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A Molecular Screening and Characterization of an Alkaloid Capsaicin in Capsicum Chinense By Esi-Ms

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ABSTRACT: Capsaicin ($C_{18}H_{27}NO_3$) is an alkaloid compound believed to be found only in peppers. Capsaicin is responsible of their characteristic hot taste or pungency. The level of hotness depends on the concentration of capsaicin in the fruit and is variable between species, among varieties within species, among plants within varieties, among fruits of the same plant, and among different parts of the same fruit [1]. DNA barcoding is a novel system designated to provide rapid and accurate and automatable species identification by using short, standardized gene regions as internal species tags. ‘‘DNA barcoding gap’’ is present between intra- and interspecific variations, using multiple accessions per species. The adequate rate of variation, easy amplification and alignment, were identified as a portion of the plastid matKgene as a universal DNA barcode for flowering plants.The short DNA sequence is generated from standard region of genome known as marker. For taxonomists, DNA barcoding assists in identification by expanding the ability to diagnose species by including all life history stages of an organism.matK is one of the most promising one for a plant barcode. ESI-MS determine that the crude extract of acetone and acetonitrile samples of Capsicum chinense reveal that all the samples has shown the presence of capsaicin by detecting the molecular weight 305.41. The large number of capsaicinoids found in pepper fruit extracts [2] can provide indications of possible occurrence of similar number of capsaicinoids in pepper fruit extracts.

KEYWORDS: Capsaicinoids, Capsaicin, vanillyl alcohol moiety, Bhutjolokia, phenyl-propanoids

I. INTRODUCTION:

Capsicum chinense belongs to the family Solanaceae, is an important vegetable and is widely used for culinary purposes due to its extreme flavour and red colour. The HabenaroChilli is very aromatic and it is known as World hottest chilli. Capsicum species are cultivated all over the world and produced at an average of 40 Million tons per year [3]. The genus Capsicum is native to tropical and humid areas in Central and South America. It is commercialized as a fresh product, dry crushed pepper, paprika oleoresin, or pepper paste[4].A group of carotenoids including violaxanthin, cap-santhin, crytocapsin, zeaxanthin, β-apo-8’carotenal, cryptoxanthin, and β-carotene was detected in Pepper seed oil (PSO). The unique keto carotenoids capsanthin and cryptocapsin provide the brilliant red color; while β-carotene, zeaxanthin, and violaxanthin impart the yellow-orange color. Capsicum chinense attracted the attention of the world why because it contain the compound Capsaicin, a metabolite that has many medicinal purposes such as stress relief, pain relief and also to control obesity [5-7].

It has some common names like ‘Bhutjolokia, Naga King Chilli, Naga Jolokia’ (Capsicum chinenseJacq.) is a hot pepper variety native to India which has received the attention of the global scientific community due to its high capsaicinoid concentration.The evolution of the principal capsaicinoids in “Naga Jolokia” peppers had a different behavior with respect to literature reports. Investigation reveals that these changes in the content can be attributed to each pepper genotype.

Capsicum chinense is economically useful crops which can be easily propagated through Plant tissue culture, it is selectively high-yield cultivar resistant to pests, disease and abiotic stresses and to improve their fruit quality as well as quantity[8]. Capsaicinoids are compounds which are responsible for the pungency in pepper that are synthesized by condensation of a common Vanillylmoicity and variable fatty acid [9].The Alkaloid Capsaicin has a lot of commercial value on the World market which has high significance in medicinal field as well as for production of cosmetic purpose.

There is always demand in the market for a good organic product and nowadays

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people keep eye on the product which have natural essence instead of a chemicals. Likewise, there is always a constant demand for flavourings in the form of powder, which are utilized more easily in the food industry. Powdered flavourings have many advantages over aqueous extracts, such as their low humidity, which allows their direct use in dry mixes and seasoning, compact packaging, easier handling and transport, and also for longer shelf life[10,11].

Capsiate (4-hydroxy-3-methoxybenzyl (E)-8-methyl-6-nonenoate), dihydrocapsiate (4-hydroxy-3-methoxybenzyl 8-methylnonanoate) and nordihydrocapsiate (4-hydroxy-3-methoxybenzyl 7-methyloctanoate) [12, 13] have been isolated in pepper fruit extracts. Capsaicinoids are nonvolatile alkaloids, which are chemically acid amides of C_9–C_{11} branched-chain fatty acids and vanillylamines. The main compounds are capsaicin (C) (trans-8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (DHC) (8-methyl-N-vanillylnonanamide), which generally represent around 77%–98% of the total capsaicinoid content. Some other related compounds, such as nordihydrocapsaicin (n-DHC), homocapsaicin (h-C), or homo-dihydrocapsaicin (h-DHC), are also present in minor amounts among the over 20 other reported compounds[14, 15].

Fundamental chemical structure and the biological activity of these compounds are very similar to the capsaicinoids. Unlike capsaicinoids, capsainoids are synthesized by the condensation of a variable fatty acid and a vanillyl alcohol moiety, addition to that it is non-pungent, producing the same biological effects but without the undesirable irritation caused by the pungency, turning into molecules with potential applications in areas such as medicine[16].

For many number of years, the association between nutrition and health has been gained popularity, and therefore, increased importance has been given to diets based on antioxidant-rich vegetables and fruits [17]. The capsaicinoids contents were determined in the pericarp and placenta of the Capsicum fruits, showing that these phenyl-propanoids were mainly localized in the placenta. Quercetin, luteolin, myricetin, kaempferol, and apigenin are present in pepper fruits[18-20].These secondary metabolites exhibit pharmacological effects such as analgesia, anticancer, antioxidant and anti-obesity activities[21].The synergistic activity of these compounds and vitamin C and vitamin E are responsible for the potent antioxidant capacity of peppers which is associated to the protection of the human body against arteriosclerosis, osteoporosis, diabetes, cancer, and coronary diseases [22]. The ascorbate and glutathione contents were higher in pepper fruits which has the greater contents of capsaicinoids [23].

