DETERMINATION OF 2-CHLOROETHYL PHOSPHONIC ACID (CEPA) CONTENT IN LATEX STIMULANT BY USING HEAD-SPACE GAS CHROMATOGRAPHY WITH FLAME IONISATION DETECTOR (HS-GC-FID)

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ABSTRACT

2-chloroethylphosphonic acid (CEPA) is a well-known stimulant commonly used to improve latex production by the rubber tree (Hevea brasiliensis). It is often known as Ethephone but also available commercially as 'Ethrel', 'Florel', 'Cerone', 'Prep', 'Bromoflor', 'Flordimex', 'Camposan', 'Etheverse', and 'Tomathrel'. Some rubber latex stimulant producers claim that they produced good stimulants and met the CEPA content as declared on the packaging. The claim was made based on the analysis done on the starting ingredient added in the mixture or finished product using Thin Layer Chromatography (TLC) technique which is a semi-quantitative method based on visual comparisons or spot intensity matching. CEPA rapidly decomposed into ethylene under alkaline and high-temperature environments. The conventional practice in the laboratory to quantify CEPA was by indirect titration of dihydrogen phosphate with sodium hydroxide. The objective of this study was to develop a rapid, easy, and both qualitative as well as a quantitative method for analysing CEPA content in latex stimulants using Gas chromatography (GC) with a Flame Ionisation Detector (FID). The ethylene gas evolved from the sample was detected and quantified using this technique which was based on the headspace sampling. In this test method, weighed quantities of the latex stimulants sample were dissolved in 2.5M NaOH solution in vials and introduced into the headspace oven. The ethylene generated was directly injected into the GC via a capillary tube enclosed in a heated transfer line. The separated components were detected and quantified by the detector. The working range for a linear calibration curve of this method is from 0.2% to 5.0% of active ingredients in the stimulant. In order to establish a reproducible GC method using a headspace sampler, some works were carried out by analysing a series of stimulants with different concentrations of CEPA ranging from 1.5% to 5.0%. Then, method validation activities were also conducted by determining the accuracy/recovery (99.39% to 128.85%), working range and limit of detection (LOD) of the test method (0.7%).

Keywords: headspace, gas chromatography, 2-chloroethylphosphonic acid (CEPA), latex, stimulant,

INTRODUCTION

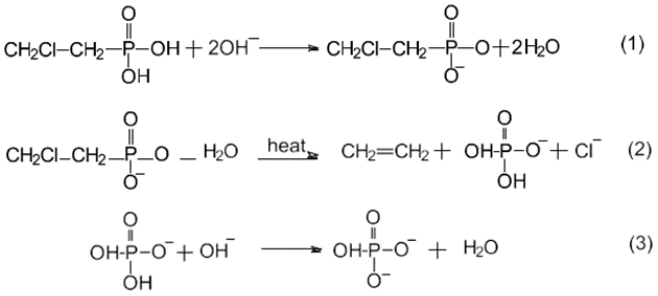
Natural rubber *(Hevea brasiliensis)* latex is obtained from its bark. The latexis divided into three layers after ultracentrifugation: C-serum, lutoids, and rubber particles (Zhang, 2009). A variety of compounds will improve latex flow when applied to the bark, right below the tapping location. 2-chloroethylphosphonic acid (CEPA), also known as Ethephon, Ethrel, Florel, Cerone, and Etheverse is a plant growth regulator that is frequently used. CEPA is used commercially to prolong the flow of latex from the rubber tree after tapping (yield stimulation) (Audley, 1976). The use of CEPA as stimulants has revolved around rubber, palm oil, fruits, and food industry. Abraham et. al. has studied the stimulant action of CEPA and acetylene on the yield of *Hevea brasiliensis.* This study revealed that Ethrel produces a high yield and is a promising stimulant, especially for systems with low tapping intensity (Abraham et. al, 1971). For many years, this stimulant boost latex yield by delaying the plugging index which lengthens latex flow

time after tapping (Budiasih et.al, 2020). Other studies have also indicated that utilizing the stimulant system will reduced routine frequency of tapping (R. Lacote et.al 2010, M.S Traore et.al, 2011)

