



Attenuated Total Reflectance - Fourier Transform Infrared (ATR-FTIR) Spectroscopy: A Tool to Determine Groundnut Seed Quality

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ABSTRACT

Background: ATR-FTIR spectroscopy is proved to be a simple, reliable, rapid and easy-to-use technique to assess the groundnut seed quality. Groundnut seeds are highly sensitive to deterioration mainly due to their chemical composition and moisture content. The high oil content and fatty acids of groundnut seeds reduces the seed viability well in advance due to lipid peroxidation process, which is the most frequent cause of seed ageing. As a result, not only lipid peroxidation, but also a series of reactions that produce toxic products occur.

Methods: Eleven groundnut seed lots were tested for their seed germination potential as well as the oil quality parameters, ATR-FTIR analysis, antioxidant enzyme activities and physiological seed quality parameters.

Result: The results indicated that the FTIR spectra of the seed lots having higher germination (>70%) exhibited deviations at different wave numbers within the range of 4000-400 cm⁻¹ when compared with seed lots having lower germination (<70%). The peak intensity was found to follow an increasing trend to a decrease in germination percentage of the seed lots. The seeds lose its viability, the activity of antioxidant enzyme decreases leading to an alteration in free fatty acid, acid value, saponification value, iodine value and peroxide value. Finally, the study concluded that the changes in seed germination from 92 to 12 per cent and their corresponding enzyme activity are closely correlated with FTIR spectral data. Hence, ATR FTIR may be used as a tool to predict the quality of the groundnut seeds during storage.

Key words: Fourier transform infrared spectroscopy, Groundnut seed lots, Oil quality, Seed germination.

INTRODUCTION

ATR-FTIR spectroscopy is a simple, reliable and rapid method to determine the seed quality. Over the past few decades, FTIR spectroscopy serving as a reliable analytical tool in the fat and oil industries and become an attractive alternative to traditional methods, being a rapid and easy technique to assess the oil quality (Li *et al.*, 2019). The spectral range of the middle infrared region (MIR) of FTIR spectroscopy, from 4000 to 400 cm⁻¹, has been commonly used for the identification of organic groups, based on their characteristic, fundamental vibrations as well as for the quantitative evaluation of components if adequate calibrations were complemented (Rodriguez and Allendorf, 2011). The FTIR-MIR spectroscopy with attenuated total reflectance (ATR) or transmission cell accessories has been used to authenticate, identify or classify fats and oils (Ozen *et al.*, 2003), the ATR accessory being a better choice due to its ability to handle easily liquid samples, requiring minimum preparation. It is also an excellent tool for quantitative analysis, because the intensities of the spectral bands are proportional to concentration. It is useful to distinguish oils from different botanical origins using specific wave number domains coupled with non-supervised classification techniques (Lai *et al.*, 1994; Rusak *et al.*, 2003).

Groundnut having rich oil content with major fatty acid like oleic (18:1), linoleic (18:2) and linolenic (18:3) fatty acid

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chain. The fatty acids are under the risk of auto oxidation during storage. This resulted in loss of oil quality by formation of free fatty acid and thus ultimately affects the seed viability. In all the oilseeds, the seed viability is negatively correlated with free fatty acid content of the seed. In order to take appropriate decision in the process of groundnut seed storage, biochemical changes associated with lipids need

to be identified at the early stage itself. If it is possible, by giving appropriate mid-storage correction treatment we can delay the process of ageing or we can quickly dispose the stored seeds for sowing or oil extraction purpose.

At present to assess the quality of the stored groundnut seed, seed germination test is being followed. It's a time consuming process and further, though this test it is not possible to determine the status of the ageing related process occurrence in the stored seeds. Further, the results also will represent the status of groundnut seeds 10 days before.

Hence, there is a need to develop simple and rapid method for periodical examination of groundnut seed quality during storage. In that case, ATR-FTIR spectroscopy assessment could be used as one of the promising method which would give a precise result on groundnut seed quality in a short period. The main objective of this experiment is to evaluate ATR-FTIR spectroscopy as a rapid tool to examine or monitoring the groundnut seed quality during storage.

