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Rapid Detection Method to Quantify Linamarin Content in Cassava

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Abstract

There are a number of natural remedies against cancer. Cassava (*Manihot esculenta* Crantz) has been proven to be a natural remedy against cancer due to the cyanogenic compounds it contains such as linamarin. A rapid and simple liquid chromatography-mass spectroscopy (LC-MS) method was developed to identify and quantify linamarin in Cassava. The method was developed using various Cassava (*Manihot esculenta* Crantz) extracts. Developed application is not limited to Cassava, but can be extended to other types of linamarin containing plant materials as well. Linamarin was quantified by the LC-MS system, with the mobile phase being H_2O:CH_3CN, 8:2, and a reverse phase C18 column. This method was utilized to determine the concentration of linamarin in leaf and tuber of *M. esculenta*. Linamarin concentrations from different types of extraction methods such as acidified water, acidified methanol, cryocooling and hot water were studied. The acidified methanol extraction yielded higher amounts of linamarin in its intact form compared to other methods and was therefore determined to be the most suitable sample preparation method to determine linamarin concentrations in Cassava. This LC-MS based linamarin detection method was shown to be sensitive, efficient, cost effective, and highly reproducible.

Keywords: Cancer; Chemotherapy; *Manihot esculenta*; Linamarin; Linamarase

Introduction

Cancer is the third most lethal disease in the world [1]. Natural medication plays a vital role in the search for novel chemotherapeutic agents against cancer [1] because, it is enriched by the Ayurvedic, Siddha and folk medicine. Cassava is one such example with anti-cancer properties which has been used for treatment of cancer for decades in folk medication [2].

The anti-cancer properties of Cassava are thought to stem from the cyanogenic compounds it contains. There are two major types of cyanogenic compounds in the cassava plant, Linamarin and Lotaustralin derived from L-Valine and L-isoleucine amino acids [3-5]. These compounds are harbored by the plant as a defense mechanism against herbivore attacks (Figure 1). Disruption of cells during such attacks results in liberation of sugar and HCN by the decomposition of cyanogenic glucosides [6,7]. Linamarin and Lotaustralin are the key molecules that possess anticancer properties in Cassava. Among these two, linamarin is the abundant cyanogen since plants only produce lotaustralin in minor amounts [5,8]. Hydrogen cyanide, which is produced by the hydrolysis of linamarin by the enzyme linamarase, is the key compound responsible for cytotoxic activity [5,8,9]. The investigation of cyanogenic compounds, especially linamarin, is important and has attracted great attention. Cyanogenicity, nutraceutical value, food safety, and toxicity of linamarin are the most important areas worth investigating.

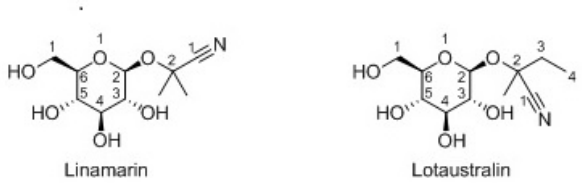

Figure 1: A) Linamarin structure; B) Lotaustralin structure.

A number of methods have been developed to identify linamarin. These methods have their own pros and cons and the detection can be done only up to a certain limit (~10 ppm) [4]. Various methods of linamarin determination in cassava incorporate the use of linamarase or sulfuric acid in the hydrolysis of linamarin, followed by the spectral determination of the formed hydrogen cyanide [8,10-12]. A spectrophotometric method (minimum detection concentration was 10 ppm) requires many reagents, including hazardous chemicals such as pyridine. The other widely used detection method developed by Haque et al. is a simple picric assay kit for cyanide identification [7]. This qualitative assay kit was based on the picrate paper kit produced in 2000 by the Hidayat et al. [13]. Microtiter plates and densitometry are some other methods used in the detection of cyanogenic compounds [4,14]. Of these two methods, the densitometric method is the more accurate, since the minimum detection limit is 20 ppb, but both methods were associated with reproducibility problems and frequently produced false positive results [14,15]. Curtis et al. published a gas chromatographic identification method for cyanide and carbonyl compounds using a PID (photoionization detector), and in 2007, Bacala and Barthet published a similar GC identification method for linastatin and neolinastatin, using a mass detector [4,6,16].

