DETERMINATION OF 5-HYDROXYMETHYL FURFURAL IN APPLE JUICE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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BEING A PROJECT SUBMITTED TO THE DEPARTMENT OF SCIENCE LABORATORY TECHNOLOGY, COLLEGE OF SCIENCE AND TECHNOLOGY, ADAMAWA STATE POLYTECHNIC, YOLA. IN PARTIAL FUFILMENT OF THE REQUIREMENT FOR THE AWARD OF NATIONAL DIPLOMA IN SCIENCE LABORATORY TECHNOLOGY.

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APPROVAL PAGE

This is to certify that this project work entitled "Determination of 5-hydroxymethyl furfural in apple juice using high performance liquid chromatography has been prepared in accordance with the Regulations governing the preparation and presentation of projects in Adamawa State Polytechnic,Yola and approved as meeting the requirements for the award of National Diploma in Science Laboratory Technology.

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DEDICATION

We dedicate this research work to God Almighty, the creator of the universe and the finisher of our faith.

ACKNOWLEDGEMENT

Our profound gratitude goes to Almighty God, for his mercy and guidance upon our life and for seeing us through the period of our study and project work for which we are truly grateful.

We are also grateful to our beloved and kind hearted supervisor Mrs. Maryam Usman Aminu, whose wealth of knowledge we have greatly benefitted from. May God Almighty bless and reward her abundantly in all her endeavors.

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Finally, our special thanks goes to all lecturers and the entire students of Science Laboratory Technology, Adamawa State Polytechnic, Yola.

ABSTRACT

The goal of this work was to develop a simple and rapid preparation for 5-HMF analysis in apple juice. 5-Hydroxymethyl furfural [5-HMF] regularly occurs in foods and in apple juice beverages. The risk of 5-HMF associated with apple juice consumption has not been systematically studied, so that this study will provide the first quantitative risk assessment of 5-HMF for consumers of apple juice beverages. The chromatography conditions showed good separation. 5-HMF was determined in all of the examined samples. Based on our research 4 samples of apple juice were used to determine the presence of 5-HMF. 100% apple juice had less 5-HMF value. With 18.8mg/kg while 50% apple juice samples concentration of 5-HMF was higher than maximum prescribed value by food drug Agency [F.D.A]. with 48.5mg/kg.

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5-HMF = Five Hydroxymethyl Furfural	1
WHO = World Health Organization	1
IFFPs = International Federation of Fruit juice Producers	1
EU = European Union	1
HPLC = High Performance Liquid Chromatography	2
MERKC = Mackellar Electro kinetic Capillary Chromatography	8
GC = Gas Chromatography	8
LC = Liquid chromatography	8
UVS = Ultra-violet Spectroscopy	8
SDS = Sodium Dodecyl Sulphate	8
LA = Levulinic Acid	9
LOQ = Limited Quantification	9

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

5-hydroxymethyl furfural (5-HMF) is an organic compound formed by the dehydration of certain sugars. It is white low-melting solid (although commercial samples are often yellow) which is highly soluble in both water and organic solvents.

In recent years, there has been a great concern in the apple juice industry on the quality of apple juice products. The study of 5-HMF in food stuff has received special attention because it has been found to exhibit mutagenic and DNA strand-breaking activities (Akkaya and Karatas, 2013). Besides the cytotoxic and nephrotoxic effects, carcinogenic and genotoxic effect are among the activities attributed to presence of 5-HMcF in apple juice (lee et,al 2014).

The toxological effects of 5-HMF is still undergoing investigation. In fact the International Federation of Fruit Juice Producers (IFFPs) suggested the maximum concentration of 5-HMF in fruit juice as 5-10mg/l and 25mg/l for fruit concentrates, (Gasper and Lucenna, 2009). On the other hand, the codex and alimentary of the World Health Organization (WHO), and the European Union (EU, Directive 110, 2001) have established the maximum concentration of 5-HMf to be 50mg/kg.

5-HMF is an aldehyde which can be used as an indicator of apple juice quality, since the presence of HMF is considered as an indicator of quality determination.

It is a characteristic of flavor compound of the Maillard reaction, and a result of complex series of reaction between amino acids and reducing sugars (Hexoses).