CEPA is stable in aqueous solutions with pH values less than 3.5, although breakdown occurs at higher pH levels due to ethylene decomposition. It is also vulnerable to UV rays. (Royal Society of Chemistry, 1987). Previously, a Thin Layer Chromatographic (TLC) technique was developed and described for the estimation of CEPA, a constituent of Ethrel in palm oil. However, it showed relatively low sensitivity and the development of the chromatogram was also relatively long (Yong, 1971). Later, the methylated phosphonic acid compound was identified using gas chromatographic techniques for ethephon residue analysis by using a flame photometric detector. Simplified extraction and clean-up procedures described in published papers are time-consuming. (Ernst, 1976). A rapid and simple testing procedure based on the amount of ethylene produced from ethephon at pH values of 12-14 has been developed. This test method is suitable for routine analysis of ethephon residues in agricultural products, obviating the need for time-consuming extraction, clean-up, and derivatization stages (Hunter, 1978, Hemmerling, 1997, Krautz, 1990).

Under alkaline and high temperature circumstances, ethephon decomposes to ethylene and dihydrogen phosphate. The quantity of released ethylene or titration dihydrogen phosphate with sodium hydroxide can be used as indirect methods for determining ethephon. The CIPAC (Collaborative International Pesticide Analytical Council) titrimetric method for determining ethephon in pesticide formulations gave the following sequence reactions as in Figure 1.

Figure 1. Sequence reactions during determination of ethephon in pesticide formulations according to CIPAC titrimetric method: neutralization (Equation 1), thermal decomposition of ethephon (Equation 2), and titration of dihydrogen phosphate formed (Equation 3). (Su-Hsiang Tseng, 2000)



The majority of latest prior research (Chamkasem, 2017, de Souza et al., 2019, Vemula et al., 2020, Maragou and Balayiannis, 2020, Chowdhury et al., 2020) have revolved around the determination of CEPA in fruits and pesticide formulation using various chromatographic techniques including gas chromatography but none for latex stimulant sample. Therefore, the objective of this study was to develop a rapid, easy, qualitative, and quantitative method for analysing CEPA content in latex stimulants using Gas chromatography (GC) with a Flame Ionisation Detector (FID) using a headspace sampler. Headspace sampler attached to gas chromatography with flame ionisation detector (HS-GCFID) is a sampling technique that involves indirect determination of volatile constituents in liquid or solids by analysing the vapour phase in thermodynamic equilibrium in the close system.

Additional objectives of the study were to produce a reproducible GC method using a headspace sampler and validate the test method produced. A series of stimulants with different concentrations of CEPA were evaluated using HS-GCFID and the test method developed was validated through evaluating several method validation parameters which include linearity, accuracy/recovery and detection limit.

MATERIALS AND METHODS

Materials

Test samples including commercially available latex stimulants were purchased from Malaysian markets.

Reagents

CEPA (Ethephon) was purchased from Sigma-Aldrich with a purity of ≥ 98.0% while Acetic acid (glacial) from Merck with a purity of 100%.

Methods

a. Preparation of standard solution

100 mg of CEPA standard was weighed and dissolved with dilute nitric acid at pH 3 and mark up to 10 ml in a volumetric flask and marked as stock solution. Standard solutions were prepared by diluting the stock solution to produce a series of standards of 0.2%, 0.5%, 1.0%, 2.5%, and 5.0% respectively.

b. Calibration curve

Calibration curve of the CEPA was established by measuring the concentration of series of CEPA solutions using headspace gas chromatography. Five different concentrations of CEPA 0.2%, 0.5%, 1.0%, 2.5%, and 5.0% respectively, were prepared and analysed. About 0.1 ml of known concentration of the standard solution and 0.1 ml of 2.5M sodium hydroxide (NaOH) were added into the headspace vial. The vial was quickly sealed and placed on the vortex mixer for homogenisation of standard solution prior to introduction of mixture into the headspace oven for 25 minutes at 70 °C. Then, the standard solutions were analysed by gas chromatography with flame ionisation detector. A calibration curve was constructed by plotting ethylene peak area against the known CEPA concentration.

c. Preparation of samples for CEPA determination

The sample was prepared by taking 0.1 ml of latex stimulant sample and 0.1 ml of 2.5M sodium hydroxide (NaOH) was added into the headspace vial. The vial was quickly sealed and placed on the vortex mixer for homogenisation of sample mixture prior to introduction of the sample into the headspace oven for 25 minutes at 70°C. Then, the samples were analysed by gas chromatography with flame ionisation detector.