MATERIALS AND METHODS

Raw materials

Groundnut seed lots of VRI 8 variety with different levels of seed germination (12 to 92%) were collected from Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore, India and used for this study (Table 1). The eleven seed lots have been presented as L₁ to L₁₁ in this paper. Before initiating the experiment, the genetic and physical purity of the seed materials was tested and confirmed to be 100 percent pure.

Oil content

The oil was extracted individually from 11 seed lots by following cold press method of extraction (Cakaloglu, 2018). The oil sample was used for further oil quality analysis.

Qualitative analysis of groundnut oil

Chemical properties like acid value (AV), free fatty acid (FFA), saponification value (SV), peroxide value (PV) and iodine value (IV) of oil extracted from eleven groundnut seed lots were estimated as per AOCS Protocols Cd 18-90, Ca 5a-40, Cd 3-25, Cd 8-53, and Cd 1-25, respectively (AOCS, 1998).

ATR-FTIR analysis

Infrared spectra were recorded on an FTIR spectrometer (Nicolet is10, Germany) equipped with an ATR accessory. The ATR-FTIR spectra were obtained against the background of air spectrum. Background scan and the extracted groundnut oil were sequentially measured at room temperature (25°C) from 4000 to 400 cm⁻¹ with a scanning time of 64 seconds and 8 cm⁻¹ resolution. After every scan of a sample, a background of new reference air spectrum was performed. Also, the ATR plate was cleaned with a soft tissue soaked in acetone to remove any residues from previous samples. The ATR cleanliness was assessed by comparing the obtained background spectrum to the

previous one. All spectra were processed with the computer software program Spectrum for Windows XP Professional (Germany). The assignment of the recorded bands to the specific functional groups was done by comparison with data reported by previous studies on groundnut seeds.

Antioxidant enzymes assay

Preparation of enzyme extract

To assess the activity of antioxidant enzymes viz., superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) enzyme extract was prepared from the eleven groundnut seed lots by adopting procedures detailed below. Briefly, one gram of seed sample from each lot was frozen using liquid nitrogen and then samples were ground with 10 ml of extraction buffer (0.1M phosphate buffer, pH of 7.5 containing 0.5 mM EDTA, 100 µl mercaptoethanol, 1 g of polyvinyl pyrrolidone). Then, the extract was centrifuged for 30 min at 20,000 rpm at 40°C. The supernatant was used for quantifying the CAT (Aebi, 1984), GR (Goldberg and Spooner, 1983) and SOD enzyme activity (Giannopolitis and Ries, 1977).

Physiological and biochemical quality of groundnut seed lots

The groundnut seed lots were tested for different physiological seed quality parameters such as speed of germination, germination percentage, root length, shoot length, dry matter production and vigour index as per ISTA seed testing protocols (ISTA, 2019). The electrical conductivity of seed leachate was measured by following the method of Presley (1958). The dehydrogenase enzyme activity of all the seed lots was measured by following the method of Kittock and Law (1968).

Statistical analysis

The experiment was executed in a completely randomized design with three replications. The means were separated using Tukey's multiple range test using the SAS program [Statistical Analysis System (SAS) Version 9.2, SAS Institute Inc., USA] and the mean values are presented with standard deviation.

Table 1: Details of seed lots and notation used in the experiment.

Seed lots	Germination (%)
L ₁	92
L ₂	90
L ₃	85
L ₄	81
L ₅	77
L ₆	72
L ₇	66
L ₈	48
L ₉	34
L ₁₀	22
L ₁₁	12

RESULTS AND DISCUSSION

ATR-FTIR analysis

ATR-FTIR analysis is more useful to assess the unsaturation index of the oil samples. Unsaturation index is closely linked with hydrocarbon chain length (Naumann *et al.*, 1991). The shifting of high to low peak intensity observed at the particular range of wave number is the indication for a higher degree of saturation in oils (Vongsivut *et al.*, 2013).