The aim of the study was to know about the compound Capsaicin which is present in the Capsicum chinense, DNA barcoding is a system for fast and accurate species identification that makes ecological system more accessible by using short DNA sequence instead of whole genome and is used for eukaryotes and also to know about its efficiency through ESI-MS. Other than the compounds value, it has lot of Medicinal purpose which is predominantly used in the commercial market for pharmaceutical benefits as well as for the cosmetic products.

II. MATERIALS AND METHODS:

DNA Barcoding

DNA barcoding is a technique which helps in species identification and it can be performed by using DNA sequences from a small fragment of the genome, with the aim of contributing to a wide range of ecological and conservation studies in which traditional taxonomic identification is not practical.DNA barcoding has many applications in various fields like preserving natural resources, protecting endangered species, controlling agriculture pests, identifying disease vectors, monitoring water quality, authentication of natural health products and identification of medicinal plants(www.barcoding.si.edu).

According to Lahayeet al.[24] “A suitable barcode must exhibit high interspecific but low intraspecific divergence.” This marker is different for various species like CO1 cytochrome c oxidase 1 for animals, matK for plants and Internal Transcribed Spacer (ITS) for fungus.An effective DNA barcode in a genebank setting is very accurate to the species level - within the plant materials of interest. Generally, in order to be effective, it need not be universally adaptable to all plant families, all genebanks or all collections, but to the extent possibly provides an unambiguous identification.

Procedure of DNA barcoding

The process of DNA barcoding involves two basic steps: First step is to build a barcode library of identified species and second is matching the barcode sequence of the unknown sample with the barcode library (known as sequence alignment)

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for its identification. It requires ecological expertise in selecting one or several individuals per species as reference samples in the barcode library. The specimens should go through lab processes that are tissue sampling and DNA processing and sequencing to generate DNA barcode in form of chromatogram. Chromatogram is visual representation of DNA sequence produced by the sequencer. This barcode can be stored in database for future use or can be used as query sequence to be compared with sequence already present in database [25].

DNA Sequencing

DNA sequencing of the rbcL, COI or ITS amplicon is required to determine the nucleotide sequence that constitutes the DNA barcode.A single, good-quality barcode from the forward strand is sufficient to identify an organism. The primers used in this experiment incorporate a universal M13 primer sequence.In the first cycle of PCR, the M13 portion of the primer does not bind to the template DNA. However, the entire primer sequence is covalently linked to the newly-synthesized DNA and is amplified in subsequent rounds of PCR. Thus, the M13 sequence is included in every full-length PCR product. This allows a sequencing center to use universal forward and reverse M13 primers for the PCR-based reactions that prepare any rbcL, COI, or ITS amplicon for sequencing.

Maturase K (matK) gene

matK is a plant plastidial gene. It encodes for an organelle intron. The coding region of matK gene is approximately 1570 bp in length (Neuhaus and Link, 1987).DNA sequences of the gene 'matK' differ among plants of different species, but are nearly identical in plants of same species.matK gene evolves more rapidly in contrast to other plastid genes, despite underlying transcriptional and functional constraints. The matK gene is very useful in DNA barcoding for the identification of plant families [26-28]. The very high evolutionary rate of matK has made it usable in phylogenetics reconstructions at high taxonomic levels, such as order of family, and sometimes also at low taxonomic levels, such as Genus or Species [29-31].

ELECTROSPRAY IONIZATION (ESI)

Experimental Implementation

Electron ionization where the process explicitly concerns the conversion of neutral molecules into ions. The principal outcome of the electrospray process is the transfer of analyte species, generally ionized in the condensed phase, into the gas phase as isolated entities. Instrument used – LC-MS 2020, Mobile phase – HPLC grade Acetonitrile:Milli 'Q' water (50:50). The flow rate is 0.2 ml/min. m/z Scan range – 50 - 500 and it is positive mode of segment. For injection, 5 µl of the crude sample is taken without any dilution, crude sample of acetone and acetonitrile extract which helps to find out the compounds at their molecular weight. The sample which is injected into the LC-MS 2020, it is fully automatic machine, which gives the result within minutes.

A solution of the analyte is passed through a capillary which is held at high potential.When an analyte is transferred from solution to the gas phase via ESI, the analytical process has been done based on the procedure. In addition, a flow of bath gas is usually applied to the interface [32] to promote droplet evaporation; controlled heating of the interface provides an alternative approach.Electrospray ionization is the ion source of choice to couple liquid chromatography with mass spectrometry.Among the numerous operating parameters in ESI-MS, the electrospray voltage has been identified as an important parameter to consider in ESI LC/MS gradient elution.

III. RESULTS & DISCUSSION

Isolation of DNA and assessment of purity

In Capsicum species, the isolation of genomic DNA and subsequent PCR amplification were complicated due to abundance of polyphenolics, polysaccharides, RNA and other secondary products. Total genomic DNA was extracted from the mother plant and in vitro grown leaves of Capsicum chinense using CTAB method and confirmed with agarose gel electrophoresis (Table 1) &(Plate 1a).After the isolation of DNA and purification, it has been further studied for DNA barcoding.

Screening of primers for the assessment of genetic uniformity

Genetic similarities of Capsicum chinense of in vitro grown plants were compared with mother plant of Capsicum chinense using the molecular markers. The matK gene is very useful in DNA barcoding for identification of plants.

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DNA barcoding and sequencing in Capsicum chinense

In the presence study, a series of indels and unique substitutions were identified although these were not only toutilized to examine intraspecific diversity or evaluated for use as DNA barcodes. It is to test the robustness of those markers for purposes of barcoding the members of the C.chinense.After the DNA sequencing, it is essential to run a BLAST (online), which helps to find the alignment of nucleotides by using the molecular marker matK gene. Two samples such the mother plant and the in vitro grown plant were compared by matKgene sequencing and the alignment of the nucleotides which clearly showed the similarity between the two samples and it is reliably identified as Capsicum chinense (Fig 1a,1b,1c & 2a,2b,2c).