5-HMF is particular is not present in fresh fruit, but it is naturally created in sugar containing foods during heat treatments. Formation of 5-HMF arise with processing and is especially dependent on the storage conditions, temperature and PH. Suitable conditions for the formation of 5-HMF are high concentration of saccharides (mainly Hexoses), lower pH value, presence of organic acid, low water activity and the concentration of 5-HMF also increases during the heating or storage process. (Lanselotmatra,2003). It is commonly found in honey, apple juice, coffee and dried fruit.

Food products are subjected to thermal treatments to obtain desirable sensory properties of texture features, assure microbiological safety and climate enzymatic activities (Kowaska, 2013). 5-HMF is one of the most known Amadori compounds found during the thermal treatment of carbohydrate containing food as a result ofMaillard reaction. Therefore, 5-HMF is generally known as an indicator of quality deterioration as a result of excess heating or storage in a wide range of foods (Mandoza,2002).

The following factor influence the formation of 5-HMF in food

- Carbohydrate content
- Thermal treatment
- Water activity
- Long term storage
- Use of metallic container and
- Physiochemical properties of pH and total acid

Different analytical techniques have been investigated for the determination of 5-HMF. Among the different techniques available, colorimetric and chromatography are most commonly used. High performance liquid chromatography (HPLC) method is widely used in 5-HMF analysis. This is because it is easier and faster than others. It consists of only sample dilution and

filtration. High performance liquid chromatography (HPLC) will be used as an analytic to determine the level of 5-HMF in different samples of apple juice. The information obtained in this research will be compared with the maximum 5-HMF limits established by the world health Organization (WHO).

1.2 STATEMENT OF THE PROBLEM

The processing of apple juice industries is being faced with a great task of trying to maintain the quality of the juice they produce. 5-HMF is one of the main things which cause deterioration in apple juice quality due to high temperature during processing and storage. If the level of 5-HMF is higher than the maximum limit given by the World Health organization (WHO), it could lead to carcinogenic effects. This makes the determination in apple juice industry highly necessary. High Performance Liquid Chromatography is a simple content and precise method that can be used for determining the 5-HMF, level in apple juices. In this work therefore, the researchers determine the level of 5-HMF in apple juices using High Performance Liquid Chromatography.

1.3 AIM AND OBJECTIVES

The aims and objectives of this research are:-

i. To determine the level of 5-HMF in locally processed apple juice using High Performance Liquid Chromatography (HPLC).

ii. To determine the presence of 5-HMF in industrially produced apple juice using High Performance Liquid Chromatography (HPLC).

iii. To compare the level obtained in the samples with that of the World Health Organization (WHO).

1.4 SIGNIFICANCE OF THE STUDY

The determination of 5-HMF will be of immense benefits to many fruit juice industries. By producing their information on their content in raw-material

and finished juice products, food borne diseases as a result of consuming deteriorated apple juice products will be reduced.

1.5 SCOPES OF THE STUDY

The scope of this study included:-

i. Sample collection
ii. Washing of samples
iii. Cutting of fresh apples
iv. Blending of apples
v. Sieving of juice
vi. Determination of the level of 5-hydroxymethyl furfural.
vii. Comparing the result obtained with that of the World Health Organization.

1.6 RESEARCH QUESTIONS

This study attempted to provide answers to the following questions

i. What is the level of 5-HMF present in locally processed apple juice?
ii. What is the level of 5-HMF present in industrial apple juice?
iii. How does the level of 5-HMF in apple juice sample compare with that of the World Health Organization (WHO).

1.7 DEFINITION OF TERMS

i. 5-hydroxymethyl furfural: is an organic compound formed by the dehydration of certain sugars.
ii. Apple is an edible fruit
iii. Apple juice: is processed apple fruit, its extract as the given name apple juice.
iv. High Performance Liquid Chromatography (HPLC): is a Technique that is used to separate, identify and quantify each components ina mixture.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 APPLE JUICE

Apple juice is a fruit juice that is made by maceration and pressing of an apple. The resulting expelled juice may be further treated by enzymatic and centrifuge classification to remove the starch and pectin, which holds fine particulate in suspension, and then pasteurized for packaging in glass, metal or aseptic processing system containers or further treated by dehydration process.

