded in the 4000–700 cm^{-1} range at scanning speed of 2 $mm.s^{-1}$ with a resolution of 4 cm^{-1} at room temperature (25 °C) and relative humidity of 30%.

Oil samples (2 µL) were coated on the KBr discs to form thin liquid films for infrared spectrometry analysis. The sample measurements were replicated 5 times with 4 scans each for a total of 20 spectra, and then the average chart was taken as a last sample spectrum. The background air spectrum, water vapor and CO2 interference were subtracted from these spectra.

4. Results

4.1. Sample Preparation and DNA Extraction

Total DNA was extracted from both olive plant and oil successfully, and after performing PCR reaction the amplicons were observed on agarose gel electrophoresis stained with “Syber green” since it is more sensitive and the bands appear more intense than the gels stained with ethidium bromide (Figure 1). In microsatellite studies, we should expect generally two alleles for each microsatellite amplification (in this case, as we hypothesized that the used olive cultivar is Carolea cv. and DCA 17 should provide 117 bp and 143 bp alleles and in contrary 164 bp and 199 bp for DCA9). Recording missing allele could be the result of either the preferential amplification of one of the two alleles in oil-derived DNA templates, or to the excess of degradation of the DNA template of the missed allele, that limited the production of a sufficient number of copies of that allele to be detected.

Figure 1. Agarose Gel Separation of DNA Recovered From Olive oil and Leaf Amplified with the Microsatellites DCA17 and DCA9. Lanes 2-5 are Amplified with DCA17, and Lanes 6-9 are Amplified with DCA9.

Lanes 1 and 10 ladder (100 bp); lanes 2 and 6 leaf, lanes 3, 4, 5, 7, 8, and 9 oil, the gel stained by the Syber green

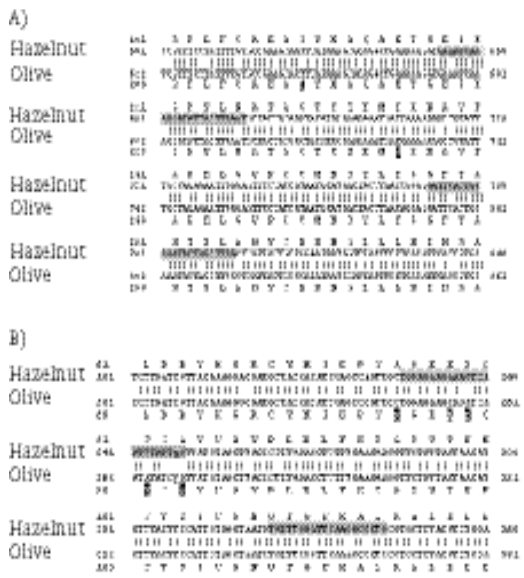
Table 1. List of Primer Pairs Used for Olive Oil Identification

Primer ID	For. 5 ’→3’	Rev. 5 ’→3’	Fragment length (bp)	reference
ssrOeUA-DCA9	AATCAAAGTCTTCCTTCCATTTCG	GATCCTTCCAAAAGTATAACCTCTC	163	Sefc et al., 2000 (28)
ssrOeUA-DCA17	GATCAAATTCTACCAAAAATATA	TAATTTTTGGCACGTAGTATTGG	117	Sefc et al., 2000 (28)
*rbcL***z1**	GCCAGTTGCTGGAGAAGAAAG	TCCAAATACATTACCCACAATGG	143	Present study
*rbcL***z2**	GAAATCAAAGGGCATTACTTGAAT	CCAAGCTAGTATTTGCAGTGAATC	118	Present study
*rbcL***z3**	TGGAGAAGAAAGTCAATTTATTGC	CAGGGCCTTGAATCCAAATA	55	Present study
SBE_*rbcL***G**	TGGAGAAGAAAGTCAATTTATT		23 (hazelnut)22 (olive)	Present study
SBE_*rbcL***C**	TAAGGGGTAAGCTACATAAG		21 (hazelnut)20 (olive)	Present study

4.2. Chloroplast DNA Analysis

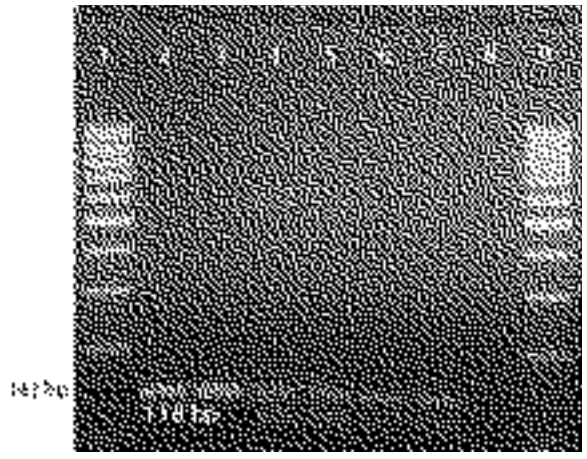
The PCR analyses were performed with the designed primers (Figure 2) in single species-derived oils and mixture of olive: hazelnut oils in the ratio of 50:50, 70:30, and 30:70 v:v). As expected, that specific primer designed for hazelnut did not anneal the olive DNA, and those primers were enough selective to identify the contamination of olive oil with hazelnut oil (Figure 3).