In the present study, the FTIR spectra wave number and peak intensity was found to follow an increasing trend with decrease in germination percentage of groundnut seeds. The results of FTIR spectra wave number ranging from 3367 to 3422 cm^{-1} indicated the stretching vibration of C=O ester groups and the wave number ranging from 3007 to 3009 cm^{-1} belongs to stretching vibration of olefinic double bonds, and highest degree of unsaturation while, the wave

number 2853 to 2925 cm^{-1} indicated symmetric stretching vibration of C-H of aliphatic CH_2 group. The wave number range of 1645 to 1746 cm^{-1} indicated the stretching vibration of ester carbonyl functional groups (C=O) of the triglycerides and fatty acids and the wave numbers ranging from 1377 to 1465 cm^{-1} indicated bending symmetric vibration of C-H bonds of CH_2 and CH_3 aliphatic groups. The wave numbers in the range of 1116 to 1118 cm^{-1} indicated the stretching vibration of C-O ester groups and the wave numbers 720 to 722 cm^{-1} indicated the over lapping of CH_2 rocking vibration and the out of plane vibration of Cis-HC=CH- group of disubstituted olefins and oxidation to alcohols (Table 2 and Fig 1).

The results of present study are in accordance with the findings of Muniyappan *et al.* (2019), who reported that the FTIR spectrum of aged sesame seed oil showed a prominent peak at 1745 cm^{-1} . The appeared peak indicated the content

Table 2: Functional groups of volatile organic compounds associated with different wave number regions of FTIR spectroscopy analysis of accelerated aged groundnut seeds.

Wave number (Cm^{-1})	Functional group	Mode of vibration
3367-3419	C=O (ester)	Stretching vibration of C=O ester groups
3007-3009	=C-H (trans and cis)	Stretching vibration of olefinic double bonds and highest degree of unsaturation
2924-2925	-C-H (CH_3 , CH_2)	Symmetric stretching vibration of C-H of aliphatic CH_2 group
2852-2854	-C-H (CH_3 , CH_2)	Symmetric stretching vibration of C-H of aliphatic CH_2 group
1745-1747	-C=O (ester, acid)	Stretching vibration of ester carbonyl functional groups (C=O) of the triglycerides
1642-1658	-C=O (ester, acid)	Stretching vibration of fatty acids and esters
1460-1465	-C-H (CH_3 , CH_2)	Bending vibration of C-H of CH_2 and CH_3 aliphatic groups
1377-1383	-C-H (CH_3)	Bending symmetric vibration of C-H bonds of CH_3 groups
1116-1119	-C-O	Stretching vibration of C-O ester groups
720-722	-C-H; -HC=CH- (cis)	Over lapping of CH_2 rocking vibration and the out of plane vibration of Cis-HC=CH- group of disubstituted olefins and oxidation to alcohols