The results proved and assured that the samples are belonging to the same genus and species, it is not having any genetic diversity and variation by studying the DNA samples of the mother plant and the in vitro grown plant of Capsicum chinense. In DNA isolation and PCR amplification, the samples Mother plant and in vitroly grown plant which do not show any difference in genetic variation, which clearly denotes that in vitro propagation which helps for true-to-type and it also help to maintain totipotency for generation to generation of culture. Furthermore, the samples were studied through DNA barcoding and sequencing which clearly denotes that the samples mother plant and in vitro grown plant which are similar were analyzed through BLAST.

Electrospray Ionization (ESI)

For ESI-LC-MS study, crude extract five samples of Capsicum chinense were taken, such as callus, leaf, shoot, fruit and seed. In Electrospray ionization, on the basis of the compounds molecular weight the samples showed the peak in all the sample extract, especially in fruit and seed, the molecular weight of the Capsaicin is 305.41188 g/mol,IUPAC ID – 8 methyl-N-Vanillyl-trans-6-nonenamide, Formula: $C_{18}H_{27}NO_3$. It is in positive mode so, +1 is added to the molecular weight of the capsaicin, so, molecular mass – 306, which denotes the presences of Capsaicin in all the samples. The highest peak which denotes the presences of capsaicin in callus, leaf, fruit, seed at 306 m/z (Plate 2a,2c,2d,2e) and the small peaks before the highest peak are called daughter ions (Plate 2b,2c). The ESI of fruit and seed, which shows the high level of complexity at 344 m/z, which indicates the higher amount of elements or substance are added to the analyte (Plate 2d, 2e).

The vertical axis shows the intensity (%), and the horizontal axis which denotes the mass of ratio (m/z). The ESI-MS shows in vitro leaf and shoot, which denotes enormous daughter ions. This study was aimed to develop and validate a selective and sensitive HPLC-ESI/MS (TOF) analytical method, allowing the identification and accurate quantification of capsaicin and dihydrocapsaicin, the two major capsaicinoids in Capsicum fruits.Results show that the HPLC-ESI/MS (TOF) method developed is capable of resolving adequately capsaicin, DMBMO, and dihydrocapsaicin when present in a combined standard solution, with retention times of 11.6, 14.0, and 15.2 min respectively.

The PSD spectrum of capsaicin showed a specific pattern of the precursor ion at m/z 306.2 and two derivative ions at m/z 182.1 and 137.0 as fragment ion that corresponds to that of the 8-methyl-6-nonenamide and 2-methoxy-4-methylphenol moieties, respectively. The signal at m/z 306.2 was detected from placenta, pericarp and seed regions, respectively. Especially, the signal at m/z 306.2 of placenta region was the highest. The ratio of signal intensity of the placenta, the pericarp and the seed was 33:10:1. From these MS spectra, it was concluded that capsaicin was most existing at placenta region, semi-quantitatively. The capsaicin was found to be more dominantly distributed in the placenta than pericarp and seed [33].

IV. CONCLUSIONS

Capsaicin is very essential compound for the present generation because it is very useful for the pharmacological development. Capsaicin is available only in the capsicum plant species. The pungency of this vegetable is caused by two groups of chemical compounds known as capsaicinoids and capsainoids[34].Capsaicin is used as food additive and also in medicine as a counter-irritant [35]. Capsaicin has been attributed to have pharmacological effects since ancient times, but its specific applications are yet to be determined including its use in the gastrointestinal tract, for weight-loss and it's an analgesic activity [36]. Capsaicin applies selective effects on the peripheral part of the sensory nervous system and relieves pain by depleting the neurotransmitter of painful impulses known as substance P from the sensory nerve terminals[37]. Capsaicinoid and Capsaicin

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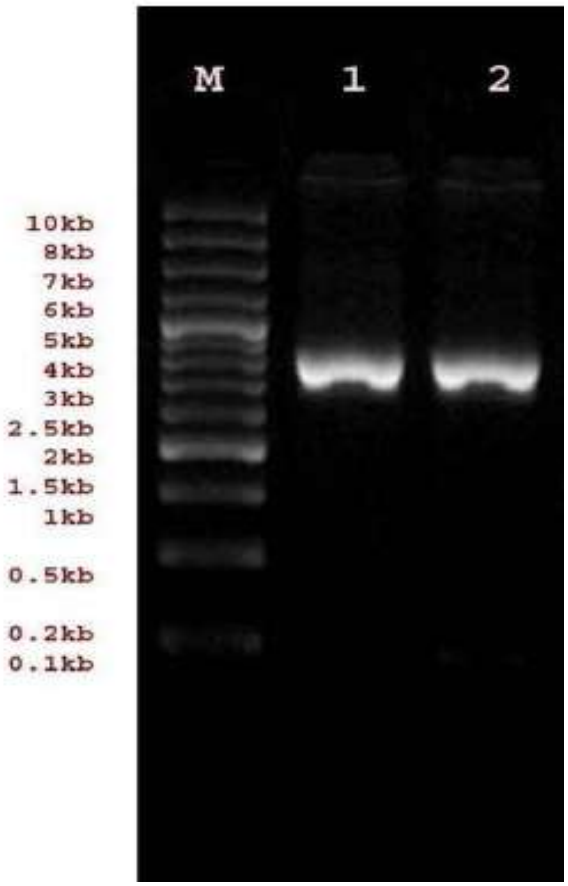
compounds have been widely studied and are currently used in the food industry, for medical purposes, as pharmaceuticals, in defensive sprays and also for agricultural purposes. Techniques such as matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) instruments have been used for the analysis and detection of the compounds. The degree of pungency depends on the Capsicum species and cultivars, and the capsaicin and dihydrocapsaicin contents can be affected by different factors such as the developmental stage of the fruit and the environmental growth conditions [38].