These methods are well tuned to detect produced HCN due to the heat cleavage of the cyanide group but not based on the actual amount of linamarin [6]. Furthermore, the cyanogenic compound amygdalin, contained in some fruit and nut samples, was determined using a polyclonal antibody based immunosorbent assay in 2006 [4,17]. But these methods are sensitive for all types of cyanogenic compounds and is not specific to linamarin. Based on the above mentioned fundamentals, few methods have been developed during the last couple of decades to identify and quantify linamarin content. Methods developed include the immobilized microplate method, the beta glucosidase electrode method, and the enzyme sensitized microcentrifuge tube method [10,12,18]. All these methods are based on the detection of HCN by the enzymatic cleavage of the cyanide bond of linamarin [8]. Therefore, frequent errors arise due to the contamination of samples with other types of cyanogenic compounds which go on to produce false results.

In order to reduce the incompatibilities that occur during research, a quick and a reliable analytical method to determine exact concentrations of linamarin coupled with an efficient sample preparation is needed. Therefore, a cost effective, fast, highly sensitive, and reproducible LC-MS based method was developed to identify and quantify linamarin in natural samples.

Materials and Methods

Plant materials

Freshly harvested tuber and leaves of cassava were selected for the isolation process. The tuber was thoroughly washed with running tap water and peeled. Both tuber and leaves were cut in to small pieces and used for extraction.

Extraction procedure

Leaves (5.0 g) and bark (5.0 g) were taken for the isolation process. Four types of extraction methods were used; namely, "normal" extraction, acid extraction, hot water extraction, and extraction after freezing in liquid nitrogen (cryocooling). Both methanol (MeOH: Sigma Aldrich, USA) and water were used as the extractors except in the hot water extraction where only water was used.

Normal extraction: Plant materials were treated as before with 50 mL of extracting solvent (water or methanol), but with no addition of acid.

Acid extraction: Plant materials (5 g each) were placed in 100 mL Erlenmeyer flasks and 50 mL of extracting solvent (water or methanol) were added to each. The pH was adjusted to 2 by adding concentrated hydrochloric acid (Fluka, USA).

Hot water extraction: Plant materials were placed in 100 mL flasks and 50 mL of water at 80°C was added. The mixture was kept for 20 min at this temperature.

Cryocooling extraction: Plant materials were first frozen with liquid nitrogen and then 50 mL of extracting solvent (water or methanol) was added.

The material in each sample was homogenized for 1 min at high speed in a blender. The homogenates were filtered through a filter cloth to remove insoluble materials, followed by a second filtration using Whatman No. 1 filter paper. The cloudy filtrate was then centrifuged at 9000 rpm for 1 hour and the clear supernatant liquid was decanted and used for analysis directly.

Isolation of pure linamarin

The acidic aqueous methanol showed the highest efficacy of extraction and was used to isolate pure linamarin from crude cassava. Cassava peels (209.2 g) obtained from cassava root were purchased from local markets in Sri Lanka and were homogenized with 600 mL of acidified methanol (pH=2) two times for 1 min at high speed in a blender. The homogenates were filtered through a filter cloth to remove insoluble materials, followed by a second filtration using Whatman No. 1 filter paper. The cloudy filtrate was then centrifuged at 9000 rpm for 1 hr and the clear supernatant liquid was decanted and concentrated under reduced pressure and the crude linamarin extract was obtained as a brown color viscous oil (10.15 g). The residue was filtered through a plug of celite followed by flash silica chromatography with 9:1 EtOAc:MeOH, to elute pure Linamarin (0.65 g, Rf=0.6) based on a TLC coloring agent (aniline, diphenylamine, H_3PO_4) and NMR analysis. The pure linamarin was used as the linamarin standard to construct the calibration curve and to spike the crude samples (Figure 2).

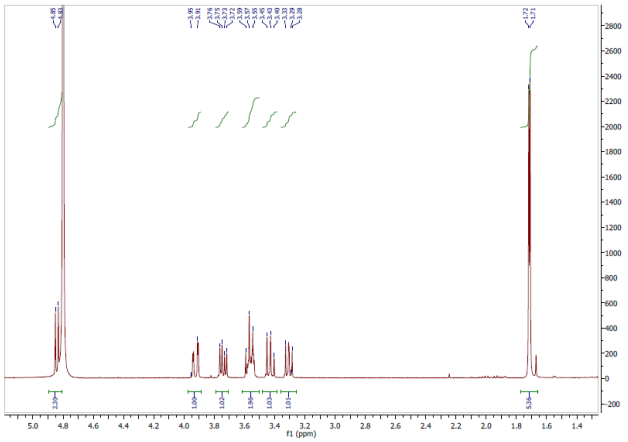

Figure 2: ^{1}H NMR spectrum of purified Linamarin.