Figure 2. BLAST Analysis of Hazelnut and Olive *rbcL* Sequences.



(A) Shows the common primer pairs (143) and (B) shows the hazelnut-specific primer pairs (118 bp). Above and below the two sequences the CDS translation is shown. The specific primer pair for hazelnut is in grey boxes. Different amino acids are evidenced in reverse color.

Figure 3. Agarose gel separation of the two chloroplast *rbcL* fragments amplified from olive (cv Frantoio), hazelnut (an anonymous genotype) and blends of the two species DNA.



Lanes 1 and 9 ladder; lanes 2-3 olive; lanes 4-5 hazelnut; lanes 6-7 olive: hazelnut 50:50 and 70:30 (v:v), respectively; lane 8 blank control (PCR mixture without DNA template)

Attempts were made to change the ratio between the two oils by reducing the contribution of hazelnut oil to < 10%, until we decided to move towards more sensitive methods of analysis such those based on single base extension.

4.3. SBE Analysis

Future SNP technology would allow direct revealing of adulteration of olive oil with oils of other species. It could be possible also to design SNP-based experiments to detect individual olive varieties when a sufficient amount of intraspecific SNPs is documented in olive. Figure 4 shows the SBE specific primers.

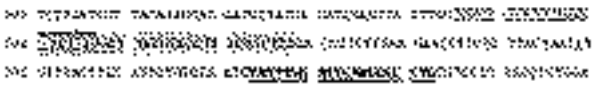


Figure 4. The primer designed on *Corylus avellana* chloroplast sequence (accession number 33113306). The primer *rbcL* z3 is in bold. SBE-*rbcL*-For and –Rev are in dark and light grey boxes, respectively. The single base extension of hazelnut oil is oversized "G" for both one-handed primers.

Figure 5 shows the clustering of AcycloPrime data obtained by plotting the TAMRA polarization values (T incorporation) against R110 ones (G incorporation) after 30 thermal cycles; hazelnut, olive, and the blend of hazelnut: olive (3: 97 and 8: 92) and the negative control (the absence of DNA template).

As expected, for hazelnut samples, the values for R110 were high (lower right) and the values for TAMRA were low, reflecting incorporation of R110 but not TAMRA. The Tamra/T scores present either in the upper right or in the upper left. Negative controls, represented by PCR without DNA template, pure olive samples (the lack of base G, hazelnut DNA absent) and failed PCR reactions are in the lower left cluster.

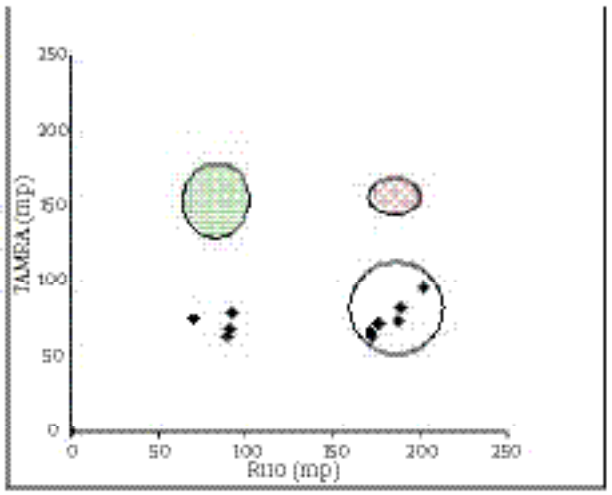
In Figure 6 the results of ABI PRISM Snapshot analysis are reported. The presence of extrapeak involved with the extension of "G" in pure hazelnut and contaminated olive oil samples with different percentages of hazelnut oil (3% and 8%) is considerable.

4.4. Spectrum Wavelength Evaluation by FTIR

Pure olive and hazelnut oils and their admixture at ratios 75: 25 and vice versa were analyzed by means of FTIR. Figure 7 exhibits the absorbance of the samples, at frequency region of 4000 – 700 cm^{-1}.

Some differences in the absorbance of the spectra of pure olive oil and hazelnut-adulterated olive oil have been revealed. The higher absorbance at frequency regions of 3007 cm^{-1} (attributed the C-H stretching), 1373 cm^{-1} (-CH3 bending), 1237, 1120, 1098, and 1032 cm^{-1} (-C-

O stretching), 1160 cm $^{-1}$ (-C-O stretching, -CH2 bending), 965 (trans-CH=CH-bending out of plane), as well as 722 (cis-CH=CH-bending out of plane) were observed for pure olive oil. However, abnormalities were recorded at frequency regions of 2922 and 2853 cm $^{-1}$ (symmetrical and asymmetrical stretching of -CH2).