(REFER TABLE, PLATE AND FIGURE)

Table 1. Estimation of DNA using UV-Spectrophotometric method

S.No	Species	A_{260}	A_{280}	Purity A_{260}/A_{280}	Concentration (ng/µl)
1.	Mother plant (Capsicum chinense)	0.067	0.038	1.8	3350
2.	In vitro grown plant (Capsicum chinense)	0.079	0.044	1.8	3950

Plate 1a.PCR profile for Capsicum chinense



M- Ladder, Lane 1- Mother plant, Lane 2- In vitro grown plant

Fig 1a. matK gene sequence of Capsicum chinense

>CC1-matK-Full length-829bp

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gtTCaAAcTcTTCGCTAttGGGtaAAAGatGCcTCTtcTTtACAtTTAtTACGATtcTttcTcCACGAATAtTgGAAT
TTGAATAGTcTTATTAcTtCAAAGAAGCCCgGtTACTCcTTTTCAAAAAAAAATCAAAgattcttCTTCTTT
TTATATAATTTTTATGTATATGAATGCGAaTCCACTTTCGTCTTTCTACGGAACCAATCTTTTCATTT
ACGATCAACATCTTTTGGAGCCCTTCTTGAACGAATATATTTCTATGGAAAAATAGAACGTCTTGT
AGAAGTCTTTGCTAAGGATTTTCAGGTTACCTTATGGTTATTCAAGGATCCTTTCATGCATTATGTT
AGGTATCAAGGAAAATCAATTCTGGCTTCAAACGGGACGTTTCTTTTGATGAATAAATGGAAATTT
TATCTTGTCAATTTTTGGAAATGTCATTTTTCTCTGTGCTTTCACACAGGAAGGATCCATATAAACC
AATTATCCAACCATTCCCGTGACTTTATGGGCTATCTTTCAAGTGTGCGACTAAATCATTCAACGGT
ACGTAGTCAAATGTTAGAAAATTCATTTCTAATAAATAATGCAATTAAAAAGTTCGATACCCTTGT
TCCAATTATTCCTTTGATTGGATCATTAGCTAAAGCACACTTTTGTACCGTATTAGGACATCCCATT
AGTAAACCGGTTTGGTCCGATTTATCAGATTCTGATATTATTGACCGATTGGGGCGTATATGCAGA
AATCTTTTTCATTATTTTAGCGGaTCTTCCAAAAAAACGACTTTATATCGAATAAAGTAtAtAcTTCG
ACTTTCttgtg

Fig.1b. Tabular representation matK gene sequence of Capsicum chinense

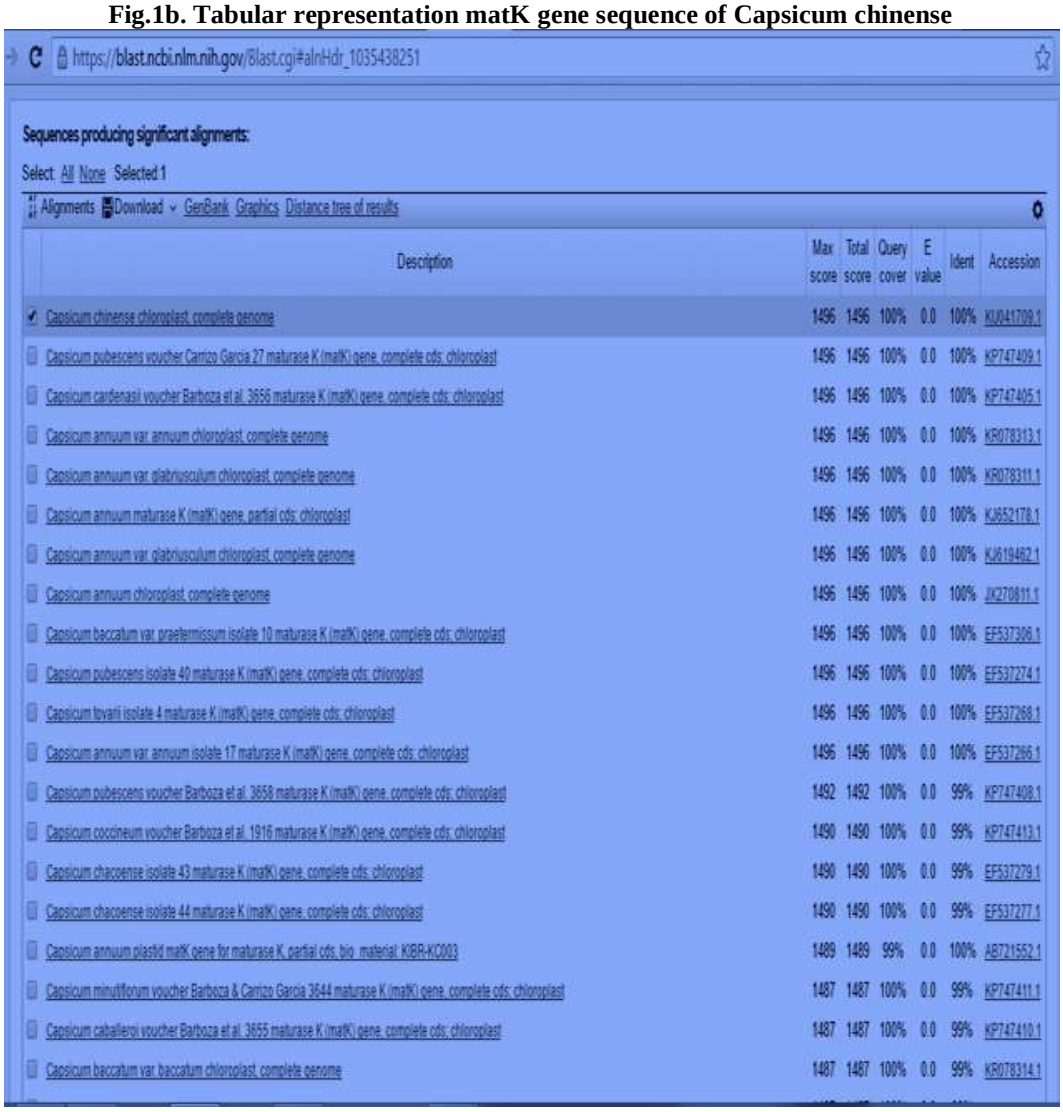

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Fig. 1c. Alignment representation of matKgene sequence in BLAST