Methods of analysis

The linamarin content of the samples was analyzed using LCMS (LC-E2695 coupled with Alliance, Mass Detector-SQD2) with isocratic elution using 8:2 H_2O:CH_3CN (GCMS grade, Sigma Aldrich, USA), and the mass spectrum was used to identify the peak corresponding to linamarin. A stainless steel (12.5 cm × 4.6 mm), Spherisorb ODS1 (5 µm), silica based, reversed-phase C_{18} column was used for analysis, with a flow rate of 2 mL/min and an injection volume of 20 µL (Column temperature 25°C). The mass spectrometer was adjusted to detect the sodium ion peak of linamarin, and peak area was used to calculate the concentration of linamarin. The linamarin peak was observed at 3.30 min with SIR m/z set at 270 (M+Na) (Figure 3).

The calibration plot, used to determine concentrations was prepared using pure linamarin, isolated from the method described previously. From a stock solution of 1.0 ppm linamarin, a dilution series with concentrations from 5 ppb to 120 ppb was prepared for this purpose.

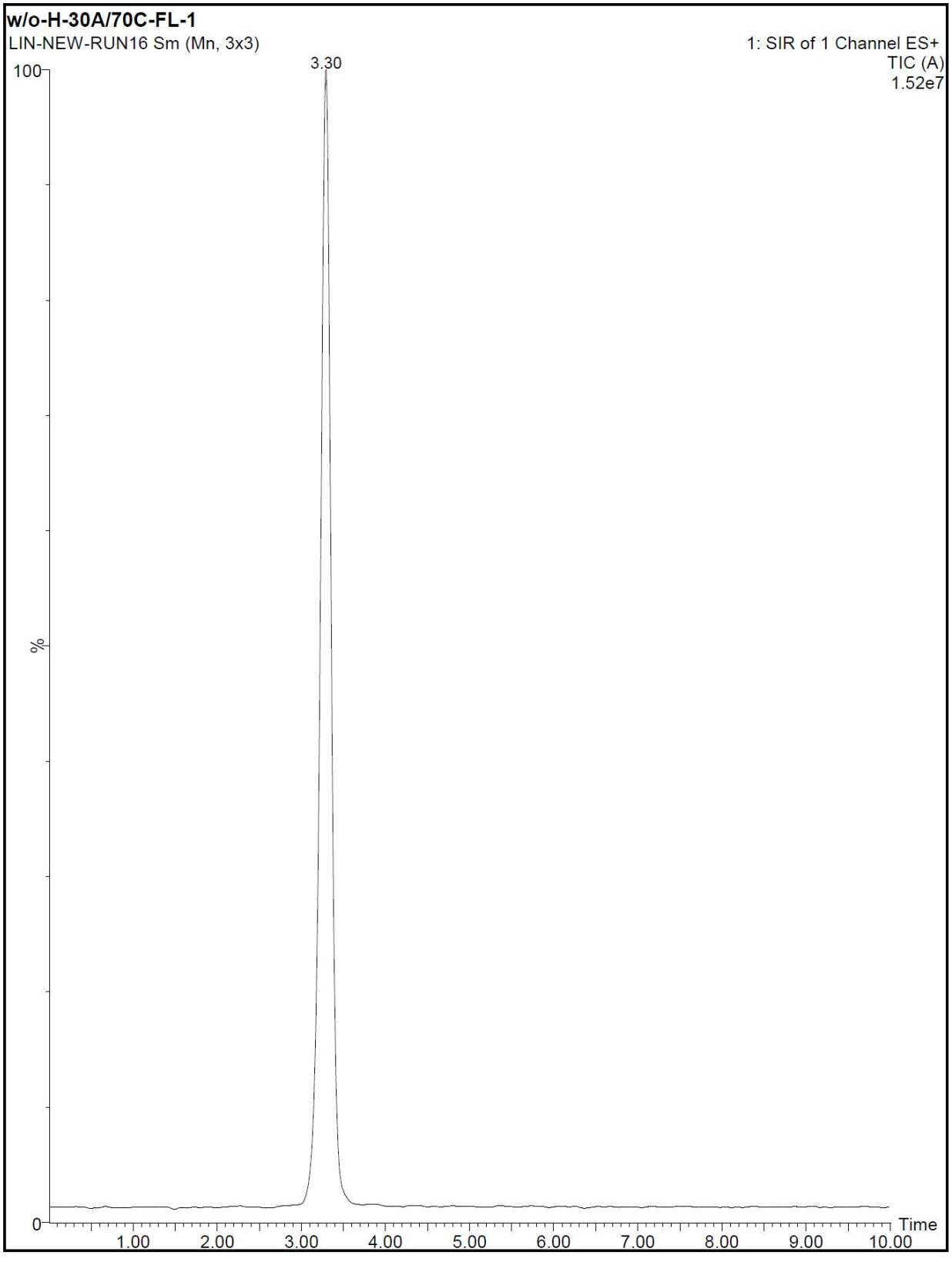

Figure 3: LCMS chromatogram of Linamarin.

Determination of matrix effect

Leaf and bark extracts were injected and the extract was selected with the range of 20 to 50 ppb concentration. Then the samples were spiked with 20 ppb concentration pure linamarin sample and the final concentrations were measured.

Results and Discussion

The results obtained from LC-MS were analyzed using Origin software. Initially, a calibration plot was drawn using the pure linamarin sample. The lower limit of detection (LLOD) of linamarin was 0.5 ppb. But the linearity of the response varied drastically from 0.5 to 5 ppb and above 5 ppb the responses were within the linear range. Therefore, the lower limit of quantitation (LLOQ) of linamarin was taken as 5 ppb. The maximum detection level was 120 ppb and a saturated column was obtained above 120 ppb concentrations. All the measurements were carried out in triplicates and the concentration values were expressed with the standard deviation (± SD). The obtained results are shown in Table 1 and the respective calibration plot is shown in Figure 4. The parameters are given in Table 2.

Concentrations of pure linamarin (ppb)	LC-MS Response (Mean)	Concentrations detected ± SD	% Deviation
0 (Blank)	120	0	0
5	83900	4 ± 1.24	-4.2
10	166776	9 ± 0.88	-5.7
15	275112	16 ± 1.62	4.8
20	363669	21 ± 1.98	4.3
25	455780	26 ± 2.04	4.8
50	884883	51 ± 0.74	2.2
60	1057644	61 ± 1.30	1.8
80	1454686	80 ± 1.02	0.8
120	2182014	121 ± 2.21	1.3

Table 1: LC-MS response (Area under the curve) relevant to Linamarin standard.

Equation	y=a+b × x		
Weight	No Weighting		
Residual Sum of Squares	1.39E+09		
Adj. R-Square	0.99965		
		Value	Standard Error
Response	Intercept	-6461.0911	6051.10109
	Slope	18153.422	113.7976

Table 2: Plotting parameters of the calibration curve prepared using Origin software.

Four types of extraction methods were analyzed, namely, "normal" extraction, acid extraction, hot water extraction, and extraction after freezing in liquid nitrogen (Cryocooling extraction) using both water and methanol as the extracting solvent. The responses for crude linamarin extracted from the leaves of *M. esculenta* with water and methanol as solvent in all four types of extraction methods is shown in Table 3 and the comparison is given in Figure 5. Table 4 and Figure 6 shows the responses and the comparison of crude linamarin extracted from the tuber of *M. esculenta* with water and methanol as solvent in all four types of extraction methods.