d. *Headspace GC set up*

Perkin Elmer Headspace GC equipped with flame ionisation detector (FID). For the headspace setting, the needle temperature was set to 90°C, transfer temperature 110°C, and HS oven temperature was set to 70°C. The GC was set with a constant helium flow rate of 30 mL/min and the air flow rate was 450 mL/min. Column used was Agilent Elite – Plot Q (30m x 0.53mm ID x 40um df). The GC oven was set to initially start at 65°C for 0.1 min with 10°C/min rates then end at 150°C with 30 min holding time. The FID temperature was set to 250°C.

e. Validation of the test method

Validation of the test method was done by performing recovery works and determination of limit of detection (LOD)

i. Accuracy/Recovery

Recovery work was carried out using CEPA standard. Standard solutions were prepared by diluting the stock solution to produce a series of standards of CEPA 0.2%, 0.5%, 1.0%, 2.5%, and 5.0% respectively. About 0.1 ml of known concentration of the standard solution and 0.1 ml of 2.5M sodium hydroxide (NaOH) were added into the headspace vial. The vial was quickly sealed and placed on the vortex mixer for homogenisation of standard solution prior to introduction of mixture into the headspace oven for 25 minutes at 70 °C. Then, the standard solutions were analszed by gas chromatography with flame ionisation detector. The recovery was calculated using the following formula:

Recovery (%) = (Observed concentration)/(Actual concentration)×100

ii. Limit of Detection (LOD)

LOD is the lowest amount of the investigated compound in a sample that can be detected but not necessarily quantified with an acceptable uncertainty. In this method, the LOD was determined based on the calculation using the standard deviation of the response and the slope method (A.Ozkan, 2011).

The LOD for this method can be expressed by the following equation:

$$LOD = \frac{3.s}{m}$$

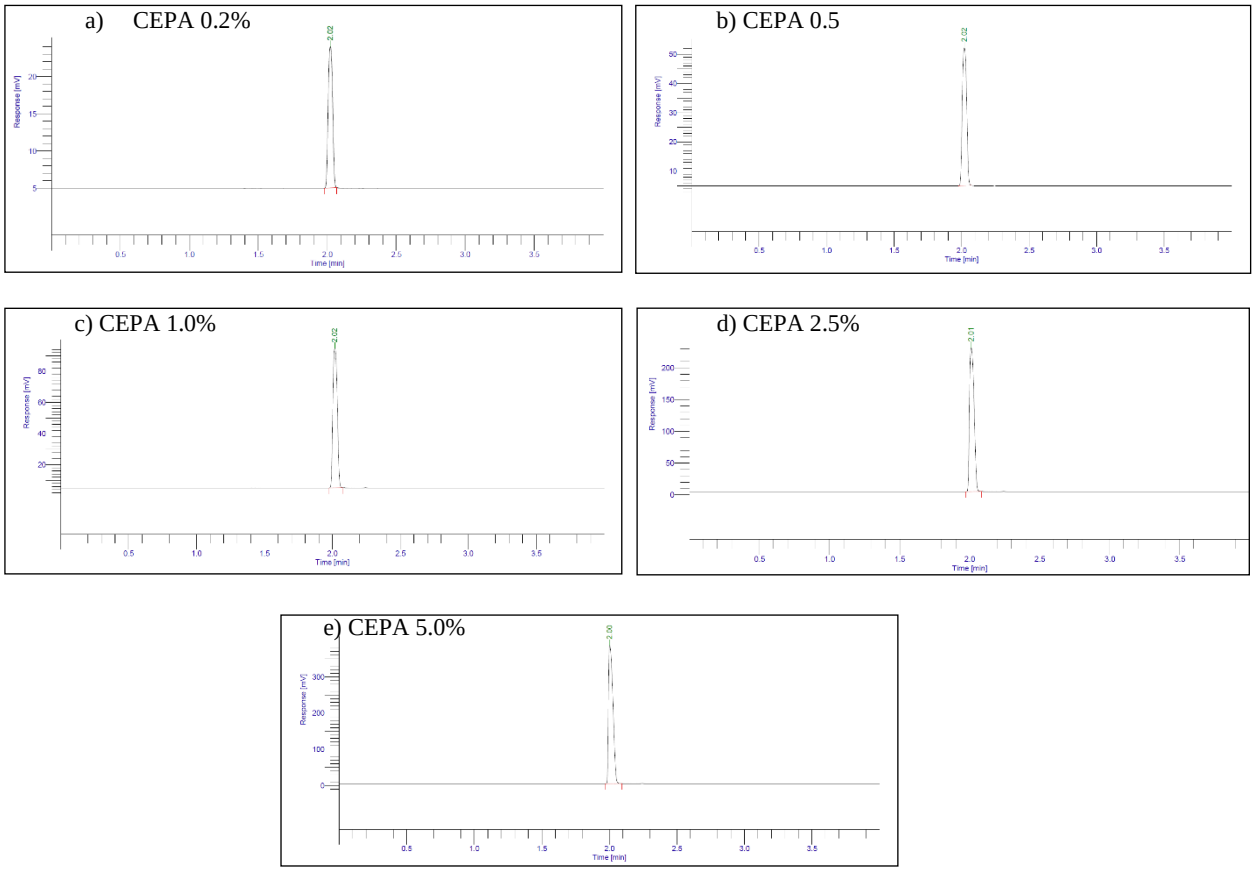
where s is the standard deviation and m is the slope of the related calibration line.