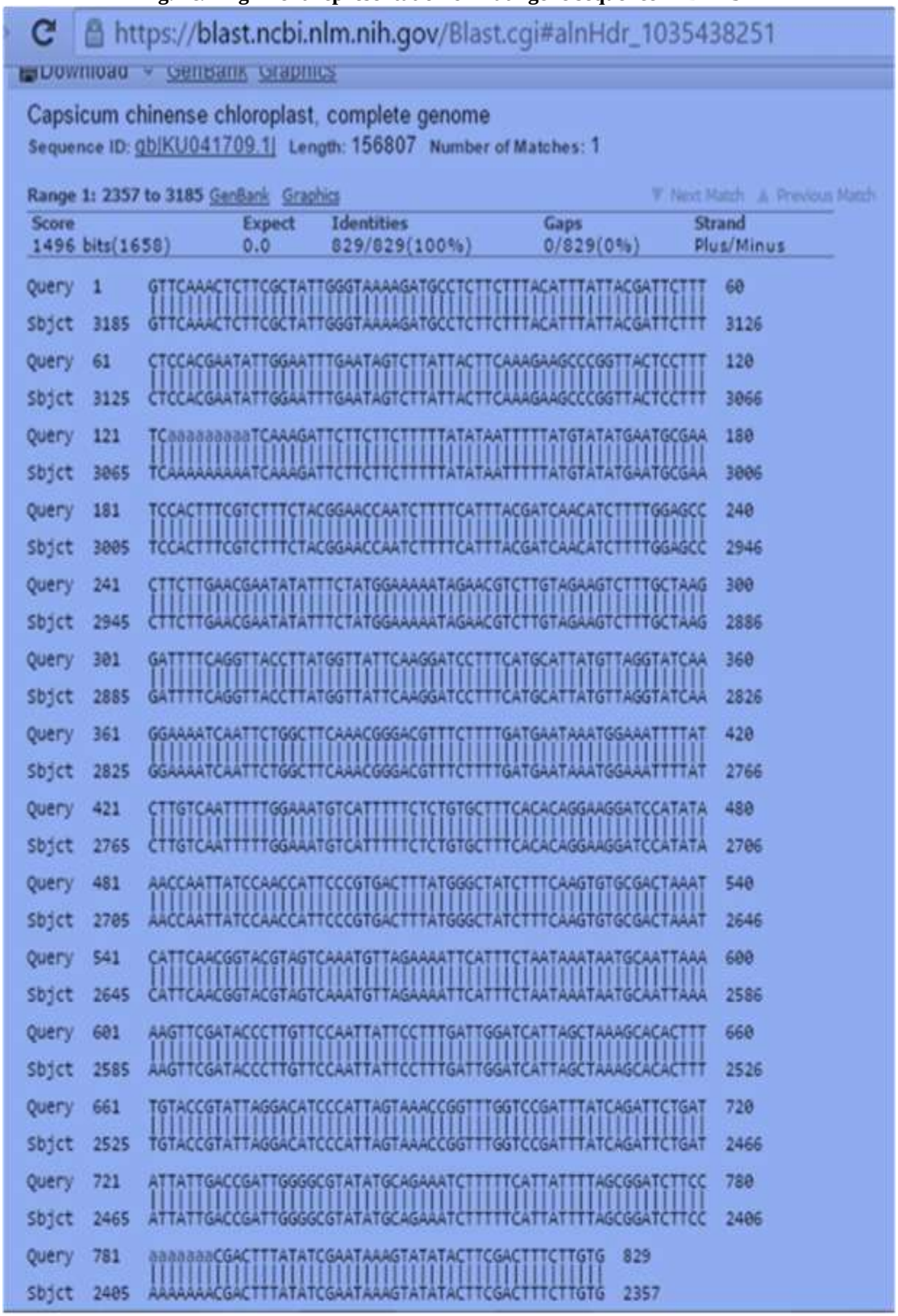

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Fig. 2a. matKgene sequence of Capsicum chinense(In vitro)

>CC2-matK-Full length-778bp
GCCtCTTCTTTACaTTTATTACGATTCTTTCTCCACGAATATTGGAatTTGaATAGTCTTATTACTTCAA
AGAAGCCCGGTTACTCCTTTTCAAAAAAAAATCAAAGATTCTTCTTCTTTTTATATAATTTTTATGT
ATATGAATGCGAATCCACTTTCGTCTTTCTACGGAACCAATCTTTTCATTTACGATCAACATCTTTT
GGAGCCCTTCTTGAACGAATATATTTCTATGGAAAAATAGAACGTCTTGTAGAAGTCTTTGCTAAG
GATTTTCAGGTTACCTTATGGTTATTCAAGGATCCTTTCATGCATTATGTTAGGTATCAAGGAAAAT
CAATTCTGGCTTCAAACGGGACGTTTCTTTTGATGAATAAATGGAAATTTTATCTTGTCAATTTTTG
GAAATGTCATTTTTCTCTGTGCTTTCACACAGGAAGGATCCATATAAACCAATTATCCAACCATTCC
CGTGACTTTATGGGCTATCTTTCAAGTGTGCGACTAAATCATTCAACGGTACGTAGTCAAATGTTA
GAAAATTCATTTCTAATAAATAATGCAATTAAAAAGTTCGATACCCTTGTTCCAATTATTCCTTTGA
TTGGATCATTAGCTAAAGCACACTTTTGTACCGTATTAGGACATCCCATTAGTAAACCGGTTTGGT
CCGATTTATCAgATTCTGAtATTATTGACCGATtGGGGCGTATATGCAGAAATCTTTTTCATTATTTT
AGCggatCTTcCAAAAAAACGACTTTtATATCGAAtAAAGT