WELL ID	EXPECTED	OBSERVED
Olive	Negative	Negative
Hazelnut	G	G
Olive + hazelnut (97:3)	G	G
Olive + hazelnut (97:3)	G	G
Olive + hazelnut (97:3)	G	G
negative control	Negative	Negative
Olive	Negative	Negative
Hazelnut	G	G
Olive + hazelnut (92:8)	G	G
Olive + hazelnut (92:8)	G	G
Olive + hazelnut (92:8)	G	G
negative control	Negative	Negative

Figure 5. Clustering of AcycloPrime data obtained by plotting the TAMRA polarization values (T incorporation) against R110 ones (G incorporation) after 30 thermal cycles (left design). The R110/G scores are represented by the cluster in the lower right, where only hazelnut samples are present. The Tamra/T scores due to aspecific primer annealing during SBE should be present either in the upper right or in the upper left. Negative controls, represented by PCR without DNA template, pure olive samples (the lack of base G, hazelnut DNA absent) and failed PCR reactions are in the lower left cluster. Right table is a short report of samples analyzed and the results obtained.

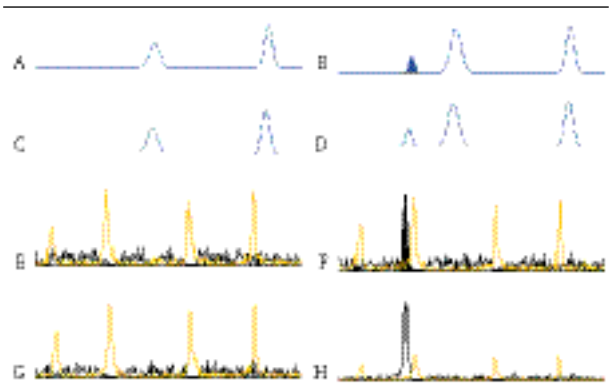


Figure 6. Analyzing the results of ABI PRISM Snapshot by GeneMapper software V.2 and GeneScan 3.7 software, separate fragments detect on Capillary Electrophoresis Platform. Figures A to D refer to Forward SBE primer; figures E to H refer to Reverse SBE primer. A) Olive, B) olive: hazelnut (97:3), C) control negative, D) hazelnut. The highlighted peaks with the red circles show the extended primers in the presence of DNA of hazelnut. E) Olive, F) olive: hazelnut (92:8), G) control negative, H) hazelnut. Black peaks in the third and fourth rows show the single extension base products, and orange peaks show the LIZ-120 size standards

The assignment of such functional groups is reported by Liang et al. (29) in adulterated walnut oil. Rohman and Che Man (30) performed FTIR analysis in discriminating virgin olive oil from other edible oils. They got the same results but the only controversy was at the frequency region 2853 with higher absorbance for olive oil. This minor discordance between results is supposed to be a function of higher/lower olive oil in admixture samples. De La Mata et al. (31) reported that FTIR uses as a semiquantification analysis can be performed suitably for screening purposes at contribution lower than 50% (w/w). Such differences at higher wavelengths are the result of thermal treatments and/or oil degradation as described by Navarra et al. (32).

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Authors' Contribution

Leila Akbari: FTIR analysis, Zohreh Rabiei: SBE analysis, Sattar Tahmasebi Enferadi: elaboration of FTIR results, Sakineh Vanaii: FTIR analysis.

Financial Disclosure

There is no conflict of interest.

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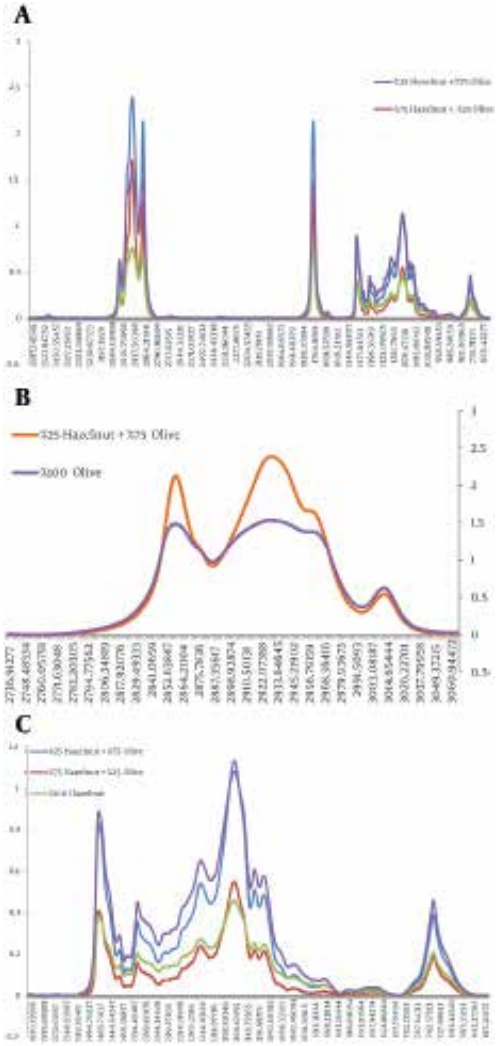

Figure 7. Total FTIR spectra of pure virgin olive and hazelnut oil and their admixture at ratio 75: 25 and vice versa at frequency regions of 4000 – 700 cm -1 (A). B and c are higher resolution of A divided in two regions; B) 3060 – 2700 cm $^{-1}$ and C) 1480 – 610 cm $^{-1}$

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