RESULTS AND DISCUSSIONS

Identification and quantification of CEPA using HS-GC-FID

In this study, the identification of CEPA peak was obtained at the retention time of 2.0 min. Complete degradation of the original compound is the main factor to ensure the reaction is thorough. From the results obtained, it is shown that the peak height and area increase with the increasing of concentration of CEPA as shown in Figure 2.

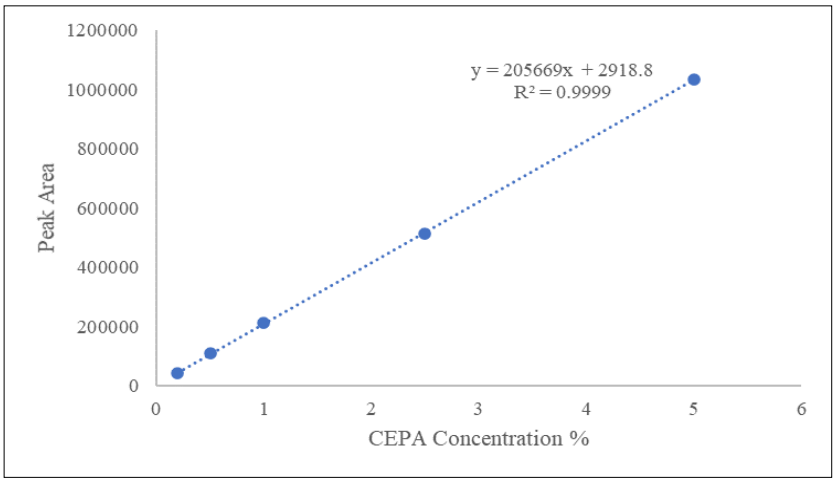
Figure 2. GC Chromatograms of CEPA standard with different concentrations.



Linearity and range

The calibration curve shown in Figure 3 was constructed from a few series of standard concentrations of CEPA. Good linearity was achieved in the concentration range of 0.2% to 5.0%. The regression equation was determined by plotting the peak area (y) against the concentration of CEPA (x): y= 205669x + 2918.8 (R^2) = 0.9999). The experiment was carried out in triplicate and shows an excellent relationship between peak area and concentration.

Figure 3. Calibration curve for CEPA.



Limit of Detection (LOD)

In this study, the determination of LOD was also established by using a linear regression approach whereby it was calculated based on triplicate analysis of CEPA standard at five different concentration levels of 0.2%, 0.5%, 1.0%, 2.5%, and 5.0%, respectively.

The LOD of 0.7% has been established for this method. Thus, the concentration of CEPA less than 0.7% should be considered as not detected.

Quantitative determination of CEPA content by substitution into the linear equation

Quantitative determination of CEPA content was performed by constructing a calibration curve using an external standard and subsequently establishing the linear regression equation. The standards used are known as "external standards" due to the reason of preparation and analysis of standard and sample were done separately. A series of standard solutions of known concentration was subjected to the determination by HS-GC-FID instrument and the peak response expressed as area was plotted against the concentration of the standard solution (Figure 3). From HS-GC-FID analysis, the percentage of CEPA in each sample was calculated by substituting the value obtained into the linear equation of y= 205669x + 2918.8. The concentration of the analyte can be determined directly from the calibration plot.