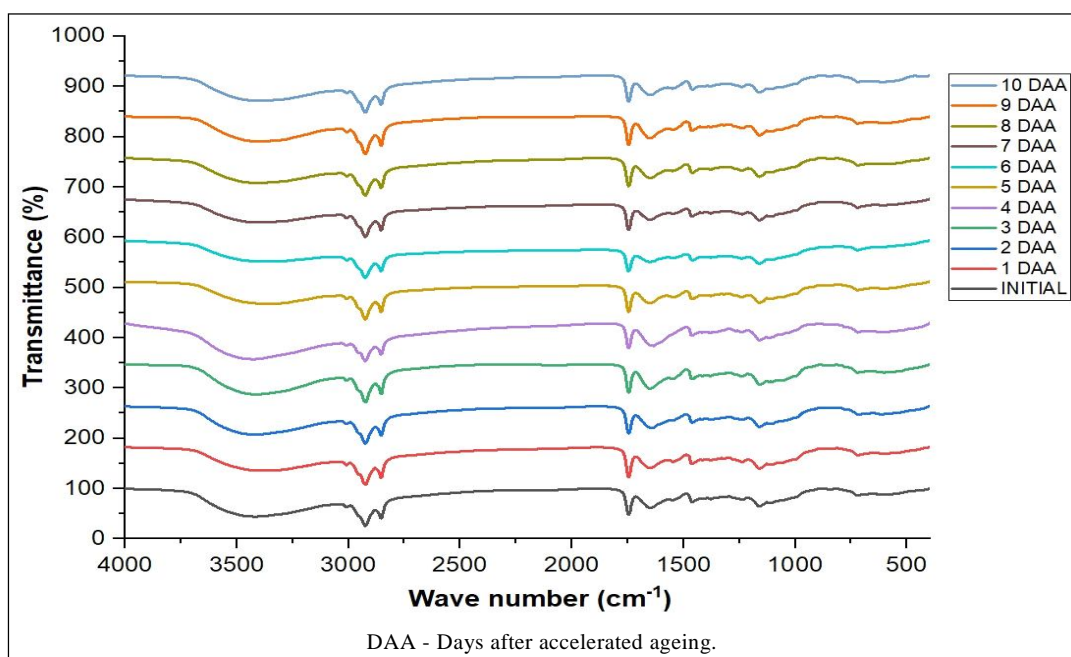


Fig 1: FTIR spectrum of accelerated aged groundnut seed lots.

of unsaturated fatty acids and total lipid content in the 4 days to 10 days accelerated aged sesame seed oil. Which gave valuable supporting information to the present study. Similarly, Shapaval *et al.* (2014) also found that in groundnut seed oil sample the peak appeared at 970 cm^{-1} which indicates the formation of trans-fat and this trans-fat formation in extracted oil sample reduced the storage potential of seeds.

Vlachos *et al.* (2006) suggesting that, this changes may be associated with the decomposition of hydroperoxides and the formation of saturated aldehydes or other secondary oxidation products such as alcohol, ketones, acids and esters. For seed samples with low germination, there were symmetrical and asymmetrical peaks observed. These results were found to be in line with iodine value and saponification value data recorded for high to low germination seed samples. Whereas acid value, FFA and peroxide value data recorded for low to high germination seed samples of this experiment.

Qualitative analysis of groundnut oil

In order to confirm the ATR-FTIR results with reference to groundnut seed quality analysis by knowing the functional group variations, qualitative analysis was done for the oil extracted from all the 11 seed lots and discussed.

Acid value (AV) is an indication on deterioration of the oilseed quality. The acid value is a measure of the amount of free fatty acids present in the oil. The acid value of the oil extracted from seed lot L_1 to L_{11} increased from 0.4 to 1.8 mg KOH g^{-1} (Table 3). Loss of viability and vigour in seed during rapid ageing may be due to increase in free fatty acids (FFA) level in the seeds. The percentage value of the FFA content of the oil extracted from seed lot L_1 to L_{11} are as follows: 0.9, 1.2, 1.4, 1.7, 1.9, 2.3, 2.5, 2.6, 2.9, 3.4 and 3.6, respectively (Table 3). The increase in FFA content of the oil from seed lot L_{11} may be due to improper handling, high

moisture content and fungal attacks which may be favourable for hydrolysis of oil in seed (Vinoth kumar, 2019).

Iodine value (IV) is a measure of the degree of unsaturation of fatty acids in triacylglycerol. Among the tested seed samples, seed lot L_1 recorded a higher iodine value (78.15) and seed lot L_{11} recorded the lowest value (32.45) (Table 3). The seed lot with more iodine value indicates that fatty acid constituents of groundnut seeds is maintained and deteriorated process not yet initiated. Further, fatty acid components have many double bonds, leading to higher stability of the oil towards oxidation. However, the seed lot with less iodine value clearly indicates that fatty acid component of groundnut seeds are affected by the deteriorative process and thus leads to increase FFA content and loss of seed quality. In this case, seed lot having higher seed viability were found to have a higher level of degree of unsaturation of fatty acids, but the loss of seed viability causes a reduction in the degree of unsaturation (Zuleta *et al.*, 2012).