2.2 APPLE JUICE INDUSTRY

In recent years, there has been a great concern in the apple juice industry on the quality of apple juice products. The study of 5-HMFin food stuffs has received special attention because it has been found to exhibit mutagenic and DNA strand-activities [AK Kaya and Karatas, 2013]. Besides that, cytotoxic, nephrotoxic and carcinogenic effects are among the activities attributed to 5-HMF [Lee, et al 2014].

Below are some companies that produce apple juice in Nigeria.

- CHIVITA Limited: A Nigeria food services manufacturer and maker of Chivita Juice Drinks Located in Lagos.
- DANSAM: Makers of brand of juice in Nigeria located in Lagos.
- LACASERA Company PLC: Offers Production of Soft Drinks and Lacasera Fruit Drinks Products, Pocated in Lagos.
- UAC Diaries: The Nigerian Fruit Drinks Manufacture and Makers of delight berries fruit drink juice product located in Lagos.
- DEAN Fruit drinks: Nigerian limited is into the manufacturing of natural and synthetic fruit drinks such as orange, Apple, Pineapple, Mixed fruit juice located in Lagos.

- ADAMA beverages limited [FARO]: also deals with the manufacture of apple and fruit juice located in Adamawa state Kofere Jimeta local government Yola e.t.c.

2.3 STORAGE OF APPLE JUICE

Fresh apple juice requires refrigeration in sealed bottles. If canned apple juice can be stored in dark, cool place, such as cupboard, to delay the degradation of the product. The appearance, texture, or taste of the juice might change over time.

Once the packaged juice is opened, it must be re-sealed tightly and refrigerated to avoid contamination from micro-organisms such as bacteria. The ideal storage temperature for apple juice is between 0- 4°C Depending on the original package of the apple juice.

2.4 5-HYDROXYMETHYL FURFURAL

Carlos and Rostelk, (2011), described 5-Hydroxymethylfurfural, as a common product of the maillard reaction and can be found in many foods, such as beverages. The structure of 5-HMF is showed below.

This compound has been shown to be a good indicator for heat processing of industrially manufactured foods. The toxological relevance of 5-HMF for humans is not yet fully elucidated. At high concentrations that are not nutritionally relevant, 5-HMF is cytotoxic and causes irritation to the eye, the upper respiratory tract and the skin. Relatively high concentrations of 5-HMF were found in some products such dried fruits and instant coffee powder.

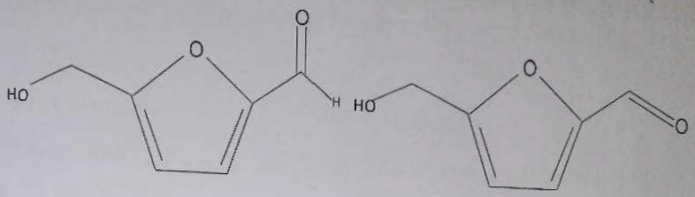
5-HMF is an indicator for spouted, adulated or products that were exposed to heat, stress or bad storage condition. On the other hand, 5-HMF is considered as irritant and is irritating to eyes. Toxological effects caused by 5-

HMF in mammals generally ranged from 50 to 5000mg/kg body with generally ranged from 500 to 5000mh/kg by weight (Hatim, 2008).

Acceptable unit for 5-HMF have been determined to establish a certain level of purity in potential solution. In general, up to Img of 5-HMF of carbohydrate is allowed in potential solutions (Anget, et, al 2008). The united kingdom have established a maximum for 5-HMF and related substance in glucose in fusion solution (1.09ml anhydrous glucose, 25ml aqueous solution), the ultra-violet absorbance of the solution of 284mm should not be exceed 0.25 which corresponds to 1,904mol/l of 5-HMF (Wies lander et, al 2007) the 5-HMF maximum specifically in the united kingdom except that 19, quantity of glucose is hydrate, 5-HMF level have been determined in food and acceptable daily intake (ADI's of 2mg) of 5-HMF level have been recommended for humans (Laiter et, al 2005) such ADI contributed a 4 or fold margin safety (Ulrich et, al 2007). A maximum of 100mg of 5-HMF per liter of syrup containing highly inverted sugar was recommended for preparation of non-alcoholic liquid (Simon Yam, 2009).