The recovery experiment was carried out to evaluate the accuracy of the method. Based on this linear equation, recovery of the known standard solution was also calculated and found to range between 99.39% to 128.85%. Although the calibration curve was linear, the recovery for 0.20% and 0.50% of standard concentration were unreliable. This again supports the LOD of 0.7% and values below LOD should be considered as not detected. The % RSD obtained from the analysis were acceptable with the recorded values of less than 1%. A satisfactory recovery and good precision were obtained from this proposed method.

Table 1. The concentration of known CEPA standard expressed in percent (%)

Replicate	Concentration standard (%)	Actual concentration Obtained (%)	Accuracy/ Recovery (%)	Average (%)	Standard Deviation	%RSD
1	0.20	0.25	126.80			
2	0.20	0.26	128.85	0.26	0.00	0.87
3	0.20	0.25	127.10			
1	0.50	0.57	113.64			
2	0.50	0.56	112.40	0.57	0.00	0.79
3	0.50	0.57	114.14			
1	1.00	1.05	105.23			
2	1.00	1.03	103.29	1.04	0.01	0.94
3	1.00	1.04	103.98			
1	2.50	2.49	99.70			
2	2.50	2.53	101.02	2.52	0.02	0.86
3	2.50	2.53	101.32			
1	5.00	4.97	99.39			
2	5.00	5.00	100.04	4.99	0.02	0.44
3	5.00	5.01	100.23			

The percentage of CEPA in commercial latex stimulants can be further confirmed by substituting the concentration obtained into the linear equation from the calibration plot. The value obtained by using this method was compared to the percentage of CEPA declared on the packaging of the latex stimulant. The concentrations of the commercial latex stimulant obtained are listed in Table 2 below. It was observed that the CEPA concentrations for both brand A and brand B were in the expected range. It can thus be said that HS-GC-FID is the precise method to evaluate CEPA concentration in latex stimulant.

Table 2. Determination of CEPA in commercial latex stimulant samples

Latex Stimulant	Concentration of CEPA (%)			
Brand A	Replicate 1	Replicate 2	Replicate 3	Average
A (1.5%)	1.42	1.46	1.45	1.47
B (2.5%)	2.42	2.46	2.52	2.47
C (5.0%)	4.62	4.64	4.69	4.63
Brand B	Replicate 1	Replicate 2	Replicate 3	Average
A (2.5%)	2.22	2.01	1.81	2.00
B (5.0%)	4.71	4.86	4.97	4.87

This project was carried out to establish a suitable method for determining the percentage of CEPA present in latex stimulants. The successful of this test method and procedure was illustrated in the form of tables and figures as shown above.

CONCLUSION

In conclusion, the HS-GC-FID method was effective and suitable to be used for the determination of CEPA content in latex stimulants. The sample was prepared by taking 0.1 ml of latex stimulant, 0.1 ml of 2.5M sodium hydroxide (NaOH) into a vial and homogenised prior to introduction into the headspace oven for 25 minutes at 70°C. The GC was set with a constant helium flow rate of 30 mL/min and the air flow rate was 450 mL/min with Agilent Elite – Plot Q (30m x 0.53mm ID x 40um df) column was used. The GC oven was set to initially start at 65°C for 0.1 minutes with 10°C/min rates then end at 150°C with 30 minutes holding time and the FID temperature was set to 250°C. The analysis time took about five minutes and the presence of CEPA can be seen after two minutes (retention time). The test method was successfully validated with an established LOD of 0.7%. Five-point calibration curve showed strong correlation with the respective R^2 value of 0.9999. The recovery of the method is good, ranging from 99.39% to 128.85%. It was observed that the CEPA concentrations for both brand A and brand B were closed with the declared concentration by the supplier. Therefore, this method is considered as accurate to determine the CEPA content qualitatively and quantitatively.

REFERENCES

Abraham, P. D., Blencowe, J. W., Chua, S. E., Gomez, J. B., Moir, G. F. J., Pakianathan, S. W., ... & Wycherley, P. R. (1971). Novel stimulants and procedures in the exploitation of Hevea. II. Pilot trial using (2-chloroethyl)-phosphonic acid (ethephon) and acetylene with various tapping systems. *Journal of the Rubber Research Institute of Malaya*, 23(2), 114-137.

Audley, B. G., Archer, B. L., & Carruthers, I. B. (1976). Metabolism of ethephon (2-chloroethylphosphonic acid) and related compounds in Hevea brasiliensis. *Archives of environmental contamination and toxicology*, 4(1), 183-200.