Saponification value (SV) is an indication of adulteration and also estimates chain length of the unsaturated fatty acids. SV is a measure of the molecular weight of components of oil. Among the various seed lots tested in the experiment, the seed lot L_1 recorded higher saponification value (188.62 mg KOH g^{-1}) and seed lot L_{11} recorded a lower value of 174.92 mg KOH g^{-1} (Table 3). Generally, the SV of any vegetable oils would give an idea about the presence of a higher or lower amount of fatty acids. This value indirectly indicates soap formation in the reaction between fatty acids and alkali. The higher peroxide value (PV) indicates the more oxidization of the oil. Peroxide value increased rapidly during storage period. The minimum PV was recorded in the oil extracted from the seed lot L_1 (7.6 mg peroxide kg of sample $^{-1}$) and seed lot L_{11} recorded a higher value of 19.8 mg peroxide kg of sample $^{-1}$ (Table 3).

Table 3: Evaluation of oil quality parameters in groundnut seed lots.

Seed lots	AV (mg KOH g^{-1})	FFA (%)	IV	SV (mg KOH g^{-1})	PV (mg peroxide kg of sample $^{-1}$)
L_1	0.4	0.9	78.15	188.62	7.6
L_2	0.6	1.2	73.78	187.94	8.1
L_3	0.7	1.4	70.96	187.25	9.5
L_4	0.8	1.7	65.12	186.58	10.4
L_5	0.9	1.9	61.34	186.13	11.2
L_6	1.1	2.3	55.85	185.22	12.6
L_7	1.2	2.5	49.72	184.28	13.8
L_8	1.3	2.6	43.46	182.56	15.1
L_9	1.5	2.9	40.14	179.42	16.7
L_{10}	1.7	3.4	37.68	177.54	18.2
L_{11}	1.8	3.6	32.45	174.92	19.8
Mean	1.1	2.2	55.33	183.68	13.0
SEd	0.13	0.11	1.31	4.07	0.20
CD (P=0.05)	0.27	0.22	2.72	8.43	0.41

AV- Acid value; FFA- Free fatty acids; IV- Iodine value; SV- Saponification value; PV- Peroxide value.

Antioxidant enzymes analysis

Antioxidant activity enzymes (CAT, SOD and GR) were analyzed in all the seed lots and have been discussed. Among the seed lots, the CAT activity was found to be maximum ($1.41 \mu\text{M H}_2\text{O}_2$ reduced $\text{min}^{-1} \text{mg}$ of protein $^{-1}$) in the seed lot of L_1 and the minimum of seed lot L_{11} ($0.39 \mu\text{M H}_2\text{O}_2$ reduced $\text{min}^{-1} \text{mg}$ of protein $^{-1}$). Similarly, the seed lot L_1 with 92% germination had the maximum SOD activity (0.73 mg of protein $^{-1}$) compared to other seed lots. The seed lot L_{11} with 12% germination had lower SOD activity (0.11 mg of protein $^{-1}$). The maximum and minimum value for GR activity were found in seed lot number one (L_1 ; $1.14 \mu\text{l}$ reduced glutathione formed $\text{min}^{-1} \text{mg}$ of protein $^{-1}$) and eleven (L_{11} ; $0.41 \mu\text{M}$ reduced glutathione formed $\text{min}^{-1} \text{mg}$ of protein $^{-1}$) (Table 4). The seed lots with higher seed germination were found to have higher CAT, SOD and GR activity.