5-HMF level has been determined in different samples obtained from honey, fruit juice preserved and upper respiratory track, and skin and mucous membrane. No positive or negative reports associating 5-HMF with cancer risk in human have been identified in available literature (FPA, 2006). However the national institute of environmental health science nominated 5-HMF for testing base on the extensive human exposure, lack of adequate data characterizing its toxicity and carcinogenicity (Miller, 2012).

This ongoing reports about 5-HMF toxicity whether in humans or animals makes the determination of 5-HMF in goods and drinks an important concern at present time before solid toxicity report can be documented.



Molecular Formula: C6H6O3

2.5 METHODS OF DETERMINIG 5-HMF

Several methods have been used in the determination of 5-HMF some of which is includes:

1. Mackellar Electro kinetic Capillary Chromatography (MERKC)
2. Gas chromatography (GC)/ Ultra-violet spectrometry analysis
3. Liquid chromatography (LC)/ Ultra-violet spectroscopy (UV)
4. High Performance Liquid Chromatography (HPLC).

2.6 MICELLESELECTRO KINETIC CHROMATOGRAPHY (MEKC)

Rosa et al, (2011) carried out the determination of 5-HMF by MEKC methodology, using caffeine as the internal standards. The optimization of the electrolyte composition was approached using 3^2 full factorial design with a central point to study the MEKC electrolyte composition was 5mmol/L sodium tetra borate (STB, pH 9,3) containing 120mmol/L sodium dodecyl sulphate (SDS), under optimal CE conditions. Separation of the investigated substances was achieved in less than 0.7mins. Quality parameters, such as linearity (R^2> 0.99), precision (RSD<5.4%), detection and qualification limits (3.37 and 11.24 mg/kg for apple juice samples) and recovery (96.37-99.5%) were obtained. The proposed methodology was successfully applied to the analysis of 5-HMF in the

apple juice samples. The analytical performance of this method made it suitable for implementation in food laboratories for the routine deterioration of 5-HMF in apple juice.

2.7 Gas Chromatography/Ultra-Violet Spectrometry

A study was done using a rapid method for the determination of Levulinic acid (LA) and 5-HMF in acid hydrolyze system of glucose based on UV spectroscopy. It was found that 5-HMF and LA have a maximum adsorption at the wavelengths of 284nm and 266nm respectively, in water medium and the adsorption of 5-HMF and La at 284nm and 266nm followed Beer's law very well. However it was found that major spectral interference species which arise in the quantification of 5-HMF and LA can be quantified with double wavelength techniques. The repeatability of the method had a reactive standard deviation of less than 4.47% for 5-HMF and 2.25% for LA, the limited quantification (LOQ) was 0.017mmol/L for 5-HMF and 4.68mmol.L for LA, and the recovery ranged from 88% to 116% for 5-HMF and from 94% to 105% for LA. The researchers found the method to be in simple rapid and accurate. And concluded that it is suitable to use in the determination of 5-HMF and LA in bio refinery area, (Mendoz et. al., 2013).

2.8 LIQUID CHROMATOGRAPHY/ULTRA-VIOLET SPECTROSCOPY

Zhang et al, (2013) made an improved analytical method for the rapid reliable and sensitive determination of 5-HMF in food. The method enabled aqueous extraction from food matrix with simultaneous clarification using Carrel I and II reagents, solid-phase extraction clean up using oasis and analysis by Liquid Chromatography-Mass spectrometry. A narrow bore column allowed fast chromatographic separation with good resolution of 5-HMF and mixture concentrations. In positive atmospheric pressure chemical ionization conditions, precursor and compound specific ions were sensitively detected in selected ion monitoring mode. Sample preparation with efficient clean-up followed by fast

chromatographic analysis allowed the analysis to be completed in <20mins. Recovery ranged between 91.8 and 94.7% for spiking levels of 0.25, 1.0 and 5.0mg/kg, 5-HMF cereal based foods. The method was shown to be successful when using liquid chromatography coupled to ultraviolet detection at 285nm.