Fig. 2b. Tabular representation matK gene sequence of Capsicum chinense(In vitro)

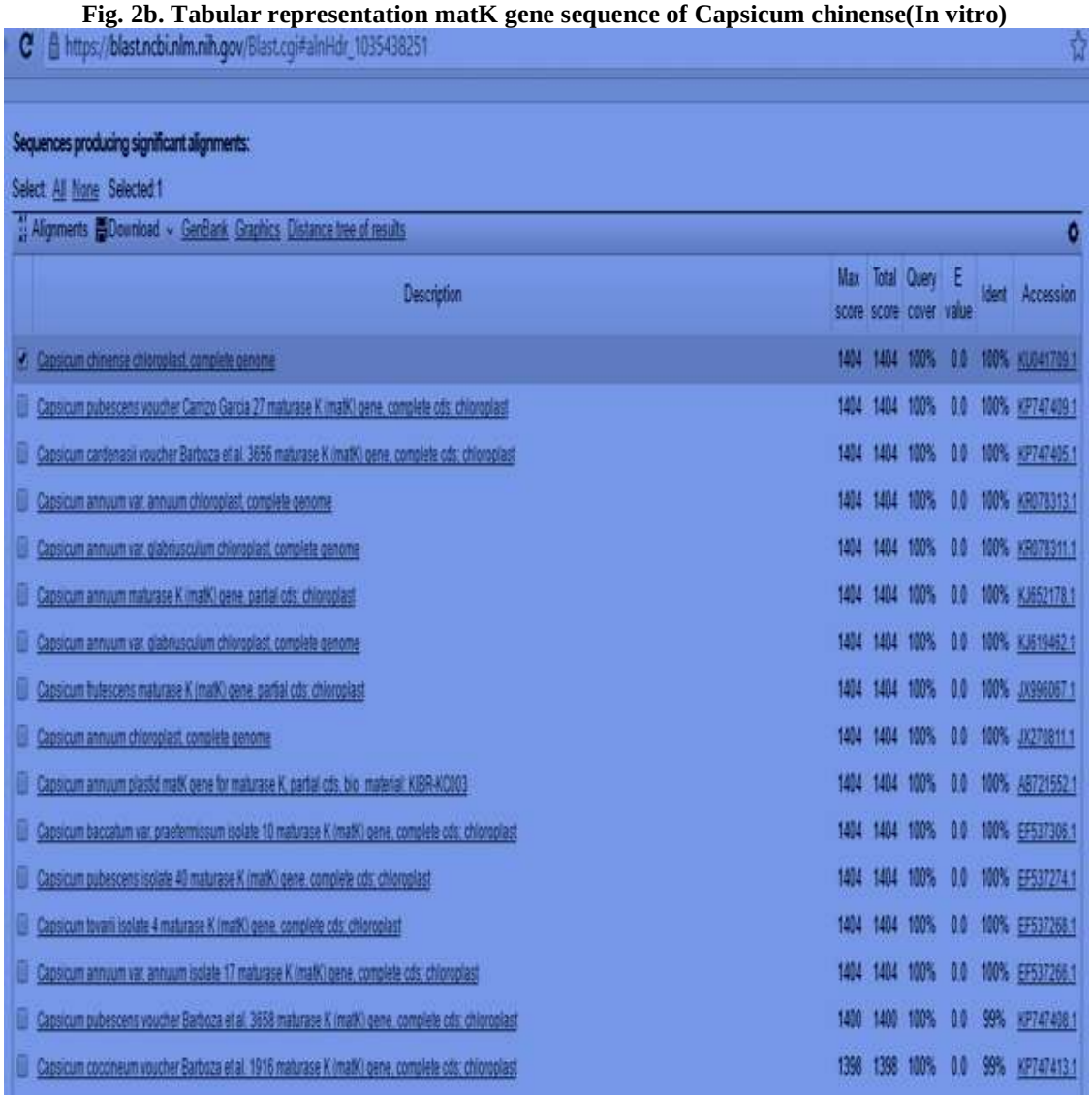

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Fig. 2c. Alignment representation ofmatK gene sequence in BLAST