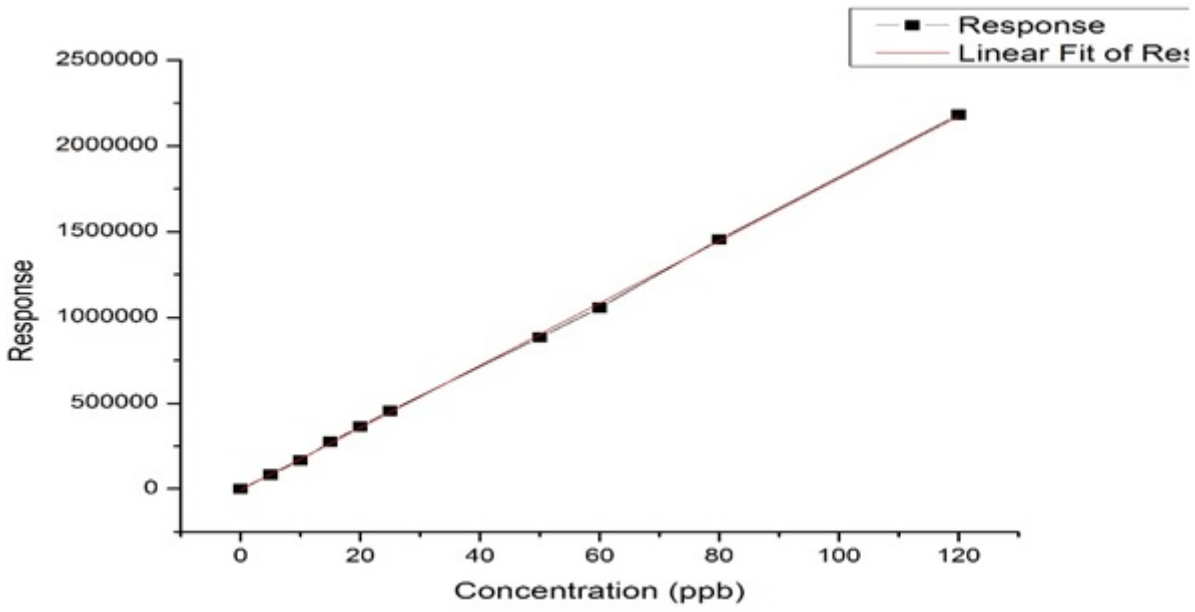

Figure 4: Linear response graph for the Linamarin standard prepared using authentic Linamarin Sigma Aldrich.

Fractions	Mean response	Concentration (ppb)
Water L	249644	14 ± 0.47
MeOH L	1547625	85 ± 3.03
Water L-N	437304	24 ± 1.00
MeOH L-N	1869775	103 ± 2.48
Water L-Acid	1619562	88 ± 3.27
MeOH L-Acid	2263861	123 ± 5.12
Water L-Heat	491256	27 ± 0.76

Table 3: The examined responses and the relevant concentrations of crude Linamarin extracted from the leaves of *M. esculenta*. Water: water was used as the extractor; MeOH: methanol was used as the extractor; L: Leaves was the plant part; TB: Tuber bark was the plant part; N: Before extraction the plant parts were cryogenized using liquid nitrogen; Acid: The extractor was acidified (pH=2) using conc. HCl before extraction; Heat: Hot water (85°C) was used as the extractor.