The SOD enzyme acts on the superoxide anion converting it to another reactive intermediate (H_2O_2) and the latter acts on H_2O_2 converting it to water and oxygen by CAT. If the superoxide anion was not efficiently scavenged in the seeds, the fatty acids would be oxidized, resulting in a decrease in oil quality. Studies have shown that seed lots with higher germination percentage had higher CAT activity. The CAT enzyme is involved in the decomposition of hydrogen peroxide (H_2O_2); if the H_2O_2 were not decomposed, it would oxidize the fatty acid present in the seeds (Kong *et al.*, 2015).

In general, the seed viability protective mechanisms are enabled by free radical and peroxide-scavenging enzymes such as CAT, SOD and GR, which facilitate the oxide reduction cycle in the living system. In the present experiment, an analysis of 11 seed lots revealed that the level of antioxidant enzymes present in the seeds was highly related to the viability potential of the groundnut seeds.

Physiological and biochemical quality analysis

The results of germination test showed that the germination values of all the eleven seed lots from L_1 to L_{11} were in the order of 92, 90, 85, 81, 77, 72, 66, 48, 34, 22 and 12 per cent, respectively (Table 1). The other physiological quality parameters like speed of germination, root length (cm), shoot length (cm), dry matter production ($\text{g } 10 \text{ seedling}^{-1}$) and vigour index observed in L_1 was the highest; while, the seed lot L_{11} possessed the lowest physiological seed quality parameters (Table 5). The data indicates the differential status of seed germination and vigour potential of the 11 seed lots included in this study. The loss of seed vigour and viability in a seed is a culmination of loss of cell membrane integrity and eventual loss in the biochemical potential of the seed, namely the activity of antioxidant scavenging enzymes. The loss of enzyme activity might have increased the degradation of carbohydrate, protein, fat, *etc.* (McDonald, 1999).

Electrical Conductivity (EC) of seed leachate as a measure of membrane integrity is considered as a good index for seed viability (Matthews, 1968) and vigour (Grabe, 1967). In the present study, the evaluation of membrane integrity in different seed lots revealed that, seed lot L_1 recorded lower value (0.592 dSm^{-1}) compared to seed lot L_{11} (1.713 dSm^{-1}) (Table 5). It clearly indicates that seed lot L_1 have higher level of cell membrane integrity than other seeds lots tested in this experiment. Similar results were also reported in groundnut seeds by Suganthi and Selvaraju (2017). Membrane integrity was mainly governed by cell wall permeability. Higher membrane integrity of seed steep water indicated higher permeability, respiration rate and metabolic activity (Francis and Coolbear, 1984). The increase in membrane integrity might be due to the loss of selective permeability of cell membrane by auto oxidation of

Table 4: Evaluation of antioxidant activity for different enzymes in groundnut seed lots.

Seed lots	CAT activity ($\mu\text{M H}_2\text{O}_2$ reduced min^{-1} mg of protein $^{-1}$)	SOD activity (mg of protein $^{-1}$)	GR activity (μM reduced glutathione formed min^{-1} mg of protein $^{-1}$)
L_1	1.41	0.73	1.14
L_2	1.37	0.65	1.08
L_3	1.32	0.62	1.06
L_4	1.24	0.54	1.01
L_5	1.05	0.50	0.94
L_6	0.92	0.48	0.93
L_7	0.84	0.34	0.88
L_8	0.65	0.28	0.76
L_9	0.54	0.21	0.67
L_{10}	0.48	0.17	0.55
L_{11}	0.39	0.11	0.41
Mean	0.93	0.42	0.86
SEd	0.02	0.01	0.02
CD (P=0.05)	0.04	0.02	0.04

CAT- Catalase; SOD- Super oxide dismutase; GR- Glutathione reductase.

Table 5: Evaluation of physiological and biochemical seed quality parameters in groundnut seed lots.