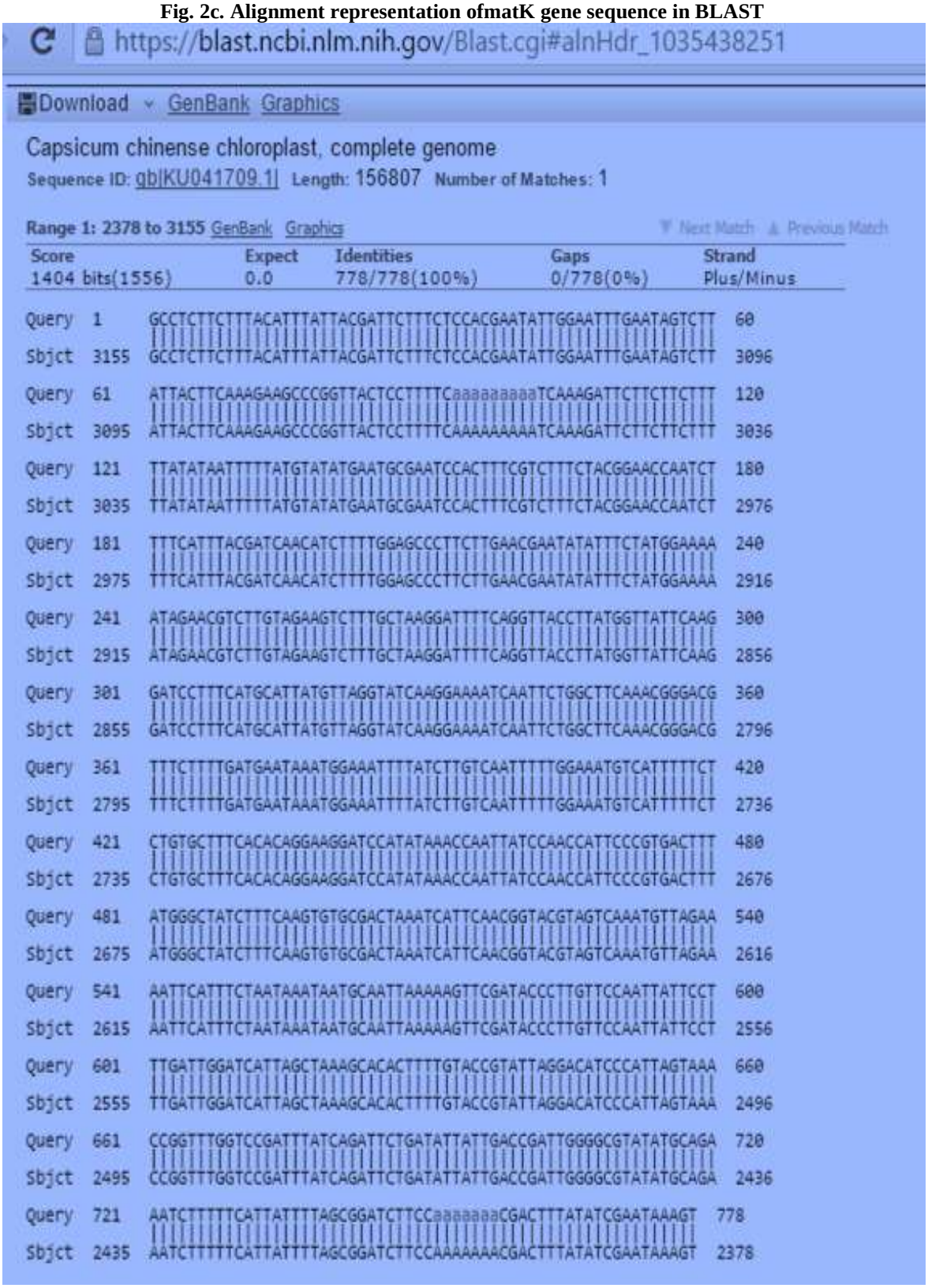

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Plate 2(a-e).ESI-MS of different extracts of Capsicum chinense