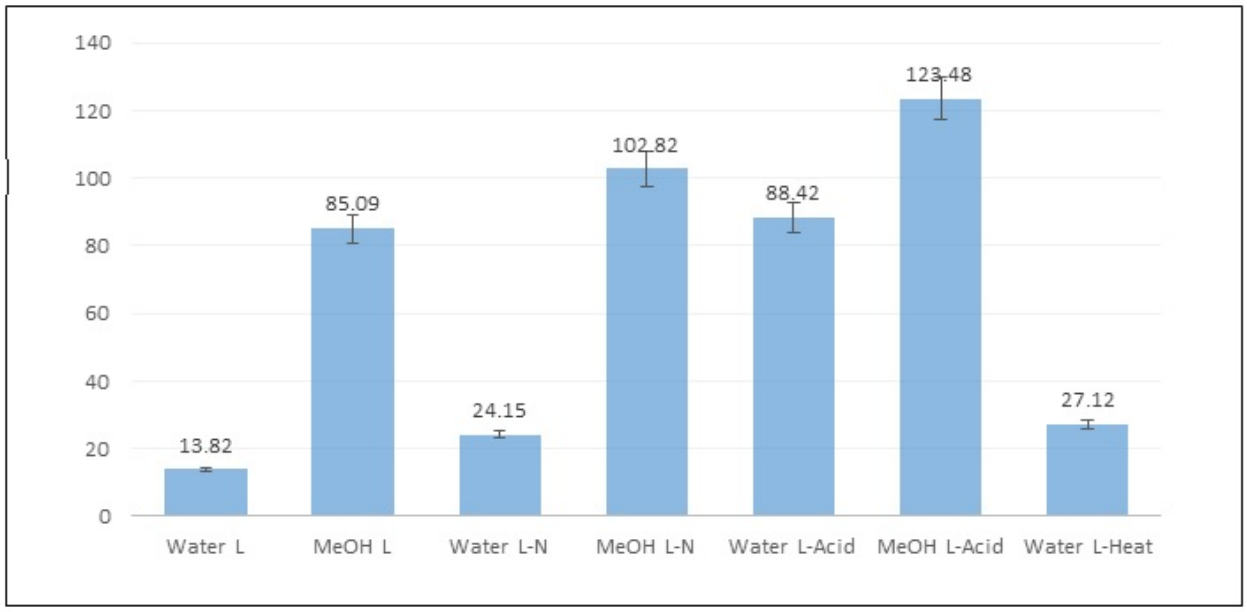

Figure 5: The comparison of the method of extraction of crude Linamarin extracted from the leaves of *M. esculenta.*

Fractions	Mean response	Concentration (ppb)
Water TB	177708	10 ± 0.21
MeOH TB	1574210	87 ± 2.41
Water TB-N	285612	16 ± 0.76
MeOH TB-N	1690716	93 ± 2.22
Water TB-Acid	1485072	82 ± 2.04
MeOH TB-Acid	2147355	118 ± 4.43
Water TB-Heat	571794	31 ± 1.21

Table 4: The examined responses and the relevant concentrations of crude Linamarin extracted from the Tuber of *M. esculenta.*

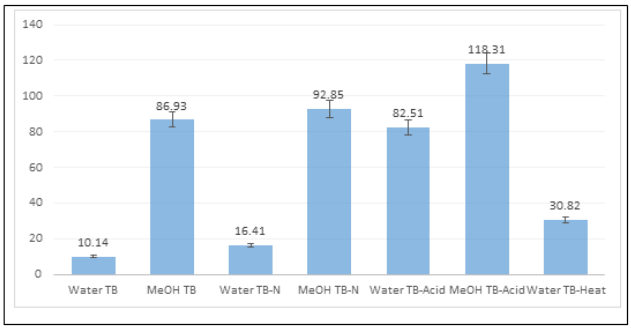

Figure 6: The comparison of the method of extraction of crude Linamarin extracted from the tuber of M. esculenta.

According to the results obtained, the maximum concentration of linamarin was shown in the leaf extract. Both leaf and tuber bark extract has shown the same concentration pattern with narrow fluctuations. The acidified methanol extract in both the leaf and tuber bark showed the highest detectable amount of linamarin while the aqueous extracts showed the lowest. The second highest linamarin concentration was detected by cryogenized methanol extract. The hot water extraction method did not result in any significant increase of linamarin, possibly due to the degradation of linamarin by heat. Therefore, sample preparation is a critical factor when determining the linamarin concentration in cassava. Based on Figures 5 and 6, it is evident that the most suitable sample preparation method to determine the linamarin in cassava plant is the acidified methanol system.

Since the crude extract of the plant material contains plant matrix, it is crucial to study any interference caused by this to the analyzed method. To determine the matrix effect on the detection of linamarin, the spiking method was introduced for both leaves and tuber barks. The results were given in Table 5.

Fraction	Initial response	Initial concentration	Spiked amount (ppb)	Final response	Final Concentration
MeOH L	452772	25.08 ± 0.50	25	922874	51.33 ± 1.32
MeOH TB	547330	30.18 ± 0.76	25	1008885	55.66 ± 1.62

Table 5: Determination of matrix effect of crude Linamarin using spiking method.

The experiment to determine the matrix effect showed that there are no significant effects of the *M. esculenta* matrix on the detectable concentration of linamarin.

Conclusion

Herein, we have described a rapid and simple liquid chromatography-mass spectroscopy (LC-MS) based method for detection of Linamarin. This method can be used to detect linamarin concentrations down to 5 ppb. To our knowledge, this is the lowest detection limit reported among published methods. Therefore, this is a very sensitive method for detecting minute amounts of linamarin present in samples. The proposed method also allows rapid detection of linamarin due to the straightforward sample preparative method (acidic methanol extract). This method can also be used for the large-scale isolation of Linamarin, which can thus be isolated with fewer purification steps, and with higher percentage yields. When compared to other existing linamarin detection methods this method is quick, inexpensive, reliable, and highly sensitive.

This study also provides an extensive comparison of the efficiency of various reported linamarin extractions methods. Our results show that the best method for isolation of linamarin is extraction with acidified methanol which could be used for sample preparation. Furthermore, this linamarin quantification method will be helpful to those who are engaged in research with linamarin and natural anticancer agents.

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