2.9 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Jalal (2010) determined 5-HMF by HPLC method in 20 samples of apple juice. The sample was extracted and the extracts were then cleaned-up on silica gel column. The 5-HMF was determined by reverse phase liquid chromatography, a C_{18} column and a photo diode array (DAD) detector, using the mixture of water as mobile phase with a flow rate of 1.0min.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 STUDY AREA

The determination of 5-HMF from apple juice was carried out in ADAMA Beverages Limited and Adamawa State Polytechnic Yola, Laboratories in Jimeta metropolis, Adamawa State.

3.2 GEOGRAPHICAL LOCATION

Adamawa state Beverages limited is located in Jimeta at longitude 9N and latitude 12E, it has a sub-Saharan climate and is located at the North Eastern part of Nigeria.

3.3 MATERIALS AND EQUIPMENTS

Three samples of industrial processed apple juice were bought from Jimeta Ultra-Modern Market Adamawa state; One (1) sample was locally processed. This sample was stored at room temperature and analyzed before the experiment date. The following reagents were bought from Northern Scientific Laboratories, Jimeta Adamawa state and all were of analytical grade

i. Acetonitrile
ii. Ethyl acetate
iii. n-Hexane (HPLC grade)
iv. Ethanol
v. Acetic acid (extra-pure grade)
vi. Anhydrous sodium sulfate
vii. Anhydrous sodium hydrogen carbonate
viii. Per chloric acid 60% and sand
ix. Hydroxymethyl furfural standard
x. Water

3.4 EXPERIMENTAL PROCEDURE

3.4.1 PROCEDURE OF LOCALLY PROCESSED APPLE JUICE

Five (5) fresh apples were washed to remove dust and dirt. Samples were cut into pieces and the seeds removed. The apples were blended for juice extraction after which the juice was sieved.

3.4.2 PREPARTION OF STOCK SOLUTION

The analysis was carried out using the method adopted by (Aryans et al, 2005).

A stock solution of 5-hydroxymethylfurfural was prepared by dissolving 5mg in 25ml of ethyl acetate, then diluting the solution to 1:50(v/v)to obtain a final 5-HMF concentration of 0.2mg/mk.

A 100ul of these stock solutions was transferred into a 10ml volumetric flask, and evaporated to dryness under a stream of nitrogen at room temperature. The residue was immediately dissolved in 10ml of water (pH 4.0) acidified with acetic acid. Working standards were prepared by appropriate dilution of these solutions with acidified water (pH4.0).

3.4.3 ANALYSIS OF 5-HMF IN APPLE JUICE

10ml of apple juice was extracted in an extraction tube with ethyl acetate extraction solvent (ethyl acetate/n-Hexane, 60:40 v/v), sodium sulfate, sodium hydrogen carbonate and sand. After extraction, the extract was cleaned up on silicagel solid-phase column (SPE, Supelco, Bellefonte, PA).

The purified extract was evaporated to dryness under a stream of nitrogen, dissolved in aqueous acetic acid solution (pH 4). The dissolved samples were filtered through a membrane filter (0,45um) and the filtrate was transferred to a HPLC vial.

CHAPTER FOUR

4.0 RESULT AND DISCUSSION

The result of the HPLC analysis in apple juice for 5-HMF is presented in the table below;

Table 4.0: Levels of 5-HMF in locally and industrially processed apple juice.

SAMPLE TYPE	NUMBER	5-HMF < 20mg/kg	5-HMF >20mg/kg	MAXIMUM (mg/kg)	MEAN/ SD
100% apple juice	1	1(100%)	0(0%)	18.8	8.16 + or – 12.8
50% apple juice	3	1(10%)	2(90%)	48.5	10.3 + or – 11.0
TOTAL	4	2(55%)	2(45%)	48.5	9.23 + or- 11.9

From the result it can be noted that the chromatography condition provided very good separation and fast analysis.

A total of 4 samples of apple juice were analyzed and 5-HMF was present in all the examined samples. In aspect of 100% apple juice, 5-HMF was found, at concentration less than 20mg/kg. Which is the maximum prescribed value by world health organization and Nigeria Legislation. The maximum level found in 100% apple juice was 18.8mg/kg while the mean content of 5-HMF was 8.16 + or – 12.8 mg/kg.