Budiasih, R., Salim, M. A., Apriani, I., Hasani, S., & Subandi, M. (2020). Effect of stimulant (ethephon) application and tapping frequency on latex production of rubber tree (Hevea brasiliensis Muell. Arg.). *Bulgarian Journal of Agricultural Science*, *26*(4), 793-799.

Chamkasem, N. (2017) Determination of glyphosate, maleic hydrazide, fosetyl aluminum, and ethephon in grapes by liquid chromatography/tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, 65(34), 7535-7541.

Chemistry., R. S. (1987). *The Agrochemicals Handbook. 2nd ed.* United Kingdom: Unwin Brothers Limited, Surrey.

Chowdhury, M. G. F., Khan, M. H. H., Miaruddin, M., Rahman, M. A., & Sabuz, A. A. (2020). Effect of ethephon on ripening and postharvest quality of tomato during storage. *Bangladesh Journal of Agriculture Research*, 45(3), 303-314.

De Souza, A. L. B., Silva, J. W. S. A., Silva, H. C. M. P., Cabral, A. G. S., Filho, C. A. A., de Lima, E. N., & de Sousa, C. E. M. (2019). A rapid and sensitive single residual method for determination of ethephon in grape by high performance liquid chromatography-tandem mass spectrometry. *Brazilian Journal of Health Review*, 2(6), 4937-4947.

Ernst, G. F., & Anderegg, M. J. (1976). Rapid gas-liquid chromatographic method for determining residues of ethephon (2-chloroethyl phosphonic acid) in tomatoes. *Journal of the Association of Official Analytical Chemists*, *59*(5), 1185-1187.

Gumustas, M., & A Ozkan, S. (2011). The role of and the place of method validation in drug analysis using electroanalytical techniques. *The Open Analytical Chemistry Journal*, *5*(1), 1-21.

Hemmerling, C., & Seidl, G. (1997). Rapid determination of ethephon residues in food with headspace gas chromotography. *Deutsche Lebensmittel-Rundschau (Germany)*, 93 : 239-242

Hurter, J., Manser, M., & Zimmerli, B. (1978). A rapid and simple method for the determination of residues of 2-chloroethylphosphonic acid (ethephon) in tomatoes, cherries, and apples. *Journal of agricultural and food chemistry*, *26*(2), 472-475.

Krautz, S., & Hanika, G. (1990). Simple and rapid determination of ethephon in fruit, vegetables, and grain by headspace analysis and gas chromatography. *Nahrung*, *34*, 569-570.

Kurtz, B. T. (1961). Calcium and Magnesium determinations by EDTA titrations. *Soil Science Society Proceeding* , 27-29.

Lacote, R., Gabla, O., Obouayeba, S., Eschbach, J. M., Rivano, F., Dian, K., & Gohet, E. (2010). Long-term effect of ethylene stimulation on the yield of rubber trees is linked to latex cell biochemistry. *Field crops research*, *115*(1), 94-98.

Su Hsiang Tseng(2000). A Rapid and Simple Method for the Determination of Ethephon Residue in Agricultural Products by GC with Headspace Sampling. *Journal of Food and Drug Analysis, 8(3)*, 213-217.

Traoré, M. S., Diarrassouba, M., Okoma, K. M., Dick, K. E., Soumahin, E. F., Coulibaly, L. F., & Obouayeba, S. (2011). Long-term effect of different annual frequencies of ethylene stimulation on rubber productivity of clone GT 1 of Hevea brasiliensis (Muell. Arg.) in south east of Côte d'Ivoire. *Agric. Biol. JN Am*, *2*(8), 1251-1260.

Vemula, M., Sreedashyam, H., Shaikh, A. S., & Upadhyayula, V. (2020). Determination of 2-chloroethylphosphonic acid and its intermediate vinylphosphonic acid from artificially ripened sapota fruit by modified dispersive solid-phase extraction cleanup and gas chromatography/mass spectrometry analysis. *Rapid Communications in Mass Spectrometry*, 34(21), e8907.

Yong, W. (1971). Determination of 2-Chloroethylphosphonic acid (Ethepon) in palm oil by Thin-Layer Chromatography. *Journal of Chromatography*, 60, 219-224.

Zhang, J. Z. (2009). Ethylene stimulation of latex production in Hevea brasiliensis. *Plant Signaling & Behavior*, 4(11), 1072-1074.