Plate 2a. ESI-MS of Callus of Capsicum chinense

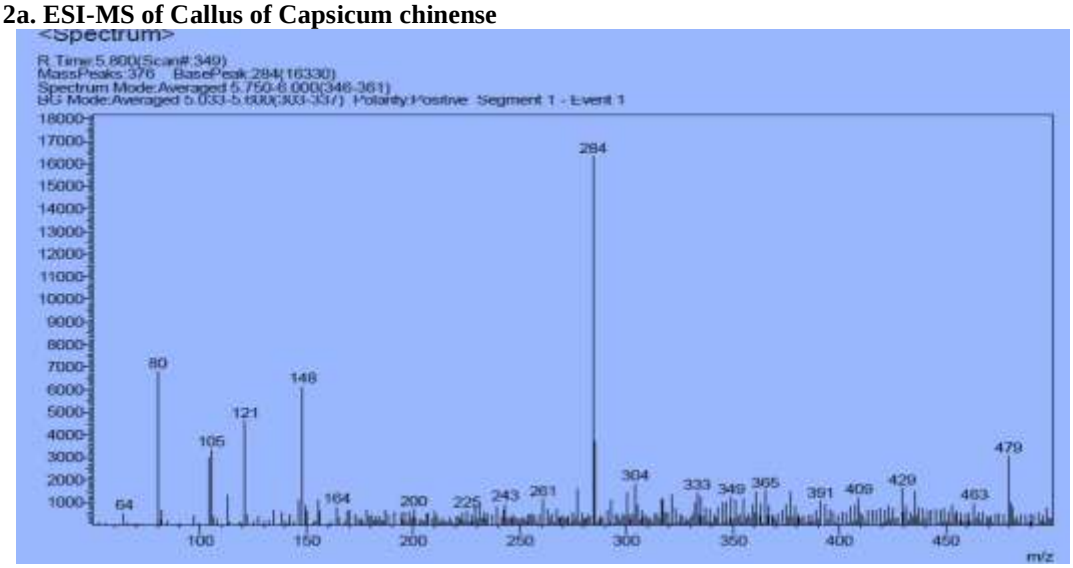

Plate 2b. ESI-MS of in vitro shoot of Capsicum chinense

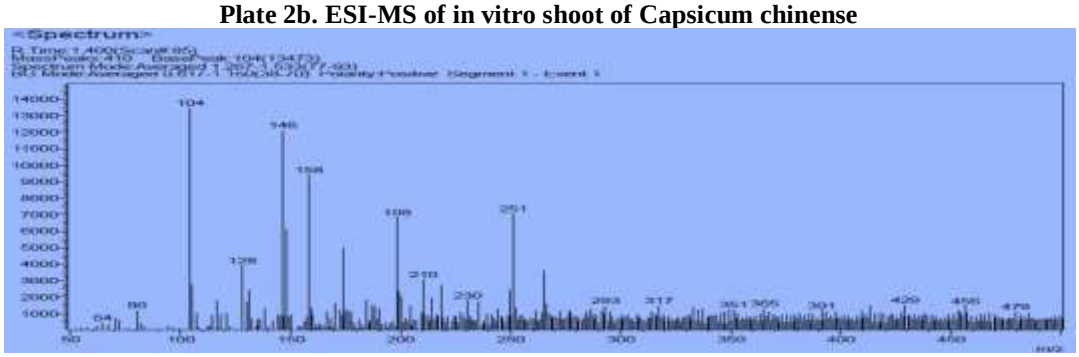

Plate 2c. ESI-MS of leaf of Capsicum chinense

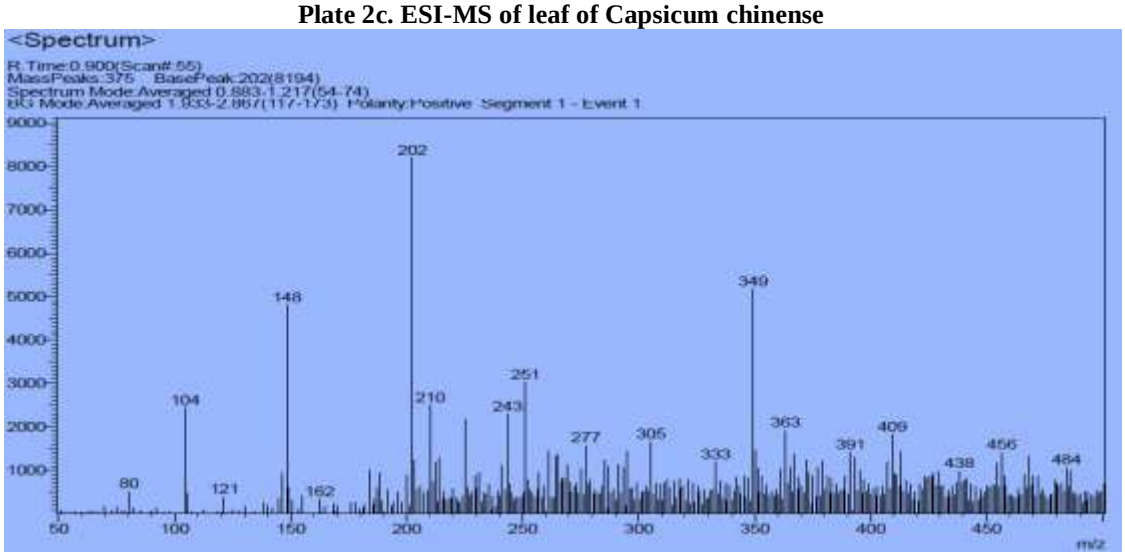

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Plate 2d. ESI-MS of fruit of Capsicum chinense

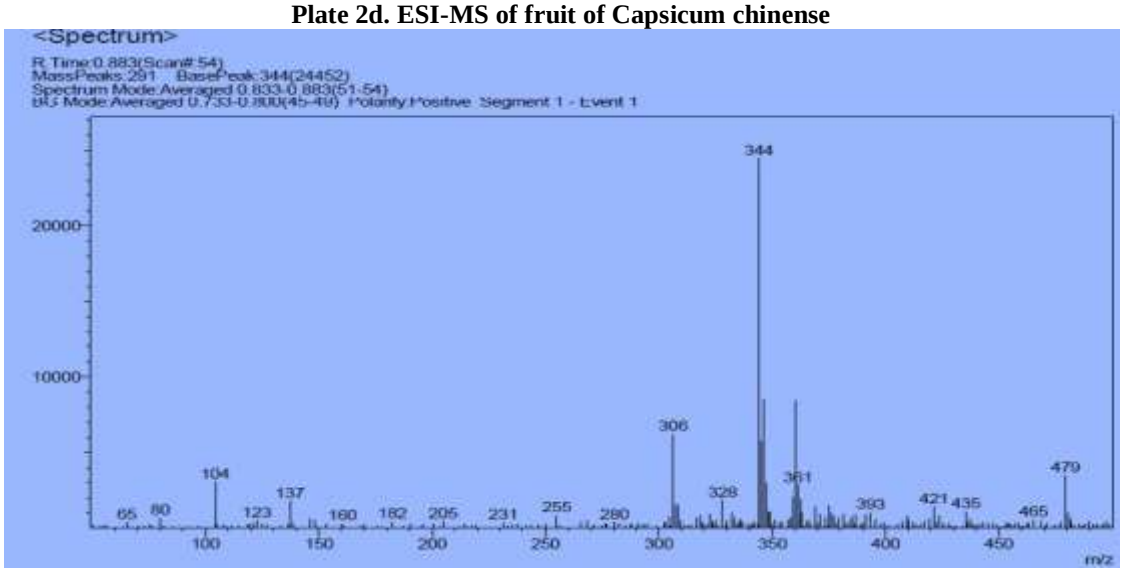
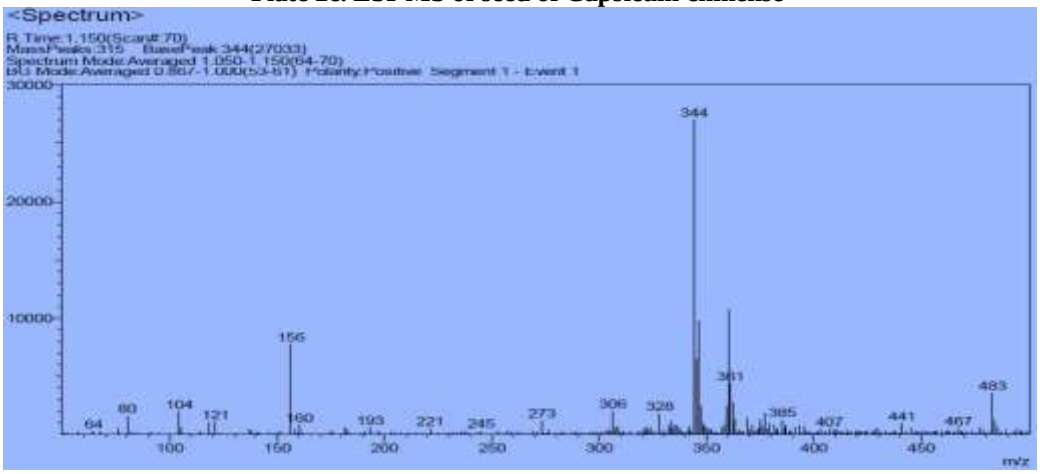


Plate 2e. ESI-MS of seed of Capsicum chinense



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