In the category of 50% apple juice, 1 sample 5-HMF had concentration below 20mg/kg. In 2 samples, 5-HMF was detected, higher than 20mg/kg, which is not in accordance with world health organization and Nigeria Legislation. The mean level of50% apple juice 10.3 + or – 11.0mg/kg, the 5-HMF found was slightly higher than in sample with 100% apple juice.

Table 4.2: World health organization limit on 5-HMF.

SAMPLE TYPE	NUMBER	5-HMF < 20mg/kg	5-HMF >20mg/kg	MAXIMUM (mg/kg)	MEAN/ SD
100% apple juice	10	9(90%)	1 (10%)	47.8	9.46 + or – 14.8
50% apple juice	10	8(80%)	2(20%)	38.5	11.5 + or – 11.0
TOTAL	2o	17(95%)	3(15%)	47.80	9.89 + or- 12.1

Based on the researched carried out by world health organization, 20 samples of apple juice were used to determine the presence of 5-HMF in the apple juices. Then the world health organization approved that 50% apple juice will be consumed more than 100% apple juices, because the 50% apple juice had less than 20mg/kg with 38.3mg/kg while 100% apple juice has maximum or more than 20mg/kg with 47.8mg/kg.

Finally, of the 4 examined samples of apple juice in (table 4.1). The 2(55%) samples content of 5-HMF was higher than maximum allowed value set by domestic legislation. Until this year, maximum level of 5-HMF in apple juice have not been prescribed by world health organization and Nigerian Legislation. Result of this study indicated the importance of further mentioning of 5-HMF in apple juice.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The result of this research work shows that 100% apple juice contain less amount of 5-HYDROXYMETHYLFURFURAL which had 18.8mg/kg while 50% apple juice had the maximum amounts of 5-HYDROXYMETHYL FURFURAL with 48.5mg/kg.

5.2 RECOMMENDATIONS

Based on the research carried out it is recommended that

Apple juice product made from 100% apples should be consumed more than 50% apple juice.

Different kind of apple juice production may be analyzed to determine the factor that enhances the formation of hydroxymethylfurfural.

Chemical analysis of all apple juice products must be carried out to ensure good quality of the product.

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APPENDIX:

CHROMATOGRAPHY FOR 5-HMF.

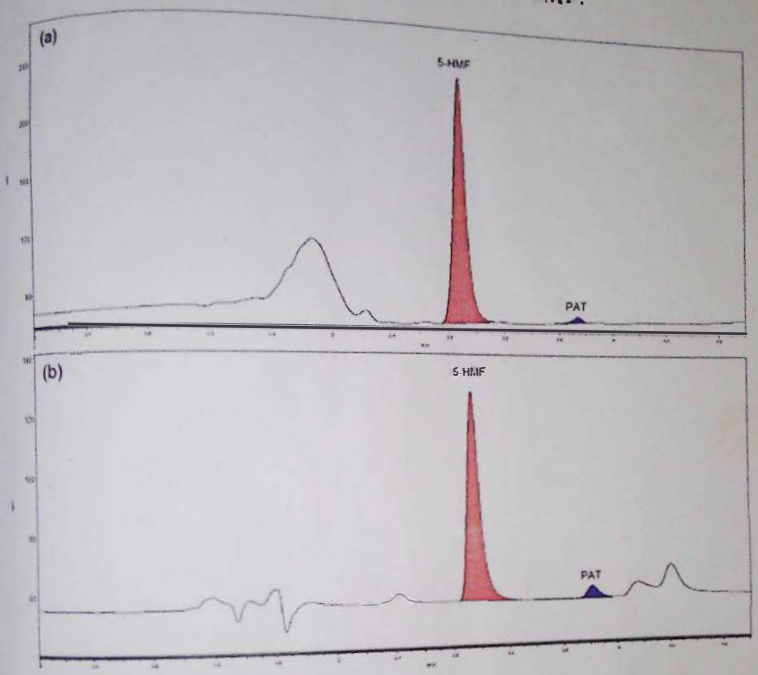

Figure 1: Typical Chromatography of (a) Apple Juice extract with 6.7mg L^{-1} 5-HMF and (b) Apple Juice extract with 0.9mg L^{-1} 5-HMF and 27ug L^{-1} PAT.