Seed lots	Speed of germination	Root length (cm)	Shoot length (cm)	Dry matter production (g 10 seedling ⁻¹)	Vigour index	EC of seed leachate (dSm ⁻¹)	Dehydrogenase activity (OD value)
L ₁	4.5	10.3	15.5	3.156	2374	0.592	2.648
L ₂	4.1	9.8	15.1	3.095	2241	0.620	2.452
L ₃	3.7	9.7	14.6	2.965	2073	0.672	2.307
L ₄	3.4	9.2	14.0	2.743	1904	0.715	2.235
L ₅	3.0	9.2	12.5	2.630	1709	0.723	2.104
L ₆	2.6	8.4	12.2	2.524	1555	0.798	1.852
L ₇	2.3	8.1	11.8	2.493	1313	0.845	1.436
L ₈	2.1	7.5	10.7	2.413	826	0.992	1.358
L ₉	1.8	5.7	9.2	2.321	507	1.202	1.108
L ₁₀	1.2	4.5	8.4	2.101	284	1.436	0.852
L ₁₁	0.8	3.2	7.7	1.923	131	1.713	0.502
Mean	2.7	7.8	12.0	2.579	1356	0.937	1.714
SEd	0.11	0.12	0.29	0.056	35.76	0.029	0.036
CD (P=0.05)	0.22	0.25	0.59	0.116	74.17	0.059	0.073

EC- Electrical conductivity.

polyunsaturated fatty acids, free radical peroxidation via auto-oxidation, lipo-oxygenase and hydrolytic damage (Doijode, 1985). Electrical conductivity of all the 11 seed lots measured in this experiment is in line with the results of ATR-FTIR and qualitative analysis of oil extracted from the groundnut seeds.

Dehydrogenase enzyme activity was measured in the form of optical density (OD) value using a spectrophotometer. The OD value is a measure of the degree of aliveness of the seed from which the sample is extracted for quantification. In this experiment, the seed lot L₁ recorded significantly higher value (2.648). While the seed lot L₁₁ recorded lower value (0.502) of dehydrogenase enzyme activity compared with other seed lots (Table 5). The dehydrogenase enzyme plays a vital role in germination and seedling emergence. Dehydrogenase enzyme is an indicator of living tissue and directly related with loss of viability and good stable metabolic marker to estimate the degree of vigour in seeds. The activity of dehydrogenase enzyme which is responsible for respiration of the seed reduced with the ageing of the seeds, which is also used as a vigour indicator (Suganthi and Selvaraju, 2017).

The experimental results of the present study suggest that the enzymatic changes with reference to the various level of seed germination ranging from 92 to 12 per cent also indicated that the activity of Catalase, super oxide dismutase, glutathione reductase and Dehydrogenase enzyme activity decreased and also closely correlated with FTIR spectra data to assess the loss of seed quality.

CONCLUSION

Fatty acids are credible indicators of groundnut seed quality. The type of chemical reactions that may occur in the oilseeds and its viability is completely dependent on the fatty acids present and its bonding patterns. In seeds with higher oil

content, lipid auto-oxidation is a phenomenon occurring during ageing which leads to undesirable changes in the fatty acid contents. Such degradation processes result in the loss of seed viability; hence, a less time consuming device or method is warranted for periodical monitoring of the changes in fatty acid composition of stored oilseeds. This kind of device or method will be useful in view of taking quick decision to prolong the storage period of oilseeds or to dispose the seeds for sowing or edible purpose. Application of ATR-FTIR spectroscopy is well-established for rapid, high-throughput, analysis of quality of various products. In this experiment feasibility and potential of ATR-FTIR spectroscopy to assess the groundnut seed quality by assessing the functional group variation associated with oils extracted from the groundnut seed was tested. As a support of ATR-FTIR results of this experiment, oil quality parameters, physiological and biochemical parameters of groundnut seeds were analysed and compared. As an outcome of this experiment, we confirmed ATR-FTIR technique is a rapid, less time consuming and reagent-free method to monitor the groundnut seed quality during storage. Further, FTIR spectroscopy will considerably improve the accuracy and reliability of fatty acid characterization, identification and thus leads to determination of quality of the oilseeds.

Conflict of interest: None.

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